Developments in Social Evolution and Virulence in Parasites

Helen C. Leggett

DPhil

Linacre, University of Oxford

Trinity 2013

Declaration

I declare that this thesis was composed by myself, that the work contained herein is my own except where explicitly stated in the text. This work has not been submitted for any degree or professional qualification except as specified.

Helen C. Leggett, Trinity 2013

For Ollie

Acknowledgements

The work presented in this thesis benefited from the comments, discussion, collaboration and encouragement of many people, including: Mike Boots, Geoff Wild, Charlie Cornwallis, Dave Hodgson, Sarah Reece, Sam Brown, Kevin Foster, Ashleigh Griffin, Britt Koskella, Janis Antonovics, Kurt & Linda Lively, Xav Harrison, Gráinne Long, Andy Morgan, and Alex Hall. This thesis would have been no-where near as rewarding without such fantastic people: Chaz – Queen of the Onion Rings Malhi, Tiffy – Tiny Taylor, Britt Koskella & Smudge, Sole & Pedro Gomez-Lopez, Leena & Ville Friman, Andy Morgan, Alex Hall, Pauline Scanlan, Daniella & Pawel Sierocinski & Scorcio, Mick Voss & Elze Hesse, Natalie Jiricny, Ben Quigley, Antonio Rodrigues, Gabe Guimond-Peron, Fred Inglis, Lee Henry, Freya Harrison, Claire El Mouden, Clara Torres-Barceló, JoãoAlpedrinha, Lorenzo Santorelli, Melanie Ghoul, Berti Fisher, Siobhan O'Brien, Sheridan Willis, Fran Tyler, Mel Smee, Chris Beirnes, Phil Doherty, Kat Roberts, Amber Teacher & Dave & Oskar, Camille Bonneaud, Floh Bayer & Leena Wilfret, Sean Meaden, Martin Grunelli, Corinna Lowry, and M.D. Sharma.

In particular, I whole-heatedly thank my supervisors, Angus and Stu, for giving me complete freedom during these four years, yet remaining firmly supportive; offering expert supervision and advice throughout; and for being there, both personally and professionally, when the going got tough. They are two in two million! They have been, and continue to be, my strength and inspiration.

I thank the Biotechnology & Biological Sciences Research Council for providing me with my studentship, Linacre College, and the University of Exeter's Cornwall Campus

for giving me premium lab and office space for two years. Even if we were flooded and struck by lightning. Twice. What are the chances?![1]

Special thanks are due to Harrison for being there when it all went wrong.

Finally, I thank my parents Colin and Dilys, and Andy and Ollie for their unwavering encouragement and support. I couldn't have done this without them.

[1] Chance of lightning striking Angus's lab twice: 1/360,000,000,000.

Publications

The following published papers have arisen from this thesis, and are presented in Chapters 2, 3, and 5:

- Leggett H.C., Buckling A., Long G. H. & Boots M. 2013. Generalism and the evolution of parasite virulence. Trends in Ecology and Evolution 28(10): 592-596
- Leggett H.C., Benmayor R., Hodgson D.J. & Buckling A. 2013. Experimental Evolution of Adaptive Phenotypic Plasticity in a Parasite. Current Biology 23: 1-4
- Leggett H.C., Cornwallis C.K. & West S.A. 2012. Mechanisms of Pathogenesis, Infective Dose and Virulence in Human Parasites. PLoS Pathogens 8(2): e1002512

The unpublished chapters 1, 4, 6 and 7 are collaborative efforts, though in each case the majority of the work is my own. I have combined chapters 1 and 7 into a single manuscript with additional contributions from S. Reece and S. Brown who helped write the manuscript. This manuscript entitled 'War and peace: social interactions in infections' is in review at *Philosophical Transactions B*, and is included in the Appendix. In chapter 4, C. Cornwallis analysed the data and S.A. West and A. Buckling contributed to the interpretation of results and the manuscript. Chapter 6 is my own work, and was supervised by A. Buckling and S. West, who contributed to the experimental design, interpretation of results and manuscript.

I contributed to three other published projects that are included in the Appendix:

∞ Hall A.R., Miller A.D., Leggett H.C., Roxburgh S.H, Buckling A. & Shea K. 2012. Diversity-disturbance relationships: frequency and intensity interact. Biology Letters 8(5): 768-771

∞ Hall A.R., Scanlan P.D., Leggett H.C. & Buckling A. 2012. Multiplicity of infection does not accelerate infectivity evolution of viral parasites in laboratory microcosms. Journal of Evolutionary Biology 25: 409-415

∞ Leggett H.C., El Mouden C., Wild G. & West S.A. 2011. Promiscuity and the evolution of cooperative breeding. Proceedings of the Royal society B Biological Sciences rspb20111627

Abstract

The study of social evolution and virulence in parasites is concerned with fitness consequences of trade-offs between parasite life history traits and interactions between parasite species and/or genotypes with their hosts. I develop our understanding of social evolution and virulence in parasites in several ways. (1) I review empirical evidence for the fundamental predictions of virulence-transmission trade-off theory and demonstrate that the fit between theory and data is primarily qualitative rather than quantitative; that parasites differ in their degree of host generalism, and this is likely to impact virulence in four ways. (2) I take a comparative approach to examine the underlying causes of an observed statistical variation in the size of parasite infectious doses across taxa, revealing that mechanisms used by parasites to infect hosts are able to explain variation in both infectious dose and virulence. (3) I formally compare data on human pathogens to explain variation in virulence across taxa, revealing that immune subversion and not growth rate, explains variation in virulence. This allows me to predict that immune subverters and not fast growing parasites are likely to cause the most virulent clinical infections. (4) Using bacteria and their naturally infecting viruses (bacterio**phage**), I take an experimental approach to investigate the consequences of coinfection for parasite life history traits, and find that viruses cultured under a mix of single infections and coinfections evolved plasticity; they killed hosts more rapidly when coinfecting, and this resulted in high fitness under both single infection and coinfection conditions. (5) I experimentally investigate how selection within and between hosts and patches of hosts affects the fitness and virulence of populations of these viruses. I find that limited host availability favours virulent, faster killing parasites with reduced transmission; suggesting high, rather than low, virulence may be common in spatially structured host-parasite communities.

Contents

Foreword

I do not provide a detailed review of the literature in the introductory chapter because I provide reviews of the relevant topics in Chapters 2-6.

“A little bit of nonsense now and then

is relished by the wisest of men.”

- *Roald Dahl*

1. Introduction

Evolvable social actions: definitions and classification

Sociality is ubiquitous throughout the domains of life (Maynard-Smith 1995; West *et al.* 2007; Bourke 2011). Classically, all social behaviours are categorised according to their impact on the reproductive success of the 'actor' and any 'recipients' (Figure 1, Hamilton 1964; Trivers 1985). The categories are: (1) *mutual benefit*, where actor and recipient mutually benefit directly from the behaviour (West, Griffin & Gardner 2007); (2) *selfishness*, where the actor gains at the expense of the recipient; (3) *altruism*, where the behaviour is detrimental to the actor but beneficial for the recipient; and (4) *spite*, where the behaviour is harmful for both actor and recipient. Examples of mutual benefit include cooperative colony founding by queen ants and cooperative foraging by pelicans (Dugatkin 2002; Clutton-Brock 2009; Bourke 2011). Selfishness is manifested in aggressive behaviours such as infanticide by incoming male domestic cats and lions (Pontier & Natoli 1999; Packer & Pusey 1984). Altruism is found in the non-reproductive zooids of marine invertebrates such as siphonophores, which aid the reproduction of the reproductive zooids (Dunn 2009). And examples of spite include the production of costly anti-competitor toxins in bacteria (Massey, Buckling & French-Constant 2004; Inglis *et al.* 2009).

Inclusive fitness theory is our most general theory of adaptation, and describes the conditions required for a gene for any of the four social behaviours to spread through a population, i.e. to undergo natural selection. A key parameter in inclusive fitness theory is genetic relatedness, which means the probability that a gene in one individual is present in another individual. Kinship is the usual reason for individuals to

share genes with above-average frequency in a population. Consequently relatedness can be understood as the chance of gene-sharing brought about by being kin, over and above the average chance (Bourke, 2011). Inclusive fitness partitions natural selection into direct and indirect efforts in which direct effects describe the impact of an individual's own genes on reproductive success; and indirect effects describe the impact of the focal individual's genes on the fitness of its social partners, weighted by genetic relatedness (Hamilton 1964, 1970). Formally, the inclusive fitness approach leads to Hamilton's rule (Hamilton 1964, 1970), which states that a trait will be favoured by natural selection when $-c + br > 0$ (where $-c$ is the direct fitness cost of the trait, b is the benefit provided to social partners by the trait and r is the genetic relatedness between the focal individual and its social partners).

Overall inclusive fitness theory has remarkable generality, versatility, and fit to data, rendering it the foremost candidate for general theory of adaptation (Trivers 1985; Bourke 2011; Bourke & Franks 1995; Lehmann & Keller 2006; West, Griffin & Gardner 2007; West & Gardner 2013). For these reasons, inclusive fitness theory will underpin the conceptual reasoning used throughout this thesis. In Leggett *et al.* (2012, appended) I explore how high relatedness favours cooperation when the major benefits of cooperating are accrued by siblings.

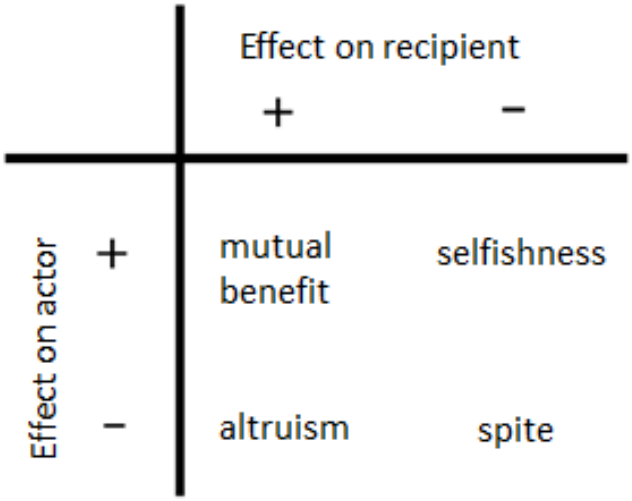


Fig. 1. A classification of social behaviours, after (Hamilton 1964; West, Griffin & Gardner 2007; Hamilton 1970)

Social and virulence evolution in microorganisms

Microorganisms can communicate and cooperate to perform a wide range of social behaviours such as dispersal, foraging, biofilm formation, reproduction, chemical warfare and signalling (reviewed by Crespi 2001; Velicer 2003; Webb, Givskov & Kjelleberg 2003; Keller & Surette 2006; Kolter & Greenberg 2006; West *et al.* 2006; Diggle *et al.* 2007; Foster, Parkinson & Thompson 2007; Hense *et al.* 2007; Williams *et al.* 2007; Brown & Buckling 2008; MacLean 2008; Popat, Crusz & Diggle 2008; West *et al.* 2007). Recent years have seen growing interest in understanding social evolution in microbes, probably for two important reasons: (1) microbes are tractable experimental systems for studying the ecology and evolution of social traits under controlled conditions in real time, (2) microbial sociality is critical to determining the

damage caused by parasites to their hosts (virulence), and hence has important applied implications (Buckling & Brockhurst 2008).

Social interactions between conspecific parasites are partly dependant on the relatedness of interacting parasites, which in turn, is predicted to affect virulence. High relatedness is generally assumed to favour less competitive interactions. However the relationship between relatedness and virulence is dependent on the social behaviour in question (Figure 2, Buckling & Brockhurst 2008). If prudent use of host resources (a cooperative behaviour) maximises the transmission success of the parasite population, decreased relatedness is predicted to cause increased host exploitation and virulence (Figure 2a; (Bremermann & Pickering 1983; Frank 1996, 1998; Hardin 1968; Nowak & May 1994). However if parasite within-host population growth rate is a positive function of the production of public goods (another cooperative behaviour), virulence is expected to increase with increasing relatedness (Figure 2b, Chao *et al.* 2000; Brown, Hochberg & Grenfell 2002; West & Buckling 2003). Furthermore, spiteful behaviours are optimised at intermediate levels of relatedness, leading to lower virulence due to the reduced growth rate of the infecting parasite population (Figure 2c, Buckling & Brockhurst 2008; Gardner, West & Buckling 2004).

These relationships can be altered by other factors such as the spatial structure of the parasites. Parasite population structure and multiplicity of infection matters since it determines the relatedness between interacting parasites, and hence the extent to which they should cooperate or exploit each other (Inglis *et al.* 2011; Griffin, West & Buckling 2004; West, Pen & Griffin 2002; Buckling & Brockhurst 2008). However there is a lack of empirical work in this area. Consequently, the main focus of this thesis

is the role of inclusive fitness on the evolution of social interactions between parasites, and the impact on virulence evolution.

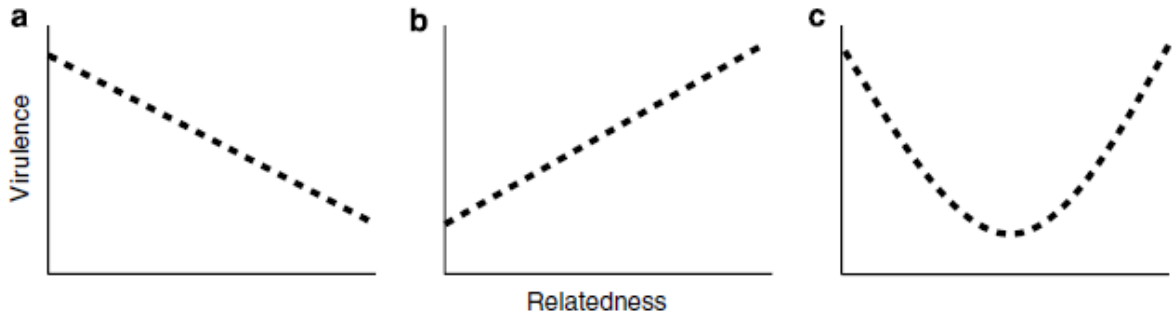


Fig. 2. Theoretical relationships between virulence and relatedness under conditions of: (a) prudence; (b) public goods cooperation; (c) spiteful interactions, after Buckling & Brockhurst (2008).

Developments in Social Evolution and Virulence in Parasites

In this thesis I will touch on and develop the above themes. Specifically:

Chapter 2: Parasites vary enormously in the range of hosts they can infect (generalism), both within and between species (Antonovics *et al.* 2013). The interplay between generalism and virulence is little studied, but likely to be crucial. I review the interaction between four factors associated with generalism – costs of generalism, multiplicity of infection, maladaptive virulence and host availability – to show that these biological details provide novel hypotheses for furthering our understanding of the evolutionary ecology of virulence.

Chapter 3: I then move to examine another context where biological details seem to matter – the infectious dose of pathogens, which varies dramatically across species. In a comparative study, I formally test the verbal argument that variation in infectious dose can be explained by the different biochemical mechanisms that pathogens use to infect hosts. I show that in pathogens where the molecules secreted to facilitate infection acted locally, the infectious dose was lower than in pathogens where the secreted molecules act more distantly; but parasite virulence shows no correlation with this local versus distant action. More generally, this highlights how broad-scale formal comparative analysis can explain variation in pathogen traits.

Chapter 4: I extend the comparative analysis above to formally test the standard assumption that increased parasite growth leads not only to greater transmission, but also greater host mortality (Frank 1996; Anderson & May 1982). I show that immune subversion, not parasite growth rate explain variation in case fatality rates among human pathogens, and answer some recent criticisms of how classical trade-off models have been relatively unsuccessful in explaining broad scale variation across parasite species.

Chapter 5: I take an experimental approach to investigate the real-time evolution of parasite life history strategies in response to coinfection. I show that viruses cultured under alternating single and co-infections evolved *de novo* adaptive phenotypic plasticity. Virulence was increased in coinfections, and this resulted in high fitness. This sheds some light on the important consequences of plasticity for the epidemiology of infectious diseases and the evolution of cooperation.

Chapter 6: I experimentally test prudence theory and the evolution of parasite virulence in viruses infecting their host bacteria. I show limited host availability and, to a lesser extent, low within-host relatedness favours virulent, faster killing parasites with reduced

transmission. This highlights the importance of studying host-parasite interactions at multiple scales and suggests high, rather than low, virulence may be common in spatially structured host-parasite communities.

2. Generalism and virulence*

Me, on the first day of my DPhil, October 2009. Polaroid taken by Alex Hall

* Published as: Leggett H.C., Buckling A., Long G. H. & Boots 2013. Generalism and the evolution of parasite virulence. Trends in Ecology and Evolution 28(10): 592-596 (appended)

Abstract

The evolution of parasite-imposed host harm (virulence) will be affected by numerous factors, not least the range of hosts that parasites can infect. Here, we consider four ways parasite host range (generalism), might directly affect observed levels of parasite virulence: (i) costs of generalism, (ii) multiplicity of infection, (iii) maladaptive virulence and (iv) host availability. Integrating parasite infectivity range with life history evolution will generate novel general hypotheses for the evolutionary ecology of virulence, as well as explicit predictions about the virulence of emerging diseases resulting from host shifts.

Introduction

Parasite virulence-transmission trade-offs

Understanding the evolution of parasite virulence (which we define as reductions in host fitness due to parasite infection) is critical to our understanding and potential control of infectious disease. A key assumption for many parasites is that virulence has negative consequences for parasite fitness because it reduces host lifespan, but is an unavoidable consequence of within-host parasite replication, which is crucial for transmission to new hosts and for avoiding host immune system clearance (Anderson & May 1981, 1982). Parasites therefore trade-off their transmission period (host life span) against their transmission rate, with the optimal level of virulence being that which maximises total transmission (Anderson & May 1982; Frank 1996). This virulence-transmission trade-off asserts three fundamental predictions: (i) that

increasing within-host parasite replication increases virulence (Figure 1a); (ii) that increasing within-host parasite replication increases the number of parasite transmission events over the duration of the infection (Figure 1b), until a point where increasing parasite replication reduces the infectious period – for example, by killing the host and preventing transmission (Figure 1c); and (iii) that increasing parasite virulence increases parasite transmission (Figure 1d) until high virulence shortens the infectious period, thereby reducing transmission (Figure 1e) (Anderson & May 1982; Frank 1996). Table S1 (appended) summarises empirical evidence for the three fundamental predictions of the trade-off theory. While these studies provide support for the parasite trade-off theory for virulence evolution (Lipsitch & Moxon 1997; Froissart *et al.* 2010; Alizon *et al.* 2009), the fit between theory and data is primarily qualitative rather than quantitative. A possible explanation for this is that the predictions of virulence theory can depend on the life-history and the mechanism used by a given parasites to infect and exploit hosts, which are not captured by classical trade-offs. Notably, the range of hosts the parasite can infect is a ubiquitous life history trait that is likely to be one of the most important determinants of virulence, but it is little studied in the discussion so far. Here we discuss how incorporating parasite host range into current theory can greatly improve our understanding and enhance the use and accuracy of testable predictions about virulence evolution.

Parasite infectivity: generalism versus specialism

Parasites vary massively in the range of hosts they can infect (generalism), both within and between species (Antonovics *et al.* 2013). Although the causes and consequences of variation in parasite infectivity ranges have been studied (Futuyma & Moreno 1988; Poulin 2007; Dybdahl & Storfer 2003; Barrett *et al.* 2009), the interplay between

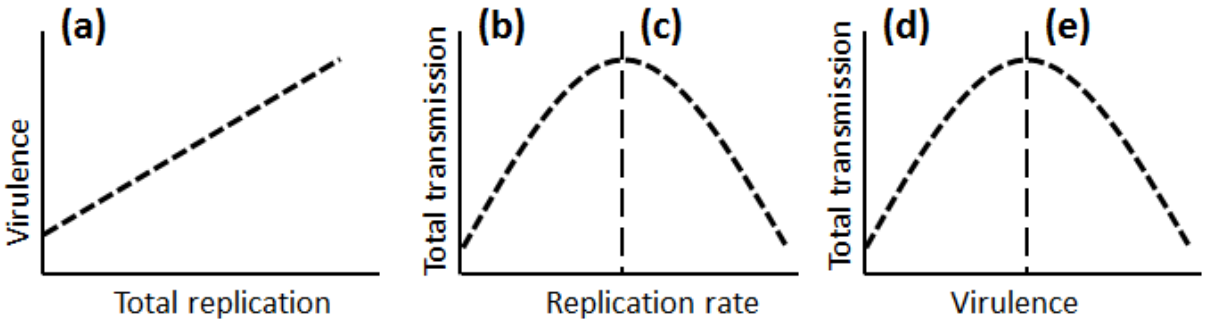


Fig. 1. Schematic of the three fundamental assumptions of parasite life history trade-off theory. First, increasing within-host parasite replication increases virulence **(1a)**. Second, increasing within-host parasite replication increases the number of parasite transmission events over the duration of the infection **(1b)**, until a point where increasing parasite replication reduces the infectious period – for example, by killing the host and preventing transmission **(1c)**. Third, increasing parasite virulence increases parasite transmission **(1d)** until high virulence shortens the infectious period, thereby reducing transmission **(1e)** (Anderson & May 1982; Frank 1998). Empirical data for these hypotheses are shown in Table S1 (appended).

parasite infectivity and virulence (as discussed above) has rarely been considered. This interplay is likely to be crucial: the virulence model discussed above assumes that high within-host replication (and therefore virulence) will lead to a high probability of infecting new hosts. However, if parasite genotypes differ in their ability to infect different host species and genotypes, parasite productivity from a host will not always be simply proportional to infectivity of new hosts. Moreover, the degree of generalism is likely to trade-off against other traits, such as growth rate, and will also affect the ecological contexts experienced by parasites, all of which may affect virulence. Note that while generalist parasites can be usefully described as parasites with low variance in infectiousness over different host genotypes and/or species, defining generalism can

be complicated when considering parasites with complex life cycles involving multiple hosts (Box 1).

Here we discuss how the degree of generalism is likely to affect the evolution and ecology of virulence. Integrating parasite generalism with life history evolution will generate novel general hypotheses for the evolutionary ecology of virulence, as well as explicit predictions about the virulence of emerging diseases resulting from host shifts (Woolhouse, Haydon & Antia 2005). We focus on four key processes: (i) costs of generalism, (ii) multiplicity of infection, (iii) maladaptive virulence and (iv) host availability. Note that a detailed discussion of the evolutionary processes that determine whether parasites evolve to be specialists or generalists and the impact of specialism on the generation and maintenance of parasite diversity, is beyond the scope of this article.

I. Costs of generalism

Natural populations of parasites often infect and transmit from multiple hosts and there are likely to be costs associated with infecting novel hosts (Woolhouse *et al.* 2001; Benmayor *et al.* 2009; Parrish *et al.* 2008). Fitness costs associated with generalism can arise for two biologically plausible reasons. First, parasite replication or transmission of generalist parasites in novel hosts might be reduced, in a scenario analogous to optimal foraging theory where resource patches vary in quality (an ecological cost to generalism) (Benmayor *et al.* 2009). Second, the ability to infect novel hosts can be associated with reduced fitness in the original hosts (an evolutionary cost to generalism) (Benmayor *et al.* 2009). As a result, a trade-off between reproduction within a given

Box 1. What is a generalist parasite? Generalism and complex life cycles

The majority of parasites are generalist parasites, capable of infecting more than one host species (Woolhouse *et al.* 2001). We define generalism versus specialism on the basis of respective low versus high variance in infectiousness across different hosts. This is in-keeping with the infectivity versus non-infectivity distinction that is advocated by phytopathology societies (Flor 1956). We suggest that while in principle generalism should be a numbers game whereby the most generalist parasites are on average the most infectious over wider host ranges; this is not necessarily the case for parasites with complex life cycles. In this scenario, it is important to consider the details of the parasite's exploitative specificity. For instance, among vectored parasites, definitions of the "vector" and "definitive host" ascribe functions to effectively two potential hosts (Elliot *et al.* 2003). Selection in both hosts affects virulence evolution (Gandon 2004), and it has been suggested that the evolution of virulence and host specialism are mediated by the same selective factors (Garamszegi 2006). Importantly this might explain why the perceived advantages of parasite specialism are outweighed by those of generalism: vectored parasites are not fixed strategy generalists, rather they are effectively phenotypically plastic specialists; and so there is no reason that we should expect them to show large fecundity costs of generalism.

In some sense, generalist and vectored parasites can be more specialised than specialist and directly transmitted parasites. For example, for the agent of plague, *Yersinia pestis,* the conditions it faces within the flea (vector) digestive tract are unique from those encountered in the mammalian host. Consequently the development of a transmissible infection requires a distinct subset of genes, with none of the tested mammalian virulence factors being required in fleas (Hinnebusch 2005). This idea can be extended to biological- and mechanical- vector transmission: since mechanically-vectored parasites simply persist, for example, on the mouthparts of their vectors, and do not *per se* infect and exploit their vector's resources, they require fewer specialisations for effective transmission than biologically-vectored parasites. Similarly, facultative pathogens, such as *Vibrio cholera* and *Staphylococcus aureus*, which tend to express virulence-associated genes only under the high density conditions associated with infection (Brown *et al.* 2012; Miller *et al.* 2002; Chan *et al.* 2004), can be viewed as more specialised than purely opportunistic pathogens - characterised as organisms that become pathogenic following a perturbation to their host - such as *Pseudomonas aeruginosa*, and *Streptococcus pneumonia*, who are "super-generalists" capable of infecting across different environments and hosts, and thus might be less specialised and even maladapted to their hosts.

host and increasing host range is predicted, such that specialist parasites replicate faster (causing more virulence), and transmit more than generalist parasites. Empirical evidence does indeed suggest that more-virulent parasites have narrower host ranges than less-virulent parasites (Agudelo-Romero & Elena 2008; Garamszegi 2006). It is also important to emphasise that these costs of generalism also apply *within* species, i.e. the ability to infect multiple host genotypes (Legros & Koella 2010; Poullain *et al.* 2008), not just different species.

If parasites adapt to infect heterogeneous hosts, it is plausible that costs of generalism will affect virulence evolution such that parasites cannot replicate well (and cause high virulence) in multiple hosts (Regoes *et al.* 2000; Gandon 2004). For instance, if a parasite grows rapidly in one host to overcome host defences, but in an alternative host the same fast growth kills the host rapidly, virulence can be optimal in one host and suboptimal in another. This has been shown in a study of viruses across multiple plant species, where viruses with lower host ranges were found to be more prevalent (Malpica *et al.* 2006). However no costs of generalism were detected among Tobacco etch potyvirus infecting four natural hosts (Bedhomme *et al.* 2012). Similarly there is a positive correlation between avian malaria host range and prevalence, which suggests that a 'jack-of-all-trades can be a master of some' (Hellgren *et al.* 2009). An explanation for this is that higher overall encounter rate for parasites with wider host ranges can compensate for the potentially reduced fitness of such generalist parasites in each host species (Hellgren *et al.* 2009; Rigaud *et al.* 2010).

Generalist parasites that are phenotypically plastic can have an advantage since plasticity can mitigate costs of generalism resulting from antagonistic pleiotropy (Futuyma & Moreno 1988). Mechanisms such as altering gene expression can allow parasites to change their behaviour to fit different environments (hosts), subsequently

improving the parasite's fitness (Schlichting & Pigliucci 1998). If parasites intermittently encounter new or different hosts, then evolution in the face of an important trade-off can select for plasticity (Brown *et al.* 2012; Stearns 1989; Agrawal 2001; West-Eberhard 2003). In this scenario, the benefits of plasticity must outweigh the likely costs of the machinery required to generate plasticity, and the costs of a mismatch between the "appropriate" behaviour for the host (Brown *et al.* 2012). Many parasites are plastic, for example bacteriophage T4 (Abedon 1999) and *Plasmodium chabaudi* (Reece *et. al*. 2008), - and a recent study has shown the *de novo* evolution of such plasticity after propagating a virus (bacteriophage ϕ2) for a few hundred generations on its *Pseudomonas fluorescens* host (Leggett *et al.* 2013a (appended)). Crucially, in this latter study, plasticity only evolved when the virus experienced alternating conditions: precisely the conditions predicted by theory. Moreover, in each of these examples, the organisms change their phenotypes in manners that maximise their fitness, i.e. phenotypic plasticity is adaptive. That adaptive plasticity can evolve easily in even a simple virus, suggests this behaviour is likely to be widespread and readily altered by selection.

ii. Multiplicity of infection and intra-host competitive strategies

Host-parasite interactions are rarely one-on-one. Infections of a single host with multiple parasites occur in many natural populations (Rigaud *et al.* 2010), and given their larger host range and consequent higher host encounter rate, we suggest that generalist parasites in some contexts might be more likely to experience these multiple genotype and/or species infections than specialist parasites. The specific context we

consider is isolated populations of pure specialist and generalist parasites, where specialists cannot infect certain hosts. By definition in this scenario, the chance of multiple infections is reduced for specialists. However, by contrast, a polymorphic population of specialist and generalist parasites could result in generalists experiencing fewer mixed infections; if generalists have access to hosts not available to the specialist parasites, resulting in them being more infectious overall (Gandon *et al.* 2002).

Multiple infections result in within-host parasite competition, which can select for a range of parasite strategies that have evolutionary consequences for virulence, including increased rates of host exploitation, exploitation of competitor's public goods (social cheating) and interference (Frank 1996; Buckling & Brockhurst 2008) (Box 2). If we assume that generalist parasites are more likely to experience multiple infections, then a generalist parasite might be expected to be a better competitor if their host becomes infected by a specialist parasite. However this prediction can be complicated by fecundity costs associated with generalism, as discussed above.

Multiple infections can also have important virulence effects due to their interactions with host immunity. Successful parasites must evolve life history strategies that enable them to reproduce before they die. Ideally the host immune system should multitask with ease, but multiple infections can strongly interact with immunity because the host cannot perfectly deal with multiple antigens simultaneously. This has been shown in mixed-genotype infections of the rodent malaria *Plasmodium chabaudi* (Taylor *et al.* 1998; Mideo 2009). Hence another reason why multiple infections could cause the expression and evolution of lower virulence is because there is less need for parasites to replicate quickly to avoid the host immune system. Consequently, an understanding of how parasites interact with host immunity and pathogenic outcome is critical to evaluating whether virulence is a consequence of parasite fitness trade-offs.

Furthermore, multiple infections are not limited to members of the same species, and this can also affect interactions with the immune system, as shown by a meta-analysis of mice co-infected with microparasites and helminths (Graham 2008). Given that parasites, in particular generalists, are likely to be co-infecting with other species, a better understanding of the ecology and evolution of co-infection might reveal predictable virulence outcomes (Graham 2008).

Multiple infections can have surprising effects on the requirements and mechanisms of virulence evolution itself for a number of reasons including the extent to which virulence reduces parasite transmission (trade-off theory), and the genetic diversity (or relatedness) of the parasites either competing for, or cooperating to exploit the host. Furthermore, both the dynamics of intra-host competition and mediation by the immune system appear to play a role in the outcome of competition. It is reasonable to speculate that parasites could face a trade-off between coping with the host immune system and out-competing co-infecting parasites. Since interacting with host immunity is a more constant selective pressure than multiple infections, the evolutionary outcome of virulence should depend on the prevalence of potential competitors for a given parasite species (Rigaud *et al.* 2010). We suggest that generalist parasites are more likely to experience multiple infections due to their higher host encounter rates as a function of their larger host ranges. Consequently generalist parasites might invest more in outcompeting co-infecting parasites, and evolve virulence accordingly (Box 2); while specialist parasites might invest more in surviving host immune responses.

Box 2. Intra-host competitive strategies

There is an important difference between the expression of virulence – where overall virulence can be lower due to the ecology of the infection such as within host competition in a co-infected host, and the evolution of virulence – which parasite strain is ultimately selected. The majority of intra-host competition models and empirical work focus on resource competition and posit that such competition selects for increased rates of host exploitation and consequently virulence (Anderson & May 1982; Frank 1996; Nowak & May 1994; Levin & Pimentel 19981; Bremermann & Pickering 1983; Frank 1992, 1994; May & Nowak 1995; van Baalen & Sabelis 1995). For clonal (single) infections, kin selection can favour lower rates of within-host resource use that maximise total transmission. However, social interactions between co-infecting parasites can be more direct than simple resource competition. Some parasites show increased within-host growth and virulence as a result of the production of costly public goods; here, within-host competition favours the evolution of social cheats, resulting in the expression of the evolution of lower virulence (Buckling & Brockhurst 2008; Mideo 2009). Multiple infections can favour the production of anti-competitor toxins as a result of both individual and kin-selection; virulence is likely to decrease when these are favoured. All mechanisms that increase within host competitive ability are typically associated with a fitness cost in clonal infections in terms of reduced transmission. In addition, multiple infections afford increased opportunities for genetic or sexual recombination between pathogen strains which can bring with it important adaptive advantages that modify virulence (Michod *et al.* 2008).

III. Maladaptive virulence

In many cases, virulence is clearly decoupled from parasite fitness (transmission) i.e. it is non-adaptive. This situation can arise when parasites accidentally infect hosts that do not normally transmit them. Such "spill-overs" can be lethal to the host since host and parasite have not co-evolved (Ebert 1998) however many others may go largely unnoticed because we do not see "avirulent" spill-overs. Spill-overs are predominantly noticed in humans infected by virulent diseases such as Ebola and avian influenzas

although two well-known wildlife examples are the parapoxvirus-driven ecological replacement of native red squirrels by invasive grey squirrels (Tompkins *et al.* 2003), and the crayfish plague spread by introduced American signal crayfish to native European white-clawed crayfish (Alderman 1996). We suggest that generalist parasites might be more likely to spill-over into novel hosts when their larger host ranges suddenly meet, leading to maladaptive virulence.

Generalist parasite virulence might also be comparatively maladaptive compared to specialist parasites since generalists have less time to adapt to any one host within their host infectivity range. Specialist parasites that show host preference will be more consistently exposed to selection on a particular host, and thus can adapt to evolving host defences faster than generalist parasites (Kawacki 1998). This might be particularly true for "super-generalist" opportunistic pathogens since clinical infections of these parasites are generally not directly communicable and their continuous circulation does not depend on their ability to cause disease (Brown *et al.* 2012).

IV. Host availability

Crucial to understanding optimal levels of virulence is host availability (Frank 1996), with, for instance, experiments using bacteria and viruses showing that high host availability selects for increased virulence (Messenger *et al.* 1999; Berngruber *et al.* 2013). The degree of generalism can alter the pool of available hosts, with highly specialised parasites having fewer available hosts than generalist parasites. For example, it has been proposed that theoretically high degrees of specialism may lead to a process akin to the 'self-shading' in spatial models (Boots & Sasaki 1999; Lion & Boots 2010),

where highly virulent and transmissible parasites overexploit their available hosts, resulting in selection for lower virulence (Kirchner & Roy 2002).

Conclusions

Understanding how selection drives the evolution of both parasite host range and parasite life histories is crucial for predicting the impact of parasites on their host populations. While the causes and consequences of both parasite traits have been extensively studied in isolation, their interplay has received much less attention. Here we have highlighted one aspect of this interplay: how parasite generalism might affect parasite virulence. We have focused on four specific examples. First, the costs of generalism: generalist parasite species and/or strains are less able to efficiently exploit their hosts compared to specialist parasites. Second, generalist parasites are more likely to find themselves in multiple infections: this increases within-host selection, which is closely linked to the expression and evolution of virulence. Third, generalist species or stains of parasites are more likely to infect novel hosts with whom they have not co-evolved: this can manifest as maladaptive virulence. Fourth, generalist parasites can access niches free of specialist parasites, reducing the burden of within host competition, leading to the evolution of lower virulence.

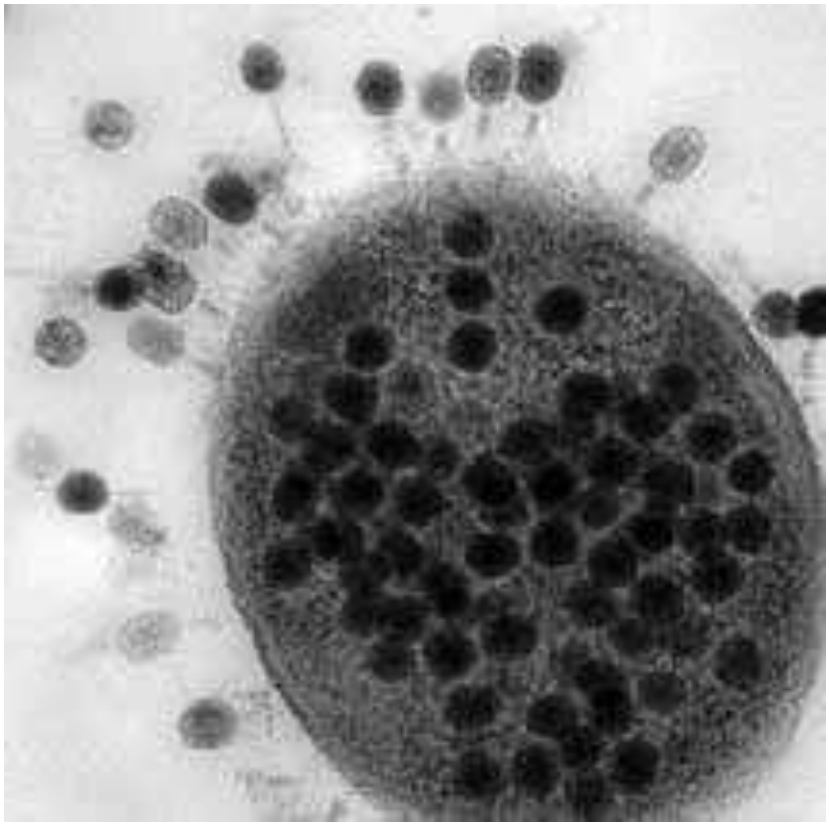
While most of our arguments are speculative, it would be straightforward to test some of the hypotheses. For example, plant viruses differ in generalism at both the species and genotype levels (Malpica *et al.* 2006; Bedhomme *et al.* 2012; Lalic *et al.* 2013), allowing for further investigation into natural correlations between virulence and generalism, taking into account phylogenies and different ecological conditions.

Furthermore, direct qualitative tests could be carried out by comparing the life histories (Benmayor *et al.* 2009) of viruses of bacteria experimentally evolved to have different host ranges (Hall *et al.* 2011 (appended)).

Understanding the virulence of emerging infectious diseases

As well as generating novel general predictions for the evolutionary ecology of virulence, integrating parasite host range and virulence evolution is likely to aid our understanding of the virulence of emerging infectious diseases (Woolhouse, Haydon & Antia 2005). As parasites become exposed to novel hosts because of increased population density, climate change and urbanisation, natural selection is likely to favour the evolution of parasite generalism (Boot & Sasaki 1999). Given that generalist parasites pose the greatest threat to disease-mediated extinction (Rigaud *et al.* 2010; Pederson & Fenton 2007), furthering our understanding of the importance of parasite generalsim on virulence evolution might be an important step in reducing the negative impact of infectious diseases. Finally, it is important to emphasise that the interaction between parasite host range and life history is likely to be reciprocal: changes in life history may in turn affect selection for generalism, i.e life history and host range coevolve. For example, high virulence might reduce the availability of susceptible hosts, imposing selection for greater host range. This interplay is clearly complex, but we hope this article might inspire more research in this area.

3. Infectious dose and virulence[†]



A bacterium having a horrible time, being infected by lots of bacteriophage.

[†] Published as: Leggett H.C., Cornwallis C.K. & West S.A. 2012. Mechanisms of Pathogenesis, Infective Dose and Virulence in Human Parasites. PLoS Pathogens 8(2): e1002512 (appended)

Abstract

The number of pathogens that are required to infect a host, termed infectious dose, varies dramatically across pathogen species. It has recently been predicted that infectious dose will depend upon the mode of action of the molecules that pathogens use to facilitate their infection. Specifically, pathogens which use locally acting molecules will require a lower infectious dose than pathogens that use distantly acting molecules. Furthermore, it has also been predicted that pathogens with distantly acting immune modulators may be more virulent because they have a large number of cells in the inoculums, which will cause more harm to host cells. We formally test these predictions for the first time using data on 43 different human pathogens from a range of taxonomic groups with diverse life-histories. We found that pathogens using local action do have lower infectious doses, but are not less virulent than those using distant action. Instead, we found that virulence was negatively correlated with infectious dose, and higher in pathogens infecting wounded skin, compared with those ingested or inhaled. More generally, our results show that broad-scale comparative analyses can explain variation in parasite traits such as infectious dose and virulence, whilst highlighting the importance of mechanistic details.

Introduction

There is huge variation across pathogen species in the number of cells required to successfully infect a host. This number is known as the 'infectious dose'. At one end of the scale, species such as *Shigella* and *Giardia lamblia* require about 10 cells to start an

infection. In contrast, species such as *Vibrio cholera* and *Staphylococcus aureus* require 10^3-10^8 cells in order for an infection to develop (Sewell 1995; Schmid-Hempel & Frank 2007; Schmid-Hemple 2011). It is unclear why infectious dose varies, with large differences occurring even between closely related pathogens (Schmid-Hemple & Frank 2007; Schmid-Hemple 2011).

Schmid-Hempel and Frank (2007) predicted that the variation in infectious dose could be explained by the different biochemical mechanisms that pathogens use to infect hosts. Pathogens secrete a number of molecules which facilitate the suppression and/or evasion of host immune responses, and hence aid parasite growth. If these molecules act locally, in the vicinity of the pathogenic cell, then only small numbers of molecules may be required for successful growth and so infections can be established from small numbers of pathogenic cells. In contrast, if the pathogenic molecules diffuse and therefore act at a distance, then large numbers of molecules may be required for evading the host immune system. In these cases greater numbers of pathogenic cells could be needed to establish an infection. However, while this prediction is consistent with anecdotal data (Schmid-Hemple & Frank 2007; Schmid-Hemple 2011), it has yet to be tested formally.

Testing infectious dose and virulence theory

Here, we test Schmid-Hempel and Frank's (2007) prediction that infectious dose is determined by whether pathogenesis is locally or distantly acting. We use data from 43 species of human pathogens across a range of enteropathogenic bacteria, protozoa, fungi and viruses. A possible problem with comparative studies across species is that closely

related species can share characters through common descent rather than independent evolution. Consequently, analysing species as independent data points can lead to misleading correlations (Harvey & Pagel 1991; Felsenstein 1985; Grafen 1989). For example, all viruses are locally acting, and so this could lead to patterns between viruses and bacteria, rather than local or distant action. We account for this potential problem of shared ancestry by using multivariate nested taxonomic models Cluttonbrock & Harvey 1977; Hadfield & Nakagawa 2010).

We then extend this work in two ways. First, Schmid-Hempel and Frank (2007; Frank & Schmid-Hemple 2008) further predicted that pathogens with distantly acting immune modulators will be more virulent, possibly because they would have a large numbers of cells in the inoculums, and higher parasite density would overwhelm the host immune system causing more harm to hosts. We therefore test whether the virulence of pathogens with distantly acting immune modulators is greater than that of pathogens with locally acting molecules. Second, we test the influence of two other factors that could affect infectious dose and virulence: mode of transmission (direct or indirect) and route of infection (ingestion, inhalation or wounded skin) (Sewell 1995; Frank 1996; Alizon *et al.* 2009; Ewald 1994). These factors could influence dose and virulence for a number of reasons, including their affect on: the extent to which virulence reduces pathogen transmission, the types of immune response they encounter; and the genetic diversity (or relatedness) of the pathogens either competing for or cooperating to exploit the host (Schmid-Hemple & Frank 2007; Schmid-Hemple 2011; Frank & Schmid-Hemple 2008; Frank 1996; Alizon *et al.* 2009; Ewald 1994; Wild, Gardner & West 2009; Lion & Boots 2010; Boots & Mealor 2007; Boots & Sasaki 1999; Bull 1994; Ewald 1991; Hamilton 1972; Bull, Molineux & Rice 1991; Ewald

1983; Nowak & May 1994; Frank 1994; Brown, Hochberg & Grenfell 2002; West & Buckling 2003; Gardner, West & Buckling 2004; Ebert & Bull 2003; Herre 1993).

Results and discussion

We found that pathogens with immune modulators that act distantly within the host have significantly higher infectious doses than pathogens with locally acting molecules, (Figure 1 and Table S2 (appended), $F_{1, 40}$=25.79, P<0.0001). This supports the prediction by Schmid-Hempel and Frank (2007) that local pathogenic action requires only a small number of molecules, and thus relatively few cells are needed to start an infection, compared to distantly acting mechanisms where a large number of diffusible molecules need to accumulate in order to overwhelm the host's immune clearance.

Contrary to the hypothesis that pathogens with distantly acting immune modulators are more virulent, we found no significant relationship between case fatality rate or severity of infection and the mechanism of pathogenesis (Table S3 and Table S4 (appended), P>0.05). However, case fatality rate was significantly negatively related to infectious dose of pathogens (Figure 2 and Table S3 (appended), $F_{1, 38}$=3.94, P=0.05). We suggest this correlation arises because, for a given dose, pathogens that are locally acting and have lower infectious doses are more likely to establish an infection. For this relationship to hold, we reasonably assume that the actual dose in natural infections is largely determined by factors such as the mode of transmission, and so does not show a strong covariance with whether a parasite acts locally or globally within the host. We attempted to collect data on mean parasite dose in different transmission modes during

natural infections so we could examine how this correlates with local/global within-host parasite action, but we were unable to obtain sufficient data.

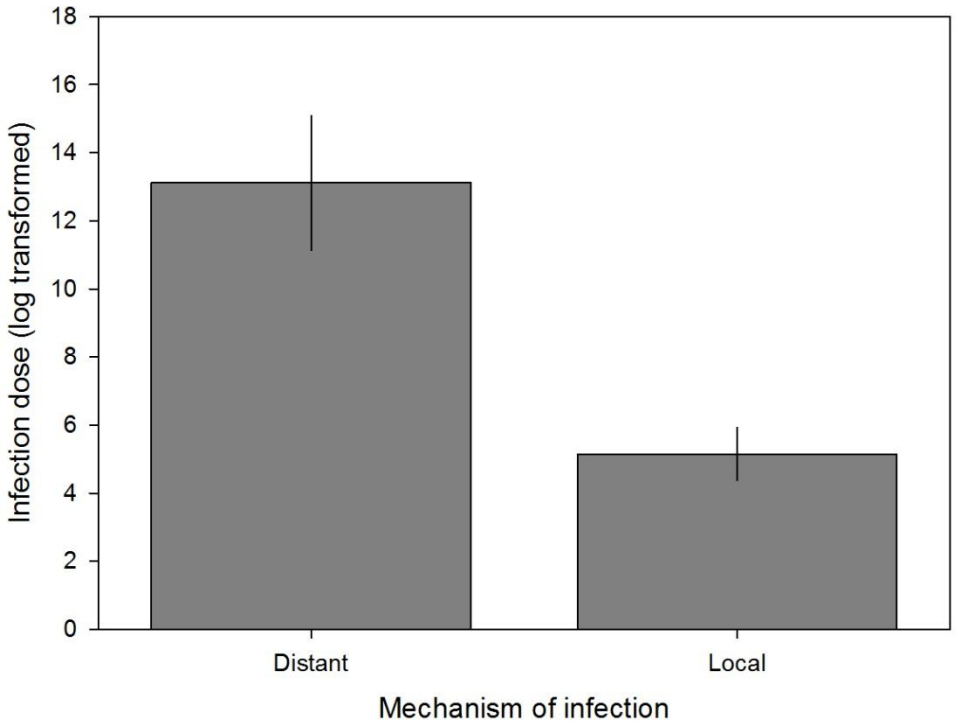


Fig. 1. Infectious dose and the mechanisms of pathogenesis used by pathogens. Means ± 1 standard error (Table S2, appended).

Explanations of negative relationship between infectious dose and virulence

There are at least two alternative explanations for the negative relationship between case fatality rate and infectious dose of parasites. First, recent theory suggests parasites might adapt to low infectious doses by evolving a higher per-parasite growth rate, causing greater host exploitation and virulence (de Roode *et. al*. 2011). However the reduction in dose in this model results from increased host resistance, and there is no

reason to assume that selection for host resistance consistently differs between global and local acting parasites.

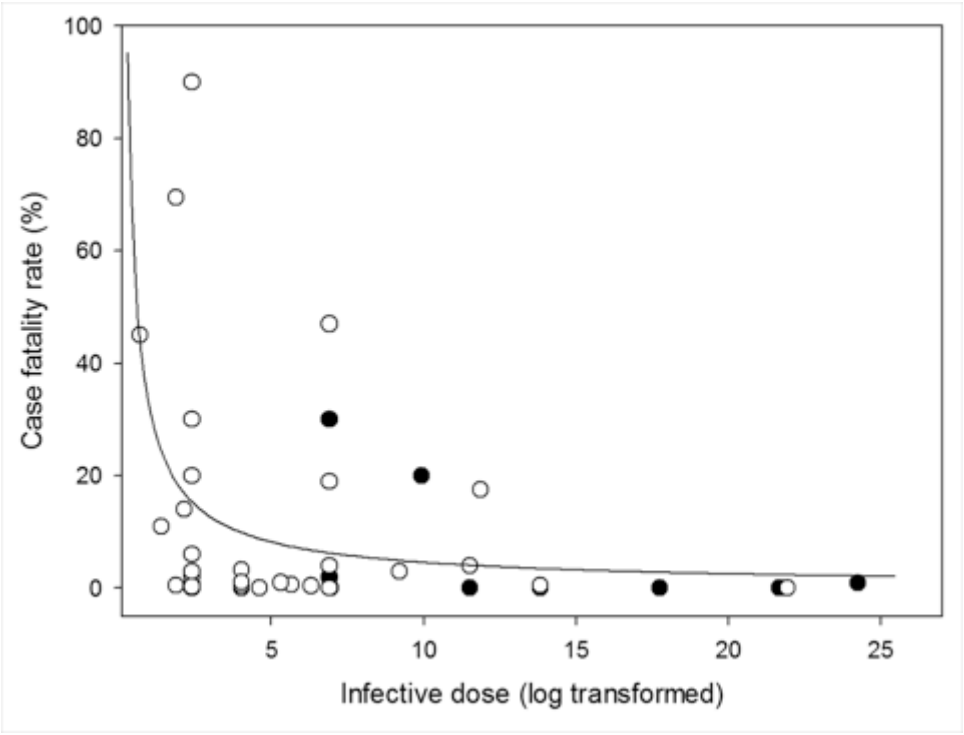


Fig. 2. Variation in case fatality rate explained by Infectious dose. Line represents a logistic curve (Table S3, appended). Black circles are pathogens with distant acting mechanisms of pathogenesis; white circles are pathogens with locally acting mechanisms of pathogenesis.

Second, a low infectious dose may reduce the incidence of multiple genotype pathogen infections since there are fewer parasites in the inoculum, which could favour higher levels of cooperation between parasites, and hence lead to greater growth and virulence (Brown, Hochberg & Grenfell 2002; West & Buckling 2003). However, the extent to which this will be of general importance will be limited by the fact that different biological details can lead to different relationships between strain diversity

and virulence. For example, when different parasite strains compete for host resources, higher strain diversity is expected to lead to greater virulence (Frank 1996, Alizon *et al.* 2009; Nowak & May 1994; de Roode *et al.* 2005, 2011; Levin & Pimentel 1981; Bremermann & Pickering 1983; van Baalen & Sebelis 1995). Alternatively, antagonistic interactions between strains, such as chemical warfare, can lead to a predicted domed relationship between strain diversity and virulence (Gardner, West & Buckling 2004). Nonetheless, it is possible that all three explanations could play a role, with their importance varying across species.

Route of infection and virulence

We found that pathogens infecting hosts through wounded skin result in significantly higher case fatality rates than pathogens inhaled or ingested (Figure 3 and Table S3 (appended), $F_{2, 26}$=5.30, P=0.01). Given that infection via wounded skin includes transmission via bites of insect vectors and contaminated water, this result supports theory on virulence-transmission trade-offs which proposes that vectors and water systems circumvent the need for an ambulatory host to transmit pathogens, selecting for the evolution of higher virulence (Ewald 1994; 1991; 1983). However, another potentially important factor is that the type of immune response that pathogens are confronted with will affect virulence. Pathogens that infect hosts through wounded skin circumvent mechanical immunity and directly enter the circulatory system. Hence, they may cause virulent systemic infections more readily than ingested or inhaled pathogens, which must overcome other anti-infection barriers such as stomach acid and mucus membranes before causing systemic infections.

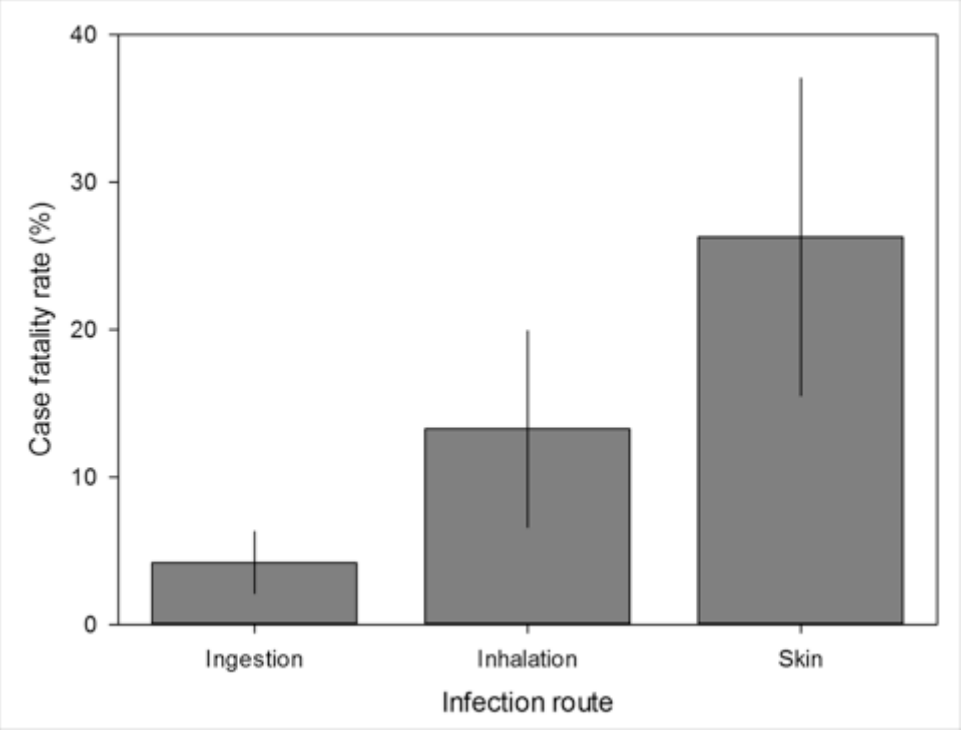


Fig. 3. Variation in case fatality rate explained by route of infection. Means ± 1 standard error (Table S3, appended).

Conclusions

More generally, our results emphasise the importance of life-history or mechanistic details for the evolution of parasite traits. Theoretical models for the evolution of parasite traits such as virulence have generally relied on simple trade-offs between virulence and transmission. These models have been able to explain variation in virulence both within species, and between closely related species with similar life histories (Boots & Mealor 2007; Bull, Molineux & Rice 1991; Herre 1993; de Roode *et al.* 2005; Ebert 1998; Kerr *et al.* 2006; Rumbaugh *et al.* 2009; Kohler, Buckling & van Delden 2009). In contrast, this body of theory has been less successful at explaining broad scale variation across species (Schmid-Hemple & Frank 2007; Frank & Schmid-

Hemple 2008; West & Buckling 2003; Ebert & Bull 2003. One possible explanation for this is that the predictions of virulence theory can depend upon the mechanisms that pathogens use to infect and exploit hosts, which are not considered in the classical models; hence our expectations of data fitting the model may be too high. If the details of how pathogens infect hosts really matter, this would limit the extent to which we would expect to find broad empirical patterns to match theory (West & Buckling 2003). Our results show that transmission, dose and virulence can be influenced by mechanistic details such as distance at which molecules act and route of infection.

Material and Methods

Infectious dose

We obtained data on the number of pathogen cells required to start an infection (infectious dose) by searching: (a) databases from the United States Food and Drug Administration (2003), Health Canada[‡], Medscape[§], the Centre for Disease Control and Prevention[**], the World Health Organisation[††]; (b) empirical studies found via keyword searches in the ISI Web of Knowledge database, PubMed and Google Scholar. Where

[‡] Health Canada (2003) Pathogen safety data sheets. Available: http://www.phacaspc.gc.ca/lab-bio/res/psds-ftss/index-eng.php.

[§] Medscape. Infectious Disease Articles. Available: http://emedicine.medscape.com/infectious_diseases.

[**] Centers for Disease Control and Prevention. Alphabetical Index of Parasitic Diseases. Available: http://www.cdc.gov/parasites/az/index.html.

[††] World Health Organization. Publications and Fact sheets. Available: http://www.who.int/research/en/

ranges or more than one estimate of infectious dose were given, we calculated the median infectious dose to use in our analyses.

We emphasise that uncertainties exist in infectious dose measurements: often they were extrapolated from epidemiologic investigations, were obtained by human feeding studies on healthy, young adult volunteers, or are best or worst estimates based on a limited data from outbreaks. Where known, we give methods of estimation in Table S1 (appended).

Classifying mechanisms of pathogenesis

We classified pathogens as having local or distant action according to the framework of Schmid-Hempel and Frank (2007). For local action, pathogens directly interact with host cells via surface-bound molecules or by injecting proteins into host cells by a type III or IV secretion systems. For example, *Yersinia entricolata/ tuberculosis* injects Yop protein into target cells via a type III secretion system, leading to cytotoxicity (Wilson, McNab & Henderson 2002), and *Ebola virus* binds to different cell surfaces and replicates leading to cell necrosis (Bray 2005).

For distant action, pathogens indirectly interact with host cells by secreting proteins that diffuse into their surroundings and only exert pathogenic effects when they bind to host cells. This may arise, for example, through immune modulators delivered by the general secretary pathway, or the type I, II and V secretary systems. For example, the well known virulence factor lysteriolysin O of *Listeria monocytogenes* and exotoxins of *Staphylococcus aureus*, are secreted via the general secretory pathway (Wooldridge 2009). We do not classify between interactions with host and immune cells

specifically since we are concerned with how far the interaction occurs from the infecting parasite, not with what cell the interaction occurs with.

Measuring virulence - case fatality rate, 'disease severity' and incidence

To capture both the short and long term consequences of pathogen infection on host health, we use case fatality rate and a 'disease severity' score to measure pathogen virulence. These are two of the three criteria used to estimate 'burden of disease' in a recent protocol for prioritising infectious disease in public health (Krause 2008).

We rated each pathogen according to its severity, as described in Table 1. We gave a score of 0 to pathogens of average importance, or pathogens for which a lack of data precluded another score. Incidence data are the estimated mean number of new cases per year in the USA. Case fatality rates are estimates of fatality without treatment or co-morbidities and represent the number of cases of a disease ending in death compared to the number of cases of the disease. We obtained data from the before-mentioned databases, plus various reports in the literature (see Table S1 in Appendix).

We emphasise that while a "case" should represent an infected individual, in practice it may involve infection of some severity, -hospitalization even. Thus overall our definition of case fatality may overestimate virulence. For example, a benign parasite that infects many hosts asymptomatically, but cause severe disease in a small proportion of hosts, may be classified as virulent. By contrast, a virulent parasite that causes disease of equal severity in its hosts may be classed as less virulent. To correct for this potential bias, we assessed whether case fatality rate is linked to incidence rate,

and examined the effects of the other variables after controlling for variation in incidence rate.

Table 1. Definition of the scores for calculating disease severity

Scores		
-1	**0**	**1**
Hospitalisation is rare. Work loss is <2 days. No persisting illnesses/ handicaps	Hospitalisation is rare. Work loss >5days is rare. Few persisting illnesses/ handicaps.	Hospitalisation is frequent, work loss of >5days is frequent. Persisting illnesses/ handicaps occur.

Adapted from Krause (2008)

Transmission mode and route of infection

We obtained data on transmission mode and route of infection using the before-mentioned databases. We classified pathogens as either direct or indirectly transmitted: direct transmission requires physical contact between an infected and susceptible host, and indirect transmission requires an agent to transfer the pathogen from an infected to a susceptible host. We classified the routes of infection used by pathogens as entry through wounded skin, inhalation, or ingestion. For example, *Bordatella pertussis* is usually spread by infected people coughing or sneezing while in close contact with susceptible others who then inhale the *pertussis* bacteria (Centers for Disease Control

and Prevention[‡‡]) (i.e. direct transmission). Where pathogens can use more than one mechanism of transmission or infection, we used the mechanism stated in the infectious dose data for our analyses.

Statistical analysis

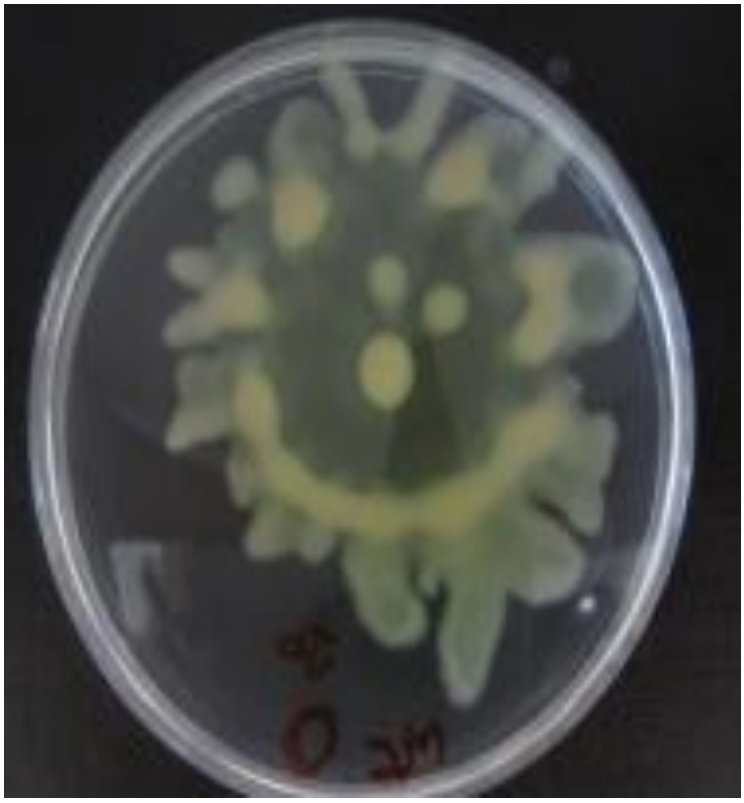
We performed three analyses. First, we tested whether infection dose (log transformed) was related to the mechanism of infection (2 level fixed factor: local or distant), infection route (3 level fixed factor: ingestion, inhalation, wounded skins) and the transmission mode of pathogens (2 level fixed factor: direct, indirect) using a linear mixed effects model (LMM) with restricted maximum likelihood estimation (REML). Second we tested if case fatality rate (% of cases resulting in death) was influenced by infectious dose (covariate log transformed), incidence (covariate log transformed), mechanism of infection, infection route and transmission mode using a generalised linear mixed effects model (GLMM) with a binomial error distribution. Finally, we analysed the severity of infection (-1, 0, 1) in relation to the same explanatory variables as the second analysis using a GLMM with an ordered multinomial error distribution.

The data Table S1 (appended) encompass a diverse range of pathogens. We obtained information on the taxonomic classification of pathogens from the National Center for Biotechnology Information (NCBI) (Sayers *et al.* 2009). We accounted for the non-independence of data arising from phylogenetic relationships between pathogens in all LMMs and GLMMs using nested taxonomic random effects structures

[‡‡] Centers for Disease Control and Prevention. Alphabetical Index of Parasitic Diseases. Available: http://www.cdc.gov/parasites/az/index.html

whereby each taxonomic level (genus, order, class and kingdom) was nested within all higher taxonomic levels (see Tables S1-4 for details, appended). We only entered genus, order, class and kingdom into models because of poor replication at other taxonomic levels. We examined the significance of fixed effects (factors and covariates) using Wald type adjusted *F* statistics and the effect with the highest P value was sequentially dropped until only significant terms ($P<0.05$) remained (Crawley 2002). Prior to all analyses covariates were Z-transformed (mean = 0, standard deviation = 1). We used the Kenward and Roger (1997) method for estimating standard errors for parameter estimates and denominator degrees of freedom since it is specifically designed for models with multiple random effects and unbalanced data, increasing the accuracy of significance tests (Kenwood & Roger 1997; Littell *et al.* 2006; Bolker *et al* 2009). We assessed the significance of random effects using log-likelihood ratio tests (LRTs) (Self & Liang 1987). All analyses were conducted in SAS version 9.2.

4. Immune subversion and virulence



The day our Pseudomonas grew a face ☺

Abstract

There is huge variation across pathogens species in virulence, the harm pathogens cause their hosts during an infection. Classic theory for the evolution of virulence is based on trade-offs between pathogen growth, transmission and virulence; and assumes higher within-host growth causes higher virulence. However, we found no support for this in a comparison of 61 human pathogens. In contrast, we found that: (1) pathogens capable of subverting the host immune system are significantly more virulent than non-immune subverting pathogens; and (2) inhaled pathogens or pathogens infecting via skin wounds are significantly more virulent than ingested pathogens. Overall, our results emphasise how virulence can be influenced by mechanistic life history details that vary across species to do with how parasites infect and exploit their hosts.

Introduction

There is huge variation across pathogen species in the harm they cause their hosts during an infection (virulence). Some bacteria, such as *Bacillus cereus* cause us mild nausea and diarrhoea for 24-48 hours (United States Food and Drug Administration (2003). In contrast, other species such as *Bacillus anthracis* kills 90-100% of their human hosts, often within 48 hours (Center for Food Security and Public Health[§§]).

Over the last 30 years there has been a proliferation of theoretical models on the evolution of virulence (Anderson & May 1981; Frank 1996, Frank & Schmid-Hempel

[§§]The Center for Food Security and Public Health: http://www.cfsph.iastate.edu/Factsheets/pdfs/anthrax.pdf

2008; Lipsitch & Moxon 1997; Bull 1994; Nowak & May 1994; Ebert & Herre 1996; Gandon *et al.* 2002; Brown, Hochberg & Grenfell. 2002; West & Buckling 2003; Buckling & Brockhurst 2008). At the core of this theory is the idea that there is a trade-off between parasite growth and host survival – higher growth rates increase transmission rate, but also increase host mortality, leading to a lower transmission period (Anderson & May 1981, 1982). Theory predicts that total transmission will be maximised at an intermediate growth rate, with host mortality rate or virulence an unavoidable consequence. This theory has been supported by a number of within species studies, which have tested the underlying assumptions or examined how virulence evolves in response to changing conditions (Lipsitch & Moxon 1997; Froissart *et al.* 2010; Alizon *et al.* 2009; Boots & Mealor 2007; Bull, Molineux & Rice 1991; de Roode *et al.* 2005; Ebert 1998, Kerr *et al.* 2006; Frank & Schmidt-Hempel 2008).

In contrast, there is a lack of evidence that the trade-off theory can explain the large differences we observe across parasite species in virulence (Herre 1993, Ebert & Bull 2003, Frank & Schmidt-Hempel 2008; Alizon *et al.* 2009, Leggett *et al.* 2013b). A possible explanation for this is that virulence is influenced by mechanistic details that vary across species, to do with how parasites infect and exploit their hosts (West & Buckling 2003; Alizon *et al.* 2009; Frank & Schmidt-Hempel 2008). For example, if traits that have large fitness benefits to the parasites (such as escape or modulation of the immune system, also cause pathogenesis, then this could lead to particularly high virulence (Frank & Schmidt-Hempel 2008). If such mechanistic details vary across species, then we might expect these to swamp any simple relationships between growth rate and virulence.

Here, we carry out a comparative study, examining variation in virulence across 61 human pathogens, comprising mostly bacteria, but also eukaryotic species and a fungus. We first examined whether virulence was correlated with maximum parasite growth rate, as assumed by virulence theory. We then examined whether virulence correlated with four life history variables that could influence the mechanism of pathogenesis: (i) whether a species can kill or survive within professional immune cells (immune subversion) (Frank & Schmidt-Hempel 2008; Gama *et al.* 2012); (ii) whether the production of virulence factors is controlled cooperatively by quorum sensing (Williams *et al.* 2000); (iii) the ability of a species to move (motility), and hence disperse between areas within a host (Josenhans & Sauerbaum 2002; Ottemann & Miller 1997); and (iv) the route of infection, which can influence the extent to which virulence decreases parasite transmission (Ewald 1991, 1994).

Results and discussion

Virulence and growth rate

Our first aim is to test whether virulence is correlated with parasite growth rate. We measured parasite virulence as the case fatality rate; the proportion of disease cases ending in host death without treatment or comorbidities. We used *in vitro* generation time as a measure of parasite growth rate, with smaller generation times implying a higher growth rate. Presumably the *in vitro* conditions are ideal for parasite growth, and so here we are discussing "maximum" growth rate, not necessarily the growth rate that trade-off theory is concerned with. Yet, all things being equal, we expect *in vitro* growth rate to be correlated with *in vivo* growth and virulence. In support of this, *in vitro*

growth measures have been shown to correlate with genomic traits associated with fast growth (such as rRNA and tRNA copy number) (Vieira-Silva & Rocha 2010). However, contrary to the standard assumption that increased parasite growth leads to greater host mortality (Frank 1996; Anderson & May 1982), we found no significant relationship between generation time and virulence (Figure 1 and Table S2 (appended, pMCMC=0.60). Furthermore, no relationship between generation time and virulence is seen when examining within groups of immune subverters (Table S3 (appended), pMCMC= 0.37) or non-subverters (Table S4 (appended), pMCMC= 0.41).

Virulence and life history details

We then examined whether parasite virulence instead correlated with a number of life history variables. Frank & Schmid-Hempel (2008) argued that mechanisms of pathogenesis associated with the immune system would be correlated with relatively high virulence, because the benefits of increased parasite growth and survival would outweigh the cost of an increased host mortality rate. We tested if virulence was correlated with whether a species is able to kill professional phagocytes or to survive and/or replicate in the intracellular milieu of these cells, termed "immune subverters" (Fortune & Rubin 2007; Stecher *et al.* 2007; Gama *et al.* 2012). We found that parasites capable of subverting the host immune system are significantly more virulent than non-immune subverting parasites (Figure 2a and Table S2 (appended), pMCMC=0.02). We found no significant difference in the generation time of immune subverting and non-immune subverting parasites (Figure S1 and Table S7, pMCMC=0.71).

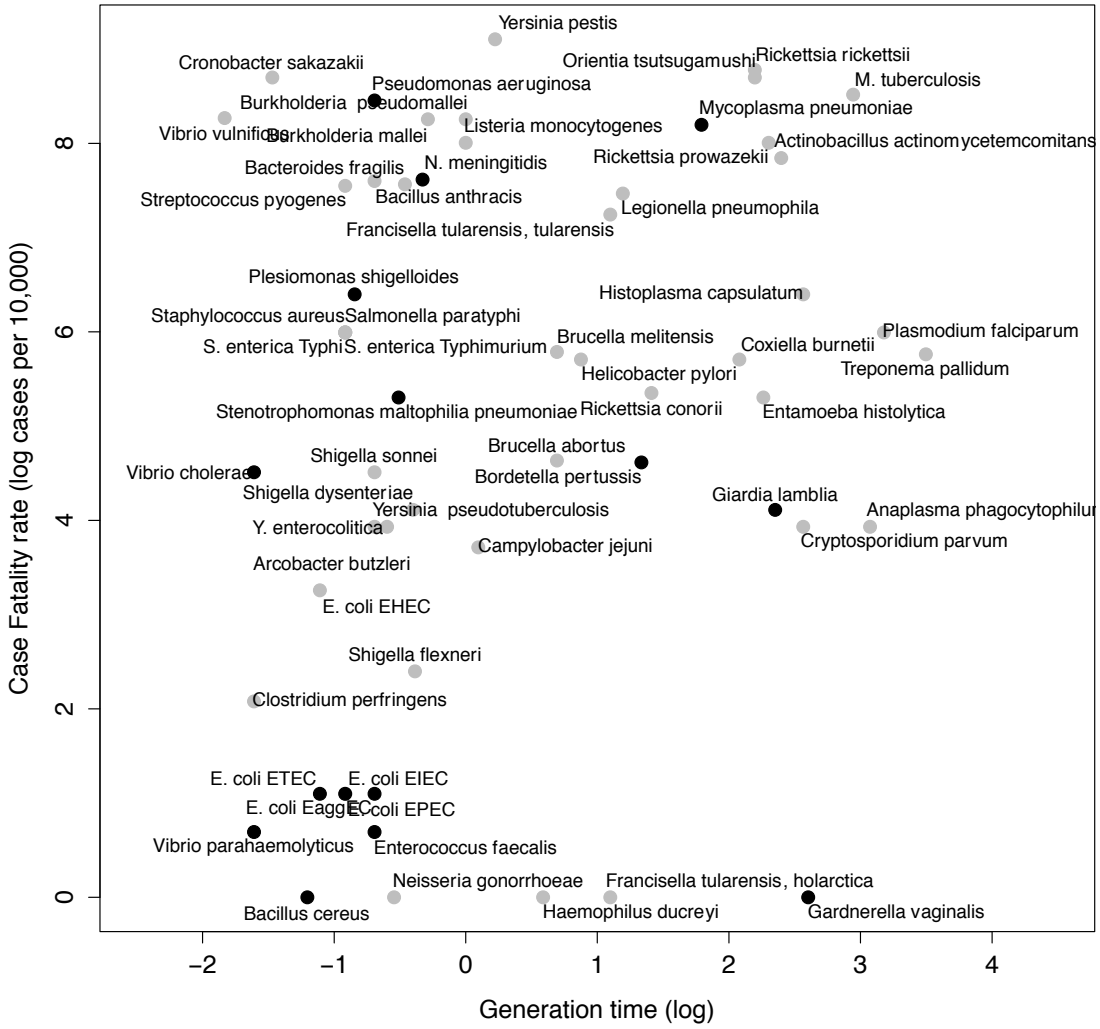


Fig.1. Virulence and maximum growth rate. Grey circles are pathogens with immune subversion, black circles are pathogens without immune subversion. We found no significant relationship between the case fatality rate and generation time.

We examined whether parasite virulence correlated with mode of transmission between hosts. Mode of transmission could matter for a number of reasons (Ewald 1983, 1994; Frank 1996). For example, it has been argued that vectors can circumvent the need for ambulatory hosts to transmit, and lead to higher strain diversity, both of

which could lead to higher virulence for vectored parasites (Ewald 1994, Frank 1996). We found that inhaled pathogens result in significantly higher virulence than ingested pathogens (Figure 2b and Table S2 (appended), pMCMC=0.0006), but there is no difference in virulence between pathogens infecting via wounded skin and ingested or inhaled pathogens (Figure 2b and Table S2 (appended), p=0.06 and 0.05 respectively). This pattern is different from our previous analysis, where pathogens infecting via skin wounds were more virulent than the other two types (Leggett, Cornwallis & West 2012 (appended)). The differences between our two studies is driven by differences in the data: we now have a larger data set with more virulent inhaled pathogens that were not included in the previous paper since we had no infectious dose data for them. Furthermore, we now exclude viruses (unlike the first paper) since we couldn't find appropriate growth rate data for viruses. There are at least three possible explanations for lower virulence of ingested pathogens. First, the type of immune response pathogens face during an infection can vary with infection route. Ingested pathogens must overcome strong anti-infection barriers such as mucus flows and stomach acid, which negatively affects pathogen viability, and subsequent virulence. Second, for vectored pathogens (which we catagorised as infecting by skin wounds), vectors negate the need for mobile hosts to transmit, potentially selecting for virulent pathogens (Ewald 1983, 1994; Frank 1996). Third, transmission via inhalation could lead to higher strain diversity, selecting for faster growth and hence higher virulence (Frank 1996).

We examined whether parasite virulence correlated with either the use of quorum sensing to regulate the production of virulence factors or motility. Quorum

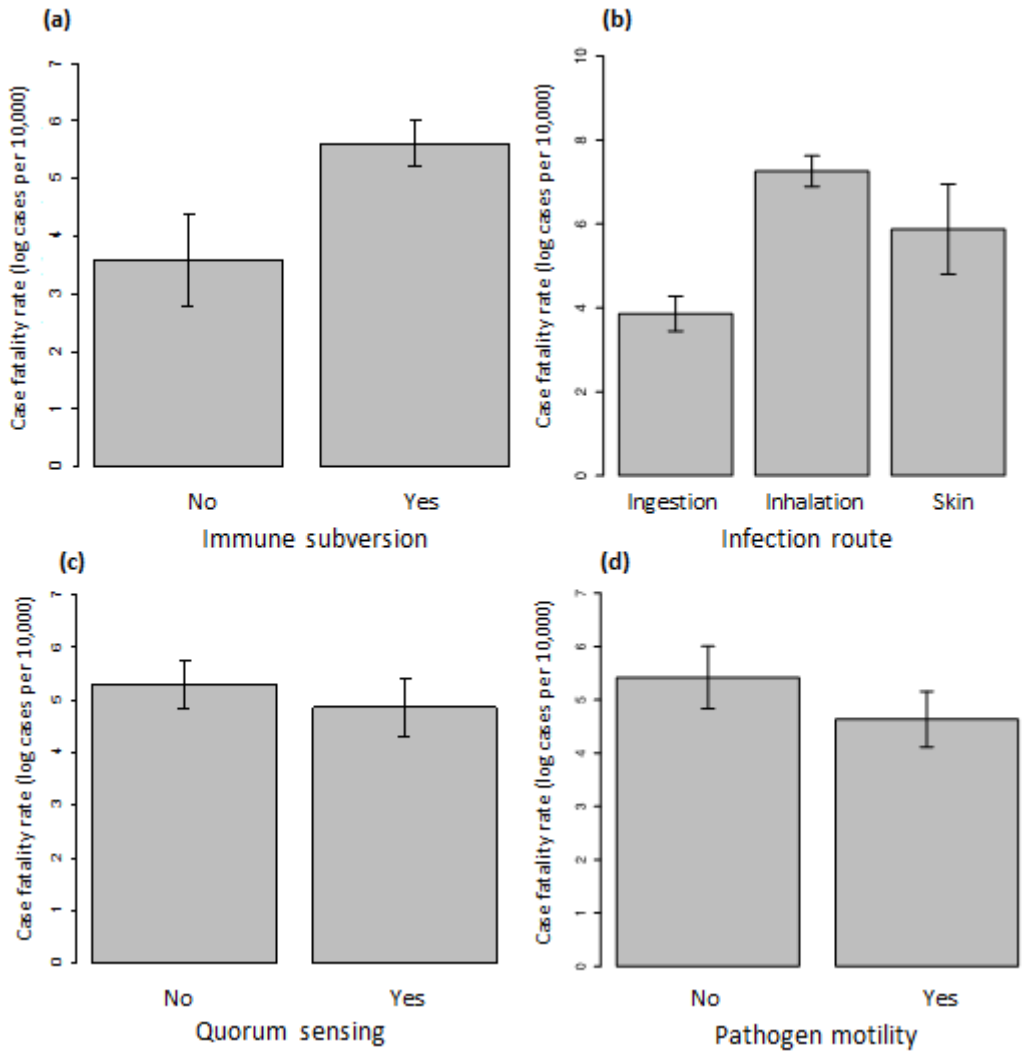


Fig. 2. Virulence and parasite life history. The case fatality rate (log number of deaths per 10,000 cases: **(a)** was significantly higher in species that subvert the immune system; **(b)** was significantly higher in species transmitted via inhalation than those transmitted by ingestion or wounded skin; **(c)** showed no correlation with whether quorum sensing was used to control the production of virulence factors; **(d)** showed no correlation with pathogen motility. See Table S2 (appended) for details.

sensing could matter because it allows pathogens to produce virulence factors more efficiently (Darch *et al.* 2012), which could either facilitate a greater attack on the host, or avoid unnecessary damage to the host (Williams *et al.* 2000; Antunes *et al.* 2010). Motility could lead to a greater virulence by facilitating dispersion and colonisation, allowing pathogens to counteract mucus flows and peristalsis (Josenhans & Suerbaum 2002; Eaton, Morgan & Krakowka. 1992; Drake & Montie 1988; Guentzel & Berry 1975; Miller; Mekalanos & Falkow 1989). Alternatively, motility could decrease virulence because flagella-mediated activation of dendritic cells is highly deleterious to bacterial survival (Salazar-Gonzalez *et al.* 2007). Flagella are also costly to make (Macnab 1992), and facilitate phagocytosis (Tomita *et al.* 1982). However, we found no significant relationship between virulence and parasite quorum sensing based regulation of virulence factor expression (Figure 2c and Table S2 (appended), pMCMC=0.98) or parasite motility (Figure 2d and Table S2 (appended), pMCMC=0.26). This is despite the fact that pathogens which use quorum sensing grow significantly faster than those that do not (Figure S2 and Table S7 (appended), pMCMC=0.008).

Other patterns

We previously found that the number of cells required to successfully infect a host (infectious dose) was significantly negatively correlated with virulence (Leggett, Cornwallis & West 2012), a pattern that we again find in this extended data set (Figure 3a and Table S6 (appended), pMCMC=0.02). Our current results suggest that this is because immune subverting parasites have both a higher virulence and a significantly lower infectious dose than pathogens that do not subvert host immunity (Figures 2a & 3b Table S6 (appended), pMCMC<0.0001). Gama *et al.* (2012) previously found that

immune subversion was the most significant factor shaping variation in infectious dose among human pathogens. Consequently, whilst it cannot be decisively proven with correlational comparative data, we suggest the correlation between dose and virulence arises due to shared causal factors, rather than one driving the other.

There have been only a limited number of formal comparative studies attempting to explain virulence across a number of pathogen species (Herre 1993; Leggett, Cornwallis & West 2012). An important example is Herre's (1993) study on fig wasp nematodes, which showed higher virulence in species where there was likely to be greater transmission. A key difference between our studies is that the fig wasp nematodes all shared very similar life histories, and so there were not the large mechanistic life history details that have been a focus of our study here. Indeed, the absence of such variation is key to Herre's study by facilitating the influence of variation in only one key variable – transmission.

Conclusions

To conclude, we have found that growth rate of pathogens didn't vary with virulence. One possible explanation for this is that our measure of growth rate *in vitro* may be irrelevant to *in vivo* growth rate. This is plausible, but seems unlikely since experimental evidence suggests the two correlate well within species (Mikonranta, Friman & Laakso 2012; Vieira-Silva & Rocha 2010). Alternatively, the effect of growth rate may be relatively small biologically in explaining variation in virulence across species relative to other life history variables. In contrast, we did find that immune subversion and infection route explained variation in virulence across human pathogens.

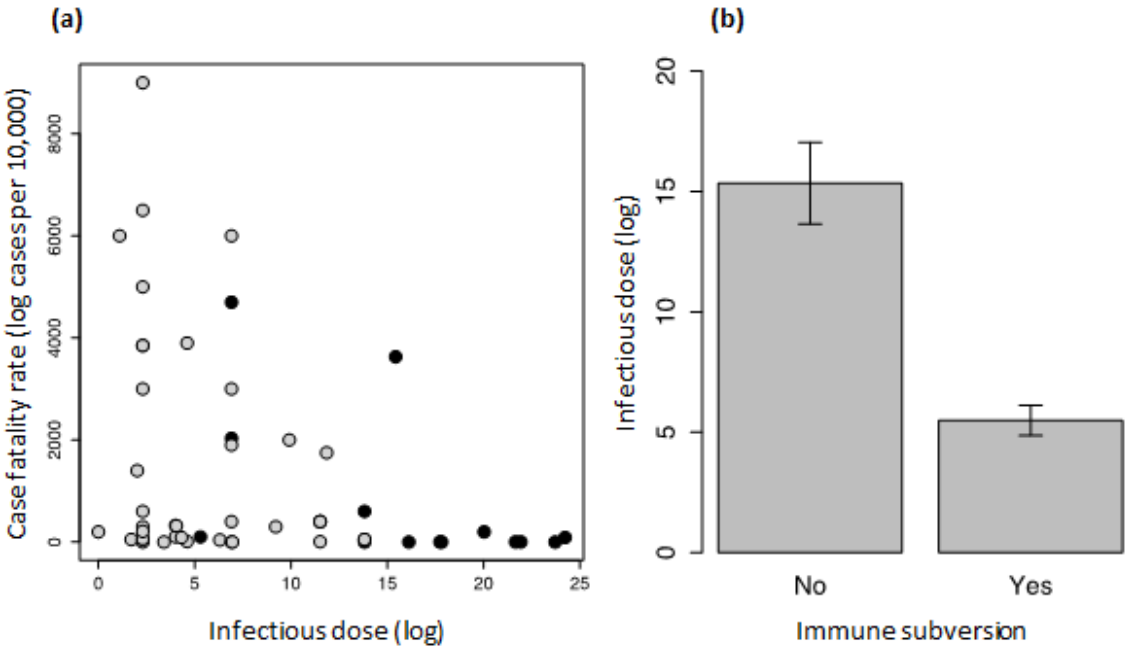


Fig. 3. Correlates of infectious dose. **(a)** The case fatality rate (log number of deaths per 10,000 cases) was significantly lower in species with a lower infectious dose (log). Grey circles are pathogens with immune subversion, black circles are pathogens without immune subversion. **(b)** The infective dose was significantly higher in immune subverters. See Table S6 (appended) for details.

Material and Methods

Collection of data from the literature

Case fatality rates are estimates of fatality without treatment or comorbidities and represent the number of cases of a disease ending in death compared to the number of cases of the disease. We classified routes of infection used by pathogens as entry through wounded skin, inhalation or ingestion. We used the route of infection described in the case fatality data for our analyses, but note that the method of exposure used in

experimental infections may not be the "natural" transmission route. We obtained data for case fatality rate, route of infection and the number of pathogen cells required to start an infection (infectious dose) by searching: (a) databases from the United States Food and Drug Administration (2003), Health Canada[***], the Centre for Disease Control and Prevention[†††]; (b) direct searches in the empirical literature using key word searches in the ISI Web of Knowledge database, Google Scholar, and Pubmed.

Growth rate data are minimal generation times (hours), which we retrieved from the supplementary material of Vieira-Silva & Rocha (2010) and Gama *et al.* (2012) with some new data from the primary literature. For interactions with the immune system, motility and quorum sensing, we first followed the supplementary material of (Gama *et al.* 2012), with new data from the primary literature. We have included all data and bibliographic references in Supplementary Materials (Table S8, appended).

Statistical Methods

First, we tested if variation in case fatality rate (transformed to deaths per 10,000 cases so all number were whole numbers for analysis) was correlated with generation times of pathogens (log transformed), immune subversion (2-level factor: yes or no), infection route (3-level factor: inhalation, ingestion and through skin), quorum sensing (2-level factor: yes or no) and pathogen motility (2-level factor: yes or no) using Bayesian linear mixed models (BMM) with Markov chain Monte Carlo estimation in MCMCglmm, R

[***] Health Canada (2003) Pathogen safety data sheets. Available: http://www.phacaspc.gc.ca/lab-bio/res/psds-ftss/index-eng.php

[†††] Centers for Disease Control and Prevention. Alphabetical Index of Parasitic Diseases. Available: http://www.cdc.gov/parasites/az/index.html

version 2.15.1 (Hadfield & Nakagawa 2010; Hadfield (2010). We modeled case fatalities as a Poisson process with a log link function. We tested each explanatory variable on their own (Table S1, appended) and then together in a multivariate analysis (Table S2, appended).

In multivariate analyses we used step-wise deletion of non-significant terms to obtain a minimum adequate model (Crawley 2002). Prior to all analyses we Z-transformed (mean = 0, standard deviation = 1) covariate fixed effects and converted two-level fixed effects to binary coding -1, 1 so that the magnitude of parameter estimates could be directly compared (Gelman 2010; Scheielzeth 2010).

We then tested if virulence was correlated with infectious dose (log transformed), and if infectious dose was correlated with infection route, generation time of pathogens, immune subversion, pathogen motility and quorum sensing using a BMM in MCMCglmm with a Gaussian error distribution (Hadfield & Nakagawa 2010; Hadfield 2010). We again first modeled explanatory variables separately and then together in a multivariate analysis.

The analyses included data from a taxonomically diverse range of pathogen species. We took into account the non-independence of data arising from the phylogenetic relationships between species by defining a nested random effects structure whereby order was nested within class, class within phylum, phylum within kingdom and kingdom within domain (Hadfield & Nakagawa 2010). Only domain, kingdom, phylum, class and order were entered into models because there was no replication within our data at the other taxonomic levels. We used inverse gamma priors for all R and G-side random effects (V = 1, nu=0.002), which produced well mixed chains that passed all convergence tests (see below) (Plummer *et al.* 2006).

We ran each analysis for 6,000,000 iterations with a burn-in of 1,000,000 and a thinning interval of 1000. This generated 5000 posterior samples that we used to calculate the posterior mode, 95% credible intervals (lower CI – upper CI), and pMCMC (number of simulated cases that are > 0 or less than 0 corrected for finite number of MCMC samples). We considered terms statistically significant when 95% credible intervals did not span 0 and pMCMC values were less than 0.05 (Hadfield 2010). We ran models with parameter expanded priors (half-Cauchy priors following (Gelman 2006): V=1, nu=1, alpha.mu = 0, alpha.V = 25^2) due to some variance components being close to 0. The inverse gamma prior led to better convergence as measured by the Gelman-Rubin statistic (see below) and produced almost identical results to equivalent frequentist models run with ASReml-R version 3 (Gilmour *et al.* 2009). We therefore used priors with V=1, nu=0.002 for all models. We imputed missing values in explanatory variables using the mean of the missing variable (Z-transformed scale = 0) so that it was possible to compare different models using DIC (Nakagawa & Freckleton 2010).

We checked the convergence of each analysis using two diagnostic tests in the R package 'coda' (Plummer *et al.* 2006). First, we ran each analysis 3 times and used the Gelman-Rubin statistic (potential scale reduction factor (PSR)) to compare within- and between-chain variance (Gelman & Rubin 1992). When convergence is met PSR <1.1 and in all our analyses PSR was less than 1.01. Second, we used Geweke's convergence diagnostic, which calculates Z scores from mean parameter estimates ± standard errors generated from the first 10% and the last 50% of the chain (Geweke 1992). If Z scores follow an asymptotically standard normal distribution the samples are considered to be drawn from a stationary distribution.

5. Adaptive phenotypic plasticity in viral parasites[‡‡‡]

[‡‡‡] Published as: Leggett H.C., Benmayor R., Hodgson D.J. & Buckling A. 2013. Experimental Evolution of Adaptive Phenotypic Plasticity in a Parasite. Current Biology 23: 1-4 (appended)

Summary

Co-infection of parasite genotypes can select for various changes in parasite life history strategies relative to single genotype infections, with consequences for disease dynamics and severity (Bull 1994; Chao *et al.* 2000; Buckling & Brockhurst 2008; Mideo 2009; Frank 1996; Nowak & May 1994; Bremermann & Pickering 1983; van Baalen & Sablelis 1995; Brown, Hochberg & Grenfell 2002; West & Buckling 2003; Gardner, West & Buckling 2004; Alizon & van Baalen 2008; Lively 2009; Choisy & de Roode 2010). However, even where co-infection is common, a parasite genotype is also likely to regularly experience single genotype infections over relatively short periods of evolutionary time, due to chance, changes in local disease transmission and parasite population structuring. Such alternating conditions between single genotype and co-infections will impose conflicting pressures on parasites, potentially selecting for facultative responses to co-infection (Choisy & de Roode 2010; Stearns 1989; Moran 1992; Agrawal 2001; West-Eberhard 2003; Thomas *et al.* 2002). While such adaptive phenotypic plasticity in response to social environment has been observed in protozoan parasites and viruses (Reece, Drew & Gardner 2008; Abedon 1999), here we show it evolving in real-time in response to co-infection under conditions where both single and co-infections are common. We experimentally evolved an obligate-killing virus under conditions of single virus infections (Single lines) or a mix of single- and co-infections (Mixed lines), and found Mixed lines to evolve a plastic lysis time: they killed host cells more rapidly when co-infecting than when infecting alone. This behaviour resulted in high fitness under both infection conditions. Such plasticity has important

consequences for the epidemiology of infectious diseases and the evolution of cooperation.

Results and discussion

We experimentally evolved an initially clonal, obligate-killing virus (bacterio**phage** ϕ2 (Buckling & Rainey 2002) of a bacteria (*Pseudomonas fluorescens* SBW25 (Rainey & Bailey 1996)) under conditions where either the densities of bacteria greatly exceeded (by five orders of magnitude) that of phage, resulting in predominantly single phage infections (Single lines), or where phage and bacterial densities were initially approximately equal, resulting in phages experiencing a mix of single- and co-infections (Mixed lines) initially, followed by predominantly co-infections as the phage population grew. We measured phage population growth rate in the phages's selection and reciprocal environments every 10 days for a total of 50 days and found no monotonic change in growth rates for either treatment, in either environment (Figure S1 (appended), linear effects of time, $P > 0.4$ in all cases). There were, however, differences in mean growth rates between treatments. Specifically, Mixed lines had lower growth than Single lines under co-infection conditions, while there was no difference in growth between treatments measured under single infection conditions (Figure 1; significant interaction between selection and assay environments; mixed-effects ANOVA with time as fixed effect, $F_{1,18.33} = 5.72$, $P = 0.028$). This reduction in growth of Mixed phages under mixed conditions may initially seem paradoxical, but is in fact consistent with adaption to co-infections: increased within-host fitness of parasites is typically associated with reduced between-host transmission and hence

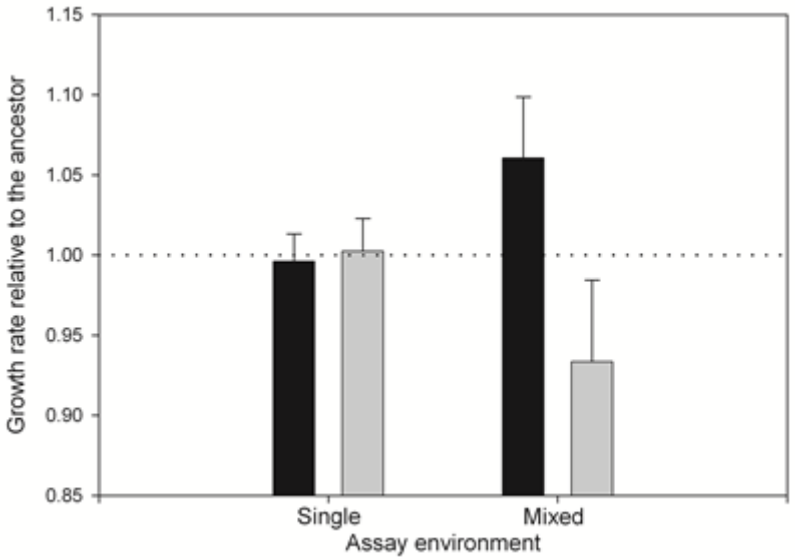


Fig. 1. Mean phage growth (multiplication rate) through evolutionary time compared to ancestral phage in single and mixed infection conditions (±SEM; n=6). Black bars: Single lines; pale bars; Mixed lines. Dashed line represents equal growth.

decreased parasite population growth rate (Bull 1994; Buckling & Brockhurst 2008; Mideo 2009; Frank 1996; Nowak & May 1994; Bremermann & Pickering 1983; van Baalen & Sablelis 1995; Brown, Hochberg & Grenfell 2002; West & Buckling 2003; Gardner, West & Buckling 2004; Alizon & van Baalen 2008; Lively 2009; Choisy & de Roode 2010).

To determine whether the evolved strategies of the Mixed lines were adaptive, we competed independent pairs of Single and Mixed evolved lines under single and co-infection conditions. We found that Mixed lines had an average threefold growth rate advantage over Single lines under co-infection conditions (Figure 2; 1-sample Wilcoxon test against relative fitness of 1, P=0.03), and no detectable difference in growth rate

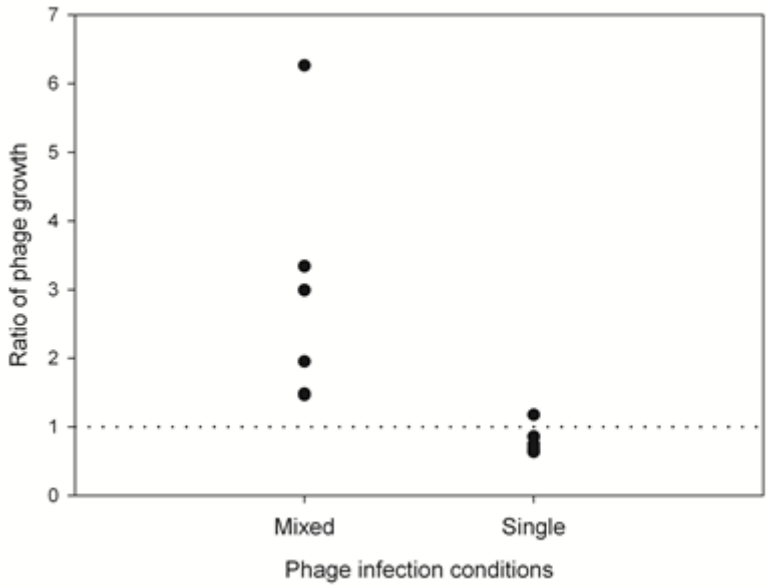


Fig. 2. Competitive growth of Mixed lines against Single lines in single and mixed infection conditions. Dashed line represents equal fitness (1). Fitness >1: Mixed lines are more fit.

under single infection conditions (Figure 2; Wilcoxon test, $P>0.05$). Reduced population growth rate of the Mixed lines therefore appears to be an adaptation resulting from selection under co-infection conditions.

We next investigated the phenotypic basis of adaptation to co-infections, and whether or not the Mixed phages had evolved adaptive phenotypic plasticity. Within-host competition resulting from co-infections can select for a range of parasite strategies including increased rates of host exploitation, interference, and exploitation of competitors' public goods (social cheating) (Buckling & Brockhurst 2008; Mideo 2009); an example of the latter being the frequently observed Defective Interfering particles in viruses (DePollo & Holland 1987; Turner & Chao 1999). It is possible that plasticity in any of these mechanisms may have evolved in the Mixed lines, but we initially investigated plasticity in the time taken to lyse hosts, because such plasticity in

response to co-infection has been shown in phage T4 and its close relatives (Abedon 1999, 2011). Like many viruses, the phage used in this study transmits by lysing its bacterial host, hence increased rates of host exploitation are likely to manifest as a faster time to lysis at the expense of a reduced viral yield (Abedon, Herschler & Stopar 2001; Wang 2006; Wang, Dykhuizen & Slobodkin 1996). Bacteriophage replication involves the production of multiple copies of the phage genetic material and protein coats, which are then packaged together to form the complete virus shortly prior to lysing the host cell (Wang, Dykhuizen & Slobodkin 1996). Earlier lysis has been shown to provide a competitive advantage during phage mixed-genotype infection (Refardt 2011), presumably because co-infecting competitors with longer lysis times will have packaged up fewer complete viruses prior to cell lysis. To investigate adaptive plasticity of lysis time, we first measured the time taken for a statistical increase in density to occur in the ancestral, Single and Mixed lines under both single and co-infection conditions. Under single infection conditions, the lysis time of ancestral, Single and Mixed lines was approximately 35 minutes (Figure 3; paired *t*- test of yield at 35 minutes versus 0 minutes, P=0.0002, P =0.0002 and P =0.001 respectively). However, under co-infection conditions, the lysis time of Mixed lines was approximately 30 minutes (Figure 3A, P< 0.0001) whereas it remained at 35 minutes for the Single lines and ancestral clone (Figure 3B, P <0.0001 and P =0.01 respectively). To formally demonstrate that there is a change in lysis time in the Mixed infection lines in response to co-infections, but not in the Single infection lines, we estimated the lysis time (the number of minutes taken to reach 50% of maximal phage density during a single synchronised growth cycle (*t*50)), for each replicate under both single and co-infection conditions. The *t*50 of Mixed lines was significantly lower than

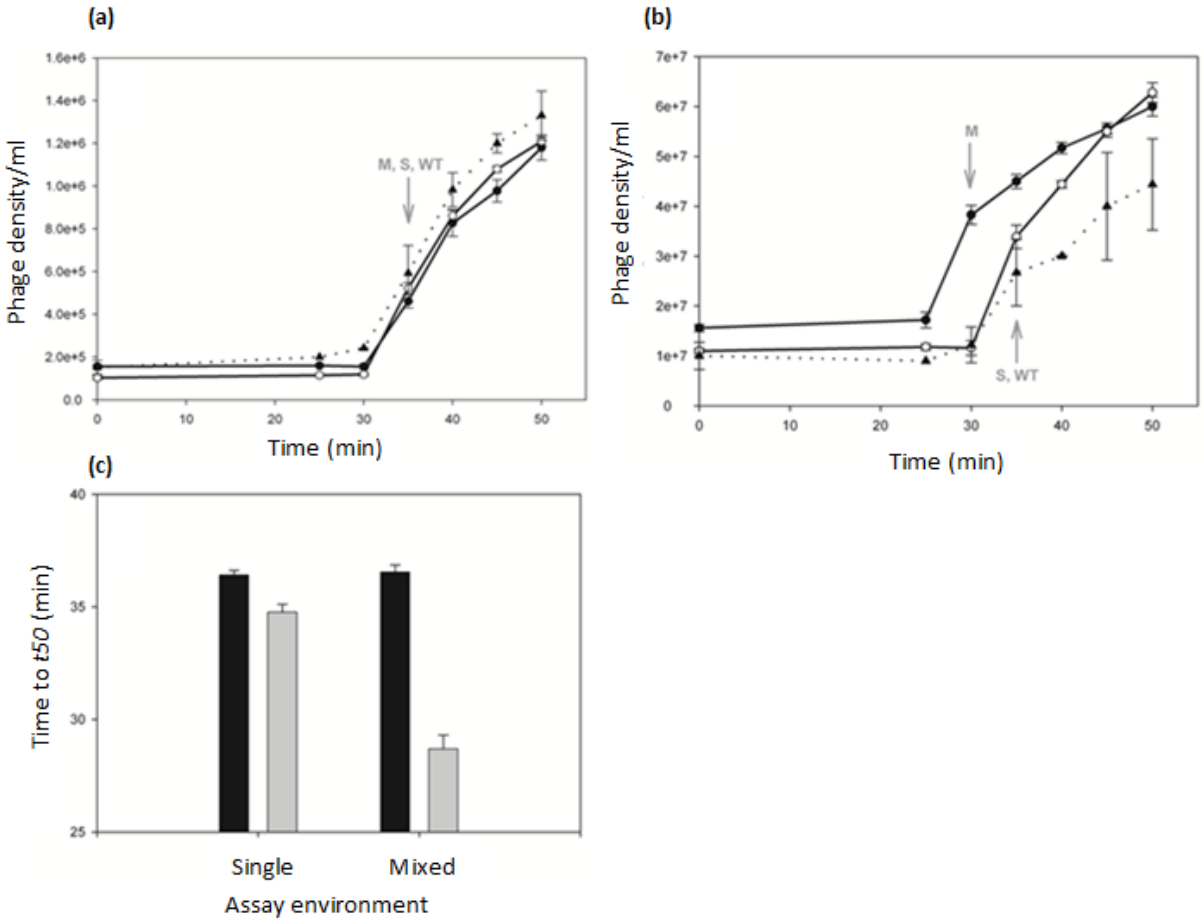


Fig. 3. Mean phage growth in **(a)** single infection and **(b)** mixed infection conditions (±SEM; n=6). Filled circles: Mixed lines (M); open circles: Single lines (S); triangles/dotted line: ancestral lines (WT). Arrow indicates the time point where phage density significantly increases. **(c)** Mean number of minutes taken for phage to reach 50% of maximal phage density (*t*50) in single and mixed infection conditions (±SEM; n=6) (C). Dark bars: Single Lines; pale bars: Mixed lines.

Single lines under mixed infection conditions, with no difference in *t*50 between the lines in single infection conditions (Figure 3C; significant interaction between selection line and assay conditions, $F_{1,20} = 59.794$, $P<0.0001$). This demonstrates that an initially clonal virus that experienced a mix of single and co-infections over few hundred generations evolved to phenotypically alter its lysis time in an adaptive manner,

depending on whether it infected bacteria by itself, or co-infected with other virus clones.

Relevance to the evolution of cooperation

Our results demonstrate that even the simplest organism can maintain individually costly cooperative behaviours in the face of social cheats. Cooperation in this context can be viewed as prudent use of hosts (killing the host relatively slowly to maximise viral yield), a strategy favoured by selection when individuals sharing the same resource (a single host) are close relatives (Frank 1996). For rapidly evolving viruses, such high relatedness can only be guaranteed if everyone has descended from the same infecting clone. In contrast, competition with other genotypes favours cheating (faster rates of host exploitation) (Frank 1996). Adaptive phenotypic plasticity in response to the number of infecting genotypes ensures viruses only cooperate under conditions of high relatedness.

Conclusions

Here we have shown that an initially clonal virus, propagated under a mix of single- and co- infection conditions, readily evolves the ability to detect the presence of co-infecting clones, and adjust their behaviour accordingly. The ease at which such adaptive plasticity evolved in our experiment suggests the behaviour is likely to be widespread amongst parasites, and readily influenced by selection. The existence of social plasticity has important implications for parasite epidemiology and virulence. First, virulence and transmission of a given parasite may differ dramatically according to

whether it is in a mixed or single genotype infection. Second, intervention strategies that aim to use avirulent social cheats ("Hamiltonian Medicine" (Foster 2005)) to outcompete virulent strains are likely to fail: parasites are likely to simply evolve to act as cheats when cheats are present, but express more virulent, cooperative phenotypes in their absence.

Experimental procedures

Selection experiment

We used a single plaque of the lytic bacteriophage SBW25 Φ2 (Buckling & Rainey 2002) to initiate all selection lines, and a single colony of *Pseudomonas fluorescens* SBW25 (Rainey and Bailey 1996) as the host bacteria. We grew bacteria and bacteria-plus-phage overnight in 6 ml King's Media B (KB) in 30 ml glass universals (shaken at 200 rpm and 28^{o}C). We isolated phages by treatment with 10% chloroform and centrifuging at 14k rpm for 2 min. We inoculated 12 KB tubes as above with approximately $5*10^{8}$ cells per ml of wild type (WT) bacteria (10% of an overnight culture shaken at 200rpm and 28^{o}C. We then added approximately $5*10^{8}$ particles per ml of phage to the first 6 replicates (Mixed phage infection lines), whereas the other 6 contained 10^{3} particles per ml of phage (Single phage infection lines). We grew the populations at 200 rpm conditions, with loose lids at 28^{o}C. After 8 hr, we isolated phages as above, stored them overnight in the fridge, and inoculated either $5*10^{8}$ or 10^{3} into fresh KB tubes, along with $5*10^{8}$ cells of ancestral SBW25. We continued the experiment for 50 transfers, with phages stored at -80^{o}C in cryotubes every ten transfers. In both treatments, phages reached densities of approximately 10^{10}/ml after 8

hr, with maximum bacterial densities of $5x10^9$/ml, hence some single- and co-infections likely occurred in both treatments. However, the frequency of co-infection was inevitably far more common in the Mixed lines. Note that we have previously established that co-infections readily occur in this system, by using marked phages (Hall *et al.* 2012 (appended)).

Population growth rate assays across environments

To investigate environment-specific adaptations, we measured population growth rates of the evolved phages under both selection conditions every ten transfers. After 8 hr of growth, we determined phage population densities by plating dilutions of each phage population onto KB agar plates with a semi-solid overlay bacterial lawn. We calculated the Malthusian growth rate as *ln*(end phage density/start phage density) (Lenski *et al.* 1991). We calculated mean growth rate of the ancestral phage from measures taken at multiple time points.

Fitness assays

To determine whether the evolved strategies were adaptive, we competed independent pairs of Single and Mixed lines. To distinguish between the lines, we attempted to select spontaneous mutants that could infect an evolved *P. fluorescens* clone. For some reason, we were only able to do this for all the Single lines, so we competed mutant/ 'marked' Mixed lines against unmarked Single lines. To attempt to control for the cost of this host range mutant marker, we also competed ancestral phages against independently marked ancestral phages. We competed pairs of phages at equal starting

ratios under both single and mixed infection conditions for 8 hr in a static incubator at 28 ^{o}C, and measured the growth of each of the populations by plating chloroform-treated populations onto separate KB agar plates with semi-soft overlay lawns of either WT or resistant bacteria. We calculated relative fitness (w) of each m line, where $w = m_1/m_2$, m_1 = Malthusian growth of the mutant; and m_2 = Malthusian growth of the competing strain. Therefore w was greater than 1 when the Mixed line grew faster. We repeated each assay three times, calculating the mean of the pseudo-replicates. We then divided each w by the mean w of the ancestral marked genotypes when competed against the ancestor, to control for the cost of the host range marker.

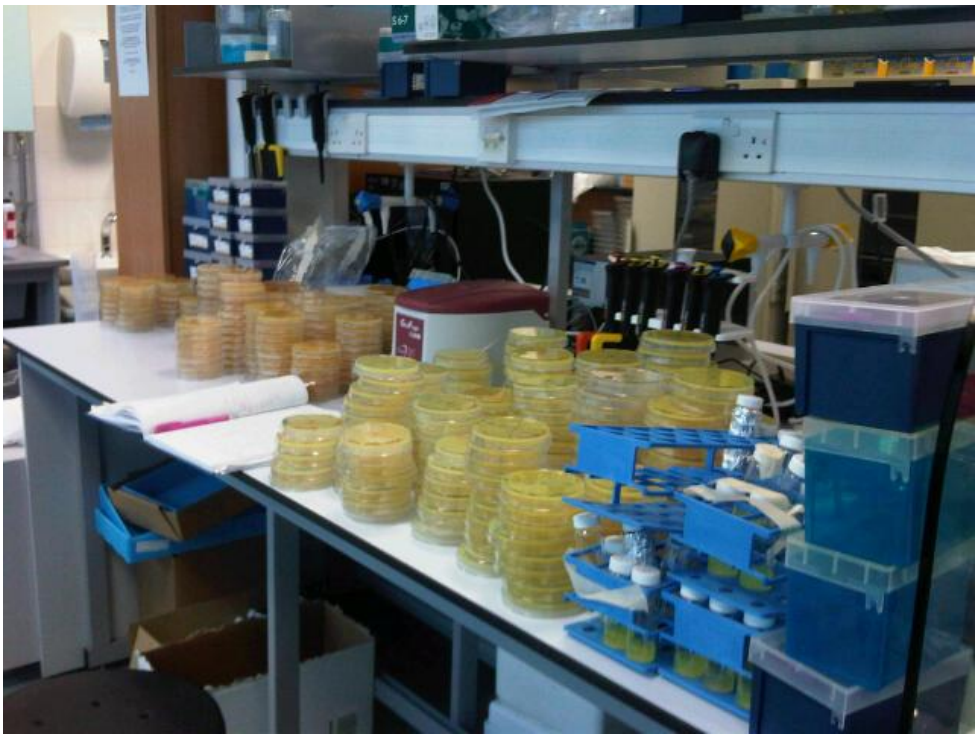
Measuring time to lysis

We measured the time taken for phage to lyse bacteria cells (latent period) in at least three phage clones in all Single and Mixed lines under reciprocal treatment conditions in a "one step growth experiment" (Ellis & Delbrück 1939). We added phages (10^5 or 10^8 for single and mixed conditions respectively) to 10^7 exponentially growing bacterial in 1 ml KB media, and measured phage density by plating onto bacterial lawns at time zero and then at 5 min intervals from 25 min (we never observed increases in density prior to 30 min in our preliminary studies). We used logistic regression of phage titres (as a proportion of their maximal density) against time, to estimate lysis time for each replicate. Our specific measure of lysis time was t_{50}: the number of minutes taken to reach 50% of maximal phage density during a single synchronised growth cycle.

Statistical analysis

We wanted to determine how mean phage growth rates varied through time for both Mixed and Single lines grown in both selection environments. We used General Linear Mixed Models with REML, with evolution treatment and time as fixed factors, and variance among population lines fitted as a random effect. Comparison of mean growth rates in mixed population assays were carried out using GLMs (with mixture as a single fixed effect), followed by post-hoc tukey pairwise comparisons. Ratios of phage growth and latent period data were not normally distributed, therefore Wilcoxon signed rank test were used. Variation in phage t_{50} was analysed as a factorial GLM with explanatory variables "evolution treatment" (Single or Mixed), "assay treatment" (single or mixed), and their interaction. Analyses were carried out using JMP or R version 2.14.0. All figures were drawn using SigmaPlot software.

6. Virulent parasites are “prudent” in space



…The inhumanity of this project

Abstract

Recent studies have suggested that parasites should be more "prudent" - less transmissible and virulent- in spatially structured host populations due to both epidemiological (host availability) and genetic (relatedness) processes. However, low transmission, the phenotype being selected for in this in context, can also be achieved by high virulence. Here we test this hypothesis, and also experimentally tease apart how host availability and parasite relatedness affect virulence evolution in a bacteria-virus system. We found that limited host availability and, to a lesser extent, low within-host relatedness favour virulent, faster killing parasites with reduced transmission. By contrast, high host availability and high relatedness favoured slower killing, more transmissible parasites. Our results highlight the importance of studying host-parasite interactions at multiple scales, and suggest high, rather than low, virulence may be common in spatially structured host-parasite communities.

Introduction

Because of the importance of infectious disease to humans, agriculture and wildlife, there is a well-developed theory on why parasites damage and even kill their hosts (termed parasite virulence). Virulence is often assumed to be a necessary consequence of host exploitation and hence transmission to new hosts (parasite fitness) (Anderson & May 1982; Bremermann & Pickering 1983; Nowak & May 1994; Frank 1996; Ebert & Bull 2003). For most parasites, virulence also negatively affects transmission success, for example if parasites can only infect from live hosts. As a result of this trade-off, an

intermediate level of host exploitation (optimal virulence) will maximise the transmission success for the parasite population. The relatedness of coinfecting parasite genotypes is also likely to affect virulence: kin selection models predict that the level of parasite virulence that maximises parasite fitness increases with decreasing relatedness of the coinfecting parasites, where parasites compete through resource competition only (Bremmerman & Pickering 1983; Nowak & May 1994; Frank 1996; but see Brown, Hochberg & Grenfell 2002, West & Buckling 2003; Gardner, West & Buckling 2004; Buckling & Brockhurst 2008 for alternative predictions when parasites produce public goods or public bads). This arises because optimal virulence is achieved only when individuals exploit host resources prudently, and is predicted to occur when relatedness between coinfecting parasite genotypes is high. When relatedness is low, within-host parasite competition causes selection for parasites to use host recourses as quickly as possible, thus increasing virulence, despite reducing population growth, an idea encapsulated by Hardin's "tragedy of the commons" (Hardin 1968; Frank 1996).

Recently, a body of work has suggested that natural selection favours relatively avirulent, prudent pathogens in spatially structured host populations, and low virulence is in turn expected to evolve because of an assumed positive relationship between virulence and transmission (Gardner & West 2009; Boots & Sasaki 1999, 2000; Read & Keeling 2003; Boots & Mealor 2007; Lion & Boots 2010). This is because of both epidemiological and genetic processes. First, when interactions are predominantly local, parasites are assumed to be constrained by the number of available hosts, therefore lower transmission is favoured to minimise the rate at which local hosts are exploited, which in turn maximises transmission to new patches of hosts (Boots & Mealor 2007; Lion & Boots 2010) (epidemiological mechanism). Second, the indirect fitness benefits

of prudent transmission are increased in structured host populations, because of the resultant increase in parasite relatedness within a patch (Wild, Gardner & West 2009; Lion & Boots 2010) (genetic mechanism). The basic prediction that spatial structure favours lowers parasite transmission has received empirical support from two recent experimental evolution studies: a moth-virus (Boots & Mealor 2007) and a bacteria-virus (Kerr *et al.* 2006) study. However, the relationship between host population structure and virulence evolution has yet to be empirically addressed.

Most models of parasite virulence in fact consider the positive relationship between virulence and transmission to be only a part of the virulence-transmission relationship: transmission is predicted to peak at a certain level of virulence and then decrease again. As such, high virulence could in principle be favoured over low virulence as a result of selection for reduced transmission (Lion & Boots 2010) (Figure 1), particularly when high virulence is also favoured because of low within-host relatedness between coinfecting strains. In other words, imprudence at one spatial scale (within-hosts) could result in prudence at another spatial scale (the patch).

Here we empirically tease apart how the epidemiological and genetic mechanisms resulting from host population structure affect virulence evolution in bacteria-virus system. Specifically, we carry out a factorial experiment in structured populations where hosts limit parasite growth or not, and parasite relatedness is either high or low. We start the experiment with a diverse population of viruses that in a previous study evolved to have high or low levels of virulence (fast – 28.69 ±0.61 min, or slow – 36.54±0.32 min killers respectively, given that they are obligate killers) (Leggett *et al.* 2013a (appended)). The fast killers have lower transmission but are more

competitive within-hosts. We hypothesise that limited host availability and low relatedness conditions should favour the more virulent (faster killing) strains.

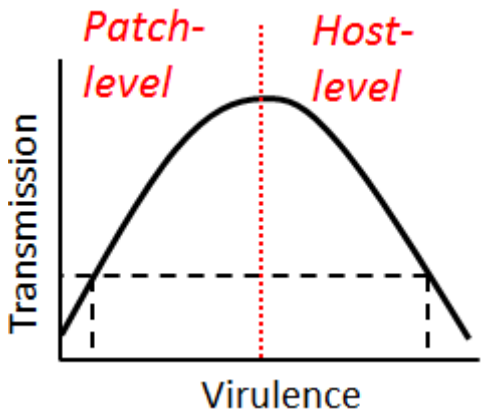


Fig. 1. The predicted domed relationship between transmission and virulence. Schematic showing how: (a) selection for prudence (low transmission) can result in either high or low levels of virulence (dashed line); (b) selection for imprudence (high virulence) at one spatial scale (within a host) can lead to prudence at another spatial scale – within a patch.

Results and discussion

We evolved 24 metapopulations, each comprising 12 patches of an obligately-killing virus (bacterio**phage** SBW25 Φ2) on its host bacterium *Pseudomonas fluorescens* for three transfers, approximately 20 phage generations. At each transfer, patches (wells in microtitre plates) within a metapopulation were mixed together and phage clones randomly selected from the mixture to seed new patches: the output of a patch was therefore dependent on its productivity (global competition). Each metapopulation was established with 12 phage populations that had previously been evolved for 300

generations, under conditions of low or variable multiplicity of infection (moi; 6 lines evolved under each treatment). Phages evolved under variable moi have a plastic lysis time (time to host killing), killing host cells faster in multiple infections, at the cost of reduced transmission to new hosts. Phages evolved under low moi had a significantly slower lysis time in multiple infections, resulting in lower within-host competiveness but increased transmission. (Leggett *et al.* 2013a). Half the metapopulations were high relatedness treatments, where patches were founded with single phage clones, while half were low relatedness, where they were founded with 2 clones. We also manipulated host availability in a fully a factorial design, such that hosts were a more or less limited resource, meaning parasite growth was relatively constrained or unconstrained (Figure 2).

Prior to the main experiment, we first established conditions where hosts were more or less limited by varying the length of time phage-bacteria metapopulations were allowed to grow. Parasite growth was relatively unconstrained over 8 hours resulting in higher relative population growth rate of the higher transmitting and less virulent populations, whereas hosts were more limited over 24 hours, resulting in higher population growth of the lower transmitting and virulent populations (Figure 3, significant effect of time*relatedness interaction $F_{1,20} = 7.84$ $P = 0.012$). We therefore transferred phage populations after either 8 or 24 hours to create conditions were parasite growth was relatively unconstrained or constrained.

At the end of the selection, we assessed the mean lysis time (*t50*) of each of the populations of phages from each of the four treatments. We found an interaction

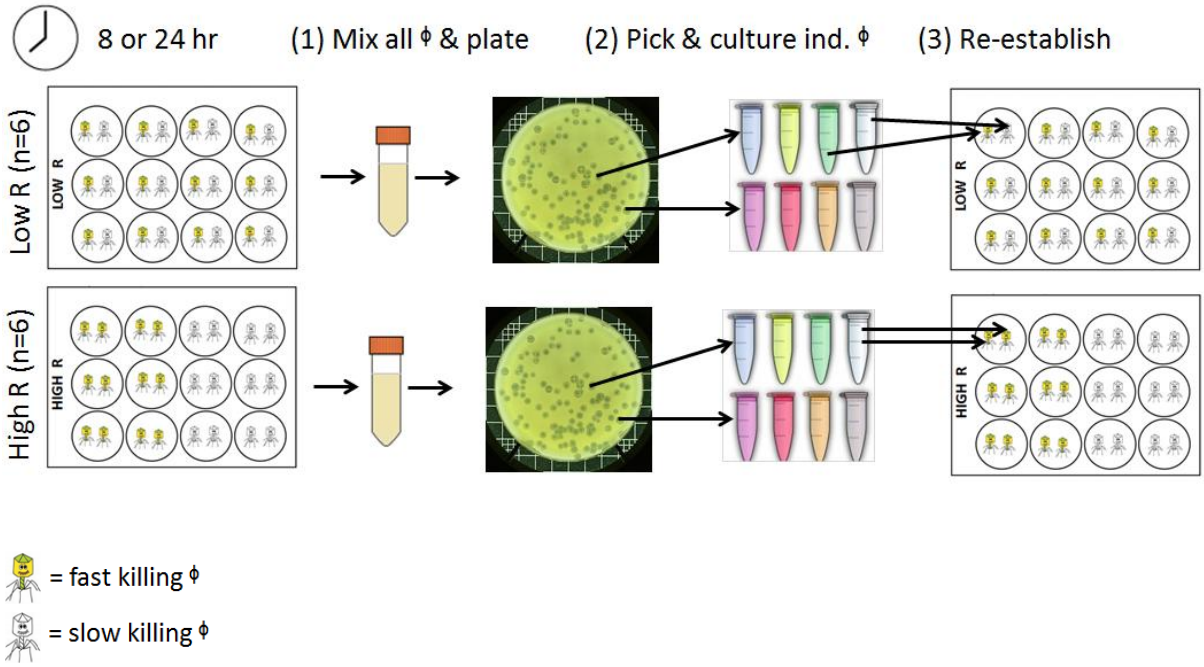


Fig.2. Experimental set up. We manipulated phage relatedness and host availability in a fully factorial design. We started the experiment with diverse metapopulations of fast and slow killing phage (Φ). Half the metapopulations were high relatedness treatments - patches (wells in microtitre plates) founded with single phage clones, while half were low relatedness - founded with 2 clones. We manipulated host availability by varying the amount of time host-phage populations were cultured: 24 versus 8 hr; hosts were a more or less limited resource, meaning parasite growth was relatively constrained or unconstrained respectively. **(1)** At each transfer, we mixed together all patches within a metapopulation and **(2)** randomly selected phage clones from the plated mixture to **(3)** re-establish new patches: the output of a patch was consequently dependent on its productivity (global competition).

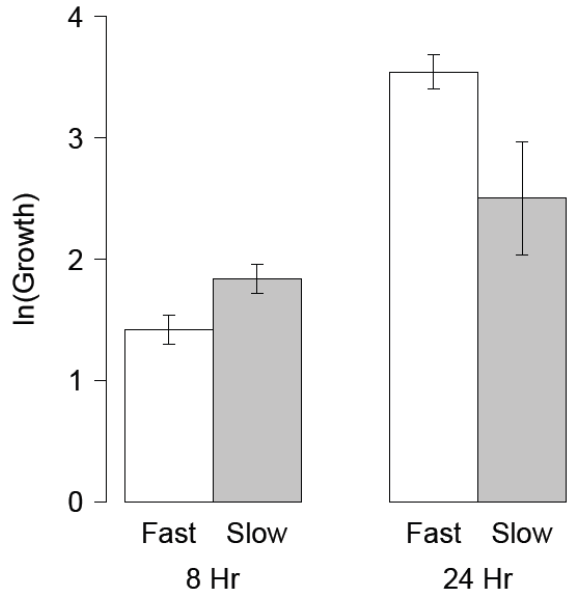


Fig. 3. Mean population growth (multiplication rate, logged) of fast- and slow-killing phage strains. Over 8 hr, host availability was less limited, resulting in higher relative population growth rate of the higher transmitting and less virulent (slow killing) populations, whereas hosts were more limiting over 24 hr, resulting in higher population growth of the lower transmitting and virulent (fast killing) populations. Data are means (n=6) ± SEM.

between host availability and relatedness (Figure 4, significant relatedness*host availability: $F_{1,14} = 37.82$, $P<0.001$), such that when host availability was low, lower lysis times were favoured regardless of relatedness, whereas where host availability was high, lysis time was lower under conditions of low relatedness. Indeed, pairwise comparisons between treatments suggest that evolved lysis times were the same except under conditions of high relatedness and high host availability, where a longer lysis time was favoured (Figure 4, Tukey's pairwise comparisons, P<0.001).

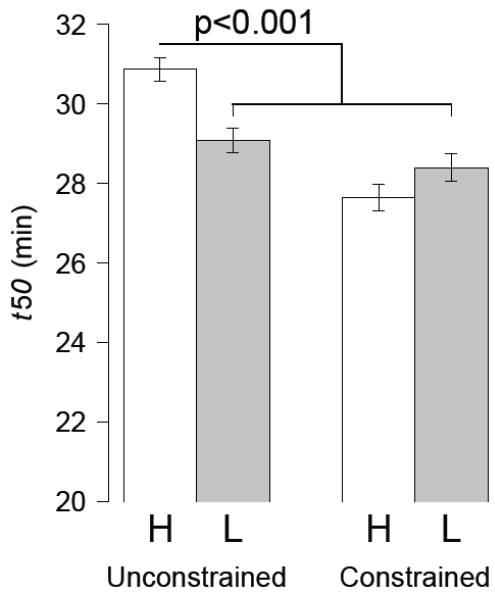


Fig. 4. Lysis time (*t50*) of populations of evolved phage lines. Phages were evolved in patches where relatedness was either high (H) or low (L), and where their growth was relatively unconstrained or constrained by unlimited or limited host availability. Data are means (n=6) ± SEM.

Consistent with previous theory and experiments (Boots & Mealor 2007; Kerr *et al.* 2006), our experiment shows that limited host availability in spatially structured environments favours lower transmitting strains. However, we show that these lower transmitting strains can also be the most virulent; selection for low transmission can therefore readily favour high virulence. Moreover, we show that within-host competition (i.e low relatedness coinfections) also favours high virulence, even when high transmission, and hence lower virulence, is favoured by limited host availability.

Previous theory and experiments investigating the role of spatial structure on the evolution of virulence, have, unsurprisingly directly manipulated spatial structure (Wild, Gardner & West 2009; Boots & Sasaki 1999, 2000; Read & Keeling 2003; Boots

& Mealor 2007; Lion & Boots 2010; Boots & Mealor 2007; Kerr *et al.* 2006). However, spatial structure can result in both epidemiological and genetic changes, both of which can affect selection for transmission and virulence. Here, we evolved all populations in spatially structured host populations, but independently manipulate the key consequence of spatial structure: host availability, which is likely to be reduced in spatially structured environments; and parasite relatedness which is likely to be increased within a patch. We show both process can have independent and important effects, although host availability appears to be more significant. It is important to emphasise that spatially structured environments *per se* do not necessarily affect virulence evolution, but only if they effect host availability and relatedness.

Previous empirical studies are broadly consistent with our results, in that spatially structured environments favour lower transmission (Boots & Mealor 2007; Kerr *et al.* 2006). However, neither of these studies explicitly investigated the life history changes that lead to reduced virulence; hence it is unclear how and why virulence changed. Additional experiments by Kerr *et al.* (2006) suggested lower transmission was accompanied by reduced competitive ability, suggesting an avirulent strategy evolved. However, this pattern was observed under conditions where host availability was both very low (high moi) and very high (very low moi), making these data extremely difficult to understand. Specifically, high productivity resulting from slow transmission should equate with high fitness when competing under conditions of high host availability.

We suggest that selection for low transmission where hosts are limited may commonly result in high rather than low virulence. This is because high or low virulence strategies can achieve the same optimal level of transmission under conditions

of high within-host relatedness; but low host availability is more likely to result in coinfections of multiple strains; potentially reducing within-host relatedness and favouring higher virulence. Thus, achieving low transmission through high rather than low virulence may be more advantageous. Note that where mixed infections results in the evolution of low virulence as a result of public goods exploitation or interference competition (Brown, Hochberg & Grenfell 2002; West & Buckling 2003; Gardner, West & Buckling 2004; Buckling & Brockhurst 2008), the situation is likely to be more complex.

Conclusions

This work highlights the important of studying host-parasite interactions at multiple scales in order to better understand the evolution of virulence. Studies of virulence evolution have typically focused on the within and between *host* level (Anderson & May 1982; Bremermann & Pickering 1983; Nowak & May 1994; Frank 1996; Ebert & Bull 2003), or more recently on the within- and between *patch* scale (Wild, Gardner & West 2009; Boots & Sasaki 1999, 2000; Read & Keeling 2003; Boots & Mealor 2007; Lion & Boots 2010; Boots & Mealor 2007; Kerr *et al.* 2006). Here, we show how consideration of all three scales - within-host, between-host (within-patch) and between-patch - alters how selection acts on virulence traits. We hope that the work will inspire theoretical work addressing the interplay between the three scales to allow more general predictions to be made.

Materials and methods

We evolved 24 metapopulations of an obligately-killing virus (bacterio**phage** Φ2) on its host bacterium *Pseudomonas fluorescens* for three transfers, approximately 20 phage generations. We embedded bacteria and viruses into microtitre plates, which imposed a metapopulation structure comprised of 12 patches (wells in microtitre plates) containing 200 μl Kings Media B (KB) and 10^2 exponentially growing ancestral bacteria. We ensured high within host competition (local competition) in all treatments by establishing high multiplicity of infection (moi) conditions (10^8 phage versus 10^2 bacteria) in each patch. Note that we have previously established that coinfections readily occur in this system by using marked phages (Hall *et al.* 2012; Leggett *et al.* 2013a (appended)). At each transfer, we mixed together all the patches within a metapopulation and randomly selected phage clones from the mixture to seed new patches: the output of a patch was therefore dependent on its productivity (global competition) (Figure 2).

Establishing high and low relatedness treatments

We established each metapopulation with 12 phage populations that we had previously evolved for 300 generations, under conditions of low or variable moi (6 lines evolved under each treatment). Phages evolved under variable moi have a plastic lysis time (time to host killing), killing host cells faster in multiple infections, at the cost of reduced transmission to new hosts. Phages evolved under low moi had a significantly slower lysis time in multiple infections, resulting in lower within-host competiveness but increased transmission (Leggett *et al.* 2013a). Thus we had 12 phage strains: 6

imprudent strains - low transmitting, fast killers; and 6 prudent strains - high transmitting, slow killers.

Half the metapopulations were high relatedness treatments, where we founded patches with 2 clones of prudent or imprudent strains, while the other half were low relatedness treatments, which we founded with 2 clones: 1 prudent and 1 imprudent clone. Thus wells came in three varieties: 2 prudent clones or 2 imprudent clones in each well for the high relatedness treatment; and 1-each of prudent or imprudent clones in each well for the low relatedness treatment. Consequently, the relatedness within patches was initially either high or low depending on the treatment, yet relatedness at the metapopulation level was initially equal across all treatments.

Establishing limited or unlimited host availability treatments

We also manipulated host availability in a fully a factorial design, such that hosts were a more or less limited resource, meaning parasite growth was relatively constrained or unconstrained. Prior to the main experiment, we first established conditions where hosts were more or less limiting by varying the length of time phage-bacteria metapopulations were allowed to grow. We cultured host-parasite communities (10^8 phage versus 10^2 bacteria in 200ul KB at 28^{o}C) for either 8 or 24 hr. We found parasite growth was relatively unconstrained over 8 hr, resulting in higher relative population growth rate of the higher transmitting and less virulent populations, whereas hosts were more limiting over 24 hr, resulting in higher population growth of the lower transmitting and virulent populations (Figure 3, significant effect of time*strain interaction $F_{1,20} = 7.84$ $P = 0.012$). We therefore transferred phage populations after

either 8 or 24 hr to create conditions where parasite growth was relatively unconstrained or constrained.

Selection experiment

We perpetuated the entire metapopulations through three serial transfers every 8 or 24 hr depending on the host availability treatment (described above). In each transfer, we combined all the subpopulations within a metapopulation to simulate global competition within spatially structured populations, by thoroughly mixing the entire metapopulations in a 10 ml falcon tube, treating with 10% chloroform and centrifuging at 14,000 rpm for 2 min to isolate the phage, then isolated individual phage clones by plating dilutions of phage populations onto KB agar plates with a semisolid overlay *P. flueorescens* lawn. We then picked individual clones (phage plaques) with sterile pipette tips, amplified them overnight in liquid KB media plus 10^6 ancestral *P. flueorescens* so they reached the same densities, then redistributed 10^8 phage into a fresh set of wells containing; reestablishing the same treatment conditions. There were four treatments in total: (i) high relatedness and unconstrained growth, (ii) high relatedness and constrained growth, (iii) low relatedness and unconstrained growth, and (iv) low relatedness and constrained parasite growth. We replicated each treatment six times (Figure 2).

Measuring time to cell lysis time

We measured the population-level time taken for phage to lyse bacteria cells in each of our 24 metapopulations in a "one-step growth experiment" (Ellis & Delbruck 1939). We

added 10^8 phage to 10^2 exponentially growing bacteria in 20 µl KB media and measured phage density by plating onto bacterial lawns at time zero and then at 5 min intervals from 25 min (we never observed increases in phage density prior to 30 min in our previous studies (Leggett *et al.* 2013a (appended)). Our specific measure of lysis time was *t50*, the number of min taken to reach 50% of maximal phage density during a single synchronized growth cycle.

Statistical analysis

We analysed variation in phage growth as a factorial generalized linear model (GLM) with explanatory variable "relatedness" (high or low) and "time" (8 or 24), and their interaction. We analysed variation in phage population lysis time *(t50)* as a factorial GLM with explanatory variables "relatedness" (high or low) and "phage growth" (unconstrained or constrained) and their interaction. We conducted pairwise comparisons of *t50* with Tukey's pairwise comparison tests using function 'glht' in package 'multcomp (Hothorn, Bretz & Westfall 2008) in R. We carried out all analyses and drew all figures using R version 2.15.3.

7. Discussion

Each of the chapters in this thesis contained their own extensive discussion. The aim of this chapter is to highlight the achievements of the preceding chapters, and to briefly discuss some emerging general points.

Chapter 2. Generalism and virulence

- Support for the virulence trade-off theory is good but not perfect – it is limited to explaining variation in virulence within species or between species with similar life histories, rather than broad scale variation across different parasite species.
- Parasites differ in their degree of host generalism – the range of hosts that parasites can infect.
- Generalism is likely to impact virulence in four ways: (i) costs of generalism, (ii) multiplicity of infection, (iii) maladaptive virulence and (iv) host availability.
- Incorporation of infectivity data may lead to better predictions of evolved virulence.

Chapter 3. Infectious dose and virulence

- Mechanisms used by parasites to infect hosts are able to explain variation in two key pathogen traits: infectious dose and virulence.

- ∞ In pathogens where the molecules secreted to facilitate infection acted locally, the number of cells required to start an infection (infectious dose), was lower than in pathogens where the secreted molecules act more distantly.
- ∞ Parasite virulence showed no correlation with local versus distant action, but was negatively correlated with infectious dose, and greater in species that infect via skin wounds.
- ∞ By showing how life history details matter, our results help explain why classic trade-off models have been relatively unsuccessful at explaining broad-scale variation in virulence.

Chapter 4. Immune subversion and virulence

- ∞ Contrary to the standard assumptions of virulence theory, in a comparative study across 61 human pathogens, there was no significant relationship between pathogen maximum growth rate and virulence, despite controlling for lots of other parasite life history variables. Rather parasites capable of subverting the host immune system are significantly more virulent than non-immune subverting parasites.
- ∞ Virulence did not vary depending upon whether a pathogen's motility, or whether it used quorum sensing to control the production of virulence factors, but fitted in with other patterns for route of infection.
- ∞ These results further emphasise how virulence can be influenced by mechanistic life history details that vary across species concerning how parasites infect and exploit their hosts. This variation can swamp simple relationships between

growth rate and virulence, hence assumptions of theory may be not useful across species.

Chapter 5. Adaptive phenotypic plasticity in a virus

- ∞ Alternating conditions between single genotype and coinfections will impose conflicting pressures on parasites, potentially selecting for plastic responses to coinfection.
- ∞ Viruses cultured under a mix of single infections and coinfections evolved *de novo* plasticity
- ∞ They killed hosts more rapidly when in coinfections than when infecting alone. This resulted in high fitness under both single infection and coinfection conditions.
- ∞ This plasticity has important consequences for the epidemiology of infectious diseases and the evolution of cooperation.

6. Virulent parasites are "prudent" in space

- ∞ Parasites are thought to be more "prudent" - less transmissible and virulent- in spatially structured host populations due to both epidemiological (host availability) and genetic (relatedness) processes. However, in this context, low transmission can also be achieved by high virulence.
- ∞ In a bacteria-bacteriophage system, limited host availability and, to a lesser extent, low within-host relatedness favoured virulent, faster killing parasites

with reduced transmission. By contrast, high host availability and high relatedness favoured slower killing, more transmissible parasites.

- This suggests high, rather than low, virulence may be common in spatially structured host-parasite communities and highlights the importance of studying host-parasite interactions at multiple scales.

2. General remarks

Some general themes have emerged from these studies:

Selfishness of exploitative strategies

In this thesis I have discussed how several evolutionary processes influence virulence. For example parasites are favoured to exploit their hosts prudently to prolong infection and avoid killing the host. Parasites also need to use some host resources to reproduce and transmit. Thus parasites face a trade-off between prudent exploitation and rapid reproduction – a life history trade-off between longevity and fecundity (Chapter 6). Other trade-offs between components of parasite fitness also affect virulence. For example, competition between coinfecting genotypes favours rapid growth to achieve greater relative competitive success within the host. Rapid growth may however lower the total productivity of the population by over exploitation of the host (Chapter 5). These evolutionary processes unanimously take both a parasite-centric viewpoint and highlight how all parasite exploitative strategies are selfish: ultimately, whether

infecting alone or in coinfections, a parasite will adjust its behaviour to maximise its potential for success.

Relevance of coevolution in host-parasite interactions

While this thesis and the majority of virulence evolution studies focus on the parasite perspective of virulence, it is necessary to emphasise that virulence evolution is likely to be affected by many other additional selective forces imposed by the ecology of the host and parasite. For example, as parasites become exposed to novel hosts because of increased population density, climate change and urbanisation, natural selection is likely to favour different life-history strategies such as increased generalism (Chapter 2). Furthermore, the expression of virulence is by no means solely determined by parasites, but rather the interactions between host and parasite genotypes are likely to be reciprocal (Read and Taylor 2001, Ebert and Bull 2003, Lambrechts *et al.* 2006, Buckling and Brockhurst 2008). For example, high virulence might reduce the availability of susceptible hosts, imposing selection for increased host range (Chapter 2).

Biological details matter

Life history trade-off theory has enjoyed great success, in terms of explaining the observed variation in virulence within species, but not between species. Given this widespread success within species, it is probable that poor empirical support across different species are not due to a misunderstanding in how selection operates, but rather it is more likely that some crucial details of the host-parasite system's biology have been overlooked. Noting that some of the biological assumptions of classical theory will not be valid in every circumstance, and developing the theory accordingly, can

dramatically improve the explanatory power of the theory. For example, relaxing assumptions about a positive correlation between growth rate and virulence and simultaneously taking into account immune subversion capabilities of parasites may allow for much improvement in the predictive power of virulence-transmission trade-off theory in explaining variation in virulence across species (Chapter 4). Using immune subversion as inspiration, and noting that biological details do appear to make a huge difference in models of virulence evolution, for example, parasite generalism (Chapter 2) route of transmission (Chapters 3 & 4), adaptive phenotypic plasticity (Chapter 5), but also bacteriocin production in Gardner, West & Buckling (2004) and siderophore production as examined by West & Buckling (2003), there is hope that developing the theory of virulence evolution will eventually achieve more broad-scale predictive success than it currently enjoys. Although, if the mechanistic details of infection vary widely between different pathogen species, this can overwhelm any simple relationship, potentially rendering assumptions of theory less useful across species. (Chapter 4)

Importance of studying selection at multiple scales

Social interactions between conspecific parasites are partly dependent on the relatedness of the interacting parasites, which in turn, is predicted to affect virulence. High relatedness is generally assumed to favour less competitive interactions, although the relationship between relatedness and virulence is dependent on the social behavior in question (Buckling & Brockhurst 2008). These relationships are also altered by other factors, not least the spatial structure of the parasites and their hosts. Host and parasite population structure matters since it determines the relatedness between interacting parasites and hence the extent to which they should cooperate or exploit each other (Inglis *et al.* 2011; Griffin, West & Buckling 2004; West, Pen & Griffin 2002; Buckling

& Brockhurst 2008). Studies of virulence evolution have typically focused on the within and between *host* level (Anderson & May 1982; Bremermann & Pickering 1983; Nowak & May 1994; Frank 1996; Ebert & Bull 2003), or on the within- and between *patch* scale (Wild, Gardner & West 2009; Boots & Sasaki 1999, 2000; Read & Keeling 2003; Boots & Mealor 2007; Lion & Boots 2010; Kerr *et al.* 2006). However, the experiment in Chapter 6 shows how consideration of all three scales - within-host, between-host (within-patch) and between-patch - alters how selection acts on virulence traits. Currently there is a lack of empirical work in this area. Addressing the interplay between these three scales of selection will allow us to makes more general predictions.

Relevance of virulence evolution to social evolution

"The most important unanswered question in evolutionary biology, and more generally in the social sciences, is how cooperative behaviour evolved and can be maintained in human or other animal groups and societies."

- Lord Robert May

In principle, we know quite a lot about why organisms cooperate with one another: inclusive fitness in the main driver of all adaptation (West & Gardner 2013). From a parasite's perspective, cooperation is an important strategy. When a virus infects in isolation, it must clone itself repeatedly within the cell. The cell contains all the material that is needed for this process to take place. Once the virus has accumulated to sufficient numbers, the virus can lyse the cell and spread to new cells. However, as demonstrated for the first time in Chapter 5, by cooperating with their clones, viruses can burst cells faster, and thereby gain a competitive advantage over other non-related

strains that may be present. Thus adaptive phenotypic plasticity in response to the number of competing genotypes ensures viruses only cooperate under conditions of high relatedness. Given that plasticity evolved *de novo* in the simplest of organisms, such behaviour is likely to be widespread in natural populations, and we should look more closely for it.

Future directions

In this thesis I have summarised current models and understanding of parasite virulence from the life-history trade-off and inclusive fitness perspective. I review and then apply and test the theory in formal comparative analysis across taxa of human pathogens and in experimental studies using a virus and its natural host bacteria. These applications show the power of simple life history analysis to highlighting interesting questions, but also highlight discrepancies between theory and data when only a few components of parasite fitness are analysed in isolation (Chapter 4). I have not addressed the host component of the host-parasite interactions. Similarly the genetics and genomic mechanisms underlying the phenotypically plastic viruses (Chapters 5 & 6) are unknown. My future work will: (a) include host evolution and components of infection ecology to investigate virulence evolution, and (b) seek to determine the genetic mechanisms underpinning the observed virulence traits.

Why we care: can we manipulate virulence and "domesticate" pathogens?

The question of why some diseases are more virulent raises the pertinent and closely related question: how can we take control of this situation once we know the answer to the first question? This is the essence of why we care about virulence evolution - we

could get evolution working in the direction we want it to go in and "domesticate" pathogens, rather than having to battle evolution as a problem.

In some cases we might want to select for virulence – for example, to generate a virus to control virulent bacterial infections or agricultural pests. But in terms of human diseases, it is generally in our interest to select for mild disease organisms. For example, in Chapter 3 I show that vectored and waterborne pathogens are among the most virulent human pathogens, possibly because these pathogens do not rely on an ambulatory host to transmit (Ewald 1994; 1991). In theory, if we clean up water supplies, we may prevent water transmission and favour natural selection towards lower virulence. There is some natural epidemic date that supports this idea: in 1991, *Vibrio cholera* broke out in Peru and within 2 years spread throughout South and Central America. Some countries had clean water supplies, others had contaminated water supplies. Within 5 years, however, when the organism invaded countries with clean water supplies, the organism evolved lower virulence (Ewald *et al.* 1998). However, while this kind of "natural experiment" is encouraging, if the plastic exploitation strategies I found in Chapter 5 are common, attempts to make microbes – during the course of their evolution – become less harmful to humans and "Hamiltonian medicine" (Foster 2005) intervention strategies that seek to use avirulent cheats to outcompete virulent strains, are likely to fail since parasites are likely to simply evolve to act as cheats when cheats are present, but express more virulent cooperative traits in their absence.

Understanding virulence also has important practical implications for enhancing the efficacy of antibiotics. For example, we could use virulent bacteriophage to significantly reduce the population size of acute antibiotic resistant bacterial infections, giving antibiotics more of a chance to clear the infection. Also, by selecting for

pathogens with lower virulence we could reduce selection pressure for antibiotic resistance: less virulent infections have fewer symptoms; therefore fewer people are treated with antibiotics, which may prolong antibiotic sensitivity. Indeed, in the Peruvian cholera outbreak discussed above, countries with clean water supplies appear to have dodged two bullets: the virulence of *V. cholera* strains decreased and antibiotic sensitivity was maintained in comparison to countries with poor water sanitation, where virulence increased and antibiotic sensitivity was reduced (Ewald 2007[§§§]).

General conclusions

To conclude, virulence evolutionary biology owes much of its success to its firm, conceptually simple, theoretical underpinnings. The theory boasts a unifying framework centred around Hamilton's rule and trade-offs between components of parasite life history. An appreciation for the subtlety of the framework is essential for: (1) development of simple, explicit models for host-parasite systems of interest; (2) ensuring rigorous theory-driven empirical research; and (3) making full use of our observations of parasite life history mechanisms to contribute to a better understanding of infections of ourselves, our agriculture and wildlife.

[§§§] Data presented by P. Ewald at TED Conference, March 2007: http://www.ted.com/talks/paul_ewald_asks_can_we_domesticate_germs.html

Bibliography

Abedon S.T (1999) Bacteriophage T4 resistance to lysis inhibition collapse. *Genet Res* 74: 1-11

Abedon S.T., Herschler T.D. & Stopar D. (2001) Bacteriophage latent-period evolution as a response to resource availability. *Appl Environ Microbiol* 67: 4233-4241

Agrawal A.A. (2001) Phenotypic Plasticity in the Interactions and Evolution of Species. *Science* 294: 321-326

Agudelo-Romero P. & Elena S.F. (2008) The degree of plant resilience to infection correlates with virus virulence and host-range. *Span J Agric Res* 6: 160-169

Alderman D.J. (1996) Geographical spread of bacterial and fungal diseases of crustaceans. *Rev Sci Tech Off Int Epizoot* 15: 603-632

Alizon S. & van Baalen M. (2008) Multiple Infections, Immune Dynamics, and the Evolution of Virulence. *Am Nat* 172(4): 150-168

Alizon S.*, et al.* (2009) Virulence evolution and the trade-off hypothesis: history, current state of affairs and the future. *J Evol Biol* 22: 245-259

Anderson R.M & May R.M (1982) Coevolution of hosts and parasites. *Parasitology* 85:

411-426

Anderson R.M. & May R.M. (1981) The Populations-dynamics of microparasites and their invertebrate hosts. *Philos Trans R Soc Lond Ser B-Biol Sci* 291: 451-524

Antonovics J.*, et al.* (2013) The origin of specificity by means of natural selection: evolved and nonhost resistance in host-pathogen interactions. *Evolution* 67: 1-9

Antunes L.C.M., *et al.* (2010) Quorum sensing in bacterial virulence. *Microbiology* 156: 2271-2282

Barrett L.G.*, et al.* (2009) Continua of specificity and virulence in plant host-pathogen interactions: causes and consequences. *New Phytol* 183: 513-529

Bedhomme S.*, et al.* (2012) Multihost experimental evolution of a plant RNA virus reveals local adaptation and host-specific mutations. *Mol Biol Evol* 29: 1481-1492

Benmayor R.*, et al.* (2009) Host Mixing and Disease Emergence. *Curr Biol* 19: 764-767

Berngruber T.W.*, et al.* (2013) Evolution of Virulence in Emerging Epidemics. *PLoS Pathog* 9: e1003209

Bolker B.M., *et al.* (2009) Generalized linear mixed models: a practical guide for ecology and evolution. *Trends Eco. Evol* 24: 127-135

Boots M. & Mealor M. (2007) Local interactions select for lower pathogen infectivity. *Science* 315: 1284-1286

Boots M. & Sasaki A. (2000) The evolutionary dynamics of local infection and global reproduction in host-parasite interactions. *Ecol Lett* 3: 181–185

Boots M. & Sasaki A. (1999) 'Small worlds' and the evolution of virulence: infection occurs locally and at a distance. *Proc R Soc B-Biol Sci* 266: 1933-1938

Bourke A F.G. (2011) *Principles of Social Evolution*, Oxford Series in Ecology and Evolution

Bourke A.F.G. & Franks N.R (1995) *Social Evolution in Ants*, Princeton University Press, Princeton

Bray M. (2005) Pathogenesis of viral hemorrhagic fever. *Curr Opin Immunol* 17: 399-403

Bremermann H.J. & Pickering J. (1983) A game-theoretical model of parasite virulence. *J. Theor. Biol.* 100: 411-426

Brown S. P. & Buckling A. (2008) A Social Life for Discerning Microbes. *Cell* 135**:** 600-603

Brown S.P., Hochberg M.E. & Grenfell B.T. (2002) Does multiple infection select for raised virulence? *Trends Microbiol* 10: 401-405

Buckling A. & Brockhurst M.A. (2008) Kin selection and the evolution of virulence. *Heredity 100*: 484-488

Buckling A. & Rainey P.B. (2002) Antagonistic coevolution between a bacterium and a bacteriophage. *Proc R Soc Lond Ser B-Biol Sci* 269: 931-936

Bull J.J. (1994) Perspective- Virulence. *Evolution* 48: 1423-1437

Bull J.J., Molineux I.J. & Rice W.R. (1991) Selection of benevolence in a host-parasite system. *Evolution* 45: 875-882

Chan W.C.*, et al.* (2004) Virulence Regulation and Quorum Sensing in Staphylococcal Infections: Competitive AgrC Antagonists as Quorum Sensing Inhibitors. *J Med Chem* 47: 4633-4641

Chao L., *et al.,* (2000) Kin Selection and Parasite Evolution: Higher and Lower Virulence with Hard and Soft Selection. *Q Rev Biol 75*: 261-275

Choisy M. & de Roode J. C. (2010) Mixed Infections and the Evolution of Virulence: Effects of Resource Competition, Parasite Plasticity, and Impaired Host Immunity. *Am*

Nat 175: 105-118

Clutton-Brock T. (2009) Cooperation between non-kin in animal societies. *Nature* 462**:** 51-57

Cluttonbrock T.H. & Harvey P.H. (1977) Primate ecology and social-organisation. *J Zool* 183: 1-39

Crawley M.J. (2002) *Statistical Computing: An Introduction to Data Analysis using S-Plus.* West Sussex: Wiley

Crespi B. J. (2001) The evolution of social behavior in microorganisms. *Trends Ecol Evol* 16**:** 178-183

Darch S. *et al.* (2012) Density-dependent fitness benefits in quorum-sensing bacterial populations. *Proc Natl Acad Sci USA* 109: 8259–8263

de Roode J.C. *et al.,* (2011) Virulence evolution in response to anti-infection resistance: toxic food plants can select for virulent parasites of monarch butterflies. *J Evol Biol* 24: 712-722

de Roode J.C. *et al.* (2005) Virulence and competitive ability in genetically diverse malaria infections. *Proc Natl Acad Sci USA* 102: 7624-7628.

DePolo N.J., Giachetti C. & Holland J.J. (1987) Continuing coevolution of virus and defective interfering particles and of viral genome sequences during undiluted passages: virus mutants exhibiting nearly complete resistance to formerly dominant defective interfering particles. *J Virol* 61: 454-464

Diggle S.P. *et al.* (2007) Cooperation and conflict in quorum-sensing bacterial populations. *Nature* 450**:** 411-414

Drake D. & Montie T.C.*et al.* (1988) Flagella, motility and invasive virulence of Pseudomonas aeruginosa. *J Gen Microbiol* 134: 43-52

Dugatkin L. (2002) Animal cooperation among unrelated individuals. *Naturwissenschaften* 89**:** 533-541

Dunn C. (2009) Siphonophores. *Curr Biol* 19**:** R233-R234

Dybdahl M.F. & Storfer A. (2003) Parasite local adaptation: Red Queen versus Suicide King. *Trends Ecol Evol* 18: 523-530

Eaton K.A., Morgan D.R & Krakowka S. (1992) Motility as a factor in the colonisation of gnotobiotic piglets by Helicobacter pylori. *J Med Microbiol* 37: 123-127

Ebert D. (1998) Evolution - Experimental evolution of parasites. *Science* 282: 1432-1435

Ebert D. & Bull J.J. (2003) Challenging the trade-off model for the evolution of virulence: is virulence management feasible? *Trends Microbiol* 11: 15-20

Ebert & Herre (1996) The evolution of parasitic diseases. *Parasitol Today* 12(3): 96-101

Elliot S.L.*, et al.* (2003) How virulent should a parasite be to its vector? *Ecology* 84: 2568-2574

Ellis E.L. & Delbrück M. (1939) The Growth of Bacteriophage. *J. Gen Physiol 22*: 365-384

Ewald P.W. (1994) *Evolution of Infectious Diseases.* Oxford, UK: Oxford University Press.

Ewald P.W. (1991) Waterborne transmission and the evolution of virulence among gastrointestinal bacteria. *Epidemiol Infect* 106: 83-119

Ewald P.W. (1983) Host-parasite relations, vectors, and the evolution of disease severity. *Ann Rev Ecol Syst* 14: 465-485

Felsenstein J. (1985) Phylogenies and the comparative method. *Am Nat* 125: 1-15

Fortune S.M. & Rubin E.J. (2007) The complex relationship between mycobacteria and macrophages: it's not all bliss. *Cell Host Microbe* 2: 5-6

Foster K.R., Parkinson K. & Thompson C.R.L. (2007) What can microbial genetics teach sociobiology? *Trends Genet* 23**:** 74-80

Foster K.R. (2005). Biomedicine - Hamiltonian medicine: Why the social lives of pathogens matter. *Science* 308: 1269-1270

Frank S. A. (1998) *Foundations of Social Evolution*, Princeton University Press: Princeton, NJ

Frank S.A. (1996) Models of Parasite Virulence. *Q Rev Biol* 71: 37-78

Frank S.A. (1994) Kin selection and virulence in the evolution of protocells and parasites. *Proc R Soc B-Biol Sci* 258: 153-161

Frank S.A. (1992) A kin selection model for the evolution of virulence. *Proc R Soc B-Biol Sci* 250: 195-197

Frank S.A. & Schmid-Hempel P. (2008) Mechanisms of pathogenesis and the evolution of parasite virulence. *J Evol Biol* 21: 396-404

Froissart R.*, et al.* (2010) The virulence-transmission trade-off in vector-borne plant viruses: a review of (non-)existing studies. *Phil Trans R Soc B* 365: 1907-1918

Futuyma D.J. & Moreno G. (1988) The Evolution of Ecological Specialization. *Annu Rev Ecol Evol Syst* 19: 207-233

Gama J.A. *et al*. (2012) Immune subversion and quorum-sensing shape the variation in infectious dose among bacterial pathogens. *PLoS Pathogens* 8: 2 e10002503

Gandon S. (2004) Evolution of multihost parasites. *Evolution* 58: 455-469

Gandon S.*, et al.* (2002) The evolution of parasite virulence, superinfection, and host resistance. *Am Nat* 159: 658-669

Garamszegi L.Z. (2006) The evolution of virulence and host specialization in malaria parasites of primates. *Ecol Lett* 9: 933-940

Gardner A., West S.A. & Buckling A. (2004) Bacteriocins, spite and virulence. *Proc R Soc Lond Ser B-Biol Sci* 271: 1529-1535

Gelman A. & Rubin D (1992) Inference from iterative simulation using multiple sequences. *Statistical Science* 7: 457-511

Gelman A. (2006) Prior distributions for variance parameters in hierarchical models. *Bayesian Analysis* 1: 515-533

Gelman A. (2010) A question about scaling. Available:

http://andrewgelman.com/2010/04/12/a_question_abou_9/

Geweke J. (1992) *Evaluating the accuracy of sampling-based approaches to calculating posterior moments*. In: Bernardo J, Berger J, Dawid A, Smith A, editors. *Bayesian Statistics.* New York: Clarendon

Gilmour A., *et al.,* (2009) *ASReml user guide:* VSN International

Grafen A. (1989) The phylogenetic regression. *Phil Trans R Soc B* 326: 119-157

Graham A.L. (2008) Ecological rules governing helminth-microparasite coinfection. *Proc Natl Acad Sci USA* 105: 566-570

Griffin A.S., West S A. & Buckling A. (2004) Cooperation and competition in pathogenic bacteria. *Nature* 430**:** 1024-1027

Guentzel M.N & Berry L.J (1975) Motility as a virulence factor for Vibrio cholera. *Infect Immun* 11: 890-897

Hadfield J. (2010) MCMC methods for multi-response generalised linear mixed models: the MCMCglmm R package. *J Stat Softw* 33: 1-22

Hadfield J. & Nakagawa S. (2010) General quantitative genetic methods for comparative biology: phylogenies, taxonomies and multi-trait models for continuous

and categorical characters. *J Evol Biol* 23: 494-508

Hall A.R.*, et al.* (2011) Host-parasite coevolutionary arms races give way to fluctuating selection. *Ecol Lett* 14: 635-642

Hall A.R., *et al.* (2012) Multiplicity of infection does not accelerate infectivity evolution of viral parasites in laboratory microcosms. *J Evol Biol* 25: 409-415

Hamilton W.D. (1972) Altruism and related phenomena mainly in social insects. *Ann Rev Ecol Syst* 193-232

Hamilton W.D. (1970). Selfish and spiteful behaviour in an evolutionary model. *Nature* 228: 1218-1220

Hamilton W.D. (1964) The genetical evolution of social behaviour, I & II. *J Theor Biol* 7: 1-52

Hardin G. (1968) The Tragedy of the Commons. *Science* 162**:** 1243-1248

Harvey P.H. & Pagel M.D. (1991) *Oxford series in ecology and evolution 1. The comparative method in evolutionary biology.* Oxford, UK: Oxford University Press

Hellgren O.*, et al.* (2009) A jack-of-all-trades and still a master of some: prevalence and host range in avian malaria and related blood parasites. *Ecology* 90: 2840-2849

Hense B. A., *et al.* (2007) Does efficiency sensing unify diffusion and quorum sensing? *Nat Rev Micro* 5**:** 230-239

Herre E.A. (1993) Population-structure and the evolution of virulence in nematode parasites of fig wasps. *Science* 259: 1442-1445

Hinnebusch B.J. (2005) The evolution of flea-borne transmission in Yersinia pestis. *Curr Issues Mol Biol* 7: 197-212

Hothorn T., Bretz F. & Westfall P (2008). Simultaneous Inference in General Parametric Models. *Biometrical Journal* 50: 346-363

Inglis R F., *et al.* (2009) Spite and virulence in the bacterium Pseudomonas aeruginosa. *Proc Natl Acad Sci USA* 106**:** 5703-5707

Inglis R.F., *et al.,* (2011) Spite and the Scale of Competition in Pseudomonas aeruginosa. *Am Nat* 178**:** 276-285

Josenhans C. & Suerbaum S. (2002) The role of motility as a virulence factor in bacteria. *Int J Med Microbiol* 291: 605-14

Kawecki T.J. (1998) Red queen meets Santa Rosalia: Arms races and the evolution of host specialization in organisms with parasitic lifestyles. *Am Nat* 152: 635-651

Keller L. & Surette M.G. (2006) Communication in bacteria: an ecological and evolutionary perspective. *Nat Rev Micro* 4: 249-258

Kenward M.G. & Roger J.H. (1997) Small Sample Inference for Fixed Effects from Restricted Maximum Likelihood. *Biometrics* 53: 983-997

Kerr B., *et al.,* (2006) Local migration promotes competitive restraint in a host-pathogen 'tragedy of the commons'. *Nature* 442: 75-78

Kirchner J.W. & Roy B.A. (2002) Evolutionary implications of host-pathogen specificity: fitness consequences of pathogen virulence traits. *Evol Ecol Res* 4: 27-48

Kohler T., Buckling A. & van Delden C. (2009) Cooperation and virulence of clinical Pseudomonas aeruginosa populations. *Proc Natl Acad Sci USA* 106: 6339-6344

Kolter R. & Greenberg E. P. (2006) Microbial sciences: The superficial life of microbes. *Nature* 441: 300-302

Krause G., Working Group (2008) How can infectious diseases be prioritized in public health? A standardized prioritization scheme for discussion. *Embo Reports* 9: S22-S27

Lalic J.*, et al.* (2013) Effect of host species on the distribution of mutational fitness effects for an RNA virus. *PLoS Genet* 7: 17

Lambrechts L., *et al.* (2006) Environmental influence on the genetic basis of mosquito resistance to malaria parasites *Proc R Soc B* 273: 1501–1506

Leggett H.C.*, et al.* (2013a) Experimental Evolution of Adaptive Phenotypic Plasticity in a Parasite. *Curr Biol* 23: 139-142

Leggett H.C. *et al.* (2013b) Generalism and the evolution of parasite virulence. *Trends Ecol Evol* 28(1): 592-596

Leggett H.C., Cornwallis C.K. & West S.A. (2012) Mechanisms of pathogenesis, infective dose and virulence in human parasites. *PLoS Pathogens* 8:2 e1002515

Leggett H.C. *et al.* (2012) Promiscuity and the evolution of cooperative breeding. *Proc R Soc B* 279: 1405-1411

Legros M. & Koella J.C. (2010) Experimental evolution of specialization by a microsporidian parasite. *BMC Evol Biol* 10: 7

Lehmann L. & Keller L. (2006) The evolution of cooperation and altruism – a general framework and a classification of models. *J Evol Biol* 19: 1365-1376

Lenski R.E., *et al.,* (1991). Long-Term Experimental Evolution in Escherichia coli. I. Adaptation and Divergence During 2,000 Generations. *Am Nat* 138: 1315-1341

Levin S. & Pimentel D. (1981) Selection of intermediate rates of increase in parasite-

host systems. *Am Nat* 117: 308-315

Lion S. & Boots M. (2010) Are parasites ‘‘prudent’’ in space? *Ecol Lett* 13: 1245-1255

Lipsitch M. & Moxon E.R. (1997) Virulence and transmissibility of pathogens: What is the relationship? *Trends Microbiol* 5: 31-37

Littell RC, *et al.,* (2006) *SAS for Mixed Models:* SAS Publishing

Lively C.M. (2009). Local host competition in the evolution of virulence. *J Evol Biol 22*: 1268-1274

Maclean R. C. (2008) The tragedy of the commons in microbial populations: insights from theoretical, comparative and experimental studies. *Heredity* 100: 233-239

Macnab R.M. (1992) Genetics and biogenesis of bacterial flagella. *Ann Rev Genet* 26: 131-158

Malpica J.M*., et al.* (2006) Association and Host Selectivity in Multi-Host Pathogens. *PLoS One* 1: 8

Massey R. C., Buckling A. & French-Constant R. (2004) Interference competition and parasite virulence. *Proc R Soc B-Biol Sci* 271: 785-788

May R.M. & Nowak M.A. (1995) Coinfection and the evolution of parasite virulence. *Proc R Soc B-Biol Sci* 261: 209-215

Maynard-Smith J.S.E. (1995) *The Major Transitions in Evolution*, WH Freeman: Oxford

Messenger S.L.*, et al.* (1999) Virulence evolution in a virus obeys a trade-off. *Proc R Soc B-Biol Sci* 266: 397-404

Michod R.E.*, et al.* (2008) Adaptive value of sex in microbial pathogens. *Infect Genet Evol* 8: 267-285

Mideo N. (2009) Parasite adaptations to within-host competition. *Trends Parasitol* 25: 261-268

Mikonranta L., Friman V-P. & Laakso J. (2012) Life History Trade-offs and Relaxed Selection Can Decrease Bacterial Virulence in Environmental Reservoirs. *PLoS ONE* 7(8): e43801

Miller J.F. Mekalanos J.J. & Falkow S. (1989) Coordinate regulation and sensory transduction in the control of bacterial virulence. *Science* 243: 916-922

Miller M.B.*, et al.* (2002) Parallel quorum sensing systems converge to regulate virulence in Vibrio cholerae. *Cell* 110: 303-314

Moran, N.A. (1992) The Evolutionary Maintenance of Alternative Phenotypes. *Am Nat* 139: 971-989

Nakagawa S. & Freckleton R. (2010) Model averaging, missing data and multiple imputation: a case study for behavioural ecology. *Behav Ecol Sociobiol* 65: 103-116

Nowak M.A. & May R.M. (1994) Superinfection and the Evolution of Parasite Virulence. *Proc R Soc Lond B* 255: 81-89

Ottemann K.M. & Miller J.F (1997) Roles for motility in bacterial-host interactions. *Mol Microbiol* 24: 1109-17

Packer C. & Pusey A.E. (1984) *Infanticide in carnivores.* In Hausfater G.H.S. (Ed.) *Infanticide.* New York: Aldine.

Parrish C.R.*, et al.* (2008) Cross-species virus transmission and the emergence of new epidemic diseases. *Microbiol Mol Biol Rev* 72: 457-470

Pedersen A.B., & Fenton A. (2007) Emphasizing the ecology in parasite community ecology. *Trends Ecol Evol* 22: 133-139

Plummer M., *et al.,* (2006) Convergence Diagnosis and Output Analysis for MCMC. *R News* 6: 7-11

Pontier D. & Natoli E. (1999) Infanticide in rural male cats (Felis catus L.) as a reproductive mating tactic. *Aggressive Behav* 25: 445-449

Popat R., Crusz S.A. & Diggle S.P. (2008) The social behaviours of bacterial pathogens. *Br Med Bull* 87: 63-75

Poulin R. (2007) *Evolutionary ecology of parasites*. Princeton University Press

Poullain V.*, et al.* (2008) The evolution of specificity in evolving and coevolving antagonistic interactions between a bacteria and its phage. *Evolution* 62: 1-11

Rainey P.B. & Bailey M.J. (1996) Physical and genetic map of the Pseudomonas fluorescens SBW25 chromosome. *Mol Microbiol 19*: 521-533.

Read A.F. & Taylor L.H (2001) The ecology of genetically diverse infections. *Science* 292: 1099-1102

Read J.M. & Keeling M.J. (2003) Disease evolution on net- works: the role of contact structure. *Proc R Soc Lond B* 270: 699–798

Reece S.E., Drew D.R. & Gardner A. (2008) Sex ratio adjustment and kin discrimination in malaria parasites. *Nature* 453: 609-614

Refardt D. (2011) Within-host competition determines reproductive success of temperate bacteriophages. *ISME J 5*: 1451-1460

Regoes R.R., *et al.* (2000) Evolution of virulence in a heterogeneous host population. *Evolution* 54: 64-71

Rigaud T., *et al.* (2010) Parasite and host assemblages: embracing the reality will improve our knowledge of parasite transmission and virulence. *Proc R Soc B-Biol Sci* 277: 3693-3702

Rumbaugh K.P., *et al*. (2009) Quorum Sensing and the Social Evolution of Bacterial Virulence. *Curr Biol* 19: 341-345

Salazar-Gonzalez R.M. *et al.* (2007) Salmonella flagellin inducers bystander activation of splenic dendritic cells and hinders bacterial replication in vivo. *J Immunol* 179: 6169-6175

Sayers E.W., *et al.* (2009) Database resources of the National Center for Biotechnology Information. *Nucleic Acids Res* 37: 3124-3124

Scheielzeth H. (2010) Simple means to improve the interpretability of regression coefficients. *Methods Ecol Evol* 1: 103-113

Schlichting C.D. & Pigliucci M (1998) *Phenotypic evolution: a reaction norm*

perspective. Sinauer Associates Incorporated

Schmid-Hempel P. (2011) *Evolutionary Parasitology: The Integrated Study of Infections, Immunology, Ecology, and Genetics.* Oxford: Oxford University Press

Schmid-Hempel P. & Frank S.A. (2007) Pathogenesis, virulence, and infective dose. *PloS Pathogens* 3: 1372-1373

Self S.G. & Liang K.Y. (1987) Asymptotic properties of maximum likelihood estimators and likelihood ratio tests under non-standard conditions. *J Am Statis Ass* 82: 605-610

Sewell D.L. (1995) Laboratory-associated infections and biosafety. *Clin Microbiol Rev* 8: 389-405

Stearns S.C. (1989) The Evolutionary Significance of Phenotypic Plasticity. *BioScience* 39: 436-445

Stecher B. *et al.* (2007) Salmonella enterica serovar typhinurium exploits inflammation to compete with intestinal microbiota. *PLoS Biol* 5: 2177-2189

Taylor L.H.*, et al.* (1998) Virulence of Mixed-Clone and Single-Clone Infections of the Rodent Malaria Plasmodium chabaudi. *Evolution* 52: 583-591

Thomas F., *et al.,* (2002) Understanding parasite strategies: a state-dependent approach? *Trends Parasitol* 18: 387-390

Tomita T. & Kanegasaki S. (1982) Enhanced phagocytic response to macrophages to bacteria by physical impact caused by bacterial motility or centrifugation. *Infect Immun* 38: 865-870

Tompkins D.M., *et al.* (2003) Ecological replacement of native red squirrels by invasive greys driven by disease. *Ecol Lett* 6: 189-196

Trivers R. L. (1985) *Social evolution.* Benjamin/Cummings, Menlo Park, California

Turner P.E. & Chao L. (1999) Prisoner's dilemma in an RNA virus. *Nature* 398: 441-443

United States Food and Drug Administration (2003) *The Bad Bug Book.* Available: http://www.fda.gov/Food/FoodSafety/FoodborneIllness/FoodborneIllnessFoodbornePathogensNaturalToxins/BadBugBook/default.htm

van Baalen M. & Sabelis M.W. (1995) The Dynamics of Multiple Infection and the Evolution of Virulence. *Am Nat* 146: 881-910

Velicer G. J. (2003) Social strife in the microbial world. *Trends Microbiol* 11**:** 330-337.

Vieira-Silva S. & Rocha E.P.C (2010) The systemic imprint of growth and its uses in ecological (meta)genomics. *PLoS Genetics* 6: e1000808

Wang I.N. (2006) Lysis timing and bacteriophage fitness. *Genetics* 172: 17-26

Wang I.N., Dykhuizen D.E. & Slobodkin L.B. (1996) The evolution of phage lysis timing. *Evol Ecol* 10: 545-558

Webb J. S., Givskov M. & Kjelleberg S. (2003) Bacterial biofilms: prokaryotic adventures in multicellularity. *Curr Opin Microbiol* 6: 578-585

West S.A. & Gardner A. (2013) Adaptation and inclusive fitness. *Curr Biol.* 23:R577-R584

West S.A., *et al.* (2007) The social lives of microbes. *Ann Rev Ecol Evol Syst* 38: 53-77

West S.A., Griffin A.S. & Gardner A. (2007) Social semantics: altruism, cooperation, mutualism, strong reciprocity and group selection. *J Evol Biol* 20: 415-432

West S.A., *et al.* (2006) Social evolution theory for microorganisms. *Nat Rev Micro* 4: 597-607

West S.A., Pen I. & Griffin A.S. (2002) Conflict and cooperation - Cooperation and competition between relatives. *Science* 296: 72-75

West S.A. & Buckling A. (2003) Cooperation, virulence and siderophore production in bacterial parasites. *Proc R Soc B-Biol Sci* 270: 37-44

West-Eberhard M.J. (2003) *Developmental Plasticity and Evolution*. Oxford University Press, Oxford: UK

Wild G., Gardner A. & West S.A. (2009) Adaptation and the evolution of parasite virulence in a connected world. *Nature* 459: 983-986

Williams P. *et al.,* (2000) Quorum sensing and the population-dependant control of virulence. *Philos Trans R Soc Lond Ser B-Biol Sci* 355: 667-680

Williams P., *et al.,* (2007) Look who's talking: communication and quorum sensing in the bacterial world. *Philos Trans R Soc Lond Ser B-Biol Sci* 362**:** 1119-1134

Wilson M. McNab R. & Henderson B. (2002) *Bacterial Disease Mechanisms: An introduction to cellular microbiology*. Cambridge University Press, Cambridge: UK

Wooldridge K. (2009) *Bacterial Secreted Proteins: Secretory Mechanisms and Role in Pathogenesis:* Caister Academic Press, Norwich: UK

Woolhouse M.E.J., Haydon D.T. & Antia R. (2005) Emerging pathogens: the epidemiology and evolution of species jumps. *Trends Ecol Evol* 20(5): 238–244

Woolhouse M.E.J*., et al.* (2001) Population Biology of Multihost Pathogens. *Science* 292: 1109-1112

Cell PRESS

Generalism and the evolution of parasite virulence

Helen C. Leggett[1,2], Angus Buckling[2], Gráinne H. Long[3], and Mike Boots[2]

[1] Department of Zoology, Oxford University, South Parks Road, Oxford, OX1 3PS, UK
[2] Biosciences, University of Exeter, Cornwall Campus, Penryn, TR10 9EZ, UK
[3] MRC Epidemiology Unit, University of Cambridge, Institute of Public Health, Addenbrooke's Hospital, Hills Road, Cambridge, CB2 0QQ, UK

The evolution of parasite-imposed host harm (virulence) will be affected by numerous factors, not least the range of hosts that parasites can infect. Here, we consider four ways that parasite host range (generalism) might directly affect observed levels of parasite virulence: costs of generalism, multiplicity of infection, maladaptive virulence, and host availability. Integrating parasite infectivity range with life-history evolution will generate novel general hypotheses for the evolutionary ecology of virulence, as well as explicit predictions about the virulence of emerging diseases resulting from host shifts.

Parasite virulence–transmission trade-offs

Understanding the evolution of parasite virulence (which we define as reductions in host fitness due to parasite infection; see Glossary) is critical to understanding, and potential control of, infectious disease. A key assumption for many parasites is that virulence has negative consequences for parasite fitness because it reduces host lifespan, but is an unavoidable consequence of within-host parasite replication, which is crucial for transmission to new hosts and for avoiding host immune system clearance [1,2]. Therefore, parasites trade off their transmission period (host lifespan) against their transmission rate, with the optimal level of virulence being that which maximises total transmission [2,3]. This virulence–transmission trade-off asserts three fundamental predictions: (i) that increasing within-host parasite replication increases virulence (Figure 1A); (ii) that increasing within-host parasite replication increases the number of parasite transmission events over the duration of the infection (Figure 1B), until a point where increasing parasite replication reduces the infectious period, for example, by killing the host and preventing transmission (Figure 1C); and (iii) that increasing parasite virulence increases parasite transmission (Figure 1D) until high virulence shortens the infectious period, thereby reducing transmission (Figure 1E) [2,3]. Table S1 in the supplementary material online summarises empirical evidence for the three fundamental predictions of the trade-off theory. Although these studies provide support for the parasite trade-off theory for virulence evolution [4–6], the fit between theory and data is primarily qualitative rather than quantitative. A possible explanation for this is that the predictions of virulence theory can depend on the life history of, and the mechanism used by, a given parasite to infect and exploit hosts, which are not captured by classical trade-offs. Notably, the range of hosts that the parasite can infect is a ubiquitous life-history trait that is likely to be one of the most important determinants of virulence, but is little studied in the discussion so far. Here, we discuss how incorporating parasite host range into current theory can greatly improve understanding of, and enhance the use and accuracy of testable predictions about, virulence evolution.

Parasite infectivity: generalism versus specialism

Parasites vary massively in the range of hosts that they can infect (generalism), both within and between species [7]. Although the causes and consequences of variation in parasite infectivity ranges have been studied [8–11], the interplay between parasite infectivity and virulence (as discussed above) has rarely been considered. This interplay is likely to be crucial: the virulence model discussed above assumes that high within-host replication (and, therefore, virulence) will lead to a high probability of infecting new hosts. However, if parasite genotypes differ in their ability to infect different host species and genotypes, parasite productivity from a host will not always be simply proportional to the infectivity of new hosts. Moreover, the degree of generalism is likely to trade off against

Glossary

Generalist parasite: parasites with low variance in infectiousness over different host genotypes and/or species.
Infectious periods: the time period during which infected hosts are able to transmit an infection to any susceptible host or vector that they come into contact with. For most parasites (excluding obligate killers), reductions in host lifespan reduce the infectious period.
Specialist parasite: parasites with high variance in infectiousness over different host genotypes and/or species.
Transmission rate: the rate at which susceptible hosts are converted into infected hosts by contact with infectious parasites. This is assumed to be related to parasite fecundity because increasing within-host parasite replication increases the number of parasite transmission events over the duration of the infection.
Virulence: the amount of harm a parasite causes its host during an infection.

Corresponding author: Leggett, H.C. (helen.leggett@zoo.ox.ac.uk).

0169-5347/$ – see front matter
 http://dx.doi.org/10.1016/j.tree.2013.07.002

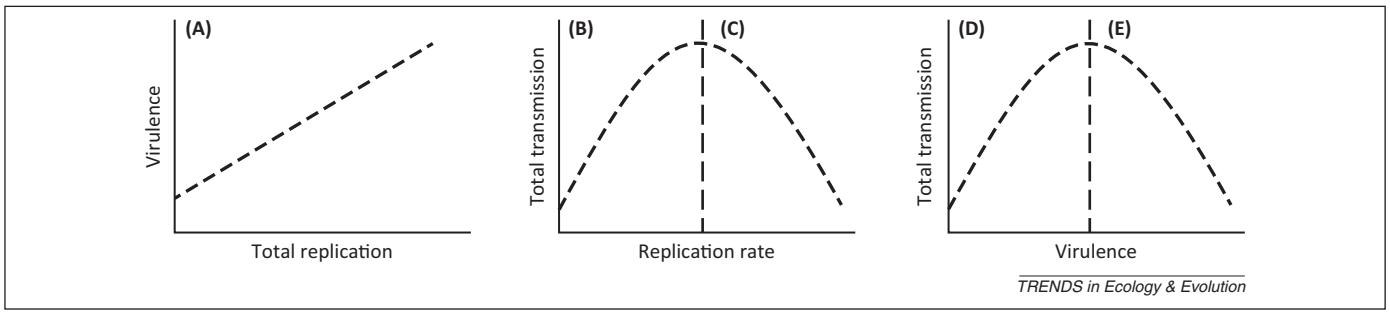


Figure 1. Schematic of the three fundamental assumptions of parasite life-history trade-off theory. First, increasing within-host parasite replication increases virulence **(A)**; second, increasing within-host parasite replication increases the number of parasite transmission events over the duration of the infection **(B)**, until a point where increasing parasite replication reduces the infectious period (e.g., by killing the host and preventing transmission) **(C)**; and third, increasing parasite virulence increases parasite transmission **(D)** until high virulence shortens the infectious period, thereby reducing transmission **(E)** [2,3]. Empirical data for these hypotheses are shown in Table S1 in the supplementary material online.

other traits, such as growth rate, and will also affect the ecological contexts experienced by parasites, all of which may affect virulence. Note that, whereas generalist parasites can be usefully described as parasites with low variance in infectiousness over different host genotypes and/or species, defining generalism can be complicated when considering parasites with complex life cycles involving multiple hosts (Box 1).

Here, we discuss how the degree of generalism is likely to affect the evolution and ecology of virulence. Integrating parasite generalism with life-history evolution will generate novel general hypotheses for the evolutionary ecology of virulence, as well as explicit predictions about the virulence of emerging diseases resulting from host shifts [12]. We focus on four key processes: (i) costs of generalism; (ii) multiplicity of infection; (iii) maladaptive virulence; and (iv) host availability. However, a detailed discussion of the evolutionary processes that determine whether parasites evolve to be specialists or generalists and the impact of specialism on the generation and maintenance of parasite diversity, is beyond the scope of this article.

Costs of generalism

Natural populations of parasites often infect and transmit from multiple hosts and there are likely to be costs associated with infecting novel hosts [13–15]. Fitness costs associated with generalism can arise for two biologically plausible reasons. First, parasite replication or transmission of generalist parasites in novel hosts might be reduced, in a scenario analogous to optimal foraging theory, where resource patches vary in quality (an ecological cost to generalism) [14]. Second, the ability to infect novel hosts can be associated with reduced fitness in the original hosts (an evolutionary cost to generalism) [14]. As a result, a trade off between reproduction within a given host and increasing host range is predicted, such that specialist parasites replicate faster (causing more virulence), and transmit more than do generalist parasites. Empirical evidence does suggest that more-virulent parasites have narrower host ranges than less-virulent parasites [16,17]. It is also important to emphasise that these costs of generalism also apply within species (i.e., the ability to infect multiple host genotypes [18,19], not just different species).

If parasites adapt to infect heterogeneous hosts, it is plausible that costs of generalism will affect virulence evolution such that parasites cannot replicate well (and cause high virulence) in multiple hosts [20,21]. For instance, if a parasite grows rapidly in one host to overcome host defences, but in an alternative host the same fast growth kills the host rapidly, virulence can be optimal in

Box 1. What is a generalist parasite? Generalism and complex life cycles

Most parasites are generalist parasites, capable of infecting more than one host species [13]. We define generalism versus specialism on the basis of low versus high variance, respectively in infectiousness across different hosts. This is in keeping with the infectivity versus noninfectivity distinction that is advocated by phytopathology societies [51]. We suggest that, although in principle generalism should be a numbers game, whereby the most generalist parasites are on average the most infectious over wider host ranges, this is not necessarily the case for parasites with complex life cycles. In this scenario, it is important to consider the details of the exploitative specificity of the parasite. For instance, among vectored parasites, definitions of the 'vector' and 'definitive host' ascribe functions to effectively two potential hosts [52]. Selection in both hosts affects virulence evolution [21], and it has been suggested that the evolution of virulence and host specialism are mediated by the same selective factors [17]. Importantly, this might explain why the perceived advantages of parasite specialism are outweighed by those of generalism: vectored parasites are not fixed strategy generalists, rather they are effectively phenotypically plastic specialists, and so there is no reason that we should expect them to show a large 'fecundity' cost of generalism.

In some sense, generalist and vectored parasites can be more specialised than specialist and directly transmitted parasites. For example, for the agent of plague, *Yersinia pestis*, the conditions it faces within the flea (vector) digestive tract are unique from those encountered in the mammalian host. Consequently, the development of a transmissible infection requires a distinct subset of genes, with none of the tested mammalian virulence factors being required in fleas [53]. This idea can be extended to biological- and mechanical-vector transmission: because mechanically vectored parasites simply persist, for example, on the mouthparts of their vectors, and do not *per se* infect and exploit the resources of their vector, they require fewer specialisations for effective transmission than do biologically vectored parasites. Similarly, facultative pathogens, such as *Vibrio cholera* and *Staphylococcus aureus*, which tend to express virulence-associated genes only under the high density conditions associated with infection [27,54,55], can be viewed as more specialised than purely opportunistic pathogens, characterised as organisms that become pathogenic following a perturbation to their host, such as *Pseudomonas aeruginosa*, and *Streptococcus pneumonia*, who are 'super-generalists' capable of infecting across different environments and hosts, and thus might be less specialised and even maladapted to their hosts.

one host and suboptimal in another. This has been shown in a study of viruses across multiple plant species, where viruses with lower host ranges were found to be more prevalent [22]. However, no costs of generalism were detected among Tobacco etch potyvirus infecting four natural hosts [23]. Similarly, there is a positive correlation between avian malaria host range and prevalence, which suggests that a 'jack-of-all-trades can be a master of some' [24]. An explanation for this is that higher overall encounter rate for parasites with wider host ranges can compensate for the potentially reduced fitness of such generalist parasites in each host species [24,25].

Generalist parasites that are phenotypically plastic can have an advantage because plasticity can mitigate costs of generalism resulting from antagonistic pleiotropy [8]. Mechanisms such as altering gene expression can enable parasites to change their behaviour to fit different environments (hosts), subsequently improving the fitness of the parasite [26]. If parasites intermittently encounter new or different hosts, then evolution in the face of an important trade-off can select for plasticity [27–30]. In this scenario, the benefits of plasticity must outweigh the likely costs of the machinery required to generate plasticity, and the costs of a mismatch between the 'appropriate' behaviour for the host [26]. Many parasites are plastic (e.g., bacteriophage T4 [31] and *Plasmodium chabaudi* [32]) and a recent study showed the *de novo* evolution of such plasticity after propagating a virus (bacteriophage ϕ2) for a few hundred generations on its *Pseudomonas fluorescens* host [33]. Crucially, in this latter study, plasticity only evolved when the virus experienced alternating conditions: precisely the conditions predicted by theory. Moreover, in each of these examples, the organisms change their phenotypes in manners that maximise their fitness (i.e., phenotypic plasticity is adaptive). That adaptive plasticity can evolve easily in even a simple virus, suggests that this behaviour could be widespread and readily altered by selection.

Multiplicity of infection and intrahost competitive strategies

Host–parasite interactions are rarely one-on-one. Infections of a single host with multiple parasites occur in many natural populations [25] and, given their larger host range and consequent higher host encounter rate, we suggest that generalist parasites in some contexts are more likely to experience these multiple genotype and/or species infections than are specialist parasites. The specific context that we consider is isolated populations of pure specialist and generalist parasites, where specialists cannot infect certain hosts. By definition in this scenario, the chance of multiple infections is reduced for specialists. However, by contrast, a polymorphic population of specialist and generalist parasites could result in generalists experiencing fewer mixed infections; if generalists have access to hosts that are not available to the specialist parasites, then the generalists will be more infectious overall [34].

Multiple infections result in within-host parasite competition, which can select for a range of parasite strategies that have evolutionary consequences for virulence, including increased rates of host exploitation, exploitation of the public goods of a competitor (social cheating) and interference [3,35] (Box 2). If we assume that generalist parasites are more likely to experience multiple infections, then a generalist parasite might be expected to be a better competitor if their host becomes infected by a specialist parasite. However this prediction can be complicated by fecundity costs associated with generalism, as discussed above.

Box 2. Intrahost competitive strategies

There is an important difference between the expression of virulence (where overall virulence can be lower due to the ecology of the infection, such as within host competition in a co-infected host) and the evolution of virulence (which parasite genotype is ultimately selected). Most intrahost competition models and empirical work focuses on resource competition and posits that such competition selects for increased rates of host exploitation and, consequently, virulence [2,3,56–62]. For clonal (single) infections, kin selection can favour lower rates of within-host resource use that maximise total transmission. However, social interactions between co-infecting parasites can be more direct than simple resource competition. Some parasites show increased within-host growth and virulence as a result of the production of costly public goods; here, within-host competition favours the evolution of social cheats, resulting in the expression of the evolution of lower virulence [35,37]. Multiple infections can favour the production of anticompetitor toxins as a result of both individual and kin selection; virulence is likely to decrease when these are favoured. All mechanisms that increase within host competitive ability are typically associated with a fitness cost in clonal infections in terms of reduced transmission. In addition, multiple infections afford increased opportunities for genetic or sexual recombination between pathogen strains, which can bring with it important adaptive advantages that modify virulence [63].

Multiple infections can also have important virulence effects due to their interactions with host immunity. Successful parasites must evolve life-history strategies that enable them to reproduce before they die. Ideally, the host immune system should multitask with ease, but multiple infections can strongly interact with immunity because the host cannot perfectly deal with multiple antigens simultaneously. This has been shown in mixed-genotype infections of the rodent malaria *P. chabaudi* [36,37]. Thus, another reason why multiple infections could cause the expression and evolution of lower virulence is because there is less need for parasites to replicate quickly to avoid the host immune system. Consequently, an understanding of how parasites interact with host immunity and pathogenic outcome is critical to evaluating whether virulence is a consequence of parasite fitness trade-offs. Furthermore, multiple infections are not limited to members of the same species, and this can also affect interactions with the immune system, as shown by a meta-analysis of mice co-infected with microparasites and helminths [38]. Given that parasites, in particular generalists, are likely to be co-infecting with other species, a better understanding of the ecology and evolution of co-infection might reveal predictable virulence outcomes [38].

Multiple infections can have surprising effects on the requirements and mechanisms of virulence evolution itself for several reasons, including the extent to which virulence reduces parasite transmission (trade-off theory), and the genetic diversity (or relatedness) of the parasites either competing for, or cooperating to exploit, the host. Furthermore, both the dynamics of intrahost competition and mediation by the immune system appear to have a role in

the outcome of competition. It is reasonable to speculate that parasites could face a trade-off between coping with the host immune system and outcompeting co-infecting parasites. Given that interacting with host immunity is a more constant selective pressure than are multiple infections, the evolutionary outcome of virulence should depend on the prevalence of potential competitors for a given parasite species [25]. We suggest that generalist parasites are more likely to experience multiple infections due to their higher host encounter rates as a function of their larger host ranges. Consequently, generalist parasites might invest more in outcompeting co-infecting parasites, and evolve virulence accordingly (Box 2), whereas specialist parasites might invest more in surviving host immune responses.

Maladaptive virulence

In many cases, virulence is clearly decoupled from parasite fitness (transmission; i.e., it is nonadaptive). This situation can arise when parasites accidentally infect hosts that do not normally transmit them. Such 'spillovers' can be lethal to the host because the host and parasite have not coevolved [39]; however, many others may go largely unnoticed because we do not see 'avirulent' spillovers. Spillovers are predominantly noticed in humans infected by virulent diseases such as Ebola and avian influenzas, although two well-known wildlife examples are the parapoxvirus-driven ecological replacement of native red squirrels by invasive grey squirrels [40], and the crayfish plague spread by introduced American signal crayfish to native European white-clawed crayfish [41]. We suggest that generalist parasites are more likely to spillover into novel hosts when their larger host ranges suddenly meet, leading to maladaptive virulence.

Generalist parasite virulence might also be comparatively maladaptive compared with specialist parasites because generalists have less time to adapt to any one host within their host infectivity range. Specialist parasites that show host preference will be more consistently exposed to selection on a particular host and, thus, can adapt to evolving host defences faster than can generalist parasites [42]. This might be particularly true for 'super-generalist' opportunistic pathogens because clinical infections of these parasites are generally not directly communicable and their continuous circulation does not depend on their ability to cause disease [27].

Host availability

Crucial to understanding optimal levels of virulence is host availability [3], with, for instance, experiments using bacteria and viruses showing that high host availability selects for increased virulence [43,44]. The degree of generalism can alter the pool of available hosts, with highly specialised parasites having fewer available hosts than generalist parasites. For example, it has been proposed that theoretically high degrees of specialism may lead to a process akin to the 'self-shading' in spatial models [45,46], where highly virulent and transmissible parasites overexploit their available hosts, resulting in selection for lower virulence [47].

Concluding remarks and future directions

Understanding how selection drives the evolution of both parasite host range and parasite life histories is crucial for predicting the impact of parasites on their host populations. Although the causes and consequences of both parasite traits have been extensively studied in isolation, their interplay has received much less attention. Here, we have highlighted one aspect of this interplay: how parasite generalism might affect parasite virulence. We have focused on four specific examples. First, the costs of generalism: generalist parasite species and/or strains are less able to exploit their hosts efficiently compared with specialist parasites. Second, generalist parasites are more likely to find themselves in multiple infections: this increases within-host selection, which is closely linked to the expression and evolution of virulence. Third, generalist species or stains of parasites are more likely to infect novel hosts with whom they have not coevolved: this can manifest as maladaptive virulence. Fourth, generalist parasites can access niches free of specialist parasites, reducing the burden of within host competition, leading to the evolution of lower virulence.

Although most of our arguments are speculative, it would be straightforward to test some of the hypotheses. For example, plant viruses differ in generalism at both the species and genotype levels [22,23,48], enabling further investigation into natural correlations between virulence and generalism, taking into account phylogenies and different ecological conditions. Furthermore, direct qualitative tests could be carried out by comparing the life histories [14] of viruses of bacteria experimentally evolved to have different host ranges [49].

Understanding the virulence of emerging infectious diseases

As well as generating novel general predictions for the evolutionary ecology of virulence, integrating parasite host range and virulence evolution is likely to aid understanding of the virulence of emerging infectious diseases [12]. As parasites become exposed to novel hosts because of increased population density, climate change, and urbanisation, natural selection is likely to favour the evolution of parasite generalism [45]. Given that generalist parasites pose the greatest threat to disease-mediated extinction [25,50], furthering our understanding of the importance of parasite generalism on virulence evolution might be an important step in reducing the negative impact of infectious diseases. Finally, it is important to emphasise that the interaction between parasite host range and life history is likely to be reciprocal: changes in life history may in turn affect selection for generalism (i.e., life history and host range coevolve). For example, high virulence might reduce the availability of susceptible hosts, imposing selection for greater host range. This interplay is clearly complex, but we hope that this article might inspire more research in this area.

Acknowledgements

We thank Stu West, Sunetra Gupta, Kevin Foster, and Sam Brown for useful discussions; BBSRC for funding; and Samuel Alizon, Sarah Reece, and two anonymous reviewers for comments that improved the manuscript.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.tree.2013.07.002.

References

1 Anderson, R.M. and May, R.M. (1981) The population dynamics of microparasites and their invertebrate hosts. *Philos. Trans. R. Soc. Lond. B: Biol. Sci.* 291, 451–524
2 Anderson, R. and May, R. (1982) Coevolution of hosts and parasites. *Parasitology* 85, 411–426
3 Frank, S.A. (1996) Models of parasite virulence. *Q. Rev. Biol.* 71, 37–78
4 Lipsitch, M. and Moxon, E.R. (1997) Virulence and transmissibility of pathogens: what is the relationship? *Trends Microbiol.* 5, 31–37
5 Froissart, R. *et al.* (2010) The virulence-transmission trade-off in vector-borne plant viruses: a review of (non-)existing studies. *Philos. Trans. R. Soc. B* 365, 1907–1918
6 Alizon, S. *et al.* (2009) Virulence evolution and the trade-off hypothesis: history, current state of affairs and the future. *J. Evol. Biol.* 22, 245–259
7 Antonovics, J. *et al.* (2013) The origin of specificity by means of natural selection: evolved and nonhost resistance in host-pathogen interactions. *Evolution* 67, 1–9
8 Futuyma, D.J. and Moreno, G. (1988) The evolution of ecological specialization. *Annu. Rev. Ecol. Evol. Syst.* 19, 207–233
9 Poulin, R. (2007) *Evolutionary Ecology of Parasites*. Princeton University Press
10 Dybdahl, M.F. and Storfer, A. (2003) Parasite local adaptation: Red Queen versus Suicide King. *Trends Ecol. Evol.* 18, 523–530
11 Barrett, L.G. *et al.* (2009) Continua of specificity and virulence in plant host–pathogen interactions: causes and consequences. *New Phytol.* 183, 513–529
12 Woolhouse, M.E.J. *et al.* (2005) Emerging pathogens: the epidemiology and evolution of species jumps. *Trends Ecol. Evol.* 20, 238–244
13 Woolhouse, M.E.J. *et al.* (2001) Population biology of multihost pathogens. *Science* 292, 1109–1112
14 Benmayor, R. *et al.* (2009) Host mixing and disease emergence. *Curr. Biol.* 19, 764–767
15 Parrish, C.R. *et al.* (2008) Cross-species virus transmission and the emergence of new epidemic diseases. *Microbiol. Mol. Biol. Rev.* 72, 457–470
16 Agudelo-Romero, P. and Elena, S.F. (2008) The degree of plant resilience to infection correlates with virus virulence and host-range. *Span. J. Agric. Res.* 6, 160–169
17 Garamszegi, L.Z. (2006) The evolution of virulence and host specialization in malaria parasites of primates. *Ecol. Lett.* 9, 933–940
18 Legros, M. and Koella, J.C. (2010) Experimental evolution of specialization by a microsporidian parasite. *BMC Evol. Biol.* 10, 7
19 Poullain, V. *et al.* (2008) The evolution of specificity in evolving and coevolving antagonistic interactions between a bacteria and its phage. *Evolution* 62, 1–11
20 Regoes, R.R. *et al.* (2000) Evolution of virulence in a heterogeneous host population. *Evolution* 54, 64–71
21 Gandon, S. (2004) Evolution of multihost parasites. *Evolution* 58, 455–469
22 Malpica, J.M. *et al.* (2006) Association and host selectivity in multi-host pathogens. *PLoS ONE* 1, 8
23 Bedhomme, S. *et al.* (2012) Multihost experimental evolution of a plant RNA virus reveals local adaptation and host-specific mutations. *Mol. Biol. Evol.* 29, 1481–1492
24 Hellgren, O. *et al.* (2009) A jack-of-all-trades and still a master of some: prevalence and host range in avian malaria and related blood parasites. *Ecology* 90, 2840–2849
25 Rigaud, T. *et al.* (2010) Parasite and host assemblages: embracing the reality will improve our knowledge of parasite transmission and virulence. *Proc. R. Soc. B: Biol. Sci.* 277, 3693–3702
26 Schlichting, C.D. and Pigliucci, M. (1998) *Phenotypic Evolution: A Reaction Norm Perspective*. Sinauer Associates
27 Brown, S.P. *et al.* (2012) Evolution of virulence in opportunistic pathogens: generalism, plasticity, and control. *Trends Microbiol.* 20, 336–342
28 Stearns, S.C. (1989) The evolutionary significance of phenotypic plasticity – phenotypic sources of variation among organisms can be described by developmental switches and reaction norms. *Bioscience* 39, 436–445
29 Agrawal, A.A. (2001) Phenotypic plasticity in the interactions and evolution of species. *Science* 294, 321–326
30 West-Eberhard, M.J. (2003) *Developmental Plasticity and Evolution*. Oxford University Press
31 Abedon, S.T. (1999) Bacteriophage T4 resistance to lysis-inhibition collapse. *Genet. Res.* 74, 1–11
32 Reece, S.E. *et al.* (2008) Sex ratio adjustment and kin discrimination in malaria parasites. *Nature* 453, 609–614
33 Leggett, H.C. *et al.* (2013) Experimental evolution of adaptive phenotypic plasticity in a parasite. *Curr. Biol.* 23, 139–142
34 Gandon, S. *et al.* (2002) The evolution of parasite virulence, superinfection, and host resistance. *Am. Nat.* 159, 658–669
35 Buckling, A. and Brockhurst, M.A. (2008) Kin selection and the evolution of virulence. *Heredity* 100, 484–488
36 Taylor, L.H. *et al.* (1998) Virulence of mixed-clone and single-clone infections of the rodent malaria *Plasmodium chabaudi*. *Evolution* 52, 583–591
37 Mideo, N. (2009) Parasite adaptations to within-host competition. *Trends Parasitol.* 25, 261–268
38 Graham, A.L. (2008) Ecological rules governing helminth-microparasite coinfection. *Proc. Natl. Acad. Sci. U.S.A.* 105, 566–570
39 Ebert, D. (1998) Experimental evolution of parasites. *Science* 282, 1432–1435
40 Tompkins, D.M. *et al.* (2003) Ecological replacement of native red squirrels by invasive greys driven by disease. *Ecol. Lett.* 6, 189–196
41 Alderman, D.J. (1996) Geographical spread of bacterial and fungal diseases of crustaceans. *Rev. Sci. Tech.* 15, 603–632
42 Kawecki, T.J. (1998) Red queen meets Santa Rosalia: arms races and the evolution of host specialization in organisms with parasitic lifestyles. *Am. Nat.* 152, 635–651
43 Messenger, S.L. *et al.* (1999) Virulence evolution in a virus obeys a trade off. *Proc. R. Soc. Lond. B: Biol. Sci.* 266, 397–404
44 Berngruber, T.W. *et al.* (2013) Evolution of virulence in emerging epidemics. *PLoS Pathog.* 9, e1003209
45 Boots, M. and Sasaki, A. (1999) 'Small worlds' and the evolution of virulence: infection occurs locally and at a distance. *Proc. R. Soc. B: Biol. Sci.* 266, 1933–1938
46 Lion, S. and Boots, M. (2010) Are parasites "prudent" in space? *Ecol. Lett.* 13, 1245–1255
47 Kirchner, J.W. and Roy, B.A. (2002) Evolutionary implications of host-pathogen specificity: fitness consequences of pathogen virulence traits. *Evol. Ecol. Res.* 4, 27–48
48 Lalic, J. *et al.* (2013) Effect of host species on the distribution of mutational fitness effects for an RNA virus. *PLoS Genet.* 7, 17
49 Hall, A.R. *et al.* (2011) Host-parasite coevolutionary arms races give way to fluctuating selection. *Ecol. Lett.* 14, 635–642
50 Pedersen, A.B. and Fenton, A. (2007) Emphasizing the ecology in parasite community ecology. *Trends Ecol. Evol.* 22, 133–139
51 Flor, H.H. (1956) The complementary genetic system in flax and flax rust. *Adv. Genet.* 8, 29–54
52 Elliot, S.L. *et al.* (2003) How virulent should a parasite be to its vector? *Ecology* 84, 2568–2574
53 Hinnebusch, B.J. (2005) The evolution of flea-borne transmission in Yersinia pestis. *Curr. Issues Mol. Biol.* 7, 197–212
54 Miller, M.B. *et al.* (2002) Parallel quorum sensing systems converge to regulate virulence in *Vibrio cholerae*. *Cell* 110, 303–314
55 Chan, W.C. *et al.* (2004) Virulence regulation and quorum sensing in staphylococcal infections: competitive AgrC antagonists as quorum sensing inhibitors. *J. Med. Chem.* 47, 4633–4641
56 Nowak, M.A. and May, R.M. (1994) Superinfection and the evolution of parasite virulence. *Proc. R. Soc. Lond. B* 255, 81–89
57 Levin, S. and Pimentel, D. (1981) Selection of intermediate rates of increase in parasite-host systems. *Am. Nat.* 117, 308–315
58 Bremermann, H.J. and Pickering, J. (1983) A game-theoretical model of parasite virulence. *J. Theor. Biol.* 100, 411–426
59 Frank, S.A. (1992) A kin selection model for the evolution of virulence. *Proc. R. Soc. B: Biol. Sci.* 250, 195–197
60 Frank, S.A. (1994) Kin selection and virulence in the evolution of protocells and parasites. *Proc. R. Soc. B: Biol. Sci.* 258, 153–161
61 May, R.M. and Nowak, M.A. (1995) Coinfection and the evolution of parasite virulence. *Proc. R. Soc. B: Biol. Sci.* 261, 209–215
62 van Baalen, M. and Sabelis, M.W. (1995) The dynamics of multiple infection and the evolution of virulence. *Am. Nat.* 146, 881–910
63 Michod, R.E. *et al.* (2008) Adaptive value of sex in microbial pathogens. *Infect. Genet. Evol.* 8, 267–285

Supplementary material

Generalism and the evolution of parasite virulence

Helen C. Leggett[1,2], Angus Buckling[2], Gráinne H. Long[3], and Mike Boots[2]

[1]Department of Zoology, Oxford University, South Parks Road, Oxford, OX1 3PS, UK

[2]Biosciences, University of Exeter, Cornwall Campus, Penryn, TR10 9EZ, UK

[3]MRC Epidemiology Unit, University of Cambridge, Institute of Public Health, Addenbrooke's Hospital, Hills Road, Cambridge, CB2 0QQ, UK

Corresponding author: Leggett, H.C. (helen.leggett@zoo.ox.ac.uk).

Table S1: Experimental evidence for the assumptions of life history trade-off theory[a]

Parasite	Host	Evidence[b]	Study
Plant-parasite interactions			
Prunus necrotic ringspot virus	Rose	A	[64]
Rice yellow mottle virus	Rice	A	[65]
Cucumber mosaic virus	Tobacco	A	[66]
Cucumber mosaic virus	Muskmelon	A, B	[67]
Papaya ringspot virus	Squash and watermelon	A	[68]
Banana streak virus	Banana	A	[69]
Beat mosaic virus	Sugar beat	B	[70]
Tomato chlorosis virus	Tomatoes	B	[71]
Hyaloperonospora parasitica	*Arabidopsis thaliana*	D	[72]
Animal-parasite interactions			
Plasmodium chabaudi	Mosquitoes	A, B, D	[73]
Pasteuria ramosa	*Daphnia* water fleas	C, E	[74]
Xenorhabdus nematophila	Nematodes	A, C, D	[75]
Myxoma	Rabbits	A, B, C, E	[76,77]
Ophryocycystis elektroscirrha	Monarch butterflies	A, B, C,	[78]
HIV	Humans	A, B, C, E	[79]
Plasmodium chaubaudi	Mice	A, B, D	[80-83]
Amblyospora dyxenoides	Mosquitoes	D	[84]
Nuclear polyhedrovirus	Gypsy moth	D	[85]
Plasmodium gallinaceum	Chickens	D	[86]

Citrobacter rodientum	Mice	D	[87]
Plasmodium falciparum	Humans	D	[88]
Corynebacterium diphtheriae	Humans	D	[89]
Human papillomavirus type 16	Humans	B	[90]
Reovirus	Mice	B	[91]
Toxoplasma gondii	Mice	A	[92]
Trypanosoma congolense	Mice	A	[93]
Deformed Wing Virus	*Varroa* mites	A	[94]
Plasmodium berghei	Mosquitoes	A-E	[95]
Bacteria-parasite interactions			
Bacteriophage f1	*Escherichia coli*	C	[96]

[a]For further discussion see [4-6,97]; [b]Letters correspond to relationships in Figure 1.

Table 1 References

64. Moury, B.t., *et al.* (2001) Survey of Prunus necrotic ringspot virus in Rose and Its Variability in Rose and Prunus spp. *Phytopathol.* 91, 84-91

65. Fargette, D., *et al.* (2002) Emergence of Resistance-breaking Isolates of Rice yellow mottle virus during Serial Inoculations. *Eur. J. Plant Pathol.* 108, 585-591

66. Shi, B.-J., *et al.* (2002) Differential Virulence by Strains of Cucumber mosaic virus is Mediated by the 2b Gene. *Mol. Plant Microbe Interact.* 15, 947-955

67. Banik, M.T., and Zitter, T.A. (1990) Determination of cucumber mosaic virus titer in muskmelon by enzyme-linked-immunosorbent-assay and correlation with aphid transmission. *Plant Dis.* 74, 857-859

68. Pacheco, D.A., *et al.* (2003) Biomass, virus concentration, and symptomatology of cucurbits infected by mild and severe strains of Papaya ringspot virus. *Scientia Agricola* 60, 691-698

69. Dahal, G., *et al.* (1998) Studies on a Nigerian isolate of banana streak badnavirus: II. Effect of intraplant variation on virus accumulation and reliability of diagnosis by ELISA. *Ann. Appl. Biol.* 132, 263-275

70. Dusi, A.N., and Peters, D. (1999) Beet mosaic virus: its Vector and Host Relationships. *J. Phytopathol.* 147, 293-298

71. Wintermantel, W.M., *et al.* (2008) Co-Infection by Two Criniviruses Alters Accumulation of Each Virus in a Host-Specific Manner and Influences Efficiency of Virus Transmission. *Phytopathol.* 98, 1340-1345

72. Salvaudon, L., *et al.* (2005) Parasite-host fitness trade-offs change with parasite identity: Genotype-specific interactions in a plant-pathogen system. *Evolution* 59, 2518-2524

73. Ferguson, H.M.*, et al.* (2003) Mosquito mortality and the evolution of malaria virulence. *Evolution* 57, 2792-2804

74. Jensen, K.H.*, et al.* (2006) Empirical support for optimal virulence in a castrating parasite. *PLoS. Biol.* 4, 1265-1269

75. Chapuis, Ã.l.*, et al.* (2012) Trade-offs shape the evolution of the vector-borne insect pathogen Xenorhabdus nematophila. *Proc. R. Soc. B-Biol. Sci* 279, 2672-80

76. Fenner F., R.F. (1965) *Myxomatosis*. Cambridge University Press

77. Dwyer, G.*, et al.* (1990) A simulation-model of the population-dynamics and evolution of myxomatosis. *Ecol. Monogr.* 60, 423-447

78. de Roode, J.C.*, et al.* (2008) Virulence-transmission trade-offs and population divergence in virulence in a naturally occurring butterfly parasite. *Proc. Natl. Acad. Sci. USA* 105, 7489-7494

79. Fraser, C.*, et al.* (2007) Variation in HIV-1 set-point viral load: Epidemiological analysis and an evolutionary hypothesis. *Proc. Natl. Acad. Sci. USA* 104, 17441-17446

80. Mackinnon, M.J., and Read, A.F. (2003) The effects of host immunity on virulence-transmissibility relationships in the rodent malaria parasite Plasmodium chabaudi. *Parasitol.* 126, 103-112

81. Mackinnon, M.J., and Read, A.F. (2004) Virulence in malaria: an evolutionary viewpoint. *Philos. Trans. R. Soc. Lond. Ser. B-Biol. Sci.* 359, 965-986

82. Mackinnon, M.J., and Read, A.F. (1999) Selection for high and low virulence in the malaria parasite Plasmodium chabaudi. *Proc. R. Soc. Lond. Ser. B-Biol. Sci.* 266, 741-748

83. Mackinnon, M.J.*, et al.* (2002) Virulence in rodent malaria: Host genotype by parasite genotype interactions. *Infect. Genet. Evol.* 1, 287-296

84. Agnew, P., and Koella, J.C. (1997) Virulence, parasite mode of transmission, and host fluctuating asymmetry. *Proc. R. Soc. Lond. Ser. B-Biol. Sci.* 264, 9-15

85. Cooper, V.S.*, et al.* (2002) Timing of transmission and the evolution of virulence of an insect virus. *Proc. R. Soc. B-Biol. Sci.* 269, 1161-1165

86. Paul, R.E.L.*, et al.* (2004) Experimental evaluation of the relationship between lethal or non-lethal virulence and transmission success in malaria parasite infections. *BMC Evol. Biol.* 4, 12

87. Wickham, M.E.*, et al.* (2007) Virulence is positively selected by transmission success between mammalian hosts. *Curr. Biol.* 17, 783-788

88. Mackinnon, M.J.*, et al.* (2008) Virulence evolution in response to vaccination: The case of malaria. *Vaccine* 26, C42-C52

89. Chen, R.T.*, et al.* (1985) Diphtheria in the United States, 1971-81. *Am. J. Public Health* 75, 1393-1397

90. Kaye, J.N.*, et al.* (1994) Viral load as a determinant for transmission of human papillomavirus type 16 from mother to child. *J. Med. Virol.* 44, 415-421

91. Keroack, M., and Fields, B.E. (1986) Viral shedding and transmission between hosts determined by reovirus L2 gene. *Science* 232, 1635-1638

92. Appleford, P.J., and Smith, J.E. (1997) Toxoplasma gondii: the growth characteristics of three virulent strains. *Acta Tropica* 65, 97-104

93. Masumu, J.*, et al.* (2006) Comparison of the virulence of Trypanosoma congolense strains isolated from cattle in a trypanosomiasis endemic area of eastern Zambia. *Int. J. Parasitol.* 36, 497-501

94. Yue, C., and Genersch, E. (2005) RT-PCR analysis of Deformed wing virus in honeybees (Apis mellifera) and mites (Varroa destructor). *J. Gen. Virol.* 86, 3419-3424

95. Pollitt, L.C.*, et al.* (2012) Costs of crowding for the transmission of malaria parasites. *Evolutionary Applications* 6, 617-629

96. Messenger, S.L.*, et al.* (1999) Virulence evolution in a virus obeys a trade-off. *Proc. R. Soc. Lond. Ser. B-Biol. Sci.* 266, 397-404

97. Sacristan, S., and Garcia-Arenal, F. (2008) The evolution of virulence and pathogenicity in plant pathogen populations. *Mol. Plant Pathol.* 9, 369-384

OPEN ACCESS Freely available online

PLoS PATHOGENS

Mechanisms of Pathogenesis, Infective Dose and Virulence in Human Parasites

Helen C. Leggett*, Charlie K. Cornwallis, Stuart A. West

Department of Zoology, Oxford University, Oxford, United Kingdom

Abstract

The number of pathogens that are required to infect a host, termed infective dose, varies dramatically across pathogen species. It has recently been predicted that infective dose will depend upon the mode of action of the molecules that pathogens use to facilitate their infection. Specifically, pathogens which use locally acting molecules will require a lower infective dose than pathogens that use distantly acting molecules. Furthermore, it has also been predicted that pathogens with distantly acting immune modulators may be more virulent because they have a large number of cells in the inoculums, which will cause more harm to host cells. We formally test these predictions for the first time using data on 43 different human pathogens from a range of taxonomic groups with diverse life-histories. We found that pathogens using local action do have lower infective doses, but are not less virulent than those using distant action. Instead, we found that virulence was negatively correlated with infective dose, and higher in pathogens infecting wounded skin, compared with those ingested or inhaled. More generally, our results show that broad-scale comparative analyses can explain variation in parasite traits such as infective dose and virulence, whilst highlighting the importance of mechanistic details.

Citation: Leggett HC, Cornwallis CK, West SA (2012) Mechanisms of Pathogenesis, Infective Dose and Virulence in Human Parasites. PLoS Pathog 8(2): e1002512. doi:10.1371/journal.ppat.1002512

Editor: Rustom Antia, Emory University, United States of America

Received May 24, 2011; **Accepted** December 15, 2011; **Published** February 16, 2012

Funding: HCL is funded by a BBSRC Docteral Training Grant (http://www.bbsrc.ac.uk/). SAW is funded by ERC (ATRQBYO: http://erc.europa.eu/). CKC was funded by The Browne Fellowship, The Queen's College Oxford. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: helen.leggett@zoo.ox.ac.uk

Introduction

There is huge variation across pathogen species in the number of cells required to successfully infect a host. This number is known as the 'infective dose'. At one end of the scale, species such as *Shigella* and *Giardia lamblia* require about 10 cells to start an infection. In contrast, species such as *Vibrio cholera* and *Staphylococcus aureus* require 10^3–10^8 cells in order for an infection to develop [1–3]. It is unclear why infective dose varies, with large differences occurring even between closely related pathogens [2,3].

Schmid-Hempel and Frank [2] predicted that the variation in infective dose could be explained by the different biochemical mechanisms that pathogens use to infect hosts. Pathogens secrete a number of molecules which facilitate the suppression and/or evasion of host immune responses, and hence aid parasite growth. If these molecules act locally, in the vicinity of the pathogenic cell, then only small numbers of molecules may be required for successful growth and so infections can be established from small numbers of pathogenic cells. In contrast, if the pathogenic molecules diffuse and therefore act at a distance, then large numbers of molecules may be required for evading the host immune system. In these cases greater numbers of pathogenic cells could be needed to establish an infection. However, while this prediction is consistent with anecdotal data [2,3], it has yet to be tested formally.

Here, we test Schmid-Hempel and Frank's [2] prediction that infective dose is determined by whether pathogenesis is locally or distantly acting. We use data from 43 species of human pathogens across a range of enteropathogenic bacteria, protozoa, fungi and viruses. A possible problem with comparative studies across species is that closely related species can share characters through common descent rather than independent evolution. Consequently, analysing species as independent data points can lead to misleading correlations [4–6]. For example, all viruses are locally acting, and so this could lead to patterns between viruses and bacteria, rather than local or distant action. We account for this potential problem of shared ancestry by using multivariate nested taxonomic models [7,8].

We then extend this work in two ways. First, Schmid-Hempel and Frank [2,3,9] further predicted that pathogens with distantly acting immune modulators will be more virulent, possibly because they would have a large numbers of cells in the inoculums, and higher parasite density would overwhelm the host immune system causing more harm to hosts. We therefore test whether the virulence of pathogens with distantly acting immune modulators is greater than that of pathogens with locally acting molecules. Second, we test the influence of two other factors that could affect infective dose and virulence: mode of transmission (direct or indirect) and route of infection (ingestion, inhalation or wounded skin) [1,10–12]. These factors could influence dose and virulence for a number of reasons, including their affect on: the extent to which virulence reduces pathogen transmission; the types of immune response they encounter; and the genetic diversity (or relatedness) of the pathogens either competing for or cooperating to exploit the host [2,3,9–28].

Author Summary

We found that mechanisms used by parasites to infect hosts are able to explain variation in two key pathogen traits: infective dose and virulence. In pathogens where the molecules secreted to facilitate infection acted locally, the number of cells required to start an infection (infective dose), was lower than in pathogens where the secreted molecules act more distantly. Parasite virulence showed no correlation with local versus distant action, but was negatively correlated with infective dose, and greater in species that infect via wounded skin. By showing how such parasite life history details matter, our results help explain why classical trade-off models have been relatively unsuccessful in explaining broad scale variation across parasite species.

Results/Discussion

We found that pathogens with immune modulators that act distantly within the host have significantly higher infective doses than pathogens with locally acting molecules, (Figure 1 and Table S2 in Text S1: $F_{1,\ 40} = 25.79$, $P < 0.0001$). This supports the prediction by Schmid-Hempel and Frank [2] that local pathogenic action requires only a small number of molecules, and thus relatively few cells are needed to start an infection, compared to distantly acting mechanisms where a large number of diffusible molecules need to accumulate in order to overwhelm the host's immune clearance.

Contrary to the hypothesis that pathogens with distantly acting immune modulators are more virulent, we found no significant relationship between case fatality rate or severity of infection and the mechanism of pathogenesis (Table S3 and Table S4 in Text S1: $P > 0.05$). However, case fatality rate was significantly negatively related to infective dose of pathogens (Figure 2 and Table S3 in Text S1: $F_{1,\ 38} = 3.94$, $P = 0.05$). We suggest this correlation arises because for a given dose, pathogens that are locally acting and have lower infective doses are more likely to establish an infection. For this relationship to hold, we reasonably assume that the actual dose in natural infections is largely determined by factors such as mode of transmission, and so does not show a strong covariance with whether a parasite acts locally or globally within the host. We attempted to collect data on mean parasite dose in different transmission modes during natural infections so we could examine how this correlates with local/global within-host parasite action, but we were unable to obtain sufficient data.

Figure 1. Infective dose and the mechanisms used by pathogens to infect hosts. Means ±1 standard error (Table S2 in Text S1).
doi:10.1371/journal.ppat.1002512.g001

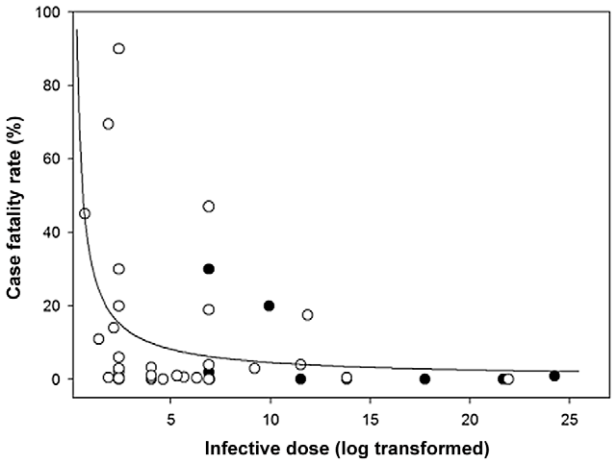


Figure 2. Variation in case fatality rate explained by infective dose. Means ±1 standard error. Line represents a logistic curve (Table S3in Text S1).
doi:10.1371/journal.ppat.1002512.g002

There are at least two possible alternative explanations for the negative relationship between case fatality rate and infective dose of parasites, although we suggest these are less likely than the above explanation. First, recent theory suggests parasites might adapt to low infective doses by evolving a higher per-parasite growth rate, causing greater host exploitation and virulence [29]. However, the reduction in dose in this model results from increased host resistance, and there is no reason to assume that selection for host resistance consistently differs between global and local acting parasites.

Second, a low infective dose may reduce the incidence of multiple genotype pathogen infections since there are fewer parasites in the inoculum, which could favour higher levels of cooperation between parasites, and hence lead to greater growth and virulence [24,25]. However, the extent to which this will be of general importance will be limited by the fact that different biological details can lead to different relationships between strain diversity and virulence. For example, when different parasite strains compete for host resources, higher strain diversity is expected to lead to greater virulence [10,11,22,29–33]. Alternatively, antagonistic interaction between strains, such as chemical warfare, can lead to a predicted domed relationship between strain diversity and virulence [26]. Nonetheless, it is possible that all three explanations could play a role, with their importance varying across species.

We found that pathogens infecting hosts through wounded skin result in significantly higher case fatality rates than pathogens inhaled or ingested (Figure 3 and Table S3 in Text S1: $F_{2,\ 26} = 5.30$, $P = 0.01$). Given that infection via wounded skin includes transmission via bites of insect vectors and contaminated water, this result supports theory on virulence-transmission trade-offs which proposes that vectors and water systems circumvent the need for an ambulatory host to transmit pathogens, selecting for the evolution of higher virulence [12,18,21]. However, another potentially important factor is that the type of immune response that pathogens are confronted with will affect virulence. Pathogens that infect hosts through wounded skin evade mechanical

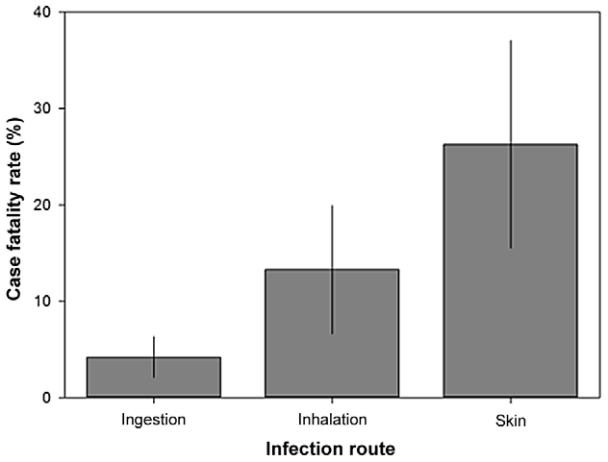


Figure 3. Variation in case fatality rate explained by infection route. Means ±1 standard error. Line represents a logistic curve (Table S3 in Text S1).
doi:10.1371/journal.ppat.1002512.g003

immunity and directly enter the circulatory system. Hence, they may cause virulent systemic infections more readily than ingested or inhaled pathogens, which must overcome other anti-infection barriers such as stomach acid and mucus membranes before causing systemic infections.

More generally, our results emphasise the importance of life-history or mechanistic details for the evolution of parasite traits. Theoretical models for the evolution of parasite traits such as virulence have generally relied on simple trade-offs between virulence and transmission. These models have been able to explain variation in virulence both within species, and between closely related species with similar life histories [15,20,28,33–37]. In contrast, this body of theory has been less successful at explaining broad scale variation across species [2,9,25,27]. One possible explanation for this is that the predictions of virulence theory can depend upon the mechanisms that parasites use to infect and exploit hosts, which are not considered in the classical models; hence our expectations of data fitting the model may be too high. If the details of how parasites infect hosts really matter, this would limit the extent to which we would expect to find broad empirical patterns to match theory [25]. Our results show that transmission, dose and virulence can be influenced by mechanistic details such as distance at which molecules act and route of infection.

Materials and Methods

Infective dose

We obtained data on the number of pathogen cells required to start an infection (infective dose) by searching: (a) databases from the United States Food and Drug Administration [38], Health Canada [39], Medscape [40], the Centre for Disease Control and Prevention [41], the World Health Organisation [42]; (b) empirical studies found via keyword searches in the ISI Web of Knowledge database [43]. Where ranges or more than one estimate of infective dose were given, we calculated the median infective dose to use in our analyses.

We emphasise that uncertainties exist in infective dose measurements: often they were extrapolated from epidemiologic investigations, were obtained by human feeding studies on healthy, young adult volunteers, or are best or worst estimates based on a limited data from outbreaks. Where known, we give methods of estimation in Table S1 in Text S1.

Classifying mechanisms of pathogenesis

We classified pathogens as having local or distant action according to the framework of Schmid-Hempel and Frank [2]. For local action, pathogens directly interact with host cells via surface-bound molecules or by injecting proteins into host cells by a type III or IV secretion systems. For example, *Yersinia entricolata/ tuberculosis* injects Yop protein into target cells via a type III secretion system, leading to cytotoxicity [44], and *Ebola virus* binds to different cell surfaces and replicates leading to cell necrosis [45].

For distant action, pathogens indirectly interact with host cells by secreting proteins that diffuse into their surroundings and only exert pathogenic effects when they bind to host cells. This may arise, for example, through immune modulators delivered by the general secretary pathway, or the type I, II and V secretary systems. For example, the well known virulence factor lysteriolysin O of *Listeria monocytogenes* and exotoxins of *Staphylococcus aureus*, are secreted via the general secretory pathway [46]. We do not classify between interactions with host and immune cells specifically since we are concerned with how far the interaction occurs from the infecting parasite, not with what cell the interaction occurs with.

Measuring virulence: Case fatality rate, 'disease severity' and incidence

To capture both the short and long term consequences of pathogen infection on host health, we use case fatality rate and a 'disease severity' score to measure pathogen virulence. These are two of the three criteria used to estimate 'burden of disease' in a recent protocol for prioritising infectious disease in public health [47].

We rated each pathogen according to its severity, as described in Table 1. We gave a score of 0 to pathogens of average importance, or pathogens for which a lack of data precluded another score. Incidence data are the estimated mean number of new cases per year in the USA. Case fatality rates are estimates of fatality without treatment or co-morbidities and represent the number of cases of a disease ending in death compared to the number of cases of the disease. We obtained data from the before-mentioned databases, plus various reports in the literature (see Table S1 in Text S1).

We emphasise that while a "case" should represent an infected individual, in practice it may involve infection of some severity, - hospitalization even. Thus overall our definition of case fatality may overestimate virulence. For example, a benign parasite that infects many hosts asymptomatically, but cause severe disease in a small proportion of hosts, may be classified as virulent. By contrast, a virulent parasite that causes disease of equal severity in its hosts may be classed as less virulent. To correct for this potential bias, we assessed whether case fatality rate is linked to incidence rate, and examined the effects of the other variables after controlling for variation in incidence rate.

Transmission mode and route of infection

We obtained data on transmission mode and route of infection using the before-mentioned databases. We classified pathogens as either direct or indirectly transmitted: direct transmission requires physical contact between an infected and susceptible host, and indirect transmission requires an agent to transfer the pathogen from an infected to a susceptible host. We classified the routes of infection used by pathogens as entry through wounded skin, inhalation, or ingestion. For example, *Bordatella pertussis* is usually

Table 1. Definition of the scores for calculating disease severity.

Scores		
−1	**0**	**1**
Hospitalisation is rare. Work loss is <2 days. No persisting illnesses/handicaps	Hospitalisation is rare. Work loss >5days is rare. Few persisting illnesses/handicaps.	Hospitalisation is frequent, work loss of >5days is frequent. Persisting illnesses/handicaps occur.

Adapted from [47].
doi:10.1371/journal.ppat.1002512.t001

spread by infected people coughing or sneezing while in close contact with susceptible others who then inhale the *pertussis* bacteria [41] (i.e. direct transmission). Where pathogens can use more than one mechanism of transmission or infection, we used the mechanism stated in the infective dose data for our analyses.

Statistical analysis

We performed three analyses. First, we tested whether minimum infective dose (log transformed) was related to the mechanism of infection (2 level fixed factor: local or distant), infection route (3 level fixed factor: ingestion, inhalation, wounded skin) and the transmission mode of pathogens (2 level fixed factor: direct, indirect) using a linear mixed effects model (LMM) with restricted maximum likelihood estimation (REML). Second we tested if case fatality rate (% of cases resulting in death) was influenced by infective dose (covariate log transformed), incidence (covariate log transformed), mechanism of infection, infection route and transmission mode using a generalised linear mixed effects model (GLMM) with a binomial error distribution. Finally, we analysed the severity of infection (−1, 0, 1) in relation to the same explanatory variables as the second analysis using a GLMM with an ordered multinomial error distribution.

The data (Table S1 in Text S1) encompass a diverse range of pathogens. We obtained information on the taxonomic classification of pathogens from the National Center for Biotechnology Information (NCBI) [48]. We accounted for the non-independence of data arising from phylogenetic relationships between pathogens in all LMMs and GLMMs using nested taxonomic random effects structures whereby each taxonomic level (genus, order, class and kingdom) was nested within all higher taxonomic levels (see Tables S2–4 in Text S1 for details). We only entered genus, order, class and kingdom into models because of poor replication at other taxonomic levels. We examined the significance of fixed effects (factors and covariates) using Wald type adjusted F statistics and the effect with the highest P value was sequentially dropped until only significant terms ($P<0.05$) remained [49]. Prior to all analyses covariates were Z-transformed (mean = 0, standard deviation = 1). We used the Kenward and Roger (1997) method for estimating standard errors for parameter estimates and denominator degrees of freedom since it is specifically designed for models with multiple random effects and unbalanced data, increasing the accuracy of significance tests [50–52]. We assessed the significance of random effects using log-likelihood ratio tests (LRTs) [53]. All analyses were conducted in SAS version 9.2.

Supporting Information

Text S1 Dataset and statistical analysis tables. Here we provide details of the pathogens included in this study and summaries of the statistical analysis: Table S1: Pathogens included in the analysis; Table S2: LMM of infection dose; Table S3: GLMM of case fatality rate; Table S4: GLMM of severity of infection.
(DOC)

Acknowledgments

We thank Sunetra Gupta, Adrian Smith, Sam Brown, Kevin Foster and Angus Buckling for helpful discussions; two anonymous referees for improvements to the manuscript.

Author Contributions

Conceived and designed the experiments: HCL SAW. Performed the experiments: HCL. Analyzed the data: HCL CKC. Contributed reagents/materials/analysis tools: HCL CKC SAW. Wrote the paper: HCL CKC SAW. All authors contributed substantially to this work: HCL CKC SAW.

References

1. Sewell DL (1995) Laboratory-associated infections and biosafety. Clin Microbiol Rev 8: 389–405.
2. Schmid-Hempel P, Frank SA (2007) Pathogenesis, virulence, and infective dose. PLoS Pathog 3: 1372–1373. doi:10.1371/journal.ppat.0030147.
3. Schmid-Hempel P (2011) Evolutionary Parasitology: The Integrated Study of Infections, Immunology, Ecology, and Genetics. Oxford: Oxford University Press.
4. Harvey PH, Pagel MD (1991) Oxford series in ecology and evolution 1. The comparative method in evolutionary biology. Oxford, UK: Oxford University Press.
5. Felsenstein J (1985) Phylogenies and the comparative method. Am Nat 125: 1–15.
6. Grafen A (1989) The phylogenetic regression. Philos T Roy Soc B 326: 119–157.
7. Cluttonbrock TH, Harvey PH (1977) Primate ecology and social-organisation. J Zool 183: 1–39.
8. Hadfield JD, Nakagawa S (2010) General quantitative genetic methods for comparative biology: phylogenies, taxonomies and multi-trait models for continuous and categorical characters. J Evol Biol 23: 494–508. doi:10.1111/j.1420-9101.2009.01915.x.
9. Frank SA, Schmid-Hempel P (2008) Mechanisms of pathogenesis and the evolution of parasite virulence. J Evol Biol 21: 396–404. doi:10.1111/j.1420-9101.2007.01480.x.
10. Frank SA (1996) Models of parasite virulence. Q Rev Biol 71: 37–78.
11. Alizon S, Hurford A, Mideo N, Van Baalen M (2009) Virulence evolution and the trade-off hypothesis: history, current state of affairs and the future. J Evol Biol 22: 245–259. doi:10.1111/j.1420-9101.2008.01658.x.
12. Ewald PW (1994) Evolution of Infectious Diseases. Oxford, UK: Oxford University Press.
13. Wild G, Gardner A, West SA (2009) Adaptation and the evolution of parasite virulence in a connected world. Nature 459: 983–986. doi:10.1038/nature08071.
14. Lion S, Boots M (2010) Are parasites "prudent" in space? Ecol Lett 13: 1245–1255. doi:10.1111/j.1461-0248.2010.01516.x.
15. Boots M, Mealor M (2007) Local interactions select for lower pathogen infectivity. Science 315: 1284–1286. doi:10.1126/science.1137126.
16. Boots M, Sasaki A (1999) 'Small worlds' and the evolution of virulence: infection occurs locally and at a distance. Proc Biol Sci 266: 1933–1938.
17. Bull JJ (1994) Perspective- Virulence. Evolution 48: 1423–1437.
18. Ewald PW (1991) Waterborne transmission and the evolution of virulence among gastrointestinal bacteria. Epidemiol Infect 106: 83–119.
19. Hamilton WD (1972) Altruism and related phenomena mainly in social insects. Annu Rev Ecol Syst. pp 193–232.
20. Bull JJ, Molineux IJ, Rice WR (1991) Selection of benevolence in a host-parasite system. Evolution 45: 875–882.

21. Ewald PW (1983) Host-parasite relations, vectors, and the evolution of disease severity. Annu Rev Ecol Syst 14: 465–485.
22. Nowak MA, May RM (1994) Superinfection and the evolution of parasite virulence. P Roy Soc Lond B Biol 255: 81–89.
23. Frank SA (1994) Kin selection and virulence in the evolution of protocells and parasites. Proc Biol Sci 258: 153–161.
24. Brown SP, Hochberg ME, Grenfell BT (2002) Does multiple infection select for raised virulence? Trends Microbiol 10: 401–405.
25. West SA, Buckling A (2003) Cooperation, Virulence and Siderophore Production in Bacterial Parasites. Proc Biol Sci 270: 37–44.
26. Gardner A, West SA, Buckling A (2004) Bacteriocins, spite and virulence. Proc Biol Sci 271: 1529–1535. doi:10.1098/rspb.2004.2756.
27. Ebert D, Bull JJ (2003) Challenging the trade-off model for the evolution of virulence: is virulence management feasible? Trends Microbiol 11: 15–20.
28. Herre EA (1993) Population-structure and the evolution of virulence in nematode parasites of fig wasps. Science 259: 1442–1445.
29. De Roode JC, Fernandez de Castillejo CL, Faits T, Alizon S (2011) Virulence evolution in response to anti-infection resistance: toxic food plants can select for virulent parasites of monarch butterflies. J Evol Biol 24: 712–722. doi:10.1111/j.1420-9101.2010.02213.x.
30. Levin S, Pimentel D (1981) Selection of intermediate rates of increase in parasite-host systems. Am Nat 117: 308–315.
31. Bremermann HJ, Pickering J (1983) A game-theoretical model of parasite virulence. J Theor Biol 100: 411–426.
32. vanBaalen M, Sabelis MW (1995) The dynamics of multiple infection and the evolution of virulence. Am Nat 146: 881–910.
33. de Roode JC, Pansini R, Cheesman SJ, Helinski MEH, Huijben S, et al. (2005) Virulence and competitive ability in genetically diverse malaria infections. Proc Natl Acad Sci USA 102: 7624–7628. doi:10.1073/pnas.0500078102.
34. Ebert D (1998) Evolution - Experimental evolution of parasites. Science 282: 1432–1435.
35. Kerr B, Neuhauser C, Bohannan BJM, Dean AM (2006) Local migration promotes competitive restraint in a host-pathogen 'tragedy of the commons'. Nature 442: 75–78. doi:10.1038/nature04864.
36. Rumbaugh KP, Diggle SP, Watters CM, Ross-Gillespie A, Griffin AS, et al. (2009) Quorum Sensing and the Social Evolution of Bacterial Virulence. Curr Biol 19: 341–345. doi:10.1016/j.cub.2009.01.050.
37. Kohler T, Buckling A, van Delden C (2009) Cooperation and virulence of clinical Pseudomonas aeruginosa populations. Proc Natl Acad Sci USA 106: 6339–6344. doi:10.1073/pnas.0811741106.
38. United States Food and Drug Administration (2003) The Bad Bug Book. Available: http://www.fda.gov/Food/FoodSafety/FoodborneIllness/FoodborneIllnessFoodbornePathogensNaturalToxins/BadBugBook/default.htm. Accessed 2 December 2009.
39. Health Canada (2003) Pathogen safety data sheets. Available: http://www.phac-aspc.gc.ca/lab-bio/res/psds-ftss/index-eng.php. Accessed 2 December 2009.
40. Medscape. Infectious Disease Articles. Available: http://emedicine.medscape.com/infectious_diseases. Accessed 2 December 2009.
41. Centers for Disease Control and Prevention. Alphabetical Index of Parasitic Diseases. Available: http://www.cdc.gov/parasites/az/index.html. Accessed 2 December 2009.
42. World Health Organization. Publications and Fact sheets. Available: http://www.who.int/research/en/. Accessed 2 December 2009.
43. ISI Web of knowledge database. Available: http://apps.isiknowledge.com. Accessed 2 December 2009.
44. Wilson M, McNab R, Henderson B (2002) Bacterial Disease Mechanisms: An introduction to cellular microbiology. Cambridge: Cambridge University Press.
45. Bray M (2005) Pathogenesis of viral hemorrhagic fever. Curr Opin Immunol 17: 399–403. doi:10.1016/j.coi.2005.05.001.
46. Wooldridge K (2009) Bacterial Secreted Proteins: Secretory Mechanisms and Role in Pathogenesis Caister Academic Press.
47. Krause G, Working group on prioritisation at the Robert Koch Institute (2008) How can infectious diseases be prioritized in public health? A standardized prioritization scheme for discussion. Embo Rep 9: S22–S27. doi:10.1038/embor.2008.76.
48. Sayers EW, Barrett T, Benson DA, Bryant SH, Canese K, et al. (2009) Database resources of the National Center for Biotechnology Information (vol 37, pg D5, 2008). Nucleic Acids Res 37: 3124–3124. doi:10.1093/nar/gkp382.
49. Crawley MJ (2002) Statistical Computing: An Introduction to Data Analysis using S-Plus. West Sussex: Wiley.
50. Kenward MG, Roger JH (1997) Small Sample Inference for Fixed Effects from Restricted Maximum Likelihood. Biometrics 53: 983–997.
51. Littell RC, Milliken GA, Stroup WW, Wolfinger RD, Schabenberger O (2006) SAS for Mixed Models SAS Publishing.
52. Bolker BM, Brooks ME, Clark CJ, Geange SW, Poulsen JR, et al. (2009) Generalized linear mixed models: a practical guide for ecology and evolution. Trends Ecol Evol 24: 127–135. doi:10.1016/j.tree.2008.10.008.
53. Self SG, Liang KY (1987) Asymptotic properties of maximum likelihood estimators and likelihood ratio tests under non-standard conditions. J Am Stat Assoc 82: 605–610.

Supporting Information

Contents

Table S1: Mechanisms of pathogenesis, infective dose and virulence of human pathogens

Pathogen	Mechanism of pathogenesis	Infective dose (number of infective particles)	Severity	Case Fatality Rate (%)	Incidence (number cases/year USA)	Route of infection	Transmission mode	Sources
Viruses								
Ebola virus	Local	5.5	1	69.5	0	Inhalation	Direct	[1-4]
Norovirus	Local	55	0	0.075	14,250,000	Ingestion	Direct	[1,5,6]
Polio viruses	Local	3*	1	11	0	Ingestion	Direct	[1,7,8]
Rotavirus Group A	Local	55	0	0.65	2,850,000	Ingestion	Indirect	[1,9-11]
Variola minor	Local	55	0	0.5	0	Inhalation	Direct	[8,12,13]
Bacteria								
Bacillus anthracis	Distant	20,250	1	20	1	Skin	Direct	[1,4,9,14-16]
Bacillus cereus	Distant	1,000,000	-1	0	27,360	Ingestion	Indirect	[4,9,17-20]
Bordetella pertussis	Local	200	1	1	6,000	Inhalation	Direct	[21-23]
Brucella abortus	Local	55	1	1.025	150	Skin	Direct	[1,9,24]

Brucella Melitensis	Local	55	1	3.25	150	Ingestion	Indirect	[1,9,25]
Campylobacter jejuni	Local	550*	1	0.4	2,850,000	Ingestion	Indirect	[1,4,8,9,15,20,26]
Clostridium perfringens	Distant	100,000	-1	0.07	607,239	Ingestion	Indirect	[4,9,20,27,28]
Coxiella burnetii	Local	10*	1	3	51	Inhalation	Indirect	[4,15,23,29]
Cryptosporidium parvum	Local	5.5	-1	0.5	1,529,000	Ingestion	Indirect	[1,4,9,26,30,31]
Escherichia coli, enteroaggregative (EAEC)	Distant	50,500,000*	0	0.025	-	Ingestion	Indirect	[26,32]
Escherichia coli, enterohemorrhagic (EHEC, serotype O157)	Local	10**	1	0.254	-	Ingestion	Indirect	[1,4,9,26,32]
Escherichia coli, enteroinvasive (EIEC)	Local	10*	0	0.025	-	Ingestion	Indirect	[4,9,26,32]
Escherichia coli, enteropathogenic (EPEC)	Local	3,367,000,000**	0	0.025	-	Ingestion	Indirect	[4,9,26,32]

Escherichia coli, enterotoxigenic (ETEC)	Distant	2,550,250,000*	0	0.025	48,710	Ingestion	Indirect	[1,4,9,26,32]
Francisella tularensis, holarctica	Local	1000	1	0	200	Ingestion	Indirect	[9,33-35]
Francisella tularensis, tularensis	Local	7.5*	1	14	200	Inhalation	Indirect	[1,4,9,15,33,34]
Helicobacter pylori	Local	10,000	1	3	1,238,918	Ingestion	Direct	[4,23]
Legionella pneumophila	Local	140,000	1	17.5	13,000	Inhalation	Indirect	[1,23,36-38]
Listeria monocytogenes	Distant	1000	1	30	2,046	Ingestion	Indirect	[1,9,20]
Mycobacterium tuberculosis	Local	10	0	0.021	14,517	Inhalation	Direct	[4,8,9,39]
Neisseria gonorrhoeae	Distant	1050	1	0	700,000	Skin	Direct	[1,8,16]
Pseudomonas aeruginosa	Local	1000	1	47	5,000,000	Skin	Direct	[40-42]
Rickettsia conorii	Local	10	0	2.1	0	Skin	Indirect	[29]
Rickettsia rickettsii	Local	10	1	20	1,125	Skin	Indirect	[1,4,29]

Rickettsia prowazekii	Local	10	1	30	2	Skin	Indirect	[4,29,43]
Salmonella enterica	Local	283.75	1	0.6	2,000,000	Ingestion	Indirect	[1,4,9,23]
Salmonella paratyphi	Local	1000	1	4	659	Ingestion	Indirect	[4,8,9,20,44]
Salmonella typhi (enterica typhimurium)	Local	100,000	1	4	1,100	Ingestion	Indirect	[1,4,8,44]
Shigella flexneri	Local	100	1	0.1	206,549	Ingestion	Indirect	[1,4,8,9,13,15,20, 26]
Staphylococcus aureus	Distant	100,000	0	0.02	185,060	Ingestion	Direct	[9,20,30]
Streptococcus A (pyogenes)	Local	1000	1	19	11,000	Inhalation	Direct	[1,9,45]
Streptococcus pneumoniae (Group A)	Distant	1000	1	2	6,177,500	Inhalation	Direct	[1,8,9]
Vibrio cholerae (serotypes O139, O1)	Distant	33,334,000,000	1	0.9	66	Ingestion	Indirect	[1,4,9,20,26]
Yersinia enterocolitica	Local	1,000,000	0	0.5	67,243	Ingestion	Indirect	[1,4,20,46]

Yersinia pestis	Local	10	1	90	13	Skin	Indirect	[1,29,47]
Fungi								
Histoplasma capsulatum	Local	10	0	6	50,000,000	Inhalation	Indirect	[4,23,48,49]
Protozoa								
Entamoeba histolytica	Local	1	1	45	-	Ingestion	Direct	[4,9,50,51]
Giardia lamblia	Local	10	0	0.6	2,500,000	Ingestion	Direct	[4,9,31]

*Data from human feeding studies; **Data from natural disease outbreaks; - unknown

Sources:

1. Centers for Disease Control and Prevention. Alphabetical Index of Parasitic Diseases. Available: http://www.cdc.gov/parasites/az/index.html. Accessed 2 December 2009.

2. Rouquet P, Froment JM, Bermejo M, Kilbourn A, Karesh W, et al. (2005) Wild animal mortality monitoring and human Ebola outbreaks, Gabon and Republic of Congo, 2001-2003. Emerg Infect Dis 11: 283-290.

3. Zampieri CA, Sullivan NJ, Nabel GJ (2007) Immunopathology of highly virulent pathogens: insights from Ebola virus. Nat Immunol 8: 1159-1164. (doi:10.1038/ni1519)

4. Health Canada (2003) Pathogen safety data sheets. Available: http://www.phac-aspc.gc.ca/lab-bio/res/psds-ftss/index-eng.php. Accessed 2 December 2009.

5. Lopman BA, Adak GK, Reacher MH, Brown DWG (2003) Two epidemiologic patterns of Norovirus outbreaks: Surveillance in England and Wales, 1992-2000. Emerg Infect Dis 9: 71-77.

6. Mead PS, Slutsker L, Dietz V, McCaig LF, Bresee JS, et al. (1999) Food-related illness and death in the United States. Emerg Infect Dis 5: 607-625.

7. Katz M, Plotkin SA (1967) Minimal infective dose of attenuated poliovirus for man. Am J Public Health Nations Health 57: 1837-1840.

8. World Health Organization. Publications and Fact sheets. Available: http://www.who.int/research/en/. Accessed 2 December 2009.

9. United States Food and Drug Administration (2003) The Bad Bug Book. Available: http://www.fda.gov/Food/FoodSafety/FoodborneIllness/FoodborneIllnessFoodbornePathogensNaturalToxins/BadBugBook/default.htm. Accessed 2 December 2009.

10. Iftekharul Islam MK, M Shamul Islam Khan (1986) Annotated Bibliography of Asian Literature on Diarrhoeal Diseases. J Diarrhoeal Dis Res 4: 165-208.

11. Fischer TK, Viboud C, Parashar U, Malek M, Steiner C, et al. (2007) Hospitalizations and deaths from diarrhoea and rotavirus among children< 5 years of age in the United States, 1993–2003. J Infect Dis 195: 1117. (doi:10.1086/512863)

12. South Dakota Department of Health. A-Z Topic Index. Available: http://doh.sd.gov/index_a_z.aspx. Accessed 2 December 2009.

13. National Institute of Allergy and Infectious disease. Health and Research Topics A-Z. Available: http://www.niaid.nih.gov/topics/pages/default.aspx?wt.ac=tnTopics. Accessed June 2011.

14. Rivera J, Cordero RJB, Nakouzi AS, Frases S, Nicola A, et al. (2010) Bacillus anthracis produces membrane-derived vesicles containing biologically active toxins. P Natl Acad Sci USA 107: 19002-19007. (doi:10.1073/pnas.1008843107)

15. Sewell DL (1995) Laboratory-associated infections and biosafety. Clin Microbiol Rev 8: 389-405.

16. Todar's Online Textbook of Bacteriology (2009). Available: http://www.textbookofbacteriology.net/index.html. Accessed 2 December 2009.

17. Wareing P, Fernandes R (2007) Foodborne bacterial pathogens. Micro-Facts: The Royal Society of Chemistry. pp. 1-167.

18. Wilson M, McNab R, Henderson B (2002) Bacterial Disease Mechanisms: An introduction to cellular microbiology. Cambridge: Cambridge University Press.

19. Fagerlund A, Lindback T, Granum PE (2010) Bacillus cereus cytotoxins Hbl, Nhe and CytK are secreted via the Sec translocation pathway. BMC Microbiol 10: 8. (doi:30410.1186/1471-2180-10-304)

20. The Institute of Food Technologists. Science reports. Available: http://www.ift.org/knowledge-center/read-ift-publications/science-reports.aspx. Accessed June 2011.

21. Paddock CD, Sanden GN, Cherry JD, Gal AA, Langston C, et al. (2008) Pathology and pathogenesis of fatal Bordetella pertussis infection in infants. Clin Infect Dis 47: 328-338. (doi:10.1086/589753)

22. Kerr JR, Matthews RC (2000) Bordetella pertussis infection: Pathogenesis, diagnosis, management, and the role of protective immunity. Eur J Clin Microbiol 19: 77-88.

23. Medscape. Infectious Disease Articles. Available: http://emedicine.medscape.com/infectious_diseases. Accessed 2 December 2009.

24. Bossi P TA, Baka A, Van Loock F, Hendriks J, Werner A, Maidhof H, Gouvras G; Task Force on Biological and Chemical Agent Threats, Public Health Directorate, European Commission, Luxembourg. (2004) Bichat guidelines for the clinical management of brucellosis and bioterrorism-related brucellosis. Eurosurveillance 9: E15-E16.

25. Federation of American Scientists. Biological Threat Agents Information. Available: http://www.fas.org/programs/bio/agents.html. Accessed 2 December 2009.

26. Kothary MH, Babu US (2001) Infective dose of foodborne pathogens in volunteers: A review. J Food Safety 21: 49-73.

27. Pollock AM, Whitty PM (1991) Outbreak of Clostridium-perfringens food poisoning. J Hosp Infect 17: 179-186.

28. Scallan E, Hoekstra RM, Angulo FJ, Tauxe RV, Widdowson MA, et al. (2011) Foodborne Illness Acquired in the United States-Major Pathogens. Emerg Infect Dis 17: 7-15. (doi:10.3201/eid1701.P11101)

29. Azad AF (2007) Pathogenic rickettsiae as bioterrorism agents. Clin Infect Dis 45: S52-S55. (doi:10.1086/518147)

30. New Zealand Food Safety Authority (2001) Microbial Pathogens Data Sheets. Available: http://www.foodsafety.govt.nz/elibrary/industry/Staphylococcus_Aureus-Science_Research.pdf. Accessed 2 December 2009

31. Vijgen SMC, Mangen MJM, Kortbeek LM, van Duijnhoven YTHP, AH V (2007) Disease burden and related costs of cryptosporidiosis and giardiasis in the Netherlands. Netherlands: Ministry of Public Health, Welfare and Sports. Available: http://www.rivm.nl/bibliotheek/rapporten/330081001.pdf. Accessed 2 December 2009

32. Rasko DA, Rosovitz MJ, Myers GSA, Mongodin EF, Fricke WF, et al. (2008) The pangenome structure of Escherichia coli: Comparative genomic analysis of E-coli commensal and pathogenic isolates. J Bacteriol 190: 6881-6893. (doi:10.1128/jb.00619-08)

33. Molins CR, Carlson JK, Coombs J, Petersen JA (2009) Identification of Francisella tularensis subsp tularensis A1 and A2 infections by real-time polymerase chain reaction. Diagn Micr Infec dis 64: 6-12. (doi:10.1016/j.diagmicrobio.2009.01.006)

34. Carlson PE, Carroll JA, O'Dee DM, Nau GJ (2007) Modulation of virulence factors in Francisella tularensis determines human macrophage responses. Microb Pathogenesis 42: 204-214. (doi:10.1016/j.micpath.2007.02.001)

35. Pechous RD, McCarthy TR, Zahrt TC (2009) Working toward the Future: Insights into Francisella tularensis Pathogenesis and Vaccine Development. Microbiol Mol Biol Rev 73: 684-711. (doi:10.1128/mmbr.00028-09)

36. Berendt RF, Young HW, Allen RG, Knutsen GL (1980) Dose-response of guinea-pigs experimentally infected with aerosols of legionella-pneumophila. J Infect Dis 141: 186-192.

37. Formica N, Yates M, Beers M, Carnie J, Hogg G, et al. (2001) The Impact of Diagnosis by Legionella Urinary Antigen Test on the Epidemiology and Outcomes of Legionnaires' Disease. Epidemiol Infect 127: 275-280.

38. Cascales E, Christie PJ (2003) The versatile bacterial type IV secretion systems. Nat Rev Microbiol 1: 137-149. (doi:10.1038/nrmicro753)

39. Cain KP, Haley CA, Armstrong LR, Garman KN, Wells CD, et al. (2007) Tuberculosis among foreign-born persons in the United States: achieving tuberculosis elimination. Am J Resp Crit Care 175: 75. (doi:10.1164/rccm.200608-1178OC)

40. Western Australia Department of Health (2007) Facts about Pseudomonas aeruginosa in Swimming and Spa Pools Environmental Health Guide. Available: http://www.public.health.wa.gov.au/cproot/1322/2/Facts_about_Pseudomonas_aeruginosa.pdf. Accessed 2 December 2009.

41. Aliaga L, Mediavilla JD, Llosa J, Miranda C, Rosa-Fraile M (2000) Clinical significance of polymicrobial versus monomicrobial bacteremia involving Pseudomonas aeruginosa. Eur J Clin Microbiol 19: 871-874.

42. Brannon MK, Davis JM, Mathias JR, Hall CJ, Emerson JC, et al. (2009) Pseudomonas aeruginosa Type III secretion system interacts with phagocytes to modulate systemic infection of zebrafish embryos. Cell Microbiol 11: 755-768. (doi:10.1111/j.1462-5822.2009.01288.x)

43. Svraka S, Rolain JM, Bechah Y, Gatabazi J, Raoult D (2006) Rickettsia prowazekii and real-time polymerase chain reaction. Emerg Infect Dis 12: 428-432.

44. Edelman R, Levine MM (1986) Summary of an international workshop on typhoid-fever. Rev Infect Dis 8: 329-349.

45. Sayers EW, Barrett T, Benson DA, Bryant SH, Canese K, et al. (2009) Database resources of the National Center for Biotechnology Information (vol 37, pg D5, 2008). Nucleic Acids Res 37: 3124-3124. (doi:10.1093/nar/gkp382)

46. Lake R, Hudson A, Cressey P (2004) Risk Profile: Yersinia enterocolitica in pork. Christchurch: Institute of Environmental Science and Research Limited. Available: http://www.foodsafety.govt.nz/elibrary/industry/Risk_Profile_Yersinia-Science_Research.pdf. Accessed 2 December 2009

47. Hacker Jr, Hentschel U, Dobrindt U (2003) Prokaryotic Chromosomes and Disease. Science 301: 790-793. (doi:10.1126/science.1086802)

48. Feigin RD (1992) Textbook of Pediatric Infectious Diseases; Ralph D Feigin JDC, editor: Saunders.

49. Shi L, Albuquerque PC, Lazar-Molnar E, Wang XT, Santambrogio L, et al. (2008) A monoclonal antibody to Histoplasma capsulatum alters the intracellular fate of the fungus in murine macrophages. Eukaryot Cell 7: 1109-1117. (doi:10.1128/ec.00036-08)

50. Yost J (2002) Amebiasis. Pediatr Rev 23: 293-294. (doi:10.1542/pir.23-8-293)

51. Sehgal D, Bhattacharya A, Bhattacharya S (1996) Pathogenesis of infection by Entamoeba histolytica. J Bioscience 21: 423-432

Table S2: Linear mixed model of minimum infection dose (log transformed)

Fixed Terms		**Parameter Estimate (β)**	**SE**	**95%LCL**	**95% UCL**	**DF**	**F**	**P**
Mechanism†	Distant	12.60	1.71	9.11	16.09	1, 40	25.79	**<0.0001**
	Local	3.60	1.06	1.41	5.80			
Infection route						2, 38	1.58	0.22
Transmission mode						1, 34	0.08	0.39
Random Terms		**Variance Component**	**SE**	**95%LCL**	**95% UCL**	**DF**	**LRT**	**P**
Kingdom†		0.00	0.00	0.00	0.00	1	0.00	1.00
Class(kingdom)†		4.20	2.96	1.51	34.35	1	3.83	**0.05**
Order (class kingdom)†		0.00	0.00	0.00	0.00	1	0.00	1.00
Genus (order class kingdom)†		0.00	0.00	0.00	0.00	1	0.00	1.00
Residual		17.57	4.08	11.68	29.38			

Note: GLMM fitted with a normal error distribution. Parameter estimates are on a log scale and significant values are shown in boldface type. LRT = log-likelihood ratio test. LCL = lower confidence limit, UCL = upper confidence limit. † denotes terms included in final model. $N_{kingdoms}$=4, N_{phyla}=11, $N_{classes}$=15, N_{orders}=23, $N_{families}$=26, N_{genera}=29 $N_{species}$=42.

Table S3: Generalized linear mixed model with Binomial error distribution of case fatality rate

Fixed Terms		**Parameter Estimate (β)**	**SE**	**95%LCL**	**95% UCL**	**DF**	**F**	**P**
Incidence rate (log transformed)						1, 32	0.51	0.48
Infection dose (log transformed)†		-0.25	0.10	-0.10	0.51	1, 38	3.94	**0.05**
Mechanism						1, 31	1.32	0.26
Infection route†	Ingestion	0.01	0.01	0.002	0.05	2, 26	5.30	**0.01**
	Inhalation	0.04	0.03	0.008	0.20			
	Wounded skin	0.18	0.14	0.03	0.63			
Transmission mode						1, 29	0.96	0.34
Random Terms		**Variance Component**	**SE**	**95%LCL**	**95% UCL**	**DF**	**LRT**	**P**
Kingdom†		0.00	0.00	0.00	0.00	1	0.00	1.00
Class(kingdom)†		1.53	1.72	0.37	144	1	1.07	0.30
Order (class kingdom)†		0.00	0.00	0.00	0.00	1	0.00	1.00
Genus (order class kingdom)†		1.88	1.15	0.75	10.33	1	7.97	**0.005**

Note: GLMM fitted with a normal error distribution. Parameter estimates are back-transformed to the probability scale and significant values are shown in boldface type. LRT = log-likelihood ratio test. LCL = lower confidence limit, UCL = upper confidence limit. † denotes terms included in final model.

$N_{kingdoms}$=4, N_{phyla}=11, $N_{classes}$=15, N_{orders}=23, $N_{families}$=26, N_{genera}=29 $N_{species}$=42.

Table S4: Generalized linear mixed model with ordered multinomial error distribution of severity of infection

Fixed Terms	**Parameter Estimate (β)**	**SE**	**95%LCL**	**95% UCL**	**DF**	**F**	**P**
Incidence rate (log transformed)					1, 32	0.00	0.99
Infection dose (log transformed)					1, 40	2.85	0.10
Mechanism					1, 36	0.06	0.80
Infection route					2, 37	0.75	0.48
Transmission mode					1, 36	2.10	0.16
Random Terms	**Variance Component**	**SE**	**95%LCL**	**95% UCL**	**DF**	**LRT**	**P**
Kingdom†	0.51	1.00	0.07	323548	1	0.40	0.53
Class(kingdom)†	0.00	0.00	0.00	0.00	1	0.00	1.00
Order (class kingdom)†	0.00	0.00	0.00	0.00	1	0.00	1.00
Genus (order class kingdom)†	0.62	0.84	0.13	368	1	0.88	0.35

Note: GLMM fitted with a normal error distribution. Parameter estimates are back-transformed to the probability scale and significant values are shown in boldface type. LRT = log-likelihood ratio test. LCL = lower confidence limit, UCL = upper confidence limit. † denotes terms included in final model. $N_{kingdoms}$=4, N_{phyla}=11, $N_{classes}$=15, N_{orders}=23, $N_{families}$=26, N_{genera}=29 $N_{species}$=42.

Supplementary Materials for Chapter 4

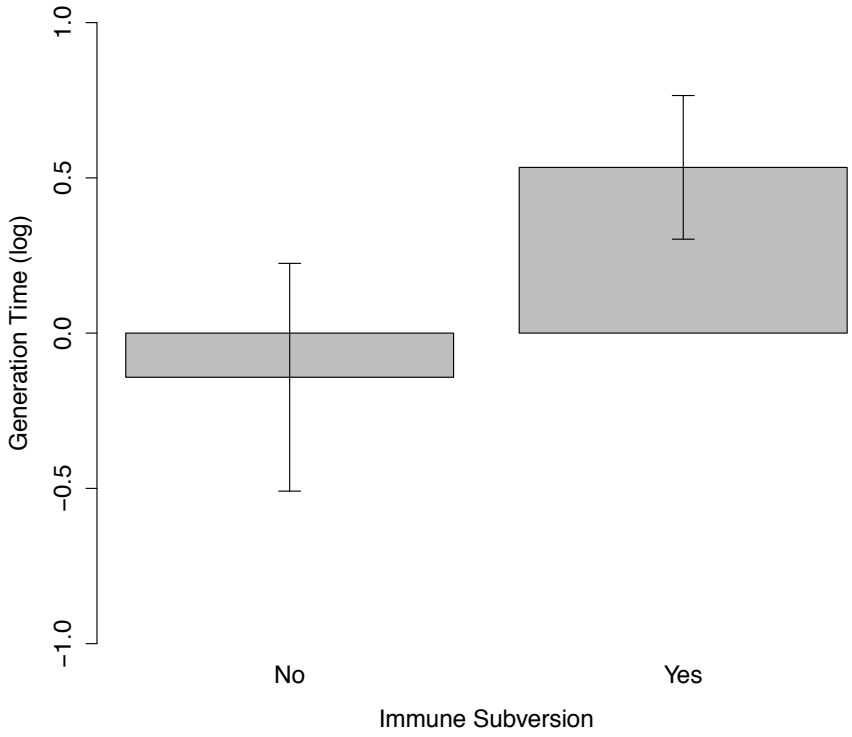


Fig. S1. Immune subversion and growth rate. We found no significant relationship between immune subversion and generation time. See Table S7

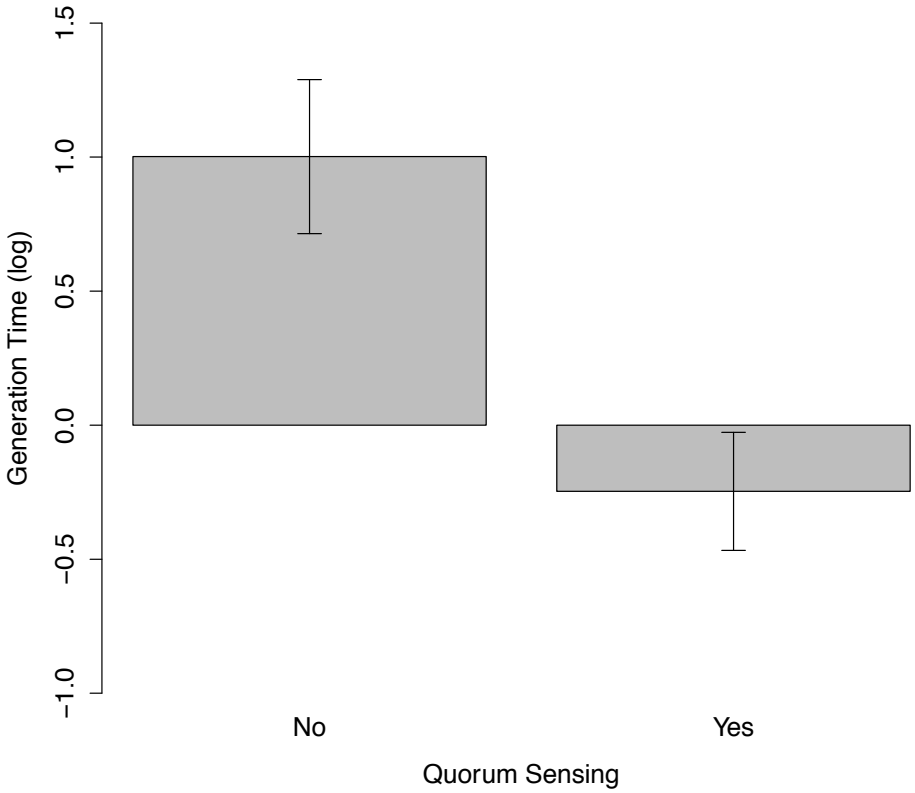


Fig. S2. Quorum sensing regulated expression of virulence factors and growth rate. The generation time of pathogens is significantly lower where quorum sensing was used to control production of virulence factors. See Table S6.

Table S1. Variation in case fatality rate (log of number of deaths per 10,000 cases) explained by variables modeled independently. All analyses control for shared ancestry of pathogens through nested taxonomic random effects structure.

Fixed effects	**Posterior mode**	**Lower CI**	**Upper CI**	**pMCMC**
Infective dose (log)	-1.29	-2.30	-0.50	**0.004**
Infection route: Ingestion	2.50	-3.33	9.55	0.12
Infection route: Inhalation	6.49	1.14	13.87	**0.04**
Infection route: Skin	4.53	-1.66	11.48	0.07
Ingestion vs inhalation	-3.30	-6.47	-1.68	**0.0002**
Ingestion vs skin	-2.01	-4.28	0.06	0.06
Inhalation vs skin	1.70	-0.68	4.73	0.08
Pathogen motility: No	3.37	-1.40	14.91	0.07
Pathogen motility: Yes	4.69	-2.49	13.90	0.08
Difference	-0.42	-2.49	1.36	0.48
Generation time (log)	0.60	-0.30	1.80	0.17
Immune subversion: No	4.96	-2.84	10.67	0.12
Immune subversion: Yes	6.78	-1.38	11.99	0.06
Difference	2.19	0.29	4.15	**0.02**
Quorum Sensing: No	4.21	-0.92	15.94	0.07
Quorum Sensing: Yes	4.21	-2.05	15.12	0.08
Difference	-0.03	-2.25	1.48	0.72

Table S2: Multivariate analysis of variation in case fatality rate (log of number of deaths per 10,000 cases).

Fixed effects	**Posterior mode**	**Lower CI**	**Upper CI**	**pMCMC**
Infection route: Ingestion	5.59	-7.07	8.31	0.60
Infection route: Inhalation	9.23	-1.85	12.94	0.08
Infection route: Skin	6.84	-5.99	9.54	0.22
Ingestion vs inhalation	-4.93	-7.60	-1.83	**0.0006**
Ingestion vs skin	-2.27	-4.41	0.19	0.06
Inhalation vs skin	2.49	-0.37	5.51	0.05
Pathogen motility: No	10.15	-6.57	8.51	0.61
Pathogen motility: Yes	11.66	-5.59	9.11	0.31
Difference	1.17	-0.80	2.86	0.26
Generation time (log)	-0.34	-1.38	1.20	0.78
Immune subversion: No	0.85	-7.47	8.52	0.62
Immune subversion: Yes	3.99	-5.27	10.38	0.17
Difference	2.61	0.38	4.14	**0.02**
Quorum Sensing: No	1.54	-7.08	8.83	0.61
Quorum Sensing: Yes	2.16	-7.42	8.64	0.62
Difference	-0.12	-1.92	1.75	0.98
Random effects	**Posterior mode**	**Lower CI**	**Upper CI**	**Heterogeneity (%)**
Domain	0.00	0.0003	116.34	0.00
Kingdom	0.00	0.0002	12.40	0.00
Phylum	0.04	0.0002	1.95	0.63
Class	0.02	0.0002	3.14	0.24
Order	0.01	0.0002	6.64	0.08
Residual variance	7.04	4.10	12.25	99.05

Table S3: Multivariate analysis of variation in case fatality rate (log of number of deaths per 10,000 cases) among immune subverting pathogens. Sample size N=39.

Fixed effects	**Posterior mode**	**Lower CI**	**Upper CI**	**pMCMC**
Infection route: Ingestion	4.38	-5.80	11.63	0.22
Infection route: Inhalation	8.35	0.25	17.14	**0.05**
Infection route: Skin	5.61	-2.55	14.86	0.09
Ingestion vs inhalation	-5.51	-9.57	-1.37	**0.002**
Ingestion vs skin	-2.50	-4.78	-0.11	**0.04**
Inhalation vs skin	2.53	-0.47	6.99	0.06
Pathogen motility: No	2.10	-6.69	9.52	0.28
Pathogen motility: Yes	3.40	-5.61	10.52	0.19
Difference	1.04	-0.88	2.38	0.31
Generation time (log)	0.57	-0.79	2.17	0.37
Quorum Sensing: No	3.54	-7.65	9.04	0.34
Quorum Sensing: Yes	5.05	-5.18	10.95	0.21
Difference	0.80	-0.74	3.02	0.22
Random effects	**Posterior mode**	**Lower CI**	**Upper CI**	**Heterogeneity (%)**
Domain	0.00	0.0002	138.28	0.00
Kingdom	0.00	0.0002	17.04	0.00
Phylum	0.00	0.0002	3.96	0.00
Class	0.05	0.0002	3.96	2.19
Order	0.00	0.0005	24.40	0.00
Residual variance	2.40	1.14	7.44	97.81

Table S4: Multivariate analysis of variation in case fatality rate (log of number of deaths per 10,000 cases) among non-immune subverting pathogens. Sample size N=15.

Fixed effects	**Posterior mode**	**Lower CI**	**Upper CI**	**pMCMC**
Infection route: Ingestion	3.13	-4.66	9.33	0.32
Infection route: Inhalation	6.79	-1.41	13.81	0.08
Infection route: Skin	4.03	-5.30	12.19	0.34
Ingestion vs inhalation	-3.02	-12.82	5.02	0.28
Ingestion vs skin	-2.51	-9.35	9.03	0.78
Inhalation vs skin	2.93	-8.26	13.51	0.26
Pathogen motility: No	-1.92	-9.79	6.37	0.68
Pathogen motility: Yes	4.11	-1.96	10.14	0.17
Difference	5.80	-1.56	11.28	0.09
Generation time (log)	-1.65	-7.18	4.81	0.41
Quorum Sensing: No	0.09	-14.77	10.97	0.92
Quorum Sensing: Yes	-4.27	-23.82	5.88	0.20
Difference	-4.59	-13.90	1.87	0.13

Random effects	**Posterior mode**	**Lower CI**	**Upper CI**	**Heterogeneity (%)**
Phylum	0.00	0.0002	228.40	0.00
Class	0.00	0.0002	195.48	0.00
Order	2.52	0.0002	86.66	88.12
Residual variance	0.34	0.0002	25.09	11.88

Table S5: Variation in infectious dose (log number of cells) explained by modeling variables independently. All analyses control for shared ancestry of pathogens through nested taxonomic random effects structure.

Fixed effects	Posterior mode	Lower CI	Upper CI	pMCMC
Case fatality rate	-1.29	-2.30	-0.50	**0.004**
Generation time (log)	-3.04	-5.32	-0.94	**0.007**
Infection route: Ingestion	7.16	-10.67	25.63	0.18
Infection route: Inhalation	0.80	-16.18	20.33	0.46
Infection route: Skin	2.57	-17.78	19.73	0.39
Ingestion vs inhalation	5.16	0.30	9.84	**0.02**
Ingestion vs skin	4.27	-0.33	8.47	0.06
Inhalation vs skin	-1.41	-6.31	4.41	0.37
Pathogen motility: No	-2.95	-11.79	20.05	0.40
Pathogen motility: Yes	1.41	-8.72	23.89	0.15
Difference	4.28	1.01	8.14	**0.02**
Immune subversion: No	13.05	-0.63	25.80	0.06
Immune subversion: Yes	2.21	-10.02	16.67	0.36
Difference	-9.32	-12.35	-6.87	**<0.0001**
Quorum Sensing: No	2.10	-15.63	17.00	0.39
Quorum Sensing: Yes	6.33	-11.39	21.21	0.15
Difference	4.28	1.38	8.39	**0.009**

Table S6: Multivariate analysis of variation in infectious dose (log number of cells).

Fixed effects	**Posterior mode**	**Lower CI**	**Upper CI**	**pMCMC**
Case fatality rate	-0.97	-1.93	-0.19	**0.02**
Generation time (log)	0.08	-1.96	2.13	0.85
Infection route: Inhalation	12.22	-6.05	28.89	0.11
Infection route: Intestine	9.08	-11.82	23.33	0.24
Infection route: Skin	10.24	-9.11	26.77	0.17
Inhalation vs intestine	4.23	-0.09	7.53	0.05
Inhalation vs skin	2.62	-1.56	5.14	0.12
Inhalation vs skin	-1.48	-5.42	1.81	0.17
Pathogen motility: No	8.37	-6.82	27.22	0.11
Pathogen motility: Yes	11.07	-4.64	29.62	0.08
Difference	1.50	-1.28	4.01	0.31
Immune subversion: No	10.43	-6.71	26.01	0.11
Immune subversion: Yes	2.46	-15.50	17.25	0.65
Difference	-9.05	-11.13	-6.05	**<0.0001**
Quorum Sensing: No	11.22	-6.61	27.96	0.11
Quorum Sensing: Yes	14.25	-2.89	31.13	0.06
Difference	3.44	1.06	6.21	**0.009**
Random effects	**Posterior mode**	**Lower CI**	**Upper CI**	**Heterogeneity (%)**
Domain	0.00	0.00	0.00	0.00
Kingdom	0.00	0.00	0.00	0.00
Phylum	0.00	0.00	0.00	0.00
Class	0.00	0.00	0.00	0.00
Order	0.02	0.0003	6.86	0.13
Residual variance	12.77	8.69	22.79	99.87

Table S7: Variation in generation time (log) explained by immune subversion and quorum sensing.

Fixed effects	**Posterior mode**	**Lower CI**	**Upper CI**	**pMCMC**
Immune subversion: No	1.49	-1.33	5.01	0.16
Immune subversion: Yes	1.82	-1.29	5.02	0.14
Difference	0.11	-0.34	0.52	0.71
Quorum Sensing: No	1.90	-0.94	5.52	0.10
Quorum Sensing: Yes	1.58	-1.50	4.93	0.18
Difference	-0.56	-1.00	-0.14	**0.008**
Random effects	**Posterior mode**	**Lower CI**	**Upper CI**	**Heterogeneity (%)**
Domain	0.00	0.0002	10.40	0.00
Kingdom	0.00	0.0002	11.40	0.00
Phylum	0.03	0.0002	3.65	2.27
Class	0.01	0.0002	1.10	1.10
Order	0.78	0.29	2.34	70.47
Residual variance	0.29	0.18	0.48	26.17

Table S8. Pathogen life history data used in analysis

Species	Immune subversion	Generation time (hr)	Case fatality rate (%)	QS- reg. virulence	Infection route	Motility	Infectious dose	Sources
Acinetobacter ADP1	0	0.5	?	0	Skin	0	?	(1-4)
Actinobacillus actinomycetemcomitans	1	11	25.5	0	Skin	0	?	(5-9)
Aeromonas hydrophila	0	0.35	?	1	Ingestion	1	50,500,000	(10-13)
Anaplasma phagocytophilum	1	21.6	1.5	0	Skin	?	?	(1,14-16)
Arcobacter butzleri	?	0.66	0.4	0	Ingestion	1	?	(1,14,17)
Bacillus anthracis	1	0.5	20	1	Skin	0	20,250	(1,10,18)
Bacillus cereus	0	0.3	0	1	Ingestion	1	1,000,000	(1,10,18)
Bacteroides fragilis	1	0.63	19.3	0	Skin	0	?	(2,19)
Bordetella pertussis	0	3.8	1	0	Inhalation	0	200	(1,20,21)
Brucella abortus	1	2	1.025	1	Ingestion	0	55	(18,22-25)
Brucella melitensis	1	2	3.25	1	Ingestion	0	55	(1,10,18,22-24)
Burkholderia pseudomallei	1	1	38.5	1	Inhalation	1	10	(10,25,26)
Burkholderia mallei	1	0.75	38.5	1	Inhalation	0	10	(1,10, 25,26)
Campylobacter jejuni	1	1.1	0.4	0	Ingestion	1	550	(1,10,18)
Clostridium perfringens	1	0.2	0.07	1	Ingestion	1	100,000	(1,10,18)

Coxiella burnetii	1	8	3	0	Inhalation	0	10	(1,10,18)
Cronobacter sakazakii	1	0.23	60	1	Skin	1	1000	(1,27-29)
Cryptosporidium parvum	1	13	0.5	0	Ingestion	1	5.5	(18,30,31)
Escherichia coli EaggEC	0	0.5	0.025	1	Ingestion	1	50,500,000	(10,18)
Escherichia coli EHEC	1	0.33	0.254	1	Ingestion	1	10	(10,18)
Escherichia coli EIEC	1	0.4	0.025	0	Ingestion	1	10	(10,18)
Escherichia coli EPEC	0	0.4	0.025	1	Ingestion	1	3.37E+09	(10,18)
Escherichia coli ETEC	0	0.33	0.025	1	Ingestion	1	2.55E+09	(10,18)
Entamoeba histolytica	1	9.6	2	1	Ingestion	1	1	(18,32,33)
Enterococcus faecalis	0	0.5	0.01	1	Ingestion	0	10,000,000	(1,10,34)
Francisella tularensis, holarctica	1	3	0	1	Ingestion	0	1000	(1,18)
Francisella tularensis, tularensis	1	3	14	1	Inhalation	0	7.5	(1,10,18)
Gardnerella vaginalis	0	13.5	0	1	Skin	0	2.00E+10	(10,34)
Giardia lamblia	0	10.5	0.6	0	Ingestion	1	10	(18,34)
Haemophilus ducreyi	1	1.8	0	0	Skin	1	30	(1,10)
Helicobacter pylori	1	2.4	3	0	Ingestion	1	10,000	(1,2,3,19)
Histoplasma capsulatum	1	13	6	1	Inhalation	0	10	(1,18,5-37)
Legionella pneumophila	1	3.3	17.5	1	Inhalation	1	140,000	(10,18)
Listeria monocytogenes	1	1	30	1	Ingestion	1	1000	(1,10,18)

Mycobacterium tuberculosis	1	19	50	0	Inhalation	0	10	(10,18,38)
Mycoplasma pneumonia	0	6	36.3	0	Inhalation	1	5,050,000	(1,10,39)
Neisseria meningitides	0	0.72	20.3	0	Inhalation	0	1000	(1,10,14)
Neisseria gonorrhoeae	1	0.58	0	0	Skin	0	1050	(1,10,18)
Orientia tsutsugamushi	1	9	60	0	Skin	0	3	(10,40)
Plasmodium falciparum	1	24	4	0	Skin	1	?	(41-43)
Plesiomonas shigelloides	0	0.43	6	0	Ingestion	1	1,000,000	(10,44)
Pseudomonas aeruginosa	0	0.5	47	1	Skin	1	1000	(1,10,18)
Rickettsia conorii	1	4.1	2.1	0	Skin	0	10	(1,18,40)
Rickettsia prowazekii	1	10	30	0	Skin	0	10	(1,10,18)
Rickettsia rickettsia	1	9	65	0	Skin	0	10	(1,10,18,45)
Salmonella enterica Typhi	1	0.4	4	0	Ingestion	1	100,000	(10)
Salmonella enterica Typhimurium	1	0.4	4	1	Ingestion	1	100,000	(1,10,18)
Salmonella paratyphi	1	0.4	4	0	Ingestion	1	1000	(1,18)
Shigella dysenteriae	1	0.67	0.6	0	Ingestion	0	10	(1,46)
Shigella flexneri	1	0.68	0.1	0	Ingestion	0	100	(2,18)
Shigella sonnei	1	0.5	0.9	0	Ingestion	0	75	(1,47)
Staphylococcus aureus	1	0.4	4	1	Ingestion	0	100,000	(1,10,18)
Stenotrophomonas maltophilia	0	0.6	?	1	Ingestion	1	5.01E+08	(1,10)

Streptococcus pyogenes	1	0.4	19	1	Inhalation	0	1000	(1,10,18)
Treponema pallidum	1	33	3.17	0	Skin	1	57	(1,10,48)
Vibrio cholera	0	0.2	0.9	1	Ingestion	1	3.33E+10	(1,10,18,34)
Vibrio parahaemolyticus	0	0.2	0.01	1	Ingestion	1	55,000,000	(1,10,34)
Vibrio vulnificus	1	0.16	39	1	Ingestion	1	100	(1,10,34)
Yersinia enterocolitica	1	0.55	0.5	1	Ingestion	1	1,000,000	(10,18)
Yersinia pseudotuberculosis	1	0.5	0.5	1	Ingestion	0	1,000,000	(1,10,34)
Yersinia pestis	1	1.25	90	1	Skin	0	10	(1,10,18)

Table S8 references

1. Vieira-Silva S. & Rocha E.P.C. (2010) The systemic imprint of growth and its uses in ecological (mata)genomics. PLoS Genetics 6: e1000808
2. Chastre, J., and Trouillet, J.L. (2000) Problem pathogens (Pseudomonas aeruginosa and Acinetobacter). *Semin Respir Infect* 15, 287-298
3. Qiu, H.*, et al.* (2012) Role of Macrophages in Early Host Resistance to Respiratory <italic>Acinetobacter baumannii</italic> Infection. *PLoS ONE* 7, e40019
4. Bhargava, N.*, et al.* (2010) Quorum sensing in Acinetobacter: an emerging pathogen. *Critical Reviews in Microbiology* 36, 349-360
5. Paturel, L.*, et al.* (2004) Actinobacillus actinomycetemcomitans endocarditis. *Clin Microbiol Infect* 10, 98-118
6. Yogev, R.*, et al.* (1986) In vitro activity of antibiotics alone and in combination against Actinobacillus actinomycetemcomitans. *Antimicrob Agents Chemother* 29, 179-181

7. Henderson, B.*, et al.* (2002) Actinobacillus actinomycetemcomitans. *Journal of Medical Microbiology* 51, 1013-1020
8. Silva, M.T. (2010) Bacteria-induced phagocyte secondary necrosis as a pathogenicity mechanism. *J. Leukoc. Biol.* 88, 885-896
9. Fong, K.P.*, et al.* (2003) luxS and arcB control aerobic growth of Actinobacillus actinomycetemcomitans under iron limitation. *Infect Immun* 71, 298-308
10. Gama, J.o.A.*, et al.* (2012) Immune Subversion and Quorum-Sensing Shape the Variation in Infectious Dose among Bacterial Pathogens. *PLoS Pathog* 8, e1002503
11. Okumura, K.*, et al.* (2011) Severe sepsis caused by Aeromonas hydrophila in a patient using tocilizumab: a case report. *J Med Case Rep* 5, 1752-1947
12. Loghothetis, P.N., and Austin, B. (1996) Variations in antigenicity of Aeromonas hydrophila strains in rainbow trout (Oncorhynchus mykiss , Walbaum): an association with surface characteristics. *Fish & Shellfish Immunology* 23, 47-55
13. United States Food and Drug Administration (2003) The Bad Bug Book. Available: http://www.fda.gov/food/foodsafety/foodborneillness/foodborneillnessfoodbornepathogensnaturaltoxins/badbugbook/default.htm. Accessed 2 December 2009.
14. Centers for Disease Control and Prevention. Alphabetical Index of Parasitic Diseases. Available: http://www.cdc.gov/parasites/az/index.html. Accessed 2 December 2009.
15. Garcia-Garcia, J.C.*, et al.* (2009) Epigenetic Silencing of Host Cell Defense Genes Enhances Intracellular Survival of the Rickettsial Pathogen *Anaplasma phagocytophilum*. *PLoS Pathog* 5, e1000488
16. Rikihisa, Y. (2011) Mechanisms of Obligatory Intracellular Infection with Anaplasma phagocytophilum. *Clin. Microbiol. Rev.* 24, 469-489
17. Vandenberg, O.*, et al.* (2004) Arcobacter species in humans. *Emerg Infect Dis* 10, 1863-1867
18. Leggett, H.C.*, et al.* (2012) Mechanisms of Pathogenesis, Infective Dose and Virulence in Human Parasites. *PLoS Pathog* 8, e1002512

19. Redondo, M.C., *et al.* (1995) Attributable mortality of bacteremia associated with the Bacteroides fragilis group. *Clin Infect Dis* 20, 1492-1496
20. Wilson M, McNab R, Henderson B (2002) *Bacterial Disease Mechanisms: An introduction to cellular microbiology*. Cambridge University Press
21. Mishra, M., *et al.* (2005) The BvgAS signal transduction system regulates biofilm development in Bordetella. *J Bacteriol* 187, 1474-1484
22. Moreno, E., and Moriyón, I. (2002) Brucella melitensis: A nasty bug with hidden credentials for virulence. *Proceedings of the National Academy of Sciences* 99, 1-3
23. Starr, T., *et al.* (2011) Selective subversion of autophagy complexes facilitates completion of the Brucella intracellular cycle. *Cell Host Microbe* 11, 33-45
24. Rambow-Larsen, A.A., *et al.* (2008) Putative Quorum-Sensing Regulator BlxR of Brucella melitensis Regulates Virulence Factors Including the Type IV Secretion System and Flagella. *J. Bacteriol.* 190, 3274-3282
25. Centre for food security and public health http://www.cfsph.iastate.edu/Factsheets/pdfs/melioidosis.pdf
26. White, N.J. (2003) Melioidosis. *The Lancet* 361, 1715-1722
27. Bowen, A.B., and Braden, C.R. (2006) Invasive Enterobacter sakazakii disease in infants. *Emerg Infect Dis* 12, 1185-1189
28. Lehner, A., *et al.* (2005) Biofilm Formation, Extracellular Polysaccharide Production, and Cell-to-Cell Signaling in Various Enterobacter sakazakii Strains: Aspects Promoting Environmental Persistence. *Journal of Food Protection* 68, 2287-2294
29. Health Canada (2003) Pathogen safety data sheets. Available: http://www.phac-aspc.gc.ca/lab-bio/res/psds-ftss/index-eng.php. Accessed 2 December 2009.
30. Plattner, F., and Soldati-Favre, D. (2008) Hijacking of Host Cellular Functions by the Apicomplexa. In *Annual Review of Microbiology*, 471-487, Annual Reviews
31. Water Research Centre: http://www.water-research.net/cryptosporidium.htm

32. Eichinger, D. (2001) Encystation in parasitic protozoa. *Curr. Opin. Microbiol.* 4, 421-426
33. Penuliar, G.M., *et al.* (2011) Mechanism of trifluoromethionine resistance in Entamoeba histolytica. *J Antimicrob Chemother* 66, 2045-2052
34. Medscape. Infectious Disease Articles. Available: http://emedicine.medscape.com/infectious_diseases. Accessed 2 December 2009.
35. Medoff, G., *et al.* (1986) Correlation between pathogenicity and temperature sensitivity in different strains of Histoplasma-capsulatum. *J. Clin. Invest.* 78, 1638-1647
36. Youseff, B.H., *et al.* (2012) Extracellular superoxide dismutase protects Histoplasma yeast cells from host-derived oxidative stress. *PLoS Pathog* 8, 17
37. Strauss, E. (1999) A Symphony of Bacterial Voices. *Science* 284, 1302-1304
38. Kramnik, I., *et al.* (2000) Genetic control of resistance to experimental infection with virulent Mycobacterium tuberculosis. *Proc. Natl. Acad. Sci. U. S. A.* 97, 8560-8565
39. O'Brien, K.L., *et al.* (2009) Burden of disease caused by Streptococcus pneumoniae in children younger than 5 years: global estimates. *Lancet* 374, 893-902
40. Stramer, S.L., *et al.* (2009) Emerging infectious disease agents and their potential threat to transfusion safety. *Transfusion* 49, 1S-29S
41. Greenberg, A.E., and Lobel, H.O. (1990) Mortality from Plasmodium falciparum Malaria in Travelers from the United States, 1959 to 1987. *Annals of Internal Medicine* 113, 326-327
42. Olliaro, P. (2008) Mortality Associated with Severe Plasmodium falciparum Malaria Increases with Age. *Clin. Infect. Dis.* 47, 158-160
43. Mutai, B.K., and Waitumbi, J.N. (2010) Apoptosis stalks Plasmodium falciparum maintained in continuous culture condition. *Malar J*, S3-S6

44. The Johns Hopkins Medical Institutions, department of pathology: http://pathology5.pathology.jhmi.edu/micro/v15n28.htm

45. Radulovic, S.*, et al.* (2002) Rickettsia-macrophage interactions: host cell responses to Rickettsia akari and Rickettsia typhi. *Infect Immun* 70, 2576-2582

46. Guerin, P.J.*, et al.* (2003) Shigella dysenteriae serotype 1 in west Africa: intervention strategy for an outbreak in Sierra Leone. *Lancet* 362, 705-706

47. Kotloff, K.L.*, et al.* (1999) Global burden of Shigella infections: implications for vaccine development and implementation of control strategies. *Bull. World Health Organ.* 77, 651-666

48. Garcia-Garcia, L.*, et al.* (2013) Epidemiology of syphilis-related hospitalisations in Spain between 1997 and 2006: a retrospective study. *BMJ open* 1, e000270

Current Biology 23, 139–142, January 21, 2013 ©2013 Elsevier Ltd All rights reserved http://dx.doi.org/10.1016/j.cub.2012.11.045

Report

Experimental Evolution of Adaptive Phenotypic Plasticity in a Parasite

Helen C. Leggett,[1,2,*] Rebecca Benmayor,[1] David J. Hodgson,[2] and Angus Buckling[1,2]
[1]Department of Zoology, Oxford University, South Parks Road, Oxford OX1 3PS, UK
[2]Biosciences, University of Exeter, Cornwall Campus, Penryn TR10 9EZ, UK

Summary

Coinfection of parasite genotypes can select for various changes in parasite life history strategies relative to single genotype infections, with consequences for disease dynamics and severity [1–14]. However, even where coinfection is common, a parasite genotype is also likely to regularly experience single genotype infections over relatively short periods of evolutionary time, due to chance, changes in local disease transmission, and parasite population structuring. Such alternating conditions between single genotype and coinfections will impose conflicting pressures on parasites, potentially selecting for facultative responses to coinfection [14–19]. Although such adaptive phenotypic plasticity in response to social environment has been observed in protozoan parasites and viruses [20, 21], here we show it evolving in real time in response to coinfection under conditions in which both single infections and coinfections are common. We experimentally evolved an obligate-killing virus under conditions of single virus infections (single lines) or a mix of single infections and coinfections (mixed lines) and found mixed lines to evolve a plastic lysis time: they killed host cells more rapidly when coinfecting than when infecting alone. This behavior resulted in high fitness under both infection conditions. Such plasticity has important consequences for the epidemiology of infectious diseases and the evolution of cooperation.

Results and Discussion

We experimentally evolved an initially clonal, obligate-killing virus (bacteriophage ϕ2 [22]) of a bacteria (*Pseudomonas fluorescens* SBW25 [23]) under conditions in which either the densities of bacteria greatly exceeded (by five orders of magnitude) that of phage, resulting in predominantly single phage infections (single lines), or phage and bacterial densities were initially approximately equal, resulting in phages experiencing a mix of single infections and coinfections (mixed lines) initially, followed by predominantly coinfections as the phage population grew. We measured phage population growth rate in the phages's selection and reciprocal environments every 10 days for a total of 50 days and found no monotonic change in growth rates for either treatment, in either environment (see Figure S1 available online; linear effects of time: $p > 0.4$ in all cases). There were, however, differences in mean growth rates between treatments. Specifically, mixed lines had lower growth than single lines under coinfection conditions, whereas there was no difference in growth between treatments measured under single infection conditions (Figure 1; significant interaction between selection and assay environments; mixed-effects ANOVA with time as fixed effect, $F_{1,18.33} = 5.72$, $p = 0.028$). This reduction in growth of mixed phages under mixed conditions may initially seem paradoxical but is in fact consistent with adaption to coinfections: increased within-host fitness of parasites is typically associated with reduced between-host transmission and hence decreased parasite population growth rate [1, 3–14].

To determine whether the evolved strategies of the mixed lines were adaptive, we competed independent pairs of single and mixed evolved lines under single infection and coinfection conditions. We found that mixed lines had an average 3-fold growth rate advantage over single lines under coinfection conditions (Figure 2; one-sample Wilcoxon test against relative fitness of 1: $p = 0.03$) and no detectable difference in growth rate under single infection conditions (Figure 2; Wilcoxon test: $p > 0.05$). Reduced population growth rate of the mixed lines therefore appears to be an adaptation resulting from selection under coinfection conditions.

We next investigated the phenotypic basis of adaptation to coinfections and whether or not the mixed phages had evolved adaptive phenotypic plasticity. Within-host competition resulting from coinfections can select for a range of parasite strategies including increased rates of host exploitation, interference, and exploitation of competitors' public goods (social cheating) [3, 4]; an example of the latter is the frequently observed defective-interfering particles in viruses [24, 25]. It is possible that plasticity in any of these mechanisms may have evolved in the mixed lines, but we initially investigated plasticity in the time taken to lyse hosts, because such plasticity in response to coinfection has been shown in phage T4 and its close relatives [21]. Like many viruses, the phage used in this study transmits by lysing its bacterial host, hence increased rates of host exploitation are likely to manifest as a faster time to lysis at the expense of a reduced viral yield [26–28]. Bacteriophage replication involves the production of multiple copies of the phage genetic material and protein coats, which are then packaged together to form the complete virus shortly prior to lysing the host cell [28]. Earlier lysis has been shown to provide a competitive advantage during phage mixed-genotype infection [29], presumably because coinfecting competitors with longer lysis times will have packaged up fewer complete viruses prior to cell lysis. To investigate adaptive plasticity of lysis time, we first measured the time taken for a statistical increase in density to occur in the ancestral, single, and mixed lines under both single infection and coinfection conditions. Under single infection conditions, the lysis time of ancestral, single, and mixed lines was approximately 35 min (Figure 3; paired t test of yield at 35 min versus 0 min; $p = 0.0002$, $p = 0.0002$ and $p = 0.001$, respectively). However, under coinfection conditions, the lysis time of mixed lines was approximately 30 min (Figure 3A; $p < 0.0001$), whereas it remained at 35 min for the single lines and ancestral clone (Figure 3B; $p < 0.0001$ and $p = 0.01$, respectively). To formally demonstrate that there is a change in lysis time in the mixed infection lines in response to coinfections, but not in the single

*Correspondence: helen.leggett@zoo.ox.ac.uk

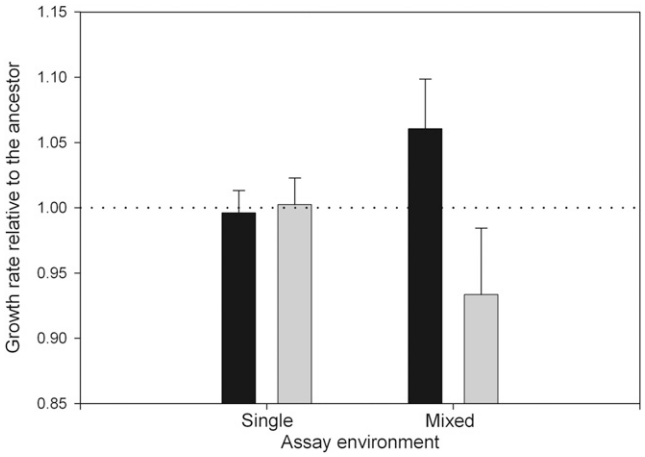


Figure 1. Mean Phage Growth through Evolutionary Time

Mean phage growth (multiplication rate) through evolutionary time compared to ancestral phage in single and mixed infection conditions (±SEM; n = 6). Black bars represent single lines; pale bars represent mixed lines. Dashed line represents equal growth.

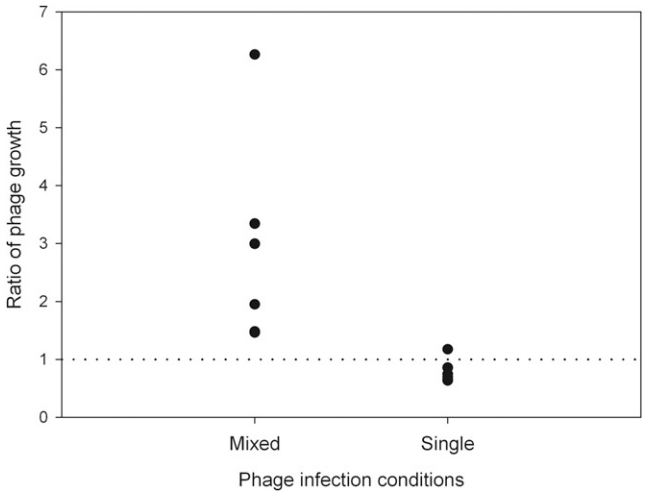


Figure 2. Growth of Mixed Lines when Competing against Single Lines

Competitive growth of mixed lines against single lines in single and mixed infection conditions. Dashed line represents equal fitness (1). Fitness > 1: mixed lines are more fit.

infection lines, we estimated the lysis time (the number of minutes taken to reach 50% of maximal phage density during a single synchronized growth cycle [*t*50]), for each replicate under both single infection and coinfection conditions. The *t*50 of mixed lines was significantly lower than single lines under mixed infection conditions, with no difference in *t*50 between the lines in single infection conditions (Figure 3C; significant interaction between selection line and assay conditions; $F_{1,20} = 59.794$, $p < 0.0001$). This demonstrates that an initially clonal virus that experienced a mix of single infections and coinfections over a few hundred generations evolved to phenotypically alter its lysis time in an adaptive manner, depending on whether it infected bacteria by itself or coinfected with other virus clones.

Our results demonstrate that even the simplest organism can maintain individually costly cooperative behaviors in the face of social cheats. Cooperation in this context can be viewed as a prudent use of hosts (killing the host relatively slowly to maximize viral yield), a strategy favored by selection when individuals sharing the same resource (a single host) are close relatives [5]. For rapidly evolving viruses, such high relatedness can only be guaranteed if everyone has descended from the same infecting clone. In contrast, competition with other genotypes favors cheating (faster rates of host exploitation) [5]. Adaptive phenotypic plasticity in response to the number of infecting genotypes ensures viruses only cooperate under conditions of high relatedness.

Here we have shown that an initially clonal virus, propagated under a mix of single infection and coinfection conditions, readily evolves the ability to detect the presence of coinfecting clones and adjusts its behavior accordingly. The ease at which such adaptive plasticity evolved in our experiment suggests the behavior is likely to be widespread among parasites and readily influenced by selection. The existence of social plasticity has important implications for parasite epidemiology and virulence. First, virulence and transmission of a given parasite may differ dramatically according to whether it is in a mixed or single genotype infection. Second, intervention strategies that aim to use avirulent social cheats ("Hamiltonian medicine" [30]) to outcompete virulent strains are likely to fail: parasites are likely to simply evolve to act as cheats when cheats are present but express more virulent, cooperative phenotypes in their absence.

Experimental Procedures

Selection Experiment

We used a single plaque of the lytic bacteriophage SBW25 Φ2 [22] to initiate all selection lines and a single colony of *Pseudomonas fluorescens* SBW25 [23] as the host bacteria. We grew bacteria and bacteria-plus-phage overnight in 6 ml King's Media B (KB) in 30 ml glass universals (shaken at 200 rpm and 28°C). We isolated phages by treatment with 10% chloroform and centrifuging at 14,000 rpm for 2 min. We inoculated 12 KB tubes as above with approximately 5×10^8 cells per ml of wild-type (WT) bacteria (10% of an overnight culture shaken at 200 rpm and 28°C. We then added approximately 5×10^8 particles per ml of phage to the first six replicates (mixed phage infection lines), whereas the other six contained 10^3 particles per ml of phage (single phage infection lines). We grew the populations at 200 rpm conditions, with loose lids at 28°C. After 8 hr, we isolated phages as above, stored them overnight in the fridge, and inoculated either 5×10^8 or 10^3 into fresh KB tubes, along with 5×10^8 cells of ancestral SBW25. We continued the experiment for 50 transfers, with phages stored at −80°C in cryotubes every ten transfers. In both treatments, phages reached densities of approximately 10^{10}/ml after 8 hr, with maximum bacterial densities of 5×10^9/ml, hence some single infections and coinfections probably occurred in both treatments. However, the frequency of coinfection was inevitably far more common in the mixed lines. Note that we have previously established that coinfections readily occur in this system by using marked phages [31].

Population Growth Rate Assays across Environments

To investigate environment-specific adaptations, we measured population growth rates of the evolved phages under both selection conditions every ten transfers. After 8 hr of growth, we determined phage population densities by plating dilutions of each phage population onto KB agar plates with a semisolid overlay bacterial lawn. We calculated the Malthusian growth rate as *ln*(end phage density/start phage density) [32]. We calculated mean growth rate of the ancestral phage from measures taken at multiple time points.

Fitness Assays

To determine whether the evolved strategies were adaptive, we competed independent pairs of single and mixed lines. To distinguish between the lines, we attempted to select spontaneous mutants that could infect an evolved *P. fluorescens* clone. For some reason, we were only able to do this for all the single lines, so we competed mutant/"marked" mixed lines

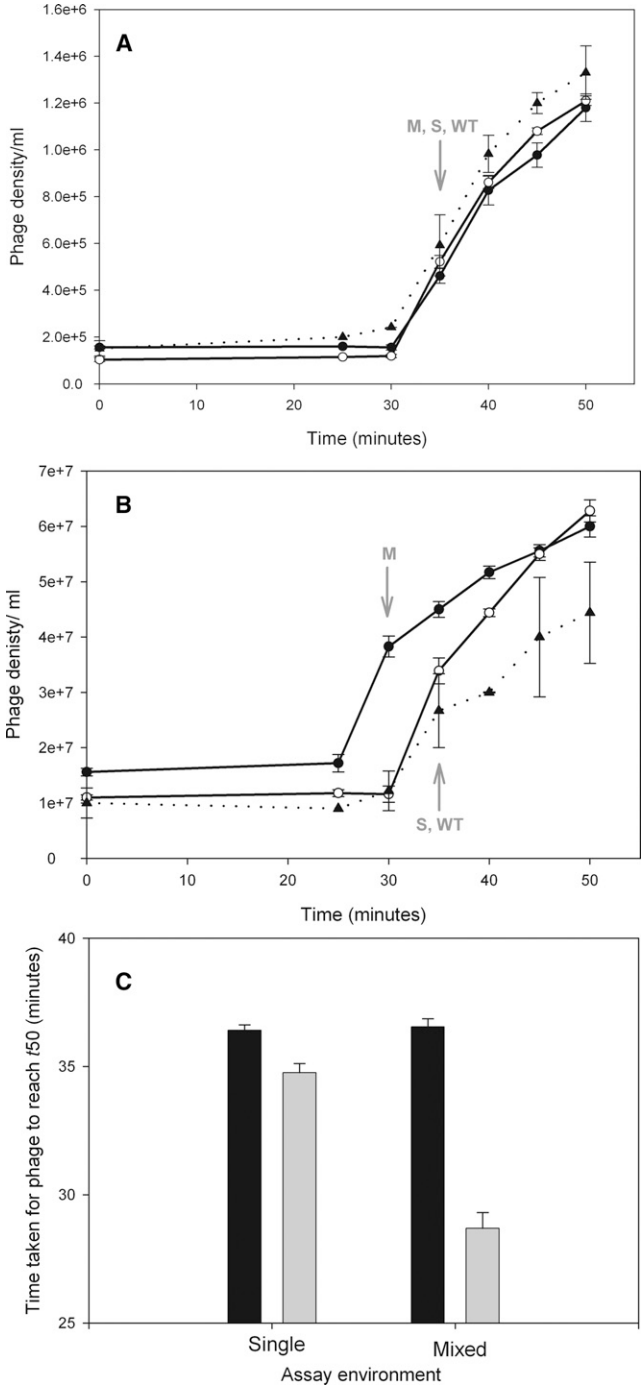


Figure 3. Short-Term Phage Growth in Single and Mixed Infections

(A and B) Mean phage growth in single infection (A) and mixed infection (B) conditions (±SEM; n = 6). Filled circles represent mixed lines (M); open circles represent single lines (S); triangles/dotted line represents ancestral lines (WT). Arrow indicates the time point when phage density significantly increases. (C) Mean number of minutes taken for phage to reach 50% of maximal phage density ($t50$) in single and mixed infection conditions (±SEM; n = 6). Dark bars represent single lines; pale bars represent mixed lines.

against unmarked single lines. To attempt to control for the cost of this host range mutant marker, we also competed ancestral phages against independently marked ancestral phages. We competed pairs of phages at equal starting ratios under both single and mixed infection conditions for 8 hr in a static incubator at 28°C and measured the growth of each of the populations by plating chloroform-treated populations onto separate KB agar plates with semisoft overlay lawns of either WT or resistant bacteria. We calculated relative fitness (w) of each mixed line, where $w = m_1/m_2$, m_1 = Malthusian growth of the mutant, and m_2 = Malthusian growth of the competing strain. Therefore, w was greater than 1 when the mixed line grew faster. We repeated each assay three times, calculating the mean of the pseudoreplicates. We then divided each w by the mean w of the ancestral marked genotypes when competed against the ancestor to control for the cost of the host range marker.

Measuring Time to Lysis

We measured the time taken for phage to lyse bacteria cells (latent period) in at least three phage clones in all single and mixed lines under reciprocal treatment conditions in a "one-step growth experiment" [33]. We added phages (10^5 or 10^8 for single and mixed conditions, respectively) to 10^7 exponentially growing bacterial in 1 ml KB media and measured phage density by plating onto bacterial lawns at time zero and then at 5 min intervals from 25 min (we never observed increases in density prior to 30 min in our preliminary studies). We used logistic regression of phage titers (as a proportion of their maximal density) against time to estimate lysis time for each replicate. Our specific measure of lysis time was t_{50}, the number of minutes taken to reach 50% of maximal phage density during a single synchronized growth cycle.

Statistical Analysis

We wanted to determine how mean phage growth rates varied through time for both mixed and single lines grown in both selection environments. We used general linear mixed models (GLMs) with REML, with evolution treatment and time as fixed factors, and variance among population lines fitted as a random effect. Comparison of mean growth rates in mixed population assays were carried out using GLMs (with mixture as a single fixed effect), followed by post hoc Tukey's pairwise comparisons. Ratios of phage growth and latent period data were not normally distributed; therefore, Wilcoxon signed-rank test was used. Variation in phage t_{50} was analyzed as a factorial GLM with explanatory variables "evolution treatment" (single or mixed), "assay treatment" (single or mixed), and their interaction. Analyses were carried out using JMP or R version 2.14.0. All figures were drawn using SigmaPlot software.

Supplemental Information

Supplemental Information includes one figure and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2012.11.045.

Acknowledgments

We thank Pedro Gomez and Andy Morgan for experimental advice and Mike Boots, Jim Bull, and two anonymous reviewers for comments on the manuscript. This work was supported by the European Research Council, the Leverhulme Trust, and NERC (UK). H.C.L. was supported by BBSRC.

Received: September 11, 2012
Revised: October 23, 2012
Accepted: November 21, 2012
Published: December 13, 2012

References

1. Bull, J.J. (1994). Perspective: virulence. Evolution *48*, 1423–1437.
2. Chao, L., Hanley, K.A., Burch, C.L., Dahlberg, C., and Turner, P.E. (2000). Kin selection and parasite evolution: higher and lower virulence with hard and soft selection. Q. Rev. Biol. *75*, 261–275.
3. Buckling, A., and Brockhurst, M.A. (2008). Kin selection and the evolution of virulence. Heredity (Edinb) *100*, 484–488.
4. Mideo, N. (2009). Parasite adaptations to within-host competition. Trends Parasitol. *25*, 261–268.
5. Frank, S.A. (1996). Models of parasite virulence. Q. Rev. Biol. *71*, 37–78.
6. Nowak, M.A., and May, R.M. (1994). Superinfection and the evolution of parasite virulence. Proc. Biol. Sci. *255*, 81–89.
7. Bremermann, H.J., and Pickering, J. (1983). A game-theoretical model of parasite virulence. J. Theor. Biol. *100*, 411–426.
8. van Baalen, M., and Sabelis, M.W. (1995). The dynamics of multiple infection and the evolution of virulence. Am. Nat. *146*, 881–910.

9. Brown, S.P., Hochberg, M.E., and Grenfell, B.T. (2002). Does multiple infection select for raised virulence? Trends Microbiol. *10*, 401–405.
10. West, S.A., and Buckling, A. (2003). Cooperation, virulence and siderophore production in bacterial parasites. Proc. Biol. Sci. *270*, 37–44.
11. Gardner, A., West, S.A., and Buckling, A. (2004). Bacteriocins, spite and virulence. Proc. Biol. Sci. *271*, 1529–1535.
12. Alizon, S., and van Baalen, M. (2008). Multiple infections, immune dynamics, and the evolution of virulence. Am. Nat. *172*, E150–E168.
13. Lively, C.M. (2009). Local host competition in the evolution of virulence. J. Evol. Biol. *22*, 1268–1274.
14. Choisy, M., and de Roode, J.C. (2010). Mixed infections and the evolution of virulence: effects of resource competition, parasite plasticity, and impaired host immunity. Am. Nat. *175*, E105–E118.
15. Stearns, S.C. (1989). The evolutionary significance of phenotypic plasticity. BioScience *39*, 436–445.
16. Moran, N.A. (1992). The evolutionary maintenance of alternative phenotypes. Am. Nat. *139*, 971–989.
17. Agrawal, A.A. (2001). Phenotypic plasticity in the interactions and evolution of species. Science *294*, 321–326.
18. West-Eberhard, M.J. (2003). Developmental Plasticity and Evolution (New York: Oxford University Press).
19. Thomas, F., Brown, S.P., Sukhdeo, M., and Renaud, F. (2002). Understanding parasite strategies: a state-dependent approach? Trends Parasitol. *18*, 387–390.
20. Reece, S.E., Drew, D.R., and Gardner, A. (2008). Sex ratio adjustment and kin discrimination in malaria parasites. Nature *453*, 609–614.
21. Abedon, S.T. (1999). Bacteriophage T4 resistance to lysis-inhibition collapse. Genet. Res. *74*, 1–11.
22. Buckling, A., and Rainey, P.B. (2002). Antagonistic coevolution between a bacterium and a bacteriophage. Proc. Biol. Sci. *269*, 931–936.
23. Rainey, P.B., and Bailey, M.J. (1996). Physical and genetic map of the Pseudomonas fluorescens SBW25 chromosome. Mol. Microbiol. *19*, 521–533.
24. DePolo, N.J., Giachetti, C., and Holland, J.J. (1987). Continuing coevolution of virus and defective interfering particles and of viral genome sequences during undiluted passages: virus mutants exhibiting nearly complete resistance to formerly dominant defective interfering particles. J. Virol. *61*, 454–464.
25. Turner, P.E., and Chao, L. (1999). Prisoner's dilemma in an RNA virus. Nature *398*, 441–443.
26. Abedon, S.T., Herschler, T.D., and Stopar, D. (2001). Bacteriophage latent-period evolution as a response to resource availability. Appl. Environ. Microbiol. *67*, 4233–4241.
27. Wang, I.N. (2006). Lysis timing and bacteriophage fitness. Genetics *172*, 17–26.
28. Wang, I.N., Dykhuizen, D.E., and Slobodkin, L.B. (1996). The evolution of phage lysis timing. Evol. Ecol. *10*, 545–558.
29. Refardt, D. (2011). Within-host competition determines reproductive success of temperate bacteriophages. ISME J. *5*, 1451–1460.
30. Foster, K.R. (2005). Biomedicine. Hamiltonian medicine: why the social lives of pathogens matter. Science *308*, 1269–1270.
31. Hall, A.R., Scanlan, P.D., Leggett, H.C., and Buckling, A. (2012). Multiplicity of infection does not accelerate infectivity evolution of viral parasites in laboratory microcosms. J. Evol. Biol. *25*, 409–415.
32. Lenski, R.E., Rose, M.R., Simpson, S.C., and Tadler, S.C. (1991). Long-term experimental evolution in escherichia coli. i. adaptation and divergence during 2,000 generations. Am. Nat. *138*, 1315–1341.
33. Ellis, E.L., and Delbrück, M. (1939). The growth of bacteriophage. J. Gen. Physiol. *22*, 365–384.

Current Biology, Volume *23*

Supplemental Information

Experimental Evolution of Adaptive Phenotypic Plasticity in a Parasite

Helen C. Leggett, Rebecca Benmayor, David J. Hodgson, and Angus Buckling

Supplemental Inventory

Supplemental Data - Figure S1

We measured phage population growth rate in the phages's selection and reciprocal environments every 10 days for a total of 50 days and found no monotonic change in growth rates for either treatment, in either environment (Figure S1; Linear effects of time: $P > 0.4$ in all cases). There were, however, differences in mean growth rates between treatments, as discussed in the main text (Figure 1).

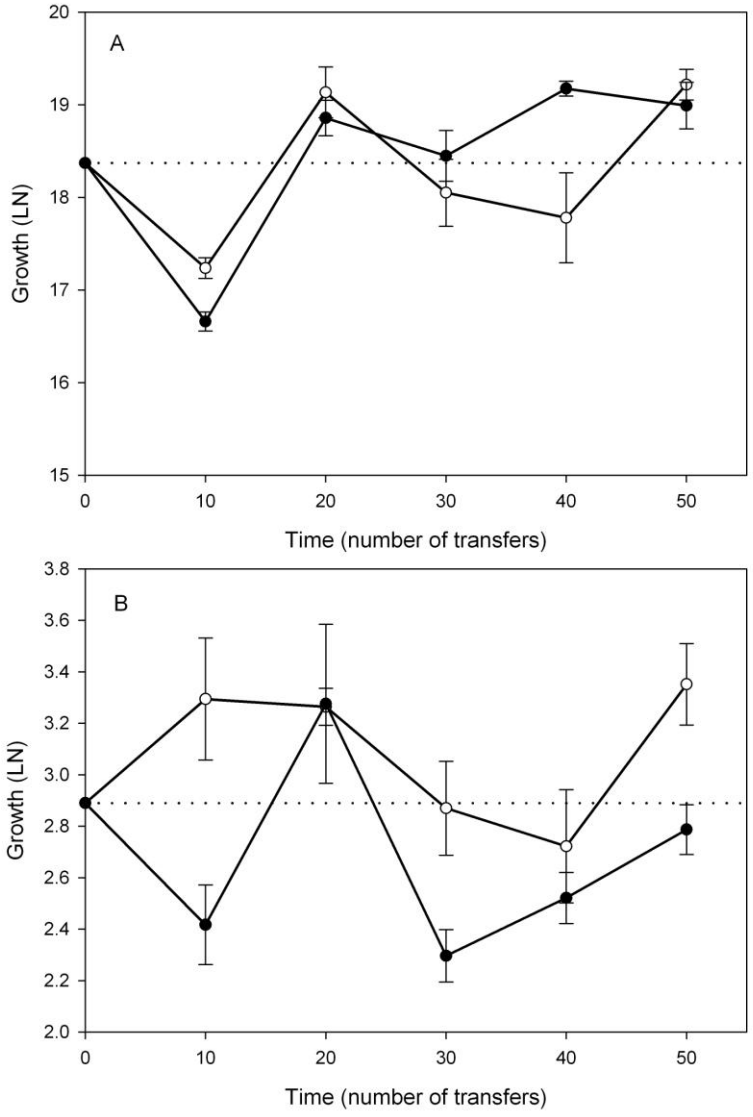


Figure S1. Mean phage growth (multiplication rate) through evolutionary time in single infection (A) and mixed infection (B) conditions (±SEM; n=6). Filled circles: Mixed lines; open circles: Single lines; dashed line: ancestral phage.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B

rstb.royalsocietypublishing.org

Review

Cite this article: Leggett HC, Brown SP, Reece SE. 2014 War and peace: social interactions in infections. *Phil. Trans. R. Soc. B* 20130365. http://dx.doi.org/10.1098/rstb.2013.0365

One contribution of 14 to a Theme Issue 'Inclusive fitness: 50 years on'.

Subject Areas:
behaviour, evolution, health and disease and epidemiology, microbiology, theoretical biology

Keywords:
plasticity, transmission, virulence, relatedness, ecology, inclusive fitness

Author for correspondence:
Helen C. Leggett
e-mail: helen.leggett@zoo.ox.ac.uk

War and peace: social interactions in infections

Helen C. Leggett[1,2], Sam P. Brown[3,4] and Sarah E. Reece[3,4]

[1]Department of Zoology, Oxford University, South Parks Road, Oxford OX1 3PS, UK
[2]Biosciences, University of Exeter, Cornwall Campus, Penryn TR10 9EZ, UK
[3]Institute of Evolutionary Biology, and [4]Centre for Immunity, Infection and Evolution, School of Biological Sciences, Ashworth Laboratories, University of Edinburgh, Edinburgh EH9 3JT, UK

One of the most striking facts about parasites and microbial pathogens that have emerged in the fields of social evolution and disease ecology in the past few decades is that these simple organisms have complex social lives, indulging in a variety of cooperative, communicative and coordinated behaviours. These organisms have provided elegant experimental tests of the importance of relatedness, kin discrimination, cooperation and competition, in driving the evolution of social strategies. Here, we briefly review the social behaviours of parasites and microbial pathogens, including their contributions to virulence, and outline how inclusive fitness theory has helped to explain their evolution. We then take a mechanistically inspired 'bottom-up' approach, discussing how key aspects of the ways in which parasites and pathogens exploit hosts, namely public goods, mobile elements, phenotypic plasticity, spatial structure and multi-species interactions, contribute to the emergent properties of virulence and transmission. We argue that unravelling the complexities of within-host ecology is interesting in its own right, and also needs to be better incorporated into theoretical evolution studies if social behaviours are to be understood and used to control the spread and severity of infectious diseases.

1. Introduction

Social acts, ranging from minor help to major self-sacrifice, are seen in all walks of life, from humans to microorganisms. It used to be generally assumed that the parasites and microbial pathogens that cause infectious diseases lived relatively independent unicellular lives, without the cooperative behaviours that have provoked interest in mammals, birds and insects [1]. However, a rapidly expanding body of research demonstrates that much of what parasites and microbial pathogens do, they do in groups. Furthermore, parasites and microbial pathogens display some amazing natural history, including behaviours described as mafia strategies, body-snatching, chemical warfare, mass suicide, suicide bombing and weapons of mass destruction (reviewed by [2–15]).

The expanding interest in understanding social evolution in parasites and microbial pathogens has likely occurred for two reasons. First, they are often well-described and tractable experimental systems for studying the ecology and evolution of social traits in real time, under both highly controlled conditions and in a 'real-world context', which for pathogens and parasites involves being exposed to the complex, changeable and hostile environments inside a host or vector. Second, sociality is a driver of the damage pathogens and parasites do to their hosts (virulence) [16,17], it shapes survival of medical interventions (such as antibiotics) [18,19], and underpins between-host transmission (e.g. [20,21]). Thus, examining the behaviours of parasites and microbial pathogens from the perspective of 'a life in society' is one of the most important issues in applied evolutionary biology. However, the mechanisms through which parasites and microbial pathogens interact with each other, the host/vector and the abiotic environment, has been largely overlooked within the evolutionary/ecological search for general principles (and their

Royal Society Publishing

empirical support) of the often-connected theories for inclusive fitness and virulence evolution.

It is our view that a 'bottom-up' approach to studying microbial pathogens and parasites is the next milestone for understanding their social behaviours and for controlling the infectious diseases they cause. In this article, our aims are to showcase recent empirical and theoretical work demonstrating that a biologically informed bottom-up view: (i) illustrates the extremely rich phenotypic landscape of parasites and microbial pathogens at the within-host scale; (ii) enables integration across levels of biological organization, from the molecular mechanisms unpinning social behaviours to population ecology, to capture the biological complexity required to explain social systems; (iii) can provide novel insight into the evolution and ecology of social behaviours in general and (iv) offers novel approaches to disease control with the potential to be more 'evolution-proof' than current therapies.

We also wish to facilitate cross-discipline communication between empirical and theoretical evolutionary ecologists and biologists in more applied disciplines such as microbiology, parasitology and biomedicine. To achieve this, we begin by providing an overview of the basic evolutionary and ecological frameworks for how social behaviours are studied (§2) and why virulence evolves (§3). The aim of these sections is to furnish readers unfamiliar to the social evolution and virulence evolution literatures, with the concepts underpinning the recent developments that form the focus of the following sections. Therefore, aficionados in ecology may wish to skip to §4. The figures and tables illustrate the concepts we discuss in the text. Most examples concern malaria (*Plasmodium*) parasites and microbial pathogens (bacteria and bacteriophage) because these groups span the taxonomic diversity of infectious disease causing organisms, and together they offer the opportunity to integrate understanding at multiple levels of biological organization, from genes and molecular pathways, to phenotypes, to epidemiology [22,23]. For brevity when discussing general concepts, we collectively refer to parasites and microbial pathogens as 'parasites' owing to their shared lifestyle of exploiting hosts.

2. Social behaviours

All organisms interact with others throughout their lives, including with family members, unrelated conspecifics and hetero-specifics. Social interactions range from extreme conflict (e.g. lethal combat) to extreme cooperation (e.g. altruistic suicide or sterility) but most interactions lie somewhere between these extremes. Social behaviours can be categorized according to their impact on the lifetime reproductive success of the 'actor' expressing a particular social phenotype and any 'recipients' impacted by the actor's phenotype (table 1) [24,45–47]. Taking a simple +/− dichotomy for both actor and recipient gives a simple four-part categorization: (i) *mutual benefit* (+/+), where the actor and recipient both gain from the actor's behaviour; (ii) *selfishness* (+/−), where the actor gains at the expense of the recipient; (iii) *altruism* (−/+), where the behaviour is detrimental to the actor but beneficial for the recipient and (iv) *spite* (−/−), where the behaviour is harmful for both actor and recipient. The pioneering work of Bill Hamilton [24,45] provided a foundation to explore how natural selection drives the spread of these four types of social behaviours through a population. The topics outlined below illustrate the key concepts involved in social interactions.

(a) Inclusive fitness: all for one and one for all

Hamilton's key insight was that genes controlling the social traits of an actor can influence the replication of gene-copies in recipients. In the case of altruistic traits, Hamilton's logic reveals a simple genetic nepotism—helping neighbours is another way of helping your own genes to reproduce, so long as they carry the helper-genes of interest. Hamilton proposed a critical metric to weight the likelihood that recipients carry the gene of interest, termed relatedness [48]. Common descent or kinship is the most common reason for interacting individuals to share genes with above-average frequency in a population. Consequently, relatedness can be understood as the chance of gene sharing among kin, above- and beyond-average probability [49]. Inclusive fitness partitions natural selection into direct and indirect effects; direct effects describe the impact of an individual's own genes on reproductive success and indirect effects describe the impact of the focal individual's genes on the fitness of its social partners, weighted by genetic relatedness [24,26,45]. Cooperation may be mutually beneficial if it directly benefits the actor as well as the recipients, for example, by increasing the success of an individual's own group (table 1). More extreme acts of altruistic cooperation may be selected if the behaviour helps recipients who are very likely to share the altruistic gene (i.e. if relatedness is high such as within families) [24,26,45]; thus indirectly propagating genes for altruism. An important point to note is that many parasite species reproduce asexually (i.e. clonally) during at least one stage of their life cycle [50], and thus each group of clonally related parasites (genotype) within an infection is expected to behave as a multicellular organism [51] because the genotype is the target of selection.

(b) Cheating: playing the system

When relatedness is low, cooperative behaviours are vulnerable to exploitation by cheats that do not contribute to collective action but still benet from the cooperative behaviours of others [6]. Cheats can proliferate under these conditions because the benefits of cooperation are shared indiscriminately, and consequently, genes for cheating will have greater tness than the genes for cooperation [31,52]. The spread of cheats through a population can in turn lead to a decline in population tness (an idea encapsulated by Hardin's 'Tragedy of the commons' [53] and by 'the Prisoners' dilemma' [54]. Empirical studies have demonstrated that cheating can indeed occur in numerous cooperative systems of microbial pathogens [7,31,55–57]. Recent years have witnessed a surge in the application of evolutionary theory to explain the ways in which cooperation is maintained (reviewed by [1,3,6,58–60]), which includes mechanisms for kin discrimination and communication.

(c) Kin recognition: deciding who to help

Relatedness is a key to understanding the direction and magnitude of selection on social traits, but what shapes relatedness? A commonly cited scenario is that social acts are expressed blindly to neighbours, who tend to be relatives simply because of incomplete mixing of individuals in populations—the population is 'viscous' [24,45]. However, altruists in this system may fall victim to 'cheats' that lack the gene for altruism. A way to avoid wasting help on cheaters is to display an altruistic or social gene and to recognize the same gene in others, and

Table 1. A classification of social behaviours, after [24–26]. These examples illustrate that the richness of social behaviours observed in multicellular organisms are mirrored in parasites. Moreover, parasite social behaviours often have consequences for the severity and transmission of disease. Note, that it is extremely difficult to quantify costs and benefits of many social behaviours, for actors and recipients, so many of these examples are yet to be fully understood.

		effect on recipient	
		+	−
effect on actor	+	mutual benefit	selfishness
		Multicellular taxa. This ranges from simple scenarios such as group cooperation providing safety in numbers, to rewarding helpers or punishing non- helpers. Group activities in the naked mole rat, *H. glaber*, reduce the risks of predation, hypothermia and starvation. Unrelated subordinate *Polistes* wasps cooperate to raise their colony's offspring because there is reasonable chance of inheriting the dominants breeding position in the near future [27]. In this case, helpers 'pay to stay' because the acquisition of breeding opportunities is a large pay-off. *Parasites*. The release of secreted public goods molecules into a sufficiently small and/or structured population can generate mutual benefits, so long as the fraction of reward returned to the actor cell exceeds the direct costs of production, and that some reward is felt by cells other than the actor. Positive interactions between species have been documented. An existing infection of *B. microti* in field voles (*M. agrestis*) appears to increase the probability of *A. phagocytophilum* infection and vice-versa [28]. Also, if mixed infections are more challenging than single infections for host immune responses, mutual benefit could occur, which may result in greater virulence.	*Multicellular taxa*. The classic example is male lions killing their predecessor's cubs when they take over a pride [29]. This brings lioness' into season and so hastens the new male's mating opportunities. Selfish acts can also be disguised as cooperation; white-winged choughs cheat by attempting to fool dominants that they are helping at the nest [30]. *Parasites*. Cheating is rife in bacterial infections; the cost of producing extracellular iron-scavenging siderophores in *P. aeruginosa* selects for non-producing cheats who can outcompete cooperators [31,32]. Because cooperation is required to efficiently acquire the iron, mixed infections can be less virulent [33]. By contrast, competition in co-infections selects for faster replication in *Plasmodium* and phage, which causes greater virulence to the host [34,35].
	−	altruism	spite
		Multicellular taxa. The classic example is the eusocial insects (including bees, ants, termites) in which a single (or few) queen monopolize the colony's reproduction [36]. All others in the colony are morphologically or behaviourally specialized to altruistically forage, guard or raise offspring. *Parasites*. Morphological castes have been described in *Philophthalmus* sp. trematodes, which produce reproductive and non-reproductive morphs. The sterile morph increases the reproductive output of relatives but only when competing strains share the host [37]. Other examples include suicide in *S. typhimurium* to facilitate the gut invasion of others [38], and in *E. coli* infected with phage to prevent parasite transmission to others [39]. Forms of suicide have been described in *Plasmodium* and Trypanosomes, which may regulate density, preventing premature death of the host/vector [15,40]. Suicidal release of a public good is not a necessary condition for altruism—the release of a costly public good into a dense and well-mixed population (minimizing the direct return to the actor cell) is likely to be an altruistic act.	*Multicellular taxa*. Soldiers of the polyembryonic parasitoid wasp *C. floridanum*, are sterile because they never reach sexual maturity. They preferentially kill other, unrelated embryos developing in their host, which frees up resources for their siblings [41]. Workers of the red fire ant *S. invicta*, kill related queens who do not have a greenbeard gene, ensuring that the greenbeard gene persists [42]. *Parasites*. Bacteria secrete anti-competitor toxins that are costly to produce (e.g. toxin release requires cell lysis in many cases). Toxin production and immunity genes are usually linked so that relatives are immune. Virulence is lower in mixed infections in caterpillar hosts compared with single infections when competing strains are susceptible to each other's bacteriocins [43]. However, the impact of spite on virulence becomes more complicated when spiteful behaviours are affected by simultaneous investments in other social traits (e.g. public goods) [44].

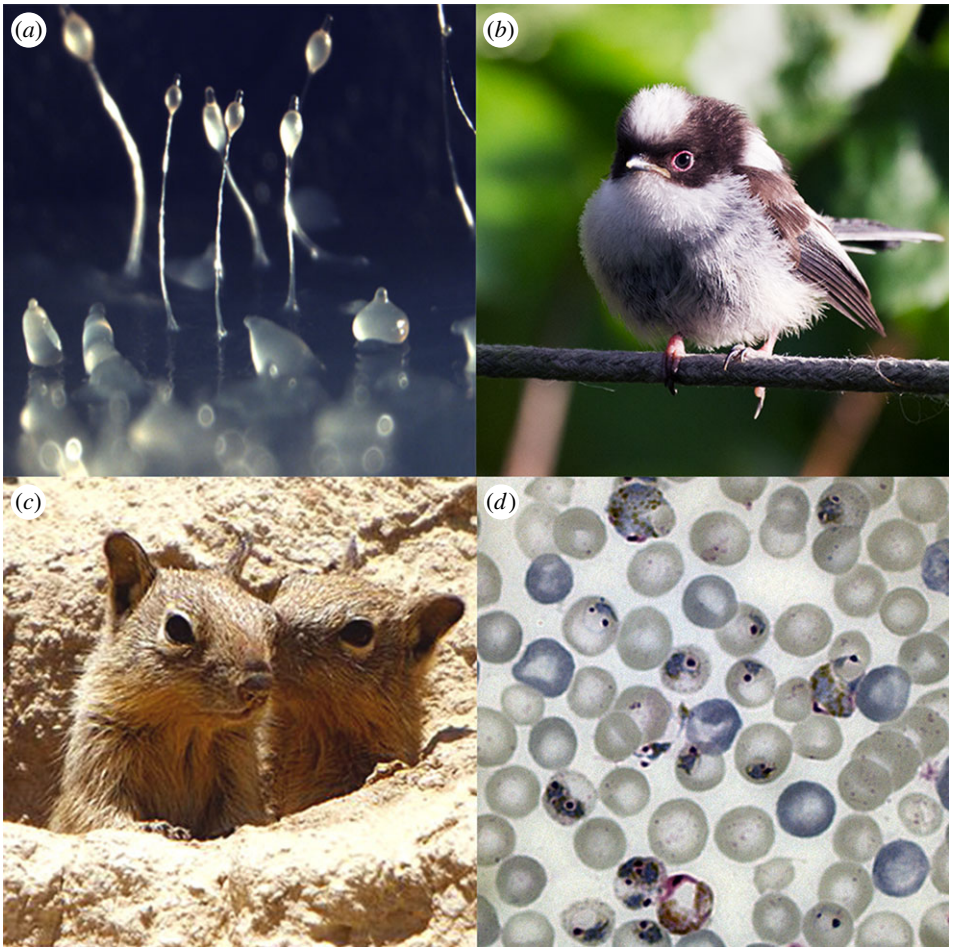


Figure 1. Examples of kin discrimination by: (*a*) direct recognition, e.g. cells of the slime mould *Dictyostelium* determine whether they are interacting with kin or non-relatives during slug and spore formation based on the sequence similarity their surface adhesion proteins [63,64] (photo credit Owen Gilbert); (*b*) indirect cues based on familiarity with individuals, e.g. long-tailed tits learn the vocalization patterns of kin during the natal rearing period [65] (photo credit Sarah Reece) or (*c*) 'armpits' which are a mixture of direct and indirect cues, e.g. ground squirrels use olfactory cues which have a genetic component and are also learnt self-referencing during development [66] (photo credit Alan Vernon). The malaria parasite, *Plasmodium chabaudi* (*d*), adjusts investment into male and female transmission stages according to how many other conspecific clones share the host, suggesting kin discrimination occurs [21] (photo credit Sarah Reece and Sinclair Stammers). The mechanism is unknown but indirect cues seems unlikely; an obvious candidate would be that parasites can infer the presence of other clones via the host immune response, but sex ratio adjustment is observed in infections before the required strain-specific responses develop.

only direct help to individuals expressing that gene—a notion popularized as a 'green beard' [61,62]. Another way to direct cooperative behaviours towards appropriate recipients is through the ability to recognize kin (figure 1). Kin discrimination can occur via: (i) direct recognition, (ii) indirect cues that convey whether a recipient is likely to be a relative or (iii) a mixture of direct and indirect information. Kin discrimination systems can require additional selective forces to maintain polymorphisms that can be used as accurate identifiers [67]. Host–parasite systems, in which genotype-by-genotype interactions and frequency-dependent selection maintain genetic variation, are candidate motors maintaining the genetic diversity required for kin discrimination (figure 1).

(d) Communication: coordinating collective action

Parasites have evolved sophisticated communication systems to coordinate behaviours across clone-mates and enable collective actions to be efficiently deployed. For example, 6–10% of all genes in the opportunistic microbial pathogen *Pseudomonas aeruginosa* are controlled by cell–cell signalling systems [68]. Coordination is especially important for behaviours that must be expressed by some, but not all individuals. For example, in cases where the suicide of some individuals benefits survivors, the suicide trait must not be expressed by all individuals otherwise there would be no survivors [15,38]. Equally, undertaking costly cooperative actions may only pay when the numbers of actors exceeds a certain threshold, and so density-sensing mechanisms are often used to ensure behaviours are only switched on at high densities ('quorum') [69]. Microbial pathogens are masters of coordinating collective actions; their quorum-sensing system enables density estimation via collectively produced diffusible molecules [69]. The recent discovery that malaria parasites secrete protein and DNA containing microvesicles that influence the sexual differentiation of other parasite cells [70,71] may be a mechanism to organize the density-dependent decisions observed in reproductive effort and sex allocation [21,72,73].

3. Virulence evolution

Parasites engage in clearly selfish acts with the hosts and vectors they exploit; here we give a brief overview of answers to the basic question of why do parasites harm the very source of their livelihoods? The development of evolutionary theory to explain virulence (parasite-induced harm to the host) has a

long history. Theories for the evolution of virulence can be categorized into four broad hypotheses [74,75] under which high virulence is attributed variously to: (i) novel host–parasite associations [76,77], (ii) transmission–virulence trade-offs [78], (iii) coincidental evolution of virulence factors [79,80] and (iv) short-term within-host evolution [81]. For many infectious diseases, social interactions among parasites and virulence are coupled, but the nature of this relationship varies according to the type of interactions involved and who the interaction partners are.

The most influential theoretical framework for virulence evolution centres on virulence being maintained as a result of an unavoidable constraint linking the benefits of transmission with the costs of virulence. In this view, virulence (measured as host death) is an unavoidable cost of the host exploitation required for transmission to new hosts [82–85]. If the costs of increasing exploitation accelerate more rapidly than the transmission benefits of increasing exploitation, then natural selection favours an intermediate level of host exploitation (optimal virulence) [78,86]. Following from this premise, the relatedness of co-infecting parasite genotypes can modulate the best or evolutionary stable strategy of virulence, depending on the nature of social interactions among co-infecting parasites (figure 2). When co-infecting genotypes have direct control over their mechanisms of host exploitation, the benefits of increased exploitation are felt by the individuals responsible, whereas the costs of virulence are shared by all, favouring greater virulence than that of parasites in single genotype infections [83–86]. By contrast, if co-infecting parasites work collectively to exploit the host (for example, via the secretion of shared extracellular digestive enzymes), then the benefits of exploitation become collectivized and mixed infections can select for 'non-producer' cheats that attenuate virulence [87]. In both scenarios, the spread of cheats (either over- or under-exploiters) undermines the productivity of the infection as a whole [53,85] but have opposite consequences for virulence.

4. Interactions in infections

The virulence–transmission trade-off models and their 'virulence-kin-selection' offshoots have been influential in the development of a vast body of subsequent theory [78]. Empirical testing has proceeded at a slower pace but only a few systems have provided support for the virulence–transmission trade-off [80,88]. A central theme emerging in the disease evolution literature is that within-host ecological dynamics are critical determinants of parasite sociality and so, virulence [80,88,89]. In the following sections, we take a mechanistically inspired bottom-up approach, viewing virulence and transmission as emergent properties of complex within-host processes and highlighting five aspects of infections that can shape parasite social behaviours: (i) public goods, (ii) mobile elements, (iii) phenotypic plasticity, (iv) spatial structure and (v) multi-species interactions. We illustrate that a better understanding of these processes brings new perspectives to the traditional 'top-down' frameworks for the evolution and epidemiology of virulence and transmission.

(a) Public goods

A central aspect of interactions between microbial pathogens is the collective engineering of their shared environment via the secretion of costly 'public goods' molecules. These molecules generate a range of benefits to any neighbouring cell that are suitably equipped to profit. For example, public goods molecules may scavenge for limiting resources (e.g. siderophores), aid in the construction of biofilms (e.g. adhesive polymers), kill competing lineages (e.g. bacteriocins) or enhance host exploitation (e.g. digestive enzymes, toxins). Because these molecules are individually costly to produce and yet return a collective benefit, they have become a focus in the study of bacterial cooperation. Among the best-studied model system for public goods cooperation is iron scavenging by secreted siderophores in the opportunistic bacterial pathogen *P. aeruginosa* (and related pseudomonads) [31,90,91]. *In vitro* studies in iron-limited environments have demonstrated that the fate of siderophore-producing 'cooperator' lineages in competition with non-producer 'cheats' is dependent on the degree of strain mixing or relatedness. When relatedness is high (each sub-population founded by a single clone), producers outcompete cheats, because the benefits of cooperation are disproportionately high for other cooperators. By contrast, when relatedness is low (e.g. each sub-population founded by multiple clones), cheats outcompete producers [31] (but see [92] for an exception driven by strong non-social selection).

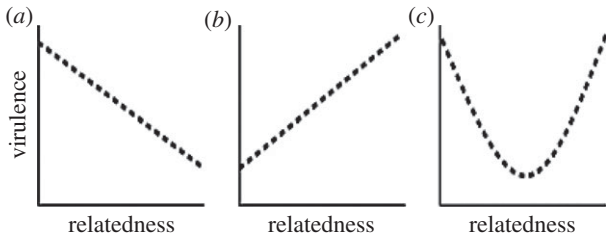


Figure 2. Theoretical relationships between virulence and relatedness under conditions of: (*a*) individual exploitation (virulence maximized at low relatedness) (*b*) collective exploitation (virulence maximized at high relatedness) (*c*) spiteful interactions, e.g. when harming competitors trades off against replication that causes virulence. (summarized by [16]).

The general applicability of a public goods framework for microbial interactions mediated by secreted factors has recently been called into question by Zhang & Rainey [92]. Q1 Here again the experimental focus was on siderophore-mediated interactions, where the authors illustrated that in certain standard laboratory experimental conditions (KB media), the production of siderophores is redundant and selected against. This result serves as a valuable reminder that the benefits of secreted molecules are undoubtedly environment dependent, and in this particular environment the secreted molecule does not provide benefits to neighbours and therefore does not function as a public good. Kummerli & Q2 Ross-Gillespie [93] responded to Zhang & Rainey [92] with an analysis of the iron content of KB, revealing that it is relatively iron-replete, ensuring that siderophore production is unlikely to provide sufficient benefit to merit the costs of production. From this, Kummerli & Ross conclude that there is no difficulty for the public goods framework, so long as the environmental context is adequately accounted for [93].

(b) Mobile elements: infectious cooperation and locus-specific relatedness

The maintenance of cooperation via a single-cell bottleneck for each sub-population (as in [31] discussed earlier) is a

very stringent condition. How is cooperation maintained under more realistic conditions that allow for some strain mixing, and more frequent interactions with cheats owing to mutation or migration? The peculiar biology of bacteria points to an intriguing role played by their molecular parasites in maintaining the cooperative phenotypes of their bacterial hosts. Bacteria are prone to infection with a diverse array of molecular parasites that are able to spread infectiously via horizontal gene transfer (HGT) through a population, bringing novel genes along for the ride [94]. Key among these molecular parasites are plasmids, vectors of many medically significant alleles, including antibiotic resistance and toxins [18]. Initial theoretical work suggested that the invasion of cheats into a population of cooperators could be prevented if the cooperative trait was encoded by an infectious conjugative plasmid [95]. In this scenario, cheats are liable to be re-programmed via infection with the cooperation-inducing plasmid. A key assumption of this model is that all plasmids carry the cooperative trait, so any act of infection will also increase cooperation, by hitch-hiking on the conjugation alleles.

But what if the social dilemma between cooperative and cheating alleles is played out at the level of the mobile element? More recent theory has pointed out that in an unstructured environment, 'cheat' plasmids will outcompete 'cooperative' plasmids for the same conditions that favour cheating chromosomal alleles over their cooperative rivals [96]; because again, the benefits of cooperation are not preferentially returned to cooperative alleles. However, the picture changes in structured populations, in which bacteria exploit discrete patches (e.g. hosts), linked by migration and/or transmission. Population structure introduces non-zero relatedness, and so the patterns of relatedness are now predicted to vary at different points of the genome depending on the rate of HGT [17,18,97]. Plasmids with high rates of HGT can readily copy themselves into neighbouring cells within a patch, and so if a cooperative plasmid gene generates benefits for neighbouring cells, it is now more likely to aid gene-copies in neighbouring cells to reproduce. In other words, highly conjugative plasmids gain a greater inclusive fitness return from helping neighbouring cells, favouring cooperative investments at these loci. Bio-informatic support for this inclusive fitness hypothesis has been demonstrated across 20 strains of *Escherichia coli*, where genes liable to experience greater HGT were more likely to code for secreted (cooperative) traits [17]. More recently, experiments show that HGT promotes plasmid-specific relatedness and selection for plasmid-encoded cooperation [98].

(c) Phenotypic plasticity: adaptive adjustment of behaviours

An important feature of parasite lifestyles is that their social environments change constantly, and so parasites have evolved mechanisms to regulate their behaviours. HGT is a form of genetic plasticity that enables the loss and gain of locally adapted alleles [18], but parasites also excel at phenotypic plasticity, extracting multiple phenotypes from one genotype. Adaptive phenotypic plasticity—the ability of an organism to change its behaviour or morphology to fit the environment—is a ubiquitous solution to the challenges of life in a changing environment. Plasticity enables organisms to maintain fitness by altering their phenotype, through mechanisms such as differential gene expression, to best suit their circumstances [99], and here we focus on how plasticity in the behaviours (life-history traits) of parasites are shaped by their social environment within the host. For example, kin discrimination is a plastic response to social circumstances. By ensuring parasites only cooperate under conditions of high relatedness, kin discrimination may maintain cooperation by ensuring that the behaviour is adaptive from an inclusive fitness perspective, by limiting the potential to be exploited by cheats. Moreover, as well as enabling organisms to respond quickly once environmental change has occurred, organisms can also respond to predictors of future environmental change which enables appropriate phenotypes to be adopted in a timely manner [100].

Typically, evolutionary biologists and parasitologists have overlooked the notion that plasticity can produce qualitative and adaptive changes to the genotype-wide social phenotypes of parasites during infections. This is because they assume that parasite responses to environmental perturbation are mostly directed at maintaining homeostasis. As a result, variation in parasite behaviours is often—and potentially incorrectly—attributed to the footprint of host regulation rather than parasites making strategic decisions. For example, when the coordinated cell cycles of the rodent malaria parasite *Plasmodium chabaudi* are perturbed, they become rescheduled during infection and return to matching the host circadian rhythm. Whether parasite cell cycles are passively scheduled by host factors with a circadian basis or by parasites actively and collectively adjusting their timing, is unclear [101]. However, evidence suggests that parasites are responsible for collectively coordinating their cell cycle schedules: synchronous and asynchronous malaria parasite species maintain their schedules in the same host environment (i.e. age–sex–strain-matched inbred mice); there are fitness benefits for parasites with cell cycles matched to the host circadian rhythm and matched infections cause greater virulence to the host [102,103]. Clearly, plasticity in parasite social behaviours complicates the understanding of within-host dynamics, but identifying to what extent parasite and/or host genes are responsible is central to interrogating their evolution.

The diversity of phenotypic plasticity in parasite social behaviours is illustrated in table 2. These traits are adjusted in response to social context and have consequences for virulence and transmission. Unfortunately, evolutionary theory has mostly ignored these behaviours, focusing instead on virulence. This is problematic because changes in virulence are achieved by changes in underlying traits (e.g. behaviours) expressed by both the host and parasites. As the social behaviours underpinning virulence and transmission are likely to be linked by genetic correlations (i.e. different traits are shaped by the same genes) and/or resource allocation trade-offs, the nature of these interactions is central to understanding and predicting virulence evolution [23]. Furthermore, when different genotypes respond to the environment in different ways (genotype-by-environment interactions or G × E), environmental change can expose (or hide) genetic variation in plasticity to natural selection [115] (figure 3). Ecological perturbations such as drugs, vaccines and host shifts are all candidate motors for constraining or facilitating evolution, depending on how the perturbation affects the amount of genetic variation underpinning parasite phenotypes. For example, genetic variation for sex ratio adjustment and reproductive effort in response to social context has been documented in malaria parasites [21,73] and these behaviours are determinants of how

Table 2. Examples of phenotypic plasticity in parasite social behaviours. That phenotypes are a product of both genotypes and the environment, and how they interact, is well known, but often the environment is viewed as obscuring the connection between genes and phenotypes. However, how social behaviours are influenced by environmental variation matters because they affect virulence and transmission. Because multiple environmental factors change simultaneously during infections and virulence and transmission phenotypes are product of multiple social behaviours, parasites can produce a wide range of adaptive phenotypes faster by plasticity than when beneficial mutations or recombination are required to generate new phenotypes.

behaviour/ trait	what happens and why?
developmental schedules	In the host blood, cycles of asexual replication in many species of *Plasmodium* are tightly synchronized; individual parasites transit through each cell cycle stage and ultimately burst out of their red blood cells in unison and at particular times of day. The duration and synchronicity of cell cycles are plastic [101]. An adaptive basis of this plasticity is yet to be established but in-host competition and host immune responses are likely drivers [104]. Disrupted *P. chabaudi* schedules result in lower virulence (anaemia; [103]) but quiescence can also help *P. falciparum* tolerate antimalarial drugs [105].
lysis time	Pi 2 bacteriophage must lyse their bacterial host (*P. fluorescens*) to transmit. They evolve a plastic lysis time in which they kill host cells more rapidly when co-infecting host cells with other phage than when infecting alone [35]. Plasticity in lysis time evolved in phage lines in mixed-infection conditions owing to the frequent variability in whether they encounter co- or single-infections in this treatment (the lysis time in single-infection conditions did not change or become plastic in response to selection). This plasticity enhances the competitive ability of phage since non-plastic phage have fewer mature propagules upon cell lysis and suggests virulence and transmission differ according to whether parasites are in single or mixed genotype infections. In addition, lysis inhibition (LIN) is a mechanism of burst-size increase and latent period extension induced by T4 bacteriophage secondary adsorption of T4-infected *E. coli* cells. This plastic growth strategy is an adaptation to environments containing high densities of T4-infected cells [106]: when T4-infected cell density is high, high densities of free phages are generated, uninfected cells are rapidly infected, secondary adsorption is likely and LIN is induced with high probability [106–109].
public goods	The production of an iron-scavenging molecule (pyoverdin) by *P. aeruginosa* bacteria is a cooperative trait. Pyverdin production per bacterium is tightly regulated by the intracellular supply of free iron, leading to decreased *per capita* production at higher cell densities and increased production in the presence of non-producing cheats. This phenotypic plasticity significantly influences the costs and benefits of cooperation. Specifically, the investment of resources into pyoverdin production is reduced in iron-rich environments and at high cell densities, but increased under iron limitation, and when pyoverdin is exploited by cheats [110,111]. Regulatory control of public goods provisioning can further protect producers from exploitation by cheats by 'metabolic prudence', limiting production to environments where the relative costs of production are minimized [57]. More globally, the regulatory control of multiple secreted factors is under the control of quorum-sensing mechanisms in numerous bacteria, including several significant pathogens [112].
reproductive effort	*Plasmodium* must replicate asexually in the vertebrate host and undergo a round of sexual reproduction in the vector. This means that resources must be divided between growth (the production of asexual stages for in-host survival) and reproduction (the production of sexual stages for transmission). *Plasmodium chabaudi* adopts reproductive restraint when facing in-host competition, which is consistent with investing in asexual replication (a key determinant of competitive ability) to gain future transmission opportunities [20,73]. *P. falciparum* also adopts reproductive restraint in response to low doses of drugs, suggesting this is a general strategy for coping with stresses encountered in the host [72].
sex allocation	In addition to the growth versus reproduction trade-off described earlier, *Plasmodium* must also divide resources between male and female transmission stages (sex ratio). Sex ratios in *P. chabaudi* and *P. falciparum* are adjusted in response to the inbreeding rate, which is determined by the number of co-infecting genotypes and their relative frequencies [21]. In single-infections, female-biased sex ratios maximize zygote production and increasing the proportion of males in mixed infections, especially if a weak competitor, maximizes representation in the zygote population.
suicide	A 'suicide trait' cannot be constitutively expressed (if everyone dies before reproducing, genes for the trait cannot be inherited). Thus, the proportion of parasites that die may be precisely adjusted in response to variation in the density and relatedness of co-infecting parasites, or noisy expression of the genes involved may ensure phenotypic variation [38,113]. The release of bacteriocins to kill competitors requires bacterial cells to lyse themselves in many species, including *E. coli* [14]. The benefits accruing to surviving kin are highest when at low density, but this is when the costs of losing group members are greatest. By contrast, *Plasmodium* experiences crowding in the vector: high parasite densities reduce per parasite productivity and elevate vector mortality so suicide in the stage infective to the vector is predicted to regulate infection intensity [114].

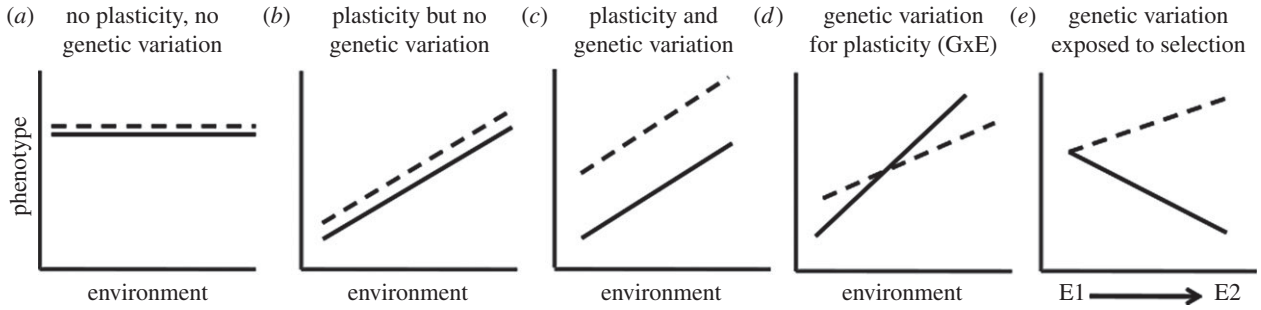


Figure 3. Phenotypic plasticity and reaction norms. In panel (*a*), phenotype does not vary with the environment and both genotypes have identical reaction norms. In panel (*b*) both genotypes are plastic and (*c*) there is also genetic variation. Panel (*d*) illustrates a genotype-by-environment interaction (G × E), where both genotypes are plastic but their phenotypic reaction norms vary. Genetic variation and G × E can complicate how much genetic variation is exposed to selection; in panel (*e*) the genotypes produce the same phenotype in environment 1 but not in environment 2, so selection can only differentiate between the genotypes in environment 2.

parasites survive drugs and overcome transmission-blocking immunity [72,116,117]. However, how G × E affects the speed that parasites could respond to selection on these behaviours is not known.

The potential for interactions between plasticity and evolution introduces yet more complications to understanding how social behaviours shape parasite fitness for two additional reasons. First, adaptive plasticity can facilitate parasite evolution simply by providing more time and/or individuals for beneficial mutations to arise because their survival is enhanced [118]. By contrast, when plasticity buffers parasites against the loss of fitness in a novel environment, the strength of selection imposed by environmental change is reduced, and so parasite evolution is constrained. Quantitative theory that makes testable predictions for the opposing effects of plasticity on rates of evolution is urgently needed. For example, the social behaviours of malaria parasites provide tolerance to drugs: plasticity in reproductive restraint helps buffer against the impact of drugs on within-host survival [72]. Thus, selection for other resistance traits (e.g. drug efflux pumps, alternative metabolic or detoxification pathways) is weakened but this clinically beneficial outcome may be undermined because the greater number of surviving parasites offers more opportunities for resistance mutations to occur. Second, while a behaviour may be plastically adjusted in response to social context, the consequences of the action can subsequently feedback to affect social context. For example, bacteriophage plastically speed-up their host lysis time phenotype in response to being in a mixed versus a single infection, i.e. they are responding to the social context encountered within their host [35]. By lysing the host cell before non-plastic phage can transmit, the plastic phage gain a competitive advantage and consequently, become increasingly more likely to interact with related phage.

It is our view that incorporating phenotypic plasticity into social evolution theory represents a milestone for bringing theoretical work and empirical observations closer. To some extent, for a few social traits (e.g. sex allocation of malaria parasites [119]) existing theory that predicts what fixed traits should be at equilibrium can also apply to plastic strategies, and so can be used to make quantitative predictions. However, analyses that incorporate phenomena specific to plasticity, such as its costs and limits, are lacking. The costs and limits of plasticity matter because they may maintain genetic variation in natural populations [120] and could offer novel disease intervention targets [23]. The importance of the costs and limits of plasticity are illustrated by parasites for which the host is an infrequent environment. For example, *P. aeruginosa* is a supreme generalist microbe, able to grow in soil, water and diverse animal and plant hosts, thanks to high investment in regulatory factors [80,121]. While the benefits of extensive and complex regulatory control are easily appreciated in its broad host range, they also raise the potential cost of making 'bad decisions', turning on genes inappropriately when faced with a novel environment. During initial human colonization, *P. aeruginosa* turns on an array of virulence factors [122,123] that cause serious damage to the host. However, many of these damaging traits are subsequently lost or turned off during within-host evolution [124], suggesting that the initial plastic responses were maladaptive. It is possible that the loss of these secreted virulence factors is owing to social interactions favouring non-producing 'cheats' that do not pay the cost of the collectively useful virulence traits [31,125]. However, the continued ability of these 'cheat' strains to persist [125] suggests that the virulence factors are redundant in the host lung, and their initial upregulation was a 'bad decision' [80].

(d) Spatial structure

A major limitation of both theoretical and experimental work is that, for simplicity, historically most microbial (especially bacterial) studies considered well-mixed groups in liquid where local spatial structure is minimal [31,126]. This view may be a reasonable approximation for taxa like malaria parasites, where social interactions appear to play-out on a host-wide scale. However, hosts are not 'a well-mixed bag' of resources and immune defences, and so the reality for many parasites is that infections are far more structured at a local (within-host) scale [127]. For example, many bacteria stick themselves to host surfaces or attach to each other, in groups called biofilms. Social interactions are most intense when individuals live side-by-side in these structured environments [128]. For example, conflict between cooperating and cheating *P. aeruginosa* is more intense in biofilms than in liquid culture [129]. In a biofilm, the presence of cheats causes a greater reduction in population growth, reduces the structural integrity of biolfilms and increases susceptibility to antibiotics [129]. However, the advantages of life in a biofilm may be tempered by a trade-off recently observed in *Vibrio cholerae*, between the benefits of being better competitors within the host and the costs of impaired ability to disperse [130].

Table 3. The potential of 'Hamiltonian Medicine': examples and limitations of proposed biomedical applications of parasite sociality.

concept	examples
cheat therapy	A strategy as simple as the introduction of a cheat (non-producer) strain can lead to direct reduction in parasite virulence, as well as a reduced bacterial population size, that may make the infection more susceptible to other intervention strategies. For example, the introduction of cheater mutants with reduced expression of secreted virulence factors into infections of the bacterial pathogen *P. aeruginosa* reduces mortality in a mouse model [142], at least in the case of simultaneous inoculation of the target wild-type and the cheater 'treatment'. The ability of cheats to increase in frequency within a wild-type infection, while simultaneously decreasing virulence has led to the idea of exploiting cheater invasion to introduce medically beneficial alleles into infections, such as sensitivity to antibiotics or a lethal toxin under the control of an inducible promoter, which when activated, would eliminate both cooperators and cheats [19]. This approach resembles phage therapy, where a live and natural enemy is administered to control an infection at a specific site, and shares the benefits of responsive dosing (the treatment can amplify at the target site, unlike chemical therapeutics). However, cheat therapies face many of the obstacles we outline in the main text—they may be vulnerable to 'reprogramming' by cooperation-inducing plasmids, they may be unable to exploit established cooperator populations owing to within-host structure, or owing to plastic phenotypic changes in the resident. Finally, rare cheats may be unable to overcome the local-adaptation advantages of established wild-type infections [92,143].
drug resistance	Drug resistance mechanisms are often thought to impose fitness costs in the absence of drugs. Experiments using malaria parasites suggest that these fitness costs include competitive inferiority, and so suppression by wild-type genotypes in mixed infections could constrain the spread of resistance [144]. However, the extent to which suppression impacts on resistance in natural infections and how this could interact with eradication programmes is unclear. This is because as parasite prevalence decreases, infections will increasingly contain highly related parasites, which are more likely to cooperate than compete. Traditional antibiotics act by killing or stopping cell division, and resistant mutants rapidly replace the original susceptible strains. Instead, if a drug attacks a cell's ability to secrete a public good that contributes to virulence (an 'anti-virulence' drug), then resistant mutants that re-evolve secretion will promote the growth of susceptible cells around them, reducing the spread of resistance. Moreover, because the susceptible cells do not pay the cost of secretion (i.e. they cheat), this puts resistant parasites at a competitive disadvantage, further reducing the spread of resistance [144,145].
evolutionary traps	An underexplored avenue concerns manipulating parasite kin recognition and communication systems to 'trick' parasites into adopting strategies that are suboptimal for their fitness and of clinical or epidemiological benefit. Evolving resistance to this type of intervention could be difficult because solutions would likely involve losing the benefit of coordinated action in untreated infections. For example, in malaria parasites, investment in asexual (which are responsible for disease symptoms) versus sexual stages is plastic. Parasites competing in mixed infections invest relatively less in sexual stages than when in single infections [73]. A drug that mimics being in a single infection (e.g. masks the cues of competition) and so, induces parasites to invest more sexual stages will result in less virulent infections, and as long as conditions are vector-free, there will be no increase in the risk of transmission to other hosts. Furthermore, the additional sexual stages will provide a stronger stimulus to the host immune system and the resulting responses could more effectively block the transmission of future malaria infections [146]. An approach to blocking transmission would be to induce mass suicide in the vector [15].

(e) Multi-species interactions

Most natural parasite communities are characterized by spatial structure, a multitude of co-infecting species and several environments to cope with. For example, the lesson from bacterial metagenomics is that thousands of species are commonly present in any one environment [128,131]. By contrast, the primary focus of parasite social evolution studies has involved examining what happens when multiple genotypes of a single species are mixed (e.g. [21,34,73,132]). Cross-species parasite social interactions are diverse: depending on the species in question, an incoming species can by excluded, facilitated or unaffected, by a resident species [133]. For example, an ongoing malaria infection can exclude conspecifics [134,135] but strongly facilitate infection by heterospefic malaria parasites. In the latter case, species preferentially infecting mature red blood cells generate anaemia to which the host responds by producing young red blood cells, which is predicted to facilitate malaria species that prefer the abundant young age class, resulting in far higher virulence than single-species infection [136]. However, the mechanisms that determine cross-species interactions are highly diverse, ranging from resource competition, interference competition (e.g. the production of antibiotics and bacteriocins), immune-mediated apparent competition and facilitation (e.g. cross-feeding on partner metabolic byproducts, immunomodulation) [137]. Together this menu of interactions contributes to the astounding diversity of communities of commensals, symbionts and parasites found within multicellular organisms.

A major challenge to unravelling the mechanisms underpinning how communities function is the necessity to combine molecular and ecological approaches to study

highly complex assemblies. A measure of the scale of the problem can be seen by the emergent ecological complexity generated by a simple two-species interaction governed by a single mechanism of metabolic exchange—a food for detoxification exchange—where a cross-feeding partner relieves a producer lineage of by-product toxicity. Recent theory has demonstrated that this simple exchange can generate mutualistic, competitive and exploitative functional relationships, and diverse spatial patternings, dependent on the exact parametrization of the molecular exchange [138]. Unravelling the complexity of these interactions—and how they affect evolution—is urgently required because the microbial communities inside vectors are being manipulated to control disease [139].

5. Why the social lives of parasites matters

Parasitism is one of the most successful modes of life, as measured by how often it evolved and how many parasitic species are presently in existence [140]. Consequently, if explaining cooperation is one of the greatest problems for evolutionary biology, then explaining cooperation in parasites is one of the key aspects of this problem. The irreducible mishmash of proximate causality of social behaviours in traditionally studied animal taxa is far more accessible for parasites, thanks to their relatively simple and manipulatable genotype–phenotype maps. Parasites make excellent model organisms thanks to their short generation times; ability to generate some real-world complexity, even in the laboratory, by studying *in vivo* infections; and well-defined, measurable, social behaviours. Moreover, the applied importance of parasites has resulted in a vast resource of tools and literature on their molecular and cellular biology, so the genetic and molecular mechanisms that underlie social behaviours can be identified and precisely manipulated [8].

Incorporating a 'bottom-up' approach provides a novel perspective on the evolution and maintenance of parasite social behaviours and provides new opportunities for theory-led experimental testing. For example, by understanding aspects of interactions in infections such as those highlighted in this article, traditional virulence evolution theory may be better reconciled with data. Research has focused on social interactions between parasites within hosts (likely owing to the greater interest in disease pathology than transmission) and so, social interactions inside vectors have been overlooked, but we expect they are equally worthy of investigation. Moreover, for parasite species whose life cycles include multiple host species or periods in the abiotic environment, quantifying how social behaviours at these different scales integrate to shape parasite fitness is also a huge challenge, and highlights the need to consider within host biology in its broader context.

The social behaviours of parasites contribute to virulence, transmission and resistance to anti-parasite drugs, as illustrated throughout the text and tables of this article. The field of 'Darwinian Medicine' aims to use ecological and evolutionary principles to inform the treatment of infections to ensure that interventions are as evolution-proof as possible, and prevent the evolution of more harmful parasites in response to anthropogenic pressures. 'Hamiltonian Medicine' is emerging as a subset of this endeavour, asking how parasite social systems and interactions might be subverted or manipulated to better control disease [9,141]. By recognizing that parasites rely on social behaviours to infect and transmit, novel strategies for treatment have been revealed (table 3).

6. Conclusion

A key strength of evolutionary biology is that theory is used to motivate experiments. Historically, this has been the case, with many empirical tests stemming from the basic virulence–transmission trade-off models and their 'virulence-kin-selection' offshoots. However, for topics such as phenotypic plasticity, empirical work is often ahead of social evolution theory and this disconnect is especially apparent in systems that have applied importance. We recognize that the complexity of within-host parasite ecology may have been off-putting for evolutionary theorists since, on the face of it, generalities seem unlikely and explaining what is going on requires deeper knowledge of the biological details of individual study systems. However, generalities do exist—such as public goods, mobile elements, phenotypic plasticity, within-host spatial structure and multi-species interactions—that will provide rewarding avenues for future theoretical and experimental research.

Acknowledgements. Thanks to A. Buckling, M. Boots and M. Bell for discussion and insight, and S. Alizon and an anonymous reviewer for useful comments on the manuscript.

Funding statement. H.L. is supported by the BBSRC and ERC. S.R. is supported by the Wellcome Trust (WT082234MA), The Royal Society, and the Centre for Infection, Immunity and Evolution (CIIE) at the University of Edinburgh. S.B. is supported by the CIIE.

References

1. West SA, Diggle SP, Buckling A, Gardner A, Griffin AS. 2007 The social lives of microbes. *Annu. Rev. Ecol. Evol. Syst.* **38**, 53–77. (doi:10.1146/annurev.ecolsys.38.091206.095740)
2. Nadell CD, Bucci V, Drescher K, Levin SA, Bassler BL, Xavier JB. 2013 Cutting through the complexity of cell collectives. *Proc. R. Soc. B* **280**, 20122770. (doi:10.1098/rspb.2012.2770)
3. Crespi BJ. 2001 The evolution of social behavior in microorganisms. *Trends Ecol. Evol.* **16**, 178–183. (doi:10.1016/S0169-5347(01)02115-2)
4. Webb JS, Givskov M, Kjelleberg S. 2003 Bacterial biofilms: prokaryotic adventures in multicellularity. *Curr. Opin. Microbiol.* **6**, 578–585. (doi:10.1016/j.mib.2003.10.014)
5. Kolter R, Greenberg EP. 2006 Microbial sciences: the superficial life of microbes. *Nature* **18**, 300–302. (doi:10.1038/441300a)
6. West SA, Griffin AS, Gardner A, Diggle SP. 2006 Social evolution theory for microorganisms. *Nat. Rev. Microbiol.* **4**, 597–607. (doi:10.1038/nrmicro1461)
7. Diggle SP, Griffin AS, Campbell GS, West SA. 2007 Cooperation and conflict in quorum-sensing bacterial populations. *Nature* **450**, 411–414. (doi:10.1038/nature0627915)
8. Foster KR, Parkinson K, Thompson CRL. 2007 What can microbial genetics teach sociobiology? *Trends Genet.* **23**, 74–80. (doi:10.1016/j.tig.2006.12.003)
9. Hense BA, Kuttler C, Muller J, Rothballer M, Hartmann A, Kreft JU. 2007 Does efficiency sensing unify diffusion and quorum sensing? *Nat. Rev. Microbiol.* **5**, 230–239. (doi:10.1038/nrmicro1600)

10. Williams P, Winzer K, Chan WC, Camara M. 2007 Look who's talking: communication and quorum sensing in the bacterial world. *Phil. Trans. R. Soc. B* **362**, 1119–1134. (doi:10.1098/rstb.2007.2039)
11. Brown SP, Buckling A. 2008 A social life for discerning microbes. *Cell* **135**, 600–603. (doi:10.1016/j.cell.2008.10.030)
12. MacLean R. 2008 The tragedy of the commons in microbial populations: insights from theoretical, comparative and experimental studies. *Heredity* **100**, 471–477. (doi:10.1038/sj.hdy.6801073)
13. Popat R, Crusz SA, Diggle SP. 2008 The social behaviours of bacterial pathogens. *Br. Med. Bull.* **87**, 63–75. (doi:10.1093/bmb/ldn030)
14. Brown SP, Inglis R, Taddei F. 2009 SYNTHESIS: evolutionary ecology of microbial wars: within-host competition and (incidental) virulence. *Evol. Appl.* **2**, 32–39. (doi:10.1111/j.1752-4571.2008.00059.x)
15. Reece SE, Pollitt LC, Colegrave N, Gardner A. 2011 The meaning of death: evolution and ecology of apoptosis in protozoan parasites. *PLoS Pathog.* **7**, e1002320. (doi:10.1371/journal.ppat.1002320)
16. Buckling A, Brockhurst MA. 2008 Kin selection and the evolution of virulence. *Heredity* **100**, 484–488. (doi:10.1038/sj.hdy.6801093)
17. Nogueira T, Rankin DJ, Touchon M, Taddei F, Brown SP, Rocha EPC. 2009 Horizontal gene transfer of the secretome drives the evolution of bacterial cooperation and virulence. *Curr. Biol.* **19**, 1683–1691. (doi:10.1016/j.cub.2009.08.056)
18. Rankin DJ, Rocha EPC, Brown SP. 2010 What traits are carried on mobile genetic elements, and why? *Heredity* **106**, 1–10. (doi:10.1038/hdy.2010.24)
19. Brown SP, West SA, Diggle SP, Griffin AS. 2009 Social evolution in microorganisms and a Trojan horse approach to medical intervention strategies. *Phil. Trans. R. Soc. B* **364**, 3157–3168. (doi:10.1098/rstb.2009.0055)
20. Mideo N, Day T. 2008 On the evolution of reproductive restraint in malaria. *Proc. R. Soc. B* **275**, 1217–1224. (doi:10.1098/rspb.2007.1545)
21. Reece SE, Drew DR, Gardner A. 2008 Sex ratio adjustment and kin discrimination in malaria parasites. *Nature* **453**, 609–615. (doi:10.1038/nature06954)
22. Mideo N *et al*. 2013 Life in cells, hosts, and vectors: parasite evolution across scales. *Infect. Genet. Evol.* **13**, 344–347. (doi:10.1016/j.meegid.2012.03.016)
23. Mideo N, Reece SE. 2012 Plasticity in parasite phenotypes: evolutionary and ecological implications for disease. *Future Microbiol.* **7**, 17–24. (doi:10.2217/fmb.11.134)
24. Hamilton WD. 1964 The genetical evolution of social behaviour II. *J. Theor. Biol.* **7**, 17–52. (doi:10.1016/0022-5193(64)90039-6)
25. West SA, Griffin AS, Gardner A. 2007 Evolutionary explanations for cooperation. *Curr. Biol.* **17**, R661–R672. (doi:10.1016/j.cub.2007.06.004)
26. Hamilton WD. 1970 Selfish and spiteful behaviour in an evolutionary model. *Nature* **228**, 1218–1220. (doi:10.1038/2281218a0)
27. Queller DC, Zacchi F, Cervo R, Turillazzi S, Henshaw MT, Santorelli LA, Strassmann JE. 2000 Unrelated helpers in a social insect. *Nature* **405**, 784–787. (doi:10.1038/35015552)
28. Telfer S, Lambin W, Birtles R, Beldomenico P, Burthe S, Paterson S, Begon M. 2010 Species interactions in a parasite community drive infection risk in a wildlife population. *Science* **330**, 243–246. (doi:10.1126/science.1190333)
29. Bertram BCR. 1975 Social factors influencing reproduction in wild lions. *J. Zool.* **177**, 463–482. (doi:10.1111/j.1469-7998.1975.tb02246.x)
30. Boland CRJ, Heinsohn R, Cockburn A. 1997 Deception by helpers in cooperatively breeding white-winged choughs and its experimental manipulation. *Behav. Ecol. Sociobiol.* **41**, 251–256. (doi:10.1007/s002650050386)
31. Griffin AS, West SA, Buckling A. 2004 Cooperation and competition in pathogenic bacteria. *Nature* **430**, 1024–1027. (doi:10.1038/nature02744)
32. Harrison F, Browning LE, Vos M, Buckling A. 2006 Cooperation and virulence in acute *Pseudomonas aeruginosa* infections. *BMC Biol.* **4**, 21. (doi:10.1186/1741-7007-4-21)
33. Meyer JM, Neely A, Stintzi A, Georges C, Holder IA. 1996 Pyoverdin is essential for virulence of *Pseudomonas aeruginosa*. *Infect. Immun.* **64**, 518–523.
34. Bell AS, De Roode JC, Sim D, Read AF. 2006 Within-host competition in genetically diverse malaria infections: parasite virulence and competitive success. *Evolution* **60**, 1358–1371. (doi:10.1554/05-611.1)
35. Leggett HC, Benmayor R, Hodgson DJ, Buckling A. 2013 Experimental evolution of adaptive phenotypic plasticity in a parasite. *Curr. Biol.* **23**, 139–142. (doi:10.1016/j.cub.2012.11.045)
36. Boomsma JJ. 2009 Lifetime monogamy and the evolution of eusociality. *Phil. Trans. R. Soc. B* **364**, 3191–3207. (doi:10.1098/rstb.2009.0101)
37. Kamiya T, Poulin R. 2013 Caste ratios affect the reproductive output of social trematode colonies. *J. Evol. Biol.* **26**, 509–516. (doi:10.1111/jeb.12062)
38. Ackermann M, Stecher B, Freed NE, Songhet P, Hardt WD, Doebeli M. 2008 Self-destructive cooperation mediated by phenotypic noise. *Nature* **454**, 987–990. (doi:10.1038/nature07067)
39. Refardt D, Bergmiller T, Kümmerli R. 2013 Altruism can evolve when relatedness is low: evidence from bacteria committing suicide upon phage infection. *Proc. R. Soc. B* **280**, 20123035. (doi:10.1098/rspb.2012.3035)
40. Pollitt LC, Colegrave N, Khan SM, Sajid M, Reece SE. 2010 Investigating the evolution of apoptosis in malaria parasites: the importance of ecology. *Parasit. Vectors* **16**, 105. (doi:10.1186/1756-3305-3-105)
41. Giron D, Dunn DW, Hardy ICW, Strand MR. 2004 Aggression by polyembryonic wasp soldiers correlates with kinship but not resource competition. *Nature* **430**, 676–679. (doi:10.1038/nature02721)
42. Keller L, Ross KG. 1998 Selfish genes: a green beard in the red fire ant. *Nature* **394**, 573–575. (doi:10.1038/29064)
43. Massey RC, Buckling A, Ffrench-Constant R. 2004 Interference competition and parasite virulence. *Proc. R. Soc. Lond. B* **271**, 785–788. (doi:10.1098/rspb.2004.2676)
44. Inglis RF, Brown SP, Buckling A. 2012 Spite versus cheats: competition among social strategies shapes virulence in *Pseudomonas aeruginosa*. *Evolution* **66**, 3472–3484. (doi:10.1111/j.1558-5646.2012.01706.x)
45. Hamilton WD. 1964 The genetical evolution of social behaviour I. *J. Theor. Biol.* **7**, 1–16. (doi:10.1016/0022-5193(64)90038-4)
46. Trivers RL. 1985 *Social evolution*. Menlo Park, CA: Benjamin/Cummings.
47. West SA, Griffin AS, Gardner A. 2007 Social semantics: altruism, cooperation, mutualism, strong reciprocity and group selection. *J. Evol. Biol.* **20**, 415–432. (doi:10.1111/j.1420-9101.2006.01258.x)
48. Gardner A, West SA, Wild G. 2011 The genetical theory of kin selection. *J. Evol. Biol.* **24**, 1020–1043. (doi:10.1111/j.1420-9101.2011.02236.x)
49. Bourke AFG. 2011 *Principles of social evolution*. Oxford Series in Ecology and Evolution. Oxford, UK: Oxford University Press.
50. Tibayrenc M, Ayala FJ. 2012 Reproductive clonality of pathogens: a perspective on pathogenic viruses, bacteria, fungi, and parasitic protozoa. *Proc. Natl Acad. Sci. USA* **109**, E3305–E3313. (doi:10.1073/pnas.1212452109)
51. Gardner A, Grafen A. 2009 Capturing the superorganism: a formal theory of group adaptation. *J. Evol. Biol.* **22**, 659–671. (doi:10.1111/j.1420-9101.2008.01681.x)
52. Dumas Z, Kummerli R. 2012 Cost of cooperation rules selection for cheats in bacterial metapopulations. *J. Evol. Biol.* **25**, 473–484. (doi:10.1111/j.1420-9101.2011.02437.x)
53. Hardin G. 1988 Tragedy of the commons. *Science* **162**, 1243–1248. (doi:10.1126/science.162.3859.1243)
54. Nowak M. 2011 *Supercooperators: altruism, evolution, and why we need each other to succeed*. New York, NY: The Free Press.
55. Dugatkin LA. 2002 Animal cooperation among unrelated individuals. *Naturwissenschaften* **89**, 533–541. (doi:10.1007/s00114-002-0379-y)
56. Chuang JS, Rivoire O, Leibler S. 2009 Simpson's paradox in a synthetic microbial system. *Science* **323**, 272–275. (doi:10.1126/science.1166739)
57. Xavier JB, Kim W, Foster KR. 2011 A molecular mechanism that stabilizes cooperative secretions in *Pseudomonas aeruginosa*. *Mol. Microbiol.* **79**, 166–179. (doi:10.1111/j.1365-2958.2010.07436.x)
58. Sachs JL, Mueller UG, Wilcox TP, Bull JJ. 2004 The evolution of cooperation. *Q. Rev. Biol.* **79**, 135–160. (doi:10.1086/383541)
59. Lehmann L, Keller L. 2006 The evolution of cooperation and altruism—a general framework and a classification of models. *J. Evol. Biol.* **19**, 1365–1376. (doi:10.1111/j.1420-9101.2006)
60. Nadell CD, Xavier JB, Foster KR. 2009 The sociobiology of biofilms. *Fems Microbiol. Rev.* **33**, 206–224. (doi:10.1111/j.1574-6976.2008.00150.x)
61. Dawkins R. 1976 *The selfish gene*. Oxford, UK: Oxford University Press.

62. Gardner A, West SA. 2010 Greenbeards. *Evolution* **64**, 25–38. (doi:10.1111/j.1558-5646.2009.00842.x)
63. Benabentos R *et al*. 2009 Polymorphic members of the lag gene family mediate kin discrimination in Dictyostelium. *Curr. Biol.* **19**, 567–572. (doi:10.1016/j.cub.2009.02.037)
64. Hirose S, Benabentos R, Ho HI, Kuspa A, Shaulsky G. 2011 Self-recognition in social amoebae is mediated by allelic pairs of tiger genes. *Science* **333**, 467–470. (doi:10.1126/science.1203903)
65. Sharp SP, McGowan A, Wood MJ, Hatchwell BJ. 2005 Learned kin recognition cues in a social bird. *Nature* **434**, 1127–1130. (doi:10.1038/nature03522)
66. Mateo JM. 2002 Kin-recognition abilities and nepotism as a function of sociality. *Proc. R. Soc. Lond. B* **269**, 721–727. (doi:10.1098/rspb.2001.1947)
67. Rousset F, Roze D. 2007 Constraints on the origin and maintenance of genetic kin recognition. *Evolution* **61**, 2320–2330. (doi:10.1111/j.1558-5646.2007.00191.x)
68. Schuster M, Lostroh CP, Ogi T, Greenberg EP. 2003 Identification, timing, and signal specificity of *Pseudomonas aeruginosa* quorum-controlled genes: a transcriptome analysis. *J. Bacteriol.* **185**, 2066–2079. (doi:10.1128/JB.185.7.2066-2079.2003)
69. Darch SE, West SA, Winzer K, Diggle SP. 2012 Density-dependent fitness benefits in quorum-sensing bacterial populations. *Proc. Natl Acad. Sci. USA* **109**, 8259–8263. (doi:10.1073/pnas.1118131109)
70. Mantel PY *et al*. 2013 Malaria-infected erythrocyte-derived microvesicles mediate cellular communication within the parasite population and with the host immune system. *Cell Host Microbe* **13**, 521–534. (doi:10.1016/j.chom.2013.04.009)
71. Regev-Rudzki N *et al*. 2013 Cell–cell communication between malaria-infected red blood cells via exosome-like vesicles. *Cell* **153**, 1120–1133. (doi:10.1016/j.cell.2013.04.029)
72. Reece SE, Ali E, Schneider P, Babiker HA. 2010 Stress, drugs and the evolution of reproductive restraint in malaria parasites. *Proc. R. Soc. B* **277**, 3123–3129. (doi:10.1098/rspb.2010.0564)
73. Pollitt LC, Mideo N, Drew D, Schneider P, Colegrave N, Reece SE. 2011 Competition and the evolution of reproductive restraint in malaria parasites. *Am. Nat.* **177**, 358–367. (doi:10.1086/658175)
74. Levin BR. 1996 The evolution and maintenance of virulence in microparasites. *Emerg. Infect. Dis.* **2**, 93–102. (doi:10.3201/eid0202.960203)
75. Margolis E, Levin BR. 2007 Within-host evolution for the invasiveness of commensal bacteria: an experimental study of bacteremias resulting from Haemophilus influenzae nasal carriage. *J. Infect. Dis.* **196**, 1068–1075. (doi:10.1086/520934)
76. Ebert D. 1998 The evolution and expression of virulence. In *Evolution in health and disease* (ed. SC Stearns). Oxford, UK: Oxford University Press.
77. Bull JJ, Ebert D. 2008 Invasion thresholds and the evolution of nonequilibrium virulence. *Evol. Appl.* **1**, 172–182. (doi:10.1111/j.1752-4571.2007.00003.x)
78. Alizon S, Hurford A, Mideo N, Van Baalen M. 2009 Virulence evolution and the trade-off hypothesis: history, current state of affairs and the future. *J. Evol. Biol.* **22**, 245–259. (doi:10.1111/j.1420-9101.2008.01658.x)
79. Levin BR, Eden CS. 1990 Selection and evolution of virulence in bacteria – an ecumenical excursion and modest suggestion. *Parasitology* **100**, S103–SS15. (doi:10.1017/S0031182000073054)
80. Brown SP, Cornforth DM, Mideo N. 2012 Evolution of virulence in opportunistic pathogens: generalism, plasticity, and control. *Trends Microbiol.* **20**, 336–342. (doi:10.1016/j.tim.2012.04.005)
81. Levin BR, Bull JJ. 1994 Short-sighted evolution and the virulence of pathogenic microorganisms. *Trends Microbiol.* **2**, 76–81. (doi:10.1016/0966-842X(94)90538-X)
82. Anderson RM, May RM. 1982 Coevolution of hosts and parasites. *Parasitology* **85**, 411–426. (doi:10.1017/S0031182000055360)
83. Bremermann HJ, Pickering J. 1983 A game-theoretical model of parasite virulence. *J. Theor. Biol.* **100**, 411–426. (doi:10.1016/0022-5193(83)90438-1)
84. Nowak MA, May RM. 1994 Superinfection and the evolution or parasite virulence. *Proc. R. Soc. Lond. B* **255**, 81–89. (doi:10.1098/rspb.1994.0012)
85. Frank SA. 1996 Models of parasite virulence. *Q. Rev. Biol.* **71**, 37–78. (doi:10.1086/419267)
86. de Roode JC, Yates AJ, Altizer S. 2008 Virulence-transmission trade-offs and population divergence in virulence in a naturally occurring butterfly parasite. *Proc. Natl Acad. Sci. USA* **105**, 7489–7494. (doi:10.1073/pnas.0710909105)
87. Brown SP, Hochberg ME, Grenfell BT. 2002 Does multiple infection select for raised virulence? *Trends Microbiol.* **10**, 401–405. (doi:10.1016/S0966-842X(02)02413-7)
88. Leggett HC, Buckling A, Long GH, Boots M. 2013 Generalism and the evolution of parasite virulence. *Trends Ecol. Evol.* **28**, 592–596. (doi:10.1016/j.tree.2013.07.002)
89. Alizon S, de Roode JC, Michalakis Y. 2013 Multiple infections and the evolution of virulence. *Ecol. Lett.* **16**, 556–567. (doi:10.1111/ele.12076)
90. Buckling A, Harrison F, Vos M, Brockhurst MA, Gardner A, West SA, Griffin A. 2007 Siderophore-mediated cooperation and virulence in Pseudomonas aeruginosa. *FEMS Microbiol. Ecol.* **62**, 135–141. (doi:10.1111/j.1574-6941.2007.00388.x)
91. Kummerli R, Griffin AS, West SA, Buckling A, Harrison F. 2009 Viscous medium promotes cooperation in the pathogenic bacterium *Pseudomonas aeruginosa*. *Proc. R. Soc. B* **276**, 3531–3538. (doi:10.1098/rspb.2009.0861)
92. Morgan AD, Quigley BJZ, Brown SP, Buckling A. 2012 Selection on non-social traits limits the invasion of social cheats. *Ecol. Lett.* **15**, 841–846. (doi:10.1111/j.1461-0248.2012.01805.x)
93. Zhang XX, Rainey PB. 2013 Exploring the sociobiology of pyoverdin-producing *Pseudomonas*. *Evolution* **67**, 3161–3174. (doi:10.1111/evo.12183)
94. Frost LS, Leplae R, Summers AO, Toussaint A. 2005 Mobile genetic elements: the agents of open source evolution. *Nat. Rev. Microbiol.* **3**, 722–732. (doi:10.1038/nrmicro1235)
95. Smith J. 2001 The social evolution of bacterial pathogenesis. *Proc. R. Soc. Lond. B* **268**, 61–69. (doi:10.1098/rspb.2000.1330)
96. Mc Ginty SE, Rankin DJ, Brown SP. 2011 Horizontal gene transfer and the evolution of bacterial cooperation. *Evolution* **65**, 21–32. (doi:10.1111/j.1558-5646.2010.01121.x)
97. Mc Ginty SE, Lehmann L, Brown SP, Rankin DJ. 2013 The interplay between relatedness and horizontal gene transfer drives the evolution of plasmid-carried public goods. *Proc. R. Soc. B* **280**, 20130400. (doi:10.1098/rspb.2013.0400)
98. Dimitriu T, Lotton C, Bernard-Capelle J, Brown SP, Linder AB, Taddei F. Submitted. Genetic information transfer promotes cooperation by increasing relatedness in bacteria.
99. Pigliucci M. 2001 *Phenotypic plasticity: beyond nature and nurture*. Baltimore, MD: John Hopkins University Press.
100. Mitchell A, Romano GH, Groisman B, Yona A, Dekel E, Kupiec M, Dahan O, Pilpel Y. 2009 Adaptive prediction of environmental changes by microorganisms. *Nature* **460**, 220–224. (doi:10.1038/nature08112)
101. Mideo N, Reece SE, Smith AL, Metcalf CJ. 2013 The Cinderella syndrome: why do malaria-infected cells burst at midnight? *Trends Parasitol.* **29**, 10–16. (doi:10.1016/j.pt.2012.10.006)
102. O'Donnell AJ, Schneider P, McWatters HG, Reece SE. 2011 Fitness costs of disrupting circadian rhythms in malaria parasites. *Proc. R. Soc. B* **278**, 2429–2436. (doi:10.1098/rspb.2010.2457)
103. O'Donnell AJ, Mideo N, Reece SE. 2013 Disrupting rhythms in *Plasmodium chabaudi:* costs accrue quickly and independently of how infections are initiated. *Malaria J.* 12, 372. (doi:10.1186/1475-2875-12-372)
104. Greischer MA, Read AF, Bjørnstad ON. 2014 Synchrony in malaria infections: how intensifying within-host competition can be adaptive. *Am. Nat.* **183**, E36–E49. (doi:10.1086/674357)
105. Witkowski B, Lelievre J, Barragan MJ, Laurent V, Su XZ, Berry A, Benoit-Vical F. 2010 Increased tolerance to artemisinin in *Plasmodium falciparum* is mediated by a quiescence mechanism. *Antimicrob. Agents Chemother.* **54**, 1872–1877. (doi:10.1128/AAC.01636-09)
106. Abedon ST. 1990 Selection for lysis inhibition in bacteriophage. *J. Theor. Biol.* **146**, 501–511. (doi:10.1016/S0022-5193(05)80375-3)
107. Abedon ST. 1992 Lysis of lysis-inhibited bacteriophage T4-infected cells. *J. Bacteriol.* **174**, 8073–8080.
108. Abedon ST. 1994 Lysis and the interaction between free phages and infected cells. In *Molecular biology of bacteriophage T4* (eds J Karam *et al.*), pp. 397–405. Washington, DC: American Society for Microbiology.
109. Abedon ST. 1999 Bacteriophage T4 resistance to lysis-inhibition collapse. Genet Res, **74**, 1–11. (doi:10.1017/S0016672399003833)

110. Kümmerli R, Jiricny N, Clarke LS, West SA, Griffin AS. 2009 Phenotypic plasticity of a cooperative behaviour in bacteria. *J. Evol. Biol.* **22**, 589–598. (doi:10.1111/j.1420-9101.2008.01666.x)
111. Kümmerli R, Brown SP. 2010 Molecular and regulatory properties of a public good shape the evolution of cooperation. *Proc. Natl Acad. Sci. USA* **107**, 18 921–18 926. (doi:10.1073/pnas.1011154107)
112. Rutherford ST, Bassler BL. 2012 Bacterial quorum sensing: its role in virulence and possibilities for its control. *Cold Spring Harb. Perspect. Med*. **2**, a012427. (doi:10.1101/cshperspect.a012427)
113. Viney M, Reece SE. 2013 Adaptive noise. *Proc. R. Soc. B* **280**, 20131104. (doi:10.1098/rspb.2013.1104)
114. Pollitt LC, Churcher TS, Dawes EJ, Khan SM, Sajid M, Basáñez MG, Colegrave N, Reece SE. 2013 Costs of crowding for the transmission of malaria parasites. *Evol. Appl.* **6**, 617–629. (doi:10.1111/eva.12048)
115. Schlichting CD, Pigliucci M. 1998 *Phenotypic evolution: a reaction norm perspective*. Sunderland, MA: Sinauer Associates Incorporated.
116. Ramiro RS, Alpedrinha J, Carter L, Gardner A, Reece SE. 2011 Sex and Death: The Effects of Innate Immune Factors on the Sexual Reproduction of Malaria Parasites. *PLoS Pathog.* **7**, e1001309. (doi:10.1371/journal.ppat.1001309)
117. Reece SE, Khan SM, Waters AP, Janse CJ, Kaczanowski S. 2012 Why are male malaria parasites in such a rush? Sex-specific evolution and host-parasite interactions. *Evol. Med. Public Health*. **2013**, 3–13. (doi:10.1093/emph/eos003)
118. West-Eberhard MJ. 2003 *Developmental plasticity and evolution*. Oxford, UK: Oxford University Press.
119. Hamilton WD. 1967 Extraordinary sex ratios: A sex-ratio theory for sex linkage and inbreeding has new implications in cytogenetics and entomology. *Science* **156**, 477–488. (doi:10.1126/science.156.3774.477)
120. DeWitt TJ, Sih A, Wilson DS. 1998 Costs and limits of phenotypic plasticity. *Trends Ecol. Evol.* **13**, 77–81. (doi:10.1016/S0169-5347(97)01274-3)
121. Stover CK *et al*. 2000 Complete genome sequence of *Pseudomonas aeruginosa* PAO1, an opportunistic pathogen. *Nature* **406**, 959–964. (doi:10.1038/35023079)
122. Duan K, Surette MG. 2007 Environmental regulation of *Pseudomonas aeruginosa* PAO1 Las and Rhl quorum-sensing systems. *J. Bacteriol.* **189**, 4827–4836. (doi:10.1128/JB.00043-07)
123. Bielecki P *et al*. 2011 *In vivo* expression profiling of *Pseudomonas aeruginosa* infections reveals niche-specific and strain-independent transcriptional programs. *PLoS ONE* **6**, e24235. (doi:10.1371/journal.pone.0024235)
124. Jiricny N, Diggle SP, West SA, Evans BA, Ballantyne G, Ross-Gillespie A, Griffin AS. 2010 Fitness correlates with the extent of cheating in a bacterium. *J. Evol. Biol.* **23**, 738–747. (doi:10.1111/j.1420-9101.2010.01939.x)
125. Bragonzi A, Paroni M, Nonis A, Cramer N, Montanari S, Rejman J, Di Serio C, Döring G, Tümmler B. 2009 *Pseudomonas aeruginosa* microevolution during cystic fibrosis lung infection establishes clones with adapted virulence. *Am. J. Respir. Crit. Care Med.* **180**, 138–145. (doi:10.1164/rccm.200812-1943OC)
126. Harrison F, Paul J, Massey RC, Buckling A. 2008 Interspecific competition and siderophore-mediated cooperation in *Pseudomonas aeruginosa*. *Isme J.* **2**, 49–55. (doi:10.1038/ismej.2007.96)
127. Grant AJ, Restif O, McKinley TJ, Sheppard M, Maskell DJ, Mastroeni P. 2008 Modelling within-host spatiotemporal dynamics of invasive bacterial disease. *PLoS Biol.* **6**, e74. (doi:10.1371/journal.pbio.0060074)
128. Mitri S, Xavier J, Foster KR. 2011 Social evolution in multispecies biofilms. *Proc. Natl Acad. Sci. USA* **108**(Suppl. 2), 10 839–10 846. (doi:10.1073/pnas.1100292108)
129. Popat R, Crusz SA, Messina M, Williams P, West SA, Diggle SP. 2012 Quorum-sensing and cheating in bacterial biofilms. *Proc. R. Soc. B* **279**, 4765–4771. (doi:10.1098/rspb.2012.1976)
130. Nadell CD, Bassler BL. 2011 A fitness trade-off between local competition and dispersal in Vibrio cholerae biofilms. *Proc. Natl Acad. Sci. USA* **108**, 14 181–14 185. (doi:10.1073/pnas.1111147108)
131. The Human Microbiome Project Consortium. 2012 Structure, function and diversity of the healthy human microbiome. *Nature* **486**, 207–214. (doi:10.1038/nature11234)
132. Balmer O, Stearns SC, Schötzau A, Brun R. 2009 Intraspecific competition between co-infecting parasite strains enhances host survival in African trypanosomes. *Ecology* **90**, 3367–3378. (doi:10.1890/08-2291.1)
133. Eswarappa SM, Estrela S, Brown SP. 2012 Within-host dynamics of multi-species infections: facilitation, competition and virulence. *PLoS ONE* **7**, e38730. (doi:10.1371/journal.pone.0038730)
134. Vardo AM, Kaufhold KD, Schall JJ. 2007 Experimental test for premunition in a lizard malaria parasite (*Plasmodium mexicanum*). *J. Parasitol.* **93**, 280–282. (doi:10.1645/GE-1005R.1)
135. Portugal S *et al*. 2011 Host-mediated regulation of superinfection in malaria. *Nat. Med.* **17**, 732–737. (doi:10.1038/nm.2368)
136. McQueen PG, McKenzie FE. 2006 Competition for red blood cells can enhance *Plasmodium vivax* parasitemia in mixed-species malaria infections. *Am. J. Trop. Med. Hyg.* **75**, 112–125.
137. Pedersen AB, Fenton A. 2007 Emphasizing the ecology in parasite community ecology. *Trends Ecol. Evol.* **22**, 133–139. (doi:10.1016/j.tree.2006.11.005)
138. Estrela S, Brown SP. 2013 Metabolic and demographic feedbacks shape the emergent spatial structure and function of microbial communities. *PLoS Comp. Biol.* **9**, e1003398. (doi:10.1371/journal.pcbi.1003398)
139. Hoffmann AA *et al*. 2011 Successful establishment of Wolbachia in Aedes populations to suppress dengue transmission. *Nature* **476**, 454–457. (doi:10.1038/nature10356)
140. Poulin R, Morand S. 2000 The diversity of parasites. *Q. Rev. Biol.* **75**, 277–293. (doi:10.1086/393500)
141. Foster KR. 2005 Hamiltonian medicine: why the social lives of pathogens matter. *Science* **308**, 1269–1270. (doi:10.1126/science.1108158)
142. Rumbaugh KP, Diggle SP, Watters CM, Ross-Gillespie A, Griffin AS, West SA. 2009 Quorum sensing and the social evolution of bacterial virulence. *Curr. Biol.* **19**, 341–345. (doi:10.1016/j.cub.2009.01.050)
143. Read AF, Day T, Huijben S. 2011 The evolution of drug resistance and the curious orthodoxy of aggressive chemotherapy. *Proc. Natl Acad. Sci. USA* **108**, 10 871–10 877. (doi:10.1073/pnas.1100299108)
144. André JB, Godelle B. 2005 Multicellular organization in bacteria as a target for drug therapy. *Ecol. Lett.* **8**, 800–810. (doi:10.1111/j.1461-0248.2005.00783.x)
145. Mellbye B, Schuster M. 2011 The sociomicrobiology of antivirulence drug resistance: a proof of concept. *mBio* **2**, e00131. (doi:10.1128/mBio.00131-11)
146. Carter LC, Mideo NM, Pollitt LC, Kafsack BFC, Llinas M, Reece SE. 2013 Stress and sex in malaria parasites: why does commitment vary? *Evol. Med. Public Health* **2013**, 135–147. (doi:10.1093/emph/eot011)
147. Kümmerli R, Ross-Gillespie A. 2013 Explaining the sociobiology of pyoverdin-producing *Pseudomonas*: a comment on Zhang and Rainey (2013). *Evolution* (doi:10.1111/evo.12311)

biology letters
Community ecology

Biol. Lett. (2012) **8**, 768–771
doi:10.1098/rsbl.2012.0282
Published online 23 May 2012

Diversity–disturbance relationships: frequency and intensity interact

Alex R. Hall[1,2,*], Adam D. Miller[3], Helen C. Leggett[1,4], Stephen H. Roxburgh[5], Angus Buckling[4] and Katriona Shea[3,*]

[1]*Department of Zoology, University of Oxford, Oxford OX1 3PS, UK*
[2]*Institute of Integrative Biology, ETH Zürich, 8092, Zürich, Switzerland*
[3]*Department of Biology, 208 Mueller Laboratory, Pennsylvania State University, University Park, PA 16802, USA*
[4]*Department of Biosciences, University of Exeter, Cornwall Campus, Penryn TR10 9EZ, UK*
[5]*Commonwealth Scientific and Industrial Research Organization Ecosystem Sciences and Sustainable Agriculture Flagship, Box 284, Canberra, Australian Capital Territory 2601, Australia*
**Authors for correspondence (alex.hall@env.ethz.ch; k-shea@psu.edu).*

An influential ecological theory, the intermediate disturbance hypothesis (IDH), predicts that intermediate levels of disturbance will maximize species diversity. Empirical studies, however, have described a wide variety of diversity–disturbance relationships (DDRs). Using experimental populations of microbes, we show that the form of the DDR depends on an interaction between disturbance frequency and intensity. We find that diversity shows a monotonically increasing, unimodal or flat relationship with disturbance, depending on the values of the disturbance aspects considered. These results confirm recent theoretical predictions, and potentially reconcile the conflicting body of empirical evidence on DDRs.

Keywords: disturbance; microcosm; *Pseudomonas fluorescens*; intermediate disturbance hypothesis

1. INTRODUCTION

Understanding how biological diversity responds to disturbance is a longstanding problem in ecology and evolution, with important ramifications for conservation and management of ecosystems [1–5]. A large body of theory predicts that ecological diversity is maximized at intermediate levels of disturbance [6,7], yet a wide range of diversity–disturbance relationships (DDRs) have been observed in empirical studies [8]. This inconsistency may arise because most studies consider a single aspect of disturbance, such as the frequency of disturbances over time, or their intensity, even though diversity probably depends on a combination of different aspects of disturbance [7]. Recent theory has formally addressed this prediction, finding, for example, that coexistence can peak at low, intermediate or high disturbance intensities depending on their frequency [9]. Here we show, using experimental populations of bacteria, that different DDRs can be observed within the same system, depending on which aspect of disturbance is considered. These findings illustrate the need to consider multiple aspects of disturbance in order to fully understand the dynamics of disturbance-prone communities.

Electronic supplementary material is available at http://dx.doi.org/10.1098/rsbl.2012.0282 or via http://rsbl.royalsocietypublishing.org.

2. MATERIAL AND METHODS

We manipulated both the frequency and intensity of disturbances (mass mortality events [10]) in populations of *Pseudomonas fluorescens* SBW25 in microcosms. Each microcosm contained 6 ml liquid King's medium B in a 28 ml glass vial, incubated without shaking at 28°C [11]. In these conditions, *P. fluorescens* rapidly diversifies into genetically distinct morphotypes: the ancestral morph (smooth, SM) grows in the broth phase; the wrinkly spreader (WS) morph grows into a biofilm at the air–liquid interface and includes numerous sub-categories; the fuzzy spreader (FS) morph grows at the bottom of the tube [11]. Crucially, different morphotypes are readily distinguished on agar plates, allowing us to quantify ecological diversity at the end of the experiment [11]. We used a fully factorial experimental design, maintaining replicate populations over 16 days and imposing 0, 1, 4, 8 or 16 disturbances (frequency manipulation) with 10, 99.9 or 99.999 per cent mortality (intensity manipulation) at each disturbance.

We enacted disturbances by removing a fraction of cells (intensity) from each microcosm before transferring the surviving cells to a fresh microcosm [10,12]. Microcosms were homogenized by vortexing prior to every disturbance, to ensure that there was no bias towards particular morphotypes. There were three replicate microcosms at each combination of disturbance intensity (10, 99.9 or 99.999%) and frequency (0, 1, 4, 8, 16 disturbances). Disturbance frequencies were chosen to be consistent with previous work [10,12]. At the end of the experiment (16 days), diversity was estimated by vortexing, diluting and plating bacteria from every microcosm onto nutrient-rich KB agar plates, before counting the number of each morphotype after 48 h incubation at 28°C [10,11].

We tested whether the diversity–intensity relationship depended on the frequency of disturbances by the interaction term in a linear model, with diversity as the response variable and frequency and intensity as factors. Diversity scores were rank-transformed to account for non-normality. To analyse the correlation between diversity and frequency in each intensity treatment, we transformed disturbance frequencies to $\log_2$(no. disturbances + 1). We tested for quadratic effects by comparing full and reduced models with *F*-tests. In some cases, it was unclear whether quadratic effects were due to unimodality or curvilinearity; to test this, we used Mitchell-Olds & Shaw tests [13,14]. WS frequencies were arcsine-transformed before analysis to stabilize the relationship between mean and variance.

3. RESULTS

The diversity–frequency relationship varied depending on the intensity of disturbances (interaction term: $F_{8,30} = 6.07$, $p = 0.0001$; figure 1*a*). At low intensity (10% mortality), there was no correlation between frequency and diversity ($F_{1,13} = 0.04$, $p = 0.84$). When disturbances were more intense (99.9% mortality), diversity was greatest at intermediate and high frequencies (quadratic term: $F_{1,12} = 34.50$, $p < 0.0001$), with a slight peak at an intermediate frequency (Mitchell-Olds & Shaw test: $p = 0.01$). At the highest intensity (99.999% mortality), diversity was unimodally related to frequency (quadratic term: $F_{1,12} = 25.92$, $p = 0.0003$), showing a clear drop at the highest frequency, meaning that diversity at the highest frequency was considerably lower at high compared with intermediate intensity (Welch's *t*-test: $t_2 = 8.28$, $p = 0.01$). Another way to describe the interaction of intensity and frequency is by the shape of the diversity–intensity relationship at different frequencies: when disturbances were rare, diversity increased with intensity (lightest grey circles in figure 1*a*). When disturbances were frequent, diversity peaked at intermediate intensity (black circles, figure 1*a*). Thus, diversity was lowest at the extremes, where intensity and frequency were both very high or both very low.

Received 28 March 2012
Accepted 30 April 2012

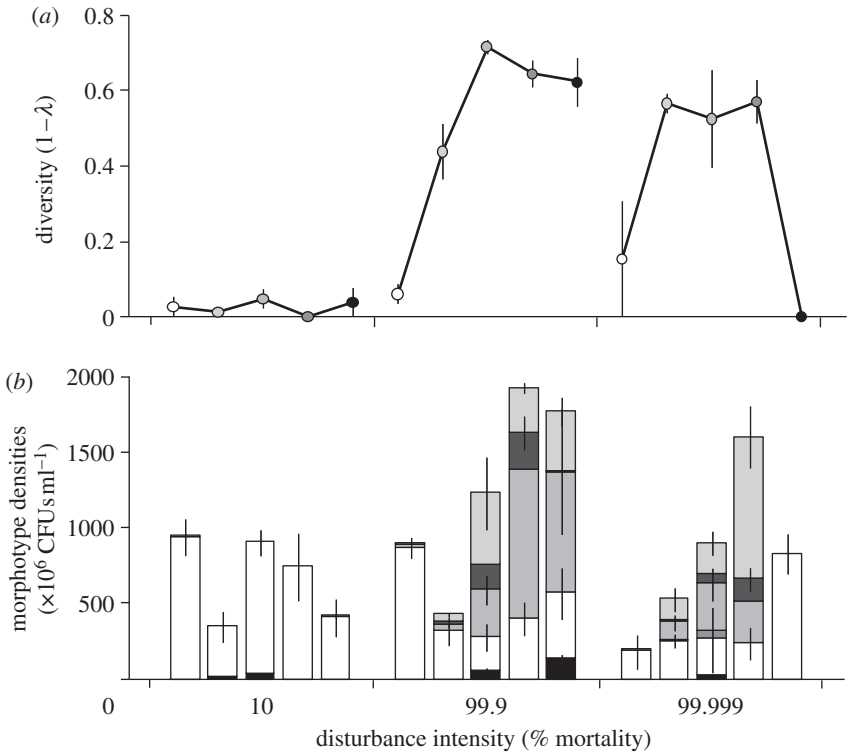


Figure 1. (*a*) Diversity, measured as the complement of Simpson's index ($1-\lambda$, where $\lambda = \sum p_i^2$ and p_i is the proportion of the *i*th morph), is shown for each of five disturbance frequencies (left to right in each series, 0, 1, 4, 8 or 16 disturbances over 16 days) at each of three disturbance intensities. Points show means ± s.e. for three replicate microcosms. (*b*) Frequencies of different morphotypes (WS, four subcategories, each a different shade of grey; SM, white bars; FS, black bars) at each combination of disturbance intensity and frequency. Bars show mean ± s.e. for three replicate microcosms at each disturbance frequency (from left to right in each group of five bars: 0, 1, 4, 8, 16 disturbances) and intensity.

Consequently, diversity can show a monotonically increasing, unimodal or flat relationship with disturbance depending on which aspect is considered.

Previous work at a single intensity (99.9%) showed that biofilm-forming morphotypes (WS) are most successful at intermediate disturbance frequencies [10,12]. Our results at 99.9 per cent intensity are entirely consistent with this pattern (figure 1*b*; quadratic term for WS frequency against disturbance frequency: $F_{1,12} = 9.03$, $p = 0.01$). We also find that increasing intensity to 99.999 per cent favours WS when disturbances are rare, but favours the broth-living morph (SM) at the highest frequency. SM was also relatively successful in low-intensity treatments. Thus, WS is most successful at intermediate frequencies and intensities of disturbance. The greater number of subcategories in WS compared with SM and FS causes total diversity to peak when the WS frequency is between 0.6 and 0.8, but we note that the same qualitative diversity patterns across disturbance treatments are obtained if we ignore WS sub-categories (frequency × intensity interaction: $F_{8,30} = 2.81$, $p = 0.019$), showing unequivocally that the frequency–intensity interaction drives the coexistence of ecologically distinct sub-populations.

4. DISCUSSION

Our results demonstrate that different DDRs can arise, even within a single empirical system, depending on an interaction between the frequency and intensity of disturbances. This is because the relative abundances of the two main morphotypes in our experimental system, SM and WS, varied nonlinearly with both increasing intensity and frequency of disturbances. Consequently, we obtained monotonically increasing, unimodal or flat DDRs, depending on which aspects of disturbance were considered, and over what range of values.

Why does long-term growth rate (a measure of fitness), as reflected by morphotype density of SM and WS, vary nonlinearly with disturbance frequency and intensity? WS, which overproduces a cellulosic polymer [15], requires a threshold population density to form a mat at the air–broth interface [16]. WS and SM can only coexist when this mat forms [11]. At high frequencies of disturbance, WS, which has a lower intrinsic growth rate than SM, is unable to reach sufficient densities to form a strong mat [10,12,16]. Similarly, if disturbances are very intense, then WS may not reach threshold densities between disturbances. However, if left undisturbed, the WS mat eventually collapses, either through invasion by SM or because general environmental degradation reduces population size [17], or both, resulting in SM again dominating the system. This breakdown should be more rapid between disturbances if disturbance intensity is low, because a smaller proportion of the degraded growth media is renewed. Thus, both diversity and the success of WS are maximized at intermediate intensities and frequencies, and the peak in diversity across frequencies will be shifted in either direction depending on intensity, and vice versa.

Since WS has a lower intrinsic growth rate, and its mat may collapse, it can be seen as a weaker competitor than SM; thus it is natural to expect SM to dominate at low frequencies and intensities.

The recent model by Miller *et al.* [9] is qualitatively consistent with our results. It predicts that a unimodal relationship between diversity and disturbance frequency can result from a single type/species dominating at high and low disturbance frequencies, and that the unimodal relationship becomes relatively flat if disturbance intensity is reduced. Crucially, while this model is not a direct representation of our experimental system, the biological mechanisms underlying DDRs are similar. In the model, the growth rates of the two species respond differently and nonlinearly to changing disturbance intensity and frequency because of differences in growth rate, survival and competitive ability. In our experimental system, WS and SM also differ in all of these traits and show different nonlinear responses to changing disturbance intensity and frequency.

The model also predicts that a unimodal DDR at intermediate intensity can become bimodal at higher intensities. At intermediate intensity, one type/species dominates at high and low frequencies and both types coexist in the middle. At higher intensity, one type dominates at high and low frequency and the other dominates in the middle, so that coexistence occurs at middle–low and middle–high frequencies where neither type is dominant, resulting in a bimodal DDR. We did not observe such bimodal DDRs in our experiments. However, the fact that unimodal DDRs for *P. fluorescens* are based on a single type dominating at high- and low-disturbance frequencies, and that WS was dominant in some microcosms at intermediate disturbance frequencies, suggests that bimodal DDRs are possible in this system, but they may only be detected when a greater range of disturbance frequencies and intensities are included in the experimental design.

The diversity–frequency relationship in this experimental system was previously modelled [10] using a modified version of Levene's [18] model that explicitly considers distinct niches, where intra-niche competition is stronger than inter-niche competition. In this model, disturbances alter the relative contributions of the two niches to total population growth, with coexistence only possible when both niches support approximately equal numbers of individuals. This model can also display qualitatively different DDRs, including bimodal relationships, if the term describing population size after disturbance is varied (see electronic supplementary material). More complex models, such as metapopulation models incorporating disturbance and extinction of a proportion of demes [19], may also show similarly complicated DDRs when both intensity and frequency of disturbance are varied, but analysis of such models is beyond the scope of this paper.

These studies illustrate that several factors influence the shape of DDRs. For instance, Kondoh [19] showed that, in a metapopulation model, productivity influences the shape of observed DDRs. Empirical research has also demonstrated that competition–colonization trade-offs in a metapopulation network of linked microcosms can generate distinct DDRs against disturbance frequency, depending on spatial scale [20]. In contrast, our data show that differing spatial scales and productivity gradients are not necessary to change the shape of DDRs. Rather, at a fixed local scale, differential responses to disturbance frequency and intensity form the biological basis for our findings. This suggests that closer attention to multiple disturbance aspects, and their interactions, may allow us to reconcile the large and conflicting body of empirical evidence on the relationship between disturbance and diversity [8].

Experimental work was funded by a European Research Council grant. K.S. and A.M. were supported by National Science Foundation grant no. DEB-0815373.

1 Chesson, P. 2000 Mechanisms of maintenance of species diversity. *Annu. Rev. Ecol. Syst.* **31**, 343–366. (doi:10.1146/annurev.ecolsys.31.1.343)
2 Levin, S. A. & Paine, R. T. 1974 Disturbance, patch formation, and community structure. *Proc. Natl Acad. Sci. USA* **71**, 2744–2747. (doi:10.1073/pnas.71.7.2744)
3 Rosenzweig, M. L. 1995 *Species diversity in space and time*. Cambridge, UK: Cambridge University Press.
4 Roxburgh, S. H., Shea, K. & Wilson, J. B. 2004 The intermediate disturbance hypothesis: patch dynamics and mechanisms of species coexistence. *Ecology* **85**, 359–371. (doi:10.1890/03-0266)
5 Sousa, W. P. 1984 The role of disturbance in natural communities. *Annu. Rev. Ecol. Syst.* **15**, 353–391. (doi:10.1146/annurev.es.15.110184.002033)
6 Connell, J. H. 1978 Diversity in tropical rain forests and coral reefs—high diversity of trees and corals is maintained only in a non-equilibrium state. *Science* **199**, 1302–1310. (doi:10.1126/science.199.4335.1302)
7 Shea, K., Roxburgh, S. H. & Rauschert, E. S. J. 2004 Moving from pattern to process: coexistence mechanisms under intermediate disturbance regimes. *Ecol. Lett.* **7**, 491–508. (doi:10.1111/j.1461-0248.2004.00600.x)
8 Mackey, R. L. & Currie, D. J. 2001 The diversity–disturbance relationship: is it generally strong and peaked. *Ecology* **82**, 3479–3492. (doi:10.1890/0012-9658(2001)082[3479:TDDRII]2.CO;2)
9 Miller, A. D., Roxburgh, S. H. & Shea, K. 2011 How frequency and intensity shape diversity–disturbance relationships. *Proc. Natl Acad. Sci. USA* **108**, 5643–5648. (doi:10.1073/pnas.1018594108)
10 Buckling, A., Kassen, R., Bell, G. & Rainey, P. B. 2000 Disturbance and diversity in experimental microcosms. *Nature* **408**, 961–964. (doi:10.1038/35050080)
11 Rainey, P. B. & Travisano, M. 1998 Adaptive radiation in a heterogeneous environment. *Nature* **394**, 69–72. (doi:10.1038/27900)
12 Benmayor, R., Buckling, A., Bonsall, M. B., Brockhurst, M. A. & Hodgson, D. J. 2008 The interactive effects of parasites, disturbance, and productivity on experimental adaptive radiations. *Evolution* **62**, 467–477. (doi:10.1111/j.1558-5646.2007.00268.x)
13 Mitchell-Olds, T. & Shaw, R. G. 1987 Regression analysis of natural selection: statistical inference and biological interpretation. *Evolution* **41**, 1149–1161. (doi:10.2307/2409084)
14 Mittelbach, G. G., Steiner, C. F., Scheiner, S. M., Gross, K. L., Reynolds, H. L., Waide, R. B., Willing, M. R., Dodson, S. I. & Gough, L. 2001 What is the observed

relationship between species richness and productivity? *Ecology* **82**, 2381–2396. (doi:10.1890/0012-9658(2001)082[2381:WITORB]2.0.CO;2)

15 Spiers, A. J., Kahn, S. G., Bohannon, J., Travisano, M. & Rainey, P. B. 2002 Adaptive divergence in experimental populations of *Pseudomonas fluorescens*. I. Genetic and phenotypic bases of wrinkly spreader fitness. *Genetics* **161**, 33–46.

16 Brockhurst, M. A., Buckling, A. & Gardner, A. 2007 Cooperation peaks at intermediate disturbance. *Curr. Biol.* **17**, 761–765. (doi:10.1016/j.cub.2007.02.057)

17 Rainey, P. B. & Rainey, K. 2003 Evolution of cooperation and conflict in experimental bacterial populations. *Nature* **425**, 72–74. (doi:10.1038/nature01906)

18 Levene, H. 1953 Genetic equilibrium when more than one ecological niche is available. *Am. Nat.* **87**, 331–333. (doi:10.1086/281792)

19 Kondoh, M. 2001 Unifying the relationships of species richness to productivity and disturbance. *Proc. R. Soc. Lond. B* **268**, 269–271. (doi:10.1098/rspb.2000.1384)

20 Cadotte, M. W. 2007 Competition–colonization trade-offs and disturbance effects at multiple scales. *Ecology* **88**, 823–829. (doi:10.1890/06-1117)

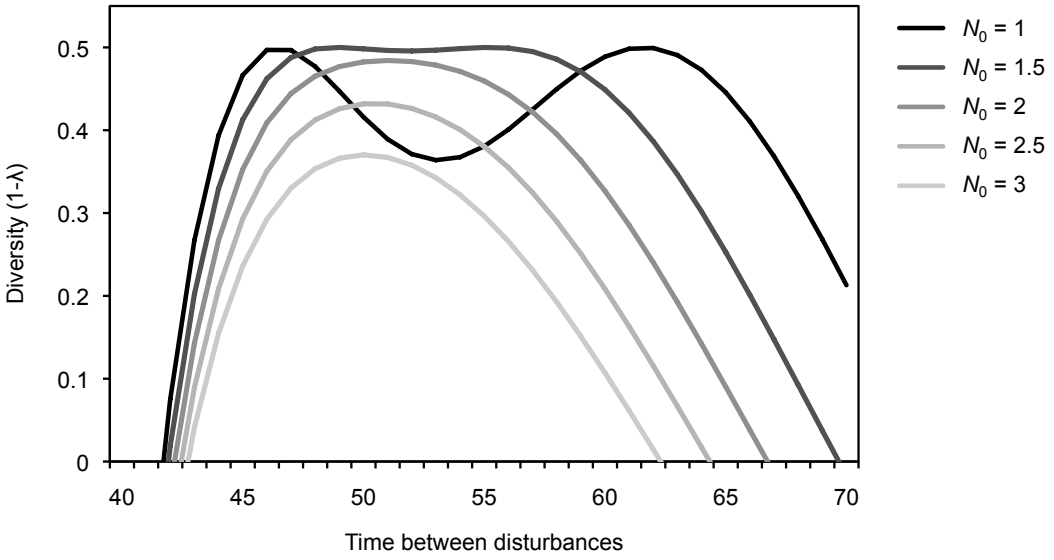
ELECTRONIC SUPPLEMENTARY MATERIAL

The diversity-disturbance relationship in the model of Buckling *et al*. (2000) varies when the term describing population size after disturbance (N_0) is changed. This model is based on a classical Levene (1953) model and describes the growth of two types at two niches. The number of individuals produced by type *i* at niche *j* as a function of time between disturbances follows a logistic growth model:

$$N_{ij}(t) = \frac{K_{ij}}{1 + a_{ij}e^{-r_{ij}(t-T_{ij})}}$$

where K is the carrying capacity at a given niche, a_{ij} is a constant (equal to (K_{ij} - $N_{ij}(0)$)/$N_{ij}(0)$, where $N(0)$ is density at time 0), r is growth rate, t is time, and T is the acclimation period before growth restarts following disturbance (Buckling *et al*. 2000).

The figure below shows the same model as Buckling *et al*. (2000) for the case where competitive ability and disturbance tolerance are positively related (parameters for type 1 in niche 1 (W_{11}): r = 0.12, K = 650, T = 0; for type 2 in niche 2 (W_{22}): r = 0.155, K = 550, T = 10), but at a range of different values for N_0 (given in the legend; in all cases N_0 is the same at both niches). Diversity is given as the complement of Simpson's index as described in the main text.



References

Buckling, A., Kassen, R., Bell, G., Rainey, P.B. 2000. Disturbance and diversity in experimental microcosms. Nature **408**, 961-964.

Levene, H. 1953. Genetic equilibrium when more than one ecological niche is available. American Naturalist **87**, 331-333.

JOURNAL OF Evolutionary Biology

doi: 10.1111/j.1420-9101.2011.02434.x

Multiplicity of infection does not accelerate infectivity evolution of viral parasites in laboratory microcosms

A. R. HALL*, P. D. SCANLAN*, H. C. LEGGETT*† & A. BUCKLING*†

*Department of Zoology, University of Oxford, Oxford, UK
†College of Life & Environmental Sciences, University of Exeter, Cornwall Campus, Penryn, UK

Keywords:

coevolution;
coinfection;
experimental evolution;
Pseudomonas fluorescens;
recombination.

Abstract

Coinfection with multiple parasite genotypes [multiplicity of infection (MOI)] creates within-host competition and opportunities for parasite recombination and is therefore predicted to be important for both parasite and host evolution. We tested for a difference in the infectivity of viral parasites (lytic phage Φ2) and resistance of their bacterial hosts (*Pseudomonas fluorescens* SBW25) under both high and low MOI during coevolution in laboratory microcosms. Results show that MOI has no effect on infectivity and resistance evolution during coevolution over ~80 generations of host growth, and this is true when the experiment is initiated with wild-type viruses and hosts, or with viruses and hosts that have already been coevolving for ~330 generations. This suggests that MOI does not have a net effect of accelerating parasite adaptation to hosts through recombination, or slowing adaptation to hosts through between-parasite conflict in this system.

Introduction

Infections with multiple parasite species or genotypes are important drivers of epidemiology and evolution (Read & Taylor, 2001; Mideo, 2009). However, evidence for any general effect of multiplicity of infection (MOI) is mixed. For example, within-host competition may select for increased (de Roode *et al.*, 2005) or decreased (Smith, 2011) parasite virulence, depending on the biological details of the interaction between competing parasite genotypes (Chao *et al.*, 2000; Buckling & Brockhurst, 2008). MOI also creates opportunities for parasite recombination or reassortment (Malmberg, 1977; Turner & Chao, 1998; Poon & Chao, 2004), which may accelerate adaptation by creating novel combinations of beneficial mutations (Fisher, 1930; Muller, 1932; Frank, 2001), especially in coevolving communities where the direction of selection changes frequently (Bell & Smith, 1987; Nee, 1989; Gandon & Otto, 2007; Morran *et al.*, 2011). However, any benefit to recombination depends on several constraints, including the form of epistasis among beneficial mutations (Kondrashov, 1988; Charlesworth, 1990; Otto & Michalakis, 1998; Otto & Lenormand, 2002), and is probably only beneficial in some coevolutionary circumstances. In this paper, we ask whether changes in parasite infectivity and host resistance during antagonistic coevolution between viruses and bacteria are affected by MOI.

In experimental communities of the bacterium *Pseudomonas fluorescens* and lytic bacteriophage SBW25Φ2, reciprocal adaptation to viral infectivity and host resistance occurs rapidly *in vitro* (Buckling & Rainey, 2002; Brockhurst *et al.*, 2007). In this system, bacteria outnumber phage in typical experimental conditions, so that coinfection is probably rare. We tested whether changes in parasite infectivity (the ability to infect a range of different host genotypes) during coevolution were affected by periodic phases of growth and replication at high (≫1) MOI. High MOI might permit recombination in the parasite population (Turner & Chao, 1998; Springman *et al.*, 2005), potentially accelerating infectivity evolution (relative to coevolving communities maintained at low MOI) by bringing together beneficial mutations from different genomes. Alternatively,

Correspondence: Alex R. Hall, Institute of Integrative Biology, ETH Zürich, CH-8092 Zürich, Switzerland.
Tel.: +41 44 632 71 13; fax: +41 44 632 12 71;
e-mail: alex.hall@env.ethz.ch
Angus Buckling, College of Life & Environmental Sciences, University of Exeter, Cornwall Campus, Penryn TR10 9EZ, UK.
Tel.: +44 1326 255 794; fax: +44 1326 371 829;
e-mail: a.j.buckling@exeter.ac.uk

recombination may provide no selective advantage if beneficial combinations of mutations appear rapidly by mutation alone. High MOI could even slow infectivity evolution if adaptation to growth in mixed infections compromises viral fitness during single infections (Turner & Chao, 1998; Chao *et al.*, 2000). We predicted that any positive effect of recombination would be most pronounced during the later stages of coevolution (after hundreds, rather than tens, of generations), because directional changes in viral infectivity slow down over time (Buckling & Rainey, 2002; Hall *et al.*, 2011a), suggesting that beneficial mutations that increase host range become less frequent, either due to a finite mutation supply rate or costs associated with a broad host range (Poullain *et al.*, 2008; Benmayor *et al.*, 2009). Thus, if recombination at high MOI increases the frequency of beneficial combinations of mutations, it may be most effective at the later stages of coevolution when such combinations are in short supply.

We measured changes in viral infectivity and host resistance over ~80 generations of host growth at either high or low MOI in a serial transfer experiment. We controlled MOI by changing the ratio of host cells to virus particles during a 2-h period of viral growth between each transfer. We did this over both the initial (0–12 transfers) and later (50–62 transfers) stages of coevolution. We also tested for an effect of MOI during phage adaptation to a nonevolving host population, to see whether any effect on phage adaptation was specific to coevolving communities. Finally, we tested whether high MOI accelerated the generation of novel viral phenotypes by screening for changes in viral infectivity over a single period of growth at high and low MOI. Results show that (i) high MOI did not cause a detectable increase in the rate at which novel phenotypes appeared and (ii) MOI had no effect on the rate of infectivity evolution at any stage of coevolution.

Materials and methods

Strains and culture conditions

Coevolving communities comprised populations of the gram-negative bacterium *P. fluorescens* SBW25 and bacteriophage SBW25Φ2 in 6 mL of nutrient-rich growth medium (M9KB: M9 salt solution supplemented with 10 g L^{-1} glycerol and 20 g L^{-1} proteose peptone) in 28-mL glass vials incubated static at 28 °C. For coevolution experiments, we transferred 60 μL of each community to a fresh microcosm every 48 h (Buckling & Rainey, 2002; Lopez-Pascua & Buckling, 2008). For evolution experiments, where phages adapted to a nonevolving population of host bacteria, we isolated a sample of phage from each microcosm (vortex 1 min, add 10% chloroform, vortex 1 min, centrifuge at 11 000 *g* 3 min) and added 60 μL to a microcosm containing wild-type (WT) bacteria reconditioned from frozen stocks (Poullain *et al.*, 2008). Between consecutive transfers, phages underwent a 2-h period of growth at either high or low MOI as described below.

Manipulating MOI

We manipulated MOI between transfers by adding the same number of phages to different numbers of the WT bacteria, followed by 2 h of growth (Fig. 1). This was achieved by periodically measuring phage population densities and adjusting the volume that was added to fixed numbers of WT bacteria, in order to keep MOI > 1 and < 1 in high and low MOI treatments, respectively. We used pilot work to determine the appropriate volumes of phages and bacteria for high/low MOI over the first four transfers, and monitored phage densities (by diluting phage stocks and spot-plating on lawns of the WT host) every two transfers for the entire experiment. For each 2-h period of growth at low or high MOI, we reconditioned WT bacteria from frozen stocks, growing them overnight in shaken KB microcosms for 24 h. The stationary-phase density of these overnight cultures, as estimated by plating on KB agar, is ~10^9 cfu mL^{-1}, and we assumed that overnight cultures made on different days were of similar density. This allowed us to maintain the correct numbers of phages and bacteria during the 2-h growth phase between transfers, and to quantify MOI at every second transfer (Fig. S1). We assumed that there were no significant fluctuations in phage density at the intervening transfers where we did not measure them.

We carried out two separate coevolution experiments, each for 12 transfers: one was initiated with WT bacteria and phage (T0–12 coevolution), and the other was initiated with bacteria and phages that had already coevolved for ~330 generations of host growth [T50–62 coevolution; (Hall *et al.*, 2011a)]. For T0–12 coevolution, ~10^8 cells of bacteria and 10^5 phage particles were added to six replicate microcosms in each treatment (high and low MOI). For T50–62 coevolution, we used bacteria and phages from a long-term coevolution experiment (Hall *et al.*, 2011a): bacteria and phages from six replicate selection lines that had already coevolved for 50 experimental transfers (~330 host generations) were used to initiate six selection lines in each MOI treatment (high and low), creating a paired design where Replicate A, MOI = high was initiated with the same hosts and viruses as Replicate A, MOI = low. We also ran six selection lines where phages adapted to a nonevolving population of hosts (T0–12 evolution): at each transfer, the bacteria were replaced with WT bacteria from frozen stocks.

To determine whether high phage-to-bacteria ratio resulted in multiple infections, we mixed WT phage with a host-range mutant phage at an approximately equal ratio at high (5×10^8 mL^{-1}) and low (10^3 mL^{-1}) total phage densities. The WT phage infects only WT bacteria, whereas the host-range mutant infects both WT bacteria and an

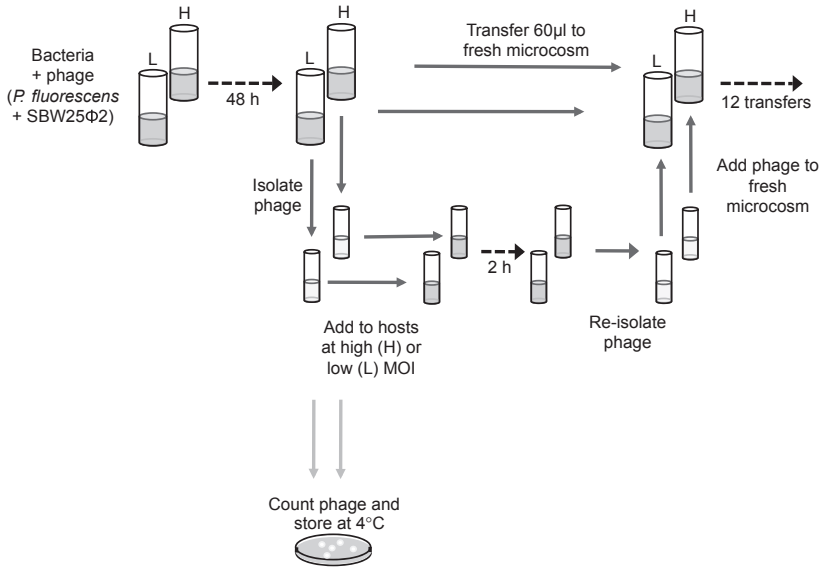


Fig. 1 Experimental manipulation of multiplicity of infection (MOI) between transfers during coevolution. Communities of bacteria and viruses were maintained for 12 transfers; between consecutive transfers we isolated viruses, grew them for 2 h with the wild-type (WT) host at either high (H) or low (L) MOI, re-isolated viruses, then added them to a fresh microcosm along with a sample from the preceding microcosm (to maintain the coevolving host population in coevolution treatments) or WT bacteria (to restore the original host population in evolution treatments). Phages in each population were counted every other transfer, allowing us to adjust dilution ratios to obtain the desired MOI in each treatment (see Fig. S1).

evolved resistant *P. fluorescens* clone (Scanlan *et al.*, 2011). Phage mixtures were allowed 10 min adsorption to WT bacteria (10^8 mL^{-1}) in 6 mL KB at 28 °C, then centrifuged for 3 min at 11 000 *g* to pellet the bacteria. Bacteria were resuspended in KB and filtered to remove free phage. Before the bacteria lysed, we diluted and plated them onto separate KB agar plates with semi-soft overlay lawns of either WT or resistant bacteria, and incubated overnight. If individual bacteria were infected by both phage genotypes, lysed cells would produce plaques on both hosts; by contrast, if bacteria were infected by a single phage genotype, lysed cells would all produce plaques on the WT host, but only those infected by the host-range mutant would produce plaques on the resistant host, resulting in a high ratio of plaque-forming units on WT plates relative to resistant-host plates. We found that this ratio was approximately twice as large at low (mean ± SE = 2.12 ± 0.33, $n = 3$ independent populations) compared with high (mean ± SE = 0.96 ± 0.06, $n = 3$ independent populations) phage densities. This is consistent with single infections at low phage-to-bacteria ratio, with approximately half of the bacteria being infected by each phage genotype, and multiple infections at high phage-to-bacteria ratio, with both genotypes represented in most infections.

In this experimental system, the density of bacteria is approximately three orders of magnitude greater than that of phage during coevolution (Morgan & Buckling, 2006), making it unlikely that MOI was > 1 other than during the 2-h inter-transfer growth phase in our high MOI treatments.

Infectivity assays

We measured infectivity of viruses from different populations and time points by streaking assays, where each phage population is streaked across a perpendicular line of a bacterial host genotype growing on an agar plate. Any inhibition of host growth after 24 h is classed as infection, and infectivity of a given population is calculated as the proportion of host genotypes infected. Host genotypes were isolated from coevolving populations at the end of the experiment by reconditioning populations from frozen by 24-h growth at 28 °C in 6 mL M9KB, dilution and plating on M9KB agar, incubation overnight, picking independent colonies and growing them overnight in liquid culture. We quantified infectivity for each phage population every two transfers and compared high and low MOI treatments using a pairwise design: each phage population was assayed against 10 host genotypes from the same community at the same time point, and against 10 genotypes from the corresponding replicate community in the opposite treatment at the same time point. For example, phages from Replicate = A, MOI = high, Transfer = 6 (A-high-T6) were assayed against 10 host genotypes from A-high-T6 and 10 host genotypes from A-low-T6; likewise, phages from A-low-T6 were assayed against hosts from A-low-T6 and A-high-T6.

Screening for novel phenotypes

To test whether high MOI increased the frequency of novel beneficial phenotypes, we tested for the appearance of phage mutants that could infect any of six resistant host genotypes isolated from a previous coevolution experiment (Scanlan *et al.*, 2011). We did this in 96 replicate phage populations that had undergone 2 h of growth at either high or low MOI with the WT host. To do this, we first generated 96 independent phage genotypes by picking 96 plaques of WT phage, amplifying for 1 h with the WT host (plaques added to $\sim 10^7$ bacteria and re-isolated after 1.5 h, yielding $\sim 5 \times 10^5$ phage per mL), before adding each phage to WT hosts at either high or low MOI in a 96-well PCR plate, growing for 2 h at 28 °C, re-isolating phage by chloroforming as above, and adding 1 μL to a lawn of exponentially growing bacteria. Each phage was tested against six resistant hosts and the wild type, the latter confirming the presence of viable phage in every sample.

Screening for recombinant phage

We expected that growth at high MOI would lead to phage recombination. To screen for recombinants, we used three pairs of phenotypically distinct phages (phage A infects host A but not host B, whereas phage B infects host B but not host A). Phage pairs and hosts were taken from a large sample of phenotypically characterized clones from a long-term coevolution experiment (Hall *et al.*, 2011a). For each phage pair, we added a mixture of the two phages to the WT host at high or low MOI in the same conditions as during transfers in our experiment. We then screened for progeny that could infect both hosts A and B by isolating phages and adding 10 μL to an agar lawn containing a mixture of the two hosts.

Statistical analyses

We tested for a difference in infectivity between high and low MOI populations using paired *t*-tests at each transfer, adjusting for multiple comparisons by sequential Bonferroni correction. We tested for a difference in sympatric infectivity (measured against hosts from the same population at the same time point) between high and low MOI treatments using a mixed effects model with arcsine-transformed infectivity as the response variable, selection line as a random effect, transfer and MOI as fixed effects.

Results

High MOI does not accelerate infectivity evolution

Transfers 0–12: Manipulating MOI between each transfer had no significant effect on viral infectivity in our experiment (paired *t*-tests, $P > 0.05$ for every comparison; Fig. 2a). Similarly, there was no consistent difference in infectivity between high and low MOI treatments with nonevolving host populations (paired *t*-tests at each transfer, $P > 0.05$ except for T2 and T10: 0.003 and 0.022 respectively – only the former is significant after sequential Bonferroni correction; Fig. 2b). The same is true when infectivity is measured against sympatric hosts only (coevolved populations – $F_{1,10} = 0.40$, $P = 0.54$; evolved populations – $F_{1,10} = 3.49$, $P = 0.09$).

Transfers 50–62: the same pattern was observed when coevolving populations were initiated with bacteria and viruses that had already spent ~330 host generations coevolving. Specifically, there was no difference in viral infectivity between phage populations that had undergone periodic phases of replication at high and low MOI (paired *t*-tests at each transfer, $P > 0.05$ for every comparison; Fig. 3). Consistent with MOI having no effect on infectivity, we found no difference between groups for infectivity against sympatric hosts ($F_{1,10} = 0.002$, $P = 0.96$).

There was no effect of MOI on the evolution of host resistance, measured for a given host population at a given time point as the proportion of bacteria–phage

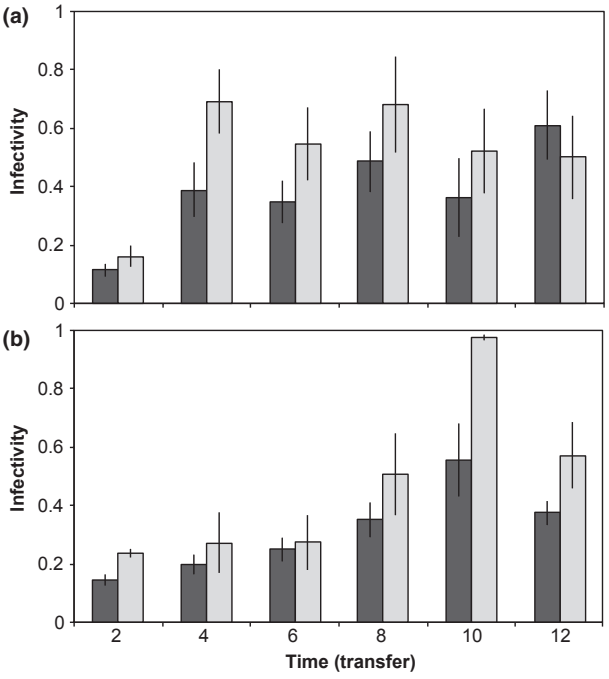


Fig. 2 Infectivity of phages from high (dark bars) and low (light bars) multiplicity of infection (MOI) treatments over time (T0–12). Infectivity of a given phage population is measured against 10 host clones from the same coevolving community at the same time point and 10 host clones from a community in the opposite (high or low) treatment. Bars show the average ± SE for six replicate selection lines in each treatment during (a) coevolution over transfers 0–12, (b) evolution over transfers 0–12.

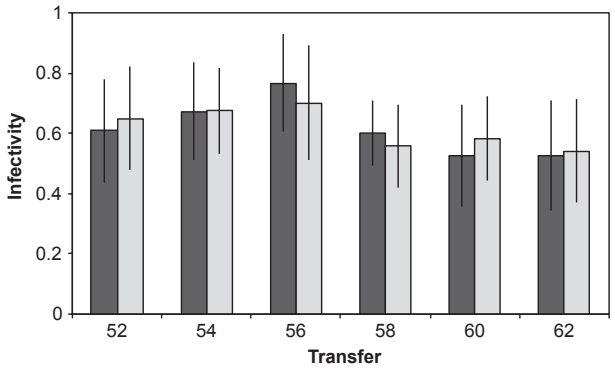


Fig. 3 Infectivity of phages from high (dark bars) and low (light bars) multiplicity of infection treatments over time (T50–62). In this experiment, coevolving communities were initiated with bacteria and phages that had already undergone 50 transfers of experimental coevolution. Infectivity is given as above; bars show mean ± SE.

interactions that did not result in infection (10 host clones tested against two phage populations). This was true for all three of our experiments (coevolution T0–12, evolution T0–12, coevolution T50–62; paired *t*-tests at each transfer are all nonsignificant after sequential Bonferroni correction; Fig. S2).

High MOI does not have a detectable effect on the generation of novel beneficial phenotypes

We grew 96 independent populations of phage at either high or low MOI before screening for novel infectivity phenotypes against six hosts that were resistant to the wild type. Each of these hosts was selected to be representative of a different phenotypic group from a wide range of host genotypes taken from a single coevolving population (Scanlan *et al.*, 2011). We did not observe any plaques after growth at high or low MOI, suggesting that the generation of novel combinations of beneficial mutations was not sufficiently greater at high MOI to be effective in our experiment.

Consistent with these findings, we did not observe hybrid progeny for any of the three phage pairs that we tested for recombination at high and low MOI. In summary, we were unable to demonstrate an effect of MOI on the appearance of recombinant phages or novel infectivity phenotypes across the limited number of host genotypes in our experiments.

Discussion

We tested for an effect of MOI on the evolution of parasite infectivity and host resistance during early and late stages of coevolution. We anticipated that our high MOI treatments would allow for recombination in the parasite population and therefore potentially accelerate the generation of novel infectivity phenotypes, leading to relatively rapid infectivity evolution during adaptation to a coevolving host population. We find no evidence that this occurred in our experiment, and separate experiments screening for novel infectivity phenotypes and hybrid progeny showed no effect of MOI. Despite this, viruses in coevolving communities of *P. fluorescens* and Φ2 undergo rapid increases in infectivity range, accumulating numerous mutations on a number of genes (Paterson *et al.*, 2010; Scanlan *et al.*, 2011), suggesting that recombination is not required for changes in the infectivity of viral parasites. Consistent with this, adaptation to bacterial hosts in our experiments resulted in phages that could infect a range of different genotypes, although host range did not differ consistently between high and low MOI treatments.

In our evolution experiments, where viruses adapted to nonevolving host populations, there was no consistent difference in infectivity between high and low MOI treatments, although at the second transfer low-MOI phages had significantly broader host ranges. This provides weak support for impaired infectivity evolution at high MOI early on, possibly due to a cost of adaptation to within-host competition that is recovered by second-site mutations later on in the experiment. However, given the lack of support for this mechanism in our other experiments, we do not take our results as indicating that high MOI impairs infectivity evolution. Directly comparing phages from evolution and coevolution treatments is not possible here because we necessarily measured infectivity against different hosts in the two experiments. However, previous work demonstrates that coevolved phages show much greater rates of genetic and phenotypic evolution than evolved phages (Poullain *et al.*, 2008; Paterson *et al.*, 2010), suggesting that any effect of MOI would in fact be more likely to be detected in the coevolution treatments.

We identify four possible scenarios that could explain our results:

1. No recombination; no effect of competition. Although recombination of related (T7) viruses has been demonstrated at high MOI (Springman *et al.*, 2005), and we found that manipulating the ratio of phage to bacteria caused a change in MOI, we cannot exclude the possibility that there was simply no recombination in our experiment. If it were also true that within-host competition was not strong enough to influence viral evolution, this would generate the observed lack of any effect of MOI on infectivity over time.
2. No recombination; undetected effects of competition. Even in the absence of recombination, within-host competition among viral genotypes could lead to changes in viral traits such as virulence or growth rate (Nee & Maynard Smith, 2000). For example, intra-host competition in RNA viruses has been implicated in the evolution of reduced viral fecundity (Dennehy & Turner, 2004). Recent data from *P. fluorescens* and Φ2 suggest that competitive interactions are also

important here: coinfecting viruses appear to evolve within-host anti-competitor mechanisms that are expressed as lower population growth rate when mixed infections are common (H.C. Leggett & A. Buckling, unpublished). We therefore suspect that within-host competition is important in *P. fluorescens*-Φ2 interactions, but does not influence the evolution of infectivity as measured here. This highlights a limitation of our study: we only tested for an effect of MOI on viral host range. Although it is well documented that this trait is under strong selection in this system (Buckling & Rainey, 2002; Brockhurst *et al.*, 2007; Hall *et al.*, 2011a) and that tradeoffs exist between population growth rates and host range (Poullain *et al.*, 2008), our experimental design does not account for changes in within-host replication independent of host range.

3. Recombination-competition balance. Turner & Chao (1998) found that viral recombination did not accelerate adaptation to a bacterial host, because the beneficial effects of recombination were countered by a cost of adaptation to intra-host competition during coinfection, meaning that derived viruses from recombinant selection lines had high fitness only during coinfection. We argue that this is unlikely to apply in our system because viruses evolved at high and low MOI do not appear to differ in fitness during single infections (H.C. Leggett & A. Buckling, unpublished), the conditions experienced by phages for most of their evolutionary history in this study, suggesting that competitive traits (interference) are not costly in our experimental scenario.
4. No benefit to recombination; no effect of competition. Recombination is predicted to accelerate adaptation by bringing together beneficial alleles, and this effect may be strongest in general for large populations and when mutations are frequent but of small effect (i.e. when N or U/s are large; (Crow & Kimura, 1965; Felsenstein, 1974). In support, Colegrave (2002) showed that the advantage of recombination in experimental populations of algae was greatest in large populations, whereas in small populations, adaptation was limited by the supply of beneficial mutations for sexual and asexual populations alike. We might therefore have predicted that, given the high mutation rate of viruses, recombination at high MOI would be advantageous. However, when mutations show positive epistasis, recombination will not accelerate adaptation because genotypes with multiple beneficial mutations will not be under-represented (Otto & Michalakis, 1998; Otto & Lenormand, 2002; de Visser & Elena, 2007). Previous work suggests that infectivity mutations in Φ2 are positively epistatic (Scanlan *et al.*, 2011): infectivity of a given host genotype often requires multiple mutations to be present simultaneously, such that individual mutations only confer infectivity in the presence of other mutations (Hall *et al.*, 2011b). We therefore argue that, given the high mutation rates of viruses, beneficial combinations of infectivity mutations were sufficiently common in coevolving populations in our experiment that recombination was not required for rapid adaptation. This scenario would lead to our observed results if intra-host competition is either ineffective [as in (1)], or has undetected effects [as in (2)].

In summary, manipulating MOI had no impact on changes in infectivity and resistance during coevolution. Although within-host competition can select for interference mechanisms in this system, this did not alter average levels of infectivity at the population level. Furthermore, we were unable to demonstrate recombination at high MOI, but given strong evidence that (i) recombination occurs in other phages (Turner & Chao, 1998; Springman *et al.*, 2005), (ii) it is important for viral evolution in general (Hendrix, 2002) and (iii) beneficial mutations in our experimental system show synergistic epistasis (Scanlan *et al.*, 2011), we suggest that recombination could have occurred in our high MOI treatments but was not required for rapid infectivity evolution. Whereas multiple infections are known to be common for some pathogens (Read & Taylor, 2001; Mideo, 2009), and comparative analysis suggests that recombination at high MOI is important for many phage species (Hendrix, 2002), key areas for future research include identifying the prevalence of multiple phage infections in nature and their role in coevolution with bacterial hosts.

Acknowledgments

We thank the editor and two reviewers for comments that helped to improve the manuscript. This research was supported by the European Research Council.

References

Bell, G. & Maynard Smith, J. 1987. Short-term selection for recombination among mutually antagonistic species. *Nature* **328**: 66–68.

Benmayor, R., Hodgson, D.J., Perron, G.G. & Buckling, A. 2009. Host mixing and disease emergence. *Curr. Biol.* **19**: 764–767.

Brockhurst, M.A., Morgan, A.D., Fenton, A. & Buckling, A. 2007. Experimental coevolution with bacteria and phage The *Pseudomonas fluorescens* – Φ2 model system. *Infect. Genet. Evol.* **7**: 547–552.

Buckling, A. & Brockhurst, M.A. 2008. Kin selection and the evolution of virulence. *Heredity* **100**: 484–488.

Buckling, A. & Rainey, P.B. 2002. Antagonistic coevolution between a bacterium and a bacteriophage. *Proc. R. Soc. Lond. B* **269**: 931–936.

Chao, L., Hanley, K.A., Burch, C.L., Dahlberg, C. & Turner, P.E. 2000. Kin selection and parasite evolution: higher and lower virulence with hard and soft selection. *Q. Rev. Biol.* **75**: 261–275.

Charlesworth, B. 1990. Mutation-selection balance and the evolutionary advantage of sex and recombination. *Genet. Res.* **55**: 199–221.

Colegrave, N. 2002. Sex releases the speed limit on evolution. *Nature* **420**: 664–666.
Crow, J.F. & Kimura, M. 1965. Evolution in sexual and asexual populations. *Am. Nat.* **99**: 439–450.
Dennehy, J.J. & Turner, P.E. 2004. Reduced fecundity is the cost of cheating in RNA virus Φ6. *Proc. R. Soc. Lond. B* **271**: 2275–2282.
Felsenstein, J. 1974. The evolutionary advantage of recombination. *Genetics* **78**: 737–756.
Fisher, R.A. 1930. *The Genetical Theory of Natural Selection*. Oxford University Press, Oxford.
Frank, S.A. 2001. Multiplicity of infection and the evolution of hybrid incompatibility in segmented viruses. *Heredity* **87**: 522–529.
Gandon, S. & Otto, S.P. 2007. The evolution of sex and recombination in response to abiotic or coevolutionary fluctuations in epistasis. *Genetics* **175**: 1835–1853.
Hall, A.R., Scanlan, P., Morgan, A.D. & Buckling, A. 2011a. Host–parasite coevolutionary arms races give way to fluctuating selection. *Ecol. Lett.* **14**: 635–642.
Hall, A.R., Scanlan, P.D., Lopez-Pascua, L.D.C. & Buckling, A. 2011b. Bacteria-phage coevolution and the emergence of generalist pathogens. *Am. Nat.* **177**: 44–53.
Hendrix, R.W. 2002. Bacteriophages: evolution of the majority. *Theor. Popul. Biol.* **61**: 471–480.
Kondrashov, A.S. 1988. Deleterious mutations and the evolution of sexual reproduction. *Nature* **336**: 435–440.
Lopez-Pascua, L.D.C. & Buckling, A. 2008. Increasing productivity accelerates host–parasite coevolution. *J. Evol. Biol.* **21**: 853–860.
Malmberg, R.L. 1977. Evolution of epistasis and advantage of recombination in populations of bacteriophage T4. *Genetics* **86**: 607–621.
Mideo, N. 2009. Parasite adaptations to within-host competition. *Trends Parasitol.* **25**: 261–268.
Morgan, A.D. & Buckling, A. 2006. Relative number of generations of hosts and parasites does not influence parasite local adaptation in coevolving populations of bacteria and phages. *J. Evol. Biol.* **19**: 1956–1963.
Morran, L.T., Schmidt, O.G., Gelarden, I.A., Parrish, R.C. & Lively, C.M. 2011. Running with the red queen: host–parasite coevolution selects for biparental sex. *Science* **333**: 216–218.
Muller, H.J. 1932. Some genetic aspects of sex. *Am. Nat.* **66**: 118–138.
Nee, S. 1989. Antagonistic coevolution and the evolution of genotypic randomization. *J. Theor. Biol.* **140**: 499–518.
Nee, S. & Maynard Smith, J. 2000. The evolutionary biology of molecular parasites. *Parasitology* **100**: S3–S18.
Otto, S.P. & Lenormand, T. 2002. Resolving the paradox of sex and recombination. *Nat. Rev. Gen.* **3**: 252–261.
Otto, S.P. & Michalakis, Y. 1998. The evolution of recombination in changing environments. *Trends Ecol. Evol.* **13**: 145–151.
Paterson, S., Vogwill, T., Buckling, A., Benmayor, R., Spiers, A.J., Thomson, N.R. *et al.* 2010. Antagonistic coevolution accelerates molecular evolution. *Nature* **464**: 275–278.
Poon, A. & Chao, L. 2004. Drift increases the advantage of sex in RNA bacteriophage Φ6. *Genetics* **166**: 19–24.
Poullain, V., Gandon, S., Brockhurst, M.A., Buckling, A. & Hochberg, M.E. 2008. The evolution of specificity in evolving and coevolving antagonistic interactions between a bacteria and its phage. *Evolution* **62**: 1–11.
Read, A.F. & Taylor, L.H. 2001. The ecology of genetically diverse infections. *Science* **292**: 1099–1102.
de Roode, J.C., Pansini, R., Cheesman, S.J., Helinski, M.E.H., Huijben, S., Wargo, A.R. *et al.* 2005. Virulence and competitive ability in genetically diverse malaria infections. *Proc. Natl Acad. Sci. USA* **102**: 7624–7628.
Scanlan, P.D., Hall, A.R. & Buckling, A. 2011. Genetic basis of infectivity evolution in a bacteriophage. *Mol. Ecol.* **20**: 981–989.
Smith, j. 2011. Superinfection drives virulence evolution in experimental populations of bacteria and plasmids. *Evolution* **65**: 831–841.
Springman, R., Badgett, M.R., Molineux, I.J. & Bull, J.J. 2005. Gene order constrains adaptation in bacteriophage T7. *Virology* **341**: 141–152.
Turner, P.E. & Chao, L. 1998. Sex and the evolution of intrahost competition in RNA virus Φ6. *Genetics* **150**: 523–532.
de Visser, J.A.G.M. & Elena, S.F. 2007. The evolution of sex: empirical insights into the roles of epistasis and drift. *Nat. Rev. Gen.* **8**: 139–149.

Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1 MOI in coevolving communities over time.

Figure S2 Host resistance at high (dark bars) and low (light bars) MOI in (A) coevolving communities over transfers 0–12, (B) evolving communities over transfers 0–12, (C) coevolving communities over transfers 50–62.

As a service to our authors and readers, this journal provides supporting information supplied by the authors. Such materials are peer-reviewed and may be re-organized for online delivery, but are not copy-edited or typeset. Technical support issues arising from supporting information (other than missing files) should be addressed to the authors.

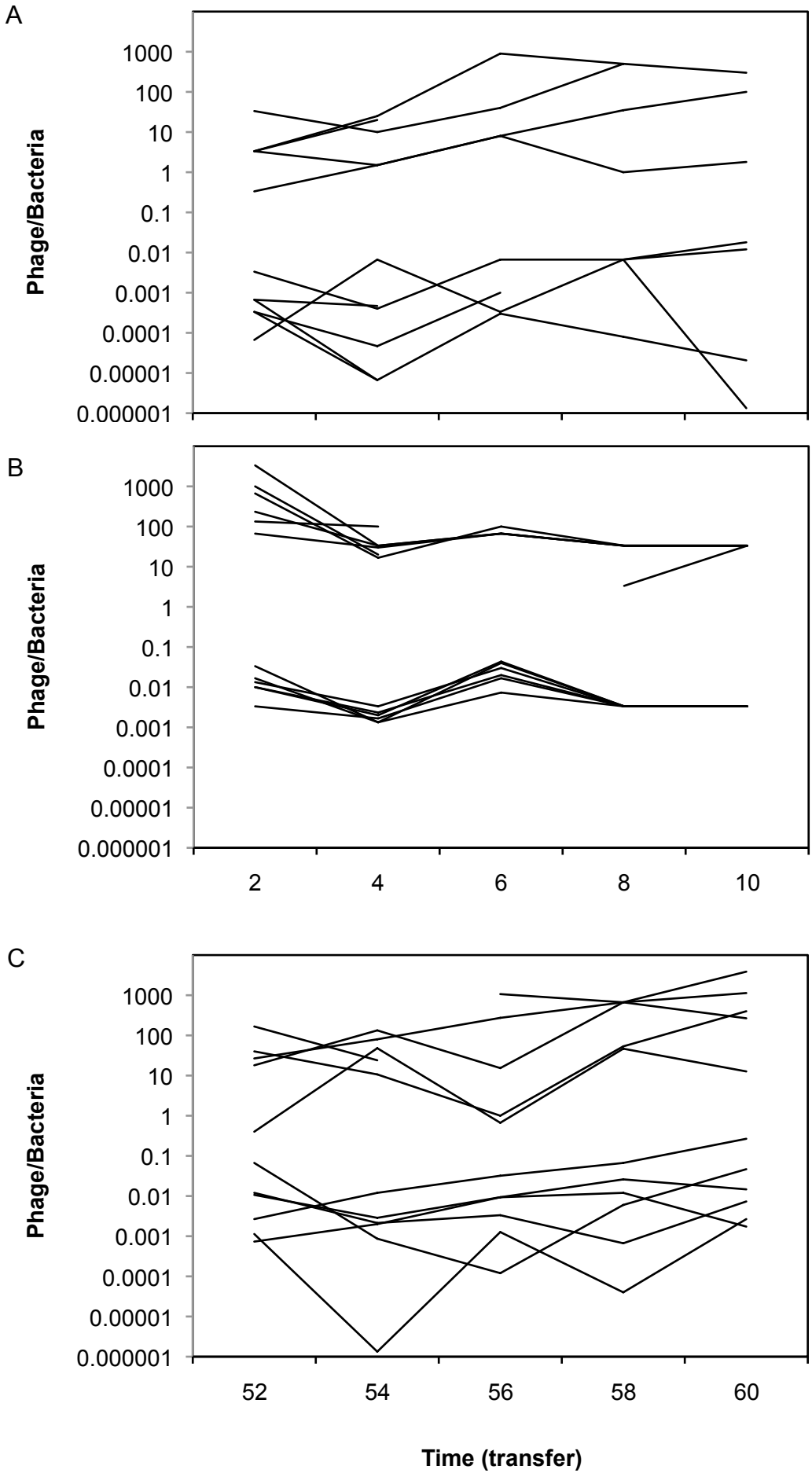
Data deposited at Dryad: doi: 10.5061/dryad.1fc37c3r

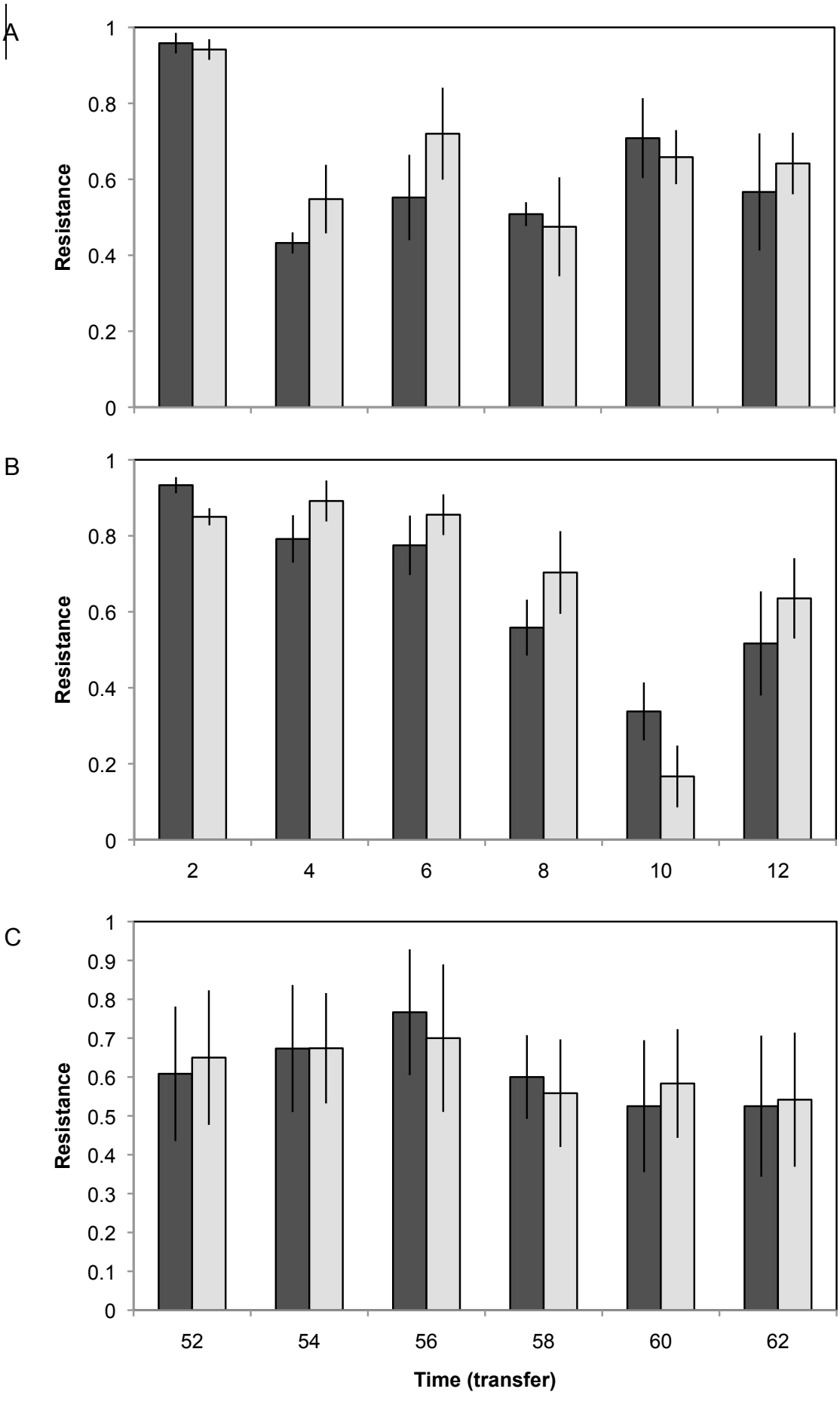
Received 15 September 2011; revised 4 November 2011; accepted 10 November 2011

SUPPORTING INFORMATION - Multiplicity of Infection does not Accelerate Infectivity Evolution of Viral Parasites in Laboratory Microcosms

Figure S1. MOI in coevolving communities over time. For each phage population, MOI is given as the ratio of phage to bacteria at the start of 2h of growth between experimental transfers, as estimated by diluting and plating phages every other transfer. Each panel shows 12 selection lines (six in each treatment: High MOI treatments consistently have MOI >> 1, while Low MOI treatments are <<1). Panels show phage during (A) coevolution over transfers 0-12, (B) evolution over transfers 0-12 and (C) coevolution over transfers 50-62.

Fig. S2. Host resistance at high (dark bars) and low (light bars) MOI in (A) coevolving communities over transfers 0-12, (B) evolving communities over transfers 0-12, (C) coevolving communities over transfers 50-62.


A
B
C
Phage/Bacteria
1000
100
10
1
0.1
0.01
0.001
0.0001
0.00001
0.000001
2
4
6
8
10
52
54
56
58
60
Time (transfer)


A
B
C
Resistance
Resistance
Resistance
1
0.8
0.6
0.4
0.2
0
0.9
0.7
0.5
0.3
0.1
2
4
6
8
10
12
52
54
56
58
60
62
Time (transfer)

PROCEEDINGS OF THE ROYAL SOCIETY B

Proc. R. Soc. B (2012) **279**, 1405–1411
doi:10.1098/rspb.2011.1627
Published online 12 October 2011

Promiscuity and the evolution of cooperative breeding

Helen C. Leggett[1,*], Claire El Mouden[1,2], Geoff Wild[3] and Stuart West[1]

[1]*Department of Zoology, University of Oxford, South Parks Road, Oxford OX1 3PS, UK*
[2]*Nuffield College, University of Oxford, New Road, Oxford OX1 1NF, UK*
[3]*Department of Applied Mathematics, The University of Western Ontario, London, Ontario, Canada N6A 5B7*

Empirical data suggest that low levels of promiscuity have played a key role in the evolution of cooperative breeding and eusociality. However, from a theoretical perspective, low levels of promiscuity can favour dispersal away from the natal patch, and have been argued to select against cooperation in a way that cannot be explained by inclusive fitness theory. Here, we use an inclusive fitness approach to model selection to stay and help in a simple patch-structured population, with strict density dependence, where helping increases the survival of the breeder on the patch. Our model predicts that the level of promiscuity has either no influence or a slightly positive influence on selection for helping. This prediction is driven by the fact that, in our model, staying to help leads to increased competition between relatives for the breeding position—when promiscuity is low (and relatedness is high), the best way to aid relatives is by dispersing to avoid competing with them. Furthermore, we found the same results with an individual-based simulation, showing that this is not an area where inclusive fitness theory 'gets it wrong'. We suggest that our predicted influence of promiscuity is sensitive to biological assumptions, and that if a possibly more biologically relevant scenario were examined, where helping provided fecundity benefits and there was not strict density dependence, then low levels of promiscuity would favour helping, as has been observed empirically.

Keywords: promiscuity; cooperative breeding; relatedness; dispersal; inclusive fitness

1. INTRODUCTION

It has been argued that monogamy or low levels of promiscuity have played a key role in favouring the evolution of cooperative breeding and eusociality. Hamilton's inclusive fitness theory [1–3] explains how cooperation can be favoured when it is directed towards relatives who also carry the gene for cooperation. Higher levels of promiscuity would decrease relatedness within family groups (figure 1*a*), which could therefore reduce selection for staying and helping in the natal family group [4]. Empirical support for this idea has come from the observations that (i) eusociality has only evolved in species with strict lifetime monogamy, and (ii) both the occurrence and amount of cooperative breeding in birds are negatively correlated with the level of promiscuity [5,7–9].

However, it is unclear whether greater promiscuity should always select against staying to help in natal groups. From an empirical perspective, some of the highest promiscuity rates ever recorded are cooperative breeders, such as the superb fairy wren and the Australian magpie [5]. From a theoretical perspective, previous predictions have been based on either verbal or heuristic models. More explicit models, which allow the action of selection to emerge as a consequence of demographic assumptions, have shown that an increased relatedness will not always favour cooperation because it can be negated by increased local competition between relatives (reviewed by West *et al.* [10] and Lehmann & Rousset [11]). Furthermore, some theoretical models make the opposite prediction, showing that because higher relatedness within groups leads to increased competition between relatives, selection can favour dispersal away from the natal group to reduce competition between relatives (figure 1*b*; e.g. [6,12–16]). Finally, a recent attempt by Nonacs [17] to explicitly model the effect of promiscuity on cooperation with population genetic simulations found that the level of promiscuity had little effect, or even a positive effect, on selection for cooperation, possibly suggesting that this is an area where inclusive fitness theory 'gets it wrong'.

Here, we use an inclusive fitness approach to model how promiscuity influences selection to stay and help in natal groups. Our aim is to consider the simplest possible scenario to explicitly examine how the level of promiscuity, through its effect on within-group relatedness, has multiple consequences. Our approach is therefore analogous to that taken by Taylor [18], when considering how limited dispersal influences cooperation. In particular, we examine how lower levels of promiscuity lead to higher within-group relatedness, which can, in turn, increase the indirect benefits of both staying to help relatives and dispersing to reduce competition among relatives [19]. In addition, we check the validity of our inclusive fitness model by comparing its predictions with that made by an individual-based model.

2. THE MODEL

We consider a population of cooperatively breeding, diploid individuals. To address the effect of promiscuity on cooperative breeding behaviour, we must assume

* Author for correspondence (helen.leggett@zoo.ox.ac.uk).

Electronic supplementary material is available at http://dx.doi.org/10.1098/rspb.2011.1627 or via http://rspb.royalsocietypublishing.org.

Received 2 August 2011
Accepted 21 September 2011

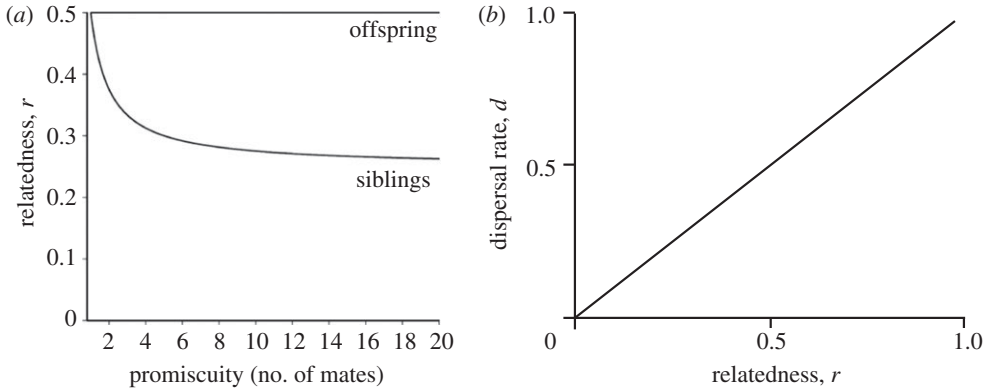


Figure 1. Promiscuity, relatedness and dispersal rate within family groups. (*a*) Promiscuity and relatedness. Promiscuity (number of mates) plotted against the mean genetic relatedness between potential helpers and both their siblings and offspring. An individual is always related to its offspring with $r = 0.5$. However, as promiscuity increases, the relatedness to siblings decreases from $r = 0.5$ to 0.25 (full-siblings to half-siblings) [4]; redrawn from [5]. (*b*) Higher relatedness within groups can lead to increased competition between relatives, which selects for dispersal away from natal groups to reduce competition between relatives [6].

that the individuals are sexual. In order to avoid complications of modelling different sexes, we assume that individuals are sexual hermaphrodites.

We assume that the population is subdivided into a very large, but fixed, number of breeding patches (say, p patches). Each patch is of equal quality and supports one adult breeder at a time, plus a large number of juvenile offspring. In nature, the cooperative behaviour of juvenile helpers can increase the breeder's survival and/or fecundity [20–22]. We focus on the former case, where the survival of the breeder is boosted by the presence of helpers. We avoid the latter case in order to avoid complications associated with the cascading effects that fecundity benefits exert on breeder fitness (more helpers result in more offspring, producing greater numbers of helpers, which in turn increases fecundity further, etc.) [23,24].

Since juvenile helpers often direct their help towards the individual(s) occupying the same patch on which helpers themselves were born (e.g. helpers help their own parents [25,26]), we assume that an offspring's tendency to help is linked to its decision to disperse. In other words, an offspring that remains on its natal patch does so in order to help the incumbent breeder, while an offspring that disperses from its natal patch will compete (without helping) on a new patch for the opportunity to breed, which occurs upon the (possible) death of a breeder. We explain our assumptions about the basis for an individual's 'tendency' to help (or 'decision' to not disperse) in greater detail below. For now, simply note that all helpers exert the same level of effort, and it is just this 'tendency' to help that varies among individuals in our model population.

Now suppose we observe the population described above at discrete, evenly spaced points in time. We assume that, between successive observations, the following series of five events occurs:

— *Mating*. Each breeder chooses exactly M mates from the global population of breeders. Mate choice occurs uniformly at random, and with replacement. Note that larger M indicates that breeders are more promiscuous.
— *Birth*. Each breeder produces a very large number (K) of offspring, and each offspring carries one chromosome pair. An offspring's 'maternally' inherited chromosome is one of the two chromosomes (chosen uniformly, at random) belonging to the local breeder, while the offspring's 'paternally' inherited chromosome is one of two chromosomes (chosen uniformly, at random) belonging to one of the breeder's M mates (chosen uniformly, at random with replacement). The local breeder, then, will be considered 'mother' to offspring produced on her patch, while the local breeder's mates will be considered potential 'fathers'. Of course, the local breeder will also be potential 'father' to offspring produced offsite, while the local breeder's mates will also be 'mother' to offspring produced on the patches they themselves occupy.
— *Dispersal*. Genes found on the chromosomes carried by an individual offspring determine d, the probability with which that individual disperses from its natal patch. We assume that maternally inherited and paternally inherited genetic information contribute equally and additively to the offspring's dispersal phenotype. Thus, we treat d as the mean of two phenotypes: one that would be produced by two identical copies of the maternally inherited gene, and a second that would be produced by two identical copies of the paternally inherited gene. We assume that offspring that disperse successfully find their new patch by choosing from among the p patches in the population uniformly at random. Note that there is a possibility that an offspring actually disperses to its natal patch, and in this case becomes a non-helping, individual-in-wait. Since offspring that do not disperse stay to help the incumbent breeder, the probability $1 - d$ quantifies an individual's aforementioned 'tendency' towards helping.
— *Helping*. Once the decision to help/disperse has been made, the direct benefit to the breeder can accrue. We assume that breeder mortality decreases at a rate proportional to the total number of helpers found on its patch immediately following dispersal. We assume that the constant of proportionality is small (say, of order $1/K$), and model the probability of breeder mortality with the function

$$\mu(\bar{d}) = \exp\{-k(1-\bar{d})\}, \tag{2.1}$$

where k is a positive constant that controls how quickly breeder mortality decays with increasing

numbers of helpers (larger k means helping is more effective at reducing mortality), and where $\bar{d}$ is the mean d among a breeder's current brood of offspring. Given this model for breeder mortality, we model the probability of breeder survival as

$$S_b(\bar{d}) = 1 - \mu(\bar{d}). \tag{2.2}$$

— *Competition for vacant patches*. If a breeder survives, then we assume it retains its patch, and breeds there again in the next time step. However, if a breeder does not survive, then we assume that competition for the vacant patch occurs among helpers (those native offspring that did not disperse) and non-helping individuals-in-wait (those non-native offspring that dispersed to the contested patch). Because a helper may compete for a breeding site more/less successfully than a non-helper owing to, for example, different territory inheritance patterns [21], costs of dispersal [27] or costs of helping [20], we introduce a positive constant c that allows the competitive ability of a non-helper to vary relative to that of a helper. When $c > 1$, a non-helper has a competitive advantage over a helper; when $c < 1$, a non-helper has a competitive disadvantage; and when $c = 1$, helpers and non-helpers are competitively equivalent. Mathematically, c acts as a competitive weight given to non-helpers, so that the terms

$$\frac{1}{\text{no. helpers} + c \cdot \text{no. non-helpers}}$$

and

$$\frac{c}{\text{no. helpers} + c \cdot \text{no. non-helpers}}$$

express the probability that a given helper and a given non-helper, respectively, secure the breeding site on which they compete.

When the competition phase is complete, all unsuccessful competing offspring die (this includes offspring competing on patches where the incumbent breeder survived), and the cycle repeats.

3. METHODS OF ANALYSIS AND RESULTS

We analysed the model using the neighbour-modulated (direct) fitness approach of Taylor and Frank [13,28,29]. For convenience, our inclusive fitness treatment assumes that the number of patches (p) and brood size (K) are both very large (ideally infinite). We make the standard suite of genetic assumptions (weak selection, additive gene action, etc.), discussed in detail elsewhere [23,28,30–33].

We show in the electronic supplementary material that, given the life-history assumptions described above, the marginal inclusive fitness of a focal juvenile with a reduced tendency to help (i.e. increased tendency to disperse) can be described by

$$\begin{aligned} \Delta W = {} & (1 - S_b)\left[\tfrac{(c-1)}{(1-d+cd)}\right] && \text{I} \\ & + \bar{R}S'_b && \text{II} \\ & + r(1 - S_b)\left[\tfrac{1}{(1-d+cd)}\right] && \text{III} \\ & - rS'_b, && \text{IV} \end{aligned} \tag{2.3}$$

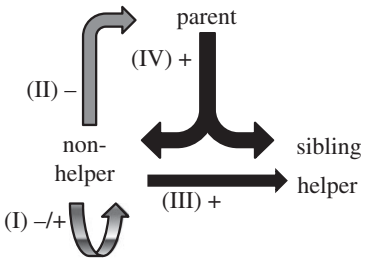


Figure 2. The costs and benefits of dispersing from the natal patch. Roman numerals relate to the lines of equation (2.3). − is a cost, + is a benefit: (I) − /+ is the direct cost/benefit associated with a decreased/increased chance of becoming a breeder; (II) − is the cost of decreasing parent survival; (III) + is the benefit of dispersing, leading to decreased competition among siblings; (IV) + is the benefit of decreasing parent survival, leading to increased chances of the helper or siblings becoming a breeder.

where S'_b is the marginal survival of the breeder (this is negative, since increased dispersal leads to reduced breeder survival), $\bar{R} = 0.5$ is the average relatedness between a juvenile and its parent, and r is the average relatedness between a focal juvenile and the average juvenile that competes on the focal juvenile's natal patch. The coefficient r can be written as $r = [(1-d)/(1-d+cd)]\ [(1/M) + 1]/4$, where M is the number of different mates a breeder has (i.e. M is the degree of promiscuity).

Equation (2.3) shows that the inclusive fitness effects of reduced helping (i.e. increased dispersal) are given by four components (figure 2):

(I) The direct fitness effect of reduced helping. That is, the difference between the focal individual's own probability of securing a breeding site in the case that it disperses (i.e. does not help), and the focal individual's own probability of securing a breeding site in the case that it does not disperse (i.e. does help). When $c > 1$, this term counts as a benefit, and when $c < 1$, this term counts as a cost.

(II) The reduction in adult breeder survival that is associated with reduced help. This term counts as a cost, since $S'_b = -k\mu < 0$.

(III) Reduced local competition due to the focal individual's increased tendency to disperse (i.e. decreased tendency to help). This is a benefit given to the average juvenile that competes on the focal juvenile's natal patch.

(IV) The benefit of increased opportunity to succeed the current breeder. When there is less help available to the breeder, adult survivorship is decreased, which in turn increases the probability that the local breeding site will become available to a juvenile competing on the patch. Again, this benefit is given to the average juvenile that competes on the focal juvenile's natal patch.

The sign of ΔW determines when reduced helping confers a selective advantage. Reduced helping is favoured when $\Delta W > 0$ and is disfavoured when $\Delta W < 0$. When $\Delta W = 0$, the population-wide level of helping is at an equilibrium. Simple algebra shows that the sign of ΔW is determined by a quadratic function of d, so it is possible to provide an exact expression for the equilibrium value

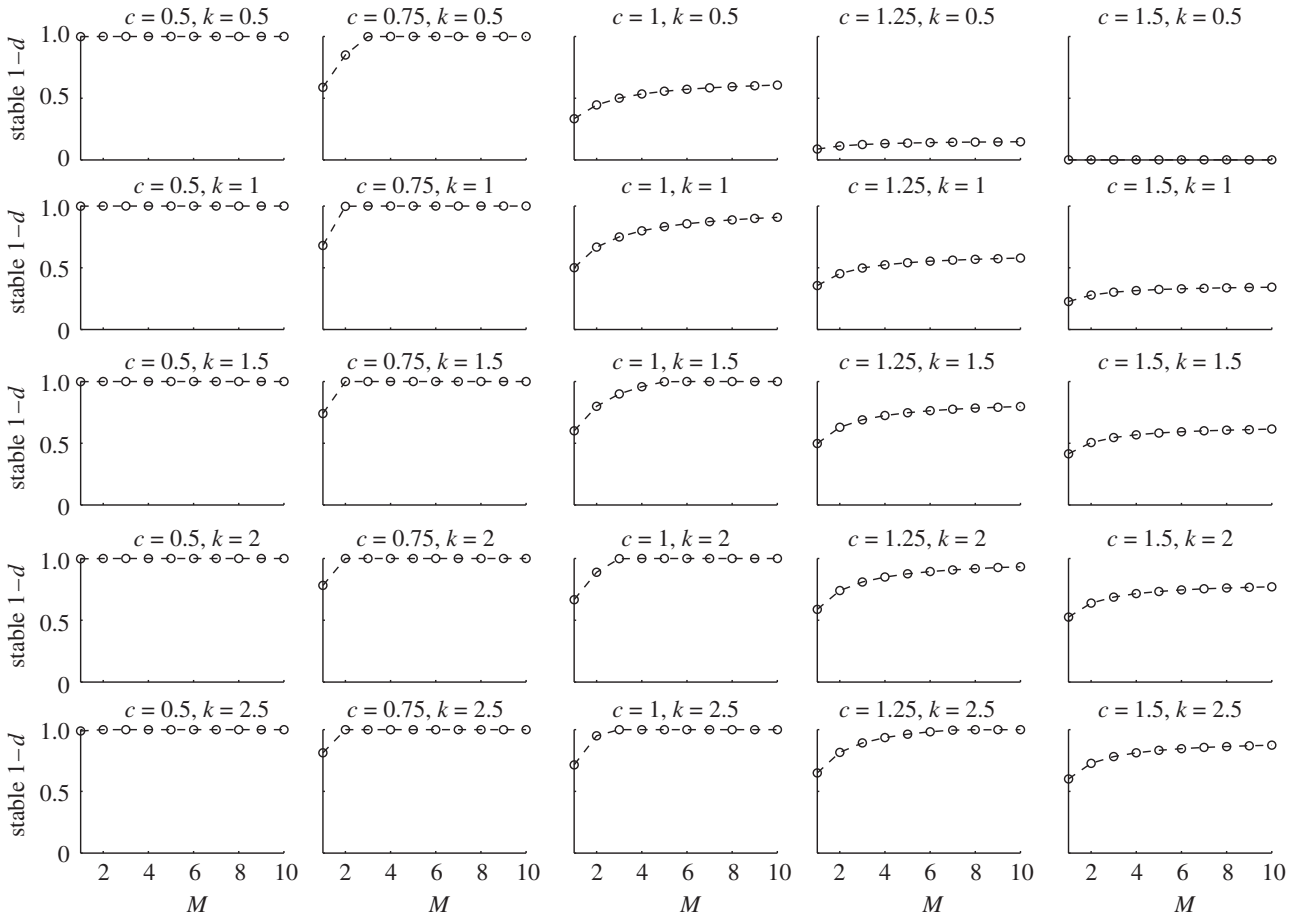


Figure 3. Helping and promiscuity. The predicted rate at which individuals stay and help ($1 - d$) is plotted against the level of promiscuity (M), for different rates of the competitive ability of non-helpers (c) and the survival benefit conferred upon breeders (k). The proportion of individuals selected to stay and help decreases with increased competitive abilities of non-helpers, c, and with decreased survival benefit conferred upon breeders, k.

of d, when it exists. That said, the expression for such a d is complicated, and its existence requires that a second complicated expression (in mathematical terms, the second expression is called the discriminant) be positive. To avoid complicated mathematical expressions that convey no biological insight, and to explore the possibility that long-term action of selection leads to non-equilibrium levels of behaviour, we turned to numerical simulation.

Our numerical procedure began by assuming a set of initial conditions that covered the entire range of phenotype space evenly. In this way, the numerical procedure simulated several evolutionary trajectories simultaneously. Any given trajectory was constructed by using the most recently observed population average d to calculate ΔW. When ΔW was positive (respectively negative) the population average d was increased (resptively decreased) by an amount that was proportional to ΔW itself. These two basic steps were repeated until all trajectories of a given simulation run were sufficiently close to one another.

We found that the predicted level of helping increased with increasing promiscuity (figure 3). Hence, the tendency to help juveniles born on the same patch increased with decreasing relatedness (from increasing promiscuity). The reason for this is that the rate of promiscuity only has consequences for lines III and IV in equation (2.3). Promiscuity does not influence either direct fitness consequences (line I) or relatedness to parent (line II). In contrast, lower levels of promiscuity lead to a higher relatedness to other helpers on a patch, which increases the indirect benefit of dispersing to reduce competition for relatives. We checked the validity of our inclusive fitness result with an individual-based simulation of the model system (see the electronic supplementary material for details of the assumptions and a version of the script used to generate simulation results), which gave the same results (figure 4).

4. DISCUSSION

Our model predicts that selection for staying to help (i) either shows no relationship or increases with higher levels of promiscuity, (ii) is decreased when non-helpers are more likely to inherit a territory (higher c), and (iii) increases when helping provides a greater survival benefit to the breeder (figure 3). In addition, we found close agreement between the predictions of our inclusive fitness analyses and an individual-based simulation (figure 4).

Our model shows that, even in a very simple scenario, the consequences of promiscuity for the level of cooperation can be far more complicated than expected from heuristic and verbal arguments (see equation (2.3) and figure 2). Previous theoretical arguments have focused on how promiscuity reduces relatedness to siblings, and hence reduces the indirect benefit of staying to help, leading to the prediction that promiscuity selects against helping (figure 1). However, when all the population level consequences of staying to help are explicitly modelled, there can also be indirect costs of staying to help. Specifically, staying to

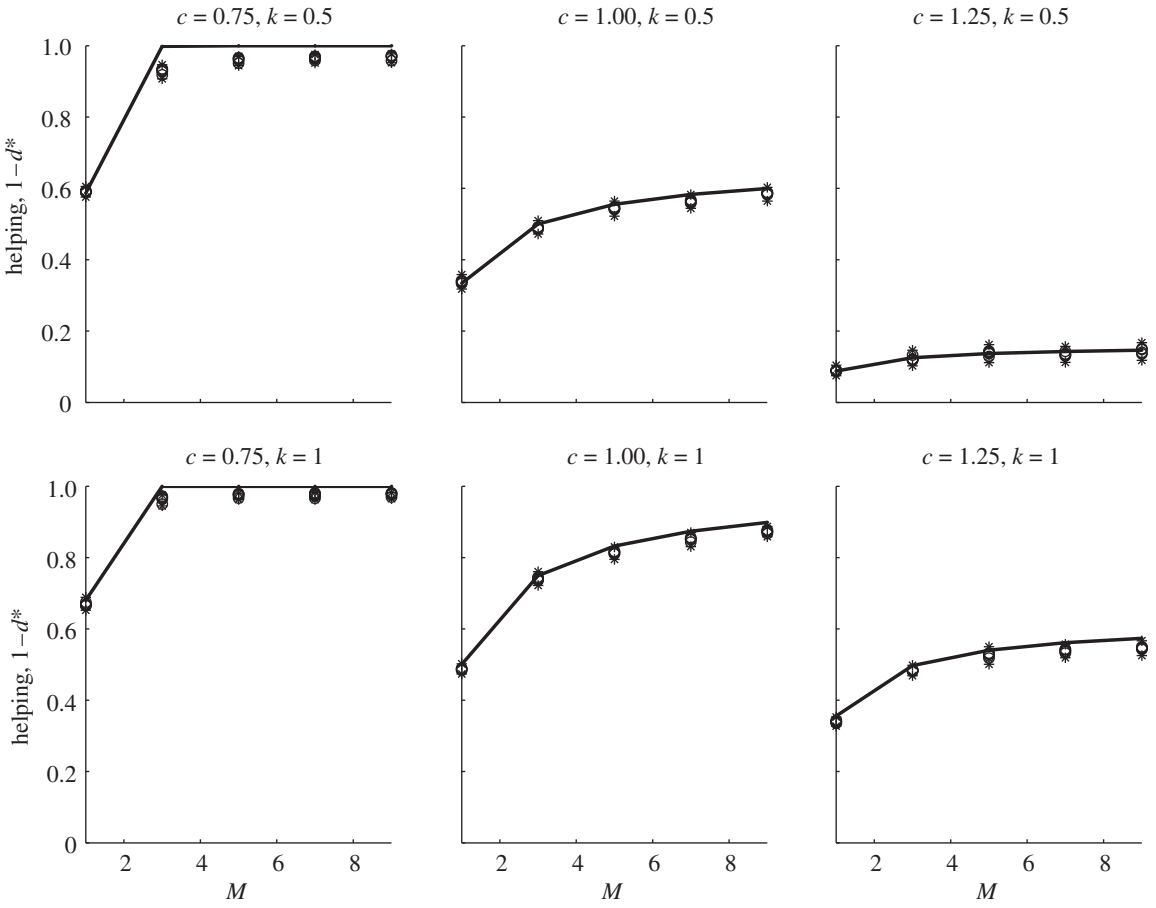


Figure 4. Inclusive fitness versus simulations. The predicted rate at which individuals stay and help ($1 - d$) is plotted against the level of promiscuity (M), for different rates of the competitive ability of non-helpers (c) and the survival benefit conferred upon breeders (k). The results obtained using inclusive fitness theory (solid lines) and individual-based simulations (dots) are in close agreement.

help increases the chance both that the breeder survives and that the helper inherits the patch if the breeder dies (lines IV and III of equation (2.3), respectively). Consequently, staying to help decreases the chance that another sibling who has stayed to help will inherit the patch.

Why does our model predict that promiscuity has either no effect or a positive effect on selection to stay and help (figures 3 and 4)? Equation (2.3) shows that in our model, staying to help has four fitness consequences: (I) direct cost of not dispersing to breed elsewhere; (II) indirect benefit of increased survival of parent; (III and IV) indirect cost of creating greater competition for non-dispersing siblings by reducing the likelihood that the breeder will die, and the helper staying to compete for the breeding spot if the breeder dies. The indirect benefit of staying to help (II) is not altered by promiscuity, because the helper is always related to their parent by $R = 0.5$. Consequently, the effect of promiscuity is driven by its indirect cost of making greater competition for siblings (III and IV). Lower levels of promiscuity lead to a higher relatedness between siblings, which favours dispersal to reduce the competition that these siblings face. Another way of looking at this is that low promiscuity does favour helping, but that the way to help siblings is to disperse away (to reduce competition), rather than to stay and help. This illustrates the general point that verbal arguments can be misleading because they focus on conspicuous traits and ignore less conspicuous consequences.

From a theoretical perspective, one of the most important points that arises from our model is that, even in a simple situation, things can be more complicated than expected. Specifically, competition between relatives can either negate or reverse predictions that follow from simple intuition. In this respect, our paper is analogous to when Taylor [18] showed that limited dispersal did not necessarily favour cooperation, because it could also lead to increased competition between relatives. A major theme since Taylor's pivotal paper has been examining exactly what kind of demographic factors lead to limited dispersal favouring cooperation (reviewed by West *et al.* [10] and Lehmann & Rousset [11]). Analogously, we are not saying that the idea that monogamy and low levels of promiscuity will favour cooperation is wrong, but rather saying that this requires certain biological assumptions, the biological relevance of which we discuss below. This illustrates Boomsma's point [7,8] that monogamy is necessary, but not sufficient for the evolution of eusociality.

A problem with our model is that by concentrating on survival benefits, it neglected one of the main aspects of helping that we had hoped to capture—the benefit to siblings from staying to help. Extending our model to include survival benefits to siblings did not change things (results not shown), because the strict density dependence means that this is cancelled out by the increased competition that it also creates. If dispersal

and helping were allowed to coevolve, we would also expect similar results, because dispersal reduces the kin-selected benefit of helping (line II of equation (2.3)), and hence selects for helping to be conditional upon not dispersing, as we have assumed [34]. We suggest that the best avenue for future research is to take an entirely different approach, and examine fecundity benefits in a population where there is not strict density dependence [23,24,35–37]. This task will be non-trivial, as even the simple scenario we considered here, with only survival effects and strict density dependence, led to a model that we had to solve numerically, rather than analytically. We suggest that this alternative approach captures a more biologically useful scenario than the one we modelled, and would lead to a negative correlation between selection to stay to help and promiscuity, as has been found in the empirical literature [5,7–9]. Noteworthy here is that the species where the benefits of helping are primarily on the survival of breeders, the superb fairy-wren, also has one of the highest levels of promiscuity [22].

Finally, our results contradict the possibility that the influence of promiscuity on the evolution of cooperation could be an area where inclusive fitness theory makes 'spurious predictions' [17]. Nonacs [17] found that a population genetic simulation predicted a flat or slightly positive relationship between promiscuity and helping, and contrasted this with arguments based on kin selection theory that promiscuity should select against cooperation (e.g. [4,5,7,8]). However, he did not also construct an inclusive fitness model, and so he was comparing scenarios with very different assumptions, rather than comparing different theoretical approaches (for a related example on the evolution of dispersal, see pp. 117–120 of [13]). We have found the same pattern as Nonacs with an inclusive fitness analysis, and also shown that when population genetic simulations are produced for the same scenario, they give the same results. This is not surprising, given the large literature showing the equivalence of different approaches [32,33,38–40]. More generally, the comparison of our simulation with inclusive fitness analyses supports the previous suggestion that the inclusive fitness approach will often be computationally simpler, and hence facilitate both the deriving of predictions and our conceptual understandings of why those predictions arise ([39], p. 671; [13], p. 120; [41], p. 119).

We thank Brad Anholt and Beth Rogers for organizing a workshop at Bamfield Marines Sciences Centre (during which this project began), Suzanne Alonzo, Peter Nonacs, Koos Boomsma, Charlie Cornwallis, Ashleigh Griffin and two anonymous referees for comments, and the BBSRC, Nuffield College, NSERC of Canada and ERC for funding.

REFERENCES

1 Hamilton, W. D. 1964 The genetical evolution of social behaviour. *Int. J. Theor. Biol.* **7**, 1–16. (doi:10.1016/0022-5193(64)90038-4)
2 Hamilton, W. D. 1970 Selfish and spiteful behaviour in an evolutionary model. *Nature* **228**, 1218–1220. (doi:10.1038/2281218a0)
3 Grafen, A. 2006 Optimization of inclusive fitness. *J. Theor. Biol.* **238**, 541–563. (doi:10.1016/j.jtbi.2005.06.009)
4 Charnov, E. L. 1981 Kin selection and helpers at the nest: effects of paternity and biparental care. *Anim. Behav.* **29**, 631–632. (doi:10.1016/S0003-3472(81) 80130-3)
5 Cornwallis, C. K., West, S. A., Davis, K. E. & Griffin, A. S. 2010 Promiscuity and the evolutionary transition to complex societies. *Nature* **466**, 969–991. (doi:10.1038/nature09335)
6 Hamilton, W. D. & May, R. M. 1977 Dispersal in stable habitats. *Nature* **269**, 578–581. (doi:10.1038/269578a0)
7 Boomsma, J. J. 2007 Kin selection versus sexual selection: why the ends do not meet. *Curr. Biol.* **17**, R673–R683. (doi:10.1016/j.cub.2007.06.033)
8 Boomsma, J. J. 2009 Lifetime monogamy and the evolution of eusociality. *Phil. Trans. R. Soc. B* **364**, 3191–3207. (doi:10.1098/rstb.2009.0101)
9 Hughes, W. O. H., Oldroyd, B. P., Beekman, M. & Ratnieks, F. L. W. 2008 Ancestral monogamy shows kin selection is key to the evolution of eusociality. *Science* **320**, 1213–1216. (doi:10.1126/science.1156108)
10 West, S. A., Pen, I. & Griffin, A. S. 2002 Cooperation and competition between relatives. *Science* **296**, 72–75. (doi:10.1126/science.1065507)
11 Lehmann, L. & Rousset, F. 2010 How life history and demography promote or inhibit the evolution of helping behaviours. *Phil. Trans. R. Soc. B* **365**, 2599–2617. (doi:10.1098/rstb.2010.0138)
12 Taylor, P. D. 1988 An inclusive fitness model for dispersal of offspring. *J. Theor. Biol.* **130**, 363–378. (doi:10.1016/S0022-5193(88)80035-3)
13 Frank, S. 1998 *Foundations of social evolution*. Princeton, NJ: Princeton University Press.
14 Gandon, S. & Rousset, F. 1999 Evolution of stepping-stone dispersal rates. *Proc. R. Soc. Lond. B* **266**, 2507–2513. (doi:10.1098/rspb.1999.0953)
15 Rousset, F. & Gandon, S. 2002 Evolution of the distribution of dispersal distance under distance-dependent cost of dispersal. *J. Evol. Biol.* **15**, 515–523. (doi:10.1046/j.1420-9101.2002.00430.x)
16 Ronce, O., Gandon, S. & Rousset, F. 2000 Kin selection and natal dispersal in an age-structured population. *Theor. Popul. Biol.* **58**, 143–159. (doi:10.1006/tpbi.2000.1476)
17 Nonacs, P. 2011 Monogamy and high relatedness do not preferentially favor the evolution of cooperation. *BMC Evol. Biol.* **11**, 9. (doi:10.5810.1186/1471-2148-11-58)
18 Taylor, P. D. 1992 Altruism in viscous populations: an inclusive fitness model. *Evol. Ecol.* **6**, 352–356. (doi:10.1007/BF02270971)
19 Wild, G. 2006 Sex ratios when helpers stay at the nest. *Evolution* **60**, 2012–2022. (doi:10.1554/06-028.1)
20 Heinsohn, R. & Legge, S. 1999 The cost of helping. *Trends Ecol. Evol.* **14**, 53–57. (doi:10.1016/S0169-5347(98)01545-6)
21 Griffin, A. S. & West, S. A. 2003 Kin discrimination and the benefit of helping in cooperatively breeding vertebrates. *Science* **302**, 634–636. (doi:10.1126/science.1089402)
22 Russell, A. F., Langmore, N. E., Cockburn, A., Astheimer, L. B. & Kilner, R. M. 2007 Reduced egg investment can conceal helper effects in cooperatively breeding birds. *Science* **317**, 941–944. (doi:10.1126/science.1146037)
23 Rousset, F. & Ronce, O. 2004 Inclusive fitness for traits affecting metapopulation demography. *Theor. Popul. Biol.* **65**, 127–141. (doi:10.1016/j.tpb.2003.09.003)
24 Wild, G., Gardner, A. & West, S. A. 2009 Adaptation and the evolution of parasite virulence in a connected world. *Nature* **459**, 983–986. (doi:10.1038/nature08071)
25 Hatchwell, B. J. 2009 The evolution of cooperative breeding in birds: kinship, dispersal and life history. *Phil. Trans. R. Soc. B* **364**, 3217–3227. (doi:10.1098/rstb.2009.0109)

26 Clutton-Brock, T. 2009 Structure and function in mammalian societies. *Phil. Trans. R. Soc. B* **364**, 3229–3242. (doi:10.1098/rstb.2009.0120)

27 Ronce, O. 2007 How does it feel to be like a rolling stone? Ten questions about dispersal evolution. *Annu. Rev. Ecol. Evol. Syst.* **38**, 231–253. (doi:10.1146/annurev.ecolsys.38.091206.095611)

28 Taylor, P. D. & Frank, S. A. 1996 How to make a kin selection model. *J. Theor. Biol.* **180**, 27–37. (doi:10.1006/jtbi.1996.0075)

29 Taylor, P. D., Wild, G. & Gardner, A. 2007 Direct fitness or inclusive fitness: how shall we model kin selection? *J. Evol. Biol.* **20**, 301–309. (doi:10.1111/j.1420-9101.2006.01196.x)

30 Taylor, P. D. 1989 Evolutionary stability in one-parameter models under weak selection. *Theor. Popul. Biol.* **36**, 125–143. (doi:10.1016/0040-5809(89)90025-7)

31 Rousset, F. & Billiard, S. 2000 A theoretical basis for measures of kin selection in subdivided populations: finite populations and localized dispersal. *J. Evol. Biol.* **13**, 814–825. (doi:10.1046/j.1420-9101.2000.00219.x)

32 Rousset, F. 2004 *Genetic structure and selection in subdivided populations*. Princeton, NJ: Princeton University Press.

33 Gardner, A., West, S. A. & Wild, G. 2011 The genetical theory of kin selection. *J. Evol. Biol.* **24**, 1020–1043. (doi:10.1111/j.1420-9101.2011.02236.x)

34 El Mouden, C. & Gardner, A. 2008 Nice natives and mean migrants: the evolution of dispersal-dependent social behaviour in viscous populations. *J. Evol. Biol.* **21**, 1480–1491. (doi:10.1111/j.1420-9101.2008.01614.x)

35 Alizon, S. & Taylor, P. 2008 Empty sites can promote altruistic behavior. *Evolution* **62**, 1335–1344. (doi:10.1111/j.1558-5646.2008.00369.x)

36 Grafen, A. 2007 Detecting kin selection at work using inclusive fitness. *Proc. R. Soc. B* **274**, 713–719. (doi:10.1098/rspb.2006.0140)

37 Taylor, P. D. & Grafen, A. 2010 Relatedness with different interaction configurations. *J. Theor. Biol.* **262**, 391–397. (doi:10.1016/j.jtbi.2009.10.012)

38 Gardner, A., West, S. & Barton, N. 2007 The relation between multilocus population genetics and social evolution theory. *Am. Nat.* **169**, 207–226. (doi:10.1086/510602)

39 Taylor, P. D. 1996 Inclusive fitness arguments in genetic models of behaviour. *J. Math. Biol.* **34**, 654–674. (doi:10.1007/BF02409753)

40 Queller, D. C. 1992 Quantitative genetics, inclusive fitness, and group selection. *Am. Nat.* **139**, 540–558. (doi:10.1086/285343)

41 Pen, I. & Weissing, F. J. 2000 Sexual selection and the sex ratio: an ESS analysis. *Selection* **1**, 59–69. (doi:10.1556/Select.1.2000.1-3.11)

Electronic Supplementary Material

Kin Selection Model

Here we show how we derived the marginal fitness of helpers (equation 3) used in the kin selection approach. We use d to denote the probability of dispersal for a focal offspring, $\bar{d}$ to denote the average level of dispersal from the natal patch, and d' to denote the population average level of dispersal. To calculate the probability that an offspring becomes a breeder, we note that a helper will share a patch with $(1-\bar{d})K$ other helpers and $d'cK$ non-helpers who dispersed to the patch. Thus, if the breeder dies, a focal helper will become a breeder with probability $[(1-d)]/[K((1-\bar{d})+d'c)]$. A non-helper will be one of $d'cK$ dispersers and will share the contested patch with $(1-d')K$ helpers. Thus if the breeder dies, all else being equal, a non-helper will become a breeder with probability $dc/[K(1-d')+Kd'c]$. Therefore, the probability of a focal offspring becoming a breeder is given by:

$$w_{AJ} = \left(1 - S_b\left(\bar{d}\right)\right)\frac{1-d}{K\left(\left(1-\bar{d}\right)+cd'\right)} + \left(1 - S_b\left(d'\right)\right)\frac{cd}{K\left(\left(1-d'\right)+cd'\right)} \quad . \qquad \text{(A1)}$$

As the notation in (A1) suggests, w_{AJ} can also be interpreted as the breeding-adult (A) component of fitness belonging to focal juvenile (J) individual.

There is also a juvenile (J) component to the fitness of a focal juvenile (J) individual, $w_{JJ} = Kw_{AJ}$. Note that this expression weights the focal individual's genetic contribution to its K locally produced offspring by 0.5, and weights the focal individual's genetic contribution to its K offspring produced on other patches by the same amount.

Similar fitness measures for adult focal individuals include, $w_{AA} = S_b(\bar{d})$ (for the breeding-adult component of fitness) and $w_{JA} = KS_b(\bar{d})$ (for the juvenile component of fitness). These fitness components are combined using the neutral reproductive values and

relative age-class frequencies, calculated as the dominant left and right eigenvectors, respectively, of the matrix,

$$W = \left(\begin{array}{cc} w_{JJ} & w_{JA} \\ w_{AJ} & w_{AA} \end{array} \right)_{d=\bar{d}=d'} = \left(\begin{array}{cc} 1-S_b(d') & KS_b(d') \\ (1-S_b(d'))/K & S_b(d') \end{array} \right) . \qquad \text{(A2)}$$

It is easy to show that the dominant eigenvalue of W in (A2) is one. It follows that the reproductive values of juveniles (J) and adults (A) are the elements of

$$\mathbf{v} = [\, v_J \;\; v_A \,] = [(1 - S_b(d'))/KS_b(d'), 1],$$

and the relative frequencies of juveniles (J) and adults (A) are the elements of

$$\mathbf{u} = [\, u_J \;\; u_A \,]^T = [\, K, \;\; 1 \,]^T .$$

Following Taylor and Frank's approach [27] we weight the juvenile and adult components of fitness to construct the fitness of a focal juvenile (J), as

$$W_J = v_J w_{JJ} u_J + v_A w_{AJ} u_J = \left(\frac{1-S_b(d')}{S_b(d')} + 1 \right) K w_{AJ} ,$$

$$= \left(\frac{1}{S_b(d')} \right) \left[\left(1-S_b(\bar{d})\right) \frac{1-d}{\left((1-\bar{d})+cd'\right)} + \left(1-S_b(d')\right) \frac{cd}{\left((1-d')+cd'\right)} \right] , \qquad \text{(A3)}$$

and the fitness of a focal adult (A) as

$$W_A = v_J w_{JA} u_A + v_A w_{AA} u_A$$

$$= \frac{1-S_b(d')}{KS_b(d')} K w_{AA} + w_{AA} = \left(\frac{1-S_b(d')}{S_b(d')} + 1 \right) w_{AA} = \frac{S_b(\bar{d})}{S_b(d')} . \qquad \text{(A4)}$$

The direction of selection acting upon the dispersal trait, d is given by the marginal fitness with respect to that trait. This is given by:

$$\frac{\mathrm{d}W}{\mathrm{d}d'} = \frac{\mathrm{d}W_J}{\mathrm{d}d'} + \frac{\mathrm{d}W_A}{\mathrm{d}d'} \qquad \text{(A5)}$$

These derivatives may be separated into partial derivatives, describing the effect of an individual's trait value, and the effect of the average trait value of its group, on the juvenile and adult class fitnesses, using the chain rule methodology of Taylor and Frank [27] (see also [28, 42]). This gives $\mathrm{d}W_J/\mathrm{d}d' = \partial W_J/\partial d + \bar{r}\,\partial W_J/\partial \bar{d}$ and $\mathrm{d}W_A/\mathrm{d}d' = \partial W_A/\partial d + \bar{R}\,\partial W_A/\partial \bar{d}$, where $\bar{r} = \mathrm{d}\bar{d}/\mathrm{d}d$ is the kin-selection coefficient of (whole-group) relatedness between a focal individual and other members of the juvenile class, and $\bar{R} = \mathrm{d}\bar{d}/\mathrm{d}d$ is the kin-selection coefficient of (whole-group) relatedness between a focal individual and members of the adult class [27, 43]. This yields an expression that is equal up to multiplication by a positive function of d' (namely $1/S_b(d')$) to the marginal fitness given in the main text (equation 3). Note that the main text uses the notation, $d' = d$.

Individual based simulation

We constructed an individual-based simulation of the model using the R2011a release of the MATLAB software package (MathWorks Inc., Boston, MA). The simulation was formulated on the same set of assumptions used in the kin-selection based model.

In our simulation, each breeder was initially assigned two random numbers that, respectively, corresponded to two dispersal phenotypes: one random number corresponded to the "maternally" inherited dispersal phenotype, and the other corresponded to the

68 "paternally" inherited dispersal phenotype. Each run of the simulation repeated the series of
69 events outlined in main text. However, in the simulation, dispersal and competition were
70 combined for the purpose of efficiency. Specifically, simulated competition for vacant
71 patches was hierarchical in nature, so that the winner of the patch was native (i.e. a helper)
72 with probability $\frac{1-Average\ d\ of\ Vacant\ Patch}{c(Population\ Average\ d)+1-Average\ d\ of\ Vacant\ Patch}$, and non-native (i.e. a non-
73 helper) from a specific foreign patch with probability
74 $\frac{Average\ d\ of\ Specific\ Foreign\ Patch}{c(Population\ Average\ d)+1-Average\ d\ of\ Vacant\ Patch}$. Once the natal patch of the winner was
75 established, the winner itself was identified by choosing randomly from among all the natal
76 offspring of that patch in such a way that the probability of an individual being chosen was
77 either $(1 - d)/(1 -$ winner's natal patch average $d)$, in the case that the winner was born on the
78 vacant patch, or d/winner's natal patch average d, in the case that the winner was not born on
79 the vacant patch.
80 Typical collections of 32 simulated evolutionary trajectories are presented as thin grey
81 lines in Figure A1. Trajectories were simulated for 3000 generations — long enough for an
82 equilibrium level of dispersal (say, d*) to become established. For each simulated trajectory
83 we fixed the number of patches at $P = 1000$, and we fixed the fecundity of each breeder at K
84 = 100 offspring. Smaller values of P and K tended to produce unacceptably large
85 fluctuations among runs; the larger values used provided us with a consistent estimate of d^*.
86 We investigated the relationship between the adaptive level of helping $(1 - d^*)$
87 predicted by simulation, and the extent of promiscuity in the model population (M). We
88 found that the predicted level of helping increased with increasing promiscuity (Figure 4).
89 Hence, the tendency to help juveniles born on the same patch increased with decreasing
90 relatedness (from increasing promiscuity).
91 Here we provide the Matlab script used to simulate the evolution of the helping trait, 1
92 – d. The particular script presented here generates 32 evolutionary trajectories, then plots

each trajectory alongside the average trajectory and an approximate 95 % confidence interval.

```
Paths=32;          % number of Paths to simulate
T=3000;            % number of generations to simulate
P=1000;            % number of patches
MutStep=0.001;     % mutation step
Data=NaN(Paths,T);

M=2;     % number of mates
K=100;   % number of offspring
c=1.5;   % parameter
k=1;   % parameter

Sb=@(d) 1-exp(-k*(1-d));

cede=632;
myStream = RandStream.create('mt19937ar','seed',cede);
          RandStream.setDefaultStream(myStream);

tic
parfor path=1:Paths
% use “for” in place of “parfor” here if missing Parallel Comp Toolbox

   % Initialize Population

    Popn=rand(2,P); % one individual per patch
                    % two chromosomes per individual

    for t=1:T
        mates=NaN(M,P); % stores index of each mate of each individual
        for i=1:P
            mates(:,i)=randsample(P,M,true); % "true"=sample w replacement
        end

        Offspring=NaN(K,2,P); % stores the genotypes of each offspring
        for i=1:P

            Fathers=randsample(M,K,true);
            PatContribs=randsample(2,K,true);
            MatContribs=randsample(2,K,true);

            for j=1:K
                Offspring(j,1,i)=Popn(PatContribs(j), mates(Fathers(j),
i));
                Offspring(j,2,i)=Popn(MatContribs(j), i);
            end

        end

        OffspringPhenotype=reshape(mean(Offspring,2),K,P);
        AvgPhenotype=mean(OffspringPhenotype);
        GlobalAvgPhenotype=mean(AvgPhenotype');

        ProbLocal=(1-AvgPhenotype)./(c*GlobalAvgPhenotype + (1-
AvgPhenotype));
        BreederSurvival=Sb(AvgPhenotype);
```

```
150          PopnNext=NaN(2,P);
151          for i=1:P
152              X=rand();
153              if (X > BreederSurvival(i))
154                  % Breeder Dies
155                  Y=rand();
156                  if (Y < ProbLocal(i))
157                      % Winner Comes from Local Patch
158                      PatchWinner=i;
159
160                      % Local Offspring Compete
161                      Winner=...
162                          randsample(1:K, 1, true, 1-
163  OffspringPhenotype(:,PatchWinner)');
164                  else
165                      % Patches Compete
166                      PatchWinner=randsample(1:P, 1, true, AvgPhenotype);
167
168                      % Offspring On Winning Patch Compete
169                      Winner=...
170                          randsample(1:K, 1, true,
171  OffspringPhenotype(:,PatchWinner)');
172                  end
173
174                  WinnersGenes=reshape(Offspring(Winner,:,PatchWinner),1,2);
175
176                  % Mutate Winner's Genes
177                  WinnersGenes=WinnersGenes + MutStep*randn(1,2);
178                  WinnersGenes(WinnersGenes <= 0) = MutStep;
179                  WinnersGenes(WinnersGenes >= 1) = 1-MutStep;
180
181                  % Update Next Gen Population Array
182                  PopnNext(:,i) = WinnersGenes';
183              else
184                  % Breeder Survives
185                  PopnNext(:,i)=Popn(:,i);
186              end
187          end
188
189      Popn=PopnNext;
190
191      % Record Data
192      Data(path,t)=mean(mean(Popn,2));
193
194      end
195  end
196  toc
197
198  MeanPath=mean(Data);
199  SE=sqrt(var(Data)/Paths);
200
201  % --- Plot Data ----
202
203  figure
204  hold on
205
206  for path=1:Paths
207      plot(1:T, 1-Data(path,:), 'Color', [0.8,0.8,0.8], 'LineWidth',0.5);
208  end
209
```

```
plot(1:T, 1-MeanPath, '-r', 'LineWidth',2);
plot(1:T, 1-MeanPath+1.96*SE, '--r');
plot(1:T, 1-MeanPath-1.96*SE, '--r');
%plot([0,T],[NumPred, NumPred], '--k', 'LineWidth',2);

axis([0,T,0,1])
xlabel('time', 'FontSize', 16, 'FontName', 'Arial')
ylabel('average level of helping (1-d)', 'FontSize', 16, 'FontName',
'Arial')
 title(['M = ', num2str(M), 'c = ', num2str(c), ' k = ', num2str(k)], ...
            'FontSize', 12)
box on
```

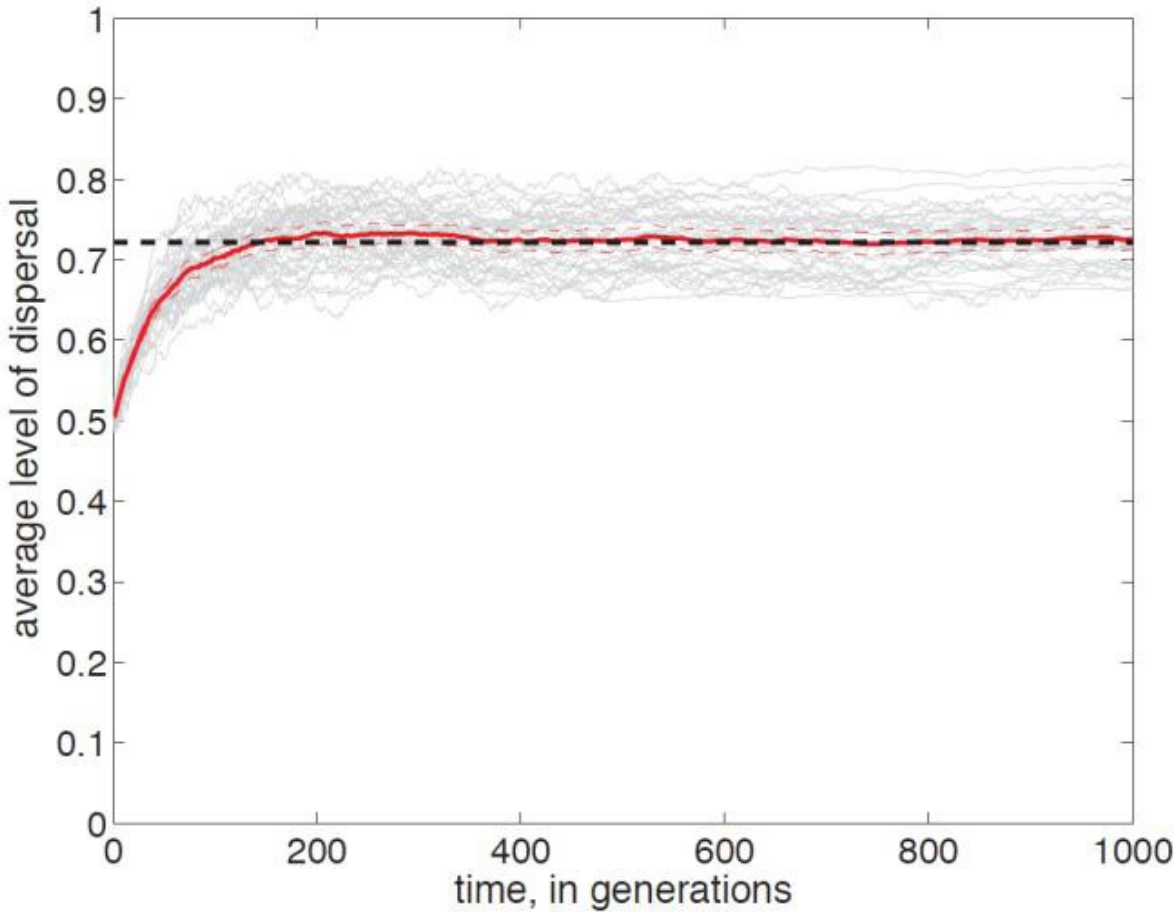


Figure A1: Level of helping over time. Typical collections of 32 simulated evolutionary trajectories are presented as thin grey lines. Red line indicates the mean of the runs. Dashed line indicates the equilibrium level of dispersal (d^*).

THE. END.