Influence of fluconazole drug-dosing regimen on the rate of adaptation and extinction in *Candida albicans*

by

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A Thesis submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirements of the degree of

MASTER OF SCIENCE

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ABSTRACT

Invasive infections caused by human fungal pathogens are a global health concern due to high mortality and morbidity rates. Many fungal infections are caused by species from the genus *Candida*. The most common, *Candida albicans*, is both a commensal member of the healthy human microbiota and a prevalent opportunistic fungal pathogen. The antifungal azole drug fluconazole (FLC) is frequently prescribed for treating *C. albicans* infections. Azole drugs are typically fungistatic—they inhibit cell growth rather than kill susceptible cells within a population. Therefore, fungal populations can potentially adapt to fluconazole through increases in drug resistance or increases in drug tolerance (the ability of some cells of a susceptible population to grow slowly at inhibitory drug concentrations). Given the limited arsenal of antifungal drugs and the difficulty in developing novel antifungals due to the close phylogenetic relationship between human and fungal cells, there is a need to design treatment strategies that prolong drug efficacy and delay the evolution of drug tolerance or resistance.

To understand the influence of fluconazole drug-dosing regimen on the rate of adaptation and extinction in *Candida albicans*, I performed nine short-term *in vitro* evolution experiments. I independently evolved twenty-four replicates from four *C. albicans* strains to 0.25 μg/mL FLC ("FLC0.25"), 1 μg/mL FLC ("FLC1"), and 16 μg/mL FLC ("FLC16"), with 1:1000 dilutions done at three transfer durations (24 h, 48 h, and 72 h). Transfer duration significantly influenced the extinction rate: all replicates evolved at all drug concentrations survived the 48 h and 72 h transfer experiments, yet the extinction rate increased sharply from low to high drug concentration with 24 h transfers. Increases in drug tolerance were very frequent regardless of the dosing regimen. In contrast, large increases in drug susceptibility occurred for higher drug concentrations- FLC1 and FLC16 evolved replicates with 48 h and 72 h transfers. Reduction in drug susceptibility and growth rates of the evolved replicates were common. Genome size changes were common in some strain backgrounds evolved to higher fluconazole concentrations and longer transfer times. Overall, we found a significant effect of the drug-dosing regimen on the phenotype and genome size and was strain background specific.

ACKNOWLEDGEMENTS

I would like to express my deepest gratitude to my supervisor, Dr. Aleeza Gerstein, for her invaluable guidance, unwavering support, and encouragement throughout my research journey. Her mentorship has been instrumental in shaping this work and inspiring me to keep going. Thank you for being so understanding throughout.

I am also very thankful to my committee members, Dr. Deborah Court and Dr. Ayush Kumar, for their insightful feedback, constructive criticism, and generous time. Their expertise and guidance have significantly enhanced the quality of this thesis.

My heartfelt thanks go to my lab mates Abdul, Javier, Aruni, and Yana, whose camaraderie, advice, and collaboration have made my research experience both productive and enjoyable. Your support during long hours in the lab and your friendship have meant the world to me.

Finally, I am grateful to my family and friends for their unconditional love and encouragement, which have sustained me throughout my academic journey.

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LIST OF ABBREVIATIONS

DDA	Disk diffusion assay
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediaminetetraacetic acid
FLC	Fluconazole
FoG_{80}	Fraction of growth within the zone where there is 80 % inhibition
OD_{600}	Optical density measured at 600 nm
RAD_{80}	Radius of zone corresponding to 80 % inhibition of growth
TE	Tris-EDTA
WHO	World Health Organization
YPD	Yeast peptone dextrose

CHAPTER 1: LITERATURE REVIEW

1.1 Introduction

The human body is inhabited by distinct microbial communities of archaea, bacteria, fungi, and viruses that shape and influence human physiology and play a crucial role in health and disease (Sender et al., 2016; Shreiner et al., 2015). Among the fungal communities, *Candida* species have been one of the leading causes of most serious fungal disease cases (Bongomin et al., 2017). *Candida albicans* is the most prevalent human fungal pathogen and the predominant cause of invasive candidiasis, accounting for approximately 70 % of infections worldwide (Boyce, 2023). It has been listed in the critical group of the World Health Organization's (WHO) fungal "priority pathogens" list (Burki, 2023).

1.1.1 Candida genus and its phylogeny

The genus *Candida* has historically been used as an umbrella term for yeasts without a conventional sexual cycle that cause infections in humans, primarily, due to a lack of clarity in their classification (Ene et al., 2021). It is a polyphyletic genus that comprises approximately 200 species spanning over 13 phylogenetically distinct clades. It also includes many closely related non-pathogenic species (Borman & Johnson, 2019). With advances in genome sequencing and phylogenetic studies, some members of the *Candida* genus have recently been renamed to better reflect the evolutionary relationships. The new nomenclature for the relevant species is mentioned in the brackets in the paragraphs that follow (Borman & Johnson, 2023; Kidd et al., 2021; Warnock, 2017, 2019).

The pathogenic species mainly belong to two distinct clades: the CTG clade and the *Nakaseomyces* group. Species in the CTG clade are classified based on their usage of an alternative CUG codon that gets translated to serine instead of leucine (Santos et al., 2011) and includes two groups. One includes *Candida albicans*, *Candida dubliniensis*, *Candida tropicalis*, and the *Candida parapsilosis* species complex (*Candida parapsilosis, Candida orthopsilosis, and Candida metapsilosis*), with a second group containing *Meyerozyma guilliermondii* (*C. guilliermondii*), *Clavispora lusitaniae* (*Candida lusitaniae*), and *Candida auris* (Gabaldón et al., 2016). The *Nakaseomyces* clade is related to other lineages that underwent a whole genome duplication and are more closely related to the non-pathogenic yeast *Saccharomyces cerevisiae*

than to *C. albicans* (Fitzpatrick et al., 2006) (Gabaldón et al., 2013) ; the primary pathogens are *Candida glabrata* (*Nakeseomyces glabratus)*, Kidd et al., 2023) and two other emergent pathogens, *Candida bracarensis* and *Candida nivarensis* (Gabaldón & Carreté, 2016).

1.1.2 Genome Plasticity and dual lifestyle of Candida albicans

C. albicans is predominantly a heterozygous diploid yeast and has a genome size of 32 Mb consisting of eight chromosome pairs (Jones et al., 2004). *C. albicans* has long been considered an asexual species that largely follows a clonal mode of reproduction (Bennett, 2015; Hickman et al., 2015). However, with the discovery of haploid and tetraploid isolates *in vitro* and *in vivo*, this species has also been found to employ a parasexual mode of reproduction (Hickman et al., 2013). Unlike conventional meiosis, the parasexual mechanism involves concerted chromosome loss due to non-disjunction during mitotic divisions (Bennett et al., 2014) (Hickman et al., 2015). This results in the formation of cells with diploid or near-diploid states from unstable cells of intermediate ploidy (Bennett & Johnson, 2003; Forche et al., 2008). Therefore, the para-sex products exhibit great genotypic and phenotypic diversity compared to their diploid parent (Hirakawa et al., 2017). In addition to karyotypic changes, the ability to undergo other genomic variations, such as inter- and intra-chromosomal rearrangements, single nucleotide variations (SNVs), copy number variations (CNVs), and loss of heterozygosity (LOH), makes the genome of *C. albicans* highly dynamic. This property of genome plasticity is hypothesized to allow *Candida albicans* to adapt to various host niches and diverse selection pressures, including exposure to antifungal drugs used to treat invasive fungal infections (Mba et al., 2022).

C. albicans is often a commensal member of the healthy human microbiota and asymptomatically colonizes different anatomical sites in the human body, including the skin and mucosal membranes of the respiratory, oral, gastrointestinal, and urogenital tracts (Bhattacharya et al., 2020; Cauchie et al., 2017; Kumamoto, 2011; Limon et al., 2017; Zhai et al., 2020). However, it can transition from harmless commensal to an opportunistic pathogen and cause superficial to life-threatening invasive infections affecting multiple organs and organ systems (Zhai et al., 2020). These infections mainly occur in susceptible individuals with immunocompromised conditions, such as those having debilitating and immunosuppressive diseases, malignancy, diabetes, medical interventions, and also due to host physiological factors (Bennett, 2015; Diba et al., 2018; Yapar, 2014). Among the approximately 20 *Candida* species

capable of causing human infections, *Candida albicans* is the most frequently recovered and, thus, the prevalent cause of commonly occurring invasive fungal infections (candidemia and candidiasis). The incidence of fungal infections is rising globally, with high rates of morbidity and mortality (30-50 %) reported annually (Yang & Berman, 2024; Enoch et al., 2017; Kullberg & Arendrup, 2015 (Yang & Berman, 2024).

1.1.3 Antifungal drugs, resistance, and tolerance

As a eukaryote, *C. albicans* shares cellular structures and many biochemical pathways with its mammalian host. Therefore, it is challenging to develop antifungal drugs specific to fungal cells, and the treatment of *C. albicans* infections is limited to three major classes of antifungal drugs: polyenes, azoles, and echinocandins (Perfect, 2017) that differ in their cellular targets and mode of action. These drugs also vary in whether they kill susceptible *C. albicans* cells (i.e., "cidal drugs": polyenes, and echinocandins) or inhibit the metabolism and growth of susceptible cells (i.e., "static drugs"; azoles). This difference may influence the population dynamics of *C. albicans* when exposed to drug.

Polyenes are associated with high levels of nephrotoxicity and other infusion-related side effects, while echinocandins are expensive to administer and do not have a broad antifungal spectrum (Perlin, 2011; Robbins et al., 2017). This has limited their clinical use to treat invasive fungal infections. On the other hand, azole antifungals, particularly fluconazole (FLC), are widely used as a first-line treatment in many infection scenarios owing to their high bioavailability, cost-effectiveness, and convenient oral and intravenous administration (Perfect, 2017). FLC acts by binding to and inhibiting the activity of the enzyme lanosterol 14-demethylase, which is involved in the biosynthetic pathway of the principal sterol component of the fungal cell membrane, ergosterol. This binding leads to the disruption of the cell membrane and the accumulation of toxic C-14α methyl sterols and elevated levels of reactive oxygen species (ROS) (Chaud et al., 2020; Pfaller et al., 2013). However, the efficacy of azole drugs can be compromised due to a weakened host defense system, pharmacokinetics and pharmacodynamics of the antifungal drugs, and distinct fungal responses that lead to treatment failure (M. C. Fisher et al., 2022).

Antifungal drug resistance, heteroresistance, and drug tolerance are among the distinct fungal responses that hinder the successful treatment of invasive fungal infections. Since azoles are typically fungistatic and inhibit cell growth rather than kill susceptible cells within the

population, the fungal populations can theoretically adapt either through increases in drug resistance (the ability of cells to grow at concentrations above the minimum inhibitory concentration, "MIC," of the drug as assessed at 24 h) or antifungal drug tolerance (the ability of a subpopulation of susceptible cells to grow slowly at concentrations above the drug's MIC that is evident when growth is assessed beyond 24 h (Berman & Krysan, 2020; Rosenberg et al., 2018; Yang & Berman, 2024). The emergence and acquisition of antifungal drug resistance is an evolutionary process. Heritable genetic changes involving small-scale changes (point mutations, duplication, inversion, translocation) and large-scale genome changes often occur in response to the selective pressure exerted by the drug (Ksiezopolska & Gabaldón, 2018). Factors influencing these changes include the microbial population size, the generation time, the mutation rate, the diversity of physiological and genetic pathways available for resistance or tolerance increases, and the fitness costs of each pathway (M. C. Fisher et al., 2022; Kukurudz et al., 2022). Some of the mechanisms known to cause resistance in *Candida albicans* include alterations in the *ERG11* gene encoding the azole drug target 14-α-demethylase, overexpression of drug efflux pumps (ABC transporters, Cdr1 and Cdr2, and Mdr1), and mutations in the *ERG3* gene involved in cellular stress response (Coste et al., 2006; L. E. Cowen et al., 2000; Leah E. Cowen & Lindquist, 2005; Dunkel et al., 2008; LaFayette et al., 2010; Marichal et al., 1999; Marr et al., 1998; D. Sanglard et al., 1995; Dominique Sanglard et al., 1997). Large-scale genomic alterations like isochromosome 5 (i5(L) formation due to duplication of Chromosome 5s left arm and increased dosage of *ERG11* and *TAC1*, as well as chromosome 3 (extra copy of the right arm encoding a ABC transporter and MDR1s transcriptional activator) and chromosome 4 trisomies, have been found in *C. albicans* fluconazole-resistant isolates (Anderson et al., 2017; Ford et al., 2015; Perepnikhatka et al., 1999; Selmecki et al., 2006, 2008). Additionally, chromosome R (Chr R) trisomy was also found to cause increased resistance to fluconazole in *C. albicans* (Li et al., 2015)

Drug-resistant *C. albicans* isolates have rarely been isolated from patients in nosocomial settings, yet the frequency of treatment failure is increasing. It has recently been found that persistent candidemia might be due to enhanced FLC tolerance levels in *C. albicans* hindering successful treatment, rather than resistance (Levinson et al., 2021; Rosenberg et al., 2018). Azole tolerance is due to phenotypic heterogeneity in which only some cells grow, although slowly in the drug. The size of the tolerant subpopulation (which is proportional to the tolerance level) depends on the strain being tested (Berman & Krysan, 2020; Yang & Berman, 2024; Yang et al.,

2023). It arises from epigenetic mechanisms and is associated with multiple signal transduction pathways, enabling tolerant cells to survive drug stress (Rosenberg et al., 2018). Some of the pathways that have been found in tolerant cells include Hsp90, calcineurin, and protein kinase C cascade (Chen et al., 2023). Notably, azole exposure induces mitotic abnormalities that lead to karyotypic changes and give rise to different ploidy products like the formation of tetraploid and aneuploid cells (Harrison et al., 2014). Therefore, these rapidly acquired genome changes (aneuploidy or copy number variations and loss of heterozygosity) likely contribute to increased tolerance but if they are beneficial to the cell or not is still a matter to delve into (Guo et al., 2024; Kukurudz et al., 2022; Todd & Selmecki, 2020; Todd et al., 2023; Yang et al., 2023)

1.1.4 Experimental evolution

Experimental evolution in model organisms has become a popular and widely used method in microbial research in the past few decades (Van den Bergh Bram et al., 2018). In eukaryotes, *S. cerevisiae* emerged as a model due to their short generation time, small genomes, and simple growth condition requirements. This made it possible to easily propagate multiple parallel replicate populations over hundreds or thousands of generations on short time scales under well-controlled conditions (K. J. Fisher & Lang, 2016). The standard setup typically involves diluting the evolving populations at regular intervals in a new medium to supply fresh nutrients and remove toxic end products. This allows for additional cell divisions, resulting in high population sizes, where evolution is driven by natural selection (Van den Bergh Bram et al., 2018). Thus, *in vitro* experimental evolution studies provide a great platform for studying adaptation due to different conditions, such as drug exposure, and to help understand how the genome evolves in response to selective pressure (Kawecki et al., 2012). Most *in vitro* experiments across all *Candida* species have been conducted primarily with fluconazole. Past short-term and long-term experimental evolution studies used an experimental framework that looked at the evolution of drug resistance to a single level of fluconazole, typically at or below the MIC, with transfers done after a set number of hours (typically every 48 h or 72 h) or were designed to study the evolution of drug resistance to a stepwise increase in fluconazole levels from low to high (either at set times or once a specific optical density has been reached; (Bing et al., 2020; Burrack et al., 2022; L. E. Cowen et al., 2000; Handelman & Osherov, 2022; Mount et al., 2018; Rybak et al., 2017).

While these studies provided valuable insights into the azole resistance mechanisms, the interplay between genetic background, constant exposure to different FLC concentrations, and their influence on selection for azole-resistant or tolerant cell populations was unknown. Genetic backgrounds provide a lens to study strain-specific variations in drug responses and thus provide insights into the trajectory of evolution. Two recent studies used *in vitro* evolution to examine the impact of drug concentration on drug response (resistance and tolerance) and genome size in *C. albicans* strains from diverse genetic backgrounds with transfers performed every 72 h. They revealed that at low drug concentrations, populations increased in resistance up to the drug concentration they were evolved in, while evolution at high drug concentrations selected for increased tolerance (Gerstein & Berman, 2020; Todd et al., 2023). Another study found that sub-inhibitory FLC concentrations led to increased resistance and supra-MICs resulted in the formation of tolerant cells (Yang et al., 2023). The resistant adapters had Chr3, Chr5, and Chr7 aneuploidies whereas ChrR trisomy was common among the tolerant adapters.

1.1.5 Drug-dosing regimen

The influence of incubation time between transfers in different genetic backgrounds has not been examined. The previous studies at different concentrations all used 72h transfer durations. We refer to the influence of incubation time and FLC concentration collectively as "dosing regimen". Moreover, very few evolution experiments report on extinction rate, which is also an important evolutionary outcome.

Physiological concentrations of FLC vary widely across different tissues and body fluids. Daily FLC doses ranging between 150-800 mg are administered to treat different types of candidiasis, yielding serum concentrations around 7.5–60.5 μg/mL (Govindarajan et al., 2023). Previous evolution studies in pathogenic bacteria used different treatment regimens and reported varying extinction frequencies among treatment types (Barbosa et al., 2018) But, to date, this area of research remains largely unexplored in human fungal pathogens. Only two studies have examined extinction rates in *C. albicans* (Kukurudz et al., 2022; Syvolos et al., 2024). Since penetration differs across body sites in the human body and dosing regimens are also variable, both factors could be very important from a clinical standpoint. Knowledge acquired from studies considering the extinction rate could be useful in designing treatment strategies based on the efficiency of the drug dosing regimen.

In this thesis, I studied the influence of fluconazole drug-dosing regimen (different concentrations of fluconazole and different times of transfer duration) on the rate of adaptation and extinction in four *C. albicans* strains belonging to different genetic backgrounds (GC75, SC5314, P78048, P75016) (Figure 1). I performed nine short-term (~ 40-generation) *in vitro* evolution experiments and evolved twenty-four independent replicates from each of the four strains to 0.25 μg/mL FLC ("FLC0.25"), 1 μg/mL FLC ("FLC1"), and 16 μg/mL FLC ("FLC16"), with 1:1000 dilutions done at three transfer times (24 h, 48 h, 72 h).

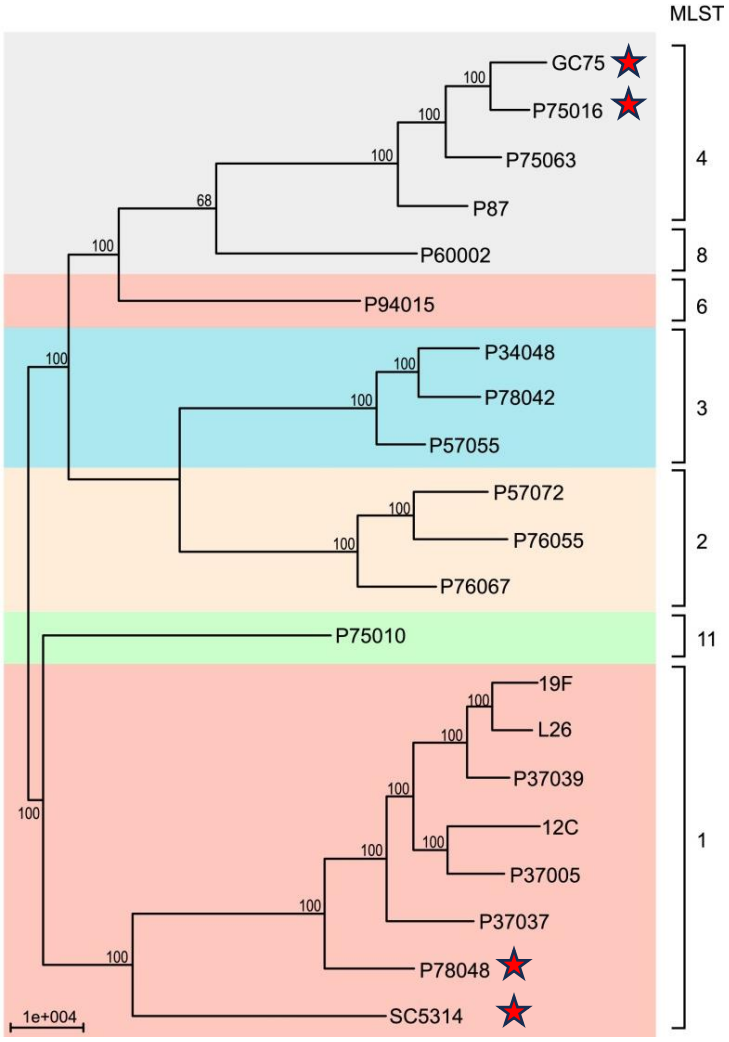

Figure 1. Phylogenetic relationship and clade assignment of the strains used in this study. Clade assignment was performed based on multilocus sequence type (MLST) analysis. Stars indicate the four strains used in our study. This figure is obtained from (Hirakawa et al., 2015).

1.2 Hypotheses

1. High drug concentrations and longer incubation times will select for drug tolerance, while high drug concentrations and shorter incubation times will select for drug resistance.
2. The evolved replicates will have a higher growth rate than their ancestors in the evolutionary drug environment.
3. Genome size changes will be commonly found on exposure to fluconazole across drug-dosing regimens.

1.3 Objectives

To test how the drug-dosing regimen influenced:

1. The rate of extinction across the four *C. albicans* strains
2. The propensity to evolve increased resistance or tolerance
3. Whether increases in resistance or tolerance correlate with increased growth rate in the evolutionary drug environment
4. The likelihood for genome size to evolve

CHAPTER 2: MATERIALS AND METHODS

2.1 Strains

Four strains of *Candida albicans* belonging to two genotypically distinct clades (clade one and clade four) were used in our study (Hirakawa et al., 2015). This included strains with the same parental MIC_{50} for fluconazole (FLC) - GC75 (MIC_{50}= 0.0125 µg/mL) (Wu et al., 2007), SC5314 (MIC_{50}= 0.0125 µg/mL) (Lockhart et al., 2002), P78048 (MIC_{50}= 0.5 µg/mL) (Wu et al., 2007), and P75016 (MIC_{50}= 0.5 µg/mL) (Wu et al., 2007). A frozen glycerol stock of each strain was streaked onto the yeast-rich standard lab medium- YPD agar plates (2% w/v peptone, 2% w/v yeast extract, 0.00016% w/v adenine sulfate, 0.00008% w/v uridine, 1.8% w/v agar, 1% w/v glucose, 0.1% v/v of each chloramphenicol and ampicillin) and incubated for 48 h at 30 ºC. For each strain, three plates were spotted randomly eight times. The 24 colonies closest to each dot were randomly chosen and inoculated into 1 mL of YPD in a deep (2 mL) 96-well culture box, followed by incubation at 30 ºC, shaking at 350 rpm overnight. From the overnight grow-up, 50 µL culture was frozen for each replicate in triplicates in 50 µL of 50% glycerol and stored at - 70 ºC. These are referred to as the ancestral replicates. Replicates evolved in fluconazole across the nine experiments with different drug-dosing regimens will be called "evolved replicates."

2.2 Experimental evolution experiments

The ancestral replicates were grown in YPD overnight. Each was then diluted 1:1000 dilution in two steps (1:100 then 1:10) into 1 mL of YPD + 0.25 µg/mL fluconazole (FLC0.25), YPD + 1 µg/mL fluconazole (FLC1), and YPD + 16 µg/mL fluconazole (YPD16) in 24 wells of two 96-well round bottom microtiter plates (Grenier, Ref 650161). For the first plate, rows A and C were used to inoculate replicates from one strain, and rows F and H were used for the second strain to try and minimize cross-contamination among strains. A similar orientation was used on a second plate for the third and fourth strains. Distilled water was added in the remaining rows. Breathe-easier membranes (Electron Microscopy Sciences, PA, United States) were used to seal the microtiter plates, after which they were incubated statically at 30 ºC for either 24 h, 48 h, or 72 h, depending upon the transfer time of the experiment.

After incubation for 24 h, 48 h, or 72 h, the culture was mixed by gently pipetting 3-5 times, followed by a two-step 1:1000 transfer into fresh YPD + FLC. This was performed for a total of four passages, i.e., ~ 40 generations of evolution. After the fourth transfer, 50 µL of culture from each evolved replicate (t4) were frozen in quadruplicates in 50% glycerol for each strain and stored at -70 ºC. One evolution experiment was performed for each combination of FLC concentration × transfer time for each strain ancestral replicates, for a total of nine evolution experiments. The experiment nomenclature is FLC concentration-transfer time, e.g., "FLC0.25-24hT" for the experiment conducted in YPD + 0.25 µg/mL FLC with transfers every 24 h. Some replicates went extinct during the 24 h transfer experiments, based on our inability to revive them from the evolved freezer stock after multiple attempts. 12 evolved replicates from each strain evolved in each drug dosing regimen were chosen for phenotypic analyses. To increase the statistical power to analyze the extinction data, a second set of evolution experiments was conducted with 24 h transfer durations.

2.3 Antifungal drug response quantification

Antifungal susceptibility testing was performed using a standardized disk diffusion assay protocol to screen for drug resistance and tolerance changes (Kukurudz et al., 2022; Salama & Gerstein, 2022). Briefly, 5 µL of the frozen ancestral and evolved strain replicates were inoculated into 500 µL of YPD in a deep 96-well culture box, covered with a breathe-easier sealing membrane followed by incubation at 30 ºC shaking at 250-350 rpm for 48 h. After incubation, the culture in each well was mixed well by pipetting 3-5 times, followed by transfer of 200 µL culture into a disposable 96-well microtiter plate. Plates were covered with a clear, non-breathable plate seal, and OD_{600} of the culture was measured in an Epoch plate reader. Based on the OD measurements, the culture was standardized to an OD_{600} 0.01 in PBS in a deep 96-well culture box. The box was covered with a lid and placed on a plate shaker, shaking at 250-350 rpm, until the culture was spread onto YPD plates.

For each replicate, 100 µL of the standardized culture was added YPD plates in duplicate. 5-mm glass beads were added to each plate and the plates were shaken for approximately 30 seconds to allow the culture to spread evenly. The beads were removed (by turning the plates upside down and emptying them into 70 % ethanol), and plates were turned upside down to sit for ~20 minutes to air dry. Once the plates were dry, an antifungal disc containing 25 µg/mL

fluconazole (Oxoid Ltd, UK) was placed in the center of each plate, followed by incubation for 48 h at 30 °C.

Photographs of the 48 h incubated plates were taken on a lightbox. Raw images were converted to 8-bit, inverted, and the brightness and contrast were adjusted using ImageJ to get bright colonies against a dark background (Kukurudz et al., 2022; Salama & Gerstein, 2022). The ImageJ processed images were used for analysis using the *diskImageR* R package, which computationally measures the drug susceptibility as RAD_{80} (the radius where an 80 % reduction in growth occurs) and tolerance as FOG_{80} (the fraction of growth above RAD_{80}) (Gerstein et al., 2016). The $RAD_{80\ and}$ FOG_{80} values reported are the average of the two technical replicates.

Table 1: Summary of the number of biological replicates of each strain across the different transfer durations for the three drug levels they were evolved to.

Drug concentration	**Strain**	**Transfer duration**		
		24hT	**48hT**	**72hT**
FLC0.25	GC75	5	1	4
	SC5314	4	2	3
	P78048	1	1	1
	P75016	1	1	1
FLC1	GC75	1	1	1
	SC5314	1	2	2
	P78048	1	1	1
	P75016	1	2	2
FLC16	GC75	4	2	4
	SC5314	4	2	4
	P78048	4	2	1
	P75016	4	2	1

2.4 Growth rate analysis

Growth rates were measured for ancestral and evolved replicates in the evolutionary drug environment in which the replicates were evolved. Briefly, 5 µL of the frozen ancestral and evolved strain replicates were inoculated into 500 µL of liquid YPD in a deep 96-well box, covered with a breathe-easy sealing membrane (Electron Microscopy Sciences, PA, United States), and incubated shaking (250 – 350 rpm) at 30 ºC for 48 h. The 48 h culture was then standardized to OD_{600} 0.02 in YPD. 100 µL of the standardized culture was added to 100 µL of YPD + FLC (0.50 µg/mL, 2 µg/mL, 32 µg/mL., as appropriate for FLC0.25, FLC1 and FLC16) to a final volume of 200 µL. Microtiter plates were covered with a clear breathe-easy sealing membrane and incubated at 30 ºC in an Epoch microplate spectrophotometer shaking continuously. OD_{600} was measured every 15 minutes for 24 h, 48 h, or 72 h, depending on the transfer time of the evolution experiment from which the strain replicates were being analyzed. Using a custom R script by Dr. Richard Fitzjohn, the spline with the highest slope from each growth curve was used to calculate the maximal growth rate per well (Syvolos et al., 2024). The average growth rate between two biological replicates of each strain for each evolution experiment was used for analysis and visualization.

2.5 Genome size estimation

Flow cytometry was used to assess the genome size of evolved replicates relative to their diploid ancestors. Twelve evolved, and ancestral replicates from all strains from two evolution experiments were analyzed on the same day. Briefly, 5 µL of the ancestral and evolved replicates were inoculated from frozen into 500 µL of liquid YPD in a deep 96-well box, covered with a breathe-easy sealing membrane, and incubated shaking at 30 ºC for 48 h. After 48 h, 10 µL was subcultured into 500 µL of fresh liquid YPD, covered with a breath-easier membrane, and incubated shaking at 30 ºC for 4 hours. 200 µL of the subculture was transferred to round bottom 96-well microtiter plates and centrifuged at 1000 x g for 5 minutes (these settings were used for all centrifugation steps). The supernatant was discarded, followed by resuspension of the pellets in 20 µL of 50:50 Tris-EDTA (TE buffer). 180 µL of cold 95% ethanol was slowly added to fix the cells in the resuspended pellet, and the plates were wrapped in aluminium foil and stored at -20 ºC. Fixed cells were typically stored for two to three weeks. The fixed culture was thawed, then

centrifuged. The supernatant was discarded, and the pellets were washed in 200 µL of TE. The plates were pelleted again and resuspended in 50 µL of 1 mg/mL RNAse solution, followed by overnight incubation at 37 ºC. The samples were then centrifuged again, the supernatant was discarded, and 50 µL of TE buffer was added to resuspend the pellets. To this, 50 µL of 1:100 sytox (Fisher Scientific): 50/50 TE solution was added, and the plates were incubated in the dark overnight. The overnight plates were centrifuged and 200 µL TE was added to the pellets. After mixing, the resuspension was added to flow tubes containing 500 µL TE. Flow cytometry was performed on an SH008S cell sorter (Sony Biotechnology Inc, San Jose, California, United States). 10,000 events were recorded for each population. The resulting data was analyzed using the PloidyPeaks R package (https://github.com/margothenry/PloidyPeaks).

CHAPTER 3: RESULTS

3.1 Extinction increases as the evolutionary drug concentration increases with 24 h transfers

To investigate how evolutionary drug concentration and transfer durations influence extinction rates in *Candida albicans* populations from different genetic backgrounds, replicates from four genetic backgrounds of *C. albicans* were passaged with 1:1000 dilutions every 24 h, 48 h, and 72 h for a total of four transfers in YPD + FLC0.25, FLC1, and FLC16. All strain replicates survived to the end of the 48 h and 72 h transfer evolution experiments. In the 24 h transfer experiments, some replicates went extinct at all drug concentrations (Figure 2), with the number that survived decreasing as drug concentration increased. In FLC0.25, all evolved replicates from SC5314, P78048, and P75016 survived, yet one-third of the GC75 replicates went extinct. In FLC1, at least one replicate went extinct in all backgrounds, with GC75 replicates again having the highest extinction rate. In FLC16, only 31 replicates survived across all strain backgrounds, with only one GC75 surviving replicate. Extinction rate was thus significantly influenced by the drug concentration and the strain background, with GC75 replicates having the highest extinction at all drug levels and SC5314 replicates the lowest (Figure 2; fixed-effect model, with the proportion of replicates that survived as the response variable and drug concentration and strain as categorical variables, p-value obtained through two-way ANOVA; $F_{2,6} = 25.63$, $p = 0.001$ for drug concentration; $F_{3,6} = 5.02$, $p = 0.045$ for strain). Replicate survival percentage was also plotted for the strains for ease of visualization (Supplementary Figure S1).

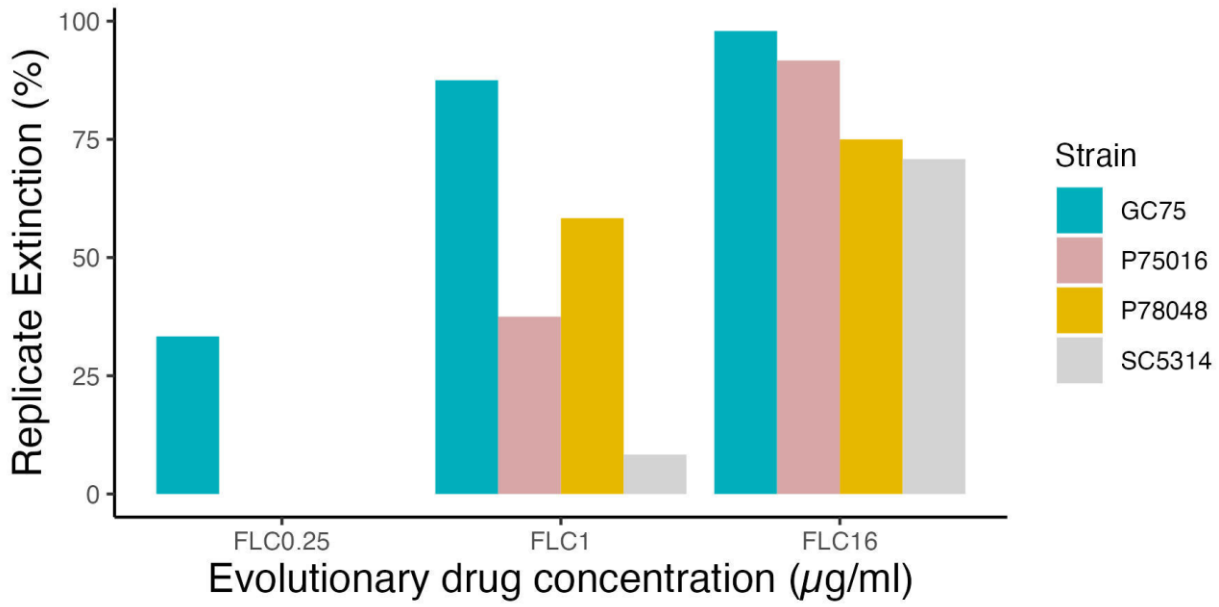

Figure 2. Differential extinction of replicates evolved to low (FLC0.25), medium (FLC1), and high (FLC16) fluconazole concentrations with transfers every 24 h. Replicate extinction percentage was calculated from 24 evolved replicates for FLC0.25 and FLC1 and 48 evolved replicates for FLC16.

3.2 Susceptibility increases at higher drug concentrations and longer transfer times

Disk-diffusion assays were performed for the evolved and corresponding ancestral replicates to test the influence of the drug-dosing regimen on the phenotype (drug susceptibility and drug tolerance) of the evolved replicates from the four genetic backgrounds. Drug susceptibility was also influenced by a significant interaction between drug concentration and transfer time (for Figure 3; linear mixed-effect model implemented using the Lmer R package (Kuznetsova et al., 2017), with the difference in evolved and ancestral RAD_{80} as the response variable, drug concentration, transfer time, and their interaction as the predictor variables, and strain as a random effect, p-value obtained through the analysis of variance test with Satterthwaite's method for degrees of freedom; drug concentration, $F_{2,559} = 15.19$, $p < 0.0001$; transfer time, $F_{2,552} = 19.73$, $p < 0.0001$; interaction, $F_{4,559} = 2.93$, $p = 0.020$).

For 24 h transfer experiments, FLC1-evolved replicates tended to increase in susceptibility, while FLC0.25-evolved replicates were generally similar to the ancestral replicates. The FLC16-evolved replicates differed by background: many P78048 replicates and SC5314 increased in susceptibility, while P75016 replicates were split, with some increasing in susceptibility and some increasing in resistance (Figure 3A). The results were somewhat different in the 48 h transfer experiments: FLC16-evolved replicates tended to exhibit increased susceptibility compared to both FLC0.25 and FLC1-evolved replicates (Figure 3B). For 72 h transfers, P78048 replicates evolved to FLC16 exhibited higher susceptibility than FLC0.25 and FLC1-evolved replicates, consistent with results from 48 h transfers. By contrast, there did not appear to be any significant changes in susceptibility overall for SC5314 evolved or GC75 evolved replicates (Figure 3C). Overall, replicates were more likely to increase susceptibility than increase resistance across the evolved replicates. The influence of the drug-dosing regimen was highly strain-specific, with P78048 and P75016 replicates demonstrating the most pronounced changes, particularly at high drug concentrations (FLC1 and FLC16) and longer transfer times (48 h and 72 h).

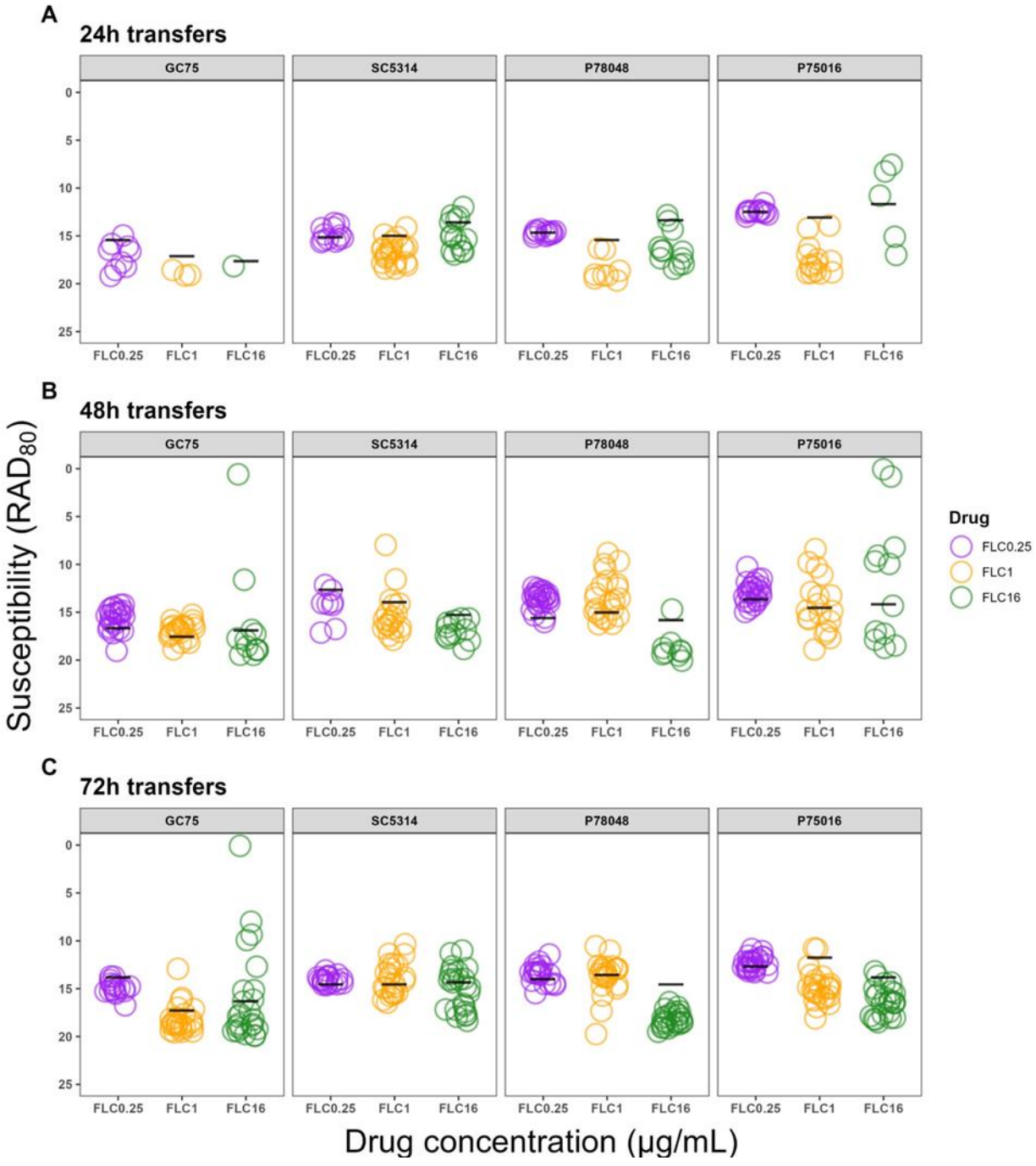
A
24h transfers
B
48h transfers
C
72h transfers
GC75
SC5314
P78048
P75016
FLC0.25
FLC1
FLC16
Susceptibility (RAD80)
Drug concentration (µg/mL)
Drug

Figure 3: Susceptibility of *C. albicans* strain replicates evolved to the drug-dosing regimen assayed on fluconazole disks. Each circle represents the mean of two technical or two biological replicates evolved to a low (FLC0.25), medium (FLC1), and high (FLC16) concentration of fluconazole with 24 h (3A), 48 h (3B), and 72 h (3C) transfer times. The black bars are the median RAD_{80} value of the ancestral strain replicates. The y-axis has been reversed to represent the resistant replicates at the top and the susceptible replicates at the bottom for easy visualization.

3.3 Tolerance increased across the drug dosing regimen

As was observed with susceptibility, tolerance was also significantly influenced by the interaction between drug concentration and transfer time (for Figure 4; linear mixed-effects model implemented using the Lmer R package, with the difference in evolved and ancestral tolerance as the response variable, drug concentration, transfer time, and their interaction as the predictor variables, and strain as a random effect; drug concentration, $F_{2,558.86} = 216.09$, $p < 0.0001$; transfer time, $F_{2,559.23} = 20.02$, $p < 0.0001$; drug × transfer time, $F_{4,558.42} = 12.06$, $p < 0.0001$). For 24 h transfer experiments, replicates evolved to FLC1 and FLC16 increased in tolerance, while FLC0.25 evolved replicates tended to decrease (Figure 4A). In the 48 h and 72 h experiment, GC75, SC5314, and P75016 replicates evolved to FLC1 and FLC16 also evolved higher tolerance than FLC0.25 evolved replicates (Figure 4B, C). While both FLC1 and FLC16 evolved P78048 replicates exhibited higher tolerance than FLC0.25 evolved replicates with 48 h transfers, with 72 h transfers, FLC16 evolved P78048 replicates had the highest tolerance across the 72 h regimen (Figure 4B, C). Overall, increases in tolerance were pervasive across the drug-dosing regimen. While replicates from most genetic backgrounds increased in tolerance, it was most pronounced in FLC16 from P78048 and P75016 replicates across the three transfer times.

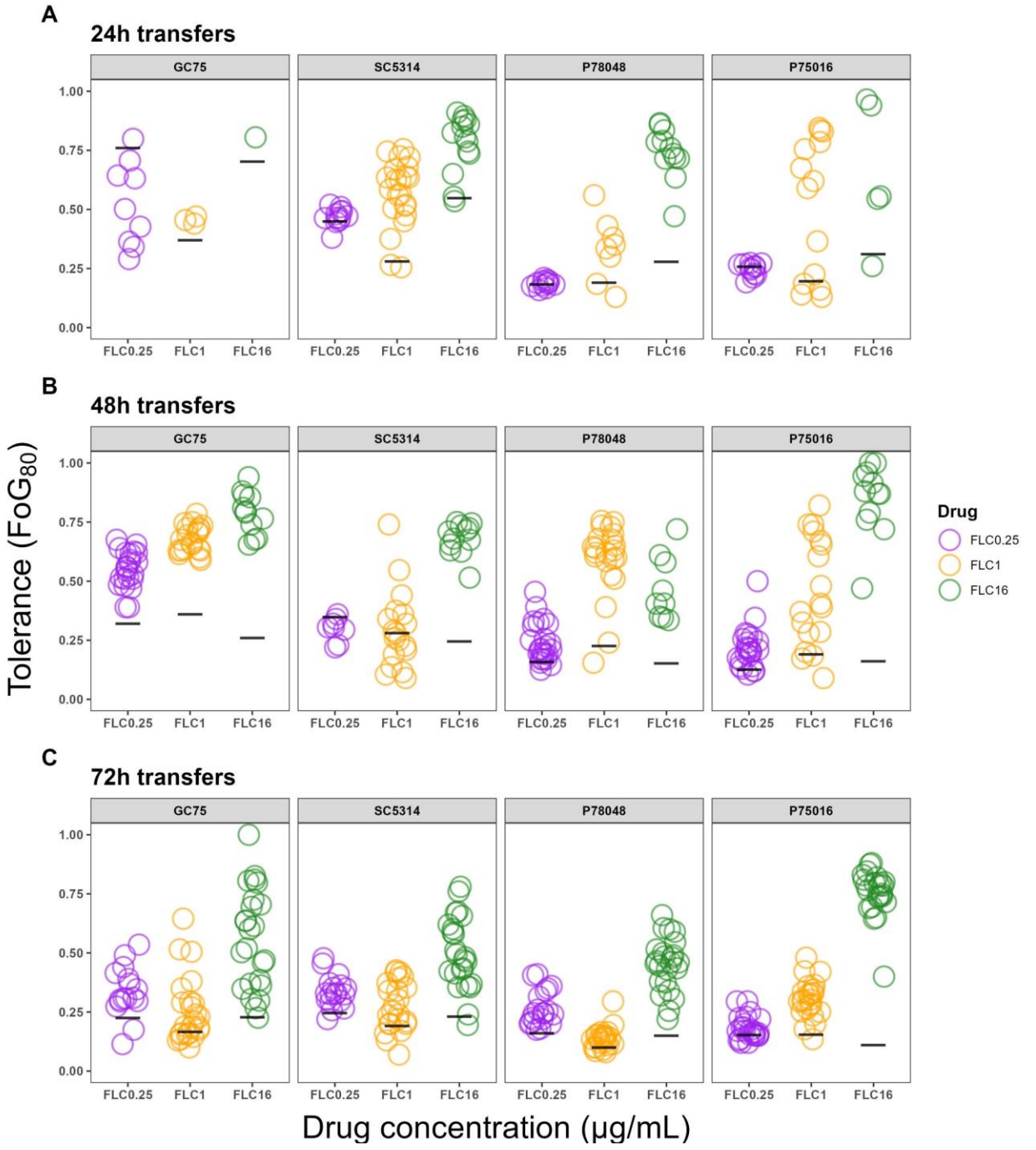

Figure 4: Tolerance of *C. albicans* strain replicates evolved to the drug-dosing regimen assayed on fluconazole disks. Each colored circle represents the mean FoG_{80} (fraction of growth) of two technical *C. albicans* strain replicates evolved to a low (FLC0.25), medium (FLC1), and high (FLC16) concentration of fluconazole across 24 h (4A), 48 h (4B), and 72 h (4C) transfer

time. The black bars represent the median FoG_{80} value of the corresponding ancestral strain replicates.

3.4 The growth rate decreases as the evolutionary drug concentration increases and transfer time increases.

The growth rate was measured for evolved replicates in the same drug concentration in which they were evolved in order to examine the influence of the drug-dosing regimen on the growth dynamics of the evolved replicates compared to their ancestors. For 24 h, 48 h, and 72 h transfer experiments, the differences in evolved and ancestral growth rates were significantly influenced by the drug concentration to which the strains were evolved (for Figure 5; Welch's ANOVA; 24 h transfers, $F_{2,19.58} = 36.72$, p-value < 0.0001; 48 h transfers, $F_{2,85.51} = 66.06$, p-value < 0.0001; 72 h transfers, $F_{2,89.64} = 62.08$, p-value < 0.0001). Evolution to FLC1 and FLC16 resulted in a significant reduction in growth rates compared to evolution to FLC0.25 with 24 h transfer time. Notably, FLC1 exhibited the most pronounced effect, resulting in the highest reduction in growth rate across the three drug concentrations (Figure 5A; Pairwise post-hoc results in Table 2). Similar to 24 h transfer experiments, FLC1 and FLC16 evolved replicates exhibited significantly reduced growth rates than FLC0.25 evolved replicates with 48 h transfers. However, the effect was indistinguishable between FLC1 and FLC16 evolved replicates (Figure 5B; Pairwise post-hoc results in Table 2). With 72 h transfers, evolution to FLC1 and FLC16 again resulted in a significant reduction in growth rates relative to FLC0.25. In contrast to 24 h transfers, FLC16 evolved replicates exhibited the most pronounced effect with 72 h transfers (Figure 5C; Pairwise post-hoc results in Table 2). Overall, evolution to higher drug concentrations (FLC1 and FLC16) and longer transfer durations resulted in the highest reduction in growth rates. We also saw variations in growth rates between the ancestral and evolved replicates of each strain across the regimen (Supplementary Figure S2).

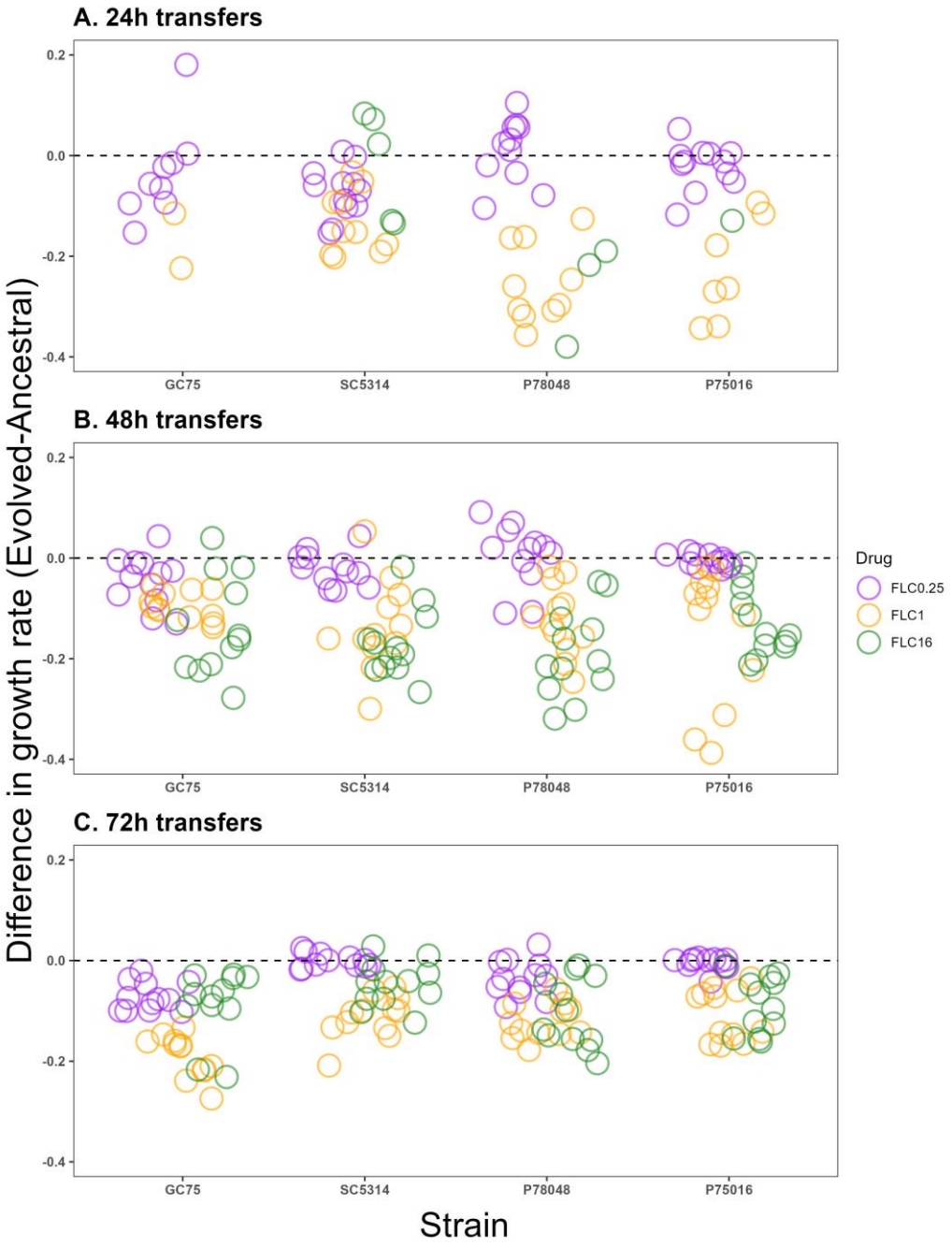

Figure 5: Growth rate measurement of four *Candida albicans* strain replicates evolved to the drug-dosing regimen. Shown here is the difference in the mean growth rate (/h) of two biological evolved and relative ancestral strain replicates evolved to a low (FLC0.25), medium (FLC1), and high (FLC16) concentration of fluconazole. Optical density was recorded every 15 min in a plate reader with constant shaking and incubation at 30 ºC for 24 h (5A), 48 h (5B), and

72 h (5C) transfer times. A negative change in growth rate represents a decrease in the growth rate of the evolved strain replicates in the evolutionary drug environment.

Table 2: Post-hoc pairwise test to compare changes in the growth rate of *C. albicans* strains across the three drug concentrations for each transfer time

Transfer Time	Drug Concentration	t-statistic	Significance Level
24 h transfer	FLC0.25 - FLC1	$t_{52} = 8.69$	$p < 0.0001$
24 h transfer	FLC0.25 - FLC16	$t_{8} = 1.69$	$p = 0.0470$
24 h transfer	FLC1 - FLC16	$t_{10} = -1.93$	$p = 0.018$
48 h transfer	FLC0.25 - FLC1	$t_{70} = 7.54$	$p < 0.0001$
48 h transfer	FLC0.25 - FLC16	$t_{74} = 10.21$	$p < 0.0001$
48 h transfer	FLC1 - FLC16	$t_{94} = 1.73$	$p = 0.150$
72 h transfer	FLC0.25 - FLC1	$t_{84} = 11.02$	$p < 0.0001$
72 h transfer	FLC0.25 - FLC16	$t_{78} = 5.11$	$p < 0.0001$
72 h transfer	FLC1 - FLC16	$t_{92} = -4.31$	$p < 0.0001$

3.5 Genome size changes were most common in higher drug concentrations and longer transfer times.

Flow cytometry was used to examine genome size changes in replicates evolved to the FLC drug-dosing regimen relative to their diploid ancestors by measuring the fluorescence intensity of the stained replicates. Evolution to FLC0.25 resulted in genome size changes in four GC75 replicates with 24 h transfers, which were fairly small and consistent with aneuploidy. Three GC75 replicates exhibited increased genome size corresponding to whole ploidy change with 72 h transfers. Interestingly, GC75 replicates evolved with 48 h transfer time and replicates from the other genetic backgrounds evolved to FLC0.25 across all three transfer times and showed no significant changes in genome size (Figure 6). Evolution to FLC1 with 24 h transfers resulted in both increases and decreases in genome size. Specifically, the two GC75 replicates that survived to the end of the

experiment decreased in genome size, as did five P78048 replicates (though one of the P78048 replicates also increased in genome size). Small increases indicative of aneuploidies were common for many SC5314 replicates, while whole ploidy changes were apparent in four P75016 replicates. For 48 h transfers, increases in genome size were pervasive, with at least one replicate among the four strains exhibiting increases consistent with whole ploidy changes. FLC1-evolved SC5314 and P75016 replicates tended to retain genome sizes similar to their diploid ancestors, GC75 replicates increased, and P78048 replicates showed both increases and decreases in genome size with 72 h transfers. These changes were small and, therefore, indicative of aneuploidies.

Evolution to FLC16 with 24 h transfers led to genome size changes only in SC5314 replicates, while the other strain replicates showed no significant change. Increases in genome size were common across all strains with 48 h and 72 h transfers but were more pronounced for GC75 and P75016 replicates.

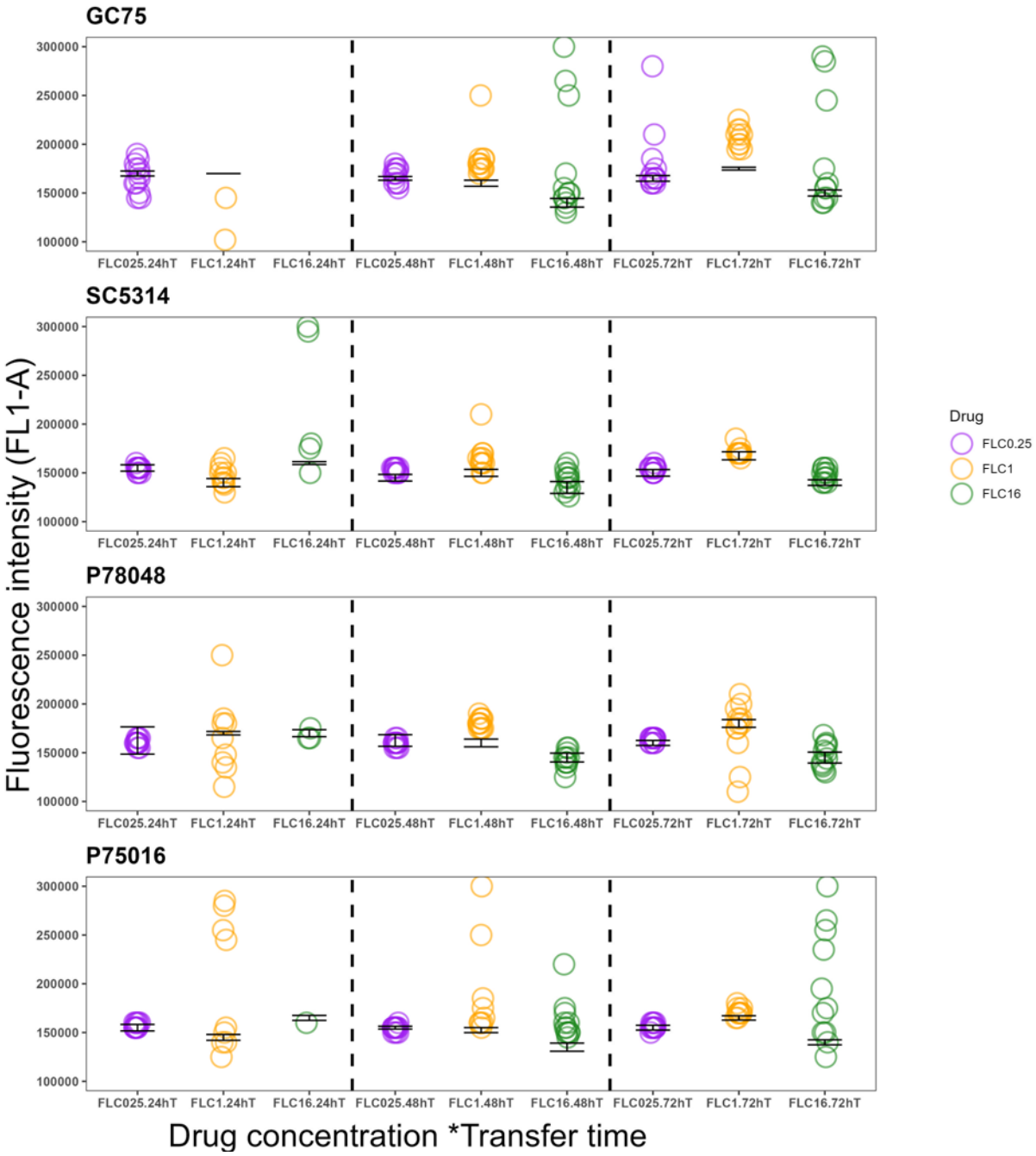

Figure 6: Genome size measurement of four *C. albicans* strain replicates evolved to the drug-dosing regimen. Each colored circle represents the fluorescence intensity (FL1-A) of the strain replicates evolved to the evolutionary drug concentration × transfer time measured using flow cytometry. The error bars represent the standard deviation in the median fluorescence intensity of the corresponding ancestral strain replicates.

CHAPTER 4: DISCUSSION

Antimicrobial resistance (AMR) is fundamentally an evolutionary process in microbial populations based on the principles of natural selection and occurs in response to the selective pressure exerted by the drug (Baquero et al., 2021). Experimental evolution is a powerful tool for testing diverse evolutionary hypotheses and studying adaptation to drugs under controlled conditions *in vitro* (Cooper, 2018; McDonald, 2019). Most experimental evolution studies in *Candida* species were conducted with the goal of studying the acquisition of drug resistance or drug tolerance and identifying the underlying genetic mechanisms in the evolved populations (Gerstein and Sethi, 2022). Hence, extinction rates have rarely been reported. In addition to adaptation, the rate of extinction is also a crucial evolutionary outcome, and it could provide valuable direction in designing therapeutic strategies or dosing regimens for treating invasive infections.

We evolved twenty-four replicates from four strains of *Candida albicans* with different genetic backgrounds to enhance our understanding of how different concentrations of fluconazole, the first-line treatment for invasive fungal infections, and transfer duration, which we are collectively referring to as the "drug-dosing" regimen, influence the rate of adaptation and extinction *in vitro*. All three drug levels (FLC0.25, FLC1, and FLC16) were higher than the ancestral MIC_{50} from strains GC75 and SC5314, while FLC1 and FLC16 were higher than the ancestral MIC_{50} from the other two strains (P78048 and P75016).

No replicates went extinct with 48 h and 72 h transfers, and extinction increased in a drug-concentration-dependent manner with 24 h transfers. This is different than (Kukurudz et al., 2022), who found extinction with both 24 h and 72 h transfers when they evolved replicate lines from eight *C. albicans* strains to an inhibitory concentration of the triazole posaconazole. The replicate survival percentage in posaconazole was variable; while no evolved replicate lines survived with 24 h transfers, the 72 h transfers resulted in approximately 50 percent survival that differed across the strain backgrounds. In line with their 72 h transfer results, extinction in our experiment was also strain-specific; GC75 replicates exhibited the highest extinction, while SC5314 showed the lowest extinction, even though both strains had the same parental MIC_{50} (0.0125 µg/mL). These findings are also similar to a recent study conducted by (Syvolos et al., 2024), who also found that the extinction rate in *C. albicans* strains increased with higher concentrations of boric acid.

Overall, we saw a strong influence of the drug-dosing regimen on the extinction rate in the evolved replicates, which was strain-specific. The rate of environmental change generally predicts extinction rates. If the rate of change exceeds the adaptation capacity of the population, it lowers the population size and allows too little time for them to adapt, thus leading to extinction (Bell & Collins, 2008; Gomulkiewicz & Holt, 1995; Lindsey et al., 2013). This suggests that under strong selection (FLC16), populations were maladapted and, therefore, went extinct. However, under low (FLC0.25) and medium (FLC1) drug concentrations, evolutionary rescue from extinction occurred due to rapid adaptation and an increase in the number of drug-resistant or drug tolerant variants in the population (Bell & Collins, 2008; Gomulkiewicz & Holt, 1995). (Jahn et al., 2017) reported that the highest extinction was found in *E. coli* populations adapted to the incremental regimen; incremental concentrations of three antibiotics- amikacin, piperacillin, and tetracycline, compared to the gradient regimen (involving passages of high drug tolerant populations into fresh gradient). They attributed the increased extinction to the rate of environmental change.

We also tested the influence of the drug-dosing regimen on the propensity of the *C. albicans* strains to evolve increased drug resistance or increased drug tolerance. Evolution in FLC0.25 resulted in increased resistance particularly in strains P78048 and P75016 with 48 h transfers. Our findings are in line with previous experiments that reported increased resistance in *C. albicans* strains evolved to drug concentrations at or below the strain's ancestral MIC (Todd et al., 2023; Yang et al., 2023). Moreover, increased resistance in bacterial species on exposure to low levels of antibiotics has also been observed*;* evolution of *S. typhimurium* and *E. coli* to sub-inhibitory levels of ciprofloxacin, streptomycin, and tetracycline selected for resistant mutants of all three antibiotics (Gullberg et al., 2011). It is possible that weak selection results in low fitness costs and, therefore, increased resistance.

We observed increased susceptibility (decreased resistance) in strains evolved to higher FLC concentrations and increased transfer times, similar to SC5314 adaptors evolved at supra-MICs by (Yang et al., 2023). This is different than (Todd et al., 2023) who found no changes in susceptibility in strains evolved to drug concentrations higher than their ancestral MIC. This is also in contrast to evolution to high fluconazole concentrations (16 µg/mL - 128 µg/mL) in *C. glabrata*, which resulted in increased resistance in most of the adaptors (Zheng et al., 2024). The adaptors also increased in tolerance at high drug concentration. This suggests that *C. albicans* is less heterogeneous than *C. glabrata* in the context of phenotypic variability as the extent of

selective pressure influenced the drug response phenotype in *C. albicans*. *C. glabrata*, on the other hand, exhibited both the phenotypes under the same selective pressure.

Increases in tolerance were common across the four strains during evolution to all three drug concentrations, particularly with 48 h and 72 h transfer times. Since tolerance is a property exhibited by a subpopulation of susceptible cells that grow slowly in supra-MIC drug concentrations that is assessed after 48 h of incubation in the presence of drug (Berman & Krysan, 2020; Rosenberg et al., 2018), we anticipated increased tolerance at higher concentrations and longer transfer times similar to the findings of (Todd et al., 2023). Interestingly, strains evolved to FLC1 and FLC16 with 24 h transfers also increased in tolerance. Similarly, SC5314 and YJB-T490 isolates passaged daily in supra-MIC fluconazole concentrations (2 to 128 μg/mL FLC) exhibited the same level and frequency of increased tolerance throughout, suggesting the presence of a drug and target interaction bypass mechanism (Yang et al., 2023). In addition to fluconazole, increased tolerance was also found when *C. albicans* strains were evolved to high concentrations of miconazole (MCZ). Increases in tolerance were dependent on the strain background and the physiological conditions (Guo et al., 2024). Interestingly, *C. glabrata* also increased in tolerance at high FLC concentrations, though the number of tolerant cells was less than the resistant cells (Zheng et al., 2024). Overall, our results also comply with past studies that found increased tolerance at high fluconazole levels.

We wanted to test if there was a correlation between change in tolerance or resistance and the growth rate of the evolved replicates in the same environment they were evolved in. Surprisingly, the evolved replicates had lower growth rates than their ancestors, and the reduction was more pronounced at higher drug concentrations (FLC1 and FLC16) and longer transfer times (48 h and 72 h). Our results are somewhat similar to what (Sun Liu-liu et al., 2023) reported when the two tolerant SC5314 adaptors obtained after exposure to a sub-inhibitory concentration of fluconazole were grown in YPD alone and YPD supplemented with the same sub-inhibitory concentration of fluconazole. The evolved adapters had reduced growth rates in both no-drug medium (YPD) and also in YPD + 0.5 μg/mL fluconazole. However, the adaptors had higher growth rates in the inhibitory concentration of fluconazole (1 μg/mL), suggesting that fitness trade-offs occur in the absence of the drug or the in same evolutionary drug environment. Therefore, we performed correlation tests for half of the evolved replicates (12 out of 24) for each strain across the drug-dosing regimen but found no significant correlation between the two phenotypic

responses (Supplementary Figure S3). Since tolerant subpopulations possess phenotypic heterogeneity, I hypothesize that some cells likely grow slower than others and reduce the average growth rate of the population.

While the mechanisms underlying increased azole tolerance have not been fully elucidated, recent studies in *C. albicans* and *C. auris* have shown the presence of aneuploid chromosomes in the azole-tolerant cells (Rasouli Koohi et al., 2023). In *C. albicans* and *C. parapsilosis,* aneuploidy has been linked to increased echinocandin tolerance (Daneshnia et al., 2023; Yang et al., 2017).The tolerant cells have susceptible MICs yet are able to grow in inhibitory concentrations of the drug. Moreover, aneuploidies have also been linked to increased azole resistance in *C. albicans*. Therefore, we performed flow cytometry analysis of our samples to assess the changes in genome size as a proxy for changes in the karyotype. Changes in genome size were widely observed, particularly for GC75 and P75016 replicates evolved to FLC1 and FLC16 with 48 h and 72 h transfer times. The changes in genome size we observed were fairly small relative to the diploid ancestors (determined by the difference in fluorescent signal intensity of the evolved and ancestral replicates), indicating that exposure to high drug concentrations also resulted in the formation of aneuploid chromosomes with longer transfer times. It is speculated that segmental or whole-chromosome trisomy of Chromosome R (ChrR) contributes to increased acquired tolerance in *C. albicans*. Most SC5314 replicates evolved to posaconazole were found to carry a partial or whole trisomy of chromosome R in addition to trisomies of Chr3 and Chr6 (Kukurudz et al., 2022). ChrR trisomy was again the most common aneuploidy in *C. albicans* adaptors evolved to subinhibitory and inhibitory concentrations of fluconazole (Yang et al., 2023). In addition to Chr3, Chr5, and Chr6 aneuploidies, ChrR trisomy was frequently found by (Todd et al., 2023). Evolution of *C. albicans* strains to increasing concentrations of miconazole resulted in ChrR trisomy in the 36 tolerant adaptors that were analyzed (Guo et al., 2024). Though we could not sequence the evolved replicates due to time constraints, I hypothesize that many of the evolved replicates in our experiments also carry a chromosome R trisomy.

Future directions

Sequencing studies are needed to pinpoint the actual karyotypic changes during evolution. We conducted a pilot restriction-assisted digestion sequencing (RAD-Seq) run to analyze the

karyotypic changes in three GC75 replicates that evolved to FLC0.25 with 24 h transfers. However, for a yet unknown reason, the library construction failed, and we recovered a few mappable reads from an initial MiSeq run. We had hoped to sequence simultaneously multiple replicates in one single run as we optimized a 96-well DNA extraction protocol that allowed us to extract DNA from 96 wells of 2-3 plates in one go.

Moreover, we employed a single drug (FLC) to study adaptation and extinction. However, in the clinic if the patient develops resistance to one antifungal, then sequential therapies are given. It would be interesting to see the influence of drug sequential application of different drugs on the rate of drug resistance and drug tolerance evolution. Also, the replicates from our experiments can be evolved to other stressors that are commonly experienced by *C. albicans* in the body to see if tolerance to the drug also leads to stress tolerance. These include oxidative stress, changes in pH, changes in osmolarity, and temperature.

Since I have studied the influence of drug-dosing regimens on adaptation in different genetic backgrounds, there are more than two variables that are interacting with one another, including the drug concentration, transfer time, and strain replicates (ancestral vs evolved). More statistical analysis is needed to tease apart the interaction using three-way interaction models.

Conclusions

Our experiments provide useful insights about the phenotype under selection when exposed to a drug-dosing regimen. Since we also considered the rate of extinction, it would be even more helpful in designing therapeutic strategies that slow down the evolution of drug resistance and drug tolerance. Overall, the influence of the regimen was strain-specific and replicates from those backgrounds showed increased tolerance and genome sizes (indicating towards aneuploidy).

REFERENCES

Abdel-Kareem, M.M., Rasmey, A.M., and Zohri, A.A. 2019. The action mechanism and biocontrol potentiality of novel isolates of *Saccharomyces cerevisiae* against the aflatoxigenic *Aspergillus flavus*. Lett. Appl. Microbiol. **68**(2): 104–111. doi:10.1111/lam.13105.

Abe-Yoshizumi, R., Kamei, U., Yamada, A., Kimura, M., and Ichihara, S. 2004. The evolution of the phenylacetic acid degradation pathway in bacteria. Bioscience, Biotechnology, and Biochemistry 68(3): 746–748.

Anderson, M. Z., Saha, A., Haseeb, A., & Bennett, R. J. (2017). A chromosome 4 trisomy contributes to increased fluconazole resistance in a clinical isolate of *Candida albicans.* Microbiology, 163(6), 856–865.

Baquero, F., Martínez, J. L., F Lanza, V., Rodríguez-Beltrán, J., Galán, J. C., San Millán, A., Cantón, R., & Coque, T. M. (2021). Evolutionary pathways and trajectories in antibiotic resistance. Clinical Microbiology Reviews, 34(4), e0005019.

Barbosa, C., Beardmore, R., Schulenburg, H., & Jansen, G. (2018). Antibiotic combination efficacy (ACE) networks for a Pseudomonas aeruginosa model. PLoS Biology, 16(4), e2004356.

Bell, G., & Collins, S. (2008). Adaptation, extinction and global change. Evolutionary Applications, 1(1), 3–16.

Bennett, R. J. (2015). The parasexual lifestyle of *Candida albicans*. Current Opinion in Microbiology, 28, 10–17.

Bennett, R. J., Forche, A., & Berman, J. (2014). Rapid mechanisms for generating genome diversity: whole ploidy shifts, aneuploidy, and loss of heterozygosity. Cold Spring Harbor Perspectives in Medicine, 4(10), a019604–a019604.

Bennett, R. J., & Johnson, A. D. (2003). Completion of a parasexual cycle in *Candida albicans* by induced chromosome loss in tetraploid strains. The EMBO Journal, 22(10), 2505–2515.

Berman, J., & Krysan, D. J. (2020). Drug resistance and tolerance in fungi. Nature Reviews. Microbiology, 18(6), 319–331.

Bhattacharya, S., Sae-Tia, S., & Fries, B. C. (2020). Candidiasis and Mechanisms of Antifungal Resistance. Antibiotics (Basel, Switzerland), 9(6). https://doi.org/10.3390/antibiotics9060312

Bing, J., Hu, T., Zheng, Q., Muñoz, J. F., Cuomo, C. A., & Huang, G. (2020). Experimental Evolution Identifies Adaptive Aneuploidy as a Mechanism of Fluconazole Resistance in *Candida auris*. Antimicrobial Agents and Chemotherapy, 65(1). https://doi.org/10.1128/AAC.01466-20

Bongomin, F., Gago, S., Oladele, R. O., & Denning, D. W. (2017). Global and multi-national prevalence of fungal diseases-estimate precision. Journal of Fungi (Basel, Switzerland), 3(4), 57.

Borman, A. M., & Johnson, E. M. (2019). *Candida, Cryptococcus* and other yeasts of medical importance. In Manual of Clinical Microbiology, 12th Edition (pp. 2056–2086). unknown.

Borman, A. M., & Johnson, E. M. (2023). Name changes for fungi of medical importance, 2020 to 2021. Journal of Clinical Microbiology, 61(6), e0033022.

Boyce, K. J. (2023). The Microevolution of Antifungal Drug Resistance in Pathogenic Fungi. Microorganisms, 11(11), 2757.

Burki, T. (2023). WHO publish fungal priority pathogens list. The Lancet. Microbe, 4(2), e74.

Burrack, L. S., Todd, R. T., Soisangwan, N., Wiederhold, N. P., & Selmecki, A. (2022). Genomic Diversity across *Candida auris* Clinical Isolates Shapes Rapid Development of Antifungal Resistance In Vitro and In Vivo. MBio, 13(4), e0084222.

Cauchie, M., Desmet, S., & Lagrou, K. (2017). *Candida* and its dual lifestyle as a commensal and a pathogen. Research in Microbiology, 168(9), 802–810.

Chaud, M. V., Rios, A. C., Alves dos Santos, C., Torqueti de Barros, C., Ferreira de Souza, J., & Ribeiro Alves, T. F. (2020). Nanostructure self-assembly for direct nose-to-brain drug delivery. In Nanomycotoxicology (pp. 449–480). Elsevier.

Chen, L., Zhang, L., Xie, Y., Wang, Y., Tian, X., Fang, W., Xue, X., & Wang, L. (2023). Confronting antifungal resistance, tolerance, and persistence: Advances in drug target discovery and delivery systems. Advanced Drug Delivery Reviews, 200, 115007.

Cooper, V. S. (2018). Experimental evolution as a high-throughput screen for genetic adaptations. MSphere, 3(3). https://doi.org/10.1128/mSphere.00121-18

Coste, A., Turner, V., Ischer, F., Morschhäuser, J., Forche, A., Selmecki, A., Berman, J., Bille, J., & Sanglard, D. (2006). A Mutation in Tac1p, a Transcription Factor Regulating CDR1 and CDR2, Is Coupled With Loss of Heterozygosity at Chromosome 5 to Mediate Antifungal Resistance in *Candida albicans*. In Genetics (Vol. 172, Issue 4, pp. 2139–2156). https://doi.org/10.1534/genetics.105.054767

Cowen, L. E., Sanglard, D., Calabrese, D., Sirjusingh, C., Anderson, J. B., & Kohn, L. M. (2000). Evolution of drug resistance in experimental populations of *Candida albicans*. Journal of Bacteriology, 182(6), 1515–1522.

Cowen, Leah E., & Lindquist, S. (2005). Hsp90 potentiates the rapid evolution of new traits: drug resistance in diverse fungi. Science, 309(5744), 2185–2189.

Daneshnia, F., Arastehfar, A., Lombardi, L., Binder, U., Scheler, J., Vahedi Shahandashti, R., Hagen, F., Lass-Flörl, C., Mansour, M. K., Butler, G., & Perlin, D. S. (2023). *Candida parapsilosis* isolates carrying mutations outside FKS1 hotspot regions confer high echinocandin tolerance and facilitate the development of echinocandin resistance. International Journal of Antimicrobial Agents, 62(1), 106831.

Diba, K., Makhdoomi, K., Nasri, E., Vaezi, A., Javidnia, J., Gharabagh, D. J., Jazani, N. H., Reza Chavshin, A., Badiee, P., Badali, H., & Fakhim, H. (2018). Emerging *Candida* species isolated from renal transplant recipients: Species distribution and susceptibility profiles. Microbial Pathogenesis, 125, 240–245.

Dunkel, N., Blass, J., Rogers, P. D., & Morschhäuser, J. (2008). Mutations in the multi-drug resistance regulator MRR1, followed by loss of heterozygosity, are the main cause of MDR1 overexpression in fluconazole-resistant *Candida albicans* strains. Molecular Microbiology, 69(4), 827–840.

Ene, I. V., Hickman, M. A., & Gerstein, A. C. (2021). The Interplay Between Neutral and Adaptive Processes Shapes Genetic Variation During *Candida* Species Evolution. Current Clinical Microbiology Reports, 8(3), 129–138.

Fisher, K. J., & Lang, G. I. (2016). Experimental evolution in fungi: An untapped resource. Fungal Genetics and Biology: FG & B, 94, 88–94.

Fisher, M. C., Alastruey-Izquierdo, A., Berman, J., Bicanic, T., Bignell, E. M., Bowyer, P., Bromley, M., Brüggemann, R., Garber, G., Cornely, O. A., Gurr, S. J., Harrison, T. S., Kuijper, E., Rhodes, J., Sheppard, D. C., Warris, A., White, P. L., Xu, J., Zwaan, B., & Verweij, P. E. (2022). Tackling the emerging threat of antifungal resistance to human health. Nature Reviews. Microbiology, 20(9), 557–571.

Fitzpatrick, D. A., Logue, M. E., Stajich, J. E., & Butler, G. (2006). A fungal phylogeny based on 42 complete genomes derived from supertree and combined gene analysis. BMC Evolutionary Biology, 6, 99.

Forche, A., Alby, K., Schaefer, D., Johnson, A. D., Berman, J., & Bennett, R. J. (2008). The parasexual cycle in *Candida albicans* provides an alternative pathway to meiosis for the formation of recombinant strains. PLoS Biology, 6(5), e110.

Ford, C. B., Funt, J. M., Abbey, D., Issi, L., Guiducci, C., Martinez, D. A., Delorey, T., Li, B. yu, White, T. C., Cuomo, C., Rao, R. P., Berman, J., Thompson, D. A., & Regev, A. (2015). The evolution of drug resistance in clinical isolates of *Candida albicans*. ELife, 4, e00662.

Gabaldón, T., & Carreté, L. (2016). The birth of a deadly yeast: tracing the evolutionary emergence of virulence traits in *Candida glabrata*. FEMS Yeast Research, 16(2), fov110.

Gabaldón, T., Martin, T., Marcet-Houben, M., Durrens, P., Bolotin-Fukuhara, M., Lespinet, O., Arnaise, S., Boisnard, S., Aguileta, G., Atanasova, R., Bouchier, C., Couloux, A., Creno, S., Almeida Cruz, J., Devillers, H., Enache-Angoulvant, A., Guitard, J., Jaouen, L., Ma, L., … Fairhead, C. (2013). Comparative genomics of emerging pathogens in the *Candida glabrata* clade. BMC Genomics, 14(1), 623.

Gabaldón, T., Naranjo-Ortíz, M. A., & Marcet-Houben, M. (2016). Evolutionary genomics of yeast pathogens in the *Saccharomycotina*. FEMS Yeast Research, 16(6). https://doi.org/10.1093/femsyr/fow064

Gerstein, A. C., & Berman, J. (2020). *Candida albicans* Genetic Background Influences Mean and Heterogeneity of Drug Responses and Genome Stability during Evolution in Fluconazole. MSphere, 5(3). https://doi.org/10.1128/mSphere.00480-20

Gerstein, A. C., Rosenberg, A., Hecht, I., & Berman, J. (2016). diskImageR: quantification of resistance and tolerance to antimicrobial drugs using disk diffusion assays. Microbiology, 162(7), 1059–1068.

Gerstein, A. C., & Sethi, P. (2022). Experimental evolution of drug resistance in human fungal pathogens. Current Opinion in Genetics & Development, 76, 101965.

Gomulkiewicz, R., & Holt, R. D. (1995). When does Evolution by Natural Selection Prevent Extinction? Evolution; International Journal of Organic Evolution, 49(1), 201–207.

Govindarajan, A., Bistas, K. G., Ingold, C. J., & Aboeed, A. (2023). Fluconazole. StatPearls Publishing.

Gullberg, E., Cao, S., Berg, O. G., Ilbäck, C., Sandegren, L., Hughes, D., & Andersson, D. I. (2011). Selection of resistant bacteria at very low antibiotic concentrations. PLoS Pathogens, 7(7), e1002158.

Guo, L., Zheng, L., Dong, Y., Wang, C., Deng, H., Wang, Z., & Xu, Y. (2024). Miconazole induces aneuploidy-mediated tolerance in *Candida albicans* that is dependent on Hsp90 and calcineurin. Frontiers in Cellular and Infection Microbiology, 14, 1392564.

Handelman, M., & Osherov, N. (2022). Experimental and in-host evolution of triazole resistance in human pathogenic fungi. Frontiers in Fungal Biology, 3. https://doi.org/10.3389/ffunb.2022.957577

Harrison, B. D., Hashemi, J., Bibi, M., Pulver, R., Bavli, D., Nahmias, Y., Wellington, M., Sapiro, G., & Berman, J. (2014). A tetraploid intermediate precedes aneuploid formation in yeasts exposed to fluconazole. PLoS Biology, 12(3), e1001815.

Hickman, M. A., Paulson, C., Dudley, A., & Berman, J. (2015). Parasexual Ploidy Reduction Drives Population Heterogeneity Through Random and Transient Aneuploidy in *Candida albicans*. Genetics, 200(3), 781–794.

Hickman, M. A., Zeng, G., Forche, A., Hirakawa, M. P., Abbey, D., Harrison, B. D., Wang, Y.-M., Su, C.-H., Bennett, R. J., Wang, Y., & Berman, J. (2013). The “obligate diploid” *Candida albicans* forms mating-competent haploids. Nature, 494(7435), 55–59.

Hirakawa, M. P., Chyou, D. E., Huang, D., Slan, A. R., & Bennett, R. J. (2017). Parasex Generates Phenotypic Diversity de Novo and Impacts Drug Resistance and Virulence in *Candida albicans*. Genetics, 207(3), 1195–1211.

Hirakawa, M. P., Martinez, D. A., Sakthikumar, S., Anderson, M. Z., Berlin, A., Gujja, S., Zeng, Q., Zisson, E., Wang, J. M., Greenberg, J. M., Berman, J., Bennett, R. J., & Cuomo, C. A. (2015). Genetic and phenotypic intra-species variation in *Candida albicans*. Genome Research, 25(3), 413–425.

Jahn, L. J., Munck, C., Ellabaan, M. M. H., & Sommer, M. O. A. (2017). Adaptive Laboratory Evolution of Antibiotic Resistance Using Different Selection Regimes Lead to Similar Phenotypes and Genotypes. Frontiers in Microbiology, 8, 816.

Jones, T., Federspiel, N. A., Chibana, H., Dungan, J., Kalman, S., Magee, B. B., Newport, G., Thorstenson, Y. R., Agabian, N., Magee, P. T., Davis, R. W., & Scherer, S. (2004). The diploid genome sequence of *Candida albicans*. Proceedings of the National Academy of Sciences of the United States of America, 101(19), 7329–7334.

Kawecki, T. J., Lenski, R. E., Ebert, D., Hollis, B., Olivieri, I., & Whitlock, M. C. (2012). Experimental evolution. Trends in Ecology & Evolution, 27(10), 547–560.

Kidd, S. E., Halliday, C. L., McMullan, B., Chen, S. C.-A., & Elvy, J. (2021). New names for fungi of medical importance: Can we have our cake and eat it too? [Review of New names for fungi of medical importance: Can we have our cake and eat it too?]. Journal of Clinical Microbiology, 59(3). American Society for Microbiology. https://doi.org/10.1128/JCM.02730-20

Ksiezopolska, E., & Gabaldón, T. (2018). Evolutionary Emergence of Drug Resistance in *Candida* Opportunistic Pathogens. Genes, 9(9). https://doi.org/10.3390/genes9090461

Kukurudz, R. J., Chapel, M., Wonitowy, Q., Bukari, A.-R. A., Sidney, B., Sierhuis, R., & Gerstein, A. C. (2022). Acquisition of cross-azole tolerance and aneuploidy in *Candida albicans* strains evolved to posaconazole. In bioRxiv (p. 2022.01.06.475277). https://doi.org/10.1101/2022.01.06.475277

Kumamoto, C. A. (2011). Inflammation and gastrointestinal *Candida* colonization. Current Opinion in Microbiology, 14(4), 386–391.

Kuznetsova, A., Brockhoff, P. B., & Christensen, R. H. B. (2017). LmerTest package: Tests in linear mixed effects models. Journal of Statistical Software, 82(13), 1–26.

LaFayette, S. L., Collins, C., Zaas, A. K., Schell, W. A., Betancourt-Quiroz, M., Gunatilaka, A. A. L., Perfect, J. R., & Cowen, L. E. (2010). PKC signaling regulates drug resistance of the fungal pathogen *Candida albicans* via circuitry comprised of Mkc1, calcineurin, and Hsp90. PLoS Pathogens, 6(8), e1001069.

Levinson, T., Dahan, A., Novikov, A., Paran, Y., Berman, J., & Ben-Ami, R. (2021). Impact of tolerance to fluconazole on treatment response in *Candida albicans* bloodstream infection. Mycoses, 64(1), 78–85.

Li, X., Yang, F., Li, D., Zhou, M., Wang, X., Xu, Q., Zhang, Y., Yan, L., & Jiang, Y. (2015). Trisomy of chromosome R confers resistance to triazoles in *Candida albicans*. Medical Mycology: Official Publication of the International Society for Human and Animal Mycology, 53(3), 302–309.

Limon, J. J., Skalski, J. H., & Underhill, D. M. (2017). Commensal Fungi in Health and Disease. Cell Host & Microbe, 22(2), 156–165.

Lindsey, H. A., Gallie, J., Taylor, S., & Kerr, B. (2013). Evolutionary rescue from extinction is contingent on a lower rate of environmental change. Nature, 494(7438), 463–467.

Lockhart, S. R., Pujol, C., Daniels, K. J., Miller, M. G., Johnson, A. D., Pfaller, M. A., & Soll, D. R. (2002). In *Candida albicans*, white-opaque switchers are homozygous for mating type. Genetics, 162(2), 737–745.

Marichal, P., Koymans, L., Willemsens, S., Bellens, D., Verhasselt, P., Luyten, W., Borgers, M., Ramaekers, F. C. S., Odds, F. C., & Vanden Bossche, H. (1999). Contribution of mutations in the cytochrome P450 14alpha-demethylase (Erg11p, Cyp51p) to azole resistance in *Candida albicans*. Microbiology (Reading, England), 145 (Pt 10)(10), 2701–2713.

Marr, K. A., Lyons, C. N., Rustad, T., Bowden, R. A., & White, T. C. (1998). Rapid, Transient Fluconazole Resistance in *Candida albicans* Is Associated with Increased mRNA Levels of CDR. In Antimicrobial Agents and Chemotherapy (Vol. 42, Issue 10, pp. 2584–2589). https://doi.org/10.1128/aac.42.10.2584

Mba, I. E., Nweze, E. I., Eze, E. A., & Anyaegbunam, Z. K. G. (2022). Genome plasticity in *Candida albicans*: A cutting-edge strategy for evolution, adaptation, and survival. Infection, Genetics and Evolution: Journal of Molecular Epidemiology and Evolutionary Genetics in Infectious Diseases, 99, 105256.

McDonald, M. J. (2019). Microbial Experimental Evolution – a proving ground for evolutionary theory and a tool for discovery. EMBO Reports, 20(8), e46992.

Mount, H. O., Revie, N. M., Todd, R. T., Anstett, K., Collins, C., Costanzo, M., Boone, C., Robbins, N., Selmecki, A., & Cowen, L. E. (2018). Global analysis of genetic circuitry and adaptive mechanisms enabling resistance to the azole antifungal drugs. PLoS Genetics, 14(4), e1007319.

Perepnikhatka, V., Fischer, F. J., Niimi, M., Baker, R. A., Cannon, R. D., Wang, Y. K., Sherman, F., & Rustchenko, E. (1999). Specific chromosome alterations in fluconazole-resistant mutants of *Candida albicans*. Journal of Bacteriology, 181(13), 4041–4049.

Perfect, J. R. (2017). The antifungal pipeline: a reality check. Nature Reviews. Drug Discovery, 16(9), 603–616.

Perlin, D. S. (2011). Current perspectives on echinocandin class drugs. Future Microbiology, 6(4), 441–457.

Pfaller, M. A., Messer, S. A., Woosley, L. N., Jones, R. N., & Castanheira, M. (2013). Echinocandin and triazole antifungal susceptibility profiles for clinical opportunistic yeast and mold isolates collected from 2010 to 2011: application of new CLSI clinical breakpoints and epidemiological cutoff values for characterization of geographic and temporal trends of antifungal resistance. Journal of Clinical Microbiology, 51(8), 2571–2581.

Rasouli Koohi, S., Shankarnarayan, S. A., Galon, C. M., & Charlebois, D. A. (2023). Identification and Elimination of Antifungal Tolerance in *Candida auris*. Biomedicines, 11(3). https://doi.org/10.3390/biomedicines11030898

Robbins, N., Caplan, T., & Cowen, L. E. (2017). Molecular Evolution of Antifungal Drug Resistance. Annual Review of Microbiology, 71, 753–775.

Rosenberg, A., Ene, I. V., Bibi, M., Zakin, S., Segal, E. S., Ziv, N., Dahan, A. M., Colombo, A. L., Bennett, R. J., & Berman, J. (2018). Antifungal tolerance is a subpopulation effect distinct from resistance and is associated with persistent candidemia. Nature Communications, 9(1), 2470.

Rybak, J. M., Michael Dickens, C., Parker, J. E., Caudle, K. E., Manigaba, K., Whaley, S. G., Nishimoto, A. T., Luna-Tapia, A., Roy, S., Zhang, Q., Barker, K. S., Palmer, G. E., Sutter, T. R., Homayouni, R., Wiederhold, N. P., Kelly, S. L., & David Rogers, P. (2017). Loss of C-5 Sterol Desaturase Activity Results in Increased Resistance to Azole and Echinocandin Antifungals in a Clinical Isolate of *Candida parapsilosis*. In Antimicrobial Agents and Chemotherapy (Vol. 61, Issue 9). https://doi.org/10.1128/aac.00651-17

Salama, O. E., & Gerstein, A. C. (2022). Differential Response of *Candida* Species Morphologies and Isolates to Fluconazole and Boric Acid. Antimicrobial Agents and Chemotherapy, e0240621.

Sanglard, D., Kuchler, K., Ischer, F., Pagani, J. L., Monod, M., & Bille, J. (1995). Mechanisms of resistance to azole antifungal agents in *Candida albicans* isolates from AIDS patients involve specific multidrug transporters. Antimicrobial Agents and Chemotherapy, 39(11), 2378–2386.

Sanglard, Dominique, Ischer, F., Monod, M., & Bille, J. (1997). Cloning of *Candida albicans* genes conferring resistance to azole antifungal agents: characterization of CDR2, a new multidrug ABC transporter gene. Microbiology (Reading, England), 143 (Pt 2), 405–416.

Santos, M. A. S., Gomes, A. C., Santos, M. C., Carreto, L. C., & Moura, G. R. (2011). The genetic code of the fungal CTG clade. Comptes Rendus Biologies, 334(8–9), 607–611.

Selmecki, A., Forche, A., & Berman, J. (2006). Aneuploidy and isochromosome formation in drug-resistant *Candida albicans*. Science, 313(5785), 367–370.

Selmecki, A., Gerami-Nejad, M., Paulson, C., Forche, A., & Berman, J. (2008). An isochromosome confers drug resistance in vivo by amplification of two genes, *ERG11* and *TAC1*. Molecular Microbiology, 68(3), 624–641.

Sender, R., Fuchs, S., & Milo, R. (2016). Revised estimates for the number of human and bacteria cells in the body. PLoS Biology, 14(8), e1002533.

Shreiner, A. B., Kao, J. Y., & Young, V. B. (2015). The gut microbiome in health and in disease. Current Opinion in Gastroenterology, 31(1), 69–75.

Sun Liu-liu, Li Hao, Yan Tian-hua, Fang Ting, Wu Hao, Cao Yong-bing, Lu Hui, Jiang Yuan-ying, & Yang Feng. (2023). Aneuploidy Mediates Rapid Adaptation to a Subinhibitory Amount of Fluconazole in *Candida albicans*. Microbiology Spectrum, 11(2), e03016-22.

Syvolos, Y., Salama, O. E., & Gerstein, A. C. (2024). Constraint on boric acid resistance and tolerance evolvability in *Candida albicans.* In bioRxiv (p. 2024.01.04.574193). https://doi.org/10.1101/2024.01.04.574193

Todd, R. T., & Selmecki, A. (2020). Expandable and reversible copy number amplification drives rapid adaptation to antifungal drugs. ELife, 9. https://doi.org/10.7554/eLife.58349

Todd, R. T., Soisangwan, N., Peters, S., Kemp, B., Crooks, T., Gerstein, A., & Selmecki, A. (2023). Antifungal Drug Concentration Impacts the Spectrum of Adaptive Mutations in *Candida albicans*. In Molecular Biology and Evolution (Vol. 40, Issue 1). https://doi.org/10.1093/molbev/msad009

Van den Bergh Bram, Swings Toon, Fauvart Maarten, & Michiels Jan. (2018). Experimental Design, Population Dynamics, and Diversity in Microbial Experimental Evolution. Microbiology and Molecular Biology Reviews: MMBR, 82(3), 10.1128/mmbr.00008-18.

Warnock, D. W. (2017). Name changes for fungi of medical importance, 2012 to 2015. Journal of Clinical Microbiology, 55(1), 53–59.

Warnock, D. W. (2019). Name changes for fungi of medical importance, 2016-2017. Journal of Clinical Microbiology, 57(2). https://doi.org/10.1128/JCM.01183-18

Wu, W., Lockhart, S. R., Pujol, C., Srikantha, T., & Soll, D. R. (2007). Heterozygosity of genes on the sex chromosome regulates *Candida albicans* virulence. Molecular Microbiology, 64(6), 1587–1604.

Yang, F., & Berman, J. (2024). Beyond resistance: antifungal heteroresistance and antifungal tolerance in fungal pathogens. Current Opinion in Microbiology, 78, 102439.

Yang, F., Scopel, E. F. C., Li, H., Sun, L.-L., Kawar, N., Cao, Y.-B., Jiang, Y.-Y., & Berman, J. (2023). Antifungal Tolerance and Resistance Emerge at Distinct Drug Concentrations and Rely upon Different Aneuploid Chromosomes. MBio, 14(2), e0022723.

Yang, F., Zhang, L., Wakabayashi, H., Myers, J., Jiang, Y., Cao, Y., Jimenez-Ortigosa, C., Perlin, D. S., & Rustchenko, E. (2017). Tolerance to Caspofungin in *Candida albicans* Is Associated with at Least Three Distinctive Mechanisms That Govern Expression of FKS Genes and Cell Wall Remodeling. Antimicrobial Agents and Chemotherapy, 61(5). https://doi.org/10.1128/AAC.00071-17

Yapar, N. (2014). Epidemiology and risk factors for invasive candidiasis. Therapeutics and Clinical Risk Management, 95.

Zhai, B., Ola, M., Rolling, T., Tosini, N. L., Joshowitz, S., Littmann, E. R., Amoretti, L. A., Fontana, E., Wright, R. J., Miranda, E., Veelken, C. A., Morjaria, S. M., Peled, J. U., van den Brink, M. R. M., Babady, N. E., Butler, G., Taur, Y., & Hohl, T. M. (2020). High-resolution mycobiota analysis reveals dynamic intestinal translocation preceding invasive candidiasis. Nature Medicine, 26(1), 59–64.

Zheng, L., Xu, Y., Wang, C., Dong, Y., & Guo, L. (2024). Parallel evolution of fluconazole resistance and tolerance in *Candida glabrata*. Frontiers in Cellular and Infection Microbiology, 14. https://doi.org/10.3389/fcimb.2024.1456907

Zhu, J., and Winans, S.C. 2001. The quorum-sensing transcriptional regulator TraR requires its cognate signaling ligand for protein folding, protease resistance, and dimerization. Proceedings of the National Academy of Sciences of the United States of America 98(4): 1507–1512. doi:10.1073/pnas.98.4.1507.

SUPPLEMENTARY FIGURES

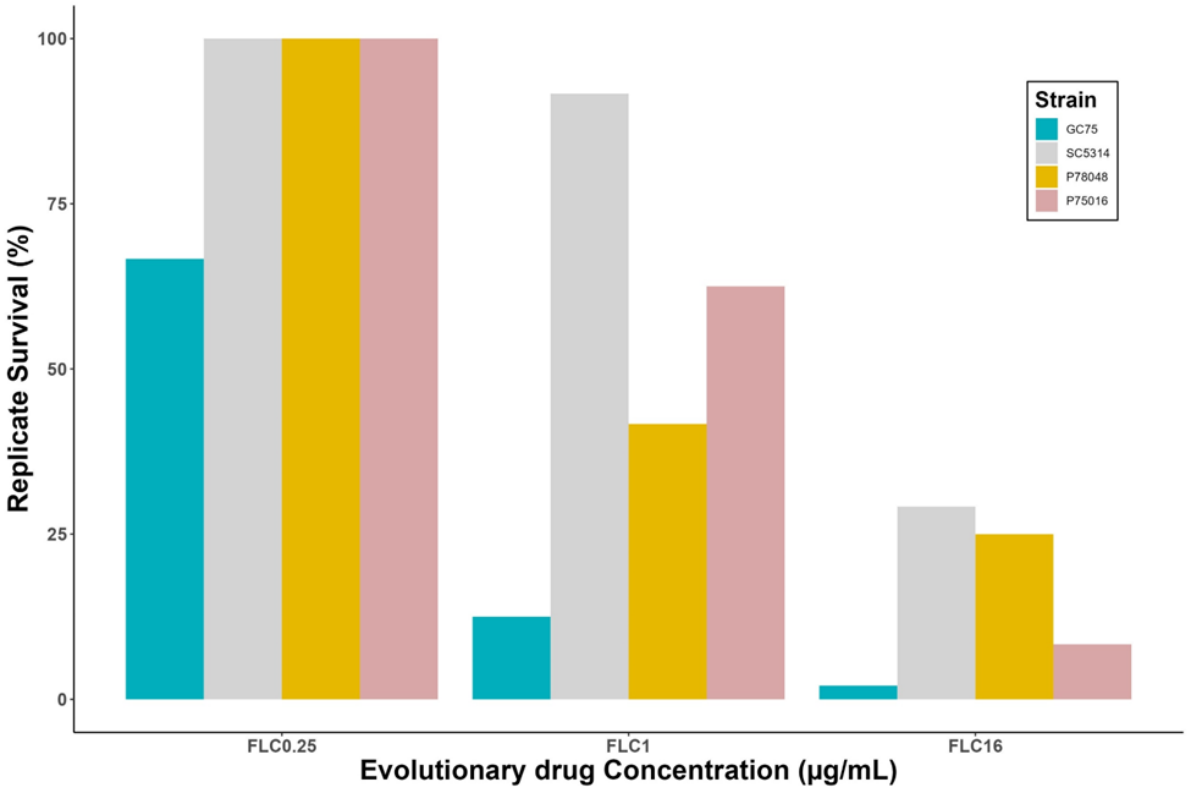

Figure S.1: Differential survival of *C. albicans* strain replicates evolved to low (FLC0.25), medium (FLC1), and high (FLC16) fluconazole concentrations with transfers every 24 h. Replicate survival percentage was calculated from 24 evolved replicates for FLC0.25 and FLC1 and 48 evolved replicates for FLC16.

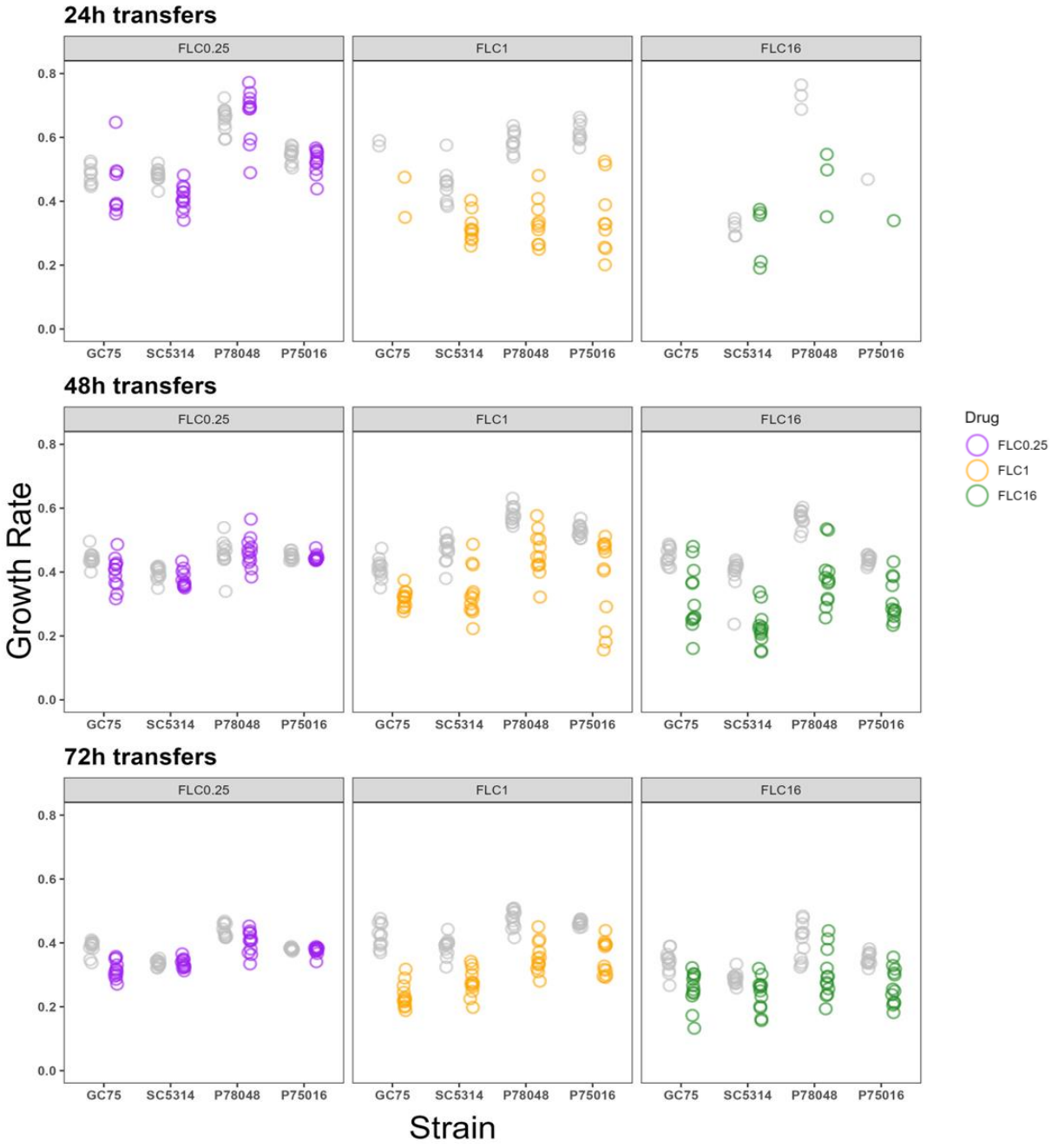

Figure S.2: Variation in growth rates of each strain's ancestral and evolved replicates across the drug dosing regimen. The gray circles represent the ancestral replicates; each colored circle represents the evolved replicates from that particular drug concentration.

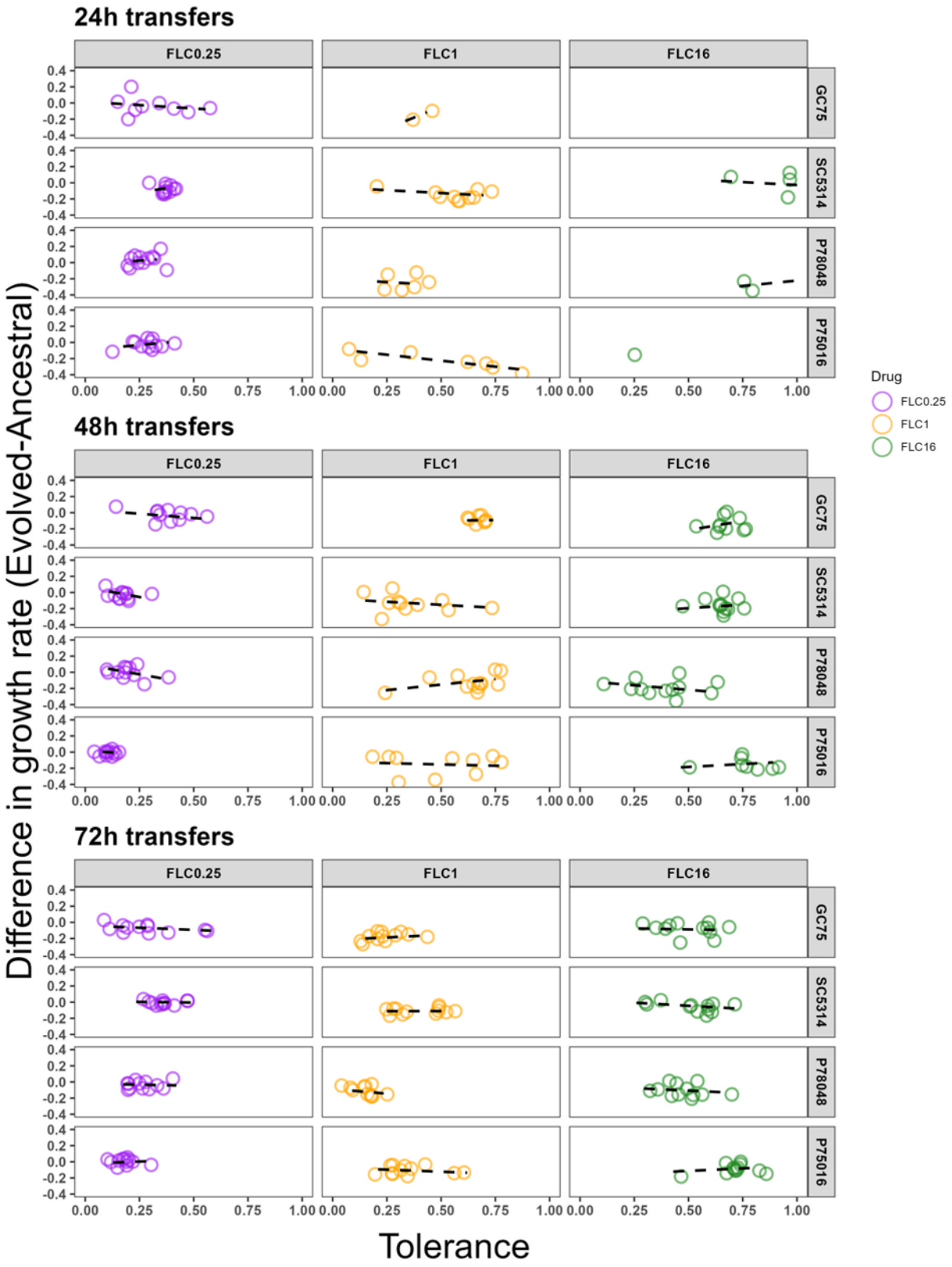
24h transfers
48h transfers
72h transfers
FLC0.25
FLC1
FLC16
GC75
SC5314
P78048
P75016
Difference in growth rate (Evolved-Ancestral)
Tolerance
Drug
FLC0.25
FLC1
FLC16

Figure S.3: Correlation between the difference in growth rate and tolerance of the evolved replicates. Differences in growth rate (evolved – ancestral) and tolerance (FoG_{20}) correlation were performed for 12 evolved replicates from each regimen**.**