

Modeling the Evolutionary Ecology of Stress Responses in Microbes

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by

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Abstract

Microbes are constantly exposed to a wide range of environmental stresses. To cope, all microbial species have developed protective mechanisms that can safeguard against potential damages due to stress. While stress protection is a beneficial contributor to microbial survival, it also carries a cost. The launch of a stress response diverts cellular resources to the synthesis of energetically expensive stress protectants. Consequently, responding and adapting to stress reduces growth in nutrient-poor environments. Hence the ability to balance allocation of available resources between stress protection and nutritional capacity in response to environmental signals is a fundamental property required for microbial survival with important consequences for species abundance and distribution in nature. This thesis deals with microbial survival strategies and their ecological impacts in two parts: First, we investigate the balance between stress-protection and nutrition as a driver of intra-species evolutionary divergence. Using a simple mathematical model we show that the protection-nutrition balance itself is sufficient to generate diversity within an initially monomorphic microbial population growing in a spatially homogeneous environment containing a single limiting resource. From experimental data we then estimate resource allocation between nutritional and stress resistant properties in glucose-limited *E. coli* chemostat populations subject to a range of environmental challenges and find that the evolutionary trajectories of multiple types can be predicted using the mathematical model. Second, we investigate the impact of inter-species differential survival strategies on microbial community structure within a *Candida* infection niche. Focusing on the lifestyles of *Candida albicans* and *Candida glabrata* under anti-fungal stress we build a mathematical model of niche competition within an infection. Calibrating the model using experimental data, we then generate predictions for the long term *Candida* ecology and find that the model is indeed predictive of equilibrium population distribution within a given infection niche.

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List of Abbreviations

ATCC	American Type Culture Collection, page 122
ATP	Adenosine triphosphate, page 29
CO ₂	Carbon dioxide, page 92
ESS	Evolutionary stable strategy, page 35
EUCAST	European Committee on Antimicrobial Susceptibility Testing, page 125
FACS	Fluorescence activated cell sorting, page 124
FLC	Fluconazole, page 88
GFP	Green fluorescent protein, page 122
MOPS	Morpholinepropane-sulfonic acid, page 125
PBS	Phosphate buffered saline, page 124
PIP	Pairwise invasibility plot, page 38
RPMI	Roswell Park Memorial Institute, page 114
RSS	Residual sum of squares error, page 70
SC	Synthetic complete medium, page 122
SPANC	<u>S</u> elf- <u>p</u> reservation and <u>n</u> utritional <u>c</u> ompetence, page 24
TCA	Tricarboxylic acid, page 92
YPD	Yeast extract peptone dextrose, page 122

Chapter 1

General Introduction

All living organisms are constantly exposed to a wide range of environmental stresses. To cope even the most primitive organisms on Earth have evolved adaptive mechanisms that can safeguard against potential damages or death under any set of environmental challenges [59] [110]. While stress protection is a beneficial contributor to survival in many environments, it also carries a cost. The launch of a stress response diverts cellular resources to the synthesis of energetically expensive stress protectants. Perhaps even more importantly, depending on the mode of regulation, expression of stress responses diverts transcription away from genes contributing to metabolism, macromolecular synthesis and vegetative growth in general. Consequently, responding and adapting to stress reduces growth and reproduction in nutrient-poor environments. Hence the ability to dynamically balance the allocation of available resources between stress protection and maximal physiological activity in response to environmental signals is a fundamental property required for survival across all forms of life. Indeed conserved regulatory mechanisms which modulate gene expression to ensure cellular functions are kept in tune with the physiological needs dictated by the environment have been documented in species ranging from plants [93] to yeast [24] to man [10].

At higher levels of biological organization the modulation of gene expression in response to the environment translates to basic biological processes related to reproduction and maintenance which ultimately determine survival fitness. Hence the defence-growth balance and its achievable settings have important ecological consequences for species abundance and distribution in nature. Given that evolutionary processes promote adaptation to the natural habitat it is not surprising that species populations and individuals possess different strategies to balance resources between fitness components. From an evolutionary perspective, diverse regulatory abilities broadens the range of nutritional and stress resistant properties within and across populations increasing the chance that at least some members will proliferate upon entry into a new habitat, be it physically or nutritionally competitive. Thus diverse intra- and inter-species stress management skills should be particularly effective for dealing with varying selection pressures in the ever changing environment over short time scales but also triggers longer term adaptations during evolution and provides a major driver for the maintenance of biodiversity on Earth.

Maintaining the right level of adaptation in a particular environment activates a complex cellular machinery which impacts the genetic, molecular and population levels simultaneously. Traditionally, research into stress response mechanisms have focused on one of these levels at a time. Whilst this has yielded major biological insights, to make further progress we need to adopt a multi-scale approach which merges all levels of life. Here we apply an integrated approach of theoretical methods and experimental systems which brings together the underlying regulatory basis of stress defence and nutrition into a population level framework. Adopting a reductionist method where the stress response mechanism is broken down into component parts we develop simple mathematical mod-

els capable of generating detailed predictions of population dynamics that can be tested against empirical data to establish the quantitative role of each participating model component in determining the stress response within a given environment. Any discrepancies between the predicted and experimental results are then resolved, either by extending the theoretical model to include other component parts or by changing the experimental set-up, thereby iteratively producing refined models and a more comprehensive view into the adaptive mechanisms under stress. The combined use of mathematical modeling and experimental analyses have been instrumental in unraveling the underlying mechanisms of complex biological processes such as nerve action potentials [50] and evolutionary genetics [34]. Hence through the formulation of appropriate physiological models and the use experimental systems we hope to elucidate the sub-cellular processes which govern higher level effects under stress in order to ultimately increase our understanding of the evolutionary and ecological consequences of stress in natural communities.

Here we focus on microbes as a means to connect the fundamental physiological basis of the balance between stress-protection and growth to its ecological impacts. There are a number of reasons for exploring stress responses in microorganisms: First, microbial experimental systems offer a tightly controlled, replicable environment to study stress responses and allows for detailed analysis of the stress-growth balance at all levels of biological organization, from the ecological to the mechanistic to the genetic. Next, microorganisms are the most abundant and diverse group of life on our planet and mediate many of the environmental processes that sustain life on Earth [116]. Finally, microbes can cause severe disease and are a major killer of hospital patients in intensive care units [92]. Hence understanding the mechanisms and dynamics of microbial stress responses is critical for our ability to control microbial survival, whether in the context of biotechnology, ecology or pathogenesis.

This thesis deals with microbial survival strategies in response to stress in two parts: In part I we study the balance between self-protection and nutrition as a driver of evolutionary divergence. Through the construction of a mathematical model we show that environmental selection pressures acting to balance resource allocation between survival functions under stress is sufficient to generate and maintain diversity within an initially monomorphic population growing in a simple, homogeneous environment. From experimental data of glucose-limited *E. coli* chemostat populations we then estimate resource allocation between protective and metabolic processes under stress and find that the evolutionary trajectories of multiple microbial types can be predicted using the mathematical model. This part is a summary of the studies "Trade-Offs Determine Evolutionary Trajectories in Laboratory Microcosms" by S. Nilsson, T. Ferenci, K. Haynes and I. Gudelj (in preparation for submission) and "Molecular and Evolutionary Bases of Within-Patient Genotypic and Phenotypic Diveristy in *Escherichia coli* Extraintestinal Infections" by Levert *et al.* [64] published in PLoS Pathogens.

In part II we investigate how different strategies in balancing stress resistance and growth influence microbial community structure within an infection niche. Focusing on the differential lifestyles of the human fungal pathogens *Candida albicans* and *Candida glabrata* under antifungal stress we build an ecological model which aims to shed light on the recent shift in species distribution within *Candida* infections. Parameterizing our model using experimental data, we generate theoretical predictions for the long term population ecology within a given infection environment and find that the ecological impact of different survival strategies between species does indeed provide an explanation for the shift in *Candida* ecology within infections. This part is a summary of the study "The Epidemiological Shift from *C. albicans* to *C. glabrata*: An Ecological Perspective"

by S. Nilsson, E. Cook, A. Smith, B. Oliver, A. Tillmann, A. Brown, K. Haynes, T. White, N. Gow and I. Gudelj (in preparation for submission).

We end the thesis with a short conclusion on how our findings in part I and II contribute to our understanding of stress responses in microbes and how our results lay the foundation for principles that could be applied to control microbial communities in practice.

Part I

Microbial Evolution of Stress Responses in the Chemostat

Chapter 2

Introduction Part I

Humans have long been fascinated by the extraordinary diversity of life on Earth. Yet, the underlying mechanisms which generate biodiversity remain poorly understood. The mystery of creation and maintenance of microbial diversity is rooted in the *competitive exclusion principle* [45] which states that the number of competing *types* that can stably coexist in a common environment cannot exceed the number of limiting resources available. Here, and throughout, by type we mean a collection of microorganisms that carry the same genotype and thereby adopt the same strategy for metabolizing available resources. Hence the competitive exclusion principle can be understood in a purely ecological context of resource competition and predicts that in a situation where multiple types compete for a single resource all but one type will eventually go extinct [53] [61]. So given the intense competition for limited resources in nature, what prevents the single best competitor from displacing all others? Actually, the evolutionary fate of any microbial type is not determined solely by its ability to compete for resources, rather, evolutionary change is a consequence of two processes; First, genetic mutations are continuously generated within populations. Next, natural selection sorts from the underlying genetic variation in an ecological context through resource competitions between micro-

bial types. Considering this interplay between genetic and ecological mechanisms which drives the evolutionary process, four principal theories have been proposed to explain patterns of diversity in nature:

1. The theory of *neutral evolution* [52] built upon the assumption that the fitness, that is the growth rate, of coexisting types in a community is equal at all times and in all places [17]. This means that no single type is ever at competitive advantage or disadvantage and thus competitive exclusion does not occur.
2. The theory of *mutation-selection balance* [44] which describes the coexistence of fit and less fit mutant types through a dynamic equilibrium where the generation of deleterious mutations arising in a population are kept at a low frequency by natural selection.
3. The theory *frequency-dependent selection* which arises through the presence of *fitness trade-offs* between *ecological niches* [97]. The niche is an abstract concept, but simply put, it defines a collection of environmental states that offer different conditions of growth in the common environment [25]. Thus different microbial types will be favoured by selection in different ecological niches thereby giving rise to trade-offs from the ability of performing one ecological function well at the cost of another. This means that the fitnesses of coexisting microbial types are decreasing functions of their own frequencies: As one type becomes more common, resource competition is more fierce among individuals of the same type than with individuals of another genotype which can exploit alternative niches in the common environment [105]. Hence environmental feedback selects for rare types so that a stable coexistence may occur at some *intermediate* frequency where the types are equally fit. Numerous experiments have shown that populations can diversify in the presence of ecological niches through, for example, spatial heterogeneity [84] where

types reside in different habitats, resource partitioning [89] where one organism excretes metabolic byproducts thereby providing ecological opportunity for the emergence of mutants with enhanced capacities to metabolize these byproducts or temporal variation [108] through differences in temporal patterns of resource requirements. Hence environmental heterogeneity allowing for ecologically differentiated populations to coexist through fitness trade-offs is considered to be a central driver of evolution and the maintenance of microbial diversity.

4. The theory of *maintenance of the fittest and the flattest* [8] which describes the coexistence of types in a peaked fitness landscape: The *fit* peak of highest absolute fitness has unfit near-mutational neighbours and the *flat* peak of lower fitness is more mutationally robust than the fit peak as shown in Figure 2.1. Mutational robustness means that while a mutant offspring will suffer a reduction in fitness relative to its parent at the local peak, the smaller this reduction the more robust this peak is to mutation [8]. At low mutation rates the fit types dominate, at high mutation rates the flat types dominate but at some *intermediate* mutation rates the fit and flat types coexist as a consequence of quasispecies-level negative frequency dependence [8].

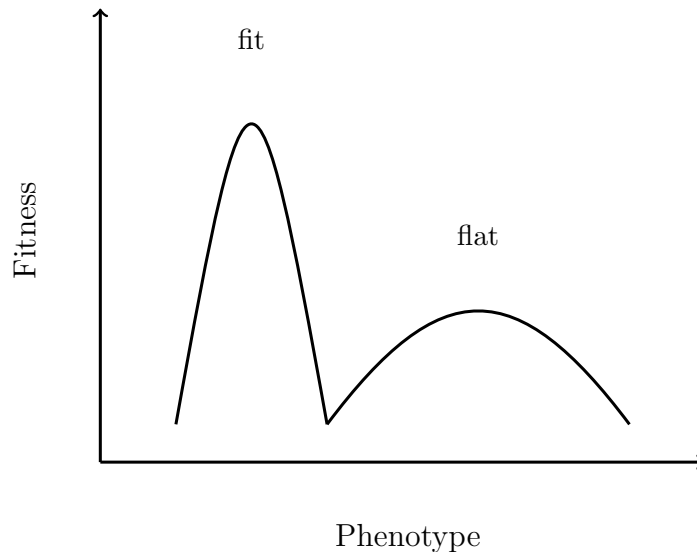


Figure 2.1: Coexistence by maintenance of the fittest and the flattest. The fit peak has the highest absolute fitness but the flat peak is more mutationally robust than the fit peak. Provided mutation rates are neither too high nor too low, a population of both fit and flat types can coexist at equilibrium.

Now consider a microbial population growing in a *chemostat*. The chemostat is an experimental device where a *single limiting resource* is fed into a culture vessel at a constant rate (see Appendix A for a detailed description of the chemostat). Under the assumptions that (i) the culture vessel is well-mixed and (ii) metabolite secretion that can be consumed by or have inhibitory effects on other cells does not occur, the chemostat represents a spatially and temporally uniform environment and thus possesses only one ecological niche. Consequently, theory predicts that microbial diversity cannot be maintained in the chemostat [101] and that over time mutations will result in genetic sweeps where the fittest type comes to dominate the population [3] [76]. However, the coexistence of multiple microbial types of different fitnesses has been detected in a number of chemostat experiments using both culturing techniques [69] and molecular population genetics [77] [89] thereby violating the competitive exclusion principle. So can the coexistence mechanisms (1)-(4) above explain such unexpected diversity? Here we investigate

a mechanism for the evolution of within-chemostat diversity based on non-uniformities in a constant environment. Instead of imposing an environmental or resource structure we consider non-uniformities inside the evolving organisms. We postulate that the evolution of microbial diversity can be explained by considering properties of alternate states that provide a major source of divergence. This can occur in the presence of trade-offs which are defined as functional relationships where a change in one trait that increases fitness is linked to a change in another trait that results in a decrease in fitness [100].

Here we focus on one of the fundamental physiological challenges for microorganisms under stress: how to balance self-preservation and nutritional competence (SPANC) [29]. Microbes possess a wide range of adaptive mechanisms at their disposal allowing them to thrive under diverse external conditions [66] and enabling them to inhabit almost every environmental niche on Earth. While stress protection is a beneficial contributor to survival in many environments [2] [7] it also carries a cost. The launch of a stress response diverts cellular resources to the synthesis of energetically expensive stress protectants. Perhaps more importantly, depending on the mode of regulation, expression of stress responses diverts transcription away from genes contributing to metabolism, macromolecular synthesis and vegetative growth in general. Consequently, responding to stress reduces growth in a nutrient-poor environment. This is the source of the SPANC trade-off that has been observed experimentally in both yeast [66] and bacteria [55].

Trade-offs play a key role in shaping evolution and yield scope for diversification through competing selective pressures working in opposite directions to balance conflicting fitness advantages. Moreover, theory predicts that the precise form of the trade-off curve crucially determines the outcome of evolution [41]. The realization that trade-off shapes matter dates back to classical life-history theory [62] [63] and recent studies have

emphasized the role of trade-off shapes in forming the fitness landscape which govern the evolutionary dynamics within an ecological setting [13] [90]. The shape of a trade-off is determined at the sub-cellular level where constraints in fundamental processes such as reproduction and maintenance directly influences the success of individuals at the population level. This is potentially problematic since, at present, many trade-offs cannot be inferred from sub-organismal processes. The shape of the SPANC balance trade-off is determined by the inherent regulatory properties which act to balance resource allocation between reproductive and protective cellular functions under stress. Hence the SPANC balance trade-off represents an ideal case study system to investigate the effects of different trade-off shapes on evolutionary outcome.

There are significant indications that variations in stress resistance are common between and even within species [29] [102]. So is resetting the SPANC balance through modulation of gene expression a major driver of such diversification? In this part we explore microbial evolution in the chemostat subject to the SPANC balance trade-off with a two-fold purpose; Firstly, to investigate whether the intra-population heterogeneity created by the SPANC balance trade-off can drive the evolution of diversity within a microbial population, and secondly, to investigate whether the shape of the SPANC trade-off determines evolutionary trajectories within a chemostat population. For simplicity and clarity, we organize this part as follows: We begin by developing a general mathematical model and study microbial evolution in the chemostat constrained by the SPANC balance. We find that the geometric shape of the SPANC trade-off determines evolutionary outcome and show that the SPANC balance itself is sufficient to generate diversity within an initially monomorphic microbial population growing in a homogenous environment containing a single limiting resource. Next, we ask whether the SPANC balance can account for the extensive variation in stress resistance observed

clinically within human extraintestinal *E. coli* infections and show that adaptation in response to environmental cues within the host does indeed promote stress resistance diversification. Finally, we use experimental data to estimate the shapes of the SPANC trade-off functions in *E. coli* chemostat populations exposed to two different environmental stresses. We generate predictions of the evolutionary dynamics and conduct short-term evolutionary experiments which confirm that trade-off shapes indeed mold evolutionary trajectories in laboratory microcosms.

Chapter 3

The SPANC Balance Drives the Evolution of Diversity in Microbes

In this chapter we develop a simple mathematical model in order to study whether the SPANC balance can drive the evolution of diversity in the chemostat. The work presented in this chapter features in the study "Trade-Offs Determine Evolutionary Trajectories in Laboratory Microcosms" by S. Nilsson, T. Ferenci, K. Haynes and I. Gudelj (in preparation for submission).

3.1 Modeling Evolution by Natural Selection

Discerning the evolutionary outcome of genetic mutations to balance resource allocation between stress protective and nutritional cellular functions in a given environment requires an approach which unifies the interplay between mutation and natural selection that shape microbial communities over time. Theoretical approaches to study evolution by natural selection began with *population genetics* [114] which studies the generation and maintenance of genetic variation by tracking population frequencies of discrete genotypes to which fixed fitnesses have been assigned. By modeling fitness as a static

quantity the population genetics approach precludes the possibility that changes in the environment alters selection on the population in the ecological setting in which evolution takes place. Hence we require an approach which allows for a feedback between evolutionary and ecological processes so that the fitness of a microbial type is neither fixed nor pre-determined but instead depends on the current state of the environment in which the type resides. Here we shall examine two different methodologies which introduce evolutionary features into an ecological framework, namely the *adaptive dynamics* method [37] and *reaction-diffusion* models [35]. Although both approaches are well suited to the study of eco-evolutionary processes providing distinct rules which describe mutation processes and the conditions that define the fate of each mutant, the two approaches differ in the coupling of evolutionary and ecological timescales: While the adaptive dynamics approach assumes that evolutionary processes occur at a time scale that is much longer than that of the ecological dynamics [37], reaction-diffusion models assume that evolutionary and ecological processes occur at comparable timescales [48]. So how do the two approaches compare when studying microbial evolution subject to the SPANC balance trade-off? In this chapter we employ both frameworks in order answer the following questions: (1) Can the SPANC balance trade-off drive the evolution of microbial diversity in the chemostat?, and (2) Do both frameworks yield the same outcome of microbial evolution constrained by the SPANC balance?

3.2 The Ecological Model

We start by building an ecological model using a series of simple assumptions regarding microbial metabolism that allows us to tease apart the population level effects of the SPANC balance.

3.2.1 Resource Consumption and Microbial Growth

Consider a microbial population growing on a single limiting resource, S , in the chemostat. We assume that the cells take up extracellular resource and convert it to adenosine triphosphate (ATP) using a simple, unbranched metabolic pathway [80]. In addition, we make the simplifying assumption that the behaviour of the entire pathway can be modeled using Michaelis-Menten kinetics of a single reaction. Hence the rate of ATP production, J^{ATP} , in the pathway is given by

$$J^{ATP} = \frac{n_{ATP}V_{max}S}{K + S},$$

where n_{ATP} denotes the number of ATP molecules produced in the pathway, V_{max} denotes the maximal uptake rate of the limiting resource and K is the Michaelis-Menten constant. Since the pathway rate represents the rate at which product is formed, which in this case is the same as the rate at which substrate is consumed, we refer to V_{max} as the maximal rate of resource uptake and to K as the measure of affinity for a resource.

If microbial growth is limited by the energetic resource, the amount of biomass formed per unit of ATP is approximately constant and does not depend on the mode of ATP production [5]. Therefore if the rate of ATP production increases, the rate of biomass formation and thus the growth rate of an organism also increases [80], which implies that the microbial growth rate can be represented as a linear function of the rate of ATP production, namely $p \times J^{ATP}$, where p is some proportionality constant. Hence we model the growth of a microbial population of density $N(t)$ at time t consuming a single

limiting resource of concentration $S(t)$ at time t in the chemostat as follows:

$$\begin{aligned}\frac{dS}{dt} &= D(S_0 - S) - \frac{V_{max}S}{K + S}N, \\ \frac{dN}{dt} &= \left(r \frac{V_{max}S}{K + S} - D\right)N,\end{aligned}\tag{3.1}$$

where $r = p \times n_{ATP}$ denotes the number of cells created per unit of resource. The constant D is the dilution rate which describes the rate of influx of the resource into the chemostat from an outside reservoir and the rate at which the content of the chemostat, including both cells and unused resource, is removed. S_0 is the concentration of resource in the input reservoir.

3.2.2 The SPANC Trade-Offs

In our model microbial types differ in the level of the core stress regulator, x , normalized to $0 \leq x \leq 1$. The SPANC trade-off is incorporated into the model by means of the following two assumptions:

1. We assume that an increase in the level of stress genes expressed leads to a decrease in the maximal uptake rate of the limiting resource V_{max} . We represent this assumption in the form $V_{max} = f(x)$ where f is an decreasing function of x .
2. We assume that there is a benefit to cell growth or survival associated with the expression of the stress gene under any given stress. Therefore the number of cells created per unit resource, r , is multiplied by $c(x)$, where c is an increasing function of x .

3.3 The Evolutionary Model: Adaptive Dynamics

We now include mutation processes into the ecological model (3.1) in order to study the effects of the SPANC trade-off on the evolution of stress responses in microorganisms. Microbes usually reproduce asexually and our model is therefore restricted to clonal reproduction. Moreover, the model takes into account both mutations in known stress regulators as well as regulators that pleiotropically affect the levels of stress regulators.

Here we adopt the adaptive dynamics approach [37] which is a set of techniques developed during the 1990's to understand the long term consequences of mutations in the traits that express the phenotype, x . Adaptive dynamics considers a *monomorphic* population of *resident* types with the same phenotypic trait, x_r , in an ecological setting while the evolutionary process is represented by a sequence of mutants from clonal reproduction with a different phenotypic trait, x_m , that successively challenge the resident population [42]. This modeling approach assumes that mutations have relatively little effect on the phenotype and occurs sufficiently infrequently that the resident population has reached dynamic equilibrium before a new mutation occurs. Hence there is a clear separation between the short ecological timescale and the slow evolutionary time scale. This allows for the incorporation of environmental feedback whereby the ability of a mutant to invade the resident population depends on the conditions set out by the resident types [42]. Whenever a mutant type manages to replace the resident, it becomes the new resident and, in turn, sets out the new conditions for invasibility of mutants thereby forming an ecological feedback loop where the fitness landscape is shaped by the current members of the resident population. A graphic illustration of the adaptive dynamics method is given in Figure 3.1.

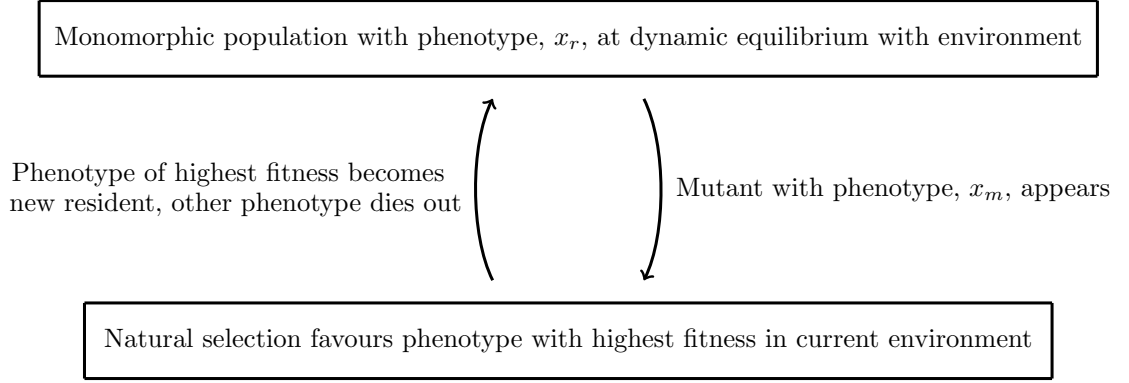


Figure 3.1: Illustration of evolution by natural selection under the adaptive dynamics framework.

3.3.1 The Adaptive Dynamics Model

Let $\Omega = (0, 1)$ denote the phenotypic trait space. Consider a resident phenotype $x_r \in \Omega$ and let N_{x_r} represents the density of cells with the resident phenotypic trait at time t . The resident population dynamics within the chemostat are given by

$$\begin{aligned}
 \frac{dS}{dt} &= D(S_0 - S) - \frac{f(x_r)S}{K + S}N_{x_r}, \\
 \frac{dN_{x_r}}{dt} &= r \frac{c(x_r)f(x_r)S}{K + S} - DN_{x_r}.
 \end{aligned} \tag{3.2}$$

The system (3.2) has two ecological equilibria; $(S_0, 0)$ which is asymptotically stable for $D \geq D_{crit}$ and $(S^*, N_{x_r}^*)$ which is asymptotically stable for $0 < D < D_{crit}$ where D_{crit} is the critical value of the chemostat dilution rate above which the microbial population will not be able to persist and eventually the chemostat will only contain the resource at concentration S_0 (See Appendix B for further details). We now assume that $0 < D < D_{crit}$ and that the resident population is at its positive ecological equilibrium $(S^*, N_{x_r}^*)$. Consider the effect of adding a rare mutant with a different phenotypic characteristic

$x_m \in \Omega$ into the resident population. The resident-mutant population dynamics are now given by:

$$\begin{aligned}\frac{dS}{dt} &= D(S_0 - S) - \frac{f(x_r)S}{K+S}N_{x_r} - \frac{f(x_m)S}{K+S}N_{x_m}, \\ \frac{dN_{x_r}}{dt} &= r\frac{c(x_r)f(x_r)S}{K+S} - DN_{x_r}, \\ \frac{dN_{x_m}}{dt} &= r\frac{c(x_m)f(x_m)S}{K+S} - DN_{x_m},\end{aligned}\tag{3.3}$$

Due to the assumption that before the mutation occurs the resident population is at its positive ecological equilibrium $(S^*, N_{x_r}^*)$ this means that after the introduction of the rare mutant in (3.3) the resident and mutant populations are close to the equilibrium point $(S^*, N_{x_r}^*, 0)$. Hence the stability of the equilibrium $(S^*, N_{x_r}^*, 0)$ determines whether or not mutants can invade; if it is unstable an initially rare mutant population can spread and eventually replace a given resident population, and if the equilibrium is stable the invading population will die out. We can determine the stability of the ecological equilibrium by calculating the eigenvalues of the Jacobian matrix, $\mathbf{J}^*$, of the resident-mutant model (3.3) evaluated at the equilibrium point $(S^*, N_{x_r}^*, 0)$:

$$\mathbf{J}^* = \begin{pmatrix} -D - N_r^* \left(\frac{f(x_r)}{K+S^*} - \frac{f(x_r)S^*}{(K+S^*)^2} \right) & -\frac{f(x_r)S^*}{K+S^*} & -\frac{f(x_m)S^*}{K+S^*} \\ N_r^* r c(x_r) f(x_r) \left(\frac{f(x_r)}{K+S^*} - \frac{f(x_r)S^*}{(K+S^*)^2} \right) & r \frac{c(x_r)f(x_r)S^*}{K+S^*} - D & 0 \\ 0 & 0 & \frac{c(x_m)f(x_m)S^*}{K+S^*} - D \end{pmatrix}.$$

Rewriting $\mathbf{J}^*$ in a way that emphasizes its block-triangular form we get

$$\mathbf{J}^* = \begin{pmatrix} \mathbf{J}_{\text{res}} & \mathbf{J}_1 \\ \mathbf{0} & \mathbf{J}_{\text{mut}} \end{pmatrix},$$

where $\mathbf{J}_{\text{res}}$ equals the upper 2×2 matrix in $\mathbf{J}^*$, $\mathbf{J}_1 = \left[-\frac{f(x_m)S^*}{K+S^*}, 0 \right]^T$, $\mathbf{0} = (0, 0)$ and $\mathbf{J}_{\text{mut}} = r \frac{c(x_m)f(x_m)S^*}{K+S^*} - D$. Because $\mathbf{J}^*$ is block triangular its eigenvalues are given by

the eigenvalues of the diagonal blocks $\mathbf{J}_{\text{res}}$ and $\mathbf{J}_{\text{mut}}$. We have already shown that the eigenvalues of $\mathbf{J}_{\text{res}}$ have negative real parts to ensure that the ecological equilibrium (S^*, N_r^*) is asymptotically stable before the mutant appears (see Appendix B for further details). Therefore, using the expression for S^* given in (B.1), the relevant eigenvalue is the single element of $\mathbf{J}_{\text{mut}}$ which is given by

$$\begin{aligned} f(x_r, x_m) &= r \frac{c(x_m)f(x_m)S^*}{K + S^*} - D \\ &= r \frac{c(x_m)f(x_m)\left(\frac{DK}{rc(x_r)f(x_r)-D}\right)}{K + \left(\frac{DK}{rc(x_r)f(x_r)-D}\right)} - D \\ &= \frac{c(x_m)f(x_m)}{c(x_r)f(x_r)} - 1. \end{aligned} \tag{3.4}$$

This eigenvalue is referred to as the *invasion fitness* and defines the expected growth rate of an initially rare mutant in an environment shaped by the resident population. Given that the invasion fitness determines the stability of the equilibrium $(S^*, N_{x_r}^*, 0)$ the evolutionary success of a mutant is given by the following set of rules: If $f(x_r, x_m) > 0$ a rare mutant will be able to invade the resident population. Alternatively if $f(x_r, x_m) < 0$ the invading mutant population will disappear. As mutants appear the population evolves in the direction of the local invasion fitness gradient, $G(x_r, x_m)$, until it reaches the neighbourhood of an *evolutionary singular type*, or equivalently, an *evolutionary singular strategy*, x^* . These are points where selection ceases to act, corresponding to maxima or minima of the fitness landscape, and are located where the fitness gradient is zero i.e.

$$G(x_r, x_m) = \left. \frac{\partial f(x_r, x_m)}{\partial x_m} \right|_{x_r=x_m=x^*} = 0. \tag{3.5}$$

Singular strategies can be classified according to two properties: convergence stability and evolutionary stability [37]. Convergence stability determines whether selection directs the population toward or away from a singular strategy, whereas evolutionary

stability determines whether a strategy can be invaded by nearby mutant types [9]. These two properties are mutually independent and hence several different evolutionary outcomes are possible [26]. A singular strategy can be both convergence stable and evolutionary stable, and therefore be the endpoint of the evolutionary dynamics; lack convergence and evolutionary stability and therefore act as an evolutionary repeller; be evolutionary stable but not convergence stable meaning that the evolutionary stable singular strategy cannot be attained through a series of small mutational steps; and, finally, be convergence stable but not evolutionary stable in which case it is called a "branching point" and can result in a protected *polymorphism* where the mutant and resident types coexist.

3.3.2 Classification of Evolutionary Outcome

The conditions for convergence and evolutionary stability can be derived using the second derivative test from basic calculus: A singular point, x^* , is an evolutionary stable strategy (ESS) if no initially rare mutant can invade, in other words, $f(x^*, x_m) < 0$ for all $x_m \neq x^*$ [37]. Since $f(x_r, x_m)$ as a function of x_m has a local fitness maximum for $x_m = x^*$, at an evolutionary stable singular strategy we have

$$\left. \frac{\partial^2 f(x_r, x_m)}{\partial x_m^2} \right|_{x_r=x_m=x^*} < 0. \quad (3.6)$$

A singular point, x^* , is convergence stable if a population of nearby phenotypes can be invaded by mutants that are even closer to the singular strategy x^* . This means that $f(x_r, x_m) > 0$ for $x_r < x_m < x^*$ and $x^* < x_m < x_r$. Near x^* the local fitness gradient is a decreasing function of x_r , thus it follows that at the singular strategy we have

$$\frac{dG(x_r, x_m)}{dx_r} = \left. \frac{\partial^2 f(x_r, x_m)}{\partial x_r \partial x_m} \right|_{x_r=x_m=x^*} + \left. \frac{\partial^2 f(x_r, x_m)}{\partial x_m^2} \right|_{x_r=x_m=x^*} < 0. \quad (3.7)$$

By definition $f(x_r, x_r) = 0$ for all x_r since the resident is at equilibrium. Thus we have

$$\left. \frac{\partial^2 f(x_r, x_m)}{\partial x_r^2} \right|_{x_r=x_m} + 2 \left. \frac{\partial^2 f(x_r, x_m)}{\partial x_r \partial x_m} \right|_{x_r=x_m} + \left. \frac{\partial^2 f(x_r, x_m)}{\partial x_m^2} \right|_{x_r=x_m} = 0,$$

and hence (3.7) can be written

$$\left. \frac{\partial^2 f(x_r, x_m)}{\partial x_r^2} \right|_{x_r=x_m=x^*} > \left. \frac{\partial^2 f(x_r, x_m)}{\partial x_m^2} \right|_{x_r=x_m=x^*}. \quad (3.8)$$

3.3.3 Adaptive Dynamics Model Results

We now attempt to classify the evolutionary outcome of the model (3.3) for different shapes of the SPANC trade-off curves. For simplicity, we restrict ourselves to SPANC trade-off functions that possess no inflection points, that is linear, concave and convex shapes of $c(x)$ and $f(x)$. In addition, we introduce the function $k(x) = c(x) \times f(x)$ which gives the geometric shape of any combination of the SPANC trade-off shapes considered (Table 3.1) and will aid in our classification.

		$c(x)$		
		linear	concave	convex
$f(x)$	linear	$k(x)$ concave	$k(x)$ concave	$k(x)$ concave
	concave	$k(x)$ concave	$k(x)$ concave	$k(x)$ concave
	convex	$k(x)$ concave	$k(x)$ concave	$k(x)$ convex

Table 3.1: Geometric shapes of $k(x) = c(x) \times f(x)$ for any combination of linear, concave and convex SPANC trade-off functions. Geometric shape was determined by computing the second derivative of $k(x)$ and verifying whether it is negative (indicating a concave function) or positive (indicating a convex function).

First, we test for evolutionary stability. Using (3.4) and (3.6) we get

$$\left. \frac{\partial^2 f(x_r, x_m)}{\partial x_m^2} \right|_{x_r=x_m=x^*} = \frac{k''(x^*)}{k(x^*)}.$$

It follows that the sign of $k''(x^*)$ determines whether a singular point is evolutionary stable. Note that the sign of $k''(x^*)$ is determined by the shape of $k(x)$ and, consequently, different shapes of the SPANC trade-off curves are expected to give different evolutionary outcomes. From Table 3.1 all combinations of the SPANC trade-off shapes considered give a concave shape of $k(x)$ ($k''(x) < 0$) except the combination of two convex trade-off curves which give a convex shape ($k''(x) > 0$). Hence all combinations of the simple trade-off shapes considered give rise to evolutionary stable singular strategies with the exception of two convex SPANC trade-offs which result in an evolutionary unstable singular strategy.

We now test for convergence stability. Using (3.4) and (3.8) we get

$$\frac{\partial^2 f(x_r, x_m)}{\partial x_m^2} < \frac{\partial^2 f(x_r, x_m)}{\partial x_r^2}, \quad (3.9a)$$

$$\frac{(k'(x^*))^2}{k(x^*)} > k''(x^*), \quad (3.9b)$$

but at a singular strategy the fitness gradient is zero:

$$\frac{\partial f(x_r, x_m)}{\partial x_m} = \frac{k'(x^*)}{k(x^*)} = 0 \quad \Rightarrow \quad k'(x^*) = 0.$$

Hence (3.9b) becomes

$$0 > k''(x^*) \quad (3.10)$$

Again (3.10) holds true for all trade-off shape combinations considered, thereby yielding convergence stable strategies, except for two convex SPANC trade-offs which do not satisfy (3.10) and are therefore not convergence stable.

Our classification reveals that the adaptive dynamics of (3.3) subject to all combi-

nations of the SPANC trade-off shapes considered, with the exception of two convex SPANC trade-offs, give rise to an evolutionary singular strategy that is both evolutionary and convergence stable. This means that once the singular strategy has been established through mutations it acts as an evolutionary endpoint that cannot be invaded by any other mutant strategy. We can demonstrate the evolutionary process over a sequence of mutations towards a singular strategy by means of a so-called pairwise invasibility plot (PIP) [20]. A PIP is a graphical representation of the sign of the invasion fitness, $f(x_r, x_m)$, for all possible values of x_r and x_m for a fixed set of model parameters and is used to identify the direction of evolutionary change over a series of mutant invasions (see Appendix C for further details on how PIPs can be used to determine the direction of evolutionary change). Here we choose to construct a PIP for two concave SPANC trade-offs in order to illustrate the march towards an evolutionary and convergence stable singular strategy as predicted by our classification above. The PIP for the concave SPANC trade-off curves given in Figure 3.2, is displayed in Figure 3.3a. For ease of interpretation a numerical simulation of the evolutionary trajectory over time is included in Figure 3.3b.

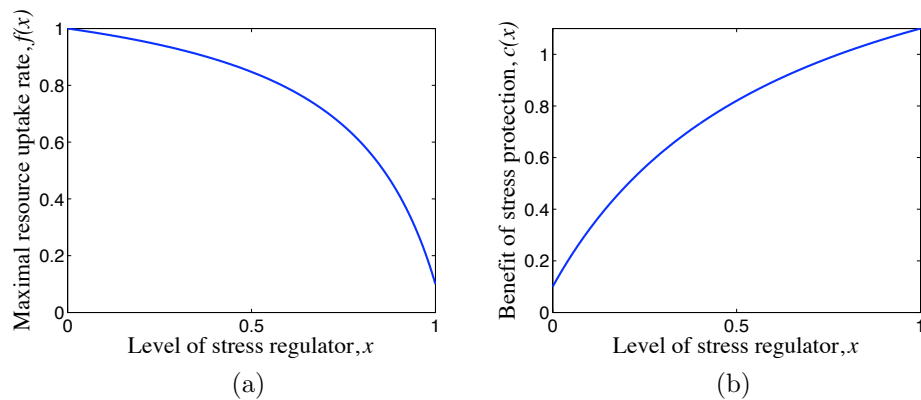


Figure 3.2: Concave SPANC trade-off curves.

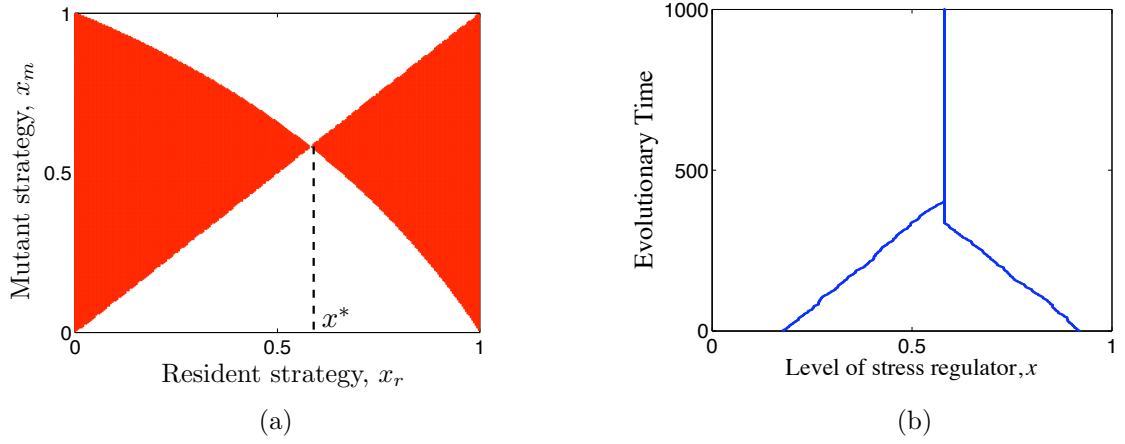


Figure 3.3: The PIP for concave $c(x)$ and $f(x)$ SPANC trade-off functions as given in Figure 3.2 indicate a singular strategy, x^* , that is evolutionary and convergence stable (a). Areas shaded in red represent the combinations of x_r and x_m for which the invasion fitness of the mutant, $f(x_r, x_m)$ as given in (3.4), is positive. MATLAB simulations of the evolutionary trajectories for two different initial resident types on opposite sides of the singular strategy, x^* , over time are given in (b). Simulations were performed using 500 phenotypic strategies uniformly spaced between 0 and 1, and the random number generator *rand* was used to generate numbers between 0 and 1 in order to decide how mutants appear over evolutionary time: if a random number ≤ 0.5 (>0.5) was generated, a neighbour of lower (higher) phenotype appears, if the invasion fitness is positive the mutant type becomes the new resident, otherwise the resident persists. Following this set of rules, after a series of invasions, concave SPANC trade-offs give rise to evolutionary trajectories that approach the ESS over time.

For convex SPANC trade-off curves the adaptive dynamics of (3.3) gives a singular strategy that is neither evolutionary nor convergence stable. This means that the singular strategy acts as an evolutionary repeller and depending on the initial value of the resident phenotypic trait, the evolutionary outcome will be either a monomorphic population of types with maximal level of stress regulator ($x = 1$) or a monomorphic population with zero level of stress regulator ($x = 0$). The evolutionary outcome of the convex SPANC trade-off functions given in Figure 3.4 is demonstrated by means of the PIP in Figure 3.5.

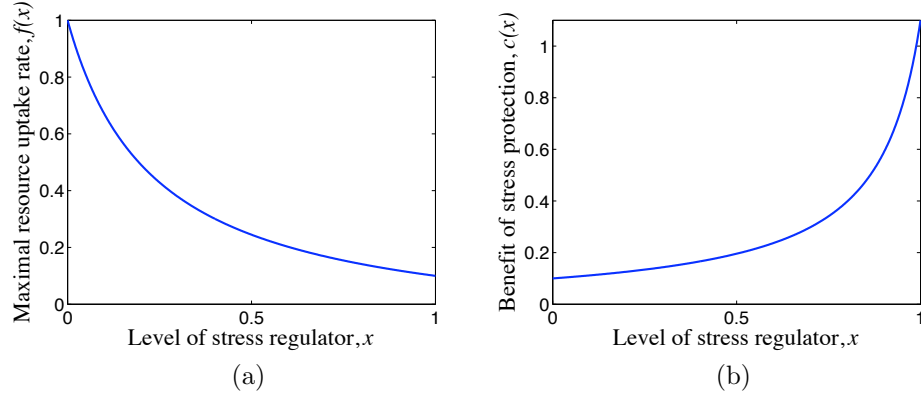


Figure 3.4: Convex SPANC trade-off curves.

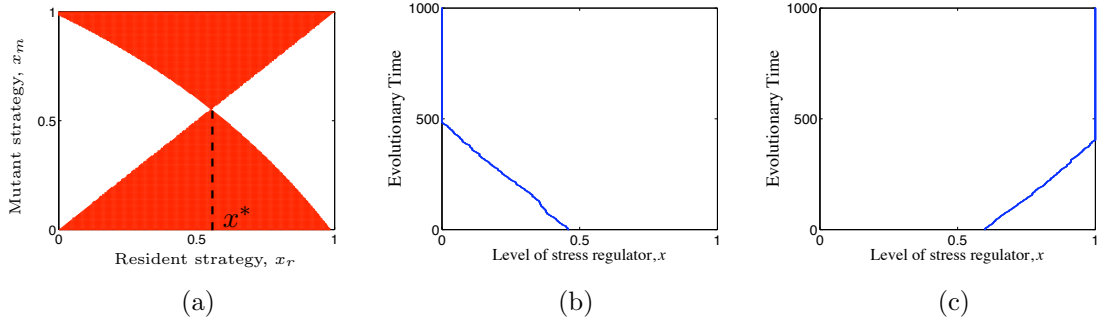


Figure 3.5: The PIP for convex $c(x)$ and $f(x)$ SPANC trade-off functions as given in Figure 3.4 indicate a singular strategy, x^* , that is neither evolutionary nor convergence stable (a). MATLAB simulations of the evolutionary trajectories for two different initial resident types on opposite sides of the singular strategy, x^* , over time are given in (b) and (c). Simulations were performed using 500 phenotypic strategies uniformly spaced between 0 and 1, and the random number generator *rand* was used to generate numbers between 0 and 1 in order to decide how mutants appear over evolutionary time: if a random number ≤ 0.5 (>0.5) was generated, a neighbour of lower (higher) phenotype appears, if the invasion fitness is positive the mutant type becomes the new resident, otherwise the resident persists. Following this set of rules, after a series of invasions, convex SPANC trade-offs only allow evolution away from the singular strategy. This means that the singular strategy is an evolutionary repeller and evolutionary outcome depends on the initial resident strategy.

So why does the adaptive dynamics of (3.3) yield a monomorphic population for all SPANC trade-off shapes considered? In order for a protected polymorphism to arise

under the adaptive framework we require that the singular strategy, x^* , is evolutionary unstable so that the resident and mutant strategies can mutually invade. This means that the expected growth rate of a rare mutant type in an environment shaped by the resident strategy is positive *and* the expected growth rate of a rare resident type in an environment shaped by the mutant strategy is positive. Thus for the evolution of a dimorphic population we require

$$f(x_r, x_m) > 0 \quad \text{and} \quad f(x_m, x_r) > 0. \quad (3.11)$$

Using (3.4) and (3.11) we get

$$\frac{c(x_m)f(x_m)}{c(x_r)f(x_r)} - 1 > 0 \Rightarrow c(x_m)f(x_m) > c(x_r)f(x_r), \quad (3.12a)$$

$$\frac{c(x_r)f(x_r)}{c(x_m)f(x_r)} - 1 > 0 \Rightarrow c(x_r)f(x_r) > c(x_m)f(x_m). \quad (3.12b)$$

(3.12a) and (3.12b) are mutually exclusive and therefore the adaptive dynamics of (3.3) does not support coexistence for *any* shape of the SPANC trade-offs curves. So does this mean that the SPANC balance cannot drive diversity in the chemostat? Actually, under the adaptive dynamics framework the evolutionary process is entirely driven by frequency-dependent ecological interactions [22]. While frequency-dependent selection is a well-established mechanism for the generation and maintenance of diversity [20], it is not the only one (see chapter 2). Hence we now consider a different modeling approach which can incorporate other coexistence mechanisms in order to establish whether the SPANC balance can drive the evolution of diversity within the chemostat by means of processes that are not considered in the adaptive dynamics framework.

3.4 The Reaction-Diffusion Approach to study Evolution

We now study the SPANC balance as a source of evolutionary divergence using an alternative modeling approach, namely through the use of reaction-diffusion equations. Here we assume that if a cell divides during asexual reproduction there is a small but prescribed probability ε of the offspring having a different value of the phenotypic trait from the parent cell. We consider the phenotypic trait, x , to be a continuous variable normalized to lie between 0 and 1 and use reaction-diffusion equations with constant diffusion coefficient, ε , to describe evolution by natural selection. The use of reaction-diffusion equations to model evolution and, in particular, to represent genetic and phenotypic population heterogeneities has a long tradition that begun with the work of Fisher [35] and Kimura [56] and has continued through to the present day in biomathematics [14] [15] [40], evolutionary biology [41] and biophysics [106] literature.

An advantage of the reaction-diffusion approach is that it incorporates the principle of evolutionary feedback; the idea that the density of a particular phenotype affects both its current environment and its competitors' likelihood of reproduction, and hence its own reproductive ability. Hence there is no phenotype that is deemed to be the fittest for all environments, instead fitness of a phenotype depends at the very least on what exists in its environment at that particular point in time which echoes evolutionary processes in nature. This is similar in nature to the adaptive dynamics method described in §3.3 with a few notable differences. Firstly, in the reaction-diffusion approach there is no physical separation of the evolutionary and the population dynamics time scales and we do not require the ecological interactions to be at equilibrium before mutations can arise. Another different feature in the reaction-diffusion formulation is that there is

a non-zero probability that a mutation can arise anywhere in the phenotypic domain $[0, 1]$ which is well suited to studying microbial evolution.

Let $N(x, t)$ denote the density of microbial types with phenotype x at time t . Then the growth of a microbial population in the chemostat is given by

$$\begin{aligned} \frac{dS}{dt} &= D(S - S_0) - \int_0^1 \frac{f(x)S}{K + S} N(x, t) dx, \\ \frac{\partial N(x, t)}{\partial t} &= \varepsilon \frac{\partial^2 N(x, t)}{\partial x^2} + \left(r \frac{c(x)f(x)S}{K + S} - D \right) N(x, t), \end{aligned} \quad (3.13)$$

subject to no-flux boundary conditions $\left. \frac{\partial N(x)}{\partial x} \right|_{x=0,1} = 0$ that restrict the newly created mutant phenotypes to the phenotypic domain $[0, 1]$.

To see how the system (3.13) arises let us consider a population of n competing distinct phenotypes, each with a different value of stress regulator expression, x , and let N_{x_i} denote the density of a type with phenotype x_i for $i = 1, \dots, n$ at time t . We assume that mutations have only small phenotypic effect and represent them in the following way. If a mutation occurs during reproduction of a cell with phenotype x_i then there is an equal probability of $1/2$ that the phenotype of the mutant offspring will either be x_{i-1} or x_{i+1} [41]. At the phenotypic boundary we assume that a parent with phenotype x_1 can only mutate into its mutational neighbour of higher phenotype (x_2) while x_n can only give rise to a mutant offspring of lower phenotype (x_{n-1}). Moreover, we assume that phenotypic values are uniformly separated by a distance $\Delta x = x_{i+1} - x_i$ and that the mutation rate from one phenotype to another is inversely proportional to the square

of the separation between phenotypes [1]. Under such assumptions (3.13) becomes

$$\begin{aligned}
\frac{dS}{dt} &= D(S_0 - S) - \sum_{i=1}^n \frac{f(x_i)S}{K + S} N_{x_i}, \\
\frac{dN_{x_1}}{dt} &= \frac{\varepsilon}{2(\Delta x)^2} (2N_{x_2} - 2N_{x_1}) + \left(r \frac{c(x_1)f(x_1)S}{K + S} - D \right) N_{x_1}, \\
\frac{dN_{x_i}}{dt} &= \frac{\varepsilon}{2(\Delta x)^2} (N_{x_{i+1}} + N_{x_{i-1}} - 2N_{x_i}) + \left(r \frac{c(x_i)f(x_i)S}{K + S} - D \right) N_{x_i}, \quad i = 2, \dots, n-1, \\
\frac{dN_{x_n}}{dt} &= \frac{\varepsilon}{2(\Delta x)^2} (2N_{x_{n-1}} - 2N_{x_n}) + \left(r \frac{c(x_n)f(x_n)S}{K + S} - D \right) N_{x_n},
\end{aligned} \tag{3.14}$$

which is readily recognized as the finite difference approximation to (3.13) where the second derivative with respect to the phenotype is approximated using a central difference scheme. Standard methods from numerical analysis could be used to show that (3.14) has solutions that converge to solutions of (3.13) in the limit $n \rightarrow \infty, \Delta x \rightarrow 0$. A schematic representation of our model in (3.13) is given in Figure 3.6.

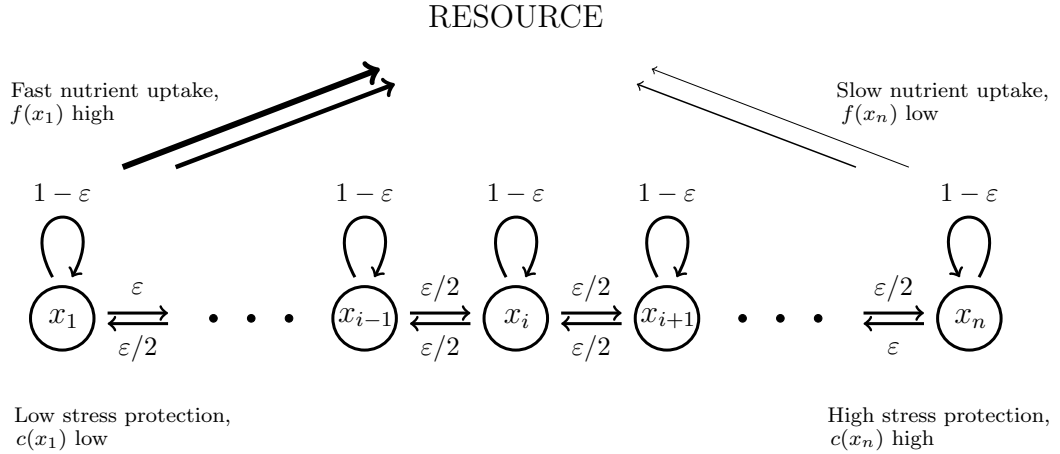


Figure 3.6: Schematic of the mathematical model in (3.13) incorporating the SPANC balance trade-off. Phenotypes are shown as nodes and mutation processes which encode how phenotypes change from parent to offspring due to a single mutational event are given by the arrows. The parameter ε on each transition arrow corresponds to the probability that a phenotypic mutation occurs. Here ε is a small quantity which embodies the idea that *most* reproduction is clonal.

Note that we use *probabilities* of mutations as *rates* of mutation in our model to dictate the temporal change in densities of different phenotypes within the chemostat. This strategy is justified here because of the high population numbers that can be used in the chemostat, however, adapting our approach to the modeling of natural communities would require a stochastic treatment of the mutation processes.

We use system (3.13) to study the long-term population heterogeneities by examining the number of local maxima in the distribution of population densities according to their phenotype. For example, an initially *monomorphic* population containing a single phenotypic cloud (Figure 3.7a) could through evolutionary and ecological dynamics of the system (3.13) give rise to a *polymorphic* equilibrium distribution of multiple phenotypic clouds (Figure 3.7b). We now fix the model parameters S_0 , K , r and D as variations in these values could cause environmental fluctuations which are a known source of divergent selection through the creation of ecological niches. Unless otherwise stated, the following parameter values were used: $S_0 = 1$ [resource unit], $K = 1$ [resource unit], $r = 1$ [resource unit/cell] and $D = 0.01$ [1/time]. Throughout, the maximal resource uptake rate is measured in [resource unit/cell/time] and all numerical simulations of (3.13) were performed in MATLAB for $n = 500$ types.

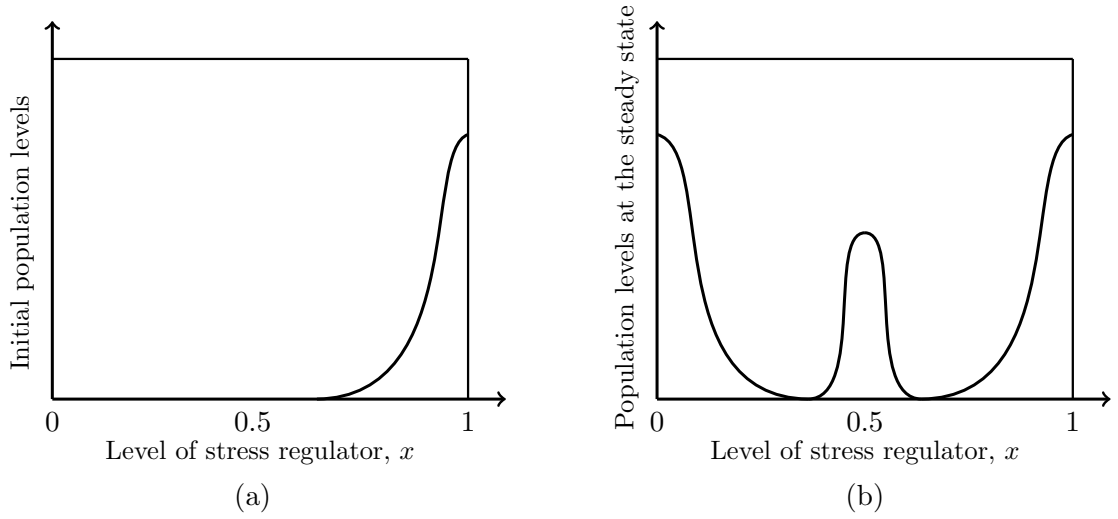


Figure 3.7: A biological interpretation of the model in (3.13): phenotypic structure of both the initial monomorphic (a) and the polymorphic steady state populations (b) can be elucidated from the number and the location of peaks in the population densities distributed according to their phenotype.

If the dilution rate of the chemostat, D , is larger than a critical value D_{crit} , the microbial population will not be able to persist and eventually the chemostat will only contain the resource at concentration S_0 (see [1] for a full proof). However, if the dilution rate lies in the range $0 < D < D_{crit}$, the microbial population will be able to persist on a single resource and converges to a unique steady state $N_x^* = (N_{x_1}^*, \dots, N_{x_n}^*)$ supported by a resource of concentration S^* . Therefore in the remainder of this section we assume that $0 < D < D_{crit}$ and explore the phenotypic structure of the steady state. Although it is possible to prove results on the global convergence of (3.13) to this non-trivial equilibrium, periodic solutions and chaotic attractors cannot be excluded (see [1] for further details). Hence we assume that the steady state that is known to exist for $0 < D < D_{crit}$ is linearly stable and verify *a posteriori* numerically via an eigenvalue computation that the equilibrium state found for a given set of SPANC trade-offs is locally and asymptotically stable.

3.4.1 Classifying Steady States

We can show mathematically that the shape of the SPANC trade-off functions $f(x)$ and $c(x)$ influences the number of phenotypic clouds present in the population at the steady state. Consider the steady-state problem of (3.13)

$$\epsilon \frac{\partial^2 N(x, t)}{\partial x^2} + \left(r \frac{c(x)f(x)S^*}{K + S^*} - D \right) N(x, t) = 0, \quad (3.15)$$

subject to the restrictions $N(x, t) > 0$ and $\frac{\partial N(x)}{\partial x} \Big|_{x=0,1} = 0$. We now define a classification function, $\gamma(x)$, given by

$$\gamma(x) = \epsilon \frac{\partial^2 N(x, t)}{\partial x^2} \frac{1}{N(x, t)} = D - r \frac{c(x)f(x)S}{K + S}, \quad (3.16)$$

which is defined whenever $N(x, t)$ is a positive solution of (3.15).

We now aim to use the properties of the function $\gamma(x)$ to classify the phenotypic diversity of (3.13) in the long term. For simplicity, we restrict ourselves to linear, concave and convex shapes of the SPANC trade-off functions, and again we make use of the function $k(x) = f(x) \times c(x)$ introduced in §3.3.3 to aid in our classification.

Lemma 1. *Assuming $k(x)$ is concave or convex then any solution of (3.15) has at most three local extrema in $[0, 1]$. Consequently, there is at most one local extremum of the steady state solution in $(0, 1)$.*

Proof. Suppose that a solution $N(x)$ of (3.15) has four, or more, local extrema $0 = x_1 < x_2 < x_3 < x_4 = 1$, so that $N'(x)$ vanishes at all of these points. This yields three zeros of $N''(x)$ in $(0, 1)$ and therefore $\gamma(x)$ too has three zeros. Now when $k(x)$ is convex, $\gamma(x)$ is

concave, and when $k(x)$ is concave, $\gamma(x)$ is convex. Consequently, $\gamma(x)$ has at least one zero in $(0, 1)$ and at most two zeros in $[0, 1]$, a contradiction. Since $N'(0) = N'(1) = 0$ we find $N''(x_0) = 0$ for some $x_0 \in (0, 1)$, whence $\gamma(x_0) = 0$. The boundary conditions provide two local extrema. $\square$

It immediately follows from Lemma 1 that any steady state solution of (3.13) has either one maxima (monomorphism), or two maxima (polymorphism) [40]. The manner in which monomorphism or polymorphism occurs is quantified below.

Proposition 1. *If $k(x)$ is strictly convex so that $k''(x) > 0$ for all $x \in [0, 1]$ then any solution of (3.15) is either monotonic or has a unique global minimum and two local maxima at $x = 0$ and $x = 1$.*

Proof. By Lemma 1 there exists a point $x_0 \in (0, 1)$ such that $N'(x_0) = 0$ or there does not. If such a point does not exist, the solution of (3.15) is monotonic. Let us suppose that such an x_0 exists. There are four different cases to consider:

1. $\gamma(x)$ has two zeros in $(0, 1)$,
2. $\gamma(x)$ has one zero in $(0, 1)$ and $\gamma(0) = 0, \gamma(1) \neq 0$,
3. $\gamma(x)$ has one zero in $(0, 1)$ and $\gamma(0) \neq 0, \gamma(1) = 0$,
4. $\gamma(x)$ has one zero in $(0, 1)$ and $\gamma(0) \neq 0, \gamma(1) \neq 0$.

In case (1), since $\gamma''(x) = -(rk''(x)S^*)/(K + S^*) < 0$, we see that $\gamma(x)$ is strictly concave and therefore has *sign pattern* $- + -$ on $[0, 1]$. Now suppose that $\gamma(x) > 0$ on an interval $I \subset (0, 1)$ and $\gamma(x) = 0$ on ∂I , if $x_0 \notin I$ then $N''(x)$ must vanish outside I , and therefore so too must $\gamma(x)$. This contradiction ensures that $x_0 \in I$. Now since $\text{sgn}(N''(x)) = \text{sgn}(\gamma(x))$ we see that $N''(x_0) > 0$ which means that $x = x_0$ is a local minimum for $N(x)$. Since $N(x)$ is monotonic on $(0, x_0)$ and $(x_0, 1)$, $N(x)$ must have a

global minimum at $x = x_0$. The boundary conditions ensure that $N(x)$ has two local maxima at the boundary of $[0, 1]$. In cases (2)-(4), the mean-value theorem ensures that $N'(x)$ cannot have a zero in $(0, 1)$, and $N(x)$ is therefore monotonic on $(0, 1)$. $\square$

Proposition 2. *If $k(x)$ is strictly concave so that $k''(x) < 0$ for all $x \in [0, 1]$ then any solution of (3.15) is either monotonic or has a unique global maximum and two local minima at $x = 0$ and $x = 1$.*

Proof. Since $\gamma''(x) = -(rk''(x)S^*)/(K+S^*) > 0$, we see that $\gamma(x)$ is strictly convex. The remainder of the proof is entirely analogous to that of Proposition 1 using the convexity of $\gamma(x)$. $\square$

A graphic illustration of the relationship between the simple SPANC trade-off shapes considered in Table 3.1, the classification function, $\gamma(x)$, and phenotypic diversity of (3.13) in the long term is given in Figure 3.8.

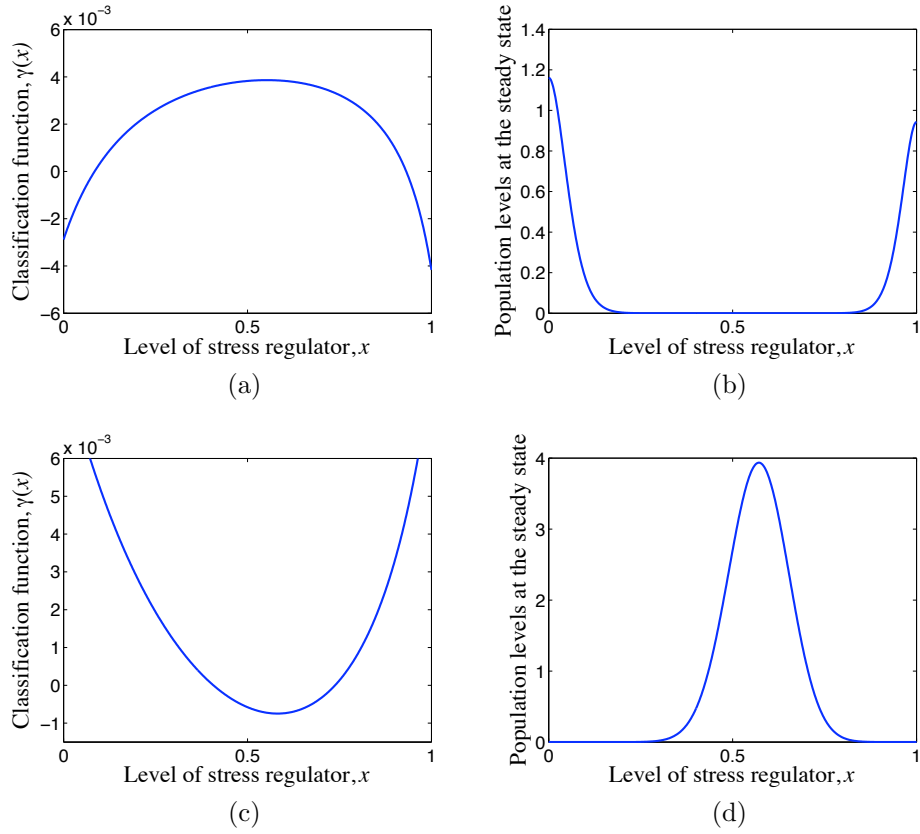
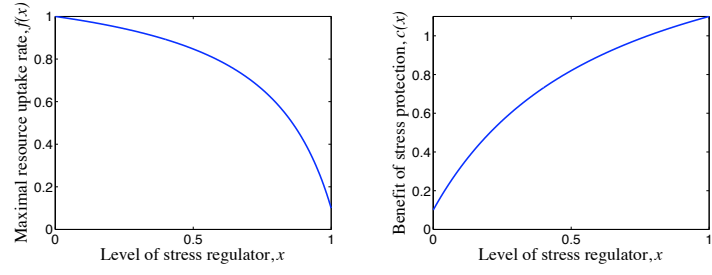


Figure 3.8: Classifying the number of phenotypic clouds in the equilibrium solution of (3.13) for linear, concave and convex SPANC trade-off functions: When $k(x)$ is convex, $\gamma(x)$ is concave (a) and the steady state solution of (3.13) supports two phenotypic clouds (b). When $k(x)$ is concave, $\gamma(x)$ is convex (c) and the steady state solution of (3.13) supports one phenotypic cloud (d).

The classification above can be extended to any SPANC trade-off shapes to support any number of phenotypic clusters in the long term, details of which can be found in the work by Gudelj *et al.* [40]. It should be emphasized that this classification of phenotypic diversity relies heavily on the shapes of the SPANC trade-off functions $c(x)$ and $f(x)$, and provides only an upper bound on the number of phenotypic clouds that exist for a given set of trade-offs. As we shall see in the next section, changes in other physical parameters such as the mutation rate and dilution rate could lead to diminishing phenotypic diversity.

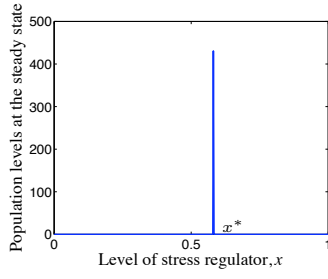
3.4.2 Steady State Dynamics

Having established mathematically the simplest SPANC trade-off shapes needed to drive phenotypic divergence in a simple resource-limiting environment in the previous section, we now investigate whether the number of phenotypic clouds supported by the steady-state structure of (3.13) is dependent on the system parameters. For example, if $f(x)$ and $c(x)$ are concave as in Figures 3.9a-3.9b, an initially monomorphic population will remain monomorphic consisting of a single phenotypic cloud situated around the partially expressed stress regulator, x^* , (Figure 3.9d) for any mutation rate ε and $0 < D < D_{crit}$. However, if $f(x)$ and $c(x)$ are convex as in Figures 3.10a-3.10b three different evolutionary outcomes are possible, crucially dependent on mutation rate: At low mutation rates an initially monomorphic population remains monomorphic consisting of a single phenotypic cloud situated around the maximal level of stress regulator at $x = 1$ (Figure 3.10e). For sufficiently high mutation rates the equilibrium population also remains monomorphic but supports a different phenotypic cloud located around the zero level of stress regulator at $x = 0$ (Figure 3.10g). Finally, for a narrow window of *intermediate* mutation rates the steady-state population is polymorphic containing two phenotypic clouds centered around the zero and the maximal level of stress regulator (Figure 3.10f).

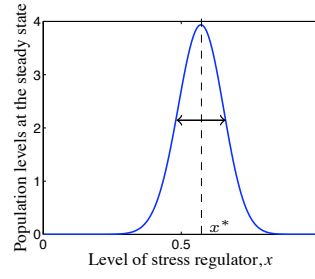


(a)

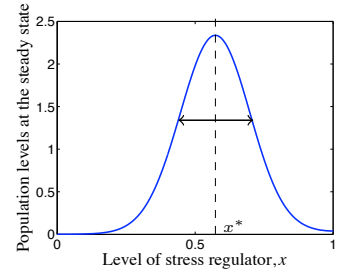
(b)



(c)



(d)



(e)

Figure 3.9: An example of the concave SPANC balance trade-offs $c(x)$ in (a) and $f(x)$ in (b) giving rise to monomorphic steady state population structures (c-e). The parameter $\varepsilon = 0$ in (c), $\varepsilon = 5 \times 10^{-6}$ in (d) and $\varepsilon = 2 \times 10^{-5}$ in (e).

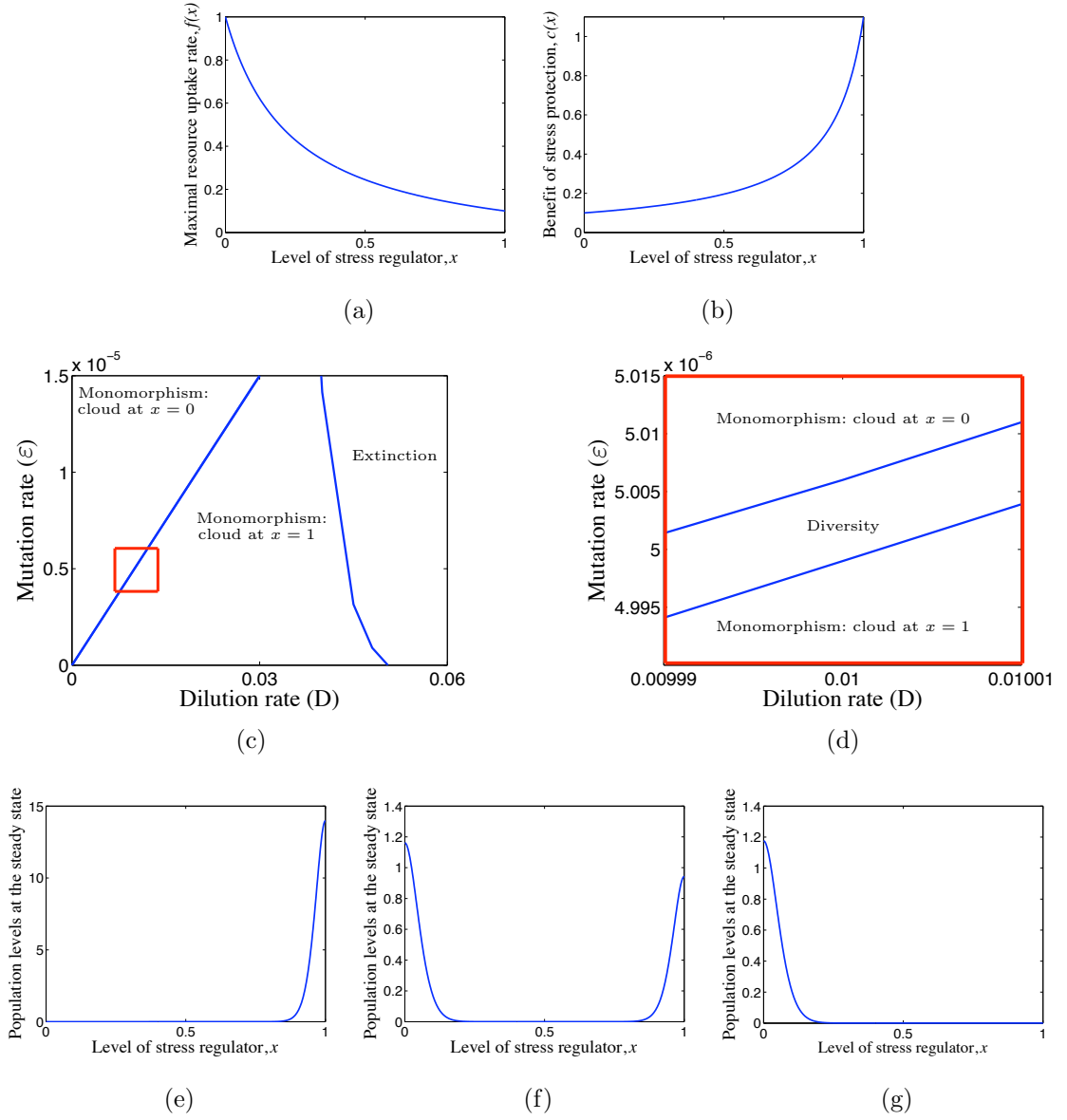


Figure 3.10: An example of convex SPANC balance trade-offs $c(x)$ (a) and $f(x)$ (b) giving rise to monomorphic (e) and (g) or polymorphic (f) steady state population structures. The structure of the steady state solution of (3.13) as a function of mutation rate (ε) and dilution rate (D) is shown in (c) and (d). The parameter $\varepsilon = 1 \times 10^{-6}$ in (e), $\varepsilon = 5 \times 10^{-6}$ in (f) and $\varepsilon = 6 \times 10^{-6}$ in (g).

The results in Figures 3.9-3.10 demonstrate that the number and the location of phenotypic clouds in the equilibrium population of (3.13) depends not only on the shape

of the SPANC trade-offs but also on the mutation rate ϵ and the dilution rate D . Note that it can be shown that the resource supply in the chemostat input reservoir, S_0 , does not affect the diversity of the chemostat population at equilibrium. We refer to [8] for a full mathematical treatment of this issue.

3.4.3 The Nature of the Observed Phenotypic Diversity

So how is diversity created and maintained in a single-resource chemostat? Can the mechanisms of coexistence maintenance, namely, neutrality, mutation-selection balance, frequency-dependent selection or a peaked fitness landscape explain the diversity we observe in our reaction-diffusion model? Starting with the neutral hypothesis we note that due to the SPANC trade-offs phenotypes differing in the level of stress regulator, x , also differ in their maximal rate of resource uptake, $f(x)$, and the associated benefit of expressing the regulator, $c(x)$, and therefore do not possess the same absolute fitness. Hence the coexistence we observe cannot be explained by fitness neutrality between phenotypes.

Next, we consider the mutation-selection balance by asking: Is mutational input from one phenotypic cluster sufficient to explain the diversity we observe in our model? Consider the convex SPANC balance trade-off functions in Figures 3.10a-3.10b for which we expect two phenotypic clouds to be maintained at steady state for an intermediate mutation rate. Now suppose we let an initially monomorphic population evolve until the two clouds have formed and then impose a *mutational barrier* (Figure 3.11) which sets the probability of mutation to zero between two phenotypes.

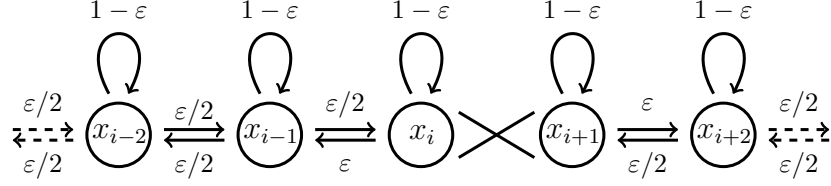


Figure 3.11: Illustration of a mutational barrier in phenotypic space.

Here we implement a mutational barrier in the middle of the phenotypic domain at $x = 0.5$ by setting $f(0.5) = 0$ in the convex $f(x)$ SPANC trade-off function in Figure 3.10a. This implies that after the two phenotypic clouds have formed, if a mutant arises at $x = 0.5$ it is set to zero and dies out so that mutations cannot feed from one cloud into the other. The $f(x)$ function that we use to investigate the effect of a mutational barrier on phenotypic diversity is given in Figure 3.12.

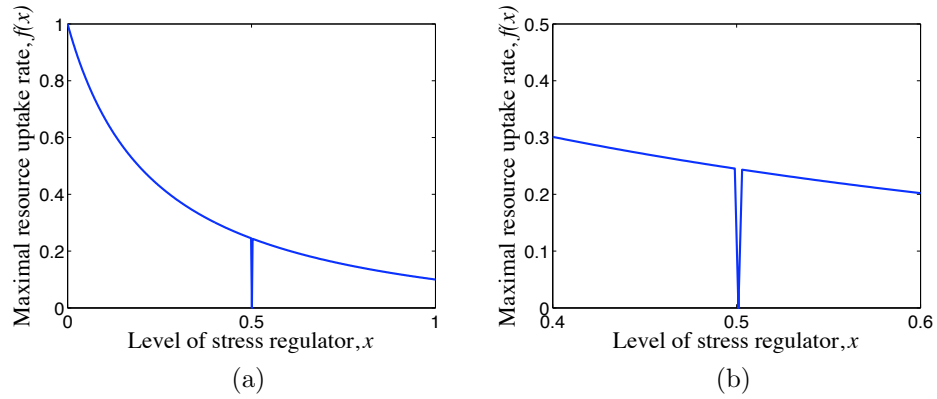


Figure 3.12: Manipulation of convex $f(x)$ function to study the effect of a mutation barrier on phenotypic diversity. Setting $f(0.5) = 0$ as shown in (a) represents a mutational barrier that is further zoomed in on in (b).

So what happens if we impose a mutational barrier between the phenotypic clouds? Simulating the system (3.13) in MATLAB subject to the convex SPANC trade-off functions in Figures 3.10a-3.10b to near equilibrium state and then implementing the $f(x)$ function in Figure 3.12 we find that the two phenotypic clouds are stably maintained

even after the imposition of the barrier as depicted in Figure 3.13. This means that the two phenotypic clouds are mutationally independent and hence a mutation-selection balance is not a full explanation for the diversity we observe in our model [8].

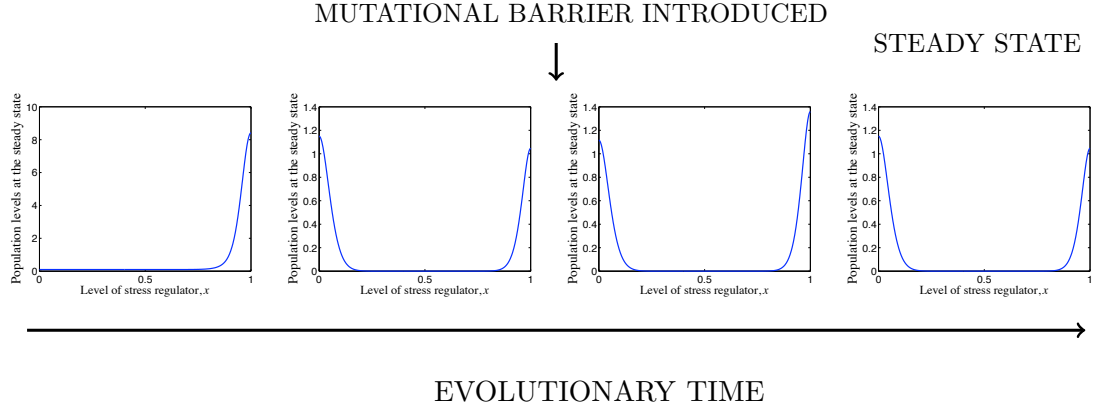


Figure 3.13: The impact of a mutational barrier at $x = 0.5$ on the equilibrium phenotypic diversity of (3.13) subject to convex SPANC trade-offs.

Note that in the case of concave SPANC trade-off functions as in Figures 3.9a-3.9b, the steady state population is always monomorphic containing a single phenotypic cloud (Figures 3.9c-3.9e). In this case, increasing the mutation rate will increase the width of the phenotypic cloud and in the limit $\varepsilon \rightarrow \infty$ the distribution of phenotypes converges to a maximal diversity state with all possible phenotypes present in equal numbers. While this is a classic example of a mutation-selection balance we do not consider this a biologically interesting diversity scenario.

The diversity we observe in Figure 3.10f is neither maintained by classical frequency-dependent selection. If it were, the resource competition between a phenotype with maximal level of stress regulator and a phenotype with zero level of stress regulator would result in a stable balance between the two [8]. However, setting the mutation rate $\varepsilon = 0$ the ecological-evolutionary system (3.13) reduces to a purely ecological system

where all types compete for a single resource. It is well known that competitive exclusion holds in such systems and that the one type with largest growth rate outcompetes all others [98]. Note that which phenotype wins, depends on the shape of the SPANC balance trade-off. For example if $f(x)$ and $c(x)$ are concave as in Figures 3.9a-3.9b an initial population containing all phenotypes will in the long term contain a single phenotype with partially expressed stress regulator at the level x^* (Figure 3.9c). On the other hand if $f(x)$ and $c(x)$ are convex as in Figures 3.10a-3.10b an initial population containing all phenotypes will in the long term contain a single phenotype at $x = 1$.

Having discarded the classical explanations of diversity maintenance, we now consider the coexistence mechanism termed "maintenance of the fittest and the flattest" [8]. Consider the fitness landscape, that is the surface of growth rates for all phenotypes, shaped by convex SPANC trade-off functions given in Figure 3.14.

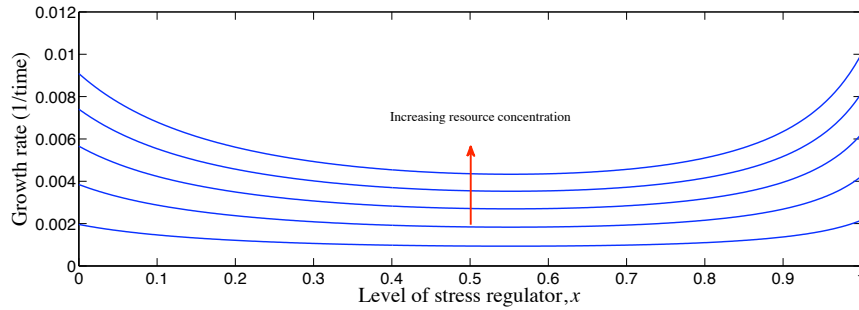


Figure 3.14: The fitness landscape shaped by the convex SPANC trade-off functions in Figure 3.10.

Convex SPANC trade-off curves translate into a fitness landscape that possesses two fitness maxima; one *fit* global maximum situated at the maximal level of stress regulator ($x = 1$) and one *flat* sub-optimal local maximum situated at the zero level of stress regulator ($x = 0$). We refer to the sub-optimal fitness peak as flat because it is more mutationally robust than the fit peak. Mutational robustness means that while a

mutant offspring type will suffer a reduction in fitness relative to its parent at the local peak, the smaller this reduction the more robust the peak is to mutation [8]. Hence at low mutation rates the fittest types (around $x = 1$) dominate while at high mutation rates the flat types (around $x = 0$) dominate, but in a region of intermediate mutation rates, the fit and the flat types are co-maintained (Figure 3.10). In this region, the mechanism of coexistence can be configured as a form of negative frequency dependence at the level of the phenotypic clouds [8]. Here we demonstrate the operation of negative frequency-dependent selection on the phenotypic clouds by simulating the system (3.13) subject to the convex SPANC trade-offs in Figure 3.10 to steady state in MATLAB and then performing an exchange of cells between the fit cloud and the flat cloud to suppress and increase the density of types within the fit cloud in turn. The resulting change in frequency of fit types as the system returns to its equilibrium state, whether increasing (a relative change in frequency above one) or decreasing (a relative change in frequency below one), was tracked by integrating over all types within the fit cloud by means of the trapezoidal rule and plotted against the initial perturbed frequency. As shown in Figure 3.15, any manipulation of the phenotypic frequencies away from the diversity state observed in Figure 3.10f will converge back to that state; types below their equilibrium value increase in frequency while those above decrease in frequency so that the fit and flat types are returned to *intermediate* frequencies when rare or common.

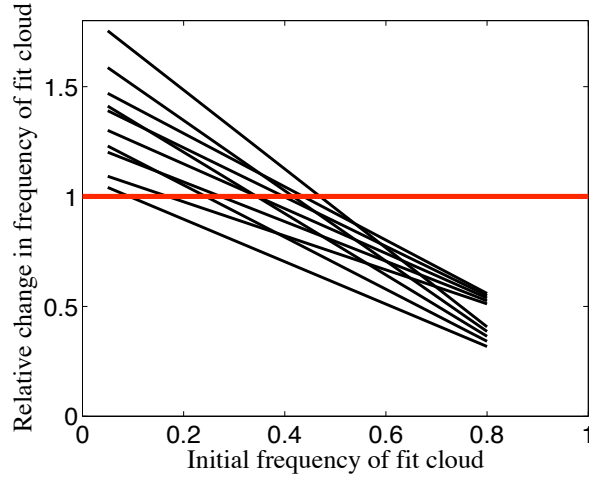


Figure 3.15: Coexistence is maintained by negative frequency-dependent selection at the level of the phenotypic clouds. Here ten tests of negative frequency dependence were conducted for ten intermediate mutation rates which allow for coexistence in the system (3.13) subject to the convex SPANC trade-off functions in Figure 3.10.

So how does the system return to its equilibrium state upon perturbation? This dynamic is mediated through resource shifts in their common environment as illustrated in Figure 3.16. If we increase the frequency of the fit cloud, that is those types with high levels of stress regulator but slow nutrient uptake, the resource concentration in the chemostat increases from its previous equilibrium value. However, at such higher resource concentrations the flat types with low levels of stress protection but high nutrient uptake are favoured and subsequently grow to decrease and eventually reset the resource level back to its equilibrium value. Similarly, increasing the frequency of the flat types momentarily decreases the resource concentration from its equilibrium value before it is returned through growth of the fit types [8]. Note that this principle of maintenance of the fittest and the flattest [8] that is mediated by the abiotic environment can be extended to explain the maintenance of any number of phenotypic clouds provided that SPANC balance trade-offs translate into a peaked fitness landscape where the local maxima differ in fitness and flatness.

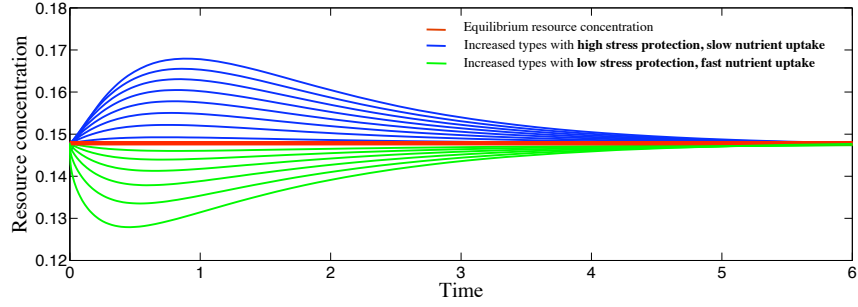


Figure 3.16: Resource dynamics in the chemostat after altering the steady state frequencies of types within the phenotypic clouds formed by convex SPANC trade-off functions. Perturbation of the equilibrium state sets the conditions for the system to return to equilibrium.

3.5 Adaptive Dynamics vs. Reaction-Diffusion Models: A Comparison

We now make a brief note on the differences between the two approaches we have adopted in this chapter to study the evolution of microbial diversity driven by the SPANC balance trade-off. Although both approaches agree that different shapes of the SPANC trade-off functions yield different evolutionary outcomes in the chemostat, we find that two convex SPANC trade-offs can support a polymorphic population in the long term under the reaction-diffusion approach (Figure 3.10) whilst a monomorphic population emerges under the adaptive dynamics framework (Figure 3.5). So why do we arrive at different evolutionary outcomes under the two frameworks? As noted in §3.3 the evolutionary process under the adaptive dynamics approach is entirely driven by frequency-dependent ecological interactions [22]. We have already established in §3.4.3 that the diversity we observe in (3.13) is not owing to classical frequency-dependent selection but through a form of negative frequency dependence at the level of the co-existing phenotypic clouds, a property that requires mutational connectivity between

individuals within a phenotypic cloud. The adaptive dynamics approach does not consider clusters of phenotypes but assumes that the number of coexisting phenotypes is finite and hence the mechanism by which the SPANC trade-offs allow coexistence in our reaction-diffusion model cannot operate under the rules of adaptive dynamics. We would like to point out here that measuring diversity by investigating the number of coexisting phenotypic clouds in the equilibrium solution of a reaction-diffusion system is consistent with the results of Maharjan *et al.* [69] where the descendants of a single ancestor radiated into phenotypic clusters with variations in metabolic strategies and global regulation after 26 days in a chemostat. Hence we argue that the use of reaction-diffusion processes to study the evolution of microbial diversity through the coexistence of multiple phenotypic clouds represents a realistic modeling approach capable of elucidating population level effects of physiological trade-offs.

Note that for concave SPANC trade-offs the single evolutionary stable strategy, x^* , derived from the adaptive dynamics of (3.3) (Figure 3.3) is in agreement with the evolutionary outcome of the reaction-diffusion model in (3.13) which supports a single phenotypic cloud around a partially expressed stressed regulator in the long term for concave SPANC trade-off functions (Figure 3.9d). This is due to the fact that concave SPANC trade-offs translate into a fitness landscape that contains only a *single* peak and hence natural selection will always favour mutant types near or at the fitness peak. Actually, it can be shown that the phenotypic location of the evolutionary stable strategy in $[0, 1]$ derived from the adaptive dynamics of (3.3) agrees with the location of the single phenotypic cloud in the equilibrium population of (3.13) for concave SPANC trade-off curves. We refer to [40] for a full treatment of this matter.

For convex SPANC trade-offs, although the adaptive dynamics approach cannot pre-

dict the emergence of a polymorphic population in the long term, we note that there is a similarity between the adaptive dynamics and the reaction-diffusion approach in that evolutionary outcome depends on a condition specific to each modeling framework: Under the adaptive dynamics approach, if the initial population resides to the left of the singular strategy, x^* , evolution yields a monomorphic population at $x = 0$ whilst if the evolutionary process starts with an initial population that resides to the right of the singular strategy we end up with a monomorphic population at $x = 1$ (Figure 3.5). Under the reaction-diffusion approach, for a given set of model parameters and any initial population structure, it is the mutation rate that determines evolutionary outcome: For low mutation rates the steady state population distribution supports a single phenotypic cloud around $x = 1$, for high mutation rates the equilibrium population supports a single phenotypic cloud located at $x = 0$ and for a narrow region of mutation rates the steady state population is polymorphic containing one phenotypic cloud around $x = 0$ and one phenotypic cloud around $x = 1$ (Figure 3.10). Hence, given that the window of mutation rates for which the reaction-diffusion model yields a diverse equilibrium population structure is narrow (Figure 3.10d), for *most* mutation rates the two modeling approaches will yield the same evolutionary outcome; a monomorphic population. However, because we are interested in the theoretical minimal set of conditions under which microbial evolution constrained by the SPANC trade-off can yield diversity in order to provide an explanation for the experimental observation of microbial diversity in the chemostat, we will use reaction-diffusion equations to study microbial evolution constrained by the SPANC balance in the remainder of this part. Finally, we emphasize our finding that the physiological SPANC balance trade-off is indeed sufficient to generate and maintain diversity in the chemostat.

Chapter 4

Molecular and Evolutionary Bases of Within-Patient Diversity in *E. coli* Infections

In this chapter we investigate microbial evolution constrained by the SPANC balance as a mechanism of diverse antibiotic stress resistance within *Escherichia coli* bacterial infections. This chapter is a summary of the collaborative study "Molecular and Evolutionary Bases of Within-Patient Genotypic and Phenotypic Diversity in *Escherichia coli* Extraintestinal Infections" by Levert *et al.* [64] published in PLoS Pathogens.

4.1 The SPANC balance in *E. coli*: The *rpoS* Response

We start by describing the regulatory network that is the source of the SPANC balance in *E. coli* bacteria. At the molecular level, stress resistance in *E. coli* involves expression of several hundred genes belonging to the general stress response which are co-regulated

by the *rpoS* gene [31]. In any given environment the selection pressure on the *rpoS* gene is due to competition between the sigma factors RpoS (σ^S) and RpoD (σ^D) for a limiting number of RNA polymerase core subunits [28]. RpoS switches the cell to stress resistance whereas RpoD is needed for vegetative growth; an imbalance between the two reduces the fitness of the bacteria in a given environment [31]. Thus there is an inverse relationship between vegetative growth and stress resistance leading to the SPANC balance in *E. coli* [55]. A graphic illustration of the SPANC balance in *E. coli* is given in Figure 4.1.

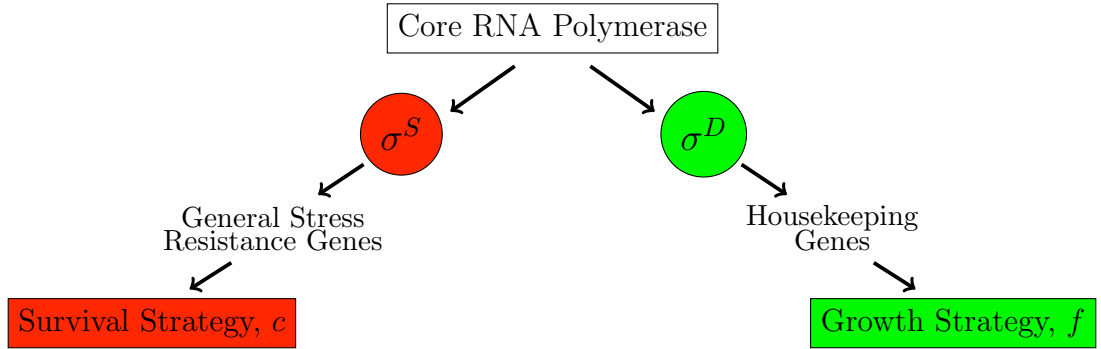


Figure 4.1: Molecular basis of the SPANC balance trade-off in *E. coli* bacteria.

4.2 A Mechanism of Within-Patient Phenotypic Diversity

The *E. coli* bacterium has the interesting characteristic of being both a widespread gut commensal of vertebrates and a versatile pathogen, thought to kill more than 2 million humans per year through both intrainestinal and extraintestinal infections [92]. It is classically assumed that such infections are caused by identical isolates originating from single clones and hence antibiotic testing is mainly based on the characterisation of a single colony from the pathogenic specimen isolation [64]. But how accurate is this

assumption? We carried out a study to investigate the level of genomic and phenotypic polymorphism among numerous *E. coli* isolates from single patients in a series of deep and severe extraintestinal infections. It was found that 8 out of 19 patients were infected by genotypically and phenotypically diverse bacteria associated with distinct levels of RpoS [64]. In a human host it is essential for microbes to manage stress and nutritional cues efficiently, so can the SPANC balance provide an explanation for the observed diversity in *E. coli* clonal isolates from a single patient? To test this hypothesis we consider the reaction-diffusion model in (3.13) which allows us to examine the role of SPANC balancing in the creation and maintenance of diversity. Assuming that *E. coli* isolates differ in their RpoS expression, x , normalized to $0 \leq x \leq 1$ so that a mutant with zero level RpoS has $x = 0$ and a mutant with maximal level of RpoS has $x = 1$, we numerically solve the system of equations (3.13) subject to an initially isogenic population at $x = 1$ and examine the equilibrium population structure for different shapes of the SPANC trade-off functions $c(x)$ and $f(x)$.

4.3 Results

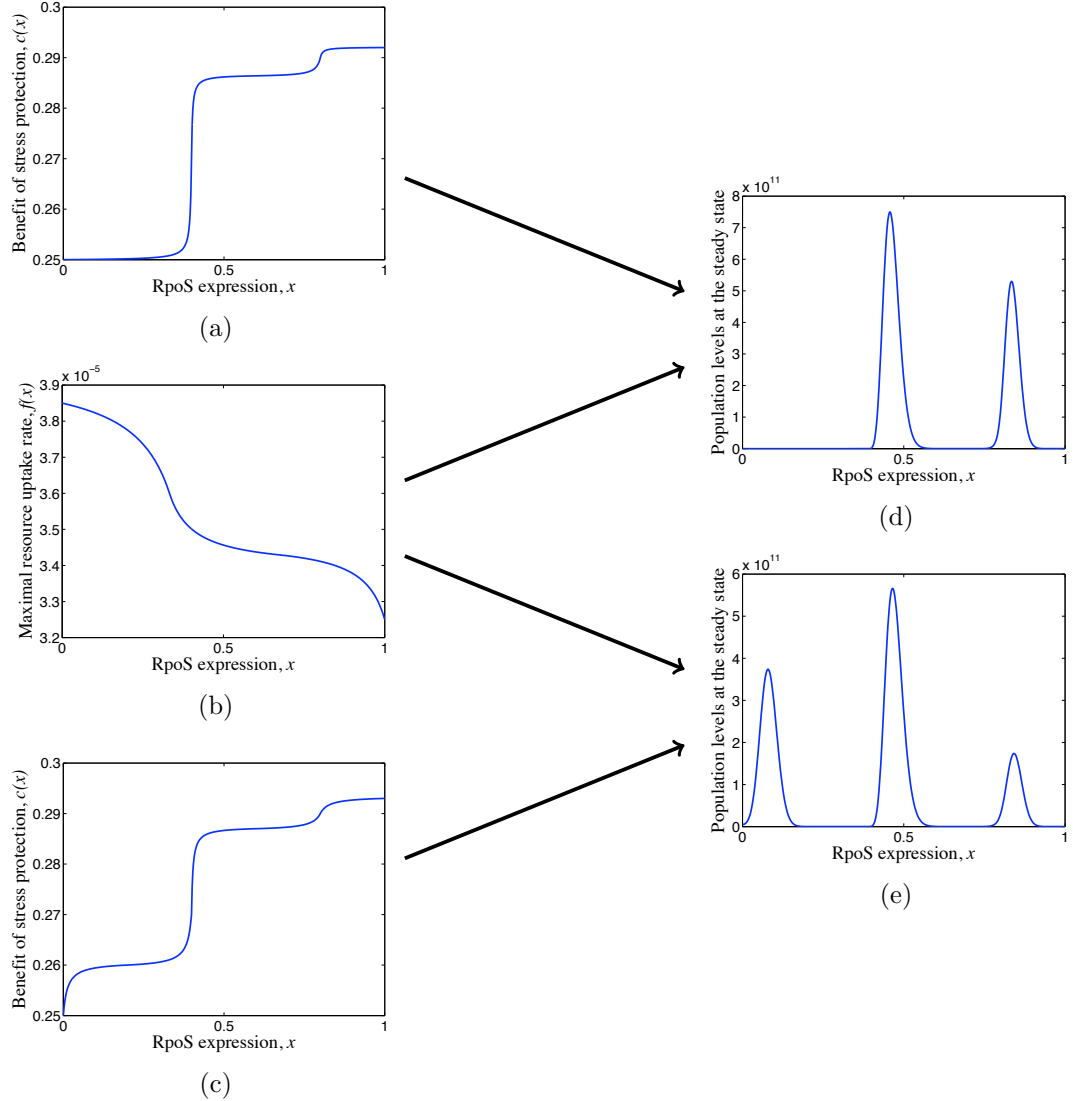


Figure 4.2: A long term population structure supporting two genotypes with distinct levels of RpoS (d) requires the benefit of stress protection and maximal rate of resource uptake to have the form illustrated in (a) and (b) respectively, while a long term population structure supporting three genotypes with distinct levels of RpoS (e) requires the benefit of stress protection and maximal rate of resource uptake to have the form illustrated in (b) and (c). Computer simulations of the model (3.13) were performed using MATLAB with the following parameters: $\epsilon = 0.325 \times 10^{-8}$, $S_0 = 1.1 \times 10^9$ picomoles, $K = 4 \times 10^6$ picomoles, $r = 237$ cells/picomole and $D = 0.0017 \text{ min}^{-1}$. The maximal uptake rate $f(x)$ is measured in picomoles/cell/min.

For an initially isogenic population with maximal RpoS expression, the steady state population of (3.13) can support two or more partial RpoS mutants at intermediate stress levels depending on the shapes of the SPANC trade-off curves $f(x)$ and $c(x)$ (Figure 4.2). For example, if mutations that further decrease the levels of RpoS in a type that already has low levels of RpoS, do not lead to a marked decrease in stress protection (Figure 4.2a) the long-term population structure supports two phenotypes with distinct RpoS levels (Figure 4.2d). On the other hand, if mutations that further decrease the levels of RpoS in a type that already has low levels of RpoS lead to a clear decrease in stress protection (Figure 4.2c) the long-term population structure supports three phenotypes with distinct levels of RpoS (Figure 4.2e). Note that the coexistence of multiple phenotypes in our model requires the assumption that changing RpoS levels from 1 to 0 induces distinct levels of stress protection. So how realistic are these shapes? Actually, stress responses to most environmental stresses involve not just RpoS but also stress specific responses whose expression is directly or indirectly affected by the RpoS level. Hence the non-linear forms of the SPANC balance trade-offs in Figure 4.2 are not unrealistic. Finally, we emphasize that our simple model in (3.13) demonstrates that the SPANC balance is indeed sufficient to create diversity in RpoS expression within an *E.coli* population, and does not require any specific effect of the immune system.

Chapter 5

Trade-Off Shapes Determine Evolutionary Trajectories in Laboratory Microcosms

In this chapter we estimate the shapes of the SPANC balance trade-offs in *E. coli* bacteria in order to investigate whether trade-off shapes have an impact on the trajectories of evolution. This chapter is a summary of the study "Trade-Off Shapes Determine Evolutionary Trajectories in Laboratory Microcosms" by S. Nilsson, T. Ferenci, K. Haynes and I. Gudelj (in preparation for submission). All experimental data presented in this chapter was generated by the Ferenci lab at the University of Sydney.

5.1 The SPANC Trade-Off Shapes in *E. coli*

It is well accepted that trade-off shapes determine the outcome of evolution. However, the experimental evidence to support this theory is so far lacking. This could be due to the fact that estimating the shape of a trade-off is a difficult task. The shape of a trade-off is determined at the sub-cellular level which directly influences the success of

individuals at the population level. This is potentially problematic since, at present, the mapping from sub-organismal to population levels remains elusive for many trade-offs. In *E. coli* bacteria it is well known that the sigma factor RpoS regulates resource allocation between stress protective and nutritional cellular functions in response to environmental cues leading to the SPANC trade-off [30] (see §4.1 for further details). Hence the RpoS-SPANC system presents an ideal opportunity to study the effect of trade-off shape on evolutionary outcome. Moreover, King *et al.* [55] have shown that the accumulation of phenotypes with distinct levels of RpoS in glucose-limited chemostat cultures of *E. coli* is affected by the nature of the environmental stress applied to the population. For example, if the environmental stress comes in the form of salt the experimental data shows replacement of the phenotypes with maximal RpoS expression by mutants with a partial phenotype, while in an acid environment mutants with zero RpoS expression were found to accumulate over time. Hence we propose that different secondary stresses impose different shapes on the SPANC trade-offs which, in turn, lead to different kinetics of mutational sweeps.

5.2 Estimation of SPANC Trade-Off Shapes

In order to test the theoretical hypothesis that the environment determines the form of a trade-off which, in turn, drives evolutionary outcome we now estimate the shapes of the SPANC balance trade-off functions in a glucose-limited *E. coli* chemostat population under two environmental stresses that according to King *et al.* [55] give rise to different evolutionary outcomes, namely osmotic (salt) and acid stress. In order to deduce the SPANC balance function shapes we use experimental data from eleven near-isogenic *E. coli* strains engineered to contain fixed but distinct intracellular concentrations of RpoS (x) thereby providing an artificial control of resource allocation between nutritional

and stress protective functions. The RpoS concentrations within the engineered strains ranging from 0 (null type strain) to 1 (wild type strain) are given in Table 5.1.

Strain	RpoS concentration
BW3709 (Null type)	0
R2	0.0035
R8	0.0370
R3	0.1626
R7	0.2235
R5	0.2245
R6	0.2700
R1	0.3087
R10	0.3836
R4	0.7235
BW2952 (Wild type)	1

Table 5.1: RpoS intracellular concentrations in the engineered *E. coli* strains used to estimate the SPANC balance trade-off functions.

We start by considering the $f(x)$ function which gives a measure of the growth functions activated through the expression of a given RpoS concentration. From experimental measurements of the maximal growth rate on glucose medium for each engineered strain we estimate $f(x)$ by finding the function which best fits the experimental data by means of a least squares procedure. Here the best fit $f(x)$ function was found by exploring different shapes of the function and choosing the form which minimizes the residual sum of squares (RSS) given by

$$RSS = \sum_{i=1}^n [f_i - \hat{f}_i]^2,$$

where n is the number of engineered strains, f_i is the estimated function and $\hat{f}_i$ are the experimental data points. The best fit $f(x)$ function relating RpoS concentration to vegetative growth in a glucose environment is given in Figure 5.1.

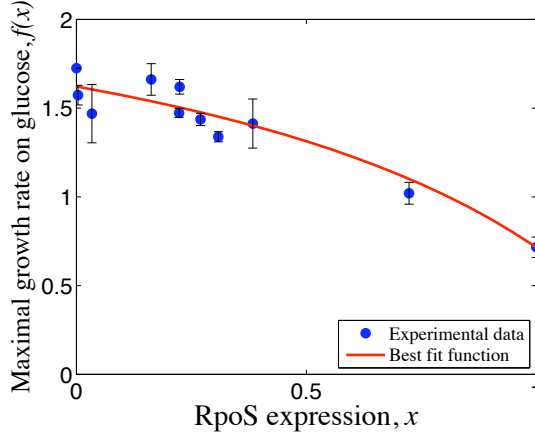


Figure 5.1: The SPANC balance $f(x)$ function for growth on glucose. Maximal growth rate is measured in units per hour. Experimental data is average of 3 replicates.

We now proceed to estimate the $c(x)$ function of the SPANC balance under osmotic and acid stress. Here we use the survival times of the engineered strains to construct the $c(x)$ function under a given stress. The survival time can be determined by calculating the area under time-survival curves up to a given time point and represents a direct measure of an organism's ability to survive the antibacterial effects imposed by the environment [33]. Hence we take the survival time to represent a measure of the protective effects of expressing a given concentration of RpoS under a particular set of stressful conditions. Here we estimate the $c(x)$ function from experimental killing curves as follows: We assume logistic growth of the initial inoculum according to the model

$$\frac{dN}{dt} = R \times N \left(1 - \frac{N}{K}\right), \quad (5.1)$$

where R is the growth rate of the population, $K = 1$ is the carrying capacity of the population and N is the percentage of viable cells where $0 \leq N \leq K$. Note that because the model in (5.1) tracks percentage viability of cells over time, when populations are exposed to a given stress the percentage of cells decrease with time and hence $R < 0$. To find the model which best describes survival over time for each engineered strain under

each stress we fit the model (5.1) to experimental data by means of a least squares procedure. Here we systematically vary the rate parameter in MATLAB to find the value of R which minimizes the residual sum of squares (RSS) between the numerical solution of (5.1) and the experimental data given by

$$RSS = \sum_{i=1}^n [N_i - \hat{N}_i]^2,$$

where n is the number of data points, N_i is the numerical solution of (5.1) and $\hat{N}_i$ is the experimental data. For brevity and clarity, the experimental killing curves and corresponding best fit models for three of the eleven strains under acid stress (pH 2.5) and osmotic stress (1.5M NaCl) are shown in Figure 5.2.

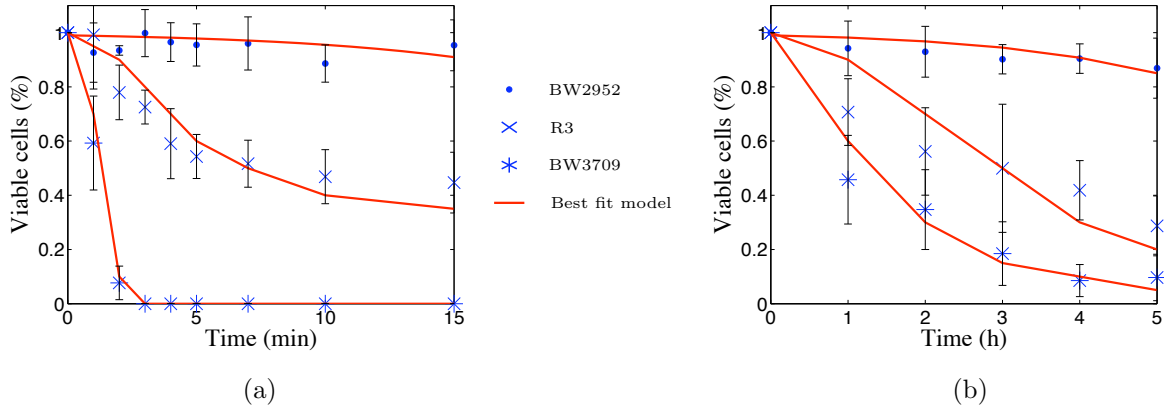


Figure 5.2: Best fit logistic model (5.1) for strains BW2952, R3 and BW3709 under acid stress (pH 2.5) (a) and osmotic stress (1.5M NaCl) (b). Simulations of the model (5.1) varying R with $K = 1$ and initial condition $N(0) = 0.99$ were performed in MATLAB by means of the solver ODE45 to find the best fit value of the rate parameter. Experimental data is average of 3 replicates.

Setting $K = 1$ in (5.1) invokes the assumption that in the long term the entire initial inoculum will eventually die out under the stress. Although this may not be a realistic assumption, particularly for the strains with high levels of RpoS, we argue

that the different survival times for each strain to die out under the dynamics of (5.1) provides a measure of survival in the long term: Under the assumption that the wild type strain with full protective effects has the greatest survival time under any stress, normalizing the survival time to extinction for all other engineered strains with respect to the wild type gives their relative survival abilities in the long term. Hence we extend the simulations of the best fit models in Figure 5.2 until the viable cell count is zero for all strains, calculate the area under each curve using the trapezoidal rule and, finally, normalize to the wild type. Plotting relative survival against RpoS expression $c(x)$ is estimated by exploring different function forms and choosing the shape which minimizes the residual sum of squares error between the function itself and the derived survival data points, analogous to the method used to find the $f(x)$ function. The best fit $c(x)$ functions under acid and osmotic stress are given in Figure 5.3.

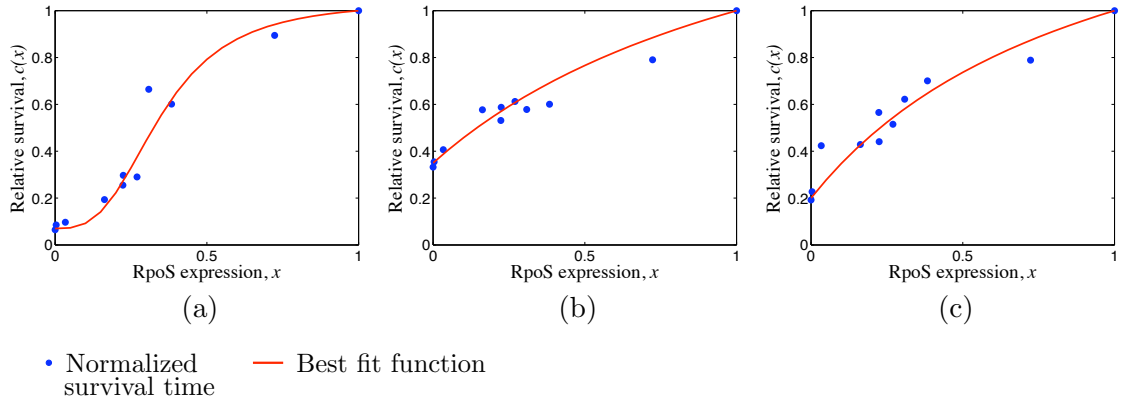


Figure 5.3: The SPANC balance $c(x)$ functions under acid stress at pH 2.5 in (a) and osmotic stress in the form of 1 M NaCl in (b) and 1.5M NaCl in (c).

We can now insert the estimated SPANC trade-off functions into our evolutionary model (3.13) to generate predictions for the mutational sweeps in an *E. coli* population under osmotic and acid stress in a glucose-limited chemostat, and subsequently, validate the model experimentally. In order to perform a chemostat experiment in practice the

environmental setting shaped by a given stress cannot be lethal or extreme enough to affect cell viability [55]. Thus in order for experimental verification of the model predictions to be possible we must consider environmental stresses that are less severe than those displayed in Figure 5.3 which do affect cell survival.

We start by considering a chemostat population exposed to osmotic stress. It is evident from Figures 5.3b-5.3c that the $c(x)$ function maintains the same shape under osmotic stress in the form of 1M NaCl and 1.5M NaCl, however, the range of the function decreases. Based on our approach to find the $c(x)$ function, a decrease in the $c(x)$ function range is an indication that the relative population survival of cells expressing low concentrations of RpoS has increased. Hence Figures 5.3b-5.3c suggest that a given stress manifests itself as a particular form of the $c(x)$ function, the range of which is dictated by the severity of the environmental stress. Based on this argument we now construct a $c(x)$ function which maintains the same form as the other functions representing osmotic stress but spans a narrower range to mimic a milder stress (0.5M NaCl) that can be applied in a chemostat experiment. A comparison of the $c(x)$ functions in decreasing NaCl concentrations is given in Figure 5.4.

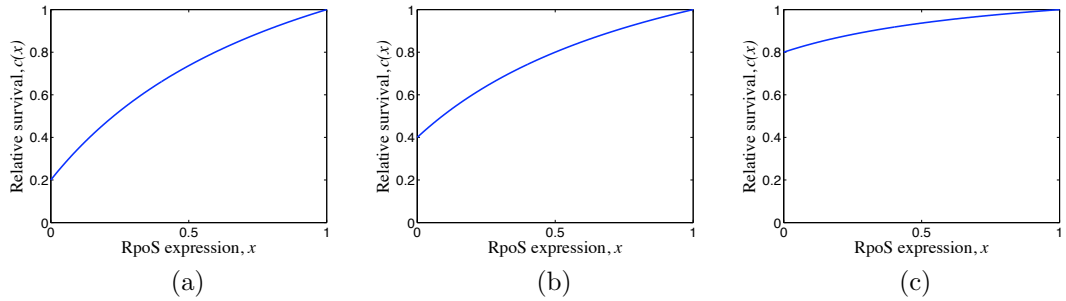


Figure 5.4: SPANC $c(x)$ functions in decreasing NaCl concentrations. The functions representing 1.5M NaCl (a) and 1M NaCl (b) are estimated from experimental data whereas the function in (c) is constructed to maintain the same shape as (a) and (b) with a more narrow range to represent a milder osmotic stress in the form of 0.5M NaCl.

Similarly, for a chemostat population exposed to acid stress we use the assumption that acidic environments induce a sigmoidal shape of the $c(x)$ function (Figure 5.3a) the range of which is controlled by the severity of the stress in order to construct a $c(x)$ function for the environmental stress of pH 6 under which mutational kinetics can be studied in a chemostat experiment. A comparison of the $c(x)$ functions representing relative survival in environments of decreasing acidic stress are given in Figure 5.5.

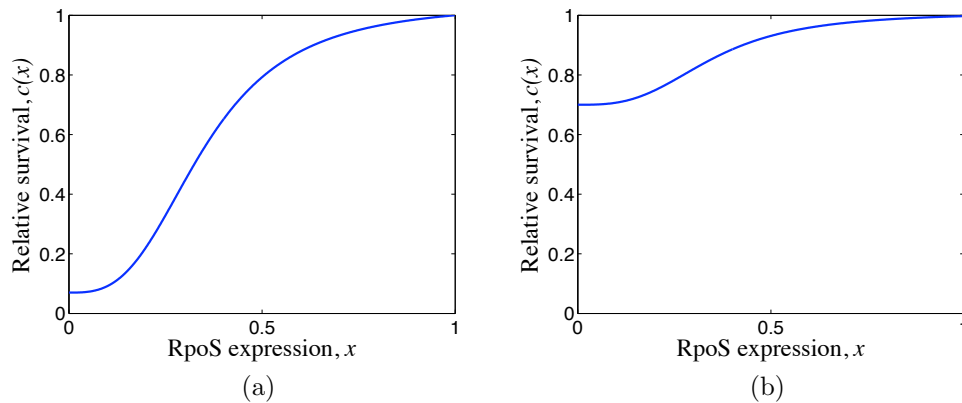


Figure 5.5: SPANC $c(x)$ functions in acidic environments of decreasing severity. The function representing pH 2.5 (a) is estimated from experimental data whereas the function in (b) is constructed to maintain the same shape as (a) with a more narrow range to represent a milder acid stress in the form of pH 6.

5.3 Model Parameterization

In order to predict the evolutionary dynamics of a chemostat *E. coli* population under osmotic and acid stress we must parameterize our evolutionary model in (3.13). Note that because $f(x)$ in Figure 5.1 actually relates RpoS expression to *maximal growth rate* measured in units $1/[\text{time}]$ instead of *maximal uptake rate* measured in $[\text{units of resource}]/[\text{cell}]/[\text{time}]$ as in chapters 3-4, our reaction-diffusion model in (3.13) must be rearranged to allow for this change in units. Hence our evolutionary model is now given

by

$$\begin{aligned}\frac{dS}{dt} &= D(S - S_0) - \int_0^1 \frac{1}{r} \frac{f(x)S}{K + S} N(x, t) dx, \\ \frac{\partial N(x, t)}{\partial t} &= \varepsilon \frac{\partial^2 N(x, t)}{\partial x^2} + \left(\frac{c(x)f(x)S}{K + S} - D \right) N(x, t),\end{aligned}\tag{5.2}$$

subject to no-flux boundary conditions $\left. \frac{\partial N(x)}{\partial x} \right|_{x=0,1} = 0$. Here K is referred to as the half-saturation constant and describes the resource concentration at which the population growth rate is at half-maximum. This is different from K in the formulation (3.13) which describes the affinity for resources in the environment. All other parameters have the same meaning as in previous chapters.

In order to generate experimentally testable predictions of the evolutionary outcome under environmental stress we must choose environmental model parameters that can be applied to the chemostat in the laboratory. Here we set the chemostat dilution rate $D = 0.1$ per hour and the concentration of glucose in the input reservoir $S_0 = 1.1 \times 10^9$ picomoles. Moreover, from [96] we set $K = 4.8 \times 10^5$ picomoles of glucose and from experimental measurements we set $r = 1.8 \times 10^2$ cells per picomole of glucose. The mutation rate (ε) is estimated by fitting the model in (5.2) to experimental data on the acquisition of *rpoS* mutations over 96 hours within a glucose-limited chemostat in the absence of secondary environmental stresses. In an environment defined only by the availability of glucose all available resources are allocated towards vegetative growth through $f(x)$ in Figure 5.1 and hence we set $c(x) = 1$ to represent an identical stress-protective ability across all *E. coli* strains. The unknown parameter ε is then estimated by systematically varying the parameter value to find the mutation rate which minimizes the residual sum of squares error (RSS) given by

$$RSS = \sum_{i=1}^n [N_i - \hat{N}_i]^2,\tag{5.3}$$

where n is the number of experimental data points, N_i is the model accumulation of mutants and $\hat{N}_i$ is the experimental data. Here ϵ was found by varying the parameter value and numerically solving the system (5.2) in MATLAB over 96 hours and stopping the simulation every 24 hours to integrate over the phenotypic clouds present in order to find the parameter value which minimizes the residual sum of squares error in (5.3). The experimental data and the best fit model of *rpoS* mutation accumulation within a glucose-limited chemostat in the absence of secondary stresses is given in Figure 5.6. For clarity, the mutational kinetics of the wild type which declines in number (Figure 5.6a) and the null type which accumulates (Figure 5.6b) in the absence of secondary stresses are displayed separately in Figure 5.6.

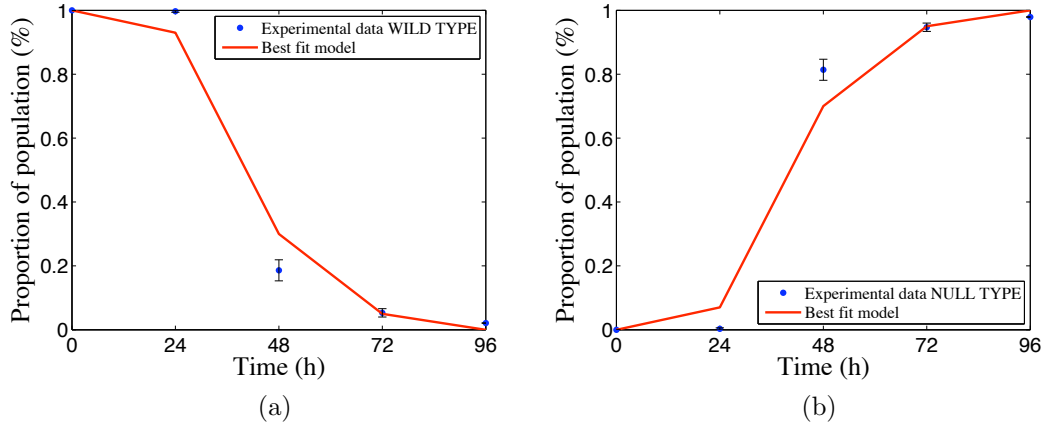


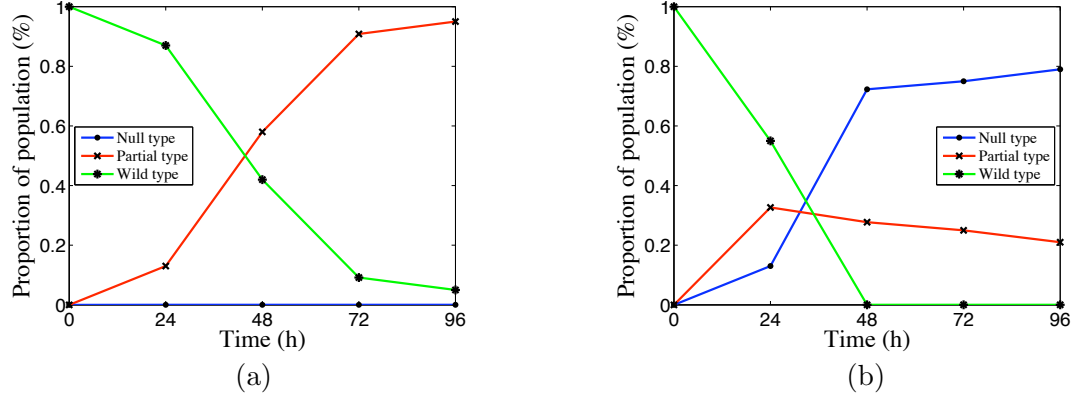
Figure 5.6: Evolutionary trajectories within a glucose-limited chemostat in the absence of secondary environmental stresses. In agreement with the experimental set-up, computer simulations of the model (5.2) were performed using MATLAB with the following parameters: $S_0 = 1.1 \times 10^9$ picomoles, $K = 4.8 \times 10^5$ picomoles, $r = 1.8 \times 10^2$ cells/picomole, $D = 0.1 \text{ h}^{-1}$ and the estimated the parameter $\epsilon = 1.082 \times 10^{-8}$. Experimental data is average of 3 replicates.

5.4 Results

We now generate model predictions for the mutational kinetics of an initially monomorphic population of wild types exposed to osmotic stress (0.5M NaCl) and acid stress (pH 6) in a glucose-limited chemostat. Starting with osmotic stress, our model in (5.2) subject to the estimated SPANC balance trade-off functions $f(x)$ in Figure 5.1 and $c(x)$ in Figure 5.4c predicts that the wild type mutant will rapidly decrease in numbers and by 96 hours the most prevalent type will be a mutant with partial RpoS expression (Figure 5.7a). Contrary to this, under acid stress the dynamics of our model (5.2) subject to the estimated SPANC balance trade-off functions $f(x)$ in Figure 5.1 and $c(x)$ in Figure 5.5b predict that by 96 hours the wild type mutant will no longer be present in the population but the null mutant with zero level RpoS expression will instead dominate the population (Figure 5.7b).

To experimentally test the above predictions, glucose-limited chemostat experiments were conducted where an *E. coli* population of wild types was exposed to osmotic or acid stress over 96 hours. By monitoring the rate of acquisitions of *rpoS* mutations we find that, as the model predicted, different stresses give rise to different evolutionary trajectories. While under the osmotic stress partial *rpoS* mutants dominate the population (Figure 5.7c), under acid stress it is the null *rpoS* mutant that is the most prevalent (Figure 5.7d).

MODEL PREDICTIONS



EXPERIMENTAL TESTS

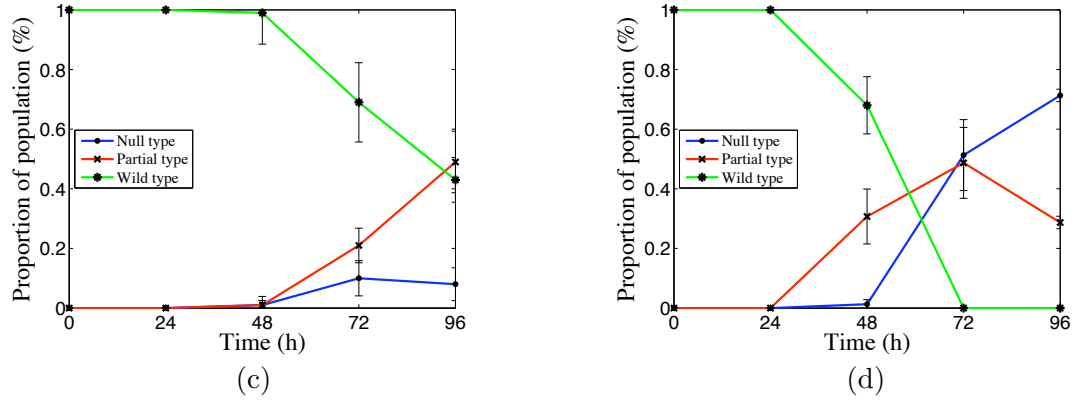


Figure 5.7: Transient dynamics depicting mutational sweeps under osmotic stress in the form of 0.5M NaCl (a) and (c) and under acid stress in an environment of pH 6 (b) and (d). In agreement with the experimental set-up, computer simulations of the model (5.2) were performed using MATLAB with the following parameters: $\epsilon = 1.082 \times 10^{-8}$, $S_0 = 1.1 \times 10^9$ picomoles, $K = 4.8 \times 10^5$ picomoles, $r = 1.8 \times 10^2$ cells/picomole and $D = 0.1 \text{ h}^{-1}$. Experimental data is average of 3 replicates.

Although there is qualitative agreement in the order in which different phenotypes appear between the theoretical predictions and the experimental tests in Figure 5.7, we note that there are discrepancies in the times at which sweeps occur: The model predicts that the wild type starts to decrease in numbers immediately (Figure 5.7a-b) whilst the experimental tests show that the wild type dominates the population for some time

before it declines in numbers (Figure 5.7c-d). A possible explanation for this discrepancy could be due to the fact that our model considers only the general stress response regulated by the gene *rpoS*. Other stress specific responses also exist which enhance bacterial survival but are not included in our model. It is possible that the expression of such stress specific genes plays an important role during the initial adaptation to a given stress [55] and that the general stress response, which we study, is a secondary effect. Hence we argue that the discrepancy in the dynamics of evolutionary trajectories between the theoretical predictions and the experimental tests could be explained by the simplicity of our model which considers only levels of the resource allocator RpoS as the determinant of the SPANC balance in a given environment. However, we emphasize that the evolutionary outcome predicted by the model under osmotic and acid stress are in agreement with the experimental results. These findings provide evidence that the environment plays a key role in shaping the SPANC trade-off functions which, in turn, shape the fitness landscape that drives the evolutionary trajectories within populations.

Chapter 6

Discussion Part I

In this part we have examined the ecological and evolutionary interactions of microbes constrained by the fundamental physiological trade-off between self-protection and nutritional competence (SPANC) imposed on microbes by their inherent regulatory and metabolic capabilities under environmental stress. Through a combination of mathematical modeling, synthetic biology and experimental microbial evolution we have developed a general model that identifies the essential ingredients integral to SPANC balancing which allow for the generation of diversity in microbial evolution and in line with previous theory [41] provides a classification of the possible evolutionary outcomes that depends on the shape of the SPANC balance trade-off.

In chapter 3 we build a simple mathematical model of microbial evolution constrained by the SPANC balance which provides strong indication that the trade-off between self-protection and nutritional competence could be a crucial driver of the creation and maintenance of microbial diversity in simple homogeneous environments containing a single limiting resource. In particular, we find that the long term population diversity will depend on an ecological, an evolutionary and a biochemical component, namely

the dilution rate, the mutation rate from clonal reproduction and the geometry of the SPANC balance trade-off. These theoretical results are an example of the coexistence mechanism termed "maintenance of the fittest and the flattest" [8] which stems from the fact that certain shapes of the SPANC balance trade-off functions translate into a peaked fitness landscape in which the most "fit" types have unfit near-mutational neighbours and the less fit "flat" types are more mutationally robust [8]. As a consequence of negative frequency dependence at the level of the peaks mediated by the abiotic environment and differences in mutational robustness we are able to find a window of mutation rates for which both fit and flat types coexist. Note that although the transition window of mutation rates for which coexistence is observed in our model is narrow (Figure 3.10), a more complex model where multiple trade-offs operate broadens the span of mutation rates through which diversity is maintained [8]. This is not an unrealistic scenario given that the intricacy of regulation of each stress response can give rise to a range of complex relationships because of the involvement of multiple components and multiple stress signals whose expression is directly or indirectly affected by the level of core stress regulator that we consider in our model. It is impossible to consider all stress responses in detail and we emphasize that the model was deliberately kept simple in order to illustrate the minimal set of conditions under which the SPANC trade-off could lead to coexistence of phenotypes with differing levels of stress regulator.

In chapter 4 we use our model to show that the SPANC trade-off supports the observed phenotypic diversity of *E. coli* bacteria within a single patient. These results have strong medical implication in terms of antibiotic therapies. The classical approach of performing antimicrobial susceptibility tests on a few (2 to 5) colonies obtained from the pathologic sample to establish antibiotic treatment may fail to detect the presence of other, more resistant isolates and could lead to therapeutic failure [83]. The high level of

resistance variability within the isolates of a single patient argues for the utilisation of a mix of numerous isolates to perform the antibiogram, or, alternatively for the realisation of simplified antibiograms with essential molecules on numerous isolates in addition to the classical antibiogram on a single isolate [64].

In chapter 5 we provide to our knowledge the first experimental verification of the classical theory which states that the precise form of a trade-off crucially determines the outcome of evolution [62] [63]. Considering the *E. coli* bacterium where the genetic and molecular basis of the SPANC balance trade-off are well understood [31] and using a synthetic biology approach we were able to undertake detailed analyses of the shape of the SPANC trade-off in *E. coli* growing on glucose under different environmental stresses. Our data shows that different environmental stresses influence the shape of the SPANC trade-off. This is agreement with a recent finding that changes to the environment in which the organisms are embedded can alter the shapes of trade-off relationships [54]. In order to experimentally test whether trade-off shapes indeed mold the evolutionary trajectories as the theory would suggest, we select the following two environmental stresses: the osmotic stress which gives rise to a concave relationship between levels of stress regulator and relative survival (Figures 5.3b-5.3c) and the acid stress which gives rise to a sigmoidal relationship between levels of stress regulator and relative survival (Figure 5.3a). In accordance with our theoretical predictions we find that in a glucose-limited environment under osmotic stress a partial loss of stress protection is selected for (Figures 5.7a and 5.7c). Contrary to this, under acid stress it is the mutants with a complete loss of stress protection that dominate the population (Figures 5.7b and 5.7d).

In conclusion, our results in this part have two important messages, firstly for understanding the evolutionary consequences of trade-offs and secondly for understanding the

generation and maintenance of evolutionary diversity. Our results show that an understanding of intracellular constraints and how they manifest themselves at the population level may serve as a solid basis for testable models and analyses of trade-offs. Hence we argue that in order to understand the mechanisms that drive evolution in nature it is imperative to adopt an integrated approach which maps the molecular bases of trade-offs in individual organisms to their ecological and evolutionary consequences at the level of populations.

Part II

Candida Epidemiology from an Ecological Perspective

Chapter 7

Introduction Part II

The human body is host to a vast diversity of microorganisms, our so called *microbiota*. These microbes are key contributors to human health [95], however, alterations to the microbiota composition can also lead to severe disease. Recent advances in molecular genetic sequencing has provided insight into the diversity of genomes that inhabit the human host and is a first step towards determining the relationship between the microbiota and healthy and diseased states [58]. However, the ecological interactions which influence species distribution and abundance within the microbiota over space and time are often overlooked. The human microbiota can be thought of as an ecological landscape which harbours numerous microbial communities structured through processes mediated by the host environment. Hence an ecological view of the interactions which drive the patterns of microbial species diversity within the human host could provide a deeper understanding into the dynamics of the indigenous microbiota and ultimately facilitate the prediction of health and disease in the human body [36] [38].

The human microbiota is distributed across the body into different habitat *niches* which offer distinct conditions of growth. From an ecological perspective, local microbial

community structure within any given niche is driven by species differences in *fitness*, determined by the set of species traits which influence growth rate. Constraints driven by limited energy means that no species can maximize all components of fitness simultaneously and hence each microbial species has evolved a different and fixed survival strategy where available resources are balanced between cellular functions to yield maximal growth rate in the natural habitat. At the population level, the underlying survival strategy emerges as a trade-off where high fitness under one set of environmental conditions induces a reduction in fitness in another ecological niche. From an ecological point of view, it is well known that natural selection eliminates all but the fittest individuals under any set of growth conditions through competitive exclusion [45], and hence any niche can support only one microbial species. Consequently, different microbial species are favoured by selection in different environments. This has important medical implications, for example, during antimicrobial therapy the reduction or elimination of target microbes within an infection niche may create selection pressure for fitter antimicrobial-resistant opportunistic pathogens to invade and cause severe disease [112]. Hence the epidemiology of resistant microbes could be explained through the ecological impact of drug administration [87].

In this part we adopt an ecological perspective of human-associated microbes and what renders them fit for survival within the human host environment in order to shed some light on the emerging epidemiological trends of *Candida* infections. *Candida* species are fungal members of the microbiota in healthy individuals. However, in immunocompromised hosts *Candida* species possess an adaptive ability to transform from harmless commensals to opportunistic pathogens that can cause superficial infections affecting the skin and mucous surfaces [49] as well as life-threatening bloodstream infections known as *candidemia* [111]. Historically, *Candida albicans* is the most commonly encountered

Candida species in medical practice accounting for more than 50 % of infections [81], however, recent years have observed a shift in the distribution of *Candida* species within infections and *Candida glabrata* is now the second or third most isolated species [6] [82]. This is an alarming development as *C. glabrata* infections are often resistant to fluconazole (FLC), the most frequently used antifungal drug, and are consequently associated with a high mortality rate [78]. So why has this epidemiological shift from *C. albicans* to *C. glabrata* taken place? Here we postulate that the selection pressure exerted by the prevalent use of FLC in clinical practice is sufficient to explain the shift from *C. albicans* to *C. glabrata* within an infection niche in an immunocompromised host. Using a combined approach of mathematical modeling and experimental methods we build a simple ecological model of *C. albicans* and *C. glabrata* competing for survival within an environment shaped by the availability of resources and the dose of FLC. Subsequently, we generate predictions for the long term competitive fates of *C. albicans* and *C. glabrata* and, finally, we verify these predictions experimentally. We find that an ecological model approach based on the fitness differences between *C. albicans* and *C. glabrata* in different environments does indeed yield a predictive methodology capable of explaining shifts in *Candida* ecology within an infection site.

This part is a summary of the study "The Epidemiological Shift from *C. albicans* to *C. glabrata*: An Ecological Perspective" by S. Nilsson, E. Cook, A. Smith, B. Oliver, A. Tillmann, A. Brown, K. Haynes, T. White, N. Gow and I. Gudelj (in preparation for submission).

Chapter 8

The Ecological Model

In this chapter we build an ecological model of niche competition within the human host.

We consider an infection environment within an immunocompromised host where the indigenous microbiota consists of the species *C. albicans* and *C. glabrata* only. For simplicity, we assume that the conditions of growth within the infection niche are determined exclusively by the concentration of a single limiting resource and the quantity of FLC present. Hence the survival fitness of each *Candida* species under any given set of environmental conditions is determined only by its abilities to compete for available resources and to resist the antifungal stress exerted by FLC. Although *C. albicans* and *C. glabrata* belong to the same genus, molecular phylogenetic analysis has revealed significant genetic differences between the two species [46] indicative of the fact that each *Candida* species has evolved a different survival strategy where available cellular resources are balanced between the two components which determine fitness in our model. Here we are interested in how these differential lifestyles perform under natural selection within any given infection niche in the long term and whether a model built on such simple assumptions is sufficient to explain the epidemiological shift from *C.*

albicans to *C. glabrata* in reality. Hence we adopt an approach where the theoretical ecological model formulated generates predictions that are experimentally testable in laboratory populations. Here we choose to study natural selection within the infection environment in a serial transfer regime [4]. Serial transfers is a laboratory version of a seasonal mode of competition where an inoculum of competitor cells is periodically provided with specified concentrations of the single limiting resource and FLC which define the niche [101]. Over time, the fittest competitor species will amplify in the inoculum by natural selection, and eventually, selective forces will establish the equilibrium population ecology that we seek within a given niche. A schematic of the serial transfer method that we use to study the competition between *C. albicans* and *C. glabrata* in any given environment is given in Figure 8.1.

TRANSFER OF N_0 CELLS

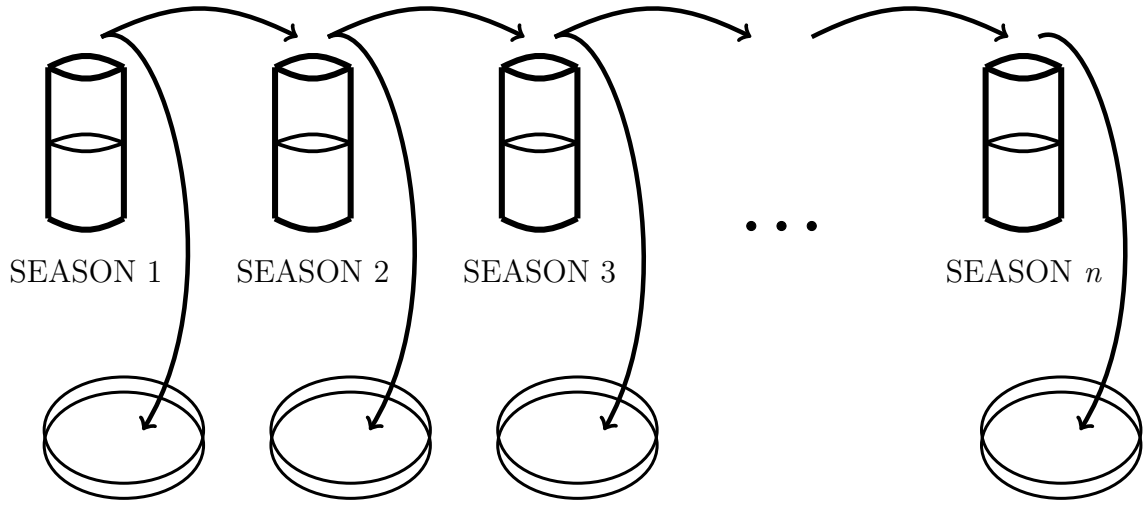


Figure 8.1: The serial transfer method in the laboratory. A initial inoculum of size N_0 cells with specified frequencies of *C. albicans* and *C. glabrata* organisms is placed in a reaction vessel with defined niche conditions. The vessel is left undisturbed until resources have been exhausted which denotes the end of the season, after which a fraction of size N_0 cells is transferred into an identical reaction vessel with the same niche conditions. Simultaneously, a fraction of cells is plated and the end-of-season frequencies of *C. albicans* and *C. glabrata* determined. Growth cycles and transfers are then repeated n times until the end-of-season frequencies remain constant between seasons indicating that the niche equilibrium population ecology has been reached.

8.1 The Mathematical Model

We now develop a mathematical formulation of the seasonal competition between *C. albicans* and *C. glabrata* in an environment defined by the concentration of a single limiting resource, S , and the concentration of FLC present. Glucose is the primary sugar found in the human host [109] and hence from here on we let glucose represent the single limiting resource.

8.1.1 Cellular Growth and Resource Consumption

We assume that cellular growth is limited by the energetic resources available and proportional to ATP production [5] according to a proportionality constant, G . Here we model resource catabolism as a two-reaction process corresponding to glycolysis and the tricarboxylic acid (TCA) cycle: First, resources are taken from the environment and are partially oxidized to form an intracellular metabolic intermediate, X_{int} , and n_1 molecules of ATP. Next, the intracellular intermediate is either completely oxidized to form carbon dioxide (CO_2) and n_2 molecules of ATP, or it passively diffuses out of the cell at rate, D , as an extracellular intermediate, X_{ext} [68] (see Figure 8.2 for an illustration of the two-reaction process considered). Under the assumption that both catabolic reactions display saturating enzyme kinetics, the rates of ATP production during glycolysis, ν_1 , and the TCA cycle, ν_2 , are given by

$$\nu_1(S) = \frac{V_1 S}{K_1 + S}, \quad \nu_2(X_{int}) = \frac{V_2 X_{int}}{K_2 + X_{int}}, \quad (8.1)$$

where V_1 is the maximal uptake rate of resources in the glycolysis pathway and K_1 is the Michaelis-Menten constant. V_2 is the maximal rate of CO_2 production in the TCA cycle pathway and K_2 its half-saturation constant.

Given that the exchange of intracellular and extracellular metabolic intermediates across the cellular boundary occurs by passive diffusion in our model, the rate at which an intracellular intermediate is converted to an extracellular intermediate is given by $D(X_{int} - X_{ext})$.

8.1.2 FLC Transport and Growth Inhibition

FLC combats *Candida* infections by inhibiting the biosynthesis of ergosterol (the major sterol in the fungal plasma membrane) leading to arrest of cell growth [113]. The first step in the FLC mode of antifungal action is entering the fungal cell. Mansfield *et al.* [70] recently reported that *C. albicans* cells import FLC by facilitated diffusion. Hence we assume that FLC import is a saturating function of the extracellular FLC concentration, FLC_{ext} , of the form

$$\text{IMPORT}(FLC_{ext}) = \frac{V_u[FLC_{ext}]}{K_u + [FLC_{ext}]}, \quad (8.2)$$

where V_u is the maximal uptake rate of extracellular FLC in the FLC transporter pathway and K_u its half-saturation constant. In the same study, Mansfield *et al.* [70] found that the intracellular FLC accumulation of *C. albicans* saturates over 24 hours, suggesting that the saturating import function is balanced by a saturating export function. Assuming that the rate of export is dependent on the intracellular accumulation of FLC [104], FLC_{int} , the export function takes the following form

$$\text{EXPORT}(FLC_{int}) = \frac{V_e[FLC_{int}]}{K_e + [FLC_{int}]}, \quad (8.3)$$

where V_e is the maximal rate of efflux in the efflux pathway and K_e its half-saturation constant.

Despite the wide use of FLC in the clinic for decades, the cellular basis for its antifungal mechanism remains poorly understood [115]. Here we assume that FLC growth suppression is the cumulative sum of the yet unknown processes which interfere with cellular metabolism to decrease growth rate. Moreover, we assume that the growth inhibitory effects of FLC depends solely on the intracellular accumulation of the drug [71]. So if FLC_{int} is the concentration of FLC within the fungal cell, we take the reduction

in growth rate, $f(FLC_{int})$, to be a number between 0 and 1 of the form

$$f(FLC_{int}) = 1 - \frac{a[FLC_{int}]^n}{b^n + [FLC_{int}]^n}, \quad (8.4)$$

where a , b and n relate to the FLC resistance properties of the fungal cell.

A schematic of the pathways we consider in our ecological model is given in Figure 8.2.

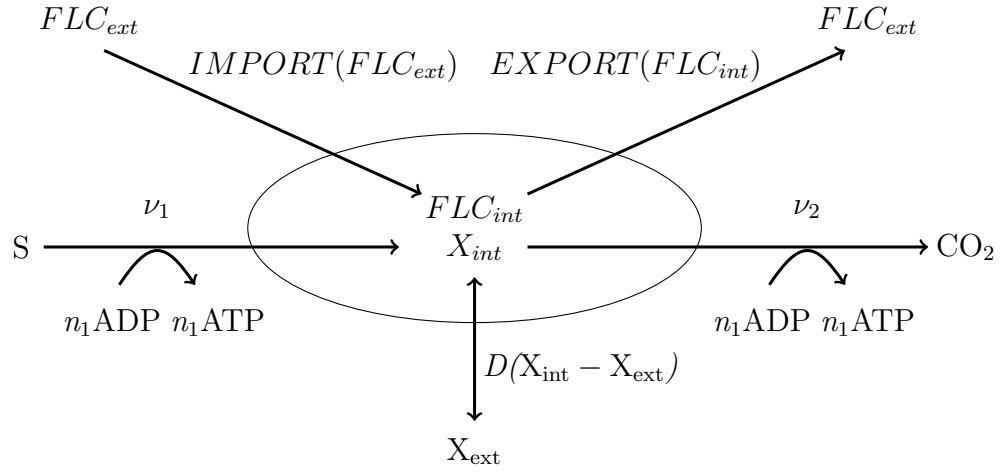


Figure 8.2: Schematic of the metabolic pathways and FLC transport considered in our ecological model.

8.1.3 The Competition Model

We now bring together the components which determine fitness in order to develop a model where the *Candida* species compete for survival under a given set of environmental conditions. Using subscripts a and g to denote parameters of *C. albicans* and *C. glabrata* respectively, the competitive dynamics within any given niche over one season of the serial transfer method are given by

$$\begin{aligned}
\frac{dS}{dt} &= -\nu_{1,a}(S)C_a - \nu_{1,g}(S)C_g, \\
\frac{dX_{ext}}{dt} &= D(X_{int,a} - X_{ext})C_a + D(X_{int,g} - X_{ext})C_g, \\
\frac{dC_a}{dt} &= G_a[\nu_{1,a}(S)n_1 + \nu_{2,a}(X_{int,a})n_2]C_a \times f(F_{int,a}), \\
\frac{dC_g}{dt} &= G_g[\nu_{1,g}(S)n_1 + \nu_{2,g}(X_{int,g})n_2]C_g \times f(F_{int,g}), \\
\frac{dX_{int,a}}{dt} &= [-D(X_{int,a} - X_{ext}) + \nu_{1,a}(S) - \nu_{2,a}(X_{int,a})]C_a, \\
\frac{dX_{int,g}}{dt} &= [-D(X_{int,g} - X_{ext}) + \nu_{1,g}(S) - \nu_{2,g}(X_{int,g})]C_g, \\
\frac{dFLC_{int,a}}{dt} &= IMPORT(FLC_{ext}) - EXPORT(FLC_{int,a}), \\
\frac{dFLC_{int,g}}{dt} &= IMPORT(FLC_{ext}) - EXPORT(FLC_{int,g}), \\
\frac{dFLC_{ext}}{dt} &= [EXPORT(FLC_{int,a}) - IMPORT(FLC_{ext})]C_a \\
&\quad + [EXPORT(FLC_{int,g}) - IMPORT(FLC_{ext})]C_g,
\end{aligned} \tag{8.5}$$

where C_a and C_g denotes the cell densities of *C. albicans* and *C. glabrata* respectively.

8.2 Model Parameterization

The purpose of our ecological model is to predict the effects of the infection environment on *Candida* ecology and verify the predictions using the serial transfer method. Hence it is important to obtain accurate parameter estimates of the processes which govern the competitive population dynamics over consecutive seasons. Here we adopt a procedure where the parameters which determine fitness in our model are first estimated for each *Candida* species in isolation and subsequently inserted into the competitive system (8.5) to generate testable model predictions which are then verified experimentally. A graphic illustration of the procedure we adopt to study the ecological interactions between *C. albicans* and *C. glabrata* is given in Figure 8.3.

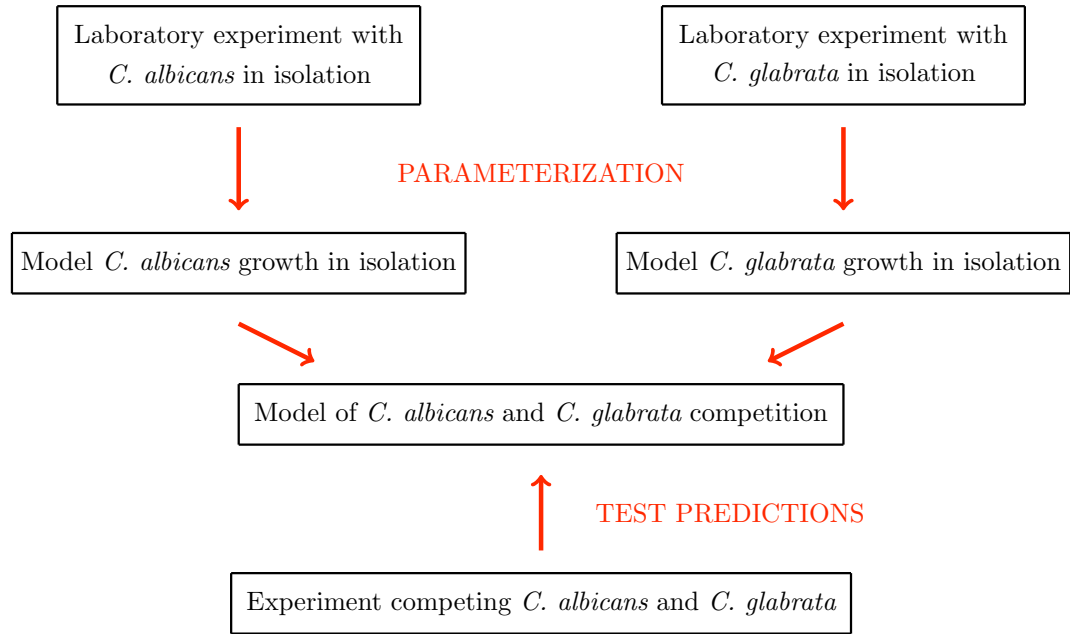


Figure 8.3: Illustration of the procedure we adopt to study the ecological interactions between *C. albicans* and *C. glabrata*.

Here the parameters which describe cellular growth, FLC import and export, and FLC growth inhibition were estimated by means of a parameterization method which fits the dynamical system (8.5) over one season to appropriate sets of experimental data. For brevity, we describe in detail only the fitting procedure performed to find the parameters which describe cellular growth in the absence of FLC for *C. albicans*. Note that all experimental data displayed in this section were generated by myself in Ken Haynes laboratory at the University of Exeter, with the exception of the experimental data of intracellular FLC accumulation (Figure 8.5) which was generated by Ted White at the University of Missouri.

In a drug-free environment, the population dynamics of *C. albicans* cells at density

C_a consuming a single limiting resource of concentration S over one season are given by

$$\begin{aligned}
\frac{dS}{dt} &= -\nu_{1,a}(S)C_a, \\
\frac{dX_{ext}}{dt} &= D(X_{int,a} - X_{ext})C_a, \\
\frac{dC_a}{dt} &= G_a[\nu_{1,a}(S)n_1 + \nu_{2,a}(X_{int,a})n_2]C_a \times f(F_{int,a}), \\
\frac{dX_{int,a}}{dt} &= [-D(X_{int,a} - X_{ext}) + \nu_{1,a}(S) - \nu_{2,a}(X_{int,a})]C_a.
\end{aligned} \tag{8.6}$$

From biochemical data on cellular respiration we set $n_1 = 2$ and $n_2 = 30$ [18] and from [68] we set $D = 10^{-12}$. The unknown parameters G_a , $V_{1,a}$, $K_{1,a}$, $V_{2,a}$ and $K_{2,a}$ can be estimated by fitting the numerical solution of *C. albicans* population growth over time, $C_a(t)$, from (8.6) to an experimental growth curve using a least squares criterion: For any set of parameters $(G_a, V_{1,a}, K_{1,a}, V_{2,a}, K_{2,a})$ we can compute the residual sum of squares (RSS) between the numerical solution and the experimental data given by

$$RSS = \sum_{i=1}^N [C_{a,i} - \hat{C}_{a,i}]^2,$$

where N is the number of data points, $C_{a,i}$ is the numerical solution of system (8.6) and $\hat{C}_{a,i}$ is the experimental data. Hence by systematically varying the combination of unknown parameters we can find the *best fit* parameter set which minimizes the residual sum of squares and therefore best describes the dynamics of *C. albicans* population growth over one season. Here the optimal parameter set was found by using the *lsqnonlin* routine from the optimization toolbox in MATLAB. Providing an initial guess for the unknown parameters, the differential equations (8.6) were integrated until resources had been exhausted using the solver ODE45. The numerical solution was then fed to the optimizer routine which performs an iterative least squares nonlinear optimization to find the set of parameters which minimizes the residual sum of squares error between the

experimental data set and the model simulation. The experimental procedure conducted to generate the *C. albicans* growth curves utilized in the parameterization of *C. albicans* population growth in the absence of FLC is given in §12.2 and the best fit model is given in Figure 8.4a and Table 8.1. All other parameters which describe niche fitness in our model were found by analogous parameterization methods, the experimental methods carried out to generate required data are described in §12.2 and §12.7 and the best fit models are given in Figures 8.4b-8.6b and Tables 8.1-8.3.

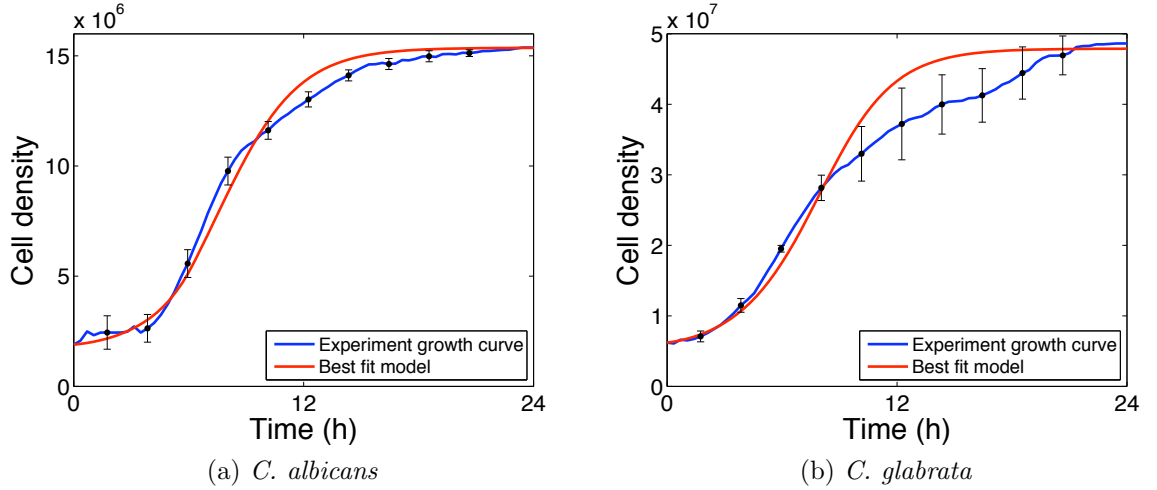


Figure 8.4: Cellular growth and resource consumption in the absence of FLC. Experimental data is average of 3 replicates. Best-fit model parameters are given in Table 8.1.

Parameter	<i>C. albicans</i> value	<i>C. glabrata</i> value	Source
G	0.0038 cell/pmol ATP	0.0118 cell/pmol ATP	Estimated
D	10^{-12} /cell/min	10^{-12} /cell/min	[68]
n_1	2 pmol ATP/pmol glucose	2 pmol ATP/pmol glucose	[18]
n_2	30 pmol ATP/pmol glucose	30 pmol ATP/pmol glucose	[18]
V_1	0.1 pmol glucose/min/cell	0.12 pmol glucose/min/cell	Estimated
K_1	3×10^6 pmol glucose	2.2×10^8 pmol glucose	Estimated
V_2	0.9 pmol $X_{int,a}$ /min/cell	0.7 pmol $X_{int,a}$ /min/cell	Estimated
K_2	1.7×10^9 pmol $X_{int,a}$	4×10^9 pmol $X_{int,a}$	Estimated

Table 8.1: The ecological model parameters for cellular growth and resource consumption in the absence of FLC.

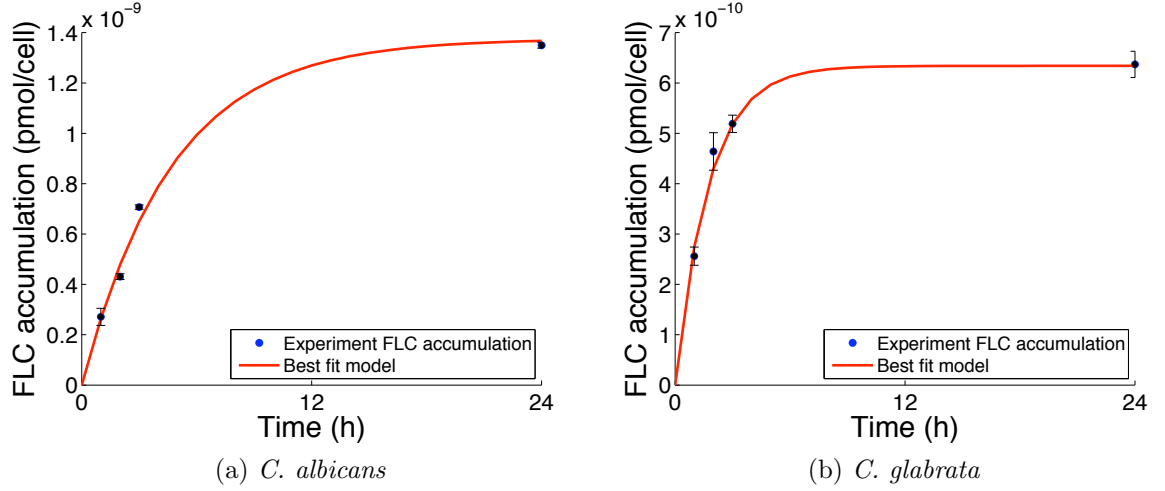


Figure 8.5: Intracellular FLC accumulation. Experimental data is average of 3 replicates. Best-fit model parameters are given in Table 8.2.

Parameter	<i>C. albicans</i> value	<i>C. glabrata</i> value	Source
V_u	6×10^{-9} pmol FLC_{ext} /min/cell	7×10^{-9} pmol FLC_{ext} /min/cell	Estimated
K_u	6×10^4 pmol FLC_{ext}	3×10^4 pmol FLC_{ext}	Estimated
V_e	1.2×10^{-8} pmol $FLC_{int,a}$ /min/cell	4.4×10^{-7} pmol $FLC_{int,g}$ /min/cell	Estimated
K_e	3.5×10^{-6} pmol $FLC_{int,a}$ /cell	5.5×10^{-5} pmol $FLC_{int,g}$ /cell	Estimated

Table 8.2: The ecological model parameters for intracellular FLC accumulation.

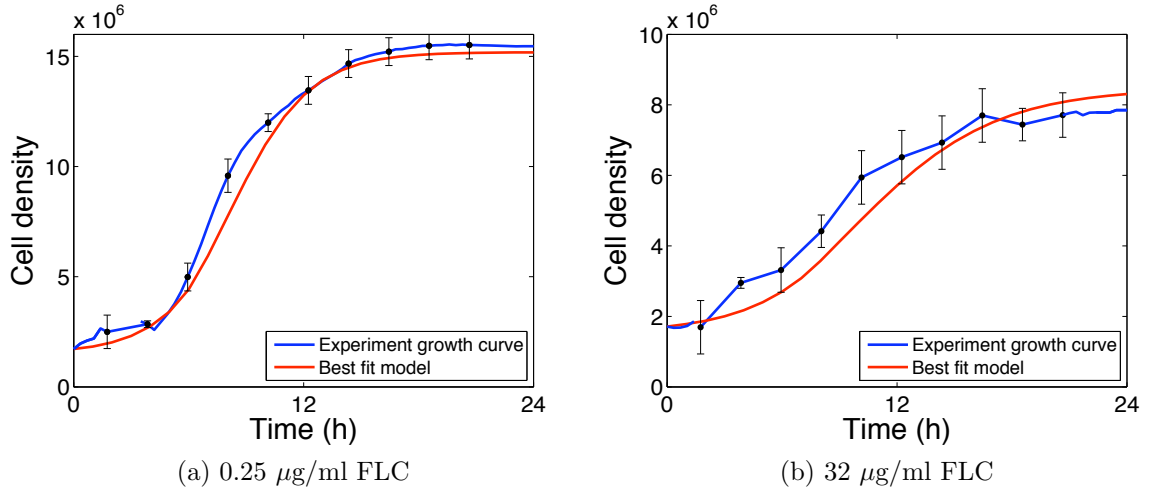


Figure 8.6: *C. albicans* growth dynamics upon administration of FLC. Experimental data is average of 3 replicates. Best-fit model parameters are given in Table 8.3.

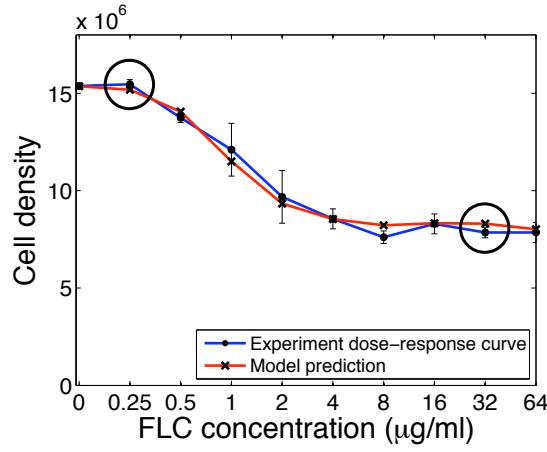
Parameter	<i>C. albicans</i> value	<i>C. glabrata</i> value	Source
a	0.5	N/A	Estimated
b	6.5×10^{-8} pmol $FLC_{int,a}$ /cell	N/A	Estimated
n	2.9	N/A	Estimated

Table 8.3: The ecological model parameters for growth upon FLC administration.

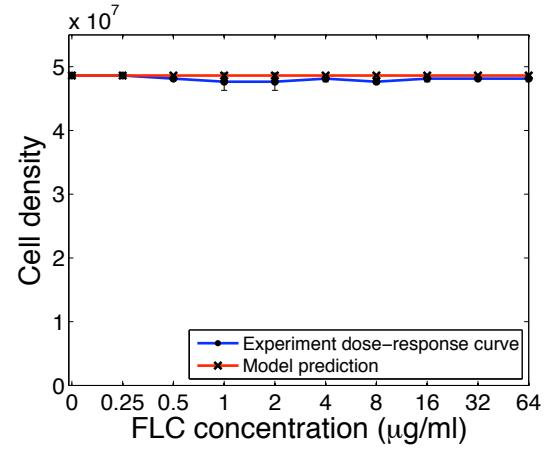
No inhibition of *C. glabrata* growth in the range of FLC concentrations tested was detected (Figure 8.7b) and hence we take $f(FLC_{int,g}) = 1$.

We have now found the parameters which determine survival fitness in our simple model. From an ecological perspective, fitness (growth rate) is environment-dependent and we now use this fact to test the predictive power of our model: If resource consumption capability and FLC stress protection sufficiently describe fitness in reality, the parameterized model should now be able to predict the population dynamics within *any* environment for each *Candida* species. Here we test this hypothesis by comparing the predicted model growth in the presence of increasing FLC concentrations to experimen-

tal FLC *dose-response curves*. Dose-response curves relate increasing drug concentrations to a measurable biological effect [47] here taken to be cell density upon resource exhaustion. We construct experimental FLC dose-response curves by performing growth assays as described in §12.2 at 2 % w/v glucose concentration in the presence of defined FLC concentrations between 0 and 64 $\mu\text{g/ml}$ and recording the cell density at 24 hours when resources had been exhausted. Note that for *C. albicans* where increasing concentrations of FLC have an effect on cell density (no effect on cell density was detected for *C. glabrata* in the range of FLC concentrations tested as shown in Figure 8.7b), the dose-response curve is of sigmoidal form (Figure 8.7a). Above we have parameterized our ecological model using two FLC concentrations near the extremes of the dosages applied to construct the dose-response curve (0.25 $\mu\text{g/ml}$ FLC and 32 $\mu\text{g/ml}$ FLC). So given that we have parameterized our model using two FLC concentrations, can our model predict the cell density for the other FLC concentrations tested and the sigmoidal behaviour in the middle of the experimental dose-response curve? Using the best fit parameters of cellular growth, intracellular FLC accumulation and growth inhibition given in Tables 8.1-8.3 we construct model dose-response curves through MATLAB simulations of growth for each *Candida* species in isolation under exactly the same conditions as the experimental dose-response curves were performed and record the model cell density upon resource exhaustion at 24 hours. Importantly, we find that the ecological model can indeed predict cell density for the range of FLC concentrations tested as illustrated in Figure 8.7.



(a) *C. albicans*



(b) *C. glabrata*

○ FLC concentrations used to parameterize ecological model

Figure 8.7: Dose-response curves at 24 hours. Experimental data is average of 3 replicates.

Given the good agreement between the model predictions of growth across environments and the experimental dose-response curves, we feel that the ecological model captures single species niche fitness well and therefore we now go ahead and consider the competition between *C. albicans* and *C. glabrata* within a given niche.

Chapter 9

Equilibrium Competition Dynamics

In this chapter we generate experimentally testable model predictions for the equilibrium population ecology yielded by the seasonal competition between *C. albicans* and *C. glabrata* within a given niche.

Analogous to the laboratory serial transfer method illustrated in Figure 8.1 we consider the ecological interactions between *C. albicans* and *C. glabrata* over a number of seasons, where the dynamics within one season are described by the system (8.5), and compute the model predictions as follows: Starting with a total population of size $N_0 = 10^6$ cells containing any given frequency, F , of *C. albicans* cells the initial conditions at the beginning of the first season are given by

$$\begin{aligned} S(0) &= S_0, & X_{int,a}(0) &= 0, \\ X_{ext}(0) &= 0, & X_{int,g}(0) &= 0, \\ C_a(0) &= FN_0, & FLC_{int,a}(0) &= 0, \\ C_g(0) &= (1 - F)N_0, & FLC_{int,g}(0) &= 0, \\ & & FLC_{ext}(0) &= FLC_{ext,0}, \end{aligned} \tag{8.7}$$

where S_0 and $FLC_{ext,0}$ denote the initial concentrations of resource and FLC respectively. We integrate system (8.5) subject to the initial conditions in (8.7) until resources have been exhausted which denotes the end of the season. Subsequent seasons are initiated by transferring a fraction of the total cell number containing N_0 cells from the previous season into a fresh environment of resource concentration S_0 and FLC concentration $FLC_{ext,0}$, and then integrating (8.5) until resources are exhausted. Denoting the beginning of each season by T_0^j while the time it takes for resources to be exhausted is denoted by T_{end}^j where j denotes the season number, the initial conditions for the $j + 1$ -st season can be written as

$$\begin{aligned}
S(T_0^{j+1}) &= S_0, & X_{int,a}(T_0^{j+1}) &= X_{int}(T_{end}^j), \\
X_{ext}(T_0^{j+1}) &= X_{ext}(T_{end}^j), & X_{int,g}(T_0^{j+1}) &= X_{int}(T_{end}^j), \\
C_a(T_0^{j+1}) &= F^j N_0, & FLC_{int,a}(T_0^{j+1}) &= FLC_{int,a}(T_{end}^j), \\
C_g(T_0^{j+1}) &= (1 - F^j) N_0, & FLC_{int,g}(T_0^{j+1}) &= FLC_{int,g}(T_{end}^j), \\
& & FLC_{ext}(T_0^{j+1}) &= FLC_{ext,0},
\end{aligned}$$

where

$$F^j = \frac{C_a(T_{end}^j)}{C_a(T_{end}^j) + C_g(T_{end}^j)}.$$

Here we integrate the competitive system (8.5) for time $T = 24$ hours over j seasons until the population frequency of *C. albicans* cells, F , no longer changes between seasons. The equilibrium value of F which resides between 0 and 1 where $F = 1$ indicates a monomorphic population of *C. albicans* and $F = 0$ indicates a monomorphic population of *C. glabrata*, gives the equilibrium *Candida* ecology within a niche defined by the resource concentration S_0 and the FLC extracellular concentration $FLC_{ext,0}$. The model predictions for the competitive fates of *C. albicans* and *C. glabrata* within a given niche

over serial transfers are given in Figure 9.1.

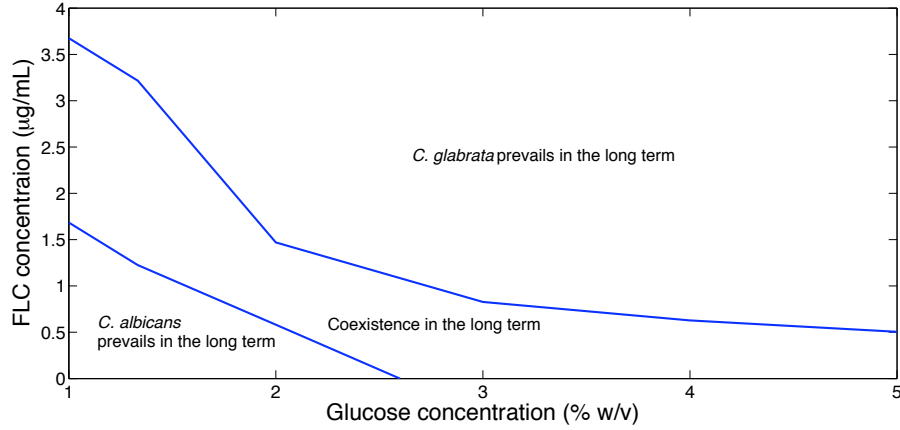


Figure 9.1: Model predictions for the equilibrium *Candida* ecology in an infection niche defined by the concentration of a single limiting resource and the concentration of FLC. Simulations were conducted at 20 glucose concentrations uniformly spaced between 1 and 5 % w/v glucose. For each glucose concentration, the initial frequency of *C. albicans* was 0.5 and the competitive system (8.5) was integrated over consecutive seasons until the equilibrium population ecology had been reached at 40 FLC concentrations uniformly spaced between 0 and 4 $\mu\text{g}/\text{ml}$.

9.1 Model Verifications: Discrete Dynamical Systems

Conducting a serial transfer regime in the laboratory is a time consuming procedure and hence we choose an alternative approach to experimentally verify the predictions in Figure 9.1. In our formulation of the serial transfer method it is only the frequencies of competitors in the fixed total population size N_0 that changes between seasons. This means that the population frequency of *C. albicans* when resources have been exhausted at the end of a competition season, F_{end} , gives the initial frequency, F_{int} , of the subsequent season, and so on. Hence the evolution of the *C. albicans* frequency, F , over multiple seasons can be described as a one-dimensional discrete dynamical system of the form

$$F_{int}^{j+1} = \mathcal{G}(F_{end}^j), \quad (8.8)$$

where $\mathcal{G}$ is a real-valued, niche-dependent function which maps any initial *C. albicans* frequency onto a sequence of iterates that determine the qualitative behavior of the system (8.5) in the long term.

We investigate the dynamics of any initial *C. albicans* frequency over multiple seasons by means of a *cobweb*. A cobweb is a graphical representations of the relation in (8.8) in the (F_{int}, F_{end}) -plane and can be used to deduce the competitive fates of *C. albicans* and *C. glabrata* in a given infection environment as illustrated in Figure 9.2 by the following method: Choose an initial *C. albicans* frequency for the first season of the serial transfer method. Draw a vertical line to the curve $F_{end} = \mathcal{G}(F_{int})$, this is the end-of-season *C. albicans* frequency after the first season. Now draw a horizontal line to the diagonal $F_{end} = F_{int}$, this line reaches the diagonal at the point (F_{int}, F_{int}) giving the initial ratio for the second season of the serial transfer method. Again draw a vertical line to the curve $F_{end} = \mathcal{G}(F_{int})$ to find the end-of-season frequency of the second season which then determines the initial ratio of the third season, and so forth until $F^* = \mathcal{G}(F^*)$ indicating that the *C. albicans* frequency no longer changes between seasons and the equilibrium population frequency of *C. albicans* , F^* , has been reached.

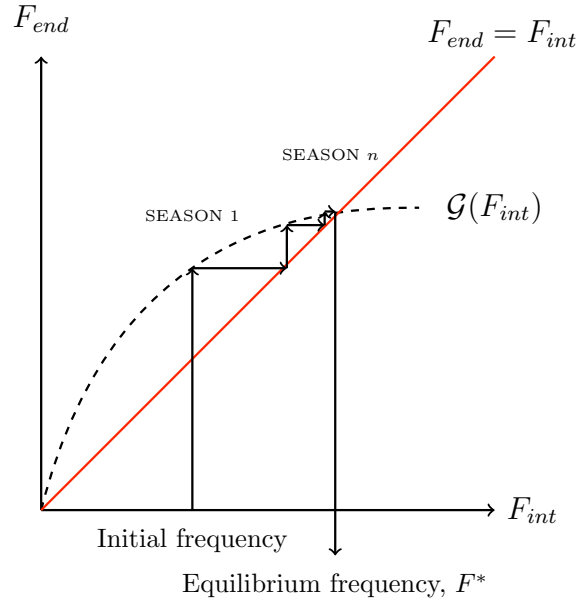
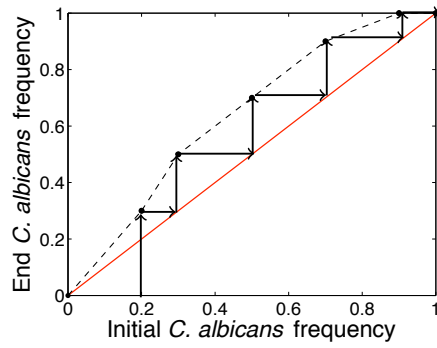


Figure 9.2: Illustration of the cobweb technique used to deduce competitive outcome between *C. albicans* and *C. glabrata*.

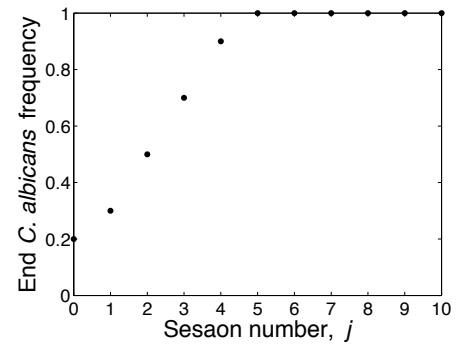
To verify the predictions in Figure 9.1, there are three possible cases of the map in (8.8) to consider: First, if the curve $F_{end} = \mathcal{G}(F_{int})$ lies above the diagonal $F_{end} = F_{int}$ for all possible values of F this means that over consecutive seasons, for any initial *C. albicans* frequency, we end every season with a higher *C. albicans* frequency than the one we started with. Thus over time F evolves towards a value of 1 indicating a monomorphic population of *C. albicans* (Figure 9.3a-9.3b). Second, if the curve $F_{end} = \mathcal{G}(F_{int})$ lies below the diagonal $F_{end} = F_{int}$ this means that for any initial population frequency the end-of-season *C. albicans* frequencies decrease over consecutive seasons and over time F marches towards a value of 0 indicating that *C. albicans* is excluded from the niche to yield a monomorphic population of *C. glabrata* (Figure 9.3c-9.3d). Finally, if the curve $F_{end} = \mathcal{G}(F_{int})$ crosses the line $F_{end} = F_{int}$, the point of crossing indicates an equilibrium where the two competitors coexist; *C. albicans* at frequency F^* and *C. glabrata* at frequency $1 - F^*$. Any initial *C. albicans* frequency above or below the

equilibrium point evolves towards the equilibrium frequency over time (Figure 9.3e-9.3f).

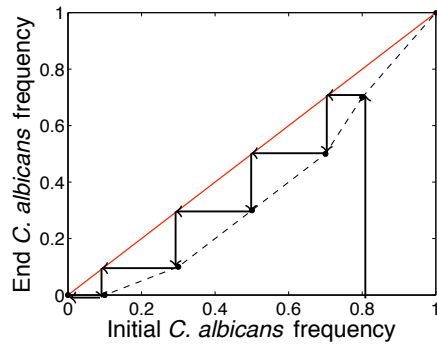
Cobweb examples of the relation in (8.8) for the three niche competition outcomes predicted by the model are given in Figure 9.3, for ease of interpretation the associated evolutionary trajectory of *C. albicans* frequency over seasons is included with each cobweb.



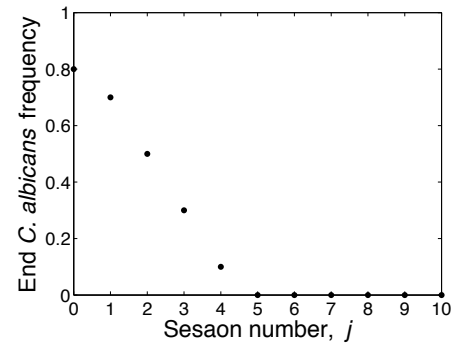
(a)



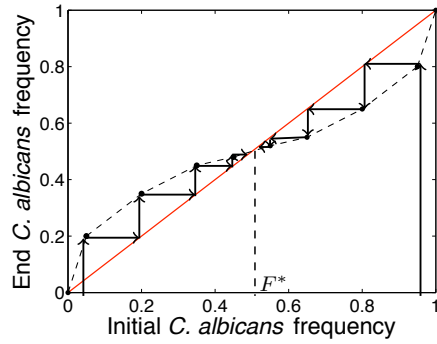
(b)



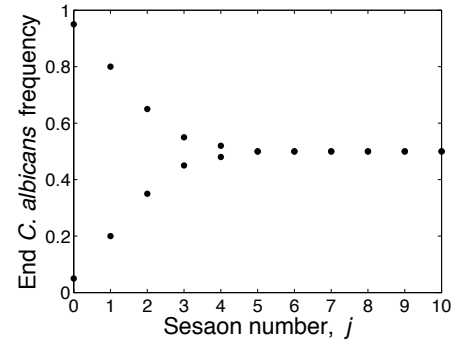
(c)



(d)



(e)



(f)

Figure 9.3: Cobwebs and corresponding trajectories of *C. albicans* population frequency over seasons in the serial transfer method. (a) and (b) For any initial *C. albicans* population frequency, the end-of-season frequency is greater and over time *C. albicans* amplifies in the population to eventually yield a monomorphic population of *C. albicans*. (c) and (d) For any initial *C. albicans* population frequency, the end-of-season frequency is lower and over time *C. albicans* is excluded from the niche to yield a monomorphic population of *C. glabrata*. (e) and (f) Any initial *C. albicans* population frequency evolves over time towards the equilibrium value, F^* , where the two competitors coexist.

9.2 Cobwebs: A Fitness Consideration

We now make a brief note of how the relation in (8.8) and its graphical representation in a cobweb relate to the concept of fitness which drives niche selection. Fitness, w , describes the growth performance of a species during direct competition within a defined environment. Hence the competitive fitness of *C. albicans*, w_a , can be estimated as the ratio of *C. albicans* population frequencies at the end of a competition season, F_{end} , and in the initial competition inoculum, F_{int} , given by $w_a = F_{end}/F_{int}$ [107]. Calculating the competitive fitness of *C. glabrata*, w_g , using the same method, the relative fitness of *C. albicans* compared to *C. glabrata*, W_{ag} , within a defined niche is given by the ratio $W_{ag} = w_a/w_g$. Hence if $W_{ag} > 1$, *C. albicans* has a growth advantage compared to *C. glabrata* under the given niche conditions. However, this is not sufficient to determine the outcome of natural selection within a given environment. Imagine, for example, that *C. albicans* has fitness advantage at low initial frequencies but a fitness disadvantage at high initial frequencies, then the two competitors will coexist through density-dependent processes at some intermediate frequency where $W_{ag} = 1$. This is equivalent to a cobweb where the mapping function $\mathcal{G}$ crosses the diagonal at some frequency F^* (Figure 9.3e). Hence only if *C. albicans* performs better than *C. glabrata* at every initial frequency between 0 and 1 ($W_{ag} > 1$) can we conclude that natural selection will favour the spread of *C. albicans* in the population. This is equivalent to a cobweb where the mapping function $\mathcal{G}$ lies above the diagonal indicating that for every initial *C. albicans* frequency, the end-of-season frequency is greater at the expense of *C. glabrata* (Figure 9.3a). If, however, *C. albicans* performs worse than *C. glabrata* at every initial frequency between 0 and 1 ($W_{ag} < 1$) then *C. glabrata* has a growth advantage and natural selection will drive *C. albicans* to competitive exclusion from the mixture. This is equivalent to a cobweb where the mapping function $\mathcal{G}$ lies below the diagonal indicating that for every initial *C. albicans* frequency the end-of-season frequency is lower (Figure 9.3c). Thus

competitive fitnesses play an essential role in predicting niche competition outcome in a serial transfer regime, however, we emphasize the importance of carrying out competition experiments and calculating relative niche fitnesses at a range of frequencies between 0 and 1 in order to deduce competition outcome using discrete dynamical systems theory.

Chapter 10

Ecological Model Results

In this chapter we experimentally construct the mapping function $\mathcal{G}$ in (8.8) for different niche environments by performing competition assays in which the two competitors are mixed at different initial frequencies in identical reaction vessels and allowed to compete until resources are exhausted at which point the end-of-season frequencies are determined (see §12.3 for a full description of the experimental procedure). Through the construction of a cobweb map we then determine the experimental niche competition outcome between *C. albicans* and *C. glabrata* and compare to the predictions in Figure 9.1. All experimental data presented in the chapter were generated by myself in Ken Haynes laboratory at the University of Exeter, starting with the experimentally constructed cobwebs given in Figure 10.1.

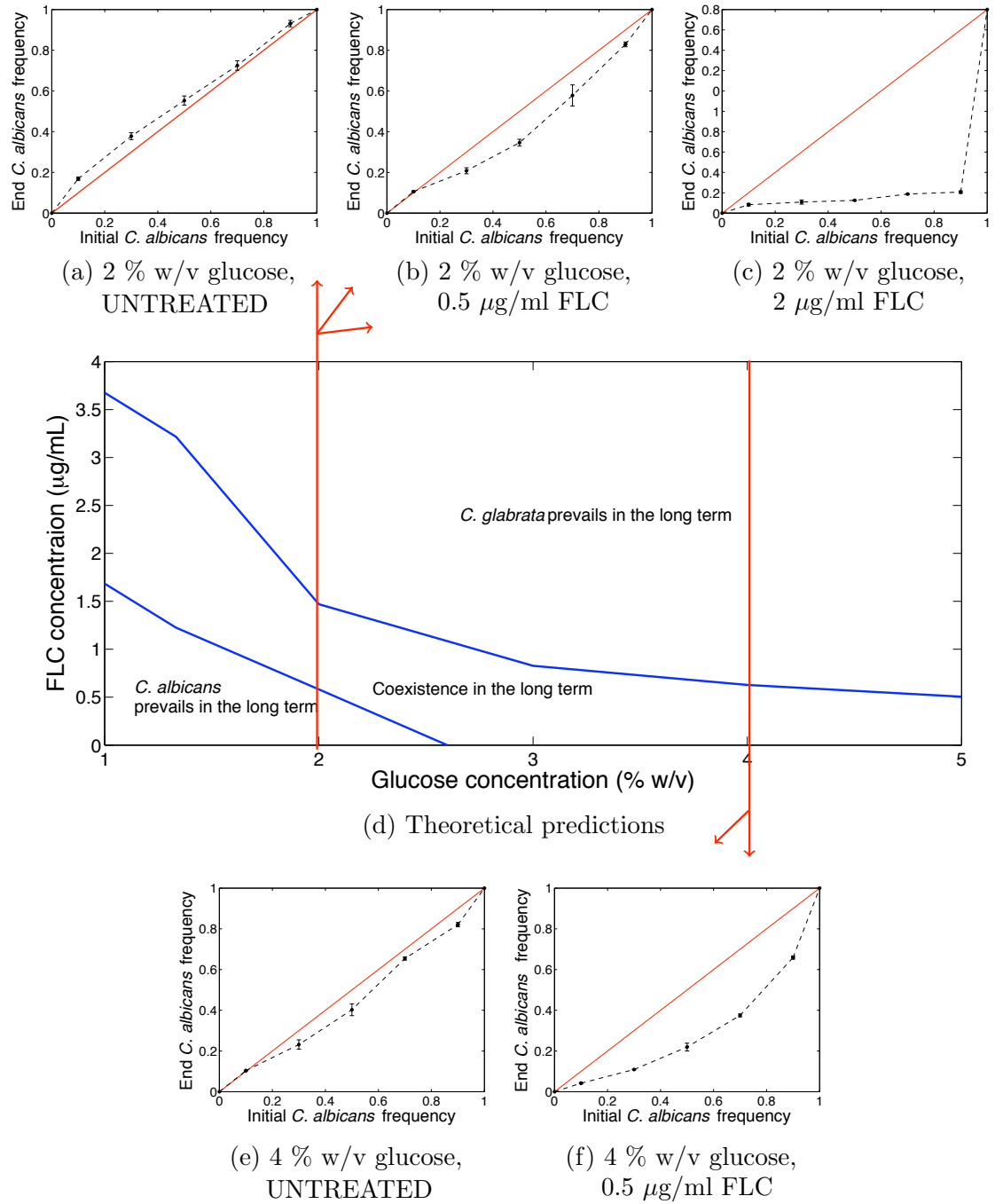


Figure 10.1: Experimental verifications of theoretical predictions in Figure 9.1. At 2 % w/v glucose concentration (a)-(c) the experimentally constructed cobwebs infer that in the absence of FLC *C. albicans* prevails in the long term (a), at 0.5 $\mu\text{g/ml}$ FLC there is coexistence between the two competitors (b) and at 2 $\mu\text{g/ml}$ FLC *C. glabrata* prevails in the long term. At 4 % w/v glucose concentration (e)-(f) the experimental cobweb maps infer that in the absence of FLC there is coexistence between the two competitors (e) and at 0.5 $\mu\text{g/ml}$ FLC *C. glabrata* prevails in the long term (f). Experimental data is average of 3 replicates.

Figure 10.1 illustrates that an epidemiological shift from *C. albicans* to *C. glabrata* indeed occurs above a threshold dose of FLC which is dependent on the resource concentration within a given infection environment. Moreover, there is qualitative agreement between the model predictions and the experimentally deduced equilibrium *Candida* ecology across niches. This suggests that population dynamics within a given niche are well represented by our ecological model and that niche selection does indeed play a role in the emerging epidemiological trends of *Candida* infections.

10.1 The Standardized Environment for Antifungal Susceptibility Testing

The experimental conditions we have used to parameterize our ecological model and verify its predictions (synthetic complete medium supplemented with glucose at 30 °C as described in §12.2-§12.3) represent common laboratory growth conditions for yeast and ensure that both *Candida* species grow as single yeast cells which facilitates species quantification at the end of a season (see §12.4 for further details). However, those conditions do not resemble the environment within a human infection. In clinical practice, therapeutic decisions on the antifungal dosage administered to patients are based on the results of antifungal susceptibility tests on patient isolates. The standardized method for antifungal susceptibility testing is performed in buffered Roswell Park Memorial Institute (RPMI) medium supplemented with glucose at 35 °C [27] which mimics the nutritional characteristics, osmotic pressure and temperature of the human *in vivo* environment [57]. It has been reported that FLC administration based on this standard *in vitro* susceptibility testing procedure correlates well with clinical response [79]. Therefore we now study the niche competition between the two *Candida* species under the

standardized conditions of antifungal susceptibility testing in order to investigate if the selective forces exerted by FLC can explain the epidemiological shift from *C. albicans* to *C. glabrata* in an environment which resembles the human host. For simplicity, we assume that although the standard medium contains substances which may enhance growth, glucose is the primary source of energy and hence resource competitions are driven by the availability of glucose only. Parameterizing the model (8.5) as described in §8.2 using experimental data derived from the methods in §12.5 (see Appendix D for the best fit models), the model predictions for the equilibrium *Candida* ecology under the standardized conditions of antifungal susceptibility testing are given in Figure 10.2.

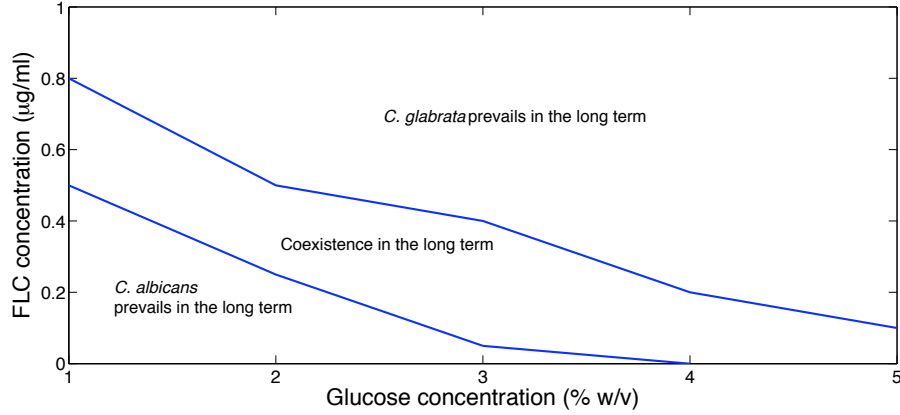


Figure 10.2: Model predictions for the equilibrium *Candida* ecology under the standardized conditions for antifungal susceptibility testing. Simulations were conducted at 20 glucose concentrations uniformly spaced between 1 and 5 % w/v glucose. For each glucose concentration, the initial frequency of *C. albicans* was 0.5 and the competitive system (8.5) was integrated over consecutive seasons until the equilibrium population ecology had been reached at 20 FLC concentrations uniformly spaced between 0 and 1 $\mu\text{g/ml}$.

The standard antifungal susceptibility protocol recommends testing in RPMI broth at 2 % w/v glucose concentration [27]. Figure 10.2 illustrates that above a threshold of $\sim 0.6 \mu\text{g/ml}$ FLC the model predicts a shift from *C. albicans* to *C. glabrata* at 2 % w/v glucose concentration. Hence we now adopt the standard methodology and experimentally study the competition between *C. albicans* and *C. glabrata* through the

construction of cobwebs at 2 % w/v glucose concentration for two FLC concentrations. Our aim here is to investigate whether niche selection mediates a shift in the equilibrium *Candida* ecology, not whether our simple model can predict the dose at which the shift occurs, hence we choose to study the long term dynamics in the absence of FLC and at a FLC concentration of 5 $\mu\text{g}/\text{ml}$ that is far above the threshold dose predicted in Figure 10.2. Note that under the standard antifungal susceptibility test conditions *C. albicans* has the ability to change its growth morphology from yeast cells to hyphal cells. This means that the frequency of *C. albicans* at the end of the growth cycle cannot be determined on a single cell basis due to excessive clumping and hence a quantification procedure based on colony counts as described §12.6 was performed. The experimentally constructed cobwebs under the standardized conditions for antifungal susceptibility testing are given in Figure 10.3.

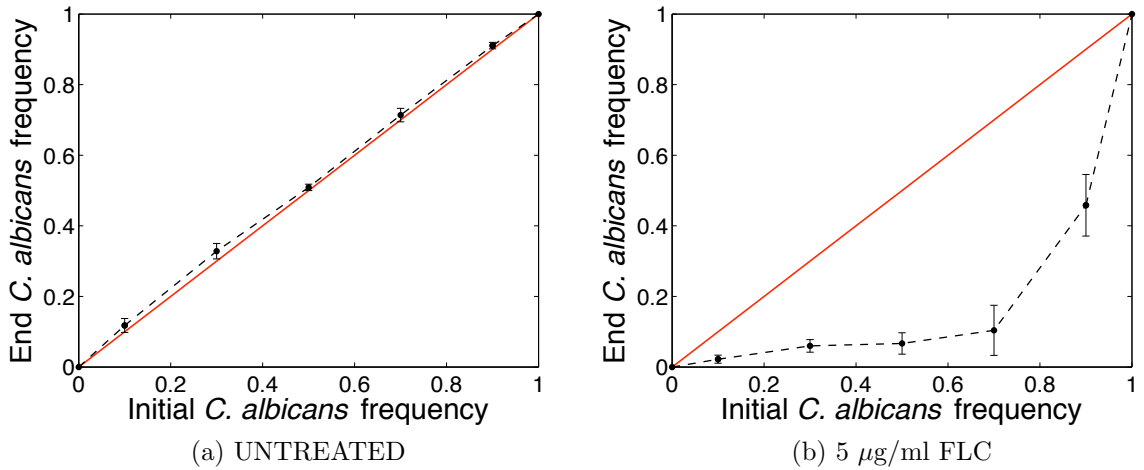


Figure 10.3: Experimental verifications of model predictions in Figure 10.2 at 2 % w/v glucose concentration under the standardized conditions for antifungal susceptibility testing. The experimentally constructed cobwebs infer that in the absence of FLC *C. albicans* prevails in the long term (a) while at 5 $\mu\text{g}/\text{ml}$ FLC *C. glabrata* prevails in the long term. Experimental data is average of 3 replicates.

Again, the predicted shift from an equilibrium population ecology consisting of *C. albicans* cells only in the absence of FLC to a monomorphic population of *C. glabrata*

cells in the presence of FLC is confirmed by the experimentally constructed cobwebs in Figure 10.3.

Chapter 11

Discussion Part II

In this part we have proposed an ecological explanation for the emerging epidemiological trends of *Candida* infections. By focusing on the competitive interactions between *C. albicans* and *C. glabrata* within an infection niche defined by the concentration of a single limiting resource and the concentration of FLC, we have developed an ecological model which qualitatively predicts the long term distribution of *Candida* species in a given niche. Our model demonstrates that survival fitness is environmentally regulated and has direct effects on the niche *Candida* ecology. Most importantly, for the two *Candida* strains considered in this study natural selection favours *C. albicans* in the absence of FLC while a threshold dose of FLC allows for the spread of *C. glabrata* within an immunocompromised host. This study provides a new perspective on the spread of FLC resistant *Candida* species in clinical practice and lays the foundation for a treatment approach where population ecology can be predicted based on ecological compatibility with the conditions in the human body.

Our study suggests that the population impact of FLC treatment is dynamic and environment-dependent. For example, increasing resource concentration within the in-

fection niche selects for *C. glabrata* and lowers the threshold FLC dose above which *C. glabrata* can invade and spread to fixation (Figure 9.1 and Figure 10.2). This is consistent with the clinical observation that infection with non-*albicans* *Candida* species such as *C. glabrata* is significantly higher in diabetic patients than in subjects with normal glucose levels [32] [85]. This model discovery paves the way for alternative treatment strategies that aim not necessarily to kill resistant organisms but rather to re-establish susceptible populations within the infection niche through alterations to the niche microenvironment. Moreover, our study highlights the importance diagnosing the infection environment, such as host glucose levels, prior to FLC treatment that could select for resistant *Candida* types and worsen clinical outcome.

The shift in population ecology from *C. albicans* to *C. glabrata* driven by the selective forces exerted by FLC involves a region of coexistence between the two species (Figure 9.1 and Figure 10.2). This is due to the fact that the experimental environment is seasonal; resources are abundant at the beginning of a growth cycle but become scarce as the population approaches its saturation density. This means that the specific environmental conditions change over the course of a growth cycle, and thus the fitness of each competitor also changes. For example, *C. albicans* has a fitness advantage at low glucose concentration whereas *C. glabrata* has an equal opposing advantage for high concentrations of glucose (Figure 9.1 and Figure 10.2). Hence a seasonal environment provides temporal niche opportunities where the competitors have complimentary fitness advantages at different stages of the growth cycle and therefore the two competitors may coexist, each having a net advantage when it is rare through frequency-dependent selection. Note that although the ecological mechanism by which *C. albicans* and *C. glabrata* coexist in our system (8.5) is a consequence of the experimental environment, environmental fluctuations which promote coexistence through fitness trade-offs between

niches are likely to operate within the human host. Indeed, co-infections of *C. albicans* and *C. glabrata* have been documented in the mouth [65], vagina [99] and in blood isolates [43]. At present, multiple-species *Candida* infections is reported to occur in a minority of patients [75], however, because different *Candida* species have similar colonial appearance in routine culture media used in clinical laboratories the frequency of mixed species infections may be underestimated. Identification of multiple-species infections has important implications from a therapeutic point of view, especially for patients with candidemia. As reported by Boktour *et al.* [11] the response rate of patients with multiple-species candidemia who received a single antifungal agent as therapy was lower than patients with multiple-species candidemia who received a combination of antifungal therapies. This is in agreement with our model which predicts the spread of FLC resistant *C. glabrata* upon FLC administration to a mixed infection and suggests that the immediate employment of a drug in response to yeast-positive blood cultures may result in poor clinical outcome without species level identification. Hence we advocate that development into fast and economic species identification of mixed infection cultures will allow further studies into how individual species respond to different drug selection pressures and ultimately promote treatment strategies to combat monomicrobial and multiple-species *Candida* infections.

Finally, our study could carry an important message for patient treatment of *Candida* infections in clinical practice. Our findings suggest that FLC selects for resistant *Candida* species within an immunocompromised host and hence the administration of prophylactic FLC to patients at risk for fungal infections may result in the spread of FLC resistant species. FLC resistant *Candida* infections dramatically reduce the probability of effective treatment and may lead to severe disease and morbidity. Although the search for new antifungal targets and drugs to combat FLC resistant *Candida* in-

fections continues we suggest that an ecological view of fungal disease opens the way for a new treatment approach. Understanding the interactions between basic physiology and the microenvironment that are required for survival within an infection niche renders predictive powers of *Candida* ecology and promotes development of alternative therapeutic treatments such as specific manipulation of the growth environment and modification of host diet [91] [103]. Hence we conclude that an ecological view of fungal disease constitutes a fruitful avenue for further study.

Chapter 12

Experimental Methods Part II

12.1 Organisms and Growth Conditions

The strains *Candida albicans* ACT1-GFP and *Candida glabrata* ATCC2001 were used. The strain *Candida albicans* ACT1-GFP strain is SBC153 [73] with pACT1-FLAG-GFP integrated at the ACT1 locus by means of positive selections using a nourseothricin resistance cassette. GFP is a green fluorescent protein. The strain *Candida glabrata* ATCC2001 is the wild type reference strain obtained from the American Type Culture Collection.

Strains were grown overnight at 30° C, at 200 rpm, in yeast extract peptone dextrose (YPD) broth (2 % w/v mycological peptone, 1 % w/v yeast extract, 2 % w/v glucose).

12.2 Cell Growth in Synthetic Complete Medium

The assay medium was synthetic complete (SC) medium (0.67 % w/v yeast nitrogen base without amino acids, 0.079% w/v synthetic complete supplement mixture (Formedium, Hunstanton, UK), 2 % w/v glucose). Sterile plastic microdilution plates containing 96

flat-bottomed wells were utilized. Stock solution of fluconazole (Pliva Pharma, Hampshire, UK) was diluted in the assay medium and dispensed in 75 μ l volumes into six replicate wells across columns 3 to 11 to yield nine two-fold serial dilutions of fluconazole. Six replicate wells in column 2 served as growth controls and were filled with 75 μ l of the assay medium. Overnight cultures of each *Candida* species were diluted appropriately in the assay medium to yield final inoculum suspensions containing 2×10^7 cells/ml. Triplicate aliquots of 75 μ l from the *C. albicans* strain suspension were dispensed across columns 2 to 11, and similarly, 75 μ l volumes from the *C. glabrata* strain suspension were dispensed in triplicate wells across columns 2 to 11. Final concentrations of fluconazole were 0-64 μ g/ml. All unused wells were filled with 150 μ l of distilled water. The plate was sealed with a transparent adhesive seal and two holes were punctured over each well by means of a sterile needle. The plate was incubated at 30° C with shaking over 24 hours and growth monitored by measuring the absorbance of the cell suspensions at 620 nm (A_{620}). Absorbance units were converted into number of cells per ml by means of calibration curves prepared for each *Candida* species.

12.3 Competition in Synthetic Complete Medium

Two assay media were used: SC medium 1 (0.67 % w/v yeast nitrogen base without amino acids, 0.079% w/v synthetic complete supplement mixture, 2 % w/v glucose) and SC medium 2 (0.67 % w/v yeast nitrogen base without amino acids, 0.079% w/v complete supplement mixture, 4 % w/v glucose). For SC medium 1 the competition experiment was performed in three fluconazole concentrations; 0, 0.5 μ g/ml and 2 μ g/ml, and for the SC medium 2 the competition experiment was performed in two fluconazole concentrations: 0 and 0.5 μ g/ml. Sterile plastic microdilution plates containing 96 flat-bottomed wells were utilized. For each competition experiment, the following procedure

was followed: Stock of fluconazole was diluted in the appropriate assay medium to yield the desired concentration and dispensed in 75 μ l volumes into triplicate wells across columns 3 to 9. Overnight cultures of each *Candida* species were diluted appropriately in the assay medium to yield final inoculum suspensions containing 2×10^7 cells/ml. Triplicate 75 μ l aliquots from the *C. glabrata* strain suspension were dispensed in column 3, and 75 μ l volumes from the *C. albicans* strain suspension were added in triplicate wells to column 9. Across columns 4 to 8 appropriate triplicate aliquot volumes were added from each *Candida* species suspension so that in a total aliquot volume of 75 μ l the (% *C. glabrata* cells : % *C. albicans* cells) across columns 4 to 8 was given by 90:10, 70:30, 50:50, 30:70, 10:90. All unused wells were filled with 150 μ l of distilled water. The plate was sealed with a transparent adhesive seal and two holes were punctured over each well by means of a sterile needle. The plates were incubated at 30° C with shaking over 24 hours. For each competition medium, quantification of *Candida albicans* ACT1-GFP frequency in each well was determined by fluorescence activated cell sorting (FACS) as described in §12.4.

12.4 FACS Quantification Procedure

Cellular fluorescence from GFP was determined quantitatively with a FACSAria flow cytometer (Becton Dickinson, CA, USA) equipped with a 20mW, 488 nm argon ion laser. All samples were suspended in phosphate buffered saline (PBS) and sonicated for 10 seconds to disperse cell clumps just prior to analysis. Typically, 10 000 cells were counted per competition sample with the following settings: forward scatter (150 V, log mode) and side scatter (200 V, log mode). GFP was detected on a 530/30 filter and sample acquisition was performed using BD FACSDiva software. Initially, a sample consisting of *C. albicans* ACT1-GFP cells only was detected and gated to contain 99-100

% of all measured events as positive for GFP fluorescence. All events occurring within the gate during subsequent analysis of competition samples were considered to be *C. albicans* ACT1-GFP cells. For any given competition sample, the frequency of gated events was calculated by means of FlowJo software and was taken to be the population percentage of *C. albicans* ACT1-GFP within the sample.

12.5 Cell Growth under the Standardized Conditions of Antifungal Susceptibility Testing

The European Committee on Antimicrobial Susceptibility Testing (EUCAST) [27] standard procedure for antifungal susceptibility testing was followed. The assay medium was RPMI-1640 (Gibco, Paisley, UK) without sodium bicarbonate and with L-glutamine buffered to pH 7.0 with 0.165 M morpholinepropane-sulfonic acid (MOPS) (Sigma-Aldrich, Dorset, UK) and supplemented with 18 g of glucose per liter to yield a final glucose concentration of 2 % w/v (RPMI-2 % glucose). The medium was sterilized by filtration. Sterile plastic microdilution plates containing 96 flat-bottomed wells were utilized. Stock solution of fluconazole was diluted in assay medium and dispensed in 100 μ l volumes into six replicate wells across columns 3 to 11 to yield nine two-fold serial dilutions of fluconazole. Six replicate wells in column 2 served as a growth controls and were filled with 100 μ l of the assay medium. Overnight cultures of each *Candida* species were diluted appropriately in the assay medium to yield final inoculum suspensions containing 1×10^6 cells/ml. Triplicate aliquots of 100 μ l from the *C. albicans* strain suspension were dispensed across columns 2 to 11, and similarly, 100 μ l volumes from the *C. glabrata* strain suspension were dispensed in triplicate wells across columns 2 to 11. Final concentrations of fluconazole were 0-64 μ g/ml. All unused wells were filled with 200 μ l of distilled water. The plate was sealed with a transparent adhesive seal and

two holes were punctured over each well by means of a sterile needle. The microdilution plate was shaken on a plate shaker for 30 seconds to ensure homogeneous distributions of the two *Candida* species within each well. The plate was incubated at 35° C without shaking over 48 hours and growth monitored by measuring the absorbance of the cell suspensions at 620 nm (A_{620}). Absorbance units were converted into number of cells per ml by means of calibration curves prepared for each *Candida* species.

12.6 Competition under the Standardized Conditions of Antifungal Susceptibility Testing

The assay medium was RPMI-2 % glucose and the competition experiment performed in two fluconazole concentrations; 0 and 5 $\mu\text{g/ml}$. Sterile plastic microdilution plates containing 96 flat-bottomed wells were utilized. For each competition experiment, the following procedure was followed: Stock solution of fluconazole was diluted in the appropriate assay medium to yield the desired concentration and dispensed in 100 μl volumes into triplicate wells across columns 3 to 9. Overnight cultures of each *Candida* species were diluted appropriately in the assay medium to yield final inoculum suspensions containing 1×10^6 cells/ml. Triplicate 100 μl aliquots from the *C. glabrata* strain suspension were dispensed in column 3, and 100 μl volumes from the *C. albicans* strain suspension were added in triplicate wells to column 9. Across columns 4 to 8 appropriate triplicate aliquot volumes were added from each *Candida* species suspension so that in a total aliquot volume of 100 μl the (% *C. glabrata* cells : % *C. albicans* cells) across columns 4 to 8 was given by 90:10, 70:30, 50:50, 30:70, 10:90. All unused wells were filled with 200 μl of distilled water. The plate was sealed with a transparent adhesive seal and two holes were punctured over each well by means of a sterile needle. The microdilution plate was shaken on a plate shaker for 30 seconds to ensure homogeneous distributions of the

two *Candida* species within each well. The plate was then incubated at 35° C without shaking over 48 hours. Each competition culture was diluted in PBS to yield a final cell density of 2×10^3 cells/ml and 100 μ l from each competition suspension was spread on CHROMagar *Candida* plates. The plates were incubated at 30° C over 72 hours. *C. albicans* appeared as green colonies while *C. glabrata* yielded colonies of pink colour. The number of colonies of each species were counted and the frequency of each *Candida* species calculated. A photograph of the CHROMagar *Candida* plates after incubation is given in Figure 12.1.

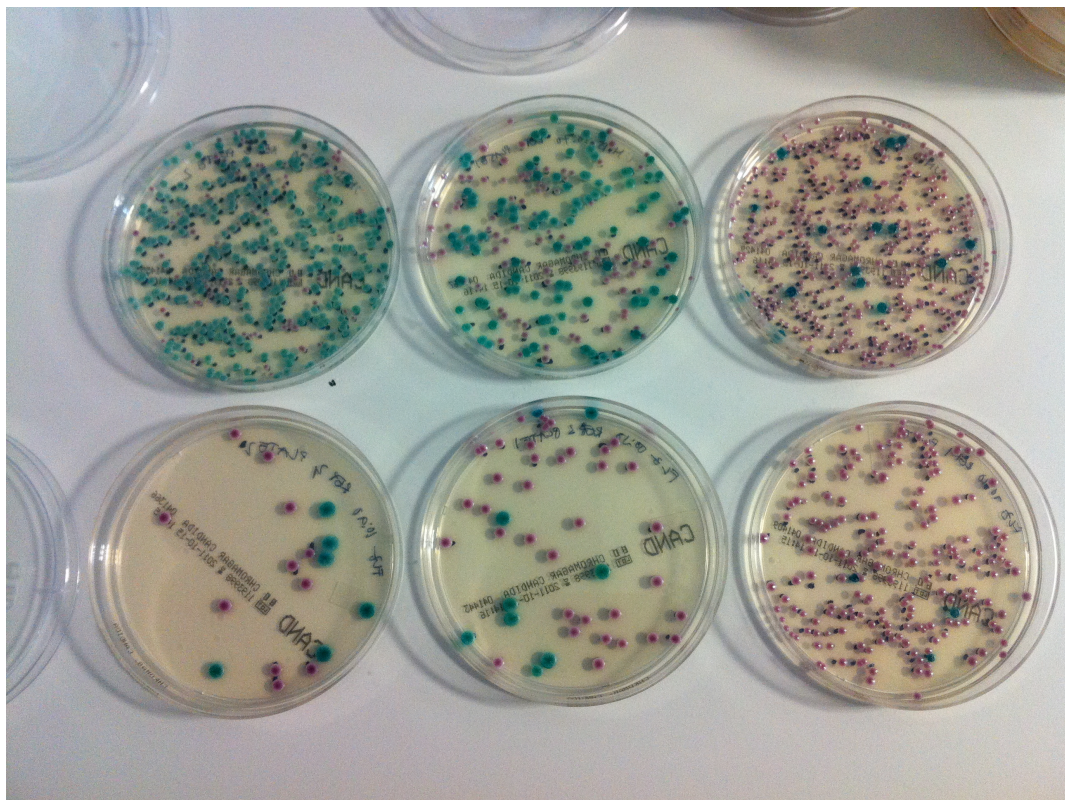


Figure 12.1: CHROMagar *Candida* plates used to verify the model predictions in Figure 10.2 under the standardized conditions of antifungal testing. Top row: Competition experiments in the absence of FLC. One replicate shown for each of the initial competition *C. albicans* : *C. glabrata* frequencies 0.9:0.1, 0.5:0.5, 0.1:0.9 (left to right). Bottom row: Competition experiments at 5 μ g/ml FLC. One replicate shown for each of the initial competition *C. albicans* : *C. glabrata* frequencies 0.9:0.1, 0.5:0.5, 0.1:0.9 (left to right).

12.7 Intracellular Fluconazole Accumulation in Fungal Cells

Measurements of the intracellular accumulation of fluconazole for both *Candida* species were performed as described in [70].

Chapter 13

General Conclusions

Microbes have a wide range of stress responses at their disposal enabling them to inhabit almost every environmental niche of Earth. Traditionally, the underlying mechanisms which modulate cellular physiology to survive under stress have been studied in isolation from the ecological consequences of stress at the level of microbial communities thereby yielding little insight into how microbes deal with stress across different scales. In this thesis we have employed a multi-scale approach that merges genetic, molecular and ecological processes and scales in order to promote an understanding of the ecological impacts of stress responses in microbes. By assuming that microbes must regulate gene expression to balance available energetic resources between two survival fitness components, namely nutrition and stress protection, we have built simple models which isolate the basic processes that drive population dynamics under stress within an experimental setting. Importantly, our findings in part I and part II indicate that the metabolic constraints imposed on microbes under environmental stress are predictive of population structure and provide an explanation for the intra- and inter-species dynamic diversity in stress tolerance within microbial communities in nature. Moreover, our results contribute to the understanding of how the environment affects microbial

community composition and function over time with significant practical implications for the management of microbial populations. For example, a better understanding of how the environment influences the emergence of stress resistance in microbes would allow research into approaches where selection pressure is applied to alter and control microbial community structure as a complement to the ongoing research into antimicrobial therapies.

The combination of mathematical modeling and laboratory microbial model systems provides a fruitful avenue for further study into the ecological consequences of stress responses in microorganisms. While building predictive and realistic models require detailed knowledge of the underlying physiological mechanisms which affect fitness traits we believe that this is achievable through strong collaboration between biologists and mathematicians. Close interdisciplinary communication promotes a comprehensive approach capable of integrating experimental data and suggesting new hypotheses of the population level impact of sub-organismal processes that can be tested and verified against empirical data. Hence we argue that further cross-disciplinary research that aims to quantify microbial stress responses in mathematical terms is not only achievable but crucial in advancing our understanding of the fundamental mechanisms which govern microbial stress responses and how these are manifested at the ecosystem scale.

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Appendix A. The Chemostat

The chemostat is an experimental set-up for studying microbial evolution in the laboratory [76]. A chemostat consists of a culture vessel to which a fresh nutrient medium is continuously added at a constant rate and from which exhausted medium and used cells are removed at the same rate, such that a constant culture volume is maintained [23]. Mechanically driven impellers ensure that the culture is well mixed and as a whole is homogeneous [51]. The medium supplies all nutrients needed for growth in excess of demand except for one, which is supplied in limiting amounts, and limits cell division within the chemostat. An equilibrium will eventually be reached in which the number of new cells created by division within the chemostat will be exactly balanced by the number of cells siphoned off in the overflow. At this point the microbial population within the vessel competes for the limiting resource at a constant growth rate where all cells are in the same physiological state. This means that equilibrium population density in a chemostat can be controlled by altering the concentration of limiting nutrient in the exogenously supplied medium and the dilution rate. Hence the chemostat offers an optimal environment for the study of microbial evolution under strictly controlled conditions [19].

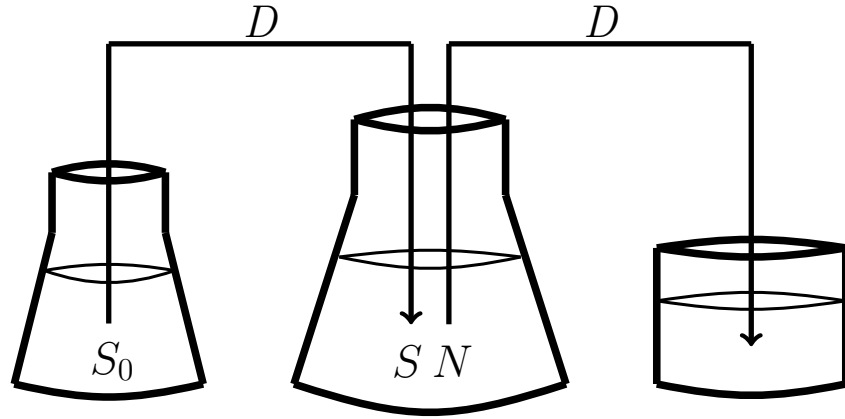


Figure A.1: The experimental set-up for the chemostat. The culture vessel contains limiting resource at concentration S and the organism under study at cell density N . S_0 represents the input concentration of limiting resource and D is the dilution rate which describes the influx of resource and the rate at which cells and unused resource are removed from the chemostat.

A theoretical basis for understanding the dynamics of competition for a limiting resource in a chemostat was originally developed by Monod [74] and further studied by Smith and Waltman [98] and Hsu *et al.* [53]. The chemostat equations are based on the following basic assumptions:

$$\begin{aligned} \text{rate of change in resource concentration} &= \text{influx} - \text{resource consumption} - \text{efflux}, \\ \text{rate of change of population size} &= \text{growth} - \text{efflux}. \end{aligned} \tag{A.1}$$

Now let $S(t)$ denote the limiting resource concentration and let $N(t)$ denote the microbial cell density in the culture vessel of volume V at time t . This means that the total amount of limiting resource and the total cell population in the culture vessel are given by $VN(t)$ and $VS(t)$ respectively. Now suppose that each microbial cell consumes the limiting resource at a resource-dependent rate $U(S)$ and reproduces at a rate $B(S)$. Assuming that the total rate of resource consumption is given by the product of resource consumption per cell and the total number of cells within the culture vessel, then using

(A.1) the equations governing total cell population and resource concentration in the culture vessel can be written as

$$\begin{aligned}\frac{d}{dt}(VS(t)) &= \overbrace{DS_0}^{\text{influx}} - \overbrace{U(S)(VN(t))}^{\text{consumption}} - \overbrace{DS(t)}^{\text{efflux}}, \\ \frac{d}{dt}(VN(t)) &= \overbrace{B(S)(VN(t))}^{\text{growth}} - \overbrace{DN(t)}^{\text{efflux}},\end{aligned}\tag{A.2}$$

where S_0 is the concentration of limiting resource in the input reservoir. Since the volume is constant we can divide equation (A.2) by V to obtain

$$\begin{aligned}\frac{d}{dt}S(t) &= d(S_0 - S(t)) - U(S)N(t), \\ \frac{d}{dt}N(t) &= (B(S) - d)N(t),\end{aligned}$$

where $d = \frac{D}{V}$ is the dilution rate measured in chemostat volumes per time.

Appendix B. Adaptive Dynamics Resident Population

Ecological Equilibria

The system (3.2) has two ecological equilibria given by $(S_0, 0)$ and $(S^*, N_{x_r}^*)$ where

$$S^* = \frac{DK}{rc(x_r)f(x_r) - D} \quad \text{and} \quad N_{x_r}^* = \frac{D(S_0 - S^*)(K + S^*)}{f(x_r)S^*}.$$

For physically reasonable values of the non-trivial steady state $(S^*, N_{x_r}^*)$ we require $S^* > 0$ so that

$$S^* = \frac{DK}{rc(x_r)f(x_r) - D} > 0 \quad \Rightarrow \quad D < rc(x_r)f(x_r) = D_{crit}. \quad (\text{B.1})$$

In addition, we assume that $N_{x_r}^* > 0$ which requires

$$S^* < S_0 \quad \Rightarrow \quad \frac{DK}{rc(x_r)f(x_r)} < S_0. \quad (\text{B.2})$$

For an ecological equilibrium to be asymptotically stable we require the eigenvalues of the Jacobian matrix evaluated at the equilibrium point to be negative. Starting with the non-trivial ecological equilibrium the Jacobian matrix, $\mathbf{J}^*$, of system (3.3) evaluated at the equilibrium point $(S^*, N_{x_r}^*)$ is given by

$$\mathbf{J}^* = \begin{pmatrix} -D - N_{x_r}^* \left(\frac{f(x_r)}{K+S^*} - \frac{f(x_r)S^*}{(K+S^*)^2} \right) & -\frac{f(x_r)S^*}{K+S^*} \\ N_{x_r}^* c(x_r) r \left(\frac{f(x_r)}{K+S^*} - \frac{f(x_r)S^*}{(K+S^*)^2} \right) & \frac{rc(x_r)f(x_r)S^*}{K+S^*} - D \end{pmatrix}, \quad (\text{B.3})$$

and its eigenvalues are given by

$$\lambda_1 = -D \quad \text{and} \quad \lambda_2 = -\frac{(-S_0(D - rc(x_r)f(x_r)) + DK)(-rf(x_r)c(x_r) + D)}{rc(x_r)f(x_r)K}.$$

Using the conditions in (B.1) and (B.2) it follows that λ_1 and λ_2 are negative whenever the non-trivial ecological equilibrium exists.

For the trivial equilibrium $(S_0, 0)$ the Jacobian matrix of (3.3) evaluated at the equilibrium point is given by

$$\mathbf{J}^* = \begin{pmatrix} -D & -\frac{f(x_r)S_0}{K+S_0} \\ 0 & \frac{rc(x_r)f(x_r)S_0}{K+S_0} - D \end{pmatrix}, \quad (\text{B.4})$$

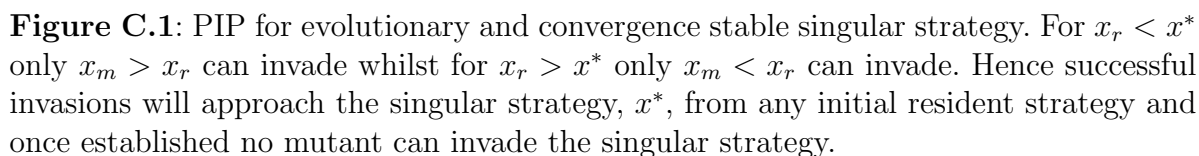
with associated eigenvalues

$$\lambda_1 = -D \quad \text{and} \quad \lambda_2 = \frac{S_0(rc(x_r)f(x_r) - D) - DK}{K + S_0}.$$

Here we require $D \geq D_{crit}$ for the equilibrium point to be asymptotically stable. This condition contradicts that of (B.1) and hence the trivial ecological equilibrium point is asymptotically stable whenever the non-trivial equilibrium $(S^*, N_{x_r}^*)$ does not exist.

Appendix C. Adaptive Dynamics Pairwise Invasibility Plot (PIP)

A pairwise invasibility plot (PIP) is used to identify the direction of evolutionary change over a series of mutant invasions. In a PIP the fitness landscape as experienced by a rare mutant correspond to the vertical lines where the resident phenotypic strategy, x_r , is constant. Hence we can determine whether a mutant strategy, x_m , can invade the resident population by calculating the sign of the invasion fitness, $f(x_r, x_m)$, at the particular values of the resident and mutant strategy: if $f(x_r, x_m) > 0$ the mutant strategy can invade and becomes the new resident, if $f(x_r, x_m) < 0$ the resident persists and the mutant strategy dies out. Here we illustrate how PIPs can be used to determine the direction of evolutionary change by means of two examples. First, consider the resident strategy, x_r , in Figure C.1. Now consider what happens when a rare mutant appears: If the mutant x_{m1} appears $f(x_r, x_{m1})$ is negative. Hence x_{m1} cannot invade the resident population and the mutant dies out. However, if the mutant x_{m2} appears $f(x_r, x_{m2})$ is positive. Hence x_{m2} can invade the resident population and becomes the new resident population. Continuing to analyze which mutant can invade the new resident population in a similar manner, we find that for $x_r < x^*$ only $x_m > x_r$ can invade and for $x_r > x^*$ only $x_m < x_r$ can invade. This is the definition of a convergence stable singular strategy which means that the singular strategy is approached through a series of invasions over evolutionary time. Moreover, due to the fact the vertical line through the singular strategy, x^* , in Figure C.1 lies entirely within an area of negative invasion fitness, this means that no mutant can invade the singular strategy once it has been established, hence the singular strategy is also evolutionary stable (ESS).



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invasions away from the singular strategy. In addition, the vertical line through the singular strategy, x^* , in Figure C.2 lies entirely within an area of positive invasion fitness, this means that mutants can invade the singular strategy and hence the singular strategy is not evolutionary stable.

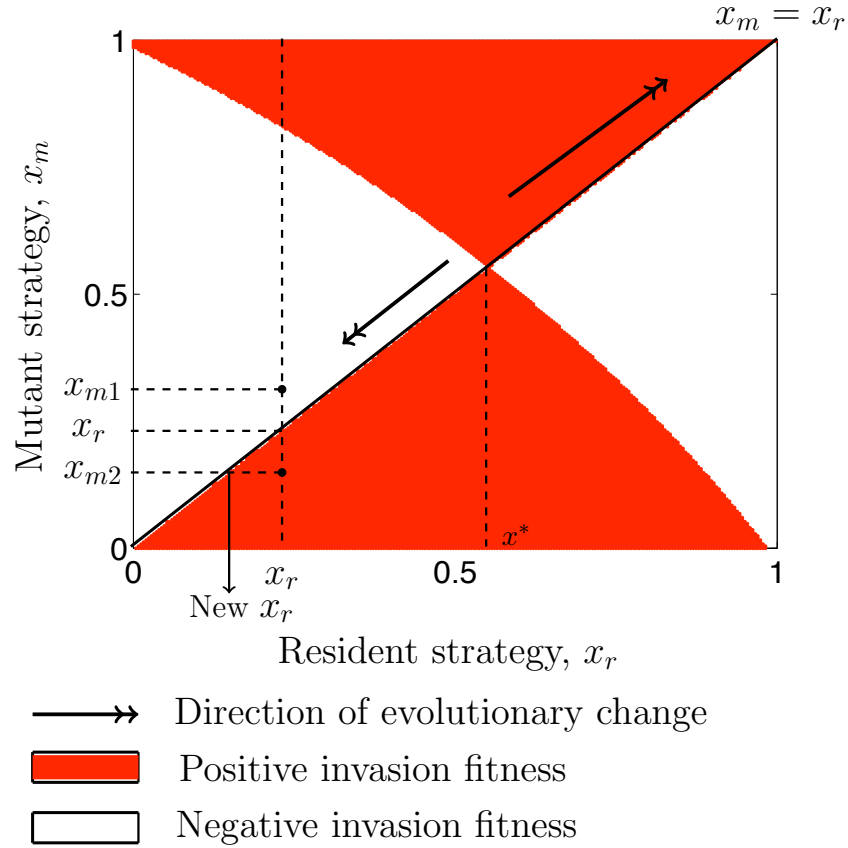


Figure C.2: PIP for evolutionary and convergence unstable singular strategy. For $x_r < x^*$ only $x_m < x_r$ can invade whilst for $x_r > x^*$ only $x_m > x_r$ can invade. Hence successful invasions will move away from the singular strategy, x^* , towards the boundary of the phenotypic domain and evolutionary outcome will depend on the initial resident strategy.

Appendix D. Model Parameterization in the Standardized Antifungal Environment

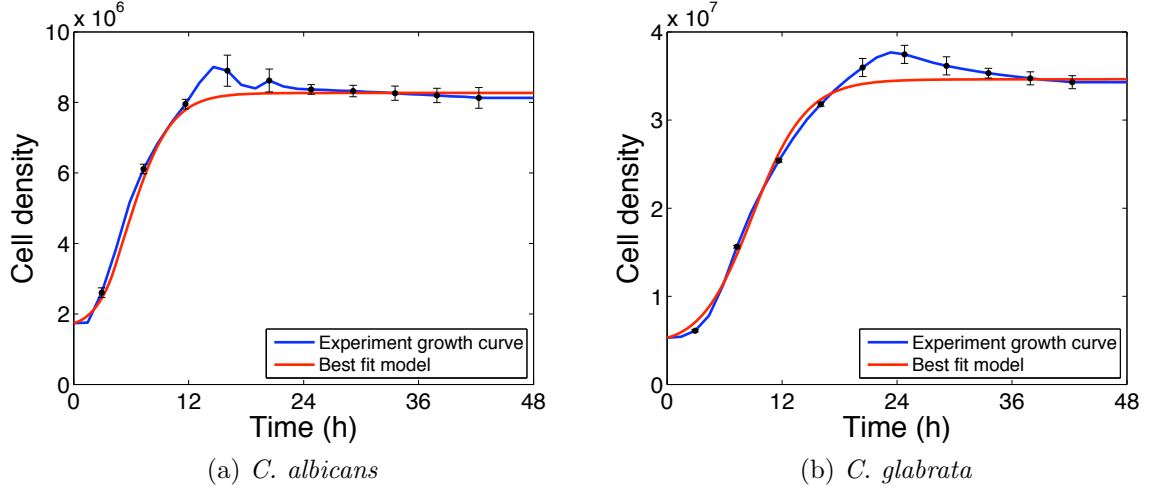


Figure D.1: Cellular growth and resource consumption in the absence of FLC under the standardized conditions for antifungal susceptibility testing. Experimental data is average of 3 replicates. Best-fit model parameters are given in Table D.1.

Parameter	<i>C. albicans</i> value	<i>C. glabrata</i> value	Source
G	0.00184 cell/pmol ATP	0.0083 cell/pmol ATP	Estimated
D	10^{-12} /cell/min	10^{-12} /cell/min	[68]
n_1	2 pmol ATP/pmol glucose	2 pmol ATP/pmol glucose	[18]
n_2	30 pmol ATP/pmol glucose	30 pmol ATP/pmol glucose	[18]
V_1	0.18 pmol glucose/min/cell	0.14 pmol glucose/min/cell	Estimated
K_1	1×10^6 pmol glucose	1.9×10^8 pmol glucose	Estimated
V_2	0.9 pmol $X_{int,a}$ /min/cell	0.5 pmol $X_{int,a}$ /min/cell	Estimated
K_2	1×10^9 pmol $X_{int,a}$	3×10^9 pmol $X_{int,a}$	Estimated

Table D.1: The ecological model parameters for cellular growth and resource consumption in the absence of FLC under the standardized conditions for antifungal susceptibility testing.

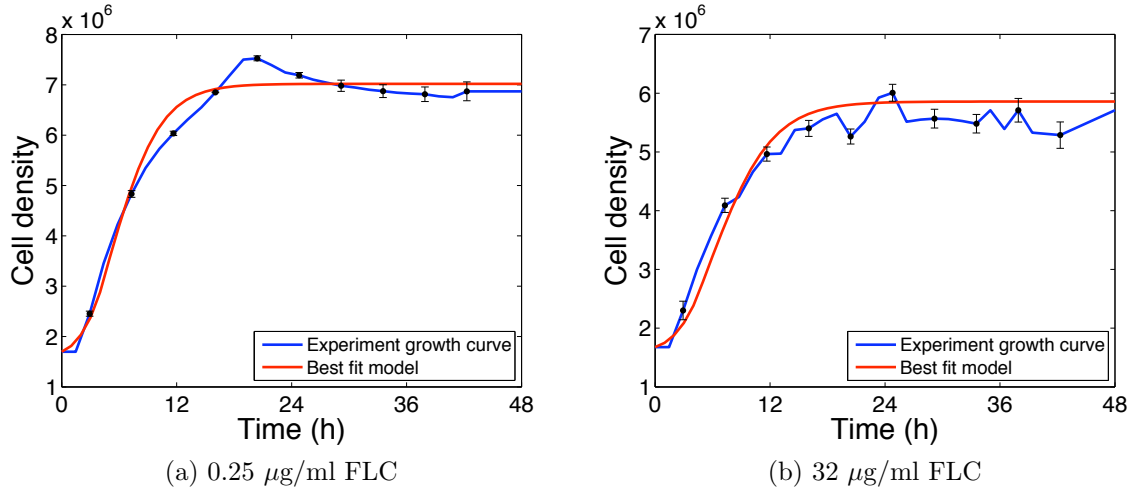


Figure D.2: *C. albicans* growth dynamics upon administration of FLC. Experimental data is average of 3 replicates. Best-fit model parameters are given in Table D.2.

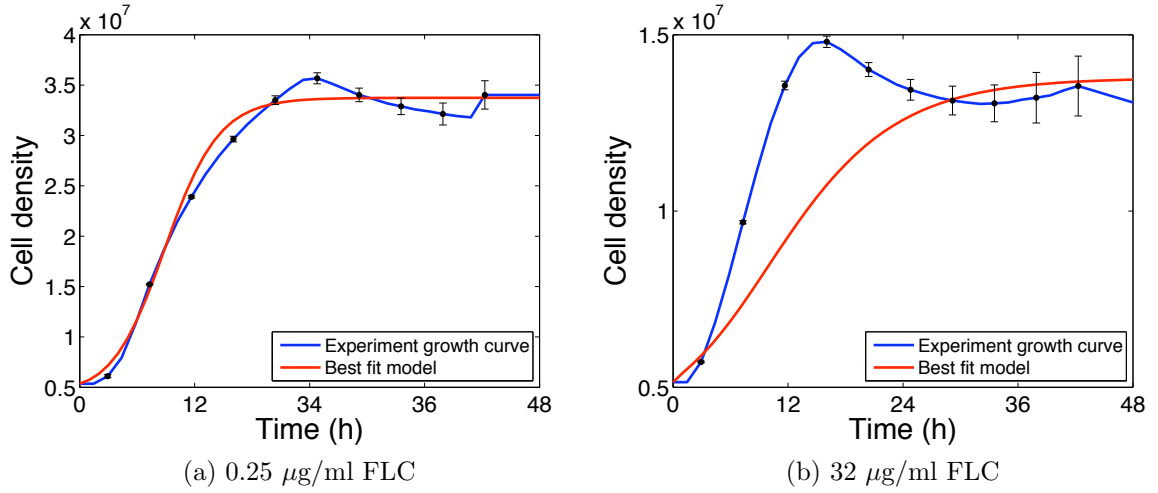
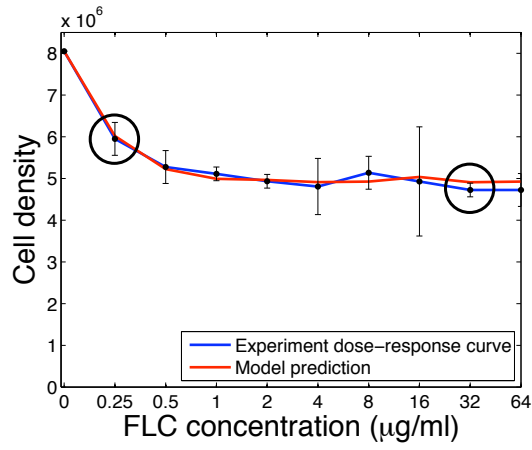


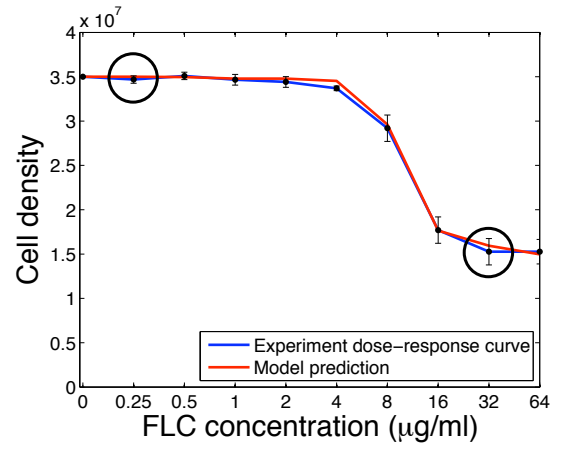
Figure D.3: *C. glabrata* growth dynamics upon administration of FLC. Experimental data is average of 3 replicates. Best-fit model parameters are given in Table D.2.

Parameter	<i>C. albicans</i> value	<i>C. glabrata</i> value	Source
a	0.36	0.75	Estimated
b	1.4×10^{-8} pmol $FLC_{int,a}$ /cell	1.5×10^{-7} pmol $FLC_{int,g}$ /cell	Estimated
n	3	1.5	Estimated

Table D.2: The ecological model parameters for growth upon FLC administration under the standardized conditions for antifungal susceptibility testing.



(a) *C. albicans*



(b) *C. glabrata*

○ FLC concentrations used to parameterize ecological model

Figure D.4: Dose response curves at 48 hours. Experimental data is average of 3 replicates.