The emergence, maintenance and demise of diversity in a spatially variable antibiotic regime

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Thesis submitted to
The Faculty of Graduate and Postdoctoral Studies

In partial fulfillment of the requirements for the
M.Sc. degree in the
Department of Biology

University of Ottawa

ABSTRACT

Antimicrobial resistance is a serious and imminent threat to human health, though its rise may be controlled with improved stewardship strategies that limit the emergence and spread of resistant strains. Motivated by theoretical models from population genetics and ecology, my M.Sc. experimentally evaluates how varying drug availability in either time or space impacts the prevalence of resistance in a population. By experimentally evolving *Pseudomonas aeruginosa* under different antibiotic selection regimes *in vitro*, I show that spatial, but not temporal, drug free refuges delay the fixation of resistance by promoting the coexistence of sensitive and resistant genotypes. Second, I establish that this polymorphism is underlain by a trade-off between resistance and growth rate in the absence of antibiotic that underpins the maintenance of diversity through negative frequency dependent selection. Third, I demonstrate that spatially varied drug selection cannot prevent the fixation of resistance because continued selection leads to the evolution of resistant types that pay smaller costs of resistance and gradually displace sensitive strains. These results provide insight into the fate of diversity under long-term selection and highlight the value of incorporating the principles of evolutionary ecology into antimicrobial resistance stewardship.

RÉSUMÉ

La résistance aux antibiotiques forme une menace grave et imminente à la santé humaine, mais l'augmentation de sa prévalence peut être contrôlée via des stratégies de gestion responsable visant à limiter l'émergence et la propagation de lignées résistantes. M'inspirant de modèles théoriques de génétique des populations et d'écologie, ma maîtrise ès sciences évalue de façon expérimentale l'effet qu'ont des variations dans le temps et dans l'espace de la disponibilité d'un médicament sur la prévalence de la résistance dans une population. En faisant évoluer *Pseudomonas aeruginosa* sous divers régimes sélectifs *in vitro*, je démontre que l'existence de refuges spatiaux libres de médicament, et non celle de refuges temporaux, retarde la fixation de la résistance en promouvant la coexistence des génotypes sensibles et résistants. Deuxièmement, j'établis qu'un compromis entre la résistance et le taux de croissance en l'absence d'antibiotique est à la base du polymorphisme qui soutient le maintien de la diversité à travers une sélection négative selon la fréquence. Troisièmement, je démontre que la sélection induite par une variation temporelle du médicament ne peut empêcher la fixation de la résistance car, lorsqu'il est continu, ce régime mène à l'évolution de types résistants, lesquels le deviennent sans compromettre leur croissance et ainsi supplantent peu à peu les lignées sensibles. Ces résultats donnent un aperçu du sort de la diversité sous une sélection à long terme et soulignent l'importance d'incorporer des principes d'écologie évolutive dans la gestion de la résistance aux antibiotiques.

ACKNOWLEDGEMENTS

I would first like to thank my thesis supervisor, Dr. Rees Kassen, whose patient guidance and support enabled me to explore and strengthen my passion for studying evolutionary biology, ecology, and infectious diseases. His great enthusiasm for biology and all of science consistently reminded me why I was so motivated to pursue research in the first place. Importantly, his appreciation for non-academic pursuits has encouraged a holistic approach to life, which I will carry in my future career paths - whatever they may be.

To past and current members of the Kassen and Rundle labs – Dr. Anita Melnyk, Dr. Jeremy Dettman, Dr. Aaron Hinz, Leah Clarke, Alana Schick, Jonathan Dench, Nick McCloskey, Nicole Filipow, Eleonore Lebeuf-Taylor, Patrick Chen, Kevin Kwok, Li Yun, and Chris Angell - you all have provided invaluable insight, advice, company and support in and out of the lab. Thank you to Dr. Alex Wong, Dr. Linda Bonen and Dr. Heather Kharouba who offered useful knowledge and input from the beginning to conclusion of my research.

I am incredibly grateful to the numerous “RAD bio-grad” students whom I have shared many beers, pizzas, Kevin’s cakes, and laughs with. Thank you for giving me the friendship and support all graduate students need to make it through. There are too many of you to list, but if we have ever shared a good laugh, then I sincerely thank you. I really cannot imagine my time in Ottawa without you all.

Lastly, but far from least, thank you to my parents and sister for your unconditional support and care with the many academic decisions and life paths I continuously contemplate and circle about. Your patience with me has been incredible. Whether it was advice in a text, phone call, FaceTime or road trip to visit - I could never have succeeded through my undergraduate or master’s degree without you.

My research was funded by NSERC-CGSM and OGS Scholarships, in combination with the Department of Biology - University of Ottawa.

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THESIS STATEMENT

Separately from my thesis, Chapter 2 was co-authored by my supervisor, Dr. Rees Kassen, for consideration as a published article in *Evolution Letters*. The manuscript style is succinct, so I have included a broader general introduction in Chapter 1, a methods section in Chapter 3, and additional discussion of conclusions in Chapter 4. Chapter 2 is written in plural first person, although all experiments and writing of this thesis was completed my myself, with help from Eleonore Lebeuf-Taylor in translating the abstract and Dr. Julian Evans in producing Figures 3 and 4.

CHAPTER 1: General Introduction

The steady rise of antimicrobial resistance (AMR) in nearly all human pathogens is a critical health threat that requires multidisciplinary attention, including from the fields of evolutionary biology and ecology. The combination of over-prescription of antibiotics in human healthcare and misuse in agriculture has resulted in strong selection favouring the evolution and persistence of drug resistant pathogens. As a consequence, our current arsenal of effective antibiotics will steadily dwindle if new drugs are not delivered to market. Managing our currently available stock of drugs to prolong their utility for as long as possible should therefore be a public health priority. Effective stewardship of antibiotics could benefit from incorporating evolutionary and ecological approaches (Nesse *et al.*, 2010). Conversely, evolutionary biologists and ecologists can arguably take greater efforts to design studies with health implications in mind to advance knowledge across research fields (Nesse & Stearns, 2008). The general aim of my M.Sc. thesis was exactly this – to investigate how established ecological and evolutionary principles of adaptation in heterogeneous environments influence drug resistance evolution and how this insight can be used to inform strategies to manage resistance. Below, I first introduce pertinent theories from population genetics and ecology, then later provide related information on AMR.

Evolutionary and ecological principles of diversity

Variation in fitness and the genetic diversity underlying it together determine the propensity of a population to adapt and persist due to natural selection (Fisher, 1930; Soule & Simberloff, 1986; Lande, 1988; Lande & Shannon, 1996). Genetic diversity, particularly in genomic regions associated with antimicrobial resistance, can increase opportunities for

adaptation to drug-imposed selection and lessen treatment efficacy. Successful treatment of infections with chemotherapeutic agents like antibiotics can therefore be frustrated by genetically diverse pathogen populations (Read & Taylor, 2001). The factors that govern the emergence, maintenance, and fate of variation of resistance profiles in pathogens remain elusive, though similar questions have been debated for some time by evolutionary biologists and ecologists for genetic diversity and biodiversity in general (Hedrick, 2006).

Genetic diversity in asexual pathogen populations may be the outcome of several processes, including transient selective sweeps, neutral variation, mutation-selection balance, and balancing selection. In the simplest case, the observation that genetic diversity exists in a population may be an artifact of time, where a "snapshot" merely captures polymorphisms during an ongoing and incomplete selective sweep of a superior genotype (Kassen, 2014; Maddamsetti *et al.*, 2015). Neutral diversity, allelic variation that is neither beneficial nor deleterious, can also contribute to the maintenance of genetic diversity (Kimura, 1983) though it cannot explain diversity at genes under strong selection for particular traits, such as those associated with drug resistance because by definition, neutral mutations have no effect on fitness. Mutation-selection balance, where mutation repeatedly introduces deleterious mutations that are then removed through negative selection, is another process capable of maintaining genetic diversity in asexual populations (Johnson & Barton, 2005). Increased mutation supply can result from both higher mutation rates and increased population sizes, so the large population sizes typically associated with bacterial pathogens imply that mutation-selection balance surely contributes some portion of genetic variation (Johnson & Barton, 2005). However, it has been suggested that a greater proportion of the genetic variation which contributes to phenotypic traits and related fitness is likely maintained through the mechanism of balancing selection on alternate alleles across heterogeneous environments in time or space

(Hedrick, 2006; Kassen, 2014). The rationale behind this claim is that most natural environments, including that of a human host, can be highly variable and so favour different types under different conditions. Balancing selection underlain by environmental variation is an appealing explanation for the maintenance of diversity because it applies very generally to both the maintenance of genetic variation within populations and species diversity in communities (Vellend & Geber, 2005). Unlike the non-selective stochastic processes of neutral variation or mutation-selection balance, balancing selection is a deterministic force whereby variation observed at loci can be linked to selection for alternate traits in varied environments (Hedrick, 2006). If balancing selection is stronger than non-selective forces generating variation, then genetic variation will be greater at these specified loci compared the rest of the genome (Hedrick, 2006).

The importance of environmental heterogeneity in promoting the emergence and maintenance of diversity has received extensive theoretical (Levene, 1953; Dempster, 1955; Levins, 1968; Felsenstein, 1976) and empirical support at a range of levels of biological organization including genetic variation (Huang *et al.*, 2015), trait variation (Mackay, 1981; Venail *et al.*, 2011), adaptive strategies (Reboud & Bell, 1997; Rainey & Travisano, 1998), and community ecology (Tilman & Pacala, 1993; Vellend & Geber, 2005). My research addresses how environmental heterogeneity impacts pathogen diversity in drug resistance, focusing on divergent selection arising from variation in antibiotic selective pressures.

Divergent selection between environments

Environments can vary through time or in space, and at different scales relative to the lifetime of an individual (Kassen, 2002). Fine-grained variation occurs when the environment changes frequently within the lifetime of an individual, whereas coarse-grained variation

involves environmental changes at scales longer than the lifetime of an individual (Levins, 1968). The form and scale of environmental variation together influence the emergence and maintenance of diversity in an evolving population, with coarse-grained, spatial heterogeneity expected to promote the evolution and maintenance of specialization through divergent selection (Kassen, 2014). Both spatial and temporal variation can generate diversifying selection, but the scale of these environmental changes will influence the extent of specialization (Levins, 1968; Kassen, 2002; Ritchie, 2009). For example, in an organism with a one week life-cycle, daily versus monthly ("fine" versus "coarse" grained) temporal fluctuations in environmental conditions may not select for the same adaptive strategy. If offspring experience different conditions than previous generations, a plastic genotype whose phenotype responds appropriately to prevailing environmental conditions during development is expected to evolve. A versatile, reversible phenotype is instead predicted to evolve if organisms experience fluctuating conditions over the course of an individual's lifespan (Kassen, 2002). Additionally, the strength of divergent selection and rate of migration between the separate environments both play a role. With low migration between environments and strongly divergent selection, specialization more readily evolves in spatially varying environments since a refuge for specializing types is provided. Migration is trivial in temporally varying environments since all lineages must repeatedly survive through each successive environment, thereby favouring a generalist strategy with a broader niche (Felsenstein, 1976; Reboud & Bell, 1997; Kassen, 2002). These general predictions of specialization under environmental heterogeneity apply across multiple levels of genetic and phenotypic variation, by which genetic associations shape habitat specific fitness.

Genetic diversity across environments

As described in Reboud and Bell (1997) and Kassen (2002), genetic polymorphisms can be supported by fitness trade-offs that evolve across environments. The trade-offs themselves stem from three genetic mechanisms. Firstly, with antagonistic pleiotropy, an allele may be beneficial in one environment but deleterious in another. Secondly, beneficial mutations that are selected in one environment may be neutral in another. Thirdly, mutations neutral in the environment of selection can accumulate through stochastic processes or by hitch-hiking alongside linked beneficial mutations, but these may be deleterious in an alternate environment. Under strong divergent selection, these genetic mechanisms mean that improvement in one trait often involves substantially less improvement, or even regress, in another, resulting in a negative genetic correlation between the two traits. If the two traits are fitness in alternative environments, the result is a fitness trade-off across environments that promotes divergence and specialization. In principle then, more genetic diversity is expected in spatially heterogeneous environments, which better enable the persistence of multiple specialized types, each adapted to a distinct patch or niche in the environment. Comparably, temporal heterogeneity should support less diversity because a single generalist genotype is favoured. Building on these very general expectations, classic population genetic models have investigated in various contexts the conditions enabling stable polymorphisms in subdivided populations (Levene, 1953; Dempster, 1955; Felsenstein, 1976).

Models for maintained polymorphisms

The analysis of the evolution of fitness trade-offs across environments discussed above suggests that spatial variation should be much more effective at maintaining genetic variation within populations than temporal variation. The conditions under which diversity can be stably maintained, however, are in fact quite restrictive. Early theoretical work on the population

genetics of selection in spatially variable environments by Levene (1953) and Dempster (1955) illustrate why. Consider a genetically diverse population composed of two specialists, each adapted to one of two patches in a spatially variable environment. A mixed population is first distributed at random into the two patches, followed by selection, and then population regulation before the cycle is repeated. The two versions of the model differ in where population regulation occurs in the sequence of events. Population regulation always occurs after selection but this happens at the level of the patch in the Levene version and at the level of the mixed population in the Dempster version (note that the Levene model is sometimes referred to as "soft selection" whereas the Dempster model is called "hard selection"). Under "Levene-type" conditions, each patch contributes a fixed number of individuals to the next generation, irrespective of the patch's relative productivity (i.e., final population size). The Dempster model, by contrast, imposes no such constraint, so that very productive patches contribute many individuals to the next generation, while less productive patches contribute relatively fewer. The impact of this subtle difference in the timing of population regulation is profound: diversity can be stably maintained by negative frequency dependent selection in the Levene model but not in the Dempster model, where the fittest type from the most productive patch eventually swamps out all other types. A polymorphism therefore can be conserved when two conditions exist – firstly, the genotypes are favoured in alternative environments, and secondly, when population regulation occurs at the local patch level (soft selection) rather than at the global population level (hard selection).

Notably, neither model, in their original form, considers the effect of on-going selection and adaptive evolution. Selection could alter any number of the models' features that impact the conditions for coexistence, including fitness trade-offs, patch productivities, or the degree of specialization. More recent studies have incorporated evolution into the Levene model (Kisdi &

Geritz, 1999; Meeûs & Goudet, 2000; Kisdi, 2001) and concluded that the extent of environmental specialization, or trade-offs, is key for maintaining genetic polymorphisms on evolutionary timescales: trade-offs must be sufficiently strong to prevent the evolution of a generalist type in order for diversity to be maintained in the long term. Interestingly there have been rather few direct experimental tests of the models' theory. *In-vitro* experiments investigating the maintenance of diversity with hard selection are scarce (Bell, 1997; Gallet *et al.*, 2017 - preprint), but indicate the value of examining fitness trade-offs between environments and frequency dependent selection on longer timescales.

Negative frequency dependence

Theory predicts that multiple specialist strategies can coexist indefinitely through negative frequency dependent selection, where a genotype's fitness is inversely proportional to its frequency in a population (Levin *et al.*, 1988; Rainey & Travisano, 1998; Rozen & Lenski, 2000). In a simple case of two types, a stable equilibrium ratio is predicted when both types are equally fit (i.e., relative fitness, or ω, = 1). A negative relationship between relative fitness and frequency of either type can ensue from some form of fitness trade-off, as exemplified in natural field populations (Smithson & MacNair, 1997) and experimental evolution studies involving cross feeding mechanisms (Rozen & Lenski, 2000; LeGac *et al.*, 2012), resource use (Friesen *et al.*, 2004; Spencer *et al.*, 2008), or spatial niche construction (Rainey & Travisano, 1998; Frenkel *et al.*, 2015). In the absence of perturbation or continued evolution, negative frequency dependence can in theory maintain a polymorphism indefinitely.

Clonal interference

The dynamics of genetic polymorphisms and associated phenotypic variation is influenced by competition between multiple arising lineages, or clonal interference (Gerrish &

Lenski, 1998). Although a frequency dependent relationship is typically interpreted as a simplified situation of two competing static types, multiple and evolving lineages may exist within each subtype. As lineages within and between subtypes compete, this ever-changing variation can interfere with selective sweeps of a superior genotype and prolong the maintenance of genetic diversity. The effects of clonal interference are particularly substantial in asexual populations since multiple beneficial mutations are unable to recombine onto a single genetic background. The superior genotype may still prevail under conditions of clonal interference but will fix more slowly, since competition with co-occurring beneficial genotypes will reduce its relative selective advantage. Classic population genetic models (Levene, 1953; Dempster, 1955; Levins, 1968) consider competition between just two types that have fixed fitness attributes. In comparison, evolution experiments must consider ongoing adaptive evolution and clonal interference arising from a steady supply of novel mutations. Past microbial evolution studies have proposed that the combination of clonal interference with negative frequency dependent selection can prevent the immediate fixation of a superior genotype (Maddamsetti *et al.*, 2015). Polymorphisms should be stable if driven by frequency dependent mechanisms, but ongoing evolution can disrupt this balance and undermine diversity in the long-term (Frenkel *et al.*, 2015). Understanding the turnover and eventual fate of genetic and phenotypic diversity is a long standing biological question (Kassen, 2014). My thesis research provides an investigation of the transitory nature of diversity under environmental heterogeneity, while also addressing the dynamics of drug resistance in bacterial populations experiencing patterned drug delivery.

Applying evolutionary and ecological principles to AMR

The mechanisms identified by population genetics and ecology for maintaining diversity with environmental heterogeneity are relevant to studying the evolution and prevalence of AMR. To start, community or hospital wide prescription patterns and heterogeneous drug delivery

within a host create temporally and spatially varying environments of drug selection. Further, pathogen strains can vary in fitness, both in terms of the level of resistance to a given drug and growth rate in drug-free conditions. There is strong evidence that resistant strains often pay a cost of resistance in terms of reduced growth rate and fitness in the absence of antibiotic (Ender *et al.*, 2004; Hurdle *et al.*, 2004; Wong *et al.*, 2012; Melnyk *et al.*, 2015), generating fitness trade-offs like those required in models for the maintenance of diversity in spatially heterogeneous environments (Levene, 1953; Dempster, 1955; Kisdi, 2001). Thus, it seems reasonable to expect that evolutionary and ecological principles for understanding the consequences of selection in variable environments might be useful for making sense of how heterogeneous drug availability in time and space influences resistance evolution.

To begin, consider that the extensive use of antibiotics in medical and agricultural sectors likely generates strong selection favouring resistant bacteria and promoting their spread (World Health Organization, 2015). In the simplest scenario of sustained drug selection in a large and well-mixed population, resistant strains are expected to sweep through the population at a rate that depends on the strength of selection imposed by the volume of drug used (Austin *et al.*, 1999). Because most resistance mutations incur a cost in terms of reduced fitness in the absence of drug, this model suggests that the prevalence of drug resistance should decrease if the antibiotic is removed from use, with sensitive genotypes that do not pay the cost of resistance replacing resistant genotypes (Levin *et al.*, 1997). It has been observed, however, that resistance can persist far longer than expected following cessation of an antibiotic (Arason *et al.*, 2002; Sjölund *et al.*, 2005; Sundqvist *et al.*, 2010). At least three genetic mechanisms could underlie persistent resistance: genetic linkage to positively selected sites, including cross resistance to other continually prescribed antibiotics, the selection of cost-free resistance mutations (Melnyk *et al.*, 2015), or reduced costs to resistance (Enne *et al.*, 2001; Sundqvist *et*

al., 2010) arising from compensatory mutations (Gagneux *et al.*, 2006; Comas *et al.*, 2012; Wong *et al.*, 2012; Melnyk *et al.*, 2015, Melnyk *et al.*, 2017). In practice, it is rare to see the complete fixation or the complete loss of resistance. Rather, sensitive and resistant genotypes often appear to coexist at varying frequencies across hospitals where drugs are continuously prescribed (Gerding *et al.*, 1991; Austin *et al.*, 1999; Lopez-Lozano *et al.*, 2000; Goossens *et al.*, 2005) and within samples collected from a single patient undergoing antibiotic therapy (Fothergill *et al.*, 2010; Levert *et al.*, 2010; Mowat *et al.*, 2011). This leads to the question – does the ecology of drug delivery play a role in preventing the spread of resistant strains?

Ecology of drug delivery in space and time

Limiting the prevalence of AMR through altered drug delivery can be approached from multiple scales, whether it be from the perspective of the broad global community, or more narrowly within an individual host. Usage by individual patients is the source that ultimately produces a global "tragedy of the commons", hence drug heterogeneity within a host must be appreciated when studying the evolution and persistence of resistance in the broader environment. Firstly, intermittent dosing of a patient can impose temporal fluctuations in drug concentrations across the body (Mackenzie & Gould, 1993; Mouton & Vinks, 1996). Secondly, the concentration of antibiotics delivered in chemotherapeutic treatment can be highly variable across different compartments (cells, organs, tissues etc.) due to different rates of drug metabolism, inactivation and diffusion in specific tissues (Nix *et al.*, 1991; Elliott *et al.*, 1995; Baquero & Negri, 1997; Joukhadar *et al.*, 2001). Sub-inhibitory concentrations in time or space may lead to inefficient pathogen clearance, consequent chronic infections, and ultimately transmission to the broader health care community. Clinically, with-in host environmental heterogeneity has been shown to be associated with diversification in pathogens (Jorth *et al.*, 2015) and theoretical models have demonstrated that drug free compartments within a host can

promote the novel evolution of resistance (Kepler & Perelson, 1998; Lipsitch & Levin, 1998). Only recently, though, have laboratory studies on AMR began to incorporate drug concentration heterogeneity in either space (Zhang *et al.*, 2011; Baym *et al.*, 2016) or time (Kim *et al.*, 2014; Fuentes-Hernandez *et al.*, 2015; Melnyk *et al.*, 2017). While epidemiological modelling approaches have compared the efficacy of spatial versus temporal drug prescription patterns across a hospital setting (Bonhoeffer *et al.*, 1997; Bergstrom *et al.*, 2004), experimental tests have yet to compare concurrently how drug availability in space versus time alters the prevalence of resistance. My thesis aims to make this comparison *in vitro* by approaching AMR using an evolutionary ecology perspective.

Evolutionary ecology of antibiotic resistance

Despite knowing that drug selection varies within patients and across communities, medical research has largely overlooked applying an ecological framework to study how spatially varying drug concentrations alter resistance evolution (Debarre *et al.*, 2009; Park *et al.*, 2015). Yet, it is easy to see parallels between models of selection in heterogeneous environments and the spectrum of resistance profiles observed in the clinic or community. For example, resistant genotypes that incur large costs of resistance resemble ecological specialists in the sense that they maintain high fitness in the presence of the drug and low fitness in its absence (Ender *et al.*, 2004; Hurdle *et al.*, 2004; Wong *et al.*, 2012; Melnyk *et al.*, 2015). A sensitive genotype very obviously has low fitness in the presence of the drug (the cell is either killed or prevented from growing) but has much higher fitness in drug free conditions, meaning we can consider it a specialist in the absence of drugs. Should both types be present in a population, then the first condition for the maintenance of diversity under spatially variable selection – a trade-off in fitness across environments – in the Levene (1953) model is satisfied. The combination of heterogeneous drug delivery plus trade-offs to resistance may therefore

contribute to the clinical observation of coexisting sensitive and resistant pathogens. On the other hand, it is possible for resistance to be 'cost-free', in the sense that a resistance mutation incurs little or no cost in the absence of drug or that the cost of a resistant mutation is compensated by second-site mutations. These situations are tantamount to being a generalist across a variable environment containing patches with or without drug (Melnyk *et al.*, 2017). Nevertheless, I am aware of only one recent study (Melnyk *et al.*, 2017) that experimentally tested the prediction that a generalist strategy of cost-free resistance should evolve under temporally varying drug selection. This study demonstrated that the resistance of *Pseudomonas aeruginosa* to ciprofloxacin following fluctuating selection between drug and no drug conditions led to the evolution of resistant genotypes with secondary mutations that provided high fitness in a drug free environment (Melnyk *et al.*, 2017). Notably, clinical evidence shows that no or low cost resistance genotypes are also found and selected for in patients (Bjorkholm *et al.*, 2001; Andersson, 2006; Gagneux *et al.*, 2006; Comas *et al.*, 2012).

Frequency dependence and resistance prevalence

Fitness costs to resistance can produce a trade-off between level of resistance and growth rate in environments with reduced drug concentrations. When such trade-offs are coupled with dispersal between environments with and without drug, it is possible to generate negative frequency dependent selection between resistant and sensitive types. Routes for dispersal of pathogenic bacteria include the movement of patients and hospital staff among wards and the community, or through circulatory and respiratory systems within a patient (Nix *et al.*, 1991; Mackenzie & Gould, 1993; Elliott *et al.*, 1995; Mouton & Vinks, 1996; Baquero & Negri, 1997; Joukhadar *et al.*, 2001). Admittedly it can be difficult to know whether the kind of population regulation required to maintain diversity indefinitely is operating in these situations. However, if dispersal is associated with some form of local population regulation, as required by

the Levene (1953) model, then in principle, it is possible for resistant and sensitive genotypes to coexist indefinitely, thereby preventing the spread of resistance.

Thesis overview and experimental details

My thesis extends the central concepts from the Levene (1953) and Dempster (1955) models to study the spread of drug resistance in populations evolved through subdivided environments with, or without antibiotic. This simple design investigates both how spatial versus temporal patterns of drug delivery may hinder the spread of antibiotic resistance and how the mechanisms of maintaining diversity develop under continued selection. Below I explain contextual details of my experimental design and their relevance to my research. The key findings of my thesis are detailed in Chapter 2 – coauthored with my supervisor Dr. Rees Kassen and submitted for consideration as published article in *Evolution Letters*. Full methodology is therefore included separately in Chapter 3, with additional conclusions and discussion of clinical implications in Chapter 4.

Approach: experimental evolution

Experimental evolution provides an elegant method for studying polymorphisms in antibiotic resistance profiles. It is an established approach for observing fundamental evolutionary processes by monitoring organisms with rapid generation times in controlled environments. For my thesis, I evolved the human opportunistic pathogen *Pseudomonas aeruginosa* in four selection regimes, each differing in their availability of ciprofloxacin antibiotic in either space or time. In contrast to the classic models of ecology and population genetics, my experiment incorporates ongoing adaptive evolution in microbial populations experiencing continued selection.

Model organism: Pseudomonas aeruginosa

P. aeruginosa is ranked among the top three resistant pathogens of concern by the World Health Organization (World Health Organization, 2017), and hence provides an appealing model organism for experimental study. More pertinent, *P. aeruginosa* is also the predominant pathogen found in fatal chronic respiratory infections of cystic fibrosis (CF) patients. Relevant to my research, it is recognized that the respiratory airway is composed of compartmentalized regions that impart diversifying selection and local adaptation (Jorth *et al.*, 2015), including regions with reduced antibiotic delivery (Aanaes *et al.*, 2011; Folkesson *et al.*, 2012). Although chemotherapeutic treatments greatly extend life expectancy, premature death ensues from chronic infections (Farrell *et al.*, 2008) that result from a combination of persistent re-infection and rapid emergence of antibiotic resistance (Folkesson *et al.*, 2012). The source of re-infection is uncertain, though is proposed to be mediated by structured regions of the lung with reduced drug penetration and immune clearance (Jelsbak *et al.*, 2007; Aanaes *et al.*, 2011; Folkesson *et al.*, 2012). In addition to spatial variability in the lung environment, daily intermittent treatment with oral fluoroquinolones during periods of exacerbation (UK CF Trust Antibiotic Working Group, 2009) cause short and long term temporal fluctuations in drug concentrations. Hence, drug resistance evolution may also be effected by temporal drug refuges produced during waning concentrations following doses. My thesis explores these two potential explanations by using selection regimes that vary in time or space in the delivery of ciprofloxacin antibiotic.

Selective pressure: ciprofloxacin antibiotic

Using ciprofloxacin as the main source of selection in my experiment is an appropriate choice since it is commonly prescribed to CF patients experiencing chronic *P. aeruginosa* infections (UK CF Trust Antibiotic Working Group, 2009). Ciprofloxacin is a fluoroquinolone antibiotic that prevents cell division by binding between DNA and gyrase or topoisomerase

proteins, thereby inhibiting their action to relieve strain on the DNA helix during unwinding in replication (Ruiz, 2003; Poole, 2005). A range of genetic targets are available to *P. aeruginosa* to reduce ciprofloxacin's inhibitory effects, with varying levels of resistance and associated costs (Melnyk *et al.*, 2017). These mutations may be specific to ciprofloxacin and alter the protein structure of DNA gyrases (examples: gyrA, gryB) and topoisomerases (examples: parC, parE), or general to a range of antibiotic classes by changing efflux pump functioning (examples: MexCD-OprJ, MexAB-OprM) (Ruiz, 2003; Poole, 2005). Various genetic targets additionally exist to bacterial pathogens to compensate costs of resistance, as exhibited both experimentally (O'Neill *et al.*, 2006; Wong *et al.*, 2012; Melnyk *et al.*, 2017) and clinically (Gagneux *et al.*, 2006; Comas *et al.*, 2012). Together, the array of numerous resistance and compensatory mutational targets provide ample opportunity for genetic variation, phenotypic variation, and consequent clonal interference in my experiment's bacterial populations.

CHAPTER 2: The emergence, maintenance and demise of diversity in a spatially variable antibiotic regime

This chapter is reproduced from:

Leale, A.M. & Kassen, R. 2017. The emergence, maintenance and demise of diversity in a spatially variable antibiotic regime. *bioRxiv.* doi: https://doi.org/10.1101/158337

Antimicrobial resistance (AMR) is a growing global threat that, in the absence of new antibiotics, requires effective management of existing drugs. Here, we explore how changing patterns of drug delivery modulates the spread of resistance in a population. Resistance evolves readily under both temporal and spatial variation in drug delivery and fixes rapidly under temporal, but not spatial, variation. Resistant and sensitive genotypes coexist in spatially varying conditions due to a resistance-growth rate trade-off which, when coupled to dispersal, generates negative frequency-dependent selection and a quasi-protected polymorphism. Coexistence is ultimately lost, however, because resistant types with improved growth rates in the absence of drug spread through the population. These results suggest that spatially variable drug prescriptions can delay but not prevent the spread of resistance and provide a striking example of how the emergence and eventual demise of biodiversity is underpinned by evolving fitness trade-offs.

Introduction

The effectiveness of antibiotic therapy to control infection is being steadily undermined by the combination of divestment in drug discovery, widespread occurrence of genetic

resistance among microbes isolated from natural environments (Nesme *et al.*, 2014), and continued evolution of resistant strains among all major pathogens (Hidron *et al.*, 2008). Managing our existing arsenal of drugs to ensure they remain effective for as many people for as long as possible is therefore an urgent public health priority.

Beyond a blanket ban on prescriptions, there is little consensus on how to delay or prevent the emergence and spread of resistance. Using multiple drugs with distinct cellular and genetic targets is often suggested as the most effective treatment but sequential use can often select for multi-drug resistant strains (Alekshun & Levy, 2007), while simultaneous delivery (as a drug cocktail) can be difficult for patients to tolerate (Tamma *et al.*, 2012). An alternative approach is to exploit the use of drug-free sanctuaries or refuges in time or space to make it more difficult for selection to fix resistant strains. Sensitive strains that do not pay a fitness cost of resistance (Melnyk *et al.*, 2015) are expected to outcompete resistant strains in drug sanctuaries, implying that resistance could be kept at manageably low levels if dispersal introduces sensitive strains at a sufficiently high rate (Felsenstein, 1976; Andow & Alstad, 1998; Austin *et al.*, 1999). Sanctuaries have been used to good effect in managing resistance in agricultural systems (Hutchison *et al.*, 2010; Carrière *et al.*, 2012) but their role in governing the spread of resistance in health settings remains unclear (Debarre *et al.*, 2009; Park *et al.*, 2015).

Theory suggests that the way sanctuaries are experienced in time and space can profoundly impact resistance evolution (Felsenstein, 1976; Bergstrom *et al.*, 2004; Debarre *et al.*, 2009; Kassen, 2014). Periodic delivery of a drug in time generates regular cycles of strong antibiotic selection followed by periods of relaxed selection when the drug is either metabolized, excreted, or not in use (Mackenzie & Gould, 1993; Mouton & Vinks, 1996). Intermittent dosing or ward-wide use generates fluctuating selection that leads to the evolution of broadly adapted resistant types with high fitness in both the presence and absence of drug (Melnyk *et al.*, 2017). Drug delivery may also be spatially variable across wards in a hospital or among compartments

(tissues or organs) within a host, generating divergent selection that can lead to the emergence of a single resistant generalist or, if selection is sufficiently strong relative to dispersal (for example, through the movement of patients and staff between hospital wards), the coexistence of more narrowly-adapted niche specialists that trade-off drug resistance with growth rate in the absence of drug (Felsenstein, 1976; Debarre *et al.*, 2009; Kassen, 2014).

To evaluate the impact of drug sanctuaries on the emergence and spread of AMR, we tracked the evolution of resistance and fitness in twelve independently evolved, isogenic lines of *P. aeruginosa* strain PA14 propagated in environments that varied through time or space with sub-inhibitory concentrations of the commonly used fluoroquinolone antibiotic, ciprofloxacin. *P. aeruginosa* ranks among the top three drug resistant pathogens globally (World Health Organization, 2017) and causes both acute infections of wounds and chronic infections of the respiratory tract, where it is a major source of morbidity and mortality in adults with cystic fibrosis (CF) (UK CF Trust Antibiotic Working Group, 2009; Folkesson *et al.*, 2012). We use drug concentrations that resemble that found in the sputum of CF patients undergoing fluoroquinolone treatment during exacerbation (Pedersen *et al.*, 1987). Controls were a permissive (PERM) environment consisting of drug free Luria-Bertrani (LB) medium and a constant selective environment (CONS) comprised of LB supplemented daily with sub-inhibitory concentrations (0.3 ug/ml) of ciprofloxacin sufficient to reduce population densities to 20% of that in the absence of drug. Temporally varying environments (TEMP) were constructed by transferring aliquots from each evolving population into either permissive (no drug) or selective (0.3ug/ml) conditions on alternating days. Spatially variable environments (SPAT) consisted of two subpopulations, or patches, one permissive and one selective, with equal volume aliquots from each mixed and redistributed into fresh drug-free and drug-supplemented media daily (see Methods and Fig. 6). The SPAT treatment resembles a well-known model in population genetics in which genetic polymorphism can be maintained through negative frequency dependent

selection, provided there is a strong fitness trade-off among genotypes across patches and population regulation occurs at the level of the local patch rather than the total population (Levene, 1953; Dempster, 1955; Kisdi, 2001; Debarre *et al.*, 2009). In contrast to previous work (Reboud & Bell, 1997; Jasmin & Kassen, 2007; Huang *et al.*, 2015; Gallet *et al.*, 2017 - preprint), we do not explicitly manipulate either fitness trade-offs or the manner of population regulation but instead allow these variables to evolve naturally over the course of the experiment.

Results and Discussion

After 20 days (~133 generations) of selection resistance failed to evolve in the absence of drug selection (Fig. 1A) but did evolve under all other conditions (Fig. 1B-D). The level of resistance was assayed by determining the minimum inhibitory concentration (MIC; the lowest drug concentration that completely inhibits growth) for eight randomly selected colonies from each evolved population. Resistance fixed in all populations experiencing drug sanctuaries in time (Fig. 1C) but not space, where 6 of 10 populations contained a mixture of resistant and sensitive colonies (Fig. 1D; note that two populations in this treatment were lost due to contamination). Notably, the least resistant genotypes in the spatially structured environment (Fig. 1D) were as sensitive to ciprofloxacin as the ancestor and evolved populations from the permissive environment (Fig. 1A), while the most resistant genotypes had similar MICs as evolved genotypes from both the constant (Fig. 1B) and temporally varying (Fig. 1C) environments. Thus, drug sanctuaries do little to prevent the initial evolution of resistance, but when spatially structured, can slow or prevent the rate at which resistance sweeps through the population. More generally, divergent selection caused by spatial heterogeneity in antibiotic concentrations can promote diversification and coexistence between resistant and sensitive genotypes, while fluctuating selection on a similar time-scale does not.

Does the diversity in resistance profiles in the spatially variable environment reflect stable coexistence, or might it simply be a transient effect reflecting a reduced overall strength of selection for resistant types (Whitlock, 2003)? Four lines of evidence point to diversity being stably maintained in the SPAT populations. First, coexistence between resistant and sensitive strains persists through an additional 20 days of selection in 8 of 10 populations from the SPAT treatment, albeit with marked fluctuations through time (Fig. 2). Second, we did not observe any evidence of sensitive genotypes persisting under temporally variable conditions, as might be expected if the primary effect of the permissive patch is to slow the rate of fixation of resistance mutations (Fig. 7). Third, we observed the expected trade-off between resistance and growth rate in the absence of drug for the eight previously isolated colonies at day 20 and an additional 8 colonies isolated at day 40 (Fig. 3A, Table 1; mixed linear analysis of covariance between growth in LB and log(MIC)*day, with population as a random effect: *slope = -0.3558, P < 0.0001*). Fourth, and most convincingly, invasion from rare experiments (see Methods) between four randomly-selected pairs of resistant and sensitive isolates from SPAT populations at day 20 and day 40 show that rare genotypes always have higher fitness than common ones, providing direct evidence for negative frequency dependent selection (Fig. 4, Table 2; mixed linear analysis of covariance between relative fitness (ω) and day*resistance frequency, with random effects of individual isolate pair nested in population: *slope = -0.41871, P < 0.0001*). These results suggest that divergent selection leads to the stable coexistence of resistant and sensitive strains through negative frequency dependent selection, consistent with predictions from models for the maintenance of polymorphism in spatially variable environments (Levene, 1953; Dempster, 1955; Kisdi, 2001).

Despite the presence of strong negative frequency dependent selection, the polymorphism does not appear to be stable in our populations on evolutionary time scales. Coexistence persists at day 40 in just three populations (Fig. 2A, B, H) whereas four populations

are fixed or nearly fixed for resistance (Fig. 2C, D, I, J) and nearly lost in two (Fig. 2F, G). The fixation or loss of resistant strains could be due to stochastic variation generated by time-lagged frequency dependent selection (Hori, 1993), perhaps associated with the daily serial transfer protocol we imposed in our experiment, or from high mutation supply rates generating complex dynamics among competing strains (clonal interference). To distinguish between these alternatives, we tracked the frequency of resistance between three independent pairs of resistant and sensitive isolates over time under spatially variable conditions (see Methods). In the absence of clonal interference we expect the frequency of resistance to tend towards an internal equilibrium without fluctuations, regardless of starting frequency. As expected, the frequency of resistant genotypes converges towards an equilibrium point between 0 and 1 within the first two days and remains relatively stable until day 8, after which frequencies diverge again, presumably as mutations are reintroduced into the population (Fig. 5). These results suggest that the fluctuations in the frequency of resistant and sensitive strains we observed in our original experiment are likely due to high mutation supply rates generating clonal interference (see also Maddemsetti *et al.* 2015), rather than stochastic effects associated with our transfer protocol.

High levels of clonal interference imply that there is a steady source of genetic variation on which selection can act, over and above the diversity supported by negative frequency dependent selection. If so, are these populations continuing to evolve or does negative frequency dependent selection act to prevent further evolution? To answer this question we focused attention on the how the trade-off between MIC and growth rate under drug-free conditions changed over the selection experiment. We find that the slope of this trade-off evolves to become shallower at day 40 than at day 20 (Table 1; log(MIC)*day interaction: *$F = 0.1221, P = 0.0382$*). Inspection of Figure 3A suggests that this effect is due to increases in the growth rate of resistant isolates in LB, rather than loss of resistance (Fig. 3A). Further support

for this interpretation comes from the lack of change in growth rate of resistant isolates in the presence of ciprofloxacin between days 20 and 40 (Fig. 3B, Table 3; log(MIC)*day interaction: F = -0.0311, P = 0.6167). These results are consistent with the selection of second-site compensatory mutations that improve growth rate under drug-free conditions without compromising resistance (Gagneux *et al.*, 2006; Comas *et al.*, 2012; Wong *et al.*, 2012; Melnyk *et al.*, 2017). As a consequence of this weakened trade-off, the internal equilibrium frequency of resistant strains (i.e., resistance frequency when $\omega = 1$) increases on average from 59% at day 20 to 87% by day 40 (Fig. 4), implying that resistant genotypes are slowly spreading through most populations. A putatively stable polymorphism on ecological time scales can thus be readily undermined by selection which, in this case, leads to the evolution of broadly-adapted genotypes that are both resistant and have high fitness under drug-free conditions and the eventual loss of sensitive genotypes.

Conclusion

Taken together, our results provide a rather bleak prognosis for the use of drug sanctuaries to manage antimicrobial resistance. Perhaps not surprisingly, given the strong selection generated by antibiotics, drug sanctuaries by themselves cannot prevent the evolution of drug resistance. Nevertheless, the manner in which drug sanctuaries are experienced by a pathogen can impact the rate at which resistance spreads in a population: while temporal variation in drug sanctuaries does little to prevent the rise of resistance, spatially distributed sanctuaries can slow the rate at which resistance fixes in a population. In line with the predictions of evolutionary theory, the strong divergent selection imposed by spatial variation in drug delivery leads to the emergence of a genetic polymorphism between resistant and sensitive strains supported by negative frequency dependent selection. In contrast with classic models of selection in spatially variable environments, however, this polymorphism is quasi-

stable in the long-term, being readily undermined by compensatory evolution that tends to generate generalist resistant strains with high fitness in the absence of drugs. In other words, even spatial variation in drug selection cannot prevent resistant strains from eventually replacing sensitive ones due to the evolution of drug resistant strains with reduced costs of resistance. The implication here should be clear: in the absence of a continual pipeline of new drugs for treating infectious disease, the best we can do is slow the rate at which existing drugs lose efficacy. The use of drug sanctuaries in space may help, for a time, but natural selection will inevitably find a way to undermine our best strategies for preventing resistance.

CHAPTER 3: Methods

Experimental evolution

A single colony of *P. aeruginosa* strain PA14 was grown overnight in Luria-Bertrani broth (LB: bacto-tryptone 10g/L, NaCl 10 g/L, yeast extract 5 g/L) and used to found 48 populations by adding 20uL into 1.5 mL of fresh media (described below). An aliquot of the progenitor was frozen in glycerol at -80^{o}C. Every 24 hours, a 20uL aliquot of overnight culture was added to 1.5 mL of fresh medium. Populations were propagated in 24-well microtiter plates and agitated using an orbital shaker (150 rpm) at 37^{o}C. Samples were frozen at -80^{o}C in glycerol every four and ten transfers, or approximately every 26 and 66 generations, respectively.

The experiment consisted of 12 replicate populations for each of four treatments growing in LB broth supplemented or not with 0.3ug/ml of the fluoroquinolone antibiotic, ciprofloxacin, for 40 days, or approximately 265 generations. This concentration of ciprofloxacin inhibits growth of the sensitive PA14 ancestor to approximately 20% of full growth in LB over a 24-hour period. Treatments were: A) a permissive environment involving daily propagation in drug-free LB (PERM); B) a constant environment with ciprofloxacin added daily (CONS); C) a temporally varying environment consisting of daily alternation in drug-free and drug-supplemented media (TEMP); and D) a spatial treatment comprised of two subpopulations, one containing drug and the other drug-free, connected through dispersal (SPAT). Dispersal was imposed by mixing 0.75mL from each pair of wells prior to transfer and inoculating a fresh pair of wells with 20uL of the combined mixture (see Fig. 6). Samples of the mixed population were frozen. Two populations from the SPAT treatment were excluded from final analyses due to contamination.

Minimum inhibitory concentration (MIC) and growth rate

We randomly selected eight colonies from each population at day 20, plus an additional eight colonies from the TEMP and SPAT treatments at day 40, by plating a sample of each population on agar and choosing those closest to an arbitrary point in the middle of the plate. For each isolate, we assayed resistance as the MIC to ciprofloxacin by first reviving frozen cultures overnight in LB media and then inoculating 100ul of dense culture into 96-well plates containing LB supplemented with 0.0, 0.10, 0.20, 0.39, 0.78, 1.56, 3.12, 6.25, 12.5, 25, 50, or 100ug/mL ciprofloxacin, respectively, and incubated on an orbital shaker at 37^{o}C. Growth was scored by reading optical density (OD) at 600 nm after 48 hours. Log-transformed MICs were used for all analyses. Resistance is defined as an MIC exceeding 2ug/mL, equivalent to 4x the ancestral MIC (0.5ug/mL).

The growth of evolved SPAT isolates relative to that of PA14 was measured in LB with and without ciprofloxacin over 24 hours. In LB, 5uL of overnight culture was added to 195uL of LB and OD600 measured every 90 minutes. The same procedure was performed for ciprofloxacin growth assays, except we used 20uL of overnight culture with 180uL of LB-ciprofloxacin media (0.3ug/mL). Gen5 software (BioTek Instruments Inc., Winooski, VT) was used to estimate maximum growth rate during exponential phase for three replicates per isolate. Growth rates are expressed relative to the mean growth rate of PA14 in LB when grown on the same 96-well assay plate.

Negative frequency dependent selection

Using reciprocal competitive invasion experiments, we estimated the strength of negative frequency dependent selection between resistant and sensitive isolates from all SPAT

treatment populations that showed evidence of coexistence from the MIC assays. We first chose four random pairs of resistant and sensitive colonies from each population, then competed each pair by mixing pure culture samples from overnight cultures at ratios of 1:9, 1:1, and 9:1 by volume and allowing them to compete for two transfers (48 hours) under the same SPAT treatment transfer protocol as in the original selection experiment. We tracked the change in relative abundance of sensitive and resistant colonies by streaking 40 randomly selected colonies on 2ug/mL ciprofloxacin agar for both the initial (0 hrs) and final (48 hrs) populations. Relative fitness, ω, was estimated using the equation:

$$\omega = (\frac{F_{final}}{F_{initial}})\text{^}(\frac{1}{doublings})$$

where $F_{initial}$ and F_{final} are the ratios of the frequency of resistant types to the frequency of sensitive types in the population, before and after competition, respectively. Doublings refers to the number of generations occurring between the initial and final measurements (~13 generations).

Dynamics of resistance

We assayed the frequency of resistance every four days for each replicate of the SPAT treatment by isolating 50 colonies at random on LB plates, streaking each colony on 2ug/mL ciprofloxacin agar, and visually inspecting each plate for growth after 24 hours at 37^{o}C.

Dynamics of invasion from rare

We chose a pair of resistant-sensitive isolates from population S4 at day 20 and two pairs from population S2 at day 40 to investigate the dynamics of resistance in the absence of clonal interference. These pairs were chosen because preliminary experiments indicated each had a different predicted equilibrium frequency (i.e., the frequency of resistance where ω = 1 in

reciprocal competitive invasion assays). Pairs of isolates were grown overnight as pure cultures and initial ratios of resistant and sensitive strains were constructed at 1:9, 2:8, 4:6, 6:4, 8:2, and 9:1 by volume, then mixtures were propagated identically to that in the original SPAT treatment for 10 days (~ 65 generations). Founding populations (day 0) and evolved populations were archived daily. The frequency of resistant individuals over time was estimated by testing the presence or absence of growth of 50 randomly selected colonies streaked on 2ug/mL ciprofloxacin agar, as described above, at days 0, 2, 4, 6, 8 and 10.

Statistical Analysis

All statistical analyses were conducted using R Studio (Version 0.99.903; https://www.rstudio.com). We used mixed linear analyses of covariance to model the trade-off between maximum growth rate in drug free media as a function of the fixed effect of log(MIC) and day, with population treated as a random effect:
$maxV = log(MIC)*day + (log(MIC) \mid population)$. A similar approach was used to estimate the frequency dependent fitness functions, with relative fitness (ω) modeled as a function of the fixed effects of day and initial frequency of resistance ($F_{initial}$) with random effects of isolate pair nested in population:
$\omega = F_{initial}*day + (F_{initial} \mid (population:pair) F_{initial}*day + (F_{initial} \mid (population:pair)$.
Evidence that coexistence is supported by negative frequency dependence exists if there is both a) statistically significant negative slope between fitness and frequency of resistance, and b) the regression line crosses the relative fitness axis (i.e., $\omega = 1$) between at a frequency between 0 and 1. Note that the *x*-intercept additionally provides an estimate of the location of the internal equilibrium frequency of resistance.

CHAPTER 4: Conclusions

Understanding the origin and maintenance of genetic and species level diversity is a central focus of evolutionary biologists and ecologists, in which the long-term fate of diversity in ever evolving populations is particularly unclear (Kassen, 2014). In a similar effort, medical research strives to understand the dynamics of resistance in evolving pathogen populations responding to strong selection by antimicrobials. My M.Sc. bridges the gap between these two fields of research by investigating how the ecology of drug delivery through drug free refuges influences the evolution and eventual prevalence of resistance.

Using experimental evolution, I evolved asexual *Pseudomonas aeruginosa* under four selection regimes, each varying in either time or space in the delivery of the antibiotic ciprofloxacin. Overall, I found that neither temporal nor spatial drug free refuges prevent the emergence of resistance, however divergent selection generated from spatial drug delivery did slow the spread of resistant types. The coexistence of resistance and sensitive types exclusively in spatially structured regimes was shown to be driven by an evolved trade-off between level of resistance and growth rate in the absence of antibiotic, with ensuing negative frequency dependence. The maintenance of susceptible types exclusively in the spatial treatment corresponds with theoretic predictions for greater diversity under spatially varying environments, compared to a single generalist strategy in temporally varying environments (Felsenstein, 1976; Kassen, 2002). Distinct from previous theoretical models (Levene, 1953; Dempster, 1955; Levins, 1968), I demonstrate that a seemingly stable polymorphism in a subdivided population can be undermined by ongoing selection and adaptation, as growth costs to resistance weaken

in my experiment and stable frequency dependent relationships shift towards the fixation of resistance.

In addition to fitness trade-offs and frequency dependence, I speculate that clonal interference frustrates selective sweeps of superior competitors and delays the eventual fixation of resistance types. This corresponds to the irregular fluctuations observed in SPAT populations, since incomplete selective sweeps would recurrently turn over existing genotypes and alter the population's stable genetic composition. Such dynamic fluctuations were not observed between individual resistant-sensitive pairs within the first few of days of selection, but instead emerged later once novel genotypes presumably arose through mutational supply. Together, these observations provide sufficient evidence that multiple, evolving genotypes were present in the SPAT populations, influencing the spread of resistant lineages. This inference is consistent with maintained polymorphisms in long term experimental evolution of *E. coli*, albeit with dramatic fluctuations in relative abundances of each polymorph (Maddamsetti *et al.*, 2015). There, it was suggested that the rich polymorphism dynamics were underlain by a combination of negative frequency dependence and clonal interference; the same mechanisms seem to be influencing the dynamics of diversity in my experiment. The gradual loss of sensitive types and anticipated fixation of resistance in my experiment is also consistent with previous studies where the eventual loss of polymorphism was observed in all but 13 of 1000 replicate yeast lineages after approximately 1000 generations (Frenkel *et al.*, 2015). The long-term fate of diversity in natural populations remains far from understood and valuable insight is to be gained from future experimental evolution studies such as mine. Continuing my selection experiment to reach thousands, rather than hundreds, of generations would provide insight into the persistence of drug sensitivity in the environment and the broader scale maintenance of diversity with continued evolution.

Experimental evolution provides a valuable methodology to observe evolutionary and ecological processes in real time, but important limitations exist in the inferences made from such studies. It can be argued that the simple experimental regimes in my research are too contrived to provide practical insight about natural populations experiencing a multitude of irregular temporal and spatial variations in the environment. In fact, I believe consideration of this argument highlights the value of laboratory evolution experiments because they offer researchers the opportunity to isolate and study how individual components of the environment influence evolutionary processes. Such specific conclusions are difficult or impossible to directly test from clinical or field data where many factors cannot be controlled. The benefits and disadvantages of experimental evolution also apply to determining the genetic targets of a specific selective pressure. Experimental evolution studies can begin with an isogenic population of a known genetic background then track the order of arising mutations under selection by a known source of selection. While this information is insightful, it is of course also unrealistic to a natural population, where the role of standing genetic variation can greatly influence adaptive evolution. Ironically, it appears that the advantages of experimental evolution approaches can often be the source for disadvantages.

The simplicity and reduced costs of laboratory studies allow for increased replication to consider the repeatability of evolution and strengthen certainty in the inferences made. In my experiment, each of the four selection regimes contained twelve independently evolved populations (10 for spatial regime due to contamination, as further discussed below). Variation in evolved levels of resistance within treatments did exist, including in the constant drug free regime (Fig. 1), but these were in range of those found by previous studies for ciprofloxacin with susceptive *P. aeruginosa* strains (Wiegand *et al.*, 2008; Llanes *et al.*, 2011; Melnyk *et al.*, 2017).

More importantly, overall trends were consistent across all replicates for each regime (Fig. 1), providing confidence in the conclusion that spatial but not temporal drug free refuges promoted the persistence of sensitive strains.

It is possible that these observations were influenced by preferential selection for mutator strains in alternate regimes. Mutator strains have evolved in previous selection experiments (Melnyk *et al.*, 2017) and in clinical CF patients (Oliver *et al.*, 2004), owing to their increased fitness under antibiotic selective pressures (Jørgensen *et al.*, 2013). I tested if higher rates of spontaneous mutations were detected more often in populations from certain regimes using the common method of a fluctuation analysis (Foster, 2006). However, an elevated mutation rate was not detected in any of the populations, verifying that evolved levels of resistance was not attributed to varied selective pressures between treatments for increased mutation rate.

The contamination of 2 of 12 SPAT populations should not impact the reliability of inferences made from other replicate populations. Cultures were set up in 24-well plates in a checkerboard pattern of lac-Z and non-lacZ marked PA14 strains, which grow blue and white in colour, respectively, on X-gal agar media. This allowed me to check for contamination every four days during the selection experiment by growing and checking the colour of the cultures on X-gal media. There is a high probability that contamination arises from an immediate neighbour well, thus the culture changes colour if the invading population has high enough fitness to reach a sufficient frequency. Further, exclusively SPAT populations were grown on a single plate, hence any low levels of contaminating genotypes would have evolved and experienced the same selective history. Colour changes were only observed in SPAT populations 1 and 12, indicating that if there was any low level of contamination in the other populations, the foreign

genotypes were inferior to those of the dominant source population. Mutations inferior to the currently dominant variant play a negligible role in clonal interference (Gerrish and Lenski 1998). Thus, we can be reasonably assured that, having excluded the two contaminated populations from the analysis, our conclusions are robust and unlikely to be strongly influenced by low levels of contamination.

Motivated by evolutionary and ecological principles of genetic and species level diversity in varied environments, my thesis exemplifies the ease at which fitness trade-offs underlying biodiversity can be modified by continued evolution, altering the long-term fate of ecological systems. Further conclusions from my thesis are also relevant for consideration in healthcare practices. It is evident that changing the ecology of drug delivery alters the spread of resistance and therefore attests to the valuable contributions evolutionary ecology should make to AMR stewardship. However, bridging principles of population genetics and ecology to real world public policy can be far from straightforward. This difficulty is particularly evident when deliberating between successful therapy of an individual patient versus long term treatment efficacy in the broader community, or when realizing the advantages and disadvantages posed by circulating sensitive strains.

I must acknowledge that the clinical significance of sensitive types persisting through spatial drug delivery is confusing for me to comprehend. On one hand, once drug therapy is halted sensitive types will predictably rise in frequency due to their greater relative fitness in the absence of drug (provided there is a cost of resistance). Consequently, later therapy with the same antibiotic may be more effective at treating infections because of the greater likelihood that the infections consist of susceptible strains. Alternatively, persisting sensitive types implies that drug therapy was imperfect in the first place and increases genetic variation, which may

facilitate evolving resistance to novel antibiotics (Read & Taylor, 2001). Because of this "double-edged sword", I currently cannot make any direct suggestions to improve drug administration practices, but insight would be gained through the following future investigations.

Firstly, genomic analyses can determine how the mutational targets of resistance and later compensatory mutations differ between the four drug delivery regimes. They can also provide important information on the genetic diversity and resultant clonal interference in not just the spatial regime populations, but all treatments. This is interesting to appreciate since genetic diversity in pathogen population can influence the progression of infection and success of drug therapy (Read & Taylor, 2001). Secondly, similar microbial evolution experiments focusing on multidrug resistance are warranted. Cyclical prescribing of different antibiotic classes (i.e., varied mutational routes to resistance) has been proposed as a strategy to extend the effective lifetime of currently used antibiotics (Bonhoeffer *et al.*, 1997; Bergstrom *et al.*, 2004; Martínez *et al.*, 2006), but my experimental design assessed only the influence of drug free environments. It would therefore be useful for future experiments to more closely resemble drug prescription practices where multiple antibiotic classes are administered either cyclically or spatially. My project's simple experimental design provides an elegant *in vitro* method to investigate multidrug resistance by simply exchanging the drug-free LB environment with a second antibiotic media.

My thesis has demonstrated that the ecological and genetic properties of pathogen populations can be drastically altered by the pattern of environmental variation experienced and that the conditions promoting diversity in populations can be readily undermined by evolution in the long term. Using drug free sanctuaries to manage AMR does not look promising, but strategic delivery in space offers a potential method to prolong the effective lifetime of our

current arsenal of usable drugs. More generally, my research exemplifies how fundamental principles in evolutionary biology can guide management of drug resistance.

FIGURES:

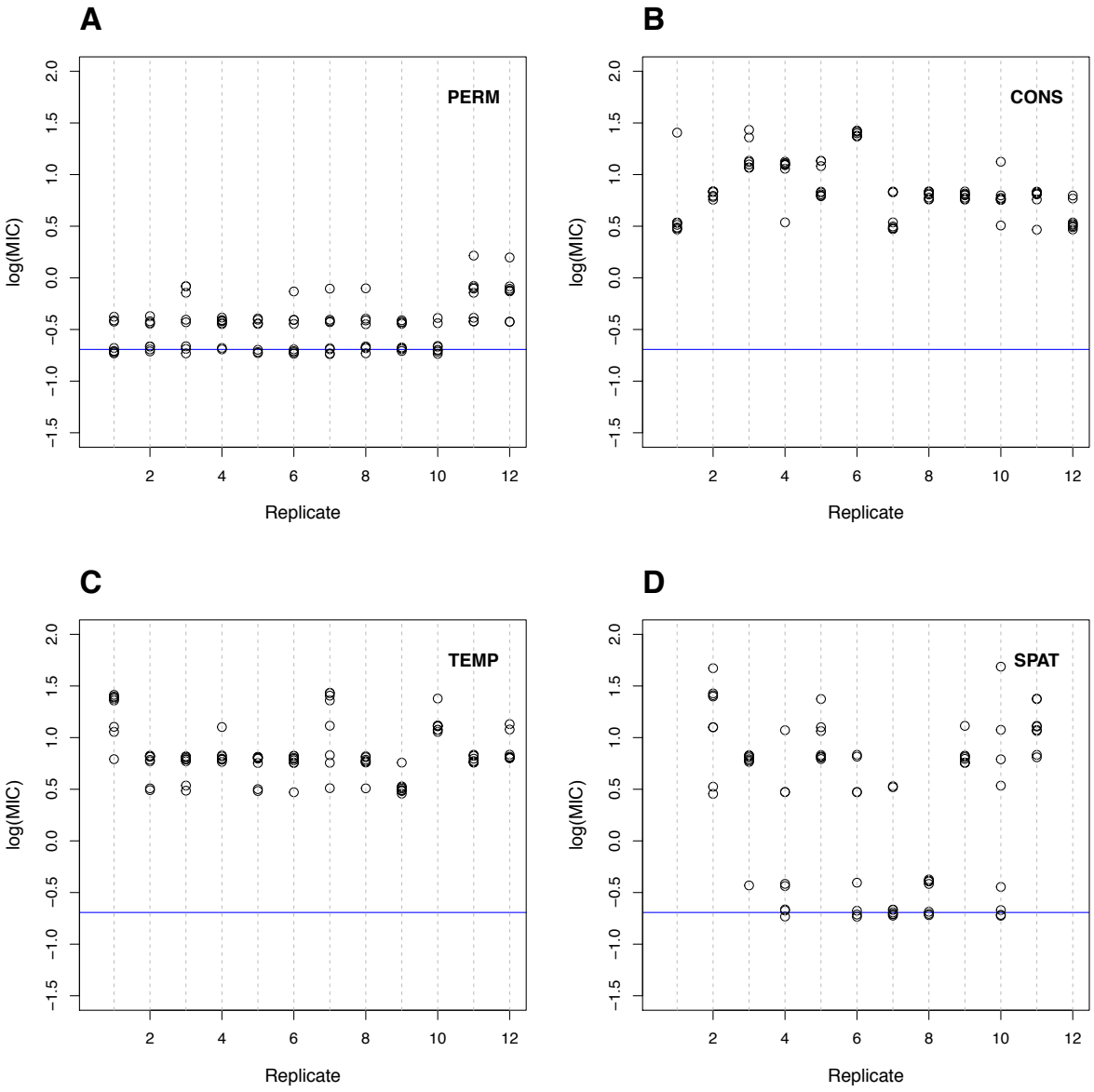

Fig. 1. Resistance, measured as the log minimum inhibitory concentration (log(MIC)) of each of eight isolates from each evolved population after 20 days of serial transfer (~133 generations). (A) PERM, **(B)** CONS, **(C)** TEMP, and **(D)** SPAT. Blue line indicates resistance level of ancestral PA14 isolate (MIC= 0.5ug/mL). An isolate is deemed resistant if its MIC > 2ug/mL (log(MIC) = 0.3). Data points are vertically jittered for clarity.

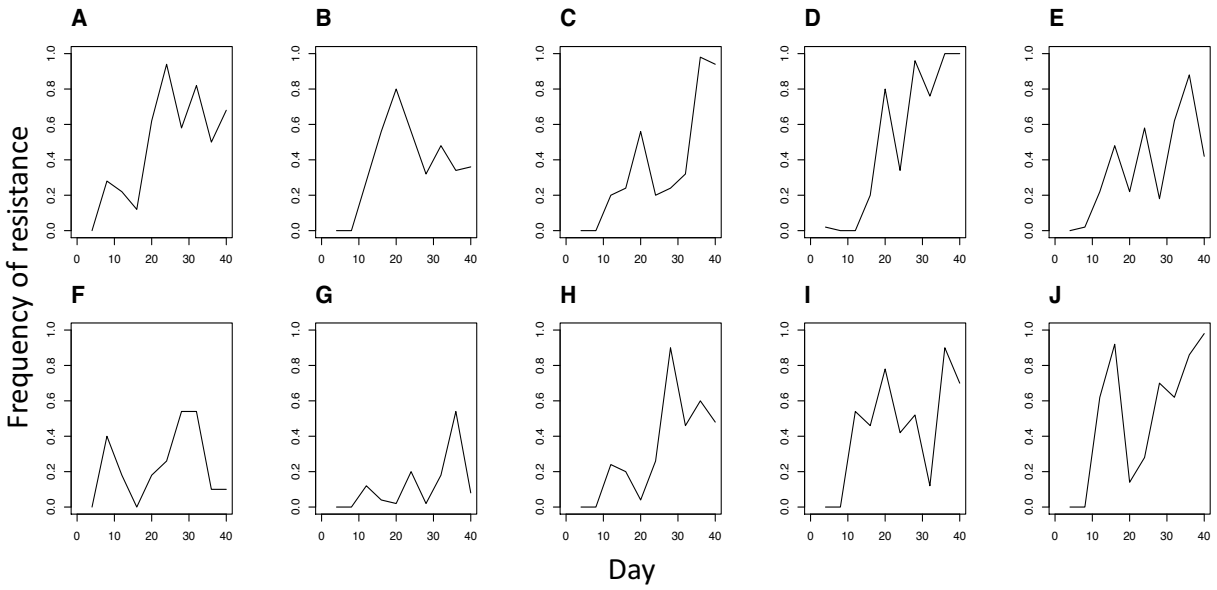

Fig. 2. Dynamics of resistance within all SPAT populations. Each panel (A-J) is an independently evolved population. Sensitive colonies were detected at all time points in all populations except populations C, D, and J where all 50 colonies were resistant by day 36 and/or 40.

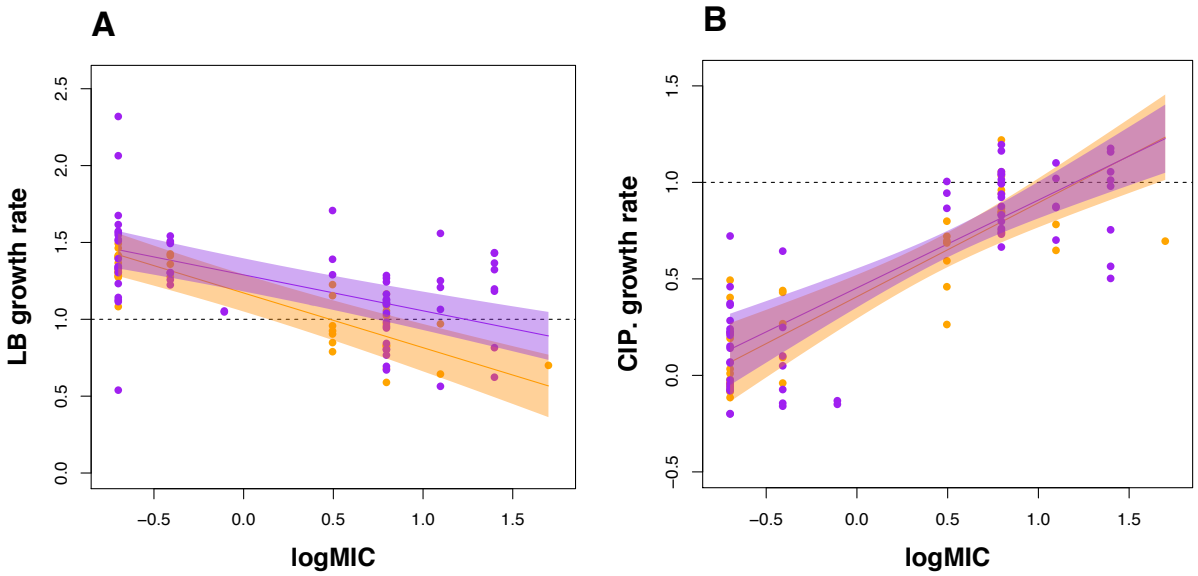

Fig. 3. The trade-off between resistance and growth rate in LB (A) and LB supplemented with 0.3ug/mL ciprofloxacin (B). Data is standardized to the growth rate of the ancestral PA14 in LB (horizontal dashed lines in each panel). Shaded areas represent 95% confidence intervals for day 20 (orange) and day 40 (purple). Only populations with both resistant and sensitive isolated colonies were included for analyses.

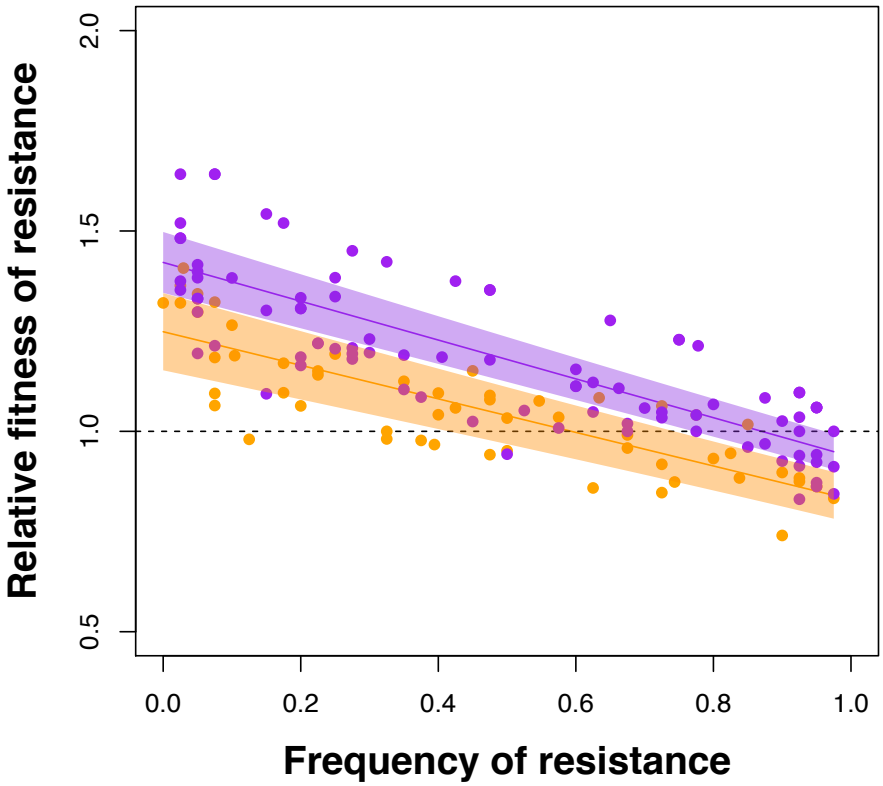

Fig. 4. Negative frequency-dependent selection between resistant and sensitive strains within populations. Fitness of a resistant colony relative to a paired sensitive colony (ω) is plotted as a function of its starting frequency. Shaded areas represent 95% confidence intervals for day 20 (orange) and day 40 (purple). The frequency of resistance at equilibrium is given by the intersection of the regression line with relative fitness (ω) = 1. Only populations with both resistant and sensitive isolated colonies were included for analyses.

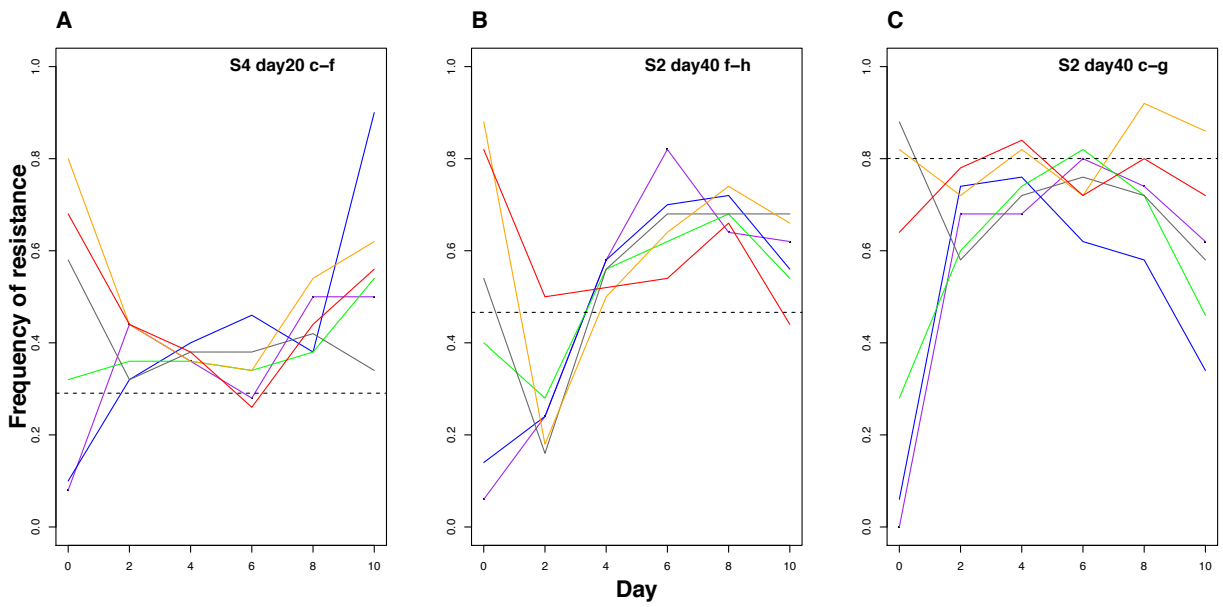

Fig. 5. Dynamics of resistant-sensitive pairs starting from different starting frequencies. Panels A-C depict the relative frequency of distinct pairs resistant-sensitive strains over time for six independent populations begun from different starting frequencies (coloured lines). Dashed axes represent predicted stable equilibrium frequencies for each individual pair estimated from initial invasion from rare experiments reported in Fig. 8.

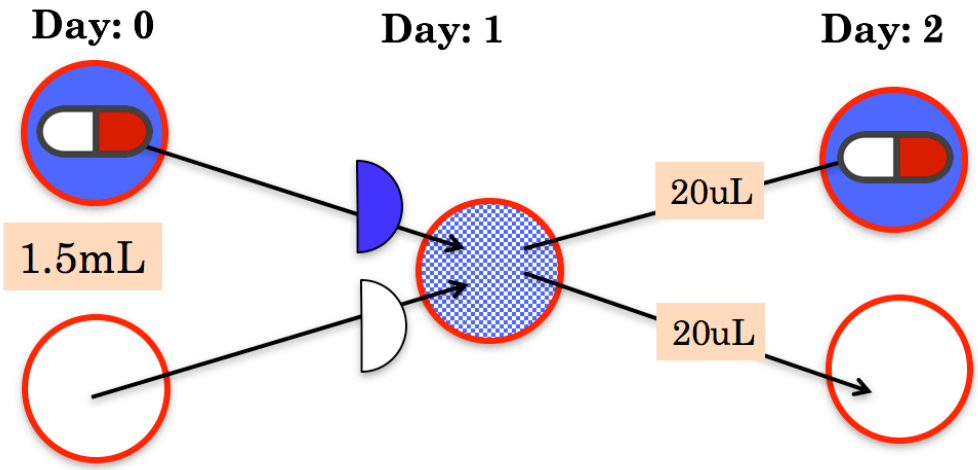

Fig. 6. SPAT selection regime. Each population consisted of bacterial cultures propagated in two separate wells of 1.5mL LB broth and 1.5 mL ciprofloxacin(0.3ug/mL), where 0.75mL of each well was combined daily and 20uL of the combined mixture (i.e., population) was then transferred again to separate wells.

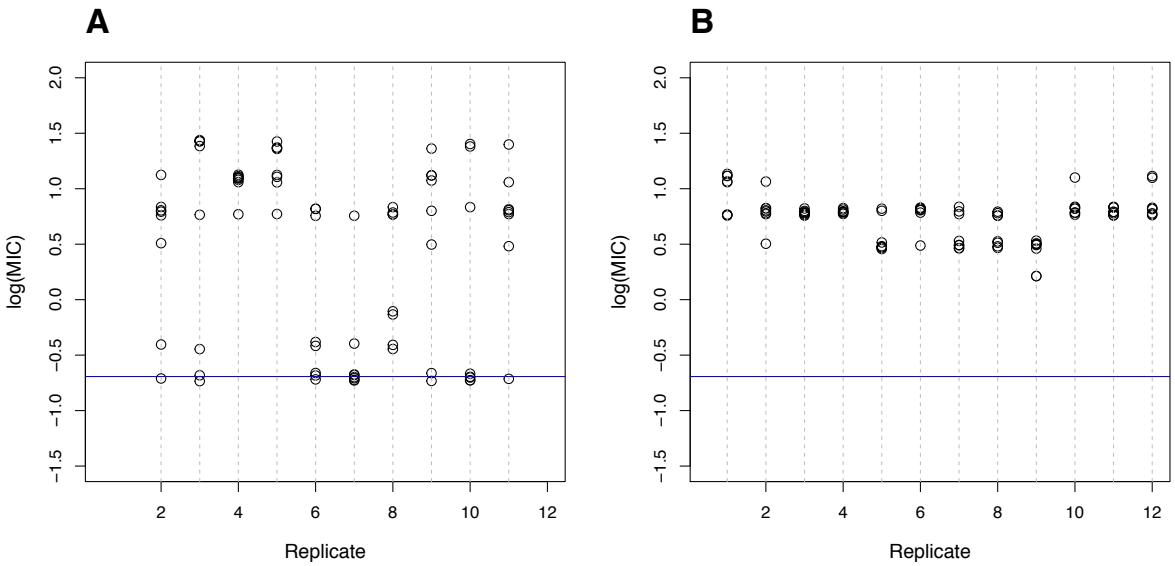

Fig. 7. Coexistence of susceptible and resistant types maintained in SPAT treatment. Evolved levels of resistance at day 40, measured as the log minimal inhibitory concentration (MIC) of ciprofloxacin, for isolated colonies for each SPAT population (**A)** and TEMP population **(B).** Blue axis indicates ancestral PA14 isolate resistance (MIC = 0.5ug/ml). Resistant isolates were defined as exhibiting an MIC exceeding 2ug/mL (log(MIC) = 0.3). Data points are vertically jittered for clarity.

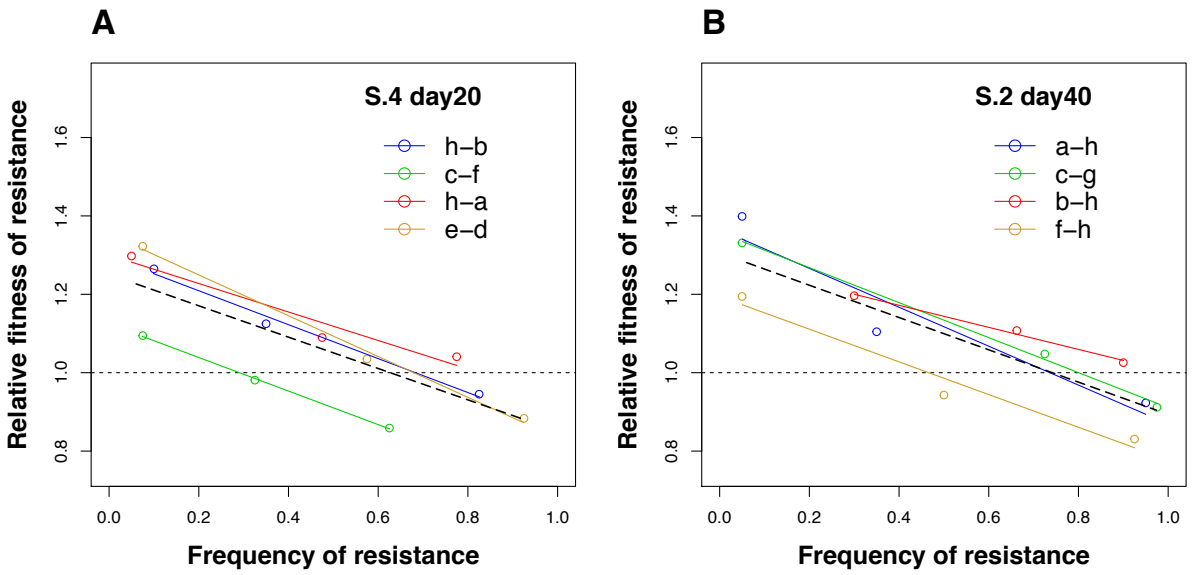

Fig. 8. Negative frequency-dependent selection for select pairs of resistant and sensitive isolates. Fitness of a resistant colony relative to its paired sensitive colony (ω) is plotted as a function of its frequency for isolates from SPAT population S4 (day 20) **(A),** and population S2 (day 40) **(B).** Solid colour lines depict linear regressions of individual resistant-sensitive isolate pairs, while pooled data regressions are shown by the dashed line. The frequency of resistance at equilibrium is given by the intersection of the regression line with relative fitness (ω) = 1.

TABLES:

Table 1: Mixed linear analysis of covariance for maximum growth rate in LB. Only populations with both resistant and sensitive isolated colonies were included for analysis.

Effect	Term	Effect	P	SD
Fixed	logMIC	-0.3558	5.38e-13	
	Day 20	1.1712	< 2.2e-16	
	Day 40	0.1178	0.02118	
	logMIC*day 40	0.1221	0.03816	
Random	Population (intercept)			0.1394
	Population (slope)			0.0104

Table 2: Mixed linear analysis of covariance for relative fitness (ω) of resistant types, with random effect of isolate pair nested in population. Only populations with both resistant and sensitive isolated colonies were included for analysis.

Effect	Term	Effect	P	SD
Fixed	initial frequency	-0.41871	< 2.2e-16	
	Day 20	1.124858	< 2.2e-16	
	Day 40	0.17295	0.005717	
	initial frequency*day 40	-0.06562	0.151243	
Random	pair : population (intercept)			0.06733
	pair : population (slope)			0.04819
	Population (intercept)			0.09806
	Population (slope)			0.04550

Table 3: Mixed linear analysis of covariance for maximum growth rate in [0.3ug/mL] ciprofloxacin. Only populations with both resistant and sensitive isolated colonies were included for analysis.

Effect	Term	Effect	P	SD
Fixed	logMIC	0.48637	2.981e-09	
	Day 20	0.40770	1.987e-12	
	Day 40	0.04540	0.3357	
	logMIC*day 40	-0.03107	0.6167	
Random	Population (intercept)			0.1262
	Population (slope)			0.1842

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