# The effects of parasite diversity on eco-evolutionary dynamics




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# Abstract

Virtually all interacting species (such as hosts and parasites) are embedded within diverse communities. However, evolutionary interactions are typically considered in a pairwise species framework. Although coevolutionary theory suggests that multiple species interactions may provide greater opportunities for diversification, the impacts of community diversity on coevolution have not been directly tested. In this thesis I synthesize the findings from recent experimental work to assess the effects of increased species diversity on the patterns and processes of host and parasite evolution. I then investigate the effects of parasite diversity on host–parasite population dynamics and evolution using the pathogen *Pseudomonas aeruginosa* and five lytic bacteriophage parasites in a brief evolution experiment. Parasite diversity was manipulated by assembling phage communities with different number of species. Phage communities suppressed host populations more rapidly but also showed reduced phage density, likely due to inter-phage competition. The evolution of resistance allowed rapid bacterial recovery that was greater in magnitude with increases in phage diversity. These results were then followed up via longer term experimental coevolution of the same host and parasite communities. Here the data showed that greater parasite diversity accelerates coevolutionary arms races and drives more diversification among lineages. Coevolution between hosts and parasite communities drove more successive increases in host resistance coupled with increasingly frequent selective sweeps at the genomic level. Consistent with this, the most rapidly evolving host genes under coevolution with parasite communities were those involved in various host resistance strategies. These results demonstrate, at phenotypic and genomic levels, how areas of high community diversity may be hotspots for rapid evolution in interacting, antagonistic species. Finally, In the face of escalating antibiotic resistance, there is now an urgent need to develop alternative antimicrobials, these results may be relevant to the application of phages as therapeutics and they are discussed in that context.

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# 1 Thesis Structure

## 1.1 Thesis Structure

### 1.1.1 Introduction; Host and parasite evolution in a tangled bank

**A. Betts**, C. Rafaluk, K. C. King, Host and Parasite Evolution in a Tangled Bank. *Trends Parasitol.* **32**, 863–873 (2016).

Based largely on my contribution to our publication in trends in parasitology earlier in 2016 this is a literature review of the relevant background information for my whole thesis.

Most hosts and parasites exist in diverse communities wherein they interact with other species, spanning the parasite–mutualist continuum. These additional interactions have the potential to impose selection on hosts and parasites and influence the patterns and processes of their evolution. Yet, host–parasite interactions are almost exclusively studied in species pairs. A wave of new research has incorporated a multispecies community context, showing that additional ecological interactions can alter components of host and parasite fitness, as well as interaction specificity and virulence. Here, we synthesize these findings to assess the effects of increased species diversity on the patterns and processes of host and parasite evolution. We argue that our understanding of host–parasite interactions would benefit from a richer biotic perspective.

I have included an addendum to root my thesis within the literature relevant to bacteria-phage interactions

### 1.1.2 Parasite diversity drives rapid host dynamics and evolution of resistance in a bacteria-phage system

**A. Betts**, D. R. Gifford, R. C. Maclean, K. C. King, Parasite diversity drives rapid host dynamics and evolution of resistance in a bacteria-phage system. *Evolution.* **70**, 969–978 (2016).

This chapter is also already published in Evolution and presents the results of a 24-hour experiment that served as the pilot for the remaining three chapters.

Host–parasite evolutionary interactions are typically considered in a pairwise species framework. However, natural infections frequently involve multiple parasites. Altering parasite diversity alters ecological and evolutionary dynamics as parasites compete and hosts resist multiple infection. We investigated the effects of parasite diversity on host–parasite population dynamics and evolution using the pathogen *Pseudomonas aeruginosa* and five lytic bacteriophage parasites. To manipulate parasite diversity, bacterial populations were exposed for 24 hours to either phage monocultures or diverse communities containing up to five phages. Phage communities suppressed host populations more rapidly but also showed reduced phage density, likely due to inter-phage competition. The evolution of resistance allowed rapid bacterial recovery that was greater in magnitude with increases in phage diversity. We observed no difference in the extent of resistance with increased parasite diversity, but there was a profound impact on the specificity of resistance; specialized resistance evolved to monocultures through mutations in a diverse set of genes. In summary, we demonstrate that parasite diversity has rapid effects on host–parasite population dynamics and evolution by selecting for different resistance mutations and affecting the magnitude of bacterial suppression and recovery. Finally, we discuss the implications of phage diversity for their use as biological control agents.

### 1.1.3 Parasite diversity accelerates coevolution

Here I test the effects of parasite community diversity on the nature of selection driving coevolutionary processes using an integrated phenotypic and genomic approach. The manuscript is in preparation for submission to Science.

Virtually all species are attacked by multiple parasites, but the evolutionary consequences of high parasite diversity are unclear. Here we used experimental coevolution of a host bacterium *Pseudomonas aeruginosa PA01* and communities of different viral parasites to investigate the effects of parasite diversity on coevolution. We show that greater parasite diversity accelerates coevolutionary arms races and drives more diversification among host lineages. Increasing parasite diversity accelerated the evolution of the host bacterium, resulting in increased resistance to parasites. Red Queen interactions in low parasite diversity communities generated negative frequency-dependent selection for phage resistance mutations. Increasing parasite diversity shifted the mode of coevolution towards host-parasite arms races that were driven by rapid selective sweeps of phage resistance mutations, most notably in LPS biosynthesis genes. Ultimately, high parasite diversity resulted in an increase in the rate of molecular evolution within host populations and genetic divergence between host populations. These results may help to explain why

regions of high parasite diversity are hotspots for rapid adaptation and speciation in interacting, antagonistic species.

### 1.1.4 The evolutionary consequences of phage cocktail diversity

This final data chapter compiles several results that have very interesting implications for the use of phages as therapeutics. The manuscript is in preparation for submission to Evolutionary Applications.

In the face of escalating antibiotic resistance, there is an urgent need to develop alternative antimicrobials such as "phage therapy" which uses viruses that infect and kill bacteria. While the clinical focus is on phage cocktails consisting of multiple viruses, most basic research uses phage monocultures. Consequently, we lack an understanding of the potential consequences of phage cocktail diversity for bacteria and phage evolution. Here, we address this gap by applying experimental evolution to the pathogen *Pseudomonas aeruginosa* and a panel of natural phage isolates, including assays with a commercially available cocktail. We hypothesised that phage diversity might influence resistance phenotypes, favouring broader resistance and we confirmed that such generalised resistance confers greater protection to an allopatric phage cocktail. The mechanisms through which generalised resistance is achieved include mucoidy, a clinically relevant phenotype associated with virulence. Finally, we show that coinfecting phages interact on a genomic level forming recombinant genotypes. This type of interaction could pose an interesting challenge to the therapeutic application of phages and we discuss how we can improve the design of phage cocktails in light of evolutionary biology.

### 1.1.5 Discussion and Future Work

This final closing chapter synthesise the findings presented in the preceding data chapters and discuss the scope for future work. I intend for these findings to lay the groundwork for a research fellowship that will ask how a focal host-parasite relationship is affected by the presence of an additional player.

The findings discussed in this thesis underline the importance of including a wider biotic context into experimental designs. Experimental investigations of host-parasite coevolution have almost entirely been explored using pairwise frameworks. Such designs are essential for a full understanding of the processes driving evolution. However, to understand the patterns we see in nature and make better predictions about them, we need to increase the complexity of our experimental systems. These experiments take an important step in that direction but are limited by their in vitro environment. To further enhance our understanding of bacteria-phage coevolution and make meaningful recommendations about phage cocktail design, similar experiments need to be conducted in vivo, during the phage therapy of an infected animal host.

*It is interesting to contemplate a tangled bank, clothed with many plants of many kinds, with birds singing on the bushes, with various insects flitting about, and with worms crawling through the damp earth, and to reflect that these elaborately constructed forms, so different from each other, and dependent upon each other in so complex a manner, have all been produced by laws acting around us.*

—Charles Darwin, On the Origin of Species (1859)

# 2

# Introduction; Host and parasite evolution in a tangled bank

## 2.1 Abstract

Most hosts and parasites exist in diverse communities wherein they interact with other species, spanning the parasite–mutualist continuum. These additional interactions have the potential to impose selection on hosts and parasites and influence the patterns and processes of their evolution. Yet, host–parasite interactions are almost exclusively studied in species pairs. A wave of new research has incorporated a multispecies community context, showing that additional ecological interactions can alter components of host and parasite fitness, as well as interaction specificity and virulence. Here, I synthesize these findings to assess the effects of increased species diversity on the patterns and processes of host and parasite evolution. I argue that our understanding of host–parasite interactions would benefit from a richer biotic perspective.

## 2.2 Host-Parasite Interactions

In nature, some hosts are attacked by communities consistently dominated by a single parasite species acting as the primary source of selection (1, 2). Other infected host species can be under simultaneous attack from predators and competitors, with communities of antagonists varying in composition across time and space (3). Multiple enemies can interact directly (e.g., competition between coinfecting parasites (4)) or indirectly via the host (e.g., parasites can increase host vulnerability to predation or reduce their competitive ability (5)), altering the evolutionary consequences across the entire community (Figure 2.1).

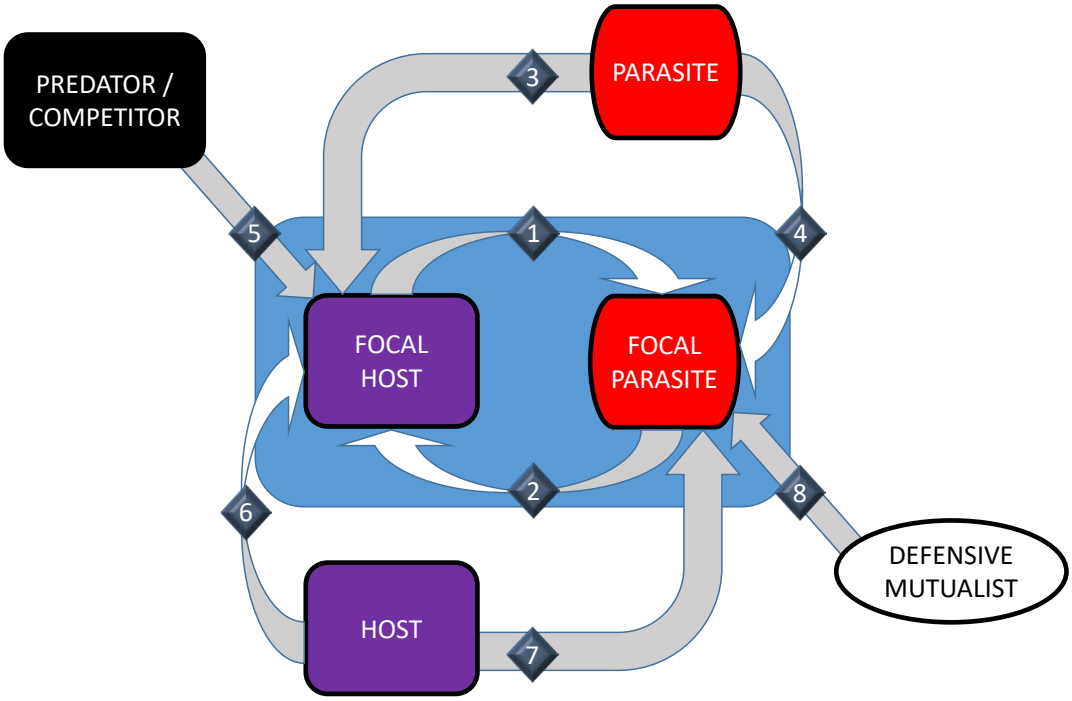



**Figure 2.1:** Focal relationship (1,2): the focal relationship between a host and a parasite, parasite growth, is a function of host population size and acts to reduce host growth. Any feedback that increases the growth of the focal parasite will decrease growth in the focal host and any feedback that increases host growth will increase parasite growth. Additional parasite species (3): an additional parasite may directly increase the growth of the focal parasite through synergistic means, perhaps a form of public goods cooperation; (4) alternatively, the new parasite may compete with the focal parasite for host resources and reduce its growth. Predator/competitor (5): a competitor or predator will decrease growth in the focal host, which will feedback into reduced growth of the focal parasite. Additional host species (6): the additional host species may increase growth of the focal host if it provides some form of social immunity; and (7) an additional host species may serve to fuel growth of the focal parasite population. Defensive mutualist (8): a defensive mutualist will reduce the growth of the focal parasite, which will feedback into increased growth of the focal host.

By sampling from natural populations over time, it is possible to make inferences about host-parasite coevolution in their full biotic context. This approach allows for time shift experiments (6), by freezing samples and comparing their infectivity or resistance with counterparts from other time points. The resultant matrix of infectivity and resistance over time allows the exploration of coevolutionary dynamics. However, as parasite diversity increases towards a "tangled bank", the coevolutionary dynamics may become much less obvious or a dominant dynamic may conceal less obvious dynamics occurring simultaneously at a lower frequency (7).

Koskella and Parr (8, 9) have sampled the microbiome of horse chestnut trees (*Aesculus hippocastanum*) alongside their parasitic bacteriophage communities over months, thus enabling the comparison of bacterial resistance between their contemporary phages and those from earlier months and across different trees. Some of the results (8) showed that from the phage's perspective, future bacteria are always more resistant. This pattern is consistent with arms race dynamics (ARD) which predict directional selection for increased resistance in hosts and increased infectivity in parasites (10). Interestingly, the same study system has previously produced patterns more consistent with fluctuating selection dynamics (FSD) (9). Under FSD, genotypes are under negative, frequency-dependent selection causing genotype frequencies to oscillate through time and producing patterns of local temporal adaptation (11). The authors provide several logical explanations for the different outcomes (8) such as the time window of the experiment being insufficient to detect a strong signal of local adaptation. However, laboratory investigations have shown that different parasites can display different coevolutionary dynamics with the same host under the same conditions (12). Such divergent evolutionary outcomes could arise as a consequence of coevolution within a wider community context where higher order ecological interactions or coevolution amongst parasites, as well as between hosts and parasites, could drive trait evolution.

When evolution occurs rapidly, the diversity of a community can influence the relative importance of ecological and evolutionary dynamics, especially in the face of multiple enemies (13). Theory predicts that if a single generalist phenotype evolves to provide resistance to multiple enemies, the ecological dynamics of a multi-enemy community may resemble a simple pairwise interaction (14). Increasing parasite diversity may allow such an outcome via diffuse coevolution, but only when there is genetic correlation between parasite resistance mechanisms and when the coevolutionary trajectories of the individual parasites are affected by the presence of others (15). If there is no genetic correlation between resistance mechanisms then only pairwise coevolution can occur allowing for independent coadaptation (16). The prevailing mode of coevolution, whether diffuse or pairwise, has implications for biological control, for example in "phage therapy" (17) where artificial communities of bacteriophage are assembled to control bacterial infection (18). If resistance to one phage confers resistance to others (19) then some of the advantages of phage communities over monocultures (20) could disappear. Such applications of biological control should be considered within a coevolutionary framework (21). Coevolutionary theory also has interesting implications for the maintenance of parasite diversity. ARD predicts the existence of universally resistant or infective hosts and parasites (22). The presence of such a 'super' generalist could result in the loss of diversity by disrupting the negative, frequency-dependent selection or kill the winner (23), dynamics which drive ecological turnover (e.g. the relative frequencies of host and parasite over time). In theory, this dynamic could facilitate the reduction of an initially diverse community of enemies to a simple pairwise interaction as some enemies dwindle towards extinction (24). For diversity to be maintained, some mitigating effect such as spatial structure (25) or fitness costs (26) must act to interrupt the rise of super hosts or parasites.

**Table 2.1:** Selection experiments demonstrating effects of additional species interactions on host and/or parasite evolution

| Additional Player | Focal Host | Focal Enemy | Trait Measured | | Result | Refs |
|---|---|---|---|---|---|---|
| | | | Host/prey | Other | | |
| | | **PARASITE** | | | | |
| Multiple phage | *Pseudomonas syringae* | Single phage | Resistance, cost of resistance | | Hosts evolving with multiple parasites parasites were no more resistant to allopatric parasites but paid a higher cost for their resistance | (44) |
| | *Pseudomonas aeruginosa* | Single phage | Resistance and breadth of resistance | | Broadest resistance range evolved in response to parasite diversity. | (4) |
| | | **PREDATOR** | | | | |
| *Tetrahymena thermophila* | *Serratia marcescens* or *Pseudomonas fluorescens* | Bacteriophage *Semad11* or Φ2 | Resistance | | Adding predators reduced resistance to parasites. | (45) |
| | *P. fluorescens* | Bacteriophage Φ2 | Resistance | Coevolutionary dynamics | Reduced signal of arms race dynamics. Reduced resistance overall due to subdivision in specialists | (46) |
| | *P. aeruginosa* | Bacteriophage *PNM* | Resistance, Virulence | | Phage + predator attenuated *P. aeruginosa* virulence less than phage alone. Greater phenotypic divergence. | (38) |
| | | **COMPETITOR** | | | | |
| *Aphis nerii* | *Danaus plexippus* (on milkweed *Asclepias* spp.) | *Ophryocystis elektroscirrha* | | Virulence, transmission | Addition of *A. nerii* increased *O. elektroscirrha* virulence and transmission. | (5) |
| | | **HOST** | | | | |
| Alternating between *Nicotiana benthamiana, Datura stramonium & Capsicum annuum* | *Nicotiana tabacum* | Tobacco etch poty-virus | | Genomics, fitness | Genomics revealed host specific mutations. Virus was fitter on sympatric hosts. No evidence of cost for generalists. | (47) |
| Distant hosts; *Brassica oleracea var. Bartolo* and *Phaseolus vulgaris var. Blanc Précoce* | Original hosts; *Solanum lycopersicum, S. melongena var. Zebrina & Geranium sanguineum var. Maverick Ecarlate* | *Ralstonia solanacearum* | | Genomics, fitness | Parasite increased in (sympatric) fitness on all hosts but biggest increase in distant hosts. Parallel evolution in a single gene led to increased fitness in beans. | (48) |
| | | **MUTUALIST** | | | | |
| *Hamiltonella defensa* | *Aphis fabae* | *Lysiphlebus fabarum* | | Infectivity | Protected hosts selected for increased infectivity. | (49) |
| *Enterococcus faecalis* | *Caenorhabditis elegans* | *Staphylococcus aureus* | | Mutualist-mediated protection | Defensive mutualist evolved increased protective ability. | (50) |
| *Wolbachia* | *Drosophila melanogaster* | Drosophila C virus | Resistance | | Host resistance allele at lower frequency in populations with defensive mutualist. | (51) |

In multi-species coinfections, parasites can interact either cooperatively (27) to the detriment of their host or competitively to detriment of one another (4). Although it is unclear to what extent these coinfections alter patterns of host and parasite evolution, experiments have shown that such interactions can have important consequences for parasite ecological dynamics (28). Exposing the three-spined stickleback *Gasterosteus aculeatus* host to the tapeworm *Schistocephalus solidus* affected the susceptibility of the host to superinfection by naturally occurring parasite communities resident in the lake where the experiment was performed. Some parasites were better able to infect hosts already infected by *S. solidus* whereas others found already infected hosts to be more resistant. These results were then confirmed by experimentation in a laboratory setting. The authors argue that further investigations manipulating parasite diversity are required to understand how inter-specific parasite interactions can shape parasite communities (29).

Combined selection by parasites and predators can alter the dynamics of host evolution and modify host phenotypes pleiotropically (30). There has been a recent effort to develop a theoretical framework to explore such interactions (31–35). These models have made links between ecological and evolutionary processes. For example, high predator density is predicted to select for lower virulence in pathogens of the prey (31), and combined selection by predators and parasites can drive the evolution of host diversity (34).

Whilst empirical evidence of the ecological effects of combined predation and parasitism is well documented (36, 37), experimental studies which also include an evolutionary perspective in a community context are still relatively uncommon (Table 2.1). Parasitism or predation of *Pseudomonas aeruginosa* (an opportunistic human pathogen) by the bacteriophage PNM or the protist *Tetrahymena thermophila* have been shown via experimental evolution (38) to select for *P. aeruginosa* phenotypes that show reduced virulence in wax moth larvae. Protist predation alone selects for reduced virulence relative to phage parasitism alone and combined selection by both results in an intermediate level of virulence between the two. Experiments have started to show the importance that pleiotropy can have for hosts and parasites (39). Detecting such effects has been made easier through advances in genomics (40) and will be crucial as experiments seek to elucidate the processes underlying coevolution with multiple parasites.

## 2.3 Bacteriophages as Biological Control Agents

We are rapidly approaching the post-antibiotic era, threatening both public health and food security. The chief medical officer Prof Dame Sally Davies warned in 2013 that the "antibiotic apocalypse" may already be under way and former prime minister David Cameron announced that "If we fail to act, we are looking at an almost unthinkable scenario where antibiotics no longer work and we are cast back into the dark ages of medicine". Unfortunately discoveries of new antibiotics are increasingly challenging (41) and there is a clear and urgent need for research into alternative antimicrobials (42). Phage therapy is one such alternative that is now actively being pursued (43).

Phage therapy is a biological control method using the natural virus parasites of bacteria (phages) to infect and kill harmful pathogens (17). There are several US phage products already licensed for use in the food industry (52) and clinical trials

for phage therapy in humans are now gaining momentum,(53, 54). Phages, are possibly the most numerous form of life on the planet (55). They are ecologically very important (56) and are responsible for killing an estimated 50% of the total bacterial population on earth every 48 hours (57). Their ability to depress bacterial populations was recognised early in the 20th century (58) when they were used to treat many diseases including staphylococcal skin infections, dysentery and the bubonic plague (59). Some of these original phage preparations are still used therapeutically in Georgia and Russia (60). However, the practice was largely abandoned in the West for the advantages that antibiotics offered, including their broad spectrum and ease of production (61, 62). Now with increasing antibiotic resistance in human pathogens (63); the advantage of antibiotics relative to phage therapy has reduced and phages are once again being considered as therapeutics (60, 64).

The use of phages as biological control agents involves applying massive doses of phage to provide effective suppression of bacterial growth (65). One of the hurdles facing modern phage therapy is the potential evolution of resistance by the target pathogen (66); phage therapy may simply replace one resistance problem with another. Researchers are keen to learn from the mistakes made with antibiotics to help prevent the evolution of resistance by using cocktails of different phage (67), and in some cases to specifically kill antibiotic resistant bacteria and restore the efficacy of existing therapeutics (17). One model by which phage therapy might work in the future involves the evolutionary training of phage (19) from a bank of quality controlled phage to quickly create a safe, effective cocktail against a target pathogen that can be redesigned upon the first signs of resistance (68).

## 2.4 Bacteria-phage Coevolution

The antagonistic coevolutionary process between bacteria and phage may be interpreted in the context of the Red Queen Hypothesis (69) driven by FSD; where species must constantly run in genetic space to maintain the same relative fitness position. Alternatively under ARD infectivity and resistance traits are under directional selection towards more infective parasites and more resistant hosts (70). Different phage isolates demonstrate the different dynamics to different extents, possibly due fitness costs of different resistance mechanisms (12), and the same phage can show different dynamics at different stages depending on how long they have been coevolving with a particular host (26).

To better understand the evolution of phage resistance, the bulk of early investigations of phage infectivity and host resistance coevolutionary dynamics focussed on a single bacterial strain coevolving with a single phage (71–74). However, the genetic diversity of hosts and parasites can have complex, yet more realistic, ecological and evolutionary consequences (75). By restricting the coevolutionary process to one host and one parasite, only pairwise coevolution between the two antagonists can be observed. However, by introducing multiple parasites or multiple hosts, diffuse coevolution may occur providing more useful insights as to how coevolution operates in natural communities as pairwise coevolution may be rare in nature (76). Pairwise coevolution can still occur in the presence of multiple parasites if the interactions between the host and the parasites are totally independent and there is no genetic overlap between the hosts resistance mechanisms to the different parasites (16). Diffuse coevolution is only said to occur when there is genetic correlation between

parasite resistance mechanisms and when the coevolutionary trajectories of the individual parasites are affected by the presence of another parasite (15).

According to the Red Queen hypothesis, diversity in bacterial host communities can be maintained by bacteriophage coevolution (9). There is evidence for this across host-parasite systems (77–79). These dynamics can prevent efficient competing genotypes or species from taking over completely (80). Given that phage infection hinges on phage-host attachment; as host density increases so does the chance of successful attachment and infection (81). Thus, if a particular species reaches a high enough density to support a phage epidemic then its population will be controlled by the phage (82) allowing less competitive bacteria to catch up. However, previous in-vitro work has suggested that bacteriophage can also destabilise communities if not all the members of the community are equally susceptible to phage attack (83). The effect of host diversity on community stability and coevolutionary dynamics has yet to be directly tested in a diverse, susceptible microbial population under phage attack.

Experimentation with multiple phages in a single host background (19, 67, 44, 84, 85) or mixed bacterial communities (23, 83, 86) has still yet to resolve directly the effects of parasite or host diversity on coevolutionary dynamics or ecological dynamics. These effects are potentially very important in an applied context for phage cocktails (87–90), where they may face diverse pathogen populations or communities (91–93). Furthermore, the extent to which diffuse or pairwise coevolution plays a role in bacteriophage communities is not known. The proposed work will compare the evolution between *P. aeruginosa* and bacteriophage communities and attempt to tackle these as yet unresolved, but fundamental issues in evolutionary biology and ecology.

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*Creationist critics often charge that evolution cannot be tested, and therefore cannot be viewed as a properly scientific subject at all.*
*This claim is rhetorical nonsense.*

— Stephen Jay Gould, Dinosaur in a Haystack (1995)

# 3

# Parasite diversity drives rapid host dynamics and evolution of resistance in a bacteria-phage system

## 3.1 Abstract

Host-parasite evolutionary interactions are typically considered in a pairwise species framework. However, natural infections frequently involve multiple parasites. Altering parasite diversity alters ecological and evolutionary dynamics as parasites compete and hosts resist multiple infection. We investigated the effects of parasite diversity on host-parasite population dynamics and evolution using the pathogen *P. aeruginosa* and five lytic bacteriophage parasites. To manipulate parasite diversity, bacterial populations were exposed for 24 hours to either phage monocultures or diverse communities containing up to five phages. Phage communities suppressed host populations more rapidly but also showed reduced phage density, likely due to inter-phage competition. The evolution of resistance allowed rapid bacterial recovery that was greater in magnitude with increases in phage diversity. We observed no difference in the extent of resistance with increased parasite diversity, but there was a profound impact on the specificity of resistance; specialized resistance evolved to monocultures through mutations in a diverse set of genes. In summary, we demonstrate that parasite diversity has rapid effects on host-parasite population dynamics and evolution by selecting for different resistance mutations and affecting the magnitude of bacterial suppression and recovery. Finally, we discuss the implications of phage diversity for their use as biological control agents.

## 3.2 Introduction

Evolution between hosts and their parasites is hypothesised to play a central role in several biological phenomena, including the maintenance of biodiversity (1) and virulence (2). Host-parasite evolutionary interactions are largely studied in a pairwise species framework, both empirically across a wide range of experimental systems (e.g.(3–7)) and theoretically (8, 9). However, in nature, most host-parasite interactions will be driven within a network of ecological interactions. For example, co-infection of a host individual by a diversity of parasite species (10) and competition between these parasites for host resources (11) are common. Evolutionary interactions between multiple parasites can also have important consequences for parasite transmission and virulence (12—15). Introducing multiple parasite species into the standard host-parasite evolutionary framework may thus provide useful insights as to how evolution operates in natural host-parasite communities (16, 17).

Here, we use experimental evolution to test for the effects of parasite diversity on host-parasite population dynamics and evolution using the bacterium *Pseudomonas aeruginosa* and its bacteriophages. *P. aeruginosa* is an opportunistic human pathogen. The strain used in these experiments (PAO1) was originally isolated from a burn wound infection from a patient in Melbourne (18). Contemporary strains of *P. aeruginosa* cause pneumonia in hospitals (19) and in particular cause mortality in patients with cystic fibrosis (20). Bacteriophages, the viral parasites of bacteria, are ideally suited to laboratory studies of evolution (21). Bacteriophages are the most numerous form of life on the planet (22). They are ecologically important as they are responsible for killing an estimated 50% of the total bacterial population on earth every 48 hours (23). Their ability to depress bacterial populations was recognised early in the 20th century (24) when they were used to treat many diseases including staphylococcal skin infections, dysentery and the bubonic plague (25).

Host resistance to bacteriophages can arise through mutation or reduced expression of the receptor molecule (26). Changes in gene expression can be coordinated by quorum sensing molecules which reduce the expression of a bacteriophage receptor in the whole bacterial population (27). Lysogenic bacteriophage are known to provide resistance against superinfection (28) and lytic phage may also be capable of excluding superinfection by their competitors. For example the pilus binding phage LUZ19 down regulates LPS genes in PAO1 upon infection (29), potentially reducing the ability of any LPS binding competitor phage from entering the same cell. However, resistance is not a binary trait: phages can infect and replicate within partially resistant cells without having a large impact on bacterial growth (30).

Early investigations of phage-bacteria evolution focussed on single bacterial strains evolving with a single phage (31—35). However, diverse phage communities exist in the human microbiome (36-–38), in the environment (39, 40), and are artificially assembled for phage therapy (41, 42). While one study showed that multiple phages can engage in within-host competition giving an advantage to fast-replicating phage (43), the presence of additional enemies (predators, parasites or competitors) in the bacteria-phage pairwise framework has been shown to affect host populations and their evolution. Adding a resistant bacterial competitor to a pairwise phage-bacteria interaction resulted in greater suppression of bacterial growth, occasionally leading to host extinction, an observation not commonly made in bacterial monocultures (44). Similarly, combining bacteriophage into three-phage communities suppresses bacterial host growth more than the constituent phages can achieve alone (45). Multiple enemies may also shape the degree to which resistance is enemy-specific in

evolved host populations. For example combining the well-studied *P. fluorescens* SBW25 — Φ2 bacteriophage system with the predator, *Tetrahymena thermophila*, can select for divergent outcomes: either specialists, whereby hosts are good at resisting either the phage or predator (46) — or for a population of generalists which can resist both (47). Phage communities have also been shown to select for the same resistance specialist strategies as the single phage that make up the community rather than a generalist phenotype (48).

In our study, we manipulated phage diversity by mixing all combinations of a panel of five naturally-isolated lytic phages with their bacterial host, *Pseudomonas aeruginosa* PAO1, and allowed them to evolve in response to those phage combinations. We predicted that, relative to phage monocultures, increasingly diverse phage communities would (i) reduce phage density due to more intense phage competition in communities, (ii) be more effective at suppressing host growth, (iii) favour general, and perhaps costly, resistance mechanisms, and thereby (iv) reduce selection for resistance to phages. We found that diverse phage communities initially acted synergistically and reduced bacterial host population growth within the first three hours of interaction. This synergy between phages eventually transitioned into antagonism as phage community size reduced and host populations recovered most efficiently in diverse phage communities. Host populations rapidly evolved resistance across all phage community treatments, a finding confirmed by whole-genome sequencing, with more generalised resistance mechanisms selected for by the five-phage community and specific resistance to phage receptors evolving against phage monocultures. Finally, we found that resistance evolved as rapidly against a diversity of parasites as against a single parasite species, all within 24 hours. Our results indicate that diverse parasite communities shape host and parasite population dynamics and evolutionary responses. We further discuss the implications of our results for applying a diversity of phages as biological control agents.

## 3.3 Materials and Methods

### 3.3.1 Strains, storage and culture conditions

*Pseudomonas aeruginosa* PAO1 was used with a panel of five, field-isolated, lytic bacteriophage; PEV2, LMA2, 14/1, LUZ7 and LUZ19. These phages have previously been used separately in evolution experiments with *P. aeruginosa*, making them ideally suited for combination into experimental communities. LUZ19 is believed to rely on type IV pili for attachment (49, 50). The receptors for PEV2 and LUZ7 (classified as N4-like phages) are unknown, however in order to attach to their hosts, these phages may rely on either LPS (51) or NrfA (52), a membrane protein with homology to TadD (53) which is involved type IV pili formation (54). LMA2 and 14/1 are related to PB1 (55) which relies on LPS for attachment (56). Isogenic bacterial stocks were prepared by inoculating 15 mL tubes containing 3 mL King's B (KB) medium (57) with twice streaked bacterial colonies from a frozen (-80°C) stock. The tubes were incubated in a MaxQ 8000 (Thermo Scientific, Waltham, MA, USA) at 225 RPM for 24 hours at 37°C. In the absence of phage but otherwise the condition of this experiment, this would allow for 4-5 bacterial doublings before stationary phase but the lysis caused by phage makes accurate estimation of doubling time almost impossible. Previous work with these phages has shown that 24 hours is sufficient for considerable phenotypic change (58, 59).

Bacteriophages were purified from bacteria by using 0.1 vol. chloroform and centrifugation at 13,000 × g to kill and pellet the bacteria. Working bacteriophage stocks were prepared from frozen isogenic aliquots; a 1 $\mu$L loop was used to inoculate a 3 mL bacterial suspension ($OD_{600}$ = 0.3). This was incubated for 24 hours at 225 RPM and 37°C before purification and filtration using a 0.22 $\mu$m syringe filter. The filtrates were diluted in M9 buffer to 1×108 plaque forming units (PFU) mL-1 and stored at +4°C for experimental use.

### 3.3.2 Experimental design

All combinations of the five phages (one, two, three, four, and five-phage communities) were prepared such that the phages were at $5\times10^5$ PFU $mL^{-1}$. Mixed phage communities were prepared to a final titre of $5\times10^5$ PFU $mL^{-1}$ with each phage present at equal densities. These phages were then mixed with fresh, diluted overnight culture in KB. Each overnight culture was prepared from a separate colony and used to inoculate each replicate to a starting concentration of $5\times10^7$ colony forming units per mL (CFU $mL^{-1}$). These mixtures were added to 96-well plates, 200 $\mu$L per well, until there were six replicates of every possible phage combination and a phage-free control (where the phage stock was substituted for M9 buffer). The six replicates were split evenly across two plates and incubated at 37°C for 24 hours.

### 3.3.3 Measuring phage growth

Phage population size was measured at the end of the experiment for every treatment by counting plaque forming units (PFU), which provides a measure of viron density, using a soft agar overlay method (e.g. (31)).

### 3.3.4 Measuring bacterial growth

The experiment was conducted in a Synergy 2 plate reader (BioTek Instruments, Winooski, VT, USA). $OD_{600}$ was measured every 2.5 minutes for the duration of the 24 hour experiment. All populations were then frozen at -80°C. Endpoint CFU counts were also performed.

### 3.3.5 Measuring resistance

Evolved populations were streaked out on KB agar and incubated overnight at 37°C in a static incubator to present single colonies. These colonies were picked, streaked out for a second time, and similarly incubated to present single colonies. This process ensured that each colony was both isogenic and free of phage. Populations evolved with a single phage type were sampled by taking eight colonies each from the 12 populations (two populations per phage type, one from each plate). Six colonies were sampled from each of the 20 populations that evolved with three-phage types (two populations per phage combination, one from each plate). The

populations evolved with five-phage types and the phage-free controls were sampled by taking six from all six replicates of each.

These bacterial colonies were separately incubated for 24 hours in 5 mL tubes containing 3 mL KB. Then 100 $\mu$L from each tube was mixed separately with 2.9 mL of soft (0.75%) KB agar and poured over the surface of a (1.5%) KB agar plate. Separate 4 $\mu$L droplets of purified phage were then applied to this soft agar lawn, allowed to dry, and incubated for 24 hours, subsequently assessed for zones of inhibition to indicate susceptibility or resistance. Zones with no visible inhibition were scored as resistant (=1), with some visible inhibition were scored as partially resistant (=0.5), and with complete inhibition were scored as susceptible (=0). These data were then analysed as the proportion of clones that were resistant or susceptible to a given phage isolate.

### 3.3.6 Whole genome sequencing of evolved bacteria

A subsample of the twice-streaked bacterial colonies scored for resistance were grown in 3 ml KB in a 5ml tube overnight at 37°C to provide the starting material for the extraction process. DNA was extracted from 1ml of culture using a Dneasy Blood and Tissue kit (Qiagen, Inc., Chatworth, California, USA), quantified using the QuantiFluor dsDNA system (Promega, Madison, WI, USA) and the salt ratio verified by Nano Drop (Thermo Scientific, Waltham, MA, USA). Library preparation and sequencing was performed at the Wellcome Trust Centre for Human Genetics (Oxford, UK) on an Illumina MiSeq in a 2 $\times$ 300 paired end reaction using v3 reagents.

In total, an ancestral clone and 17 evolved clones were sequenced: six phage-free evolved clones (one from each replicate) from 24 hours to control for possible laboratory adaptation, one from each replicate of the five-phage treatment and one from the first replicate of each one-phage population. The sequenced clones were randomly selected from those also scored for resistance. Full details of the bioinformatics are provided in the supplementary materials.

### 3.3.7 Statistical analysis

Analysis and graphing was performed using JMP 10.0 (60). All data were analysed using ANOVA without transformation . All post-hoc contrasts were t-tests with a significance threshold of 0.05. Between-treatment final phage population size significance was analysed by ANOVA. The $OD_{600}$ data were analysed by calculating the area under the curve using the trapz function within pracma in R 3.1.1 (R Core Team 2013), and then ANOVA where the same model was used on two subsets of the data; 0-3 hours and 21-24 hours. These time windows captured the initial suppression of host growth and the recovery of host populations where growth of resistant mutants had exceeded the maximum reached before the populations were supressed. For the PFU data and the $OD_{600}$ data, the number of phage types in the community was treated as a categorical explanatory variable. The resistance of bacteria evolved with single, three and the five-phage communities to the five ancestral phages were compared in two separate ANOVA tests. The resistance of evolved bacteria were compared to their sympatric ancestral phage, then to all five phage types, both allopatric and sympatric.

## 3.4 Results

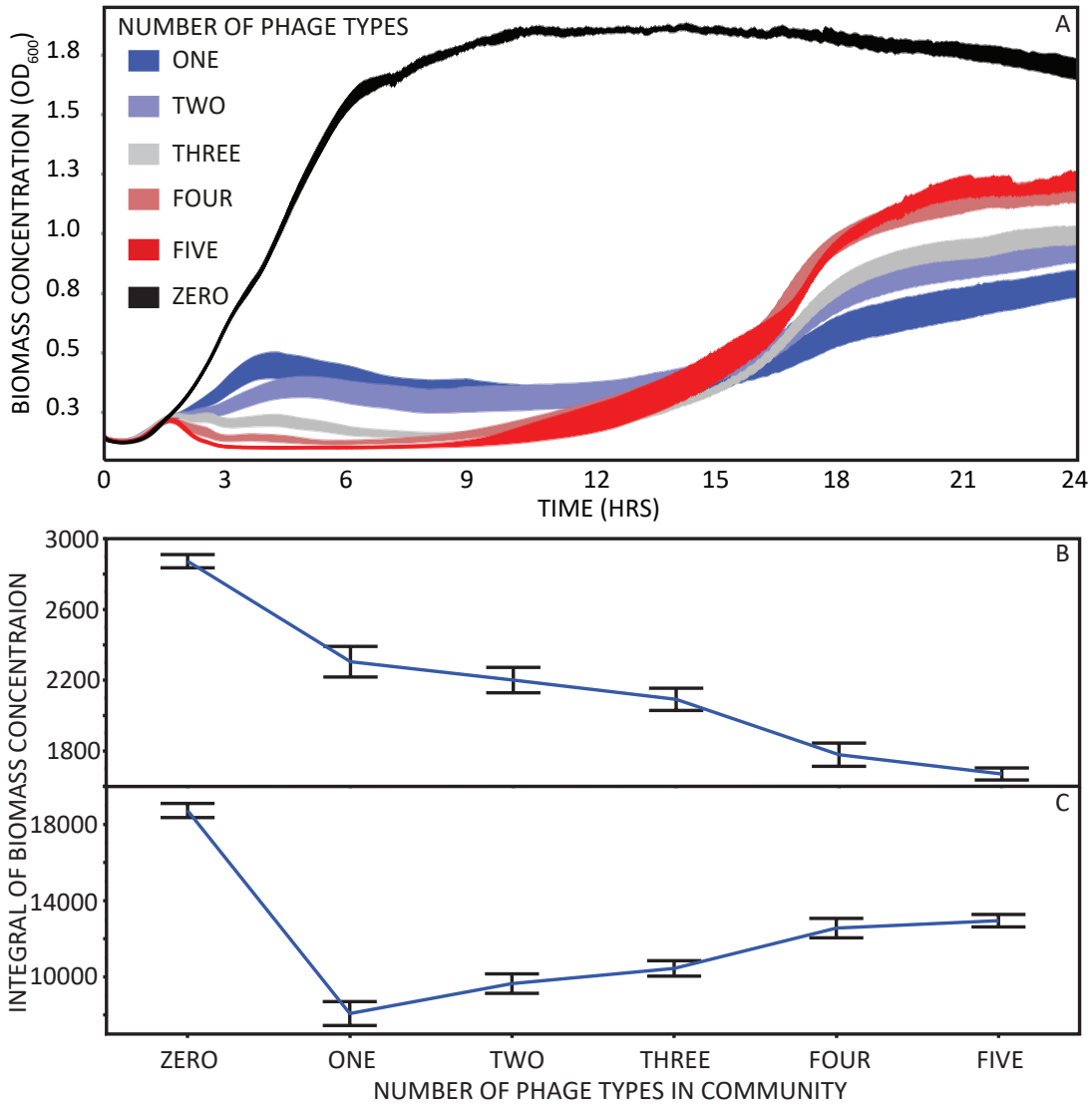



**Figure 3.1:** (A) shows the bacterial biomass concentration ($OD_{600}$) over time for the different phage containing treatments and the phage-free control. The areas under the curves for $OD_{600}$ during the first 3 (B) and last 3 (C) hours of growth split by the number of phage types in the community. The area under the curve reflects how much bacterial biomass was present within the time window. Error bars show 1 standard error.

### 3.4.1 Effects of phage diversity on bacterial growth

To measure the impact of phage diversity on bacterial population dynamics, we measured the optical density of bacterial populations that were exposed to phage relative to phage-free control populations (0-3 hours: $F_{1,191}=18.4$ $P<0.01$; 21-24 hours: $F_{1,191}=32.2$ $P<0.01$). There was a significant effect of the number of

phages both in early and late growth (0-3 hours: $F_{4,191}$=6.6 P<0.01; 21-24 hours: $F_{4,191}$=8.3 P<0.01) however the relationship between the number of phage and bacterial growth differed between the two time points (Figure 3.1). In early growth (0-3 hours), higher phage diversity was associated with a greater depression of bacterial population growth. Post-hoc contrasts revealed that bacterial growth was suppressed significantly more by the four- and five-phage treatments, which were not significantly different from one another. The three-phage treatment was the next lowest, but the one and two-phage treatments did not significantly differ. Conversely, in late growth (21-24 hours), lower phage diversity was associated with a greater depression of bacterial population growth. The one-phage treatment reduced bacterial growth more significantly than all other treatments, followed by the two-phage treatment. The three, four and five-phage treatments did not significantly differ from one another. To confirm that optical density provides an appropriate measure of population density, we performed endpoint CFU counts, which corroborated the OD data (Supplementary materials Figure 3.5).

### 3.4.2 Effects of phage diversity on phage growth

Although increasing phage diversity has the potential to increase bacterial mortality rates, increasing phage diversity also introduces the possibility for competition between phage types. To test for this effect, we measured the final phage titres (PFU $ml^{-1}$) after 24 hours incubation (Figure 3.2). There was a significant effect of phage community diversity on phage titre ($F_{4,180}$ = 4.17, P<0.01). Post-hoc contrasts revealed that the single-phage treatment had the highest mean titre, while all other phage communities were lower and not significantly different from each other.

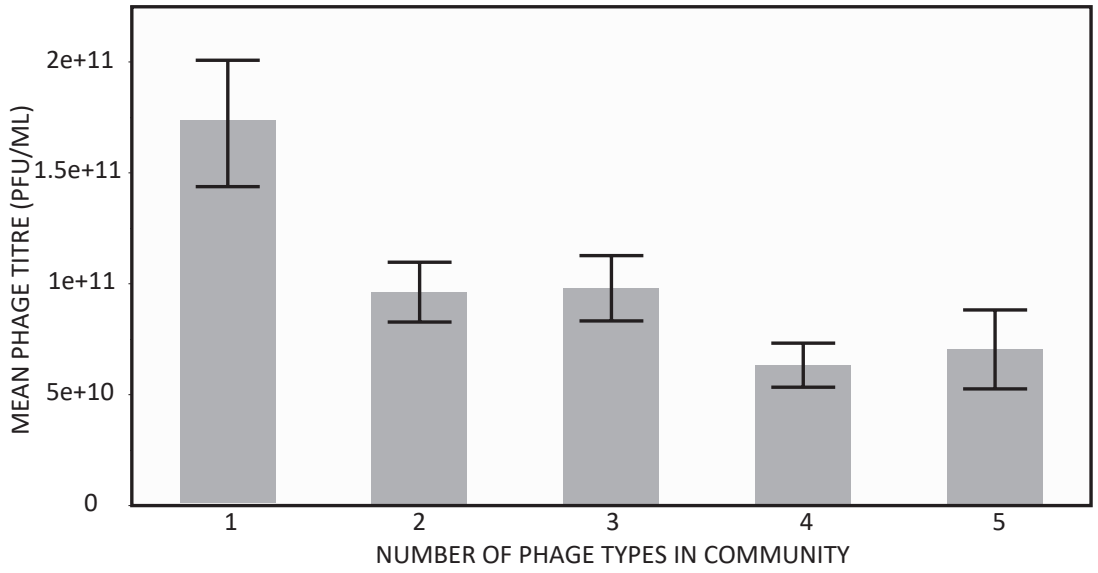



**Figure 3.2:** shows the mean phage titres measured after 24 hours where five bacteriophages were cultured in every possible combination up to a five-phage community. There were six replicates of each possible combination split across two 96 well plates. Error bars show 1 standard error.

### 3.4.3 Effects on bacterial resistance evolution

Predation by phage generates selection for phage resistance in bacterial populations; increasing phage diversity is predicted to alter both the rate and mechanisms of resistance evolution. To test for resistance evolution, we measured the ability of the five ancestral phage to infect bacterial clones that were sampled from the end of our experiment. We found phenotypic resistance across all treatments and not the phage-free control.

Firstly, we hypothesised that increasing phage diversity should decrease the rate of resistance evolution to sympatric phage (Figure 3.3). There was a significant effect of phage community diversity (single-, three- or five-phage types) on mean resistance to sympatric phage ($F_{2,605}$=80.2.58 P<0.001). However, host resistance to the five ancestral phage types differed significantly ($F_{4,605}$=73.8 P<0.001) and resistance to the ancestral phages differed depending on treatment($F_{8,605}$=12.65 P<0.001). The post-hoc comparisons (t-tests with a significance threshold of 0.05) revealed groupings amongst the five phage types. The degree of resistance in bacteria evolved against 14/1 and PEV2 differed from all others, but were not significantly different from each other. A similar pattern was revealed for bacterial populations evolved against LUZ7 and LMA2. In contrast, the degree of resistance in bacterial populations evolved against LUZ19 was significantly different from populations evolved against other phages. Within each phage type, the post-hoc contrasts revealed a significant difference between evolved bacterial resistance among the phage communities, except for those evolved against PEV2 and 14/1 where the one and three phage populations did not differ.

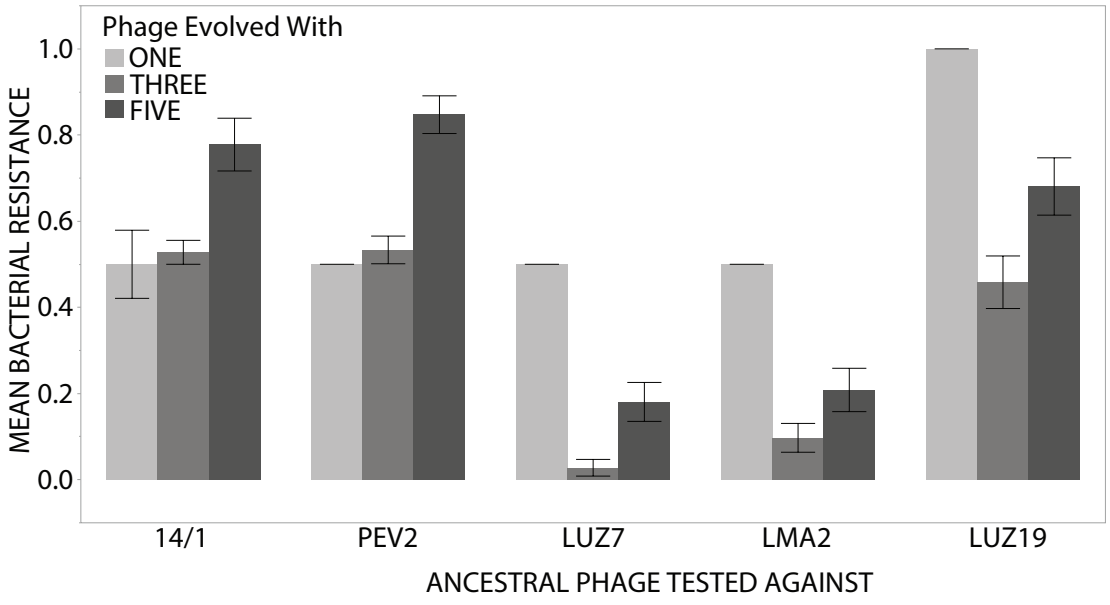



**Figure 3.3:** shows the mean bacterial resistance to the five ancestral phage types for those that had evolved with one, three and five phage types. Only sympatric data are included i.e. only bacteria evolved with PEV2 were scored against PEV2. Error bars show 1 standard error.

Secondly, we hypothesised that parasite diversity would select for more generalised resistance mechanisms. We compared the resistance of the evolved bacteria from

the single-phage treatments to all five ancestral phages (Figure 3.4). Here evolved bacterial isolates from the single, three and five-phage populations were exposed to all five phage types. The number of phage types with which bacteria had evolved significantly affected the mean resistance against the five phage types ($F_{2,1164}$=52.6 P<0.001). Nested within the single-phage populations, the different phage types evolved with selected for different levels of resistance to allopatric phage ($F_{13,1164}$=4.48 P<0.001). Post-hoc contrasts revealed similar groupings amongst the single-phage populations. Populations evolved with 14/1 and PEV2 had selected for the highest resistance against the five ancestral phage, and were significantly different from all others, but not from each other. Similarly, LUZ19, LMA2 and LUZ7 had selected for the lowest resistance to the five ancestral phage, but did not differ from each other.

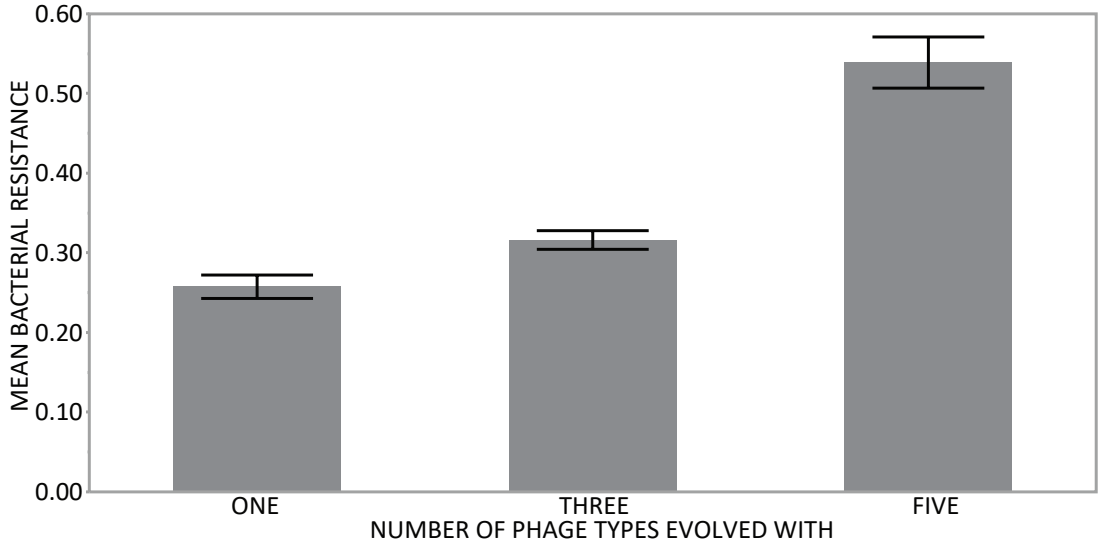



**Figure 3.4:** shows the resistance of evolved bacteria averaged across the five different ancestral phage types against which they were challenged. The data are shown separately for the bacterial populations evolved with one, three and five phages in a single community. Error bars shows 1 standard error.

To confirm that the observed changes in resistance were due to evolution and not phenotypic plasticity, we sequenced bacterial clones to look for evidence of genomic changes. All of the evolved clones possessed a single chromosomal mutation relative to the ancestor revealing that genomic evolution had occurred within 24 hours. No mutations were observed in the clones sequenced from the phage-free control populations. We observed parallel evolution in genes known to play roles in phage/bacteria interactions (e.g. LPS production). The details are summarised in Table 3.1.

## 3.5 Discussion

Here, we show that phage diversity drives significant changes in bacterial host ecology and evolution. While bacterial populations are initially depressed in size when exposed to a diversity of phages, they recover rapidly thereafter within a

**Table 3.1:** shows the mutations detected in the evolved bacteria. Each row shows the result for a single evolved clone. No mutations were detected in the phage-free lines but a single mutation was detected in every phage-evolved clones, one clone for each single-phage treatment and 6 clones from the five-phage treatment (fs–frameshift, mgd–multi-gene deletion, *–stop codon). The resistance of sequenced clones to the five ancestral phage types are also given. Clones were scored as either completely resistant (=1), partially resistant (=0.5). or completely susceptible (=0).

| EVOLUTION TREATMENT | MUTATION INFORMATION | | | | PHAGE RESISTANCE | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Gene ID | Gene | Function | Mutation | PEV2 | LUZ7 | LUZ19 | LMA2 | 14/1 |
| PEV2 | PA1959- PA2216 | - | 257 genes | mgd | 0.5 | 0.5 | 0 | 0.5 | 0.5 |
| 14/1 | PA3154 | wzy | LPS | G94* | 0.5 | 0 | 0 | 0.5 | 0.5 |
| LUZ7 | PA2911 | - | TonB receptor | L20P | 0.5 | 0.5 | 0 | 0.5 | 0 |
| LMA2 | PA2911 | - | TonB receptor | A26fs | 0 | 0.5 | 0 | 0.5 | 0 |
| LUZ19 | PA3805 | pilF | Type IV pili | R169_L170insRL | 0 | 0 | 1 | 0 | 0 |
| FIVE-PHAGE | PA3154 | wzy | LPS | W207fs | 1 | 0 | 1 | 0 | 1 |
| FIVE-PHAGE | PA3154 | wzy | LPS | A43Lfs | 1 | 0 | 1 | 0 | 1 |
| FIVE-PHAGE | PA3154 | wzy | LPS | A43Lfs | 1 | 0 | 1 | 0 | 1 |
| FIVE-PHAGE | PA0938 | wzz2 | LPS | T263M | 1 | 0 | 1 | 0 | 1 |
| FIVE-PHAGE | PA1893- PA2206 | - | 314 genes | mgd | 0.5 | 0.5 | 0 | 0.5 | 0.5 |
| FIVE-PHAGE | PA0763 | mucA | Alginate | Q192fs | 1 | 1 | 0.5 | 1 | 0.5 |

few hours. Recovery is likely due to a combination of resistance evolution and competition-induced lower phage population sizes. The greater the phage diversity in the community, the more rapidly the hosts were initially suppressed suggesting an additive effect of phages on the host population. For example, mixed phage infections can result in increased infection success if one phage affects the host in such a way that it is then more susceptible to a second phage (61).

Conversely, after less than 24 hours this synergy gave way to antagonism between phages as phage diversity resulted in a lower phage population size, enhancing bacterial recovery. Competition between phages could take place both between and within hosts. Phages of the same type could compete over attachment sites on the host cell surface (62) or modify host gene expression to prevent other phages from attaching to the same cell (63). Additionally, when there is multiple-infection, host death can occur prematurely, without phage production, as a result of damage caused by the sheer number of penetrations (64) thereby reducing both bacterial and phage population sizes. On the other hand, competition within the host could arise when phages differ in lysis time (43), and the slower phage fails to complete its life cycle. Such competition is important for bacteriophage ecology and can drive inter-phage coevolutionary arms races (65).

The increased recovery of bacterial populations evolving with the five-phage communities could also be due to early bottlenecking. Strong initial selection for resistant clones left them to grow with little competition, consequently by 24 hours they had reached a higher biomass concentration than the other treatments. Interestingly, the greater bacterial recovery observed in bacterial populations evolving with the five-phage communities relative to the single-phage populations suggests that despite the diversity of parasites, the evolution of resistance was not delayed. A diversity of phages instead applied a stronger selection pressure and allowed for greater bacterial recovery. Parasite competition affects the dynamics of infection in other systems (11, 66) but interactions between parasites can also lead to more virulent infections, for example in the case of HIV enhancing the TB epidemic (13).

Our results are consistent with the hypothesis that hosts evolving with a diverse parasite assemblage are more likely to evolve generalized resistance mechanisms,

while pairwise evolution selects for specific resistance (67). For example, a single mutation in *pilF* (involved in type IV pili formation) occurred in the bacterial population that evolved with LUZ19 (which is type IV pili-dependent phage). This mutation conferred complete resistance to LUZ19 and did not occur in any of the sampled bacterial populations subjected to attack by five-phage communities (Table 1). However, a single mutation in *mucA* was observed which seemed to confer partial resistance to multiple phage types. *MucA* represses alginate production and its inactivation is associated with conversion to mucoidy (68). Mucoid phenotypes have been shown to evolve in response to bacteriophage selection pressure in other systems (69, 70), but such a mutation was not present here in the single-phage treatments. These data will enable the formation of more developed hypotheses in our future work.

When evolved bacterial populations from all treatments were tested against all five ancestral phage, we observed the greatest breadth of resistance in those populations that had evolved with all five-phage types. This result suggests that the greater the phage diversity, the more bacterial populations are likely to evolve a broad resistance phenotype. When we focus on sympatric bacteria-phage comparisons only, we observe a strong phage-dependent effect such that for PEV2 and 14/1 combination into five-phage communities increases the amount of resistance evolved to these phage whereas the amount of resistance to LUZ7, LMA2 and LUZ19 decreases when they are combined with other phage. Interestingly, for all phage the level of evolved resistance was always lower for the three-phage communities than the five-phage communities, suggesting that there are important interactions between phages when in communities, perhaps in the form of competition for hosts (as suggested by the observed reduction in phage population size in communities) and evolution of cross-resistance (Supplementary materials Figure 3.6).

Future work will need to establish the effects of combining phage types on longer-term host-parasite coevolutionary dynamics. Here, we show widespread evolution of resistance, but our data also indicate that a degree of partial susceptibility remained. Thus, it is possible that some individuals at lower frequency remained completely susceptible (71, 72), providing the host reservoirs necessary for coevolution with phages. All the phages herein have been shown to exhibit the ability to coevolve with *P. aeruginosa* (59), and the nature of host mutations observed has interesting implications for potential long-term coevolutionary dynamics with bacteriophage communities. When coevolution was restricted to pairwise interactions in this study, mutations occurred at different loci depending on the phage type. For instance, two out of five clones showed LPS mutations with the remaining three in either type IV pili- or TonB-dependent receptors. In contrast, five out of six clones from bacteria populations evolved with the five-phage communities had mutations in LPS, and one additional mucoidy-associated mutation. When different LPS phages were combined, they can co-select for the same resistance mechanism. The LPS phages PEV2 and 14/1 both selected for genes that effect LPS production and the five-phage cocktail selected for similar mutations. Alternatively, there may be no genetic correlation in resistance to LPS phage and type IV pili; specific resistance to LUZ19 (type IV pili dependent) via a single mutation in a pili gene confers no protection to any of the other phage and so combining them might allow for diffuse coevolution. A mixture of diffuse and pairwise coevolution may take place when host populations coevolve with a diverse parasite community (73).

There are implications in our results for the application of a 'cocktail' of phage types to treat bacterial infections. In phage therapy, phages may be administered as monocultures (74) or in multi-phage communities (42). The single phage has the advantage of simpler kinetics and less regulatory burden (75), but it is limited by

its host range (76) and may only be effective against a narrow range of pathogen genotypes (77). However, the phage cocktail community can have a broader host range (78) and may delay the evolution of host resistance (79, 80). In addition to phage diversity, the phage titres are also important (81) as phage therapy is more effective with greater multiplicity of infection (82). Although our experiments were conducted in vitro and with a strain that is genetically-divergent from those of current clinical relevance, they are novel in exposing bacteria to all phage types separately and simultaneously, for a clinically-relevant period of time and for considering the effects on phage productivity. We observed that diverse phage communities did not delay the evolution of host resistance and eventually resulted in reduced phage titres. Our data thus suggest that boosting phage diversity in a cocktail might not automatically increase the efficacy of phage therapy.

In summary, we demonstrate that diverse parasite communities comprised of multiple phage types more rapidly depress host growth than the constituent phages alone and that increasing phage diversity did not delay the evolution of resistance. Our results therefore support the hypothesis that diverse parasite communities can impose stronger selection on host populations than single parasite species. Host resistance evolution also depends on the biology of individual parasite species in the community. Overall, our results demonstrate the potential for interesting ecological and evolutionary dynamics in bacteria-phage communities beyond lab investigations of pairwise phage-host interactions. Exploring the effect of parasite community diversity will therefore be a significant step towards creating host-parasite coevolutionary mosaics (5, 7) more representative of nature and testing hypotheses on host-parasite coevolution in a community context.

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## 3.7 Sequencing Supplement

### 3.7.1 Genome resequencing analysis

The whole genome sequencing analysis was performed as described in (1). Whole genome sequencing services were provided by the Wellcome Trust Centre for Human Genetics (Oxford, UK) using the Illumina Miseq platform with 300 bp paired-end reads. Initial read filtering was done using NIH QC Toolkit (2). 5' or 3' ends were trimmed if the Phred quality score was less than 20. Reads were discarded if they were shorter than 50 bp after trimming, if more than 2% of bases were ambiguous, or if more than 20% of bases had a Phred score $< 20$. BWA was used to map filtered reads were mapped to the *P. aeruginosa* PA01 reference genome (NC002516.2). Mapped reads were processed to increase the quality of the variant calling: 1) reads with multiple best hits were discarded; 2) duplicated reads were discarded using MarkDuplicates from the Picard package (http://picard.sourceforge.net); 3) reads around indels were locally realigned using RealignerTargetCreator and IndelRealigner from the GATK package to correct for misalignment; and 4) mate pairs were sorted using FixMateInformation in the Picard package. Variant calling was performed with GATK's Unified Genotyper (3) and Samtools's mpileup (4). VCFtools (vcf-annotate (5)) and GATK toolkit (VariantFiltration,(3)) were used to filter the raw variants for strand bias, end distance bias, base quality bias, SNPs around gaps, low coverage and erroneously high coverage. Variants were combined using GATK's CombineVariants (keeping any unfiltered). High quality variants not

filtered were annotated using SnpEff (6). Due to a decline in quality toward the 3' end of reads, we re-analyzed the reads by first removing 125 nt from the 3' end of the read. However, this re-analysis did not reveal any additional mutations.

Three approaches were used to detect structural variants. First, BreakDancer (7) was used to predict deletions, insertions, inversions, and translocations using deviations in the separation or orientation of mapped read pairs. Pindel (8) was used to infer deletions, short insertions, long insertions, inversions, tandem duplications, and breakpoints using a split-read approach (the output of BreakDancer was also fed to Pindel to improve its output). Finally, Control-FREEC (9) was used to detect copy number variants (CNVs). Control-FREEC finds CNVs using depth-of-coverage (normalized by GC-content). Regions of low mappability were excluded by supplying Control-FREEC with mappability tracks generated by gem-mappability (GEM library, (10)).

### 3.7.2 Summary of evolved mutations

Information about gene function was obtained from the Pseudomonas Genome Database (11).

Large deletions of 257 and 314 genes were found in the bacterial clone evolved with PEV2 and the five-phage community, respectively. These deletions almost completely overlapped (by 247 genes). Similar deletions containing have been observed in *P. aeruginosa* following phage exposure, and they may include genes which confer resistance to phage attachment (12). As single phage, both LUZ7 and LMA2 selected for different mutations in the same gene, a TonB-dependent receptor. TonB-dependent receptors are known to be important for phage attachment in some *E. coli* (13) and *Salmonella enterica* (14) phages, but also have broader regulatory functions (15) that could mediate phage resistance. Again, these mutations were not seen in the five-phage cocktail populations.

The four remaining mutations were all in two genes (*Wzy* and *Wzz2*) involved in LPS formation (16). Three different mutations were found in *Wzy*; a stop codon gain found after evolution with 14/1, a deletion of a Cytosine from six consecutive Cytosine residues with the five-phage cocktail, and the addition of a Thiamine to seven consecutive Thiamines which arose twice in two of the five-phage clones. Finally, a missense mutation was observed in *wzz2* in one of the five-phage clones, confirming the importance of LPS in the evolution of resistance to these phage.

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## 3.8 Supplementary Figures

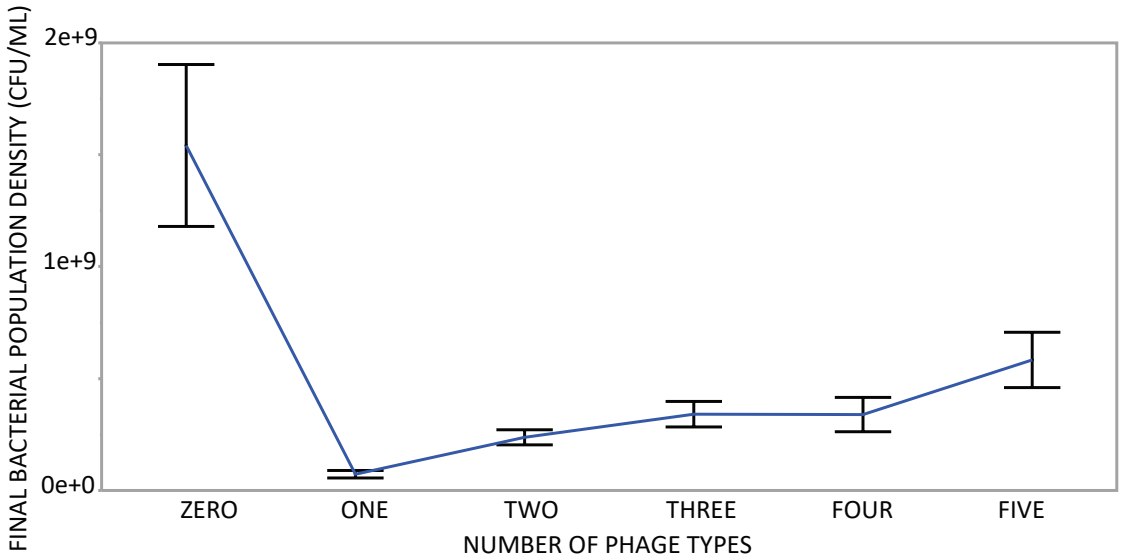



**Figure 3.5:** This figure shows the final bacterial population density measured by counting CFU per ML. These data were collected to corroborate the $OD_{600}$ data shown in Figure 1. They show the same relationship as the optical density data, with the highest bacterial density recorded in the phage-free control, then the five-phage populations. The lowest final bacterial population size was recorded with the one-phage treatment. Each error bar shows one standard error.

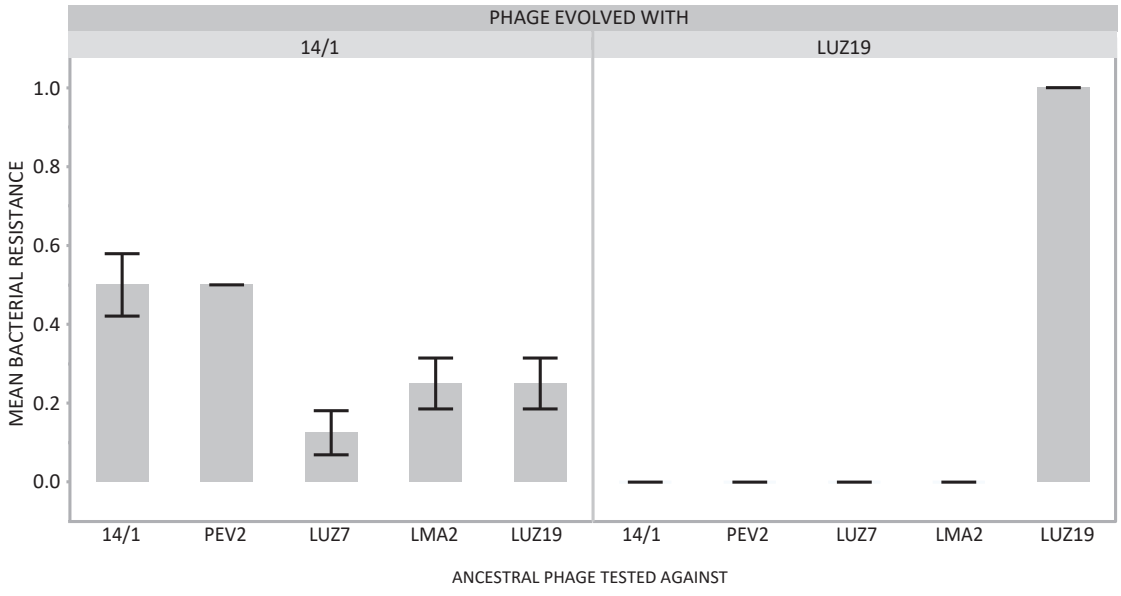



**Figure 3.6:** Demonstrates the evolution of cross resistance to multiple phage following exposure to a single phage. This figure shows the resistance evolved to all phage following evolution with 14/1 (left) or LUZ19 (right) as single-phage treatments. Evolution with 14/1 selects for intermediate resistance to 14/1 and as a result of cross resistance, 14/1 evolved bacteria also show intermediate resistance to PEV2 and a modicum of resistance to LUZ7, LMA2 and LUZ19. LUZ19 evolved bacteria showed complete resistance to LUZ19 with no cross resistance to other phage types. Error bars show 1 standard error.

*Now, here, you see, it takes all the running you can do, to keep in the same place. If you want to get somewhere else, you must run at least twice as fast as that!*

— Lewis Carroll, Through the Looking Glass (1896)

# 4

# High Parasite Diversity Accelerates Host Adaptation And Diversification

## 4.1 Abstract

Virtually all species are attacked by multiple parasites, but the evolutionary consequences of high parasite diversity are unclear. Here we used experimental coevolution of a host bacterium *Pseudomonas aeruginosa PA01* and communities of different viral parasites to investigate the effects of parasite diversity on coevolution. We show that greater parasite diversity accelerates coevolutionary arms races and drives more diversification among host lineages. Increasing parasite diversity accelerated the evolution of the host bacterium, resulting in increased resistance to parasites. Red Queen interactions in low parasite diversity communities generated negative frequency-dependent selection for phage resistance mutations. Increasing parasite diversity shifted the mode of coevolution towards host-parasite arms races that were driven by rapid selective sweeps of phage resistance mutations, most notably in LPS biosynthesis genes. Ultimately, high parasite diversity resulted in an increase in the rate of molecular evolution within host populations and genetic divergence between host populations. These results may help to explain why regions of high parasite diversity are hotspots for rapid adaptation and speciation in interacting, antagonistic species.

## 4.2 Introduction

Coevolution between hosts and parasites is hypothesized as central to numerous biological phenomena, from rapid evolutionary change (1) to the maintenance of diversity (2) and sex (3, 4). Host-parasite coevolution is traditionally investigated

in pairwise frameworks where a single parasite species infects one host species (5). However, in nature, these pairwise species relationships rarely exist in isolation as hosts are often under attack by multiple parasite species (6), and parasites must compete for hosts (7–10). Coevolution thus operates within a network of species interactions. However, it remains unknown how diverse communities shape the patterns and processes of host-parasite coevolution. Here we directly address this problem by studying how increasing parasite diversity impacts host-parasite coevolution using a microbial model system consisting of a single host bacterium, *Pseudomonas aeruginosa*, and a diverse community of 5 lytic bacteriophage that act as obligate, lethal parasites.

Diversity may be a self-reinforcing principle in community ecology (11). Populations within species rich ecosystems, such as tropical rainforests, have been shown to undergo rapid evolution (12) and we predict that increasing diversity may accelerate evolutionary change and diversification during coevolution. Increasing parasite diversity should increase the mortality within host populations, strengthening the selection for parasite resistance, suggesting that increasing parasite diversity should accelerate host evolution. Ultimately, this should facilitate increased divergence between host populations. These outcomes could be underpinned by two competing selection dynamics; fluctuating or directional selection. Either mode may be favoured by parasite diversity and have major consequences for coevolutionary dynamics.

To test these predictions, we applied an experimental coevolution approach to our *in vitro* bacteria-phage system. We exposed the host bacterium, to communities of 1-5, viral parasites (bacteriophages PEV2, LUZ19, LUZ7, 14-1 and LMA2). Phage infect bacteria by attaching to specific cell surface receptors (lipopolysaccharides (LPS), Ton-B dependent receptors or Type IV pili) and bacteria typically evolve resistance to lytic phage by modifying or deleting these attachment sites (13). Parasites, in turn, can evolve reciprocal adaptations to circumvent host resistance (14). By pooling parasites in different combinations, we can directly manipulate parasite community diversity and test for effects on the tempo and mode of host-parasite coevolution, as well as on host genomic evolution. Specifically, we experimentally coevolved host populations independently in microcosms with replicates of a five-parasite community (High Diversity) alongside every possible pairwise parasite combination (Intermediate) and a single parasite treatment with replicates of each parasite in monoculture (Low).

## 4.3 Materials And Methods

### 4.3.1 Isolates and bacteriophage characteristics

Independent isogenic lineages of *Pseudomonas aeruginosa PAO1* (referred to as the ancestors) were prepared from single colonies with a common ancestor and preserved as stocks of approximately $10^7$ CFU/ml at -80°C with 25% glycerol. Five bacteriophage types were used, three from the Podoviridae (PEV2, LUZ19 and LUZ7) and two from the Myoviridae (14/1 and LMA2). All five ancestral phage strains were stored as stocks of approximately $10^8$ PFU/ml at 4°C.

### 4.3.2 Experimental coevolution

Combinations of the five phages (one-phage monocultures, every possible two-phage combination, and a five-phage community alongside a phage-free control) were prepared such that the phages were at an inoculation titre of $5\times10^5$ PFU $mL^{-1}$ and within mixed phage communities each phage type was present in equal densities. These communities were then mixed with P. aeruginosa cultured overnight in KB. Each culture was prepared from a separate ancestral colony and used to inoculate each replicate to a starting concentration of $5\times10^7$ colony forming units per mL (CFU $mL^{-1}$). These experiments were conducted in 5 ml round bottom polystyrene test tubes with vented caps (Corning, Flintshire, UK) containing 2.5 ml of King's B (KB) culture medium (microcosms). These microcosms were incubated at 37°C and 2250×RPM orbital agitation in a MaxQ 8000 shaker-incubator (Thermo Scientific, Waltham, MA, USA). This experiment was designed to include a blocking structure, with three sequential batches each of 40 lineages. Within each batch, replicates were evenly separated across the three levels of phage diversity (One, Two and Five-phage) and the phage three control such that each treatment had 10 replicates per batch. For each replicate, a 50$\mu$l sample containing both bacteria and phage were serially transferred to a new microcosm every 24 hours. Each batch underwent ten such transfers. At each transfer, a 500$\mu$l sample was taken and stored in 50% glycerol at -80°C for subsequent analysis.

### 4.3.3 Measuring host and parasite community density

Every 24 hours samples were taken from each replicate to allow hosts and parasites to be enumerated. Samples were serially diluted and plated via a previously used soft KB agar overlay method to allow the counting of individual phage plaque forming units (PFU) on a lawn of susceptible *P. aeruginosa PAO1* from which phage population density (PFU/ml) can be estimated, these diluted samples were simultaneously plated on KB agar to display single bacterial colony forming units (CFU/ml) from which bacterial population density was estimated.

Host and parasite community densities (Fig. 1.A) were analysed using separate linear mixed effects models. We fitted the maximal model with parasite diversity and transfer as interacting fixed effects, and batch, parasite community and replicate as nested random effects. The model was simplified using stepwise model simplification based on AIC. A 2-parameter autocorrelation-moving average correlation structure was built in to the host density model. post hoc contrasts were performed using Tukey HSD tests.

### 4.3.4 Measuring parasite infectivity and host resistance

Using a sterile 1 $\mu$L inoculation loop, coevolved populations were streaked out onto KB agar. These plates were then placed in a static incubator overnight at 37°C to allow the growth of individual colonies. Some such colonies were picked with another loop and similarly streaked out and incubated to present single colonies thus enabling the isolation of phage free, isogenic bacterial populations from the evolved communities. Isolates from each replicate were used to inoculate individual microcosms incubated for 24 hours. Subsequently a 100 $\mu$L sample from each

microcosm was mixed with 2.9 mL of molten KB agar (0.75% agar) and spread evenly over the surface of a KB agar (1.5% agar) plate. These soft agar lawns were then inoculated with 4 $\mu$L droplets of purified phage which were allowed to dry, and then incubated for 24 hours. This time period allowed the formation of zones of inhibition which indicated susceptibility or resistance. Droplets which produced with no visible inhibition were scored as resistant (=1), those with some visible inhibition were scored as partially resistant (=0.5), and those with complete inhibition were scored as susceptible (=0). These data were then analysed as the proportion of clones that were resistant or susceptible to a given phage isolate.

### 4.3.5 Time shift assays: Detecting coevolution between bacteria and phages

A time-shift assay approach enabled the detection of adaptations and counter-adaptations that coevolved during the serial transfer experiment. Bacterial resistance was measured against phage from the same lineage (sympatric), isolated from past, present and future transfers, Likewise, the infectivity of phage was measured on sympatric bacteria from past, present and future transfers. Escalating coevolutionary arms races between hosts and parasites are defined by successive increases in resistance and infectivity. Deviations in linearity, especially those that give rise to local minima or maxima of infectivity and resistance, indicate fluctuating selection dynamics as with the frequency-dependent selection dynamics that underlie Red Queen coevolution. A total of 13,500 combinations of coevolved bacterial genotypes and phage communities were assayed during the time-shift comparing the resistance of bacteria from 24, 120 and 240 hours to the phage communities with which they had coevolved from 10 time points, every 24 hours between 24 and 240 hours. These data were categorised first by the diversity of phage with which they were coevolved and second by whether the contrast combined a bacterial genotype with its contemporary phage community, phage from its past or phage from its future. These data were then analysed in a linear mixed effects model in R (15) with bacterial genotype nested within replicate, within phage diversity, within batch as random effects and phage diversity crossed with phage time origin as fixed effects.

### 4.3.6 Genome resequencing analysis

Whole community DNA extractions were performed on 91 samples for sequencing. Three ancestral *P. aeruginosa* clones and 14 whole populations from the phage-free control were sequenced to enable the identification of mutations resulting from laboratory adaptation and any differences in the ancestor from the published reference sequence (NC002516.2). 15 one-phage, 10 two-phage and 12 five-phage replicates were sampled longitudinally at two time points (5 days and 10 days) so that allele frequency changes within populations could be easily observed.

Whole community DNA extractions were performed on 1ml of culture using a Dneasy Blood and Tissue kit (Qiagen, Inc., Chatworth, California, USA), quantified using the QuantiFluor dsDNA system (Promega, Madison, WI, USA) and the salt ratio verified by Nano Drop (Thermo Scientific, Waltham, MA, USA). Whole genome sequencing services were provided by the Wellcome Trust Centre for Human Genetics (Oxford, UK) using the Illumina Hiseq platform with 150 bp paired-end reads. All

mapping and variant calling was performed using CLC genomics Workbench (CLC Bio). First, 5' or 3' ends were trimmed if the Phred quality score was less than 20. Reads were discarded if they were shorter than 50 bp after trimming or if more than 2 of bases were ambiguous. Filtered reads were then mapped to the *P. aeruginosa PA01* reference genome. Mapped reads were then processed to increase the quality of the variant calling; duplicated reads were discarded and reads around indels were locally realigned. A Neighbourhood Quality Standard model was then applied to detect single nucleotide polymorphisms and a separate model was used concurrently to detect deletion/insertion polymorphisms. Variants were then filtered to remove any also present in the three sequenced ancestral clones, thus ensuring that all reported SNPs are certain to have arisen during the experiment, not before. Finally, variants were annotated using the *Pseudomonas* database (16).

### 4.3.7 Detecting genomic signatures of strong directional selection

We used PoPoolation 2 (17) to calculate the per locus $F_{ST}$ between each 10 day coevolved population and one of the three isogenic sequenced ancestors. The mapped genomes were sorted and synchronised using SAMtools (18). The $F_{ST}$ was then calculated for every SNP genomewide using the $F_{ST}$-sliding tool of PoPoolation 2. The majority of the 6.3 million loci were not under strong selection in this experiment and produced very low $F_{ST}$ values, all values $<0.05$ were excluded and with the remaining data (mean 0.174 s.d.=0.164) we calculated 3 standard deviations above the mean to give a conservative threshold $F_{ST}$ of 0.67, all values higher than this were taken to be indicators of strong directional selection driving alleles towards fixation. The number of mutations with signatures of strong directional selection were compared between treatments with a $X$ squared test.

### 4.3.8 Determining effects of parasite diversity on host evolutionary rates

The Euclidean genetic differences (the square root of pairwise differences) between each coevolved bacterial population and the ancestor was calculated using the frequency of detected variants. Indels varied in size but were each counted as just a single mutation. Mutations with a frequency of $<5\%$ were not included to avoid the possible contribution of sequencing errors. A phylogeny was produced on the resultant distance matrix using the library APE (19) in R (15). The significance of the differences in genetic distances between treatments were compared by ANOVA and post hoc contrasts were performed using Tukey HSD tests.

The Euclidean genetic distance between each coevolved host population were investigated by nMDS ordination and ANOSIM which partitions diversity within and between treatments and computes a value or $R\approx0$ when there is no difference. Secondly, the genetic distances between each coevolved host population within each diversity treatment were analysed by ANOVA followed by post hoc Tukey HSD tests.

We also performed the test for transgressive overyielding ($D_{max}$ (20)) to confirm if our diversity effect was due to diverse communities being more likely to contain phages which drive rapid evolution rather than a positive interaction between phages

in communities accelerating coevolution. We calculated $D_{max}$ using the genetic distances from the high parasite diversity treatment and the genetic distances of each of the parasites in monoculture. One-sample t-tests were performed with a Bonferroni correction to test whether each phage had a $D_{max}$ significantly greater than zero.

## 4.4 Results and Discussion

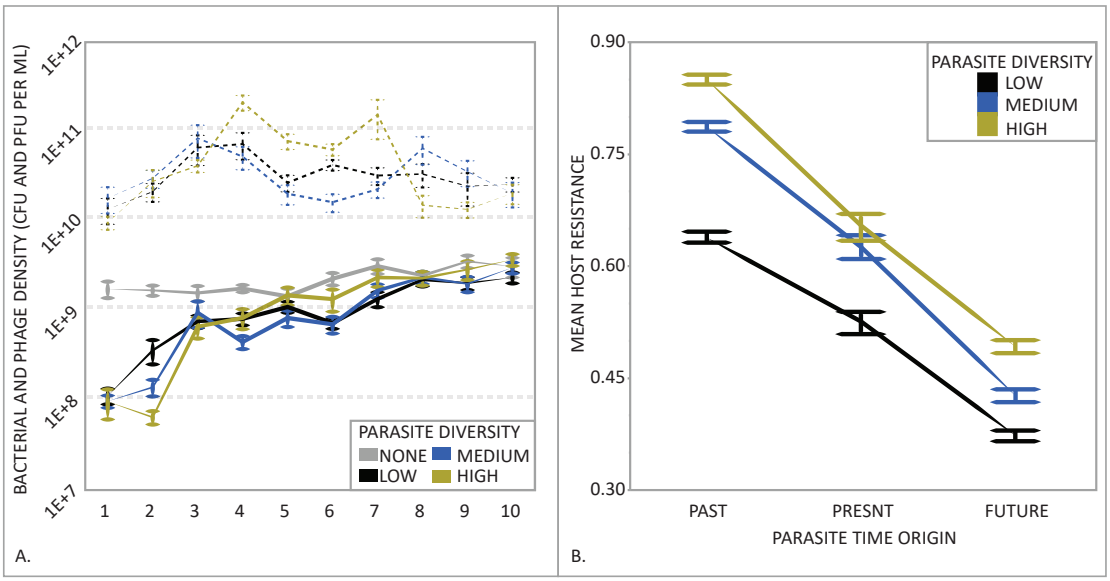



**Figure 4.1:** Ecology and Coevolution in antagonistic communities. (A.) No reciprocal oscillations in host and parasite densities over time, host population density (solid lines) and parasite community density (dashed lines) were recorded through time. Parasite densities show no trend over time whereas the host densities show significant recovery, achieving the same densities as the parasite free control. This pattern is only possible through evolution. Error bars show 1 standard error. (B.) By comparing the resistance of hosts from multiple time points to parasites from their coevolutionary past, present and future, the pattern of phenotypic change in hosts and parasites through time can be summarised. We show that higher levels of resistance have evolved in host populations coevolving with more diverse parasite communities and that coevolved parasite are more infective than their ancestors. Furthermore, hosts are always more susceptible to parasites from their future than their contemporary parasites and that they are most resistant to parasites from their past. This pattern is consistent with Arms Race coevolutionary dynamics. Error bars show 1 standard error.

Parasite densities are expected to track with host densities in a time-lagged fashion producing host-parasite population cycles over time (21). Instead, we observed that host densities increased over time whilst parasite densities remained stable (Fig. 4.1, Supplementary Text 4.6.1), suggestive of underlying coevolutionary dynamics interfering with the expected ecological dynamics (22). To directly test for antagonistic co-evolution, we performed time-shift assays that measure the resistance of hosts to their past, present and future sympatric parasites. Resistance

was always lowest to future parasites and highest to past parasites demonstrating that parasites evolved greater infectivity over time. This pattern is typical of arms race dynamics, commonly seen in in vitro bacteria-phage interactions (23, 24). However, bacterial resistance increased with parasite diversity ($F_{2,85}$=9.7, P<0.001), suggesting that parasite diversity accelerates evolution. To test this idea further, we used whole-genome sequencing to re-sequence entire bacterial populations at multiple time points throughout the experiment. This approach allowed us to detect any mutations that arose during coevolution and estimate their frequency changes within populations. We observed 515 polymorphisms across 173 genes. Parallel evolution was common: >50% of mutations occurred in <5% these genes and notably we observed 190 mutations in genes encoding LPS biosynthesis proteins (Table 4.2). We used this dataset to determine the effects of parasite diversity on host evolutionary rate. To measure the rate of bacterial molecular evolution, we calculated the Euclidean genetic distance of each population from the ancestor (Fig. 4.2A). We found that greater parasite diversity drove greater host divergence from the ancestor (ANOVA $F_{2,34}$=10.5, P<0.001; Post hoc Tukey test P <0.05), demonstrating that increasing parasite diversity leads to more rapid host evolution.

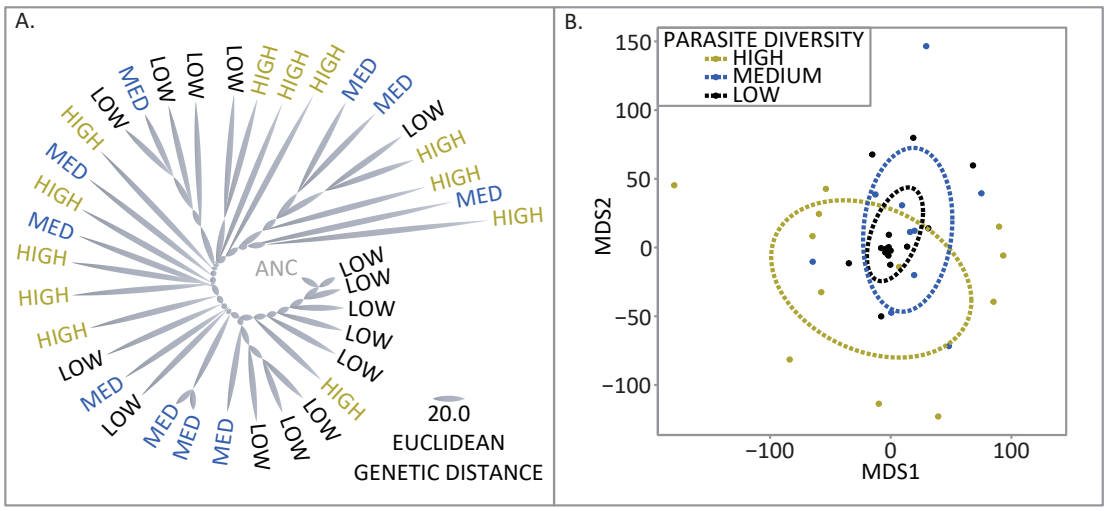



**Figure 4.2:** Parasite diversity accelerates evolutionary divergence. (A) Host allele frequencies after 10 days of coevolution were used to calculate pairwise Euclidean distances between the ancestral sequence (ANC) and each coevolved population. While coevolution with diverse parasite communities accelerated host evolution, pairwise coevolution resulted in host populations that were on average the least genetically distant from the ancestor. (B) The genetic distances between coevolved populations were ordinated by nMDS. We found greater diversification between treatments than within, and the greatest within treatment diversification was observed in the high parasite diversity treatment.

Two mechanisms could explain why diverse parasite communities accelerate the rate of host evolution. On the one hand, communities that are more diverse are more likely, by chance alone, to contain parasites that drive rapid evolution. Alternatively, it is possible that parasite diversity *per se* accelerates the rate of evolution by increasing the strength of selection for resistance. To discriminate between these mechanisms, we calculated the contribution of parasite diversity to the rate of host evolution by adopting an approach often used to determine the contribution of biodiversity to ecosystem function (25). We treated host evolutionary rate as our yield and tested for transgressive overyielding ($D_{max}$) to

compare the yield in the face of the parasite diversity with the highest yielding component parasite species in monoculture. When $D_{max}$ >0 it indicates that parasite diversity accelerated evolution more than the corresponding monocultures. Importantly, all 5 parasites contributed to increasing the rate of host evolution ($D_{max}$ >0; Bonferroni corrected one-sample t-tests P<0.05). Thus, the diversity of interactions between parasites within communities, and not merely the presence of a particular parasite, drove the rapid evolution. Diverse parasite communities imposed stronger selection, leading to faster coevolution than expected by the traditional pairwise host-parasite pairwise framework.

Evolutionary interactions between hosts and parasites are usually caricatured as being arms races, where selection favours increased resistance and infectivity (26), or Red Queen, where selection favours rare host and parasite genotypes (27). Each parasite herein follows a different coevolutionary trajectory when interacting with this host in monoculture; some coevolve by arms race dynamics whereby directional selection drives increasing infectivity over time (Fig. 4.7); while others engage in Red Queen dynamics driven by fluctuating selection (28). We therefore sought to determine if parasite diversity alters the mode of coevolution using a population genomic approach.

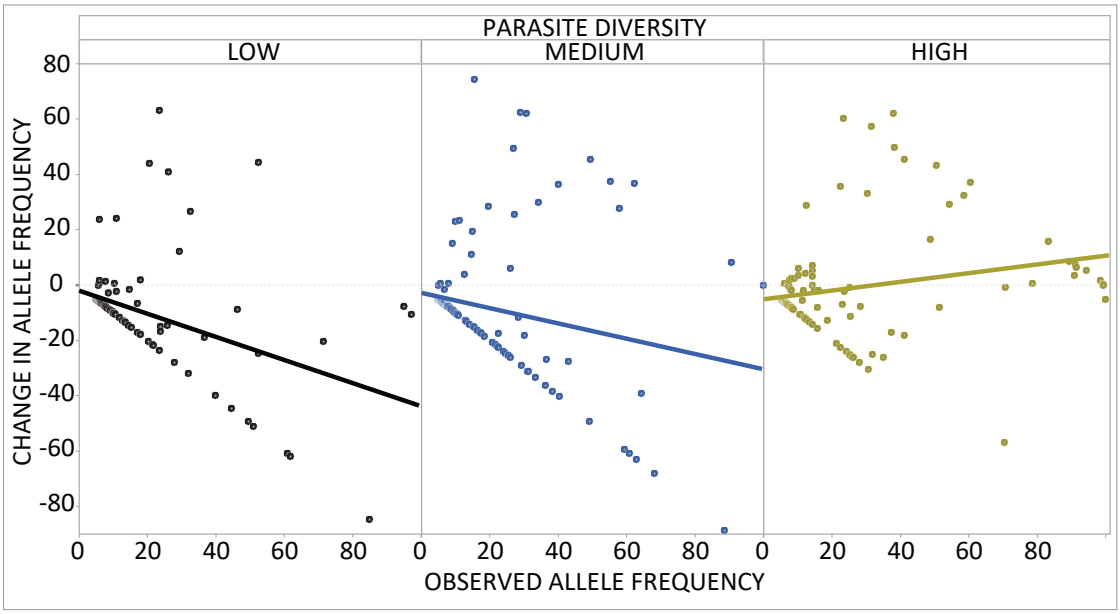



**Figure 4.3:** Parasite diversity decreases negative, frequency-dependent selection. We regressed the change in each allele's frequency with its observed initial frequency to determine the extent of frequency dependent change. We found less frequency dependence as parasite diversity increased suggesting a reduced role for red queen dynamics in the face of increasing parasite diversity.

Red Queen dynamics are dominated by negative, frequency-dependent selection where the rarest genotypes have an advantage (27). We tested whether there was evidence for these dynamics by comparing the change in frequencies of each allele between the mid- and end-points of the experiment (29). Common alleles tend to decrease in frequency over time, which is consistent with negative, frequency-dependent selection on phage resistance genes (Fig. 4.3). However, this mode of selection is less common at higher levels of parasite diversity, as we would

expect if high parasite diversity decreased the importance of Red Queen dynamics (ANCOVA $F_{2,335}=12.64$, $P<0.0001$).

In contrast to Red Queen dynamics, arms races should result in recurrent selective sweeps of parasite resistance mutations. To test for selective sweeps we calculated the $F_{ST}$ (30) per mutation between each coevolved population and a sequenced ancestor, and we set a conservative threshold $F_{ST}$ above which any mutation was deemed to be under strong directional selection (Fig. 4.4A). The data convincingly demonstrate that there was a much greater tendency towards strong directional selection as parasite diversity increased ($X^2=20$, df = 3, $P<0.001$) such that the number of fixed alleles in the high parasite diversity treatment was more than double than that under intermediate parasite diversity (Fig. 4.4B). This result shows that parasite diversity results in faster arms races and drives host resistance alleles rapidly towards fixation. Of the 29 alleles approaching fixation, 23 were in genes relating to LPS biosynthesis. It is possible that such mutations are subject to positive selection as they serve as generalist resistance mechanisms. Given that LPS is very abundant on the cell surface, phage with other ultimate receptors may bind initially to LPS, and from there probe across the cell surface until forming an irreversible attachment to their final receptor (31). In which case, mutations in LPS biosynthesis proteins could provide some resistance to a diversity of phage with different receptors.

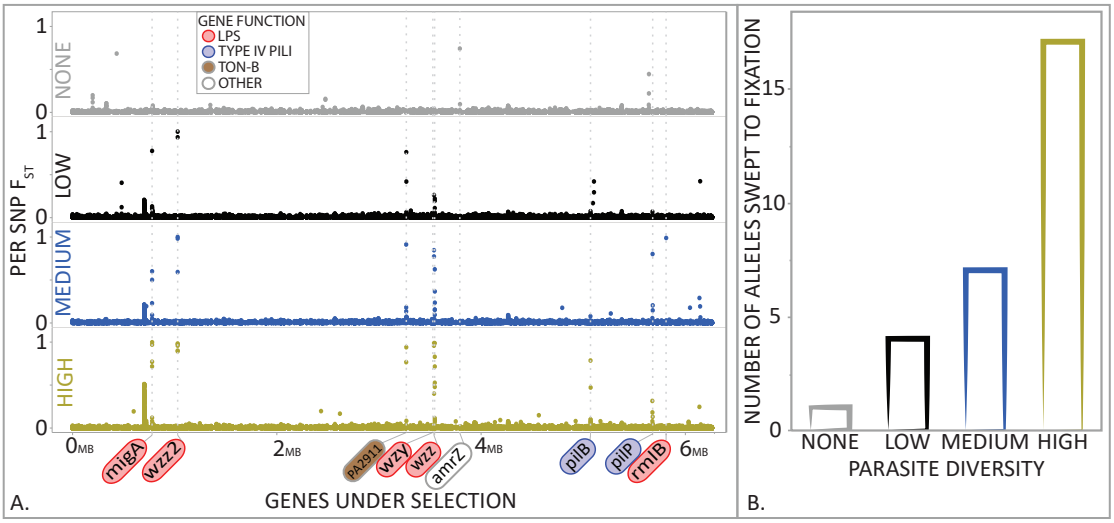



**Figure 4.4:** Parasite diversity favours Arms Race Coevolutionary Dynamics. (A) By calculating the $F_{ST}$ of all SNPs genome wide we get an indication of the strength of directional selection. We compared the $F_{ST}$ of SNPs between high, medium and low parasite diversity treatments with a parasite free control to see if parasite diversity encouraged stronger directional selection driving arms races. Genes with alleles under strong directional selection are annotated with their function, specifically LPS biosynthesis proteins, Type IV pili biosynthesis, a Ton-B dependent receptor and one other which occurred only in the control (B) The number of SNPs under strong directional selection increased with parasite diversity, with the greatest number of high $F_{ST}$ SNPs in the high parasite diversity treatments.

Finally, coevolution with communities of enemies might increase genetic divergence within host populations by selecting for different resistance mutations in hosts and driving faster evolution. Our results were consistent with the hypothesis that divergent selection on different resistance mechanisms was driven by different

parasites within communities. Although we observed greater variation in host evolutionary trajectories between treatments (Fig. 4.2B) than within treatments (ANOSIM R = 0.11 P<0.01), parasite diversity had a strongly significant effect on within-treatment variation in evolutionary trajectories (ANOVA $F_{2,213}$=122.15, P<0.001) with the greatest diversification in the high parasite diversity treatment (Post hoc Tukey test P<0.05).

In conclusion, our results highlight diverse communities as hotbeds of rapid and divergent evolution during antagonistic coevolution. We directly demonstrate that parasite community diversity drives faster arms races with hosts, leading to higher levels of host resistance. Moreover, stronger directional selection imposed by multiple parasites on several host resistance traits accelerates host population diversification at the genomic level. The idea that parasite communities can select for increased host genetic diversity has previously been suggested in the context of MHC alleles in animals (32). Similarly, here, resistant hosts have accumulated multiple mutations in different surface receptors to provide a breadth of resistance strategies. Our findings suggest why biodiversity hotspots, such as the Amazon rainforest, might contain high levels of rapid evolution with rapid speciation (33): parasite diversity fuels the diversification.

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## 4.6 Supplementary Text

### 4.6.1 Analysis of ecological data

Parasite diversity significantly affected host population density ($F_{3,45}$ =6.59, P<0.001). The highest host densities were achieved with high parasite diversity but host densities increased over time regardless of parasite diversity ($F_{1,1079}$ =160.74, P<0.001). We observed no time-dependent relationship with the parasite community densities. Indeed, the only significant factor explaining parasite densities was parasite diversity ($F_2, 43$ =6.1, P<0.01) with the high parasite diversity treatment reaching the highest parasite density (Post-hoc Tukey test P<0.05).

Accelerated resistance evolution could have important ecological consequences as parasites require susceptible hosts to complete their lifecycles (34). In the absence of any evolutionary change, we would expect parasite populations to grow with host populations. Our observation that parasite densities were stable over time despite simultaneously increasing host densities suggests feedbacks between evolutionary and ecological dynamics (35) and confirms the evolution of host resistance. That parasite densities do not subsequently decrease in the face of increasing resistance (and dilution into fresh media every 24 hours) agrees with our time shift results and could be explained by a quick coevolutionary response from the parasites allowing them to infect the new host genotypes (16).

### 4.6.2 Analysis of time-shift data

We observed that parasite diversity significantly increased the magnitude of resistance evolution over time ($F_{2,85}$ =9.7, P<0.001) with the low parasite diversity treatment selecting for the least resistance; significantly less than the intermediate (Post-hoc Tukey test P<0.05) and high parasite diversity treatments (Post-hoc Tukey test P<0.001). The data also show significant change in the ability of parasites to infect hosts over time ($F_{2,13044}$ =1766.21, P<0.0001). Within each level of parasite diversity, resistance was always lowest to parasites from the future and highest to parasites from the past (Post-hoc Tukey test P<0.0001). This pattern is typical of arms race dynamics, commonly seen in in vitro bacteria-phage interactions (17, 18). The significant interaction between parasite diversity and parasite time origin ($F_{4,13044}$ =16.99, P<0.0001) is largely explained by the differing abilities of parasite communities to overcome evolved host resistance. Interestingly, the resistance of host populations to their contemporary parasite communities were very similar (Post-hoc Tukey test P>0.2) yet the resistance of the intermediate parasite diversity coevolved hosts to past and future parasites was significantly lower (Post-hoc Tukey test P<0.05) than the high parasite diversity coevolved hosts. These results suggest a greater ability of the host populations facing high parasite diversity to evolve resistance.

### 4.6.3 Phage diversity PCR

The number of phage types detected in the samples from the end of the experiment (Fig. 4.6) were analysed by ANOVA. In several samples, different phage have fallen below the detection threshold, but not to the extent that our diversity treatments were compromised (ANOVA $F_{2,34}$ =38.73, P<0.001) the high diversity treatment remains more diverse than the medium (Post-hoc Tukey test P<0.0001), which is in turn more diverse than the low (Post-hoc Tukey test P<0.05).

The presence or absence of the five different phage types at the end of the experiment in the high parasite diversity treatment was confirmed via PCR.

Primers specific to each phage (Table 4.1) were designed and tested for potential cross amplification against the *P. aeruginosa* chromosome and the other phage types used here in Geneious 6.06 (Biomatters Ltd. Aukland, NZ). All primer pairs were designed with an amplicon size of 150bp within genes thought to encode the tail fibre proteins of each phage. PCR was performed using GoTaq green Mastermix (Promega, Madison, WI, USA) on whole community DNA extracted 1ml of culture using a Dneasy Blood and Tissue kit (Qiagen, Inc., Chatworth, California, USA).

## 4.7 Supplementary Figures And Tables

**Table 4.1:** This table gives details of the phage specific primer pairs used here and the identity of their target regions.

| Phage | Gene | Forward Primer | Reverse Primer |
|---|---|---|---|
| PEV2 | *gp53* | TGCCGAAGCATTGGTCAGAT | GACTACACCGACCAGTGGTG |
| LUZ7 | *gp056* | TGACCACGTACTCGGTGTTG | ACAAGCTGGGCCAGTTCTAC |
| LUZ19 | *gp40* | ACCCTAGCTGGTCAAGTCCT | CACCAGAGTAGTAGGCTGCC |
| LMA2 | *gp38* | TACAATCAGCAGCGCATCCA | CCGGAAAGTGTCTGCCAGAT |
| 14-1 | *gp45* | CTTAGCAGCACGACTTGGGA | CTCCCACGTCCCATTCAACA |

**Table 4.2:** Identities of genes in which mutations were particularly likely to be detected across all treatments.

| Gene Function | Number of mutations | Number of genes mutated | Genes with >10 mutations |
|---|---|---|---|
| LPS Biosynthesis | 190 | 10 | *wzy, wzz, wzz2* and *migA* |
| Type IV pili formation | 69 | 17 | *pilQ* and *pilB* |
| TonB-dependent receptor | 50 | 1 | *PA2911* |
| Other/unknown/intergenic | 241 | 146 | *PA1874* |

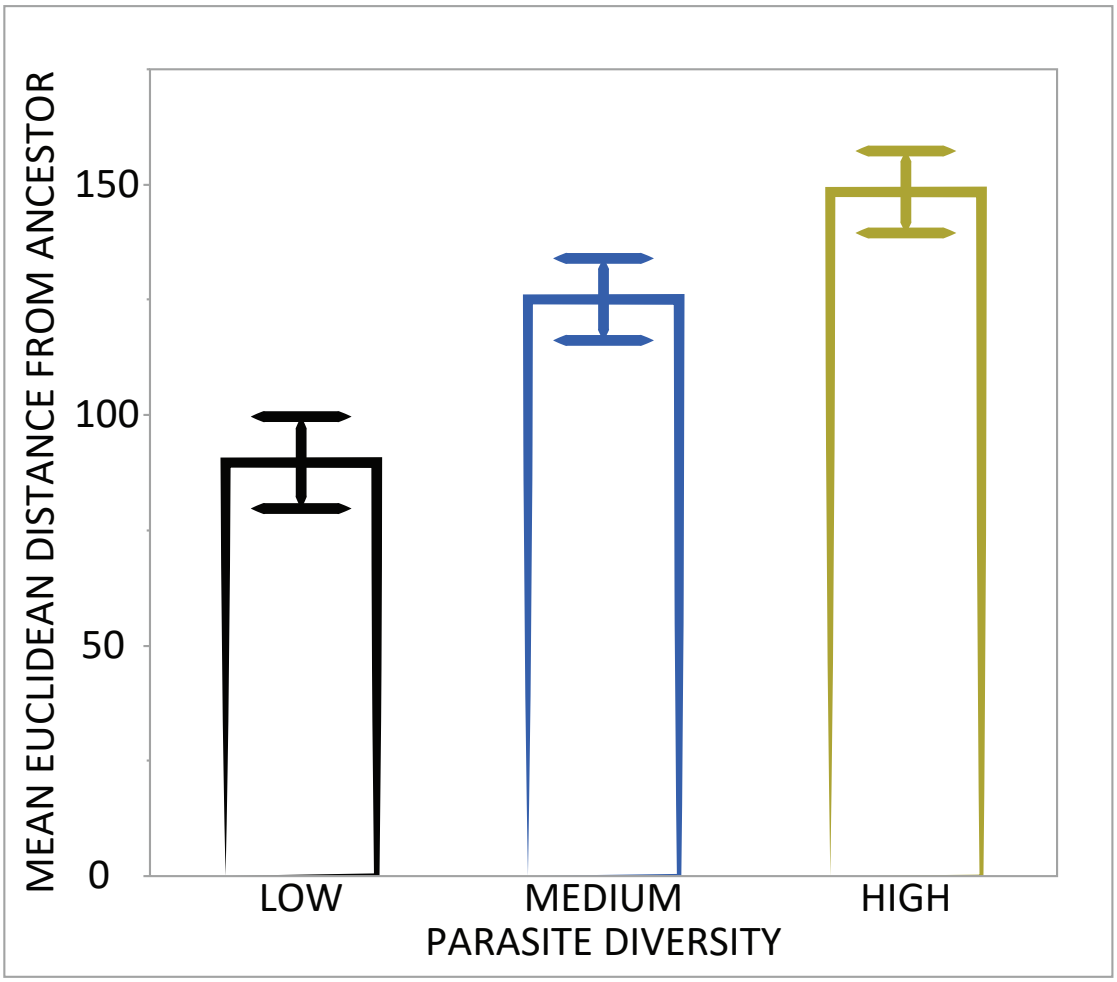



**Figure 4.5:** We compared the mean Euclidean distance between the bacterial populations that had coevolved with phage communities of different diversities. The low parasite diversity populations had the lowest mean distance from the ancestor. Error bars show 1 standard error.

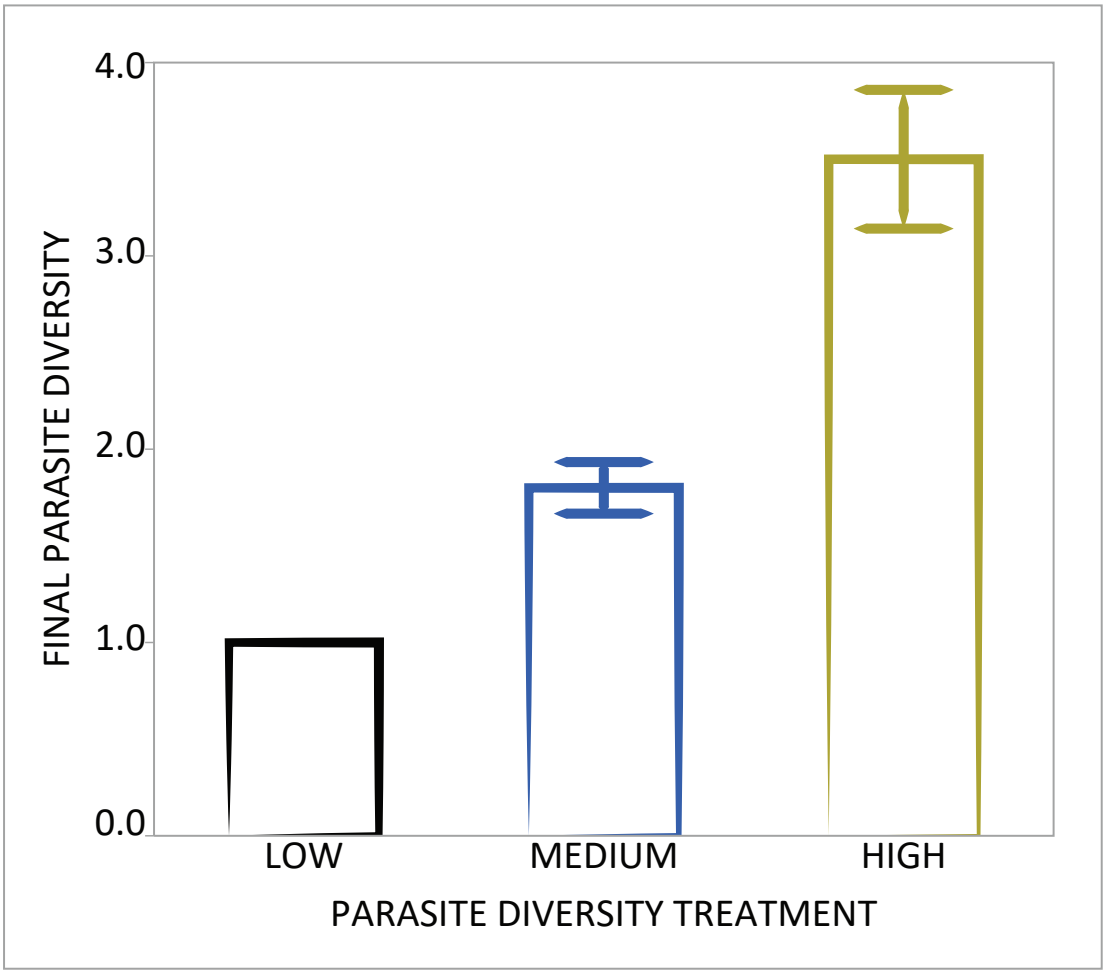



**Figure 4.6:** Shows the final diversity within the communities selected for host genomic analysis as confirmed by phage specific PCR. Error bars indicate 1 standard error.

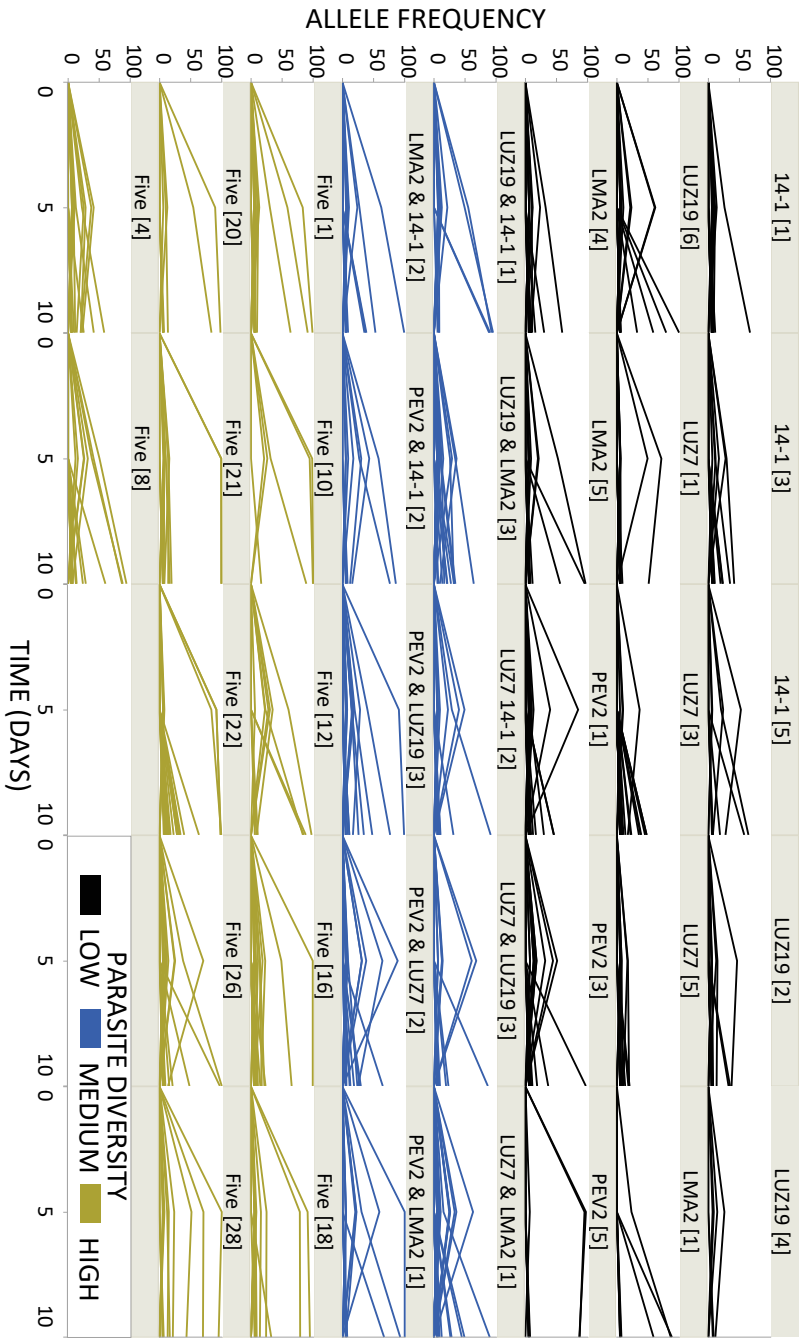



**Figure 4.7:** The frequency of each observed mutation in each sequenced population from the start, mid and endpoints of the experiments. More alleles reach higher frequencies in the high parasite diversity treatment than in the medium or low.

*The apocalyptic scenario is that when I need a new hip in 20 years I'll die from a routine infection.*

— Prof Dame Sally Davies (2013)

# 5

# The Evolutionary Consequences Of Phage Cocktail Diversity

## 5.1 Abstract

In the face of escalating antibiotic resistance, there is an urgent need to develop alternative antimicrobials such as "phage therapy" which uses viruses that infect and kill bacteria. While the clinical focus is on phage cocktails consisting of multiple viruses, most basic research uses phage monocultures. Consequently, we lack an understanding of the potential consequences of phage cocktail diversity for bacteria and phage evolution. Here, we address this gap by applying experimental evolution to the pathogen *Pseudomonas aeruginosa* and a panel of natural phage isolates, including assays with a commercially available cocktail. We hypothesised that phage diversity might influence resistance phenotypes, favouring broader resistance and we confirmed that such generalised resistance confers greater protection to an allopatric phage cocktail. The mechanisms through which generalised resistance is achieved include mucoidy, a clinically relevant phenotype associated with virulence. Finally, we show that coinfecting phages interact on a genomic level forming recombinant genotypes. This type of interaction could pose an interesting challenge to the therapeutic application of phages and we discuss how we can improve the design of phage cocktails in light of evolutionary biology.

## 5.2 Introduction

Antibiotic resistance has reached worrying levels amongst pathogens of both humans (1) and livestock (2). Even the "last line of defence", the antibiotic colistin is now facing unprecedented levels of resistance (3). For several years, researchers have been

calling for better stewardship of existing antibiotics and increased research into new or alternative antimicrobials (4). One such alternative is "phage therapy" (5), in which phages (the ubiquitous viral parasites of bacteria) are used to treat infections. To complete their natural lifecycle, phages must infect and kill bacteria, and in this way, they necessarily influence bacterial evolution by selecting for resistance in their bacterial hosts (6). For phage therapy to be viable, it is therefore imperative we understand the nature of phage resistance evolution.

The evolutionary interactions within many host-parasite systems, including bacteria and phage, have mostly been investigated in pairwise frameworks where a single parasite species attacks a single host species (7–9). Such designs are powerful tools for exploring the processes that might drive host-parasite evolutionary interactions (10, 11), hosts can be simultaneously under attack from multiple different enemies. As the diversity of interactions in experimental systems is increased through additional parasites, predators, competitors, towards what Darwin referred to as a "tangled bank", feedbacks produce outcomes that are not simply the sum of their parts (12). For example, when two parasites co-infect, they can interact antagonistically and compete for host resources (13) or they may have a synergistic effect where the presence of one parasite facilitates successful infection by a second (14). In the context of phage therapy, such antagonistic interactions must be avoided to optimise the efficacy of treatments (15).

In the phage therapy, treatments can be administered as "monophage" preparations whereby a single species of phage is applied to an infection (16). However, often treatments involve the application of multi-species "phage cocktails" (17). Cocktails may help delay the evolution of resistance (18) and can increase the potential host range of phage preparations (15). In studies reporting the efficacy of such cocktails, the evolution of pathogen resistance is considered after the cocktail has been assembled and tested (19–24). Or it is not discussed at all (21, 25, 26). Although some phage cocktails have been assembled based on cross resistance between the phages within that cocktail (16), it should be possible to apply a broader evolutionary perspective and create more evolutionarily favourable banks of phage cocktails which may reduce the probability of resistance evolving.

Despite the therapeutic use of phage cocktails, we still lack an understanding for how phage diversity might influence the nature of resistance evolution in the target bacterial hosts or any reciprocal adaptations that might arise in the phage. Here, we explored the consequences of phage diversity under experimental coevolution with a bacterial host. In this experiment, we have allowed 10 days for reciprocal adaptation to take place between the bacteria and phage communities of differing complexity, from mono-phage up to a five-phage cocktail. Firstly, we examine the effects of diversity on the evolution of a clinically-relevant mucoid phenotype. Mucoidy almost always evolves during chronic *P. aeruginosa* infection of the lungs of Cystic Fibrosis patients and it is associated with increased mortality (27). Secondly, we test how evolution with one phage cocktail affects the resistance to an allopatric phage cocktail. Such cross-resistance mechanisms have been important for the rapid spread of antibiotic resistance could also affect phage therapy outcomes. Thirdly, we examine whether coinfecting phage species interact on a genomic level and whether the outcomes of such interactions have an effect on phage infectivity. We discuss our results in the context of phage therapy and specifically how we can harness evolution to improve the design of phage cocktails.

# 5.3 Materials And Methods

## 5.3.1 Isolates and bacteriophage characteristics

A commercially available Pyophage preparation was obtained from the Eliava institute (Tbilisi, Georgia). These preparations are used in Georgia to treat infections in humans (28) and are designed to be effective against five different bacterial species, including *Pseudomonas aeruginosa*.

For the evolution experiment, isogenic stocks of *P. aeruginosa PAO1* were prepared from single colonies and preserved at approximately $10^7$ CFU/ml at -80°C with 25% glycerol. Five bacteriophage types were used, three from the Podoviridae (PEV2, LUZ19 and LUZ7) and two from the Myoviridae (14/1 and LMA2). All five phage strains were stored as stocks of approximately $10^8$ PFU/ml at 4°C.

## 5.3.2 Evolution Experiment

Monocultures of each phage (Low phage diversity), every possible two-phage combination (Medium phage diversity) and a mixture of all five phages (High phage diversity) were prepared such that the phages were at an inoculation titre of $5\times10^5$ PFU $mL^{-1}$ and within cocktails, each phage type was present in equal densities. These communities were mixed with *P. aeruginosa* cultured overnight in King's B (KB) (29). Each culture was prepared from a separate ancestral colony and used to inoculate each replicate to a starting concentration of $5\times10^7$ colony forming units per mL (CFU $mL^{-1}$). These experiments were conducted in 5 ml round bottom polystyrene test tubes with vented caps (Corning, Flintshire, UK) containing 2.5ml of KB culture medium. These microcosms were incubated at 37°C and 2250 RPM orbital agitation. This experiment was designed to include a blocking structure, with three sequential batches each of 40 lineages. Within each batch, replicates were evenly separated across the three levels of phage diversity (Low, Medium and High-phage diversity) and the phage three control such that each treatment had 10 replicates per batch. For each replicate, a 50$\mu$l sample containing both bacteria and phage were serially transferred to a new microcosm every 24 hours. Each batch underwent ten such transfers. At each transfer, a 500$\mu$l sample was taken and stored in 50% glycerol at -80°C for subsequent analysis.

## 5.3.3 Isolation of phage-evolved bacterial genotypes

Using a sterile 1$\mu$L inoculation loop, coevolved populations were streaked out onto KB agar, these plates were then placed in a static incubator overnight at 37°C in to allow the growth of individual colonies. Some such colonies were picked with another loop and similarly streaked out and incubated to present single colonies thus enabling the isolation of phage free, isogenic bacterial populations from the evolved communities. Five bacterial genotypes were isolated from every replicate microcosm every 24 hours to give a total of 4500 genotypes for phenotypic testing.

### 5.3.4 Selection for Mucoidy

Each of the 4500 isolated bacterial genotypes was scored for the presence/absence of a mucoid phenotype. We have previously observed that mucoidy can evolve very rapidly in the presence of phage and can be mediated by a SNP in genes such as *mucA* (30). The phenotype is very distinctive and results in the excessive production of a translucent slime, which covers an area much larger than a typical colony. These data were categorised first by the diversity of phage with which they were coevolved and then analysed in a linear mixed effects model in R (31) with bacterial genotype nested within replicate, within phage diversity level, within batch as random effects and phage diversity as a fixed effect.

### 5.3.5 Resistance to allopatric Phage

Each bacterial genotype was tested for its resistance against six phage genotypes isolated from the Pyophage cocktail. Isolated bacterial genotypes from 24 hours, 120 hours and 240 hours phage exposure were used to inoculate individual microcosms incubated for 24 hours. Subsequently a 100$\mu$L sample from each microcosm was mixed with 2.9 mL of molten KB agar (0.75% agar) and spread evenly over the surface of a KB agar (1.5% agar) plate. These soft agar lawns were then inoculated with 4$\mu$L droplets of each purified phage which were allowed to dry, and then incubated for 24 hours. This period allowed the formation of zones of inhibition that indicated susceptibility or resistance. Droplets which produced with no visible inhibition were scored as resistant (=1), those with some visible inhibition were scored as partially resistant (=0.5), and those with complete inhibition were scored as susceptible (=0). These data were then analysed as the proportion of clones that were resistant or susceptible to a given phage isolate.

These data were categorised first by the diversity of phage with which they were coevolved and second by the length of time they had undergone coevolution. These data were then analysed in a linear mixed effects model in R (31) with bacterial genotype nested within replicate, within phage diversity level, within batch as random effects and phage diversity crossed with length of coevolution as fixed effects.

### 5.3.6 Detecting inter-phage recombination

Whole genome sequencing services were provided by the Wellcome Trust Centre for Human Genetics (Oxford, UK) using the Illumina HiSeq 4000 platform with 150 bp paired-end reads. We selected one of the five-phage community samples for an in depth recombination analysis. Using CLC genomics Workbench (CLC Bio, Aarhus, Denmark), 5' or 3' were trimmed if the Phred quality score was less than 20 and reads were discarded if they were shorter than 50 bp after trimming or if more than 2 of bases were ambiguous.

Filtered reads were then de novo assembled into contigs using SPAdes 3.10.1 (32). The contigs were then blasted (33) against the NCBI database (34) and a single 74 kb contig was identified with >90% identity to two of the phage from the 5-phage community, PEV2 and LUZ7. This contig was then aligned with the reference genomes for the five phages which formed the cocktail using Kalign

(35). This alignment was then inspected in MEGA7 (36) and the final quality controlled alignment was then analysed using RDP4 (37). A phylogeny showing the evolutionary relationships between the five phage types and the assembled contig was produced using the FastNJ tool built into RDP4 and a full exploratory recombination analysis was performed.

## 5.4 Results

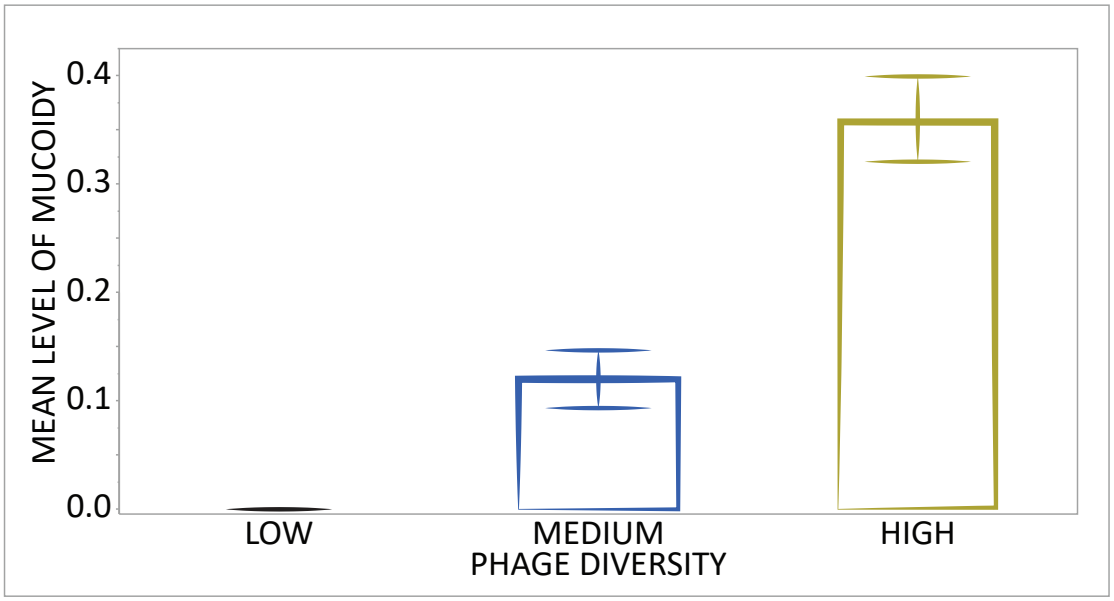



**Figure 5.1:** This plot shows how the frequency of mucoid phenotypes that evolved during coevolution increased with the diversity of the coevolving phage community. Error bars show 1 standard error.

### 5.4.1 Selection for Mucoidy

Mucoidy was observed in the medium and high phage diversity populations after 24 hours coevolution but not in the low phage diversity treatment (Fig. 5.1). No mucoid phenotypes were observed at later time points. There was a significant effect of phage diversity on the level of mucoidy ($F_{2,447} = 39$ $P<0.001$). All three phage diversity treatments were significantly different from each other (Post-hoc Student's T test $P<0.05$).

### 5.4.2 Resistance to allopatric Phage

We observed that parasite diversity significantly affected the level of resistance evolved to allopatric phage (Fig. 5.2, $F_{2,447} =52.6$, $P<0.01$), the high phage diversity treatment selected for the greatest resistance and the low phage diversity selected

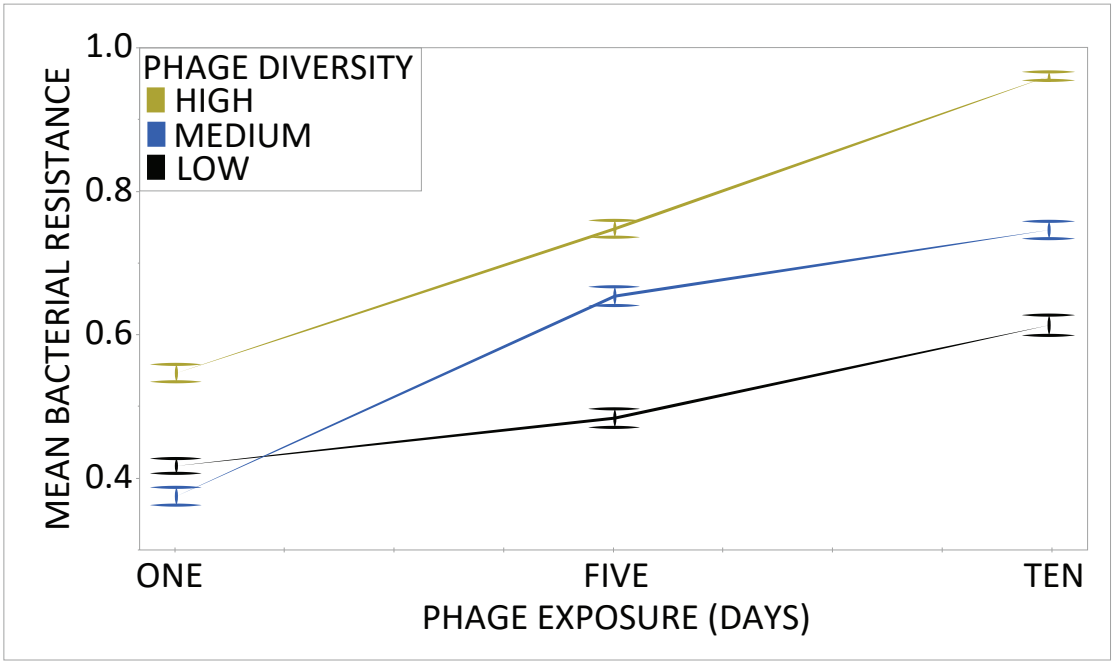



**Figure 5.2:** This figure illustrates how coevolution over time with phage preparations with different levels of diversity affects resistance to phage from a commercially available phage preparation. Increased prior exposure to our experimental phage cocktails increases allopatric resistance and the higher the phage diversity, the greater the evolved resistance. Error bars show 1 standard error.

for the least resistance (Post-hoc Student's T test $P<0.01$). The data also show significant change in the level of evolved resistance as coevolution progressed ($F_{2,7644}=839.6$, $P<0.01$). Within each level of parasite diversity, resistance increased as the length of phage exposure increased (Post-hoc Student's T test $P<0.01$). The significant interaction between parasite diversity and time ($F_{4,7644}=49.8$, $P<0.01$) is largely explained by increasing divergence between the levels of resistance in the low and medium phage diversity populations. These populations were not significantly different after 24 hours but were very different after 5 days (Post-hoc Student's T test $P<0.01$) and remained so after 10 days (Post-hoc Student's T test $P<0.01$).

### 5.4.3 Detecting Recombination

The phylogenetic topology produced from the whole genomic alignments reflected what is known of the taxonomy of these phage, correctly grouping the Myoviridae and the Podoviridae and it grouped the assembled contig closely with the phage LUZ7 (Fig. 5.3).

Five separate recombination detection methods significantly supported a recombination event between the phages LUZ7 and PEV2 in the formation of the phage genotype being investigated ($P<0.01$, Table 5.1).

**Table 5.1:** This table shows the results of a full exploratory recombination analysis performed on the assembled phage genome in RDP4 with five detection methods strongly supporting a signficant recombination event between LUZ7 and PEV2.

| Major Parent | Minor Parent | Detection Method | P value |
|---|---|---|---|
| *LUZ7 (NC_013691)* | *PEV2 (NC_031063)* | GENECONV | <0.01 |
| | | Maxchi | <0.01 |
| | | Chimaera | <0.01 |
| | | SiSscan | <0.01 |
| | | 3Seq | <0.01 |

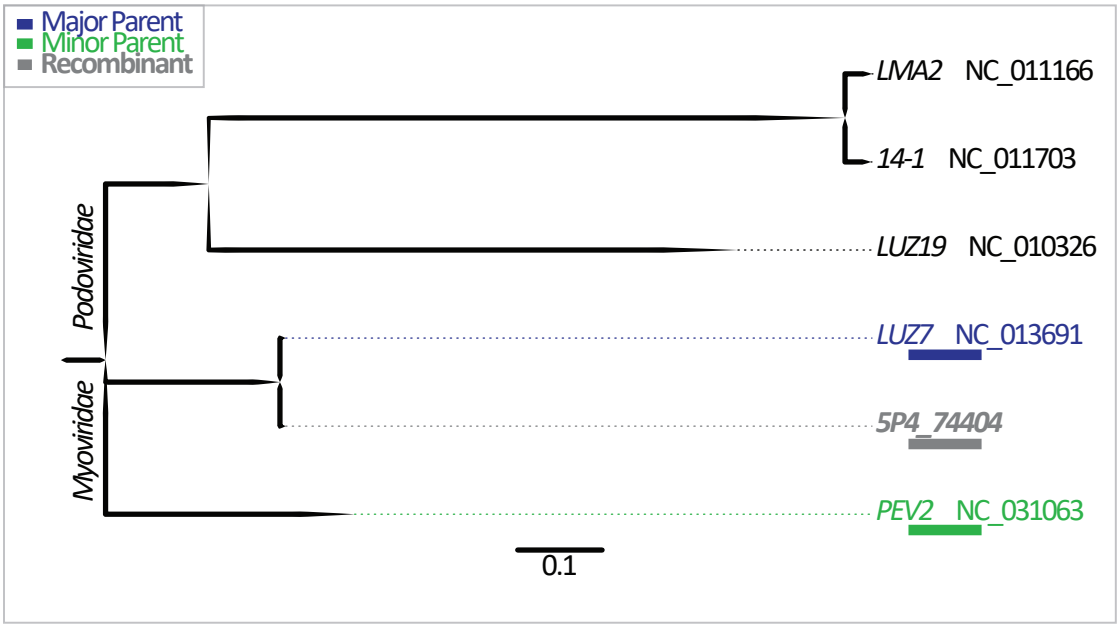



**Figure 5.3:** A phylogeny built using the FastNJ tool built into RDP4 showing the relationship between the five ancestral phage species and a regombinant genoype that arose during experimental coevolution.

## 5.5 Discussion

Phage cocktails can have important evolutionary consequences for both phages and their bacterial hosts. Different phage types within cocktails can interact, and we show they have the potential to recombine during coinfections producing novel genotypes that hosts must resist. The diversity within cocktails here also favoured the rapid emergence of a mucoid phenotype that confers generalized resistance to never before encountered phage clinical treatments. These two outcomes might have opposing consequences for the effectiveness of phage therapy as a suitable alternative to antibiotics.

Recombination between phages has long been recognised as a prominent feature of bacteriophage evolution (38, 39), and such recombination events can produce phage genotypes which differ substantially in their infectivity (40). Recombination between

different phage genotypes of the same strain has been observed during the long term (180 days or 13,000 phage generations) experimental coevolution with its bacterial host (41). However, an understanding of the potential influence of between strain recombination within phage cocktails is limited, perhaps due to the assumption that it could not occur within the time scale of treatment. However, host-parasite antagonism can drive very rapid evolution (42) and here we see strong evidence of significant recombination occurring quite rapidly between different phage strains (within 10 days). This represents a powerful evolutionary mechanism that could influence the evolutionary arms races that take place between hosts and parasites on a time scale that may be relevant in the context of phage therapy. The Red Queen Hypothesis explains that hosts evolve to have sex to benefit from recombination and fend off parasites (43). This theory could potentially also be applied to parasite populations facing the evolution of host resistance (44). In this case hosts can evolve resistance extremely rapidly (30) and reciprocally we see the evolution of recombinant parasites. This property may in fact be a bonus of phage diversity within cocktails, as increasing diversity increases the possibility for recombination to produce novel infectious genotypes to challenge the bacterial target.

On the other hand, a potential disadvantage of phage diversity is that is seems to favour the evolution of a mucoid bacterial phenotype. Mucoid phenotypes evolved rapidly in the presence of phage cocktails but not phage monocultures. However, phage cocktails only selected for transient mucoidy as populations also lost this phenotype very quickly and it did not reappear later in coevolution. Mucoid conversion as a result of phage imposed selection is known to have a high fitness cost for *Pseudomonas* (45), it makes sense that mucoidy could be replaced by a less costly resistance mechanism. In a clinical context conversion of *Pseudomonas* to a mucoid phenotype is associated with the chronic lung infections which affect cystic fibrosis (CF) patients (46). Given that phages have also long been known to be a component in the selective environment of the CF lung (47) it is possible that phage selection plays a role in the selection for this phenotype during such chronic infections (45). These data unambiguously demonstrate that it is not just the presence of phages that favour mucoidy but also the diversity of those phages. In practice, it might be possible to protect against such an outcome by including in cocktails phages that can break down the alginate secreted by bacteria (48), thereby reducing the advantage conferred by mucoidy. Nevertheless, phage diversity can substantially effect the nature of resistance phenotypes in vitro. Thus, we should consider likely resistance mechanisms when designing phage cocktails to limit selection for potentially dangerous resistance phenotypes.

We find that greater cocktail diversity can generate bacterial genotypes more resistant to a second, allopatric phage cocktail. We show that the resistance mechanisms evolved against monocultures of phage are sufficient to convey partial resistance to phage from an allopatric cocktail, but that coevolution with a diverse phage cocktail can provide almost complete resistance to an allopatric phage cocktail. Such broad resistance could evolve if the diversity of phage receptors available in *P. aeruginosa* were limited, which it may be as the majority of *Pseudomonas* phage which have been recently characterised seem to rely on LPS or type IV pili (49–53). In which case, any resistance mutation would be likely to convey a modicum of resistance to an allopatric cocktail. In gram negative bacteria, such as *P. aeruginosa*, phages attached to the surface features LPS, protein receptors, pili and flagella (54). The cocktail used in our coevolution experiment contains phage that select for resistance mutations in LPS, type IV pili and an outer membrane protein (30). Therefore, it may be that the unknown phages in the allopatric cocktail share the same surface receptors. If phage therapy were to be used on chronic infections requiring multiple applications of phage longitudinally, then the

effectiveness of each application might be strongly influenced by the composition of previous cocktails. Each cocktail could select for resistance mutations that are also effective against the next treatment.

In conclusion, our results highlight how an evolutionary perspective could benefit phage therapy. Phage therapy is undergoing a renaissance (55) and resistance to phage readily evolves in vitro (30), it therefore seems prudent for phage therapy research to explore how to limit the potential impact of the evolution of resistance. A first step to achieving this could be by applying a wider evolutionary rationale when phage cocktails are being designed. By considering how resistance to one phage affects resistance to another, and investigating not only cross-resistance between phage within cocktails (16), but also the cross resistance between phages in different cocktails intended for use in the same environment. Such data could then be used to organise treatments in such a way as to force the greatest mutational burden on bacteria requiring them to accumulate the maximum number of resistance mutations to survive. Furthermore, characterisation of the biology of different resistant phenotypes that emerge to different phage could be used to estimate the cost of resistance (56) for the bacteria to each phage. Thus enabling an evolutionarily enlightened approach to cocktail design in which cocktails are not only be assembled based on non-overlapping resistance profiles but also based on maximising the cost of resistance. This could ensure that when resistance evolves, the bacteria are forced into a less favourable region of their adaptive landscape (57) where they pay the maximum fitness cost possible for resistance, ideally creating trade-offs which leaving them more vulnerable to host immune systems or the next phage cocktail. This approach is limited by the context dependence of fitness cost measurements (58), therefore further study *in vivo* is required to explore how this approach could be implemented.

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# 6

# Discussion, Conclusions and Future Work

## 6.1 Key Findings

Each of the presented chapters includes an in depth discussion of the data presented within, here I will highlight the key findings and discuss some of their wider implications.

### 6.1.1 Parasite diversity drives rapid host dynamics and evolution of resistance in a bacteria-phage system

- Bacteria evolve can evolve resistance to parasite communities very quickly (within 24 hours).
- Parasites within communities can interact antagonistically, competing for hosts.
- Different parasites favour different resistance mechanisms in their host.

### 6.1.2 Parasite diversity accelerates coevolution

- Parasite diversity drives faster, more divergent evolution.
- Rapid host-parasite coevolution disrupts the population density oscillations that one might expect from a purely ecological process.
- High parasite diversity imposes stronger directional selection and shifts interactions away from negative frequency dependence in favour of Arms Race Dynamics.

### 6.1.3 The evolutionary consequences of phage cocktail diversity

- Coinfecting phage can recombine within hosts during antagonistic coevolution to produce novel genotypes.
- Phage diversity can favour the evolution of clinically relevant mucoid phenotypes.
- Coevolution with more diverse phage cocktails can select for broad resistance that is effective against allopatric phage cocktails

## 6.2 Discussion

The initial pilot evolution experiment presented in Chapter 3 demonstrated how the complexity within parasite communities can influence the nature of resistance evolution, with parasite diversity favouring generalised resistance mechanisms. The rapid emergence of resistance differentially affected the ecological dynamics within host-parasite communities of differing diversities. This experiment highlighted some important issues which merited further investigation. In particular, higher order interactions between parasites in communities produce evolutionary outcomes which differ from those that might be observed in pairwise investigations between parasite monocultures and their hosts. Yet the vast majority of our experiments which have advanced our understanding of host-parasite interactions are based on such pairwise frameworks. If we are to use our experimental data to make more accurate predictions about diverse natural systems with many parasite species we must then build higher diversity into our experimental designs.

These initial results emphasised the possibility that parasite diversity could have important long term consequences for host-parasite ecology and evolution. If parasites in communities differ in the resistance phenotypes for which they select, and parasites in communities compete for hosts, then it is logical that coevolution with parasite communities will follow divergent trajectories from coevolution with members of those communities in pairwise frameworks. This possible eventuality was then explored via experimental coevolution in Chapter 4.

Coevolution between hosts and parasites can be very fast. As these data show, rapid change in the composition of host populations from susceptible to resistant genotypes necessarily impacts ecological dynamics i.e. by reducing parasite growth. Effectively this allows that when coevolution is sufficiently rapid, the classic ecological predictions about host-parasite densities oscillating through time begin to break down. Relative densities of both species could in fact remain more stable over time, as the populations of both players subdivide into genotypes of different infectivity and susceptibility ranges. Under such conditions, it makes perfect sense that rapid coevolution could be detected purely from ecological data as a result of eco-evolutionary dynamics and the resultant disruptions in the oscillations predicted under purely ecological processes. Interestingly, such density oscillations between bacteria and phage have been observed between bacteria and phage in marine environments (1) but are not seen in the mammalian gut (2–4). This could be indicative of different coevolutionary rates in different environments, although to my knowledge this has never been directly tested.

Evolutionary rates were explored here using allele frequency data from the coevolution experiment. The data unambiguously demonstrate that host evolution is more rapid in the presence of more diverse coevolving parasite communities. The phenotypic data support this and also show that the host phenotypes selected for are more resistant to infection. These results together with the in depth analysis of the underlying type of selection indicate that parasite diversity drives stronger arms races with hosts. These result allows some insight into why such swift evolution can be observed in parasite rich biodiversity hotpots but also have potentially very important implications for the design of biological controls.

These experiments have shown that parasite diversity accelerates host evolution and shifts coevolution away from arms race towards red queen dynamics. Although I have not previously speculated on why the dynamics have shifted in this way, I believe we have enough signal within the collected data to build a circumstantial case. In the 24 hour experiment we sequenced clones that have evolved resistance to a diverse phage communities and compared these mutations to those observed to combat phage monocultures. The data clearly show that monocultures select for resistance mutations specific to each parasite whereas communities favour broader resistance phenotypes driven primarily by selection for mutations in genes encoding LPS biosynthesis. This was the first evidence from the data that suggested LPS mutations might serve as generalised resistance mechanisms capable of resisting many types of phage. As stated previously (Chapter 5), it may be that LPS serves as an initial or supporting source of attachment that enables phages to cling to their hosts whilst they search across the surface for their final receptor to which they can bind and infect the host. This makes sense given the data we have collected as phages that we know bind to surface proteins or type IV pili can also be resisted by single nucleotide polymorphisms in LPS biosynthesis genes.

Furthermore, populations which have a high frequency of LPS biosynthesis mutations following coevolution with phage communities are more resistant to very diverse allopatric phage communities, supporting the idea that LPS biosynthesis mutations confer broad resistance. If LPS biosynthesis mutations can then be viewed as generalised resistance mechanisms, we might then expect coevolution with parasite communities to favour directional selection promoting LPS biosynthesis mutations. In which case the shift from red queen could be explained by increased selection for generalised resistance phenotypes driven by selective sweeps for LPS biosynthesis mutations in the face of increasing phage diversity.

The implications of these results for biological controls are supported by some findings in Chapter 5. Increasing phage diversity favoured the evolution of a clinically problematic mucoid phenotype, increased resistance to allopatric phage. In phage therapy, preparations of multiple phage species (phage cocktails) are often administered rather than simple phage monocultures. The best justification for this is to increase the host range of the treatment (5), more phage species means a greater probability that at least one of them will be effective against the bacteria causing infection. However, it seems that should treatment fail, the consequences for bacterial resistance evolution could potentially make the pathogen more problematic. It could be important for those designing phage cocktails to investigate the likely resistance phenotypes to ensure that there are no dangerous consequence such as increase virulence. Furthermore, if multiple rounds of treatment are required, it may be necessary to develop cocktails with minimal cross resistance to reduce some of the problems caused by rapid resistance evolution.

Unfortunately, for phage therapy there are still scientific and regulatory challenges that stand as huge obstacles in the face of the wider therapeutic application of phages.

These data show that bacteria can evolve resistance to phage very quickly. The data also show this resistance may also be overcome following a coevolutionary response from a phage. However, it is difficult to convince clinicians that phage evolution may be a good thing even if it allows continued efficacy, as effectively it means that the dose, pharmacokinetics and pharmacodynamics may all change during treatment as phages replicate and mutate in response to the selection imposed by the patient's own immune system, the host bacteria and interactions between phages in the cocktail.

Furthermore, despite cocktails apparently being the preferred option by clinicians, the data from these experiments have confirmed several disadvantages of cocktails relative to monocultures including: reduced phage titres due to competition between coinfecting phage, accelerated resistance evolution by the bacteria and the selection for potentially dangerous resistance phenotypes such as mucoidy. Ostensibly the greatest advantage of phage cocktails is increased spectrum of effect allowing more bacterial genotypes to be treated with a single preparation. This is very desirable if the pathogen causing the infection has not been identified. However, if the target bacteria is known, these data suggest that a phage monoculture might be more effective. As technology improves, particularly DNA sequencing and genotyping technologies, rapid identification infecting pathogens might become more feasible in a clinical setting. This could allow clinicians to move away from broad spectrum antimicrobials in favour of more specific therapeutics like phage monocultures.

## 6.3 General Conclusions

These findings underline the importance of including a wider biotic context into experimental designs. Experimental investigations of host-parasite coevolution have almost entirely been explored using pairwise frameworks. Such designs are essential for a full understanding of the processes driving evolution. However, to understand the patterns we see in nature and make better predictions about them, we need to increase the complexity of our experimental systems. These experiments take an important step in that direction but are limited by their in vitro environment. To further enhance our understanding of bacteria-phage coevolution and make meaningful recommendations about phage cocktail design, similar experiments need to be conducted in vivo, during the phage therapy of an infected animal host.

## 6.4 Future Work; Surviving the Antibiotic Apocalypse

We now understand how infectious bacteria and their phages interact in test tubes. Such in vitro tests paired with longitudinal sampling of clinical infections confirm that infectious bacteria and phages can evolve together over time, revealing increases in the ability of phages to destroy bacteria or in the ability of bacteria to resist. However, we lack an understanding for how bacteria might evolve resistance to phage therapy (or how phages themselves might evolve) in an infected animal host. Thus, I propose to develop an in vivo experimental system (Figure 6.1) to directly test hypotheses on the evolutionary interactions between pathogenic bacteria and therapeutic phages. This work will allow me to make robust predictions on the

outcomes of bacteria-phage evolution for host health with major consequences for the application of phage therapy in a post-antibiotic world.

Larvae of the wax moth (*Galleria mellonella*) are natural parasites of honeybee colonies; they tunnel through honeycomb eating pollen, wax and stored honey. Bacterial infections of wax moths are common, and may include multiple species of bacteria simultaneously including *Pseudomonas aeruginosa*, which is the primary microbe that will be used in these experiments. *P. aeruginosa* is also an opportunistic human pathogen and causes particularly high mortality in patients suffering from cystic fibrosis. It has long been suggested as a phage therapy target due to its propensity towards high levels of antibiotic resistance. The phage that will be used here are all natural isolates whose coevolutionary dynamics with *P. aeruginosa* have now been well characterised *in vitro* making them ideally suited for these experiments.

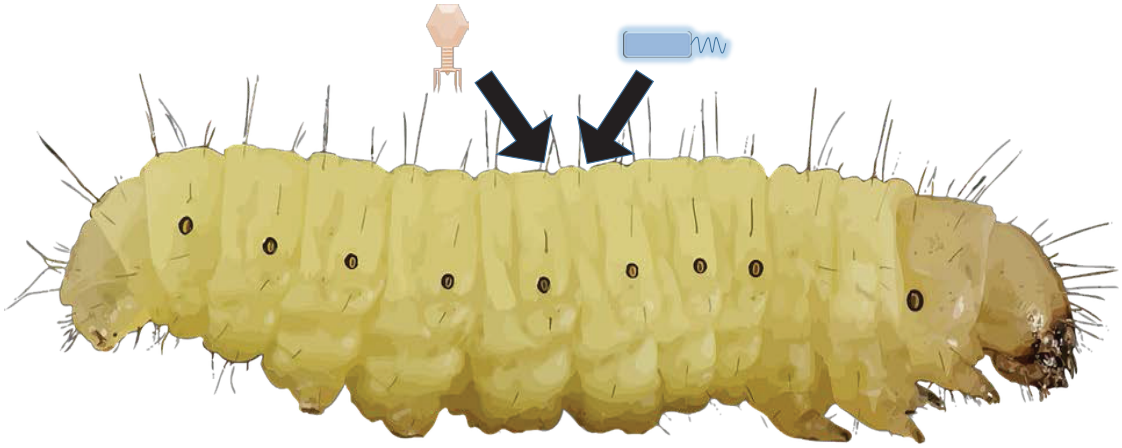


**Figure 6.1:** Wax moth larvae will be injected with both bacteria and phage. Samples will be taken from the insect will be taken over time so that the dynamics of infection can be tracked and coevolution between the bacteria and phage can be observed

### 6.4.1 Summary of Proposed Research

An experimental evolution approach will be used to test how a natural host environment can impact upon bacteria and phage evolution during phage therapy, with a focus on three key questions;

- How do bacteria and phage evolve within an animal host?
- How does a phage cocktail affect phage-bacteria evolution during host therapy?

- What are the effects of phage therapy on the evolution of polymicrobial infections?

Much of our knowledge of bacteria and phage evolution is under in vitro conditions and is focussed on pairwise interactions between single species of bacteria and phage. My PhD has extended this classical framework by studying the patterns and processes of bacteria-phage evolution in the context of diverse phage communities. We now know, for instance, that bacterial pathogens can evolve resistance to multiple phages simultaneously, but that phages can reciprocally fight back. This proposed fellowship will go much further, by including a more realistic, diverse system with: (i) an animal host dimension, so we can assess consequences of bacteria-phage evolution for host health, and (ii) polymicrobial infections, a reality of many hospital-acquired infections. Here, bacteria will be monitored as they adapt to host immune systems, phage, and to competition with other bacteria.

This system will be used to characterise the infection evolutionary dynamics in wax moth larvae undergoing phage therapy using phage preparations of differing diversity and in the presence of secondary bacterial infection by a competing microbe. By sampling the infections over time, the epidemiological consequences can be simultaneously considered alongside the evolutionary consequences. Here, the survival of infected larvae, the levels of bacteraemia and the level of viraemia will be contrasted with phenotypic measures (e.g. virulence and resistance) from the samples taken over time and used to track the evolution of phage and bacterial virulence and resistance during a single infection. These experiments will be extended with serial transfers of evolving communities between infected individuals to sample the long-term dynamics when infections are transmitted between hosts. These samples will be analysed by next-generation sequencing to elucidate the genomic basis underlying microbial and bacteriophage adaptation.

### 6.4.2 Concluding Remarks

Given the unprecedented decline in antibiotic efficacy due to the evolution of pathogen resistance, and the concomitant surge in interest in alternative therapeutics, an enhanced understanding of the evolutionary consequences of phage therapy is timely and significant. Moreover, addressing this gap in our understanding of phage therapy will be essential to informing policy as decisions about where to invest in future antimicrobials are made. There is no theoretical or empirical precedent for investigating the evolutionary dynamics that take place during phage therapy of animals or polymicrobial infections. Yet these dynamics are incredibly important in determining the outcome of drug-resistant bacterial infections and the trajectory of disease evolution in host populations. In addition to characterising these interactions, these experiments will help to develop a conceptual framework for phage therapy through collaboration with theoreticians in Oxford (Prof. Mike Bonsall, Prof. Sunetra Gupta) and thus stimulate further research into phage therapy whilst encouraging a more holistic, progressive perspective in the development of antimicrobial strategies.

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