ENVIRONMENT-DEPENDENT CAUSES AND CONSEQUENCES OF MUTATION IN *SACCHAROMYCES CEREVISIAE*

by

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DEDICATION PAGE

For my teachers.
My debts are impossible to repay…but I will try.

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ABSTRACT

Environmental effects on mutation have been documented for many years but have concentrated on agents that directly interact with DNA. Mutation research in its early history investigated a variety of more mundane environmental factors at levels that inhibited biological function and attempted to characterize their mutagenicity. This thesis revisits these old questions armed with more modern methods. It consists of one review chapter and three experimental chapters. The review chapter proposes that biological organization itself acts to direct mutation pressure, and that many mutations are context dependent within this organization. Experimentally, I performed an approximately 1,500-generation mutation accumulation (MA) experiment using the budding yeast *Saccharomyces cerevisiae* as an evolutionary genetic model. This thesis investigates the rates and distribution of effects of new mutations on fitness when they accumulate under a moderate salt stress. The first experimental section describes the production of the MA lines, measures the diploid fitness traits mitotic growth rate and sporulation, and uses changes in fitness among replicate lines to infer mutation parameters affecting these traits. Mutation rate estimates for these traits were roughly doubled in the salt stress treatment. The proportion of beneficial mutations was high for mutations affecting sporulation in both MA treatments but zero for growth rate. Measurements of haploid viability and haploid growth rate on strains derived from the diploid MA lines were used to infer mutation parameters. Mutation rates affecting haploid growth were ten-fold higher in our salt-line derivatives than those derived from the non-stress treatment. Variance component analysis identified a large fraction of genetic variation arising from differences among haploids within the same tetrad. This component was significantly larger in the salt MA treatment than the non-stress treatment. MA lines were subjected to a novel weak-acid stress. Mutation rate estimates were 38-fold higher in the salt MA treatment when lines were tested under acid stress. Cross-environmental genetic correlation for growth in acid stress versus standard media was significantly different between the two MA treatments suggesting that both MA environment and test environment are important factors when considering mutational effects on fitness.

LIST OF ABBREVIATIONS AND SYMBOLS USED

A	Adenine
α	gamma distribution scale parameter
ANOVA	Analysis of Variance
β	gamma distribution shape parameter
BM	Bateman-Mukai
bp	base pair(s)
C	Cytosine
CpG	Cytosine Guanine dinucleotide
E(hs)	average heterozygous effect
E(s)	average hemizygous effect
G	Guanine
GFP	Green Fluorescent Protein
GpC	Guanine Cytosine dinucleotide
GSH	reduced glutathione
GSSG	oxidized glutathione
HEPA	High Efficiency Particulate Air
HO	hydroxyl radical
H_2O_2	hydrogen peroxide
HOG	High Osmolarity Glycerol
HSA18	Human chromosome 18
HSA19	Human chromosome 19
MA	Mutation Accumulation
ML	Maximum Likelihood
MMR	Mis-Match Repair
N_e	effective population size
N_g	effective number of genes
NO	Nitrous Oxide
OD	Optical Density
P_B	Proportion of Beneficial mutation
PCR	Polymerase Chain Reaction
Pol δ	Polymerase delta
Pol ε	Polymerase epsilon
Pol η	Polymerase eta
PCNA	Proliferating Cell Nuclear Antigen
ρ^0	mitochondrial deficient
ρ^+	mitochondrial competent
REML	Restricted Maximum Likelihood
ROS	Reactive Oxygen Species
roGFP	redox sensitive Green Fluorescent Protein
SNP	Single Nucleotide Polymorphism
T	Thymine

TSS	Transcription Start Site
U	Genome-wide mutation rate
UV	Ultra Violet
YPD	Yeast extract, Peptone, Dextrose
YPG	Yeast extract, Peptone, Glycerol

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CHAPTER 1 INTRODUCTION

A historical sketch

Mutation biology is more than one hundred years old. The term was introduced by Hugo deVries to describe sudden changes he observed in the evening primrose *Oenothera* (Auerbach, 1976). It became a subject of modern study through work performed with fruit flies by Hermann Muller in the early twentieth century. Muller (1928) devised a scheme by which mutations arising on the second chromosome of *Drosophila* could be shielded from the effects of natural selection using a system of 'balancer' chromosomes. By reducing natural selection, mutations could accumulate in laboratory populations on this shielded chromosome. After a period of time, the balancer was removed and the subject chromosome brought together with a second copy of itself, allowing newly formed recessive mutations to be expressed in the homozygous condition. Muller identified differences between fly strains as well as effects of temperature on mutability establishing both a genetic and environmental basis of mutation.

The period following Muller's seminal study was an extremely active time for mutation research. Many studies followed that attempted to characterize mutational effects ascribed to common environmental factors, such as temperature, during the period of mutation accumulation (MA). Results from these studies are somewhat conflicting (see Auerbach, 1976). A different set of environmental factors soon became the focus of much mutation research. These followed from the demonstrations that mutations were formed as a result of treatment with X-radiations (Muller, 1927), eventually earning Muller the Nobel Prize in Physiology or Medicine in 1946. Studies of this period were not limited to biophysical explanations. Another Nobel-winning study solved the

standing dispute as to whether mutations were induced by their specific environment or arose independently. Luria and Delbrück (1943) utilized the properties of the Poisson distribution to explain how phage resistance arose independently from the environment in which they were beneficial. Population geneticists meanwhile were actively attempting to measure mutation parameters in natural populations (e.g., Dobzhansky and Wright, 1941).

Concurrent with these studies was the demonstration that DNA was genetic material (Avery et al., 1944). This confirmation allowed for the beginning of the study of the biochemistry of mutation, eventually leading to the identification of numerous factors that physically interact and modify DNA. DNA damage is now recognized as a major intermediate step in many mutation processes (Baer et al., 2007).

The next major advance in the study of new mutations entering populations was the work of Angus John Bateman (Bateman, 1959) and Terumi Mukai (Mukai, 1964). Bateman developed a mathematical framework to estimate rates and effect sizes of new mutations from divergent MA populations. Mukai used this technique to estimate spontaneous mutation parameters on fitness characters in *Drosophila*. Bateman's technique has been succeeded by more powerful statistical models to estimate mutation parameters but the same basic MA approach to measure new mutations entering populations is still used today (Halligan and Keightley, 2009).

A eukaryote for all seasons: Saccharomyces cerevisiae *and mutation biology*

The budding yeast *Saccharomyces cerevisiae* is one of the dominant model organisms in biology. It has arguably contributed more to our understanding of

eukaryotic cells than any other organism. It has also contributed significantly to our understanding of genetics, from the molecular to population levels. As a model, it has several advantages. For genetic studies, the primary advantage is that *S. cerevisiae* can be cultured asexually as either diploids or haploids and that the four haploid products of an individual meiotic event can be isolated. This allows for the direct observation of meiotic segregation, a particularly useful feature for studying mutation.

S. cerevisiae has been used to study mutation since the middle of the twentieth century. Ogur et al. (1960) studied the dependence of temperature on the appearance of mutations to respiratory deficiency, the so-called petite phenotype. This phenotype is usually associated with mutations in the mitochondrial genome but nuclear petites do exist (Hutter and Oliver, 1998). Loss of respiratory function and reversion at specific loci were also studied in response to temperature by Schenberg-Frascino and Moustacchi (1972) who found that periods of exposure to high temperature increased mutations in actively growing haploid yeast.

S. cerevisiae has more recently contributed to our knowledge of genome-wide mutation processes. The genome of *S. cerevisiae* consists of sixteen chromosomes with a total length approximately twelve million bases and containing approximately six-thousand protein-coding genes (Goffeau, 1996). Zeyl and DeVisser (2001) first calculated genome-wide mutation parameters for genes affecting fitness in *S. cerevisiae*, and a number of others have subsequently studied additional aspects of genome-wide mutation in *Saccharomyces*. Wloch et al. (2001) assayed fitness in haploids immediately after a short period of mutation accumulation in diploids in an attempt to investigate mutational effects of single mutations. Joseph and Hall (2004) performed MA over

approximately 1000 mitotic generations, concluding that a surprising number of mutations were of beneficial effect. These studies all utilized asexual growth rate as the measure of yeast fitness. More recently, other phenotypic traits have been investigated as fitness measures including sporulation (Hill and Otto, 2007; Hall and Joseph, 2010), post-meiotic viability, and haploid growth rate (Hall and Joseph, 2010). Large-scale sequencing of haploid *S. cerevisiae* MA lines has also been performed to characterize mutation processes at the molecular level (Lynch et al., 2008).

Thesis overview

This thesis investigates aspects of environmental sources of mutation rate variability. In some ways it resembles studies performed by early geneticists but utilizes modern methods that allow inferences of mutation affecting fitness-related traits across a whole genome. It also uses more a sophisticated mathematical approach that allows estimation of the distribution of mutational effects and the proportion of beneficial mutations. It begins with a review paper (chapter two) outlining the hypothesis that mutation rates are subject to environmental influences that are a product of biological organization itself. I examine how levels of biological hierarchy affect mutation rates by effectively filtering out environmental information through a phenotype-environment continuum. For example, mutation rates at nucleotide positions are subject to neighborhood effects, so that surrounding nucleotides exert an influence on a target site. Another example, from a higher level of organization, is the mutagenic influence of damaged tissue on undamaged tissue adjacent to it, otherwise known as bystander effects. Chapter Three, the first experimental chapter, describes the generation of a series of yeast

mutation accumulation lines in one of two treatments. One treatment consists of lines cultured under benign conditions, while the other treatment is cultured under a moderate salt stress. I measure two fitness characters, one asexual and one sexual and infer rates of mutation and distribution parameters for each treatment. Chapter Four extends the analysis in the previous chapter to post-meiotic characters and addresses the effect of meiosis on the fitness of haploids generated in each of the two MA treatments. Chapter Five is the final chapter presenting original research. It addresses the role of stress in the assessment of fitness of newly generated mutations. We use the diploid MA lines generated in Chapter Three and subject them to a mild weak-acid stress. We then infer rates and distribution of effects for both MA treatments in this new environment and compare it to those we obtained under benign assay conditions in Chapter Three.

CHAPTER 2 THE INTERNAL ECOLOGY OF MUTATION

Mutation is justifiably heralded as the ultimate source of genetic variation and as such is one of the four recognized mechanisms of evolutionary change. Despite its importance and nearly 100 years of experimental investigation, many important aspects await a full understanding. Evolutionary geneticists are concerned with understanding both the rates and distributions of effects for new mutations. Molecular geneticists have focused on disentangling the mechanisms underlying the different classes of mutation and the conditions under which they take place. Both fields acknowledge that there is a strong genetic basis controlling the incidence of mutation and that the mutation rate itself has thus been subject to natural selection and hence can evolve over time. Many excellent reviews of these aspects exist (e.g., Baer et al., 2007; Drake, 2007). Here I review various internal structures in organisms and their roles in generating new mutations. I use these examples to formulate a hypothesis which emphasizes environmental factors as a significant source of mutational input for genetic systems.

Early studies of mutation were attempts by the pioneering geneticists at measuring the rates and factors influencing the incidence of mutation. The genetic basis of the mutation rate was discussed at length in a classic paper by Sturtevant (1937) and spawned the study of mutation rate evolution. Here I wish to highlight two points raised by Sturtevant. The first, and generally appreciated, is that given the genetic basis for mutation rate variability, and that most mutations will be on average deleterious, selection should cause a decrease in the mutation rate over time. Secondly, and perhaps less fully appreciated, Sturtevant proposes that even if "external factors" cause mutation,

these should still drive directional selection for decrease in the mutation rate. Explicit in his discussion is the idea that the environment external to the organism could potentially act in some way to generate mutations. I extend this view and define the environment as anything external to the site of mutation. This review will summarize the relevant data regarding various internal sources of mutation rate variability.

I present a telescopic view of the hierarchical structure of organisms, focusing on large multi-cellular eukaryotes, as these organisms have the most levels of hierarchy. Where appropriate I utilize results from studies in unicellular species but limit these to eukaryotes. I also choose to discuss effects on nuclear DNA only. Both of these boundaries for discussion were chosen solely for the sake of brevity. Many relevant findings and similarities exist to those discussed throughout the large literatures that deal with the mutation biology of both prokaryotes and the organellar genomes of eukaryotes.

I consider each hierarchical level as a distinct environment type in which a mutable site exists. From the lowest levels of structure in organisms to the highest, I ask the questions: What physical factors affect the probability that any given site will mutate, and to what extent does each level contribute to the overall probability of change? Again for brevity I limit the discussion to physical factors while acknowledging the additional importance of temporal factors. Temporal bases for differences in mutation can arise in several demonstrated ways including differences in cell-cycle phase (Stamatoyannopoulos et al., 2009) as well as in the number of replicative cycles such as those used to explain male mutation bias in animals. The majority of discussion in this paper will attempt to summarize the current literature to address the first question above, namely the factors affecting mutability of a site. The second question of determining the

relative importance of these factors over evolutionary time is recognized as a largely empirical one and is one of great importance. One possible approach to resolving this question will be discussed. Lastly, I summarize recent discussions of the evolutionary relevance of internal factors affecting mutation.

Another set of boundaries is placed to limit the discussion. I here will only consider levels above that of a DNA strand and up to what is typically regarded as a large multi-cellular individual. Below the primary structure of DNA, biochemical features such as the availability of nucleotide resources may influence the generation of new mutations (Desler et al., 2007). Many well established examples describe features at higher levels of organization including lineage-specific biases, male mutation biases in animals and plants, biases ascribed to life-history differences such as annual versus perennial strategies in plants (Petit and Hampe, 2006). I would also like to note the possibility that in humans similar biases might exist through higher level factors such as culture, e.g. via diet. These supra-individual levels deserve a separate treatment and may be just as important as the factors discussed here in the relevant organisms. Given the aforementioned limitations to the discussion, I begin by discussing factors influencing the rate of mutation from the lowest levels of biological organization up through factors present at the highest levels considered here.

The Nucleotide Neighborhood

The observation that certain nucleotide changes are dependent on the sequence context within which they take place has been recognized for over thirty years (Coulondre et al., 1978). Context dependence has been broadly investigated at the

molecular level (reviewed in Rogozin and Pavlov, 2003). Few of the mechanisms behind these context-specific changes (see Table 1 in Rogozin and Pavlov, 2003) are understood. The best known example is the high mutability of CpG dinucleotides. Cytosines that precede a guanine can undergo spontaneous deamination to thymine when methylated. The large volume of sequence data generated in the last decade has enabled the detection of neighbour-dependent effects on a genomic scale. Zhao and Boerwinkle (2003) examined nearly 2.6 million polymorphic sites (SNPs) in the human genome and detected a strong bias for transitions at CpG dinucleotides. They also noted a complex pattern of biases for transversions, where the incidence of a polymorphism increased with increasing AT content flanking the SNP. This pattern for transversions has also been observed in mice (Zhang and Zhao, 2004) and cattle (Jiang et al., 2008). Plant genomes show similar features. AT content at flanking sites is correlated with transversions in plastid genomes of various species (Morton et al., 1997; Savolainen et al., 1997; Yang et al., 2002). This was also found in the nuclear genomes of *Arabidopsis* (Zhang and Zhao, 2004; Morton et al., 2008) and to a lesser extent maize (Morton et al., 2006).

The studies investigating nucleotide context have also considered sites at further distances from polymorphic sites. The difference attributed to neighboring nucleotide effects extended up to 200bp to each side of the SNP in one study (Zhao and Boerwinkle, 2003). The effect of local AT content on transition frequency has been discussed in recent years. Morton et al. (2006) found no significant effect due to the next nearest base while controlling for the identity of the nearest neighbor in the nuclear genome of maize. This contrasts with the influence of AT content at next-neighboring sites in the *Arabidopsis* nuclear genome (Morton et al., 2008). The influence of AT content at

further distances on SNP frequency has been investigated. A general positive correlation has been found in humans (Fryxell and Moon, 2005; Zhao and Jiang, 2007), chimps (Jiang and Zhao, 2006) and *Arabidopsis* (Morton et al., 2008). Elango et al. (2008) recently completed an extensive study of regional AT context on CpG transitions by making comparisons between human, chimp and baboon sequences. They compared the number of CpG substitutions to the number of GpC substitutions. GpC substitutions are used as a control as they are comprised of the same bases as CpG sites but are believed to occur primarily due to replication errors and not through a methyl-cytosine deamination mechanism. At CpG sites, the deamination of methylated cytosine is preceded by a temporary melting of the DNA duplex. The weaker AT pair bond leads to the situation where regions with high AT content (thus low GC) are more prone to deamination due to the lower energetic cost of melting duplex DNA in these regions. Using this mechanistic rationale, the authors proposed a 'distance-decaying hypothesis', where the effect of AT vs GC content diminishes with increasing distance away from the substitution site. Their analysis detected a significant effect on the CpG substitution rate but not the GpC substitution rate from the local AT content up to 2000 base pairs away. Hodgkinson et al. (2009) recently noted that humans and chimps have a larger than expected number of shared SNPs that cannot be accounted for by simple (immediate) context effects, strand asymmetry (see below), other known mutational biases, ancestral polymorphism or natural selection.

The Different DNA Strands Exhibit Mutation Biases in Several Contexts

Various activities of DNA metabolism have been investigated for differences affecting the incidence of mutation between the two strands in a DNA molecule. Those identified include differential activity of replicative polymerases, unequal repair and lesion bypass, and transcription-associated processes. Here I treat four demonstrated examples of differential mutation occurring on one strand over the other.

i) Differential Polymerase Replication Bias: Lower replication error rates have been observed on the lagging strand in yeast (Pavlov et al., 2002). Current models of eukaryotic DNA replication propose that two separate DNA polymerases are responsible for the majority of copying at replication forks (Kunkel and Burgers, 2008; Nick McElhinny et al., 2008). Polymerase delta (Pol δ) and polymerase epsilon (Pol ε) have been implicated as the major active polymerases for the lagging and leading strands respectively. The propensity for two different polymerases to act on the different strands of a replication fork provides a mechanism for a strand-specific bias, given the unique error signatures of these two polymerases (Kunkel and Burgers, 2008 and references therein). Distinctive error signatures for yeast expressing a modified Pol δ were found using a reporter gene placed in various orientations about a yeast origin of replication (Nick McElhinny et al., 2008). This signature depended on whether the reporter was on the leading or lagging strand and is consistent with Pol δ acting as the primary enzyme acting on the lagging strand. It remains to be established whether this is a general feature of eukaryotic replication or is particular to the yeast system these authors investigated.

ii) Mismatch Repair (MMR) Bias: An additional explanation for the observed differences between replicative strands is that MMR acts unequally between the two, acting preferentially on the lagging strand. Pavlov et al. (2003) investigated this

possibility by measuring mutation rate strand bias in a series of yeast mutants deficient in MMR functions and compared them to MMR proficient strains. The strand bias detected in a *ura3-29* reversion assay was reduced for each case in deletion mutants lacking a particular MMR gene. These authors propose that MMR may be more efficient on the lagging strand due to the prevalence of gaps between adjacent Okazaki fragments and/or the protein machinery responsible for the post-replicative processing of these gaps. These features could act as strand discrimination signals, a necessary marker for proper MMR. More recently, Kow et al. (2007) identified components of the MMR apparatus that act differentially between the leading and lagging strands. They identified differential activity of the two MutS-like complexes, MutSα and MutSβ. These complexes are responsible for the detection of mismatches and loops in DNA post replication. MutSα primarily detects single-base mismatches and single-base loops, while MutSβ detects larger loops. Kow et al. demonstrated, using a single-stranded oligonucleotide transformation assay, that MutSα activity is greater on the lagging strand than on the leading strand while MutSβ has equal activity on both strands. These authors note that their findings are consistent with the earlier findings of Pavlov et al. (2003) and may help to explain the increased mutation on lagging strands when MMR is impaired.

iii) Lesion Bypass: There is also an apparent strand bias in the ability of DNA polymerase eta (Pol η) to bypass DNA lesions in association with replication. Early work on the bypass of UV damage in human cell extracts indicated that Pol η bypassed UV damage primarily on the leading strand template (McGregor et al., 1999). More recent work in yeast suggests that an interaction with the processivity factor PCNA is required

for Pol η-mediated bypass of lesions on the lagging strand but is not required for bypass on the leading strand (Abdulovic et al., 2007).

iv) Transcription: Polak and Arndt (2008) have investigated the pattern of nucleotide substitution in intronic and intergenic regions in relation to their position along coding sequences. Using genome-wide alignments of human, chimpanzee, and rhesus monkey, these authors detected several regional patterns including strand asymmetries. This was done by comparing the rate of a particular substitution to the rate of the reverse complement substitution on the same strand. Along the whole length of coding sequences, there were four pair substitutions that showed a strand asymmetrical rate of substitution on the nontemplate strand: A→G/T→C, C→G/G→C, A→T/T→A, G→T/C→A. The A→G/T→C ratio also showed the opposite bias when the authors measured it upstream of the transcription start site (TSS) which the authors attribute to antisense transcription. A more localized bias was observed for the C→T/G→A transitions where the C→T transition rate is increased by about 20% up to 1kb downstream of the TSS. This local difference in the ratio of C→T/G→A transitions disappears at about 1.5 kb from the TSS. Although the localized asymmetry of these substitutions remains to be thoroughly explained, the authors of this study suggest that it may be a result of this portion of the non-transcribed strand existing in the ssDNA state for longer periods and thereby being exposed to increased DNA damage.

Nuclear Structure and Dynamics Underlie Patterns of Change

The organization of genomes into distinct units raises the possibility that mutation occurs at different rates among the chromosomes of eukaryotes. The early work of Rabl

and Boveri (cited in Spector, 2003) established the existence of chromosome domains or territories in plant and nematode nuclei respectively. Chromosome domains are regions in the interphase nucleus where specific chromosomes reside (Spector, 2003). For plants, yeast and *Drosophila*, chromosomes are typically found in a 'Rabl' configuration, where the telomeres and centromeres of chromosomes are found at opposite ends of the nucleus. For vertebrates, it appears that interphase chromosomes occupy distinct domains that are radially arranged in the nucleus (Folle, 2008). These domains appear to be evolutionarily conserved among primates (Tanabe et al., 2002) but also show some variation due to cell type, shape, quiescence, functional status or transformation (Folle, 2008).

Over 30 years ago it was proposed that the architecture of the nucleus could greatly influence the rate of DNA damage and thereby mutation on different chromosomes. The 'bodyguard hypothesis' (Hsu, 1975) stated that chromosomes, or portions thereof, that resided near the nuclear periphery would be exposed to greater concentrations of DNA-damaging agents produced or transduced from the cytoplasm. These regions would act to shield the more transcriptionally active genetic material lying in the nuclear interior. More recent work appears to refute the specific features of this hypothesis (Gazave et al., 2005) but the idea that the physical locations of chromosomes contribute to the extent of genetic damage due to intra-nuclear environmental differences remains valid. Gazave et al.(2005) determined the locations of human chromosomes 18 (HSA18) and 19 (HSA19) in the nuclei of cells of a human fibrosarcoma cell line. They confirmed that the gene-poor HSA18 typically resides at the nuclear periphery while the more gene-dense HSA19 is found in the nuclear interior. These authors then proceeded to assay for DNA damage repair functions in concentric nuclear regions after treatment

with DNA damaging agents. In separate experiments, both peroxide treatment and UV-C-irradiated cells showed increased induction of repair processes concentrated at the nuclear centre. Elevated repair function could conceivably match increased rates of damage in regions of differential damage. To investigate the long-term genetic consequences of their distinct nuclear location, the authors then measured the synonymous substitution rates of both HSA18 and 19. They found that synonymous substitution rates were higher for HSA19 than 18 and further proposed that higher GC content on this chromosome may predispose it to increased rates of damage and ultimately mutation.

The Cellular Environment: a Crucible for Genomes

An important early event in the genesis of many mutations is the production of a damaged DNA molecule. It is increasingly apparent that the prevalent kind of damage is the chemical modification of bases and of the sugar-phosphate backbone that arise as a result of normal metabolic processes within the cell. The single most common DNA lesion in eukaryotic cells is the disruption of the DNA backbone commonly referred to as a single strand break. The frequency of these is estimated at 10^4- 10^5 cells/day in mammalian cells (Degtyareva et al., 2008; Roldán-Arjona and Ariza, 2008). Similarly, the number of DNA base excision events has been estimated at ~10^4 for human cells (Baute and Depicker, 2008). Base excision repair is the primary pathway responsible for the removal of DNA adducts due to reactive oxygen species (ROS). ROS are produced in several cellular locations and perform various roles in metabolism. The chief sources for ROS are mitochondria, plastids and peroxisomes (Roldán-Arjona and Ariza, 2008).

In animal cells, mitochondria are believed to account for approximately 90% of total cellular ROS production (Kim et al., 2006). Oxidative damage is thus a persistent threat to the integrity of DNA and originates within the context of the cellular environment.

Generally speaking, cells are reducing environments separated by membranes against more oxidizing conditions. The redox poise of human cells is estimated to be ~-250 mV with respect to the cell exterior (Hancock, 2005). Different cell compartments also display differing redox potentials dependent on their functional characteristics (Kemp et al., 2008). Oxidative damage to cellular components takes place in this reducing environment through the action of strong ROS. These can be formed in various ways including production by various organellar chemistries (Table 2.1), by specific oxidase and peroxidise enzymes (Roldán-Arjona and Ariza, 2008) as well as by interaction of cellular components with penetrating agents such as radiation (Ridley et al., 2009). Contrary to the early adherents to the 'target theory' of mutagenesis, modern interpretation of induced mutation posits that most pre-mutagenic damage occurs when the surrounding cell absorbs the bulk of the ionizing energy, causing a cascade of oxidation capable of damaging proteins, lipids and nucleic acids (Roldán-Arjona and Ariza, 2008). This interpretation has consequences for the understanding of damage progression due to ROS formed through sources of normal metabolism.

The persistent threat of oxidation to cellular constituents is also consistent with the large number and variety of anti-oxidant molecules involved with the control of the cellular redox environment. A large literature exists on these systems (see reviews by Oktyabrsky and Smirnova, 2007; Herrero et al., 2008). Here I present a broad overview and discuss features of antioxidant defense that are most pertinent to the protection of

Table 2.1: Cellular Sources of Reactive Oxygen Species (ROS). From Herrero et al., 2008; Roldán-Arjona and Ariza, 2009

Source	Reactive Species	Notes
Mitochondrion	Superoxide Radical	short-lived, formed by leakage from electron transport chain, dismutation forms H_2O_2
Plastid	Singlet Oxygen	short-lived
	Superoxide Radical	produced from photosynthetic electron transport
Peroxisome	Superoxide Radical	
	Hydrogen Peroxide	relatively long-lived, partial reduction forms the strong oxidant hydroxyl radical
NADPH oxidase	Superoxide Radical	enzymatic production of ROS, produces 'oxidative burst' in plant pathogen attack

genetic material. Cellular anti-oxidants can be broadly classified as enzymatic or small molecule defences (Table 2.2). These components of cellular redox regulation are often associated with one another forming a complex regulatory network that modulates the cellular environment. The tripeptide glutathione acts as a major redox control node in most compartments in eukaryotic cells. The reduced form (GSH) exists in concentrations as large as 10mM in the cytosol of mammalian cells, while the oxidized form (GSSG) is present in an estimated 3000-fold lower concentration (López-Mirabal and Winther, 2008). Changes in the redox balance of this couple result in a large number of downstream changes in cell physiology in concert with the direct effects of damage due to ROS (Hancock, 2005). Glutathione has been known to play a role in the protection of biological molecules for many years (Biaglow et al., 1983) and is invoved in the regulation of apoptosis (Pallardó et al., 2009). Changes in the glutathione profile in the nucleus through cell cycle progression have been measured (Markovic et al., 2007). GSH levels are highest in the nucleus during $S/G_2/M$ and are lower in G_0/G_1. The precise reasons for redistribution are unclear but may be involved in regulating changes in protein activity in a more reducing environment. It is noteworthy however, that the changes in GSH distribution are also consistent with a protective role at times when nuclear material may be more exposed.

Cellular stress leads to a general increase in the production of ROS (Shao et al., 2008; Locato et al., 2009). At critical levels, the production of ROS is believed to trigger apoptotic pathways (Shao et al., 2008). Below the apoptotic thresholds, the increased ROS concentrations can act to damage cellular constituents as mentioned and can trigger diverse cellular signaling pathways. Given the enormous load of oxidative DNA damage

Table 2.2: Cellular antioxidant systems. From Rubartelli and Sitia, 2009

Enzymatic Defence	*Small Molecule Defence*
Superoxide Dismutase	Glutathione
Glutathione Peroxidase	Vitamin C
Catalase	Vitamin E
Peroxiredoxins	Carotenoids
Heme oxygenase	Flavonoids
Glutathione Synthases	

that eukaryotic cells face, it seems reasonable that induced levels of ROS due to cellular stress would lead to correlated levels of genomic damage. One strategy cells could use to counter increasingly damaged genomes could be the induction of increased repair capacity. Even if inducible DNA repair function does take place, there are reasons to suspect that it cannot always compensate for increased levels of damage. The first is based on Ockham's Razor. Arguments made to explain why the mutation rate in organisms has evolved to a particular level but not lower propose that selection for lower mutation rate is balanced by costs of increasing the accuracy of repair. If inducible repair can act upon increased levels of damage due to stress, why would it not act constitutively? One possibility is that the induced repair function is only cost effective under stress conditions. Another possibility is that the rate is balanced by positive selection for beneficial mutations. Each of these is plausible but requires additional assumptions to be made.

The second set of reasons indicating an incomplete compensation is based on empirical research. A large number of applied stresses on organisms result in genomic instability. In some cases the applied stresses can clearly act in a manner that directly affects DNA. An example of this kind is the production of pyrimidine modifications that result from exposure to some kinds of UV radiation. In many other cases the mechanistic bases are unclear and may best be explained by a general stress-induced mechanism. An extensive older literature describes the efforts of early mutation researchers to detect changes in mutation rates in organisms under environmental stresses (reviewed in Auerbach, 1976). In these experiments there are conflicting results with some authors finding an effect (e.g. Muller, 1928) while others failed to find any (e.g. Timoféeff-

Ressovsky, 1934). Modern interpretation of these findings is additionally complicated by the since-discovered phenomenon where so-called hidden genetic variation can be released in adverse conditions. Despite this possibility, some cases are only readily explained by an induced mutagenesis. These include the generation of chromosomal deficiencies and rearrangements (Randolf, 1932), and mutations to respiratory deficiency (i.e. mutations to the petite phenotype) in yeast (Ogur et al., 1960).

If a general relationship exists between cellular stress and mutation, we should expect to see highly correlated levels of oxidative stress and mutation in single cell systems. Persistent oxidative stress in mutant yeast was found to be responsible for numerous chromosomal instabilities (Degtyareva et al., 2008). Real time monitoring of the redox state in living cells has only recently become available (Mullineaux and Lawson, 2008). A recent study makes use of a redox-sensitive GFP (roGFP) that allows the monitoring of redox conditions in single yeast cells (Yu et al., 2009). These authors exposed yeast cultures to heavy metals and found that roGFP fluorescence showed similar emission when cells were exposed to lead and arsenic as when exogenous H_2O_2 was applied. The mutation rate at the *CAN1* locus was correlated with metal concentration in the medium except at the highest levels of arsenic where cell death was highest. Intriguingly, the addition of the free-radical scavenger thiourea to metal-exposed cultures decreased levels of both roGFP fluorescence and *CAN1* mutation rate. This suggests that heavy-metal-induced mutagenesis acts through a ROS-mediated pathway. Whether this pathway is via increased levels of DNA damage that are incorrectly repaired remains to be seen, but it seems to be a strong possibility.

Intercellular Interactions Induce Mutation: Lessons from the Radiation-Induced Bystander Effect

Towards the middle part of the 20th century, two competing hypotheses explaining the action of radiation on genetic material were discussed. The 'target' theory held that mutation took place after direct interaction between the radioactive particles and genetic material (Auerbach, 1976). The proposed alternative was that mutation could arise by direct action of the radio particle on the genes and also through the generation of reactive intermediates. In one synthetic version, the idea of a radio-suseptible target was expanded to include the shell of water surrounding the genetic material (Hutchinson, 1961). The size of this shell was determined by the diffusibility and lifespan of reactive chemical species, such as H_2O_2 and OH, produced when ionizing radiation interacted with water in the vicinity of DNA. In recent years, understanding of the interaction between ionizing radiation and the local environment of DNA has increased in scope due to the discovery of the so-called bystander effect. The bystander effect refers to the expression of radiation-damage-induced phenotypes present in cells not exposed to radiation, but whose neighbor cells were exposed. A related effect is the persistence of these phenotypes across cell generations. These effects have been measured at distances up to a millimetre from the irradiated site (Belyakov et al., 2005). These phenotypes include characteristics usually associated with radiation damage, including genomic instability. Some of these phenomena are induced through direct physical contact and mediated by intercellular connections while others can be induced by culture of cells in medium taken from irradiated cultures. This latter finding suggests that diffusible signals mediate the bystander response in some cases. The particular nature of these signals

remains unknown but a great deal of evidence points to the involvement of ROS (Morgan, 2003; Chen et al., 2009). Bystander effects have been extensively demonstrated in mammalian cells and tissue models (see Matsumoto et al., 2009 for reviews) and more recently in other diverse organisms. Müller et al. (2006) treated cells of the sponge *Suberites domuncula* with UV-B radiation and H_2O_2 and induced DNA damage. When these cells were transferred to shared medium with untreated cells, they induced apoptosis in the untreated cells. The authors identified nitric oxide (NO) as the diffusible signal that initiated the cell death. A bystander effect was detected in the fission yeast *Schizosaccharomyces pombe* (DeVeaux et al., 2006). Mutation rates in bystander cells were increased following co-culture with cells exposed to various stresses including electron beam irradiation, UV irradiation, heat, and antibiotic treatment. Based on the response seen irrespective of the particular stress, these authors propose that this bystander effect is a general stress response in *S. pombe*. Bystander effects were not observed in *Caenorhabditis elegans* gonad upon exposure to a heavy ion microbeam (Sugimoto et al., 2006). These authors propose that worm germ line may be somehow protected from bystander effects, or alternatively, that the lack of nitric oxide (NO) signalling system prevents bystander effects in worms.

A central role for the mitochondrion is emerging in the propagation of a bystander signal inducing DNA damage. Kim et al. (2006) investigated the role of mitochondria in the radiation-induced genetic instability of hamster cell lines. They found that unstable clones had greater levels of total cellular ROS than the parental cell line from which they were derived. Using mtDNA depleted (ρ^0) mammalian cells and normal cells (ρ^+) treated with inhibitors of mitochondrial respiratory function, Chen et al. (2008) found a

decreased response in DNA damage levels in normal bystander cells after exposure to the treated cells medium. Decreased DNA damage in bystander cells was also observed when inhibitors of the enzyme NO synthase and inhibitors of mitochondrial calcium uptake were added prior to irradiation of normal cells. These authors conclude that both NO and superoxide radicals of mitochondrial origin are important early signals in the bystander response detected in their system. Another study by the same group (Chen et al., 2009) used a similar approach and found that levels of ROS and gene mutation were increased in bystander cells exposed to medium from irradiated ρ^+ cells but not from similarly treated ρ^0 cells.

Bystander effects were initially identified as a direct response to ionizing radiation exposure. Some evidence now indicates that similar effects are part of a more general intercellular stress response. Bystander-associated DNA damage has been observed as a result of UVC irradiation, physical wounding, alterations of media pH, and treatment with SDS (Sokolov et al., 2007). A recent observation that bystander effects arise in human cells exposed to UVA but not UVB (Whiteside and McMillan, 2009) is consistent with the view that the genotoxic mode of action of UVA arises by ROS-mediated damage and not the production of premutagenic photoproducts. In addition, effects are more severe when bystander cells are stressed by sodium chloride (Han et al., 2007; Zhu et al., 2008). This group measured increased levels of DNA damage in bystanders when they were cultured in higher concentrations of NaCl. They propose that the high salt exacerbates bystander factor-induced oxidative stress and that NO seems to be involved in the signaling pathway. The increasing generality of bystander-like

phenomena clearly demonstrates that genomic stability is partly a function of the local cellular environment.

The Mutational Environment of a Multicellular Individual

Early work investigating bystander effects inferred the presence of factors present in blood plasma that could induce radiation exposure effects when plasma was transferred from an irradiated individual to a non-irradiated recipient. So-called clastogenic factors (Morgan, 2003) induce chromosomal aberrations when plasma from irradiated subjects is mixed with blood lymphocytes of unirradiated donors. This implied that there were systemic effects that resembled typical bystander phenomena. More recently in an in vivo study, radiation-shielded spleen tissue from mice whose heads were exposed to X-rays show significantly higher levels of DNA double strand breaks than unirradiated controls (Koturbash et al., 2008).

While intense radiation treatments may represent a special case, evidence from the field of whole organism stress biology suggest that some systemic responses to stress could lead to increased levels of DNA damage and repair. Stress is known to activate a series of conserved hormonal responses in animals and in many cases these lead to oxidative stress at the cellular level (Kassahn et al., 2009). Studies of exercise physiology in humans have generally shown increases in levels of oxidative stress and DNA damage during intense exercise. While most studies show such a relationship, some show conflicting results (reviewed by Reichhold et al., 2009). Somewhat more controversial, some studies have investigated links between psychological stress in humans and altered mutational susceptibility. Associations have been found between various groups

described as stressed and levels of DNA damage (Fischman et al., 1996; Silva et al., 1999; Dimitroglou et al., 2003; Kontogianni et al., 2007), antioxidant defense (Lesgards et al., 2002) and DNA repair capacity (Kiecolt-Glaser et al., 1984; Cohen et al., 2000; Forlenza et al., 2000; Kontogianni et al., 2007). No difference in DNA damage and repair levels were found in lymphocytes between schizophrenic patients and healthy controls (Psimadas et al., 2004). Results from exercise and psychological stress studies, while at times conflicting some show general patterns. High-intensity stress seems to show greater induction of damage and subsequent repair. Physiological adaptation is likely important in removing the detrimental effects of these stressors when they are prolonged and when subjects are not naïve to exposure.

All of the findings above relate to the induction of premutagenic biochemistries that arise in somatic tissues. In the evolutionarily relevant case, can the physiological state of the soma affect mutation in the germ-line? Separation of germ and soma is thought to arise as a way to protect the integrity of genomic information from environmental damage and mutations arising from excessive cell divisions. While these are likely important, some evidence suggests that this protection may be incomplete. Increased numbers of cross-overs and chromosomal aberrations are found in the sperm of laboratory mice exposed to stress (Daev, 2007). In another study, mutation frequencies were also increased in the sperm of inbred mice when exposed to airborne pollutants (Yauk et al., 2008). Treated mice were exposed to industrial-quality air with elevated levels of particulates, metals and polycyclic aromatic hydrocarbons. Control mice were exposed to the same air HEPA filtered. Treated mice in this experiment showed elevated levels of DNA adducts in lung tissue but not in testes. Higher levels of DNA strand

breaks as well as a 1.6-fold higher mutation frequency at a reporter gene were found in the sperm of treated mice as compared to controls. Additional study is required to address the potential for the mutagenic effect of stress to enter the germ line, but we should be cautious when assuming that environmental stress has little or no penetrance for mutation in germ tissues.

Discussion

The rate of mutation is arguably the most important parameter in evolutionary genetics. This importance necessitates a thorough understanding of factors known to influence it over various scales. New genomic approaches have allowed for much greater insight into the genetic variability between organisms and in some cases there is significantly more variation than could previously be accounted for (Hodgkinson et al., 2009). Like most polygenic characters, the mutation rate is governed by both genetic and environmental control. The genetic basis underlying the frequency of mutation is evident when one considers the vast repertoire of cellular machinery dedicated to DNA synthesis/repair and damage avoidance. Much of this machinery represents the response of organisms to the deleterious effects of mutation over evolutionary time. Environmental effects on the incidence of mutation have been known since the observation of the effects of X-rays on *Drosophila* and have been expanded to include a wide variety of exogenous and endogenous agents. Despite this, some authors question the long-term contributions of environmental factors affecting the rate of mutation (Metzgar and Wills, 2000; Sniegowski, 2001; Metzgar, 2007). This review addresses an intermediate viewpoint, where the physical features of organisms are considered as

nested environments of the heritable sites. This view may seem somewhat contrived at first glance, but it is important to consider that each level in the hierarchy at one time in evolutionary history likely represented the highest, most external extant environment.

When surveying the diverse factors affecting the mutation rate detailed above, we can broadly group each into one of two input classes (see Baer et al., 2007 Figure 1). The first class are those that arise due to replication errors via processes primarily at the levels within the sub-nuclear machinery responsible for DNA copying. The second class are inputs due to DNA damage are mediated through higher levels by their direct or cascading affects on cellular metabolism. Partitioning inputs into these two classes (Table 2.3) resembles the classical distinction of mutation phenomena as either spontaneous or induced. It has long been noted that this classical division is somewhat artificial (Auerbach, 1976) and the most recent observations suggest a further eroding of its biological reality. While these input classes may also be somewhat artificial, they are a significant improvement over the dichotomy of induced versus spontaneous mutation.

Several reviews have emphasized the long-term biases observed at the lower levels of organization discussed here and pertaining mostly to replicative mutation (Ellegren et al., 2003; Yampolsky and Stoltzfus, 2008; Nishant et al., 2009). These have been investigated mechanistically as well as through long time-scale DNA sequence change measures. Damage-induced mutation has been addressed mostly through mechanistic studies investigating the biochemical properties of DNA and its modifying enzymes, both in vitro and in vivo. The long-term importance of damage-induced mutation has been discussed previously in various guises. Lobashev several decades ago developed a physiological hypothesis of mutation (see Daev, 2007 for discussion).

Table 2.3: Mutation input classes for structural contexts described

Structural Context	*Input Class*	*Notes*
Nucleotide Neighbourhood	Replicative	nucleotide properties believed to influence local interactions
Strand	Damage/Replicative	various possible sources, differential recruitment of modifying enzymes to different strands; differential exposure to damage between strands
Chromosome Territory	Damage/Replicative?	source unknown
Cytoplasm	Damage	endogenous byproducts of metabolism cause the majority of cellular damage
Cell Neighbourhood/Tissue	Damage?	ROS/RNS implicated, but may be acting as signal only
Multicellular Individual	Damage?	some evidence of increased incidence of damage

Similar ideas have been proposed in the context of bacterial mutagenesis (Lenski and Mitler,1993) but could also be relevant for eukaryotes.

Given the lack of investigations into the long term changes of DNA sequence for damage-based mutational inputs, how should we interpret the relative importance of each class for biological systems? In order to address this question it will be necessary to evaluate each within a common framework. Whole-genome sequencing of individual genomes has been carried out in yeast that have undergone mutation accumulation (Lynch et al., 2008). This approach, combined with use of treatments designed to manipulate levels of genotoxic stress, will allow research of mutation across both damage-induced and replication (S-phase) induced contexts within a single experimental design. Such an experiment would allow for a single analytical framework, similar to the ANOVA approach used by Smith and Eyre-Walker (2003) in which the relative contributions of each source could be evaluated. Answering the question of which sources of mutational input are most important could have important implications for our understanding of evolutionary change.

The importance of mutation biases in affecting evolutionary outcomes has been repeatedly debated throughout the history of evolutionary biology (Lynch, 2007). The prevailing view is that mutation acts as a weak pressure opposing selection. This has been questioned recently on several grounds. Stoltzfus and Yampolsky (2009) have argued that the role of mutation as the origin of novelty has important consequences that are overlooked in the 'opposing pressures' view. In their population genetic model (Yampolsky and Stoltzfus, 2001), they observe biased fixations of alleles strongly

influenced by mutation bias under several conditions. A notable exception is the case when there is pre-existing standing variation in the population. Lynch (2007) also proposes that mutation biases are important in shaping genome evolution. He advocates the view that the relative strength of selection on many genomic features will be weak due to the combined influence of the magnitude of selection and the low effective number of genes (N_g) in the population. For many genomic scenarios, he predicts that the probability of finding a particular allele is driven primarily by the mutation rate to that allele. Both of the arguments above in support of the orienting of new variation merit further study. In the case of Stoltzfus and Yampolsky, despite the importance of novelty, defining a novel allele is not straightforward in many cases given the various mutational and recombinatorial avenues available. Lynch restricted his argument by considering only genomic features under conditions of low N_g and small selection coefficients. Should complex characters such as morphology, life-history traits, or fitness show similar parameter values? These will determine the general importance of mutation biases for evolution.

Organisms are a dangerous place. For genetic information to persist over time numerous complex conditions must be kept, many of which we have only become aware of quite recently and many undoubtedly await discovery.

CHAPTER 3 MUTATION ACCUMULATION UNDER SALT-STRESS IN YEAST. I. RATES AND EFFECTS OF OMNIGENETIC CHANGE IN DIPLOID FITNESS

3.1 ABSTRACT

Environmental components of mutation rate variation have been studied for nearly a century. Most attention has focused on mechanistic actions of radiations and other agents that directly interact with nucleic acids while many widespread but less direct genotoxic stresses are more poorly understood. We performed an approximately 1500 generation mutation accumulation study in the model eukaryote, *Saccharomyces cerevisiae* to investigate the rate and distribution of effects of mutations affecting fitness that occur in a moderately stressed organism. We grew fifty replicate lines in a high salt medium alongside a set of control lines cultured for an equivalent number of generations on identical medium without additional salt and compared fitness traits of both sets of lines to the ancestral genotype. Maximum likelihood analysis of two fitness measures, maximal growth rate and sporulation, indicates that the rate of mutation is roughly doubled in the high salt-stressed lines for both fitness traits. We also find a high proportion of positive effect changes for sporulation in both treatments but not for growth rate. Phenotypic measures are commonly used to infer genome-wide mutations in complex traits without a precise understanding of their material basis, in particular the contributions due to epigenetic phenomena. We use the term 'omnigenetic' to describe the combined action of DNA variation and stable expression patterns on phenotypes acted on by evolutionary forces.

3.2 Introduction

Mutation creates heritable variation, the source of raw material for evolution. Owing to its importance, the conditions under which it takes placed have been extensively studied in the roughly hundred years of modern mutation research. Hermann Muller contributed greatly in early studies that identified effects of both genotype and environment on mutability in fruit flies (e.g., Muller, 1928). The discovery of a genetic basis of mutation rates spawned a whole field of research characterizing mutation rate evolution. Environmental induction of mutations was an active area of early research and focused extensively on effects due to radiations, temperature (reviewed by Lindgren, 1972) and a range of other abiotic factors (see Auerbach, 1976). While many classic mutagens were discovered in these early studies, many factors showed conflicting results that were and continue to be difficult to interpret. One generality that arose from early studies was the dichotomy of spontaneous versus induced mutation. Despite its widespread use, this classification has been previously recognized as somewhat artificial (Auerbach, 1976). An illustration of this is the modern recognition that many mutagenic compounds are naturally produced within living cells during normal metabolic processes. Environments that influence these processes may affect mutability without directly interacting with DNA.

Experimental studies of mutation rates and effects have been largely investigated in laboratory populations of model organisms (see Baer et al., 2007; Halligan and Keightley, 2009 for reviews). A common approach is a kind of divergence experiment called mutation accumulation (MA). This involves the parallel propagation of a number of independent evolutionary lines derived from a single genotype that are raised in a

reduced selection environment. Inferences of mutation can be made by examining the resulting lines after a period of MA and by comparison to the ancestral, mutation-free condition.

The yeast *Saccharomyces cerevisiae* represents one of the best studied model organisms, both generally in terms of its genetics and cell biology as well as specifically in the range of mutation accumulation studies where it has been used. Zeyl and DeVisser (2001) first measured genome-wide mutation rates affecting fitness in diploid yeast. Their fitness measure, asexual mitotic growth rate, represents one of the best studied phenotypes in *S. cerevisiae*. Growth rate was chosen because of its strong association with total fitness along with its property of being a large mutational target, having many genes that can affect this trait. Subsequent studies have investigated genome-wide mutation for a range of other characters, including haploid growth (Wloch et al., 2001; Hall and Joseph, 2010), haploid viability (Hall and Joseph, 2010), and sporulation (Hill and Otto, 2007; Hall and Joseph, 2010). Like many of the historical studies of mutation, these recent studies have left us with perhaps as many questions as answers regarding basic mutation parameters in evolving populations. Mutation rates affecting growth rate seem to be low but estimates vary by nearly an order of magnitude (Zeyl and DeVisser, 2001; Hill and Otto, 2007). Rates affecting sporulation were found to be much larger than those affecting growth rate (Hill and Otto, 2007; Hall and Joseph, 2010), with a corresponding decrease in the average size of mutational effect. Other mutation parameters have been estimated in these studies, such as those describing the overall distribution of effects as well as the proportion of mutations that are beneficial (see also Dickinson, 2008) with little consensus.

In this study we address the same general questions regarding the basic parameters of genome-wide mutation affecting fitness in the yeast *S. cerevisisae* while addressing an additional question. Here we ask, what are the mutational consequences of growth in a sub-optimal fitness i.e., stressful environment? We propagated a series of MA lines where one half of the lines were cultured in a high sodium chloride (NaCl) environment, while the other half were propagated in a standard yeast culture environment. High NaCl has numerous effects on *S. cerevisiae* and is one of the best studied stress environments. It broadly fits into two classes of stress, osmotic stress, and ionic stress. Osmotic stress in yeast activates the high osmolarity glycerol (HOG) signaling pathway (Hohmann, 2008) resulting in an increase in the concentration of intracellular glycerol mitigating the effects of high extracellular osmolyte. Additional downstream events in this pathway affect numerous transcriptional responses including the transient cessation of the cell cycle and protein synthesis. Ionic stress results in the activation of additional ion transport mechanisms that act to restore balance across the cell membrane. It has been shown that NaCl can induce oxidative stress and DNA damage in cultured mammalian cells (Kültz and Chakravarty, 2001; Zhang et al., 2004) as well as sensitizes cells to other mutagenic treatments (Han et al., 2007; Zhu et al., 2008). DNA damage responses are also involved in the generalized yeast stress response (Gasch and Werner-Washburne, 2002), a program of transcriptional responses induced by a wide range of stressful environments.

In this study we describe the fitness affects of MA in diploid *S. cerevisiae* over approximately 1500 generations in a stressful environment and focus on two diploid fitness measures, mitotic growth rate and sporulation. We use our fitness measures to

infer mutation parameters using a maximum likelihood approach. We compare our results to previous studies that have examined similar fitness related traits in *S. cerevisisae.* Our results indicate that the mutation rate is roughly doubled when mutations accumulate in a high salt environment compared to lines that accumulated mutations in a standard yeast laboratory environment.

3.3 METHODS

Yeast strains and mutation accumulation

We used a diploid isolate of *Saccharomyces cerevisiae* PP271.1, a W303-like strain to inoculate a batch culture in 200 ml YPD. This culture was grown for 48 hrs at 28° C while shaking and constitutes the ancestral population. Aliquots (0.75 ml) of the liquid culture were taken and added to an equal volume of 40% glycerol and frozen at -80° C. This population was used to initiate a series of mutation accumulation (MA) lines cultured on solid YPD media. Lines were separated into one of 2 treatments. Fifty lines were cultured on normal YPD and fifty lines were cultured on YPD made with 1.0M NaCl. This level of NaCl was chosen because it slows the growth of this strain by approximately 50% at 28°C (data not shown). Replicate lines were routinely bottlenecked by streaking a haphazardly chosen colony using a sterile swab onto a fresh media plate. Prior to transfer, colonies were photographed using a camera (COHU 4915-2000, Cohu Inc., USA) mounted on a stereo dissection microscope (Olympus SZH-ILLK, Olympus Optical Co. Ltd.., Japan) and images were used later in the estimation of generation number (Appendix A). All cultures were grown at 28°C in a single growth cabinet with plates shuffled at each transfer with respect to their position within the

chamber and to their neighboring plates. Lines cultured on YPD were transferred to fresh medium daily for 75 days. Lines cultured on salt were transferred every 2 days to fresh medium for 81 transfers over 162 days. Media for both treatments was made as a single batch prior to the addition of NaCl to the salt medium to ensure otherwise uniformity of environments in the experimental treatments. During the early course of MA on the salt medium, we observed a marked decline in the growth rates of colonies in this treatment group. At transfer 26 we changed the level of NaCl to 0.9M to alleviate the level of stress on these lines. Salt lines were propagated at this level of salt for the remainder of the MA phase. During the course of MA, two lines of the YPD treatment and 3 lines of the salt treatment went extinct. Using the information from the colony sizes at each transfer, we calculated the average effective population size (N_e) by taking the harmonic mean for lines in each treatment. Assuming that each transfer starts from a single cell and that the population doubles until the average population size at transfer is reached, both YPD and salt treatments had a N_e of approximately 10.5 over the course of MA.

Upon completion of approximately 1500 generations, lines were expanded into late log-phase populations by inoculating 5ml liquid culture tubes of YPD and salt YPD with lines from the appropriate treatment. Aliquots of these cultures were frozen and stored in 20% glycerol at -80° C. During the expansion of the MA lines into larger populations prior to freezing, a single line of the YPD treatment became contaminated with bacteria and was excluded in further analyses. We tested all remaining lines from both treatments for respiratory deficiencies i.e., the petite phenotype by plating on YPG, a non-fermentable carbon source. We found five lines in the YPD MA lines that could not grow on YPG while all high salt MA lines could grow on this medium. We excluded

lines showing the petite phenotype from further study in this work. Accounting for line extinctions, contaminants and respiratory deficiencies, we recovered 42 MA lines from YPD and 47 MA lines from the salt treatment with which to conduct this study.

Fitness assay layout

We performed all of our fitness assays in a 96-well microplate format. A series of inoculum source templates were constructed to expedite inoculation of replicate fitness assay cultures. We devised a series of randomized arrangements such that a pair of plates contained a complete set of surviving MA lines along with 20 replicate ancestral cultures. Within each plate-pair, each plate contained 25 lines from each treatment and 10 ancestral replicates for a total of 60 cultures per plate. These 60 cultures were randomly assigned to the 60 positions within the interior of a plate. This arrangement of wells on the margin of the plate containing no cultures was made owing to the observation of growth differences at plate edges. Using 96-well PCR plates (Greiner Bio-One,USA) we added 50µl of sterile 20% glycerol to each well. We then added 50µl of culture from each MA or ancestral line stock to the appropriate well on each plate pair. This procedure was repeated for 5 separate plate pair randomizations to reduce effects due to positional peculiarities during the subsequent fitness assays. After cultures were added to wells and mixed by pipetting, plates were covered with plastic sealers (Greiner Bio-One, USA) and frozen at -80°C.

Asexual fitness assay

We quantified asexual fitness in yeast lines by measuring their maximal growth rate. Master template plates were used to inoculate 96-well assay plates (Microtest 96, BD Falcon). 150µl of YPD was added to each well of the 96-well plate. We then used a flame-sterilized 96-pin apparatus to inoculate the growth plate from the inoculation plate. Each plate was dipped twice into each assay plate to increase the inoculum size. Growth plates were then incubated at 28±0.5° C with shaking in a plate-reading spectrophotometer (Genios, Tecan Group Ltd., Switzerland). Optical density (OD) measurements were taken at set intervals over a 48-hr period and recorded directly into a spreadsheet program using the program XFluor^{TM} (Tecan Group Ltd.). Maximal growth rates were estimated by finding the maximal slope of the log-transformed OD values. We used a sliding-window procedure to find the region of maximal growth for each curve. We used Hill and Otto's (2007) method, where the window size chosen was the one that minimized the between-replicate variance. In our experiment this corresponded to the growth rate in 7-hr windows. Two complete sets of measurements were taken, each consisting of the same 5 plate-pair randomizations. The first of these was taken in 2007 and the second in 2009, resulting in a total of 10 point estimates for each extant MA line and 200 ancestral point estimates. In order to reduce environmental variability in the data, we generated a standardized data set that removed artifactual effects we were able to detect in the raw data. We found positional patterns within plates as well as an effect of individual plates on ancestral genotype growth rates. We used this information to remove the effects of these factors on the measurements for each MA line estimate, reducing the environmental variation in the data (Appendix A).

Sexual fitness assay

We measured sexual fitness as rate of sporulation. Using the same inoculum templates as used in the asexual fitness assay, we sporulated MA lines in 96-well microplates. We inoculated a complete set of MA lines and twenty ancestral replicates across a pair of plates into 150µl presporulation medium (1% yeast extract, 2% peptone, 1% potassium acetate, 0.05% dextrose) by 96-pin inoculation. Plates were incubated at room temperature for 48hrs before cells were washed with sterile distilled H_2O and resuspended in 150µl sporulation medium (1% yeast extract, 1% potassium acetate). Culture densities were measured by spectrophotometry and incubated at room temperature for 48hrs with shaking. Cells were then observed and photographed using an inverted compound microscope (Zeiss Axiovert 200M). Sporulation rates were obtained by counting a minimum of 200 cells per replicate and scoring tetrads versus unsporulated cells. All counts were done in a blind fashion, with the line identity unknown to the counter. This was repeated four times each with a different set of line-well arrangements giving a total of 100 ancestral sporulation rate estimates and five MA line estimates for each extant MA grande line. As for the asexual estimates, we standardized our sporulation estimates to reduce sources of environmental variability. Correction of MA line sporulation rates came after detection of effects due to individual plates and culture density on ancestral genotype sporulation.

Statistical analyses

We used randomization tests performed in Mathematica 8.0 (Wolfram Research, Inc.**)** to test for changes in means, variances, skew and kurtosis between MA treatments.

Measures were randomly assigned to a treatment to produce 10,000 data sets and we asked what the probability was of obtaining the original difference between treatments.

We used the Mixed Procedure in SAS version 9.2, (SAS Institute Inc., Cary, NC, USA) to calculate among-line variances by restricted maximum-likelihood. We performed likelihood ratio tests to ask whether among-line variances differed from zero. Two times the difference in log-likelihoods between the full model and a model without the MA treatment effect should have a chi-square distribution with 1 d.f. We halved the resulting *P*-values as the test is one-tailed.

Likelihood ratio tests were also used to determine whether among-line variance differed between treatments. We compared a full model in which among-line variance was modeled separately for each treatment to a reduced model in which only one estimate of among-line variance was calculated for both treatments. Both the full and reduced models allowed within-line variance to vary between treatments. This test is two-tailed.

Genetic correlations among treatments for each trait were estimated using the 'type=unr' covariance option in SAS. Likelihood ratio tests were used to evaluate the hypothesis that the genetic correlation differed from zero and differed from one by constraining the estimate of the correlation using the PARMS statement. The tests for r_g equal to zero is two-tailed while the test for r_g equal to one is one-tailed. Confidence intervals for the estimate of genetic correlation were estimated by examining the profile likelihoods. A test of significance on the cross-trait genetic correlation between MA treatments was performed by generating two-thousand randomized data sets and asking what was the probability of obtaining the original correlation.

Mutation parameter estimation

We obtained estimates on mutation parameters for both traits using the Bateman-Mukai method of moments (BM; Bateman, 1959; Mukai, 1964) and that developed by Keightley (mlgenomeu v.2.08; Keightley, 1994; Keightley and Ohnishi, 1998) based on maximum likelihood (ML). The BM method uses information on the changes in mean and among-line variance to estimate bounded values of mutation rates and average effects. It assumes that all mutations are detrimental and of equal effect. Keightley's ML analysis utilizes more information as it takes trait values for each MA line into account. It assumes a Poisson distributed number of mutations among the replicate lines and a gamma distribution of mutational effects. It uses the gamma distribution reflected about zero to estimate the proportion of positive effect mutations. Analyses were run on the glooscap cluster at the Atlantic Computational Excellence Network (ACEnet). We found ML parameter estimates by holding the gamma distribution shape parameter (β) and the proportion of positive effect mutations (P_B) constant for each run and allowing the program to find the ML values of the gamma distribution scale parameter (α) and the mutation rate (U). Confidence intervals for the estimates were obtained by examining profile likelihood curves and taking the parameter values at -2 log likelihood values in each direction from the maximum as the lower and upper bounds of the interval.

3.4 RESULTS

Fitness differences in MA treatments

We measured the diploid fitness components growth rate and sporulation in lines derived from a common ancestor that had accumulated mutations in normal and stressful

environments. We found a statistically significant reduction in mean growth rate between ancestral and MA replicates in each environment (ancestor vs. YPD MA: P = 0.0021; ancestor vs. salt MA: P =0.00029) but no difference between the two MA treatments (P =0.6; Table 3.1, Figure 3.1A). Compared to the ancestor, total variance in growth rate increased by a factor of 2.25 in the YPD MA treatment (P = 0.00008) and by 2.09 in the salt MA treatment (P = 0.0005), but the MA environments did not differ (P = 0.7; Table 3.1). The among-line component of standardized variance was significantly greater than zero for both YPD (43%) and high salt (16%) environments (Figure 3.1B; likelihood-ratio test, one-tailed $P < 0.0001$ for both). The among-line variance differed between MA environments (likelihood-ratio test, two-tailed P = 0.045).

Compared to the ancestor, mean sporulation declined by 0.43 standard deviations in the YPD lines $(0.0188/(0.0019)^{1/2}$; P=0.011) and by 0.49 standard deviations in the salt lines (P=0.031), but no difference was found between the two MA treatments (P=0.8; Table 3.1). Compared to the ancestor, total variance in sporulation increased by a factor of 2.46 in the YPD treatment (P = 0.00008) and by 4.85 in the salt treatment (P = 0.0005), and the MA environments differed (P = 0.00014; Table 3.1). The among-line component of standardized variance in sporulation was significantly greater than zero for both YPD (48%) and salt (68%) environments (Figure 3.1B; likelihood-ratio test, one-tailed $P < 0.0001$ for both). The among-line variance did not differ between MA environments (likelihood-ratio test, two-tailed P = 0.74).

Correlation between growth and sporulation

We observed correlations between growth rate and sporulation for both YPD-MA and salt MA lines (Figure S7). The correlation between corrected growth and sporulation for the YPD-MA lines was 0.89 (P<0.0001). The correlation for salt MA lines was 0.55 (P<0.027). No significant difference was found between the MA treatments (P = 0.18).

Mutation parameter estimates

We calculated mutation rates and effect parameters for each character and MA treatment using the Bateman-Mukai and maximum-likelihood (ML) (Table 3.2; Figures S5, S6) approaches.

Our ML estimates of the genome-wide mutation rate for yeast accumulating mutations affecting growth rate on YPD was found to be 0.00017 mutations/genome/generation. When lines accumulated mutations under salt stress, the rate was roughly doubled to 0.00037 mutations/genome/generation. The estimate of the average heterozygous effect for lines grown on YPD was roughly twice that of those grown on salt, 0.055 to 0.028 respectively. Our ML estimate of the gamma distribution shape parameter (β) was lower for salt MA lines, indicating more mutations of smaller effect within this treatment. The estimated proportion of positive effect mutations (P_B), affecting growth rate, in both MA environments was 0.

Table 3.1: Summary statistics of yeast asexual and sexual fitness measures after ~1500 generations of mutation accumulation

		N	Mean	Variance	Skew	Kurtosis
Growth Rate						
	Ancestor	200	0^{A}	0.001^{A}	-0.9^{A}	5.2^{A}
	YPD MA	416	-0.0111^{B}	0.00225^{B}	-1.4^{B}	7.2^{B}
	Salt MA	366	-0.0129^{B}	0.00209^{B}	-1.2^{C}	8.3^{C}
Sporulation						
	Ancestor	99	0^{A}	0.0019^{A}	-0.12^{A}	2.5^{A}
	YPD MA	206	-0.0188^{B}	0.00468^{B}	-0.87^{B}	4.5^{B}
	Salt MA	196	-0.0213^{B}	0.00921^{C}	-0.24^{C}	3.2^{C}

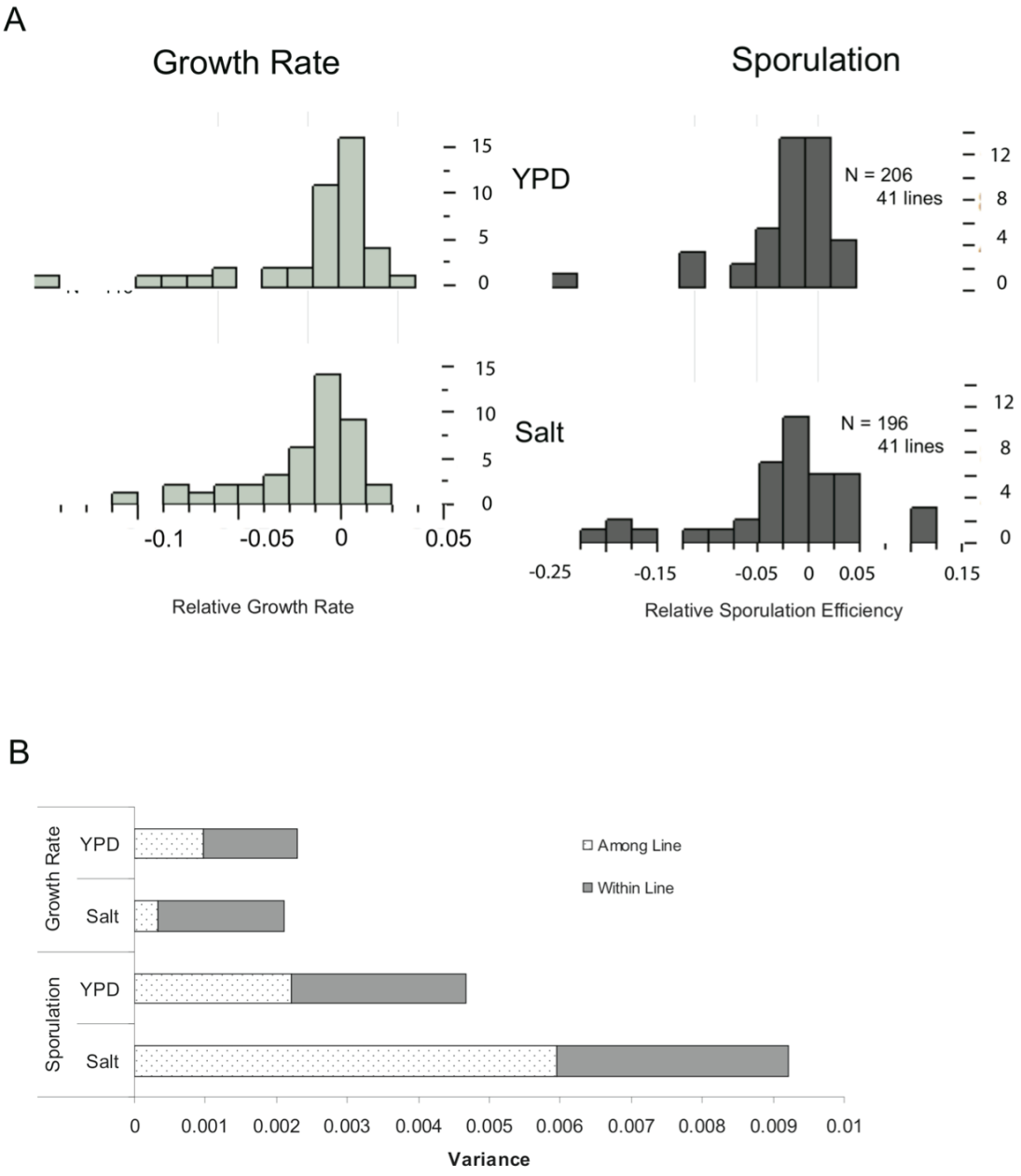

Figure 3.1: Phenotypic measures of fitness traits in after ~1500 generations of mutation accumulation in *S. cerevisiae*. A. Distributions of diploid fitness line means. B. Among-line variance in growth rate and sporulation.

Sporulation mutation rate estimates are at least 2 orders of magnitude higher than rates affecting growth. Conversely, effect sizes are approximately 2 orders of magnitude smaller than for effects on growth rate. Estimates of the shape parameter β are much smaller for sporulation, indicating that the distribution of effects on sporulation is highly leptokurtic. The proportion of positive effect mutations (P_B) is higher for sporulation than growth rates in both MA treatments relative to the proportion found affecting growth rate. Salt MA lines have a mutation rate affecting sporulation twice as high as MA lines that were grown on YPD, 0.14 to 0.073 respectively. These estimates may not represent the true ML values however, as the likelihood surface is very flat near the maximum value found and may increase further. The average heterozygous effect on sporulation was slightly higher in salt-MA lines, 0.00041 to 0.00034 for lines accumulating mutations in YPD. β was found to be less than or equal to 0.005 for both MA treatments. We were unable to investigate smaller values of β due to limitations arising from the amount of computation time required for these sets of parameter combinations. The ML estimates of P_B affecting sporulation were found to be 0.3 and 0.4 for lines accumulating mutations on YPD and salt respectively.

The likelihood profiles obtained presently are somewhat irregular compared to those seen in previous studies. They will be further examined for the published version of this work.

Table 3.2: Estimates of mutation parameters after ~1500 generations of mutation accumulation on YPD and salt

		Bateman-Mukai		Maximum Likelihood			
		Umin	***smax***	***U***	***E(hs)***	***β***	***P_B***
Growth Rate	YPD MA	0.000085	0.087	0.00017 *(0.00012-∞)*	0.055 *(0-∞)*	3.0 *(0-∞)*	0 *(0-0.26)*
	Salt MA	0.00032	0.026	0.00037 *(0.00026-∞)*	0.028 *(0-∞)*	1.7 *(0-16.2)*	0 *(0-0.25)*
Sporulation	YPD MA	0.00011	0.12	0.073 *(0.00012-∞)*	0.00034 *(0-∞)*	≤0.005 *(0-∞)*	0.3 *(0-0.53)*
	Salt MA	0.000049	0.28	0.14 *(0.0007-∞)*	0.00041 *(0-0.64)*	≤0.005 *(0-2.8)*	0.4 *(0.26-0.55)*

3.5 Discussion

Fitness effects of mutation accumulation under high salt

We found effects on our fitness traits due to MA but few significant differences between the two MA treatments. Significant changes in mean growth rate have not always been detected in MA studies using non-mutator strains of diploid yeast. Zeyl and DeVisser (2001) found no detectable differences in fitness for their 31 grande non-mutator lines compared to their ancestor after ~600 generations of MA. Significant fitness differences were detected for 151 lines after ~1000 generations of MA from a completely homozygous ancestor in Joseph and Hall's (2004) study as well as their subsequent work after a further ~1000 generations using the same lines (Hall et al., 2008). Hill and Otto (2007) found a decrease in growth rate in their 78 MA lines that accumulated at a small effective population size but an increase in growth rate in their 50 lines that accumulated at a large effective size. These results illustrate the difficulty in measuring and comparing fitness changes after MA in yeast. Such measures will depend critically on several factors including the number of generations of MA, the effective size of the population, the number of replicate lines, and the genetics of the ancestral strain.

Mutation parameter estimates for lines accumulating on YPD

Our ML mutation parameter estimates show similarities to other estimates using similar methods in yeast. Our best estimates for the mutation rate affecting growth rate for MA on YPD is roughly between the values obtained by others using similar methods (Hill and Otto, 2007; Hall and Joseph, 2010; Table 3.3). As is typical with these approaches, we find that we can only place a firm lower bound on our estimate. Our ML

estimate of the average effect size in this treatment is also similar in magnitude to previous work, again with a large confidence interval. The shape parameter of the gamma distribution suggests, somewhat differently from Hill and Otto (2007), who found a highly leptokurtic gamma distribution (β<1) best fit their data. Our best estimate of β=2 still implies a large number of small effect mutations affecting growth particularly when the broadly overlapping confidence intervals between the studies are taken into account.

Our results indicate a high proportion of positive effect mutations affecting sporulation but not growth rate. This is in contrast with Hill and Otto (2007) whose best estimate of P was 0 for both asexual and fitness and Hall and Joseph (2010) who estimated a relatively high proportion of positive effect changes for both traits (Table 3.3). Dickinson (2008) reported approximately 25% beneficial changes in competition-based growth rates after 4800 generations of mutation accumulation. The 30% estimate of P_B affecting sporulation in our YPD MA treatment is higher than those observed in previous studies. A common explanation for the high proportion of beneficial changes observed is that the yeast genotypes being investigated are far from their fitness optimum, invoking Fisher's (1930) geometrical model of adaptation. In our case, Fisher's model would suggest that our starting genotype was relatively well adapted to our conditions for asexual mitotic growth but poorly adapted to the sporulation environment we used. It is also important to note that other explanations have been offered (see Hall et al., 2008).

Mutation parameters for lines accumulating in high salt

Our ML estimates show several interesting trends with respect to the MA environment. The most striking observation is the roughly two-fold increase in the mutation rate for

both growth rate and sporulation in the salt treatment compared to the YPD (Table 3.2). Many hypotheses have been put forth regarding the adaptive significance of a stress-induced increase, particularly in regards to the adaptive mutation controversy. The simplest explanation, we believe, is entirely non-adaptive. It is that stress disrupts the normal fidelity mechanisms that work to preserve genomic information. This possibility was raised as a specific possibility with regards to the adaptive mutation controversy (Lenski and Mittler, 1993). This also provides a basis from which to consider an environmental component to the mutation rate, viewed as a quantitative character. Sturtevant (1937) recognized such 'external factors' in his discussion of selective forces acting to drive down the mutation rate. In the context of our experiment we are able to observe the mutational effects arising from an external factor, i.e., high salt, because the effects of selection have been reduced.

The doubling of the mutation rate affecting growth rate between MA treatments is accompanied by a decrease in the average effect size and a more leptokurtic distribution. The biological interpretation of this is unclear. One possibility is that the frequencies of particular kinds of mutations that occur in high salt conditions may differ from those that take place in regular YPD but such an explanation is only speculative in light of the current analysis. For sporulation, the increased mutation rate is accompanied by a larger average effect size for lines that accumulated in salt. This difference in the response of the two traits to the two MA environments could arise in several ways. The genetic architecture of the two traits may differ in important ways. Previous studies of MA affecting growth rate and sporulation (Hill and Otto, 2007; Hall and Joseph, 2010) as well as our results here show that there is a strong genetic correlation between the two

Table 3.3: ML mutation parameter estimates for fitness-related traits in *Saccharomyces cerevisiae*

Fitness Trait	U (*10^{-5} mut/genome/gen)	$E(hs)$	β	P_B	Reference
Growth	5.5	0.217			Zeyl and DeVisser, 2001*
	30	0.017	∞	0	Hill and Otto, 2007
	6.3	0.061		0.125	Hall and Joseph, 2010
	17	0.055	3.0	0	this study**
Sporulation					
	1300	0.0046	0.01	0	Hill and Otto, 2007
	→∞	→0		0.2	Hall and Joseph, 2010
	7300	0.00034	≤0.005	0.3	this study**

* growth by competition assay
** YPD MA treatment

traits, consistent with significant pleiotropy. It is known however, that there are sporulation specific genes (Gerke et al., 2006). The presence of such genes should decrease any correlation that we find, assuming that they are targets of mutation in our lines.

Mutation parameters and the effects of selection bias

Despite our attempts to minimize the efficiency of selection it is not possible to eliminate it entirely. In the most severe case, lethal mutations that arise during colony growth are lost from our experimental populations and are therefore underrepresented in any analysis. In less severe cases, mutations that alter growth rate should cause uneven distributions of mutant and non-mutant cells within colonies, depending on when they take place as well as the severity of the mutational effect. These eneven distributions will cause estimations of mutation parameters to be biased in particular ways. Such biases have been taken into account in previous yeast MA studies (Zeyl and DeVisser, 2001; Joseph and Hall, 2004) where mutation parameters have been corrected.

Our estimates of mutation parameters affecting growth rate presented here are uncorrected estimates and thus may be biased due to selection during colony growth. Mutation parameters affecting sporulation should be less affected as sporulation is not directly selected during the MA phase and could only be affected by the pleiotropic effects for genes affecting both growth and sporulation. Despite these sources of bias, the central conclusion of this work, that salt stress causes an increase in the mutation rate,

should be qualitatively unaffected by selection bias. The rationale for this is as follows. If selection does not differ between the two MA treatments then estimates of parameters in each treatment will be affected similarly and our finding of a two-fold increase in the mutation rate should only be minimally affected. If selection is stronger in salt, we would expect our estimates to be biased in different ways. Selection should remove some fraction of the deleterious mutations affecting growth rate and add some fraction that are beneficial. This would lead to a biased estimate that depends on the proportion of positive versus negative mutations. If as expected, negative mutations are much more prevalent than positive, we should observe fewer mutations in our MA lines than were actually generated during MA. Stronger selection in our salt MA treatment should therefore cause a stronger downward bias in the mutation rate than the YPD treatment. The third possibility is that selection is stronger in our YPD treatment than in salt. We believe this to be highly unlikely and do not consider it further. Even allowing for greater selection bias in the salt MA treatment, the estimated mutation rate is nearly twice under salt MA than in the YPD treatment, and therefore we are confident in concluding that MA in high salt casuses more mutations to occur than in YPD.

Selection bias may affect our other estimated parameters as well. Estimates of the average effect size will be biased in different ways depending on the proportions of positive and negative effect mutations and whether selection affects negative or positive mutation differently. For example, if P_B=0, then only large effect negative mutations will be removed by selection causing an underestimate in the average effect size. At the other extreme, if P_B =1, then large positive effect mutations will be overrepresented and we will overestimate the average effect size. Selection can also bias P_B. Selection removes

negative effect mutations and enriches positive effect mutations in replicate lines. This will cause an overestimate in P_B due to selection. As mentioned, methods for correcting for selection bias have been used in previous studies. Corrections for our estimates made here will be pursued further in the publication versions of this work.

Mutation estimates based on heritable phenotypic changes should be considered 'omni-genetic'

This study uses phenotypic measures to infer mutational changes across a whole eukaryotic genome, a common approach in MA studies of fitness. A common caveat to inferring genetic mutation in these studies is that the phenotypic changes observed could be due to other heritable features of organisms, in particular epigenetic phenomena (Hill and Otto, 2007). We acknowledge this possibility but wish to place a different emphasis on what has been done. In measuring phenotypes, we capture the total effect of heritable changes among our lines. This total effect is the combined action of canonical mutation in concert with extant epimutation. We use the term omnigenetic change to describe these combined influences and suggest that all MA studies that have not directly measured changes in DNA have actually measured this total effect. This may in part explain some of the peculiarities observed in these studies, such as the highly leptokurtic distributions of mutational effects found in some studies but not in others. Further work is needed to examine this possibility. One difficulty that arises when viewing genetic and epigenetic variants together in this way is the relative stability and rate of occurrence of each type of change. DNA sequence changes are stable and the rate of back-mutation is generally thought to be very low. They have been directly measured for one yeast MA

experiment by Lynch et al. (2008) who measured a mutation rate of 0.32 mutations per cell division. We currently have few data on the rate of generation or persistence of epialleles affecting fitness in yeast or any organism. This remains an important question to be addressed.

Environmental stress and mutation

Our study directly addresses the question of the effect of a moderate stress on mutability of fitness characters in budding yeast. Our most striking finding is the two-fold increase in the rate of omnigenetic change in the high salt-stress treatment. Environmental stress and mutation has mostly been investigated in bacteria (Galhardo et al., 2007) but there is also some evidence in eukaryotes (Goho and Bell, 2001; Capp, 2010). Explanations for these phenomena have typically focused on adaptive explanations, where higher mutation rates have some benefit that allows mutators to spread throughout a population (Galhardo et al., 2007). It is well established that many mutations arise via a two-step process of damage followed by incorrect repair (Baer et al., 2007). The induction of increased damage through production of harmful metabolic by-products provides a plausible, non-adaptive explanation for this phenomenon. Such a mechanism is now thought to contribute to the generation of antibiotic resistance in bacteria (Kohanski et al., 2010). Many others have proposed ways in which a general increase in mutability associated with stress could occur (e.g. Lenski and Mittler, 1993; Goho and Bell, 2000; Capp, 2010). While both adaptive and non-adaptive explanations remain viable, we believe that non-adaptive explanations are simpler and require fewer assumptions. We emphasize that our study does not address which of these might be

more important in stress-induced mutagenesis but rather demonstrates and quantifies a case of context-dependent mutations on fitness in a model eukaryote.

The mutation rate is a fundamental evolutionary genetic parameter and its value is critical to many important phenomena including the setting of the molecular clock, explaining rates of adaptation, the origin and maintenance of sex, and the maintenance of genetic variation. We may need to address the possibility that this trait is elastic in nature. Periods where the fit of the organism to its environment is good generally lead to low levels of environmentally induced mutation whereas when the fit is poor, the mutation rate is elevated and there is an influx of new genetic variation into the system. The extent to which mutability is affected by such a process in natural systems remains an important and unexplored possibility.

CHAPTER 4 MUTATION ACCUMULATION UNDER SALT STRESS IN YEAST. II. MUTATIONAL EFFECTS ON POST-MEIOTIC FITNESS

4.1 ABSTRACT

The fitness consequences of new mutations are an important link in our understanding of the genotype-phenotype-environment interrelationship. To investigate this, we have generated a series of diploid mutation accumulation (MA) lines in the budding yeast *Saccharomyces cerevisiae* over a period of approximately 1500 mitotic generations under two environmental treatments. One group accumulated mutations under standard culture conditions while the other was exposed to a moderate salt stress for the period of MA. In this work we examined the effects of these MA conditions on the post-meiotic fitness measures spore viability and haploid growth rate in a common, non-stressful environment. Haploid viability did not differ significantly between MA treatments. Haploid growth rates, in contrast, were reduced due to MA and reduced more severely in the salt treatment. Salt MA haploid growth rates were also more variable and skewed toward lower fitness than either the non-stress treatment or our ancestor-derived replicates. Maximum-likelihood analysis of mutation parameters indicated that salt stress increased mutation rates for both characters. In both MA treatments we estimated that 45% of mutations affecting haploid viability were beneficial. Conversely, we found that for haploid growth rates our non-stress treatment showed no evidence for beneficial changes while we estimated that 15% of changes in our stress treatment were positive. We discuss our high proportion of beneficial mutations in light of other similar findings. Variance component analysis on haploid growth rate measures identified a large proportion of variation between siblings of the same meiotic event, particularly in our stress treatment. This represents to our knowledge the first estimation of newly generated, post-meiotic mutational variation in any organism.

4.2 INTRODUCTION

An advantage of diploidy over haploidy is often ascribed to robustness, as a second genome copy allows tolerance of gene alteration. One way in which this is accomplished is through the dominance-recessivity relationship of alleles, where new mutations have reduced effect on the phenotype due to the presence of a fully functional second copy. Another advantage of diploidy lies in the capacity to repair damage to one copy by using the undamaged copy as a template. Despite these advantages, many eukaryotes spend a significant amount of time in haploid stages. Many eukaryotes exist predominantly as haploids and some groups of land plants have their dominant multi-cellular life stage in haploidy. Even for large elaborate diploid organisms, if they reproduce sexually, they must survive a crucial pre-fertilization haploid stage. Sexual organisms are therefore characterized in part by the alternation of ploidy, and by the associated cycle of masking and unmasking of recessive alleles. The transition between ploidy phases therefore is an important stage for many species at which mutational variation can be expressed.

Mutation accumulation (MA) is the most direct experimental approach to quantifying the input of genetic variation into populations. Few studies have allowed mutations to accumulate in diploids, and then gone on to measure fitness effects in the haploid state. In many organisms such a study is impossible or nearly so owing to short-lived or reduced haploid stages combined with current technical limitations. *Saccharomyces cerevisiae* provides the advantages that they can be cultured asexually as either diploids or haploids and that the meiotic products are immediately accessible. Wloch et al. (2001) performed mutation accumulation in diploid yeast, induced meiosis, and measured subsequent growth rates of haploid spores. Hall and Joseph (2010) also measured haploid growth rates and viability along with diploid growth rates and

sporulation effeciency, after a much longer period of MA. They found a greater than seven-fold larger difference in the mutational heritability of haploid growth rates over diploid rates, consistent with the unmasking of recessive alleles.

In this study, we utilize a series of previously generated (Chapter 3) MA lines of *S. cerevisiae* to investigate the environmental context of MA on post-meiotic fitness components. We estimate mutation rates and distribution parameters on haploid viability and growth rates compare them to other fitness components previously studied.

4.3 METHODS

Yeast Strains and Culture

Mutation accumulation (MA) lines were fully described earlier (Chapter 3). Briefly, we cultured a diploid W303-like diploid isolate of *Saccharomyces cerevisiae* for approximately 1500 mitotic generations under mutation accumulation conditions. An initial population was divided into 50 lines that were restreaked on YPD agar while a second group of 50 lines was restreaked on high-salt YPD agar. Generations were counted by obtaining digital images of colonies prior to restreaking and using colony area as a proxy for cell number based on an empirically derived standard curve. Lines were frozen at -80°C after MA was completed.

Sporulation and Haploid Isolation

We inoculated 3ml of presporulation medium (1% yeast extract, 2% peptone, 1% potassium acetate, 0.05% dextrose) and incubated cultures for 3 days shaking in the dark at room temperature. Cells were centrifuged at 1500g for 5 minutes, washed with an equal volume of sterile distilled water, centrifuged again, and resuspended in 3ml of

sporulation medium (1% yeast extract, 1% potassium acetate). Cells were returned to the shaker for 3 days prior to tetrad dissection. After incubation, a 100µl aliquot of sporulated culture was spread in a narrow streak on a 2% peptone, 1% dextrose agar slab. Tetrads were identified by microscopic examination, picked and haploids separated using a Narishige MN-151 micromanipulator (Narishige Co., Ltd. Tokyo, Japan). We picked 5 tetrads per MA line. After dissection, the slab was transferred to a fresh YPD plate and incubated at 27°C until colonies appeared. After this incubation, slabs were photographed for later scoring of spore viability. To confirm that our dissections produced haploids, we scored mating types of our newly isolated strains (see Supplemental Methods). Confirmed haploids were grown in replicate 0.75ml cultures at 27°C to which was added an equal volume of 40% glycerol. Cultures were then frozen at -80°C.

A total of 38 MA lines were sporulated and had haploids successfully dissected, 17 lines from the YPD MA treatment and 21 lines from the salt MA treatment. During culture, one YPD line and three salt lines were contaminated, subsequently lost, and were included in only one of the fitness measures studied. Alongside the sporulation and dissection of a single or pair of MA lines from each treatment we performed the same procedure on the ancestral genotype from the MA experiment. This was performed for two reasons. First, our ancestral strain may have had some amount of heterozygosity for which we did not account prior to this. This variation could be expressed among our MA lines but will be treated as residual variation that is present in the ancestor. Second, due to the labor involved in haploid isolation, we could only isolate a few strains at a time. Therefore, environmental differences from one episode of haploid isolation to another

could have confounded effects we might ascribe to mutation. By culturing lines from each treatment along with the ancestor in parallel, we reduced the effects of different isolation environments on our subsequent analyses.

Fitness Assays

We scored two measures of post-meiotic fitness. Haploid viability was assessed from scoring the number of haploid lines that were recovered from each dissected tetrad. We obtained viability measures from five tetrads each from seventeen YPD lines, twenty-one salt MA lines, alongside a total of forty-three dissected tetrads from the ancestral strain. Viability was scored from digital images of dissection plates after incubation.

Maximum haploid growth rate was measured by growth curve analysis. Growth curves were generated by measuring changes in optical density (OD) of liquid cultures grown in the central 60 wells of a 96-well microplate as previously described (Chapter 3). To maximize the available mutational information, we included haploids derived from each possible diploid parent on every microplate. Inclusion of haploids as representative of each diploid parent was done by random assignment. To make efficient use of space within plates, we grew an additional set from one MA treatment. This was balanced by including an additional set from the opposite treatment on the next growth plate. Either eight or ten haploid strains derived from the ancestral genotype were also included, depending on the MA set combination on a given plate.

Haploid strains were recovered from their frozen cultures and arrayed according to their prescribed plate assignment in a 96-well microplate containing YPD agar. These agar plates were incubated at room temperature in the dark for 3 days prior to inoculation

of the assay plates. Cells were transferred between microplates using a flame-sterilized 96-pin apparatus. We used an intermediate dilution step to prevent high initial OD readings resulting from the transfer of excessive numbers of cells by the pin apparatus directly from some agar-supported cultures to liquid. Specifically, cells were transferred from the agar plate to a sterilized, custom-made aluminum 96-well plate containing 150µl of YPD broth. From this plate, cells were transferred to a 96-well cell culture assay plate (BD Falcon) using the pin apparatus. Two separate dips of the apparatus were made from the dilution plate to the assay plate to ensure an adequate inoculum.

Plates were incubated and measured using a Tecan Genios (Tecan Group, USA) plate-reading spectrophotometer. Plates were incubated at 28±0.5°C with intense shaking between measurements. Measurements were taken every 10 minutes over a 48-hour period. OD measurements were subtracted from blank media wells, $\log_2$-transformed and used to calculate slopes of varying window sizes as previously described (Chapter 3). We chose a 7-hr window as the best size based on several criteria including minimization of error variance, maximization of variance ascribed to differences among parental diploids, as well as maximizing the overall likelihood of the model. These variance component estimates were calculated using restricted maximum likelihood (REML) in JMP 9.0.2 statistical software (SAS Institute Inc., Cary, NC, USA.). Similar to previous analyses, we detected growth differences in the haploids derived from the ancestor based on position within a plate. We used this information to correct our measurements of MA haploids relative to the ancestor-derived haploids.

Statistical Analyses

For both viability and haploid growth rate, we used randomization tests implemented in Mathematica 8.0 (Wolfram Research, Inc.) to test for differences in distribution properties between MA treatments. We randomly reassigned each observation to a MA treatment generating 10,000 randomized data sets for growth rate and 1000 randomized sets for viability and then calculated the probability of obtaining our original data. We looked for changes in mean, variance, skew and kurtosis in each MA treatment group compared to the ancestor values as well as to each other.

Variance components were calculated using restricted maximum likelihood in the MIXED procedure in SAS software (version 9.2, SAS Institute Inc., Cary, NC, USA) under the following nested model:

Haploid Growth Rate = MA Treatment + Diploid Parent (MA Treatment) + Tetrad (Diploid Parent, MA Treatment) + Haploid Strain (Tetrad, Diploid Parent, MA Treatment) + error

We used likelihood-ratio tests to ask whether null models constraining the different variance components to zero differed from the unconstrained model. Parameters were constrained using the "parms" statement. Tests to determine whether the variance component differed from zero were one-tailed. We tested for differences between MA treatments in the estimated variance components. In this case our null models were obtained by estimating only a single variance component between both MA treatments. We compared these using likelihood ratios to models that calculated separate variance components for each MA treatment. These tests were two-tailed.

Mutation Parameter Estimation

We used the program mlgenomeu (v.2.08; Keightley, 1994; Keightley and Ohnishi, 1998) based on maximum likelihood (ML) to estimate mutation parameters affecting haploid viability and haploid growth rate. For viability we submitted raw data of the number of viable spores produced by each tetrad we dissected. For haploid growth rate we collapsed each measure into a single average per diploid line. We took groups of randomly selected ancestor-derived haploids averaged in the same way as the MA lines to generate our ancestor block of data.

We also used the Bateman-Mukai (BM) method of moments to calculate bounded estimates of mutation parameters (Lynch and Walsh, 1998). This method assumes an equal-effect distribution of effects and that all mutations have negative effects. Both of these assumptions are overly restrictive, given more recent findings but we include them here for comparison to older studies. BM utilizes information on the among-line variance of replicate MA lines. In this study we were able to partition variance attributed to mutation further than just diploid MA line divergence by considering variation between haploid strains within single tetrads. We therefore calculated BM estimates according to two methods. Method A utilized the variance component attributed only to differences between diploid parental lines. Method B utilized the total variance minus the within-haploid line residual variance. This results in method B capturing variance attributed to differences between parents, tetrads within parents and haploid strains within tetrads.

4.4 RESULTS

Haploid viability differed between ancestors and the salt MA treatment

Mean haploid viability was measured as 2.3 spores/tetrad among our ancestral replicates. Our YPD and salt MA lines had mean viabilities of 2.1 and 1.8 spores/tetrad, respectively. Randomization tests on our viability data indicated that the only significant difference between our three treatment groups was between the ancestral group and the salt MA treatment. We found differences between means (P=0.022) and variances (P=0.036) of these two groups. The higher-order moments skew and kurtosis did not differ between these groups. No differences in means, variances, skew or kurtosis were found between the YPD line haploid viabilities and either the ancestor replicates or salt MA line replicates. Despite the lack of significant changes in mean and variance of viability between our YPD treatment and ancestor and salt treatments, there is a shifting of the modal class between the three treatments (Figure 4.1A). Ancestor dissections produced four viable tetrads more than either of the other treatments and this was the most common outcome of ancestral dissections. Three viable spores were nearly as common. YPD lines produced four and two viable spores most commonly with nearly equal frequency. Two viable spores was the most common outcome of salt line dissections while four surviving spores was the most uncommon case for this treatment.

Haploid growth rates differed between MA treatments

Properties of growth-rate distributions (Figure 4.1B) were also investigated by randomization tests. Haploid lines showed significantly slower growth on average than ancestor haploid replicates for both YPD (P=0.001) and salt (P<0.0001). Salt MA haploids also grew more slowly than YPD lines (P=0.0002). Variance differences between the treatments were also found. YPD lines did not differ in variance from

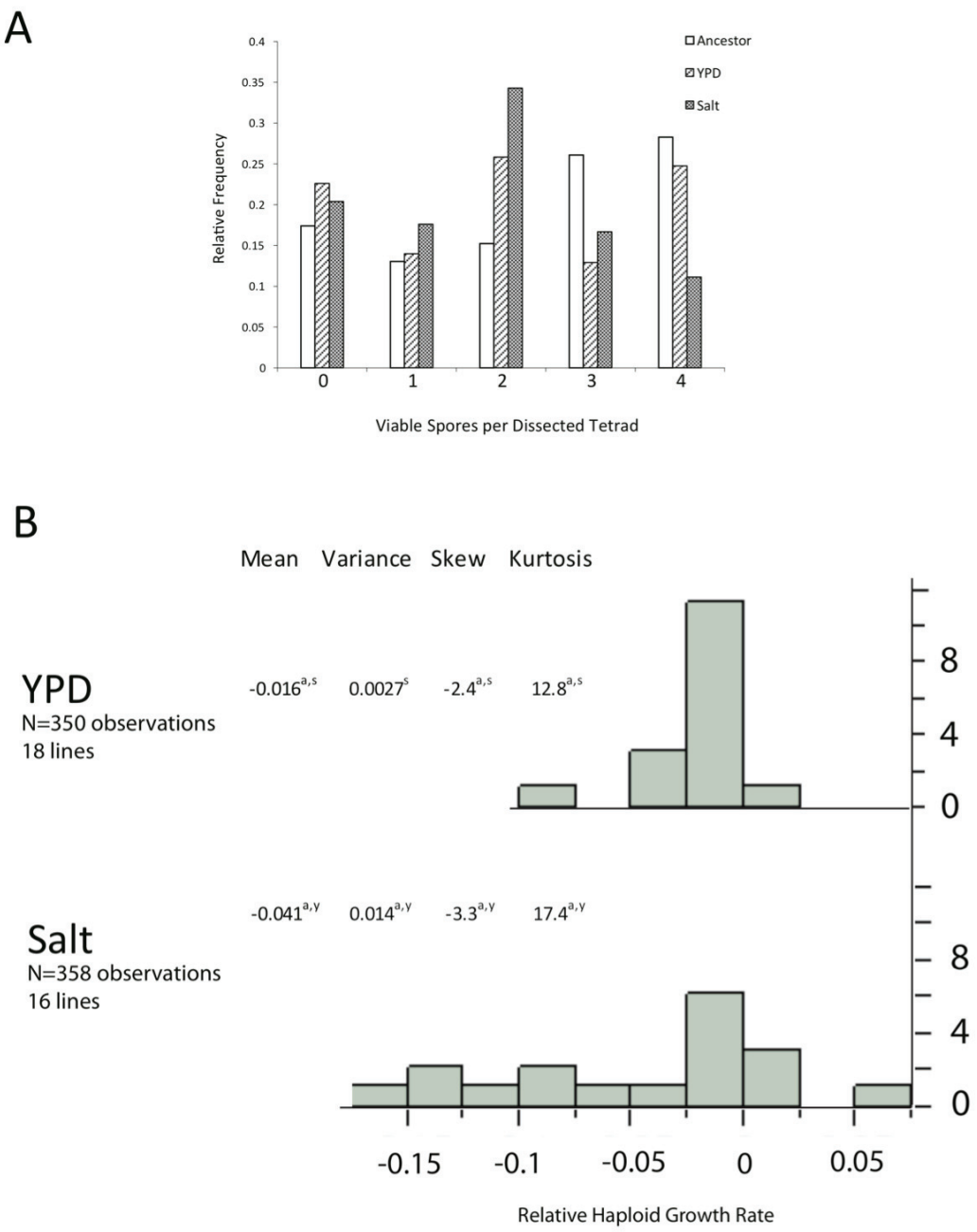

Figure 4.1: Post-meiotic fitness measures. A. Spore viability. B. Haploid growth rate. Histograms show distribution of line means. Distribution parameters are for all observations. Letters denote differences from the treatment indicated. a=Ancestral; y=YPD; s=Salt

control haploid lines (P=0.29) but salt-MA derived haploids showed significantly higher variance than both (Ancestral vs Salt, P<0.0001; YPD vs Salt, P<0.0001). Higher moments of the distributions of haploid growth rates were also investigated and in every case significant differences (P<0.0001) were found between all treatment groups. The salt-MA haploids had significantly lower skew than the YPD MA lines which were in turn significantly lower than the control ancestral lines. Similarly, kurtosis was lowest in the ancestral replicates, higher in the YPD, and again higher still in the salt-treatment.

Salt MA lines had a larger component of within-tetrad variance

We partitioned the total variance in our haploid growth rate measures into four components plus error (Figure 4.2). For both MA treatments, variance components ascribed to diploid parent (likelihood-ratio tests: YPD, P=0.0004; salt, P=0.009) and within-tetrad haploid strain (YPD, P=0.0002; salt, P<0.0001) were all significantly greater than zero, while the variance that could be ascribed to differences among tetrads of the same parent were not different from zero (P=1). Furthermore, the variance among haploid strains within a tetrad was significantly greater in the salt-MA lines compared to the YPD-MA lines (likelihood-ratio test, P=0.01). We also found significantly greater residual variance in the salt MA treatment than in the YPD (P=0.008).

Mutation parameter estimation

We used the Keightley maximum likelihood (ML) method and the Bateman-Mukai (BM) method of moments to estimate various parameters of newly arising

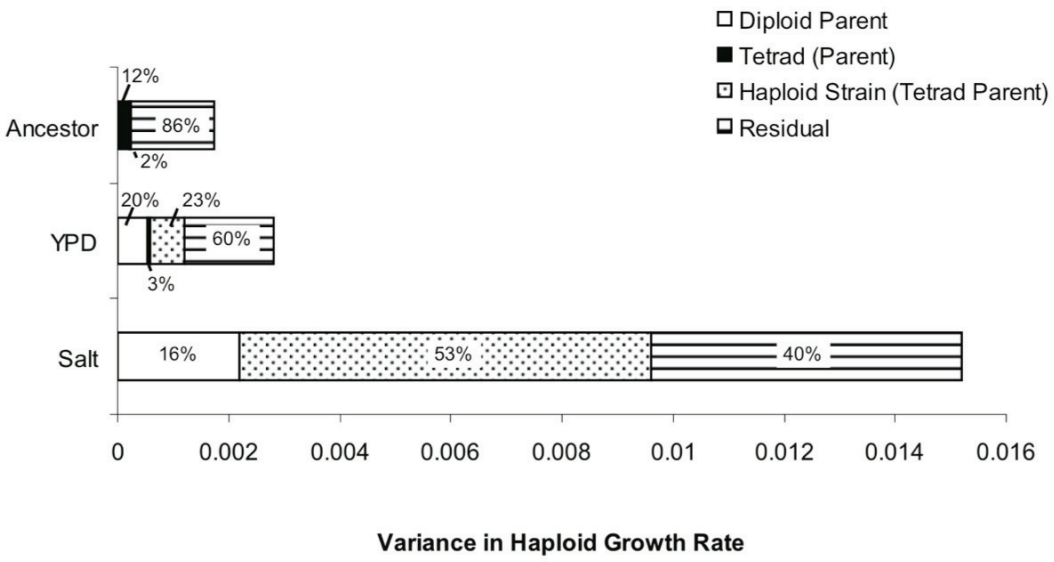

Figure 4.2: Variance component analysis of haploid growth rate

mutations (Table 4.1, Figures S8, S9). Our ML estimate of the mutation rate affecting haploid growth in our YPD lines was 4×10^{-5} mutations/genome/generation (hereafter, mut/genome/gen) under an equal-effects model with no beneficial mutations. In our salt lines the ML estimate of the mutation rate was an order of magnitude higher, 51×10^{-5} mut/ genome /gen, under a model with 15% beneficial mutations and a distribution of effects best described by a gamma-distribution shape parameter value of 38. Our BM estimates were calculated using two methods. Method A estimated a lower-bound mutation rate for YPD lines of 31.6×10^{-5} and a maximum effect (s_{max}) of 0.034. Method B generated estimates of 17.7×10^{-5} mut/genome/gen and a maximum effect of 0.06. BM estimates of our salt MA-derived haploid growth rates were calculated as 48.5×10^{-5} mut/ genome /gen and maximum effect of 0.054 for method A and 11.4×10^{-5} mut/ genome /gen with a 0.23 maximum effect for method B.

4.5 Discussion

Genome-wide mutation parameters affecting haploid viability

Maximum-likelihood estimates of mutation parameters affecting haploid viability indicated that parameters were generally similar between the YPD and salt-stressed MA lines. Mutations affecting this trait are generally frequent, of large effect, and show an almost equal probability of being beneficial or detrimental. Our parameter estimates differed substantially from those found by Hall and Joseph (2010). They assessed viability of 147 MA lines and compared it to 37 ancestral replicates. Their ML estimates found that mutation rates affecting this trait were generally low, and with a low proportion of beneficial mutations. The average spore viability of their ancestral replicates was much higher than ours (91% versus 58% in our study). The low viability

Table 4.1: Genome-wide mutation parameters affecting post-meiotic fitness characters

	MA		Bateman-Mukai		Maximum Likelihood			
	Treatment		U_{min} (×10^{-5} mut/gen/gen)	s_{max}	Shape	U (×10^{-5} mut/gen/gen)	P	E(a)
Haploid Viability								
	YPD		11	1.8	∞	1170	0.45	> ∞
					0-∞	(35-36000)	(0-0.55)	(0.0062-∞)
	Salt		23	1.4	60-80	2800	0.45	0.12
					(0.01-80)	(38-22000)	(0-0.5)	(0.008-1.03)
Haploid Growth Rate		method						
	YPD	A	31.6	0.034	∞	4	0	> ∞
		B	17.7	0.06	≤0.01 -∞	4-2900	0-0.45	0.00033-∞
	Salt	A	48.5	0.054	38	51	0.15	0.07
		B	11.4	0.23	≤0.01 -∞	44-9300	0.01-0.45	0.00054-∞

of spores in our ancestral control lines may partly explain the large proportion of positive effect changes in our ML estimation if our ancestral genotype is far from its optimal fitness.

These findings represent some of the most extreme values for mutation parameters of any trait estimated by ML in yeast to date. Several explanations for this are possible. One possibility is that our data deviate from assumptions implicit in the ML method we implemented. This method assumes that there is a normal distribution of environmental effects, the mean and variance of which are estimated from our control line data. In our controls, the mean and variance are approximately equal to each other (μ=2.3 spores/tetrad; σ^2=2.1) suggesting a non-normal distribution. Our measure of haploid viability, the number of living spores produced by a given tetrad dissection, can only produce discrete outcomes of zero through four surviving spores and therefore cannot strictly follow a normal distribution. We chose to proceed because we are unsure of the sensitivity of the method to violations of this assumption. Another methodological issue with our ML estimation of haploid viability mutation parameters is the relatively small numbers of measures we are using in this analysis. The high proportion of positive-effect mutations could possibly be a result of sampling error given the relatively low number of tetrads we dissected per MA line and the relatively few MA lines we have used. Another possibility, given the high variability of viable tetrads among our ancestral replicates is that the ancestral genotype is particularly sensitive to environmental perturbations. These issues strongly suggest that caution be used in interpreting our MA estimates of mutation parameters affecting haploid viability.

Mutation parameters affecting haploid growth rate

We estimated mutation parameters affecting haploid growth rate in our two MA treatment groups by ML and BM methods. The mutation rate estimate in our salt lines is an order of magnitude larger than that found in our YPD lines. Previously we found that mutation rates affecting diploid growth and sporulation were each roughly doubled in the salt treatment relative to the YPD treatment (Chapter 3). Our estimates for haploid growth therefore reflect the largest difference in mutation rate we have found for any trait between our two groups of MA lines. This finding is consistent with the uncovering of recessive mutations that were compensated for by another intact copy in the diploid state.

A further interesting finding from the ML analysis is the estimated proportion of beneficial changes (P_B). We found that in our YPD treatment that a model with zero percent positive changes maximized the likelihood while 15% of mutations in the salt treatment were beneficial. For all fitness traits we have measured here and previously, we found a difference in P_B only for sporulation between our YPD and salt lines. Thirty percent of changes affecting sporulation were "beneficial" (i.e., caused an increase) in YPD lines while forty percent were beneficial in our salt lines. The fifteen percent difference in beneficial changes in haploid growth we observed between the MA treatments is notable in several ways. First, along with sporulation, it is only the second instance where we observed a difference in the proportion of beneficials between our MA treatments. Second, the direction of change of P_B is the same for both traits, where more beneficial changes are observed in our salt-stressed MA lines. Third, unlike the case of sporulation, for haploid growth we estimated that P_B was zero in our YPD lines but non-zero in our salt treatment.

Several possibilities were suggested by Hall and Joseph (2010) to account for the high proportion of beneficial mutations found in their study. The first possibility is that their starting genotype could be far from its fitness optimum, such that many large effect mutations were potentially available. Second, that antagonistic pleiotropy could have resulted in a large proportion of mutations positively affecting growth rate if these same mutations negatively affected other traits. Third, they propose that beneficial mutations are less recessive than deleterious mutations, such that they are more strongly expressed for any given mutation. They conclude in their study that pleiotropic effects from conditionally beneficial mutations on diploid growth rate were unlikely to explain the high proportion of beneficials they observed. Instead, they suggest that a sub-optimal ancestral genotype combined with higher recessivity of harmful mutations best explain their results.

We have summarized our results along with previous studies of yeast MA where at least two fitness components have been measured (Table 4.2). The proportion of positive-effect changes affecting haploid viability in our study are the highest estimates found to date. Our ancestral average of 58% viability (2.3 spores per dissected tetrad) is quite low for the W303 genotype, a genetic background commonly used in laboratory studies. This low value may therefore best represent a low initial fitness scenario, where more beneficials can be observed when there is more 'room for improvement' in the phenotype. This explanation is consistent with Hall and Joseph's (2010) finding of zero proportion of beneficials in haploid viability, where they measured 91% average viability among their ancestral replicates, a relatively high initial fitness. A high proportion of

Table 4.2: Maximum likelihood estimates of genome-wide mutation parameters affecting fitness traits in *Saccharomyces cerevisiae**

Trait	MA Environment	# MA lines	# generations MA	Gamma Distribution Shape	U ($\times 10^{-5}$ mut/gen/gen)	P	E(hs or s)	Reference
Diploid	YPD	78	800	∞	30	0	0.017	Hill and Otto, 2007**
Growth Rate	YPD	151	1012		6.3	0.125	0.061	Hall and Joseph, 2010
	YPD	42	1503	3.0	17	0	0.055	Kozela and Johnston, unpublished
	Salt	42	1573	1.7	37	0	0.028	Kozela and Johnston, unpublished
Sporulation	YPD	78	800	0.01	1300	0	0.0046	Hill and Otto,2007**
Efficiency	YPD	147	1012		→∞	0.2	→0	Hall and Joseph, 2010
	YPD	42	1503	≤0.005	≥7300	0.3	0.00034	Kozela and Johnston, unpublished
	Salt	45	1573	≤0.005	≥14000	0.4	0.00041	Kozela and Johnston, unpublished
Haploid	YPD	147	1012		5.05	0	0.47	Hall and Joseph, 2010
Viability	YPD	17	1503	∞	1170	0.45	→ ∞	this study
	Salt	21	1573	80	1900	0.45	0.14	this study
Haploid	YPD	130	1012		12.6	0	0.79	Hall and Joseph, 2010
Growth Rate	YPD	16	1503	∞	4	0	→ ∞	this study
	Salt	18	1573	38.0	51	0.15	0.07	this study

*ML analysis of Zeyl and DeVisser (2001) was performed assuming P=0 and therefore not included here

**all data from Hill and Otto (2007) is from SMA experiment

beneficial changes were also found when we assessed changes in sporulation efficiency after MA (Chapter 3). Similarly, Hall and Joseph (2010) also estimated 20% of the changes in their lines increased sporulation. In contrast, Hill and Otto (2007) estimated no positive-effect mutations affecting sporulation. The average sporulation found by Hill and Otto for their ancestral strain was 67.5%. Hall and Joseph counted only four-spore tetrads in their sporulation assay and found 27% of cells formed a full tetrad. In our sporulation assay, we counted total tetrads regardless of the number of spores observed and found only 23.3% tetrad formation in our ancestor. Despite only having three studies to directly compare, it appears that there is a negative relationship between initial sporulation efficiency of an ancestral yeast strain and the proportion of beneficial changes estimated by ML. This again is consistent with a high proportion of beneficial changes when initial fitness is far from an optimum.

Growth rate assays have only shown two instances where a positive P_B value has been found. Hall and Joseph's diploid growth rate analysis showed 12.5% to be beneficial and we found 15% beneficial changes in haploid growth in our salt MA treatment. Growth rate assays are somewhat more difficult to interpret than viability and to a lesser extent sporulation due to uncertainty in what constitutes an optimal phenotype. In other words, we have little basis for knowing how far growth rate measures taken in our microplate environment may be from an optimal phenotype. Our finding that fifteen percent of changes are beneficial in haploid growth is intriguing. This suggests that an organism's fitness to its MA environment also needs to be considered as an independent factor influencing P_B. In our salt lines we found that the ML estimate of positive-effect changes was zero for diploid growth rate, contrasting with the 15% found for haploid

growth rate. One possibility is that some proportion of salt-stress-induced beneficial mutations is recessive. Another possibility is that the haploid growth rate has a unique set of genes from diploid growth and that this set of genes are poorly adapted to the assay environment. To explain our results with this possibility, we would need some explanation for salt treatment's qualitatively different P_B estimate compared to the YPD MA-treatment estimate in this study and Hall and Joseph's (2010) estimate. This could arise possibly if the mutation rate in non-salt stress MA was below the threshold of detectability given the opportunity for mutations to accumulate. This could also arise if the spectrum of mutations induced by salt-stress somehow affected genes specific to haploid growth. While we cannot rule out a unique set of genes affecting haploid growth, we think this possibility is unlikely to fully explain our results and instead suggest the possibility of recessivity of some beneficials.

Biases affecting mutation parameters

Selection bias will affect our mutation parameter estimates in similar ways as those discussed (Chapter 3) to the extent that selection on diploid growth during MA affects genes controlling our haploid measures. Growth from a dissected tetrad is a major component of haploid viability as we have defined it here. Haploid growth rates as measured by changes in optical density are likely to also be affected by unintended selection that occurred during the MA phase of this study. In addition to the general ways selection can bias our mutation parameter estimates as discussed (Chapter 3), an additional source of bias is generated in the way we have treated our haploid growth rate measures. As mutations accumulated in diploids, selection can act on them only as the

combined action of selection (*s*) on the new mutation and the degree of dominance (*h*) of the new mutation. Once we have generated haploid lines, we remove any effects of dominance altogether. For our mutation parameter analysis however, we pool our haploid measures into average values of haploid growth rate for each diploid MA line. This averaging procedure artificially inflates the dominance coefficient. For example, in the simplest case of one mutation in an MA line, the numerical average of the haploid line with the mutation and the line without the mutation is exactly half the sum of the two values, equivalent to a dominance coefficient of one half. In a true diploid organism, the dominance coefficient will be somewhere between zero and one-half and so we have effectively over-estimated it. By effectively increasing the value of dominance in our lines, our ML analysis of mutation effect sizes (the product of *hs*) will be upwardly biased.

Salt-stress MA drives divergence between haploid siblings

Our variance-component analysis (Figure 4.2) provides insight into the generation of variation at a previously uninvestigated scale. As expected, replicate haploids that were derived from our diploid ancestor show almost complete residual variation attributable to environmental effects (86%). The lack of any variation but environmental suggests that there was little or no heterozygosity present in our founding genotype that affects haploid growth. The divergence between the diploid parents in haploid growth rate reflects the hemizygous effects of mutations in our MA experiment. Divergence is approximately four times greater in our salt lines. This finding is interesting in light of our previous results with diploid growth rate, where we found that among-line variance

was greater in our YPD lines (Chapter 3). This difference could be explained if many of the mutations we observe in our haploid growth assay are recessive and do not detectably affect diploid growth rates.

We found nearly no variation in haploid growth rate that could be attributed to differences among tetrads of the same parent. Part of this component could potentially have come from individual differences between diploid cells during any stage of the experimental procedure propagated by non-Mendelian mechanisms such as cytoplasmic inheritance or persistent epigenetic states. This suggests that such mechanisms are likely of minimal importance in explaining the pattern of variance observed.

Two features of this analysis are striking when we compare our two MA treatments. The first is that the total variation in haploid growth is much greater in our salt-MA lines. This seems to be a result of increased contributions from every component in our model except differences between tetrads, including higher residual variance. Residual variance is roughly equal between our ancestral replicates and our YPD MA lines, suggesting a similar basis. More residual variance in our salt MA group could be a result of differing genotype-by-environment interactions but it is difficult to make any conclusions without further information.

The second feature of note is the large component of within-tetrad differences between haploids. This component is more than ten times greater in terms of actual variance, and more than twice as great (53% in salt versus 23% in YPD) in terms of proportional variance for each MA treatment. We believe this to be the first attempt at partitioning newly generated, combined segregational and recombinational variance from a diploid organism directly into its haploid state. This significant change in proportional

variance in our salt-stress treatment makes it tempting to speculate on the biological implications. A link between recombination and DNA repair has been known for some time as some DNA repair mechanisms utilize the recombinational machinery to correct damage. A great deal of early research in the long-term propagation of laboratory microbe populations indicated that episodes of sex had a 'rejuvenating' effect on cultures (Bell, 1988). Our results are consistent with a hypothesis that meiosis itself can act to concentrate genes into more-fit and less-fit genotypes, making natural selection on haploids more effective. This is a variant of the idea that is usually formulated as a mechanism to explain the maintenance of sexual reproduction, where genome reduction combined with syngamy produce more variant genotypes upon which selection can act more effectively. For *S. cerevisiae*, where intra-tetrad matings are common (G. Bell, pers. comm.), the advantage of meiosis itself in purging mutations may outweigh the costs associated with inbreeding. This may be particularly true under stressful conditions, as our results would seem to suggest. More work to address this is clearly required.

Our current analysis provides no information as to the kinds of mutations that enter our populations in either treatment group. Whole-genome sequencing of haploid yeast MA lines was performed by Lynch et al. (2008). They found that most mutations were single-base-pair changes and small indels. While these are important first attempts at characterizing mutation accumulation in yeast, we are unsure how or whether they could be extrapolated to the predominant diploid state of *S. cerevisiae.* The recent finding that stress in yeast can induce an increase in aneuploidies and chromosomal rearrangements (Chen et al., 2012) is also pertinent, in that mutation spectra may be

fundamentally different under adverse, low-fitness conditions. By combining modern genome-wide molecular characterizations along with the organismal fitness-based approach used in this study, we may begin to make significant headway in our understanding of some fundamental biological phenomena that have heretofore remained elusive.

CHAPTER 5 MUTATION ACCUMULATION UNDER SALT STRESS IN YEAST. III. MUTATIONAL CONSEQUENCES IN A NOVEL WEAK-ACID STRESS ENVIRONMENT

5.1 ABSTRACT

Stress is a near ubiquitous feature of the struggle for existence. For evolutionary studies it is often defined as suboptimal fitness. While selection acts to move populations towards optimality in a given environment, the nature of environmental stochasticity can critically affect the dynamics of adaptive evolution. We have used a series of mutation accumulation (MA) lines of the yeast *Saccharomyces cerevisiae* to investigate the fitness consequences of new mutations when assayed in a reduced-fitness environment. We grew replicate cultures of MA lines under acetic acid stress at pH 4.5 and measured maximal growth rates using changes in optical density of cultures and compared our two MA treatments to each other and to their mutation-free progenitor. We found mean changes in growth rate between our salt-stress MA treatment and both the benign (YPD) MA treatment and the ancestor, which did not differ from each other. We estimated mutation parameters using maximum likelihood and found differences between our MA treatments in comparison to estimates from a previous study where fitness was assessed under standard culture conditions. Under mild acid stress, our YPD MA lines exhibited a decreased mutation rate, increased average effect, and more platykurtic distribution of effects than the same lines tested under benign conditions. In contrast, our salt MA lines exhibited a higher rate of mutation, smaller average effect, and more leptokurtic distribution of effects when tested under acid stress. We also estimated that nineteen percent of mutations were beneficial in our salt MA lines while less than one in a

thousand were beneficial in our YPD lines in the new environment. Cross-environmental genetic correlations indicated that the mutations affecting growth in acid were significantly different in our salt MA lines than in our YPD lines. These results suggest that mutational processes influenced by environmental factors can affect the genetic basis of fitness in a context-dependent fashion and may be particularly relevant when organisms are challenged by suboptimal fitness environments..

5.2 Introduction

The rates and fitness effects of new mutations are fundamental parameters in the study of evolution. Studies that characterize these properties tend to be difficult, expensive and laborious, such that we have relatively few data in only a handful of species from which to draw broad conclusions. The distribution of fitness effects of new mutations has recently attracted more attention as the number of relevant studies has reached the need for a broad overview (see Eyre-Walker and Keightley, 2007 for review). One general finding to emerge is that the fitness consequences of a new mutation depend on the particular environment in which it is tested. The typical experimental scheme is to produce a series of mutation accumulation (MA) lines and then assay fitness in different environments. For example, Shabalina et al. (1997) estimated that mutational pressure on competitive ability in fruit fly MA lines was approximately two percent per generation when traits were assayed under harsh but nearly zero under benign conditions. Similarly, Rutter et al. (2010) found that average fruit production in *Arabidopsis thaliana* was reduced when MA lines were tested in the field compared to the greenhouse.

The budding yeast *Saccharomyces cerevisiae* is one of the best-studied eukaryotic systems at several of levels of biological organization. A number of MA studies have been conducted in *S. cerevisiae* attempting to measure the rate and distribution of effects of new mutations affecting fitness. We are interested in characterizing the degree to which the environmental context affects the fitness consequences of new mutations. One hypothesis, based on Fisher's geometrical theory of adaptation, predicts that the proportion of beneficial mutations should be high when an initial genotype is relatively unfit (see Hall and Joseph, 2010; Rutter et al., 2010), as is expected to be the case in a novel environment.

For microorganisms, assaying fitness of MA lines in natural environments may be particularly challenging. An alternative and more tractable approach is to assay fitness in a harsh laboratory environment to dissect fitness differences to particular environmental challenges. Weak-acid stress in microorganisms has been relatively well studied due to its use in food preservation. Weak acids at inhibitory concentrations for *Saccharomyces* cause an accumulation of protons within the cytosol leading to disruption of a number of cellular processes (Mira et al., 2010).

We have previously generated a series of diploid MA lines of *S. cerevisiae* (Chapter 3) over a period of approximately 1500 cell generations under two environmental conditions. One set of lines accumulated mutations under standard culture conditions while the other accumulated mutations under a moderate salt stress. Here we test for fitness as measured by maximal diploid growth rate in our two sets of MA lines under conditions of weak-acid stress. We use these data to estimate mutation parameters for genes affecting fitness under this novel and stressful environment.

5.3 METHODS

Mutation Accumulation and Yeast Culture

Generation of mutation accumulation (MA) lines was previously described (Chapter 3). Briefly, we obtained a single isolate of a W303-like diploid *Saccharomyces cerevisiae* strain. From this isolate, we generated a large ancestral population that was in turn used to generate 100 initial MA lines. Fifty lines were propagated on standard yeast culture medium YPD and another 50 lines on YPD made with high salt (0.9-1.0M; this molarity reduced growth rate by one-half). The YPD lines were transferred daily and the high salt lines every two days until each had been propagated for approximately 1500 cell generations. The ancestor and all surviving MA lines were frozen in 20% glycerol at -80°C.

Fitness assay

We grew diploid yeasts in 96-well microplates to assess fitness in the novel stress environment. Diploid yeasts were arranged in wells according to the scheme previously described for our diploid lines. We randomly assigned 25 lines from each treatment to a random location on one plate and the remaining 25 to a second plate so that a pair of plates constituted the entire set of extant diploid lines. Extinct lines and petites were assigned places but not inoculated and are not considered further. Cultures were excluded from the exterior of the plate due to previously detected growth differences on the outer edge. Ten wells per plate were assigned to the ancestral genotype.

MA lines were first grown for 3 days at room temperature in the dark on YPD agar in 96-well plates according to the specified arrangement. Cells were then transferred with a flame-sterilized 96-pin apparatus to an aluminum dilution plate containing 150µl of assay medium. Cells were transferred from this dilution plate by four dips of the pin apparatus into 150µl of assay medium in a 96-well cell culture plate (BD Falcon). Our assay medium was made by acidifying standard YPD broth with glacial acetic acid to pH 4.5. After acidification, the medium was filter sterilized and used immediately. After inoculation of the assay plate, the edges of the plate were sealed with laboratory film and the plate then transferred to the spectrophotometer (Tecan Genios) for incubation and optical density (OD) measurement.

Plates were incubated at 28±0.5°C with intense shaking between measurements. Measurements were taken every 20 minutes for 48 hours. After incubation, the average OD value of blank wells containing only media was subtracted from the OD of experimental wells containing yeast. We excluded all resulting values that were smaller than 0.02 and $\log_2$-transformed the resultant values. We calculated slopes of these over the 48-hour time course of the experiment. Maximum slopes were calculated over a series of window sizes but we chose to analyze these data at the 7-hour window size to facilitate comparison to the previous study of diploids in standard YPD (Chapter 3). Unlike previous analyses, we found only suggestive but non-statistically significant effects ($0.05< P <0.1$) arising from positions within plates. We therefore used raw values of growth rates in 7-hr windows in further analyses. In total we obtained non-zero growth rate values for n=60 measures for our ancestral genotype, n=125 for YPD MA, and n=123 for salt MA lines.

Statistical Analyses

We used randomization tests implemented in Mathematica (Wolfram Research, Inc.) as previously described (Chapter 3) to determine differences in growth rate distribution parameters between MA treatment groups. Variance component estimates were performed as before using the MIXED procedure in SAS software (SAS Institute Inc.). Comparisons were made to data generated in a previous study where the same MA lines were grown in standard YPD (Chapter 3). For these comparisons, we used raw growth rate measures over the 7-hour window size used in both YPD and acid test environments. The smaller sample size in this study (n=3 for each extant grande MA line) allowed for a larger influence of zero-value measures than in previous studies. We observed a larger number of these in our salt-MA lines (n=18) than in the YPD lines (n=1). We are unable to ascribe these to mutational or non-mutational effects; therefore all statistical analyses were performed with zero-growth rate measures excluded.

We used the MIXED procedure in SAS software to calculate the cross-environmental genetic correlation between growth in standard YPD and in low pH for each of the two MA treatments. Point estimates of the correlation were calculated using the 'type =UNR' covariance option. Lower and upper bounds on our estimates were generated by constraining the correlation using the 'PARMS' statement and evaluating the likelihood against the unconstrained model using likelihood ratio tests with one degree of freedom.

Mutation parameter estimation

We analysed our data using the program mlgenomeu (v.2.08; Keightley, 1994; Keightley and Ohnishsi, 1998) which utilizes maximum likelihood (ML) to estimate mutation parameters including mutation rates, distribution of effects and the proportion of positive-effect mutations. We constrained the program to particular values of β (the gamma distribution shape parameter) and P_B (the proportion of positive-effect mutations) and allowed the program to estimate U (the genome-wide mutation rate) and α (the gamma distribution scale parameter) that maximized the likelihood given our data. We then repeated this procedure over a wide range of β and P_B to find the overall maximum likelihood estimates of all four parameters. Confidence intervals were obtained by examining profile likelihoods and taking the parameter values at -2 log likelihoods above and below the maximum. We also estimated mutation rates and average effects using the Bateman-Mukai method (Lynch and Walsh, 1998), which assumes that all mutations are harmful and are of equal effects.

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5.4 RESULTS

Acid stress more severe in salt MA lines

Randomization tests performed on non-zero growth rate measures (Figure 5.1) showed no significant difference between our mean ancestral measures and our YPD MA lines (P=0.50). Differences in variances between these two groups were also not significant (P=0.17). Only higher-moment differences were different between ancestral genotype and YPD MA replicates (skew, P<0.0001; kurtosis, P<0.0001). Mean salt growth rate measures were significantly lower than both our ancestral (P=0.0004) and

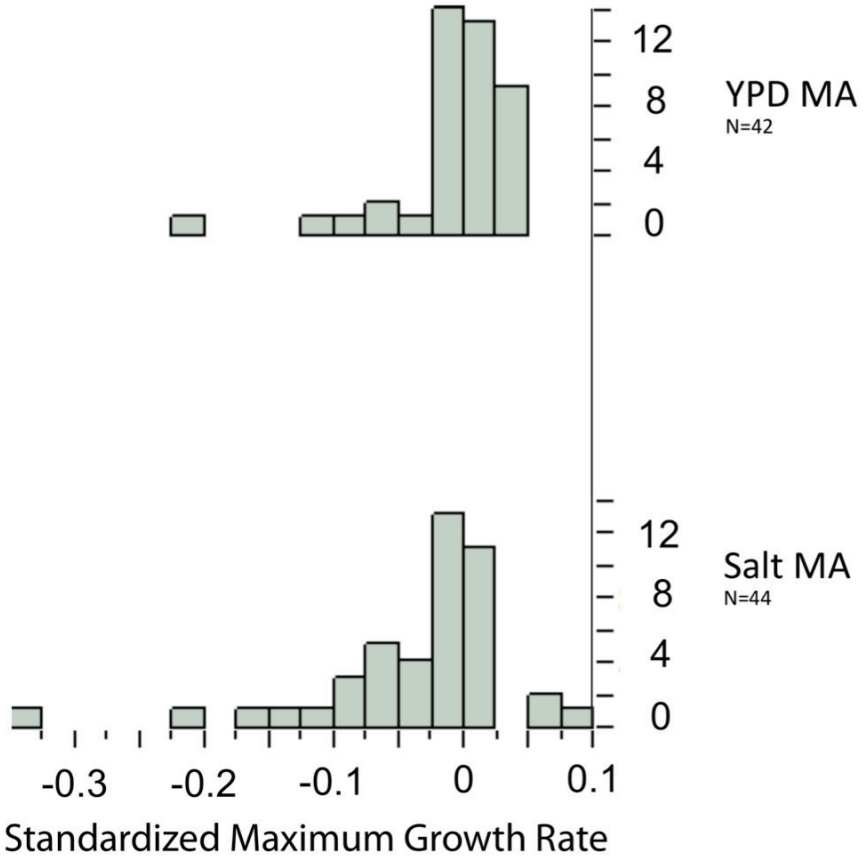

Figure 5.1 Maximal growth rates of yeast MA lines in acidic media. Zero-values are excluded. Values are corrected against the mean ancestral growth rate.

YPD MA (P<0.0001) measures. The salt MA treatment also showed significantly greater variance than the ancestral replicates (P=0.0082) but only marginally higher variance than the YPD treatment (P=0.056). Skew and kurtosis were also significantly different in the salt treatment than in both the ancestral and YPD MA lines (P<0.0001 in each case).

Among-line variance generated by MA revealed in growth under acid stress

We estimated the components of variance in growth rate measures in both MA treatment groups by restricted maximum-likelihood (REML) using a mixed model approach. Likelihood-ratio tests indicated significant among-line variance in both MA treatments (Figure 5.2; P<0.0001 for both YPD MA and salt MA). We also asked whether the proportion of among-line variance differed between the two MA groups. In this case we compared a model where standardized among-line variance components were estimated separately by MA treatment to a null model where they were estimated as a single among-line variance. A likelihood-ratio test between these two models indicated that the proportion of among-line variance did not differ between MA treatments (P=0.48).

Cross-environment genetic correlation higher in YPD MA lines

We estimated the genetic correlation for growth in standard YPD as measured in a previous study (Chapter 3) versus growth in low pH (this study) for both MA treatments (Table 5.1; Figure S10). YPD-MA lines had a correlation of 83% with a lower bound of 61% and an upper bound of 96%. Salt-MA lines had a correlation of 5%, which was not significantly

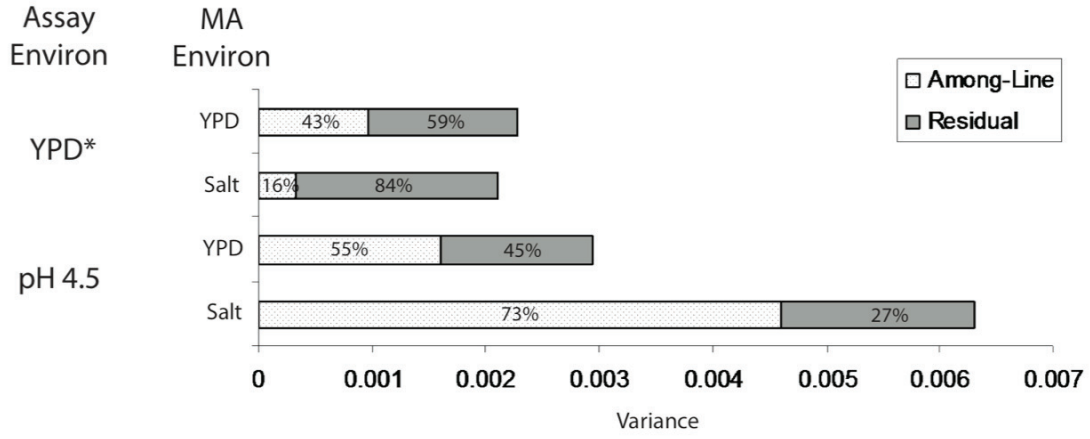

Figure 5.2 Variance components for diploid yeast MA lines grown in acidic media
*data from Chapter 3

Table 5.1: Cross environment genetic correlation for growth rate in YPD versus acidified YPD

MA Treatment	Correlation	Lower Bound	Upper Bound
YPD	0.83	0.61	0.96
Salt	0.05	-0.36	0.45

different from zero (likelihood-ratio test, P=0.8) with a lower bound at -36% and an upper bound 45%.

Genome-wide mutation parameters for genes affecting fitness under acid stress

Mutation parameters were estimated by the Bateman-Mukai (BM) method of moments and by ML (Table 5.2; Figure S11). We calculated the BM lower bound mutation rate as 1.04×10^{-5} mutations/genome/generation and upper bound mutational effect as -0.318 for our YPD lines. The BM estimates for our salt-treatment group are 18.9×10^{-5} mut/genome/gen and a maximum effect of -0.124. We estimated a ML mutation rate for genes affecting growth under acid stress in our YPD MA lines as 10.0×10^{-5} mut/genome/gen with an average mutational effect of 0.091 and a proportion of positive-effect mutations P_B of 0.1%. In our salt MA lines, we found ML estimates of 382×10^{-5} mutations/genome/generation with an average size of 0.0086, 19% of which were of positive effect.

5.5 Discussion

MA in high salt reduces growth in acidic medium

We were only able to detect overall changes in mean and variance of growth rate measures between our salt MA lines and either of the YPD MA or ancestral lines. Two distinct possibilities can explain this result. First, it may be that the relatively low level of replication in this experiment reduced the power of the test and we were only able to detect a significant difference in the larger growth rate difference between ancestor and

Table 5.2: Mutation parameters for diploid growth rate in acidified YPD

Test Environment	MA Treatment	Bateman-Mukai		Maximum Likelihood			
		U_{min} (*10^{-5} mut/gen/gen)	s_{max}	Gamma Distribution Shape	U (*10^{-5} mut/gen/gen)	P	E(hs)
YPD pH 4.5	YPD	1.04	-0.32	35 (0.01-40)	10 (10-690)	≤0.001 (0-0.25)	0.091 (0.0015-0.093)
	Salt	18.9	-0.12	0.1 (0.01-60)	382 (35-4100)	0.19 (0.05-0.35)	0.0086 (0.00088-0.077)
YPD*	YPD	8.5	0.087	3.0 *(0-∞)*	17 *(12-∞)*	0 *(0-0.26)*	0.055 *(0-∞)*
	Salt	32	0.026	1.7 *(0-16.2)*	37 *(26-∞)*	0 *(0-0.25)*	0.028 *(0-∞)*

* Data from Chapter 3

salt MA. If this is the case, then MA affects growth in a mildly acidic new environment, with MA under salt stress causing more severe fitness declines than YPD MA. The other possibility is that there is no actual effect of YPD MA on growth in an acidic medium relative to the ancestor. This would be surprising for two reasons. First, we found that YPD significantly affected growth with YPD as the test environment and for which we had ten measurements per line (Chapter 3). Second, previous studies in a variety of organisms have generally found that MA lines tested in either stressful (Shabalina, et al., 1997; Szafraniec et al., 2001) or natural (Rutter et al., 2010) environments tended to have reduced fitness compared to measures taken under less stressful conditions. It remains possible, however, that in the mild acid stress used here, the YPD MA lines did not differ substantially from the ancestral strain. Additional replicate measures may be required to show a difference in means and variances between these two treatments if they do in fact exist.

Another line of evidence is highly suggestive that mutational effects were present among all our MA treatments under acidic growth. Our estimates and significance tests of differences in the higher moments of the distributions of acid growth rates indicate that there were differences between MA treatments. Skew and kurtosis differed significantly between all of the MA treatments. Despite the clear results of our significance tests, it is somewhat challenging to resolve how changes in skew and kurtosis of fitness measure distributions reflect mutational changes in our MA populations. There are general trends in cases where we find significant differences in both skew and kurtosis for fitness measures we have measured here and previously (Chapters 3, 4; Table 5.3). For growth rate measures we have studied to date, we find that ancestral skew values are small and

Table 5.3: Higher order moments of fitness measure distributions

Moment	MA Treatment	Fitness Measure				
		Diploid Growth				
		YPD	YPD pH4.5	Haploid Growth	Sporulation	Haploid Viability
Skew	Ancestor	-0.9	-0.2	-1.3	-0.1	-0.4
	YPD	-1.4	-1.4	-2.4	-0.9	0
	Salt	-1.2	-1.9	-3.2	-0.2	0.1

		Diploid Growth				
		YPD	YPD pH4.5	Haploid Growth	Sporulation	Haploid Viability
Kurtosis	Ancestor	5.2	2.8	9.8	2.5	1.8
	YPD	7.2	6.4	12.8	4.5	1.6
	Salt	8.2	9.1	17.4	3.2	2.1

negative, YPD MA skew values are more negative, and that salt MA skew values are usually the lowest. This is consistent with the expectation that most new mutations are harmful and would shift the distribution to the side of lowered fitness. Kurtosis values also show a consistent increasing trend from ancestor to YPD MA to salt MA. Kurtosis is generally thought to represent the 'peakedness' of a distribution, but it is also affected by the shapes of the tails. The biological basis of this trend is less certain than in the case of skew. The trends for non-growth-rate characters are less obvious. For sporulation, we observe that the YPD MA treatment has the most extreme value of both skew and kurtosis, followed by the salt treatment, followed by the ancestor. Again it is not clear how these should be interpreted in terms of mutational effects on sporulation. The measures we have obtained for skew and kurtosis on haploid viability are based on relatively few measures and should be interpreted with caution.

The genetic basis of line divergence depends on both MA and fitness assay environments

Our variance component analysis indicates that lines in both MA treatments have diverged significantly. These results are somewhat similar to our results for diploid growth in YPD where we also found evidence for significant line divergence. In the case of growth in YPD at normal pH, we found that the proportional among-line variance was greater for our YPD MA lines (Chapter 3). We found no significant difference in the proportional among-line variance between MA treatments for growth in acidic YPD. This discrepancy led us to investigate the basis of line divergence in each of the two assay environments between the two MA treatments. By calculating the cross-

environmental correlation, we ask to what extent are the same MA lines responsible for the observed growth rates in each environment. We interpret our relatively high value of 83% in our YPD MA treatment to indicate that genes that affect growth in normal YPD also affect genes in acidic YPD. A very different situation seems to exist in our salt MA lines. In this case, the very low correlation of 5% suggests that there is only a negligible common genetic basis for the line divergence seen in each assay environment. One interpretation is that, for salt MA lines, there are two classes of genes that have been differently affected by the MA treatment. One of these affects growth at normal pH and the other that affects growth at low pH.

Salt MA stimulates genetic changes favorable in an acidic environment

Our estimates of mutation parameters for genes affecting growth in acidic YPD are interesting in several respects. Our initial prediction, based on Fisher's geometrical theory of adaptation (Fisher, 1930), was that a larger proportion of beneficial changes would be observed in our MA lines when tested in a suboptimal environment. This is only weakly supported by our findings here. We do see a small, non-zero value for beneficial changes in our YPD MA lines; however given the broad confidence ranges in our ML estimates we do not believe that they are meaningfully different from zero.

Beneficial changes to genes affecting growth in mildly acidic medium were estimated to occur 19% of the time in our salt MA lines. This was also accompanied by a nearly forty-fold increase in the rate of mutation with an approximately ten-fold decrease in the average effect size of each change. This is the second character where we observe

a large fraction of beneficial changes in our salt MA lines but not in our YPD lines when tested in the same environment. For haploid growth rate, we estimated zero beneficials present in our YPD lines but 15% in our salt MA lines (Chapter 4). It is still difficult to conclude that Fisher's model cannot explain our results. At present we have uncertainty in terms of how far from an optimal genotype our ancestral strain may be. *Saccharomyces* can be relatively tolerant to acidic conditions and can produce acetic acid under fermentative conditions (Mira et al., 2011). We have little information with which to evaluate possible explanations for this high proportion of positive effect changes in our salt MA lines.

As with the previous results of this work (Chapters 3, 4), selection during colony growth may play a role in biasing our estimates of mutation parameters in certain directions. The extent to which this takes place will depend on the shared genetic basis of colony growth and growth in our high acid test environment. Our results addressing the cross-environmental genetic correlation suggest that the genes affecting growth in each environment differ based on the MA environment. Determining how these influence selection bias will require further study and will be addressed in the publication versions of this work.

The ML estimates of mutation parameters from this study compare interestingly to those where our lines were tested under more benign conditions. We estimated that beneficials were only present at a high proportion in the circumstance of our salt MA lines tested in our acidic test environment. This finding is consistent with our results on the difference in cross-environmental genetic correlation between our MA treatments. In each case we observe a unique set of mutational effects for one treatment in a particular

condition. It is possible that different classes of mutations accumulated in our different MA environments. It is hypothetically possible that fitness effects depend on mutation spectra which are subject to environmental variation. For example, if segmental duplications are more prevalent under stressful MA, then we may see increased copy number variation via gene amplification. In our case, if genes involved with proton extrusion were subject to amplification, it might provide the necessary scenario to explain our results. While this is highly speculative, it is an empirical question and wholly testable. Recent advances in sequencing prowess promise characterization of mutation spectra under differing conditions and should provide some clarification.

CHAPTER 6 CONCLUSIONS

This thesis investigates aspects of mutation biology with respect to the complex relationship between genetics and the environment. It begins with a review (Chapter 2) that considers hierarchical biological structure and the influences of this structure on mutability. Chapter 3 provides the basis for the remainder of the thesis by describing the generation of a series of mutation-accumulation lines which are utilized in subsequent chapters. Chapter 3 also describes the estimation of mutation parameters for genes affecting the two diploid fitness components growth rate and sporulation. We find that mutation accumulation under salt stress causes an approximately two-fold increase in mutation rates affecting both fitness components. We also estimate a high proportion of beneficial mutations affecting sporulation but not diploid growth rate. Chapter 3 ends with a discussion that clarifies the basis of phenotypic measures used to infer mutation properties. Many studies have identified epigenetic mechanisms as factors influencing phenotypes. As the phenotypes we have studied here are polygenic quantitative traits, it is prudent to consider that epigenetic regulation may play a role in explaining any of our results. I introduce the term 'omnigenetic' to account for our inability to distinguish genetic and epigenetic sources as the basis for the phenotyes we have measured. Although the term is not used explicitly in the subsequent chapters, it should be regarded as the most accurate basis for all phenotypes we have measured here. Chapter 4 describes how a salt-stress MA treatment affects the two post-meiotic fitness components haploid viability and growth rate. Mutations affecting these traits show larger differences between the MA treatments than is observed in diploid fitness traits. In particular, the

mutation rate affecting haploid growth rate is estimated to be more than ten times higher in our salt MA treatment. Intriguingly, we find a large amount of genetic variation ascribed to differences between haploid spores from the same meiotic event, especially in our salt MA treatment. The basis of this is unknown, but may be involved in the clearance of harmful mutations. As far as I am aware, this is the first measurement of this kind of variation in an MA study. Chapter 5 is the final data chapter and asks about the fitness consequences of salt stress MA when yeast are challenged in new stressful environments. Our mixed-model analysis of the cross-environment genetic correlation suggests that growth rate in regular YPD has a similar genetic basis as growth in acidic media for our YPD MA lines. Lines that accumulated mutations under salt-stress on the other hand show no correlation. This suggests that there are dependencies between the MA environment and the fitness assay environment, the causal bases of which can only be speculated.

Fitness traits in general show decreases in average value and increased variability, hallmarks of the average deleterious effect of genetic change. The accumulaton of mutations under stressful and non-stressful conditions shows significant effects of salt-stress on mutability in *S. cerevisiae*. Mutation rates are typically elevated under salt stress but are sometimes accompanied by an increase in beneficial change. Finally, mutations generated in non-stress conditions exhibit a high correlation in fitness effect in two environments, while mutations generated under salt stress show no such correlation. Thus, environmental conditions affect the rate and spectrum of newly arising mutations as well as their fitness consequences in other environments.

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APPENDIX A Supplementary Material Chapter 3

Estimation of the number of generations during mutation accumulation

We used colony area as a proxy for population number by deriving the empirical relationship between the two for the ancestral genotype. This was used as an initial guide for tracking the approximate generation number during the course of MA. We could not rule out that the MA treatments themselves may have affected the relationship between the two with the possible result that one treatment would undergo many more divisions than the other and thus skewing our estimates of mutation parameters. We therefore wished to directly address the effect of the mutation accumulation conditions we used in our experiment on the derived relationship between colony area and population size.

We generated 6 additional lines from the frozen ancestral population and cultured three on YPD and three on 1.0 M NaCl YPD in an identical fashion to our main MA experiment. At different time points (Figure S1), we measured colonies at the relevant sizes picked in our main MA experiment. One group of colonies was chosen in the early phase of the experiment while a second group of colonies was chosen in a later phase, in an attempt to detect any effect with a significant latency.

After a colony was haphazardly chosen and restreaked on a fresh plate to continue the MA procedure, additional colonies from the same plate were chosen for analysis. These colonies were measured for colony area from images taken using a CCD camera and stereo dissecting microscope (Olympus). The photographed colonies were subsequently excised from the agar with minimal disruption of the colony and placed in 100µl of fixative (3.7% formaldehyde in PBS). Colonies were stored at 4°C until cell counts were taken. Just prior to counting, samples were briefly sonicated to disrupt cell clumps. We used the Countess® automated cell counter (Invitrogen) to count cells in these excised colonies according to the manufacturer's instructions. Accurate readings from this device depend on the concentration of cells in a given sample. Large colonies that contained cell numbers beyond the reliable limit of the machine were diluted by an appropriate factor (10X or 100X). Small colonies that contained numbers of cells below the reliability limit of the machine were concentrated by centrifugation and resuspension

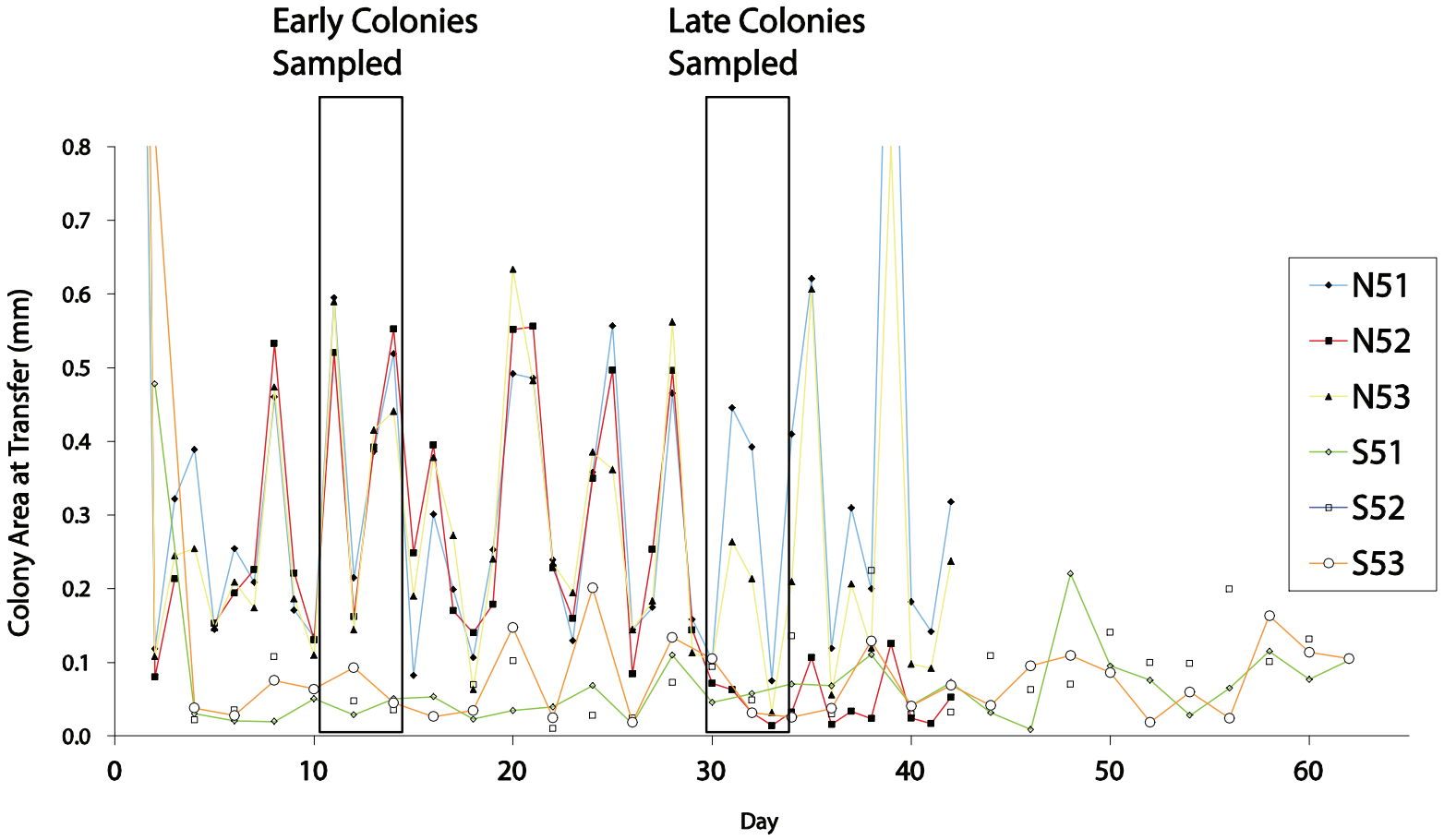

Figure S1: Additional lines propagated by MA procedure. YPD lines (N) are solid symbols. Salt lines (S) are open symbols.

in one-fourth the volume, prior to counting. We plotted cell number against colony area for these colonies (Figure S2) and fitted curves of several different forms to establish the relationship between the two measures. We used randomization tests to test for differences between YPD and high salt treatments. We also asked whether colonies picked early or late in the experiment differed for both treatments. No significant differences were detected between MA treatments or between early or late colonies. We then pooled our data together in order to determine a single relationship between colony area and cell number. We fit several different curves to our data but decided to use a quartic function fit through zero. This curve was chosen because it produced no discernible pattern upon visual inspection of residual values. The relationship was determined to be:

$$\text{Cell Number} = 2.3*10^6\text{Area} - 4.6*10^5\text{Area}^2 + 1.1*10^7\text{Area}^3 - 3.3*10^6\text{Area}^4$$

This relationship was then used to derive colony population sizes for colonies we transferred during the main MA experiment. Colony population values Figure (S3, S4) during MA were then used to estimate population parameters including generation number and effective population size during the main experiment.

Removal of Environmental Effects during Fitness Assays

All analyses to remove environmental effects were performed using JMP 9.0.2 (SAS Institute Inc.) statistical software. We used the ancestral genotype that was replicated 10 times on each assay plate as the standard with which to investigate environmental effects during our assay. We investigated several factors, including position within a plate, individual plate, year (growth rate only) and culture density (sporulation only). To investigate plate position, we created a coordinate system that allowed us to assign a numerical position to every well on a plate. For culture density, we used OD measurements taken after the presporulation cultures had been transferred to

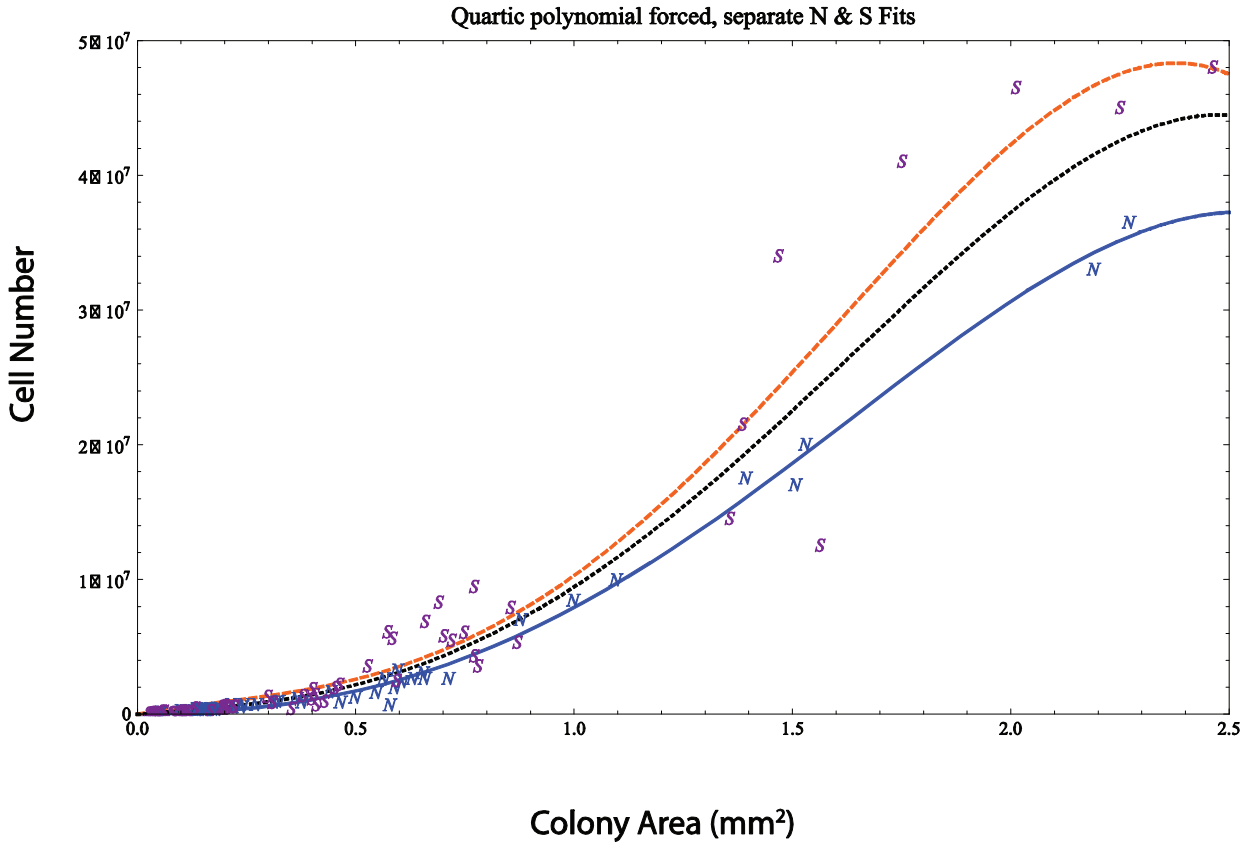

Figure S2: Quartic fit of the number of cells in a colony in a given area. Colonies were sampled from early and late time points in Figure S1. Blue (N) : YPD colonies; Orange (S): salt colonies; Black: all colonies.

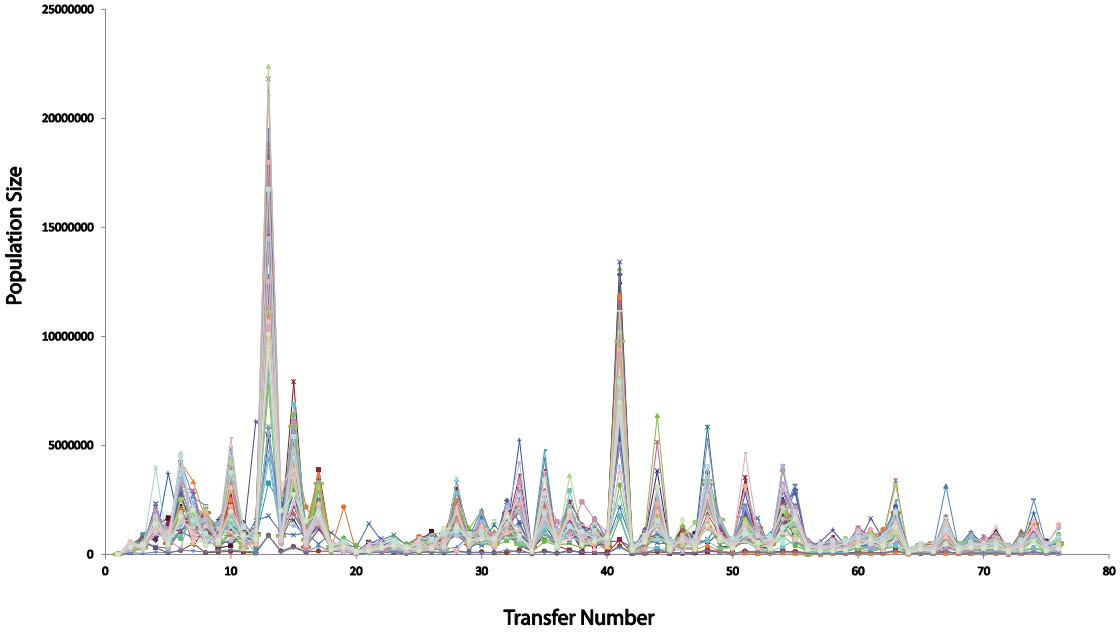

Figure S3: Population size at transfer for YPD MA treatment.

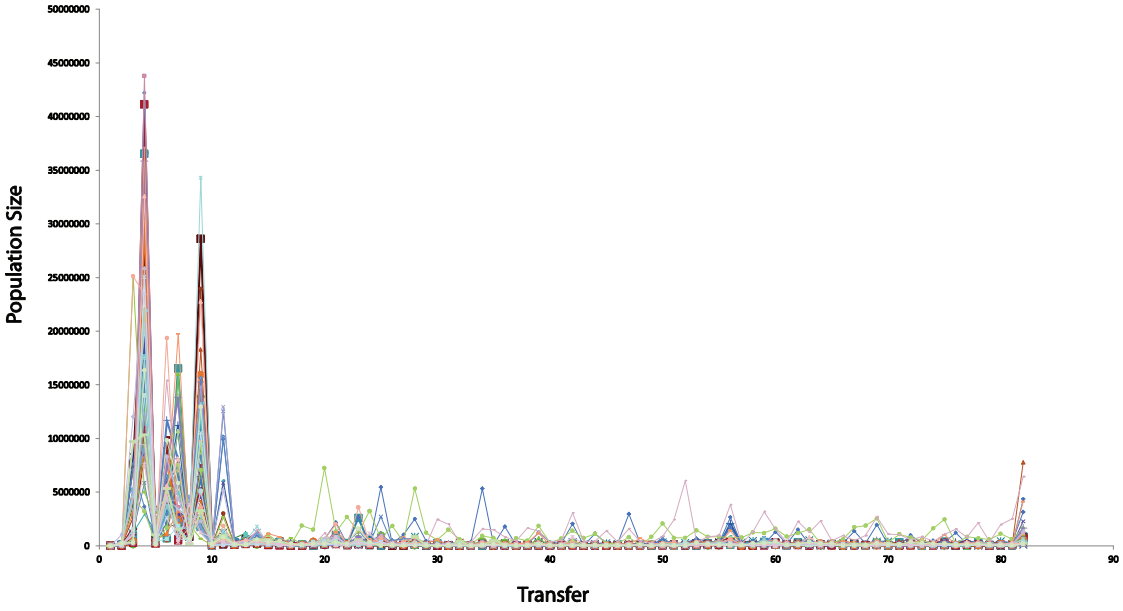

Figure S4: Population size at transfer for salt MA treatment.

sporulation medium. We investigated a series of models using ANOVA to determine which factors significantly affected the fitness of the ancestor.

We found that the following model best described the growth of the ancestor:

$$\text{Growth Rate} = x + y + x^2 + \text{plate} + \text{error}$$

We found the following model best described sporulation in the ancestor:

$$\text{Sporulation} = \text{plate} + \text{optical density} + \text{error}$$

We then asked JMP to calculate a predicted value for each well in each experiment based on these models. We then subtracted these predicted values from the actual values we obtained for our MA line replicates to arrive at values corrected for environmental effects on our fitness measures.

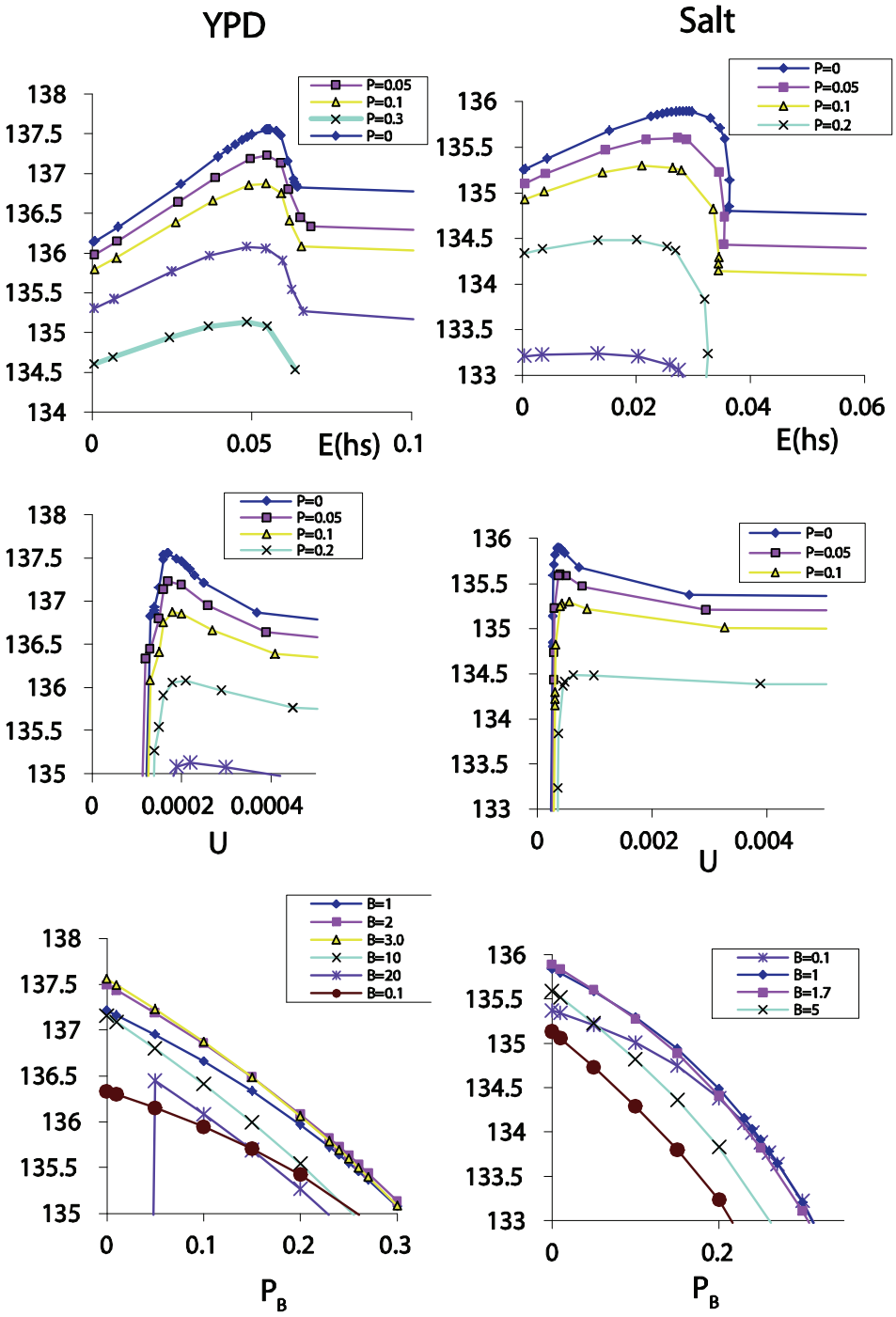

Figure S5: Profile likelihoods for mutation parameters affecting diploid growth rate

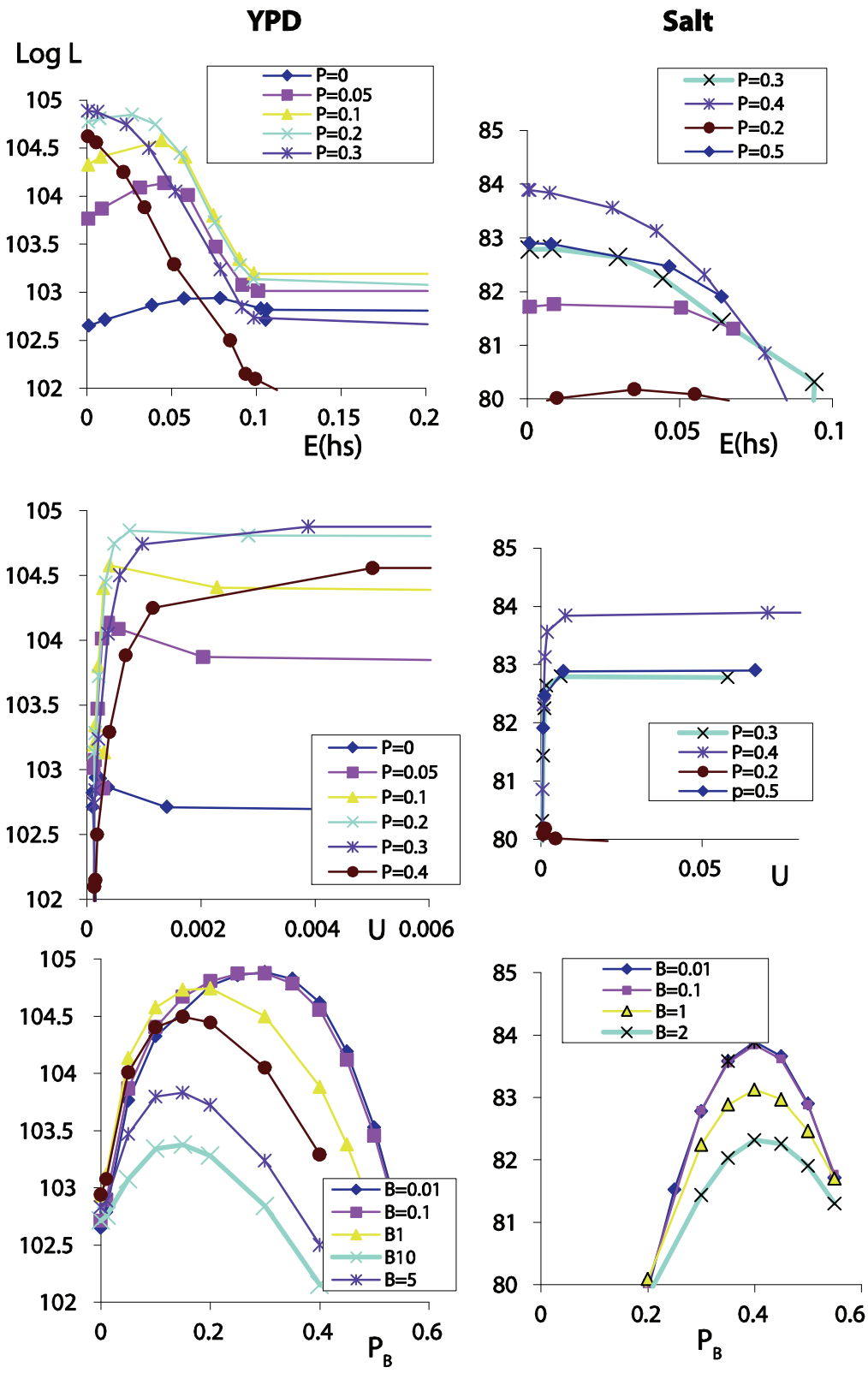

Figure S6: Profile likelihoods for mutation parameters affecting sporulation efficiency

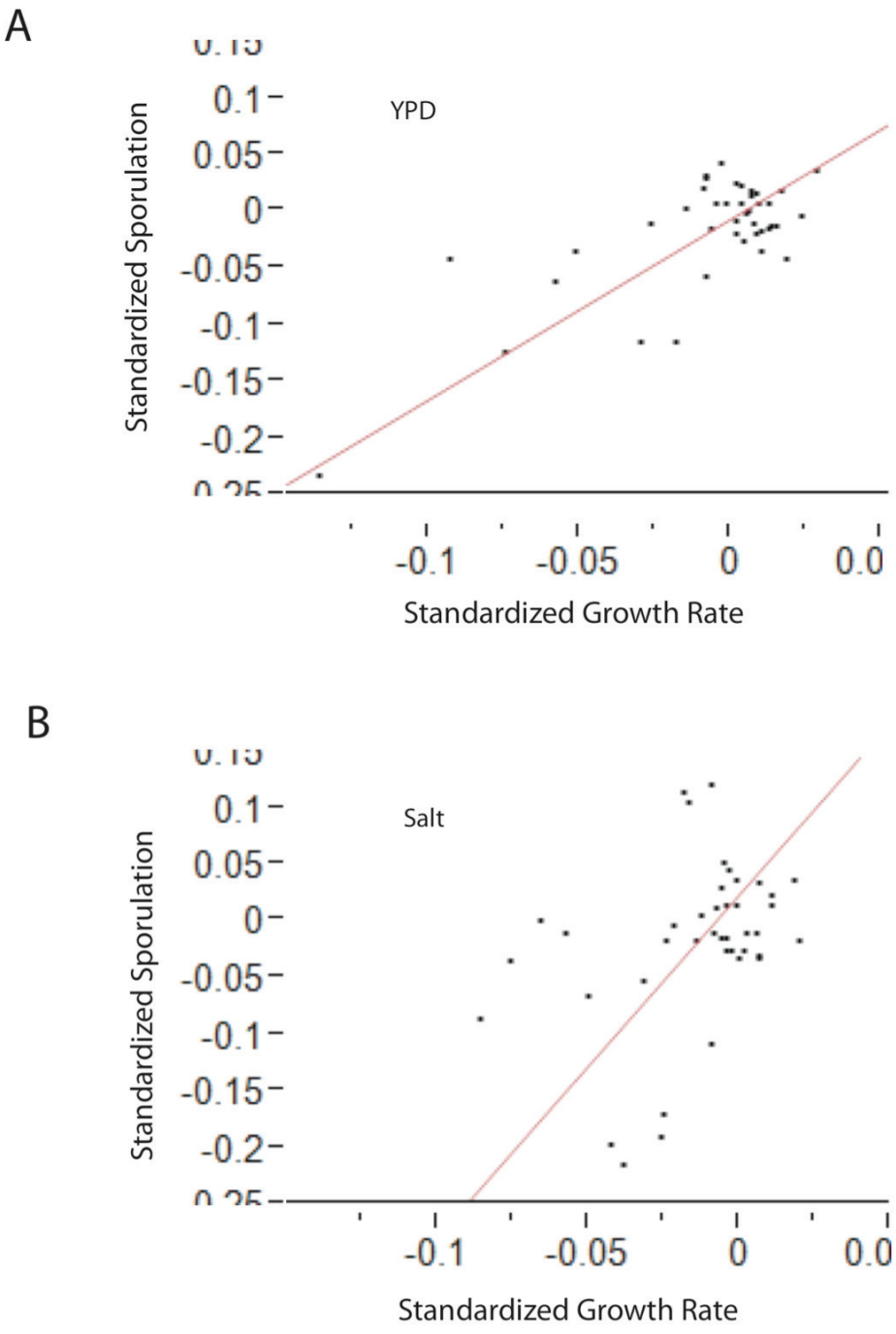

Figure S7: Average sporulation versus growth rate for diploid grande MA lines. A. YPD treatment. B. Salt treatment. Plotted lines are orthogonal regressions performed in JMP (v.9.02) statistical software.

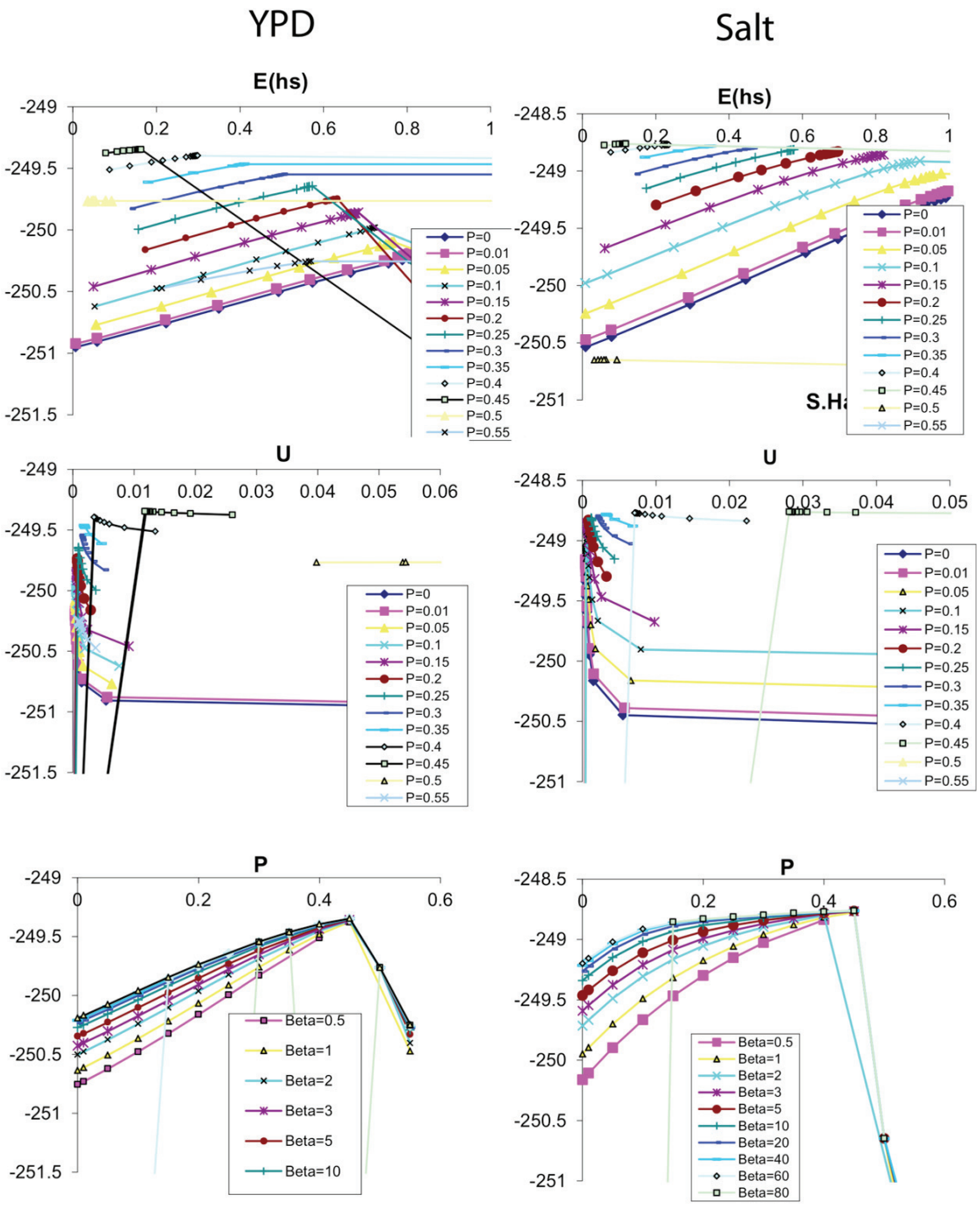

Figure S8: Profile likelihood curves for maximum likelihood analysis on mutation parameters affecting haploid viability.

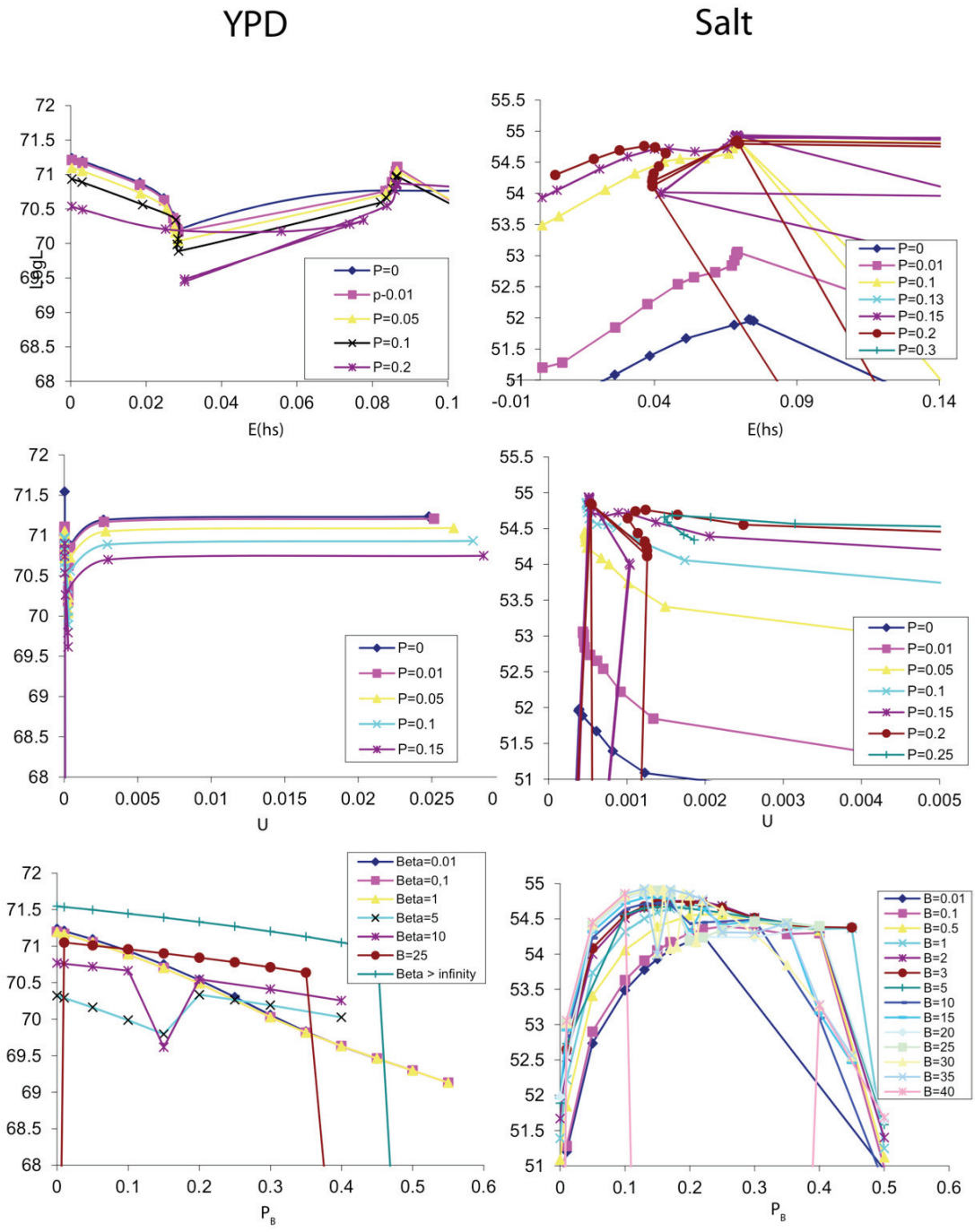

Figure S9: Profile likelihoods for maximum likelihood analysis on mutation parameters affecting haploid growth rate.

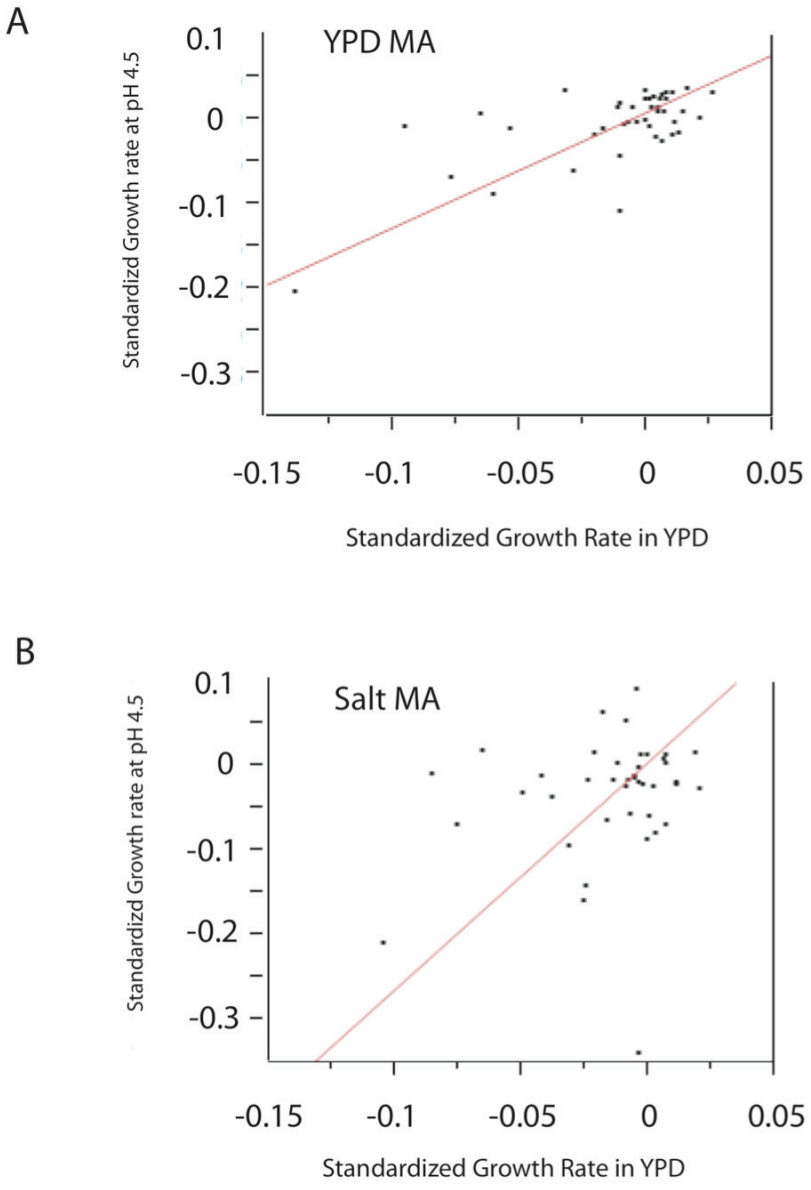

Figure S10: Average growth in acidic media versus growth in YPD for diploid MA lines. A. YPD treatment. B. Salt treatment. Plotted lines are orthogonal regressions performed in JMP (v.9.02) statistical software.

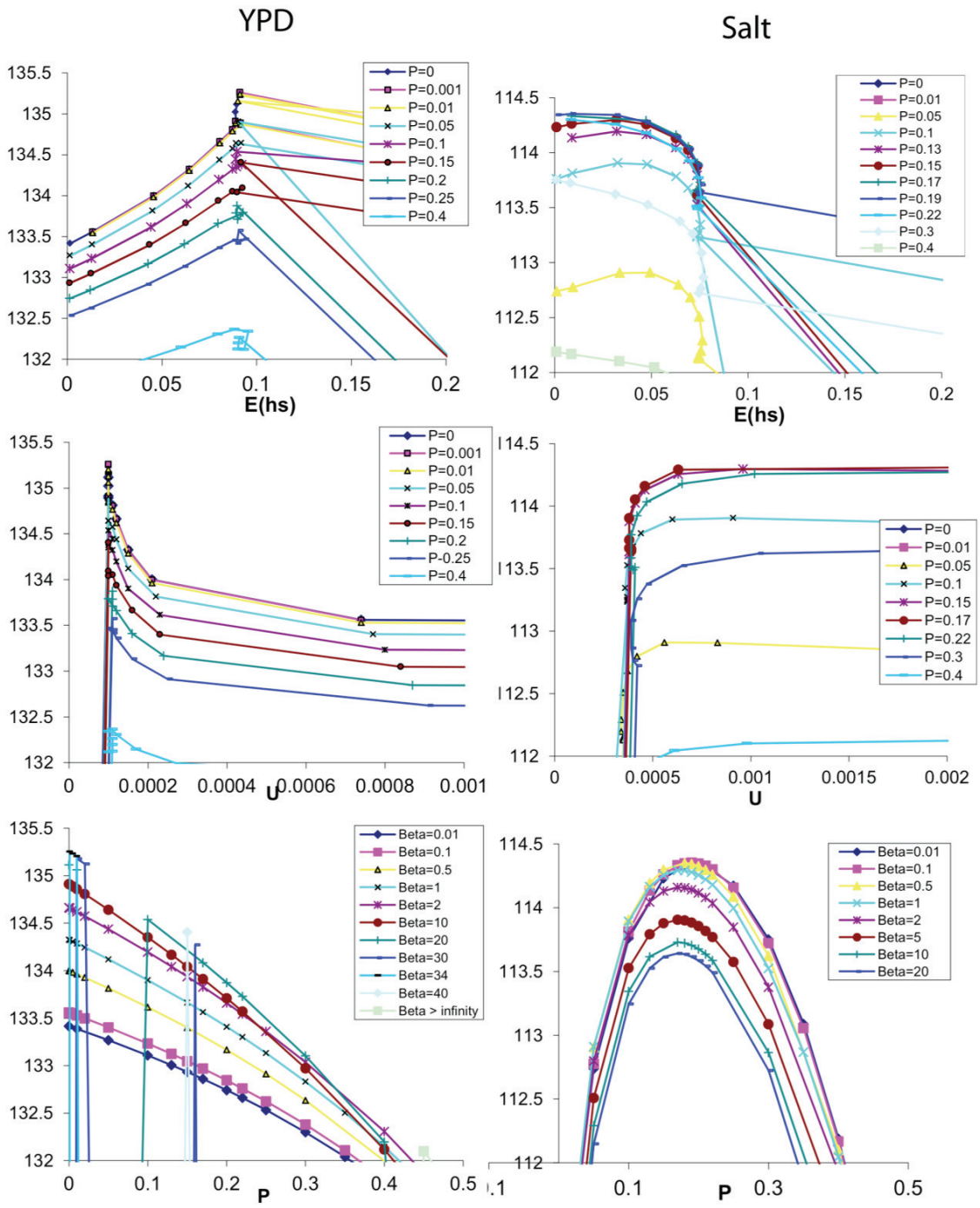

Figure S11: Profile likelihoods of maximum likelihood analysis on mutation parameters affecting maximum growth rate in acidic medium, pH 4.5.