Evolution of both host resistance and tolerance to an emerging bacterial pathogen
Camille Bonneaud, Luc Tardy, Mathieu Giraudreau, Geoffrey E. Hill, Kevin J. Mcgraw, Alastair J. Wilson

To cite this version:
Camille Bonneaud, Luc Tardy, Mathieu Giraudreau, Geoffrey E. Hill, Kevin J. Mcgraw, et al.. Evolution of both host resistance and tolerance to an emerging bacterial pathogen. Evolution Letters, Wiley Open Access 2019, 3 (5), pp.544-554. 10.1002/evl3.133. hal-02468855

HAL Id: hal-02468855
https://hal.umontpellier.fr/hal-02468855
Submitted on 10 Feb 2020

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L’archive ouverte pluridisciplinaire HAL, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d’enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.
Evolution of both host resistance and tolerance to an emerging bacterial pathogen

Camille Bonneaud,1,2 Luc Tardy,1 Mathieu Giraudet,1,3,4 Geoffrey E. Hill,5 Kevin J. McGraw,3 and Alastair J. Wilson1

1Centre for Ecology and Conservation, University of Exeter, Penryn, Cornwall TR10 9FE, United Kingdom
2E-mail: c.bonneaud@exeter.ac.uk
3School of Life Sciences, Arizona State University, Tempe Arizona 85287
4Current address: Centre for Ecological and Evolutionary Research on Cancer, UMR CNRS/IRD/UM 5290 MIVEGEC, 34394 Montpellier, France
5Department of Biological Sciences, Auburn University, Auburn, Alabama 36849

Received May 21, 2019
Accepted July 29, 2019

Understanding how hosts minimize the cost of emerging infections has fundamental implications for epidemiological dynamics and the evolution of pathogen virulence. Despite this, few experimental studies in natural populations have tested whether, in response to disease emergence, hosts evolve resistance, which reduces pathogen load through immune activation, or tolerance, which limits somatic damages without decreasing pathogen load. Further, none has done so accounting for significant natural variation in pathogen virulence, despite known effects on host responses to infection. Here, we investigate whether eastern North American house finches (Haemorhous mexicanus) have evolved resistance and/or tolerance to their emerging bacterial pathogen, Mycoplasma gallisepticum. To do so, we inoculated finches from disease-exposed and disease-unexposed populations with 55 distinct isolates of varying virulence. First, although peak pathogen loads, which occurred approximately eight days postinoculation, did not differ between experimentally inoculated finches from disease-exposed versus unexposed population, pathogen loads subsequently decreased faster and to a greater extent in finches from exposed populations. These results suggest that finches from exposed populations are able to clear the infection through adaptive immune processes. Second, however, finches from exposed populations also displayed lower symptom severity for a given pathogen load, suggesting that a damage-limitation mechanism, or tolerance, has accompanied the evolution of immune clearance. Our results highlight that resistance and tolerance should be seen as complementary, not alternative, defense strategies: the evolution of resistance benefits from the concomitant evolution of tolerance mechanisms that protect against the damage of immune activation, whereas the evolution of tolerance without resistance will risk runaway selection on pathogen virulence.

KEY WORDS: Coevolution, house finch, infectious disease, Mycoplasma gallisepticum, tolerance, virulence.

Impact Summary
Emerging infections can have devastating impacts on their hosts, yet how hosts naturally evolve to deal with novels infections remains poorly understood. In particular, it remains unclear whether hosts evolve to become more resistant by mounting an immune response that will clear the infection, or whether they evolve to become more tolerant by lessening the symptoms of the infection. Understanding how hosts evolve to respond to infection is made all the more challenging by the fact...
that, for a given disease, there is natural variation in the level of aggression (i.e., virulence) of the infectious agents in circulation. Because more virulent pathogen variants are likely to give rise to stronger host responses, variation in virulence will impact our measurements of host responses to infection. As a result, if we want to understand how hosts evolve to deal with novel infections, we need to measure these responses against a range of pathogen variants that differ in their level of virulence.

In this article, we take advantage of a naturally emerging infection in a wild North American songbird (house finches) to test whether hosts evolve resistance or tolerance. We experimentally infect house finches with 55 pathogen variants of differing virulence, and compare the response of finches from disease-exposed populations that are known to have evolved in response to infection with that of finches from disease-unexposed populations, which remain susceptible to the infection. We show that, relative to finches from disease-unexposed populations, finches from disease-exposed populations have evolved to be able to clear the infection through an immune response, and to limit the damages due to the infection. Thus, resistance and tolerance should be seen as complementary, rather than opposing, defense strategies.

Hosts can alleviate the costs of infection by evolving two distinct—although not necessarily mutually exclusive—strategies (Kover and Schaal 2002; Schneider and Ayres 2008). They can evolve resistance, which serves to reduce the establishment of infectious pathogens and/or to clear pathogens following establishment (Boots and Bowers 2004; Janeway 2005), and they can evolve tolerance. The latter serves to mitigate somatic damage caused by the infection without reducing pathogen load (Råberg et al. 2009; Medzhitov et al. 2012; Råberg 2014). Whether and when hosts evolve resistance, tolerance, or both in response to emerging pathogens have far-reaching consequences for predicting virulence evolution and epidemiological dynamics (Miller et al. 2006), as well as for the design of novel pharmacological treatments (Vilaplana et al. 2013; Soares et al. 2017). Despite this, few experiments have been performed to investigate the relative importance of resistance versus tolerance in evolved responses to naturally emerging pathogens.

Hypotheses based on the evolution of resistance versus tolerance make contrasting predictions. First, if resistance has evolved, hosts will be better at clearing the infection and so show reduced pathogen load during an infection relative to nonevolved hosts (Miller et al. 2005, 2006; Råberg et al. 2007). Second, if tolerance has evolved, hosts will be better able to mitigate the impact of an increasing pathogen load. Under the widely used “range tolerance” concept (Little et al. 2010), this would be detected as a shallower negative regression of fitness on pathogen load (Simms 2000). Alternatively, assuming that loss of fitness can be directly attributed to clinical symptom severity, we would predict a weaker positive regression of symptoms severity (rather than fitness) on pathogen load (Råberg et al. 2007; see Box 1). Given these predictions, it is important to reiterate that damage-limitation mechanisms could evolve in conjunction with resistance (e.g., by limiting immunity or initiating repair) (Restif and Koella 2004; Howick and Lazzaro 2017). Thus, resistance and tolerance mechanisms need not be mutually exclusive, and evidence for the evolution of one is not necessarily evidence against evolution of the other.

There have been few experimental tests of the predictions for the evolution of resistance and tolerance in response to naturally emerging pathogens, and the handful of studies to date have yielded rather mixed conclusions. For example, strong evidence for the evolution of resistance comes from observations of the epidemic of myxoma virus in European rabbits (Oryctolagus cuniculus) in Australia (Kerr and Best 1998; Kerr et al. 2015). Following initially dramatic population declines, at seven years post-outbreak, rabbits from disease-exposed populations displayed mortality rates of only ~25% in response to experimental infection. In contrast, mortality rates of over 88% were found in unexposed wild and domestic rabbits (Marshall and Douglas 1961). Subsequent work confirmed that reduced mortality was mediated by the evolution of innate and cellular immune responses leading to significantly reduced pathogen loads (Best and Kerr 2000). By contrast, the endemic Hawaiian bird, Hawai‘i ʻAmakihi (Chlorodrepanis virens), was suggested to have evolved tolerance to Plasmodium relictum, following the pathogen’s introduction to the archipelago around the 1930s (Van Riper et al. 1986). Experimentally infected ‘Amakihi from high-altitude sites (where lower temperatures limit mosquito numbers and malaria parasite development; Van Riper et al. 1986; LaPointe et al. 2010), displayed significantly higher mortality and weight loss than did individuals from low-altitude sites. Crucially, however, there was no significant difference in pathogen load (Atkinson et al. 2013).

Here, we test the role of resistance versus tolerance in evolutionary responses of North American house finches (Haemorhous mexicanus) to the emerging, conjunctivitis-causing bacterium Mycoplasma gallisepticum, following its jump from poultry in 1994 (Dhondt et al. 1998; Nolan et al. 1998). Several previous experiments on this system have yielded apparently contradictory conclusions. Bonneau et al. (2011) concluded that resistance had evolved from standing genetic variation within 12 years of
**Box 1: Definitions, predictions, and implications of key terms**

Typically, it is assumed that resistance and tolerance represent mutually exclusive evolutionary responses to emerging pathogens (see “typical” predictions). However, there is no inherent reason why the two mechanisms cannot operate in tandem, which changes the predictions for each (see “generalized” predictions). Note that predictions may be stated in terms of “fitness” rather than proxies such as “clinical symptom severity.” The latter are more readily measured but carry implicit assumptions (e.g., that fitness declines with increasing symptom severity). The choice of the response variable also has implications for the distinction between point and range tolerance (see Fig. B1)

| Definition | Predictions | Assumptions/Implications |
|------------|-------------|--------------------------|
| **Resistance**: Resistant hosts are better able to clear the infection than nonresistant hosts. (Note that we consider here resistance via immune activity) | **Typical**: Resistant hosts display reduced pathogen load relative to nonresistant hosts, which results in reduced clinical symptoms but not in lower symptom severity for an equivalent pathogen load. **Generalised**: Resistant hosts display reduced pathogen load relative to nonresistant hosts, which results in reduced clinical symptoms. | Resistance and tolerance are mutually exclusive evolutionary strategies. |
| **Tolerance**: Tolerant hosts are better able to mitigate the cost of infection than nontolerant hosts. | **Typical**: Tolerant hosts display reduced symptom severity relative to nontolerant hosts, but carry equivalent (i.e., not significantly different) pathogen load. **Generalised**: Tolerant hosts display reduced symptom severity conditional on pathogen load (which may/may not be equivalent to nontolerant hosts). | Resistance and tolerance can cooccur within the same evolutionary response. |
| **Range tolerance**: Tolerant hosts display reduced rate of symptom worsening as pathogen load increases than nontolerant hosts. | The slope of the regression between clinical symptoms and pathogen load is reduced for tolerant hosts relative to nontolerant ones. Usually estimated from symptom severity observed across a range of nonzero pathogen loads. | If the measure of clinical symptom severity is assumed to equal 0 in the absence of infection (i.e., pathogen load = 0), range and point approaches to characterizing tolerance are equivalent (Fig. B1). If this condition cannot be assumed biologically (e.g., because symptoms are nonspecific) and/or is not imposed analytically, empirical conclusions about tolerance may appear to differ depending on whether a slope or point approach is taken (see Fig. B1 legend). |
| **Point tolerance**: Tolerant hosts display lower clinical symptoms severity for a given pathogen load. | Tolerant hosts display reduced clinical symptoms for a given pathogen load relative to non-tolerant hosts. Usually estimated from a symptom severity at a single nonzero pathogen load. | Note that if fitness rather than symptom severity is used, point tolerance approaches carry an implicit assumption of equal y-intercepts (i.e., fitness in the absence of infection) that is unlikely to hold true. Violation of this assumption will bias conclusions about tolerance. |

**Figure B1.** Schematic figure of the regression of symptoms severity on pathogen load for two host genotypes. (A) Host genotypes differing in tolerance, with the gold genotype being more tolerant to infection (i.e., symptoms severity increases more slowly with increasing pathogen load). If we assume that symptoms severity is equal to 0 in the absence of infection (i.e., pathogen load = 0), then any evidence of point tolerance (dots) will be equivalent to a difference in range tolerance (i.e., slopes). (B) and (C) For point tolerance to be distinct from range tolerance, regression slopes of symptoms on pathogen load need to have intercepts that can differ from 0. Hypothetically, host genotypes could then differ either (B) in range tolerance (i.e., slopes), but not in point tolerance (gray dot), or (C) in point tolerance (dots), but not in range tolerance. It is difficult to imagine that infection-specific symptom severity is >0 in the absence of infection. Nonspecific indicator of health, on the other hand, could vary among uninfected individuals (as of course could fitness). Nevertheless, if the severity of specific symptoms is used, we suggest that evidence of point but not range tolerance (or vice versa) is an artefact of the sampling regime.
outbreak: finches from disease-exposed populations displayed reduced pathogen load following infection with a virulent, contemporary 2007 isolate. By contrast, Adelman et al. (2013) concluded that tolerance had evolved: finches from disease-exposed populations in 2010 had similar pathogen load, but reduced symptoms (conjunctival swelling) relative to finches from unexposed populations and following inoculation with a low-virulence bacterial isolate collected at epidemic outbreak (i.e., in 1994). Further experiments are clearly needed to understand the relative roles of resistance and tolerance in the response to this emerging pathogen. In addition, because previous support for tolerance evolution arises in part from a lack of significant differences in pathogen load (as would be predicted under resistance evolution; Råberg et al. 2009), here we use a greater number of host individuals and of pathogen isolates of varying levels of virulence to reduce the possibility of type II error.

We conducted a large-scale infection experiment using 112 naïve house finches from disease-exposed (N = 53) and unexposed (N = 59) populations, and 55 bacterial isolates collected from the epidemic outbreak (1994) and during the subsequent 20 years (until 2015). After emergence near Washington D.C. in 1994, M. gallisepticum spread throughout the entire eastern U.S. range of house finches within three years, killing millions of birds (Fischer et al. 1997; Dhdndt et al. 1998). Although it later spread through much of the native western range (between 2000 and 2010; Duckworth et al. 2003; Dhdndt et al. 2006), some populations remain unexposed to date (e.g., in Arizona; Staley et al. 2018). We have shown previously that the virulence of M. gallisepticum, defined as the amount of damage done to the house finch host, has increased over the course of the epidemic (Bonneaud et al. 2018; Tardy et al. 2019). Furthermore, we have shown that house finches from exposed populations display less severe symptoms than those from unexposed populations (Bonneaud et al. 2018). In this study, we test the key contrasting predictions set out above to determine whether this host evolutionary response is principally attributable to changes in resistance or tolerance.

First, if finches from exposed populations have evolved resistance, we would expect them to display lower pathogen loads during infection than birds from unexposed populations (i.e., populations that have not evolved resistance). Specifically, because resistance to M. gallisepticum is thought to be mediated through the ability to mount a cell-mediated immune response (Bonneaud et al. 2012b), given evolved resistance, finches from exposed populations are expected to show reduced pathogen load from approximately two weeks postinfection (i.e., the time required to mount a pathogen-specific immune response). By contrast, if tolerance alone has evolved, we predict no differences in pathogen load over the course of the month-long infection experiment between finches from the two populations. Further, we would expect that the relationship between symptom severity and pathogen load will be shallower in birds from exposed populations (range tolerance; Råberg et al. 2007), although reduced symptoms for a given pathogen load have also been given as evidence of (point) tolerance (Graham et al. 2011) (see Box 1).

Methods

CAPTURE AND HOUSING

Wild hatch-year house finches from populations that have never been exposed to M. gallisepticum (unexposed populations) were captured in urban areas and in suburban parks (see Bonneaud et al. 2018) in Arizona over a two-week period of the summer 2015. We trapped, weighed, and banded each bird with a numbered metal tag for individual identification (N = 171; 93 males and 78 females). They were then immediately transported by car to an aviary at Arizona State University, where they were housed for the remainder of the experiment. On arrival, we sampled blood from each bird by brachial venipuncture (60 µL of whole blood) and also took a choanal swab. A lack of prior infection was confirmed for each bird by screening blood plasma samples for anti-M. gallisepticum antibodies using a serum plate agglutination assay (Luttrell et al. 1996). Absence of current infection was verified using the choanal swabs in PCR amplification of M. gallisepticum DNA (Roberts et al. 2001a). No prior or current M. gallisepticum infections were detected (as expected given no documented reports of M. gallisepticum from this area of Arizona; Staley et al. 2018). They were then allowed to acclimate in the aviary for >1 month, with ad libitum food and water. During this time, although none of the birds displayed any sign of infection with other diseases, all were treated prophylactically for Trichomonas gallinae with carmidazole (Spartrix, Janssen/Elanco) and Isospora spp with sulfadimethoxine in the first 40 days of captivity.

During the same time period, we also caught hatch-year house finches from populations known to have been exposed to M. gallisepticum since the disease outbreak (i.e., currently maximally 20 host generations; exposed populations). All eastern house finch populations were exposed to M. gallisepticum within three years of outbreak, meaning that there is little variation in exposure duration among them (Dhdndt et al. 1998). These were captured from urban areas and suburban parks in Alabama (see Bonneaud et al. 2018). Birds were similarly banded, weighed, and sampled for blood and choanal swabs (N = 131). They were then immediately transported by car to aviaries at Auburn University, where they were housed separately in the same conditions as in the aviaries in Arizona and tested for prior and current infection as described above. Birds positive for either test were released immediately and not used for the study. In this way, we ensured that individuals from exposed populations used in the study had not themselves been previously infected with the pathogen. These remaining individuals (N = 53; 24 males and 29 females) underwent
>30-day quarantine period with ad libitum food and water, and during which they were treated prophylactically for *Trichomonas gallinace* and *Isospora* infections (see above). They were then transported in an air-conditioned vehicle to the aviary at Arizona State University. Care was used to minimize travel time (<30 h), movement, and stress to the birds; food and water was provided ad libitum throughout the trip and the birds were regularly checked for any signs of distress or injury.

Following arrival at Arizona State University, 112 birds (53 birds from the exposed populations and 59 birds from the unexposed populations) were haphazardly selected for use in the present study (the remaining 112 individuals being used in another experiment). They were then allowed to acclimate in the aviary, with ad libitum food and water, for >1 month prior to experimental inoculation.

**EXPERIMENTAL INOCULATION**

We haphazardly inoculated each of the birds with one of 55 *M. gallisepticum* isolates sampled over the course of the epidemic. We elected to use a large number of isolates in this paired design so that differences between exposed and unexposed host populations can be interpreted as averaged across any isolate-specific effects. This study is thus designed to draw maximally general inference on between-host population differences in response to *M. gallisepticum* infection. It is not designed to fully characterize differences among pathogen isolates, an aim that would be better served by using fewer isolates replicated across multiple hosts per population. Isolates were originally obtained from naturally infected, wild-caught house finches by swabbing the conjunctiva of a symptomatic bird and placing the swab in SP4 growth medium. Isolates were collected over a 20-year period and obtained from various urban and suburban sites in eight different States in the eastern United States (mainly from Alabama; Bonneaud et al. 2018). Isolates were administered via 20 µL of culture containing 1 × 10^4 to 1 × 10^6 color changing units/mL of *M. gallisepticum* in both eyes. To quantify conjunctival swelling, we photographed the right and left eyes at 0, 6, 13, and 25 days postinoculation (dpi) from a standardized distance. We then measured the average area of the conjunctiva swelling across the two eyes and at each day as: the area of the outer ring minus the area of the inner ring at 6, 13, or 25 dpi—the area of the outer ring minus the area of the inner ring at 0 dpi (see Staley et al. 2018). Measurements were blind with respect to the isolate inoculated and the population of origin of the bird. The experiment was stopped at 35 dpi and all birds were euthanized. Protocols were approved by Institutional Animal Care and Use Committees of Auburn University (protocol # PRN 2015–2721) and of Arizona State University (protocol #15-1438R), and by Institutional Biological Use Authorizations to Auburn University (# BUA 500).

**PATHOGEN LOAD**

Bacterial load was measured by quantitative amplification of *M. gallisepticum* DNA from pooled conjunctival and tracheal swabs obtained at 8, 14, 21, and 28 dpi. DNA was extracted using a QIAGEN DNeasy® Blood and Tissue Kit according to the manufacturer’s standard protocol (Qiagen, Germany). For each sample, we ran a multiplex quantitative PCR of the *M. gallisepticum*-specific gene mgc2, which encodes a cytidylyltransferase, and the house finch recombination-activation gene rag1, using an Applied Biosystems™ StepOnePlus™ Real-Time PCR system (Applied Biosystems, USA) (Tardy et al. 2019).

Each reaction contained: 2 µL of sample genomic DNA template, 1 µL each of 10 µM mgc110-F/R, and rag1-102-F/R primers (total 4 µL), 0.5 µL each of 10 µM Mgc110-JOE and Rag1-102-6FAM fluorescent hydrolysis probes (total 1 µL), 10 µL of 2× qPCRBio Mix HI-ROX (PCR BIOSYSTEMS) and 3 µL Nuclease-free water (Ambion®, USA). Reactions were then run at 95°C for 3 min, followed by 45 cycles of 95°C for 1 s and 60°C for 20 s. Samples were run in duplicate alongside serial dilutions of plasmid-based standards (range of standards for mgc2: 1.6 × 10^8 – 1.6 × 10^3 copies; range of standards for rag1: 8.0 × 10^7 – 8.0 × 10^2 copies). Amplification data were exported to LinRegPCR version 2017.1 for calculation of individual reaction efficiencies and quantification of low-amplification samples (Ruijter et al. 2009; Tuomi et al. 2010); between run variation was normalized using Factor qPCR version 2016.0 (Ruijter et al. 2015), with plasmid standard serial dilutions used for factor correction.

**STATISTICAL ANALYSES**

All statistical analyses were conducted in R 3.3.2 (Team RC 2016) using linear mixed effect models fitted in lme4 (Bates et al. 2015), and figures were made using ggplot2 (Wickham 2009). We previously showed that the probability of developing conjunctivitis following experimental inoculation did not differ between birds from exposed versus unexposed populations, but the former subsequently displayed less severe symptoms (Bonneaud et al. 2018). To test the roles of resistance and tolerance in this host evolutionary response, we restricted our analyses only to individuals that became symptomatic (N = 83) and further removed 3 individuals that died during the course of the experiment due to incomplete data. We thus analyzed data from 80 symptomatic individuals (N = 34 birds from exposed populations each inoculated with a distinct isolate and 46 birds from unexposed populations inoculated with 1 of 45 isolates (one isolate from 2007 was inoculated into two birds from unexposed populations)). When fitted as response variables, pathogen load was transformed using natural logarithm, whereas peak conjunctival swelling was square root transformed to ensure normal residuals. In total, we
run three different models. First, we investigated the effects of year of pathogen sampling and whether finches were obtained from disease-exposed or unexposed populations on peak pathogen loads (ln transformed) using a mixed linear model, with pathogen isolate fitted as a random intercept. Second, to test for evidence of population differences in the rate of pathogen clearance, we ran a mixed effects model with ln(pathogen load) as the response term, host population (exposed vs. unexposed), dpi (as a continuous covariate), and their interaction as explanatory terms. Bacterial isolate identity was included as a random intercept. Bird identity was included as an additional random term to account for repeated measures of loads over the course of the experiment. Third, we also used a mixed model to test for population differences in the association between pathogen load and clinical symptoms severity. Here peak conjunctival swelling (square root transformed) was the response term, with host population, peak pathogen load (square root transformed), and their interactions as fixed effects. Bacterial isolate identity was again included as a random intercept, but not individual bird identity (because each bird was only represented once). We note that square root transformations can stabilize variance and, in this case, that risks removing (or at least reducing) any signature of a population \times pathogen interaction on symptom severity. Because this interaction is key to our hypothesis testing (i.e., we predict a steeper regression of symptom severity on pathogen load in unexposed populations if range tolerance has evolved), we reran this second analysis using un-transformed conjunctival swelling data. As conclusions were unaltered, we elected not present that analysis here (but see results in Supplementary Results and Fig. S1). In addition, we tested whether evidence of point tolerance might be a manifestation of range tolerance by rerunning the third model, but forcing the intercept at 0, as would be expected under the assumption that with no pathogen there are no symptoms.

Results

PATHOGEN LOAD

Over the course of the experiment, the median peak bacterial load observed across all four measures of all individuals used was 78 bacteria per host cell. There was marked variation around this median (IQR of 42–154, total range of 1–522 bacteria per host cell), arising in part from effects of bacterial isolate identity. Specifically, the mixed model analysis revealed that isolate identity explained 19% of the variance in peak load. Further, as expected, year of pathogen sampling showed a substantial positive effect on peak pathogen load, with later isolates achieving higher peak load than early isolates (mixed GLM; linear estimate \pm SE = 4.68 \pm 1.15, \chi^2 = 4.99, df = 1, P = 0.025; quadratic estimate \pm SE = -2.53 \pm 1.15, \chi^2 = 4.97, df = 1, P = 0.026). Finally, however, peak loads were similar in birds from exposed and unexposed populations on average (population effect (unexposed relative to exposed) \pm SE = 0.004 \pm 0.26, \chi^2 = 0.0003, df = 1, P = 0.99). This latter result is not sufficient to distinguish the roles of resistance and tolerance in the evolutionary response to infection.

The absence of a population difference in peak load is not incompatible with evolved resistance if pathogen loads are peaking prior to the time when genetically resistant birds are able to mount an effective immune response. Indeed, we found here that bacterial loads were highest (on average) in birds from both populations at 8 dpi and thereafter declined significantly on average (mixed GLM; main dpi effect: estimate \pm SE = -0.07 \pm 0.009, \chi^2 = 54.7, df = 1, P < 0.0001). However, there was a significant population by dpi interaction (estimate \pm SE = 0.07 \pm 0.02, \chi^2 = 13.9, df = 1, P < 0.0002), with birds from exposed populations clearing the pathogen approximately three times faster than those from unexposed populations (Fig. 1). Differential clearing rates are such that birds from exposed populations have a fourfold lower bacterial load than birds from unexposed populations by 28 dpi. These findings support the hypothesis that genetic resistance through acquired immunity has evolved in exposed populations.

CONJUNCTIVAL SWELLING AS A FUNCTION OF PATHOGEN LOAD

The key symptom of *M. gallisepticum* infection in house finches is conjunctivitis, which, when severe, causes blindness and death in the wild through starvation or predation (Roberts et al. 2001b; Kollias et al. 2004; Adelman et al. 2017). Using the area of conjunctival swelling to measure clinical symptom severity, we found no obvious support for the hypothesis that tolerance, as

![Figure 1. Changes in pathogen load over the experiment. We show pathogen load (log-transformed) at 8, 14, 21, and 28 dpi for birds from exposed and unexposed populations. Raw values are shown as triangles (exposed) or circles (unexposed populations); lines are predicted from the model (solid = exposed; dashed = unexposed), with SEs represented by ribbons.](Image 321x575 to 561x722)
Figure 2. Association between pathogen load and clinical symptom severity. We show peak conjunctival swelling (square root-transformed; in pixels) as a function of peak pathogen load (square root-transformed) for birds from exposed and unexposed populations. Raw values are shown as triangles (exposed) or circles (unexposed populations); lines are predicted from the model (solid = exposed; dashed = unexposed), with SEs represented by ribbons. Boxplot show the median and range peak conjunctival swelling and peak pathogen load for each population. Birds from exposed populations displayed significantly lower peak conjunctival swelling than those from unexposed populations (estimate ± SE = -1.13 ± 0.54, χ² = 4.36, df = 1, P = 0.037), but equivalent peak pathogen load (see Results).

Figure 3. Association between pathogen load and clinical symptom severity with intercept forced at 0. For birds from exposed and unexposed populations, we show peak conjunctival swelling (square root-transformed values) as a function of peak pathogen load (square root-transformed). Raw values are shown as triangles (exposed) or circles (unexposed populations); lines are predicted from the model (solid = exposed; dashed = unexposed), with SEs represented by ribbons.

measured by the regression slope of peak symptom severity on peak pathogen load, has evolved. Across all individuals used, the mean measure of peak conjunctival swelling was 64.4 ± 34.0 pixels, and swelling increased with peak pathogen load as expected (mixed GLM; pathogen load main effect: estimate ± SE = 0.21 ± 0.052, χ² = 15.30, df = 1, P < 0.0001). However, the slope of this regression did not differ between exposed versus unexposed populations (population × peak pathogen load interaction effect: estimate ± SE = -0.073 ± 0.10, χ² = 0.52, df = 1, P = 0.47) (Fig. 2). Nonetheless, birds from exposed populations did have 24% lower clinical symptom severity for any given pathogen load, which is predicted under the point tolerance concept (population main effect: estimate ± SE = 1.30 ± 0.50, χ² = 6.88, df = 1, P = 0.009). We reran this analysis using the integral of pathogen load rather than peak pathogen load as our predictor variable, but results were qualitatively unchanged (see Fig. S1). Thus, our results suggest that mechanisms to limit immune damage have evolved in tandem with resistance.

POINT VERSUS RANGE TOLERANCE

Although the evidence above is consistent with the concept of point rather than range tolerance, the apparent distinction between the two might be an artefact of whether asymptomatic hosts are sampled (see Box 1). For example, because in our study all hosts were infected, the regression slopes of pathogen loads on conjunctival swelling had intercepts in excess of zero. However, it may be more reasonable to assume that with no pathogen, there are no symptoms, and as a consequence the regression needs to originate at 0 (see Fig. B1). Where this reasonable assumption is made, any difference in points will derive from a difference in slope. To test this possibility, we reran the model presented above, but wherein we force the intercept to be 0. As expected, doing so generates a significant difference in the slopes, with finches from exposed populations showing reduced slope as expected under range tolerance (mixed GLM; population × pathogen load interaction effect: estimate ± SE = -0.15 ± 0.06, χ² = 4.9, df = 1, P = 0.027: Fig. 3). These results suggest that there is no distinction between point and range tolerance, and any apparent evidence of point tolerance is in fact evidence of range tolerance (Box 1 and Fig. B1).

Discussion

To test whether house finches from disease-exposed populations have evolved resistance or tolerance to infection to the emerging bacterial pathogen *M. gallisepticum*, we conducted an inoculation experiment of house finches from disease-unexposed and exposed populations using isolates collected over a 20-year period from epidemic outbreak and differing in virulence. We found that birds from exposed and unexposed populations had comparable peak pathogen loads, which were maximal at 8 dpi in birds from both populations. However, thereafter birds from previously exposed populations cleared the pathogen more rapidly and to a greater
extent during our experiment. That bacterial loads only started
to differ between exposed and unexposed finch populations af-
after 14 dpi is consistent with our prior evidence that evolved
finches clear *M. gallisepticum* through cell-mediated immunity
(Bonneaud et al. 2012b). We interpret these patterns as evidence
that, in the exposed populations, hosts have evolved resistance in
response to the emerging pathogen *M. gallisepticum*.

In contrast to the evidence supporting the hypothesis of
evolved resistance, evidence for the evolution of tolerance in the
exposed finch population was more ambiguous. Notably, the gra-
dient of the regression of symptom severity on pathogen load
was comparable in birds from exposed and unexposed popula-
tions. In other words, because we did not observe the predicted
shallower regression slope for finches from exposed populations,
our results are ostensibly inconsistent with the hypothesis that
range tolerance to *M. gallisepticum* has evolved (sensu Little et al.
2010). Nonetheless, on average, birds from exposed populations
did exhibit lower clinical symptoms for a given pathogen load,
which suggests that a “tolerance” mechanism has evolved to limit
damage (i.e., symptom severity). Because we found a difference
in reaction norm intercept, but not slope, between unexposed
and exposed finch populations, strictly our results would more
consistent with the evolution of “point” than “range” tolerance
(see, e.g., Graham et al. 2011 and references therein for further
discussion).

That said, we suggest that the distinction between point and
range tolerance might be rather artificial, at least when symp-
tom severity rather than fitness is used on the y-axis, as we do
here (see Box 1). Most notably, if one makes the intuitive as-
sumption that potential hosts are asymptomatic prior to infection,
then any regression slope of symptom severity on pathogen loads
must intercept zero. And, where this is the case, any significant
point difference must result from a difference in slopes. Thus,
ostensible evidence of point tolerance might be a manifestation
of range tolerance, but wherein the range of pathogen loads fail
to include zero (we stress this is not necessarily true when fitness
used as variation in intercepts is expected even among uninfected
individuals). In support, when we forced the regression slopes
of symptom severity on pathogen load for the two host popula-
tions through zero, we found a significant difference between the
slopes: finches from exposed populations showed reduced slope
as expected under range tolerance.

Semantics over the definition and labels of tolerance notwith-
standing, what is clear is that any change in tolerance that has oc-
curred has been accompanied by a change in resistance. This novel
finding based on a large-scale inoculation experiment using >50
isolates collected throughout the epidemic helps to clarify previ-
ous ambiguity in this system. For instance, using a low-virulence
1994 isolate, Adelman et al. (2013) concluded a significant role
for tolerance, but not resistance, because inoculated finches from
exposed populations displayed comparable loads than those from
unexposed populations, but lower peak eye lesion scores. By con-
trast, Bonneaud et al. (2011) used a more virulent 2007 strain and
found population differences in pathogen load, consistent with the
evolution of resistance. We now know that there is substantial
among-isolate variation in peak pathogen load (this study), and
that differences in symptom severity between exposed and unex-
posed host populations are more apparent under infection with
late-epidemic bacterial isolates (Bonneaud et al. 2018). It there-
fore seems likely that studies performed on a restricted subset of
isolates (typically 1 isolate) will provide an incomplete picture of
host evolutionary responses to selection.

We would be surprised if our key finding that both resis-
tance and tolerance have evolved in response to the emerging
pathogen were not general. The overarching implication is that
although resistance and tolerance can be viewed as distinct host
defense strategies, this does not mean they must be either mech-
anismically independent or mutually exclusive. Indeed, immune
cells are increasingly recognized as playing a dual role in resis-
tance and damage-limitation processes in the broad sense (Wynn
and Vannella 2016; Kubes 2018). Clearly then there is value in
future studies addressing the role of evolved damage-limitation
mechanisms that curtail and resolve immune responses to pre-
vent autoimmunity, remove cellular debris and stimulate tissue
repair and regeneration (Wynn and Vannella 2016). The results of
our study highlight that future tests of resistance versus tolerance
evolution in response to naturally emerging pathogens require: (i)
inoculations with sufficient numbers of pathogen isolates taken
from varying time points of the host–pathogen interaction and
varying in virulence; and (ii) analyses of pathogen load over a
sufficient infection duration to encompass the consequences of
both innate and adaptive immune processes. Comparisons of the
results presented here with those published previously on the
house finch system (Adelman et al. 2013), including by ourselves
(Bonneaud et al. 2011), suggests that failure to do so is likely to
lead to reduced coherence regarding host responses to emerging
pathogens.

Not distinguishing between the contributions of resistance
and tolerance to evolved host defense will negatively impact the
ability to predict coevolutionary dynamics. For instance, although
resistance is implicated in antagonistic host–pathogen coevolution
(Gandon et al. 2003; Gandon et al. 2008), it is often noted that
the emergence of tolerance should benefit both parties, allowing
interactions to evolve toward commensalism (Roy and Kirchner
2000; Miller et al. 2006). In fact, we argue this latter prediction is
likely contingent on the assumption that virulence is a by-product
of pathogen replication rates rather than a direct target of selection
on pathogens (Anderson and May 1982; Ebert 1998; Mackinnon
and Read 1999a,b; Gandon et al. 2003; Miller et al. 2006). In this
case, tolerance alleviates the cost of virulence, thus allowing the
pathogen to evolve high replication rates without causing damage to coevolved (tolerant) hosts. However, in *M. gallisepticum* and many other diseases (e.g., respiratory tract infections with aerosol transmission), increased symptom severity may itself drive higher transmission (Horn et al. 2002). If so, the evolution of host tolerance will actually impose selection for increased damage (and so transmission) in coevolved (tolerant) hosts.

In the current context, *M. gallisepticum* requires virulence because transmission occurs through ocular fluid exudates (Dhondt et al. 2007), and so depends on the bacterium causing a misdirected inflammatory response to disrupt the mucosal surface of the conjunctiva and respiratory tract (Gaunson et al. 2000; Lam and DaMassa 2000; Ganapathy and Bradbury 2003; Gaunson et al. 2006). Given that high virulence is broadly expected to favor the evolution of host resistance, while low virulence should favor tolerance (Restif and Koella 2003), it is intuitive that obligately virulent pathogens should lead to the evolution of resistance. In finches, resistance to *M. gallisepticum* via an effective cell-mediated immune response does seem to have evolved, but has likely been accompanied by the ability to resist the pathogen-driven activation of an inflammatory response (Bonneaud et al. 2012a; Adelman et al. 2013). This interpretation is consistent with our finding that the slopes of the relationships between pathogen load and clinical symptoms severity (i.e., “range” tolerance) were equivalent between finches from disease-exposed and unexposed populations, but those from the latter displayed higher symptoms overall.

In conclusion, we provide evidence that house finches have evolved resistance following the infectious outbreak of the bacterial pathogen, *M. gallisepticum*, with finches from disease-exposed populations likely reducing pathogen load through acquired immune processes (Bonneaud et al. 2011). Further, however, we also found evidence to suggest that the ability to tolerate infection and limit damage caused by the pathogen has evolved in tandem with resistance. Thus, while tolerance and resistance have been widely conceptualized as evolutionary alternatives (Råberg 2014), presumably because of their differing implications for host–pathogen coevolution, from a host perspective they are better viewed as complementary strategies that are likely to evolve together to fight infection and reduce damage.

**ACKNOWLEDGMENTS**

This research was supported by a Natural Environment Research Council standard grant to C.B. and A.W. (NE/M00256X). We thank A. Russell, the Associate Editor K. Lythgoe, and two anonymous referees for helpful discussion and/or constructive comments on the manuscript. We thank M. Staley for growing and shipping the pathogen isolates, M. Cook for assisting with bird captures in Arizona, A. Santos, W. R. Hood, and the undergraduates in the Hood lab for assisting with bird captures in Alabama, and A. K. Ziegler for assisting with the experiment in Arizona. The authors declare no conflicts of interest.

**AUTHOR CONTRIBUTIONS**

CB conceived and designed the study. GEH, KJM, MG, and CB obtained the animals and/or bacterial isolates. MG, KJM, and LT conducted the experiment. LT conducted the molecular work. CB and AW analyzed the data and wrote the paper.

**DATA ARCHIVING**

Data reported in this paper have been deposited in Dryad Digital Repository (https://doi.org/10.5061/dryad.4711h78).

**LITERATURE CITED**

Adelman, J. S., L. Kirkpatrick, J. L. Grodio, and D. M. Hawley. 2013. House finch populations differ in early inflammatory signaling and pathogen tolerance at the peak of mycoplasma gallisepticum infection. *Am. Nat.* 181:674–689.

Adelman, J. S., C. Mayer, and D. H. Hawley. 2017. Infection reduces anti-predator behaviors in house finches. *J. Avian Biol.* 48:519–528.

Anderson, R. M., and R. M. May. 1982. Coevolution of hosts and parasites. *Parasitology* 85:411–426.

Atkinson, C. T., K. S. Sáilí, R. B. Uzzurrum, and S. I. Jarvi. 2013. Experimental evidence for evolved tolerance to avian malaria in a wild population of low elevation Hawai’i ‘Amakihi (Hemignathus virens). *Ecohealth* 10:366–375.

Bates, D., M. Maechler, B. Bolker, and S. Walker. 2015. Fitting linear mixed-effects models using lme4. *J. Stat. Softw.* 67:1–48.

Best, S. M., and P. J. Kerr. 2000. Coevolution of host and virus: the pathogenesis of virulent and attenuated strains of myxoma virus in resistant and susceptible European rabbits. *Virology* 267:36–48.

Bonneaud, C., S. Balenger, A. F. Russell, J. Zhang, G. E. Hill, and S. V. Edwards. 2011. Rapid evolution of disease resistance is accompanied by functional changes in gene expression in a wild bird. *Proc. Natl. Acad. Sci. U SA.* 108:7866–7871.

Bonneaud, C., S. L. Balenger, G. E. Hill, and A. F. Russell. 2012a. Experimental evidence for distinct costs of pathogenesis and immunity against a natural pathogen in a wild bird. *Mol. Ecol.* 21:4787–4796.

Bonneaud, C., S. L. Balenger, J. Zhang, S. V. Edwards, and G. E. Hill. 2012b. Innate immunity and the evolution of resistance to an emerging infectious disease in a wild bird. *Mol. Ecol.* 21:2628–2639.

Bonneaud, C., M. Giraudet, L. Tardy, M. Staley, G. E. Hill, and K. J. McGraw. 2018. Rapid antagonistic coevolution in an emerging pathogen and its vertebrate host. *Curr. Biol.* 28:2978–2983.

Boots, M., and R. G. Bowers. 2004. The evolution of resistance through costly acquired immunity. *Proc. R. Soc. Lond. Ser. B-Biol. Sci.* 271:715–723.

Dhondt, A. A., D. L. Tessaglia, and R. L. Slothower. 1998. Epidemic mycoplasmal conjunctivitis in house finches from Eastern North America. *J. Wildl. Dis.* 34:265–280.

Dhondt, A. A., A. V. Badyaev, A. P. Dobson, D. M. Hawley, M. J. L. Driscoll, W. M. Hochachka, and D. H. Ley. 2006. Dynamics of mycoplasmal conjunctivitis in the native and introduced range of the host. *Ecohealth* 3:95–102.

Dhondt, A. A., K. V. Dhondt, D. M. Hawley, and C. S. Jennelle. 2007. Experimental evidence for transmission of Mycoplasma gallisepticum in house finches by fomites. *Avian Pathol.* 36:205–208.

Duckworth, R. A., A. V. Badyaev, K. L. Farmer, G. E. Hill, and S. R. Roberts. 2003. First case of Mycoplasma gallisepticum infection in the western range of the house finch (Carpodacus mexicanus). *Auk* 120:528–530.

Ebert, D. 1998. Evolution - experimental evolution of parasites. *Science* 282:1432–1435.

Fischer, J. R., D. E. Stallknecht, M. P. Luttrell, A. A. Dhondt, and K. A. Converse. 1997. Mycoplasmal conjunctivitis in wild songbirds: The
spreading of a new contagious disease in a mobile host population. Emerg. Infect. Dis. 3:69–72.

Ganapathy, K., and J. M. Bradbury. 2003. Effects of cyclosporin A on the immune responses and pathogenesis of a virulent strain of Mycoplasma gallisepticum in chickens. Avian Pathol. 32:495–502.

Gandon, S., M. Mackinnon, S. Nee, and A. Read. 2003. Imperfect vaccination: some epidemiological and evolutionary consequences. Proc. R. Soc. Lond. Ser. B-Biol. Sci. 270:1129–1136.

Gandon, S., A. Buckling, E. Decaestecker, and T. Day. 2008. Host-parasite coevolution and patterns of adaptation across time and space. J. Evol. Biol. 21:1861–1866.

Gaunson, J. E., C. J. Philip, K. G. Whithar, and G. F. Browning. 2000. Lymphocytic infiltration in the chicken trachea in response to Mycoplasma gallisepticum infection. Microbiology 146:1223–1229.

Gaunson, J. E., C. J. Philip, K. G. Whithar, and G. F. Browning. 2006. The cellular immune response in the tracheal mucosa of Mycoplasma gallisepticum in vaccinated and unvaccinated chickens in the acute and chronic stages of disease. Vaccine 24:2627–2633.

Graham, A. L., D. M. Shuker, L. C. Pollitt, S. Auld, A. J. Wilson and T. J. Little. 2011. Fitness consequences of immune responses: strengthening the empirical framework for ecomimmunology. Funct. Ecol. 25:5–17.

Hornef, M. W., M. J. Wick, M. Rhen, and S. Normark. 2002. Bacterial strategies for overcoming host innate and adaptive immune responses. Nat. Immunol. 3:1033–1040.

Howick, V. M., and B. P. Lazzaro. 2017. The genetic architecture of defense as resistance to and tolerance of bacterial infection in Drosophila melanogaster. Mol. Ecol. 26:1533–1546.

Janeway, C. 2005. Immunobiology: the immune system in health and disease. Garland Science, New York, NY.

Kerr, P. J., and S. M. Best. 1998. Myxoma virus in rabbits. Rev. Sci. Tech. Oie. 17:256–268.

Kerr, P. J., J. Liu, I. Cattadori, E. Ghedin, A. F. Read and E. C. Holmes. 2015. Myxoma virus and the leporipoxviruses: an evolutionary paradigm. Viruses 7:1020–1061.

Kollia, G. V., K. V. Sydenstricker, H. W. Kollias, D. H. Ley, P. R. Hosseni, V. Connolly, and A. A. Dhomt. 2004. Experimental infection of house finches with Mycoplasma gallisepticum. J. Wildl. Dis. 40:79–86.

Kover, P. X., and B. A. Schaal. 2002. Genetic variation for disease resistance and tolerance among Arabidopsis thaliana accessions. Proc. Natl. Acad. Sci. USA. 99:11270–11274.

Kubes, P. 2018. The enigmatic neutrophil: what we do not know. Cell Tissue Res. 371:399–406.

Lam, K. M., and A. J. DaMassa. 2000. Mycoplasma gallisepticum-induced release of macrophage inflammatory protein-1 beta from chicken monocytes-macrophages. J. Comp. Pathol. 122:35–42.

LaPointe, D. A., M. L. Goff, and C. T. Atkinson. 2002. Bacterial strategies for overcoming host innate and adaptive immune responses. Nat. Immunol. 3:1033–1040.

Marshall, I. D., and G. W. Douglas. 1961. Studies in the epidemiology of infectious myxomatosis of rabbits. VIII. Further observations on changes in the innate resistance of Australian wild rabbits exposed to myxomatosis. J. Hyg. 59:117–122.

Nolan, P. M., G. E. Hill, and A. M. Stoehr. 1998. Sex, size, and plumage redness predict house finch survival in an epidemic. Proc. R. Soc. Lond. Ser. B-Biol. Sci. 265:961–965.

Räberg, L. 2014. How to live with the enemy: understanding tolerance to parasites. PLoS Biol. 12:9.

Räberg, L., D. Sim, and A. F. Read. 2007. Disentangling genetic variation for resistance and tolerance to infectious diseases in animals. Science 318:812–814.

Räberg, L., A. L. Graham, and A. F. Read. 2009. Decomposing health: tolerance and resistance to parasites in animals. Philos. Trans. R. Soc. B-Biol. Sci. 364:37–49.

Restif, O., and J. C. Koella. 2003. Shared control of epidemiological traits in a coevolutionary model of host-parasite interactions. Am. Nat. 161:827–836.

Restif, O., and J. C. Koella. 2004. Concurrent evolution of resistance and tolerance to pathogens. Am. Nat. 164:E90–E102.

Roberts, S. R., P. M. Nolan, and G. E. Hill. 2001a. Characterization of mycoplasma gallisepticum infection in captive house finches (Carpodacus mexicanus) in 1998. Avian Dis. 45:70–75.

Roberts, S. R., P. M. Nolan, L. H. Lauerman, L. Q. Li, and G. E. Hill. 2001b. Characterization of the mycoplasmal conjunctivitis epizootic in a house finch population in the southeastern USA. J. Wildl. Dis. 37:82–88.

Roy, B. A., and J. W. Kirchner. 2000. Evolutionary dynamics of pathogen resistance and tolerance. Evolution 54:51–63.

Ruijter, J. M., C. Ramakers, W. M. Hoogaaars, Y. Karlen, O. Bakker, M. J. van den Hoff, and A. F. Moorman. 2009. Amplification efficiency: linking baseline and bias in the analysis of quantitative PCR data. Nucleic Acids Res. 37:12.

Ruijter, J. M., A. Villalba, J. Hellemaans, A. Untergasser, and M. van den Hoff. 2015. Removal of between-run variation in a multi-plate qPCR experiment. Biomed. Detect. Quant. 5:10–14.

Schneider, D. S., and J. S. Ayres. 2008. Two ways to survive infection: what resistance and tolerance can teach us about treating infectious diseases. Nat. Rev. Immunol. 8:889–895.

Simms, E. L. 2000. Defining tolerance as a norm of reaction. Evol. Ecol. 14:563–570.

Soares, M. P., L. Teixeira, and L. F. Moita. 2017. Disease tolerance and immunity in host protection against infection. Nat. Rev. Immunol. 17:83–96.

Staley, M., C. Bonneau, K. J. McGraw, C. M. Vleck and G. E. Hill. 2018. Detection of Mycoplasma gallisepticum in house finches (Haemorhous mexicanus) from Arizona. Avian Dis. 62:14–17.

Tardy, L., M. Giraudet, G. E. Hill, K. J. McGraw, and C. Bonneau. 2019. Contrasting evolution of virulence and replication rate
in an emerging bacterial pathogen. Proc. Natl. Acad. Sci. USA. https://doi.org/10.1073/pnas.1901556116
Team, R. C. 2016. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.
Tuomi, J. M., F. Voorbraak, D. L. Jones, and J. M. Ruijter. 2010. Bias in the C-q value observed with hydrolysis probe based quantitative PCR can be corrected with the estimated PCR efficiency value. Methods 50:313–322.
Van Riper, C., S. G. Van Riper, M. L. Goff, and M. Laird. 1986. The epizootiology and ecological significance of malaria in hawaiian land birds. Ecol. Monogr. 56:327–344.
Wickham, H. 2009. ggplot2: elegant Graphics for data analysis. Springer, New York, NY.
Wynn, T. A., and K. M. Vannella. 2016. Macrophages in tissue repair, regeneration, and fibrosis. Immunity 44:450–462.

Associate Editor: K. Lythgoe

Supporting Information
Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1: Association between pathogen load and clinical symptom severity.