Microbial adaptation and evolutionary rescue in multi-stress environments

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Contents

Abstract

EN: The capacity of populations to undergo rapid evolution to survive in the face of a single stressor has been well-established. However, it is not known if such rapid evolution is possible in multi-stressor environments. Here, we studied the dynamics of adaptation and evolutionary rescue in populations of *Pseudomonas fluorescens* exposed to an antibiotic (tetracycline) and salt (NaCl). Populations were grown in a two-dimensional landscape consisting of gradients of tetracycline and salt and were either isolated or connected to other populations via global dispersal. All populations were then presented with three separate lethal challenges: tetracycline, salt, and tetracycline + salt. Populations were more likely to be rescued in the face of a stressor when historically exposed to that stressor. But, adaptation to a stressor was slowed by the presence of the second stressor. Populations were less likely to be rescued in the face of a stressor when historically exposed to a second stressor. Dispersal either promoted or reduced likelihood of rescue depending on the stress challenge. No populations were able to be rescued when confronted with lethal doses of both stressors simultaneously. Thus, both adaptation and evolutionary rescue dynamics are altered when multiple types of stress are present. The presence of multiple disparate stressors in combination reduce both adaptation and the likelihood of evolutionary rescue when confronted with a lethal level of stress.

FR: Il est maintenant bien établi que les populations peuvent démontrer une capacité à évoluer rapidement face à un stress unique. Cependant, il n'est pas connu si cette évolution rapide est possible dans un environnement à stress multiples. Nous avons donc étudié les dynamiques d'adaptation et de rescousse évolutive dans des populations de *Pseudomonas*

fluorescens exposées à la fois à un antibiotique (tétracycline) et à du sel (NaCl). Les populations, élevées dans un environnement bi-dimensionnel consistant en des gradients de tétracycline et de sel, furent soit isolées, soit connectées aux autres populations par dispersion globale. Toutes les populations furent soumises à 3 traitements différents: tétracycline, sel, et tétracycline + sel. Les populations ayant été exposées à un stress donné par le passé furent plus susceptibles d'être secourues face à ce stress. Cependant, l'adaptation au stress furent ralentie par la présence d'un second stress. Les populations furent moins susceptibles d'être secourue face à un stress donné si elles avaient été exposée à un stress différent par le passé. La dispersion soit augmenta soit décru la probabilité d'être secourue, selon le traitement. Aucune population ne fut capable d'être secourue quand confrontée avec des doses létales des 2 stress simultanément. Donc, les dynamiques d'adaptation et celles de rescousse évolutive sont altérées quand plusieurs stress sont présents. La présence de multiples facteurs de stress disparates combinés réduit à la fois l'adaptation et la probabilité de rescousse évolutive face à un niveau de stress mortel.

Preface

Acknowledgements

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Introduction

Description and Rationale for Studying Stressors

Stressors have long been the subject of study in both ecological and evolutionary contexts. In general, a stressor is any form of environmental condition that limits the survival or growth of an organism (1). Stressors exist in the form of both biotic stress (ex. competitors, predators, and parasites (2)) and abiotic stress (ex. chemical or environmental conditions leading to starvation, desiccation, oxidative damage, etc. (1)), which may exist in different combinations and quantities depending on a wide array of variables including ecosystem type, season, surrounding land cover, and species composition. Because stressors are so prevalent in the environment, they form an integral part of ecosystems. Taking stressors into account when examining ecological questions helps to inform intra- and inter-species dynamics.

Stressors exert different pressures on organisms depending on their mechanism of action and the organism involved. For example, antibiotics may affect a wider or more narrow range of bacteria depending on their targets within the cell: tetracycline can affect a broad spectrum of bacteria because it targets the ribosome and inhibits protein synthesis (3), while penicillin and other β-lactam antibiotics only affect gram-negative bacteria because they target the enzymes that synthesize the cell wall (4). However, all organisms must induce specific physiological responses in order to survive exposure to a certain level of stress. As such, organisms that exhibit a stress-tolerant phenotype gain a fitness advantage when exposed to stress. From an evolutionary perspective, stress applies pressure on populations that then may cause selection for organisms that are best able to tolerate or adapt to the stressors that are present. The capacity for organisms to adapt to any given stress may determine the fate of their respective populations if

the stress is strong enough to drive the population to extinction. Populations may adapt to stress over time, or they may be rescued in the face of a single lethal stress event in a process known as evolutionary rescue (5,6).

A stressor can be defined in a microbial context as “any environmental variable leading to a decrease in bacterial growth or competitive ability“ ((7). Microbes are important targets to consider in the context of stress because they both play vital roles in nutrient cycling in the environment and also impact other organisms directly when they act as pathogens. As such, stressors that impact microbial growth and survival can likely have effects that cascade to other organisms. Stressors come in many forms, but from an environmental perspective, human land use has greatly increased the diversity and the quantity of stressors that enter surrounding ecosystems. Approximately 85,000 chemicals are currently listed in the Environmental Protection Agency’s chemical substances inventory, with about 3,000 of these chemicals being produced at high volumes each year (8). Nearly 95% of these high-volume chemicals are missing at least one form of environmental assessment, while about half of them are lacking any form of assessment at all. It has been found that human land use, particularly urban, agricultural, and industrial land use, is associated with diverse chemical inputs into the environment (9–13). Each type of land use tends to be associated with effluent containing specific types of stress; for example, urban land cover has high levels of impervious land compared to other types of land cover, and consequently, urban runoff into the surrounding environment tends to have higher levels of nutrients, heavy metals, and salts (reviewed in (14)). Agricultural land use, because of fertilization, irrigation, and animal husbandry, is associated with increased nutrient and pesticide input (13) into the surrounding environment. Multiple types of human activity are associated with antibiotic input into the environment, and sub-inhibitory concentrations of antibiotic have

been found in sewage effluent, lakes, rivers, and soils (15,16). However, although the complexity and intensity of stressors in the environment continues to rise, the degree to which organisms may be able to adapt to complex stress conditions is largely unknown. The goal of this review is to synthesize the work that has been done on multi-stress adaptation in bacteria. I set out to determine what areas of research still need to be explored to gain a more comprehensive picture of how organisms may adapt to multi-stress systems, particularly in the context of common anthropogenic stressors.

Although there are a wide variety of compounds that can be inhibitory or lethal to microbes, microbes have many strategies by which they can adapt to stress. Some of these strategies, such as biofilm formation, involve phenotypic change induced by altering gene expression without changing the underlying genes themselves (17). Often, though, genetic changes can be necessary for individuals, and thereby populations, to survive in the face of a stress challenge. Some of the most well-known forms of stress resistance in bacteria, such as antibiotic resistance, are at least partly dependent on the emergence, transfer, and selection of antibiotic resistance genes that allow cells to more efficiently remove or inactivate active antibiotic compounds (ex. tetracycline resistance genes, reviewed in (3)). Genetic changes such as these that confer a direct benefit in the face of stress are selected upon and may become fixed within a population over time, thereby promoting survival of the population in the face of continued exposure to the same stress. The likelihood of any beneficial mutation to become fixed within a population is dependent on a number of factors, including population size and incidence of clonal interference. With large population sizes, mutations that confer a benefit have a lower probability of fixation because of clonal interference (18), but the probability for multiple different stress-tolerant mutants to arise is higher. These mutants must then compete to become

fixed within the population in a process known as clonal interference (18). When clonal interference occurs, it may delay or prevent the fixation of stress-resistant mutations within the population.

Rationale for studying microbes

Bacteria are ideal for studying adaptation in response to stress primarily because their short generation times allow us to examine evolutionary responses over reasonable experimental time scales. Beyond this, though, bacterial stress responses and stress adaptation have been studied in both clinical and ecological contexts. In a clinical context, bacterial adaptation to antibiotics is necessitating the use of complex mixtures of antibiotics in order to reduce the likelihood that resistance prevents treatment; however, multi-resistant variants have already begun to emerge to the point that some researchers state that a post-antibiotic era is already here (19–21). In an ecological context, bacteria play a vital role in nutrient cycling in all types of environment (ex. (22,23)). If bacterial populations are decimated or restructured by acute or chronic stress, the impacts on bacteria may then affect nutrient cycling and food web processes. As such, in this review, I focused on the means by which microbes may adapt and evolve in order to survive multi-stress challenges. In order to provide a more comprehensive picture, I first offer an overview of microbial single-stress adaptation as context for more complex interactions when multiple stressors are present. This single-stress overview benefits from several prior reviews that have described bacterial adaptation to stress (7,24–30) as well as studies that offered additional overviews of stress adaptation mechanisms within their texts (31,32). Evolutionary rescue (ER) and dispersal are not discussed in the scope of this review, as there has been no prior work on multi-stressor impacts on either; however, these are discussed further in Chapter 1.

Review of Literature

Literature Selection

To obtain articles suitable for this review, we conducted a search on Web of Science in February 2019. The terms included in the search were ("multiple stressor*" OR "stressors") AND (evolution* OR adapt*) AND (bacteri*), which yielded a total of 313 papers, which was reduced to 149 after a general scan for relevance regarding bacterial adaptation to stressors. Reviewing abstracts and introductions of this initial list resulted in a final list of 25 papers that specifically focused on multiple stressors in combination. These papers encompassed a variety of subtopics within the overarching theme of microbial evolution and adaptation to multiple stressors. These were then further subdivided according to the stress administration treatment; namely, sequential vs. simultaneous application of stress treatments, and pre-adapted or novel stressor (Table 1). These papers, and the references therein, were used to create this review.

Microbial Adaptation to Single Stressors

The microbial response to stress is conserved across domains and includes both general and stress-specific responses. There is some overlap between these two types of response, and links between the general and specific stress responses help to promote survival and adaptation against a given stress. Both types of stress response involve the redirection of cellular resources away from growth and division and towards repair, protection, and mitigation mechanisms. The mechanisms associated with the general stress response are induced regardless of the type of stress involved, while those activated within the available stress-specific responses will vary depending on the mechanisms of action of the stressor.

The Microbial General Stress Response

The general stress response involves the sigma response factor RpoS and the SOS response. Some of the primary purposes of the general stress response are to promote survival within a cell and to mitigate DNA damage, so mechanisms induced include DNA double-strand break repair, suppression of cell division, and redirection of cellular resources (25,27,33–35). In addition to this, the general stress response also recruits error-prone DNA polymerases (25,27,29,32), which may result in mutations that can be selected upon in the presence of a stressor.

One specific mechanism by which microbes may adapt to stressful environments regardless of the type of stress imposed is through adaptive, or stress-induced, mutagenesis (36,37). There are several different hypotheses as to how stress-induced mutagenesis (SIM) works, although the existence of SIM is itself contested (29,38). One hypothesis of SIM is that it is a product of genetic drift; namely, that stress is a rare event, and that genes that specifically induce DNA replication in response to stress are subject to weaker selection than genes responsible for DNA replication under standard conditions (7). Another is that a stressor may increase the general mutation rate in a population, which increases the likelihood that a resistant mutant will emerge and come to dominate the population as the stress continues (7,29). A third contesting hypothesis is that the presence of a stressor increases the mutation rate within a stressed organism particularly at sites related to responses to the stressor (i.e., nonrandomly in genomic space) (27,39–42). The third of these is, notably, the most difficult to investigate, as it is difficult to decouple the emergence of a mutation and the conditions that select for it (29). The genetic drift hypothesis, as the authors of the hypothesis note, also cannot explain how stress-related DNA repair mechanisms evolved (7). Increases in mutation rate as a whole in response to stress, however, have been observed in several stress scenarios. Adaptive mutagenesis in the

context of increased mutation rate has been observed in bacteria in response to starvation, hypoxia, pH stress, osmotic stress, and antimicrobials (25,27).

The process of stress-induced or adaptive mutagenesis allows bacteria to acquire permanent stress tolerance in inhospitable environments (26). However, one subtype of adaptive mutagenesis, termed adaptive amplification, is more easily reversed when the stressor is no longer present. This form of genetic change involves making one or more copies of a gene related to a stressor, which allows one gene to retain its original function and the other to change freely (28). This allows the organism to adapt with a lower risk of loss of function due to the presence of the original gene. The replicated gene may be inactivated once the stress is no longer present. Both adaptive amplification and stress-induced mutagenesis more generally have been tied to the general stress response (25,28,32); however, since the mutations that arise due to SIM are selected for under the specific stress environment encountered (7,27,29), stress-induced mutagenesis is implicated in stress-specific responses as well.

Microbial Stress-specific Responses

Stress-specific programming can be categorized based on the type and mechanism of action of the stressor being used. Some common categorizations of stressor include heat, osmotic, oxidative, acidic, heavy metal, alkali, and butanol (29,31). Each of these categories does require some of the same genetic programming in order to promote survival, and the general stress response is induced in addition to the specific response; however, each category of stress also requires specific cellular machinery to be activated in order for an organism, and consequently a population, to survive. Our review of multi-stressor systems focuses on the mechanisms involved in responses to osmotic and oxidative stressors, as antibiotics are frequently classed as oxidative stressors because their end stage effects include the generation of

reactive oxygen species (25). These two classes of stressors are the stressors that we selected for the multi-stressor adaptation and evolutionary rescue experiment presented in Chapter 1; however, examples of other types of stress may be found in (31,43).

Responses to osmotic stress are partially organism-dependent, but frequently involve either synthesis or aggregation of biocompatible solutes within the cell. These solutes, which among others, include KCl, proline, glutamate, and glutamine, help to preserve osmolarity with the surrounding environment while still retaining biological function (24,44). In extreme cases, such as in halophilic organisms that survive in brine environments, populations adapt to and thrive in extreme saline conditions by modifying protein structures with acidic residues (45). These residues interact with water and salt ions and allow proteins to remain soluble in the face of extremely saline conditions (44,45). The evolutionary pathway needed to acquire such protein adaptation has been determined to have been possible via rapid acquisition of independent steps, including an intermediate phenotype in which an organism's enzymes remain active under both high and low salt conditions (44). From a genetic standpoint, there are various means by which an organism may become halotolerant or halophilic, but in extreme halophiles (who can tolerate upwards of 4M NaCl), one primary adaptation allowing for salt tolerance involves increased activity in a novel form of mercuric reductase (45), an enzyme that reduces mercury to a nontoxic form.

Many types of anthropogenic stressors are categorized as oxidative stressors for bacteria. Several classes of antimicrobial may be considered oxidative stressors, as the end stages of these antimicrobial effects include the generation of reactive oxygen species that result in the death of the cell; however, many antibiotics may also be thought of as ribosomal stressors as their cellular targets specifically include subunits of the ribosome (25,46). Tetracycline, for example, targets

the 30S and 50S subunits of the microbial ribosome, which inhibits protein synthesis and leads to the death of the cell (3). More generally, though, oxidative stressors are a broad class that all induce specific cellular defense responses. The multidrug efflux protein systems SoxRS-AcrABtolC and MexXY-OprM are induced by oxidative and nitrosative stressors, and MexXY-OprM is specifically induced in antimicrobials that target the ribosome (25). Adaptation to antibiotics, specifically, is often dependent on various antibiotic resistance genes, which may be located on the plasmid or in the chromosome (3). For example, tetracycline resistance may involve one or more of twelve known resistance genes depending on the family and genus of the bacteria in question (reviewed in (3)). These genes have to do with antibiotic efflux, ribosome protection, and detoxification mechanisms. In addition to this, exposure to a single antibiotic has been found to select for multi-antibiotic resistance (MAR) in gram-negative bacteria, which has been linked to a specific locus termed *marA* (3,47).

Multiple Stressors and Stressor Interactions

Although single stressors generally have well-defined effects, when combined in a single medium, multiple stressors together may have either interacting or non-interacting effects (48). Interactions between stressors can impact the concentration and the degree to which they partially or completely inhibit populations. On a two-dimensional stress gradient, non-interacting stressors will generally cause populations to be limited by the more severe stressor (38). Interactions between stressors will cause populations to grow differently depending on whether the stressors inhibit or enhance their effects on the organisms (38,49). Inhibitory effects between stressors mean that the stressor combination is less effective at suppressing bacterial growth or survival than would be expected based on the concentrations of the two stressors. The main type of inhibitory effect between stressors is antagonism, where the effect of both stressors stresses

the population to the point where they are less effective than one stressor alone (1+1 is less than 1) (38) or less effective than an additive effect (1+1 is less than 2) (49). Several recent studies examining suppressive effects between stressors have focused on stressor combinations that include antibiotics, since combinatorial therapies are being investigated by researchers as a means of reducing the likelihood of antibiotic resistance. One such study examining multi-antibiotic pairings found that about 5% of 2-antibiotic combinations exhibited suppressive interactions, while around 17% of three-antibiotic combinations were suppressive (50). Exacerbatory effects between stressors can also occur, such that combinations of stressors can be additive (1+1 is 2) or synergistic (1+1 is greater than 2) (38,51). The type of interaction that two stressors may have depends on whether cellular components and mechanisms required to alleviate each type of stress are common or disparate. Common demand for resources such as ATP often results in an additive effect, while disparate concentrations of cellular resources may result in a synergistic effect.

It is generally difficult to predict what type of interaction two stressors will have without testing them directly. Reviews and meta-analyses across domains on stressor combination experiments that have been conducted thus far have found the majority of stressor pairs tested to be synergistic (52–54); however, these results have been contested by other studies, and some have suggested that synergistic effects are severely overrepresented in the literature (49). As such, the extent to which these effects are consistent is not fully known. In addition to this, it has been suggested that stressor interactions are largely species- and context-specific (54), and within bacteria, there have been few studies on stressor interactions, especially over evolutionary timescales.

Complex Stress Environments May Inhibit or Induce Microbial Stress Adaptation

Adaptation to single stressors has been well-studied in microbes, but multi-stress environments impose additional levels of complexity that have only recently begun to be examined. In addition to stressors interacting with one another, as described above, stressors in combination have been found to impose different selective pressures than single stressors alone (16). Complex stress environments select for different mutations than single-stress environments (16,31) and reduce selection strength on loci associated with adaptation to single stressors alone (16,55). It has been suggested that the genes important in multi-stress responses, termed the environmental stress response (ESR), comprise a genetic program of upregulation of around 300 genes and downregulation of around 600 genes (56) that are associated with protection against stress and synthesis of proteins. The main regulators of these genes are also associated with the general stress response. Only a small fraction of the genes involved in the ESR program correlate with increased survival in the face of a single stress; instead, they appear to be related to the adaptive stress response. The ESR has been implicated in cross-protection between disparate stressors, but its role in other complex stress reactions such as collateral sensitivity has not been established (Figure 1). The role of stress-specific programming in survival and adaptation in complex stress scenarios has also not been determined.

Cross-protection, also known as cross-resistance, occurs when the presence of one stressor provides resistance against a second stressor. Cross-protection has been observed in several stressor pairs (31). The fact that many stressor pairs that exhibit cross-protection share mechanisms of action (such as in osmotic-osmotic pairings) suggests that some aspects of the cross-protective stress responses required for survival in the face of both stressors in a cross-protective pair must be shared. Cross-protection has only been observed in specific scenarios (ex.

(31,43)). Many cross-protection studies tend to examine sequential stress rather than simultaneous stress, such that a population only encounters one stressor at a time. Such studies have observed cross-protection between osmotic-osmotic stressor pairs and osmotic-n-butanol stressor pairs (31,43). However, when examining the order in which stressors were applied, multiple studies found that cross-protection was only observable when stressors were applied in the order that the experimental organism would have experienced these stressors in nature, and not when the order of stressors was reversed (31,57–59).

There has been limited investigation thus far into the mechanisms underlying cross-resistance. It has been found that exposure to a mild stress increases survival probability against a lethal level of stress primarily because of protein synthesis requiring the general stress response (56). However, in combinations of differing stressors, it has not been determined the extent to which cross-resistance is dependent on general or specific stress response programming (Figure 1). Additional transcriptome work examining the genetic underpinnings of cross-resistance and collateral sensitivity has also found that resistant strains have similar expression profiles, with 15-20 genes offering the highest prediction accuracy (43). It has also been suggested that adaptive mutagenesis induced by antibiotic exposure can play a role in some cross-resistance stress scenarios (27,60) since increases in mutation rate would increase the likelihood that a multi-stress-tolerant variant would emerge. However, in the transcriptome study, fewer than 10 mutations were fixed in each resistant strain (43), and it is uncertain whether the emergence of these mutations was driven by adaptive mutagenesis. Thus, while the mutations implicated at least partly explain cross-resistance and collateral sensitivity, there is still a significant portion of these mechanisms that remain unknown.

Collateral sensitivity occurs when acquired resistance to one stressor slows or reduces the capacity of an organism or population to adapt to a second stressor. Collateral sensitivity, like cross-protection, has been observed in multiple stressor pairs. *E. coli* exposed to NaCl treatments and KCl treatments were both found to exhibit collateral sensitivity to $CoCl_2$ (43). Both NaCl and KCl may be classified as osmotic stressors, while $CoCl_2$ is classified as a heavy metal stressor; however, it is unknown if all osmotic stressors correlate with collateral sensitivity in all heavy metal stressors.

The mechanisms underlying collateral sensitivity are also partially understood. It has been hypothesized that collateral sensitivity may be due to the necessity of resource partitioning by a cell in an attempt to mitigate one stress or the other; for example, ATP may be required for cellular responses to stress (38) such as multidrug efflux pumps or ion pumps, and a cell must direct ATP towards one response or the other (Figure 1). Directing resources towards one specific stressor response reduces the amount of resources available to respond to the second stressor.

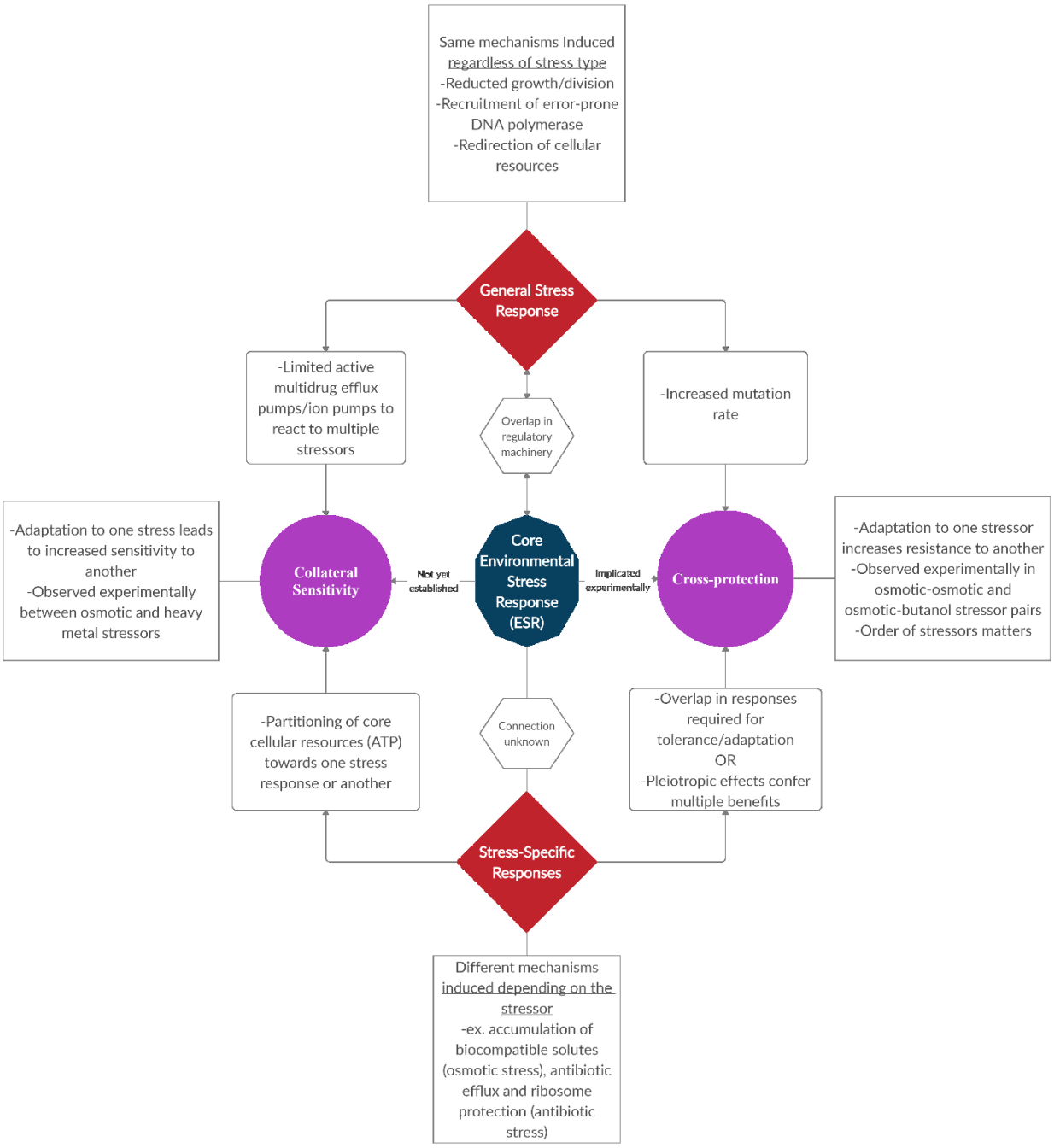

Figure 1. Concept map of stress responses and how they relate to multi-stress adaptation, based on the current available literature

In experiments that have examined complex stress thus far, there have been mixed results as to whether collateral sensitivity, cross-protection, or neither is most likely to occur, even with the same stressor pair. In one study examining stressor combinations of antibiotic and phage in *E. coli*, antibiotic-antibiotic, and phage-phage, cross-resistances were found to be common, while antibiotic-phage cross-resistances were rare (61). In addition, an experiment on the effect of pre-exposure to antibiotics on the emergence of phage resistance found that any mutagenic effects that could have led to an increase in phage resistance were small or nonexistent compared to the strong negative effect that antibiotics had on *E. coli* population size (62). However, another study examining coselection under antibiotic and phage stress in *P. fluorescens* found that enhanced phage resistance emerged under the multi-stress scenario to the point that the phage went completely extinct (63). In experiments examining other stressor combinations, *Saccharomyces cerevisiae* exposed to H_2O_2 exhibited protection against later exposure to H_2O_2 but did not protect against a challenge with menadione (64). Another experiment examining *Pseudomonas aeruginosa* epidemic strains isolated from lungs of patients with cystic fibrosis that were historically exposed to oxidative stress, antibiotics, and immune response exhibited greater antibiotic resistance than reference strains when faced with any of three antibiotic challenges (65). A study focusing on soil isolates from polluted environments found that bacterial and fungal communities that had developed metal tolerance did not exhibit significantly different tolerance to secondary types of stress (66). Some researchers, in addition to examining stressor combinations themselves, have also begun to investigate the effects of multi-stressor exposure on the timing of resistance evolution. One experiment that exposed *E. coli* and *S. typhimurium* simultaneously to an herbicide and an antibiotic found that populations exposed to both stressors were able to evolve antibiotic resistance more rapidly than bacteria exposed to the antibiotic

alone (67). By contrast, populations of *Pseudomonas fluorescens* simultaneously exposed to a sublethal level of antibiotic and the predator *Tetrahymena thermophila* were found to evolve predator defense more slowly than populations only exposed to the predator (16,68).

We have much to learn about microbial adaptation to multi-stress environments. Although some studies have begun to explore how coselective pressures influence the capacity of organisms to adapt to stress, it is unclear if stressor pairs of similar types to those that have already been studied would show consistent effects of collateral sensitivity or cross-resistance. In addition, it is not fully known how different levels of exposure to each stressor in a complex stress environment impact the likelihood of adaptation to the other stressors over an extended period of selection. The impact of prior multi-stress exposure on the likelihood of populations to undergo evolutionary rescue in the face of a lethal level of stress has also yet to be examined in an experimental setting. This observation is the motivation for chapter 1 of this thesis.

Conclusion

This review sought to ascertain how much is already known about microbial adaptation to complex stress challenges. Microbial responses to single stressors are well-established, and many of the different means by which microbes may adapt to stressful environments have been described from the cellular responses required to the genes that must be upregulated, amplified, or selected upon. However, based on the literature reviewed here, when multiple stressors are in play, selective pressures are different than when only one stressor is present. The type, concentration, and timing of stressors, as well as the organism experiencing the stress, all influence whether collateral sensitivity, cross-resistance, or no interaction at all will emerge over time. Because of this, much of what is known about multi-stress adaptation is specific to the contexts of the experiments that have been conducted. There is still much to be determined

regarding microbial adaptation to complex stress, including adaptation to stressor combinations that have not yet been tested, and the degree to which the results of multi-stress experiments are generalizable to stressors of similar categories and mechanisms of action.

The following research chapter was designed to test the effects of a two-dimensional gradient of chronic complex stress on the capacity of *Pseudomonas fluorescens* populations to adapt to each kind of stress involved. The objectives of the experiment were to 1) determine how prior exposure to differing levels of two kinds of stressor impact the likelihood of evolutionary rescue following lethal doses of each stressor, 2) determine if threshold levels of prior exposure to each stressor are required and promote rescue following lethal levels of combined stress, 3) examine the dynamics of bacterial populations as they respond to single and bi-stressor environments, and 4) examine the effects of dispersal on both evolutionary rescue and adaptation in multi-stressor environments.

Tables

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		Study Type			Stressor Method Applied/Reviewed			Stressor Type(s)								
Author	**Yr.**	**Experiment**	**Survey**	**Review**	**Seq.**	**Simul.**	**Indep.**	**Temp.**	**Nutr.**	**Osmo.**	**Acid.**	**Oxi./Ribo**	**Butanol**	**H. Metal**	**Biotic**	**Other**
Total		15	1	9	13	13	5	9	7	12	5	15	1	4	6	8
Allen et al.	2017	x					x					x			x	
Alvarez-Ordonez et al.	2015			x	x	x		x		x	x	x				x
Arias-Sanchez, Allen, and Hall	2018	x			x							x			x	
Azarbad et al.	2016	x			x					x				x		x
Beppler et al.	2017	x				x						x				
Cairns et al.	2017					x						x			x	
Dettman et al.	2013	x	x			x				x		x				
Dragosits et al.	2013	x			x					x	x	x	x			
Fitzgerald et al.	2017			x			x	x	x	x	x	x				
Hastings	2007			x			x	x	x							
Hiltunen, Ayan, and Becks	2015	x				x			x						x	
Hiltunen et al.	2018	x				x						x			x	
Horinouchi et al.	2017	x			x					x	x			x		x
Kurenbach et al.	2018	x				x						x				x
Maclean, Torres-Barcelo, and Moxon	2013	x				x		x		x						
Materna et al.	2012	x				x		x		x						
Mathieu et al.	2016	x			x							x				
Paerl and Otten	2013			x	x	x		x	x	x		x			x	x
Petrosino et al.	2009	x			x				x			x				
Poole	2012			x					x			x				x

Sayed et al.	2014	x		x	x		x		x			x	
Schimel, Balser, and Wallenstein	2007		x	x			x						x
Steinberg, Sturzenbaum, and Menzel	2008		x	x	x				x	x	x		
Vauclare et al.	2014		x	x	x	x	x	x	x			x	x
Wright	2004		x	x		x							

Table 1. References retrieved in the Web of Science literature search, as categorized by reference type, stressor administration, and stressors included. Papers where multiple administration types are marked are reviews that describe multiple types of stress within their text. The papers listed, and the references therein, form the basis of the multi-stress portion of this review.

Chapter 1: Adaptation and Evolutionary Rescue of *Pseudomonas fluorescens* in response to two distinct stressors

Introduction

Anthropogenic alteration of ecosystems is now widespread and is exposing many populations to multiple stressors. Stressors are environmental variables that act on organisms by reducing growth or competitive ability (1). Rapid increases in the concentrations of stressors can reduce population fitness and drive the rapid decline in population abundances. In this case, populations may respond in several ways. To survive continued environmental deterioration, populations must make use of various mechanisms in an attempt to cope with stress. One such possible mechanism is evolutionary rescue (ER), where "genetic adaptation allows a population to recover from demographic effects initiated by environmental change that would otherwise cause extirpation" ((2), p. 1). Evolutionary rescue has been shown in the lab (3–6) and in mesocosm systems (7), as well as in single-species populations (4,5) and in complex communities (6,7).

Evolutionary rescue has been repeatedly shown in single stressors such as salt (4), antibiotics (5), pH (7), and pesticides (8). Several factors promote evolutionary rescue in populations and communities: prior exposure to sublethal levels of a stressor and high population abundance increase the likelihood of rescue (4,9,10), although exposure to a high level of the stressor can decrease the likelihood of rescue by reducing population size and the appearance of beneficial mutation (10). Dispersal is another important mediating factor for evolutionary rescue. Immigration can allow gene flow between populations and allow adapted individuals to spread from one population to other populations across a gradient of stress (4,11). In addition, dispersal

has been shown to increase the probability of fixation of resistance mutations while minimizing negative fitness costs associated with antagonistic pleiotropy (12). However, dispersal has also been found to have negative impacts on evolutionary rescue in cases where populations are locally adapted to their conditions. In these cases, increased dispersal can produce a mismatches between organismal genotypes within a population and environmental conditions, resulting in a reduction in overall population fitness (11). In single-stress scenarios, an intermediate level of dispersal has been found to optimize the trade-off between the beneficial effects of gene flow and the negative effects of migration load (13).

Organisms are rarely exposed to one stressor at a time – more commonly, there are suites of stressors that may have differing impacts and interactions depending on their mode of action. Stressors are commonly classed into categories depending on the type of stress they exert upon the cell; some common categories include starvation, acidification, osmotic stress, oxidative stress, heat stress, and heavy metal contamination, and other categories (see (14,15)). Populations may have the capacity to adapt to certain combinations of stressors when beneficial pleiotropic effects or cross-stress protection between stressors occurs. Cross-stress protection, where adaptation to one stress promotes resistance to a later secondary stress, has been observed in microbes in several different combinations of stressor, including butanol and osmotic stress, acid and osmotic stress, and oxidative and osmotic stress (14). There is a generalized stress response that microorganisms use to tolerate exposure to both individual and complex stress that is mediated by the sigma factor RPoS (16). This stress response is instigated by DNA damage, and the mechanisms involved in this response modulate cellular metabolism, recruit DNA polymerase, reduce cell division, promote biofilm formation, and induce mutagenesis (reviewed in (17)). The mutagenic stressor response is implicated in both the general stress response (17)

and in an single-stress response referred to as stress-induced mutagenesis, or SIM (1,16–23). SIM has been associated with antibiotic resistance, and even low levels of antibiotic exposure have been found to correlate with increased mutation rate in antibiotic resistance- and tolerance-associated genes (20). SIM has also been implicated in other stress systems (reviewed in (23)).

The capacity for populations and communities to adapt to simultaneously applied coselecting stressors has only recently begun to be examined in the lab. Coselecting environments have been found to slow adaptation in some contexts (24) and to lead to the evolution of resistance to both stressors with no evidence of disruptive selection, defined as selection against intermediate trait values, in others (25). The degree to which cross-resistance, where acquired resistance to one stressor increases resistance to another, or collateral sensitivity, where acquired resistance to one stressor increases sensitivity to another, are generalizable across stressor types, and the degree to which adaptive capacity can be predicted based on stressor combinations and concentrations, is still unknown. We conducted an experiment to test the effects of gradients of two coselective stressors, tetracycline and salt, on adaptation and evolutionary rescue in populations of the bacterium *Pseudomonas fluorescens*. Based on studies that have been conducted on simultaneous stressors (24,25) and sequential stressors (26,27), we hypothesized that i) the presence of a second stressor will inhibit adaptation to the first stressor, and ii) dispersal will promote adaptation to all forms of stress. Extending what we know of rescue in single-stressor populations and metapopulations, we also hypothesized that i) ER will be most likely in single-stressor challenges when populations have been exposed to a sublethal level of the challenge stressor and a minimal level of a second stressor, and ii) evolutionary rescue will be most likely in a bi-stressor ER challenge when populations have been historically exposed to sublethal levels of both stressors.

Methods

Experimental design

The isogenic line SBW25 of *P. fluorescens* was selected as our study organism due to its well-established use for experimental evolution (28–30) and evolutionary rescue (5) research. Populations were grown along a selection gradient of two common stressors, salt and tetracycline (31–33). *P. fluorescens* was grown in the inner 60 wells of 96-well plates containing 120 uL of King's B medium containing no stressors in the top left corner of the plate, increasing concentrations of salt down the rows of the place (the vertical gradient of 0, 20, 40, 60, 80, and 100 g/L). The highest concentration of salt completely inhibited growth as determined in growth assays conducted in July and August 2018. We increased the concentration of tetracycline along the column of the plate (i.e. the horizontal axis with each column allocated 0, 0.5 ug/mL, 1 ug/mL, 2.5 ug/mL, 5 ug/mL, 7.5 ug/mL, 10 ug/mL, 15 ug/mL, 25 ug/mL, 50 ug/mL, 100 ug/mL). Again, the highest concentration completely inhibited growth, as determined in growth assays conducted in August 2018. Transfers of 1% total well volume were made to fresh plates every 48 hours. Optical density at 660 nm was taken for every plate immediately post-transfer and after 24 hours of growth. The experiment was maintained for 20 transfers, or approximately 130 generations, as the doubling time for *P. fluorescens* in batch culture has been determined to be 1.35 hours (34). One set of plates experienced no dispersal, and the other set of plates experienced global dispersal upon each transfer. For the global dispersal condition, to maintain the same 1% population transfer used in the non-dispersal condition, a pooled sample of each plate was created in pipette basins using a 12.5 uL sample of each experimental well in the plate and 6750 uL King's B medium. 0.6 uL was taken from this pooled sample to inoculate each well of the next transfer of the plate, while 0.6 uL of inoculum for the plate was taken from the

corresponding well of the previous plate. Plates were kept at 27°C and shaken at 120 RPM on an orbital shaker for the duration of the experiment. All combinations of treatments were replicated eight-fold. Dispersal was halted for the final three transfers of the selection phase (transfers 18-20).

After 20-transfers, 1% of culture from each well was transferred to the corresponding well of a new plate with media supplemented for evolutionary rescue assays. 1% of inoculum was taken for each of three ER assays conducted, for a total of 8 replicates of each combination of historical stressor exposure and dispersal condition for each assay. One single-stress assay tested rescue in response to an initially lethal level of tetracycline (100 ug/mL), while the other single-stress assay tested rescue in response to a lethal level of salt (80 g/L). The bi-stressor ER assay tested rescue in response to lethal levels of both stressors in combination (100 ug/mL tetracycline + 80 g/L salt). Rescue in populations was measured as change in OD_{660} as compared to the blank wells after 24-hours. The threshold ΔOD for rescue was set at 0.1 as a stringent measure of rescue; this value was selected to ensure that all rescued populations exhibited a substantial amount of growth, as a blank or non-viable well exhibited an OD less than 0.02 and a fully saturated well generally exhibited an OD of around 1.3. As such, all values >0.1 were scored has having grown during the period of the assay.

Statistical analyses

All analyses were conducted in R 3.5.1 (35). For Phase 1 of the experiment, we used a generalized additive model (GAM; package mgcv (36)) to infer the effects of each of our treatments on population growth over time. Because we expected to observe adaptation to each stressor over time, the model was fitted with time as well as the interaction terms between time and each stressor as smoothed terms. To test for the impact of each stressor alone on OD,

tetracycline and salt were each included as smoothed terms. An interaction term for tetracycline x salt was also included to determine if an effect of the two stressors together on OD was observable beyond the effect of each stressor alone. The effect of dispersal was measured by adding dispersal as a parametric term and by fitting models for both the no dispersal and global dispersal conditions.

For Phase 2 of the experiment, effects of historical exposure (tetracycline, salt, and the interaction of the two) on likelihood and degree of rescue, measured as ΔOD, for each ER challenge (tetracycline, salt, and both) were determined via ANCOVA. To test for differences due to dispersal in the frequency of ER included the interaction with dispersal and each historical stressor alone and in combination.

Results

Beginning of experiment

At the start of the experiment a concentration of 25 ug/mL of tetracycline, or 80 g/L of salt, was sufficient to completely inhibit growth of *P. fluorescens* over a 24-hour period (Fig. 1). When the two stressors were combined, growth was observed at 10ug/ml of tetracycline and 40g/L of salt. These are substantially lower concentrations than when salt and tetracycline were tested alone.

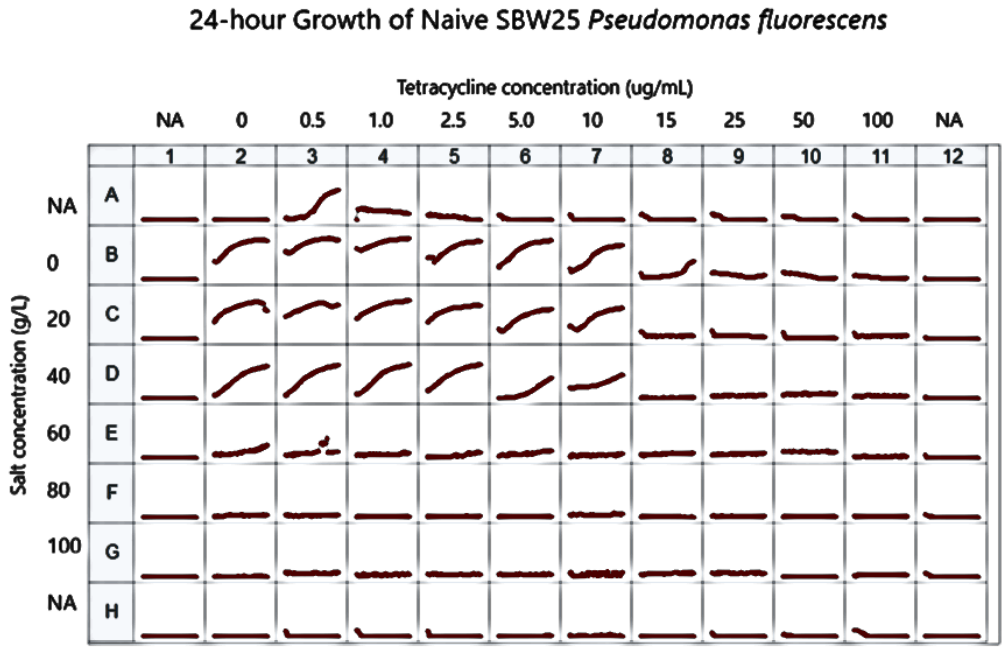


Figure 1. The initial susceptibility of naïve SBW25 *P. fluorescens* in response to a cross-gradient of tetracycline and salt. Within each box, the horizontal axis represents time, while the vertical axis represents OD. NA rows indicate uninoculated blank wells. A concentration of 25 ug/mL of tetracycline alone, and a concentration of 80 g/L of salt alone, was enough to completely inhibit growth over a 24-hour period. When the two stressors were combined, lower concentrations of salt and tetracycline were able to completely inhibit growth.

Phase 1

Over the course of 20 transfers, populations of *P. fluorescens* were able to adapt to the highest level of tetracycline in the absence of salt or when salt levels were low (20 g/L). Populations were able to grow in salt levels of up to 60 g/L at the beginning of the experiment, but only populations exposed to 40 g/L were able to persist over the full duration of the experiment. (Fig. 2). Populations were initially able to grow in tetracycline levels up to 5 ug/mL

with no inhibition and in levels up to 15 ug/mL with a moderate degree of inhibition. Over the course of the experiment, in the absence of salt, populations were able to adapt to even the highest level of tetracycline (100 ug/mL).

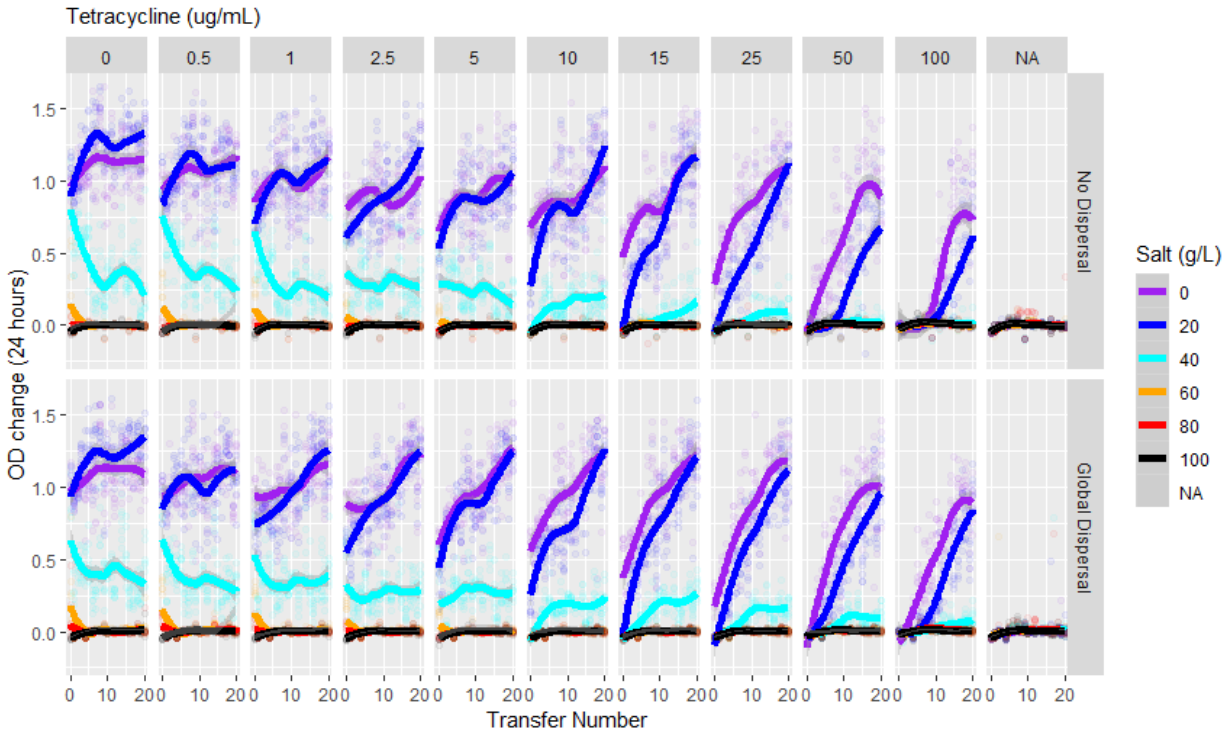


Figure 2. Growth curves in phase two of the experiment. Time series of 24-hour growth responses of *P. fluorescens* populations (n=8 per treatment condition) in response to increasing tetracycline (0-100 ug/mL) and/or salt (0-100 g/L) concentrations. 1% of cultures were transferred to a new plate every 48 hours for a total of 20 transfers. 24-hour change in OD was taken as a measure of growth for each plate at each transfer.

Both time and the interaction between time and each stressor had significant effects in our GAM, and populations either collapsed over time or adapted to the constant concentration of stressor (Table 1). However, time and its interactions with other treatments had relatively small

effect compared to the other effects included in the model. Out of all the terms, salt had the most significant effect in our model, with an F value nearly twice as large as the next highest value in the no dispersal condition, and over twice as large as the next highest value in the global dispersal condition. Tetracycline had the next highest F value, with a value around twice that of the next largest term. The interaction between tetracycline and salt had the third highest F value in our model.

Combining the two stressors inhibited growth to a greater degree than either of the stressors alone, and the interaction between tetracycline and salt had a significant impact on OD (Table 1). Upon examining the differences in rates of increase in ΔOD early in the time-series (between transfer 1 and transfer 5), we found that the presence of tetracycline or salt slowed or inhibited adaptation to the second stressor (Supplementary Figure 1; Supplementary Table 1). In addition, populations adapted to tetracycline and salt more quickly when dispersal was present, but adaptation was slower in both dispersal treatments as the concentration of salt stress increased to 40g/L. Dispersal increased growth at 40g/L of salt even in the presence of 50ug/ml of tetracycline.

Phase 1

Parametric terms		***Estimate***	***Std. Error***	***t-value***	***p-value***
	Intercept	0.299916	0.001285	233.4	0
	Dispersal	0.019267	0.001817	10.6	0
Smoothed Terms		***EDF***	***RefDF***	***F***	***p-value***
No dispersal	S(Time)	18.531	18.9721	121.03	0
	S(Salt)	4.996	5.000	15861.85	0
	S(Tetracycline)	7.584	8.001	998.85	0
	Interaction(Time*Salt)	23.547	24.800	165.24	0
	Interaction(Time*Tetracycline)	26.677	34.959	34.26	########
	Interaction(Salt*Tetracycline)	24.417	24.963	423.58	0
Global dispersal	S(Time)	18.547	18.974	224.44	0
	S(Salt)	4.996	5.000	17515.65	0
	S(Tetracycline)	7.543	7.966	697.64	0
	Interaction(Time*Salt)	23.721	24.848	242.33	0
	Interaction(Time*Tetracycline)	27.675	36.109	34.72	########
	Interaction(Salt*Tetracycline)	23.943	24.874	266.03	0

Table 1. GAM model effects for each treatment and interaction term over Phase 1 of the experiment. Dispersal was included as a parametric term, and all other terms were smoothed. Although the extent to which each term impacted adaptation differed, all terms and interactions had a highly significant impact on the degree of growth and adaptation of populations over time.

Phase 2

No population of *P. fluorescens* was underwent ER in the presence of both stressors at concentrations lethal to the initial population; this was true regardless of the prior concentration of salt, tetracycline or dispersal history. However, populations were able to be rescued in the face of lethal levels of a single stressor alone (Fig. 3). In the tetracycline challenge, 101 populations out of 480 rescued in the no dispersal condition, and 193 out of 480 populations rescued in the

global dispersal condition. In the lethal salt challenge, 64 populations out of 480 rescued in the no dispersal condition, and 4 populations out of 480 rescued in the global dispersal condition. ER was promoted by a higher level of historical exposure to the stressor used in the Phase 2 challenge (i.e., populations exposed to a higher historical level of tetracycline were more likely to be rescued in the tetracycline challenge) (Table 2). However, ER was also promoted by a lower level of historical exposure to the stressor not used in the Phase 2 challenge. In both the tetracycline and salt lethal challenges, historical exposure to salt had one of the most significant impacts on degree of ER, though the effect direction was opposite from one challenge to the other. In the lethal tetracycline model, historical salt exposure had the largest F value, while in the lethal salt model, it had the third largest F value (Table 2). Historical tetracycline exposure had a much more significant effect in the lethal tetracycline challenge than in the lethal salt challenge, as its F value was second largest in the lethal tetracycline model but was only fourth largest in the lethal salt model. Similarly, the interaction of historical tetracycline and salt was much more significant in the lethal tetracycline model. The effect of dispersal on rescue was dependent on the stressor presented in the lethal challenge. Dispersal was the most significant factor in the lethal salt challenge, with an F statistic around twice as large as the next largest term. In the lethal tetracycline challenge, dispersal had only the fifth largest F value. In addition, in the lethal tetracycline challenge, rescue was promoted by global dispersal (Fig. 3a), while in the lethal salt challenge, rescue was most frequent in the absence of dispersal (Fig. 3b).

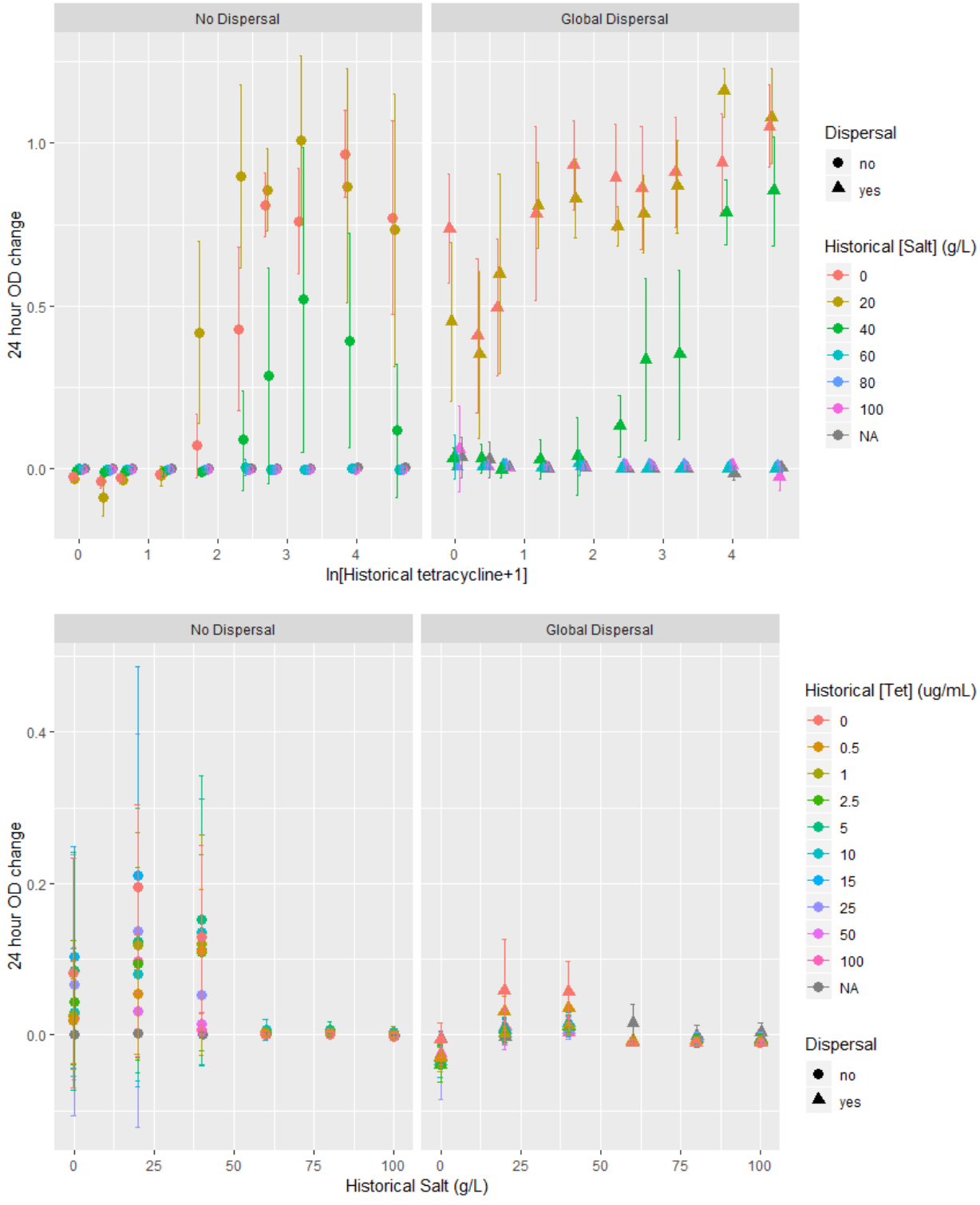


Figure 3. Phase 2 lethal single-stressor evolutionary rescue challenges. Points represent the mean of 8 populations exposed to the same conditions, while bars represent 95% confidence

intervals. 24-hour optical density (OD) change is representative of each population's ability to grow in a medium containing initially lethal levels of a stressor. Historical tetracycline (x-axis, upper panels) is the concentration of tetracycline (ug/mL) to which populations were exposed in Phase 1 of the experiment, while historical salt (x-axis, lower panels) is the concentration of salt (g/L) to which populations were exposed. Populations in the left panels experienced no dispersal, while populations in the right panels received partial inoculum from the previous transfer's corresponding well and partial inoculum from all other wells/treatment conditions on the previous transfer's plate. Upper panels represent the lethal tetracycline (100 ug/mL) challenge, while lower panels represent the lethal salt (80 g/L) challenge. No populations were able to be rescued in the face of lethal levels of both stressors (threshold value: 24-hour OD change ≥ 0.1), so the corresponding graph is not presented here.

Phase 2

Tetracycline ER challenge	***Df***	***F statistic***	***p-value***
tetra	1	128.6	< 1e-04
salt	1	819.55	< 1e-04
disp	1	78.3	< 1e-04
tetra:salt	1	110.72	< 1e-04
tetra:disp	1	0.01	0.9217
salt:disp	1	80.4	< 1e-04
tetra:salt:disp	1	3.61	0.05788
Salt ER challenge	***Df***	***F statistic***	***p-value***
tetra	1	5	0.02562
salt	1	32.27	< 1e-04
disp	1	76.93	< 1e-04
tetra:salt	1	1.99	0.15898
tetra:disp	1	3.05	0.08118
salt:disp	1	37.75	< 1e-04
tetra:salt:disp	1	0.96	0.32631

Table 2. ANCOVA for lethal single-stressor evolutionary rescue challenges. The degree to which each variable influenced the likelihood of rescue depended on the specific rescue challenge, but all single variables and at least one form of interaction did have a significant impact on rescue in likelihood and/or degree.

Discussion

Factors impacting evolutionary rescue

In this study, we tested whether exposure to stressors alone and in combination affect the likelihood of ER. We found that populations that had experienced selection at higher levels of tetracycline and lower levels of salt were more likely to undergo ER in the face of a lethal tetracycline challenge. In the case of the lethal salt challenge, populations were more likely to undergo ER when they were historically exposed to an intermediate level of salt and lower levels of antibiotics. In addition, the interaction of the two stressors when both were present reduced the likelihood of ER beyond the effects of each stressor alone. This provides support for our hypothesis that ER is less likely in the presence of two stressors. However, our results confirm previous work (4,9,10) that found that prior sublethal exposure to a stressor increases the likelihood of ER.

There are several factors that influence the likelihood of ER in single-stressor scenarios. Population size, genetic diversity, migration, and a low rate of stress all increase the probability of rescue (3). In the context of our multi-stressor scenario, there is a trade-off between maintenance of a larger population size from which populations may be able to be rescued and increased selection strength for resistant or tolerant types along each axis of stress. Larger populations are more likely to find a solution to stress when confronted with a lethal challenge (9), because they may contain higher standing variation (5,37,38), and a greater probability of beneficial mutations arising.

Over the course of phase 1, some populations were able to evolve resistance to the highest level of tetracycline, but resistance to salt only remained possible at an intermediate level throughout the duration regardless of the presence of tetracycline. Populations above 25 ug/mL

of tetracycline, the minimum inhibitory concentration (MIC) in our experiment, were required to develop tetracycline resistance over the course of phase 1; however, the minimum selective concentration (MSC) for antibiotics has shown to be much lower than the MIC (39–42); thus, populations that persisted through selection under any level of tetracycline in phase 1 were expected to have a higher likelihood of ER in the lethal tetracycline challenge in phase 2. Combined with the effects of population size on the likelihood of ER, we initially expected populations exposed to sublethal levels of tetracycline to be the most likely to undergo ER. However, by the end of phase 1, supralethal levels of tetracycline had only a small negative impact on 24-hour growth when no salt was present. As such, populations exposed to lethal or supralethal levels of tetracycline and lower levels of salt were the most likely to be rescued in the phase of lethal tetracycline in phase 2.

In the case of adaptation to the salt stressor in phase 1, populations were able to tolerate or adapt to a maximum of 40 g/L of salt; populations exposed to 60 g/L of salt and no tetracycline initially persisted, but all populations at 60 g/L salt or higher collapsed as the experiment continued. Populations persisting at 20 g/L and 40 g/L of salt were likely required to promote osmoprotective responses and/or adapt through mutations enhancing cytosol saline responses and protein structure, as organisms surviving in NaCl concentrations around 0.5 M (29.22 g/L) are considered slightly halophilic and those surviving in 0.5-2.5 M (29.22 g/L-146.1 g/L) are considered moderately halophilic (22). The increased likelihood of populations exposed to low-to-moderate levels of salt to be rescued in the face of a lethal salt challenge were likely a result of this adaptation, while the increased likelihood of populations exposed to lower levels of tetracycline may have been an effect of population size, or else, an effect of collateral sensitivity

where adaptation to tetracycline increased demand for cellular resources (43), reducing the amount of available resources below the amount needed to survive a lethal salt challenge.

Multi-stress adaptation dynamics over long-term selection

Over the long-term complex selective environment provided in Phase 1 of our experiment, populations were able to adapt to supralethal levels of tetracycline and an intermediate level of salt, and the presence of salt slowed adaptation to tetracycline. This supports our hypothesis that the presence of a second simultaneous stressor would inhibit adaptation to the first, as has been found in other work (24). Multi-stress systems can slow or inhibit the emergence of resistance to an individual stressor by reducing the selection strength on loci that would confer resistance to the single stress alone, thus reducing the likelihood that a beneficial mutation will become fixed (24,44–46). Complex stressor environments also often select on different mutations than single stressors. Unless a mutation either improves the general stress response, or else has pleiotropic effects that confer a benefit against multiple kinds of stress, multi-stress resistance is unlikely to emerge. Several stressors have been shown to promote resistance in microorganisms against a second disparate stressor, a phenomenon known as cross-resistance or cross-protection (14,15,43). Cross-resistance has been observed between osmotic-osmotic stressor pairs and n-butanol-osmotic pairs (14,15), and changes in selection under multiple stress has been observed in antibiotics and heavy metals, antibiotic and phage, and phage and resource limitation (reviewed in (25)). However, prior work examining coselective stress and cross-resistance has shown mixed results even within the same stressor pair. Antibiotic-phage experiments have shown both collateral sensitivity due to population effects (27). and cross-resistance due to increased mutation rate (25) under different experimental designs. As expected because of the disparate mechanisms of the two stressors, we

found no evidence of cross-stress protection between tetracycline and salt, as populations exposed to both stressors generally grew and adapted more poorly than populations in a lower level of the second stress, or in one stressor alone.

Effects of dispersal

We observed mixed effects of dispersal in Phase 2 of our experiments, with outcomes depending on the lethal condition imposed. In the tetracycline challenge, dispersal extended the range of ER into populations exposed to a lower level of tetracycline in Phase 1. In the salt challenge, dispersal reduced the likelihood of ER even in populations historically exposed to sublethal levels of salt. As such, the positive and negative effects of dispersal likely had differing importance depending on the stressor involved in the lethal challenge. Dispersal has been shown to facilitate gene flow and to spread beneficial alleles (ex. resistance genes) across populations (11); however, when populations are locally adapted to their environments, dispersal can increase migration load in a system and cause mismatches between organisms and environmental conditions. In our tetracycline ER challenge, where the global dispersal condition increased ER, the likely spread of tetracycline resistance alleles outweighed any negative effects of migration load. In our salt ER challenge, where more populations were able to be rescued in the no dispersal condition, migration load likely outweighed any beneficial effects of gene flow between populations. This difference in dispersal effect may be partially due to the ease of transferring antibiotic resistance genes in bacteria. Some tetracycline resistance genes are known to be exchanged by horizontal gene transfer in addition to arising *de novo* (47), while there is little evidence for horizontal transfer of salt resistance genes. Because of this, tetracycline resistance may be able to fix quickly in a population even if the number of resistant organisms dispersed into the population is low. By contrast, if salt resistance can only become fixed in a

population through propagation of organisms that acquired resistance *de novo*, a pooled global dispersal sample may have diluted resistant populations too much for resistance to become fixed in later transfers. There was also possibly an additional impact of population size in the dispersal condition, as the inoculum for dispersal transfers comprised half of the corresponding well on the previous plate and half of a pooled sample of all populations in the previous plate. If plates exhibited low population density in several wells due to either stressor alone or both together, it may be the case that the population transfers for the global dispersal condition were slightly less than the 1% target for which we aimed, which would have made them less likely to rescue than the no dispersal condition. It may be the case that the impacts of dispersal on ER depend on the magnitude of migration rate and the identity of the stressor; however, this conclusion requires more research with a wider array of stressors combinations and dispersal rates.

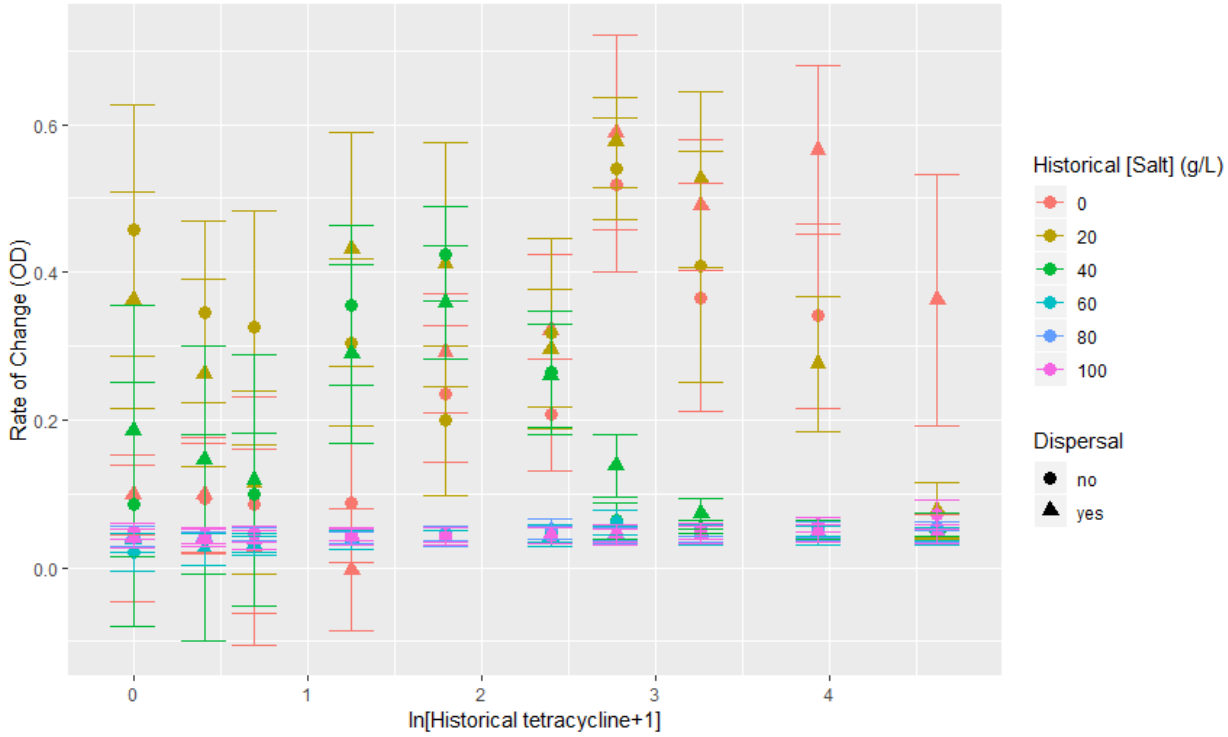
Future work

Because populations exposed to 60 g/L of salt or higher collapsed over the course of phase 1 of our experiment, it could be useful to refine the salt gradient to include a smaller range of concentrations to observe the evolutionary dynamics that happen just below the threshold of collapse. It would also be interesting to repeat this experiment with 1) a stressor pair with a history of cross-resistance to see if cross-resistance mechanisms can more effectively promote rescue in a dual-stressor system, such as n-butanol and salt and 2) a microorganism (such as an extremophile) that has a greater capacity to be rescued in the face of hypersaline environments, such as yeast (*S. cerevisiae*). More generally, these types of mechanisms should continue to be studied on larger scales and with more complex combinations of stressors. Since the ideal outcome would be to understand how and when microbial communities in the environment may be able to adapt and rescue in the face of increasingly complex combinations of stressors.

Additional multi-stressor experiments could be conducted in the future in larger mesocosm systems containing microbial communities sampled from the natural environment (48). These communities should be tested against a complex adaptive landscape consisting of the types of stressor cocktails that currently exist in different types of land use. By studying complex systems such as these, we will be able to gain an understanding of how organisms may be able to adapt and rescue in the future as land use and pollutant distributions continue to change.

Our study highlights the effects of stressor mixtures on adaptation, and on evolutionary rescue in bacterial populations. Two stressors in combination together slowed adaptation to each other in phase 1 of our experiment, which then led to a reduced probability of evolutionary rescue when confronted with a lethal level of either stressor alone. This result was likely due to a mixture of reduced population sizes and limited cellular resources such as ATP, which must be directed towards one stress response pathway or the other. Since mixtures of pollutants are common in soils and aquatic ecosystems, it is important to continue research on evolutionary rescue in complex multi-stressor contexts.

Supplementary Figures and Tables



Supplementary Figure 1. Rate of change in OD for populations (n=8) of *P. fluorescens* exposed to gradients of tetracycline and salt. Points represent the mean change in ΔOD from Transfer 1 to Transfer 5, the error bars represent 95% confidence intervals; higher values thus represent a treatment whose growth in increasing more quickly. Circular points represent populations in the no dispersal condition, while triangular points represent populations in plates with global dispersal.

Phase 1 Rate of Increase: Transfers 1-5	***Df***	***F statistic***	***p-value***
tetra	1	7.05	0.008065
salt	1	387.78	< 1e-04
disp	1	4.27	0.038972
tetra:salt	1	0.81	0.369641
tetra:disp	1	4.49	0.034373
salt:disp	1	8.41	0.003813
tetra:salt:disp	1	10.84	0.001031

Supplementary Table 1. ANCOVA for differences in rate of change in ΔOD for all treatment conditions (n=8 for each treatment).

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Concluding Remarks

Given the wide variety of environmentally and clinically relevant microbial stressors present in the environment, it is likely that microbes are frequently exposed to complex multi-stress pressures and challenges that make knowledge about multi-stress selection and evolutionary rescue pertinent in the real world. There has been limited investigation thus far into microbial adaptation in the face of complex stress, and there has been even less research on evolutionary rescue dynamics when multiple stressors are present. We studied long-term adaptation and evolutionary rescue in the face of a gradient of two common anthropogenic stressors, tetracycline and salt, to elucidate multi-stress adaptation and rescue mechanisms that are likely to come into play in real-world scenarios. Given the studies that have been conducted on multi-stress systems thus far, we expected the two stressors in combination to be likely to

inhibit adaptation, as many stressor combinations implicated in cross-resistance are stressors of the same type. Tetracycline and salt, as ribosomal and osmotic stressors, respectively, were likely to require separate stress response pathways, and we expected bacteria to exhibit collateral sensitivity between the two in both long-term adaptation and rescue scenarios.

The results of the experiment presented in Chapter 1, show that chronic stress influences the likelihood of a population undergoing evolutionary rescue. The interaction of two lethal stressors proved to be too inhospitable for populations to be rescued even when pre-exposed with both stressors. Historical exposure to both stressors together reduced the likelihood of populations undergoing evolutionary rescue in the face of a lethal level of one stressor alone compared to what was expected based on the level of rescue to each stressor alone. These extend our understanding of multi-stress adaptation and evolution, an avenue of research that has only recently begun to be explored. The results of this experiment also offer several possible directions for future research.

One of the most plausible explanations for our results is that the presence of a secondary stressor above a certain threshold dose reduced population sizes, which reduced genetic diversity within and thereby reduced the likelihood of evolutionary rescue. Because population size likely played so significant a role in our experiment, it will also be important to conduct further experiments examining multi-stressor adaptation and evolutionary rescue where population sizes are controlled. The role of population size in promoting evolutionary rescue is well-established (6,69) and previous work that has specifically tested culture volume as a proxy for population size in single-stress scenarios has shown that large cultures exposed to the same stressor challenge are more likely to be rescued than small cultures (69)

Our experiment also presented bacteria with two disparate stressors that affect different mechanisms of cell function. Tetracycline has been shown to stress cells by affecting the ribosome (3), while salt causes stress by disrupting cellular osmotic balance (24). Prior literature has shown mixed results regarding adaptation to coselective stress, with some experiments showing emergence of cross-resistance and some showing collateral sensitivity or no interaction. The results of previous experiments examining resistance associations, when taken together, seem to support the idea that cross-resistance may be more likely in stressor combinations of the same type or that operate on similar mechanisms of action, while cross-resistance between differing stressor types is rare (ex. (31,61)). The results of Phase 1 of our experiment supports this idea, as we observed no evidence of cross-resistance between populations exposed to both tetracycline and salt. Rather, populations exposed to one stressor tended to evolve resistance to the second stressor more slowly, as has been observed in another selection study examining disparate stressor pairs (16). To fully gain an understanding of the generalizability of these results, more work should be done examining long-term coselective dynamics under a wide range of stressor pairs and combinations. It will be especially important to expand the amount of data on the dynamics of adaptation between stressor pairs of the same type (ex. osmotic-osmotic and antibiotic-antibiotic), which have been shown to exhibit cross-resistance in at least some scenarios and stressor pairs of different types (ex. antibiotic-biotic stress), which have shown mixed results.

To our knowledge this is the first experiment to examine evolutionary rescue in a multi-stressor context, and similarly to multi-stress adaptation, it may be the case that cross-resistance plays a more dominant role in promoting rescue in other stress scenarios. As such, it will be

important to expand our understanding of different factors influencing stress adaptation and likelihood of rescue in different contexts.

To begin to scale our results to an ecologically relevant contexts (e.g, with interspecific interactions), it will be important to conduct experiments in semi-natural (e.g. mesocosms) and natural environments (e.g. in-pond or lake experiments). *P. fluorescens* is a well-established study organism for evolutionary studies (70), and it has a history of use in experiments examining antibiotic resistance under antibiotic alone (69) and under coselection with antibiotic and phage (ex. (71,72)). However, *P. fluorescens* has a limited level of halotolerance: a study examining salt adaptation in Pseusomonads found that a strain of *P. fluorescens* was able to tolerate 0.68 M (40. g/L) NaCl by accumulating biocompatible solutes within the cell (73). Because other microbial species can exhibit greater or lesser degrees of adaptation to different stressors, *P. fluorescens* may not be fully representative of all microbes' adaptive capacities. Beyond this, although we used an isogenic line to study de novo adaptation to stress, this decision prevented us from studying the effects of standing genetic variation or species interactions on the likelihood of rescue in a multi-stress or environment (e.g.(74)). Communities with more diverse mutants or species compositions may be more likely to have one or more stress-tolerant types and therefore be more likely to exhibit multi-stress adaptation and evolutionary rescue. It is too early to draw any definitive conclusions regarding the general implications of our experiment; the study of evolutionary rescue in multi-stressor contexts is only just starting. However, our study provides some baseline results for future work.

Fulfillment of Objectives

Overall, this thesis set out to determine 1) the capacity of microbes to adapt to complex stress, 2) the factors influencing the likelihood of adaptation, 3) the capacity of microbes to undergo evolutionary rescue after selection in complex stress environments, and 4) factors that increase or decrease the likelihood of rescue in complex stress environments. We were able to ascertain the degree to which prior work had already established a knowledge base regarding multi-stress interactions and adaptation as well as the existing knowledge gaps. By doing so, we were able to design and conduct a long-term, two-stress, selection experiment and evolutionary rescue assay. In addition, we conducted the first multi-stress evolutionary rescue assays that tested both the capacity for microbes to be rescued in the face of lethal levels of multiple stressors and the effects of historical exposure on the likelihood of evolutionary rescue.

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