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Rapid Antagonistic Coevolution in an Emerging Pathogen and Its Vertebrate Host

Highlights

- We adopt a time-shift design to test for host-pathogen coevolution in a vertebrate
- House finches evolved to develop sub-lethal symptoms in response to inoculation
- Concurrently, their bacterial pathogen has responded by increasing its transmission
- We demonstrate that host resistance and pathogen virulence have coevolved

Authors

Camille Bonneaud, Mathieu Giraudeau,
Luc Tardy, Molly Staley,
Geoffrey E. Hill, Kevin J. McGraw

Correspondence

c.bonneaud@exeter.ac.uk

In Brief

Bonneaud et al. provide a rare demonstration that hosts and pathogens evolve in response to each other. House finches have evolved increased resistance to their emerging pathogen, while the pathogen has evolved increased virulence in response. Our results have implications for pathogen evolution in response to resistance through antibiotics.



Rapid Antagonistic Coevolution in an Emerging Pathogen and Its Vertebrate Host

Camille Bonneaud,^{1,5,*} Mathieu Giraudeau,^{1,2} Luc Tardy,¹ Molly Staley,^{3,4} Geoffrey E. Hill,³ and Kevin J. McGraw²

¹Centre for Ecology and Conservation, University of Exeter, Penryn, Cornwall TR10 9FE, UK

²School of Life Sciences, Arizona State University, Tempe, AZ 85287-4501, USA

³Department of Biological Sciences, Auburn University, Auburn, AL 36849-5414, USA

⁴Present address: Department of Biology, Loyola University Chicago, Chicago, IL 60660, USA

⁵Lead Contact

*Correspondence: c.bonneaud@exeter.ac.uk

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SUMMARY

Host-pathogen coevolution is assumed to play a key role in eco-evolutionary processes, including epidemiological dynamics and the evolution of sexual reproduction [1–4]. Despite this, direct evidence for host-pathogen coevolution is exceptional [5–7], particularly in vertebrate hosts. Indeed, although vertebrate hosts have been shown to evolve in response to pathogens or vice versa [8–12], there is little evidence for the necessary reciprocal changes in the success of both antagonists over time [13]. Here, we generate a time-shift experiment to demonstrate adaptive, reciprocal changes in North American house finches (*Haemorrhous mexicanus*) and their emerging bacterial pathogen, *Mycoplasma gallisepticum* [14–16]. Our experimental design is made possible by the existence of disease-exposed and unexposed finch populations, which were known to exhibit equivalent responses to experimental inoculation until the recent spread of genetic resistance in the former [14, 17]. Whereas inoculations with pathogen isolates from epidemic outbreak caused comparable sub-lethal eye swelling in hosts from exposed (hereafter adapted) and unexposed (hereafter ancestral) populations, inoculations with isolates sampled after the spread of resistance were threefold more likely to cause lethal symptoms in hosts from ancestral populations. Similarly, the probability that pathogens successfully established an infection in the primary host and, before inducing death, transmitted to an uninfected sentinel was highest when recent isolates were inoculated in hosts from ancestral populations and lowest when early isolates were inoculated in hosts from adapted populations. Our results demonstrate antagonistic host-pathogen coevolution, with hosts and pathogens displaying increased resistance and virulence in response to each other over time.

RESULTS AND DISCUSSION

A key challenge in testing host-pathogen coevolution lies with measuring reciprocal changes in the success of interacting hosts and pathogens across time, thereby ruling out parallel evolution driven mainly by other factors, including random genetic drift [5–7]. The most effective method for testing such reciprocal changes in success is through a time-shift experiment, which pairs up hosts and pathogens sampled at different time points of their interaction [18]. Such an approach was used in an exceptional demonstration of coevolution in a crustacean host, *Daphnia major*, and its bacterial endoparasite, *Pasteuria ramosa*: on pairing revived hosts and pathogens sampled from different layers of pond sediments (i.e., different time points in their interaction), pathogens from a given layer were found to be more infective to hosts from the same layer than were pathogens from earlier or later layers [5]. Demonstrating coevolution between vertebrate hosts and their pathogens is non-trivial, because immobilizing hosts in time and subsequently reviving them is impossible. One option is to capitalize on recently separated host populations that have diverged largely as a result of differences in exposure to a specific pathogen [14, 17]. By experimentally infecting hosts from previously exposed versus unexposed populations with pathogens sampled from initial outbreak and over subsequent evolutionary time, it is possible to generate a time-shift experimental design in a vertebrate system.

Here we adopted this approach using wild-caught North American house finches (*Haemorrhous mexicanus*) from eastern and western U.S. populations that have, versus have not, been exposed to the bacterial pathogen *Mycoplasma gallisepticum*, since its jump from poultry and emergence in eastern populations in 1994 [14, 19, 20]. Although exposed (eastern) and unexposed (western) populations are separated in space, and we fully acknowledge that space is not time, we can use historical differences in the exposure of eastern and western populations to *M. gallisepticum* as a surrogate for time in this case. First, eastern populations originated from western-caught birds that were released into the wild relatively recently (~60 years ago) and that then rapidly spread throughout the eastern United States [21, 22]. Second, previous experiments revealed that (1) birds from eastern and western populations displayed equivalent gene expression responses to inoculation with *M. gallisepticum* in 2000, before resistance spread in the



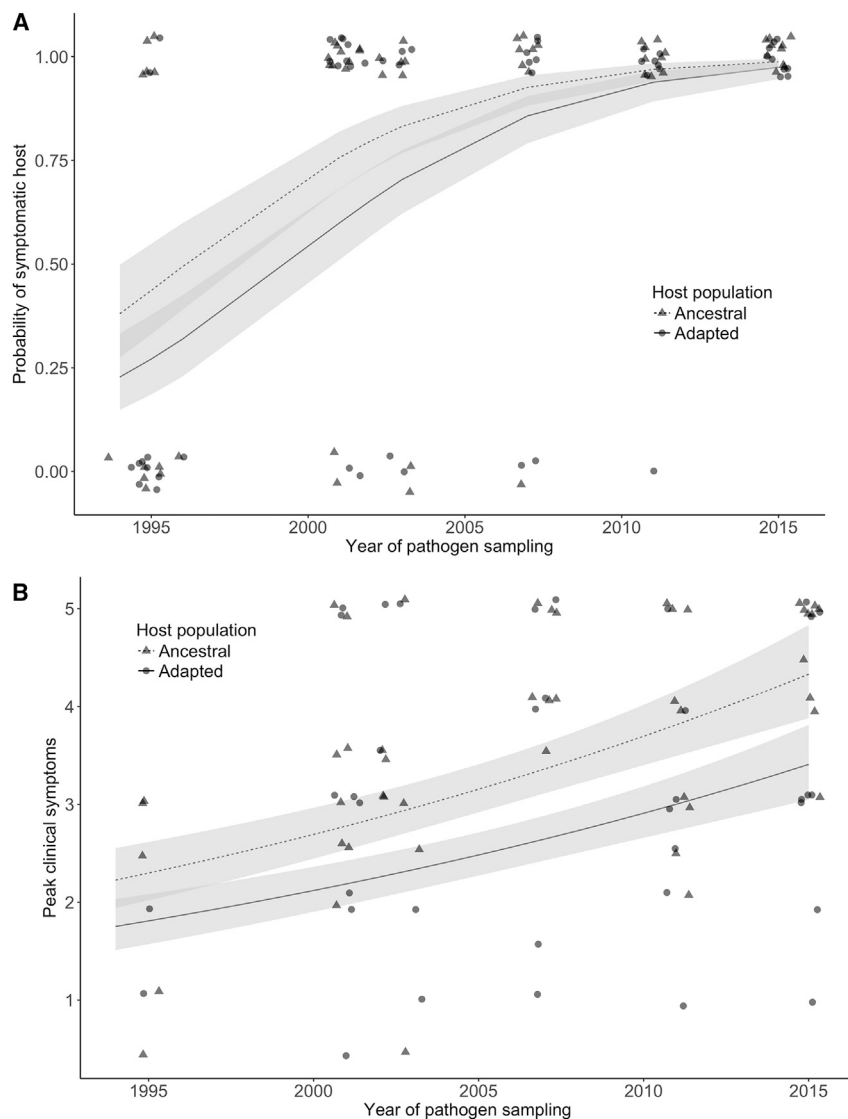


Figure 1. Outcome of Host-Pathogen Interactions across Host Populations and over Time

(A) Probability that hosts developed clinical symptoms as a function of the year of pathogen sampling for both host population types (ancestral and adapted). Gray triangles, ancestral host population; dots, adapted host population. Points represent the raw values of symptom development coded as 0 or 1.

(B) Peak clinical severity in symptomatic hosts as a function of the year of pathogen sampling in hosts from ancestral and adapted populations. Gray triangles, ancestral host population; dots, adapted host population. Points represent the raw values of severity of the peak clinical symptoms (score: 0–5). In both figures, lines are predicted from the models (dashed, ancestral host population; solid, adapted host population), with SEs represented by the ribbons.

exposed eastern populations [14], and (2) these transcriptional responses then diverged as exposed eastern populations evolved genetic resistance (i.e., by 2007) [14]. Thus, as far as responses to *M. gallisepticum* are concerned, eastern and western finch populations were equivalent at least until 2000 (i.e., 6 years post-outbreak) but began to diverge soon after, following widespread resistance in eastern exposed populations. This divergence was mediated through the evolution of resistance to pathogen-induced immune suppression and the ability to mount a protective cell-mediated immune response [17]. Consequently, here we generated a “time-shift” experimental design by inoculating birds from exposed (hereafter adapted) and unexposed (hereafter ancestral) populations with 55 bacterial isolates sampled over the course of the epidemic, from 1994 (i.e., initial outbreak), through the spread of host resistance (i.e., by ~2007) [14], to 2015 (i.e., when the experiment was performed).

A first step in testing for coevolution is to analyze general changes in the outcome of host-pathogen interactions measured

as changes in infection severity [23, 24]. The primary symptom of *M. gallisepticum* infection in house finches is conjunctivitis, which, when severe, causes blindness and death in the wild through starvation or predation [25–27]. Peak symptom severity followed a zero-inflated distribution, with 26% (29/112) of inoculated finches remaining asymptomatic. Consequently, we analyzed peak symptoms using a 2-part hurdle model [28]. The first part tested whether inoculation with pathogen isolates sampled from varying time points during the epidemic had differential effects on the probability of the infection overall, and whether there were differences for hosts from adapted versus ancestral populations. In support of pathogen evolution, we found that the probability of hosts developing

conjunctivitis increased as a function of the year of pathogen sampling (Figure 1A; 1st part of hurdle model: mixed effect logistic regression, estimate \pm SE = 0.4 ± 0.1 , $z = 3.5$, $p < 0.0005$), but did not differ significantly between hosts from adapted versus ancestral populations (estimate \pm SE = 1.1 ± 0.7 , $z = 1.6$, $p = 0.11$). The second part of the model then tested for differences in the severity of the infection in symptomatic hosts. In support of both pathogen and host evolution, we found that the peak clinical severity of symptomatic hosts also increased when inoculated with pathogens sampled later in the epidemic (Figure 1B; 2nd part of hurdle model: linear mixed effect model, estimate \pm SE = 0.03 ± 0.01 , $\chi^2 = 11.6$, degrees of freedom [df] = 1, $p < 0.001$) and was, as expected, significantly higher in hosts from ancestral populations (estimate \pm SE = 0.2 ± 0.1 , $\chi^2 = 4.2$, $df = 1$, $p = 0.041$). Together, these results demonstrate that (1) pathogen virulence has increased over the course of the epidemic, and (2) although hosts from adapted and ancestral populations were similarly likely to become infected, those from the former were subsequently better able to resist the infection. Further, given that hosts

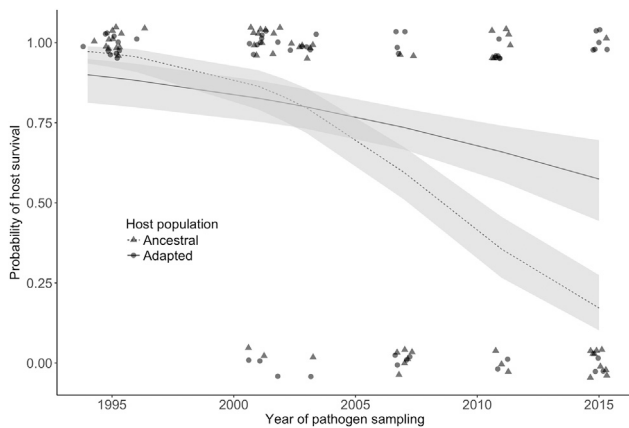


Figure 2. Adaptive Host Evolution in Response to the Pathogen

Host fitness was estimated as the probability of maintaining eyesight as a function of the year in which the pathogen inoculated was sampled. *M. gallisepticum* kills its finch host when eye swelling is sufficiently severe that it leads to blindness and so starvation or predation [27]. The probability that hosts maintained sufficient eyesight to survive declined over time (mixed effect logistic regression, pathogen year: estimate \pm SE = -0.2 ± 0.06 , $z = -3.3$, $p = 0.001$), but the degree to which it did so was less in hosts from adapted populations (see text for statistics). Lines are predicted from the model (dashed, ancestral host population; solid, adapted host population), with SEs represented by the ribbons. Gray triangles (ancestral host population) and dots (adapted host population) represent the raw values coded as 0 or 1.

from the two populations did not differ significantly in the probability of being infected but that birds from exposed eastern populations displayed reduced symptoms, we hypothesize that resistance has evolved to clear the pathogen rather than to prevent its establishment. In support, we have previously shown that resistance evolved through the ability to prevent pathogen-induced immune suppression and mount a protective cell-mediated immune response [14, 17].

Although these results display the hallmarks of coevolution, demonstrating that this is the case requires testing whether the observed effects have measurable consequences for metrics of fitness in both host and pathogen. For a house finch host infected with *M. gallisepticum*, failure to minimize conjunctival swelling and/or to clear the infection will result in blindness, which in the wild leads to death through starvation or predation [25, 27]. Our metric of the fitness consequences of infection for hosts, therefore, is whether or not they reached symptom severity tantamount to death in the wild (see STAR Methods). We found significant support for adaptive changes in the host population in response to the pathogen over time. Overall, 30% (34/112) of birds developed symptoms that would have led to death in the wild, with 70% (24/34) doing so within 2 weeks of inoculation. Whereas hosts from adapted and ancestral populations were both likely to maintain sight when inoculated with pathogen isolates sampled early in the epidemic, hosts from adapted populations were 3 times more likely to maintain sight (and so survive in the wild) when inoculated with later isolates than hosts from ancestral populations (Figure 2; mixed effect logistic regression: pathogen year \times host population interaction: estimate \pm SE = -0.2 ± 0.1 , $z = -2.0$, $p = 0.048$). Thus, hosts from adapted populations were significantly less likely to develop lethal swelling than hosts from ancestral populations, with the

divergence in symptom severity occurring when birds were inoculated with isolates sampled after the spread of host resistance.

These results are not easily explained by processes other than the evolution of genetic resistance for 3 reasons. First, we found no evidence to suggest that responses to inoculation differed between birds sampled from urban areas versus suburban parklands, which would be expected if ecology played a significant role in our results (see STAR Methods). These results corroborate our previous findings, which showed that body condition had no impact on responses to experimental inoculation with *M. gallisepticum* [29]. Second, our results are unlikely to be explained by maternal effects because (1) there is no evidence that avian mothers can confer sufficiently long-lasting protection to their offspring [30], and (2) circulating antibodies, which they can deposit in the eggs, do not confer protection against *M. gallisepticum* [31, 32], largely because this bacterium can hide within host cells [33]. In addition, because non-resistant birds are immuno-suppressed by *M. gallisepticum* and so cannot mount a protective immune response [17, 34, 35], any potential maternal effect would require the prior evolution of genetic resistance. Third, we ensured that all birds used in the experiment were captured within 3 months post-fledging and had no prior exposure to the pathogen, meaning that population differences cannot be explained by secondary responses to infection [12, 36] (see STAR Methods). Taken together, our results strongly suggest that house finches have evolved genetic resistance in direct response to the pathogen.

In the case of the pathogen, the finding that later isolates induced more severe clinical symptoms (see above; Figure 1) suggests evolutionary changes in the pathogen in response to the host. But again, evidence for coevolution requires testing whether these changes are adaptive. From the perspective of the pathogen, evidence of coevolution thus requires that pathogen fitness varies as a function of the relative time shift between host and pathogen, with fitness increasing when the pathogen is sampled progressively later in the epidemic relative to the host, and decreasing as the host is sampled progressively later in the epidemic relative to the pathogen. For example, late pathogen isolates (e.g., from 2015) inoculated in hosts from ancestral populations will generate a positive time shift of +20 years and should be most fit, whereas early pathogen isolates (e.g., from 1995) inoculated in hosts from adapted populations in 2015 will generate a negative time shift of -20 years and should be least fit. Pathogen fitness is measured as the product of infection duration in its current host and transmission rate to an uninfected host, thereby approximating R_0 [37, 38]. Although this estimate accounts neither for any fluctuations in transmission probability over the course of an infection nor for natural ecological variation, such as in host contact rate [39], it provides us with a robust comparison of pathogen fitness among isolates under standardized laboratory conditions. We considered infection duration as the number of days over which experimentally inoculated hosts showed sub-lethal symptoms, and transmission rate as 1 divided by the number of days taken to transmit to an uninfected sentinel (as determined by PCR; see STAR Methods). Correlation analyses suggest that infection duration (Spearman's rank correlation: $r_s = 0.16$, $p = 0.085$) and time to transmission (Spearman's rank correlation: $r_s = -0.24$, $p = 0.037$) tend to vary as a

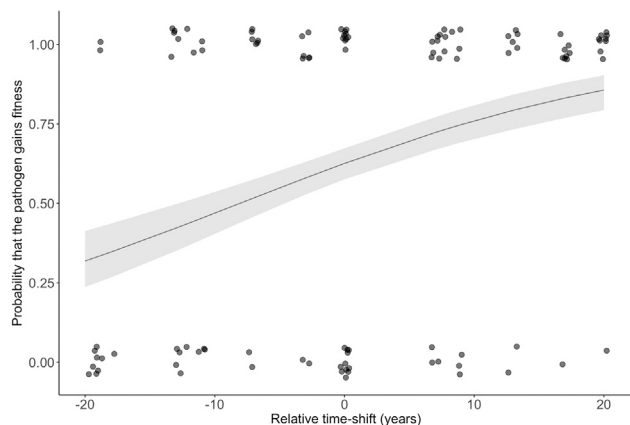


Figure 3. Adaptive Pathogen Evolution in Response to the Host

We show the probability that pathogens achieved a fitness >1 (i.e., gained fitness; see STAR Methods) as a function of the relative time shift (in years) between antagonists. Years were considered negative when non-contemporary pathogen isolates (i.e., those sampled before 2015) were inoculated in hosts from adapted populations, and positive when post-outbreak isolates (i.e., obtained after 1996) were inoculated in hosts from ancestral populations. The number of years was 0 when hosts from adapted populations were inoculated with contemporary isolates sampled in the same year (i.e., 2015), and when hosts from ancestral (unexposed) populations were inoculated with isolates obtained at epidemic outbreak (i.e., at pathogen emergence in the previously unexposed finch host). Negative values of relative time shift therefore show the impact of host resistance on pathogen fitness, whereas positive values show the impact of increased virulence on pathogen success. The line is predicted from the model with the SE represented by the ribbon. Gray dots represent the raw values of fitness coded as 0 (i.e., fitness <1) or 1 (i.e., fitness ≥ 1).

function of the relative time shift between pathogen and host, indicating that both are likely to contribute to evolutionary changes in pathogen fitness.

Overall, the median pathogen fitness was 2, with lower and upper quartiles of 0 and 7, respectively. For pathogens to successfully transmit to a secondary host and therefore gain fitness, our measure of fitness needs to be >1 [40, 41]. Isolates obtained a fitness value of <1 in 38% (i.e., 42/112) of birds, indicating a failure to gain fitness during the experiment. In 69% of these cases (i.e., 29/42), isolates failed to establish an infection in the experimentally inoculated host, in 19% of cases (i.e., 8/42) they failed to transmit to the sentinel, and in the remaining 12% of cases (i.e., 5/42) they transmitted only after inducing death in the experimentally inoculated host. Because our values of pathogen fitness followed a zero-altered gamma distribution (ZAG), they were analyzed using a 2-part hurdle model [28]. In support of coevolution, we found that the probability that pathogen fitness was ≥ 1 (i.e., the pathogen gained fitness [40, 41]) increased as a function of the relative time shift between pathogen and host (Figure 3; 1st part of hurdle model: mixed effect logistic regression, estimate \pm SE = 0.2 ± 0.1 , $z = 2.5$, $p = 0.012$), although there was no additional effect of the time shift on quantitative variation in fitness for those that gained fitness (i.e., had fitness values ≥ 1) (2nd part of hurdle model: mixed effects model with gamma errors, estimate \pm SE = 0.01 ± 0.01 , $\chi^2 = 0.8$, $df = 1$, $p = 0.37$). These results indicate that *M. gallisepticum* has also evolved over the course of

the epidemic to increase its chances of gaining fitness in direct response to changes in the house finch host.

In conclusion, we show that host resistance and pathogen virulence have evolved adaptively in response to each other over the course of the epidemic. These findings of antagonistic coevolution are highly unlikely to arise by chance through random processes such as drift. Our results have at least three further implications. First, they corroborate our previous demonstration that resistance has spread in house finches in response to *M. gallisepticum* [14] and provide an explanation for contrary evidence suggesting a role of host tolerance in this system [42]. Most notably, our results suggest that apparent evidence for tolerance is likely to be an artifact of inoculation with an early, non-virulent 1994 isolate: inoculation with later-epidemic isolates are required to generate the differences between ancestral and adapted populations predicted under resistance. Second, it has been recently hypothesized that incomplete immunity against *M. gallisepticum* protects hosts against secondary infections with low virulence isolates, thereby favoring the evolution of increasing virulence [12]. This hypothesis assumes that the selective consequences of secondary infections are sufficiently high to drive the evolution of pathogen virulence. Our findings that resistance has evolved in the host population in response to infection counter this assumption, because the selective consequences of primary infections provide a more parsimonious explanation for virulence evolution. Finally, that both host and pathogen fitness have increased over time suggests that this coevolution is currently driven by directional selection (i.e., arms race) rather than by fluctuating selection [43]. Future studies will allow us to test whether this arms race transitions into fluctuating dynamics as the coevolutionary interaction becomes more established [44], or whether we are seeing the initial stage of fluctuating dynamics with a periodicity in excess of the duration from outbreak to the present day (i.e., currently maximally 20 host generations). Either way, given our demonstration of antagonist coevolution, we are now in a position to understand more precisely the specific phenotypic and genomic changes in both hosts and pathogens. For example, we know relatively little about the physiological and immunological mechanisms underlying host resistance in wild systems [17]. Similarly, the mechanisms under selection in pathogens as resistance spreads in their vertebrate hosts are unclear. Addressing these issues is fundamental if we are to understand the fate and impact of emerging infectious diseases.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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AUTHOR CONTRIBUTIONS

C.B. conceived the study and designed the experiment. G.E.H., K.J.M., M.S., M.G., and C.B. obtained the animals and/or bacterial isolates. M.G., K.J.M., and L.T. conducted the experiment. L.T. conducted the molecular work. C.B. analyzed the data and wrote the paper.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and Virus Strains		
<i>Mycoplasma gallisepticum</i> isolate 1994_1	This study	N/A
<i>Mycoplasma gallisepticum</i> isolate 1995_1	This study	N/A
<i>Mycoplasma gallisepticum</i> isolate 1995_2	This study	N/A
<i>Mycoplasma gallisepticum</i> isolate 1995_3	This study	N/A
<i>Mycoplasma gallisepticum</i> isolate 1995_4	This study	N/A
<i>Mycoplasma gallisepticum</i> isolate 1995_5	This study	N/A
<i>Mycoplasma gallisepticum</i> isolate 1995_6	This study	N/A
<i>Mycoplasma gallisepticum</i> isolate 1995_7	This study	N/A
<i>Mycoplasma gallisepticum</i> isolate 1996_1	This study	N/A
<i>Mycoplasma gallisepticum</i> isolate 2001_1	This study	N/A
<i>Mycoplasma gallisepticum</i> isolate 2001_2	This study	N/A
<i>Mycoplasma gallisepticum</i> isolate 2001_3	This study	N/A
<i>Mycoplasma gallisepticum</i> isolate 2001_4	This study	N/A
<i>Mycoplasma gallisepticum</i> isolate 2001_5	This study	N/A
<i>Mycoplasma gallisepticum</i> isolate 2001_6	This study	N/A
<i>Mycoplasma gallisepticum</i> isolate 2001_7	This study	N/A
<i>Mycoplasma gallisepticum</i> isolate 2001_8	This study	N/A
<i>Mycoplasma gallisepticum</i> isolate 2001_9	This study	N/A
<i>Mycoplasma gallisepticum</i> isolate 2001_10	This study	N/A
<i>Mycoplasma gallisepticum</i> isolate 2002_1	This study	N/A
<i>Mycoplasma gallisepticum</i> isolate 2002_2	This study	N/A
<i>Mycoplasma gallisepticum</i> isolate 2002_3	This study	N/A
<i>Mycoplasma gallisepticum</i> isolate 2002_4	This study	N/A
<i>Mycoplasma gallisepticum</i> isolate 2003_1	This study	N/A
<i>Mycoplasma gallisepticum</i> isolate 2003_2	This study	N/A
<i>Mycoplasma gallisepticum</i> isolate 2003_3	This study	N/A
<i>Mycoplasma gallisepticum</i> isolate 2003_4	This study	N/A
<i>Mycoplasma gallisepticum</i> isolate 2003_5	This study	N/A
<i>Mycoplasma gallisepticum</i> isolate 2003_6	This study	N/A
<i>Mycoplasma gallisepticum</i> isolate 2007_1	This study	N/A
<i>Mycoplasma gallisepticum</i> isolate 2007_2	This study	N/A
<i>Mycoplasma gallisepticum</i> isolate 2007_3	This study	N/A
<i>Mycoplasma gallisepticum</i> isolate 2007_4	This study	N/A
<i>Mycoplasma gallisepticum</i> isolate 2007_5	This study	N/A
<i>Mycoplasma gallisepticum</i> isolate 2007_6	This study	N/A
<i>Mycoplasma gallisepticum</i> isolate 2007_7	This study	N/A
<i>Mycoplasma gallisepticum</i> isolate 2011_1	This study	N/A
<i>Mycoplasma gallisepticum</i> isolate 2011_2	This study	N/A
<i>Mycoplasma gallisepticum</i> isolate 2011_3	This study	N/A
<i>Mycoplasma gallisepticum</i> isolate 2011_4	This study	N/A
<i>Mycoplasma gallisepticum</i> isolate 2011_5	This study	N/A
<i>Mycoplasma gallisepticum</i> isolate 2011_6	This study	N/A
<i>Mycoplasma gallisepticum</i> isolate 2011_7	This study	N/A

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
<i>Mycoplasma gallisepticum</i> isolate 2011_8	This study	N/A
<i>Mycoplasma gallisepticum</i> isolate 2011_9	This study	N/A
<i>Mycoplasma gallisepticum</i> isolate 2015_1	This study	N/A
<i>Mycoplasma gallisepticum</i> isolate 2015_2	This study	N/A
<i>Mycoplasma gallisepticum</i> isolate 2015_3	This study	N/A
<i>Mycoplasma gallisepticum</i> isolate 2015_4	This study	N/A
<i>Mycoplasma gallisepticum</i> isolate 2015_5	This study	N/A
<i>Mycoplasma gallisepticum</i> isolate 2015_6	This study	N/A
<i>Mycoplasma gallisepticum</i> isolate 2015_7	This study	N/A
<i>Mycoplasma gallisepticum</i> isolate 2015_8	This study	N/A
<i>Mycoplasma gallisepticum</i> isolate 2015_9	This study	N/A
<i>Mycoplasma gallisepticum</i> isolate 2015_10	This study	N/A
Chemicals, Peptides, and Recombinant Proteins		
<i>Mycoplasma gallisepticum</i> Antigen	Charles River Laboratories	Catalogue # 10100760
<i>Mycoplasma gallisepticum</i> SPA positive serum	Charles River Laboratories	Catalogue # 10100703
<i>Mycoplasma gallisepticum</i> SPA negative serum	Charles River Laboratories	Catalogue # 10100511
Glycerol	VWR	Catalogue # BDH1172-1LP
Thallium acetate	VWR	Catalogue # AA11102-14
<i>Mycoplasma</i> broth base	Sigma-Aldrich	Catalogue # F6797-500G
Tryptone	VWR	Catalogue # 97063-386
Peptone	VWR	Catalogue # 61001-506
Dextrose	Sigma Aldrich	Catalogue # D9434-250G
CMRL 1066 media	Thermofisher	Catalogue # 11530037
L-Glutamine	VWR	Catalogue # 0131-0100
Yeast extract	VWR	Catalogue # 97064-368
Yeastolate	VWR	Catalogue # 90003-510
Fetal bovine serum	VWR	Catalogue # 71004-562
Phenol red 1%	VWR	Catalogue # RC5725-16
Penicillin G sodium salt	VWR	Catalogue # 97061-280
Deposited Data		
Dryad Digital Repository	Dryad	https://doi.org/10.5061/dryad.5g5n8n2
Experimental Models: Organisms/Strains		
House finches (<i>Haemorhous mexicanus</i>) wild-caught in Alabama and in Arizona in 2015	This study	N/A
Oligonucleotides		
13R: 5' GCTTCCTTGCGGTTAGCAAC 3'	Sigma Aldrich	[45]
14F: 5' GAGCTAATCTGTAAGTTGGTC 3'	Sigma Aldrich	[45]
Software and Algorithms		
R software	https://www.r-project.org	[46]

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Camille Bonneaud (c.bonneaud@exeter.ac.uk).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Protocols were approved by Institutional Animal Care and Use Committees (IACUC) of Auburn University (permit # PRN 2015-2721) and of Arizona State University (permit # 15-1438R), as well as by Institutional Biological Use Authorizations to Auburn University (# BUA 500), and the University of Exeter's ethics committee. All birds were born within the calendar year (N = 117 males and 107 females).

Wild house finches from populations that have never been exposed to *M. gallisepticum* (ancestral populations) were captured in ecologically distinct urban areas and in suburban parks ([47, 48]; see below for rationale) in Arizona over a two-week period of the summer 2015. Birds (N = 171; 93 males and 78 females) that had hatched in the spring 2015 were trapped, weighed and banded with a numbered metal tag for individual identification. They were then immediately transported by car to aviaries at Arizona State University, where they were housed for the remainder of the experiment. On arrival, we obtained a blood sample from all birds using brachial venipuncture (60 μ L of whole blood) and a choanal swab. A lack of prior infection with *M. gallisepticum* since hatching was confirmed by screening blood plasma for anti-*M. gallisepticum* antibodies using a serum plate agglutination (SPA) assay with antigen prepared from *M. gallisepticum* (Charles River Laboratories, USA) [49]. Briefly, 10 μ L of plasma was added with 1 drop of *M. gallisepticum* plate antigen. The plate was gently rocked to ensure complete mixing and then allowed to stand for 2 min, at which time results were read. Positive (agglutination) and negative (no agglutination) control serum were included in each test as a reference. A lack of current infection was verified by performing endpoint PCR using *M. gallisepticum*-specific primers (Forward: 5' GCTTCCTTGCGGTTAGCAAC 3'; Reverse: 5' GAGCTAATCTGTAAAGTTGGTC 3') on DNA extracted from choanal swabs followed by agarose gel electrophoresis. DNA was extracted by placing the swab in 100 μ L of sterile nuclease-free water at 100°C for 10 min, followed by –20°C for 10 min, and a final centrifugation step of 13,000 rpm for 5 min. PCR conditions were 94°C for 5 min, then 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s, and lastly a 5-min extension at 72°C [45]. Negative results for both SPA and PCR tests were expected as *M. gallisepticum* has never been documented in the area of Arizona in which sampling was conducted [16].

During the same time period, wild house finches from populations that have been exposed to *M. gallisepticum* since disease outbreak (adapted populations) were captured in urban areas and in suburban parks in Alabama (see below for rationale). Upon capture, birds (N = 131) that had hatched in the spring 2015 were trapped, and similarly banded and weighed, and a blood sample (~60 μ L) and a choanal swab taken for analyses. 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. All individuals were examined for anti-*M. gallisepticum* antibodies indicating prior exposure using the serum plate agglutination assay, and their current infection status determined with the choanal swabs [45, 49]. Birds positive for either test were released immediately, while the remaining birds (N = 53; 24 males and 29 females) underwent a 30-day quarantine period, before being transported by car to the aviary at Arizona State University. It is important to note that we contrasted responses to inoculation by eastern exposed populations with western unexposed populations and did not include populations exposed to the disease for varying lengths of time for 2 reasons. First, all eastern populations were exposed to *M. gallisepticum* within 3 years of outbreak, meaning that there is little variation in exposure duration among eastern populations [19]. Second, we cannot meaningfully inoculate birds from more recently exposed western populations with isolates sampled from the East, given that western birds and western isolates have evolved in isolation from eastern isolates [50].

Following arrival at Arizona State University, birds from all populations were assigned to treatment groups. Each bird from an adapted population was housed in a cage with one bird from an ancestral population in 53 randomly-assigned female-male pairs. The remaining 118 finches from ancestral populations were housed in cages as 54 randomly-assigned female-male pairs and 5 male-male pairs. The birds were then allowed to acclimate for one month prior to experimental onset and provided with *ad libitum* food and water throughout. None of the birds displayed any sign of infection with other diseases and all birds were similarly treated for infection by *Trichomonas gallinae* and *Coccidia spp* in the first few weeks of captivity.

METHOD DETAILS

All 53 birds from the adapted populations, and one bird randomly selected from each of the 59 pairs containing only birds from ancestral populations, were inoculated with 1 of 55 *M. gallisepticum* isolates sampled over the course of the epidemic (N total inoculated = 112; 4 isolates were each inoculated into 2 birds from ancestral populations, and 1 isolate was inoculated into 2 birds from adapted populations). 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 containing SP4 base (final concentration in medium: 0.004% thallium acetate, 3.5g/L Mycoplasma broth base, 1g/L tryptone, 5.35 g/L peptone, 5g/L dextrose), 5% CMRL 1066 media with 0.72 mM L-glutamine, 5.75 g/L yeast extract, 2 g/L yeastolate, 17% fetal bovine serum, 0.002% phenol red and 1.86 g/L Penicillin G. Cultures were placed at 37°C until evidence of growth was observed based on a change in pH (i.e., phenol red indicator changing from red to yellow). Successful isolation of *M. gallisepticum* in culture was confirmed based on a lack of observable turbidity and PCR-based testing of DNA extracted from cultures [45]. Stocks of each isolate were made by placing 500 μ L of culture into 500 μ L of new growth medium containing 50% glycerol and then stored at –80°C. All isolates were obtained in Lee County, Alabama (USA), with the exception of: 1994_1 (Virginia), 1995_1 (Tennessee), 1995_2 (Georgia), 1995_3 (Kentucky), 1995_5 (Pennsylvania), 1995_6 (Missouri), and 1996_1 (Ohio). In preparation for inoculation, an aliquot of each isolate was grown in new SP4 growth media at 37°C. None of the isolates inoculated had been passaged in culture more than 4 times [51]. They were then administered via 20 μ L of culture containing 1×10^4 to 1×10^6 color changing units/mL of *M. gallisepticum* in both eyes. The second bird of each pair was used as a sentinel to measure transmission rate and were not subject to inoculation; all sentinels were from ancestral populations in order to measure transmission rates to susceptible hosts [14, 17].

We monitored the development of clinical symptoms in the experimentally-inoculated bird by visually scoring eye lesion severity at both eyes (0–5 scale [26]) at 3, 6, 8, 14, 21, 25, 28 and 34 days post-infection (dpi). Eye scores were based on previous definitions [26], briefly: 0 = normal eye; 1 = pink conjunctiva, signs of watering of the eye, and slight periorbital swelling; 2 = pink conjunctiva, excess

watering of the eye with signs of mucoid discharge and moderate periorbital swelling; 3 = red conjunctiva, excess watering with discharge, feather loss around periorbital ring, significant swelling with irritation; 4 = red conjunctiva, excess watering with discharge, feather matting extending below the eye, severe swelling with significant irritation; 5 = red to purple conjunctiva, extreme swelling with eye barely visible at best, heavy feather loss and matting over entire face. The scorer had no knowledge of which birds were in which treatment at any point during the experiment. Peak symptoms severity was then determined as the maximum score reached by the experimentally-inoculated bird over the course of the experiment. Infection duration in the primary host was scored every ~3 days post-inoculation (dpi), and considered null when the pathogen failed to cause clinical symptoms, and lethal when symptoms reached the score that is known to cause blindness and mortality through predation or starvation in the wild [25–27]. For hosts whose symptoms remained sub-lethal, infection duration was estimated as the duration of the experiment + 1 day (i.e., 36 days); such an approach will tend to under-estimate fitness differences between isolates and hence provide more conservative estimates of fitness differences between time points. Transmission rate to the sentinel was measured by amplification of *M. gallisepticum* DNA from conjunctival and tracheal swabs [45] obtained at 2, 3, 4, 5, 6, 7, 8, 11, 14, 17, 20, 23, 26, 29, 32 and 35 dpi. The experiment was stopped at 35 dpi and all birds were euthanized as stipulated by home office licensing.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analyses

All statistical analyses were conducted in R 3.3.2 [46] using lme4 [52], and figures were made using ggplot2 [53]. All analyses considered the effect of host habitat at site of capture (i.e., urban areas versus suburban parklands) as a fixed effect, but this effect was removed in all cases because it failed to improve the fit of the model (see [Host habitat effects](#) below). Both peak symptoms and pathogen fitness followed zero-inflated distributions and were therefore analyzed using 2-parts hurdle models [28]. In the analysis of peak symptoms, the hurdle model comprised a mixed effect logistic regression model on peak symptoms values of 0 versus non-zero with a logit link function (part 1; $N = 112$ birds), followed by a mixed effect GLM on the non-zero values of log-transformed peak symptoms (part 2; $N = 83$ birds). In both steps, peak symptoms were fitted as the response term (0 or non-zero in the former; >0 values in the latter), year of pathogen sampling, host population and their interaction were fitted as explanatory terms, and pathogen isolate as a random effect. In the analysis of pathogen fitness, fitness values followed a zero-altered gamma distribution (ZAG). Part 1 therefore used a mixed effect logistic regression with a logit link function on whether the isolate failed to gain fitness (i.e., fitness < 1) or succeeded (i.e., fitness ≥ 1) ($N = 55$ isolates in 112 birds); part 2 used a linear mixed effect model with gamma error structure and log link function on the values of fitness ≥ 1 ($N = 46$ isolates in 70 birds) [28]. In both steps, fitness was fitted as the response term (fitness < 1 versus fitness ≥ 1 in the former; fitness ≥ 1 values in the latter), the time gap between antagonists (in years) was fitted as the explanatory term, and with host habitat at site of capture nested within host population and pathogen isolate as random effects. Finally, changes in host fitness over time were analyzed using a mixed effect logistic regression with blindness (no/yes) as the dependent variable, with the relative time-shift between pathogen and host (see above), host population and their interaction as explanatory terms, and with pathogen isolate, its year of sampling and host habitat at site of capture were included as random effects ($N = 112$ birds). All non-significant interactions were removed from the final models.

Host habitat effects

Birds were captured in ecologically distinct urban and suburban parkland habitats in both Alabama and Arizona. House finches from more urban areas are known to be lighter and more susceptible to other infectious pathogens [47, 48]. As a consequence, if ecology has a significant effect on patterns of host resistance, we would expect birds from urban and parkland habitats to show dissimilar responses to inoculation. Further, if ecology represents the primary driver of differences in resistance, then we would expect responses by birds from the same habitat type to be more similar to each other, irrespective of whether they were from Alabama or Arizona. By contrast, if resistance is genetically-determined, we would expect habitat to have no influence on our results [14, 17].

Overall, in Alabama populations, 11 birds came from urban areas and 42 birds came from two suburban parklands, while in Arizona populations, 17 experimentally inoculated birds came from urban areas and 42 came from two suburban parklands (birds in urban areas are less common and so harder to catch). To test for evidence of ecological impacts on our results, we included habitat type as a 2-level factor as a main effect in all analyses (suburban park versus urban), and where possible/relevant, as an interaction term with host population (Alabama versus Arizona) and year of pathogen sampling (see below). Z statistics are provided for logistic regression analyses, while χ^2 tests denote the use of the anova function in R to compare mixed model analyses that include versus exclude terms of interest (df are presented when >1).

First, in the analysis of peak symptoms, we found no effect of habitat on the probability that hosts developed conjunctivitis (estimate = 0.1 ± 0.9 , $z = 0.15$, $p = 0.88$). (Habitat effects could not be determined in interaction with host population due to failure of the model to converge). Nor did habitat have any effect on quantitative values of peak symptoms, either as a main effect (estimate = -0.02 ± 0.1 , $\chi^2 = 0.02$, $p = 0.88$), or in interaction with host population ($\chi^2 = 0.02$, $p = 0.89$), or in a 3-way interaction additionally including year of pathogen sampling ($\chi^2 = 1.0$, $df = 3$, $p = 0.81$). Second, habitat also failed to influence our estimate of host survival probability (estimate = 0.7 ± 0.6 , $z = 1.3$, $p = 0.26$), and we found no evidence for an interaction between habitat and host population ($z = -0.8$, $p = 0.40$), or for a 3-way interaction additionally including year of pathogen sampling ($z = -1.1$, $p = 0.92$). Finally, we found no evidence to suggest that pathogens gained differential fitness as a function of whether their host was from suburban parkland or from urban areas (effect on probability of gaining fitness: estimate = -1.3 ± 1.3 , $z = -1.0$, $p = 0.32$; effect on quantitative

variation of fitness (fitness ≥ 1): estimate = 0.3 ± 0.2 , $\chi^2 = 1.9$, $p = 0.17$). (Tests of interaction effects with host population and year of pathogen sampling are not relevant in these 2 analyses because neither were fitted as fixed effects in the original model; see main text).

Together, these results have two important implications. First, they provide no evidence to suggest that ecology has an impact on host resistance and pathogen success in this system, thus corroborating our previous findings of the role of genetic resistance in population differences to experimental inoculation with *M. gallisepticum* [14, 17]. Second, by extension, our results are therefore unlikely to be explained by any unmeasured phenotypic differences between host populations.

DATA AND SOFTWARE ACCESSIBILITY

The accession number for the data reported in this paper is Dryad Digital Repository: <https://doi.org/10.5061/dryad.5g5n8n2>. The data include lists of individual hosts and bacterial isolates, year of pathogen sampling, host infection status and severity, and estimates of host and pathogen fitness.