Understanding the Evolution of Emerging Bacterial Pathogens in Response to Host Resistance

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I certify that all material in this thesis which is not my own work has been identified and that no material has previously been submitted and approved for the award of a degree by this or any other University.

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Abstract

Understanding the evolution of parasites and hosts following a host-shit event is increasingly recognised as being of great importance to public health and the clinical/veterinary sciences in predicting the behaviour and evolutionary consequences of emerging infectious diseases. Microbial pathogenesis and virulence are remarkably complex traits, and only by considering them in the context of their hosts can we begin to unravel key questions as to how and why disease emerges and persists. The period immediately following a host-shift event, where a pathogen circulating in one host species successfully jumps into another is critical – whether such outbreak events “burn out” or become endemic, and what the ramifications of this might be are difficult to model and predict.

That microbial pathogens and their hosts are in a close coevolutionary relationship has been evident since the early days of our understanding of disease, but it is only relatively recently that the ecological, molecular, genomic and bioinformatic tools all required to understand the subject have become widely available and applicable. In the work presented within this thesis, we utilise an exceptionally well monitored and studied novel host-pathogen interaction – that of the avian bacterial pathogen *Mycoplasma gallisepticum* and its recently infected novel host the House Finch (*Haemorhous mexicanus*). Approximately 25 years ago this pathogen jumped from its established host in chickens into the wild passerine finch species, triggering an epidemic which has been well monitored from the outset

We aim to address how host-pathogen coevolution drives particularly the evolution of microbial virulence. Our current understanding of such host-

pathogen interactions within an evolutionary context centres around the mathematical and ecological framework of the “Trade-off hypothesis”, but many of the assumptions linking pathogen virulence, transmission and replication have been difficult to test or to integrate with our modern understanding of how microbial virulence is manifested on a molecular level. In exploring these issues throughout this text, we consider that this work has progressed our understanding in this field and goes some way towards this integration of evolutionary theory and more descriptive classical microbiology / molecular biology.

Acknowledgements

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Thanks to Dr. Andrea Dowling, for getting me started in working with *Mycoplasma* and Dr. Mathieu Giraudeau for his expertise in working with House Finches, as well as Dr. Stineke van Houte and Dr. Uli Klumper for their advice on biofilms, and Dr. Manmohan Sharma, Dr. Samantha Kay and Dr. Karen Moore for their expertise in working with DNA and RNA over the course of this work. Finally, thanks to the Undergraduate and Masters students who I have worked with over the course of this project; to Karen Carpenter, Joycelyn Tanner, Joe Money and Amer Townsend, I wish you the best going forwards.

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Statement of Contribution

The work in this thesis was made possible through a NERC-funded experimental infection study conceived and designed by my supervisor, Dr Camille Bonneaud, using a library of pathogen isolates collected over a 20-year period by Prof Geoffrey Hill (Auburn University, USA), and conducted by Dr Mathieu Giraudeau (U. Exeter) in the facilities of Prof Kevin McGraw (Arizona State U., USA). Luc Tardy assisted with the experimental infection in Arizona, developed and conducted the molecular and microbiological assays, performed the statistical analyses and wrote the chapters, with supervisory support. Supplementary data of relevance to this thesis, but not analysed by Luc Tardy, are included as an Appendix in chapter 4.

Chapter 1: Introduction

Understanding the evolution of pathogen virulence and coevolution within host-pathogen systems is critical to furthering our ability to predict the potential development of both endemically circulating and emerging infectious diseases, with implications to public health policy and clinical/veterinary sciences. As human population growth and demographic changes continue, and the impacts of climate change becoming more evident on a global ecological scale, the risk of novel emerging infectious diseases (EIDs), particularly from zoonotic sources is set to only increase if left unchecked (Petersen *et al*, 2018). EIDs, for example the coronavirus outbreaks of Severe acute respiratory syndrome (SARS) and Middle East respiratory syndrome (MERS), and of recent and ongoing ebolavirus outbreaks have garnered substantial public interest and concern in recent years, highlighting the fact that research into the drivers and ramifications of EID emergence will be increasingly of critical importance to public health (Watkins, 2018).

Despite the efforts of an extensive research community working across myriad host-pathogen systems, key questions remain unanswered and theory largely untested in our understanding of disease emergence and evolution, owing largely to a scarcity of well-defined research on recent EIDs for which these very evolutionary processes have been left to develop *in natura* without human intervention; rightly administered in regards to the fact that those well studied pathogens would often have severe public health or commercial ramifications if left alone. This fact has meant that finding relevant host-shift and host-pathogen systems alone can be complex, with a large focus being confined to laboratory phage-bacteria study systems and viral evolution in plants and insects, whilst

detailed research concerning of the arguably more complex co-evolutionary aspects arising in vertebrate host-pathogen systems has been scarce (Alizon *et al,* 2009).

Over the course of the work presented herein and going forwards, we aim to address the above caveats to our current understanding by using a uniquely well-studied vertebrate host-pathogen system, uncomplicated by any intervention since its emergence; that of the avian bacterial pathogen *Mycoplasma gallisepticum* and its novel host in the House Finch (*Haemorhous mexicanus*). As shall be elaborated further upon later, some 25 years ago *M. gallisepticum* jumped from its normal host in chickens (*Gallus gallus*) and into the wild passerine Finch species – well studied in it's own right, where it was quickly diagnosed and tracked, with data on its prevalence and severity collected on a massive scale. Additionally, bacterial isolates from wild birds collected from the outset out of epizootic provide us with an extensive library of experimentally valuable samples, which has allowed for the design and execution of experiments to study a vertebrate host-pathogen coevolutionary processes at an unprecedentedly fine-scale temporal resolution.

Before, however, we move to a detailed discussion of the system, we include here below a brief background concerning the history and current state of research in regards to the evolution of virulence and the wider co-evolutionary processes in host-pathogen interactions.

The evolution of virulence – an introduction to pathogen-host interactions and evolutionary theory

Defining Virulence

Scientific understanding of why pathogens exhibit virulence; i.e. why they cause morbidity over the course of an infection and/or often ultimately the death of their hosts has been an area of interest from the earliest stages of germ theory development, with the concept of differential virulence between strains of newly identified pathogens present from the earliest days of its inception (Pasteur *et al,* 1881). However, despite this long history of recognition, providing a concrete definition of virulence remains surprisingly difficult, with the term used often in different contexts or to mean different things depending on the field of research and often conflated with the arguably similar term “pathogenicity”. Within the fields of clinical microbiology and invertebrate pathology a classical consensus stemming primarily from the 1970s emerges, where “pathogenicity” is defined as a qualitative ability or potential for an organism to cause disease (often defined itself by fulfilment of Koch’s postulates), being applied to broad groups of hosts and pathogens, and “virulence” being thusly derived as the quantifiable disease causing power of an organism or the degree of pathogenicity exhibited by this organism within a narrow host grouping (Aizawa, 1971; Young & Barger, 1971). In contrast, within plant pathology these definitions may be more vague, for example stating “virulence” to be the relative capacity of an infective agent to cause damage in a host (Casadevall & Pirofski, 1999), or even reversed so that “virulence” becomes the qualitative term describing where an agent causes disease and “pathogenicity” becomes the quantitative measure of such (Bos & Parlevliet, 1995). Such distinctions in the definitions of simple terms really do

matter in accurately and correctly discussing the subject of virulence and wider, the topic of host-pathogen interactions at large. In our case and utilised throughout this work we endorse the use of the clinical definition above; that pathogenesis is the qualitative ability or potential to cause disease while virulence relates to the quantifiable and relative elements of this trait, for example through measured mortality rates, or sub-lethal measures such as degree of inflammation at a site of infection and/or weight loss in infected hosts.

The avirulence hypothesis and early concepts in the evolutionary biology of pathogens

Early evolutionary theory in regard to pathogen virulence was largely dominated by the "avirulence hypothesis", arising from "the law of declining virulence and advancing parasitism" postulated by Theobald Smith at the turn of the 20th century (Smith, 1904). Based on his experience as a clinical microbiologist and work on "Texas Cattle-fever" (later identified as the apicomplexan *Babesia bovis*) and "rabbit septicaemia bacillus", Smith theorised that for obligate parasites evolution drives a closer and closer association between a pathogen and its host leading towards mutualism and an associated loss of virulence over time. Since virulence expression leads to host morbidity and mortality, he argued, and these in turn would reduce the lifetime transmissibility of a parasite through either host death or immune clearance, selection should over time act to reduce virulence and thus maximise what we would now term R_0, the number of secondary hosts infected within a primary hosts infective lifetime, or broadly "transmissibility". This is theory which we now consider defunct and supplanted in large-part by the trade-off hypothesis which emerged later on in the latter half of the 20th century and which we shall elaborate upon shortly. Hints however to the development of

our understanding of pathogen evolution can be found in Smith's work; although his concept of an idealised pathogen tending towards mutualism over time appears today to offer little explanation for those which maintain their virulence over long-associations with hosts, for example *Mycobacterium tuberculosis*, and is arguably naively optimistic in its projections, in his 1904 paper coining "the law of declining virulence and advancing parasitism" Smith writes "… our thesis implies in addition to the natural decline of virulence also a gradual rise in virulence whenever the resistance of susceptible individuals is raised on a very large scale". He then posits that such increases should occur due to factors which increase host-resistance and resistance heterogenicity and variation in host populations, namely via incomplete vaccination and population mixing arising from host-migration. These predictions are largely in line with some of those made by the trade-off hypotheses which came to supplant his work, an interesting point in evaluating Smiths theories and an indication perhaps of what theory in the field of pathogen evolution would eventually become.

Modern theory – the trade-off hypothesis and paradigm

Regardless of whether our view of his theory is now oversimplified, Smith's "avirulence hypothesis" stood for most of the 20th century as the commonly expressed dogma within the study of pathogen evolution with limited, although not non-existent opposition. A primary point of contention which would grow in prominence was the implied link between the age of a host-pathogen association and its virulence; that old associations should result in avirulence whilst novel hosts to a disease would experience much greater mortality and morbidity arising from the maladaptation of the parasite. Gordon H. Ball wrote in 1943 an early commentary on this point, arguing that the amounting evidence from the ever-

more accurate taxonomies of various pathogens, and the finding from various cross-infection studies using generalist pathogens strongly indicated that this idea did not hold to biological reality, particularly referencing some early works with avian malarias which had shown low virulence in novel hosts or lasting virulence in known long-term associations contrary to the predictions of what had come to be termed the “conventional wisdom” (Ball, 1943).

Along with the rapid progress in molecular biology and genetics over the course of the early to mid-20th century, and the coupled development of the Modern Synthesis in evolutionary theory, a greater legitimacy and emphasis on mathematical modelling in the biological sciences would gain prominence over time (Méthot, 2012). In their seminal works, the two-part “Population biology of infectious diseases” by Roy Anderson and Robert May, published in 1979 (Anderson & May, 1979; May & Anderson, 1979), would apply mathematical logic and modelling techniques to address the shortcomings of the avirulence hypothesis, in what would come to be termed the “transmission-virulence trade-off hypothesis” of pathogen evolution (Anderson & May, 1982).

Central to this idea is virulence is explained as an obligate function of pathogen transmission as some degree of host damage or dysregulation by a pathogen is maintained by selection in order to optimise transmission of an infection from host-to-host. Therefore, virulence is not a fixed trait for pathogens but one which is constantly in flux around an equilibrium defined by host-population structures and susceptibility to infection, including degrees of resistance and immune clearance in a much more complex, yet mathematically expressible relationship (Anderson & May, 1979; May & Anderson, 1979). This schematisation of host-pathogen population dynamics, an example of which is

illustrated below in Fig. 1., has provided a framework in which hypothesis about pathogen virulence and transmission dynamics can tested.

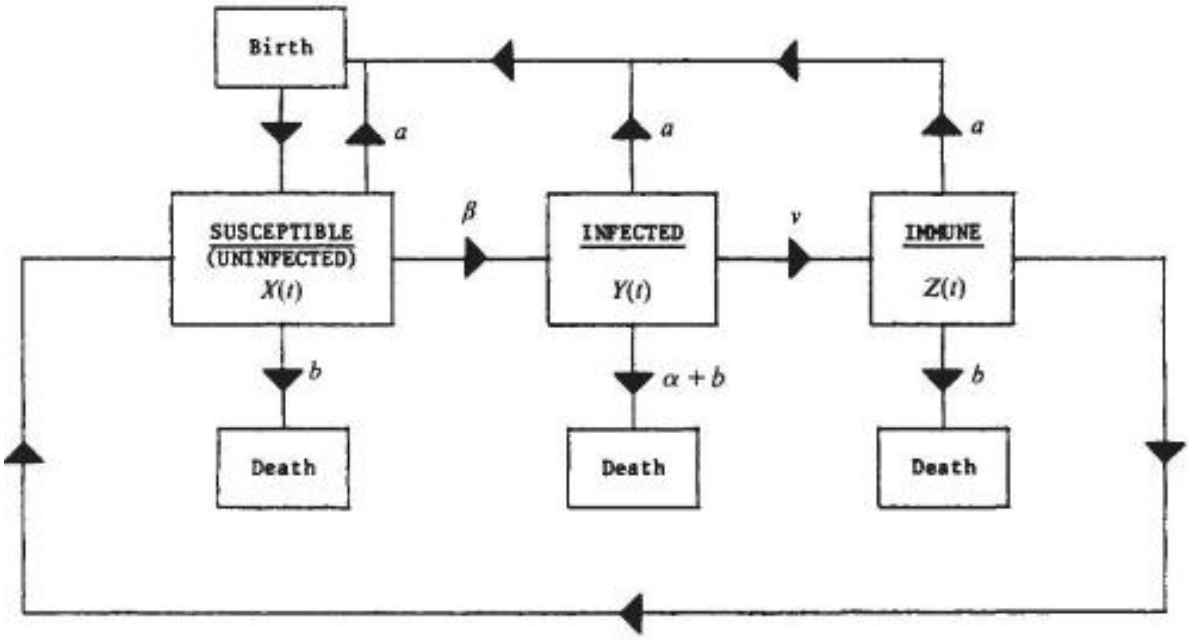

Fig. 1. A schematic representing the demographic and biological variables in effect for a horizontally transmitted pathogen, where **X** are susceptible hosts, **Y** are infected hosts and Z are immune hosts and the equilibrium between these groups is set by the rate variables a – host birth-rate, **b** – background mortality rate, **α** - mortality due to infection, **v** – recovery of hosts from infection, **β** – Transmission rate between infected and susceptible hosts and **γ** – Loss of immunity. Amended from Anderson, R. M., May, R. M., 1979, Population biology of infectious diseases: Part I, *Nature,* 1979, 280 Fig.3.

Through this integrative approach, the trade-off hypothesis provides not only an explanation for pathogen virulence in a wider biological context, but also has predictive power and suggests mechanistic basis for testing a number of key questions in the study of host-pathogen co-evolution of significant importance. An important implication of the theory is that it poses questions in relation to clinical therapeutics and the rise of drug-resistance, an issue of critical societal

importance. Under the trade-off hypothesis, increased host-resistance, and subsequently faster pathogen clearance, is suggested to select for faster replicating and more virulent strains of the pathogen to evolve over time (Porco *et al*, 2005). Whether naturally occurring through host immune-processes or induced via therapeutic treatment of infections, this predicted increase in pathogen virulence in response to host-resistance is increasingly well understood under the framework of the trade-off hypothesis. When *Myxoma* virus was introduced as a biocontrol agent to control Australian rabbit populations the virulence of the introduced strains was found to decrease rapidly at first, only to increase again later as a result of evolving host immunity (Best & Kerr, 2000), and laboratory experiments conducted under the trade-off framework to test this link seem to confirm this assumption; measurements of the evolution of virulence in naïve and immunised mice exposed to the murine malaria *Plasmodium chaubaudii* indicated that parasite lines evolved in immune hosts ultimately produced higher parasitaemia infections than those evolved in mice more susceptible to infection (MacKinnon & Read, 2004). This latter experiment also exemplifies a key assumption made in the study of pathogen evolution in measuring parasitaemia as a proxy of virulence – that pathogen replication rate and virulence are proportional, with more pathogen replication resulting in greater host-exploitation and damage.

3 – Evolution and Mechanisms of Microbial Virulence

Virulence manifests as a proportional effect of Pathogen replication

Although the trade-off hypothesis offers an explanation for why virulence emerges – i.e. depending on transmission dynamics some host exploitation is

strictly required by a parasite, with subsequent damage and dysregulation manifesting as observed pathogen virulence, this does not by itself explain why virulence varies between parasite lineages and, although it posits an “optimal virulence” maxima where transmission is maximised this alone does not describe adequately what evolutionary processes drive virulence towards this optimal value. It has long been observed that pathogens in serial passage *in vivo* often gain in virulence (Ebert, 1998), suggesting that this selection cannot only occur in response to host-immunity. Developing theory on these processes requires an understanding of the multi-hierarchical nature of selection occurring in the multi-stage and often complex life cycles of successful pathogens; i.e. that there is not only between-host selection, where high fitness corresponds to the most transmissible lineages, but also within-host selection where pathogen lineages will compete within a single infection.

If we consider that in nature, even for a single pathogen species, infections are not commonly caused by a single infective cell or particle but rather an inoculum population containing multiple parasite lineages, then the basis for this becomes clear. In order to maximise fitness on an inter-host level a single parasite lineage must come to dominate the transmissible population of different parasite lineages over the course of an infection (Bose *et al,* 2016). Since the most obvious way to do this is to outnumber the competition within-host, selection on this level should in theory act to increase replication rates up to a point. Thus, if faster replication is selected for, and is coupled to host-exploitation, virulence subsequently increases. Of note, even if a truly clonal parasite is used to infect a host with just one parasite lineage, given the rapid generation time of most pathogens in an infection both neutral and diversifying selection processes

generate standing variation within the population (Didelot *et* al, 2016) on which this selection for increased replication rate could occur.

Social Theory and pathogen evolution

At this point, having considered how selection for faster replication may occur within a host we lead naturally into a brief discussion of social-theory in microbial evolution. This burgeoning field aims to investigate how interactions between microbes effects their behaviour through both co-operative and antagonistic processes and how such this leads to community structure, as well as it's implications for evolutionary processes in such conditions. In regard to pathogen virulence these theories suggest a role to play for not only inter-specific microbial interactions, for example between invading pathogens and host microbiota (Bäumler *et al,* 2016; Koskella *et al,* 2017), but also makes predictions for the kind of intra-specific selection that might occur over the course of a single infection. A great array of microbes have evolved quorum sensing apparatus – typically two-component sensory systems, to enable them to detect the community composition of other microbes around them, and for pathogens this can be employed to better survive and proliferate within infected hosts (West *et al,* 2007). Many of the molecular effectors of virulence, discussed later in this chapter, can be classed as "public goods", in the sense that their production benefits not only the source bacteria but also those around it. Siderophore production for example helps sequester free iron away from the host and makes it available to pathogens as a cofactor in their growth, but are secreted and, operating outside of the cell which produced them provide a more dispersed benefit to cells over a larger area. Because of this, evolutionary strategies like "cheating", where a cell produces no public good effectors themselves but gains

the benefit of those around them have evolved, and in order to minimise the amount of these “cheaters”, many microbes have evolved counterstrategies, for example regulating their production of these virulence factors based upon their surrounding community composition and evolving higher specificity to effectors so that only highly-related neighbours will benefit (Meyer *et al,* 1997; Griffin *et al,* 2004; Smith *et al, 2005*).

Although the networks of co-operative and antagonistic behaviours between microbes highlighted by social theory can be complex, we can derive from it many hypotheses relevant to studying the evolution of pathogen virulence. Notably, much recent research has shown that relatedness between bacteria can impact virulence and its evolutionary trajectory. For *Pseudomonas aeruginosa* and *Staphylococcus aureus*, quorum sensing enables cooperation between closely related bacteria and leads to greater host-exploitation and virulence (Rumbaugh *et al,* 2009; Pollitt *et al,* 2014). The exact relationship between relatedness and virulence however is likely subject to the particulars of community composition, scale, and the type of interactions which occur between microbes (West *et al,* 2007; Buckling *et al,* 2008)

The role of host-resistance in increasing pathogen replication rate

Complicating the picture discussed above of intra-host competition between lineages is the issue of host immunology and resistance. Since infection with parasites, particularly those which manifest severe virulence, incurs a fitness cost to hosts there is in novel host-pathogen interactions a tendency for host resistance to quickly co-evolve along with pathogen virulence. Typically, this will result in faster immune clearance of the pathogen, shortening infection times and reducing the window for onwards transmission of the parasite. Along with the

selection pressure to increase pathogen replication rates arising from the stochastic intra-host competition mentioned above, the effect of host-resistance is expected to compound further upon this effect to some extent but is also a perhaps more complex relationship than is obvious. Host-resistance or immunity, in increasing rates of clearance and shortening the duration of infection may select for pathogen lineages which are capable of faster replicating to a transmissible level and also causing the necessary virulence manifestation to transmission to occur depending on its route. However, pathogens lineages which are too virulent are also more likely to be cleared by the increased host immune processes under these models, and this would result in a selective pressure favouring parasite lineages of intermediate virulence in response to host-resistance (Antia, 1994).

Although this above point presents some conveniently testable hypotheses in linking virulence to pathogen replication and host-resistance, developments from the large body of work building on the Transmission-virulence trade-off model continues to challenge the underlying assumptions presented therein. In regard to the effect of host-resistance, it has become clear from both modelling and empirical approaches that, far from being an easily parameterised variable, resistance is in fact a complex and multi-facetted trait. Firstly, we should consider that resistance, in an otherwise viable host-pathogen interaction, arises from often complex host immunological-processes which can vary both between individuals and within individuals based on life-history (reviewed in Ashley *et al,* 2012). Ageing, for example, appears to play a role in the behaviour of innate immune processes; as infection history develops and cell damage mount over a hosts lifespan, the expression of innate immune pathogen recognition ligands and other innate-immunity signalling genes can increase, resulting in in-turn

higher levels of pro-inflammatory molecules and higher inflammatory responses in aged hosts compared to younger individuals (Shaw *et al,* 2013). On shorter timescales, seasonal conditions can produce similar heterogenicity in host immune-responses – for example, pro-inflammatory cytokines are downregulated in responses to LPS during simulated shorter days lengths (i.e. simulating winter conditions) in Siberian hamsters, alongside more general changes in host sickness behaviours, and these are thought to be associated with the changing fitness costs to mounting immune-responses as energy requirements and resource availability vary in the wider ecological context (Bilbo *et al,* 2002). That immune-system activity – and subsequently host-resistance to pathogens, might not be a constant parameter as formulated by a simple model of the trade-off hypothesis presents significant challenges to its applicability in understanding pathogen-evolution without some modification. If as stated above, resistance should select for faster-replicating and thus more virulent pathogen strains up to a value optimal for onwards transmission, then the shifting patterns of host immune activity and its subsequent effectiveness in clearing pathogens could have large implications in the strength or direction of such selection.

A key modification to the trade-off hypothesis which has been made in redress to these shortcomings is to integrate it with the concept of immunopathology (Graham *et al,* 2005; Day *et al,* 2007; Long *et al,* 2011), which goes some way to include the energetic and physiological costs of resistance, and how these might impact host-pathogen coevolution. Reasoning that virulence for many infections is not driven solely by pathogen elements, but also manifests from self-damage caused by host immune-processes allows for consideration of how the relative damage of an infection caused either directly by a pathogen or indirectly by the host response can alter the fitness parameters of virulence

evolution. A mathematical approach to this delineates host immune-responses to infection into those which cause either an independent immunopathology, i.e. self-damage not scaling to pathogen resource exploitation, or a proportional immunopathology, and posits that this can have drastically different effects on the evolution of pathogen virulence. Such models predict that when independent immunopathology causes high host-mortality – for example characteristically for influenza (Xu *et al,* 2004), selection should favour more exploitative, and thus virulent pathogen strains, since lessening exploitation would have little effect on lowering mortality and thus extending the time-frame for transmission. Conversely, where immunopathology scales to pathogen exploitation, pathogen transmissibility and fitness can be increased by lowing directly pathogen-driven virulence effecting host-mortality, leading to selection for less exploitation as a mechanism to reduce host immune activation and immunopathology driven mortality (Day *et al,* 2007).

Tolerance – further complicating the picture of host-defences to infection

Related to the above concept of immunopathology, it is important to consider further that host defensive responses to infection can be broadly defined into the two broad categories of resistance and tolerance. From an evolutionary standpoint, hosts can adopt two strategies for dealing with infection. Resistance relates to those mechanisms which directly combat pathogens by either blocking infection and providing some degree of protective-immunity, e.g. by genetic mutation of receptors required for pathogen colonisation/invasion, or by facilitating faster clearance of pathogen by altering the specificity of immune-responses; the effect of either mechanism being to lower pathogen loads in a resistant host compared to one with no evolved resistance. Alternatively,

mechanisms of tolerance can evolve, where pathogen load is unaffected but immunopathology and thus an infections manifest virulence, is reduced (Schneider *et al,* 2008; Medzhitov *et al,* 2012). Importantly, the relationship between tolerance and resistance evolution is likely distinct depending on the exact mechanistic basis at play, with these mechanisms possible trading off against one another in effect. They can be intrinsically linked and antagonistic to each other, for example in the production of microbe killing reactive oxygen species by phagocytic cells in the innate immune response, which provide resistance to pathogens by increasing rates of clearance but incur large immunopathological costs though oxidative stress (Lambeth *et al,* 2007a; Lambeth *et al,* 2007b) and thus reduce tolerance. Alternatively, tolerance mechanisms can exist which act largely independently of resistance, for example through systemic changes in host-metabolism or the increased expression of host-repair genes (Schneider *et al,* 2008). The potentially huge complexity of this aside, the point of this remains that both resistance and tolerance are capable of influencing the evolutionary trajectory of pathogens, though with perhaps different effects, through their integration into such models which include immunopathological parameters, and are as such vital to our understanding of virulence evolution.

It is thus evident that the relationship between virulence, pathogen replication, transmission and host resistance is clearly a complex one, likely to vary depending on the exact biology of the species involved. Furthermore, although evolutionarily perhaps the most intuitive driver of virulence, microbial virulence is clearly a multifaceted trait with numerous causes which cannot simply be explained by this linkage to pathogen replication. In the following section we address some of the molecular mechanisms which affect virulence upon an

infected host, whether that is dependent and strictly proportional to pathogen load or not.

Virulence Factors and the Damage-Response framework of microbial pathogenesis and virulence

From a clinical and descriptive microbiology perspective, much of the host-damage manifested by infecting pathogens occurs via the molecular effects of what is loosely termed “virulence-factors”. This umbrella term defines a myriad of compounds, proteins and other pathogen components and traits which act as effectors of damaging host-responses, often acting to subvert host-resistance and immunological mechanisms to the pathogens benefit. These individual virulence factor components can have multiple functions within an infection, varying between hosts and even sites of infection, and encompass a field of study far too vast to detail in anything nearing completion; however, for our purposes a brief overview given here with some defining concepts and important examples should suffice.

Largely an artefact of the historical argument that microorganisms exhibiting pathogenicity, i.e. “pathogens”, must have some intrinsic qualitative difference to environmental or commensal microbes, defining what is and what isn’t a virulence factor has proven to be a complicated issue. Since many pathogens are now known to be opportunistic, or to only exhibit pathogenic behaviour under certain conditions, the line between pathogenic and non-pathogenic microorganisms has been significantly blurred. A modern concept which has recently developed to address these issues and provide a framework in which to discuss virulence-factors is that of the “Damage-Response” framework of microbial pathogenesis (Casadevall & Pirofski, 2003). Rather than

focusing entirely on the biology of the microorganism, this approach considers instead that virulence factors are defined from the interactions of specific microbial components with host-factors and responses and allows for the contextualisation of pathogenesis and virulence on both an organismal and molecular level. Under this framework individual microbial species and strains can be associated with a given damage-response curve in a host species (fig. 2 below); an expression of how its virulence manifests under different host-response strengths.

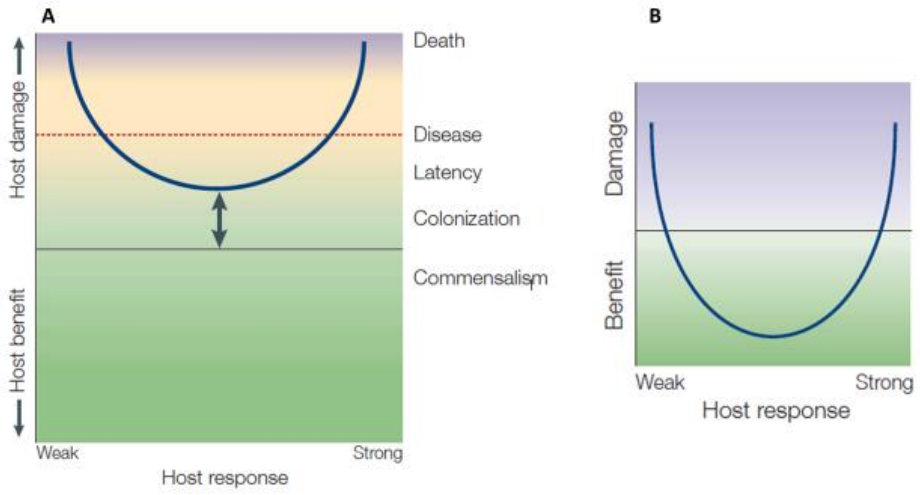

Fig. 2 : **A)** A damage-response plot for a typical pathogen – virulence is limited at intermediate host-responses, but where host responses are either weak (e.g. in immunocompromised hosts) or strong (e.g. in immune over-activation) virulence causes increased morbidity and mortality. **B)** The damage-response curve of a microbe which causes disease only in context of weak or overly strong host-responses but is otherwise beneficial. This curve might relate best to commensal gut microbiota, which when kept in check by normal host-responses contributes

to host-health. Amended from Casadevall, A., Pirofski, L., 2003, The Damage-Response Framework of Microbial Pathogenesis, *Nature Reviews Microbiology,* 2003, 17, Fig.1. and Fig.2.C

Applying this concept to host-pathogen interactions, a virulence factor becomes defined as any microbial behaviour or component which biases the host-response variable to induce greater virulence, and this approach resolves some of the issues of defining virulence factors otherwise (Casadevall & Pirofski, 2009). For example, bacterial endotoxin is a classic virulence-factor, but is also an often-essential membrane component of bacteria which may or may not exhibit pathogenic behaviours; the damage-response framework allows it to be regarded as a virulence-factor under specific circumstances as an infective agent within the context of a host response, but not, for example when the microbe exists in a free-living environmental state. This idea can also be reasonably extended to encompass a more fine-detail exploration of how and why virulence is manifested in an evolutionary context. In the case of virulence selection mediated by replication rates detailed above it becomes arguable that for example a mutation in one of a microbes DNA-replication machinery genes, or some other which increases the efficiency of host resource exploitation and thereby increases the speed or frequency of replication becomes a virulence-factor, whereas its wild-type or the population norm variant of a gene is not. Within this context, the definition of virulence as the relative damage or harm caused to the host, with emphasis on the word “relative”, combined with the quantisation of how we can contextually define a virulence-factor goes some way to combine the evolutionary and molecular aspects of our understanding of the subject.

We have spent some time here on this discussion of the damage-response framework because, in the authors opinion, this provides perhaps the best way forwards with which to understand the mechanistic basis of pathogen virulence traits of particular importance to this thesis; that of replication rates as discussed above, microbial immunomodulation and immunoevasive strategies, and the capacity of microbes to form biofilms and their architectural determinants of virulence, to be reviewed briefly below.

Microbial Immunomodulation of hosts

Microbial pathogens have evolved many strategies in order to both evade host immune processes and to co-opt them to further their infective lifecycles. The immunomodulatory capabilities of microbial pathogens are diverse and can target both humoral and cell-dependant immunity, as well as many facets of the innate immune system, as we shall now discuss in brief.

On encountering an infective agent, “acute-phase” innate immunity is the first line of defence for susceptible hosts, beginning with the non-specific recognition of non-self cells or particles and occurring concurrently with the initial infection and colonisation of the pathogen. At this stage, interactions between microbes and host cells are primarily based on those occurring between the immediately visible cell (or capsid/envelope in the case of viruses) surface components of the pathogen and host cells and other secreted host immune effectors. As part of the normal innate-response which evolved early in Metazoa (Buchmann, 2014), these pathogen surface Pathogen-associated molecular pattern (PAMP) molecules can be recognised by host cell membrane proteins, broadly termed Pattern Recognition Receptors (PRRs), for example the recognition of Gram-negative bacterial lipopolysaccharide by host Toll-like

receptor 4 and CD14 PRRs (Zanoni *et al,* 2011), leading to phagocytosis of the bacteria. Simultaneously opsonisation, where PAMPS interact with secreted innate immunity effectors of the complement system may occur, enhancing their recognition by host cellular defences or directly killing parasite cells through lysis and the complement cascade (Gasque, 2004).

Ideally from the host immune perspective this results in either direct killing of pathogens or phagocytosis and endosomal destruction of the infective agent before an serious infection can be established, with a wider immune response to the infection induced via a complex network of cell-to-cell and cytokine signalling pathways. At the same time however, pathogenic microbes can employ a huge variety of means to evade or hijack these processes.

Immunoevasion

Staphylococcus aureus is a particularly notable human pathogen which exhibits significant immunoevasive capabilities against host immune processes, possessing a variety of genes encoding virulence factors for this purpose (Foster, 2005). Staphylococcal complement inhibitor (SCIN) is an early expressed and secreted protein which binds to C3 convertase, an integrating part of the complement cascade, blocking opsonisation of the bacteria and inhibiting its complement mediated cell-lysis and phagocytosis. Similarly, Chemotaxis inhibitory protein of staphylococci (CHIPS), also expressed early on by the bacteria, acts to affect downstream signalling from activated immune cells and inhibit neutrophil chemotaxis towards sites of infection (Rooijakkers *et al,* 2006). The effect of these for *S. aureus* is to generally inhibit the host pro-inflammatory response and dampen immune responses to the site of infection in general, a

predominately immunoevasive strategy; but this is not always the case for all immunomodulating pathogens.

Immunomodulation and the Induction of Pro-inflammatory Signalling

In discussing the induction of pro-inflammatory processes, a useful example can be found in recent work on *Staphylococcus epidermidis* which also goes to exemplify the contextual nature of virulence depending on host biology and infection site. *S. epidermidis* is typically regarded as a commensal and normal part of the human skin microbiota (Grice *et al,* 2009), but can cause disease in the immunocompromised (Blum & Rodvold, 1987) and is the leading cause of late-onset neonatal sepsis (Dong *et al,* 2015). In such cases infection with *S. epidermidis* becomes particularly invasive within the respiratory and pulmonary systems of susceptible hosts where the bacteria bias the host-response to promote signalling of pro-inflammatory cytokines. Expression of pro-inflammatory inducing virulence factors by *S. epidermidis* such as cytolytic phenol-soluble modulins (PSMs) (Cheung *et al*, 2014) cause upregulation of host TNF-α, IL-6 and IL-8, as well as chemoattractants which lead to a runaway inflammatory response and subsequently high virulence expression in these tissues (Dong *et al*, 2019). PSMs as virulence factors are themselves interesting in the regards that they appear to have originated as part of the commensal colonisation of *S. epidermidis* as a non-pathogenic skin microbiota being involved in biofilm formation on epidermal surfaces, only evolving to contribute to invasiveness and pathogenesis later (Cheung *et al,* 2014), again highlighting the complexity of defining microbial virulence-factors in isolation.

Biofilm formation and the importance of infection architecture in disease

The above example of *S. epidermidis* also hints at the role played by the physical state and microenvironment which pathogens exist in over the course of infection. For our work, the capability of microbes to form biofilm structures is an important area of investigation. Many, if not most bacteria in the environment (Dunne, 2002) and within the context of infections (Segura *et al*, 2016) live in this sessile form as a part of biofilms, and this appears to have major, though not clear-cut implications for microbial pathogenesis and virulence. As with the staphylococcal examples discussed above, biofilm formation has been linked to virulence for many pathogens, particularly those causing persistent infections (Costerton *et al,* 1999). Biofilm production creates static sites of microbial growth, where either commensal or pathogenic microbes can live within structured multi-species communities or as mono-culture populations, and effect many of the dynamics between microbes and hosts. Consisting of a complex extracellular matrix of secreted polysaccharide, proteins and nucleic acids, biofilms appear to be major modulators of host-responses during infection but determining the virulence-factor mechanisms and basis of this has remained challenging; whether biofilm production enhances or inhibits virulence seems to vary on a case to case basis. For *Pseudomonas aeruginosa* biofilms are strongly linked to increased virulence and persistence in cystic fibrosis patients, where it is a noted and severe opportunistic pathogen (Phillips & Schultz, 2012), whereas for *Streptococcus suis* infecting pigs it appears to bias infections towards low-virulence commensalism (Vötsch *et al,* 2018). This apparent variability in the function of biofilms as relates to microbial virulence is clearly an important area of study if we are to understand the biology of individual pathogens and may arise as a consequence of complex evolutionary processes which can only be adequately understood by considering

such structures. Previous work indicates that the structural and environmental heterogeneity of biofilms can create complex diversifying selection processes in such structures, as illustrated in Fig. 3 below. Changing exposure to host immune factors and nutrients along the diffusive gradients generated within biofilms can affect microbial replication and the expression of virulence factors, and as populations stratify along these gradients selection pressures are expected to change substantially (Steenackers *et al,* 2016).

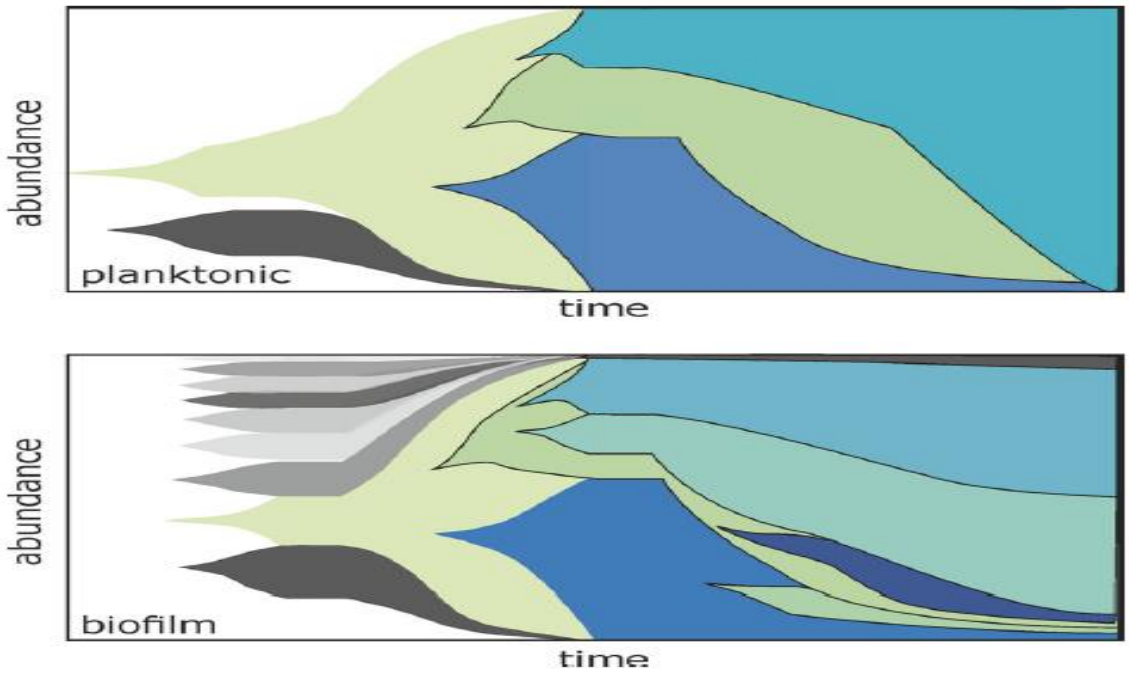

Fig. 3. Population stratification and microenvironmental variation in biofilms allows for greater diversification and maintenance of diverse lineages/genotypes within a microbial population. Different colours represent new lineages arising from radiation of starting lineages in either planktonic or biofilm forming cultures. Amended from Martin, M., Hölscher, T., Dragoš, A., Cooper, V. S., Kovács, A. T., 2016, Laboratory Evolution of Microbial Interactions in Bacterial Biofilms, *Journal of Bacteriology,* 2016, 198; 19

The examples given above of microbial virulence-factors and determinants are, as mentioned, only scratching the surface of the rich and complex ways in which microbes interact with their hosts and host-environments over the course of an infection and affect each other through hard to predict co-evolutionary processes which require often-difficult experimental approaches to adequately study and address in any detail. In our study system of the *M. gallisepticum* – House finch's epizootic we are able to begin to address many issues concerning co-evolution; due to the recent nature of this host-shift and novel-interaction, many of the complexities of this relationship are simplified in that the confounding effects from unobserved and unmeasured long-term host-pathogen co-evolution are here absent, giving us an unparalleled opportunity to study host-pathogen co-evolution in a wild vertebrate host. Below, we detail a little of the evolutionary history and pathogenesis of Mycoplasmas in general and a brief history of the House finch, leading into a more in-depth discussion of the system at large.

4 – Background of the *M. gallisepticum and H. mexicanus* host-pathogen system

Mycoplasma

Mycoplasma spp. belong to a diverse genus of *Mollicutes* class bacteria and are amongst the smallest known prokaryotes in terms of both physical size and genomic content (Maniloff, 1983), with typical cell-diameters of around 0.3μm and genomes ranging from only around 600-1700 kilobases (Razin, 1992). Although modern phylogeny of the taxa suggests an origin for the clade within the Gram-positive bacteria (Thomson *et al*, 2011), they are considerably dissimilar from the typical structural form of this grouping as well as from the Gram-negative bacteria,

lacking entirely a defined cell wall with neither typically prominent peptidoglycan or lipopolysaccharide components and instead possessing a lipid-bilayer membrane with associated lipoproteins (Razin, 1992), in many ways resembling that of typical eukaryotic membrane architectures. By this interesting confluence it should perhaps therefore be unsurprising that by-and-large members of this genus are found in close association as either as parasites or symbiotes of eukaryotes; in fact they appear to be near-ubiquitous across the Metazoa, found in a great number of host-species (Sumithra *et al*, 2013), and have even differentiated within individual hosts down to the level of specific tissue-trophisms – notably between species, for example *M. pneumoniae* and *M. genitalium* infecting *Homo sapiens,* but even down to the within species strain-level in some *Mycoplasma spp.*(Kleven *et al,* 1975). This unusual physiology and wider ecology of Mycoplasmas has since interested microbiologists and clinicians from the discovery of the microorganisms.

First identified at the end of the 19th C. as the aetiological microbe causing bovine peripneumonia (Nocard *et al*, 1898), but misclassified as a fungal infection at the time due to its atypical appearance, Mycoplasmas are commonly associated with disease and, despite a paucity of identifiable “classical” toxins, appear to possess complex immunomodulatory and immunoevasive capabilities though the actions of various lipoproteins and their derivatives (Chopra-Dewasthaly *et al,* 2017; Saeki *et al,* 2018, *but see* Christodoulides *et al*, 2018 for a recent in-depth review). For example, in *Mycoplasma gallisepticum* and other Mycoplasmas infecting poultry antigenic variation caused by the expression of variable length haemagglutinin pMGA / VhlA adhesins by the bacteria mask these major surface epitopes to help to avoid immune detection and clearance and to promote colonisation and cell-invasion by the bacteria (Benčina *et al*, 2001,

Pflaum *et al.* 2018). Upon successful establishment of an infection, Mycoplasmas then cause pathogenic and cytotoxic damage to their hosts through further action of the immunomodulatory lipoproteins and lipopeptides discussed above, and directly from the production of hydrogen peroxide and superoxide radicals generated during bacterial metabolism and growth (Parrott *et al,* 2016). The inflammation caused by these processes then aid in the transmission of the most well-studied Mycoplasmas – for example in *M. pneumoniae* by aerosolisation of the bacteria caused by coughing and sneezing (Brown *et al,* 2016), or by causing conjunctivitis and fomite deposition of the bacteria in *M. gallisepticum* infecting birds (Dhondt *et al,* 2007).

M. gallisepticum is of particular interest as the focus of a large part of this body of work. One of several *Mycoplasma spp.* found in poultry – particularly infecting chickens and turkeys, *M. gallisepticum* causes chronic respiratory disease and sinusitis in these respective hosts, with other symptoms including coughing, sneezing and nasal discharge and, notably, conjunctivitis in chickens (although the latter is considered a minor clinical indicator), with potentially severe and commercially important effects on weight, growth and egg-laying as well as relatively high mortalities of up to 30% in complex outbreaks (Stripkovits *et al,* 1996). Transmission occurs either horizontally between birds through direct contact or via fomites, or vertically, at least in chickens, via invasion through infected tissues into the oviduct and subsequently ova (Levisohn *et al*, 2000). Despite the observed virulence of *M. gallisepticum,* in many cases infections are seen to be sub-clinical or asymptomatic, with the continued circulation of disease attributed to mycoplasma's immunoevasive capabilities, e.g. though antigenic-variation of immunodominant lipoproteins (Bencina, 2002), and as for many other pathogenic *Mycoplasma spp.,* the apparent ability to form biofilms (Feng *et al*,

2018; Raymond *et al,* 2018, McAuliffe *et al*, 2006), enabling more long-term chronic infections in some cases rather than the typically observed acute respiratory disease (Chen *et al*, 2012).

The molecular biology of *M. gallisepticum* pathogenesis having been touched upon briefly above and will further explored throughout the text, but a comprehensive review of *Mycoplasma* virulence is beyond the scope of this introduction and indeed still a rich vein of research across evolutionary, clinical and veterinary sciences. Suffice to say, as with the other Mycoplasmas associated with disease, the mechanisms by which *M. gallisepticum* manifests pathology are diverse, complex and often unique to the genera.

The House Finch (Haemorhous mexicanus)

The House Finch is a small, brightly coloured songbird found throughout N. American ranges through the continental United States and Mexico. Native to the southwestern US and Mexican Sonoran Desert biomes, the species has an interesting story itself and has been utilised as a model organism for studies in population genetics (Wang *et al,* 2003), as well as for understanding colouration, signalling and stress responses in birds (Hill *et al,* 1994, McGraw *et al,* 2000). With males displaying attractive vibrant red and yellow colouration, individuals were commonly traded as pets for much of the 19th and 20th centuries, transplanting the species from its native range to locations throughout the east coast of America and Hawaii. In 1940 federal protection of the species made this trade illegal, and the subsequent release of individuals from dealers in Long Island, New York resulted in the establishment of the first eastern U.S. populations of House finches (Aldrich *et al*, 1978). Spreading rapidly throughout this new range and supplanting a number of other species within its ecological

niche (Wootton, 1987), the House finch soon became a familiar site at bird-feeders throughout the country. As such, the geographical spread and monitoring of *Haemorhous mexicanus* has yielded a great deal of data through citizen science projects such as the Cornell Lab of Ornithology associated Project Feederwatch (www.feederwatch.org) on the species behaviour and ecology, and this has facilitated it's use as a model organism in the aforementioned fields.

The M. gallisepticum – House Finch Epidemic

As populations of *H. mexicanus* continued to grow within this eastern-range, more and more contact between wild House finches and commercial and domesticated species would be expected and subsequently the risk of a disease spill over from one into the other more likely. In the U.S. state of Maryland c.1994 this pathogen host-shift event did indeed occur, with *M. gallisepticum* making the jump from commercial broiler chicken flocks into the previously uninfected House finch (Lay *et al,* 1996, Dhondt *et al*, 1998, Delaney *et al,* 2012) , Fig. 4.. Although a number of studies have identified that all circulating House finch *M. gallisepticum* isolates appear to be descended from a single common ancestor (Tulman *et al,* 2012; Delaney *et al,* 2012), the low competency of chicken-derived isolates subsequently measured in house finches suggests some pre-adaptive mutation of *M. gallisepticum* circulating in chicken hosts to have predisposed the host-shift to occur, indicating that the event cannot just be due to greater contact between host-species alone (Staley *et al,* 2018)..

Fig. 4. (Left Panel): *H. mexicanus* (House Finch) exhibiting severe symptoms of mycoplasmal conjunctivitis. **(Right Panel):** Healthy house finch. Copyright Hill, G. E., amended from Bonneaud *et al*, 2011)

Causing severe conjunctivitis and upper respiratory tract infections and proving highly virulent and contagious within this novel host, *M. gallisepticum* spread rapidly throughout the eastern House finch population, causing a precipitous decline in the number of birds recorded and mortality estimated to be in the tens to hundreds of millions of individuals (Nolan *et al,* 1998, Hochachka *et al,* 2000). Despite this initially high observed virulence, follow up studies showed a decrease in House finch *M. gallisepticum* (HFMG) prevalence in a relatively short space of time. For example in Auburn Alabama some 60% of the House finch population was symptomatic for *M. gallisepticum* infection in late summer 1996, but this had decreased to 9% just two years later in the same season of 1998 (Roberts *et al*, 2001). This shift from an epidemic disease ecology to one resembling more endemicity within the eastern House finch population suggests immediately some degree of host and pathogen adaptation to one another with,

in theory, selection processes acting on both host-resistance and pathogen virulence.

Following the host-shift event, phenotypic selection on physical attributes in the species was evident very quickly from studies of pre- and post-epidemic eastern population birds, with birds surviving infections found to be smaller and, in the case of males more brightly coloured than average (Nolan *et al,* 1998). Interestingly however, a recent large-scale analysis of single-nucleotide-polymorphisms in eastern populations pre and post- epidemic indicated no significant genetic signature within the house finch genome attributable the outbreak (Shultz *et al,* 2016). This finding was in stark contrast to other work which suggests strong diversifying selection on immune-function associated major histocompatibility complex (MHC) loci pre- and post- epidemic (Hawley *et al,* 2012), and the clear emergence of resistance in response to endemic *M. gallisepticum* within a relatively short space of time, evident in the differential immune expression and responses of susceptible western and resistant eastern population birds to infection post-2007 (Bonneaud *et al*, 2011, 2012).

Additionally, recent findings strongly support the hypothesis that tolerance has evolved in *H. mexicanus* in response to the epizootic alongside resistance, with birds from historically exposed populations exhibiting lesser disease symptoms at a given bacterial load than those from unexposed populations, but also clearing *M. gallisepticum* infection at a higher rate due to evolved resistance (Bonneaud *et al,* 2018; Bonneaud *et al,* 2019). As previously discussed, the role of tolerance in pathogen evolution is only starting to be well studied, but it seems likely that continued research in this area will reveal a more complete picture of how real-world coevolutionary processes are likely to occur.

Regarding adaptation of *M. gallisepticum* itself to its novel host in the House finch, previous work has shown that despite the decreasing prevalence of *M. gallisepticum* in house finch populations, the bacteria itself has evolved to have higher virulence on average over time (Hawley *et al,* 2010; Hawley *et al*, 2013, Bonneaud *et al, 2018*), with rapid genomic changes to a number of virulence and regulatory genes, notably including substantial mutations to virulence-associated lipoproteins and. Intriguingly, these studies have also shown that substantial losses of CRISPR (clustered regularly interspaced short palindromic repeat) sequences and associated *cas* genes has occurred in *M. gallisepticum* over the epizootic (Tulman *et al,* 2012; Delaney *et al,* 2012). involved primarily in phage-defence but with potential further ramifications to regulatory pathways (Sorek *et al,* 2008; Bozic *et al,* 2019).

These studies of the co-evolutionary processes between *M. gallisepticum* and *H. mexicanus* have provided some preliminary evidence to support broadly the virulence-transmission trade-off hypothesis. Some previous work has shown that a trade-off does appear to present itself between the virulence, host-recovery and transmission rates of the pathogen in this novel host, with both higher virulence and rates of immune-clearance predictive of faster-transmission between hosts (Williams *et al,* 2014), although firm conclusions from this study are hard to make due to the limited library of *M. gallisepticum* isolates inoculated into birds during the experiment. For evolutionary studies such as this, limiting the number of isolates used can often fail to capture the variation within time points and over large enough time-scales to make strong conclusions as to the evolutionary trajectory of pathogens. It is generally considered that early isolates of pathogens taken after a host-shift can exhibit great variability in their virulence

due to maladaptation to their novel host (Ebert, 1999), and this can be a major bias when using small isolate libraries.

Despite these typical issues of sample size – particularly regarding the number of *M. gallisepticum* isolates used in a normal experiment, such studies are helping to build a large set of data of value in answering some of these key questions in regards to the evolution of pathogen traits and the evolutionary trajectories of emerging infectious diseases, and highlight the applicability of the *M. gallisepticum – H. mexicanus* model system within these fields. Due to the spatial separation of western and eastern House finch populations, and with minimal contact and gene flow between these populations it has been possible to study the responses to infection with various *Mycoplasma* isolates in both "resistant" and "non-resistant" birds from either population, and the large library of *M. gallisepticum* isolates collected from wild House finches over the last 25 years has insights into both host and pathogen adaptation over time. Using such time-shift experiments it has recently been shown that co-evolution between *M. gallisepticum* and *H. mexicanus* has clearly occurred over time in an antagonistic process; *M. gallisepticum* fitness being tied to whether it is inoculated into a "contemporary", "past" or "future" type host and indicating that virulence has evolved adaptively in response to host-resistance (Bonneaud *et al,* 2018). However, despite this relevance and applicability of the system to testing such hypotheses, clearly some contention remains over the extent of adaptation of *M. gallisepticum* to its novel host, and conversely in regard to the evolution of resistance in the House finch and its impacts on the overall co-evolution occurring in the host-pathogen interaction. Over the course of this body of work, we will aim to address many of these issues and intend to showcase the *M. gallisepticum* –

H. mexicanus model system as one of great potential for answering such key questions in host-pathogen evolutionary biology.

Overview of Chapters

Chapter 2 will address two of the underlying assumptions of the trade-off hypothesis; that i) Pathogen virulence will increase with host-resistance over time in a novel host-pathogen interaction following a host-shift and ii) Virulence is underpinned by pathogen-replication – i.e. that the damage a pathogen causes to it's host is proportional to the rate at which is can replicate and ergo the pathogen load in an infection. We utilise a large collection of samples taken during an *in vivo* experimental infection study, with concurrent observational and quantitative data on pathogen virulence to trace both how virulence as a trait overall has evolved over the epidemic and how replication rates, by contrast have changed over the same time-period.

Chapter 3 considers the role of biofilm formation in *M. gallisepticum* infections and its relation to virulence, as well as its own evolution as a trait. This study represents, to this authors knowledge, the first on such a scale considering the evolution of biofilm formation in a novel pathogen-host system directly, and of its discussion within the larger framework of the trade-off hypothesis.

Chapter 4 reports on the results of a transmission study conducted using the *M. gallisepticum – H. mexicanus* system. Here we measure the rate of transmission between experimentally infected birds and secondary sentinel hosts in shared enclosures in order to reveal whether transmission rate has changed over the course of the epidemic and if, as expected under the virulence-transmission

trade-off hypothesis, higher virulence isolates of *M. gallisepticum* will transmit faster than those manifesting lower virulence *in vivo*.

Chapter 5 concludes the body of this thesis, with a brief exploration of the effects of host-resistance on transmission and virulence in a secondary host. The large-scale population structure of *H. mexicanus,* containing populations which have had long-term exposure (and evolved resistance) to *M. gallisepticum*, and others which have never had circulating *M. gallisepticum*, allow us to test whether passage through either resistant or non-resistant primary hosts immediately affects infectivity and virulence in secondary hosts following transmission.

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Chapter 2: Host resistance increases virulence but not replication rates in an emerging pathogen

Abstract

There is growing evidence that antibiotic treatment selects for pathogens that are more harmful to hosts and hence more virulent. Yet how virulence evolves in response to host resistance in natural populations, and by which mechanism, remains unclear. The virulence-transmission trade-off hypothesis posits that any decrease in within-host persistence, for example through host immune clearance, should favour faster-transmitting pathogens, with increased virulence assumed to arise from the increased replication rates required for faster transmission. Here we use the natural outbreak of the bacterial pathogen, *Mycoplasma gallisepticum*, in house finches (*Haemorhous mexicanus*) from the United States, in a rare test of the consequences of host resistance evolution for the evolution of both pathogen virulence and replication rates, as well as the role of replication rates in shaping virulence. We inoculated non-resistant finches from disease-unexposed populations with 55 isolates collected over the 20 years of the epidemic. We show that replication rates increased from outbreak through to the spread of resistance but reached a plateau after hosts had become resistant. In contrast, virulence (measured as body mass loss and symptom severity) increased linearly throughout the epidemic. In addition, we found a significant association between replication rates and symptom severity only when considering isolates sampled before the spread of resistance. Taken together, our results suggest that replication rates do not underpin virulence and that virulence and replication rates may be under independent selection as a result of host resistance. Further, they suggest that replication rates may be a strong

predictor of pathogen virulence at the start of an epidemic, while traits such as immune manipulation subsequently shaping virulence once hosts have become resistant.

Introduction

Understanding the evolution of pathogen virulence in response to host resistance is central not only to managing pathogenesis, but also to predicting the long-term efficiency of our therapeutic approaches (Boots, 2015, Read et al., 2015, Restif, 2009). There is growing evidence that host resistance, either through host immunity (natural or vaccine-prompted) or antibiotic treatment, selects for pathogens that are more virulent (i.e. cause more harm to hosts) (Barclay et al., 2012, Gandon et al., 2001, Mackinnon and Read, 2004a). Yet the underlying mechanisms of virulence evolution remain to be clarified. The classic prediction is that host resistance will select for increased replication rates that will enable the pathogen to transmit more quickly and escape before it is cleared (Porco et al., 2005). Increased virulence then arises because intensified pathogen growth accelerates host mortality (de Roode et al., 2008, Doumayrou et al., 2013, Fraser et al., 2007). Despite theoretical predictions of an association between virulence and replication rates (Alizon et al., 2009, Alizon and Michalakis, 2015), empirical evidence is surprisingly limited (Ebert, 1994, Mackinnon and Read, 2004c, Dwyer et al., 1990). Resolving the role of variation in replication rates in shaping variation in virulence will, however, require experimental contrasts to be made across a large number of isolates of a given pathogen that differ in virulence.

Assumptions of virulence differences shaped by variation in pathogen growth are based largely on experimental infections of mice with the rodent malaria model *Plasmodium chabaudi*. Such studies have shown a significant positive

association between parasite multiplication rates and measures of both morbidity (anemia and weight loss) and mortality (Ferguson et al., 2003, Mackinnon and Read, 1999a, Mackinnon and Read, 1999b, Mackinnon and Read, 2004c, Timms et al., 2001). Consistent findings emerge from correlational studies of *P. falciparum*, *P. vivax* and *P. malariae* and *P. ovale* in humans, with asexual parasitemia found to be positively associated with virulence (McKenzie et al., 2001, McKenzie et al., 2002, Simpson et al., 2002, Collins and Jeffery, 2002); reviewed in (Mackinnon and Read, 2004b). In contrast, correlations made across studies failed to detect an effect of pathogen load in explaining virulence difference among a range of diseases in both humans and plants (Froissart et al., 2010, Leggett et al., 2017). Yet, whether this is due to the fact that virulence differences are driven by other processes than replication rates, or to the difficulty of comparing symptoms and measures of pathogen load across studies of different pathogens, remains to be determined.

The epidemic of the bacterium, *Mycoplasma gallisepticum*, in house finches (*Haemorhous mexicanus*) from the eastern US offers a rare opportunity to test the role of replication rates in shaping changes in virulence among isolates of a same pathogen, in response to the spread of host resistance. *M. gallisepticum* first emerged in house finches after a single host shift from poultry, giving rise to a severe conjunctivitis outbreak that killed millions and selected for genetic resistance in hosts in only 12 years (Bonneaud *et at,* 2011; Bonneaud *et al*, 2012). Indeed, while finches from exposed and unexposed populations initially displayed equivalent responses to experimental inoculation, these responses subsequently diverged as the former evolved the ability to resist pathogen-induced immune-suppression and mount a protective cell-mediated immune response (Bonneaud *et al*, 2012). Using a time-shift experimental design, we

recently showed that virulence evolved adaptively in response, with the severity of eye swelling in symptomatic host increasing significantly as isolates were sampled later in the epidemic.

Here we experimentally contrast virulence and growth patterns among 55 *M. gallisepticum* isolates collected throughout the epidemic and known to differ in their level of virulence. To control for confounding effects of host resistance on measures of virulence, we inoculated isolates to house finches from populations that have never been exposed to the pathogen and hence are unable to mount a protective immune response. We previously showed that isolates collected after the spread of resistance were three times more likely to cause host mortality than isolates collected before the spread of resistance (Bonneaud *et al,* 2018). We now further quantify differences in virulence as the amount of eye swelling and mass loss throughout the infection. To quantify bacterial load, we develop a new qPCR methodology that allows us to precisely estimate the number of cell copies of both the host and the pathogen that we obtained in ocular/tracheal swabs. We then test whether changes in virulence over the course of the epidemic were accompanied by parallel changes in pathogen growth, and whether any potential association between virulence and replication differs before and after the spread of host resistance.

Results

Virulence

We found that the severity eye swelling differed as a function of the year of pathogen sampling, with later isolates causing significantly greater eye swelling than earlier isolates (linear mixed model; year effect: estimate $\pm$ se = 1.4 $\pm$ 0.4,

χ^2 = 21.3, p <0.002; Figure 1). Birds lost more body mass when inoculated with isolates sampled later in the epidemic (linear mixed model; year effect: estimate ± se = -0.05± 0.02, χ^2 = 10.4, df = 1, p = 0.001; Figure 2), independently of their body mass at inoculation (year × initial mass interaction effect: χ^2 = 2.1, df = 1, p = 0.15).

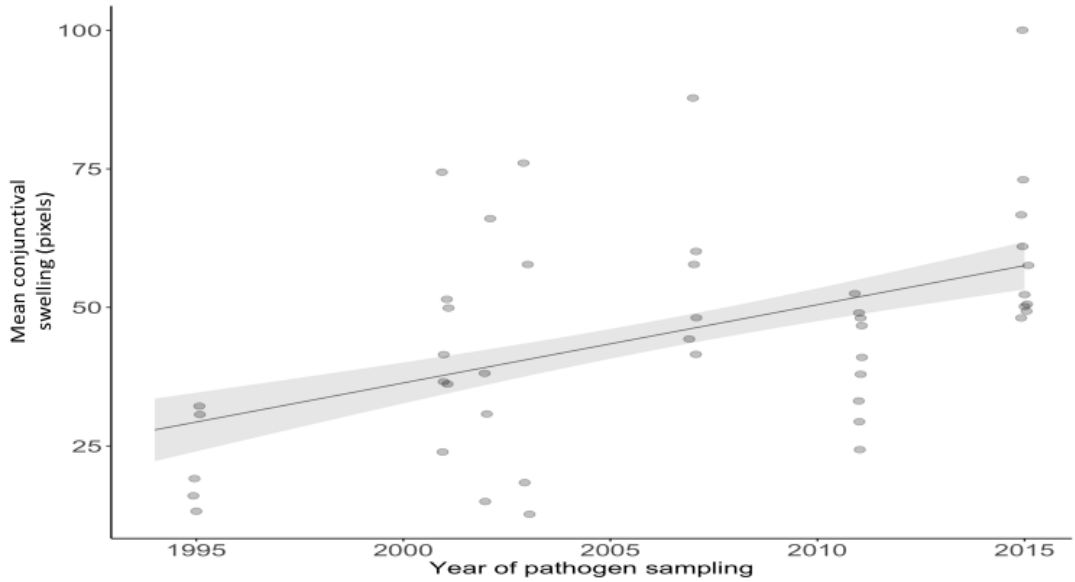

Figure 1. Change in eye swelling severity over time. Quantitative measure of eye swelling as a function of the year of pathogen sampling. Points represent raw values; the line is predicted from the model with the standard error represented by the ribbon.

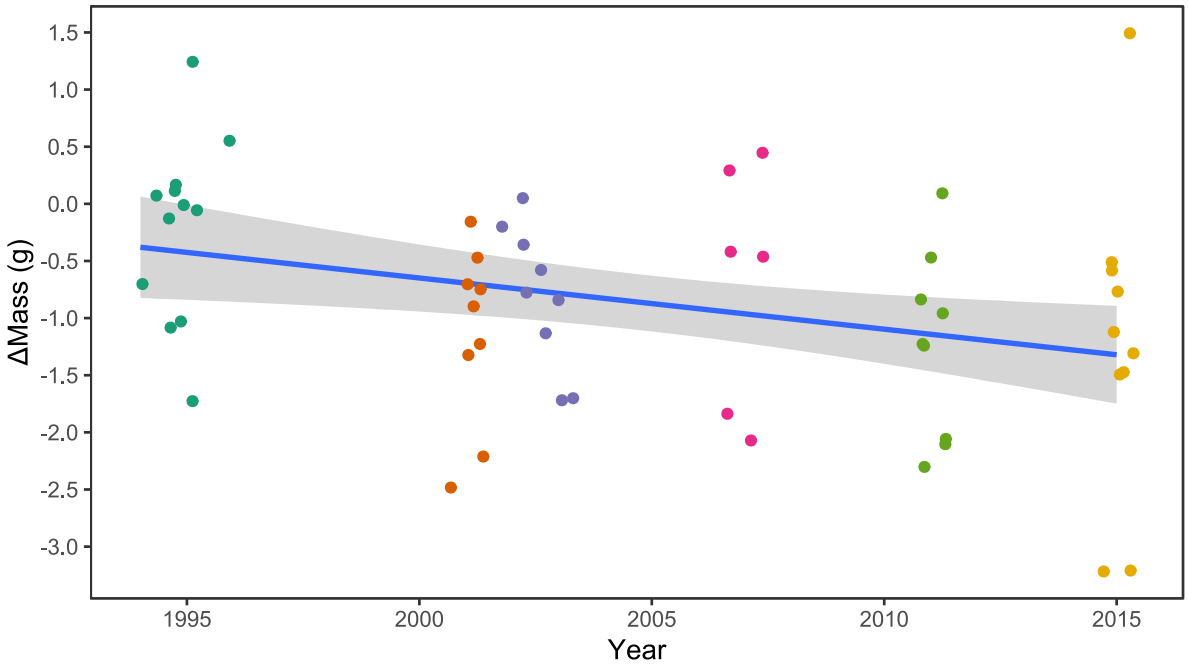

Figure 2. Change in mass loss due to infection over time. Body mass change as a function of the year of pathogen sampling. Points represent raw values, line is predicted from the model with standard error represented by ribbon.

Bacterial load

Overall, there was a significant quadratic relationship between peak bacterial load at the site of infection and year of pathogen sampling (linear mixed model; year effect: estimate ± se = 0.1 ± 0.03, χ^2 = 25.4, df = 1, p < 0.0001; year^2 effect: estimate ± se = 7.7 ± 1.3, χ^2 = 5.8, df = 1, p = 0.016). Indeed, bacterial load increased with year of pathogen sampling for isolates collected between 1994 and 2007 (pre-resistance), but then plateaued for isolates obtained after 2007 (i.e. post-resistance) (Figure 3). As a result, while bacterial load differed significantly over time among isolates sampled before the spread of host resistance (linear mixed model; year effect: estimate ± se = 0.2 ± 0.07, χ^2 = 6.9, df = 1, p = 0.009), isolates sampled after the spread of host resistance achieved

similar bacterial loads over the course of the infection (linear mixed model; year effect: estimate ± se = -0.007 ± 0.05, χ^2 = 0.02, df = 1, p = 0.89).

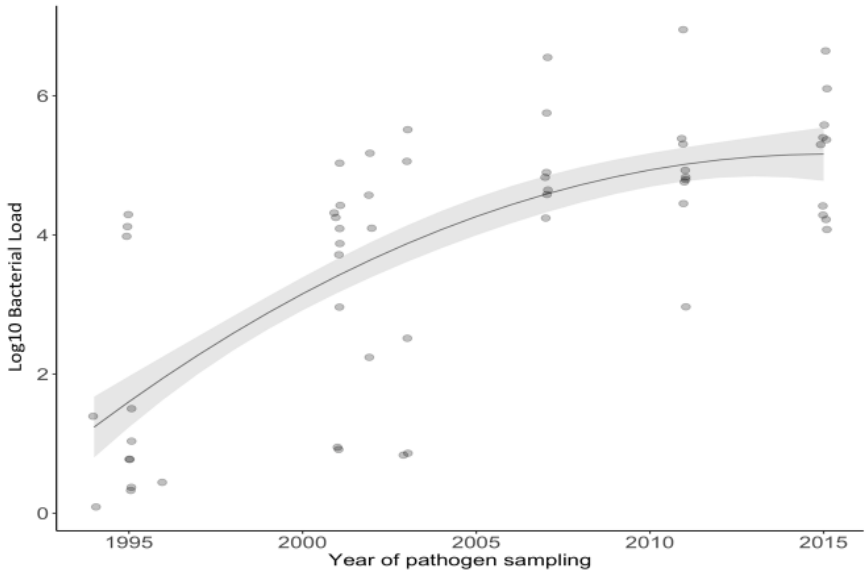

Figure 3. Change in replication rates of *M. gallisepticum* isolates sampled over time. Replication rates increase with time but exhibit a curvilinear trend, plateauing later in the epizootic.

Association between virulence and bacterial load

There was a significant effect of year of pathogen sampling on the association between bacterial load and eye swelling (linear mixed model; year × bacterial load interaction effect: χ^2 = 12.7, df = 6, p = 0.049). The association between bacterial load and eye swelling differed significantly between isolates sampled before vs. after the spread of host resistance (linear mixed model; sampling period × bacterial load interaction effect: χ^2 = 32.5, df = 1, p < 0.0001; Figure 4). Indeed, while there was a positive association between bacterial load and eye

swelling among isolates sampled before the spread of resistance (linear model; bacterial load effect: estimate ± se = 8.2 ± 2.6, $t_{1,18}$= 3.1, p < 0.006), this association was lost when considering isolates sampled after the spread of resistance only (linear model; bacterial load effect: estimate ± se = -0.8 ± 3.8, $t_{1,24}$= -0.2, p = 0.83). Results were qualitatively similar for mass change over the course of the experiment: there was a significant effect of year of pathogen sampling on the association between bacterial load and mass change (linear mixed model; year × bacterial load interaction effect: χ^2 = 32.8, df = 6, p < 0.0001). While there was a negative association between bacterial load and mass change in isolates sampled before the spread of host resistance, this association disappeared when considering isolates sampled post-resistance (linear mixed model; sampling period × bacterial load interaction effect: χ^2 = 31.0, df = 1, p < 0.0001).

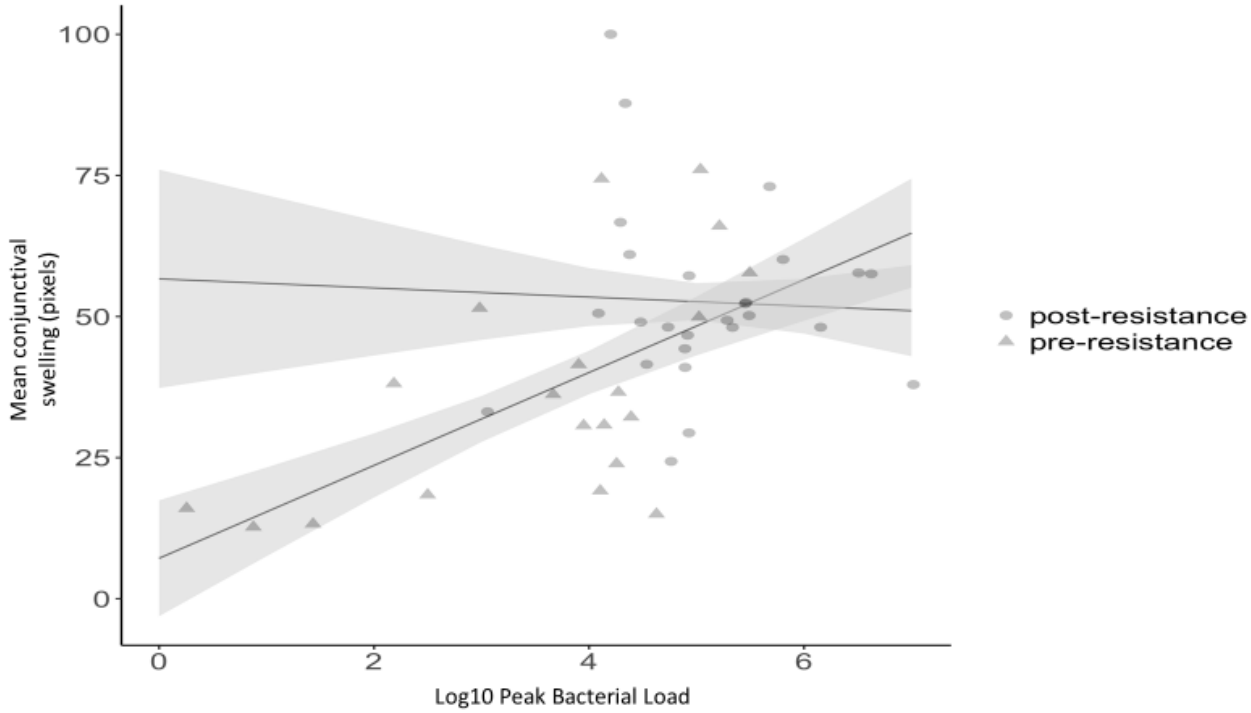

Fig. 4. The association between bacterial replication and virulence. For pre-resistance isolates, virulence increases linearly with replication rate, whereas for post-resistance isolates no such significant association is evident.

Discussion

Our results show that eye swelling and mass loss increased positively over the course of the epidemic, with isolates sampled after the spread of host resistance displaying significantly more severe eye swelling and greater mass loss than isolates sampled before the spread of resistance. In contrast, pathogen load displayed a quadratic relationship with year of isolate sampling, such that replication rates increased linearly from outbreak (1994) to the spread of resistance (2007), but subsequently plateaued after the spread of host resistance (2007-2015). In addition, we found significant associations between our measures of virulence, eye swelling and mass change, and pathogen load, but only in isolates sampled before the spread of host resistance. Indeed, eye

swelling and mass loss increased with increasing pathogen load in pre-resistance isolates, but these associations were lost in post-resistance isolates. Taken together, our results suggest that increases in pathogen virulence in response to host resistance are unlikely to have been mediated by increases in replication rates and that, following the spread of resistance, replication rates might have been under stabilizing selection.

It is important to note that our lack of support for an association between virulence and replication rates in this system does not, however, bring into question the value of the virulence-transmission trade-off for understanding and predicting virulence evolution. At the heart of the trade-off hypothesis is the idea that increases in pathogen fitness (R0), which represents the number of secondary infections arising from a single infected host, can be achieved by accelerating between-host transmission or by lengthening the duration of infection within the host, but not both (Alizon & Michalakis, 2015, Anderson & May, 1982, Ewald, 1983). This is because greater transmission rates require greater exploitation of the host and hence greater virulence (Anderson & May, 1982, Ewald, 1983). While models of virulence evolution have used changes in replication rates as the mechanism underlying differences in resistance, a positive correlation between virulence and transmission (or a negative one between infection duration and transmission) can still arise even if virulence is mediated by processes other than changes in pathogen growth.

M. gallisepticum pathogenesis is primarily dictated by the bacterium's ability to bind host tissues and to evade and manipulate the host immune system (Szczepanek *et al.*, 2010a, Szczepanek *et al.*, 2010b, Staley & Bonneaud, 2015, Chambaud *et al.,* 1999). Colonization occurs via the mucosal surfaces of the conjunctiva and upper respiratory track. This process first requires that *M.*

gallisepticum erodes/disrupts the mucosal barrier and epithelium by manipulating the host immune system into mounting a misdirected inflammatory response (d'Hauteville *et al.,* 2002, Hornef *et al.,* 2002, Szczepanek *et al.,* 2010a, Szczepanek et al., 2010b, Ganapathy & Bradbury, 2003, Gaunson *et al.*, 2000, Gaunson *et al.*, 2006, Lam & DaMassa, 2000), and that the bacterium then attaches to host tissues, by instance by binding to proteins of extracellular matrix (May et al., 2006). For example, chickens experimentally inoculated with a virulent strain of *M. gallisepticum* (Rlow) displayed a greater up-regulation of pro-inflammatory cytokines, which are responsible for local and systemic inflammation and can give rise to tissue destruction and local necrosis, than chickens inoculated with a more attenuated strain (GT5) (Mohammed *et al,* 2007). The subsequent persistence of *M. gallisepticum* then depends on the bacterium's ability to evade and suppress other component of immunity known to play a role in controlling *M. gallisepticum* infection (Gaunson *et al,* 2006). Chickens infected with *M. gallisepticum* displayed lower T-cell activity 2 weeks post-inoculation than controls (Ganapathy and Bradbury, 2003, Gaunson et al., 2000), as well as lower humoral responses against other pathogens *(Matsuo et al.*, 1978, Naylor *et al.*, 1992). This is consistent with the finding that, following experimental inoculation, house finches from exposed populations that had evolved resistance were able to up-regulate the expression of genes associated with acquired immunity (cell-mediated immunity), whereas finches from unexposed/susceptible populations instead displayed evidence of a suppression of protective immune processes (Bonneaud *et al*., 2011, Bonneaud *et al.,* 2012). Our results of increase virulence, but not of increased pathogen growth, therefore suggest that host resistance selected for immune escape/manipulation processes rather than increased replication rates.

The lack of association between measures of virulence (eye swelling and mass loss) and pathogen load in post-resistance isolates suggest that, following the spread of host resistance, pathogen replication rates were in fact under selection not to increase. Evolutionary stasis may occur if replication rates were already optimized for the post-resistance host environment. Alternatively, stasis may result from evolutionary constraints if there was an evolutionary trade-off between replication rates and the trait underlying increased virulence after the spread of host resistance (for e.g. immune avoidance/manipulation mechanisms). Further work will be required to better understand the links between different pathogen traits and how they evolve in a changing host environment.

Finally, our results support some of the conclusions drawn from the study of the myxomatosis outbreak that was triggered in an attempt to eradicate European rabbits from Australia in the 1950s. Soon after the release of highly virulent isolates of the virus, over 95% of the rabbit population are thought to have died. The eradication attempt was, however, unsuccessful because rabbits evolved genetic resistance from standing variation within a few years only. Although viral virulence initially dropped, the spread of host resistance was found to be followed by a rise in the frequency of more virulent viral isolates, leading to the same pattern of increased virulence in response to host resistance that we find in this study. It is important to note that while the myxoma and the *M. gallisepticum* outbreaks differ in the direction of virulence evolution right at disease outbreak (i.e. with virulence decreasing initially in the former but increasing in the latter), both patterns of virulence evolution remain consistent with theoretical predictions. Indeed, in the case of the myxoma outbreak, extremely virulent viral isolates were repeatedly introduced in the rabbit

population until they finally succeeded in sparking off an epidemic. However, given the disproportionality high level of virulence of the virus and the very high susceptibility of the rabbit population, there would have been strong selection initially for reduced virulence, at least until resistance spread in the rabbits. A similar scenario would have occurred when *M. gallisepticum* reached western US house finch populations after having circulated within eastern US finch populations for at least 6 years. The rapid increase in virulence found in our study suggest that the isolates that would have reached the unexposed/susceptible western populations would have been more virulent than those found at outbreak in eastern populations, such that there would have been strong selection for an initial decrease in virulence. While the specificities of different systems/populations mean that virulence evolution will not necessarily follow the same evolutionary trajectory right at outbreak, the concordance of patterns of virulence evolution in response to host resistance found in both the myxoma and the *M. gallisepticum* systems suggest that virulence evolution is predictable when pathogens are faced with increasingly resistant hosts and that virulence will increase over time in such circumstances.

Methods

Capture and housing

Wild house finches from populations that have never been exposed to *M. gallisepticum* were captured in Arizona in the summer 2015. Birds (N = 118, 64 males and 54 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 µL 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 assay (Luttrell et al., 1996), and a lack of current infection was verified using the choanal swabs in PCR amplification of *M. gallisepticum* DNA (Roberts et al., 2001). This was expected as *M. gallisepticum* has never been documented in the area of Arizona in which sampling was conducted (Staley et al., 2018). We used 53 individuals in another experiment, and all remaining 118 birds 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.

Experimental inoculation

One bird randomly selected from each of the 59 pairs was inoculated with 1 of 56 *M. galliseptum* isolates sampled over the course of the epidemic. Isolates were administered via 20 µL of culture containing 1×10^4 to 1×10^6 color changing units/mL of *M. galliseptum* in both eyes. None of the isolates had been passaged in culture more than 3 times (Papazisi et al., 2002). The second bird of each pair remained uninfected and used in another study. We monitored the development of clinical symptoms in the experimentally-inoculated bird by visually scoring eye lesion severity at both eyes (0–5 scale; (Kollias et al., 2004)) at 3, 6, 8, 14, 21, 25, 28 and 34 days post-infection (dpi). For those birds that developed symptoms, we photographed the right and left eyes at 0, 6, 13 and 25 days post-inoculation (dpi). We then quantified the area of the conjunctiva swelling at each day as the area of the outer ring minus the area of the inner ring (see Staley et al 2017). Measurements of photographs were done blindly with respect to the isolate inoculated. Mean eye swelling severity was then determined as the mean value reached by the experimentally inoculated bird over the course of the experiment. All birds were weighed (±0.01 g) at the start and at the end of the experiment using a top-pan balance. The experiment was stopped at 35 dpi and all birds were euthanized as stipulated by home office licencing. Protocols were approved by Institutional Animal Care and Use Committees (IACUC) of Auburn University (permit # PRN 2015-2721) and Arizona State University (permit #15-1438R), and by Institutional Biological Use Authorizations to Auburn University (# BUA 500).

Bacterial load

No significant effect of any variation in inoculum dose was found on pathogen load or conjunctival swelling at any time-point subsequently sampled in this

experiment (Fig. 6 and Fig.7.). Furthermore, we investigated the appropriateness of our measure of pathogen replication rate by comparing it to pathogen load determined at each sampling time point (Fig.8).

We re-designed and optimised a qPCR assay for the single-copy *M. gallisepticum mgc2* and *H. mexicanus rag1* genes(Grodio et al., 2008), with the aim of improving amplification specificity and efficiency. Notably, our assay includes: (1) shorter amplicons for faster amplification; (2) primers and probes designed for near identical thermodynamics between *mgc2* and *rag1* in order to promote high efficiencies in multiplex reactions; (3) lower background signal due to refined fluorophores and quenching. We designed oligonucleotide probes and primers from consensus assemblies of *M. gallisepticum mgc2* gene sequences (Genbank IDs: CP003513.1, CP003512.1, CP003511.1, CP003508.1, CP003507.1, CP003509.1, CP003506.1, CP003510.1) and of *H. mexicanus* (and other closely related *Haemorhous*/*Carpodacus* species) *rag1* gene sequences (Genbank IDs: EU165349.1, EU165350.1, KJ455991.1, KJ455990.1, KJ455989.1, KJ455986.1, KJ455985.1, KJ455992.1, KJ455988.1), using Geneious™R8 v.8.1.8 (Kearse et al., 2012) and Primer3 (Untergasser et al., 2012), and checked for specificity with Primer BLAST (Ye et al., 2012) (see Table 1 for sequences and details of oligos). Standard curves for both *mgc2* and *rag1* amplicons were produced by cloning of approx. 600bp gene fragments from *M. gallisepticum* strain R_{Low} and from an uninfected Arizona population control bird respectively into separate pCR™2.1 plasmid vectors in *Escherichia coli*, using an Invitrogen™ TA Cloning™ Kit according to manufacturer standard protocols. Plasmids containing target sequences were validated by restriction endonuclease analysis and checked for specific binding of internal primers by PCR and gel electrophoresis. 10-fold dilution series of plasmids containing either *mgc2* or *rag1* target sequences were

quantified using a Qubit™ dsDNA HS Assay Kit, and their accuracies and efficiencies further verified by qPCR as individual and multiplexed reactions. The final range of standards used in experiments was approx. $1.6x10^8 – 1.6x10^3$ *mgc2* target copies and $8.0x10^7 – 8.0x10^2$ *rag1* target copies (Fig. 5). Limits of detection and quantification were determined by extinction dilution of standards (Forootan et al., 2017), with limits of quantification set to a threshold coefficient of variation (CV) of 35%, representing absolute values of 28 target copies for *mgc2* and 18 target copies for *rag1*.

Further validation of plasmid standards was made by comparison of amplification efficiencies between plasmid DNA and genomic DNA template serial dilutions, as well as with genomic DNA spiked with known quantities of plasmid DNA. First, we calculated the cycle-based efficiency of plasmid and genomic DNA template serial dilutions. StepOne™ Software v2.3 (ABI) reported typical efficiency values of 92.7% and 91.7% respectively for *rag1* amplification from plasmid DNA and genomic DNA templates and 96.4% and 94.3% respectively for *mgc2* amplification from plasmid DNA and genomic DNA templates. Additionally, LinRegPCR calculated mean efficiencies (across multiple runs and replicates) for *mgc2* from genomic DNA at 83.3% (n=1137), for *mgc2* from plasmid DNA at 83.2% (n=128), for *rag1* genomic DNA at 85.3% (n=1137) and for *rag1* from plasmid at 85.2% (n=128). N.B. LinRegPCR typically produces lower efficiency values than other methods due to differences in efficiency calculations and the greater variation in efficiencies observed when measuring reactions independently. Second, we conducted genomic DNA and plasmid spike tests. Amplification efficiencies of *mgc2* and *rag1* from either plasmid or genomic DNA was further verified by spiking dilutions of genomic DNA with known quantities of plasmid DNA targets. 10-fold dilutions of house finch or

M. gallisepticum genomic DNA were measured independently and when spiked with known quantities of pCR2.1-rag1 or -mgc2 plasmid (827 *rag1* and 1709 *mgc2* copies respectively). Accuracy was determined by linear regression of observed copy number against expected copy number (genomic DNA + plasmid) in each reaction. *rag1* amplification from genomic DNA spiked with plasmid DNA observed *vs*. expected copy number coefficient = 1.03 $\pm$ 0.04, *mgc2* amplification from genomic DNA spiked with plasmid DNA observed *vs*. expected copy number coefficient = 1.00 $\pm$ 0.006, indicating that amplification efficiencies are analogous between plasmid or genomic DNA templates.

Bacterial load was measured from conjunctival and tracheal swabs obtained at 8, 14, 21 and 28 dpi by quantifying the number of *M. gallisepticum mgc2* gene copies and the number of house finch *rag1* gene copies using a re-designed qPCR assay (Grodio et al., 2008) (Table 2 and Supplementary Methods for assay design and validation). The gene *mgc2* encodes a cytadhesin protein and is present at one copy/*M. gallisepticum* genome, while the recombination-activating gene *rag*1 is present in two copies/diploid house finch cell (Grodio et al., 2008). Pathogen load is then determined as the number of *M. gallisepticum* cells divided by the number of house finch cells to control for variation in sampling efficiency (Grodio et al., 2008). DNA was extracted using a QIAGEN DNeasy® Blood and Tissue Kit according to the manufacturer's standard protocols. Multiplex qPCRs for *mgc2* and *rag1* were then conducted using an Applied Biosystems™ StepOnePlus™ Real-Time PCR system. Sample concentrations were determined by comparison to standard curves of both *mgc2* and *rag1* amplicons. These amplicons were produced by cloning of approx. 600bp gene fragments into separate pCR™2.1 plasmid vectors in *Escherichia coli*, using an Invitrogen™ TA Cloning™ Kit according to manufacturer standard protocols. The final range

of standards used was approx. $1.6x10^8$ – $1.6x10^3$ *mgc2* target copies and $8.0x10^7$ – $8.0x10^2$ *rag1* target copies. Each multiplex qPCR reaction was run in a final volume of 20 µl and contained: 2µl of either plasmid standard or sample genomic DNA template, 1 µl each of 10 µM mgc110-F/R and rag1-102-F/R primers (total 4ul; Table 2), 0.5 µl each of 10 µM Mgc110-JOE and Rag1-102-FAM fluorescent hydrolysis probes (total 1 µl), 10 µl of 2X qPCRBIO Probe Mix HI-ROX (PCR BIOSYSTEMS) and 3 µl Nuclease-free water (Ambion®). Cycling conditions were as follows: 95°C for 3 minutes for initial denaturing of template DNA, followed by 45 cycles of 95°C for 1 second and 60°C for 20 seconds for primer and probe binding and amplification of target DNA. Samples and standards were measured in duplicate in each run with a negative control of elution buffer. All data was exported to LinRegPCR v.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 normalised using Factor qPCR v.2016.0 (Ruijter et al., 2015), with standard series presented as between-run replicates.

Statistical analyses

All statistical analyses were conducted in R 3.3.2 (Team, 2016) using lme4 (Bates et al., 2015), and figures were made using ggplot2 (Wickham, 2009). Pathogen load data were log-transformed to fit a normal distribution. To determine how virulence and replication rates evolved over the course of the epidemic, we ran linear mixed effects models, with year of pathogen sampling (year) as the explanatory term and pathogen isolate as the random effect. We included either mean eye swelling, body mass at the end of the experiment (i.e. 34 dpi), or peak pathogen load as the response term. For changes in body mass, we included

initial body mass at inoculation in interaction with year as explanatory terms. To determine how virulence and replication rates differ between isolates sampled before and after the spread of host resistance, we ran the same 3 models but this time including the sampling period of the pathogen (i.e. pre- or post-resistance) as the explanatory term. We tested for differences in virulence over the course of the infection using linear mixed effect models that included either eye swelling or body mass were as the response variable, the day of the infection (dpi) and either the year of pathogen sampling (year) or the pathogen sampling period (pre- *vs*. post-resistance) and their interactions as the explanatory terms, and with pathogen isolate as the random term. Body mass at inoculation was included in the model of body mass change. Tests of the association between virulence and pathogen load were run as linear mixed effect models with pathogen load and either the year of pathogen sampling (year) or the pathogen sampling period (pre- *vs*. post-resistance) and their interaction as the explanatory terms, and with pathogen isolate as the random term. We included either mean eye swelling or body mass change between inoculation and 34 dpi as the response variable.

Name	Sequence	Target	Use
Rag1-608-F	5’-TCATCCTGCTGTCTGTCTGG-3’	608bp fragment of *rag1* from *H.mexicanus* genomic DNA	Construction of pCR™2.1 – *rag1* plasmid for standard curves
Rag1-608-R	5’-GATCCGATTCATCAGCCAGC-3’		
Rag1-102-F	5’-GCCCTCCTACCAGGTTATCA-3’	Internal sequence of pCR™2.1-*rag1* plasmid	Amplification of *rag1* target for probe binding in qPCR
Rag1-102-R	5’-TGGCAGTCCTGATAGTCCAT-3’		
Rag1-102-FAM	5’-[6FAM]-TTGAGTGGAAACCTC[+C][+C][+C]TGA-[BHQ1]-3’	Internal sequence of Rag1-102 amplicon	Quantification of Rag1-102 amplicon in multiplex qPCR
Mgc2-597-F	5’-GGTGCTGGGTTGATTGTTGT-3’	597bp fragment of *mgc2* from genomic *M. gallisepticum* DNA	Construction of pCR™2.1 – *mgc2* plasmid for standard curves
Mgc2-597-R	5’-GTGATTAAACCCACCTCCAGC-3’		
Mgc110-F	5’-AATGCCACCAAGACCAAACT-3’	Internal sequence of pCR™2.1-*mgc2* plasmid	Amplification of *mgc2* target for probe binding in qPCR
Mgc110-R	5’-CAGCTTTATTTCCCATCGGC-3’		
Mgc110-JOE	5’-[JOE]-[+A]ACCAAGACCAGGTTTC[+A]GAC-[BHQ1]-3’	Internal sequence of Mgc2-110 amplicon	Quantification of Mgc2-110 amplicon in multiplex qPCR

Table 1: Primers and probes used in the quantification of bacterial load. 6FAM/JOE- fluorophore moieties for probes. BHQ1 – Black-hole quencher 1. [+N] – modified LNA base.

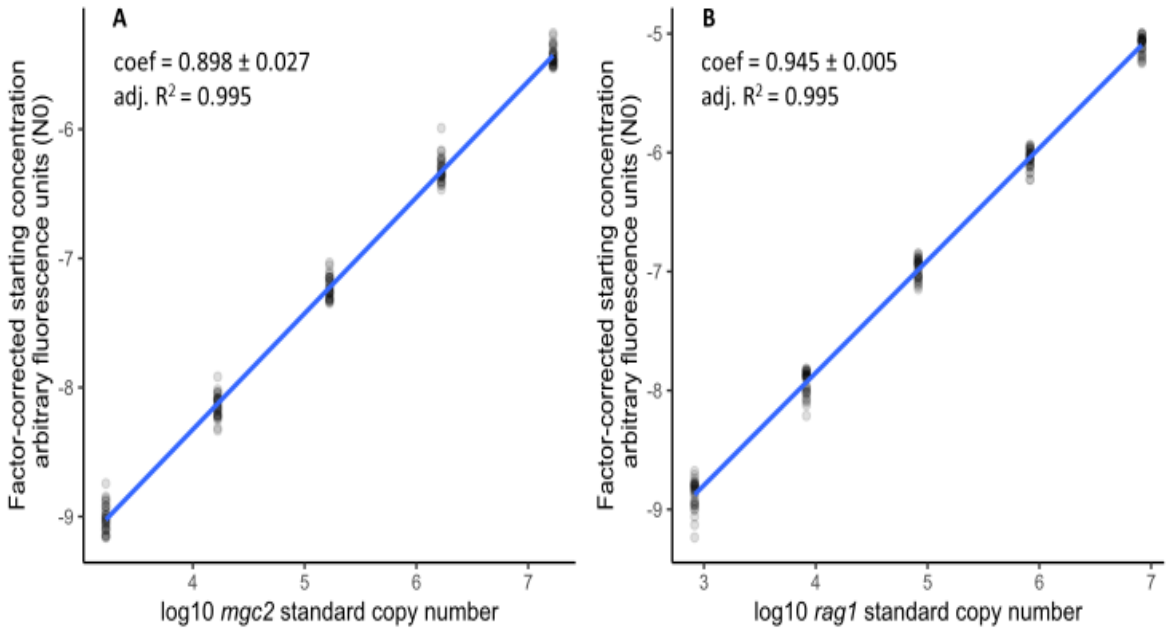

Figure 5. Linearity of **(A)** *mgc2* and **(B)** *rag1* standard curves from linear regression of standard dilution series (n=32) copy number against factor-corrected starting concentrations calculated using LinRegPCR.

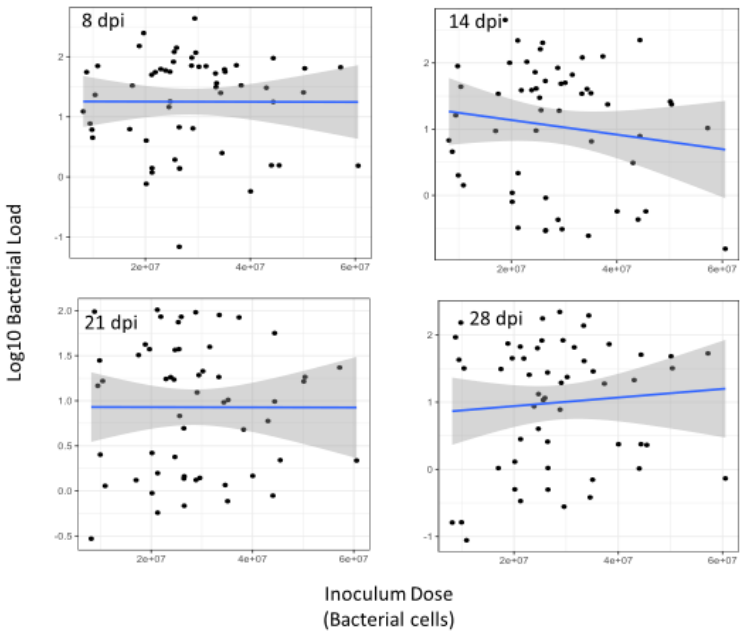

Fig. 6. The effect of inoculum dose size on measures of bacterial load. Top row – 8 dpi, 14, dpi. Bottom row – 21 dpi, 28 dpi. Dose size effect was non-significant at every time point. Linear regressions of log10 Bacterial Load ~ Inoculum size, -1.78e-10 +/- 8.95e-09 (8 dpi), -1.10e-08 +/- 1.07e-08 (14 dpi), -9.20e-11 +/- 8.16e-09 (21 dpi), 6.34e-09 +/- 1.06e-08 (28 dpi).

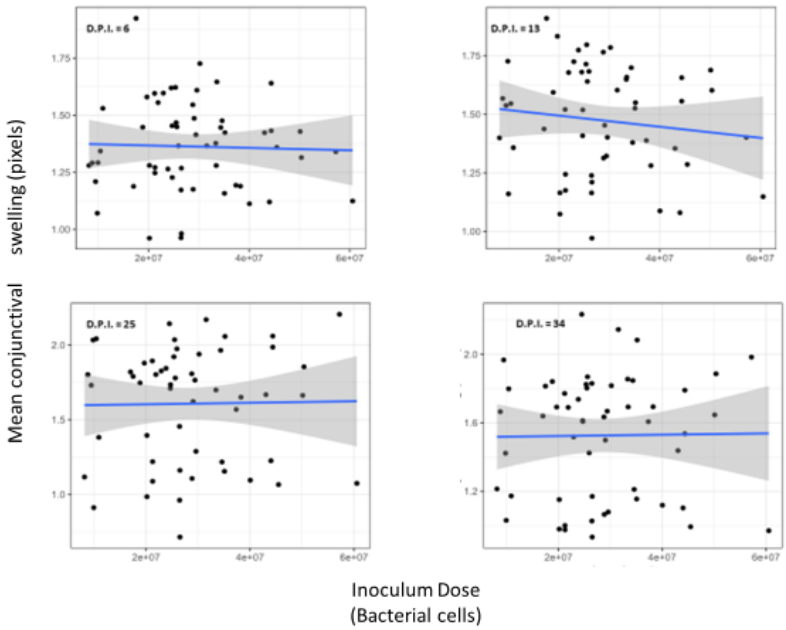

Fig. 7. The effect of inoculum dose size on measures of virulence (as conjunctival swelling). Top row – 6 dpi, 13, dpi. Bottom row – 25 dpi, 34 dpi. Dose size effect was non-significant at every time point. Linear regressions of log10 conjunctival swelling (pixels) ~ Inoculum size, coef = -5.24e-10 +/- 2.25e-09 (6 dpi), -2.37e-09 +/- 2.59e-09 (13 dpi), 5.02e-10 +/- 4.34e-09 (25 dpi), 3.82e-10 +/- 4.04e-09 (34 dpi).

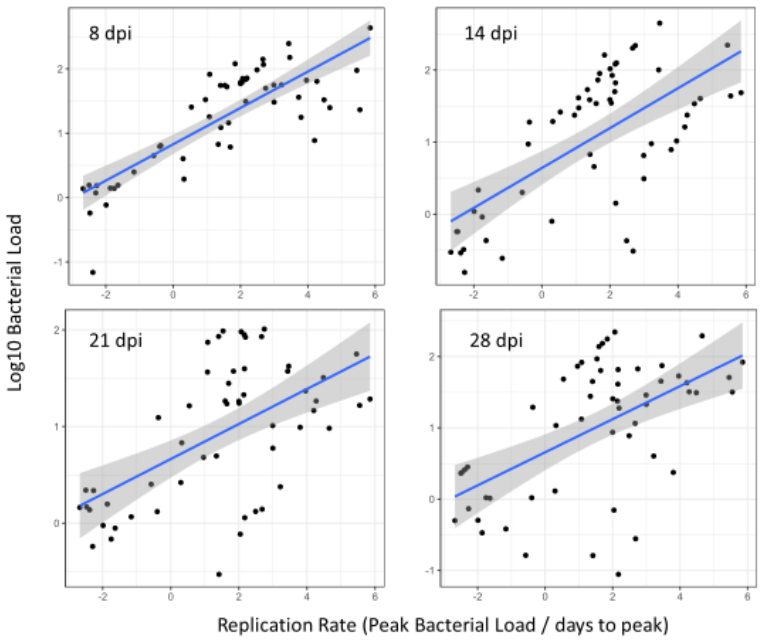

Fig. 8. The association of replication rate to bacterial load measured at 8, 14, 21 and 28 dpi. Replication rate is highly predictive of bacterial load at all time points. coef = 0.28 +/- 0.028, $F_{1, 54}$ = 102.8, p = $4.18e^{-14}$ (8 dpi), coef = 0.28 +/- 0.043, $F_{1, 53}$ = 40.72, p = $4.45e^{-8}$ (14 dpi), coef = 0.18 +/- 0.36, $F_{1, 53}$ = 25.61, p = $5.36e^{-6}$ (21 dpi), coef = 0.0041 +/- 0.0019, $F_{1, 53}$ = 4.83, p = 0.0324 (28 dpi).

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Chapter 3: Evolution of biofilm formation ability in an emerging bacterial pathogen

Abstract

Microbial life *in natura* is often restricted to growth on solid strata in the form of biofilms. In this sessile form of living, microbial species and communities can be found in structurally and demographically complex structures and the structural, metabolic and genetic heterogenicity present in these biofilms could have major implications for microbial evolutionary processes as well as large impacts on host responses in the case of pathogenic infecting microbes. Additionally, the role of biofilms and its relationship with microbial pathogenesis and virulence in an evolutionary context is largely unknown. In this chapter we use the *M. gallisepticum* – *H. mexicanus* system to study how a bacterial pathogens ability to form biofilms has evolved in response to growing host resistance, and whether this has played a role in how other traits, i.e. the pathogens virulence, have changed over time. Low-passage isolates of *M. gallisepticum* sampled across the epizootic and used concurrently in an *in vivo* study experimental infection were grown *in vitro* and allowed to form biofilms. These were then stained with crystal violet in order to assay the abundance of biofilm produced by each isolate. Using this data, we show that the biofilm forming capability of *M. gallisepticum* isolates has decreased on average over time and that these decreases have occurred concurrently with the increasing average virulence of the pathogen, suggesting that the loss of biofilm forming ability in *M. gallisepticum* has occurred in response to host-resistance and selection to maintain or increase its virulence in its novel host.

Introduction

Understanding the mechanisms by which pathogens mitigate host immune defences is critical to predicting pathogenesis and virulence evolution. Theory predicts that host resistance via immune activity will select for pathogens that can transmit rapidly before they are cleared by the immune system (MacKinnon & Read, 1999; Mackinnon & Read, 2004). Since faster transmission rate requires greater levels of host exploitation and damages, host resistance should select for more virulent pathogens. Such increases in virulence can occur, for example, as a result of increasing rates of pathogen replication within the host, or as a consequence of immune manipulation and escape (Foster, 2005). While there is accumulating empirical evidence for the evolution of increasing virulence in response to host resistance (Chapter 1), less is known about the mechanisms mediating virulence evolution.

One mechanism by which bacterial pathogens can persist in hostile host environments and either escape and/or modulate host immunity, is through forming biofilms (Vuong *et al,* 2004). Biofilms consist of aggregated bacteria that are embedded within a complex matrix of extracellular polymeric substances and adhered to a solid surface (Dunne, 2002). The extracellular polymeric substances at the surface of biofilms can form a physical barrier that protects against host immunity (Domenech *et al,* 2013). For example, *Staphylococcus aureus* in biofilms are shielded from phagocytosis by macrophages, most likely as a result of the size and/or physical complexity of the biofilm (Thurlow *et al,*2011). In addition, biofilm formation can disrupt and modify immune processes. For example, in *Pseudomonas aeruginosa* and *Candida albicans*, biofilm formation is associated with strong, but ineffective inflammatory responses (Alhede *et al,*

2014). However, although phagocytes can mount an oxidative burst response against *P. aeruginosa* biofilm, they become immobilised following contact and can no longer migrate away (Jesaitis *et al,* 2003). *S. aureus* biofilms can also cause macrophage dysfunction and apoptosis upon physical contact (Cerca *et al,* 2006), most likely as a result of exposure to toxic by-products of bacterial metabolism or lytic toxins (Hanke & Kielian, 2012). *C. albicans* biofilms, on the other hand, do not trigger a reactive oxygen response and instead appear to have an immunosuppressive effect, with monocytes becoming entrapped in the biofilm ultrastructure (Chandra *et al,*2007; Katragkou *et al*, 2010; Xie *et al,* 2012). That biofilms can be used as a means of protection against the immune system is evidenced by the fact that immune activity can stimulate biofilm formation. For example, the presence of polymorphonuclear neutrophils has been shown to increase biofilm formation in *P. aeruginosa* by a factor of 2-3.5 times (Walker *et al,* 2005; Parks *et al,* 2009). Although biofilms have been shown to protect bacterial pathogens against host immunity, by acting as a physical barrier and/or by modifying the immune response, the role of biofilm formation in driving virulence evolution in response to host resistance remains to be determined.

The emergence of the bacterial pathogen, *Mycoplasma gallisepticum*, in a wild North American songbird, the house finch (*Haemorhous mexicanus*), offers a rare opportunity to test how the role of biofilm formation ability in pathogen virulence evolution in response to the spread of host resistance. *M. gallisepticum* jumped from poultry into house finches in 1994, giving rise to a severe conjunctivitis outbreak that killed millions (Delaney *et al,* 2012; Dhondt *et al,* 1998). Faced with the intensity of this selection event (Gates *et al*, 2018), disease-exposed house finch populations evolved genetic resistance in less than 12 years (Bonneaud *et al*, 2012). The evolution of host resistance, in turn, gave rise to an adaptive

increase in pathogen virulence (Bonneaud *et al*, 2018; Chapter 1). While virulence increased linearly over the course of the epidemic, replication rates increased from outbreak through to the spread of resistance but reached a plateau after hosts had become resistant (Chapter 1). This indicates that increasing replication rates do not underpin increasing virulence in this system, and that virulence and replication rates may be under independent selection as a result of host resistance.

Here, we investigate the biofilm formation ability of 55 distinct *M. gallisepticum* isolates collected from epidemic outbreak, throughout the initial spread of host resistance and afterwards to present day (1994-2015). We have shown previously that these isolates differ in virulence, as measured by increased conjunctival swelling and putative host mortality (Chapter 1 of this thesis). In this study, we test whether biofilm formation mediates, at least in part, the evolution of increasing pathogen virulence in response to the spread of host resistance. To do so, we measured the amount of biofilm produced *in vitro* by each of the isolates for which measures of virulence were taken *in vivo* during an experimental inoculation of non-resistant house finches (see Chapter 1). This allowed us to test: (i) how biofilm formation ability has changed over the course of a naturally-evolving epidemic; and (ii) the association between pathogen virulence and biofilm formation ability in the face of increasing host resistance.

We predict that, if biofilms formation has been under selection as a means of protection against host resistance, then the amount of biofilm produced by isolates should increase over the course of the epidemic and should be positively associated with measures of virulence. In contrast, if another pathogen trait drives the adaptive increase in virulence over time but trade-offs with biofilm formation, then biofilm production should decrease over time and should be

negatively associated with measures of virulence. Alternatively, if biofilm formation plays no role in pathogen evolution in this system, then there should be no change in the amount of biofilm produced over time and no association with measures of virulence. Finally, given the commonly-assumed role of pathogen load in shaping virulence (see Chapter 1), we also test for any association between pathogen load and amount of biofilm produced.

Results

45 out of 47 isolates (96%) were found to have produced a detectable level of biofilm *in vitro*. The average amount of biofilm produced, measured by optical density was A_{595} 0.023 We found a negative linear association between amount of biofilm produced and year of pathogen sampling, with late epidemic isolates forming significantly less biofilm than earlier ones (linear model: estimate ± se = -0.091 ± 0.034, $F_{1,44} = -2.69$, p = 0.010; Fig. 1). Furthermore, we found significant negative associations between biofilm formation and the two measures of virulence. The amount of biofilm produced decreased significantly with increasing conjunctival swelling of the host *in vivo* (linear model: estimate ± se = -0.021 ± 0.006, $F_{1,44} = -3.94$, p < 0.0003; Fig. 2). Furthermore, the amount of biofilm produced was significantly lower in hosts that would have died in the wild as a result of infection (linear model: estimate ± se = -1.39 ± 0.47, $F_{1,44} = -2.97$, p < 0.005; Fig. 3). Finally, we found no association between the amount of biofilm produced and the total pathogen load achieved over the course of an infection *in vivo* (linear model: estimate ± se = -0.00014 ± 0.00018, $F_{1,44} = -0.80$, p = 0.43).

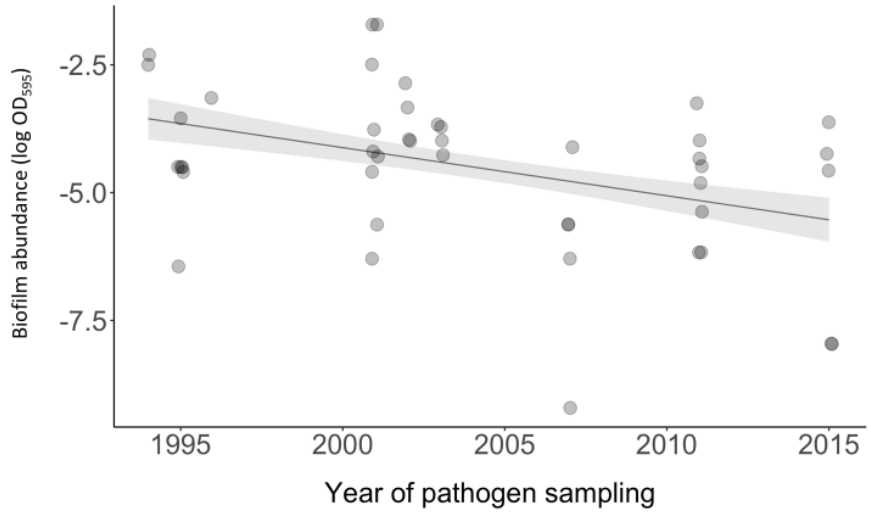

Figure 1. Changes in biofilm production over the course of the epidemic. We show changes in the amount of biofilm produced as pathogen isolates were sampled progressively later in the epidemic. Points represent raw values; the line is predicted from the model with the standard error represented by the ribbon.

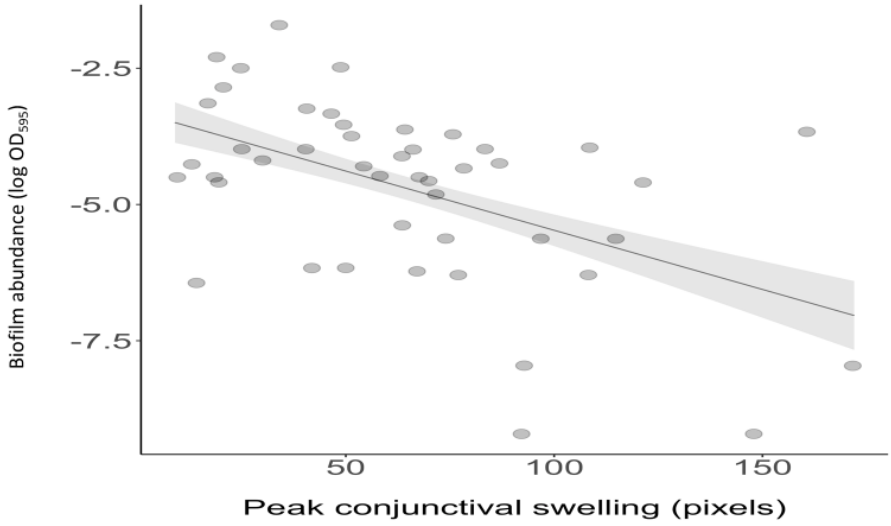

Figure 2. Association between biofilm formation and conjunctival swelling. We show the amount of biofilm produced (ln transformed) as a function of mean conjunctival swelling (in pixels), with greater amounts of biofilm produced being associated with lower virulence. Points represent raw values; the line is predicted from the model with the standard error represented by the ribbon.

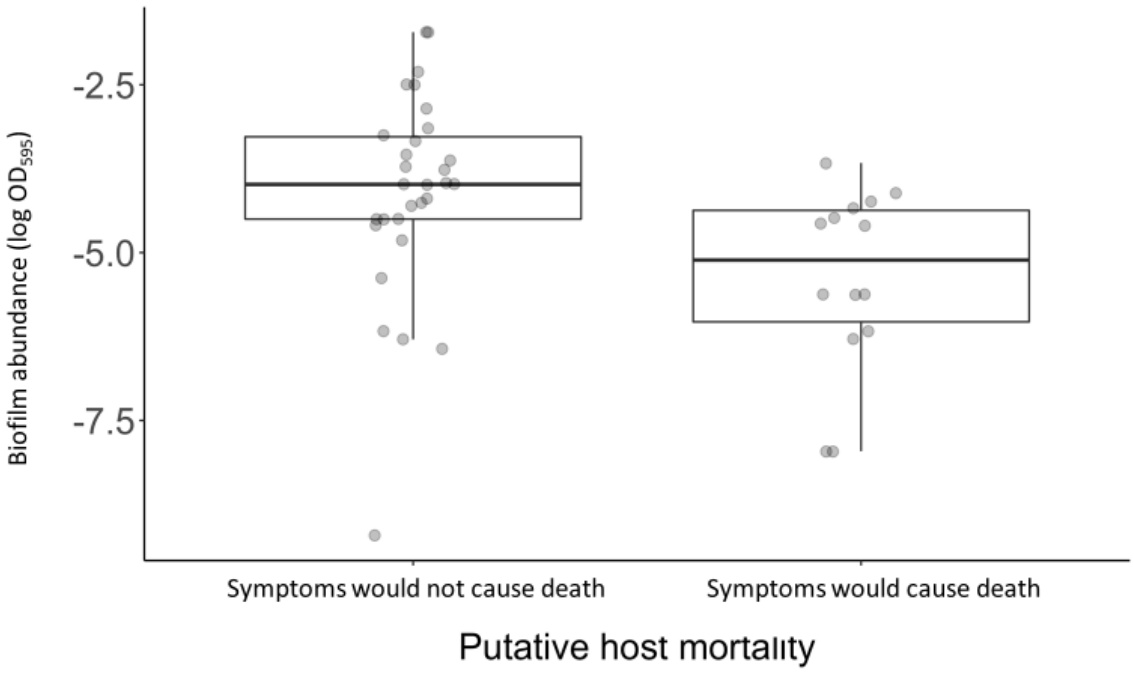

Figure 3. Association between biofilm formation and host mortality. Boxplot diagram showing amount of biofilm produced (ln transformed OD595) for isolates that have versus have not caused putative host mortality. Depicted are the median and range transmission rates; points represent raw values.

Discussion

By taking advantage of a large repository of bacterial isolates of known virulence *in vivo* that were collected over the course a natural epidemic since initial outbreak and over the next 20 years, we were able to test key questions about the role of biofilm formation ability in the evolution of pathogen virulence in response to host resistance. We found that the amount of biofilm produced decreased significantly over time and was negatively associated with two measures of virulence (conjunctival swelling and putative mortality). The amount of biofilm produced, however, was not associated with the total bacterial load achieved at the site of infection *in vivo*, indicating no association between biofilm

formation ability and pathogen replication rate. Our findings suggest that another mechanism has driven the evolution of increasing virulence in response to host resistance and that this mechanism trade-offs with biofilm formation ability. What this mechanism is and how it trades-off with biofilm formation, however, remains to be determined.

The link between biofilm formation and virulence has primarily been investigated through a comparison of chronic versus acute infections. For instance, *Pseudomonas aeruginosa* biofilms appear to be instrumental in the establishment of chronic respiratory infections of cystic fibrosis patients (Starkey *et al*, 2009). Furthermore, *Neisseria meningitidis* biofilm forming isolates are more commonly associated with asymptomatic infections in “carrier” individuals and less prevalent in the highly virulent invasive infections causing bacterial meningitis (Yi *et al,* 2004). Finally, in porcine hosts, the capability of *Streptococcus suis* isolates to form biofilms appears to bias infections towards a chronic persistent state (Wang *et al*, 2011; Vötsch *et al,* 2018). Our finding of a negative association between amount of biofilm produced and virulence is consistent with these findings and suggest that mechanisms that favour more aggressive pathogens may also lessen their stealth behaviour.

Unravelling the relationship between biofilm formation and virulence in *M. gallisepticum*, particularly in the context of emergent resistance and tolerance in the House finch (Bonneaud *et al,* 2018; Bonneaud *et al,* 2019), will require further work aimed at understanding the immunopathological consequences and mechanisms underpinning them. In the well characterised *Pseudomonas aeruginosa* system, biofilm production is known to have myriad effects across both innate and secondary immunity processes. For example, *P. aeruginosa* biofilms appear to be highly activating of innate immune effectors, largely through

the activation of neutrophils, but also difficult for these defences to attack efficiently (Moser *et al*, 2017). This is thought to lead to immunopathology in cystic fibrosis patients due in large part to the long-term activation of the oxidative-burst neutrophil response and it's associated "bystander" host-damage (Kolpen *et al,* 2014). In *Mycoplasma pulmonis* at least, a similar phenomenon in the activation of the complement system appears to occur. *M. pulmonis* biofilms appear to provide structural protection from the effects of host complement and gramicidin innate immune effectors, whilst still being highly-activating of these immune responses (Simmons *et al,* 2007). In contrast, the evolution of resistance to *M. gallisepticum* by *H. mexicanus* has been shown to have been mediated by cell-mediated immunity, and the downregulation of innate (inflammatory) immunity genes, including those involved in complement cascade activation in unexposed finch populations on infection with a 2007 *M. gallisepticum* isolate (Bonneaud *et al,* 2012). Squaring this data with our results here and within the context of immunopathology and resistance/tolerance evolution will require further work. However, we consider broadly that perhaps selection to increase or maintain virulence (linked to transmission), in response to the lower immunopathology in resistant/tolerant birds has led to a shift in the immunomodulatory behaviours of *M. gallisepticum*. If the mechanisms by which resistance/tolerance has evolved are focused on a narrow set of immune-responses, there may be the potential for *M. gallisepticum* to in turn evolve anti-resistance/tolerance strategies by shifting how it interacts with these host-immune processes.

The lack of any significant association between biofilm formation and pathogen replication found in this experiment is perhaps not surprising. Previous studies attempting to link replication and biofilm formation have indicated a complex and hard to predict relationship between the two. In *Pseudomonas*

aeruginosa it has been suggested that so long as the microenvironment around cells is the same, the growth kinetics between planktonic and sessile biofilm cells should be constant (Bakke *et al,* 1994). On the other hand, a number of studies have shown either decreased or increased growth rates in biofilms relative to planktonic cells measured under various conditions (van Loosdrecht *et al*, 1990). Perhaps the takeaway from this research is that these contrasting findings on the relationship between biofilms and microbial replication appear as a result of the complicated and variable structural and environmental conditions of biofilms, as well as differences in the biology of primary (biofilm forming) and secondary (growing in existing biofilms) cells (Rice *et al,* 2000). Developing assays for biofilm formation which adequately address the methodological issues in making accurate and biologically relevant measurements of biofilm forming capability are critical to furthering our understanding of their importance for both environmental and pathogenic microbes.

Although we acknowledge these obvious limitations and others presented by the study presented herein, we consider that this work should contribute to our understanding of select on biofilm formation. That *in vivo* biofilm formation may be quite different in character to the behaviour of *M. gallisepticum* in *in vitro* infection conditions is a clear issue, but we would nevertheless suggest that the propensity of our isolates to form biofilms under the conditions used should bear some relevance to its biology within the host. *in vivo* studies of biofilm formation which could resolve this are notoriously difficult and full of confounding factors, making it difficult to control for or design experiments which can produce the amount of data needed to draw evolutionary conclusions. We hope that follow up work from this study will help to address such issues, for example by identifying

biofilm associated genetic and transcriptional biomarkers which might facilitate indirect determination of *M. gallisepticum* biofilm formation *in vivo.*

Methods

Biofilm formation culture and crystal violet staining

Low passage inoculum stocks of *M. gallisepticum* as used in the *in vivo* experimental infection above where inoculated into 10ml of Mycoplasma Broth media supplemented with Mycoplasma selective supplement G (OXOID) and concentrations standardised to an OD595 of 0.01. 10-fold dilutions of standardised cultures were made using fresh media and added in triplicate to each of 2 rounded bottom 96-well culture plates. Each plate contained in triplicate a negative control of media from the same batch as used for initial cultures and a positive control/standardisation factor of R_{Low}, a widely studied and consistently high-biofilm producing *M. gallisepticum* isolate from poultry (Chen *et al*, 2012). Plates were sealed and incubated for 72hrs at 37C and 5% CO2.

Plates were removed from the incubator and media gently aspirated from each well, washed twice with 1x PBS solution and left to dry upside-down on paper for 5 minutes. When dry, 200ul of 100% MeOH was added to each well to fix and permeabilise biofilms and left to incubate for 10 minutes at room temperature. MeOH was then aspirated from wells and plates left to dry face up for 10 minutes to remove any trace solvent. When dry, 150ul of 0.5% Crystal Violet solution was added to each well and plates were left to incubate at room temperature for 45 minutes to ensure thorough staining of biofilms. Plates were then washed extensively 6x with sterile d.H20 to remove any unbound CV and left to dry for 10 minutes. To dislodge biofilms and dissolve bound CV we added

200ul of 30% Acetic Acid to each well and placed plates on a rotating mixer at 120 rpm for 20 minutes. 100ul of solvent from each well was then transferred into corresponding wells on a flat-bottom 96-well plate for spectrophotometry, the OD595 of each well were measured and mean values calculated for each *M. gallisepticum* isolate on each plate. Between plate normalisation was performed using factor-correction in Factor (Rutjer *et al*, 2006), with mean R_{Low} values per plate used as the standardisation factor.

Experimental inoculation

Wild house finches from populations that have never been exposed to *M. gallisepticum* were captured in Arizona in the summer 2015. Birds (118, 64 males and 54 females) that had hatched in the spring 2015 were brought back to aviaries at Arizona State University and housed independently. 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 assay (Luttrell et al. 1996), and a lack of current infection was verified using the choanal swabs in PCR amplification of *M. gallisepticum* DNA (Roberts, Nolan, and Hill 2001). Birds were randomly inoculated with 1 of 55 *M. gallisepticum* isolates sampled over the course of the epidemic (see Chapter 1). The experiment was stopped at 35 dpi and all birds were euthanized as stipulated by home office licencing. Protocols were approved by Institutional Animal Care and Use Committees (IACUC) of Auburn University (permit # PRN 2015-2721) and Arizona State University (permit #15-1438R), and by Institutional Biological Use Authorizations to Auburn University (# BUA 500).

Virulence

To quantify the size of the conjunctiva and so severity of conjunctival swelling, we photographed the right and left eyes at 0, 6, 13 and 25 days post-inoculation (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 (Staley *et al,* 2018). Measurements of photographs were done blindly with respect to the isolate inoculated. Finally, eyes were also inspected visually on days 3, 6, 8, 14, 21, 25, 28 and 34 post-infection: infection is considered lethal when the conjunctiva is red to purple, and the eye is difficult to see and produces discharge – such symptoms, with little or no vision possible, are thought to have caused the death of millions of infected finches due to starvation or predation (Adelman *et al,* 2017; Kollias *et al,* 2004; Roberts *et al,* 2001).

Pathogen load

Bacterial load was measured from conjunctival and tracheal swabs obtained at 8, 14, 21 and 28 dpi by quantifying the number of *M. gallisepticum mgc2* gene copies and the number of house finch *rag1* gene copies (Tardy et al submitted). Pathogen load was then determined as the number of *M. gallisepticum* cells divided by the number of house finch cells to control for variation in sampling efficiency (Grodio *et al,* 2008). DNA was extracted using a QIAGEN DNeasy® Blood and Tissue Kit according to the manufacturer's standard protocols. Multiplex qPCRs for *mgc2* and *rag1* were conducted using an Applied Biosystems™ StepOnePlus™ Real-Time PCR system. Each multiplex qPCR reaction was run in a final volume of 20 µl and contained: 2µl of either plasmid

standard or sample genomic DNA template, 1 µl each of 10 µM mgc110-F/R and rag1-102-F/R primers (total 4ul; Table 2), 0.5 µl each of 10 µM Mgc110-JOE and Rag1-102-FAM fluorescent hydrolysis probes (total 1 µl), 10 µl of 2X qPCRBIO Probe Mix HI-ROX (PCR BIOSYSTEMS) and 3 µl Nuclease-free water (Ambion®). Cycling conditions were as follows: 95°C for 3 minutes for initial denaturing of template DNA, followed by 45 cycles of 95°C for 1 second and 60°C for 20 seconds for primer and probe binding and amplification of target DNA. Samples and standards were measured in duplicate in each run with a negative control of elution buffer. All data was exported to LinRegPCR v.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 normalised using Factor qPCR v.2016.0 (Ruijter *et al,* 2015), with standard series presented as between-run replicates.

Statistical analyses

All statistical analyses were conducted in R 3.3.2 (Team 2016) and figures were made using ggplot2 (Wickham, 2009). In all analyses, the amount of biofilm produced *in vitro* was natural log transformed to fit a normal distribution. We conducted 4 linear models with amount of biofilm produced as the response term. To test how biofilm formation evolved over the course of the epidemic, we fitted year of pathogen isolate sampling as the explanatory term. To test for associations between biofilm formation and virulence, we included either peak conjunctival swelling or putative host mortality as the explanatory term. Finally, we tested the association between biofilm formation and pathogen load by fitting peak pathogen load as the explanatory term.

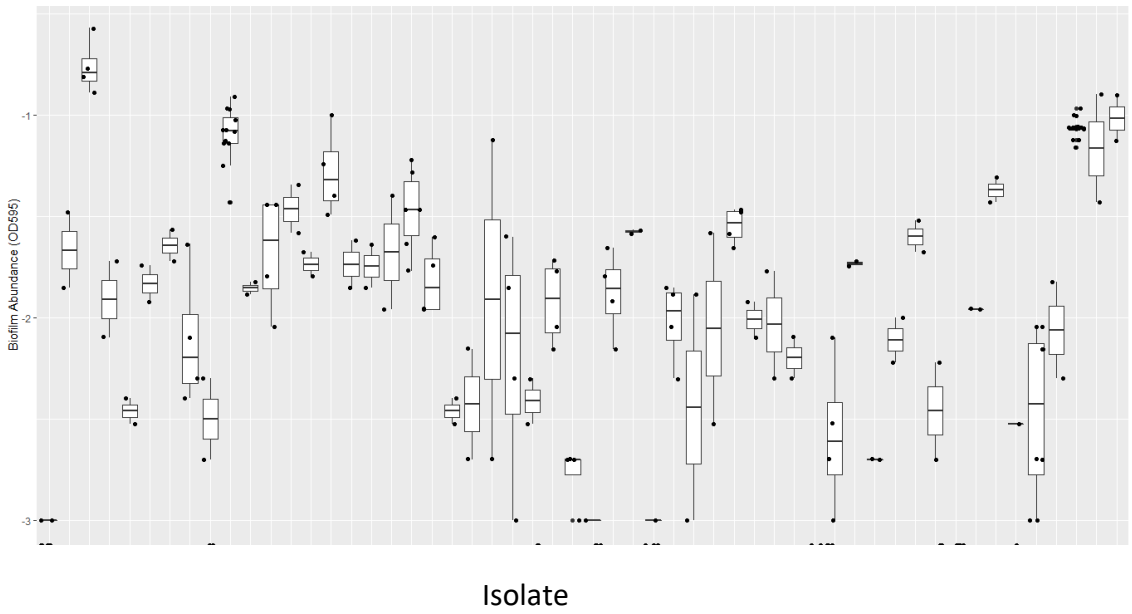

Fig. 4. Variation in biofilm abundance between biological replicates of *M. gallisepticum* isolates used in this experiment. Variation in biofilm abundance was more pronounced for some isolates than others, and this variation is likely an important aspect of *M. gallisepticum* biofilm formation, possibly arising due to stochastic differences in growth and expression causing population stratification from growth of polyclonal isolates in culture.

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Chapter 4: Positive association between virulence and transmission rate in an emerging bacterial pathogen

Abstract

The virulence-transmission trade-off hypothesis posits that for pathogenic microbes, the rate of transmission from host-to-host should exist in an equilibrium state with the degree of virulence manifested by the pathogen upon the host. Under this framework it is assumed that virulence is a function of transmissibility, insofar as its evolutionary function is to cause host exploitation and dysregulation sufficient for an infecting parasite population to reach transmissible levels and to open a successful physiological route for this transmission to occur. In this experiment, we utilise observational data and samples collected from a large scale *in vivo* transmission experiment to determine whether virulence and transmission are so linked in the *Mycoplasma gallisepticum* – House finch host-pathogen model system. We inoculated 59 birds from a susceptible population with no prior *M. gallisepticum* exposure with low-passage isolates collected across the epizootic. Each of these inoculated birds was then housed with an uninfected sentinel. Time of transmission for each isolate was determined by PCR detection of *M. gallisepticum* in the sentinel from samples taken throughout the experiment. Our data shows that transmission rates have evolved over the course of the epizootic to be more rapid, with later isolates transmitting to secondary hosts faster than isolates sampled early in the epizootic. We then show that this increased transmission rate is explained by the increasing virulence of isolates across the same time-frame, supporting the assumed linkage of virulence and transmission in the trade-off hypothesis.

Introduction

The evolution of host resistance to infection should give rise to strong selection pressures that shape the evolutionary trajectory of the pathogen. The hypothesis that links host resistance and pathogen evolution is the virulence-transmission trade-off hypothesis (Alizon *et al,* 2009; Anderson & May, 1982; Ewald, 1983; Gandon *et al*, 2001). Under this hypothesis, increasing host resistance as a result of immune clearance is predicted to select for increasing rates of host exploitation and damage (i.e. virulence (Fraser *et al*, 2007; Ebert, 1998; Mackinnon & Read, 1999b; Frank, 1996)), which enable the pathogen to transmit to other hosts before being cleared (Porco *et al,* 2005; Gandon & Michalakis, 2000). Since greater damages reduce infection duration by hastening host death, this hypothesis therefore predicts a negative association between infection duration and transmission rate (hence the 'trade-off'), but a positive association between pathogen virulence and transmission rate (Agnew & Koella 1997; Ferguson & Read 2002; Mackinnon *et al,* 2002; Mackinnon *et al,* 2008; Mackinnon & Read 1999a, 2003; Paul *et al,* 2004; Salvaudon *et al,* 2005; Wickham *et al*, 2007), with host resistance driving the evolution of increasing virulence and transmission rate. While the virulence-transmission trade-off hypothesis is increasingly proving to be a powerful framework for predicting pathogen evolution (Alizon *et al*, 2009; Alizon & Michalakis 2015; Kerr *et al,* 2017), we still lack experimental tests of the impacts of host resistance on the relationship between virulence and transmission rates (Barclay et al. 2012; Mackinnon and Read 2004).

Evidence for a link between virulence and transmission have, nevertheless, been found in a variety of systems (Ferguson *et al*, 2003; Jensen *et al*, 2006; Mackinnon & Read 1999a, 2003; Mackinnon *et al,* 2008; Wickham *et al*, 2007;

Agnew & Koella 1997; Paul *et al*, 2004; Salvaudon *et al,* 2005), even though the precise shape of this association remains unclear. For instance, an experimental infection of monarch butterflies (*Danaus plexippus*) with three clones of the protozoan parasite *Ophryocystis elektroscirrha* found that higher parasite burden led to greater host mortality and mating success (i.e. virulence) (de Roode *et al,* 2008). Among the infected females that did reproduce, however, higher maternal parasite load resulted in a greater deposition of parasites on eggs and plant leaves, thus suggesting a positive (and linear) association between levels of virulence and transmission. Similarly, a study of HIV-infected patients found that the duration of the asymptomatic phase decreased with increasing viral load, and that there was a positive association between viral load and transmission potential, estimated as the expected number of individuals infected over the entire infectious period (Fraser *et al,* 2007). In this case, however, transmission potential reached maximal levels for intermediate levels of viral load, suggesting a positive (but non-linear) association between virulence and transmission. Whether virulence and transmission rate remain positively associated in the face of increasing host resistance, however, remain to be tested.

The epidemic of the bacterial pathogen, *Mycoplasma gallisepticum*, in house finches (*Haemorhous mexicanus*) offers a rare opportunity to test how the association between pathogen virulence and transmission rate have evolved in response to the spread of host resistance. *M. gallisepticum* emerged naturally in eastern North American house finches after a single host shift from poultry, which gave rise to a severe conjunctivitis outbreak that killed millions (Delaney *et al,* 2012; Dhondt *et al,* 1998). Faced with the intensity of this selection event (Gates et al. 2018), disease-exposed house finch populations evolved genetic resistance in less than 12 years (Bonneaud et al. 2011). The evolution of host resistance, in

turn, gave rise to an adaptive increase in pathogen virulence, with pathogen fitness increasing significantly as the pathogen was sampled progressively later in the epidemic relative to the host (Bonneaud et al. 2018; Tardy *et al*, 2019).

Here, we inoculated 55 distinct *M. gallisepticum* isolates collected from epidemic outbreak, throughout the initial spread of host resistance and afterwards to present day (1994-2015) into non-resistant houses finches from disease unexposed populations. We have shown previously that isolates sampled later in the epidemic gave rise to increased conjunctival swelling and putative host mortality than isolates obtained earlier (Tardy *et al*, 2019). In this study, we measured transmission rate to uninfected sentinels from unexposed populations. Using wild finches from non-resistant populations allows us to test for genetically-determined changes in pathogen virulence and transmission rate without the confounding effects of protective immune activity (Barclay *et al*, 2012; Kerr *et al*, 2017; Mackinnon & Read, 2004). Evolutionary inferences can be made about the pathogen because the 55 bacterial isolates used were collected at random from naturally-infected finches and had exhibited variation in symptom severity in infected birds, therefore composing a representative sample library of the isolates circulating during the epidemic (Bonneaud *et al,* 2018). In addition, using a large number of such isolates obtained across the epidemic protects against the effect of outlying isolates and allows for a test of the precise way in which pathogen traits have changed over time in response to host resistance. Our approach therefore produces a novel test of: i) how pathogen transmission rate has changed over the course of a naturally-evolving epidemic; and ii) the association between pathogen virulence and transmission rate in the face of increasing host resistance.

Results

Transmission to the uninfected sentinel by the end of the experiment 35 days post-inoculation (dpi) occurred in 76% of trials (N = 45). The rate of transmission was measured as 1 divided by the number of days to the transmission event. We found a significant effect of year of pathogen sampling on transmission rate, with isolates sampled later in the epidemic transmitting faster to uninfected sentinels than those sampled earlier (linear model: estimate ± se = 0.035 ± 0.012, $F_{1,43}$ = 2.93, p = 0.005; Fig. 1). As predicted by the virulence-transmission trade-off hypothesis, we found a positive association between the severity of conjunctival swelling and transmission rate (linear model: estimate ± se = 0.011 ± 0.004, $F_{1,42}$ = 2.60, p = 0.013; Fig. 2). Thus, isolates that were more virulent (i.e. caused greater conjunctival swelling) also transmitted faster. Furthermore, there was a significant difference in transmission rates between isolates that caused or not conjunctival swelling severity that, in the wild, would lead to death (linear model: estimate ± se = 0.040 ± 0.16, $F_{1,43}$ = 2.54, p = 0.015; Fig. 3). Indeed, isolates that caused putative host mortality also transmitted faster than those that did not. However, when considering only those isolates that caused putative mortality, we did not find the expected negative association between mortality and transmission rate. In fact, isolates that killed their primary host faster transmitted more slowly than isolates that took longer to kill their host (linear model: estimate ± se = 0.027 ± 0.022, $F_{1,19}$ = 2.51, p = 0.021). Nevertheless, this effect disappeared when we considered only those isolates that transmitted to sentinels before causing putative mortality in their primary host (linear model: estimate ± se = 0.0039 ± 0.0019, $F_{1,16}$ = 2.08, p = 0.054). In other words, while isolates that caused putative mortality in the primary host did transmit faster than those that

did not, we found no evidence that mortality rate in the former group was associated with greater transmission rates. Concerning degree of conjunctival swelling at time of transmission, we calculated a mean swelling of 26.02 mm^2 with a SD error of +/- 9.31 mm^2.

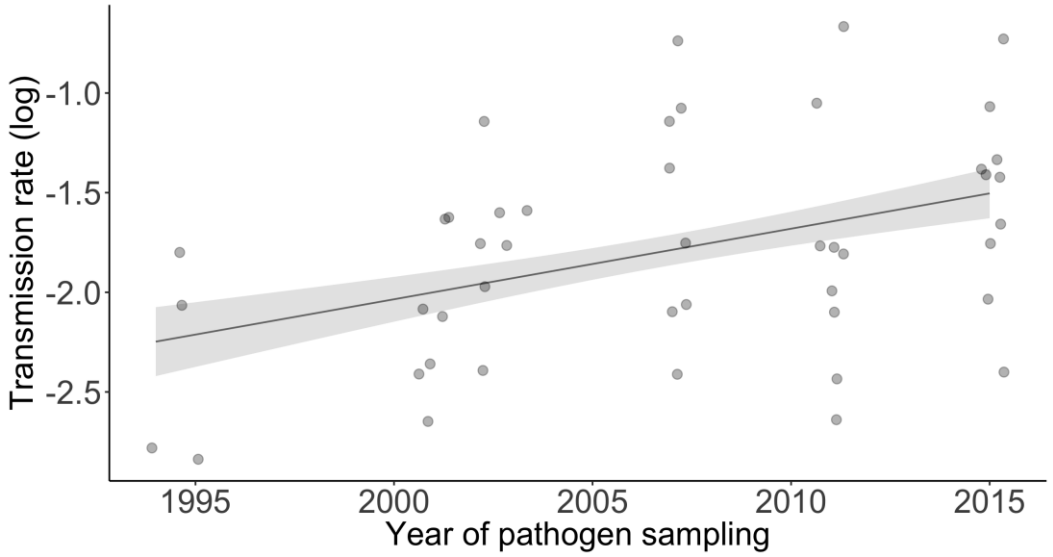

Figure 1. Changes in transmission rate over the course of the epidemic. We show changes in transmission rate from experimentally-inoculated host to uninfected sentinel as pathogen isolates were sampled progressively later in the epidemic. Points represent raw values; the line is predicted from the model with the standard error represented by the ribbon.

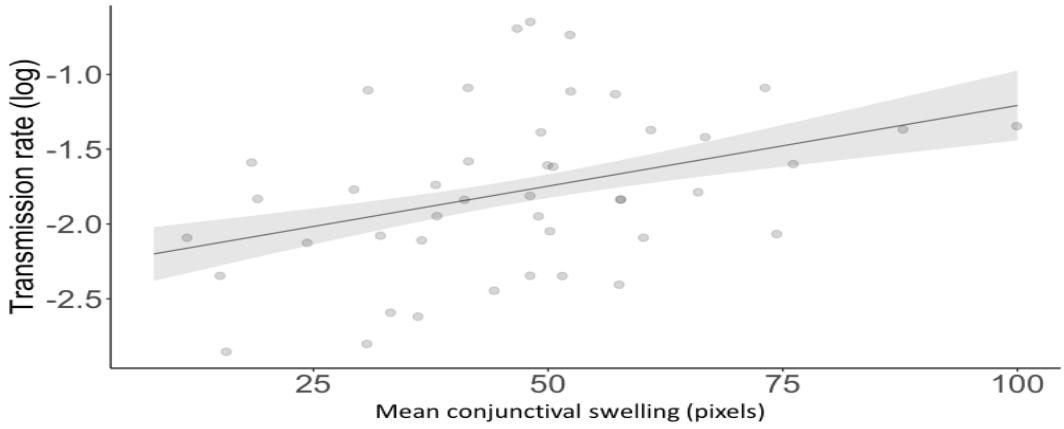

Figure 2. Association between transmission rate and conjunctival swelling. We show transmission rates to an uninfected sentinel house finch as a function of mean conjunctival swelling (in pixels), with faster transmission (i.e. lower transmission rate) being associated with greater virulence. Points represent raw values; the line is predicted from the model with the standard error represented by the ribbon.

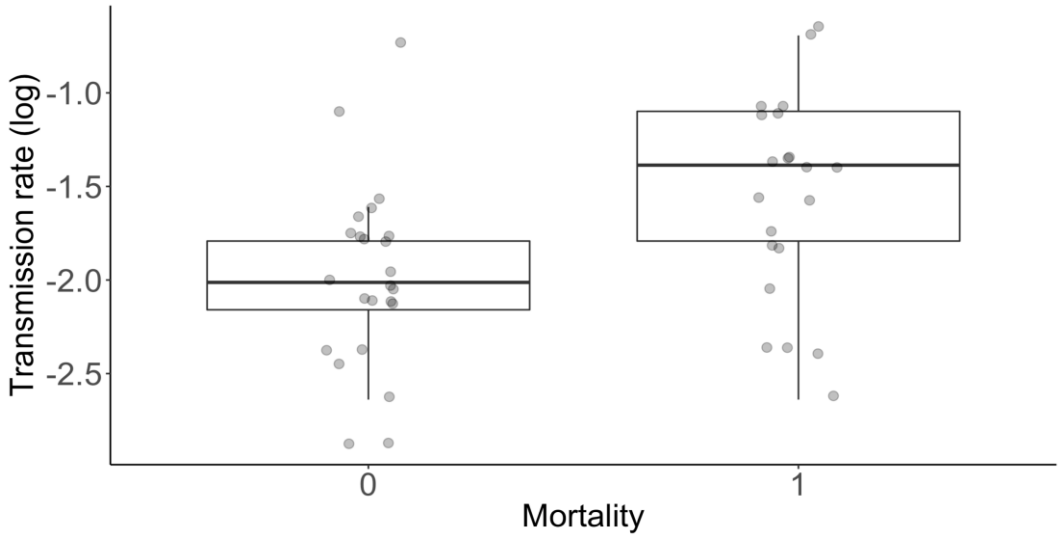

Figure 3. Association between transmission rate and host mortality. Boxplot diagram showing transmission rates to uninfected sentinel house finch for isolates that have caused versus have not scored virulence equating to putative host mortality. Depicted are the median and range transmission rates; points represent raw values.

Discussion

By conducting a natural transmission experiment using a large number of isolates of bacterial pathogen, *M. gallisepticum*, sampled from epidemic outbreak (1994-5) and over the course of the subsequent 20 years (to 2015), we showed that pathogen transmission rate decreased over time, indicating that the pathogen

evolved to transmit faster. Furthermore, we found positive associations between two measures of virulence (eye swelling and putative mortality) and transmission rate. Indeed, isolates that caused more severe conjunctival swelling or putative host mortality were those that transmitted fastest. Our result therefore indicate that, in this system, pathogen virulence and transmission rate are positively associated and that both have increased in response to the spread of host resistance.

Few studies have tested the link between virulence and transmission rate directly and even fewer have used direct measures of transmission rate. For instance, in the rodent malaria parasite, *Plasmodium chabaudi*, genetic relationships between virulence and transmission were investigated by testing the association between virulence and replication rate (as measured parasitaemia) and between transmission and replication rate. Furthermore, in this experiment transmission was measured as either the density of sexual forms (gametocytes) in the blood or the proportion of mosquitoes infected after taking a blood-meal from the mouse (Mackinnon & Read, 1999a). Such indirect measures, however assume a close causal association with the actual trait, and therefore also make inferences about the underlying mechanisms of that trait. As a result, whether virulence and transmission are necessarily positively linked remains to be tested more broadly.

In this study, we have found further evidence to support the basic underlying assumption of the trade-off hypothesis that transmission and virulence are fundamentally linked, with the former being at least in part determined by the latter. In addition, we consider that our experiment here has a notable limitation in that transmission was measured only from an infected individual to a single sentinel in each cage, with the possibility that more significant host-heterogenicity

and variation in sentinel susceptibility than expected might skew our results. The alternative design, however, of having multiple sentinels per cage would, despite the potentially greater accuracy also convolute the experiment in that it would be hard to discern transmission from primary (inoculated) to secondary (sentinel) versus from secondary to secondary hosts.

Despite supporting the overall assumption of a functional link between virulence and transmission posited by the trade-hypothesis, we do not address here the underlying mechanistic cause of this linkage; although increased fomite deposition resulting from the increased conjunctival swelling in high virulence infections seems the obvious underlying cause this remains to be thoroughly tested. A previous study showed that *M. gallisepticum* fomites could be a major source of transmission, but remained viable on inorganic surfaces for less than 24 hours (Dhondt *et al*, 2007). A future experiment could attempt to quantify viable *M. gallisepticum* isolates of varying virulence in such fomite depositions and test their environmental persistence to discover if selection on either of these traits underlies the relationship between transmission and virulence observed here. Alternatively, this relationship might be explained by a decrease in the infective dose for more virulent isolates, i.e. do more virulent isolates transmit at a lower threshold? Although the former experiment will be difficult to address, we anticipate that determining the infective dose during each of our transmission events recorded in this experiment should be quite simple, applying our qPCR method (outlined in Chapter one) to sentinel samples collected on the day of transmission as determined here.

Methods

Capture and housing

Wild house finches from populations that have never been exposed to *M. gallisepticum* (i.e. that have not evolved genetic resistance) were captured in variety of urban and suburban sites in Arizona, in the summer 2015 (N = 118, 64 males and 54 females). *M. gallisepticum* has never been recorded in the sampling area despite continuous monitoring (Staley *et al*, 2018). Using birds that have not had the opportunity to evolve protective immune responses to *M. gallisepticum* is essential to measuring virulence and transmission rate without the confounds of the capacity for immune clearance (Kerr *et al,* 2017). Birds 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 indoor aviaries at Arizona State University's Tempe campus, where they were housed for the remainder of the experiment. On arrival, we obtained a blood sample from all birds using brachial venipuncture (60 µL 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 assay (Luttrell *et al,* 1996), and a lack of current infection was verified using the choanal swabs in PCR amplification of *M. gallisepticum* DNA (Roberts *et al,* 2001). Although none of the birds displayed any sign of infection with other diseases, all birds were prophylactically medicated for infection with *Trichomonas gallinae* with carnidazole (Spartrix, Janssen/Elanco) and *Isospora spp* with sulfadimethoxine over 40 days. The birds were then allowed to acclimate for one month prior to experimental onset and provided with *ad libitum* food and water throughout.

Experimental inoculation and transmission rate

The birds were divided into 2 groups of 59 birds. Each of the 55 *M. gallisepticum* isolates sampled over the course of the epidemic was inoculated into one bird selected at random from the 59 birds of the first group (30 males and 29 females), with 5 isolates (one from 1994, two from 1995, and two from 2007) each inoculated in two birds. 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 8 different States in the eastern US (mainly from Alabama). Isolates were administered via 20 µL of culture containing 1×10^4 to 1×10^6 colour changing units/mL of *M. gallisepticum* in both eyes. Following inoculation, all 59 birds were maintained in separate cages and co-housed with one bird from the second group in 54 randomly-assigned female-male pairs and 5 male-male pairs. Birds from the second group thus served as an uninfected sentinel. Transmission rate to the sentinel was measured by amplification of *M. gallisepticum* DNA from conjunctival and tracheal swabs (Roberts *et al,* 2001) obtained at 2, 3, 4, 5, 6, 7, 8, 11, 14, 17, 20, 23, 26, 29, 32 and 35 days post-inoculation (dpi). All birds had access to *ad libitum* food and water throughout the duration of the experiment. The experiment was stopped at 35 dpi and all birds were euthanized as stipulated by home office licencing. 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.

Virulence

To quantify the size of the conjunctiva and so severity of conjunctival swelling, we photographed the right and left eyes at 0, 6, 13 and 25 days post-inoculation (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 (Staley *et al*, 2018). Measurements of photographs were done blindly with respect to the isolate inoculated. Weighted averages of conjunctival swelling at time of transmission were calculated using quantitative conjunctival swelling data as above, where swelling on day of transmission was calculated from the linear interpolation of its two nearest data-points (e.g. swelling at 11 dpi interpolated from linear change between 6 dpi. and 13 dpi).

Finally, eyes were also inspected visually on days 3, 6, 8, 14, 21, 25, 28 and 34 post-infection: infection is considered lethal when the conjunctiva is red to purple, and the eye is difficult to see and produces discharge – such symptoms, with little or no vision possible, are thought to have caused the death of millions of infected finches due to starvation or predation (Adelman *et al,* 2017; Kollias *et al*, 2004; Roberts *et al,* 2001).

Statistical analyses

All statistical analyses were conducted in R 3.3.2 (Team 2016) and figures were made using ggplot2 (Wickham, 2009). In all analyses, transmission rate was natural log transformed to fit a normal distribution. We analysed changes in transmission rate over time using a linear model with transmission rate (log) as the response term and with year of pathogen sampling as the explanatory term.

Associations between transmission rate and virulence were analysed similarly with transmission rate (log) as the response term and with either quantitative measures of conjunctival swelling or putative host mortality as the explanatory term. Finally, for those isolates that caused putative host mortality, we tested the association between infection duration in the primary host and transmission rate to secondary host using a linear model with transmission rate (log) as the response term and days to putative mortality as the explanatory term.

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Appendix 1

Models examining the associations between bacterial load, replication rate and transmission rates are presented here (see Bonneaud, C., Tardy, L., Hill, G. E., McGraw, K. J., Wilson, A., Giraudeau, M., Experimental evidence for stabilizing selection on pathogen virulence, *in review*).

Methods and Results

Associations were measured using linear models with log transmission rate as the response term against explanatory terms of peak pathogen load, total pathogen load (the integral of peak pathogen load over the 34 day experiment), and replication rate (Peak pathogen load / days to peak pathogen load, see Chapter. 2).

Our results suggest no association between pathogen and transmission rate using either peak bacterial load or its integral over the experiment as explanatory variables (Peak pathogen load coef = $0.88e^{-4}$ +/- $0.41e^{-3}$, $F_{1, 41}$ = 0.22, p = 0.82, Total pathogen load coef = $0.60e^{-5}$ +/- $0.58e^{-4}$, $F_{1, 41}$ =0.1, P = 0.92). Similarly, no association between replication rate and transmission was found in these data (Replication rate coef = $0.20e^{-2}$ +/- $0.87e^{-2}$, $F_{1, 43,}$ = 0.24, p = 0.82)

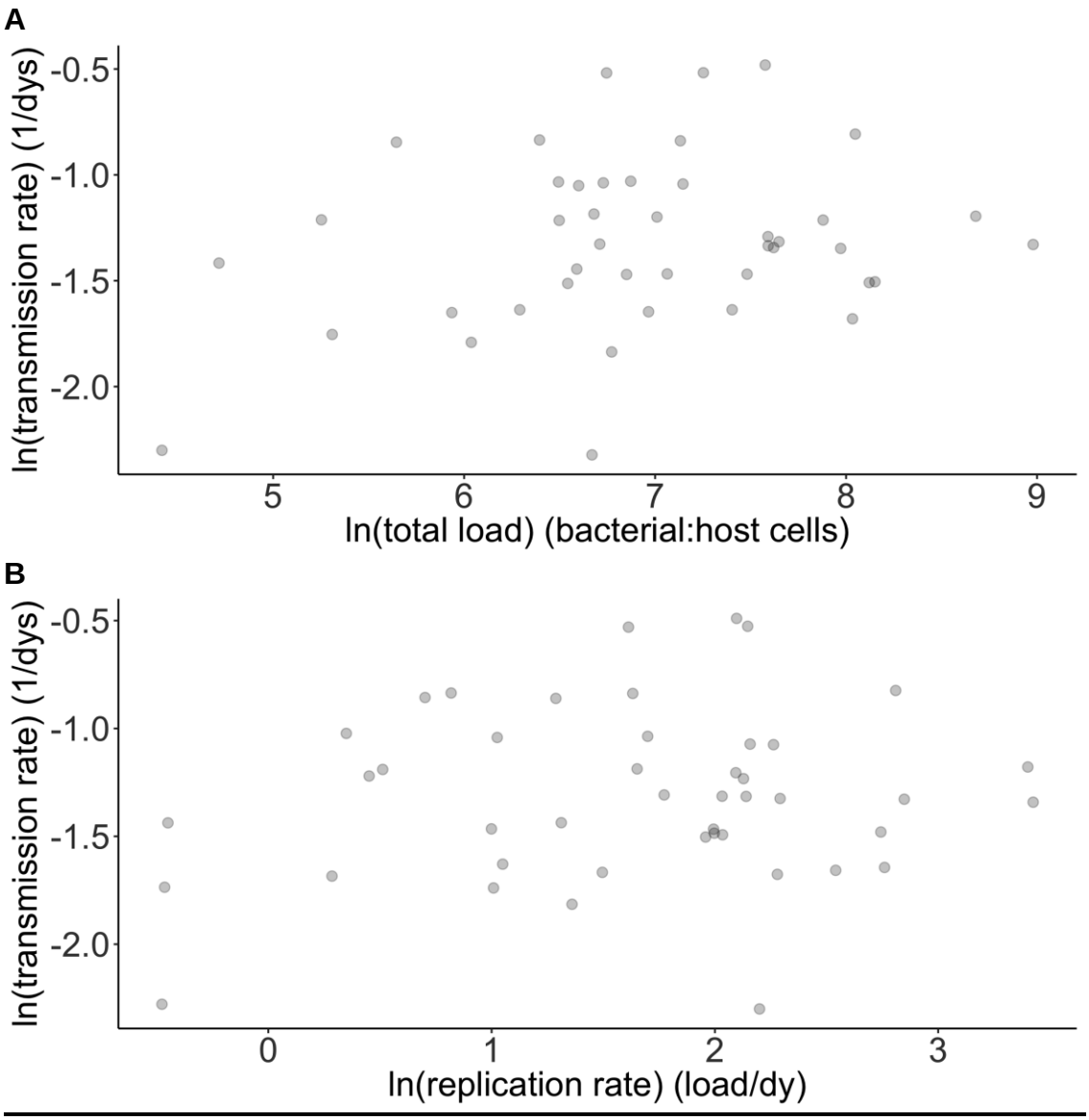


Fig. 1. Association between transmission rate and pathogen load. **(A)** Transmission rate as a function of pathogen load. **(B)** Transmission rate as a function of pathogen replication rate. Points represent raw data.

Chapter 5: Effect of resistance on pathogen transmission rate and virulence in secondary hosts

Abstract

Under the trade-off hypothesis we expect that host-resistance should select for higher virulence pathogens over time, as increased clearance should weed out those pathogen lineages which fail to reach transmissible levels at a sufficient rate. The result of this is that *in vivo* passage of an infectious pathogen in a resistant host will result in higher virulence lineages being transmitted preferentially onwards into new hosts, biasing the pathogen population in such secondary towards more virulent and faster transmitting lineages. In this experiment, we utilise samples from a large-scale experimental infection study in which the transmission rates of various *Mycoplasma gallisepticum* isolates and their virulence in primary and secondary hosts was measured. Birds from both susceptible (unexposed) and resistant (exposed) populations were inoculated with *M. gallisepticum* isolates from across the epizootic and housed alongside susceptible sentinel birds. Transmission rates and probabilities were determined by PCR detection of *M. gallisepticum* in these secondary (sentinel) birds from regularly taken samples during the experiment. Isolate virulence in these secondary hosts was also determined by measures of peak conjunctival swelling in the sentinels. Our results suggest that a single passage in a resistant versus naive primary host does not have a significant differential effect on either the probability of transmission occurring or on the virulence manifest by *M. gallisepticum* in a secondary infection in a susceptible host, indicating that the

selective effects of resistance on pathogen evolution may not be evident within a single infection.

Introduction

Host resistance to infectious diseases can have profound effects on pathogen evolution. Theoretical models predict that pathogens will maximise their success by maximising the number of secondary hosts that they infect over the time span of an infection. Because faster transmission to secondary hosts comes at the cost of greater exploitation rate of the primary host, which accelerates host death (i.e. virulence), pathogens should evolve a level of virulence that optimises the trade-off between infection duration and transmission rate. By clearing the pathogen, host immune activity will thus reduce the infectious period, thereby favouring pathogens that are able to transmit faster and are hence more virulent. While there is some experimental support for an effect of host resistance on pathogen virulence evolution, the speed at which resistance selects for increased virulence remains unknown. In particular, whether the infection of a primary host that is resistant is sufficient to increase pathogen virulence in secondary infections remains to be tested.

The impact of the level of resistance of the primary host on the virulence of secondary infections has been measured in the context of ‘leaky’ vaccines. Vaccines are considered leaky when they prevent host death from infection without preventing pathogen establishment, replication or transmission. Indeed, such vaccines will alleviate the mortality costs of high virulence, thus allowing increased virulence to evolve unchecked. For example, when chickens that were either unvaccinated or vaccinated with a leaky vaccine, were inoculated with

highly lethal strains of Marek's Disease Virus (MDV), transmission to an uninfected sentinel occurred successfully in the latter group only, with all the unvaccinated chickens dying before transmission (Read et al 2015). Furthermore, vaccination was found to have increased the transmission rate of a slightly less virulent strain of MDV. Together these results suggest that vaccination can allow the transmission of pathogens that would normally be too lethal to transmit, and that resistance can have a selective impact on pathogen virulence in the course of a single infection. Whether this finding is generalisable, in particular in the context of natural host responses to infection, remains to be determined.

The epidemic of the bacterial pathogen, *Mycoplasma gallisepticum*, in house finches (*Haemorhous mexicanus*) allows us to test the selective impact of host resistance over the course of single primary infection on the level of pathogen virulence during a secondary infection. *M. gallisepticum* emerged naturally in eastern North American house finches after a single host shift from poultry, which gave rise to a severe conjunctivitis outbreak that killed millions (Delaney et al. 2012; Dhondt *et al,* 1998). This intense selection event led to the evolution of resistance in disease-exposed house finch populations in less than 12 years (Gates et al. 2018; Bonneaud et al. 2011). The evolution of host resistance was found to have given rise, in turn, to an adaptive increase in pathogen virulence, with pathogen fitness increasing significantly as the pathogen was sampled progressively later in the epidemic relative to the host ((Bonneaud et al. 2018), Tardy et al).

Here, we inoculated 55 distinct *M. gallisepticum* isolates collected from epidemic outbreak, throughout the initial spread of host resistance and afterwards to present day (1994-2015) into resistant houses finches from disease- exposed

populations and into non-resistant (susceptible) ones from disease-unexposed populations. Each isolate was inoculated into at least one resistant and one susceptible house finch, and each experimentally inoculated finch was separately co-housed with one uninfected sentinel originated from unexposed populations. Using non-resistant sentinels allows us to test for changes in pathogen virulence and transmission rate without the confounding effects of protective immune activity (Barclay et al. 2012; Kerr et al. 2017; Mackinnon & Read 2004). Furthermore, using a large number of isolates obtained across the epidemic and of differing level of virulence (Bonneaud *et al,* 2018) protects against the effect of outlying isolates and allows test of the precise way in which pathogen traits have changed in response to the level of resistance of the primary host. Thus, our approach allows a novel test of the impact of variation in resistance in the primary host on: (i) pathogen transmission rate to secondary hosts; and (ii) the level of pathogen virulence in secondary hosts.

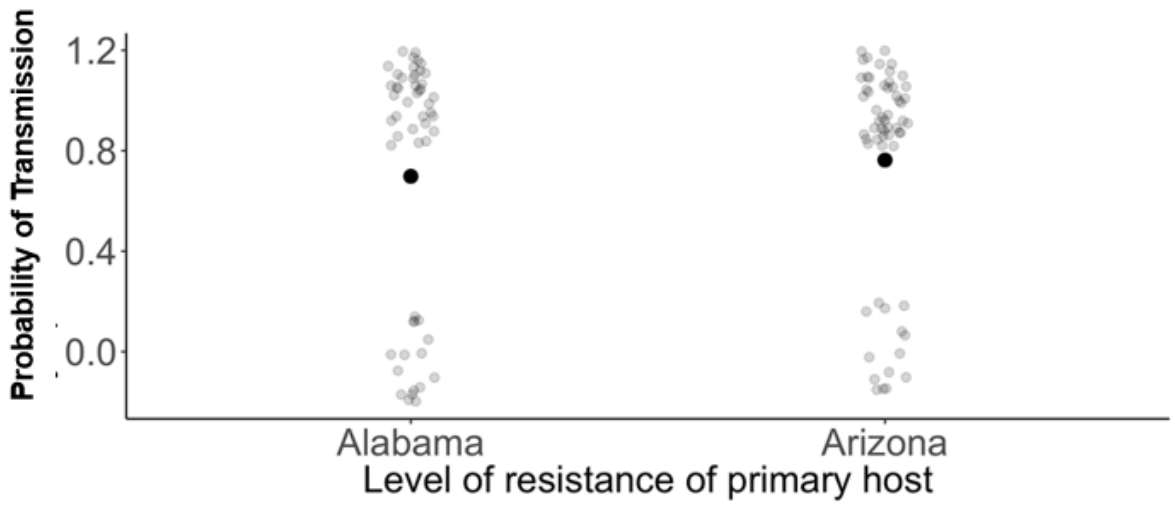
Results

First, we tested the effect of the level of resistance of the primary host on the pathogen's transmission rate to secondary host. The time to transmission from experimentally-infected primary hosts to uninfected sentinels varied between 2 and 23 days, with an average of 7 days. We found no effect of the level of resistance of the primary host on the time to transmission to secondary hosts (estimate $\pm$ se = -0.06 $\pm$ 0.12, χ^2 = 0.28, df = 1, p = 0.60). Second, we tested the effect of the level of resistance of the primary host on the pathogen's virulence in secondary host. Peak symptom severity in sentinel birds followed a zero-inflated distribution, with 27% (30/112) of inoculated finches remaining asymptomatic.

Consequently, we analysed peak symptoms using a 2-part hurdle model (Zuur & Ieno 2016). The first part tested whether the level of resistance of primary hosts had differential effects on the probability of the infection in secondary hosts overall. However, we found no evidence for an effect of the level of resistance of the primary host on the probability of that secondary hosts developed conjunctivitis (Figure 1A; 1st part of hurdle model: mixed effect logistic regression, estimate $\pm$ se = 1.2 $\pm$ 1.1, z = 1.06, p = 0.29). The second part of the model then tested for differences in the severity of the infection in secondary hosts that were symptomatic. We found only a non-significant marginal effect of the level of resistance of the primary host on the peak clinical severity of symptomatic secondary hosts, with peak severity increasing following infection of resistant primary hosts (Figure 1B; 2nd part of hurdle model: linear mixed effect model, estimate $\pm$ se = 0.17 $\pm$ 0.09, χ^2 = 3.4, df = 1, p = 0.067).

Tests of the symptom severity and resistance/tolerance to infection between Arizona and Alabama populations was investigated in Bonneaud *et al,* 2018.

A



B

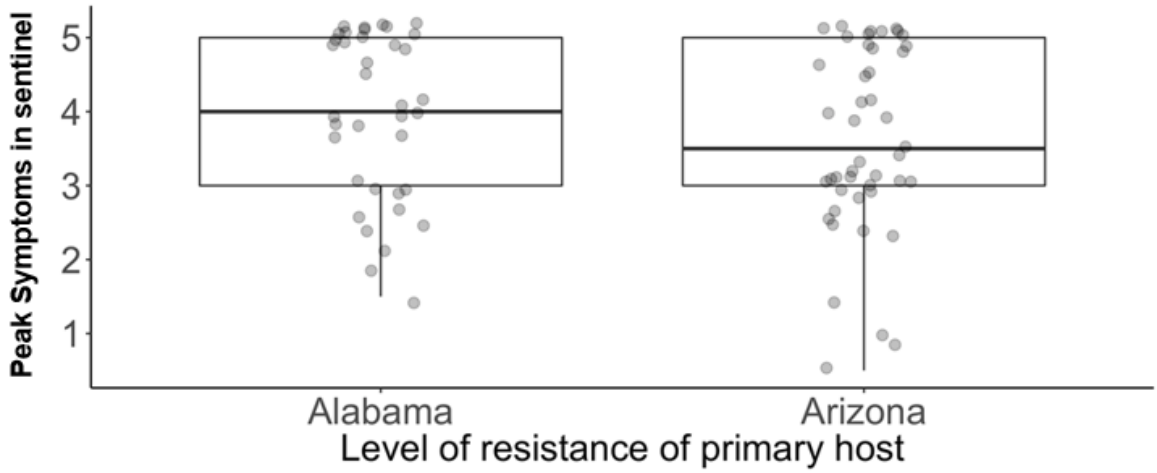


Figure 1. Symptom severity of sentinel. (**A**) We show the probability that the secondary host develops clinical symptoms of conjunctival swelling as a function of the level of resistance of the primary host. Grey points represent raw values; the larger blacks are predicted from the model. Grey points offset from actual for clarity of image. (**B**) Boxplot diagram showing the conjunctival swelling score in secondary host as a function of the level of resistance of the primary host. Depicted are the median and range symptoms score; points represent raw values.

Discussion

That we observed no significant different in either the probability of transmission or the peak virulence manifested in sentinels attributable to primary (inoculated) host resistance is perhaps not overly surprising given the complexity of the potential underlying mechanisms of these traits. The most parsimonious explanation for this null result is that simply one *in vivo* passage is insufficient for detectible differences in pathogen virulence in response to either the presence or absence of host resistance to emerge. Studies which have looked at the effect of limited *in vitro* versus *in vivo* passages on pathogen virulence, roughly approximating a non-resistant versus resistant environments have returned mixed findings. *Salmonella enterica* serovar Typhimurium virulence has been shown to increase in secondary infections following single passaging in mice, whilst decreasing in subsequent *in vitro* passaging (Mastroeni *et al*, 2011), whilst for *Mycobacterium tuberculosis* passaged in mice and guinea pigs no such increase in virulence over limited time-scales was observed (Converse *et al,* 2010).

Alternatively, we might construe the lack of expected increases in transmission probability and virulence for isolates passaged in resistant hosts to be linked phenomena, suggesting a trade-off in virulence, it's underlying mechanisms and transmissibility and infectivity. Our previous work has suggested that as host-resistance has increased over the epizootic, *M. gallisepticum* has evolved increased virulence and replication rates (Chapter 2), and the rate at which it transmits to secondary hosts (Chapter 4). *M. gallisepticum* also appears to be losing the ability to form biofilms over time in response to host resistance, potentially as a mechanism to maintain and increase its virulence

(Chapter 3). With this in mind, it may be that our lack of significant findings in this single-passage experiment reflect a scenario in which, although the rate at which transmission occurs will increase by selection in the resistant host, the infectivity of these more virulent lineages is somehow reduced. Principally, a lack of ability for more virulent isolates to produce biofilms may be reducing their rate of successfully establishing an infection, with lineages within a transmitted population which might express lower virulence but more colonisation factors (i.e. biofilm) being more successful on encountering the secondary host. The immunoevasive properties of biofilm formation have been studied in a number of host-pathogen systems, and some innate and cell-mediated host-immune processes are known to be inhibited by pathogens which readily form biofilm (Rolides *et al,* 2015); ergo we might posit that the lack of ability to form biofilms makes these more virulent lineages of *M. gallisepticum* in our experiment more likely to be detected and controlled or cleared by the early immune-responses of the secondary hosts. In the future, it might be possible to sequence the metagenome of our transmitted *M. gallisepticum* at the before, at the point of initial transmission, and then later within the secondary host, thereby unravelling the population genetics of what might be occurring at this transmission bottleneck.

Methods

Capture and housing

Wild house finches from populations that have never been exposed to *M. gallisepticum* (i.e. that have not evolved genetic resistance) were captured in variety of urban and suburban sites in Arizona, in the summer 2015 (N = 118, 64

males and 54 females). *M. gallisepticum* has never been recorded in the sampling area despite continuous monitoring (Staley *et al,* 2018). Using birds that have not had the opportunity to evolve protective immune responses to *M. gallisepticum* is essential to measuring virulence and transmission rate without the confounds of the capacity for immune clearance (Kerr et al. 2017). Birds 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 indoor aviaries at Arizona State University's Tempe campus, where they were housed for the remainder of the experiment. On arrival, we obtained a blood sample from all birds using brachial venipuncture (60 µL 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 assay (Luttrell et al. 1996), and a lack of current infection was verified using the choanal swabs in PCR amplification of *M. gallisepticum* DNA (Roberts et al. 2001). Concurrently, during the summer of 2015, wild house finches from adapted populations where *Mycoplasma gallisepticum* has circulated from the outset of the epizootic, were captured in urban and suburban parts of Alabama. They were similarly screened for current or prior *Mycoplasma gallisepticum* infection via serum plate agglutination and PCR assays (Luttrell *et al,* 1996; Roberts *et al,* 2001), and birds testing positive for either current or prior infection were released immedietly. The remainder (N = 53; 24 males and 29 females) were transported to and housed seperately to those collected from unexposed Arizona populations at the indoor aviary facilities of Arizona State University. Although none of the birds displayed any sign of infection with other diseases, all birds were prophylactically medicated for infection with *Trichomonas gallinae* with

carnidazole (Spartrix, Janssen/Elanco) and *Isospora spp* with sulfadimethoxine over 40 days, following which birds from both populations were allowed to acclimate for one month prior to experimental onset and provided with *ad libitum* food and water throughout.

Experimental inoculation and transmission rate

Birds were then assigned to groups so that all 53 birds from the resistant Alabama population would be housed with one bird from the unexposed Arizona population, making (N = 53) randomly assigned male-female pairings. The remaining 118 unexposed population finches were then divided into experimental and sentinel male-female pairs and 5 male-male pairs (total N = 59)

One of 55 *M. gallisepticum* isolates sampled over the course of the epidemic was inoculated into one bird selected at random from both groups. In the unexposed-unexposed paired group containing 59 birds (30 males and 29 females), 5 isolates (one from 1994, two from 1995, and two from 2007) were each inoculated in two birds. All 53 Alabama-caught resistant birds from the resistant-unexposed paired group were similarly inoculated with one of the 55 isolates selected at random.

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 8 different States in the eastern US (mainly from Alabama). Isolates were administered via 20 µl of culture containing 1×10^4 to 1×10^6 colour changing units/mL of *M. gallisepticum* in both eyes. Following inoculation, all 59 birds were maintained in separate cages and co-housed with one bird from the second group in 54 randomly-assigned female-

male pairs and 5 male-male pairs. Birds from the second group thus served as an uninfected sentinel. Transmission rate to the sentinel was measured by amplification of *M. gallisepticum* DNA from conjunctival and tracheal swabs (Roberts *et al,* 2001) obtained at 2, 3, 4, 5, 6, 7, 8, 11, 14, 17, 20, 23, 26, 29, 32 and 35 days post-inoculation (dpi). All birds had access to *ad libitum* food and water throughout the duration of the experiment. The experiment was stopped at 35 dpi and all birds were euthanized as stipulated by home office licencing. 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.

Virulence

To quantify the size of the conjunctiva and so severity of conjunctival swelling, we photographed the right and left eyes at 0, 6, 13 and 25 days post-inoculation (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 (Staley *et al,* 2018). Measurements of photographs were done blindly with respect to the isolate inoculated. Finally, eyes were also inspected visually on days 3, 6, 8, 14, 21, 25, 28 and 34 post-infection: infection is considered lethal when the conjunctiva is red to purple, and the eye is difficult to see and produces discharge – such symptoms, with little or no vision possible, are thought to have caused the death of millions of infected finches due

to starvation or predation (Adelman *et al,* 2017; Kollias *et al,* 2004; Roberts *et al,* 2001).

Statistical analyses

All statistical analyses were conducted in R 3.3.2 (Team 2016) and figures were made using ggplot2 (Wickham, 2009). Transmission rate was natural log (ln) transformed to fit a normal distribution. We analysed the effect of the level of resistance of the primary host on transmission rates to secondary hosts using a linear mixed model with transmission rate (ln) as the response term, with the level of resistance of the primary host (resistant *versus* non-resistant) as the explanatory term, and with isolate identity as the random term. Peak symptoms in secondary hosts followed a zero-altered gamma distribution (ZAG) and was therefore analysed using 2-parts hurdle model (Zuur & Ieno 2016). Part 1 used a mixed effect logistic regression with a logit link function on whether the secondary host failed or not to developed symptoms; part 2 used a linear mixed effect model with gamma error structure and log link function on the values of symptoms scores > 0 (Zuur & Ieno 2016). In both steps, symptoms score was fitted as the response term (score = 0 *versus* score >0 in the former; scores > 0 values in the latter), the level of resistance of the primary host (resistant *versus* non-resistant) was fitted as the explanatory term, and pathogen isolate identity as a random effect.

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Concluding statement

The work conducted under this project and presented above has, by employing an integrative molecular microbiology approach furthered both our understanding of the co-evolution of *M. gallisepticum* and its novel host in the house finch, and more generally has provided a system in which to test some key assumptions of the virulence-transmission trade-off hypothesis. We find strong support for many of the key underlying assumptions of the trade-off hypothesis, including the predicted linkage between virulence and transmission (Chapter 4) and the increase in pathogen virulence in response to emerging host-resistance (Chapter 1). However, our research also highlights that many of the assumptions we might make about the molecular underpinnings of these traits can be often oversimplified. An assumption of virulence being driven solely by pathogen replication, and of the later being thus a decent proxy for the former, was shown in Chapter 1 to hold perhaps for only the early stages of co-evolution in novel host-pathogen associations, with selection for increased replication easing off after significant host resistance emerges whilst virulence continues to increase.

In Chapter 2 we investigated whether a complex trait such as biofilm formation might contribute to this unexpected observation, indicating as we suspected that this dis-linkage between virulence and replication could perhaps be due to selection shifting to optimise the immune-manipulative strategies employed by *Mycoplasmas*. That we saw a decrease in biofilm forming capability in *M. gallisepticum* over time was quite opposite to our initial predictions; we would have expected that host-resistance might select for enhanced immunoevasion and utilising biofilms to produce a protective environment. Our finding contrary to this is in interesting one; raising the prospect that we are in

fact observing a selection against biofilm formation as a pro-inflammatory immunomodulatory strategy, increasing the immuno-visibility of late epizootic *M. gallisepticum* isolates in order to maintain the high virulence needed for optimal transmission.

This observation also helps to contextualise our results in Chapter 5 in which we detected no significant increase in either transmission probability or virulence in a secondary infection in response to host resistance. Although we postulate that the most likely explanation for this is simply that a single passage in a resistant host provides insufficient time for selection to generate a discernable signal, the secondary explanation mentioned – that the adaptations in *M. gallisepticum* which arise in response to host resistance might have some detrimental affects on its ability to initially infect and colonise after transmission, also bears further consideration.

In addressing this, the author considers that future work should focus on the molecular biology of *M. gallisepticum* infection dynamics, particularly regarding transmission traits, on a more granular scale. For example breaking transmission down into stages of i) escape from the primary host, ii) ability to persist as fomites and iii) ability to infect and colonise a secondary host, would allow for relatively simple experiments to be designed targeting specific biological determinants at each stage. One way to approach this work would certainly be to apply a genomic, transcriptomic and gene-expression focused direction of study; identifying genes which have accumulated mutations or which are differentially expressed under the different conditions found at these more defined stages. We are confident that, in addition to its important findings presented in this thesis, this project has further helped to lay the groundwork for such future undertakings.