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The limit to evolutionary rescue depends on ploidy in yeast exposed to nystatin

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Abstract

The number of copies of each chromosome, or ploidy, of an organism is a major genomic factor affecting adaptation. We set out to determine how ploidy can impact the outcome of evolution, as well as the likelihood of evolutionary rescue, using short-term experiments with yeast (*Saccharomyces cerevisiae*) in a high concentration of the fungicide nystatin. In similar experiments using haploid yeast, the genetic changes underlying evolutionary rescue were highly repeatable, with all rescued lines containing a single mutation in the ergosterol biosynthetic pathway. All of these beneficial mutations were recessive, which led to the expectation that diploids would find alternative genetic routes to adaptation. To test this, we repeated the experiment using both haploid and diploid strains and found that diploid populations did not evolve resistance. Although diploids are able to adapt at the same rate as haploids to a lower, not fully inhibitory, concentration of nystatin, the present study suggests that diploids are limited in their ability to adapt to an inhibitory concentration of nystatin, while haploids may undergo evolutionary rescue. These results demonstrate that ploidy can tip the balance between adaptation and extinction when organisms face an extreme environmental change.

Key words: evolutionary rescue, adaptation, ploidy, yeast, *Saccharomyces cerevisiae*, experimental evolution

Introduction

In the face of a large environmental change, species must adapt to persist. By knowing the factors that affect adaptation, we can begin to predict or influence a population's likelihood of survival. Here, we investigate the impact of genome copy number (ploidy) on the response to an extreme environmental shift, which requires short-term evolutionary rescue. Evolutionary rescue occurs when a population is saved from eventual extinction by genetic adaptation to a new environment (Carlson et al. 2014), a topic relevant for both conservation efforts and pathogen control. Because the extent to which new mutations are masked or expressed depends on ploidy, the rate of adaptation to a new environment and the probability of evolutionary rescue depend strongly on ploidy level (Otto and Whitton 2000; Unckless and Orr 2020; Uecker 2017). While the diploid phase dominates the life cycles of many animals and plants, the haploid phase is more extensive in several groups of algae (e.g., *Ulothrix* and *Chlamydomonas*) and fungi (e.g., *Schizosaccharomyces*), as well as many protists and bacteria (Bell 1982). Beyond this coarse categorization, many species experience substantial selection as both haploids and diploids (Bell 1982), including arrhenotokous animals with haploid males and diploid females (e.g., Hymenoptera (bees, ants, and wasps) and Thysanoptera (thrips)), sexual species that alternate between free-living haploid and diploid phases (e.g., many algae and fungi), and even predominantly diploid species that experience haploid selection among gametes or gametophytes (both in plants and animals, Immler and Otto 2018). Similarly, ploidy is a major factor determining the chance that pests and pathogens evolve resistance to antimicrobial treatments, with pests and pathogens also ranging from typically haploid (e.g., *Candida auris* and *C. glabrata*), typically diploid (e.g., *Candida albicans*), to alternating haploid and diploid generations (e.g., *Cryptococcus neoformans*) (Du et al. 2022). Therefore, understanding the impact of ploidy on evolutionary rescue is important for predicting the potential of both species persistence in a changing environment and antibiotic or pesticide resistance evolution. Biocides are also prime examples of the types of rapid environmental changes that necessitate evolutionary rescue while also making rescue less likely by limiting the number of available mutations, both by lowering population size (Marrec and Bitbol 2020) and by making certain evolutionary trajectories inaccessible because multiple mutations are required (Lindsey et al. 2013).

Following an extreme environmental shift, ploidy affects the probability of evolutionary rescue in a declining population (Unckless and Orr 2020; Uecker 2017). This is largely through the effect of dominance because recessive mutations will not be 'seen' by selection when they initially arise in

heterozygous form in diploids and therefore have a smaller chance of rising to high frequency in a population compared to partially dominant or fully dominant mutations (Orr and Otto 1994). Indeed, starting from the same mutant frequency, diploid adaptation is expected to be slower, and adaptive changes are less likely to establish unless dominant (Fisher et al. 2018; Otto and Whitton 2000). Consistent with this expectation, long-term evolution experiments using yeast (*Saccharomyces cerevisiae*) have found a bias towards dominant mutations during diploid adaptation (Marad et al. 2018). On the other hand, if haploids and diploids have the same per base-pair mutation rate, diploids acquire twice the number of mutations per individual, which may allow diploids to adapt sooner (e.g., Otto and Whitton 2000), especially when adaptation is limited by mutational availability (Orr and Otto 1994). Experiments performed using yeast also support this prediction. In large populations, adaptation is faster in haploids because mutations are not limiting, but when population sizes are decreased, the haploid advantage is lost (Zeyl et al. 2003). Using the probability of evolutionary rescue from Uncklesss and Orr (2020), rescue is more likely in haploid populations at low dominance levels, but in diploids at high dominance levels (Fig. S1). The diploid advantage at high dominance levels arises from assuming an equal population size and hence double the number of mutational targets but disappears if the number of haplotypes is the same as in haploids. Importantly, rescue is not expected in diploids if the dominance of beneficial mutations is too low relative to the rate at which the population is declining. Thus, whether rescue is more or less likely in diploids than in haploids is an empirical question, depending on the distribution of dominance among potential rescue mutations. Complicating matters, the spectrum of mutations (including both type and location) can differ between haploids and diploids (Sharp et al. 2018), which could help or hinder evolutionary rescue in either ploidy. Much of the relevant experimental work has been done with bacteria (e.g., studies of antibiotic resistance evolution reviewed in MacLean et al. 2010) or haploid eukaryotes (e.g., haploid yeast in Bell and Gonzalez 2009 or primarily haploid *Chlamydomonas reinhardtii* in Lachapelle and Bell 2012, for more examples see Bell 2017). One study using effectively polyploid bacteria concludes that rescue is unlikely from de novo mutations when those mutations are recessive but uses introduced rescue mutations instead of allowing for spontaneous mutation (Sun et al. 2018). Here, we present a direct study of the effects of ploidy on evolutionary rescue following a severe environmental shift.

We investigate how ploidy affects the ability of the yeast *Saccharomyces cerevisiae* to undergo evolutionary rescue in response to a high concentration of the fungicide nystatin. We model our design after a previous experiment in this system performed exclusively with haploids (Gerstein et al. 2012), which found in each population an adaptive allele in one of four genes of the ergosterol biosynthesis pathway. The mutations in three of these genes are likely to be complete (*ERG3*, *ERG6*) or partial (*ERG5*) loss of function mutations as the genes are non-essential in the genetic background tested (S288C) and deletion strains for each gene also have increased nystatin resistance (Gerstein et al. 2012; Te Welscher et al. 2008; Bhattacharya et al. 2018). Gerstein et al. (2012) additionally found a single adaptive mutation in the gene *ERG7* (Phe699Leu), which is unlikely to represent a null mutation as this gene is essential in the genetic background tested. Other mutations associated with increased resistance to nystatin are null mutations in *ERG2* (Te Welscher et al. 2008), *ERG4* (Bhattacharya et al. 2018), *KES1* (in text as *OSH4*) (Beh et al. 2001; Georgiev et al. 2013), *OSH2* (Beh et al. 2001), *NCP1* (in text as *CPR1*) (Daum et al. 1999), *SLK19* (Kennedy and Bard 2001), *VHR2* (Kennedy and Bard 2001), and *YEH2* (Mullner et al. 2005); a missense mutation in *PMA1* (in text as *KTI10*) (Zink et al. 2005); unspecified mutations in *HEM3* (in text as *OLE2*) (Bard 1972), and *IPT1* (in text as *KTI6*) (Zink et al. 2005), as well as overexpression of *OSH6* (Wang et al. 2005). The adaptive alleles found by Gerstein et al. (2012) were all recessive in the diploid state (Gerstein et al. 2014), and the other known resistance mutations are untested in a heterozygous diploid state. The only likely dominant known resistance mutation is overexpression of *OSH6*, but this mutation is only known to confer resistance to a low concentration of nystatin (1 μmol/L) and on solid medium (Wang et al. 2005) instead of the liquid medium we use in this experiment. The recessive alleles would not be sufficient to rescue diploid populations of yeast, as they first appear in heterozygotes, but there may well be other beneficial mutations in this environment, and our previous work may have missed beneficial alleles with sufficient heterozygous expression to confer resistance.

Diploids, aided by their larger genome size and therefore higher number of mutations per cell, may explore a wider range of potentially adaptive mutations, finding some that are at least partially dominant. We therefore expected diploid yeast to access alternative adaptive paths to haploids, albeit with a lower rescue probability due to the recessivity of many rescue mutations. Supporting this idea is a similar study performed by Anderson et al. (2004) that evolved both haploid and diploid yeast to the drug fluconazole, for which candidate pathways to drug resistance are known. At low concentrations of the drug, mutations in *PDR1* and *PDR3* were favoured, and diploids were able to adapt faster than haploids due to increased mutation availability and therefore decreased waiting time. All mutations found in diploids were dominant (29/29) while only about half of those found in haploids were dominant (12/29) when tested in a heterozygous diploid background. At high concentrations of the drug, however, recessive mutations in *ERG3* (one of the genes also implicated in nystatin resistance) are favoured, and diploids were found to adapt slower than haploids (Anderson et al. 2004). The authors concluded that diploids likely required two mutational events to occur (a mutation in *ERG3* followed by a second mutation that rendered the first mutation in *ERG3* homozygous) in order to acquire resistance, slowing their adaptation. In the current study, we set out to determine the mutations involved in the evolutionary rescue of diploid populations to a high concentration of nystatin. We found that diploid populations did not genetically adapt within the short time course of our evolution experiments, even though we observed over a hundred cases of rescue in haploid populations over this same time period. These results have implications for the efficacy of nystatin when applied

to fungal pathogens, which include *Candida albicans*, a common human fungal pathogen that is predominantly diploid (Hickman et al. 2013), and *S. cerevisiae*, for which clinical strains are mostly either diploid or of higher ploidy (Zhu et al. 2016).

Materials and methods

Strains

Except where noted, we used the S288C background, using the strains BY4741 (*MAT***a** *his3*Δ1 *leu2*Δ0 *met15*Δ0 *ura3*Δ0), BY4739 (*MATα leu2*Δ0 *lys2*Δ0 *ura3*Δ0) (Open Biosystems, now Horizon Discovery, Cambridge, UK) and a diploid produced by mating the two (BY4741 × BY4739). To assess the sensitivity of the results to strain background, one mutant acquisition experiment was performed using the W303 background, using the haploid strains MJM64 (*MAT***a***-YCR043C::KANMX STE5pr-URA3 ade2-1 his3*Δ*::3xHA leu2*Δ*::3xHA trp1-1 can1::STE2pr-HIS3 STE3pr-LEU2*) and MJM36 (*MATα-YCR043C::HPHB STE5pr-URA3 ade2-1 his3*Δ*::3xHA leu2*Δ*::3xHA trp1-1 can1::STE2pr-HIS3 STE3pr-LEU2*) constructed by McDonald et al. (2016), and a diploid produced by mating MJM64 × MJM36 (OLY075). The diploid was generated following the procedures of McDonald et al. (2016) with slight modifications; using YPAD (YPD + 40 mg/L adenine sulfate) in place of YPD, incubating the matings overnight, excluding the PBS buffer step, and performing the selection step twice.

Mutant acquisition in deep well boxes

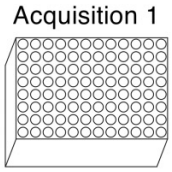
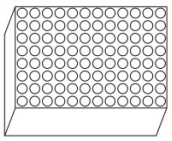
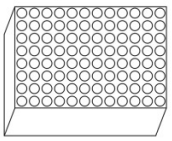
In total, three mutant acquisition experiments, similar to Gerstein et al. (2012), were performed, plus two flask experiments conducted at large population size (Fig. 1). For acquisition experiments 1 and 2, the strains were first grown from frozen on YPD plates at 30°C for three days. To produce stationary phase cultures for use in the mutant acquisition phase, single colonies were inoculated into 150 μL of YPD in 100-well honeycomb plates used with the Bioscreen C Microbiological Workstation (originally from Thermo Labsystems, now Oy Growth Curves Ab, Turku, Finland). Acquisition experiment 1 used 191 colonies of the diploid strain and 191 colonies of the haploid strains, split between mating types (*MAT***a**: 96, *MATα*: 95), alternating between diploid and haploid strains throughout the honeycomb plates. Acquisition experiment 2 used 286 colonies of the diploid strain, 47 colonies of *MAT***a**, and 48 colonies of *MATα*, with any given plate containing either all diploids (three plates) or half *MAT***a** and half *MATα* (one plate). The plates were incubated for 24 h in the Bioscreen C machine at 30°C with maximum, continuous shaking. Note that all liquid medium throughout this paper was supplemented with ampicillin at a final concentration of 0.04 mg/mL to prevent bacterial growth.

The following day, the honeycomb plates were visually assessed to confirm full growth in each well. A 1/100 dilution was performed in a final volume of 1 mL of YPD + 4 μmol/L nystatin ('YPDnystatin4') in deep well boxes. We estimate that each diploid population well contained ∼7 × 10^5 cells (based on hemocytometer counts). The same general map was used for the deep well boxes as for the honeycomb plates. Aluminum lids were used to prevent cross-contamination while sampling and plastic lids were added on top to protect the aluminum lids. The boxes were incubated at 30°C, shaking at 200 rpm. The growth of all strains was inhibited under these conditions, with no observable positive growth in the absence of a resistant mutant within 48 h (Fig. S2).

The wells were checked for growth by visual examination every 24 h. Small, individual clumps were typically observed at the bottom of the well prior to the yeast covering a larger, circular area, so a well was considered to have growth if there was a patchy covering of cells or a circle of at least roughly 3 mm in diameter. On the first day that growth was observed in a well, the growth was recorded, and the culture was frozen in 15% glycerol at −80°C. In acquisition experiment 1, potential rescue strains were sampled from 42 *MAT***a** wells, 86 *MATα* wells and 90 diploid wells over the course of 12 days. As discussed further below, the putatively rescued diploid strains did not grow on fresh nystatin and/or showed signs of contamination for this and subsequent acquisition experiments. In acquisition experiment 2, 16 *MAT***a** strains, 48 *MATα* strains, and 100 diploid strains were collected over the course of 10 days. For two wells from acquisition experiment 1 and three wells from acquisition experiment 2, the wells were not sampled despite growth, and these wells are excluded from the paper entirely.

Acquisition experiment 3, performed in the W303 background, used similar methods to acquisition experiment 1, with exceptions described here. The strains were originally struck from frozen on YPAD plates and grown for only two days. SC (supplemented with adenine) was used instead of YPD for growth in the honeycomb plates. SC was formulated using 20 g/L of dextrose, drop-out mix complete (D9515, US Biological, Salem, MA, USA), and yeast nitrogen base including ammonium sulfate (Y2025, US Biological, Salem, MA, USA), according to the manufacturer's instructions. This medium was supplemented with an additional 57 mg/L of adenine sulfate. Pregrowth in the honeycomb plates alternated between strains with 155 diploid colonies, 80 *MAT***a** colonies and 165 *MATα* colonies. SC + 4 μmol/L nystatin ('SCnystatin4') was used in place of YPDnystatin4 in the deep well boxes. Diploid initial inoculum was estimated as ∼7.4 × 10^5 cells per well. Pilot experiments indicated that mutants would be difficult to isolate in the *MATα* background (the initial stock was later found to be respiratory-deficient), so 80 additional wells of this strain were added to the deep well boxes, using the same pre-growth culture as for one other well, giving a total of 245 wells of the *MATα* strain. In the analysis, we restrict ourselves to considering only one of these two replicates because they are not independent. If neither replicate grew, we counted that as no growth (63 cases). If one of the two replicates grew (13 cases) or both replicates grew (4 cases), we chose one at random to analyze and discarded the other (by chance, we analyzed 7 out of the 13 populations where only one replicate grew). The map of the deep well boxes was slightly modified from that of the honeycomb plates, although still alternating between strains. Over the course of seven days, 77 *MAT***a** strains, 34 *MATα* strains and 121 diploid strains were collected.

Fig. 1. Visual representation of all mutant acquisition experiments. The first two experiments were performed in 96-well deep well boxes using the BY strains (BY4741, BY4739 and BY4741 × BY4739) and YPDnystatin4 as the medium. The third acquisition experiment was performed similarly except that it used W303 strains (MJM64, MJM36, and OLY075) and SCnystatin4. The final acquisition experiments investigated a much larger population size (roughly 100-fold greater), being performed in flasks instead of deep well boxes and again using the BY strains and YPDnystatin4. Design type refers to whether adjacent wells were of different (alternating) or the same (split) ploidy level (see Materials and methods).


	Acquisition 1	Acquisition 2	Acquisition 3	Flask (x2)
Medium:	YPD + 4 μM nystatin	YPD + 4μM nystatin	SC + 4 μM nystatin	YPD + 4μM nystatin
Strains:	BY	BY	W303	BY
Design:	alternating	split	alternating	-----
Dip Pop Size:	~7x10^5	~7x10^5	~7.4x10^5	~7x10^7
Pop Num:				
*MAT***a**	96	47	80	1
MATα	95	48	165	1
diploid	191	286	155	10

Confirming nystatin resistance

All populations frozen from the acquisition experiments were tested for resistance to nystatin. Populations were pregrown from frozen by inoculating 975 μL of 0.5 μmol/L nystatin (in YPD for acquisition experiments 1 and 2, SC for acquisition experiment 3) with 25 μL of frozen culture in deep well boxes according to a randomized map. Aluminum lids were added to the boxes, and they were incubated at 30°C, shaking at 200 rpm for 72 h, after which almost all wells had full growth as judged by visual inspection. From these pregrowth cultures, 200 μL was transferred to a 1.5 mL tube and stored at 4°C.

Confirmation assays for resistance were conducted by measuring growth in YPDnystatin4 (for acquisition experiments 1 and 2) or SCnystatin4 (for acquisition experiment 3) of all populations on three different days using the Bioscreen C, which automatically measures optical density at 420–580 nm ("OD") in 100-well honeycomb plates. Honeycomb plates filled with 148.6 μL of nystatin medium were inoculated with the cultures kept at 4°C according to a new random map for each growth assay. The culture tubes were vortexed until fully resuspended, and 1.5 μL was transferred to the appropriate well. All tubes were returned to 4°C when inoculation was completed. OD was measured automatically using the wideband filter at 30 min intervals for 72 h from cultures growing at 30°C with medium continuous shaking.

We used a generous cutoff for resistance based on OD after 72 h of growth ("OD72"): half-way between OD72 measured for populations with no observable growth and the OD72 of the most resistant of the populations. Only if OD72 for the majority of assays (at least two out of three) was above this half-way point did we consider a putative rescue strain truly resistant. All data manipulations and analyses were performed in R version 4.3.1 (R Core Team 2023), including use of the packages *ggplot2*, *emmeans*, *dplyr*, and *ggpubr* (Wickham 2016; Lenth 2024; Wickham et al. 2023; Kassambara 2023).

Based on initial testing, we knew that our collection of haploid populations included some nystatin-resistant mutants, so the highest OD72 reflected a truly resistant strain. Initial tests were found to be unreliable due to the degradation of our nystatin stock solutions, similar to the degradation of nystatin observed during the acquisition experiments (see section "Nystatin efficacy over time"), so we only report here the results of assays that were performed with newly diluted nystatin stock solutions. A single cutoff was used for both acquisition experiment 1 and 2 for each assay day because they were tested in the same medium and always assayed together. Because acquisition experiment 3 used a different medium and the assays were conducted on different days, the cutoffs were recalculated.

Our original intention was to determine the genetic basis of diploid adaptation. In tests conducted before these confirmation assays, we found eight potential diploid mutants, which we whole-genome sequenced[1]. In six of these strains, no coverage was observed for the deletion mutations expected to be heterozygous in our diploid lines. These were *his3*Δ1 and *met15*Δ0 (consistent with the BY4741 *MAT***a** haploid) in five strains and *lys2Δ0* (consistent with the BY4739 *MATα* haploid) in one strain (Fig. S6). Subsequent mating assays, conducted by plating alongside tester haploid strains (*his1-123*) of known mating type on plates where only mated cells would grow, confirmed the presence of haploids of a single mating type in each of these six populations, as well as at a low frequency in one of the other two. We also observed that the mating type was always found with the unlinked auxotrophic marker from the same haploid strain, not the mating partner, providing evidence for contamination rather than haploidization of the diploid strain; this cosegregation would only be expected to occur half of the time

[1] "Whole genome sequencing of potential diploid mutants" in Supplementary File 1.

if it were true haploidization. Five of the sequenced haploid contaminants carried non-synonymous mutations at three independent sites in *ERG3*, likely explaining their nystatin resistance (Fig. S7; see details in Table S2). The remaining putative diploid population, which did not contain haploid cells according to the mating assay, did not pass the cutoff for resistance in the confirmation assays.

Conducting confirmation assays with the above cutoff for resistance, 19 out of 632 diploid populations (3%) showed signs of rescue. Given our whole genome sequencing results, we critically examined all potentially resistant diploid populations and followed each up with plating assays for both mating type and auxotrophy. Haploid contaminants were detected by their ability to mate as well as their inability to grow on medium lacking histidine (if *MAT***a**) or lacking lysine (if *MATα*). Contamination by non-*Saccharomyces* microbes was detected by unusual colony morphology followed by visual inspection under a microscope. Contamination was found in all cases[2]. These 19 populations are omitted from the analyses presented here but including them does not qualitatively change any of the statistics presented.

Mutant acquisition with larger population sizes

Because we had such difficulty isolating resistant diploid strains using relatively small diploid population sizes ($\sim 7 \times 10^5$ cells inoculated) in our original acquisition experiments, we conducted two mutant acquisition experiments using higher population sizes (Fig. 1). In each, we first struck BY4741, BY4739, and BY4741 × BY4739 from frozen onto YPD plates and allowed them to grow for three days at 30°C. We then inoculated 10 mL of YPD in separate culture tubes from 10 diploid colonies and one of each haploid mating type. These tubes were allowed to grow for 24 h on a rotor at 30°C before 1 mL was used to inoculate 99 mL of YPDnystatin4 in 250 mL flasks (24 flasks in total). We estimate that this inoculum contained $\sim 7 \times 10^7$ diploid cells (based on hemocytometer counts).

The flasks were covered with aluminum foil and incubated at 30°C, shaking at 200 rpm. The flasks were checked every 24 h by visual examination for growth. When growth was observed (as a noticeable lightening of the culture colour and loss of clarity when compared to a flask containing no yeast), 500 μL of culture was sampled and frozen at −80°C in 15% glycerol. All flasks in the first experiment showed growth by day 10. In the second experiment, two flasks became contaminated by non-yeast cells by the end of the experiment and are omitted from the analysis. By day 10, six of the other eight had growth and the other two were slightly cloudy. Resistance to nystatin was then assayed as described for the first two acquisition experiments with cutoffs being recalculated because these assays were performed on different days.

Nystatin efficacy over time

Many diploid cultures appeared to undergo evolutionary rescue in nystatin, but growth was typically only observed late in the acquisition experiment, and most of these populations did not exhibit resistance when regrown in nystatin. We thus tested whether the effective concentration of the drug was decreasing over the duration of the acquisition experiments, allowing for growth of non-resistant cells. We also tested whether the presence of dead yeast cells altered the efficacy of nystatin. Dead cells were obtained by growing cultures of BY4741 × BY4739 in 1 mL of YPD in 1.5 mL tubes overnight at 30°C, shaking at 200 rpm. The next day, these tubes were placed in a heat block at 95°C for 10 min. Tests indicated that this amount of time was sufficient to kill all of the cells. On the first day of the experiment (day 0), 990 μL of YPD, YPDnystatin4, SC, and SCnystatin4 were aliquoted into deep well boxes, with half of all nystatin-containing wells also receiving 10 μL of heat treated cells, giving a total of 16 wells per type (base medium, base medium with nystatin, and base medium with nystatin and dead cells) per box. Ten μL of live BY4741 × BY4739, grown overnight in 1 mL of YPD, was used to inoculate 18–20 wells of each medium type split evenly across boxes on days 0, 4, and 8 of the experiment. On day 12, there was not enough live culture to inoculate all wells. Many control (YPD or SC) wells and one SCnystatin4 + dead cells well were not inoculated and excluded from the analysis. The other wells were inoculated with as much as possible, ranging from 2 to 10 μL of culture. The fact that fewer cells were used on day 12 is conservative with respect to our results below. Between inoculation days, the BY4741 × BY4739 stock culture was stored at 4°C while the deep well boxes were incubated at 30°C, shaking at 200 rpm. The boxes were visually inspected for growth (a patchy covering of cells or a circle of at least roughly 3 mm in diameter) every 24 h for 16 days, excluding days 10 and 11. Wells in which growth was observed before inoculation, implying contamination, were omitted from analysis (eight wells). The observations in media containing nystatin were statistically tested using a generalized linear model with a binomial error distribution and logit link function performed using the *glm* function in the package *stats* in R (R Core Team 2023). Models with and without interaction terms were compared with likelihood ratio tests run using the *anova* function, and no interactions significantly improved the model, so they were all dropped. The single effects were tested by comparing a model containing all single effects to ones with individual effects dropped.

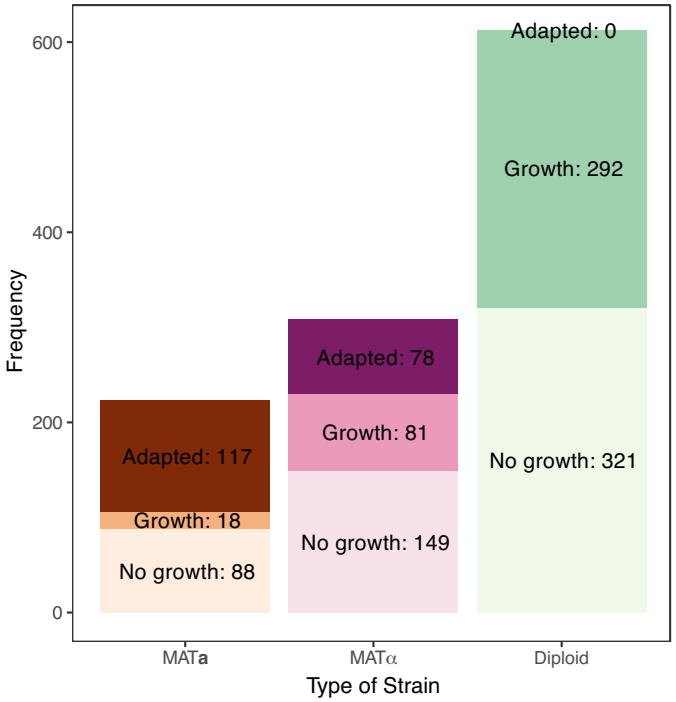
Results

Ploidy played a substantial role in the likelihood of evolutionary rescue. Including all acquisition experiments, we found that none of the 613 inoculated diploid wells (excluding those found to harbour contamination[3]) or flasks underwent evolutionary rescue, compared with 117 out of 223 *MAT***a** wells (53%) and 78 out of 308 *MATα* wells (25%) (Fig. 2). These numbers are based on tests of resistance to nystatin performed by following the growth of the populations for 72 h in their original medium type (either YPDnystatin4 or SCnystatin4) in the Bioscreen C. For each of the three acquisition experiments in deep well boxes, strain type influenced

[2] "Further testing of potential diploid mutants" in Supplementary File 1.

[3] "Further testing of potential diploid mutants" in Supplementary File 1.

Fig. 2. Stacked bar plot indicating the outcome for all inoculated wells over the three acquisition experiments in deep well boxes. The total height of the bar is proportional to the total number of wells inoculated of that strain type. The two darker bars together are proportional to the total number of wells that had growth in the acquisition experiments. The darkest coloured bar is proportional to the number of wells where resistance evolved based on the confirmation assays (excluding the populations verified to have contamination). No resistant diploid populations were found, while a large proportion of the haploid populations that grew in the acquisition experiments were reliably resistant to nystatin.



the proportion of populations that grew in the acquisition phase that were determined to be resistant (χ^2 contingency test using the *chisq.test* function in the package *stats* in R [R Core Team 2023] with a simulated *p*-value based on 10 000 replicates: acquisition experiment 1: $\chi^2 = 57.39$, $p < 1.00 \times 10^{-4}$; acquisition experiment 2: $\chi^2 = 108.78$, $p < 1.00 \times 10^{-4}$; acquisition experiment 3: $\chi^2 = 193.31$, $p < 1.00 \times 10^{-4}$, Fig. S3). *MAT***a** consistently had the highest proportion of resistant mutants among the wells that grew in the original acquisition experiments (1: 27/42 = 0.64; 2: 14/16 = 0.88, 3: 76/77 = 0.99), followed by *MATα* (1: 29/86 = 0.34; 2: 35/48 = 0.73, 3: 20/34 = 0.59). None of the fully diploid populations tested grew in confirmation experiments (Fig. 2 and Fig. S2). These results indicate that ploidy restricts the ability of yeast populations to undergo evolutionary rescue under these conditions. All underlying raw data and analyses can be found in Zenodo (doi:10.5281/zenodo.10947969).

Rescue in larger populations

One reason for a population to fail to undergo evolutionary rescue is that potentially adaptive mutations are rare and do not occur in that population. To help determine whether mutational opportunity was a limiting factor preventing evolutionary rescue in diploids, we conducted two mutant acquisition experiments with roughly 100-fold more initial cells (inoculation used 1 mL of overnight culture compared to 10 μL used in the main acquisition experiments). In total, 18 diploid populations, as well as two populations of each haploid type, were exposed to 100 mL of YPDnystatin4 in 250 mL flasks.

Increasing initial population size roughly 100-fold was not sufficient to allow for beneficial mutations to occur in diploids during these short adaptation experiments. In the first experiment, all populations grew within 10 days of inoculation, with the *MAT***a** and *MATα* populations growing on day 3, and the diploid populations growing on days 8–10. In the second experiment, the *MAT***a** and *MATα* populations grew on day 2 and six out of eight non-contaminated diploid populations grew on days 9 and 10. The other two flasks were slightly cloudy but did not exhibit full growth by day 10. In confirmation assays, no diploid population was deemed resistant when tested in YPDnystatin4 (Fig. S4). On the other hand, haploid populations showed reliable rescue as demonstrated by growth in confirmation assays, consistent with the smaller volume experiments. We note that many mutations allowing evolutionary rescue will have arisen during the initial clonal expansion of each line, prior to exposure to nystatin (as noted also by Gerstein et al. 2012). Indeed, one haploid control population showed growth in the confirmation assays, consistent with preexisting genetic variation for resistance, although the growth occurred later (after 24 h) than with the evolutionarily rescued haploid lines (growth to near stationary phase within 12 h).

Mutant coverage

The absence of adaptive evolution among diploids is unlikely to be due to a small number of replicates being tested. We modelled the accumulation of mutations during the growth of a population from a single cell struck out on a plate to the portion of liquid that is used in the inocula for the acquisition experiments. Using the mutation rate estimate of 1.67×10^{-10} from Zhu et al. (2014), we expect 1.2 nucleotide-changing mutations per site within the genome, combining all deep well acquisition experiments and the flask experiment[4]. When we multiply this number of mutations per site over the average length of an open reading frame, we expect to have sampled ~1300 non-synonymous or nonsense mutations for each gene within the genome. Based on these numbers, if a one-step dominant rescue mutation were available in the diploids, we likely would have observed the evolution of full resistance. We know that two-step mutations could confer resistance if they generate two mutant alleles at recessive resistance loci (Gerstein et al. 2014), but the calculations in the Supplementary Material indicate that there is a <0.0004 chance per gene that any such secondary mutation would have arisen over the course of the experiment if the first of these was not selectively favoured. Even when considering that there are 15 known genes for which recessive mutations are likely to cause nystatin resistance

[4] Supplementary Files 2 and 3 are a *Mathematica* file used for these calculations and a pdf copy.

(excluding overexpression mutations in *OSH6* as these mutations are expected to be dominant), this overall probability remains <0.006. Thus, this experiment primarily captures single-step rescue mutations.

Nystatin degradation

Based on the observation that many populations grew in the acquisition experiments but were not resistant when retested in nystatin[5] (Fig. 2), we hypothesized that nystatin was losing efficacy over the course of the acquisition experiments (lasting 7–12 days). Both YPDnystatin4 and SCnystatin4 showed degradation of nystatin efficacy over time (Fig. S5). This was observed as a loss of the ability to inhibit the growth of BY diploids for four days from inoculation, tested in a generalized linear model ($df = 3$, $p < 10^{-15}$). The presence or absence of dead yeast cells in the medium did not have a significant effect on this loss of efficacy ($df = 1$, $p = 0.056$) but the base medium did ($df = 1$, $p = 0.0043$), with SC allowing more growth overall than YPD.

Discussion

In this study, we have provided an example where ploidy alters the probability of evolutionary rescue. Diploids were generally not able to adapt to a high concentration of the fungicide nystatin in the short time course of the experiment, with none of 613 inoculated wells (excluding those found to have contamination) showing evolutionary rescue, as compared with 201 out of 531 haploid wells. Although we expected rescue to be less common in diploids, given previous work demonstrating that resistance mutations in haploids were recessive (Gerstein et al. 2014), it was possible that diploids would explore beneficial mutations not observed in the haploids, as has been seen in long-term evolution experiments with tetraploid populations (Scott et al. 2017). We conclude that there is a genetic limit to adaptation for diploids in this environment with few if any simple one-step dominant rescue mutations available. There are many differences between haploids and diploids that could cause the observed difference in their ability to undergo evolutionary rescue. Factors that can favour haploid rescue include larger population size, more beneficial mutations in haploids, and low dominance of potentially beneficial mutations (Otto and Whitton 2000). These differences will be discussed in turn below.

In yeast, haploids have smaller cells and therefore larger population sizes for the same volume of inoculum, approaching half of the cell volume and double the population size in many environments (Mable 2001). Larger population sizes in haploids should correspond to a larger number of mutations in these populations, but this is countered by diploids having twice the genome per cell, and therefore twice the number of mutational targets. To determine whether our diploid starting population sizes were too small to allow evolution, we exposed 18 diploid populations to nystatin in flasks containing 100-fold more medium and initial inoculum. None of these populations underwent evolutionary rescue (Figure S4). At this population size ($\sim 7 \times 10^7$ cells), we expect to have sampled over 1100 mutations per gene across all of the flasks, leading us to conclude that initial population size is not the limiting factor[6].

The fitness effect of potentially beneficial mutations may differ between haploids and diploids, even between haploids and homozygous diploids, which are often treated as equivalent because they both carry only a single allele. Resistance mutations acquired in haploids in the same selective environment as the one used in this study (Gerstein et al. 2012) were shown to have different effect sizes in haploid and homozygous diploid backgrounds, with haploids having higher fitness (Gerstein 2013). In nystatin, these effect size differences may arise as a consequence of the geometrical differences between haploid and diploid cells. Nystatin acts by binding to ergosterol in the yeast membrane, making the membrane more permeable to ions, sugars and metabolites, resulting in cell death (Carrillo-Munoz et al. 2006). Because surface areas are higher for diploids than haploids (Mable 2001), their sensitivity to nystatin may differ. In addition, because resistance mutations primarily arose in the ergosterol biosynthesis pathway (Gerstein et al. 2012), diploids may suffer a substantial fitness cost due to decreased stability of their larger cell membrane, counteracting the benefits of resistance. Nevertheless, ergosterol mutations still confer a large fitness benefit to diploids in nystatin when present in homozygous form (Gerstein 2013), suggesting that the difference in rescue probability between haploids and diploids is not due to differences in the costs of mutations.

Another way in which the effect of potentially beneficial mutations can differ between ploidies is through their dominance. Many new mutations are recessive. In haploids, they can be 'seen' by selection even when rare. In diploids, however, rare recessive beneficial alleles will not often spread to high enough frequency for homozygotes to be common (Orr and Unckless 2008), and sex by random assortment is very unlikely to combine these rare alleles. Natural yeast perform a version of selfing wherein mating is most common between gametes from a single diploid individual. This mechanism is more likely to produce an adapted, homozygous individual (Uecker 2017). Because reproduction is strictly asexual in our experiment, diploids must, however, acquire a second mutation (either another new mutation or a loss-of-heterozygosity event) in order to gain any advantage from a recessive allele (Mandegar and Otto 2007). Based on our results, we infer that there are no dominant or semi-dominant rescue mutations in this environment. This places a limit to evolutionary rescue on diploids at a lower concentration of the drug than for haploids. These results will not necessarily generalize to other environments because they depend on the nature of available adaptive mutations and the exact effects of the environment on the ancestral type. However, Agrawal and Whitlock (2011) find that among yeast knockout mutations, alleles of large (homozygous) effect tend to be more recessive. This pattern was observed for large effect alleles that were deleterious in a benign environment, but these may be the same large effect mutations that are able to confer high resistance to fungicides, as many antibiotic resistance mu-

[5] "Strain Differences" in Supplementary File 1.

[6] "Mutant Coverage" in Supplementary File 1.

tations carry a cost in benign environments (Melnyk et al. 2015).

In our short-term evolutionary rescue experiments, exposure to a high concentration of the fungicide nystatin appears to have exceeded the limit of genetic adaptation possible in diploids, but not in haploids. Anderson et al. (2004) performed a similar experiment at high concentrations of another antifungal drug (64 and 128 μg/mL fluconazole), in which the ancestral yeast were able to undergo seven to nine doublings but were unable to proliferate further. In their experiment, diploids evolved resistance more slowly than haploids but eventually all replicate diploid populations evolved heritable resistance (minimum inhibitory concentrations of 256 μg/mL fluconazole). Thus, diploids were not limited in their ability to undergo evolutionary rescue under their experimental conditions. Importantly, we have similar total numbers of cells in our inocula (accounting for the initial growth that was observed in fluconazole), so that the contrast between observing 100% rescue (Anderson et al. 2004) and our result of 0% rescue must reflect a difference in limits to evolutionary rescue and not a difference in experimental power. While certain two-step mutations would rescue diploid populations in our experiment (such as mutations in both copies of an ergosterol pathway gene), they would require much larger populations than those used. In contrast, Anderson et al. (2004) observed patterns consistent with such two-step mutations. The difference in observed two-step mutations likely reflects a difference in the selective environment. Because yeast undergo several generations in fluconazole before arresting growth, there is the opportunity for weakly dominant resistant mutations carried by heterozygotes to increase in frequency in the populations, making loss of heterozygosity more likely for one of these mutations.

Resistance to nystatin, and other polyenes, like amphotericin B, in clinical strains of the usually diploid fungal pathogen *Candida albicans* is relatively rare but occasionally observed (Carolus et al. 2020; Ksiezopolska and Gabaldón 2018). The mechanism of resistance to nystatin is largely similar between *C. albicans* and *S. cerevisiae*, involving changes to the ergosterol biosynthesis pathway (Carolus et al. 2020; Ksiezopolska and Gabaldón 2018). When tested, all mutations that conferred resistance were found to be homozygous, implying that resistance is recessive in *Candida* as well (Sanglard et al. 2003; Martel et al. 2010; Jensen et al. 2015). Indeed, resistance in this species is usually observed only after long-term fungicide treatment (Dick et al. 1980; Kelly et al. 1996; Martel et al. 2010; Jensen et al. 2015; Dhasarathan et al. 2021). By contrast, predominantly haploid species of *Candida* are not as susceptible to polyenes, potentially because of the rapid appearance of resistance mutations (Ksiezopolska and Gabaldón 2018).

Exact environmental conditions can also change the types of mutations that are adaptive. A previous study found that diploids were able to adapt at the same rate as haploids to a lower concentration of nystatin (0.6 μmol/L) over a longer period of time (140 generations) (Gerstein et al. 2011) under conditions that allowed growth of the initial strains (i.e., not an evolutionary rescue experiment). It is possible that the larger number of generations in that experiment provided the opportunity for strains to get the kinds of two-step mutations that seem to be necessary for resistance to high concentrations of the drug. However, initial whole-genome sequence data from these strains found no mutations in either *ERG3* or *ERG6* (data not shown), the most commonly used genes in haploids at high concentrations of nystatin (Gerstein et al. 2012). Instead, the ability of the diploids to evolve in Gerstein et al. (2011) suggests that different, and potentially more dominant, mutations may be available at lower concentrations of nystatin that are not sufficient to provide resistance to higher concentrations, as can be predicted by theory (Anciaux et al. 2018). Alternatively, the same resistance alleles may have different dominance at different concentrations of the drug, something that is observed for herbicides (Roux et al. 2005). In other evolution environments, such as the carbon source raffinose, higher ploidy yeast (tetraploids) are able to adapt faster than either haploids or diploids (Selmecki et al. 2015). These results indicate that the exact nature of the selective environment is an important factor in determining how ploidy impacts adaptation.

In our experiments, deterioration of the nystatin environment allowed diploids to grow sooner than they were able to adapt genetically. Nystatin can kill most actively growing cells (Snow 1966, though we will have lower efficiency as we did not subject our populations to nitrogen starvation), but phenotypic heterogeneity (without an underlying genetic basis) in the ability to persist in the presence of antibiotics is a known phenomenon (e.g., Balaban et al. 2004). These "persister" types remain sensitive to the antibiotic; however, when re-tested. Such persistence could explain the presence of nystatin-sensitive populations among those that grew in the acquisition experiments. The populations may persist at low numbers while the concentration of nystatin is high enough to be inhibitory and then grow once the efficacy of nystatin has dropped below some threshold. This is consistent with the observation that diploids tend to grow on the later days of the acquisition experiments, which is also when we observe growth of non-resistant haploid populations (Fig. S2). Follow up experiments evaluating the efficacy of nystatin over time indicate that nystatin likely lost efficacy by this time (Fig. S5), allowing the growth of lower tolerance strains. While we conclude that full resistance to 4 μmol/L nystatin was not exhibited by any of the diploids assayed, it is possible that the diploids did evolve low levels of resistance that improved their ability to persist or to grow once nystatin became less effective.

We find that evolutionary rescue is not always possible and that the limits can depend strongly on the ploidy of the organism in question. These results have implications for conservation. For example, among algae, we might expect evolutionary rescue in the face of climate change to change the relative proportions of species with a haploid phase (haplonts or haploid-diploid species) relative to those where mitosis occurs only in the diploid phase (diplonts, such as *Fucus* and diatoms). As another example, more attention may be needed for pollutants/toxins that require exposed organisms to adapt using recessive mutations, because of the risk that evolutionary rescue will fail. There are also implications for disease management where, by investigating the genetic basis of po-

tential resistance to our treatments of choice (antibiotics, pesticides), we can make informed decisions about timing and dosage. Considerations of ploidy and dominance of potential beneficial mutations should be included in informed biological management strategies.

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Data availability

Data generated and analyzed during this study, as well as the scripts used for analysis, are available in the Zenodo repository (doi:10.5281/zenodo.10947969, https://zenodo.org/records/10947970) and the National Center for Biotechnology Information Sequence Read Archive (accession No. PRJNA1112093, https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1112093).

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Supplementary material

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