# Experimental adaptive radiation with respect to substrate metabolism

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## Abstract

The ecological theory of adaptive radiation is the main theoretical framework that has been used to explain the evolution of biodiversity on the planet Earth. Despite its broad acceptance in the scientific community, the theory of adaptive radiation cannot explain the pattern by which biodiversity evolves in a given environment and as a result could not predict the outcome of evolution. The objective of my PhD research was to show that the pattern of metabolic specialization in the laboratory can be predicted from biochemical principles and then be used to interpret the pattern found in natural populations.

In the second chapter I started by documenting the pattern of specialization found in isolates from wild yeast populations. We found geographical variation of substrate use at continental, regional, and local scales. Isolates from Europe and North America could be distinguished on the basis of the pattern of yield across substrates. Two geographical races at the North American sites also differed in the pattern of substrate utilization. Substrate utilization patterns were also geographically correlated at local spatial scales. Pairwise genetic correlations between substrates were predominantly positive, reflecting overall variation in metabolic performance, but there was a consistent negative correlation between categories of substrates in two cases: between the core diet and the ancillary diet, and between pentose and hexose sugars. Such negative correlations in the utilization of substrate from different categories may indicate either intrinsic physiological trade-offs for the uptake and utilization of substrates from different categories, or the accumulation of conditionally neutral mutations. Divergence in substrate use accompanies genetic divergence at all spatial scales in *S. paradoxus* and may contribute to race formation and speciation.

In chapter 3, I conducted an experiment to test that the pattern of metabolic specialization among experimental populations of wild yeast could be predicted based on the biochemical properties of the environment and the cells living there. Briefly, I used experimental populations of yeast and evolved them in 12 defined cultures where each culture was supplemented with a single-carbon-substrate. These substrates belonged to 3 different metabolic pathways therefore; I could test the effect of local adaptation on alternative substrates with similar or different metabolic pathways. My results show that local adaptation to a given substrate did not consistently affect growth on other substrates in the same pathway. Individual lines that adapted more successfully than the average tended to be superior on other substrates in the same pathway. I found no evidence for trade-offs between substrates on the same pathway. The indirect response of substrates on other pathways, however, was consistently negative, with little correlation between direct and indirect responses. The conclusion therefore, is that the grain of specialization in this case is the metabolic pathway, and that specialization appears to evolve through mutational degradation. This is the first time that the evolution of specialization could be predicted by the biochemical properties of the selection environment.

In chapter 4, I investigated the consequences of metabolic adaptation of yeast populations for selection in subsequent stressful environments. Since, many species including bacteria, yeast, and fruit fly have general stress response systems that govern resistance against wide variety of stressors we hypothesized that evolutionary exposure of yeast populations to a prior stressful environment might facilitate adaptation of such populations to subsequent stressors in future.

Surprisingly, I found that prior stress by starvation impairs the physiological response but enhances the evolutionary response to subsequent stress by a different agent. The physiological response may be impaired because selection for growth through serial transfer is antagonistic to

selection for individual integrity. The evolutionary response may be enhanced because chronic sublethal stress increases the genomic mutation rate. Our result shows that the two are negatively correlated because they are inversely related to the severity of the prior stress: the physiological response to a subsequent stressor is more impaired and the evolutionary response more elevated by more severe prior stress. This interpretation is consistent with all our results, although it must await explicit demonstration of the mechanisms responsible. Our results further suggest that the evolutionary rescue of populations threatened by successive unrelated stresses is a two-part process. The populations are at heightened risk in the short term, but, should they survive, are more likely to survive in the longer term. The fate of populations in environments subject to a series of shocks will depend on both physiological and evolutionary processes, whose interaction must be understood in order to predict the likelihood of rescue.

## Résumé

La théorie écologique du rayonnement adaptatif est la théorie principale proposée pour expliquer comment la biodiversité sur la planète Terre a évolué. Malgré son acceptation dans la communauté scientifique, la théorie de la radiation adaptative ne peut pas expliquer la manière par lequel la biodiversité évolue dans un environnement donné, et, par conséquent, ne peut pas prédire les résultats de l'évolution dans ces environnements. L'objectif de ma thèse de doctorat est de démontrer que le modèle de spécialisation métabolique dans un laboratoire peut être prédit à partir de principes biochimiques et ensuite utilisé pour interpréter les structures trouvées dans les populations naturelles.

Dans le deuxième chapitre, j'ai commencé par documenter le modèle de spécialisation trouvé dans les isolats de populations de levures sauvages. Nous avons trouvé de la variation géographique de l'utilisation de substrat à l'échelle continentale, régionale et locale. Les isolats provenant de l'Europe et l'Amérique du Nord pourraient être distingués sur la base de leurs préférences de substrats. Deux races géographiques sur les sites nord-américains diffèrent aussi dans le mode d'utilisation de substrat. Les modes d'utilisation de substrat ont également été géographiquement corrélés à des échelles spatiales locales. Les corrélations génétiques par paires entre substrats étaient majoritairement positifs, reflétant la variation globale de la performance métabolique, mais il y avait une corrélation négative systématique entre catégories de substrats dans deux cas: entre le régime de base et le régime accessoire, et entre les pentoses et hexoses. Ces corrélations négatives dans l'utilisation de substrat à partir de différentes catégories peuvent indiquer soit des compromis physiologiques intrinsèques pour l'adoption et l'utilisation de substrats de différentes catégories, ou l'accumulation de mutations conditionnellement neutres.

La divergence de l'utilisation de substrats accompagne la divergence génétique à toutes les échelles spatiales dans *S. paradoxus* et peut contribuer à la race la formation et de la spéciation.

Dans le chapitre 3, j'ai testé le modèle de spécialisation métabolique chez les populations expérimentales de levures sauvages pour voir si on peut prédire leur spécialisation métabolique sur la base des propriétés biochimiques de l'environnement et les cellules qui y vivent. Brièvement, j'ai utilisé des populations expérimentales de levure pour les évoluer dans 12 cultures définies où chaque culture a été complétée avec une seule source de carbone. Puisque ces substrats appartenaient à 3 voies métaboliques différentes je pouvais tester l'effet de l'adaptation locale sur des substrats alternatifs avec les voies métaboliques semblables ou différents. Mes résultats montrent que l'adaptation locale à un substrat donné n'affecte pas toujours la croissance sur d'autres substrats dans la même voie. Les lignes individuelles qui se sont adaptées avec le plus de succès avaient tendance à être supérieure à d'autres substrats dans la même voie. J'ai trouvé aucune preuve de compromis entre les substrats sur la même voie. La réponse indirecte de substrats sur d'autres voies, cependant, était toujours négative, avec peu de corrélation entre les réponses directes et indirectes. Par conséquent, la source de la spécialisation dans ce cas est la voie métabolique, et la spécialisation semble évoluer à travers la dégradation de mutation. Ceci est la première fois que l'évolution de la spécialisation est prédite par les propriétés biochimiques de l'environnement de sélection.

Dans le chapitre 4, j'ai étudié les conséquences de l'adaptation métabolique des populations de levures pour la sélection dans des environnements stressants. Puisque de nombreuses espèces, y compris les bactéries, les levures et la mouche du vinaigre ont des systèmes de réponse au stress généraux qui régissent la résistance contre une grande variété de facteurs de stress, nous

émettons l'hypothèse que l'exposition évolutive des populations de levure à un environnement stressant pourrait faciliter l'adaptation de ces populations aux facteurs de stress ultérieurs.

Étonnamment, j'ai trouvé qu'être exposé par le stress par voie de famine altère la réponse physiologique, mais améliore la réponse au stress évolution ultérieure par un agent différent. La réponse physiologique peut être altérée parce que la sélection pour la croissance à travers le transfert de série est antagoniste à la sélection de l'intégrité individuelle. La réponse évolutive peut être améliorée parce que le stress sublétale chronique augmente le taux de mutation génomique. Notre résultat montre que les deux sont en corrélation négative parce qu'ils sont inversement proportionnels à la gravité de la contrainte préalable: la réponse physiologique à un stress ultérieur est plus altérée et la réponse de l'évolution plus élevé de contrainte avant plus sévère. Cette interprétation est compatible avec tous nos résultats, même si elle doit attendre la démonstration explicite des mécanismes responsables. Nos résultats suggèrent en outre que le sauvetage de l'évolution des populations menacées par des contraintes indépendantes successives est un processus en deux parties. Les populations sont à risque accru à court terme, mais, si elles survivent, sont plus susceptibles de survivre à long terme. Le sort des populations dans des environnements soumis à une série de chocs dépendra à la fois des processus physiologiques et évolutifs, dont l'interaction doit être comprise afin de prédire la probabilité de sauvetage.

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At last but not least, I am most grateful to my parents, brother, family and friends for their unconditional love. I shall proudly dedicate this thesis to my father, Prof. Nozar Samani, my beautiful mother Saeideh and my handsome brother Alireza.

## Contribution of authors

The research in this thesis which is presented in 5 chapters (including the Summary chapter) was predominantly conducted by me. I primarily developed the research hypothesis, designed, implemented and conducted the surveys and experiments, analysed the data, and wrote the documents and manuscripts in this thesis. In particular, chapter 1 which was a short review of the literature was written by me and edited by Dr. Graham Bell. In the chapter 2 the field survey and collection of samples was conducted by myself, Kyra Bonasia (McKelvey), Dr. Austin Burt, Dr. Vassiliki Koufopanou, Dr. Thomas Bell and Dr. Christian Landry. The lab work was conducted by me and Dr. Graham Bell. Data analysis was performed by me, Dr. Graham Bell and Dr. Etienne Low-Decarie. The manuscript was written in collaboration with all the authors.

In chapters 3 and 4, I designed and conducted the experiments. Data analysis was performed in collaboration with Dr. Graham Bell and we both wrote the manuscripts.

## Statement of originality

The Manuscripts presented in this thesis (chapters 2-4) are novel contributions to scientific knowledge in fungal ecology and evolution, evolution of metabolic specialization, and the evolutionary rescue of stressed populations.

In chapter 2 we extend our understanding of metabolic variation in microbial populations by investigating the use of carbon substrates by random isolates of wild yeast (*Saccharomyces paradoxus*) from two local populations, one in North America and the other in Europe. Our enquiry intended to characterize the potential diet of wild yeast populations, the amount of standing genetic variation in substrate utilization, and the pattern of trade-offs among substrates or groups of substrates. We showed that divergence in substrate use accompanies genetic divergence at all spatial scales in *Saccharomyces paradoxus* and may contribute to race formation and speciation.

In chapter 3 the objective of our experiment was to estimate the grain of specialization that evolves when a population is restricted to a single defined substrate. We explored the consequences of metabolic adaptation of experimental populations in the laboratory in relation to alternative metabolic environments and discovered that the grain of specialization in this case is the metabolic pathway, and that specialization appears to evolve through mutational degradation. This is the first time that the evolution of specialization is defined by the biochemical properties of the selection environment.

In chapter 4 we investigated the consequences of metabolic adaptation of yeast populations for selection in subsequent stressful environments. Surprisingly, we found that prior stress by starvation impaired the physiological response but enhanced the evolutionary response to

subsequent stress by a different agent. Our result showed that the physiological and evolutionary responses were negatively correlated because they are inversely related to the severity of the prior stress. Our finding suggests that the evolutionary rescue of populations threatened by successive unrelated stresses is a two-part process. The populations are at heightened risk in the short term, but, should they survive, are more likely to survive in the longer term. The fate of populations in environments subject to a series of shocks will depend on both physiological and evolutionary processes, whose interaction must be understood in order to predict the likelihood of rescue.

# Chapter 1: Introduction (Mechanisms of metabolic trade-offs)

Adaptation to any particular way of life may be accompanied by loss of adaptation to ancestral or alternative conditions, thereby creating a degree of specialization. Generalists evolve when populations are exposed to variable conditions in space and time (Kassen, 2002). In contrast, if populations experience a specific environmental condition for a long time during which individuals compete for a limited resource, the direct response to selection would be in the form of a higher capability to capture and process that particular resource. If adaptation to such conditions is coupled with loss of adaptation to ancestral or alternative conditions selection would also lead to an antagonistic response giving rise to specialists (Kassen, 2002). This is the ecological theory of adaptive radiation in a nut shell (Schluter, 2000). Since its construction by Simpson (1944, 1953), Lack (1947), and Dobzhansky (1951) the ecological theory of adaptive radiation remains the main explanation for adaptive radiation (Schluter, 2000). Despite its broad acceptance in the scientific community the ecological theory of adaptive radiation is unable to answer a wide range of questions (MacLean, 2005; Prosser *et al.*, 2007; Kassen, 2009; Saxer *et al.*, 2010). For example, it is unable to provide clear insights into the genetics of adaptive radiation. More specifically, despite its generality it is unable to shed light in the genetic basis of adaptive radiation and specialization, nor does it provide any mechanism for the evolution of trade-offs at the molecular level. Consequently, it lacks the potential to provide predictive insights into the pattern of adaptive radiation and specialization (Kassen, 2009).

In this thesis I propose a mechanism for the evolution of trade-offs as the antagonistic response to selection which provides a predictive power for the pattern of diversification and evolution of generalists and specialists in specific environments.

**The antagonistic response to selection evolves through functional interference and mutational degradation.** Ecological specialization evolves if adaptation to a given selection pressure is accompanied with an antagonistic response. The antagonistic response to selection (trade-offs) may evolve by two mechanisms. First, functional interference: this occurs when increase in fitness in one environment results in an equivalent fitness decline in another environment. Second, mutational degradation: this occurs when the accumulation of mutations that are neutral in the residence environment act as deleterious mutations when transferred to another environment (Bell, 2008).

The rate of the flux of a biochemical pathway is usually limited by the enzyme with the slowest reaction rate called the rate-limiting enzyme. The rate-limiting enzyme in a metabolic pathway is usually the first enzyme and/or the one at a branching point. A beneficial or deleterious mutation in the first rate-limiting enzyme could impose an opposite competitive effect on consumption rates of other substrates. Similarly, beneficial or deleterious mutations in a branching point enzyme might cause a shift in the mode of metabolism by blocking one branch and thereby directing the flux through the other branch of the pathway (Rausher, 2013). Thus, I speculate that mutations in rate-limiting enzymes potentially lead to functional interference (pleiotropic effect) and should be a cause for ecological specialization.

Antagonistic response to selection could also evolve due to the accumulation of mutations that are neutral in the environment of selection but cause a loss-of-function in some alternative environment (Bell, 2008). These are called conditional loss-of-function mutations. I hypothesize that a conditional loss-of-function mutation (LFM) in any of the steps of a metabolic pathway can result in the inability to consume the upstream substrates and therefore evolving some

degrees of specialization. Therefore, mutational degradation resulting from accumulation of conditional LFMs could be the other cause of specialization. I further hypothesize that specialization due to occurrence of conditional LFMs should evolve more often among substrates that are located on different metabolic pathways. For example, microbial lines that were selected on fructose should more often lose their ability to metabolise proline than mannose because, fructose and proline are metabolised through different pathways while enzymes involved in metabolising fructose and mannose are almost identical. Additionally, I predict that mutational degradation should occur more frequently than functional interference as a consequence of specific metabolic adaptation because the target size for conditional LFMs is much larger.

My purpose in this chapter is to test my hypotheses by reviewing the literature on the evolution of antagonistic response to selection in experimental evolution of metabolism using microbes.

**How often do trade-offs evolve in experimental evolution?** Evolution of trade-offs have been observed among different model organisms and in a variety of phenotypic traits and environments. These studies include trade-offs between nutrition and stress resistance (King *et al.*, 2006; Oakley *et al.*, 2014), $CO_2$ uptake affinity at different $CO_2$ concentrations (Collins *et al.*, 2006), photosynthetic versus heterotrophic growth (Bell & Reboud, 1997; Reboud & Bell, 1997; Kassen & Bell, 1998), mixotrophs specializing into pure autotrophs and heterotrophs (Troost *et al.*, 2005), variable pH environments (Bradley S. Hughes *et al.*, 2007), growth rate versus yield (Novak *et al.*, 2006), growth versus resistance to antibiotics (Björkman *et al.*, 2000; Perron *et al.*, 2006), morphological evolution in threespine stickleback (Barrett *et al.*, 2009), social gene epistases and associated trade-offs (Sinervo *et al.*, 2008), social behaviors versus

growth rate (Velicer *et al.*, 1998), oxidative stress resistance and DNA repair (Torres-Barceló *et al.*, 2013), growth components versus resistance to pathogens (Rigby *et al.*, 2002; Luong & Polak, 2007; Vijendravarma *et al.*, 2009), viral predators (Lenski, 1988), growth at high versus low temperatures (Bennett *et al.*, 1992; Bennett & Lenski, 1997, 2007; Carrière & Boivin, 2001), local adaptation and flowering time variation in plants (Kover *et al.*, 2009; Leinonen *et al.*, 2013), between the sexual and asexual phases of the yeast life cycle (Zeyl *et al.*, 2005), between different parasite hosts (Ebert, 1998; Turner & Elena, 2000; Duffy *et al.*, 2006; Spor *et al.*, 2009), amang growth substrates (Velicer *et al.*, 1998; Cooper & Lenski, 2000; Cooper, 2002; Zhong *et al.*, 2004; Ostrowski *et al.*, 2005; Maughan *et al.*, 2006; Wenger *et al.*, 2011; Hong & Nielsen, 2013; Kvitek & Sherlock, 2013; Carroll *et al.*, 2014; Samani *et al.*, 2015), and parasitoid resistance and competitive ability (Jessup & Bohannan, 2008).

Kassen (2014) reviewed literature on the evolution of trade-offs in microbial systems which measured the fitness of selected lines in both selection and alternative environments through a full reciprocal transplant assay. He found that adaptation as the direct response to selection was accompanied by a cost in 35 of the 40 pairs of selection/alternative environments that were tested (Table S1, data obtained from Kassen (2014)). Kassen (2014) argues that adaptation in the selection environment coevolves with a trade-off because fitness increase was larger in the environment of selection than in the alternative environment.

It appears that local adaptation is ubiquitously accompanied by some kind of trade-off among a wide variety of organisms and environments. These trade-offs however, could be asymmetrical among pair of traits (Kassen, 2014). Kassen (2014) suggests that genes involved in the expression of for example two traits could have different patterns of pleiotropic fitness effects on each other thus giving rise to asymmetric responses to divergent selection. Lee *et al.* (2009) also

put forward a hypothesis for the existence of asymmetry in the evolution of trade-off for adaptation to C1 versus multi-C substrates in *Methylobacterium extorquens AM1* bacterium which emphasizes the difference between the number of genes involved in each of the traits. The trait that has more genes involved in its regulation and expression has a larger mutational target size for conditional LFMs to emerge in addition to a larger cost due to the expression of a larger number of enzymes. This could be another reason for the asymmetrical evolution of trade-offs among multiple environments.

**Which of mutational accumulation or functional interference makes the largest contribution to costs of adaptation in selection experiments?** The contributions of functional interference and mutational degradation to the antagonistic response can be evaluated by regressing growth in the ancestral or alternative conditions on growth in the environment of selection. The slope of this relationship reflects functional interference, while its elevation reflects mutational degradation(MacLean & Bell, 2002). Moreover, if during adaptation the decline in the alternative environments occurs immediately and equal to the degree of adaptation in the environment of selection, this is usually due to antagonistic pleiotropic effects of the mutations that are being selected (Cooper & Lenski, 2000). Another method to discern which mechanism is responsible for the observed trade-off is to select the evolved lines in an alternative environment. If the increase in fitness in the alternative environment was accompanied by an immediate and equal loss of fitness in the original environment of selection then antagonistic pleiotropy is the causal mechanism of the trade-offs. However, if fitness improvement when selecting in the alternative environment is not accompanied by any loss of fitness in the original

environment of selection then conditional LFMs are responsible for the observed trade-offs (Reboud & Bell, 1997; Bell, 2008).

Studies investigating the emergence of trade-offs in experimental systems of metabolic activity in microbes are very limited. I found twenty five studies that have used experimental evolution of metabolism in microbes to test for the occurrence of mutational accumulation and functional interference. Several of these studies (Ciriacy & Breitenbach, 1979; Bell & Reboud, 1997; Reboud & Bell, 1997; Funchain *et al.*, 2000; Cooper *et al.*, 2001; MacLean & Bell, 2002; Ostrowski *et al.*, 2007; Lee *et al.*, 2009; Warringer *et al.*, 2011; Zörgö *et al.*, 2012; Kvitek & Sherlock, 2013; Leiby & Marx, 2014) found evidence for the occurrence of mutational accumulation as an indirect consequence of adaptation to specific carbon substrates as sole carbon source. Some other (Cooper & Lenski, 2000, 2010; Takemoto & Liao, 2001; Lunzer *et al.*, 2002; Zhong *et al.*, 2004; Liao & Laufs, 2005; Jasińska *et al.*, 2007; Lee *et al.*, 2009; Hong & Nielsen, 2013; Jasmin & Zeyl, 2013; Rausher, 2013; Carroll *et al.*, 2014) found antagonistic pleiotropy to be the source of the observed trade-off in their experiments while three studies (Travisano & Lenski, 1996; Jasmin & Kassen, 2007; Jasmin & Zeyl, 2013) found positive pleiotropy for the utilization of other carbon substrates as a consequence of specific adaptation to other carbon sources.

Anderson et al., (2013) investigated local adaptation in the field among recombinant inbred lines and parental lines of *Boechera stricta* (*Brassicaceae*) exposed to their parental environment. Interestingly, they showed that 2.8% of the genome exhibited antagonistic pleiotropy and 8% contributed to conditional neutrality. Their finding supports my prediction that the genomic target size for conditional LFMs is much larger than the genomic target size for mutations exhibiting functional interference. However, among the twenty five studies that I found twelve

studies found evidence for conditional LFMs and another twelve found evidence for functional interference as the mechanism for evolved trade-offs.

**Is there any evidence in the literature supporting metabolic specialization through mutational accumulation by emergence of conditional loss-of-function mutations in unused metabolic pathways or functional interference by mutations in rate-limiting enzymes?** All the above mentioned studies that found mutational accumulation as the mechanism for the observed trade-offs were able to directly (by sequencing) or indirectly (by phenotypic assays briefly described in the previous section) confirm the emergence of conditional LFMs in unused metabolic traits (pathways) in the populations as the indirect and sometimes direct response to selection.

Cooper *et al.*, (2001) observed loss of ribose utilization among *E-coli* populations that were selected on glucose limited medium and attributed this cost to antagonistic pleiotropy. The molecular analysis of their lines however, showed that loss of ribose catabolism was due to the deletion of part or all of the ribose operon (*rbs* genes). They were able to confirm that the improvement of growth in glucose environment was due to the emergence of deletion mutations in ribose operons and thus attributed these mutations to antagonistic pleiotropy. Hottes *et al.*, (2013) conducted a meta-analysis of 144 conditions using data from seven studies, and found that in almost all of the examined conditions (139/144) loss-of-function mutations were the cause of adaptation as the direct response to selection. Kvitek & Sherlock (2013) also observed selection of loss-of-function mutations in a variety of regulatory pathways as the direct response to selection in a constant environment leading to the evolution of ecological specialists. Loss-of-

function mutations in regulatory and catabolic pathways could therefore become fixed in the populations as the direct response to selection in constant environments causing specialization while as a second attribute contribute to a cost of adaptation.

A few of the studies that found antagonistic pleiotropy as the source of the trade-off in their experiment have identified the responsible mutation(s).These studies support the evolution of functional interference by mutations in rate-limiting enzymes. For instance, (Liao & Laufs, 2005) showed that the emergence of loss-of-function mutations in a given rate-limiting enzyme caused a decline in the biochemical activity of other related molecules or studies that showed restricting the rate-limiting step of one pathway resulted in an increase in the activity of other pathways (Takemoto & Liao, 2001; Liao & Laufs, 2005; Jasińska *et al.*, 2007). Modeling the flux control of branched metabolic pathways, Rausher (2013) showed that beneficial mutations in the gene of a branching enzyme can reduce the flux in the counterpart branch. Carroll *et al.* (2014) investigated the basis of trade-offs during adaptation to methanol and succinate as a sole source of carbon and energy using *Methylobacterium extorquens AM1*. They found that a loss-of-function mutation in formate-tetrahydrofolate ligase (*ftfl* ), was consistently responsible for growth improvement in succinate selected lines while it is costly because the cells lose their ability to metabolise methanol. Formate-tetrahydrofolate ligase has been shown that act as rate-limiting step in methanol catabolic pathway for the conversion of formate to serine (Strong & Schirch, 1989). Methanol when provided as the sole carbon source will be converted to formate. Formate can then pursue two branching pathways: one branch catalyses formate to serine by formate-tetrahydrofolate ligase to account for the structural requirements of the cells. After that a fraction of serine will be converted to pyruvate, through which will enter the ATP-producing pathways required for propagation and growth of the cells. The other branch converts formate to

formylmethanofuran, which will eventually enter the respiration pathway via Acetyl-CoA. Being a rate-limiting enzyme, Formate-tetrahydrofolate ligase controls the rate of the flux of formate into the two branches described above (Strong & Schirch, 1989). If methanol is provided as the sole carbon source both of these branches must be active in order for the cells to produce serine (and eventually other amino acids using serine as the initial substrate) and ATP molecules to propagate and grow. A loss-of-function mutation in the formate-tetrahydrofolate ligase would therefore cripple cells whose only carbon source is methanol. Succinate, on the other hand, can directly enter the tricarboxylic acid cycle to produce energy. Succinate enters a variety of other pathways to account for the production of other necessary biomolecules including serine. Cells whose only source of carbon and energy is succinate would have no need for the formate-tetrahydrofolate ligase activity. Therefore as shown by Carroll *et al.*, (2014) a loss-of-function mutation in this enzyme consistently emerges among succinate selected lines improving growth in succinate while ceasing growth of these cells when solely grown on methanol. The loss-of-function mutation observed in formate-tetrahydrofolate ligase would presumably shift the flux of formate molecules into the production of formylmethanofuran, thus providing an additional flow of substrates to the respiration pathway leading to higher production of ATP and thus improving growth among succinate selected lines.

My research program had three main components. The first (chapter 2) documents the pattern of specialization found in isolates from wild yeast populations. The second (chapters 3) explores the consequences of metabolic adaptation of experimental populations in the laboratory in relation to alternative metabolic environments. And the third (chapter 4) investigates the consequences of metabolic adaptation of yeast populations for selection in subsequent stressful

environments. The overall goal of the research is to show that the pattern of metabolic specialization in the laboratory can be predicted from biochemical principles and then used to interpret the pattern found in natural populations.

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# Chapter 2: Metabolic variation in natural populations of wild yeast

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## Abstract

Ecological diversification depends on the extent of genetic variation and on the pattern of covariation with respect to ecological opportunities. We investigated the pattern of utilization of carbon substrates in wild populations of budding yeast *Saccharomyces paradoxus*. All isolates grew well on a core diet of about 10 substrates and most were also able to grow on a much larger ancillary diet comprising most of the 190 substrates we tested. There was substantial genetic variation within each population for some substrates. We found geographical variation of substrate use at continental, regional and local scales. Isolates from Europe and North America could be distinguished on the basis of the pattern of yield across substrates. Two geographical races at the North American sites also differed in the pattern of substrate utilization. Substrate utilization patterns were also geographically correlated at local spatial scales. Pairwise genetic correlations between substrates were predominantly positive, reflecting overall variation in metabolic performance, but there was a consistent negative correlation between categories of substrates in two cases: between the core diet and the ancillary diet, and between pentose and hexose sugars. Such negative correlations in the utilization of substrate from different categories may indicate either intrinsic physiological trade-offs for the uptake and utilization of substrates from different categories, or the accumulation of conditionally neutral mutations. Divergence in substrate use accompanies genetic divergence at all spatial scales in *Saccharomyces paradoxus* and may contribute to race formation and speciation.

*Keywords*: Microbial metabolic diversity, Metabolic trade-offs, Ecological diversification, Evolution, Genetic variation, *Saccharomyces paradoxus*

## Introduction

Populations adapt to altered conditions of growth through natural selection, provided that there is genetic variation in fitness. Adaptation may be caused either by the sorting of standing genetic variation or by the cumulative selection of successive beneficial mutations (Barrett & Schluter, 2008). The primary source of variation, and thereby the balance between sorting and cumulative selection, depends in part on the size of the population, because this will govern the number of beneficial mutations that arise during a given period of time. Very large asexual populations can adapt through mutation alone (Bell 2008). Experimental populations of unicellular microbes such as bacteria, phytoplankton and yeasts can steadily improve when cultured in a novel environment (Kawecki *et al.*, 2012a) and may evolve new metabolic capabilities (Bell 2013; Blount et al. 2008). Conversely, lineages that have diverged ecologically over hundreds of millions of years may evolve similar phenotypes after a brief period of laboratory culture (Gravel *et al.*, 2012). Animals and plants are larger and hence less abundant. The size of a local population is difficult to define because its limits are unclear, but the steep decline in maximum abundance with body size (Peters & Wassenberg, 1983) implies that the local population of animals and plants experiencing altered conditions often comprises only a few hundred or a few thousand individuals. The rate of supply of beneficial mutations will be correspondingly low, and most evolutionary change will be based on standing variation (Barrett & Schluter, 2008).

The relation between body size and abundance has led evolutionary studies of microbes to concentrate on mutation supply as a source of variation, whereas studies of animals and plants have emphasized standing variation. Microbial experimental evolution often involves the study of natural selection in populations descending from a single clone. Consequently, the quantity of standing variation for ecologically relevant traits in natural populations of microbes has rarely

been systematically investigated. Indeed, before the genomics era industrially or clinically relevant bacteria and yeasts were often identified by the range of substrates they metabolized, implying that variation within species is inconsequential (Garland & Mills, 1991; McGinnis *et al.*, 1996; Praphailong *et al.*, 1997; Konopka *et al.*, 1998). Detailed surveys of the filamentous fungus *Trichoderma,* however, have detected variation in substrate use among strains of the same species (Kubicek *et al.*, 2003; Druzhinina *et al.*, 2006; Hoyos-Carvajal *et al.*, 2009). More recently, Deng et al. (2014) found evidence for metabolic diversification within species in microbial communities of the Baltic sea.

Metabolic variation has been documented both within and among populations of wild yeast (Schacherer *et al.*, 2009). Geographically isolated populations of *Saccharomyces paradoxus* are genetically and phenotypically distinct (Leducq *et al.*, 2014), whereas wild populations of its sister species, the domestic yeast *Saccharomyves cerevisiae,* are less varied and the entire species expresses only about as much genetic variation as a single population of *S. paradoxus* (Liti *et al.*, 2009). Within local populations, a previous survey of *S. paradoxus* found that the genetic difference between isolates was correlated with their distance apart (Koufopanou *et al.*, 2006), although others were unable to detect such a relationship (Tsai *et al.*, 2008). The pattern of phenotypic variation in microbial populations can now be characterized more easily and extensively than hitherto by the use of high-throughput phenotyping techniques (Konopka *et al.*, 1998). Warringer et al. (2011) measured the growth of 86 isolates from five species of *Saccharomyces* sensu stricto yeasts and found extensive variation both between and within species with respect to almost 200 ecologically relevant conditions, including carbon substrates, nitrogen sources, nutrients, toxins and physical factors (Warringer *et al.*, 2011).

Natural selection will act to eliminate variation through the fixation of a metabolically superior generalist type, if such a type exists. Some degree of specialization may be actively maintained by diversifying selection, however, if there is negative genetic correlation such that the ranking of fitness of genotypes differs among substrates. This might be caused by functional interference between physiologically incompatible processes, or by the mutational degradation of inactive pathways, resulting in a 'trade-off' between the utilization of substrates or sets of related substrates. Specialization will tend to evolve when genotypes compete for depletable resources (MacLean et al. 2005; Doebeli and Dieckmann 2000; Geritz et al. 1998; Martin and Pfennig 2012). The range of specialized types that can be maintained by divergent selection will then depend primarily on the pattern of trade-offs between alternative substrates. Experiments with microbes have identified trade-offs between traits in as a cause of diversification (Gudelj *et al.*, 2010), but the extent of metabolic variation and the pattern of trade-offs in natural populations of microbes remain poorly understood.

In this report, we extend the investigation of metabolic variation in microbial populations by investigating the use of carbon substrates by random isolates of wild yeast (*Saccharomyces paradoxus*) from two local populations, one in North America and the other in Europe. This species is sexual, but outcrossing occurs very infrequently and populations are almost completely homozygous (Johnson *et al.*, 2004; Tsai *et al.*, 2008). Our enquiry was intended to characterize the potential diet of wild yeast populations, the amount of standing genetic variation in substrate utilization, and the pattern of trade-offs among substrates or groups of substrates. The presence of trade-offs in substrate utilization would indicate the potential for ecological diversification within and among natural populations.

## Materials and methods

### Sites

Wild yeast is known to grow on oak trees (Bowlesa and Lachance, 1983; Naumov et al., 1998). We collected samples from the bark of oak trees at North American and European sites. The North American site included stands of white oak (*Quercus alba* L.) in old-growth forest in the McGill University nature reserve at Mont St-Hilaire, Quebec (referred to as 'MSH'). The European site consisted of scattered oaks (*Quercus robur* L.) in parkland on the Silwood Park campus of Imperial College London (referred to as 'Silwood'). The Silwood isolates had previously been characterized genetically by Johnson et al. 2004. GPS coordinates of the locations of the isolates are available with the data (electronic supplementary material; Tables1-2).

### Isolates

Samples were collected using bark punches or putty, and yeast isolated by the selective culture procedure of Sniegowski et al. (2002). *Saccharomyces* sensu stricto yeasts were identified through PCR amplification of the ITS region and species identity confirmed through sequencing of CEN9 (Johnson et al., 2004; Bensasson et al., 2008). We chose 22 isolates of *S. paradoxus* from MSH and 23 isolates from Silwood at random from about 200 available from each site, subject to the constraint that no two isolates came from the same tree.

## Substrates

We used 96-well Biolog plates PM1 and PM2A (BIOLOG, Inc., Hayward, CA) to provide 190 carbon substrates. One well on each plate contains no carbon source.

## Survey technique

From frozen stocks, isolates were grown in YPD (yeast extract peptone dextrose) for two growth cycles of about eight generations each. 1.5 ml of each isolate was pelleted in an Eppendorf tube, washed twice with 0.75 ml minimal medium (yeast nitrogen base with no carbon source), re-suspended in 1 ml minimal medium and starved for 4h. Following Biolog protocol, 0.25 ml of this culture was added to 20 ml IFY-0 medium (BIOLOG Inc., Hayward, CA), 0.32 ml dye mix D (tetrazolium, BIOLOG Inc., Hayward, CA ) and distilled water to 25 ml. The Biolog plates were then inoculated with 0.1 ml per well of this suspension. Two replicates of each isolate were grown, plus a blank uninoculated plate. The plates were kept in incubators at 28˚ C. Optical density (OD) was scored on a plate reader at 590 nm after 24h, 48h and 72h of growth.

Tetrazolium measures the reducing power of the NADH supply of cells generated through catabolic pathways such as glycolysis and citric acid cycle. Since it might not necessarily indicate the amount of growth on a given substrate, a supplementary experiment was conducted to validate the use of tetrazolium to detect metabolic activity and yield. Four PM1 and four PM2A plates were inoculated as above, using tetrazolium dye, while four PM1 and four PM2A plates were inoculated without using tetrazolium. These plates were incubated at 28˚ C and optical density (OD) was scored on a plate reader at 590 nm after 24h, 48h and 72h of growth.

### Yield calculation

We estimated the yield in any given well as yield = (OD of inoculated well after 72 hours of growth) – (OD of corresponding well on uninoculated plate). For any given isolate, the pattern of yield can be expressed in a standard fashion as the deviation of yield for a substrate from the average of yield over all substrates for that isolate: standardized yield = (yield of focal isolate on given substrate) – (mean yield of focal isolate overs all substrates).

The average inoculum density over all strains was 5465 cells $mL^{-1}$ (st dev 2462) for the Silwood survey. The average yield of an isolate over all substrates was weakly and negatively related to inoculum density ($r^2 = 0.15$ for OD after 72h); hence, yield was not adjusted for inoculum density.

Statistical analyses were conducted using the R language (R Development Core Team, 2009). Data and analysis scripts are available in the electronic supplementary material. Pathways in which each substrate occurs were identified using KEGG: Kyoto Encyclopedia of Genes and Genomes (Ogata et al., 1999).

## Results

### Diet

We found that OD measurements with and without addition of tetrazolium were highly correlated after 72h of growth ($R^2 = 0.76$; Fig. S1), showing that OD accurately estimates biomass yield on a given carbon source. Almost every substrate (185/190) could be utilized by at

least one isolate (t-test compared to value for water, test-wise $P < 0.01$). The rank distribution of yield for the 50% of substrates with the highest mean scores is shown in Figure 1 and these substrates are the basis of all further analysis. The other 95 substrates are very poorly utilised, giving OD readings that barely exceed the blank.

A group of 10 substrates that are used efficiently by all isolates from both sites forms a prominent shoulder at the upper end of the distribution (Fig. 1). This core diet consists of the sugars glucose and fructose, their epimers (galactose, mannose) and combinations (sucrose, turanose and palatinose). It also includes intermediary metabolites derived from these sugars (a-methyl-D-glucoside, pyruvic acid, acetic acid). The remaining substrates constitute an ancillary diet for which yield scores fall roughly linearly with increasing rank ($r^2 = 0.86$ for linear regression of yield on rank across both sites). All MSH isolates grew well on maltose, but the Silwood isolates did not, and maltose is thus not included in the core diet.

The yield on each of these 95 substrates is highly correlated between the two surveys ($r^2 = 0.86$). This correlation is not solely driven by the difference between core and ancillary diet; the surveys are correlated, though less strongly, even within the ancillary diet ($r^2 = 0.41$).

## Variation among isolates

The amount of genetic variation in yield after 72h growth for each substrate in the core diet and the ancillary diet is shown in Figure 2. The average genetic coefficient of variation over all substrates is 0.20 at MSH and 0.16 at Silwood. Isolates varied significantly (main effect of isolate, F-test, test-wise $P < 0.05$) in yield on 13 substrates at both sites (all but turanose are from the ancillary diet); 11 substrates had significant genetic variance for MSH isolates only and 23 for Silwood isolates only. The genetic variance for given substrates is correlated between the

two surveys, although less strongly than the mean ($r^2 = 0.17$). Genetic variance is also correlated with mean yield ($r^2 = 0.16$).

### Covariation of diet

Correlations in yield between substrates may arise even for random data, depending on the overall quantity of genetic variance ('house-car paradox' (Zuk *et al.*, 1996)). The prevalence of positive correlations among substrates that we observed will thus be generated in part by the substantial genetic variance found in both populations. The observed distribution of pairwise correlations between substrates can be compared with the corresponding distribution obtained from randomizing the yield of each substrate among isolates. This breaks up any correlation between substrates among isolates, and provides a null hypothesis with which the observations can be compared. Over all substrates, the average genetic correlation between substrates is very weakly positive (mean $r = 0.04$ with $P < 0.001$ for both MSH and Silwood sites) and the distribution does not exhibit extreme values outside the range expected from the permutation test provided by the null hypothesis. Hence, the hypothesis that growth on random substrates will be negatively correlated among random isolates is rejected by our observations. Within the whole range of substrates, however, two contrasts with substantial negative correlations could be identified.

The first contrast is between substrates belonging to the core and ancillary diets (Fig. 3). Both within populations at each site and between isolates from different sites, yield on pairs of substrates from the same diet group (core or ancillary) are on average positively correlated (mean pairwise correlation core-core $r = 0.37$, $sd = 0.24$, $P < 0.001$ and ancillary-ancillary mean r =

0.04, sd = 0.46, $P < 0.001$), but yield on substrates from different groups is negatively correlated (mean r = -0.13, sd = 0.32, $P < 0.001$, test using permutation of values in one of the categories).

The second contrast is between pentose and hexose sugars (Figure 4). Yield on sugars with the same number of carbon atoms is positively correlated (pentose-pentose mean r = 0.77, sd = 0.14, $P< 0.001$, hexose-hexose mean r = 0.06, sd = 0.48, $P<0.1$); whereas yield on sugars with different numbers of carbon atoms is negatively correlated (pentose-hexose mean r = -0.16, sd = 0.40, $P<0.005$).

## Continental dietary variation

Isolates from the two localities have different patterns of substrate utilization (effect of location in ANOVA on principle component 1, $P < 0.001$, 100% proper classification when using classification algorithms such as Linear Discriminant Analysis, Fig. 5) and in this sense form distinct ecotypes. The sites are differentiated most strongly along PC1, whereas isolates within a site are differentiated along PC2.

Some substrates have consistently higher yield at one of the two sites (Figs. 1 and 5). When grouping by substrate type, North American strains have consistently higher yield than European strains on substrates in the pentose phosphate pathway, such as 2−deoxy−D−ribose, D−arabinose, L−arabinose, D−ribose, D−xylose and L−lyxose. Nevertheless, this is not accompanied by a consistent increase in their capacity to metabolize other substrates found in the pentose and glucuronate interconversion pathway (L−arabitol, D-arabitol and xylitol lead to lower yield in North American strains). This difference in the capacity to utilize pentoses appears to be one of the main differences between the sites (Fig. 5).

There are other substrates that were preferred (higher than average standardized yield on these substrates) by many isolates at one locality but by few or none at the other (Fig. 6). Most Silwood isolates preferred substrates that are metabolized through the alanine, aspartate and glutamate pathway (L−aspartic acid and b−methyl−D−galactoside; some isolates could also utilize D-alanine) and through the galactose pathway (dulcitol (galactitol), D-tagatose and stachyose), while few if any of the MSH isolates could metabolize these substrates.

### Regional and local dietary specialization

Within each site, the geographical distance between isolates explains part of the genetic variance in the pattern of substrate utilization (Mantel test, $r = 0.16$, $P = 0.02$ for European site and $r = 0.28$, $P = 0.002$ for the North American site; see Legendre & Fortin 2010). Despite this geographic variation in substrate utilization, patterns of utilization of substrates were not sufficiently different to allow the differentiation between isolates (0% consistent proper classification when using classification algorithms) and no consistent pattern between sites was evident either among the substrates that varied most between isolates at each location or among the substrates that varied most with latitude and longitude.

The MSH sample includes isolates from two major geographical races that are found in different areas of the mountain. These clades also display different patterns of substrate use (Fig. 5).

## Discussion

### Breadth and variation of diet

Most of the efficiently utilized substrates in the core diet are sugars. The ancillary diet is quite extensive, however, including about half the substrates tested. Wild yeast is able to grow, albeit inefficiently, on the random mixtures of substrates which it is likely to encounter in natural environments. Our surveys confirm that there is genetic variation in metabolic capacity within natural populations of a eukaryotic microbe. Isolates varied consistently in the amount of metabolic activity for several substrates, and isolates from the two sites could be distinguished on the basis of their pattern of substrate utilization.

### Geographical variation in substrate utilization

There is geographical variation of substrate use in *Saccharomyces paradoxus* at continental, regional and local scales. The divergence of substrate utilization between North American and European isolates is associated with the genetic divergence of European and North American populations documented by previous genetic surveys of isolates from MSH, Silwood and other sites (Koufopanou *et al.*, 2006). In a similar fashion, wild *Saccharomyces cerevisiae* ecotypes have specialized on vineyards and oaks separately, with oak ecotypes found within vineyards but not vice-versa (Hyma & Fay, 2013). In *S. paradoxus*, this divergence has gone some way towards speciation; the European and North American populations were in fact distinguished as *S. paradoxus* and *S. cariocanus* until recently. The MSH site happens to straddle the boundary between two North American races of *S. paradoxus*, and isolates can be assigned to one or the other by genetic criteria (Leducq *et al.*, 2014). Moreover, it has been recently shown that these two races are partially reproductively isolated (Charron *et al.*, 2014). As with continental

variation, regional genetic divergence is associated with consistent differences in the pattern of substrate use. Finally, there is evidence for spatial variation of substrate use at both localities on scales of tens or hundreds of meters.

## Purifying and diversifying selection for substrate utilization

Genetic variation for substrate utilization may be maintained through the balance between deleterious mutation and purifying selection or through diversifying selection arising either from functional interference in substrate utilization or from mutational degradation of unused metabolic pathways.

First, the local variation in metabolic performance that we have found might be attributable in part to the balance between mutation and purifying selection. This seems quantitatively plausible because the average amount of genetic variation in yield is comparable with the amount of genetic variation in fitness estimated in natural populations of other kinds of organism. The advance in fitness per generation, by the fundamental theorem of natural selection, is equal to the standardized variance of fitness ($SV_A$, additive variance divided by the square of the mean). This is the square of the genetic coefficient of variation, which has an average value of $SV_A = 0.04$ at MSH and 0.02 at Silwood. An experimental study of fitness in a natural population of the annual herb *Impatiens pallida* at Mont St-Hilaire gave an estimate of $SV_A = 0.03$ (Schoen *et al.*, 1994). Other studies of birds and plants suggest that $SV_A$ is usually in the region of 0.01 – 0.1 in natural conditions of growth (Bell 2008).

An alternative explanation for the maintenance of diversity is that variation is protected by diversifying selection. The ability to use the whole range of available substrates may be constrained by intrinsic physiological trade-offs, functional interference, among different kinds

of substrates, such that the enhanced ability to utilize some is necessarily accompanied by reduced ability to utilize others. Alternatively, if some substrates are lacking for a long period of time, the ability to consume them is not maintained by selection. Mutations in the genes that govern consumption will then be neutral, and will tend to accumulate over time. Variation in substrate availability over sites will then lead to a spatial pattern of specialization. Such trade-offs are evaluated through the genetic (among-isolate) correlations between substrates or kinds of substrate. The prevalence of positive correlation caused by overall genetic variance is not necessarily inconsistent with an underlying tendency for correlations to be negative for isolates with equivalent overall performance.

We found two trade-offs that we were able to identify with confidence because they involve consistent negative correlations between substrates in broad pre-defined categories. One involves the interference between substrates in the core diet and those in the ancillary diet, while the other involves the two main types of sugars, hexoses and pentoses. This might in principle lead to the divergence of two or more ecotypes. The trade-off between pentose and hexose utilization, indeed, appears to be one of the drivers of divergence between the Silwood population, which may have lost some of its pentose utilization function, and the MSH population, which may have specialized on pentose utilization. In laboratory strains of *Saccharomyces cerevisiae* bearing mutations in the pentose phosphate pathway, growth on hexoses is not compromised (Lobo & Maitra, 1982) while growth on gluconate has led to the isolation of strains with increased ability to utilize gluconate at the expense of their ability to grow on glucose (Cadière *et al.*, 2011).

To distinguish between purifying and diversifying selection as explanations of variation, the crucial prediction is that substrate use by isolates will correspond with substrate availability at sites if variation is adaptive. Opulente et al. (2013) showed that metabolic patterns in 448 strains

of the genus *Saccahromyces* were partially predictable from the environments where the strains occurred. Further progress in understanding the maintenance of metabolic variation in microbial populations is likely to depend on elucidating the availability of substrates in natural habitats and relating it to the metabolic specialization of resident strains.

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## Figure captions

Fig. 1 Rank distribution of yield among substrates. The barplot shows yield for the 95 most efficiently utilized substrates in the MSH (black) and Silwood (grey) surveys. 10 substrates are clearly identified by the ability of isolates to reach higher yields on these substrates. The core diet can also be identified by the fact that all isolates in both locations achieve higher yield on these substrates (Fig. 6).

Fig. 2. Variance of growth among substrates. The histogram bar indicates the square root of the among-isolates variance component ("genetic standard deviation") for 72h scores in the MSH (black) and Silwood (grey) surveys. Substrates are in the same order as in Figure 1. Asterisk denotes test-wise significance at $P < 0.001$ (experiment-wise $P < 0.1$).

Fig. 3 Genetic correlation coefficient between pairs of substrate grouped by diet category (mean value with standard deviation). The Core/Core and Ancillary/Ancillary groupings express correlation coefficients between pairs of substrates from the core diet and ancillary diets respectively, whereas the Core/Ancillary grouping expresses correlation coefficients between a substrate from the core diet and a substrate in the ancillary diet.

Fig. 4 Genetic correlation coefficient between pairs of sugars grouped by number of carbon (mean value with standard deviation). Pentoses and hexoses are compared together (two values at left) or with one another (value at right).

Fig. 5. The divergence of yield among isolates, summarized by projection on the two dominant principle component axes. Mean value for each isolate is presented. MSH isolates are shown as triangles, race B as solid triangles and race C as hollow triangles, and Silwood isolates by grey

squares. Ellipses show 95% confidence intervals. The ordination of the ten substrates that contribute most to PC 1 are shown as red lines.

Fig. 6. Number of isolates from MSH (black) and Silwood (grey) preferring given substrates. The criterion for preference is that the yield deviation from mean yield across all substrates is positive, i.e. yield on target substrate > mean yield across substrates. Substrates in the core diet are preferred by all isolates from both locations. Substrates from the ancillary diet that differ in preference between sites are grouped according to the site with most isolates preferring the substrate, and ordered by the difference in isolate preference between sites.

Supplementary Fig. 1. Growth of PM1 and PM2A plates that were inoculated with cell mixture supplemented with and without tetrazolium were highly correlated after 72h of growth (R2 = 0.7556).

## Table Captions

Table 1. GPS coordinates of MSH trees.

Table 2. GPS coordinates of Silwood trees.

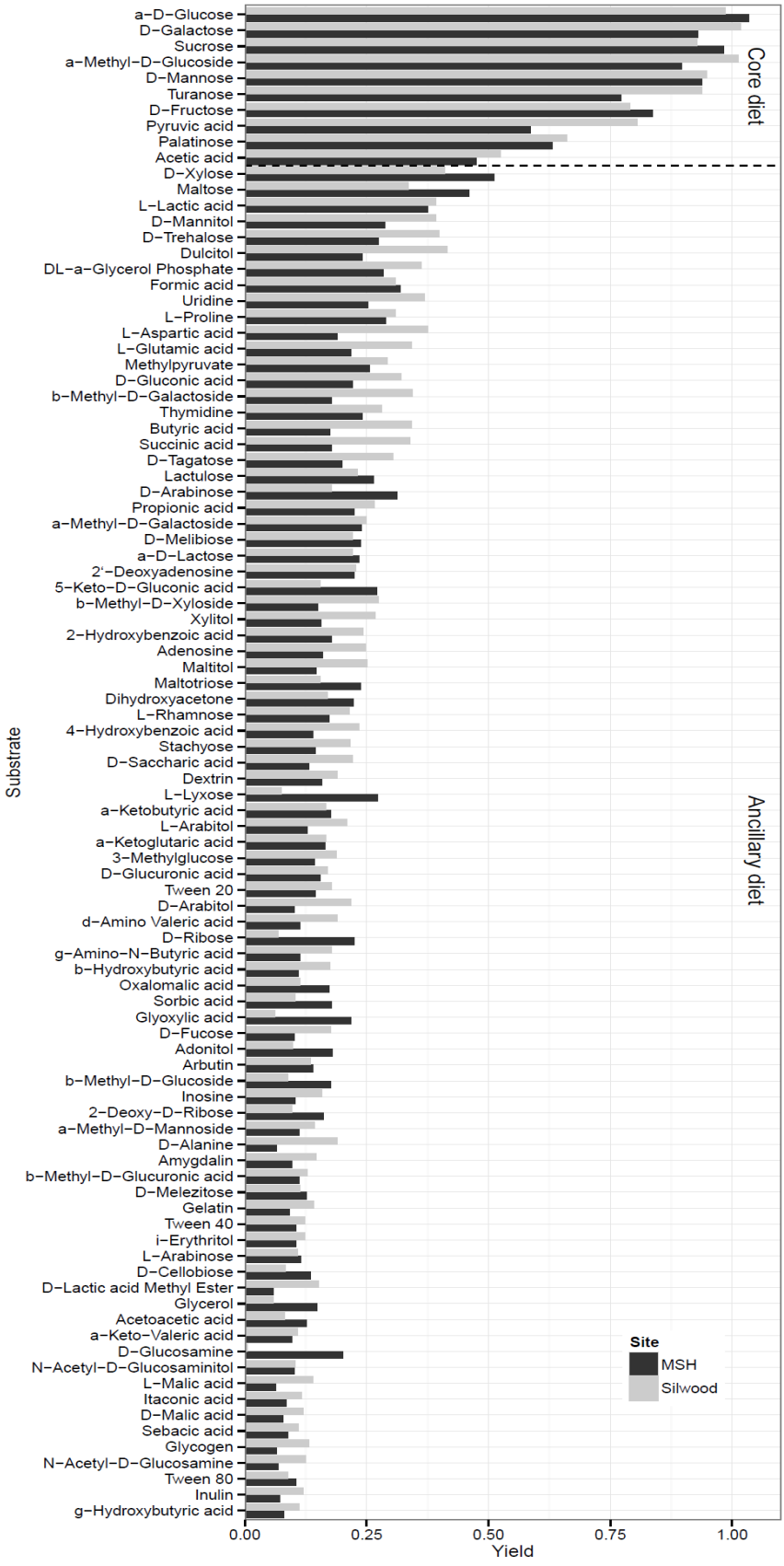

a−D−Glucose
D−Galactose
Sucrose
a−Methyl−D−Glucoside
D−Mannose
Turanose
D−Fructose
Pyruvic acid
Palatinose
Acetic acid
D−Xylose
Maltose
L−Lactic acid
D−Mannitol
D−Trehalose
Dulcitol
DL−a−Glycerol Phosphate
Formic acid
Uridine
L−Proline
L−Aspartic acid
L−Glutamic acid
Methylpyruvate
D−Gluconic acid
b−Methyl−D−Galactoside
Thymidine
Butyric acid
Succinic acid
D−Tagatose
Lactulose
D−Arabinose
Propionic acid
a−Methyl−D−Galactoside
D−Melibiose
a−D−Lactose
2'−Deoxyadenosine
5−Keto−D−Gluconic acid
b−Methyl−D−Xyloside
Xylitol
2−Hydroxybenzoic acid
Adenosine
Maltitol
Maltotriose
Dihydroxyacetone
L−Rhamnose
4−Hydroxybenzoic acid
Stachyose
D−Saccharic acid
Dextrin
L−Lyxose
a−Ketobutyric acid
L−Arabitol
a−Ketoglutaric acid
3−Methylglucose
D−Glucuronic acid
Tween 20
D−Arabitol
d−Amino Valeric acid
D−Ribose
g−Amino−N−Butyric acid
b−Hydroxybutyric acid
Oxalomalic acid
Sorbic acid
Glyoxylic acid
D−Fucose
Adonitol
Arbutin
b−Methyl−D−Glucoside
Inosine
2−Deoxy−D−Ribose
a−Methyl−D−Mannoside
D−Alanine
Amygdalin
b−Methyl−D−Glucuronic acid
D−Melezitose
Gelatin
Tween 40
i−Erythritol
L−Arabinose
D−Cellobiose
D−Lactic acid Methyl Ester
Glycerol
Acetoacetic acid
a−Keto−Valeric acid
D−Glucosamine
N−Acetyl−D−Glucosaminitol
L−Malic acid
Itaconic acid
D−Malic acid
Sebacic acid
Glycogen
N−Acetyl−D−Glucosamine
Tween 80
Inulin
g−Hydroxybutyric acid
Substrate
Core diet
Ancillary diet
Site
MSH
Silwood
0.00
0.25
0.50
0.75
1.00
Yield

**Figure 1**.

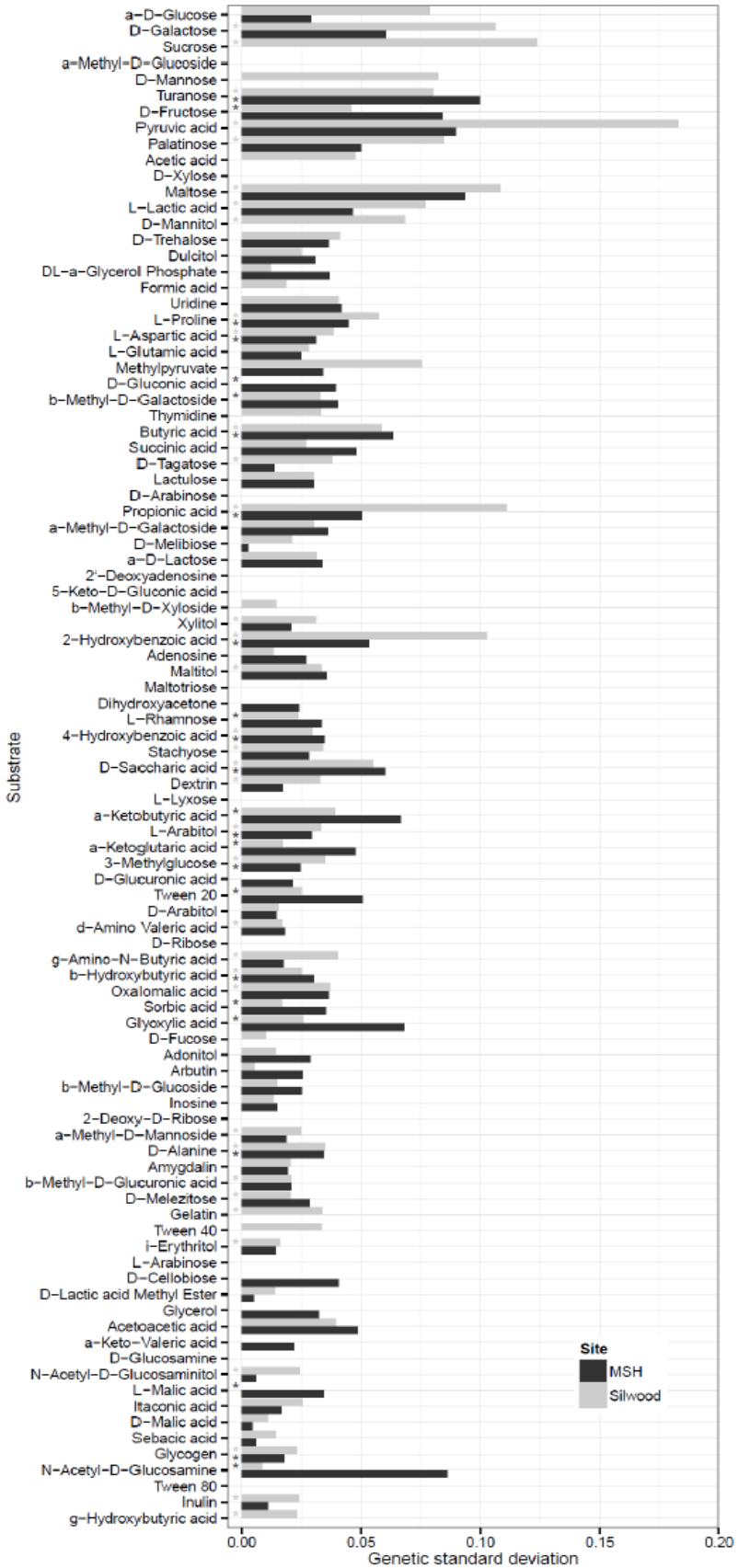

a−D−Glucose
D−Galactose
Sucrose
a−Methyl−D−Glucoside
D−Mannose
Turanose
D−Fructose
Pyruvic acid
Palatinose
Acetic acid
D−Xylose
Maltose
L−Lactic acid
D−Mannitol
D−Trehalose
Dulcitol
DL−a−Glycerol Phosphate
Formic acid
Uridine
L−Proline
L−Aspartic acid
L−Glutamic acid
Methylpyruvate
D−Gluconic acid
b−Methyl−D−Galactoside
Thymidine
Butyric acid
Succinic acid
D−Tagatose
Lactulose
D−Arabinose
Propionic acid
a−Methyl−D−Galactoside
D−Melibiose
a−D−Lactose
2'−Deoxyadenosine
5−Keto−D−Gluconic acid
b−Methyl−D−Xyloside
Xylitol
2−Hydroxybenzoic acid
Adenosine
Maltitol
Maltotriose
Dihydroxyacetone
L−Rhamnose
4−Hydroxybenzoic acid
Stachyose
D−Saccharic acid
Dextrin
L−Lyxose
a−Ketobutyric acid
L−Arabitol
a−Ketoglutaric acid
3−Methylglucose
D−Glucuronic acid
Tween 20
D−Arabitol
d−Amino Valeric acid
D−Ribose
g−Amino−N−Butyric acid
b−Hydroxybutyric acid
Oxalomalic acid
Sorbic acid
Glyoxylic acid
D−Fucose
Adonitol
Arbutin
b−Methyl−D−Glucoside
Inosine
2−Deoxy−D−Ribose
a−Methyl−D−Mannoside
D−Alanine
Amygdalin
b−Methyl−D−Glucuronic acid
D−Melezitose
Gelatin
Tween 40
i−Erythritol
L−Arabinose
D−Cellobiose
D−Lactic acid Methyl Ester
Glycerol
Acetoacetic acid
a−Keto−Valeric acid
D−Glucosamine
N−Acetyl−D−Glucosaminitol
L−Malic acid
Itaconic acid
D−Malic acid
Sebacic acid
Glycogen
N−Acetyl−D−Glucosamine
Tween 80
Inulin
g−Hydroxybutyric acid
Substrate
0.00
0.05
0.10
0.15
0.20
Genetic standard deviation
Site
MSH
Silwood

**Figure 2**.

**Figure 3.**

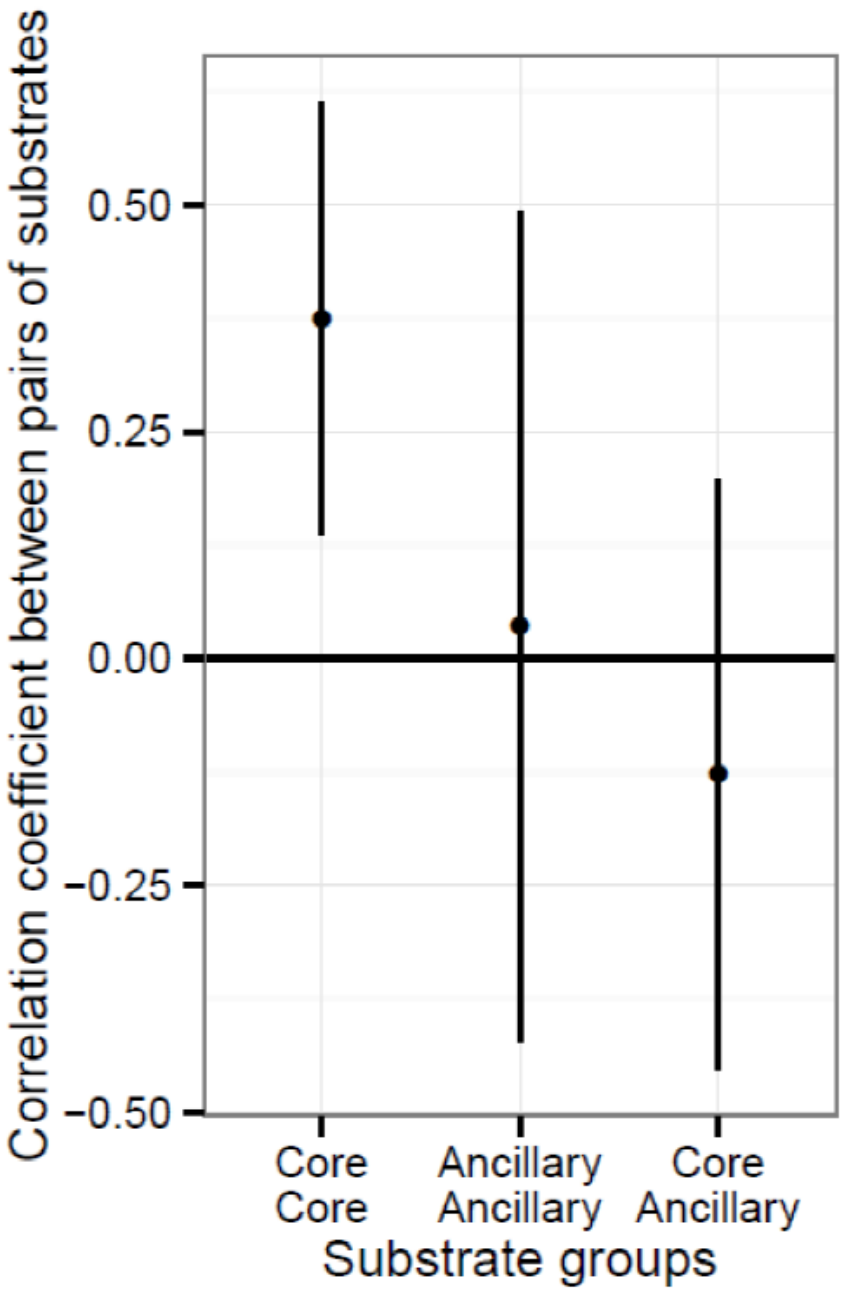
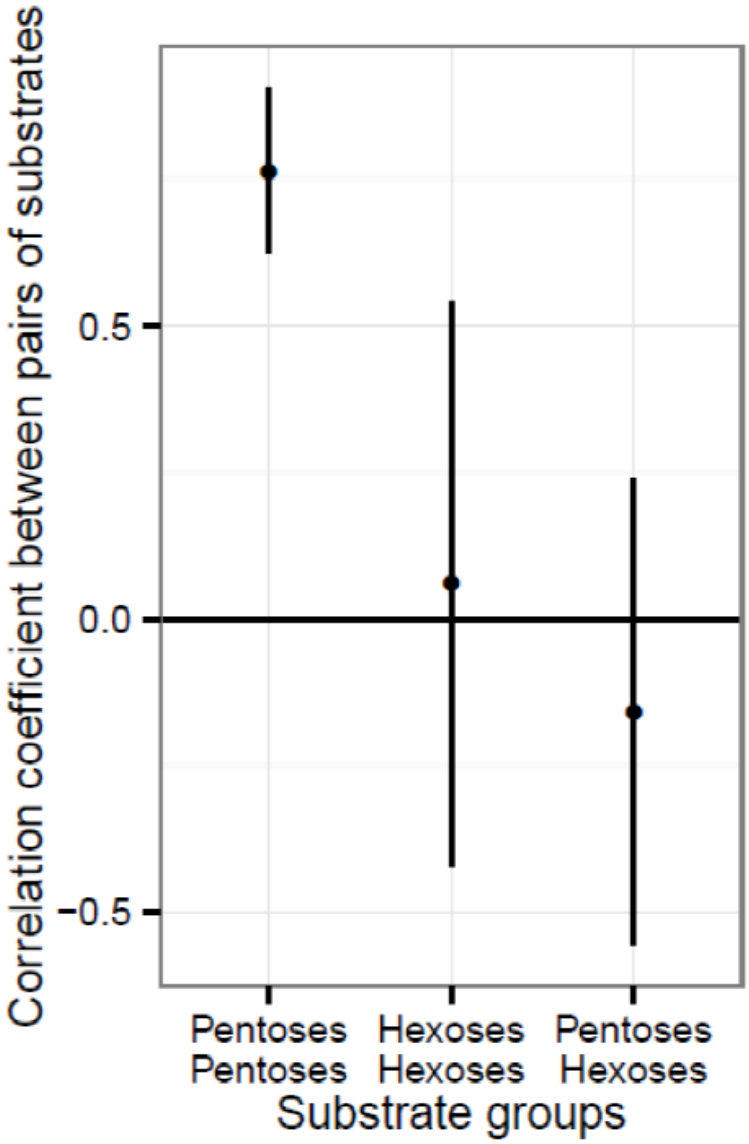
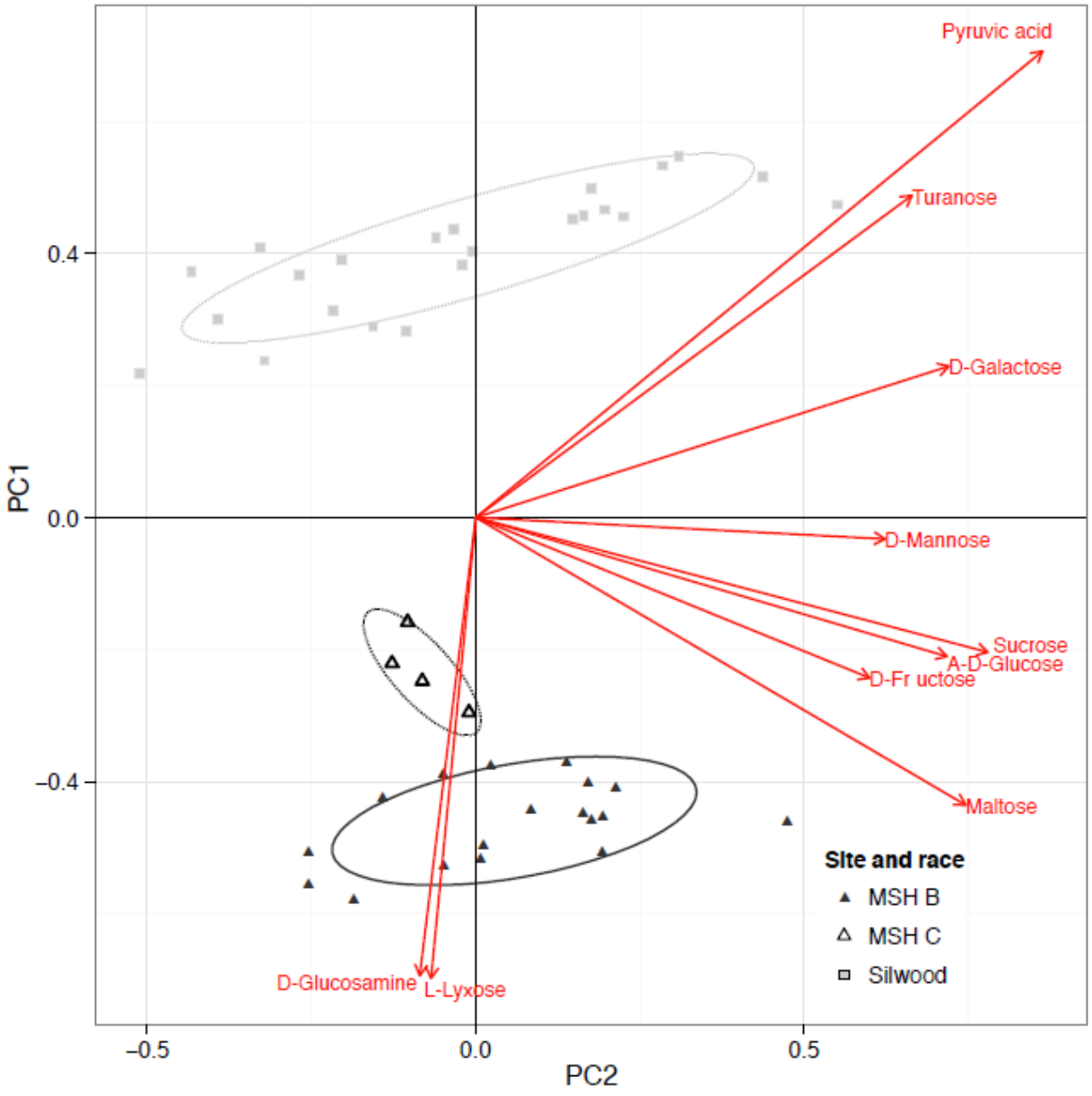

Correlation coefficient between pairs of substrates
0.50
0.25
0.00
−0.25
−0.50
Core
Core
Ancillary
Ancillary
Core
Ancillary
Substrate groups

**Figure 4.**


Correlation coefficient between pairs of substrates
0.5
0.0
−0.5
Pentoses Pentoses
Hexoses Hexoses
Pentoses Hexoses
Substrate groups

## Figure 5.


Pyruvic acid
Turanose
D-Galactose
D-Mannose
Sucrose
A-D-Glucose
D-Fr uctose
Maltose
D-Glucosamine
L-Lyxose
Site and race
MSH B
MSH C
Silwood
PC1
PC2
0.4
0.0
−0.4
−0.5
0.0
0.5

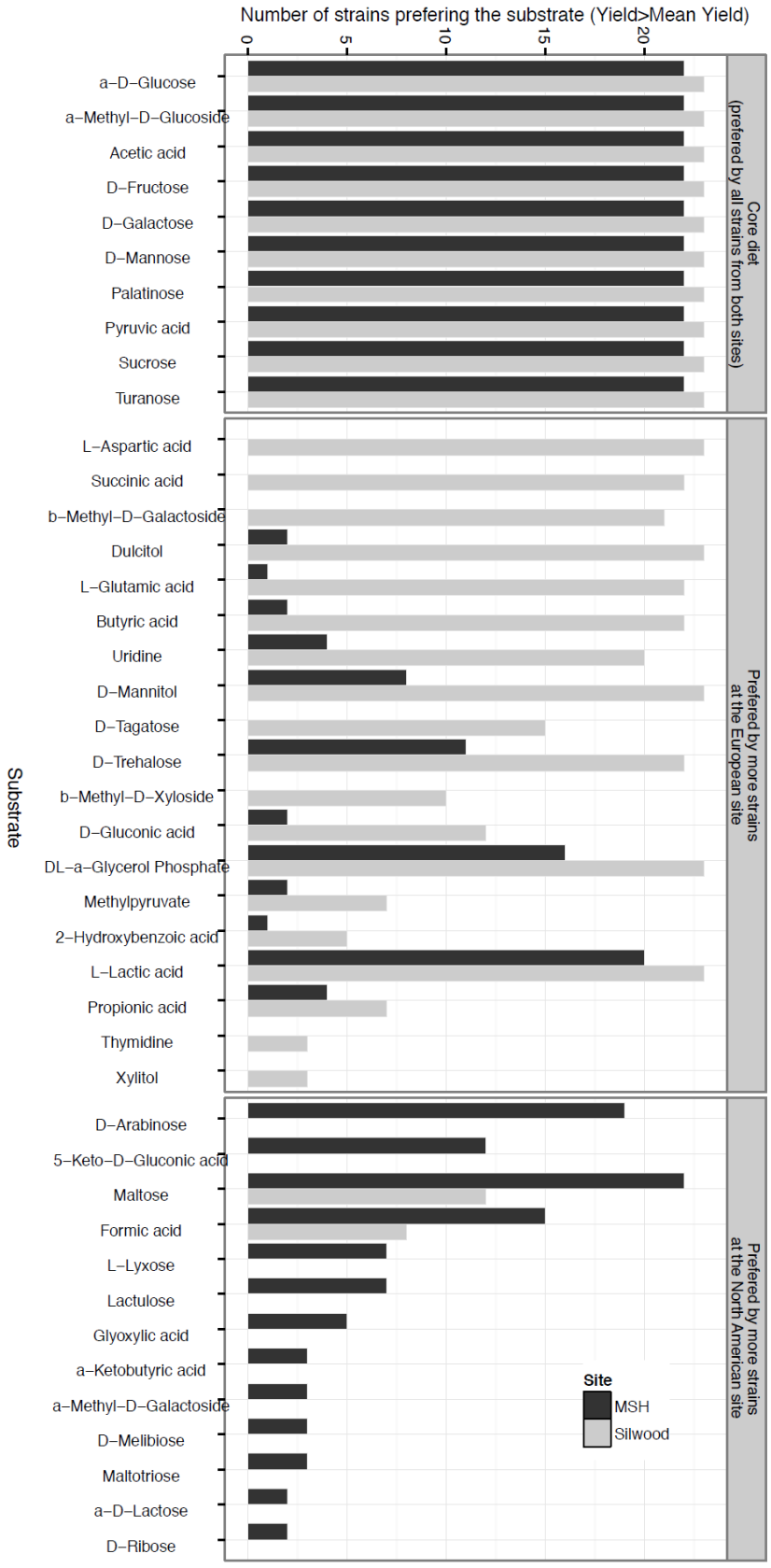
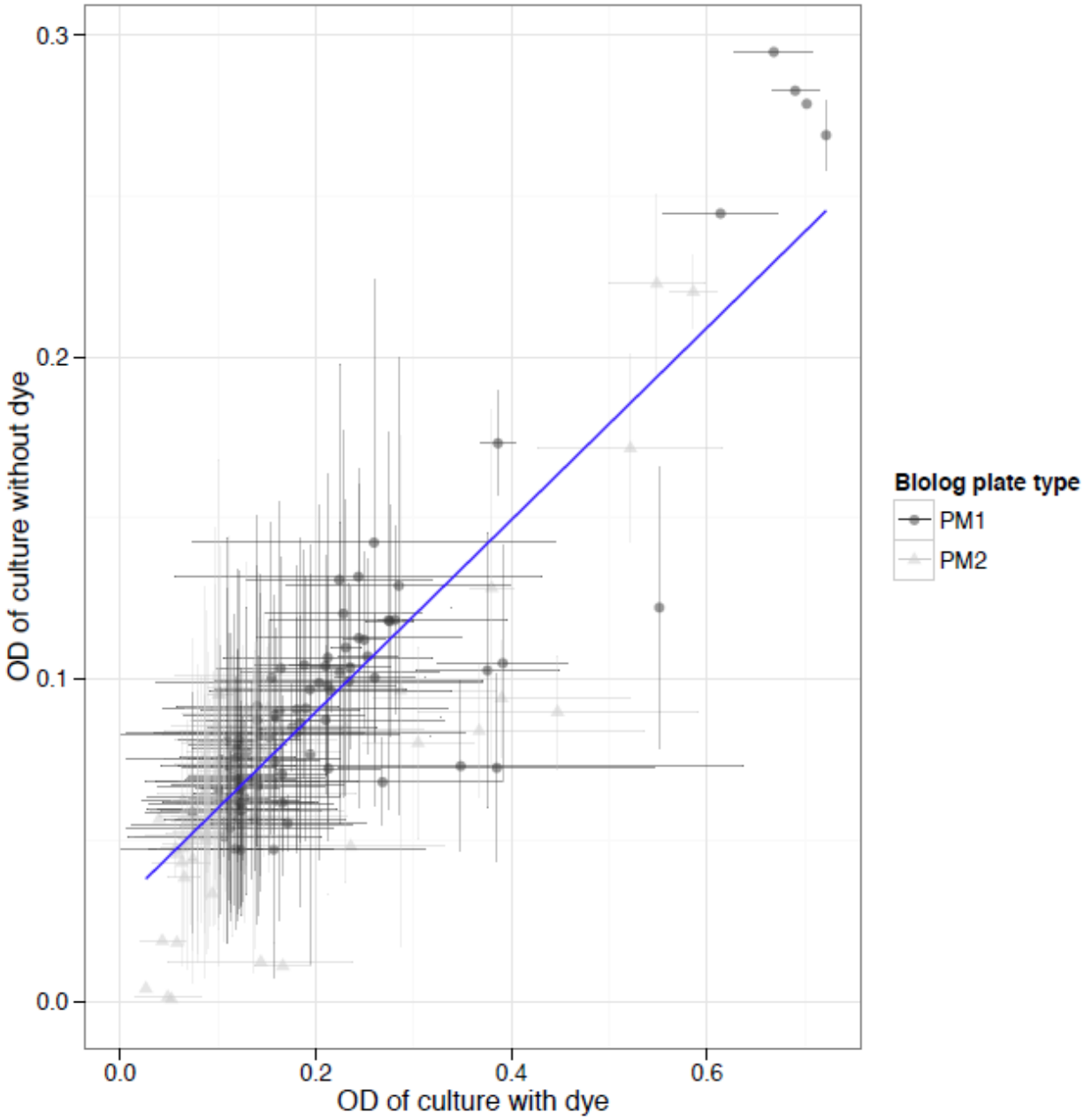

Number of strains prefering the substrate (Yield>Mean Yield)
0
5
10
15
20
Substrate
a–D–Glucose
a–Methyl–D–Glucoside
Acetic acid
D–Fructose
D–Galactose
D–Mannose
Palatinose
Pyruvic acid
Sucrose
Turanose
L–Aspartic acid
Succinic acid
b–Methyl–D–Galactoside
Dulcitol
L–Glutamic acid
Butyric acid
Uridine
D–Mannitol
D–Tagatose
D–Trehalose
b–Methyl–D–Xyloside
D–Gluconic acid
DL–a–Glycerol Phosphate
Methylpyruvate
2–Hydroxybenzoic acid
L–Lactic acid
Propionic acid
Thymidine
Xylitol
D–Arabinose
5–Keto–D–Gluconic acid
Maltose
Formic acid
L–Lyxose
Lactulose
Glyoxylic acid
a–Ketobutyric acid
a–Methyl–D–Galactoside
D–Melibiose
Maltotriose
a–D–Lactose
D–Ribose
Core diet
(prefered by all strains from both sites)
Prefered by more strains
at the European site
Prefered by more strains
at the North American site
Site
MSH
Silwood

**Figure 6**.

## Supplementary figure 1.



## Table 1: GPS coordinates of MSH trees

| site | Strain # | tree # | latitude | longitude | elevation (m) |
|---|---|---|---|---|---|
| Burned hill | 139 | 3004 | 45°32'29.58"N | 73°9'55.20"W | 252 |
| Burned hill | 148 | 3006 | 45°32'29.65"N | 73°9'54.36"W | 265 |
| Burned hill | 604 | 3012 | 45°32'29.11"N | 73°9'53.25"W | 259 |
| Dieppe | 23 | 567 | 45°33'40.25"N | 73°10'28.42"W | 363 |
| Dieppe | 44 | 826 | 45°33'39.82"N | 73°10'29.85"W | 350 |
| Dieppe | D1S11 | not labeled | 45°33'40.54"N | 73°10'29.76"E | - |
| Dieppe | D2S35 | 823 | 45°33'40.43"N | 73°10'29.24"W | - |
| Dieppe | D2B12 | 822 | 45°33'40.29"N | 73°10'28.35"W | - |
| East hill | 620 | 850 | 45°32'46.2"N | 73°08'34.2"W | 274 |
| Lake hill | 475 | 846 | 45°32'22.8"N | 73°08'59.8"W | 258.2 |
| Lake hill | 483 | 3070 | 45°32'09.0"N | 73°08'55.2"W | 127.4 |
| Lake hill | 498 | 3077 | 45°32'20.1"N | 73°08'57.0"W | 269.8 |
| Lake hill | 544 | 839 | 45°32'17.1"N | 73°08'55.8"W | 231.1 |
| Nature center | 95 | 578 | 45°32'22.30"N | 73°9'23.49"W | 193 |
| Nature center | 503 | 830 | 45°32'21.55"N | 73°9'23.10"W | 176 |
| Nature center | 584 | 575 | 45°32'21.03"N | 73°9'23.64"W | 177.6 |

## Table 2: GPS coordinates of Silwood trees

| region | site | tree | latitude | longitude | elevation (m) |
|---|---|---|---|---|---|
| Windsore park | A | Q14.4 | 51°25'15.40"N | 0°38'15.70"W | 55 |
| Windsore park | A | Q15.1 | 51°25'15.26"N | 0°38'11.40"W | 54 |
| Windsore park | B | Q31.4 | 51°24'40.56"N | 0°37'7.18"W | 46 |
| Windsore park | B | Q32.3 | 51°24'39.70"N | 0°37'6.00"W | 46 |
| Windsore park | A | Q4.1 | 51°25'12.87"N | 0°38'16.35"W | 56 |
| Windsore park | B | Q43.5 | 51°24'39.83"N | 0°37'10.54"W | 46 |
| Windsore park | C | Q59.1 | 51°24'54.65"N | 0°37'35.56"W | 58 |
| Windsore park | A | Q6.1 | 51°25'11.25"N | 0°38'13.56"W | 60 |
| Windsore park | C | Q62.5 | 51°24'54.74"N | 0°37'29.69"W | 58 |
| Windsore park | C | Q69.8 | 51°24'57.59"N | 0°37'34.64"W | 64 |
| Windsore park | C | Q74.4 | 51°24'58.02"N | 0°37'12.67"W | 73 |
| Windsore park | C | Q95.3 | 51°24'57.88"N | 0°37'16.46"W | 74 |
| Windsore park | C | Q89.8 | 51°25'0.45"N | 0°37'13.11"W | 75 |
| Silwood | D | S36.7 | 51°24'34.82"N | 0°38'35.71"W | 66 |
| Silwood | D | T18.2 | 51°24'40.68"N | 0°38'39.42"W | 64 |
| Silwood | D | T21.4 | 51°24'39.43"N | 0°38'42.48"W | 66 |
| Silwood | B | T26.3 | 51°24'28.32"N | 0°38'39.37"W | 68 |
| Silwood | D | T32.1 | 51°24'37.01"N | 0°38'36.11"W | 62 |
| Silwood | F | T62.1 | 51°24'51.30"N | 0°39'15.87"W | 67 |
| Silwood | F | T68.2 | 51°24'53.80"N | 0°39'9.09"W | 66 |
| Silwood | C | T76 (T76.6) | 51°24'44.70"N | 0°38'29.13"W | 62 |
| Silwood | A | T8.1 | 51°24'25.59"N | 0°38'49.64"W | 61 |
| Silwood | B | W7 | 51°24'29.01"N | 0°38'36.74"W | 69 |
| Silwood | C | Y6.5 | 51°24'35.85"N | 0°38'21.69"W | 69 |
| Silwood | C | Y7 | 51°24'35.85"N | 0°38'21.69"W | 69 |

## Connecting statement between chapters 2 and 3

In the previous chapter we documented the pattern of specialization found in isolates from wild yeast populations. Our results showed geographical variation of substrate use at continental, regional, and local scales among populations of wild yeast, *Saccharomyces paradoxus*. Pairwise genetic correlations between substrates were predominantly positive, reflecting overall variation in metabolic performance, but there was a consistent negative correlation between categories of substrates in two cases: between the core diet and the ancillary diet, and between pentose and hexose sugars. Such negative correlations in the utilization of substrate from different categories may indicate either intrinsic physiological trade-offs for the uptake and utilization of substrates from different categories, or the accumulation of conditionally neutral mutations. In the next chapter, we evaluate the evolution of metabolic specialization among experimental population of *S. paradoxus*. The goal of this experiment is to elucidate a mechanistic process through which populations evolve to a specialist or a generalist state.

# Chapter 3: Experimental evolution of the grain of metabolic specialization in yeast

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Pedram Samani & Graham Bell. (in prep.). Experimental evolution of the grain of metabolic specialization in yeast.

## Abstract

Adaptation to any given environment may be accompanied by a cost in terms of reduced growth in the ancestral or some alternative environment. Ecologists explain the cost of adaptation through the concept of a trade-off, by which gaining a new trait involves losing an older trait. Two mechanisms have been invoked to explain the evolution of trade-offs in ecological systems, mutational degradation and functional interference. Mutational degradation occurs when a gene coding a specific trait is not under selection in the resident environment; therefore it may be degraded through the accumulation of mutations that are neutral in the resident environment but deleterious in an alternative environment. Functional interference evolves if the gene or a set of genes have antagonistic effects in two or more ecologically different traits. Both mechanisms pertain to a situation where the environment of selection and the alternative environment are ecologically very different. To test this hypothesis, we conducted an experiment in which twelve experimental populations of wild yeast were each grown in a minimal medium supplemented with a single substrate. We chose twelve different carbon substrates that were metabolized through similar and different pathways in order to represent a wide range of ecological conditions. We found no evidence for trade-offs between substrates on the same pathway. The indirect response of substrates on other pathways, however, was consistently negative, with little correlation between the direct and indirect responses. We conclude that the grain of specialization in this case is the metabolic pathway, and that specialization appears to evolve through mutational degradation.

***Keywords:*** local adaptation, cost of adaptation, selection, trade-off, mutation accumulation, functional interference, wild yeast, metabolic specialization, reciprocal transplant assay, metabolic pathway.

## Introduction

Economic and ecological communities are structured by the pattern of specialization. In economic communities this constitutes the division of labour, reflecting how different kinds of employment are distributed among people. The analogous feature of ecological communities is the extent to which individuals are restricted to particular sites or diets, or resistant to particular parasites or predators. In either case, the ideal type is the perfect generalist who is capable of flourishing in all employments or thriving in all sites more successfully than any rival. In practice, this ideal state is seldom or never attained, at least for any considerable period of time (Kawecki, 1994; Gilchrist, 1995; Meijden, 1996; Wei *et al.*, 2015). In human societies a division of labour is enforced by law, or emerges spontaneously from the increased productivity made possible by devoting exclusive attention to a narrow range of tasks. Among other organisms the processes that favour specialization are different, but nonetheless invoke the greater fitness of specialists, and conversely the inferiority of generalists, over some part of the range of conditions available to the population. This constitutes a cost of adaptation: enhanced performance in the ambient conditions of growth is associated with regress in the ancestral conditions, or in some defined alternative conditions of interest (Stearns, 1989; Elena & Lenski, 2003; Kawecki *et al.*, 2012b).

Ecologists explain the cost of adaptation, and thus the evolution and maintenance of specialization, through the hypothesis, derived from economics, of a trade-off, meaning that increased fitness over some more or less restricted range of conditions entails reduced fitness, relative to rivals, in conditions outside this range. There are two well-understood causes of trade-offs. The first is that alternative morphological structures, physiological processes or behaviors may be incompatible. For example, a limb with the form of a hinged lever can produce

either a slow, powerful stroke or a rapid, weak stroke, depending on the position of the fulcrum relative to the point of application of force; speed and strength are inversely related by the laws of mechanics. Secondly, any feature that is never used will tend to deteriorate. This is because loss-of-function mutations in the genes which encode it are neutral so long as it does not contribute to fitness (Bell, 2008). There are numerous examples of both functional interference (Cooper & Lenski, 2000, 2010; Lunzer *et al.*, 2002) and mutational degradation (Reboud & Bell, 1997; MacLean & Bell, 2002; Ostrowski *et al.*, 2007; Behe, 2010) from experiments in which a population has been exposed to a novel environment.

Any population with access to several substitutable resources may evolve into a single broad generalist, or into a mixture of narrow specialists, or towards some intermediate position, depending on the trade-offs between alternative competencies. Trade-offs are more likely to occur when alternative resources require different and exclusive competencies, and are least likely to occur when they can be exploited in a similar manner with the same equipment. This suggests that they can be predicted by organizing resources hierarchically, from the most to the least inclusive categories. The most inclusive metabolic categories utilize resources so different that they require completely different cellular machinery, such as heterotrophic and photoautotrophic growth. Within such broad categories there may be sub-systems consisting of linked metabolic pathways, such as fermentation and respiration, that are incompatible because a given substrate molecule can be transformed through only one or the other. At a finer scale, there are individual metabolic pathways, such as glycolysis or the tricarboxylic acid cycle. The finest scale of all consists of an individual substrate that is metabolized at some point along a given pathway. The leading ecological attribute of a population is the 'grain of specialization' that represents its position on this hierarchy.

**Specilization among and within metabolic pathways.** Natural populations of microbes encounter a diversity of potential substrates, metabolized through several different pathways, and may evolve a greater or lesser degree of specialization (Samani *et al.*, 2015). The evolution of the grain of specialization can be studied experimentally by restricting the diet to a single substrate and measuring the growth of a lineage on this and on other substrates, on the same or on different metabolic pathways, in subsequent generations. The position of a target substrate on a metabolic pathway can be used to predict trade-offs as the result of how functional interference and mutational degradation are expected to act on another substrate on the same or on a different pathway.

The general principle governing the degree of functional interference is that the more similar are two tasks the less likely they are to interfere with one another. Hence, adaptation to a target substrate, supplied as sole carbon source, is less likely to interfere with the utilization of another substrate on the same pathway and more likely to interfere with the utilization of a substrate on a different pathway.

Mutational degradation may affect any non-target substrate. It is always likely to reduce growth on substrates belonging to another pathway if, as a result of the experimental treatment, this pathway is no longer active. Hence, growth on off-pathway substrates is expected to be reduced as the indirect response to the exclusive provision of a single substrate. For substrates on the same pathway, the likelihood of degradation will depend on the position of the target substrate and the topology of the pathway. If a given substrate is supplied in excess of the rate at which it can be processed by the existing cellular machinery, the direct response to selection will be an improvement in this machinery through functional complementation, for example by the modification of transport, regulatory or catabolic pathways. Overall growth will depend on the

flux along the pathway as a whole, however, which may be constrained by the capacity of downstream steps in the pathway, which may act as bottlenecks preventing any improvement from being fully implemented. Hence, according to Bell (2007), the next successful mutation will involve the substrate immediately upstream of the furthest downstream bottleneck. More generally, we expect the indirect response to be positive for substrates downstream of the target substrate. Upstream substrates, on the other hand, are no longer used, and the machinery responsible for their metabolism will eventually deteriorate through mutational degradation. This will be expressed as a negative indirect response for substrates upstream of the target substrate. For example, in the simple linear pathway A→ B → C the provision of B as the sole substrate is expected to lead to enhanced growth on C and reduced growth on A. In a branched pathway where metabolism may proceed from A to either B or C, adaptation to either downstream substrate may lead to loss of function for both the alternative and the upstream substrate. In a cyclical pathway, any given substrate can both be transformed into and generated from any other substrate, either directly or indirectly. The indirect response should therefore be positive for all substrates on the pathway.

In order to demonstrate a cost of adaptation it must first be established that adaptation to a given substrate has in fact occurred, and then that growth on alternative substrates has been compromised. This is done most convincingly through a reciprocal transplant assay in which every line is assayed on every substrate. The results show whether there is interaction between selection and assay environments; whether there is a positive direct response to selection; and finally whether the direct response varies among replicate selection lines. If these conditions are satisfied, the cost (or benefit) of adaptation will be expressed through the indirect response to selection, which we predict will be:

1. negative for off-pathway substrates through mutational degradation or functional interference;
2. negative for upstream substrates on the same pathway through mutational degradation;
3. positive for downstream substrates, including all substrates in a cyclical pathway, through functional complementation.

In this report, we shall describe an experiment in which populations of wild yeast, *Saccharomyces paradoxus*, were each grown on a single substrate, constituting the sole source of carbon and energy. The objective of the experiment was to estimate the grain of specialization that evolves when a population is restricted to a single defined substrate.

## Materials and methods

**Ancestor.** We used a single wild-type strain of *Saccharomyces paradoxus* collected from the Gault Nature Reserve of McGill University at Mont St-Hilaire, Quebec, as the ancestor for this experiment.

**Substrates.** We selected isogenic yeast populations on substrates belonging to three kinds of pathway; linear, cyclical, and branched. For each pathway we chose three substrates, plus a fourth substrate belonging to a different pathway.

(a) Linear pathway: Glycolysis is an example of a linear pathway. We chose raffinose, fructose, and pyruvate, as the three substrates located on this pathway: raffinose is the furthest upstream substrate, fructose is intermediate, and pyruvate is downstream in the

pathway. We chose aspartate as the fourth substrate: it is catabolized through the TCA cycle after conversion to oxaloacetate.

(b) Branched pathway: glutamate, proline, and citruline are catabolised through a complex and branched pathway to tricarboxylic acid (TCA) cycle intermediary metabolites such as fumarate and ketoglutarate. We chose xylose as the fourth substrate: it is converted to xylulose-5-phosphate and is then metabolised through the pentose phosphate pathway.

(c) Cyclical pathway: succinate, fumarate, and malate are the three substrates that participate in the TCA cycle to generate energy in aerobic metabolism. We used melibiose as the fourth substrate: it is hydrolysed to glucose and galactose and subsequently metabolized through glycolysis.

Each set of four substrates was treated as a separate experiment in assays and analysis.

**Natural selection.** Selection lines were cultured in 12-well plates containing minimal medium supplemented with 3% w/v of each of the carbon sources, using 12 replicates per treatment. Lines were transferred every 7 days and assayed and stored frozen every ten transfers.

**Reciprocal transplant assay.** Inoculation procedures were performed at the CIAN robotics/automation core facility (McGill University) on a BiomekFX liquid handler and an ORCA robotic arm controlled by the SAMI software (Beckman, Mississauga, Canada). The response to selection was evaluated by a reciprocal transplant assay in which each selection line was grown in each of the four substrates chosen for a given pathway. The response variable was maximum yield (as optical density, OD), which corresponds with growth at transfer. Each

pathway was evaluated by a separate assay. Each of the three assays comprised 4 selection environments x 4 assay environments x 12 replicate lines per selection environment x 2 replicate cultures of each line. In addition, we recorded the yield of the ancestor in all 12 assay environments. Two complete assays were conducted, the first after 24 cycles and the second after 42 cycles.

**Analysis of the reciprocal transplant assay.** The direct and indirect responses to selection can be evaluated from a reciprocal transplant assay in which all evolved lines are grown on all substrates. This reciprocal transplant assay generates a matrix of scores for all combinations of selection environment and assay environment. If populations have become specifically adapted to the environment in which they were selected, scores along the leading diagonal of the matrix (in which the assay environment corresponds to the selection environment) will be consistently greater than scores in off-diagonal cells. This will generate a statistical interaction between selection and assay environments that can be used to evaluate the occurrence of specific adaptation.

The results of the assay can then be used to answer two questions. The first is whether growth on a particular substrate is greater among lines that have been propagated on that substrate than among lines that have been propagated on other substrates. This expresses the improvement in a given assay environment that has been caused by natural selection in that environment, relative to any improvement that might be caused in that environment by selection in other environments. This is the sense in which 'local adaptation' is usually understood (Kawecki & Ebert, 2004). The second question is whether lines that have been propagated on a given substrate grow better

on that substrate than on other substrates. This shows whether the improvement of a line in the environment of selection is consistently associated with its deterioration in other environments. This is often called the 'cost of adaptation' (Levins, 1968).

To evaluate the extent and the cost of adaptation, the overall response R(ijk) of the k-th replicate line subjected to the j-th selection treatment and grown in the i-th assay conditions can be partitioned as follows:

$$R(ijk)- A(i) = [R(i..) – A(i)] + [R(ij.) – R(i..)] + [R(ijk) – R(ij.)]$$

where the subscript dots indicate averaging. The three components of the overall response are as follows.

1. The first term on the right-hand side is the general response associated with a given assay environment, estimated as the difference between the average of all selection treatments in a given assay environment, R(i..), and the growth of the ancestor in that environment, A(i). This is attributable to common features of the selection environments.

2. The second term is the specific response to the selection treatment, estimated from the difference between the average of replicate lines subjected to this treatment, R(ij.), and the average of all selection treatments in this assay environment, R(i..). If i = j this is the specific direct response, which expresses local adaptation, using replicate selection lines to test its significance. If $i \neq j$ it is a specific indirect response in a given assay environment. The degree of local adaptation to the j-th selection environment relative to the i-th assay environment is evaluated by comparing R(jj.) with R(ji.), which corresponds to the 'local vs foreign' comparison of Kawecki & Ebert (2005). The cost of

adaptation in the i-th assay environment is evaluated by comparing R(jj.) with R(ij.), which corresponds to the 'home vs away' comparison of Kawecki & Ebert (2005).

3. The third term is the deviation of the growth of each line, R(ijk), from the average of lines subjected to a particular selection treatment and assayed in a particular environment, R(ij.). Functional interference is expressed by a negative correlation among replicate lines between the deviations for a given assay environment and those for the environment of selection, given that the direct response is positive (local adaptation has evolved) and varies among lines (potentially leading to different degrees of maladaptation).

This interpretation of a reciprocal transplant assay can be summarized like this: the specific direct response expresses the degree of local adaptation, while the specific indirect response expresses the cost of adaptation, whose source can be identified from the line-based estimates.

## Results

**Analysis of variance.** The selection x assay environment interaction after 24 cycles was significant only for the linear pathway ($F(9, 368) = 12.5$, $P < 0.001$). After 42 cycles, however, the selection x assay environment interaction was highly significant in the linear pathway ($F(9, 368) = 6.6$, $P < 0.001$), the branched pathway ($F(9, 368) = 6.3$, $P < 0.001$) and the cyclical pathway ($F(9, 368) = 7.1$, $P < 0.001$).

**Direct response to selection.** The general response was positive in all cases at cycle 24. It had further increased in all lines by cycle 42, at which point average growth (over all substrates, as OD) had increased by a factor of 6.6. Substrates differed widely in the magnitude of the

advance: those where the ancestor already grew well, especially fructose and raffinose , showed only modest gains of 20 – 58%, whereas those where the ancestor grew poorly advanced by as much as 3 order of magnitude above the ancestral value. Hence, the general response reduced the variation of growth among substrates, roughly halving the coefficient of variation from 1.64 in the ancestors to 0.59 in the selection lines at cycle 42.

Local adaptation is evaluated by the specific direct response, averaged over replicate lines, which was positive for 8/12 substrates at cycle 24 and for 10/12 substrates at cycle 42 (Figure 1), with 7/10 estimates being significant at cycle 42 (t-test for departure from 0, $P < 0.05$). The response also became larger (more positive) for 9/12 substrates over this interval, the exceptions being raffinose, fructose and (marginally) fumarate. The average overall direct response was +0.029 (se 0.022, $t = 1.33$ for departure from 0, $df = 11$, $P = 0.21$) at cycle 24, and was +0.079 (se 0.024, $t = 3.29$ for departure from 0, $df = 11$, $P < 0.01$) at cycle 42. Hence, there is good evidence of local adaptation, which may have strengthened between cycles 24 and 42 (difference between average overall responses, $t = 1.53$, $df = 22$, $P = 0.15$). These overall growths of the lines represent advances above the growth of the ancestor of 26% at cycle 24 and 150% at cycle 42.

**Variance among lines.** The variation of growth among lines when assayed in the environment of selection expresses the degree of divergence of the direct response. Estimates of the among-line variance component were positive for all substrates and significant ($F(11, 12) = 4.4$, , $P < 0.01$) for all except raffinose and xylose. The standard deviation (square root of the among-line variance component) of the direct response increased with the mean ($r = 0.51$, $df = 10$, $0.05 < P < 0.1$), showing that replicate lines diverged more in environments where growth increased more. This was attributable entirely to the specific direct response ($r = +0.58$, $df = 10$, $P = 0.05$) and not at all to the general response ($r = 0.08$, $df = 10$, $P > 0.5$).

Estimates of the among-line variance component were positive for all combinations of different selection and assay substrates except one (glutamate assayed in xylose) and significant ($F(11, 12) = 2.81$, $P < 0.05$) for 25/36 combinations.

**The indirect response to on-pathway substrates.** The specific indirect response to substrates on the same metabolic pathway was on average about zero (18 estimates, mean = +0.0036, se = 0.0104 at cycle 24; 18 estimates, mean = +0.009, se = 0.020 at cycle 42). The sign of the observed values did not consistently correspond with prediction (Table 1). The lack of fit with prediction also applied when the three pathways were examined separately.

**The indirect response to off-pathway substrates.** There are 18 estimates of the indirect response for off-pathway substrates, 9 from the indirect response to selection for on-pathway substrates and 9 from the indirect response to selection for the off-pathway substrates themselves (Figures 2a-d). 12/18 were negative at cycle 24 (7/18 significant at $P < 0.05$, t-test for departure from 0) and all 18/18 were negative at cycle 42 (mean -0.0564, se 0.0074, $t = -7.6$, $P < 0.001$; 6/18 significant at $P < 0.05$, t-test for departure from 0).

**The correlated response of replicate lines.** Each pathway provided 12 correlations of the indirect with the direct response to selection on a given substrate. Most of these (29/36) are positive (Table 2); none of the negative correlations are significant at $P < 0.05$. There may be a tendency for off-pathway correlations (mean 0.282, se 0.102) to be somewhat lower than on-pathway correlations (mean = 0.491, se = 0.088; one-tail t-test; $t = -1.63$, $df = 17$, $P < 0.06$).

## Discussion

**The general response.** The single ancestral strain was isolated from oak bark and subsequently maintained on solid agar in petri plates using rich medium (yeast extract peptone dextrose, YPD) in the laboratory. To investigate the evolution of specialization, this strain was grown in well plates with liquid minimal medium supplemented with a single carbon substrate. Although we intended to study how lines adapted specifically to each substrate, the environment of selection had many features common to all lines and all substrates that differed from anything previously experienced by the ancestor, either in the wild or subsequently in domestication. The outcome was a rapid general response to these new conditions that was expressed regardless of the particular substrate supplied. This mirrors a more extensive experiment in which the bacterium *Pseudomonas fluorescens* was cultivated on 95 carbon substrates (MacLean & Bell, 2002). When lines cultivated on a given substrate were tested on the other substrates, they grew better than the ancestor in the great majority of cases, which the authors attributed, as we do, to common environment. In both experiments, a positive general response was detected because several differential factors were used and the outcome was evaluated by a full reciprocal transplant assay. Should only a single factor be used, or should lines be tested only in the environment of selection, it would not be possible to separate the general and specific responses to selection.

**The grain of specialization.** Specialization evolves through loss of function rather than through gain of function. Imagine a population of microbes in which all individuals are generalists equally capable of metabolizing a wide range of substrates. A mutation arises in a certain lineage that confers the ability to utilize one of these substrates more efficiently, and this lineage consequently displaces all of its competitors. This process does not result in specialization,

however, but merely in the evolution of a more efficient generalist. The case is different if there is a trade-off, such that the modified lineage, as the consequence of its newly acquired proficiency, loses the ability to metabolize certain other substrates, or metabolizes them less efficiently than its competitors. The population now consists of two types with somewhat different patterns of substrate metabolism, and may thereafter maintain this degree of specialization through divergent natural selection.

Adaptation to a particular substrate through gain-of-function mutations is a direct response to selection. Any loss of adaptation to other substrates that evolves concomitantly is an indirect response to selection that constitutes a cost of adaptation. Consequently, the crucial process in the evolution of specialization is a negative indirect response to selection reflecting loss of function caused by a trade-off of some kind. In our experiment, there was a consistently negative response to off-pathway substrates, whereas the direction of response to substrates on the same metabolic pathway was neither strong nor predictable. This suggests that the grain of specialization is the metabolic pathway, rather than the individual substrate.

This conclusion is consistent with well-established trade-offs between major metabolic systems such as autotrophy versus heterotrophy (Reboud & Bell, 1997) or fermentation versus respiration (Novak *et al.*, 2006; Frank, 2010). It is also broadly consistent with metabolic surveys of natural populations of wild yeast. (Samani *et al.*, 2015) found that wild isolates are capable of metabolizing a wide range of substrates, with no evidence for narrow specialists nor for generally negative correlations between substrates. There was, however, evidence for trade-offs between certain categories of substrates. Growth on a dozen or so core substrates, which are metabolized efficiently by all strains, was negatively correlated with growth on ancillary substrates, whose utilization is more erratic. There was also a negative correlation between

growth on hexose and pentose sugars. Experimental populations propagated on a mixture of substrates did not evolve narrow specialization to each individual substrate, but instead evolved as incomplete overlapping generalists, such that each genotype in a diverse population was capable of efficiently metabolizing many but not all of the available substrates (Barrett *et al.*, 2005). In all these cases, neither specialization nor generalization proceeded to the limit, but rather displayed an intermediate, coarse-grained degree of specialization.

**The source of specialization.** Two replicate populations exposed to the same conditions of growth may adapt at different rates or to different extents as the result of chance events such as the order of substitution of beneficial mutations. The idiosyncratic variation among replicate lines has been used to identify the cause of trade-offs. If those populations that have adapted most successfully to their new conditions are systematically the least successful in other conditions then it can be inferred that one specialized function interferes with others. Conversely, if the degree of superiority of replicate populations in their new conditions is unrelated to their degree of inferiority in other conditions it can be inferred that the trade-off is caused by the effect of disuse, through the accumulation of conditionally deleterious mutations. Hence, the historical divergence of replicate lines can be used to identify the source of the trade-off responsible for a negative specific indirect response.

Correlations between replicate lines in our experiment were generally positive, however, although those involving off-pathway substrates were somewhat weaker. We conclude that selection on a single substrate will usually improve performance on other substrates in the same pathway, so that there is no trade-off and hence no evolution of specialization at this level. Substrates on different pathways are weakly correlated or uncorrelated. The exception may prove the rule: there is a strong positive correlation ($r > 0.9$) between pyruvate and aspartate.

The two may be linked, however, through the conversion of pyruvate to oxaloacetate by pyruvate carboxylase, followed by the reversible transamination of oxaloacetate to aspartate through aspartate transaminase, an anaplerotic reaction that was not anticipated when the experiment was designed. If these correlations are discounted, none of the off-pathway comparisons are significant, and the specific indirect response to off-pathway substrates is economically explained by mutational degradation.

## Conclusion

Local adaptation to a given substrate did not consistently affect growth on other substrates in the same pathway. Individual lines that adapted more successfully than average tended to be superior on other substrates in the same pathway. We found no evidence for trade-offs between substrates on the same pathway. The indirect response of substrates on other pathways, however, was consistently negative, with little correlation between direct and indirect responses. We conclude that the grain of specialization in this case is the metabolic pathway, and that specialization appears to evolve through mutational degradation.

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## Figure captions

Figure 1. Direct response to selection as a representation for local adaptation. a) at cycle 24. b) at cycle 42. Y-axis is the direct response to selection measured by optical density averaged over replicate lines. Error bars are the standard error of the mean.

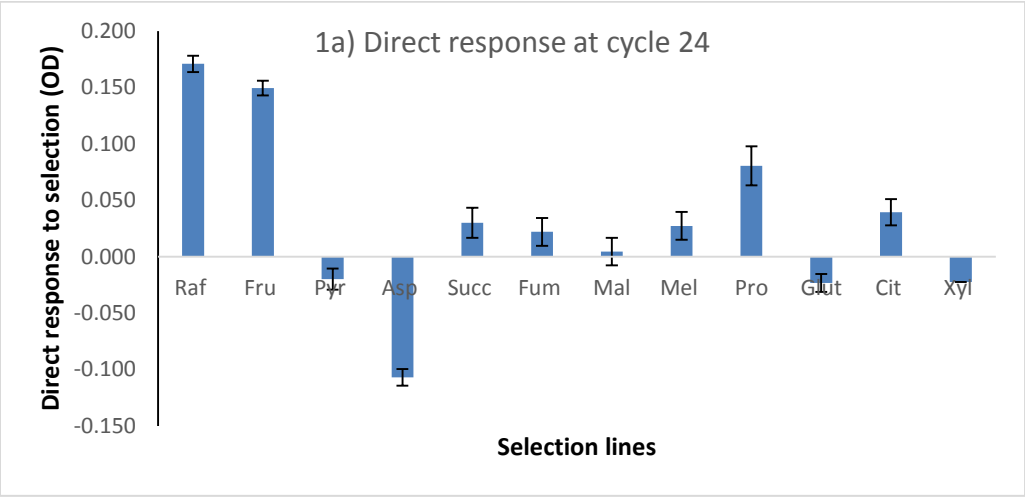
Figure 2. Indirect response for off-pathway substrates a) Indirect response to off-pathway substrates from selection on the on-pathway substrates at cycle 24. b) Indirect response to off-pathway substrates from selection on the on-pathway substrates at cycle 42. c) Indirect response to on-pathway substrates from selection on the off-pathway substrates at cycle 24. d) Indirect response to on-pathway substrates from selection on the off-pathway substrates at cycle 42. Error bars are standard error of the mean.

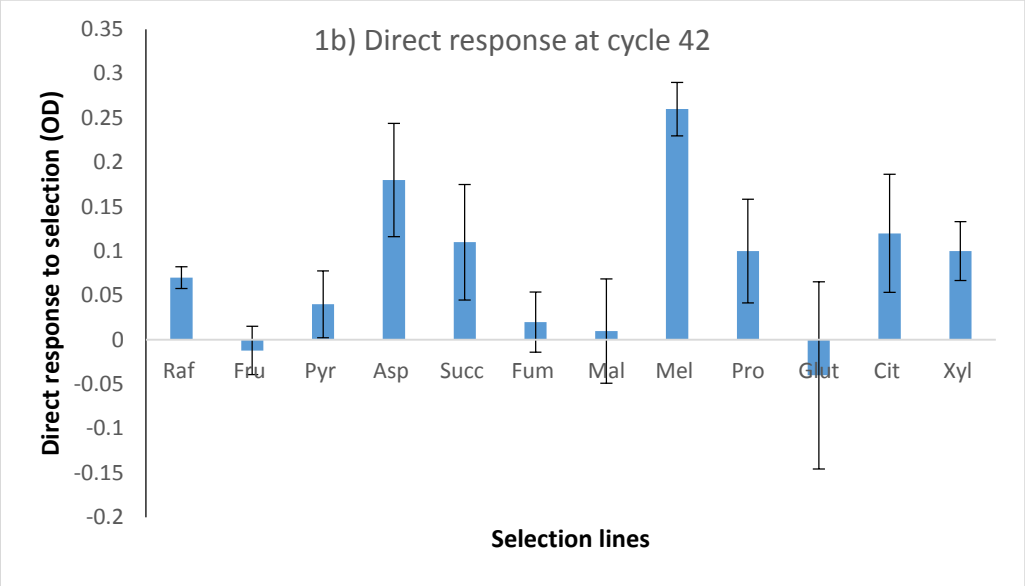
## Table captions

Table 1. Indirect response to selection to on-pathway substrates.

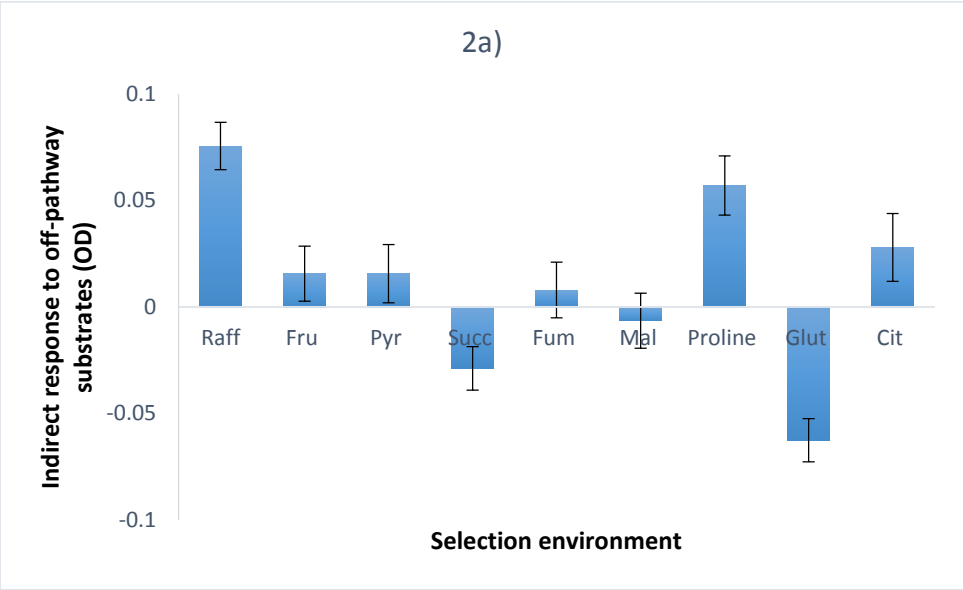
Table 2. Correlations of the direct with the indirect response to selection.

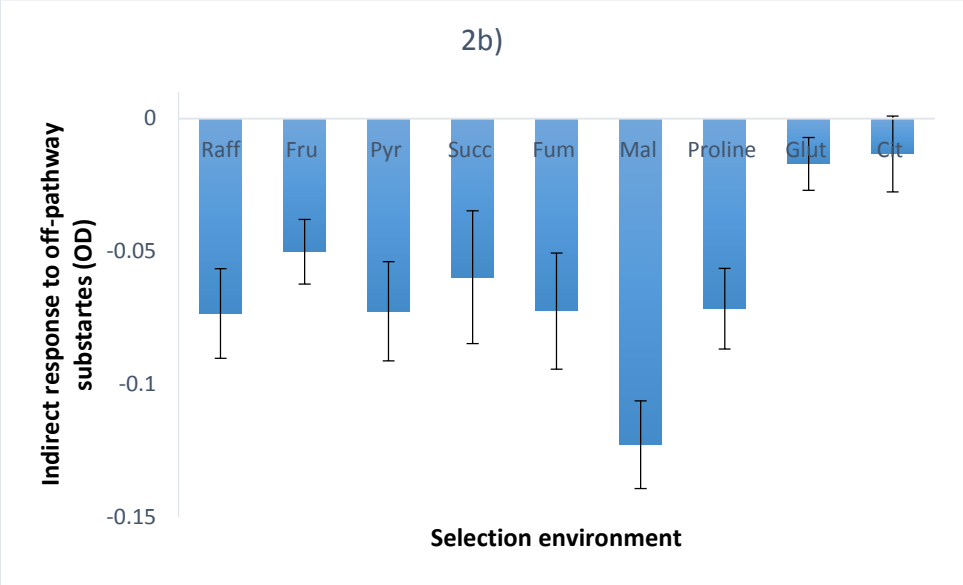
## Figure 1a-b.

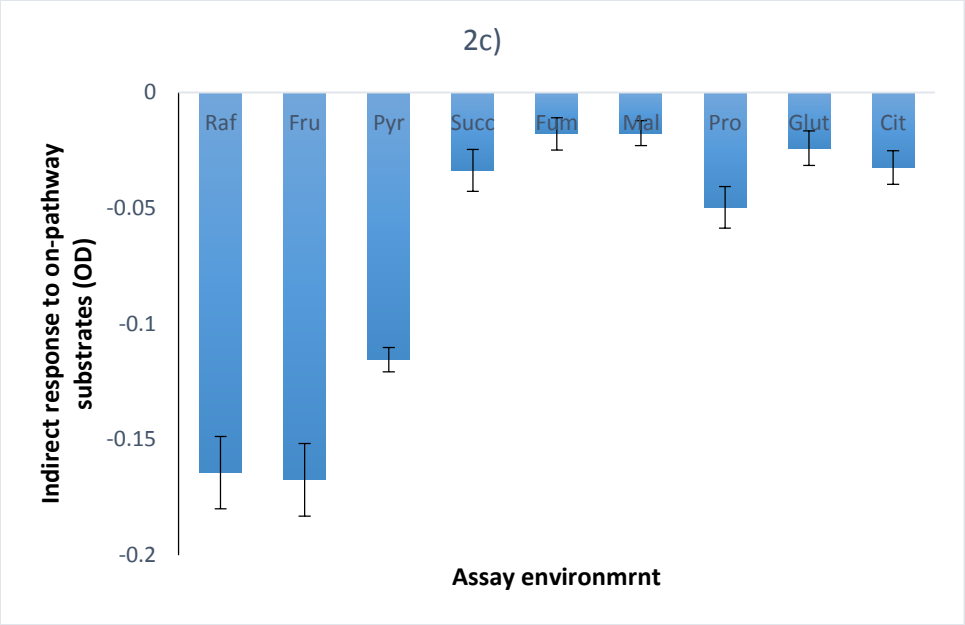

1a) Direct response at cycle 24
Direct response to selection (OD)
0.200
0.150
0.100
0.050
0.000
-0.050
-0.100
-0.150
Raf
Fru
Pyr
Asp
Succ
Fum
Mal
Mel
Pro
Glut
Cit
Xyl
Selection lines

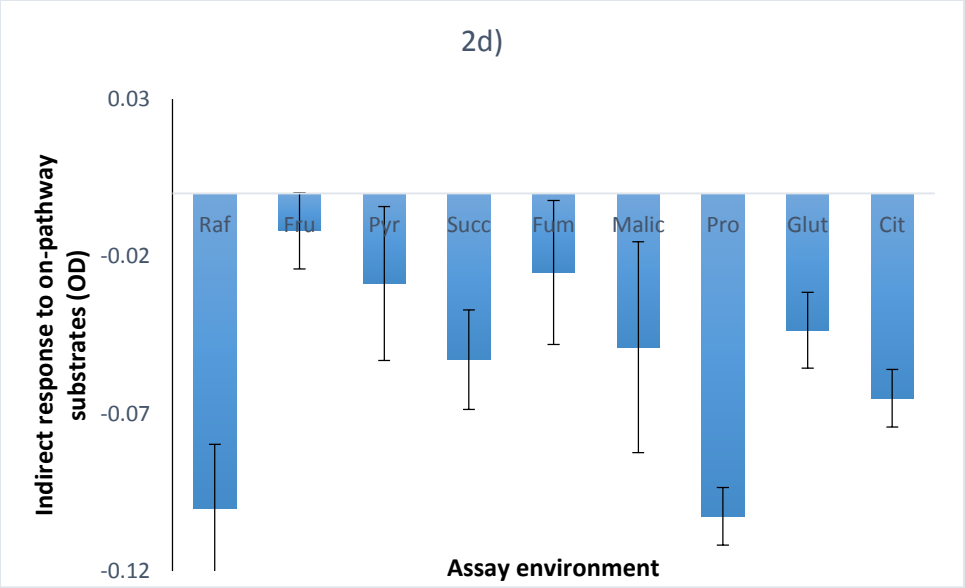

1b) Direct response at cycle 42
Direct response to selection (OD)
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
-0.05
-0.1
-0.15
-0.2
Raf
Fru
Pyr
Asp
Succ
Fum
Mal
Mel
Pro
Glut
Cit
Xyl
Selection lines

## Figure 2a-d.


2a)
0.1
0.05
0
-0.05
-0.1
Indirect response to off-pathway substrates (OD)
Raff
Fru
Pyr
Succ
Fum
Mal
Proline
Glut
Cit
Selection environment


2b)
0
-0.05
-0.1
-0.15
Indirect response to off-pathway substartes (OD)
Raff
Fru
Pyr
Succ
Fum
Mal
Proline
Glut
Cit
Selection environment


2c)
0
-0.05
-0.1
-0.15
-0.2
Indirect response to on-pathway substrates (OD)
Raf
Fru
Pyr
Succ
Fum
Mal
Pro
Glut
Cit
Assay environmrnt


2d)
0.03
-0.02
-0.07
-0.12
Indirect response to on-pathway substrates (OD)
Raf
Fru
Pyr
Succ
Fum
Malic
Pro
Glut
Cit
Assay environment

## Table 1.

| Cycle | substrate selection | substrate assay | predicted indirect response | observed indirect response at cycle 24 ± Std-error | observed indirect response at cycle 42 ± Std-error |
|---|---|---|---|---|---|
| 24 | Raffinose | Fructose | + | +0.215 ± 0.011 | +0.0442 ± 0.007 |
| 24 | Raffinose | Pyruvate | + | +0.0663 ± 0.012 | -0.0016 ±0.011 |
| 24 | Fructose | Raffinose | + | +0.093 ± 0.016 | +0.0744 ± 0.008 |
| 24 | Fructose | Pyruvate | + | +0.069 ± 0.016 | -0.0076 ± 0.018 |
| 24 | Pyruvate | Raffinose | - | -0.0995± 0.018 | -0.0441 ± 0.025 |
| 24 | Pyruvate | Fructose | - | -0.1969 ± 0.018 | -0.0207 ± 0.024 |
| 24 | Succinate | Fumarate | + | -0.0067 ± 0.009 | +0.0409 ± 0.034 |
| 24 | Succinate | Malate | + | -0.0037 ± 0.009 | +0.0475 ± 0.036 |
| 24 | Fumarate | Succinate | + | +0.0307 ± 0.013 | -0.0513 ± 0.014 |
| 24 | Fumarate | Malate | + | +0.0168 ± 0.012 | -0.0087 ± 0.03 |
| 24 | Malate | Succinate | + | -0.0270 ± 0.013 | -0.0049 ± 0.018 |
| 24 | Malate | Fumarate | + | +0.0027 ± 0.012 | -0.0381 ± 0.014 |
| 24 | Proline | Glutamate | + | +0.0203 ± 0.01 | -0.0067 ± 0.02 |
| 24 | Proline | Citruline | + | +0.0570 ± 0.017 | -0.0028 ± 0.02 |
| 24 | Glutamate | Proline | + | -0.0569 ±0.009 | -0.0440 ± 0.02 |
| 24 | Glutamate | Citruline | + | -0.0639 ±0.008 | -0.0532 ± 0.011 |
| 24 | Citruline | Proline | - | +0.0260 ± 0.014 | +0.0512 ± 0.027 |
| 24 | Citruline | Glutamate | - | +0.0271 ± 0.009 | +0.0895 ± 0.029 |

## Table 2.

| Selection line | Assay environment | Correlation | P-value |
|---|---|---|---|
| Raff | Fru | 0.5987 | **0.0397*** |
| Raff | Pyr | -0.2854 | 0.3686 |
| Raff | Asp | -0.5482 | 0.065 |
| Fru | Raff | 0.3235 | 0.305 |
| Fru | Pyr | 0.1409 | 0.6624 |
| Fru | Asp | -0.1445 | 0.6542 |
| Pyr | Raff | 0.1277 | 0.6926 |
| Pyr | Fru | -0.0466 | 0.8856 |
| Pyr | Asp | 0.9504 | **<.0001*** |
| Asp | Raff | 0.0816 | 0.8009 |
| Asp | Fru | -0.5092 | 0.0909 |
| Asp | Pyr | 0.9298 | **<.0001*** |
| Succ | Fum | 0.9605 | **<.0001*** |
| Succ | Malic | 0.9542 | **<.0001*** |
| Succ | Mel | 0.5087 | 0.0912 |
| Fum | Succ | 0.7246 | **0.0077*** |
| Fum | Malic | 0.9471 | **<.0001*** |
| Fum | Mel | 0.4275 | 0.1657 |
| Malic | Succ | 0.4701 | 0.123 |
| Malic | Fum | 0.9133 | **<.0001*** |
| Malic | Mel | 0.1264 | 0.6955 |
| Mel | Succ | 0.3242 | 0.3039 |
| Mel | Fum | 0.4106 | 0.1849 |
| Mel | Malic | 0.2724 | 0.3917 |
| Pro | Glut | 0.6035 | **0.0377*** |
| Pro | Cit | 0.0808 | 0.8029 |
| Pro | Xyl | -0.0137 | 0.9662 |
| Glut | Pro | 0.5596 | 0.0585 |
| Glut | Cit | 0.5452 | 0.0668 |
| Glut | Xyl | 0.3646 | 0.2439 |
| Cit | Pro | 0.3921 | 0.2074 |
| Cit | Glut | 0.813 | **0.0013*** |
| Cit | Xyl | -0.0499 | 0.8775 |
| Xyl | Pro | 0.8659 | **0.0003*** |
| Xyl | Glut | 0.5091 | 0.091 |
| Xyl | Cit | 0.5732 | 0.0514 |

## Connecting statement between chapters 3 and 4

In chapter 3, I conducted an experiment to test that the pattern of metabolic specialization among experimental populations of wild yeast could be predicted based on the biochemical properties of the environment and the cells living there. I found no evidence for trade-offs between substrates on the same pathway. The indirect response of substrates on other pathways, however, was consistently negative, with little correlation between direct and indirect responses. The conclusion therefore, is that the grain of specialization in this case is the metabolic pathway, and that specialization appears to evolve through mutational degradation. In chapter 4, in order to investigate the consequences of metabolic adaptation of yeast populations for selection in subsequent stressful environments, I used the lines that were evolved in the previous chapter along with the ancestral line (preserved frozen in -80 ℃) and selected them in 4 different stressful environments. I also propagated these lines in glucose, as control for any features of the second round of selection other than the specific stressors employed. Our finding suggests that the evolutionary rescue of populations threatened by successive unrelated stresses is a two-part process. The populations are at heightened risk in the short term, but, should they survive, are more likely to survive in the longer term.

# Chapter 4: Evolutionary rescue of experimental yeast populations after prior exposure to starvation

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## Abstract

Extinction in stressed populations might be prevented if adaption leads to persistence. This process is called evolutionary rescue. The likelihood of rescue depends on ecological and genetic factors. We know that evolutionary rescue is more probable in populations that had prior exposure to lower doses of the same stressor. In this study we hypothesized that due to the presence of generalized stress response in many organisms the probability of rescue will be increased by prior exposure to other stressors. We tested this hypothesis by using experimental populations that had previously experienced and adapted to long periods of starvation. These populations were selected on four unrelated stressors: high temperature, high pH, alcohol and salt. We also propagated these lines in glucose, as control for any features of the second round of selection other than the specific stressors employed. We found that evolutionary history of adaptation to starvation imposes a contrasting effect on the physiological and evolutionary responses of populations to alternative stressors that they may encounter. Such populations when exposed to new stressors demonstrate a higher risk of extinction however, if they survive the initial exposure to the new stressors, they are more likely to survive in the longer term. We conclude that evolutionary rescue of populations subject to successive stressful conditions depends on the interaction between physiological and evolutionary processes.

*Keywords: evolutionary rescue, stressors, wild yeast, evolutionary history,*

## Introduction

Growth declines when a population accustomed to benign conditions is exposed to a stressful environment. If the stress continues for generations the population may become extinct or it may adapt to the new environment through natural selection. Adaptation causing persistence is called "evolutionary rescue" (Gomulkiewicz & Holt, 1995). Evolutionary rescue by natural selection depends on a variety of factors such as population size, rate of environmental change, the strength of selection, and history of stress (Lynch *et al.*, 1991; Burger & Lynch, 1995; Bell & Collins, 2008; Orr & Unckless, 2008). In this article we report an investigation of the evolutionary history of stress, and in particular how experimental populations responded to novel stressful conditions when they were first exposed to starvation for hundreds of generations.

For the past few decades, studies using several model organisms have shown that there are general stress response systems that activate similar physiological responses to many different stressors. These studies have shown that when bacterial cells perceive a wide variety of stressors such as prolonged starvation, osmotic stress, low pH, low temperature, heat shock, and oxidative stress a group of transcription initiation factors activate a group of genes so as to protect cells against the perceived stress as well as against other stressors that cells have not yet encountered (Lange & Hengge-Aronis, 1991; McCann *et al.*, 1991; Völker *et al.*, 1994; Sledjeski *et al.*, 1996; Muffler *et al.*, 1996, 1997; Yildiz & Schoolnik, 1998; Miura *et al.*, 1998; Suh *et al.*, 1999; Hecker & Völker, 2001; Price *et al.*, 2001; Petersohn *et al.*, 2001; Hengge-Aronis, 2002; Hülsmann *et al.*, 2003; Hecker *et al.*, 2007). Similarly, in budding and fission yeast scientists have discovered a general environmental stress response that actively protects cells against many stressors (Chen *et al.*, 2003b; Berry & Gasch, 2008). Consequently, exposure to one stressor may

confer protection against a variety of stressors through expression of generalized stress response systems.

The existence of generalized stress response systems implies that populations that have evolved resistance to one stressor may be resistant to other stressors as well. This would constitute a correlated response to a novel stressful environment that has evolved as the result of selection in the original stressful environment. Several studies have used experimental evolution to examine this phenomenon in *Drosophila*. Populations of *Drosophila* were propagated in a given stressful environment and then tested in other stressful conditions, quantitatively or qualitatively different from the original conditions. Many such studies showed that selecting for resistance to one stressor leads to resistance to other stressors, indicating the existence of a common genetic basis for resistance to a variety of stressors in *Drosophila* (e.g. Bubliy & Loeschcke, 2005; A A Hoffmann & Parsons, 1993; A. a. Hoffmann & Parsons, 1989; A. A. Hoffmann & Parsons, 1989; Service & Rose, 1985; Telonis-Scott, Guthridge, & Hoffmann, 2006; Zwaan, Bijlsma, & Hoekstra, 1995; for a comprehensive review see Samani, 2010). This implies that if populations are exposed to new stressful environments they may become extinct unless rescued by the evolution of resistance.

It has been shown that the likelihood of rescue increases significantly if populations were previously exposed to lower doses of the same stressor (Samani & Bell, 2010; Bell & Gonzalez, 2011; Gonzalez & Bell, 2013). This is due to the positive genetic correlation that exists between mutations conferring resistance to lethal stress and those mutations that were fixed while the population was selected under sub-lethal doses of the same stressor. Genetic correlation, therefore, might be an important factor in determining the evolutionary rescue of stressed populations.

Genetic correlation may evolve when one gene or a group of genes influence the same phenotypic traits. The existence of generalised stress response in organisms could therefore lead to positive genetic correlation among different stressors, as has been documented my many physiological and evolutionary studies (Hecker & Völker, 2001; Chen *et al.*, 2003a; Bijlsma & Loeschcke, 2005; Hecker *et al.*, 2007; Berry & Gasch, 2008). This suggests that the likelihood of rescue in novel stressful environments may be increased by prior exposure to other stressors.

Rescue after exposure to a new stressor may be attributable either to a short-term physiological response or to a longer-term evolutionary response. In the first place, exposure to a prior stressor may activate a physiological response, often in the form of a generalized stress response. The activated generalized stress response after exposure to the first stressor may confer resistance to a second stressor. This will result in a lower frequency of extinction immediately after exposure to the second stressor. The rescue of populations exposed to the second stressor is an indirect response to selection on the first stressor.

The second possibility is that the second stressor persists, so that the population grows slowly and eventually either becomes extinct or becomes adapted through the selection of individuals that show a superior stress response, whether general or specific. In this case, prior evolutionary exposure to a first stressor may induce a long-term response if the genetic modifications that confer resistance to the first stressor facilitate the subsequent evolution of resistance to the second stressor. The long-term response will be the elevation of growth rate through natural selection during several generations after exposure to the second stressor. Rescue therefore, is the direct response to selection imposed by the second stressor, which in turn reflects an indirect response to the first stressor.

The short-term and long-term responses are both consequences of the activity of generalized stress response systems. Hence, we predict that the magnitudes of the short-term and long-term responses will be positively correlated. We further hypothesize that if a more severe stress (i.e. a stress that causes a greater depression of growth) both induces and requires a higher general level of stress resistance, the effect of prior exposure on the frequency of rescue will vary with the severity of both the prior and subsequent stressors.

The growth of evolved populations may also vary among different kinds of prior stressor. Populations that are adapted to more severe stressors should be more resistant to any subsequent stressor. This implies that the non-evolving ancestral line and lines that were maintained in comparable permissive conditions should show less resistance to a subsequent stressor than lines that were previously exposed to the prior stressor and became adapted to it. Thus, we expect rescue to be more frequent among lines that have previously become adapted to stressful conditions than in the ancestor or in comparable lines propagated in permissive conditions.

We tested these hypotheses by using experimental populations that had previously evolved the ability to utilize refractory substrates on which growth was initially very poor. These populations have all experienced long periods of starvation during the course of adaptation. One set of populations was maintained in fructose, representing permissive conditions in which growth is high from the start and does not improve substantially thereafter. The ancestor was stored frozen. The experimental populations, each with a history of starvation, were exposed to four unrelated stressors: high temperature, high pH, alcohol and salt. The populations that survived initial exposure were then propagated with the stressor for about 20 generations. They were also propagated in glucose, as a control for any features of the second round of selection

other than the specific stressors employed. This design enables us to evaluate both the short-term and long-term effects of exposure to a different prior stressor.

## Materials and methods

**Initial evolution experiment.** Our ancestral population was a wild Saccharomyces paradoxus strain isolated from Mont Saint-Hilaire, 40 km east of Montreal, Canada. We previously used this wild strain to establish 12 single-substrate lines (SSLs) each having been selected for metabolising a single carbon source: raffinose (raf), fructose (fru), pyruvate (pyr), aspartate (asp), succinate (suc), fumarate (fum), malate (mal), melibiose (mel), proline (pro), glutamate (glu), citruline (cit), and xylose (xyl). The SSLs were cultured in 12-well plates containing minimal medium supplemented with 3% w/v of their corresponding carbon source, using 12 replicates per treatment. Substrate lines were incubated at 28 ℃ and transferred every 7 days with an inoculum size of 5% of culture volume for 42 transfers, equivalent to about 180 generations (Samani & Bell, in prep). The ancestral line was stored at -80 ˚C. Fructose is a benign environment in which growth was initially high and did not improve thereafter; the other substrates are stressful in different degrees.

**Short-term evolution in novel stressful environments.** At the end of the initial selection experiment, all SSLs and 12 replicates of the ancestral line were grown in 12-well plates containing minimal medium supplemented with 2% w/v glucose for two consecutive transfers at 28 ℃. This ensured a common physiological state before exposure to a novel stressor. We then established 5 sets of new selection lines, with each line growing in a base medium containing minimal media supplemented with 2% w/v glucose. Four sets of these selection lines were each

exposed to a novel stressor: 20% ethyl alcohol, high temperature (38 ℃), pH 10, and 80 g/L NaCl. The fifth set was not exposed to any stressor and therefore represented a control for any features of selection other than the four specified stressors. Inoculation procedures were performed at the CIAN robotics/automation core facility (McGill University) on a BiomekFX liquid handler and an ORCA robotic arm controlled by the SAMI software (Beckman, Mississauga, Canada).

The treatments thus comprised a set of prior stressors crossed with a set of subsequent stressors. The prior stressors were 11 single substrates, together with a benign substrate (fructose) and the stored ancestor. The subsequent stressors were the four named above, with glucose as a non-stressful control. There were 12 replicates of each treatment combination: the replicates are independent selection lines for the substrates, and replicate colonies for the ancestor. All populations were exposed to the subsequent stressor, and afterwards transferred every 7 days with an inoculum size of 5% of culture volume for 4 transfers.

**Growth assay.** The number of populations becoming extinct was recorded at the end of transfers 1 and 4. Optical density (OD) of persistent selection lines at transfers 1 and 4 were measured at 620nm immediately after inoculation (time 0), and at 24h and 72h after inoculation. Yield was estimated as the difference between OD at 72h and initial OD for the 4 stressful regimes and as the difference between OD at 24h and initial OD for the glucose control.

**Statistical analysis.** We used JMP®, Version 11, SAS Institute Inc., Cary, NC, (1989-2007) to conduct the statistical analysis.

## Results

### Short-term response: extinction of lines and physiological response of persistent lines at transfer one.

**Overall frequency of extinction in stressful environments.** We observed no extinction events among SSLs when cultured in glucose medium, confirming the permissiveness of the glucose environment. Some SSLs became extinct being exposed to the stressful environments for the first time (transfer one; Table 1). Extinction events over all stressors were more frequent among SSLs than among fructose lines (Fisher's exact test, $p < 0.05$) or the ancestor (Fisher's exact test, $p < 0.01$).

**Frequency of extinction in relation to stressor.** The frequency of extinction varied among SSLs over all stressors combined (chi-square test, $p< 0.001$). Extinction was most frequent at high pH and in alcohol medium.

**Frequency of extinction in relation to selection history.** Extinction frequency over all stressors varied among selection lines (Chi-square test, $p < 0.01$). Ancestral, fructose and raffinose lines had the fewest extinction events at transfer one (Fisher's exact test, $p < 0.05$ for all three comparisons), each having a single extinction over all stressors.

**Physiological response to novel stressors.** We define the physiological response to a given stressor as the difference between the average yield of selection lines and ancestor in the same stressor at transfer one. The effect of evolutionary history (i.e. prior stressor) on the yield of persistent selection lines at their first exposure to novel stressful environments was evaluated by separate single-factor ANOVAs followed by post-hoc Tukey-Kramer HSD tests (Figure 1). The

effect of evolutionary history on yield was significant for high temperature ($F(11, 132) = 7.46$, $p < 0.001$); alcohol ($F(11, 119) = 3.29$, $p = 0.0006$); NaCl ($F(11, 127) = 12.71$, $p < 0.0001$); pH ($F(11, 93) = 2.44$, $p = 0.01$); and glucose ($F(11, 132 = 6.01$, $p < 0.0001$).

## Long-term response: improvement of growth after short-term evolution in stressors.

**Adaptation to the subsequent stressor.** To test whether or not adaptation occurred during the four growth cycles of the experiment, the growth of lines between transfer 4 and transfer 1 was compared using single-factor ANOVAs (Table S1). Adaptation to the four stressful environments occurred frequently among SSLs, whereas none improved during the 4 cycles of selection in glucose.

55% (24/44) of the SSLs selected in the 4 stressors showed significant increase in yield during the four transfers while the rest of the persistent SSLs showed no significant change during this short-term evolution. The only SSLs that declined in any of the environments were raffinose lines in the glucose environment ($F(1, 22) = 6.77$, $p = 0.01$). The ancestor adapted to only two of the environments, high temperature ($F(1, 22) = 14.95$, $p < 0.001$) and NaCl ($F(1, 22) = 31.75$, $p < 0.0001$), while significantly decreasing in yield in alcohol ($F(1, 22) = 23.62$, $p < 0.0001$), and glucose ($F(1, 22) = 48.97$, $p < 0.0001$). Furthermore, the fructose lines failed to adapt to any of the environments, while declining in two of the environments, alcohol ($F(1, 22) = 13.54$, $p = 0.001$) and glucose ($F(1, 22) = 26.13$, $p < 0.0001$). The probability of adaptation was significantly lower in fructose selected lines than other selection treatments (Fisher's exact test, $p < 0.05$). Moreover, fructose and ancestral lines declined in yield during short-term evolution in

stressful environments more frequently than other selection lines (Fisher's exact test, $p = 0.01$, $p < 0.05$ respectively).

**Improvement in relation to stressor.** We calculated the difference between yield in transfer 4 and transfer 1 as the long-term (evolutionary) response to selection in the stressors. This is the growth improvement caused by the evolutionary rescue of selection lines in the four stressful environments. The long-term response in every stressor over all SSLs was significantly larger that the long-term response in glucose medium (comparison with response to glucose medium using Dunnett's test; $p < 0.0001$ for all four comparisons). Long-term response to selection in stressful environments was significantly higher in SSLs compared to ancestor and fructose lines ($F(1, 430) = 28.72$, $p < 0.0001$). If we take pH and temperature with 64% and 52% extinction rates as the most severe and NaCl and alcohol with 10% and 3% extinction rates as the least severe stressors at transfer 4 (extinction rate in high pH and temperature was significantly higher than in NaCl and alcohol, Fisher's exact test, $p < 0.0001$), long-term response to selection in the four stressors although insignificant was negatively correlated with the severity of the stressors ($F(1, 2) = 8.32$, $p = 0.1$, with an $R^2$ of 0.81, Figure 2).

**Improvement in relation to selection history.** There was significant variation among SSLs in their long-term response to evolution in stressors ($F(11, 363) = 4.72$, $p < 0.0001$). The response to selection in stressful environments among SSLs was negatively correlated with the growth of the ancestor in the single substrates ($R^2 = 0.54$, slope = -0.23; $F(1, 11) = 11.63$, $p < 0.01$) showing that lines from more stringent selection environments improved more during evolution (Figure 3).

We compared the long-term responses of SSLs, ancestor, and fructose lines in every stressor (Figures 4a-d). The effect of evolutionary history on the response to selection on glucose was insignificant but was significant among SSLs excluding fructose for all four stressors: temperature ($F(10, 55) = 2.45$, $p = 0.01$), alcohol ($F(10, 120) = 2.36$, $p = 0.01$), NaCl ($F(10, 97) = 2.25$, $p < 0.05$), and pH ($F(9, 35) = 2.26$, $p < 0.05$).

### Relation between short-term and long-term responses

The short-term (physiological) and long-term (evolutionary) responses of selection lines in novel stressors were negatively correlated in all environments (Figures 5a-e). All of the correlations are significant at $p < 0.05$ except in high pH environment.

## Discussion

We investigated the effect of evolutionary history on evolutionary rescue of experimental yeast populations. We used experimental populations that were previously selected to utilize poor carbon sources therefore, had experienced chronic starvation stress for hundreds of generations. We exposed and selected these populations to new stressful environments for four cycles. Although only about 20 generations were available for adaptation, we detected both short-term and long-term responses to selection in all four stressful environments, permitting detailed tests of our hypotheses. We used the ancestral line and permissive (fructose) selection lines as the basis of comparison with single-substrate lines.

The main hypothesis tested by our experiment is that prior exposure to a particular stressor will predispose populations to resist another stressor more effectively, either through short-term (physiological) or through long-term (evolutionary) responses or both (Lange & Hengge-Aronis, 1991; McCann *et al.*, 1991; Völker *et al.*, 1994; Muffler *et al.*, 1997; Sledjeski *et al.*, 1996; Muffler *et al.*, 1996; Yildiz & Schoolnik, 1998; Miura *et al.*, 1998; Suh *et al.*, 1999; Petersohn *et al.*, 2001; Price *et al.*, 2001; Hecker & Völker, 2001; Hengge-Aronis, 2002; Chen *et al.*, 2003b; Hülsmann *et al.*, 2003; Hecker *et al.*, 2007; Berry & Gasch, 2008). Subsidiary hypotheses predict that more stringent selection environments would induce greater stress resistance, and that populations adapted to these conditions will be more resistant to more severe stresses (Samani & Bell, 2010; Bell & Gonzalez, 2011; Gonzalez & Bell, 2013).

These hypotheses were decisively rejected for the short-term response. Indeed, there was a clear trend in the opposite direction: our single-substrate selection lines (SSLs) actually had a much higher frequency of extinction than either their ancestor or comparable lines previously propagated in a benign (fructose) environment. The SSLs that survived their initial exposure to the novel stressor also grew less than ancestor and fructose lines. Hence, far from protecting populations against a novel stressor, prior exposure to a different stressor substantially increased the risk of extinction.

The success of the short-term response to a stressor is governed by the speed and effectiveness with which cells can modify their current phenotype to counteract the adverse effect of the stressors on their physiology. In immotile organisms such as yeast, the dominant aspect of adaptive phenotypic plasticity is acclimation, the reconfiguration of the physiological state of the organism through a variety of processes such as changes in enzyme activity and the synthesis or degradation of specific proteins and intermediary metabolites (Hoffman & Parsons, 1991). One

important agent of acclimation is the generalized environmental stress response, a common gene expression system in which yeast cells up- and down-regulate about 900 genes to protect the cell against environmental damage (Gasch *et al.*, 2000; Causton *et al.*, 2001). This system is induced by stress and upregulates genes involved in carbohydrate metabolism, cell stress, and energy generation; at the same time, a wide group of genes that are engaged in cell growth and protein synthesis are repressed (Gasch *et al.*, 2000; Causton *et al.*, 2001). This defensive strategy will promote survival rather than growth and cell division.

The way in which our SSLs were propagated may have created conflicting demands on the cells. On the one hand, it was necessary for them to resist the stress, to avoid dying; on the other hand, it was necessary for them to grow, in order to avoid being washed out by serial transfer. The generalized stress response incorporates a fundamental antagonism between survival and growth. The lineages most likely to survive chronic starvation under serial transfer, therefore, are not those with a superior generalized stress response, but rather those with a damaged response, permitting growth, coupled with an enhanced ability to metabolize a particular substrate. This explains the high frequency of extinction among the SSLs when challenged with a subsequent stressor, and is consistent with the unimpaired ability of the ancestor and the fructose lines to survive.

Our main hypothesis correctly predicts the effect of prior stress on the long-term response to a subsequent stressor. SSLs often improved substantially within four growth cycles and showed no decline during this time. The growth of ancestral lines, on the other hand, declined in at least two environments and improved only in the high-pH environment. Fructose lines failed to improve in any of the stressors, and actually grew more slowly in alcohol and glucose environments.

Unlike the physiological response, there is no well-characterized evolutionary response to stress that would lead to greater evolvability and thereby the survival of the lineage, rather than the individual cell. The rate of response to selection in an asexual population with little standing genetic variation will depend primarily on the mutation supply rate, which is proportional to the product of population size and the mutation rate. The SSLs have historically smaller yield than the fructose lines, so population size cannot explain the difference in response. The mutation rate is a more likely candidate, because it has been observed that chronic sublethal stress may cause an increase in the variance of fitness (Goho & Bell, 2000), presumably through mutagenesis (Bjedov *et al.*, 2003; Galhardo *et al.*, 2007). Moreover, the generalized stress response system in yeast upregulates mutagenesis in response to non-DNA-damaging chronic stress such as starvation or heat shock (Shor *et al.*, 2013). This may be specifically targeted to the response system itself, since prolonged and constant proteotoxic stress in budding yeast may induce mutations in the genes that govern the response (Shor *et al.*, 2013). Our results show that SSLs that have previously experienced chronic stress adapt more rapidly to novel environmental stressors than their ancestors, which had no such exposure, and those lines that experienced comparable but benign conditions of growth (the fructose lines) in their recent evolutionary past. This pattern is consistent with an elevated rate of stress-induced mutagenesis caused by chronic starvation in the SSLs.

We have found that prior stress by starvation impairs the physiological response but enhances the evolutionary response to subsequent stress by a different agent. The physiological response may be impaired because selection for growth through serial transfer is antagonistic to selection for individual integrity. The evolutionary response may be enhanced because chronic sublethal stress increases the genomic mutation rate. The two are negatively correlated because they are

inversely related to the severity of the prior stress: the physiological response to a subsequent stressor is more impaired and the evolutionary response more elevated by more severe prior stress. This interpretation is consistent with all our results, although it must await explicit demonstration of the mechanisms responsible. It suggests that the evolutionary rescue of populations threatened by successive unrelated stresses is a two-part process. The populations are at heightened risk in the short term, but, should they survive, are more likely to survive in the longer term. The fate of populations in environments subject to a series of shocks will depend on both physiological and evolutionary processes, whose interaction must be understood in order to predict the likelihood of rescue.

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## Figure captions

Figure 1. The effect of evolutionary history on physiological responses in novel stressful environments. Selection treatments not connected by the same letter are significantly different at their physiological response in a given stressor (post-hoc Tukey-Kramer HSD test, $\alpha < 0.05$). a) pH, b) NaCl, c) Alcohol d) Temperature e) Glucose. Error bars are standard error of the mean for each SSL.

Figure 2. Long-term response to selection in the four stressful environments is negatively correlated with the severity of the stressors represented by the number of extinction events at transfer 4.

Figure 3. The response to selection in stressful environments among SSLs was negatively correlated with the growth of the ancestor in the single substrates ($R^2 = 0.54$, slope = -0.23).

Figure 4. The effect of evolutionary history on the long-term response to selection in stressful environments. Selection treatments not connected by the same letter are significantly different at their response to selection on a given stressor (post-hoc Tukey-Kramer HSD test; $\alpha < 0.05$). a) pH, b) NaCl, c) Alcohol d) Temperature.

Figure 5. Correlation between physiological response and evolutionary response of selection lines in novel stressors. a) pH ($R^2 = 0.14$, slope = -0.89). b) alcohol ($R^2 = 0.46$, slope = -0.84). c)

temperature ($R^2$ = 0.37, slope = -1.16). d) NaCl ($R^2$ = 0.65, slope = -0.52). e) glucose ($R^2$ = 0.71, slope = -0.51). R2 and the formula of the regression line are displayed in each graph.

## Table captions

Table 1. Number of extinctions over all stressors at transfer 1.

Table S1. Single factor ANOVA comparing growth at T4 with growth at T1 of persistent selection lines.

## Figure 1a-e

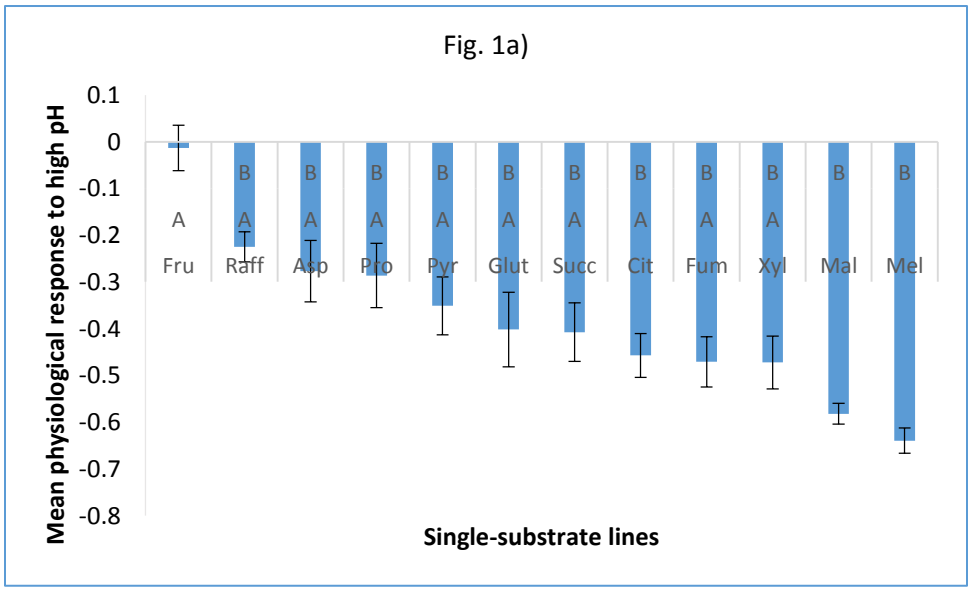


Fig. 1a)
Mean physiological response to high pH
0.1
0
-0.1
-0.2
-0.3
-0.4
-0.5
-0.6
-0.7
-0.8
A B B B B B B B B B B
Fru Raff Asp Pro Pyr Glut Succ Cit Fum Xyl Mal Mel
Single-substrate lines

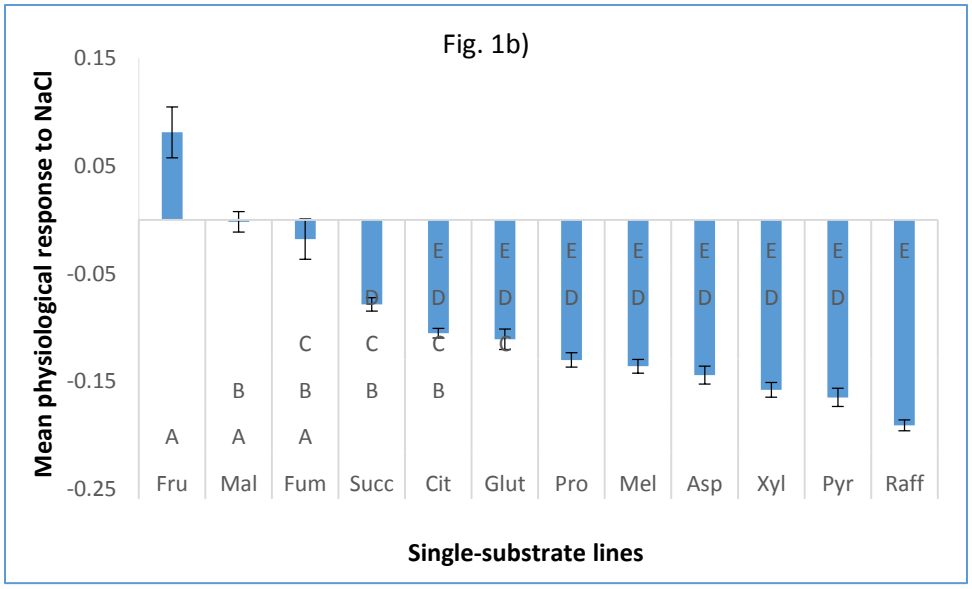


Fig. 1b)
Mean physiological response to NaCl
0.15
0.05
-0.05
-0.15
-0.25
A B C B E E E E E E E
Fru Mal Fum Succ Cit Glut Pro Mel Asp Xyl Pyr Raff
Single-substrate lines

Fig. 1c)

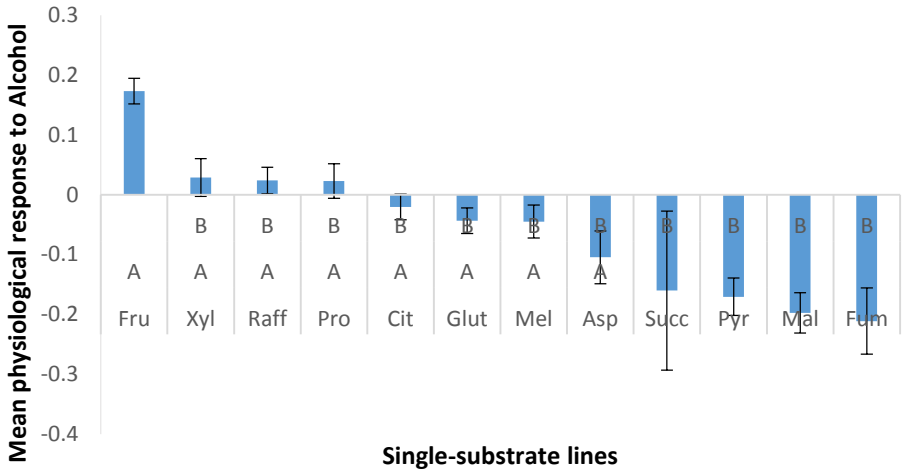
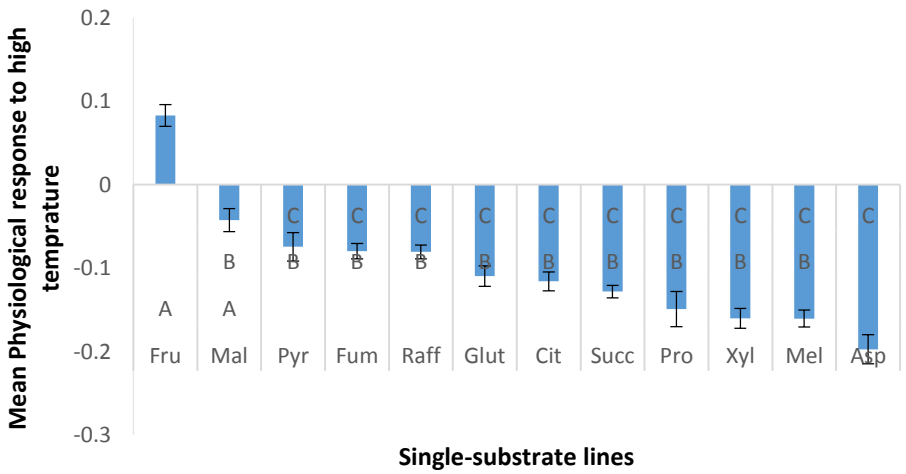

Mean physiological response to Alcohol
0.3
0.2
0.1
0
-0.1
-0.2
-0.3
-0.4
A
B
A
B
A
B
A
B
A
B
A
B
A
B
A
B
A
B
A
B
A
B
Fru
Xyl
Raff
Pro
Cit
Glut
Mel
Asp
Succ
Pyr
Mal
Fum
Single-substrate lines

Fig. 1d)


Mean Physiological response to high temprature
0.2
0.1
0
-0.1
-0.2
-0.3
A
B
A
C
B
C
B
C
B
C
B
C
B
C
B
C
B
C
B
C
B
C
B
C
Fru
Mal
Pyr
Fum
Raff
Glut
Cit
Succ
Pro
Xyl
Mel
Asp
Single-substrate lines

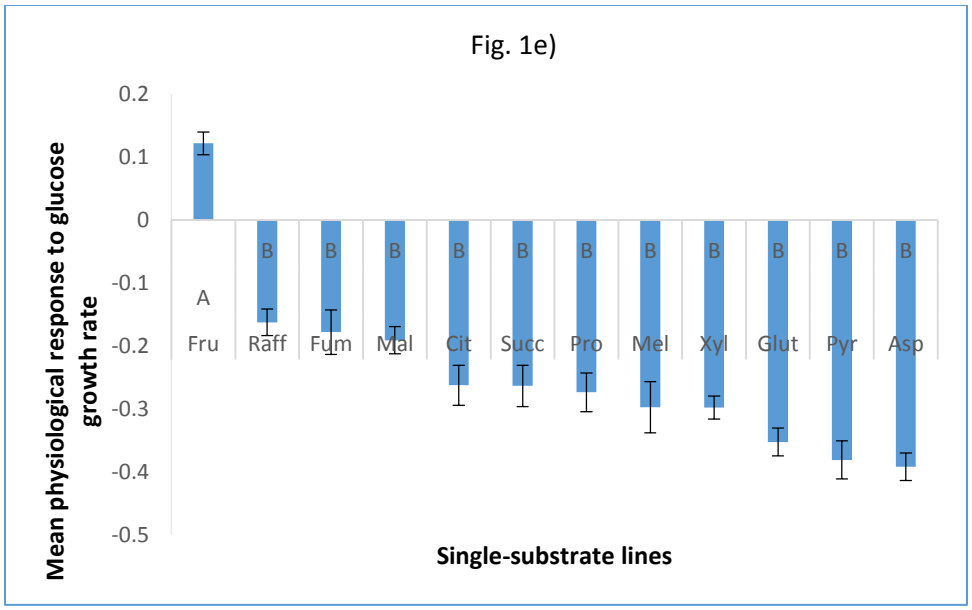


Fig. 1e)
Mean physiological response to glucose growth rate
0.2
0.1
0
-0.1
-0.2
-0.3
-0.4
-0.5
A
B
Fru
Raff
Fum
Mal
Cit
Succ
Pro
Mel
Xyl
Glut
Pyr
Asp
Single-substrate lines

## Figure 2.

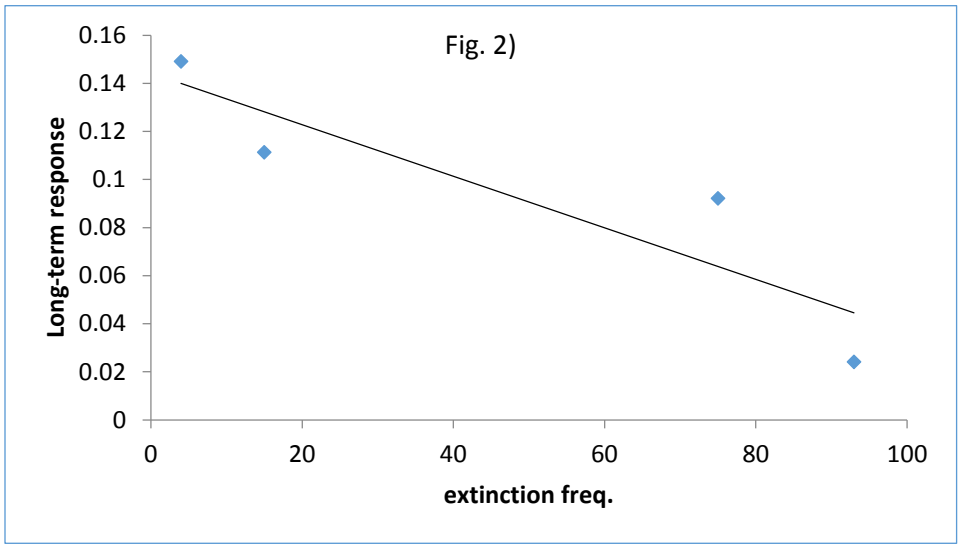


Fig. 2)
Long-term response
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
0
20
40
60
80
100
extinction freq.

## Figure 3.

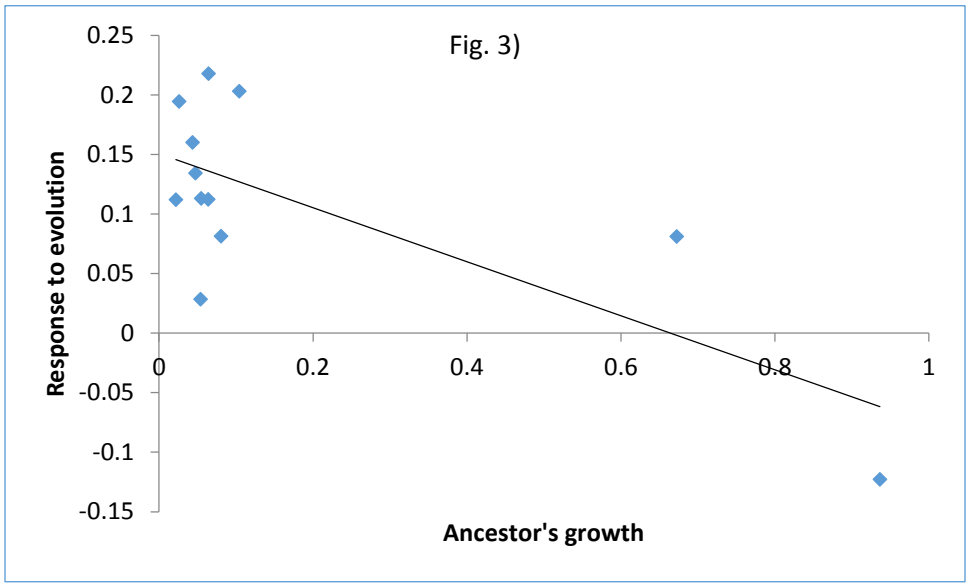


Fig. 3)
Response to evolution
Ancestor's growth
0.25
0.2
0.15
0.1
0.05
0
-0.05
-0.1
-0.15
0
0.2
0.4
0.6
0.8
1

## Figures 4a-d.

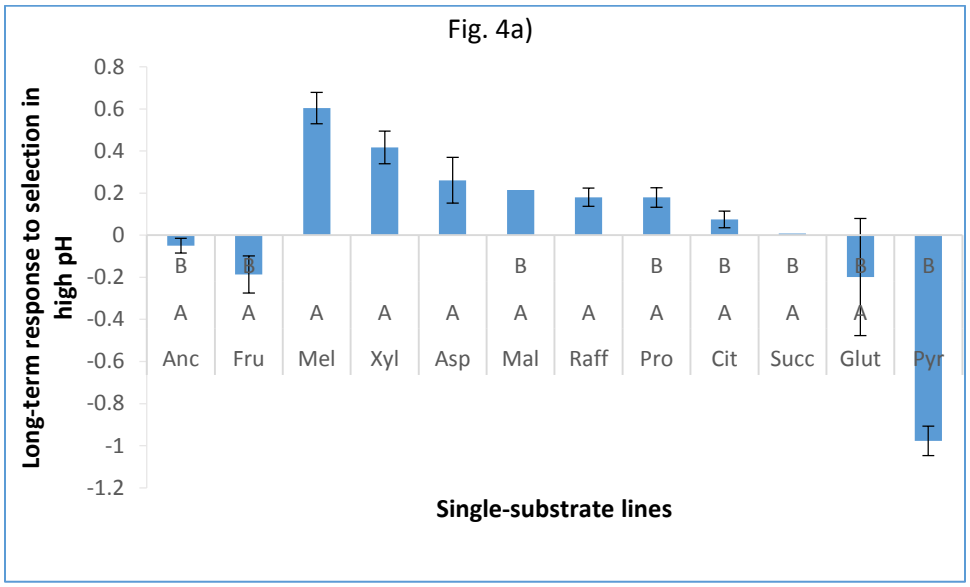


Fig. 4a)
Long-term response to selection in high pH
0.8
0.6
0.4
0.2
0
-0.2
-0.4
-0.6
-0.8
-1
-1.2
B
A
Anc
A
Fru
A
Mel
A
Xyl
A
Asp
B
A
Mal
A
Raff
B
A
Pro
B
A
Cit
B
A
Succ
B
A
Glut
B
Pyr
Single-substrate lines

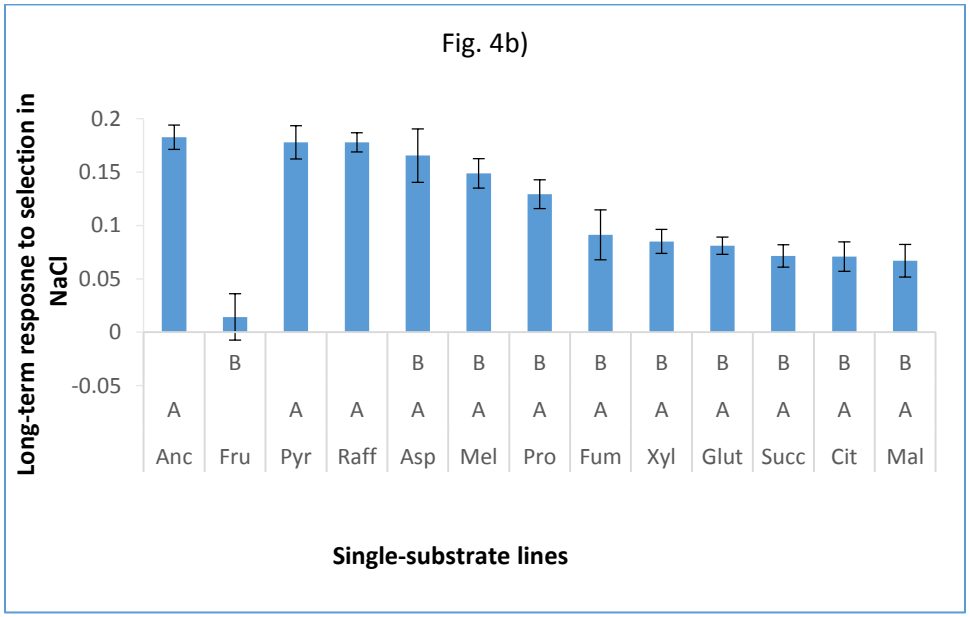


Fig. 4b)
Long-term resposne to selection in NaCl
0.2
0.15
0.1
0.05
0
-0.05
B
A
Anc
B
Fru
A
Pyr
A
Raff
B
A
Asp
B
A
Mel
B
A
Pro
B
A
Fum
B
A
Xyl
B
A
Glut
B
A
Succ
B
A
Cit
B
A
Mal
Single-substrate lines

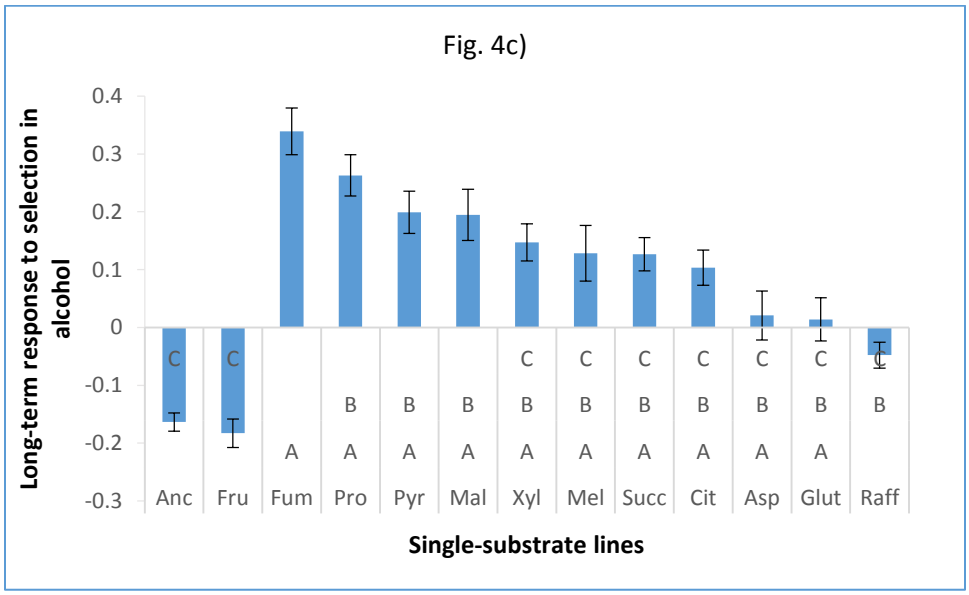


Fig. 4c)
Long-term response to selection in alcohol
0.4
0.3
0.2
0.1
0
-0.1
-0.2
-0.3
C C A B A B A B A C B A C B A C B A C B A C B A C B B
Anc Fru Fum Pro Pyr Mal Xyl Mel Succ Cit Asp Glut Raff
Single-substrate lines

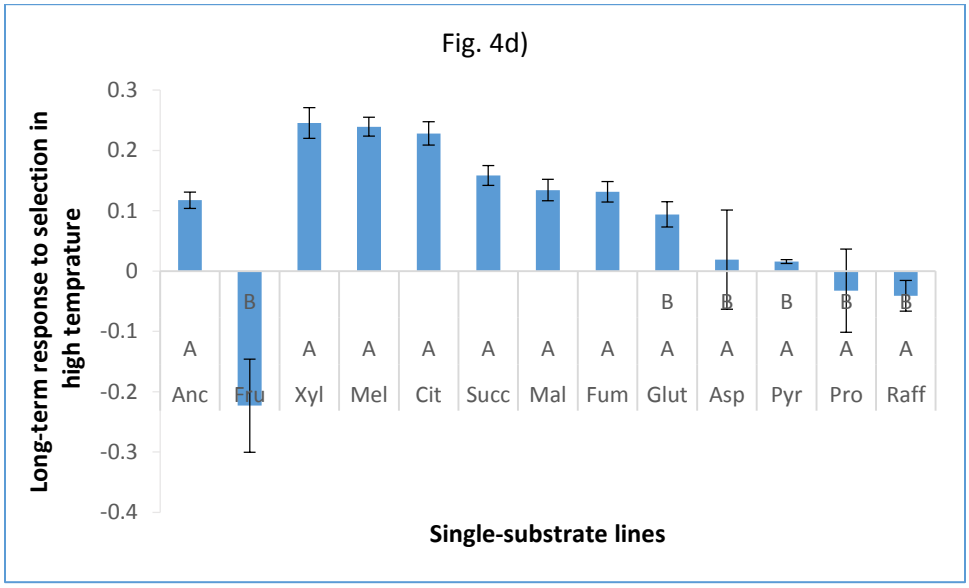


Fig. 4d)
Long-term response to selection in high temprature
0.3
0.2
0.1
0
-0.1
-0.2
-0.3
-0.4
A B A A A A A A B A B A B A A
Anc Fru Xyl Mel Cit Succ Mal Fum Glut Asp Pyr Pro Raff
Single-substrate lines

## Figures 5a-e.

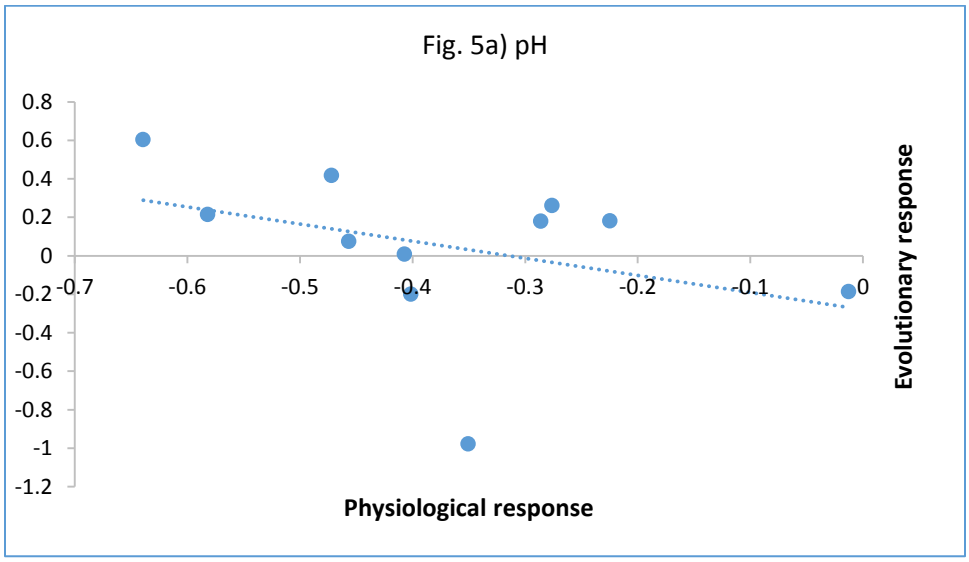


Fig. 5a) pH
0.8
0.6
0.4
0.2
0
-0.2
-0.4
-0.6
-0.8
-1
-1.2
-0.7
-0.6
-0.5
-0.4
-0.3
-0.2
-0.1
0
Evolutionary response
Physiological response

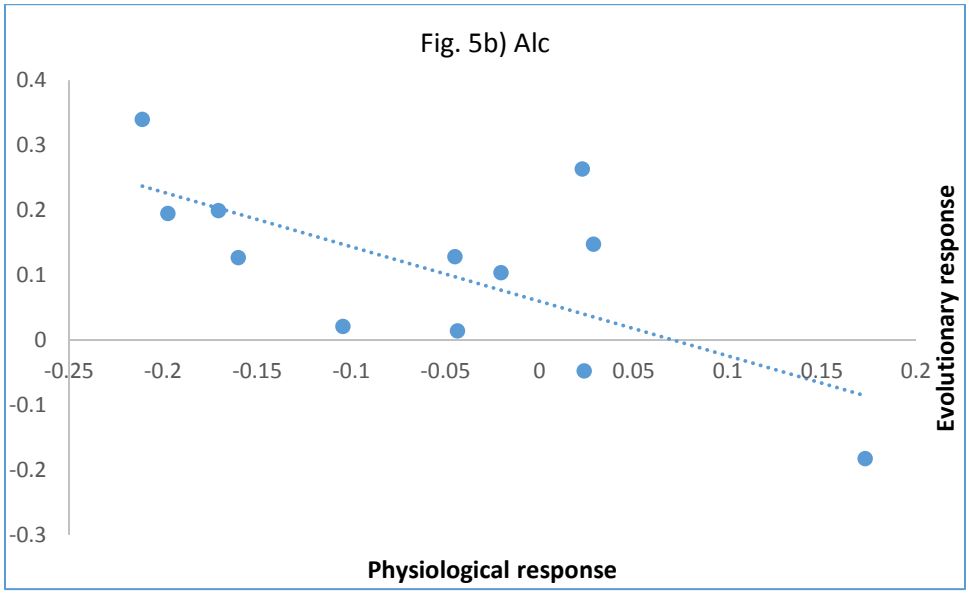


Fig. 5b) Alc
0.4
0.3
0.2
0.1
0
-0.1
-0.2
-0.3
-0.25
-0.2
-0.15
-0.1
-0.05
0
0.05
0.1
0.15
0.2
Evolutionary response
Physiological response

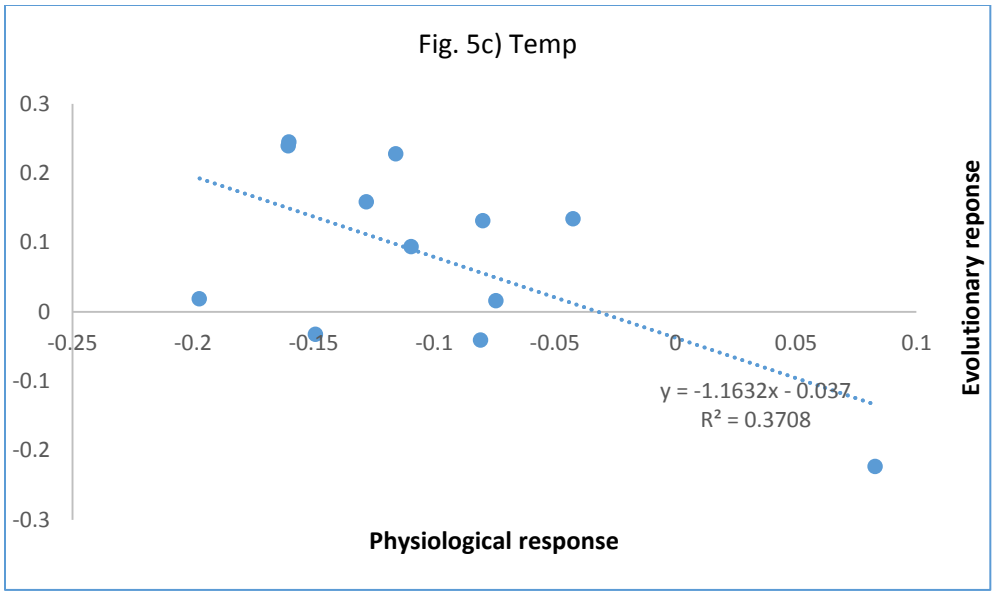


Fig. 5c) Temp
0.3
0.2
0.1
0
-0.1
-0.2
-0.3
-0.25
-0.2
-0.15
-0.1
-0.05
0
0.05
0.1
y = -1.1632x - 0.037
R² = 0.3708
Evolutionary reponse
Physiological response

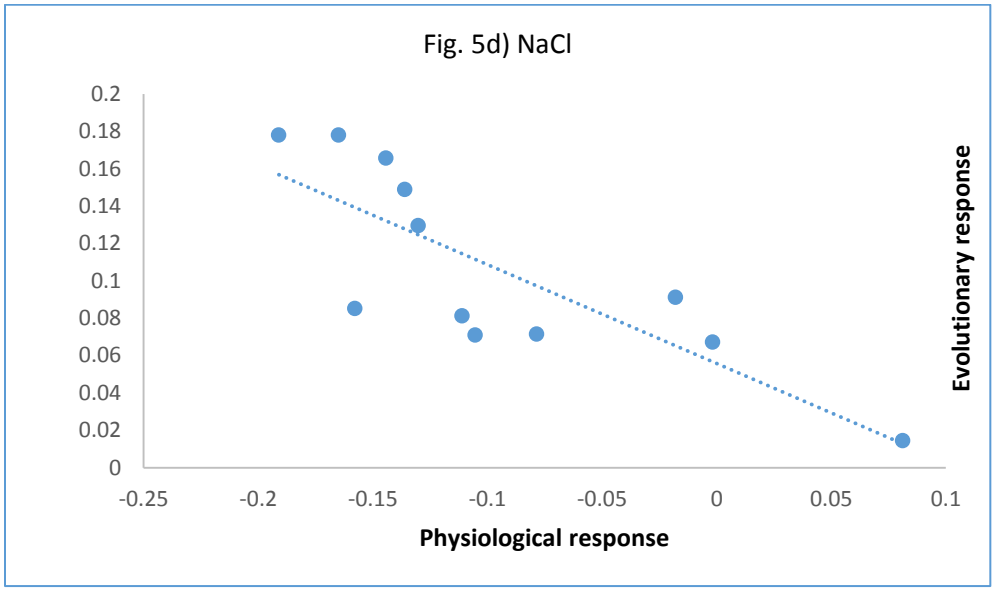


Fig. 5d) NaCl
0.2
0.18
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
-0.25
-0.2
-0.15
-0.1
-0.05
0
0.05
0.1
Evolutionary response
Physiological response

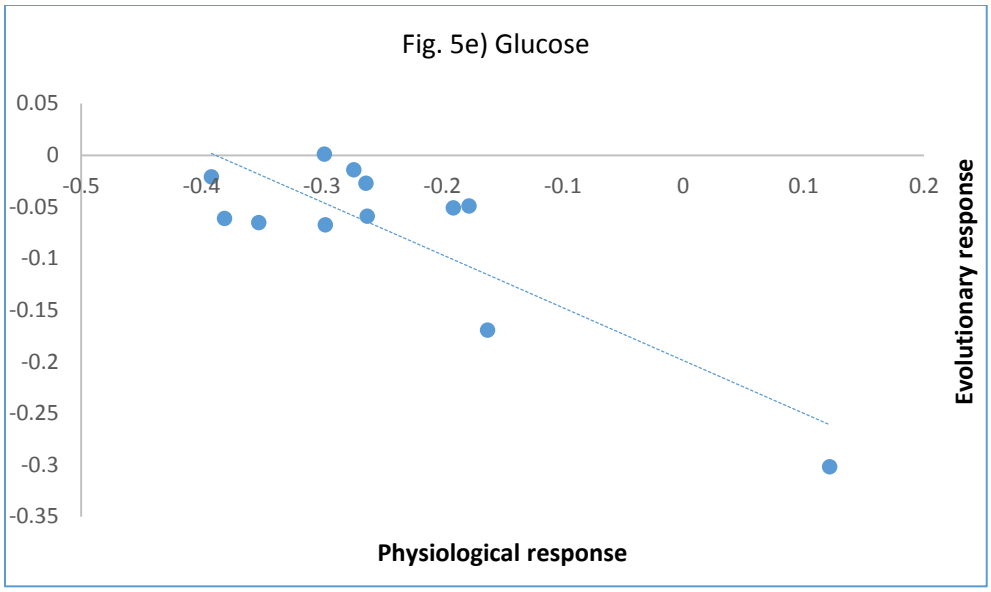


Fig. 5e) Glucose
0.05
0
-0.05
-0.1
-0.15
-0.2
-0.25
-0.3
-0.35
-0.5
-0.4
-0.3
-0.2
-0.1
0
0.1
0.2
Evolutionary response
Physiological response

## Table 1.

| selection lines | Number of extinct lines over all stressors |
|---|---|
| *Raff* | *1* |
| *Fru* | *1* |
| *Pyr* | *7* |
| *Asp* | *7* |
| *Succ* | *8* |
| *Fum* | *10* |
| *Malic* | *7* |
| *Mel* | *6* |
| *Pro* | *2* |
| *Glut* | *4* |
| *Cit* | *2* |
| *Xyl* | *2* |

## Table S1.

| Selection treatment | Stressor | Mean T1 ± stdev | Mean T4 ± stdev | Adaptation (ANOVA) |
|---|---|---|---|---|
| Ancestor | Temp | 0.6±0.06 | 0.71±0.085 | $F(1, 22) = 14.95$, $p < 0.001$ |
| Raffinose | Temp | 0.52±0.06 | 0.45±0.15 | Insignificant |
| Fructose | Temp | 0.7± 0.09 | 0.65 ± 0.11 | Insignificant |
| Pyruvate | Temp | 0.52 ± 0.12 | 0.43 ± 0.19 | Insignificant |
| Aspartate | Temp | 0.4 ± 0.12 | 0.45 ± 0.33 | Insignificant |
| Succinate | Temp | 0.47 ± 0.05 | 0.62 ± 0.08 | $F(1, 18) = 24.7$, $p < 0.0001$ |
| Fumarate | Temp | 0.52 ± 0.06 | 0.66 ± 0.11 | $F(1, 18) = 13.14$, $p < 0.002$ |
| Malate | Temp | 0.55 ± 0.1 | 0.63 ± 0.1 | $F(1,18) = 2.9$, $p = 0.1$ |
| Melibiose | Temp | 0.44 ± 0.07 | 0.68 ± 0.1 | $F(1, 17) = 37.07$, $p < 0.001$ |
| Proline | Temp | 0.45 ± 0.15 | 0.59 ± 0.09 | $F(1,15) = 4.14$, $p = 0.05$ |
| Glutamate | Temp | 0.48 ± 0.08 | 0.58 ± 0.16 | $F(1,15) = 2.76$, $p=0.11$ |
| Citruline | Temp | 0.48 ± 0.08 | 0.67 ± 0.08 | $F(1, 16) = 23.55$, $p = 0.0002$ |
| Xylose | Temp | 0.44 ± 0.08 | 0.66 ± 0.11 | $F(1, 15) = 22.21$, $p = 0.0003$ |

| Selection | Stressor | Mean T1 ± | Mean T4 ± | Adaptation (ANOVA) |
|---|---|---|---|---|

| treatment | | stdev | stdev | |
|---|---|---|---|---|
| Ancestor | Alcohol | 0.73 ± 0.02 | 0.57 ± 0.11 | F (1, 22) = 23.62, p < 0.0001, decline |
| Raffinose | Alcohol | 0.76 ± 0.16 | 0.71 ± 0.12 | Insignificant |
| Fructose | Alcohol | 0.91±0.15 | 0.72±0.09 | F (1, 22) = 13.54, p = 0.001, decline |
| Pyruvate | Alcohol | 0.56± 0.18 | 0.71± 0.09 | F (1, 17) = 4.74, p < 0.05, |
| Aspartate | Alcohol | 0.63±0.28 | 0.6±0.14 | Insignificant |
| Succinate | Alcohol | 0.57 ± 0.27 | 0.71 ± 0.13 | F (1, 20) = 2.38, p = 0.13 |
| Fumarate | Alcohol | 0.36 ± 0.35 | 0.7 ± 0.22 | F (1, 22) = 8.27, p < 0.01 |
| Malate | Alcohol | 0.54 ± 0.22 | 0.69 ± 0.14 | F (1,21) = 3.72, p = 0.06 |
| Melibiose | Alcohol | 0.69 ± 0.17 | 0.71 ± 0.12 | insignificant |
| Proline | Alcohol | 0.76 ± 0.2 | 1.02 ± 0.18 | F (1, 22) = 11.33, p = 0.002 |
| Glutamate | Alcohol | 0.69 ± 0.15 | 0.77 ± 0.11 | insignificant |
| Citruline | Alcohol | 0.69 ± 0.14 | 0.74 ± 0.11 | insignificant |
| Xylose | Alcohol | 0.76 ± 0.22 | 0.91 ± 0.17 | F (1, 22) = 3.46, p = 0.07 |

| Selection treatment | Stressor | Mean T1 ± stdev | Mean T4 ± stdev | Adaptation (ANOVA) |
|---|---|---|---|---|
| Ancestor | NaCl | 0.24 ± 0.09 | 0.43 ± 0.07 | $F(1, 22) = 31.75$, $p < 0.0001$, |
| Raffinose | NaCl | 0.05 ± 0.03 | 0.22 ± 0.06 | $F(1, 21) = 67.03$, $p < 0.0001$ |
| Fructose | NaCl | 0.35 ± 0.14 | 0.34 ± 0.08 | insignificant |
| Pyruvate | NaCl | 0.08 ± 0.05 | 0.24 ± 0.09 | $F(1, 18) = 22.64$, $p = 0.0002$ |
| Aspartate | NaCl | 0.01 ±0.06 | 0.28 ±0.12 | $F(1, 16) = 18.07$, $p = 0.0006$ |
| Succinate | NaCl | 0.17 ± 0.04 | 0.24 ± 0.05 | $F(1, 20) = 14.35$, $p = 0.001$ |
| Fumarate | NaCl | 0.23 ± 0.13 | 0.32 ± 0.08 | $F(1, 22) = 4.45$, $p < 0.05$ |
| Malate | NaCl | 0.24 ± 0.07 | 0.31 ± 0.09 | $F(1, 22) = 4.61$, $p < 0.05$ |
| Melibiose | NaCl | 0.11 ± 0.04 | 0.26 ± 0.09 | $F(1, 19) = 25.52$, $p < 0.0001$ |
| Proline | NaCl | 0.11 ± 0.05 | 0.25 ± 0.08 | $F(1, 16) = 21.63$, $p = 0.0003$ |
| Glutamate | NaCl | 0.13 ± 0.07 | 0.22 ± 0.06 | $F(1, 20) = 9.45$, $p = 0.006$ |
| Citruline | NaCl | 0.14 ± 0.03 | 0.21 ± 0.08 | $F(1, 21) = 7.25$, $p = 0.01$ |
| Xylose | NaCl | 0.09 ± 0.05 | 0.20 ± 0.06 | $F(1, 16) = 20.74$, $p = 0.0003$ |

| Selection treatment | Stressor | Mean T1 ± stdev | Mean T4 ± stdev | Adaptation (ANOVA) | Note |
|---|---|---|---|---|---|

| | | | | | |
|---|---|---|---|---|---|
| Ancestor | pH | 0.85 ± 0.09 | 0.81 ± 0.19 | insignificant | One replicate in T4 grew about 30 % of the mean. |
| Raffinose | pH | 0.62 ± 0.22 | 0.83 ± 0.16 | F (1, 20) = 6.13, $p < 0.02$ | |
| Fructose | pH | 0.84 ± 0.32 | 0.91 ± 0.08 | insignificant | |
| Pyruvate | pH | 0.49 ± 0.39 | 0.16 ± 0.08 | F (1, 10) = 1.41, $p = 0.26$ | |
| Aspartate | pH | 0.57 ± 0.37 | 0.71 ± 0.32 | F (1, 15) = 0.62, $p = 0.44$ | |
| Succinate | pH | 0.44 ± 0.31 | 0.95 | F (1, 5) = 2.33, $p = 0.18$ | Only one replicate survived at T4 |
| Fumarate | pH | 0.38 ± 0.26 | 0 | - | 6 replicates survived at T1, complete extinction at T4 |
| Malate | pH | 0.27 ± 0.11 | 0.43 | F (1, 5) = 1.97, = | Only one |

| | | | | | |
|---|---|---|---|---|---|
| | | | | 0.21 | replicate survived at T4 |
| Melibiose | pH | 0.21 ± 0.15 | 0.83 ± 0.19 | F (1, 10) = 38.45, p = 0.0001 | |
| Proline | pH | 0.56 ± 0.43 | 0.72 ± 0.19 | insignificant | |
| Glutamate | pH | 0.13 ± 0.07 | 0.22 ± 0.06 | F (1, 20) = 9.45, p = 0.006 | |
| Citruline | pH | 0.39 ± 0.29 | 0.75 ± 0.43 | F (1, 11) = 2.83, p = 0.12 | |
| Xylose | pH | 0.37 ± 0.36 | 0.74 ± 0.29 | F (1, 15) = 4.82, p = 0.04 | |

| Selection treatment | Stressor | Mean T1 ± stdev | Mean T4 ± stdev | Adaptation (ANOVA) | Note |
|---|---|---|---|---|---|
| Ancestor | Glucose | 0.99 ± 0.04 | 0.85 ± 0.05 | F (1, 22) = | |

| | | | | | |
|---|---|---|---|---|---|
| | growth rate | | | 48.97, p < 0.0001 | |
| Raffinose | Glucose growth rate | 0.83 ± 0.15 | 0.66 ± 0.17 | F (1, 22) = 6.77, p = 0.01 | |
| Fructose | Glucose growth rate | 1.11 ± 0.12 | 0.81 ± 0.16 | F (1, 22) = 26.13, p < 0.0001 | |
| Pyruvate | Glucose growth rate | 0.61 ± 0.21 | 0.55 ± 0.12 | insignificant | |
| Aspartate | Glucose growth rate | 0.59 ± 0.15 | 0.60 ± 0.14 | insignificant | |
| Succinate | Glucose growth rate | 0.73 ± 0.23 | 0.67 ± 0.19 | insignificant | One replicate at T4 is devastated if omitted mean T4 = 0.72 ± 0.09 |
| Fumarate | Glucose growth rate | 0.81 ± 0.24 | 0.74 ± 0.09 | insignificant | |
| Malate | Glucose | 0.79 ± 0.15 | 0.74 ± 0.17 | insignificant | |

| | | | | | |
|---|---|---|---|---|---|
| | growth rate | | | | |
| Melibiose | Glucose growth rate | 0.69 ± 0.28 | 0.63 ± 0.14 | insignificant | |
| Proline | Glucose growth rate | 0.72 ± 0.21 | 0.70 ± 0.21 | insignificant | |
| Glutamate | Glucose growth rate | 0.64 ± 0.15 | 0.57 ± 0.21 | insignificant | |
| Citruline | Glucose growth rate | 0.73 ± 0.22 | 0.68 ± 0.15 | insignificant | |
| Xylose | Glucose growth rate | 0.69 ± 0.13 | 0.69 ± 0.18 | insignificant | |

# Chapter 5: Summary

The ecological theory of adaptive radiation is one of the chief explanations that has been put forward to explain how biodiversity evolves on Earth. Despite its broad acceptance in the scientific community, however, the theory of adaptive radiation cannot by itself explain the pattern by which biodiversity evolves in a given environment and as a result does not directly predict the outcome of evolution in a given environment (MacLean, 2005; Prosser *et al.*, 2007; Kassen, 2009; Saxer *et al.*, 2010). The objective of my PhD research was to show that the pattern of metabolic specialization in the laboratory can be predicted from biochemical principles and then used to interpret the pattern found in natural populations.

I presented this thesis in four main chapters. In the first chapter I briefly discussed the limitations of the ecological theory of adaptive radiation, proposing a hypothesis, on the mechanisms by which trade-offs through mutational accumulation and functional interference may evolve in experimental populations, to help to overcome some limitations of the theory of adaptive radiation. I further tested my hypothesis by reviewing the literature that have investigated the sources of trade-offs in experimental evolution of metabolism using microbes. I found compelling evidence for accumulation of conditional loss-of-function mutations in unused metabolic pathways to be the cause for metabolic specialization (Ciriacy & Breitenbach, 1979; Bell & Reboud, 1997; Reboud & Bell, 1997; Funchain *et al.*, 2000; Cooper *et al.*, 2001; MacLean & Bell, 2002; Ostrowski *et al.*, 2007; Lee *et al.*, 2009; Warringer *et al.*, 2011; Zörgö *et al.*, 2012; Kvitek & Sherlock, 2013; Leiby & Marx, 2014). I also found supportive evidence for mutations in rate-limiting enzymes to cause functional interference (antagonistic pleiotropy)

leading to specialization and adaptive radiation (Takemoto & Liao, 2001; Liao & Laufs, 2005; Jasińska *et al.*, 2007; Rausher, 2013; Carroll *et al.*, 2014).

The second chapter documented the pattern of specialization found in isolates from wild yeast populations. We found geographical variation of substrate use at continental, regional, and local scales. Isolates from Europe and North America could be distinguished on the basis of the pattern of yield across substrates. Two geographical races at the North American sites also differed in the pattern of substrate utilization. Substrate utilization patterns were also geographically correlated at local spatial scales. Pairwise genetic correlations between substrates were predominantly positive, reflecting overall variation in metabolic performance, but there was a consistent negative correlation between categories of substrates in two cases: between the core diet and the ancillary diet, and between pentose and hexose sugars. Such negative correlations in the utilization of substrate from different categories may indicate either intrinsic physiological trade-offs for the uptake and utilization of substrates from different categories, or the accumulation of conditionally neutral mutations. Divergence in substrate use accompanies genetic divergence at all spatial scales in *S. paradoxus* and may contribute to race formation and speciation.

Chapters 3 explored the consequences of metabolic adaptation of experimental populations in the laboratory in relation to alternative metabolic environments. The goal of this experimental chapter was to rigorously test the hypothesis that I presented in chapter 1. I used experimental populations of yeast and evolved them in 12 defined cultures where each culture was supplemented with a single-carbon-substrate. These substrates belonged to 3 different metabolic pathways therefore; I could test the effect of local adaptation on alternative substrates with similar or different pathways (compared to the local selection environments). I discovered that

local adaptation to a given substrate did not consistently affect growth on other substrates in the same pathway. Individual lines that adapted more successfully than the average tended to be superior on other substrates in the same pathway. I found no evidence for trade-offs between substrates on the same pathway. The indirect response of substrates on other pathways, however, was consistently negative, with little correlation between direct and indirect responses. The conclusion therefore, is that the grain of specialization in this case is the metabolic pathway, and that specialization appears to evolve through mutational degradation. This is the first time that the evolution of specialization is defined by the biochemical properties of the selection environment.

Chapter 4 investigates the consequences of metabolic adaptation of yeast populations for selection in subsequent stressful environments. Surprisingly, we found that prior stress by starvation impaired the physiological response but enhanced the evolutionary response to subsequent stress by a different agent. Our result showed that the physiological and evolutionary responses were negatively correlated because they are inversely related to the severity of the prior stress: the physiological response to a subsequent stressor is more impaired and the evolutionary response more elevated by more severe prior stress. This interpretation was consistent with all our results, although it must await explicit demonstration of the mechanisms responsible. Our finding suggests that the evolutionary rescue of populations threatened by successive unrelated stresses is a two-part process. The populations are at heightened risk in the short term, but, should they survive, are more likely to survive in the longer term. The fate of populations in environments subject to a series of shocks will depend on both physiological and evolutionary processes, whose interaction must be understood in order to predict the likelihood of rescue.

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