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Emily Rego March 29th, 2021

Investigating *in vivo and in vitro* trade-offs of antifungal resistance in *Candida albicans*

by

Emily Rego

Dr. Meleah Hickman
Advisor

Department of Biology, Emory College

Dr. Meleah Hickman

Advisor

Dr. Levi Morran

Committee Member

Dr. Shonna McBride

Committee Member

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An abstract of
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of Emory University in partial fulfillment
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Abstract

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Biological trade-offs occur when an adaptive trait comes at the cost of another. In microbes, it has historically been thought that resistance to antifungals and antibiotics comes with costs to traits such as growth and virulence. However, recent work demonstrates that antibiotic resistance can often come at no cost to the organism, suggesting there may not be trade-offs due to resistance. As well, it remains largely unknown if there are trade-offs with drug resistance in fungal pathogens such as Ca*ndida albicans.* Here, I measured the growth rates and virulence of caspofungin resistant *C. albicans* in the absence of antifungal. Our results show that caspofungin resistant *C. albicans* did not have attenuated *in vitro* growth rates or virulence in a *C. elegans* host compared to susceptible *C. albicans.* I also measured the minimum inhibitory concentrations (MIC) of *C. albicans* to another common antifungal fluconazole to determine if evolution in caspofungin conferred an advantage in cross-adaptation. Interestingly, evolved tetraploids, but not diploids were better adapted to fluconazole than ancestral *C. albicans.* Together, this work demonstrates that caspofungin resistance does not at a cost to growth and virulence in *C. albicans* and that long term evolution in caspofungin in tetraploids allows for adaptation to fluconazole.

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Introduction

Trade-offs have been discussed extensively in biology, with discourse ranging back to the 18th century with Goethe's Law of Compensation. Goethe proposed that, "In order to spend on one side, nature is forced to economize on the other side," an idea that Darwin cited 150 years ago in the *The Origin of Species* (Ferenci, 2016; Lenoir, 1987). In modern day, biological trade-offs occur when an adaptive trait comes at the cost of performing a different trait (Basra et al., 2018). In the 1980's, considerable discussion of trade-offs began, stimulated by the observations that plants' resistance to herbivores had decreased resources available for growth (Coley et al., 1985). The researchers reasoned that limitations on energy expenditure and resources serve as the source for these trade-offs (Lenormand et al., 2018). However, within the decade, trade-offs would be applied to resistance to herbicides, pesticides and antimicrobials. Now, with the growing problem of antimicrobial resistance, investigating trade-offs due to antimicrobial resistance has become critical.

In bacteria, important characteristics like metabolism, biofilm formation, virulence, and tolerance to stressors are constrained by trade-offs (Ferenci, 2016). In *E. coli,* antibiotic resistance can come at a cost to nutrient accessibility due to decreased membrane permeability to both antibiotics and resources (Ferenci, 2016; Phan & Ferenci, 2013). Such trade-offs are due to structural constraints, a limitation in which structural changes to a protein alter function and are inherent to many biological processes (Ferenci, 2016) Though trade-offs are near omnipresent in bacteria, special interest has been given to trade-offs in which resistance comes at the cost of another function due to the burgeoning issue of antibiotic resistance. Resistance to antibiotics has been linked to slower growth rates and attenuated virulence in the absence of drug (Schulz Zur Wiesch et al., 2010). For example, quinolone resistant *E. coli* demonstrate attenuated growth rate

and decreased production of virulence factors in the absence of antibiotic (Bhatnagar & Wong, 2019; Cepas & Soto, 2020; Vila et al., 2002).

However, new research shows that trade-offs are context dependent and there is not always a cost to antibiotic resistance. Costs may be mitigated by compensatory mutations, that is, mutations that alleviate the cost incurred by a mutation at another site (Melnyk et al., 2015; Poon & Chao, 2005). In addition, it may be that resistance to antimicrobials does not occur at a cost to the organism's other functions. For example, research using *Pseudomonas aeruginosa*, a bacteria that can commonly causes respiratory and urinary tract infections, has shown resistance mutations in the *gyrA* and *parC* genes actually have higher expression of virulence genes (Bodey et al., 1983; Cepas & Soto, 2020). Therefore, trade-offs between antimicrobial resistance and other traits are likely dependent on the organism and antimicrobial being investigated. For example, in *E. coli* ceftazidime resistance came at a cost to growth rate, however, here was no significant trade-off between growth and resistance to in *E. coli* to ciprofloxacin (Basra et al., 2018). For this reason, more empirical studies regarding trade-offs associated with antimicrobial resistance are needed.

A cost to drug resistance has been considered in bacteria, but less is known about trade-offs in eukaryotic pathogens. The opportunistic fungal pathogen *Candida albicans* is a valuable organism for investigating trade-offs associated with drug resistance. *C. albicans* is typically a commensal yeast that colonizes many parts of the human body including the skin, GI tract and urogenital area, but can transition to a disease-causing pathogen (Romo & Kumamoto, 2020). Common infections include oral thrush and vaginal yeast infections, however in immunocompromised individuals *C. albicans* can present significant problems (Cole et al.,

1989). The ability to treat *Candida* infections is restricted by the limited number of antifungals in comparison to antibiotics. There are three main classes of antifungals used in the treatment of *Candida* infections: azoles, echinocandins and polyenes, all of which clinical isolates have demonstrated resistance to (Prasad et al., 2019). Currently, bloodstream *Candida* infections impact more 250,000 patients per year, work in understanding *C. albicans* resistance to antifungals, is imperative (Kullberg & Arendrup, 2015).

One of the most common drugs used for treatment is the echinocandin caspofungin. Caspofungin targets 1,3-B-D-glucansynthase which synthesizes a polymer in the *C. albicans* cell wall (Kurtz & Douglas, 1997). Resistance to caspofungin occurs through single point mutations in the *FKS1* gene (Pfaller et al., 2019). However, questions remain if caspofungin resistance allows for better adaptation to other antifungals. Due the limited classes of antifungals, multidrug resistance, defined as the resistance of a microbe to several antimicrobials that have different targets, poses a large threat (Arendrup & Patterson, 2017; Magiorakos et al., 2012). Other *Candida* species have demonstrated cross adaptation between echinocandins and a commonly used antifungal fluconazole (Pham et al., 2014). Fluconazole targets *ERG11* which is involved in the synthesis of ergosterol, a component of the plasma membrane and a different target than caspofungin (Zhou et al., 2018). In *Candida glabrata,* clinical isolates approximately 9% were resistant to only fluconazole, 2.7% were resistant to at least one echinocandin, and 1.5% were resistant to both fluconazole and at least one echinocandin (Pham et al., 2014). Therefore, further investigation needs to be done regarding cross-adaptation to caspofungin and fluconazole in *C. albicans.*

The trade-offs associated with caspofungin resistance in *C. albicans* are also not well understood. There are few studies investigating the trade-off between caspofungin resistance and growth rates in *Candida* species (Ben-Ami et al., 2011; Sharma et al., 2020). In *C. albicans*, that *fks1* mutants have lower growth rates compared to caspofungin susceptible wild-type strains in the absence of drug (Ben-Ami et al., 2011). However, more work has been done investigating, the impact of antifungal resistance on the production of virulence factors and *in vivo* virulence in *Candida* species but with contradictory results. One study found that resistance to micafungin, an echinocandin, occurs at no cost to biofilm formation, phospholipase, and aspartyl protease activities in *C. albicans* (El-Houssaini et al., 2019). However, a small number of *in vivo* studies indicate there are trade-offs between echinocandin resistance and virulence in *Candida* species (Angiolella et al., 2008; Ben-Ami et al., 2011). With much of this work *in vivo* being done using murine models, other organisms that would allow for a less limited sample size should be considered.

Most studies exploring trade-offs between caspofungin resistance and growth rate or virulence use only diploid *C. albicans* (Ben-Ami et al., 2011). Though traditionally diploid, *C. albicans* can exist as an array of ploidy states including a pseudo-stable tetraploid state with high loss of heterozygosity (LOH) rates as well as frequent concerted chromosome loss (Hickman et al., 2015). Ploidy plays an important role in the relationship between antifungal exposure and genome instability. Recent work demonstrates that tetraploids adapt faster to caspofungin and with higher levels of resistance compared to diploids (Avramovska et al., 2021). Therefore, when examining if there are trade-offs between caspofungin resistance and growth rates or virulence, including both diploid and tetraploids *C. albicans* is crucial.

Here, I investigated if there are trade-offs between caspofungin resistance and other traits in diploid and tetraploid *C. albicans* by comparing growth rates of caspofungin susceptible and resistant isolates in the absence of antifungal drugs. Our results demonstrate that in the absence of caspofungin, there is not a trade-off between growth rate and resistance regardless of ploidy. I also examined if evolution in caspofungin enabled adaptation to the antifungal fluconazole and observed that evolved tetraploids were better adapted to fluconazole compared to ancestral tetraploids. However, evolved diploids had significantly lower fluconazole MIC than ancestral diploids, suggesting ploidy may play an important role in purging deleterious mutations. Finally, using *C. elegans* as a host, I assess if caspofungin resistance leads to attenuated virulence measured by host survival and reproduction rates. *C. elegans* serve as a valuable host due to their conserved innate immune system, large brood size, and the ability for pathogens to be introduced through their diet (Irazoqui et al., 2010; Kim, 2002). I observed there is not a trade-off between caspofungin resistance and virulence in a *C. elegans* host. Overall, the results of our study suggest that resistance to caspofungin come at a cost to growth rate and virulence in the absence of antifungal, questioning a universal cost of resistance.

Materials and Methods

a.) Yeast strains, media and experimental evolution

Evolved strains were obtained as part of an experimental evolution described in (Avramovska et al., 2021). Briefly, 72 replicate diploids and 72 replicate tetraploids were inoculated in YPD, grown at 30°C for 24 hours before being normalized to 0.05 OD. Cultures were then inoculated in casitone (0.9% bacto-casitone, 0.5% yeast extract, 1% sodium citrate,

2% glucose) with 100 ug/mL of antibiotics streptomycin and ampicillin and 0.25 ug/mL of caspofungin. Replicates were evolved in this static concentration of caspofungin for 60 days.

b.) Microbroth dilution assay

Microbroth dilution assays were performed similar to as described (Avramovska et al., 2021). In short, 10 uL of ancestral and evolved lines were inoculated into 490 uL of casitone media and incubated at 30°C shaking for 48 hours. Cultures were then standardized to $1x10^3$ cells/mL and 100 uL added to 100 uL of plates with round-bottom 96 well plates containing a concentration gradient of fluconazole or caspofungin. Plates were covered with BreatheEasy tape and incubated at 30°C for 24 hours. The OD at 24 hours was read using the BioTek ELx808 plate reader and the MIC50 was calculated as when the ratio of the drug concentration over the no drug was below 50%.

c.) Growth rate and analysis

Ancestral and evolved lines were inoculated in 200 uL of YPD (1% yeast extract, 2% bactopeptone, 2% glucose, 0.004% adenine, 0.008% uridine) in a 96-well block and grown at 30°C while shaking for 48 hours. A 1:10 dilution was then performed with 50 uL of culture into 450 uL casitone with 100 ug/mL of antibiotics streptomycin and ampicillin to prevent bacterial growth. Cultures were grown for 48 hours at 30 °C while shaking. Cultures then diluted 1:200 into YPD media into a round-bottom 96 well plate. OD600 was read every 15 minutes using the BioTek ELx808 plate reader. Growth rates were calculated using a R-script in which the growth rate was the spline with the highest slope from each individual well (Gerstein et al., 2012).

d.) C. elegans strains and media

C. elegans glp-4 strain were used for liquid virulence assays where each microtiter well needed a singular adult host. glp-4 strain have germline deficiencies at the restrictive temperature (25 °C) impeding progeny production (Rastogi et al., 2015). N2 (wildtype) were used for the lineage expansion assay. Both strains were maintained on NGM plates with lawns of seeded *E. coli* (OP50). Every 4 days *C. elegans* were moved to a new NGM plate seeded with *E. coli*. glp-4 *C. elegans* were stored at 15°C and N2 *C. elegans* at 20°C.

e.) Seeding plates for liquid virulence assay and lineage expansion assay

A 10uL of glycerol stock of caspofungin resistant evolved replicate (diploid 63) and the diploid ancestor (MH84) were inoculated in YPD for 24 hours shaking at 30°C. The optical density of the cultures was measured at 600nm (OD_{600}) and then concentrated to 3 OD. Simultaneously, a single colony of *E. coli* (OP50) was inoculated into 50mL of LB and incubated at 30°C for 24 hours while shaking. The culture was the pelleted and diluted to 200 mg/mL. The uninfected control treatments consisted of NGM plates seeded with 31.25 uL *E. coli* and concentrated with water to a total volume of 250 uL per plate. The *Candida* treatments comprised of NGM plates seeded with 6.25 uL of caspofungin susceptible (diploid ancestor MH84) or caspofungin resistant (diploid ancestor MH84) along with 31.25 uL of *E. coli* and concentrated with water to a total volume of 250 uL per plate.

f.) Population synchronization for liquid virulence assay and lineage expansion assay

C. elegans populations were synchronized similarly to previously with some alterations (Smith & Hickman, 2020). To synchronize populations, *C. elegans* and eggs were washed off of NGM plates using M9 buffer, transferred to a 15 mL conical, and pelleted by centrifugation

(1900 rpm for 2 minutes). M9 was removed and 25% bleach wash diluted with water was added and mixed via inversion. Eggs were collected by centrifugation (1900 rpm for 2 minutes) and 25% bleach wash was removed, washed once more with M9, and resuspended in 1 mL of M9. 20 uL was pipetted onto a concave slide to determine the egg concentration and ~100 eggs were added to each control plate or *Candida* plate.

g.) Liquid virulence assay

C. elegans were exposed to either *E. coli* or *E. coli* and *C. albicans* strain for four days. Following exposure, *C. elegans* were washed off plates with M9 buffer, pelleted through centrifugation and M9 removed, and washed with 2% bleach and inverted to mix. The tube was then centrifuged, and 2% bleach was pipetted off and washed with M9 again before mixing and suspending in 1 mL of M9. *C. elegans* were then pipetted onto M9 plates and dried under the hood for approximately an hour. A single adult host was transferred to a each well which contained 100 uL of media (79% M9, 20% BHI, 10 ug/mL cholesterol in ethanol, 90 ug/ml of kanamycin). 96 *C. elegans* were added to 48 wells containing media with 4 ug/ml caspofungin and 48 wells containing media without antifungal. *C. elegans* survival was monitored for 6 days. *C. elegans* were recorded as dead when they did not respond to being poked with a pick.

h.) Lineage expansion assay

The lineage expansion assay was performed as previously detailed (Feistel et al., 2020). Briefly, a single L4 *C. elegans* was transferred to NGM plates with 300 uL of seed of either *E. coli* or *E. coli with C. albicans*. Plates were incubated at 20°C for 5 days. The plates were then washed with M9 off the plate and into 15 mL conical and placed in the 4°C for 1 hour for the *C.*

elegans to fall to the bottom of the tubes. 20 uL was then taken from each tube and counted six times to determine the population size of the plate.

i.) Statistical analysis

Statistical analysis was performed using GraphPad Prism 8 software. Unpaired Mann-Whitney U-test was used to determine significant of differences in growth rates and in population size in lineage expansion assays. The relationship between the caspofungin MIC and fluconazole MIC of evolved populations was determined by a linear regression model. Differences in survival curves of the liquid virulence assay was determined by Log-Rank Mantel Cox test.

Results

Evolved diploid and tetraploids have varied resistance to caspofungin

Caspofungin resistance is defined by the clinical breakpoint at the minimum inhibitory concentration (MIC) of 1 ug/mL (Santos et al., 2014). Therefore, 72 evolved diploids and 72 evolved tetraploids were then classified as either caspofungin sensitive (MIC < 1 ug/mL) or resistant (MIC $\geq$ 1 ug/mL) (Figure 1a and 1c). As hypothesized more tetraploids (38.8%) were caspofungin resistant than diploids (15.3%). To establish that caspofungin resistant lines grow better in caspofungin than susceptible lines, I measured the growth rates of resistant and susceptible lines in the presence of caspofungin. I expect that caspofungin resistant diploids and tetraploids will have higher growth rates than susceptible diploids and tetraploids in the concentration of caspofungin (0.25 ug/mL) they were evolved in for 60 days. Diploid resistant-lines had growth rate average of 0.39 compared to susceptible at 0.08, an approximately 5-fold-difference (Fig. 1b). This is similar to tetraploids in which resistant lines average growth rate was

0.39 in contrast to 0.11 of susceptible lines, an approximately 3.5-fold difference (Fig 1d). As predicted, the caspofungin resistant diploids and tetraploids *C. albicans* grew significantly better than caspofungin susceptible yeast (Fig. 1b and Fig. 1d). I therefore concluded that caspofungin resistant replicates are better adapted to caspofungin then susceptible replicates allowing for further investigation of how this relationship changes in the absence of caspofungin.

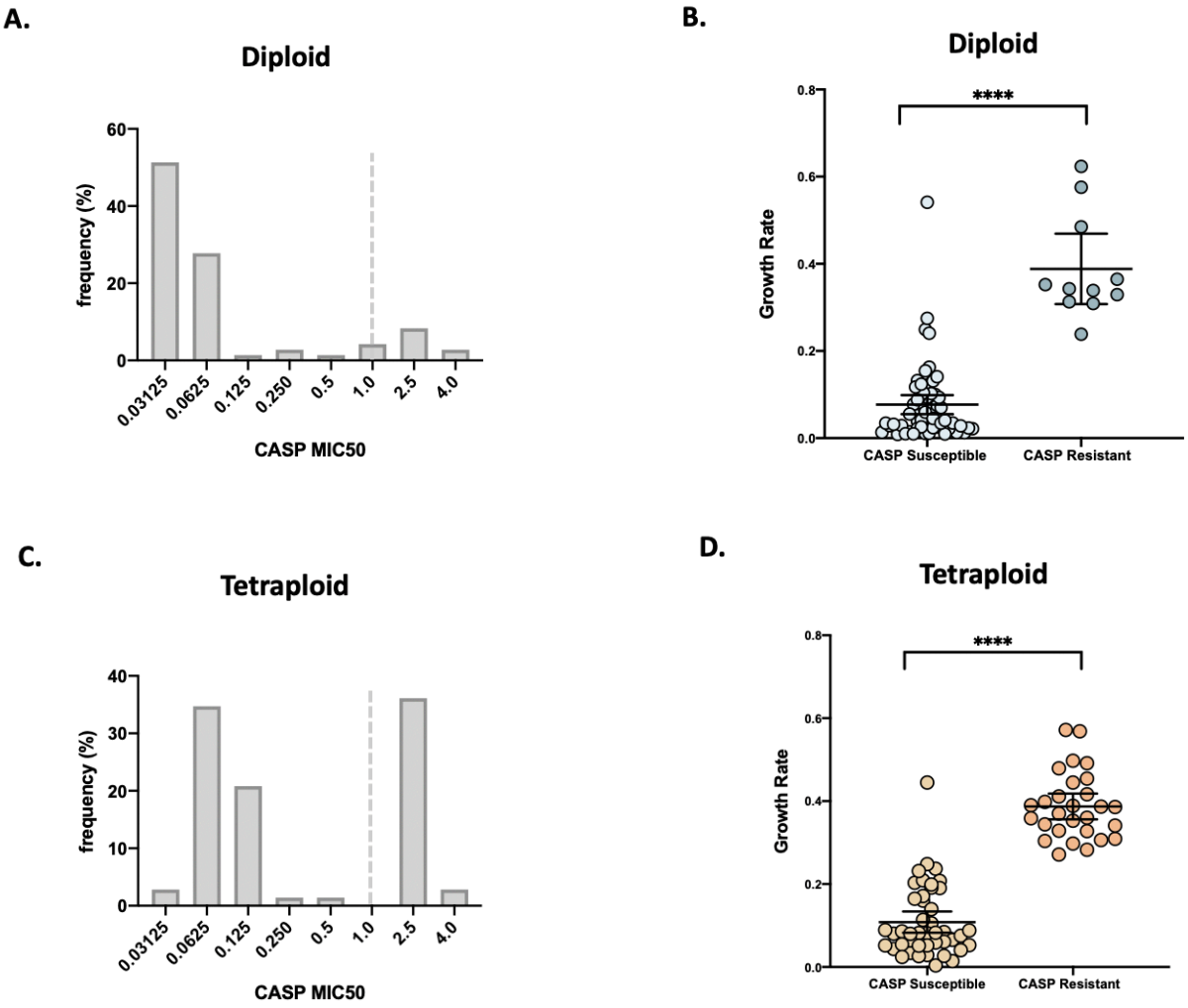


Figure 1: Caspofungin resistant isolates grow significantly better than sensitive populations in the presence of caspofungin

A) The minimum concentration of caspofungin that inhibits 50% of growth for 72 evolved diploids. The grey line at the point of clinical resistance (1 ug/ml) separates the caspofungin susceptible (MIC50 <1) and resistant (MIC50 ≥1) diploid *C. albicans.*

B) The growth rate of caspofungin susceptible (light blue, n=61) and resistant (blue, n=11) diploids in the concentration of caspofungin they were evolved in (0.25 ug/ml). The black line represents the mean and error bars are at the 95% confidence interval. Statistical comparison is between resistant and sensitive *C. albicans* and represents a Mann-Whitney U-test (****, p<0.0001).

C) The minimum concentration of caspofungin that inhibits 50% of growth for evolved tetraploids. Clinical resistance is indicated by grey dashed line and separates the caspofungin susceptible (MIC50 <1) and resistant (MIC50 ≥1) tetraploid *C. albicans.*

D) Growth rates of caspofungin susceptible (yellow, n=44) and resistant (orange, n=28) tetraploids in the concentration of caspofungin they were evolved in (0.25 ug/ml). The black line represents the mean and error bars are at the 95% confidence interval. Statistical comparison same as in 'B'.

Resistance to caspofungin does not lead to decreased growth rate in the absence of antifungal

C. albicans with resistance mutations in *FKS1*, a gene that encodes for the drug target of caspofungin, have attenuated growth rates compared to wild-type yeast (Ben-Ami et al., 2011). To test if there is a trade-off to growth in the absence of drug with caspofungin resistance, I measured the growth rates of caspofungin susceptible and resistant *C. albicans* in the absence of caspofungin. If there is a growth trade-off associated with being caspofungin resistant, I predict to observe lower growth rates for caspofungin resistant diploids and tetraploids. Diploid resistant lines had growth rates average of 0.41 compared to susceptible at 0.36 (Fig. 2a). In tetraploids, there was no difference in average growth rate between resistant lines and susceptible lines, with growth rates of 0.39 and 0.37 respectively (Fig. 2b). Therefore, we observed no trade-off between caspofungin resistance and growth rate for either ploidy. In addition, caspofungin resistant diploids grew significantly better ($p < 0.05$, Mann-Whitney U-test) than susceptible lines. This does not support the prediction that tetraploids may experience less of a cost to resistance than diploids due to shedding delirious mutations through concerted chromosome loss. Altogether, these results suggest that there is not a cost to growth rates that occur with caspofungin resistance.

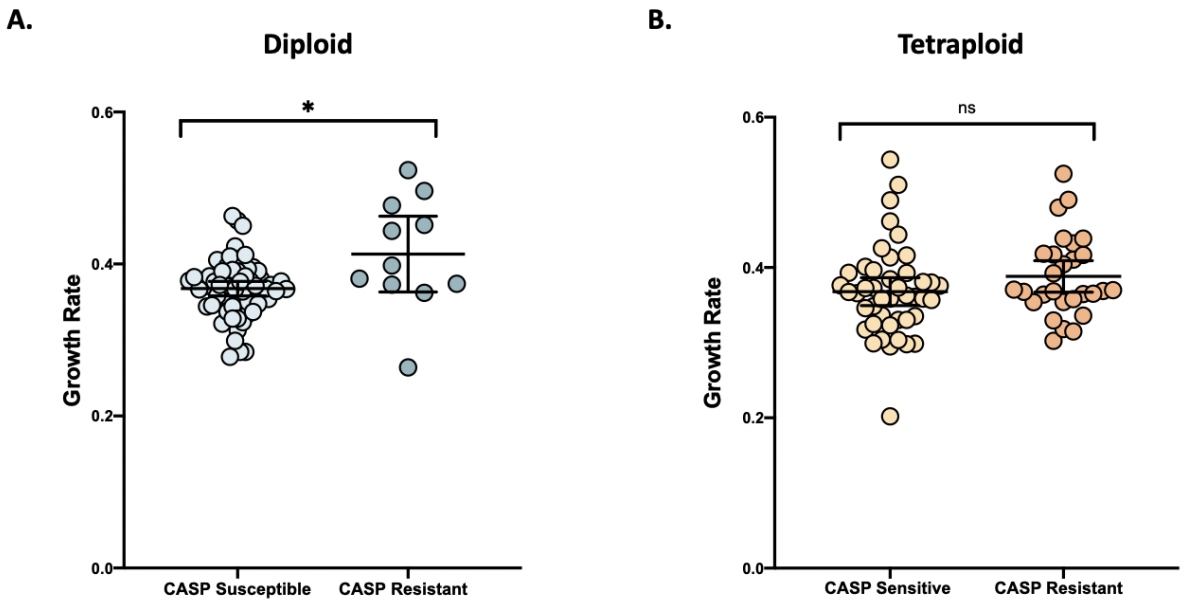


Figure 2: Caspofungin resistant *C. albicans* do not have attenuated growth in the absence of caspofungin

A) Growth rate of caspofungin susceptible (light blue, n=61) and resistant (blue, n=11) diploids in the absence of antifungal. The black line represents the mean and error bars are at the 95% confidence interval. Statistical comparison is between resistant and sensitive *C. albicans* and represents a Mann-Whitney U-test (*, p<0. 05).

B.) Calculated growth rate of tetraploid caspofungin susceptible (yellow, n=44) and resistant (orange, n=28) *C. albicans* in the absence of antifungal. Black horizontal lines represent the mean. Statistical comparison is the same as 'A' and error bars represent the 95% CI.

Evolved diploids are less resistant to fluconazole than ancestral C. albicans

After demonstrating that caspofungin resistant *C. albicans* do not have attenuated growth rates in the absence of caspofungin, we considered the effects of long-term evolution in caspofungin on the ability to adapt to other antifungals. Due to caspofungin being mutagenic, evolved diploids and tetraploids may experience increased ability to adapt to fluconazole, which has a distinct drug target from caspofungin (Zhou et al., 2018). Therefore, to determine if *C. albicans* evolved in caspofungin were more resistant to fluconazole than their ancestors, we

compared fluconazole MIC50 of evolved diploids and tetraploids to their ancestors. The average MIC50 in fluconazole of evolved diploids (0.63 ug/ml) was significantly less than the ancestral diploid MIC50 (1.0 ug/ml) in fluconazole ($p<0.0001$ Mann-Whitney U-test). As well, the majority (approximately 60%) of the evolved diploids had fluconazole MIC50s less than the ancestral diploid (Fig. 3a). However, in tetraploids the average MIC50 in fluconazole of evolved tetraploids was 0.99 ug/ml and was significantly more than the ancestral tetraploid (0.5 ug/ml) with approximately 40% of all evolved replicates with fluconazole MIC50s greater than their ancestor ($p<0.05$, Mann-Whitney U-test, Fig 3c). Therefore, though evolved tetraploids are more fluconazole resistant than their ancestors, diploids are not.

However, due to previous findings in other *Candida* species that resistance to echinocandins may allow for resistance to fluconazole, I also considered if resistance to caspofungin (CASP MIC50 ≥ 1) aids in cross adaptation to fluconazole (Pham et al., 2014). I compared the caspofungin and fluconazole MIC50s of all evolved yeast and to determine if there was an association between resistance to caspofungin and fluconazole. If cross-adaptation is occurring, then high caspofungin MIC will positively correlate to high fluconazole MIC. For both diploids and tetraploids, there was not a significant relationship between resistance to caspofungin and to fluconazole (Fig. 3b and Fig. 3d). This indicates that these caspofungin-resistant *C. albicans* strains do not demonstrate cross adaptation to fluconazole.

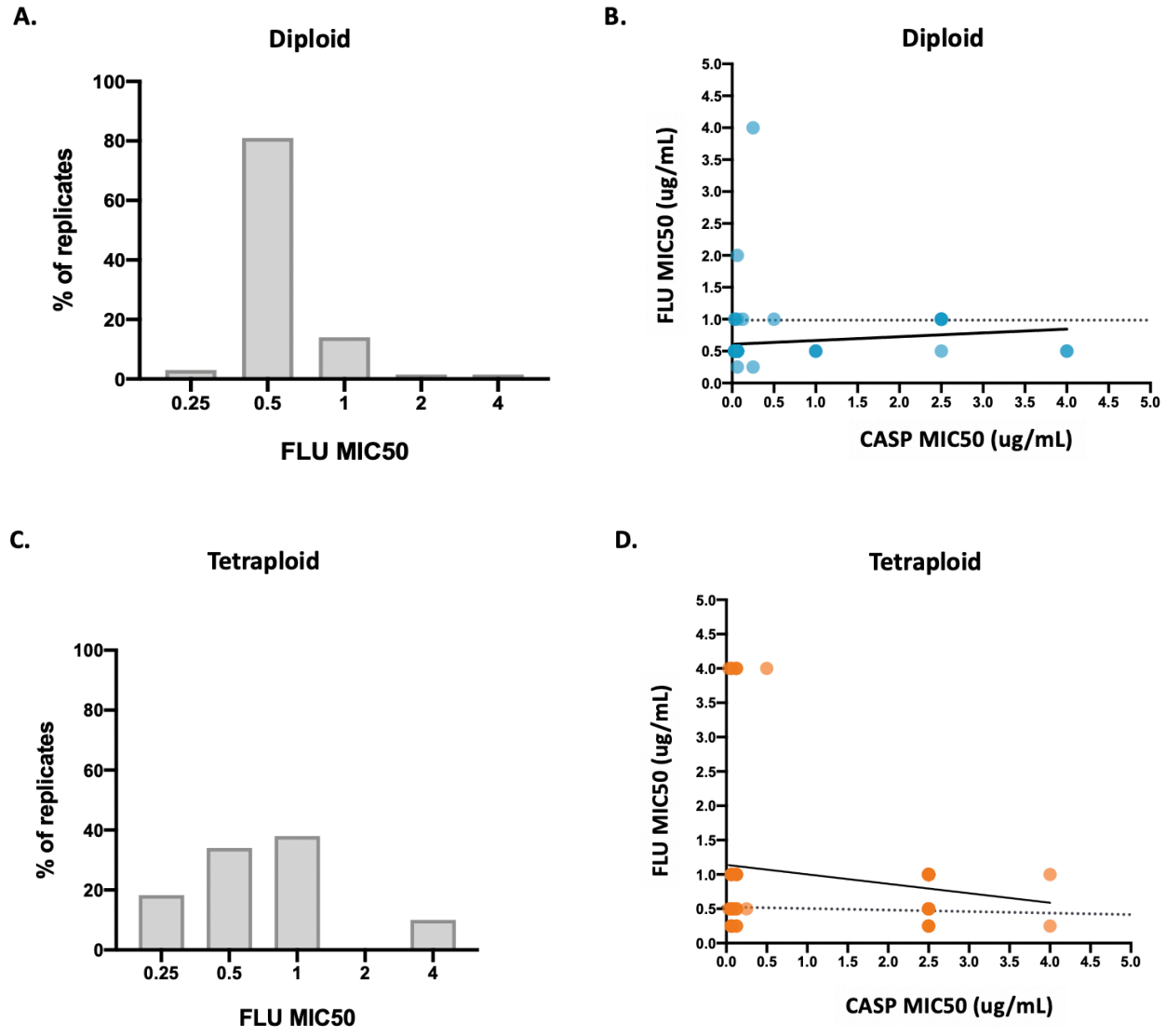


Figure 3: Evolution in caspofungin leads to higher fluconazole MIC in tetraploids, but not diploids

A) The minimum concentration of fluconazole that inhibits 50% of growth for 72 evolved diploids. The grey line is at the ancestral resistance (1.0 ug/mL) of diploid *C. albicans.*

B) The MIC50 in fluconazole (y-axis) by the MIC50 in caspofungin (x-axis) by of all evolved diploids. Black line represents the line of best fit determine by linear regression (y=0.059x+ 0.6). Slope of linear regression line is not significantly different from zero (ns, p>0.05). Grey dashed line represents the fluconazole MIC50 of ancestral populations that have not been exposed to caspofungin (FLU MIC50 = 1 ug/ml).

C) The minimum concentration of fluconazole that inhibits 50% of growth for 72 evolved tetraploids. The grey line represents the MIC of ancestral of tetraploid *C. albicans* (0.5 ug/mL).

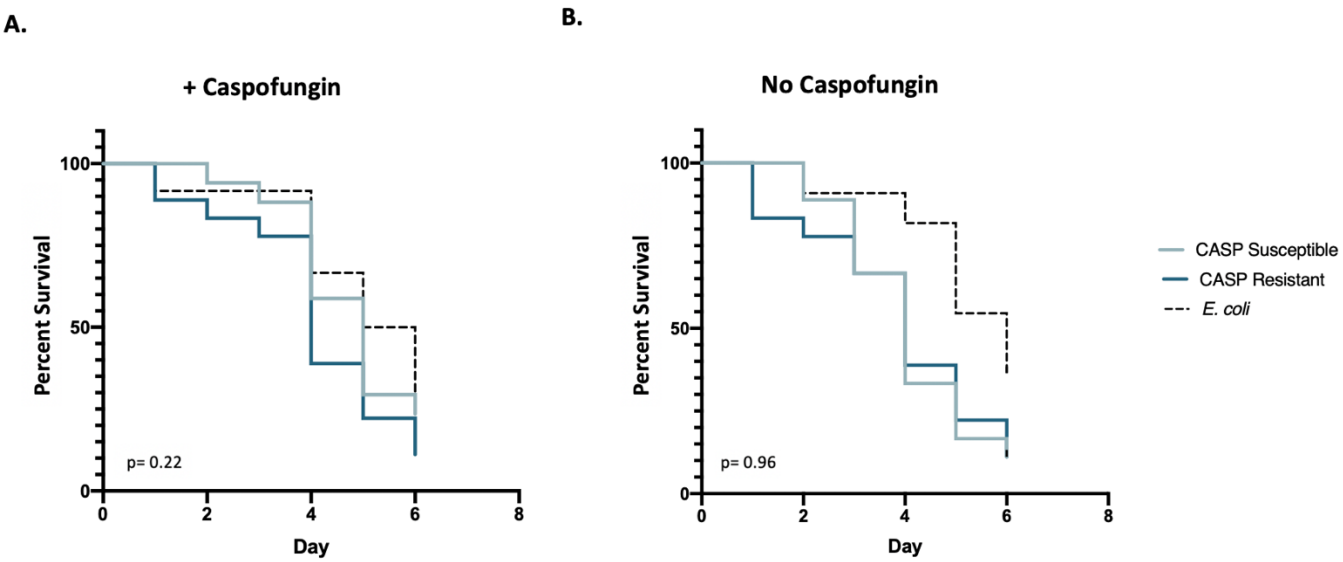
D) The MIC50 in fluconazole (y-axis) by the MIC50 in caspofungin (x-axis) by of all evolved diploids. The black line represents the linear regression determined line of best fit which is not significantly different from zero (y= -0.138x + 1.139, ns, p>0.05). Grey dashed line represents the fluconazole of MIC50 of ancestral populations (FLU MIC50 = 0.5 ug/ml).

There is not a trade-off between caspofungin resistance and virulence in C. albicans

Due to previous studies in bacteria demonstrating that virulence does not always come at a cost to antibiotic resistance, I investigate if resistance to caspofungin comes at a cost to virulence in *C. albicans* (El-Houssaini et al., 2019). To investigate if there is a trade-off, I developed a liquid virulence assay to record host survival rates in the presence and absence of caspofungin. Previously, conventional survival assays on solid media do not allow for measuring virulence in the presence of antifungal, solid media with antifungal treats the *C. elegans* but not the infection (Feistel et al., 2020). The assay was designed similar to previous work that demonstrated that treatment with caspofungin could treat Candida infections in *C. elegans* (Breger et al., 2007). Therefore, I predict that hosts infected with caspofungin susceptible *C. albicans* will survive better in media with caspofungin equal to resistant *C. albicans* MIC50. I observed that hosts infected with susceptible yeast have higher survival rates than host infected with resistant yeast, however the difference is not significant ($p > 0.05$, Mantel-Cox test, Fig. 4a).

To test if there is a trade-off between caspofungin resistance and virulence, *C. elegans* were infected with caspofungin susceptible or resistant *C. albicans*. If caspofungin resistance comes at a cost to virulence, I anticipate that *C. elegans* infected with caspofungin resistant yeast will have higher survival rates compared to host infected with caspofungin sensitive yeast. Uninfected *C. elegans* survived significantly better than hosts infected with caspofungin susceptible and resistant *C. albicans* ($p < 0.05$, Mantel-Cox test, Fig. 4b). Though *Candida* infected *C. elegans* had significantly lower survival rates than uninfected *C. elegans*, I observed no significant difference ($p > 0.05$, Mantel-Cox test, Fig. 4b) between survival rates between

hosts infected with caspofungin resistant and those infected with caspofungin susceptible lines which contrasts our previously mentioned predictions. To establish that there is not a trade-off between caspofungin resistance and virulence in the absence of antifungal, I also performed a lineage expansion assay. In contrast to the liquid killing assay, lineage expansion measures virulence in differences in population growth from a single founder *C. elegans* and has been utilized previously by the lab (Feistel et al., 2020). The average lineage produced by host infected with susceptible *C. albicans* was 2139 progeny compared to average of 1839 produced by single founder hosts infected with resistant *C. albicans* (Fig. 4). The viable population size for hosts infected with susceptible and resistant *C. albicans* was not significantly different ($p>0.05$, Mann-Whitney U-test). Therefore, in the absence of caspofungin, both the liquid virulence assay and the lineage expansion demonstrate there is not a trade-off between resistance to caspofungin and virulence in *C. albicans.*



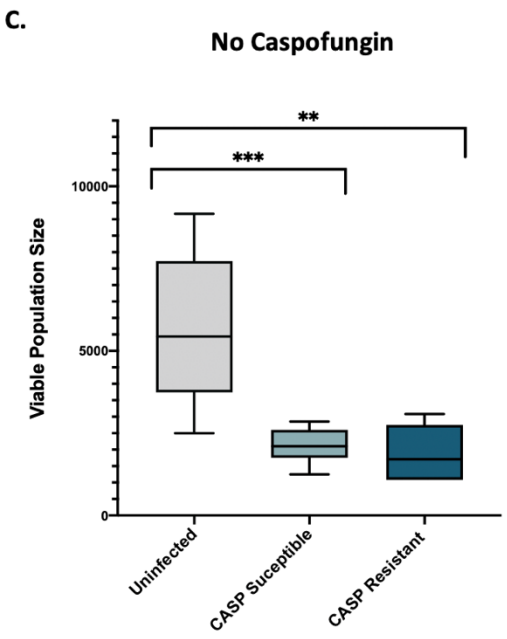


Figure 4: Survival curves of *C. elegans* infected with caspofungin susceptible and resistant *C. albicans* in the presence and absence of caspofungin

A) Survival curves for *C. elegans* populations that are uninfected (only exposed to *E. coli,* grey, n=11) or infected with caspofungin susceptible (light blue, n=18) and resistant (dark blue, n=18) *C. albicans* in media containing 8 ug/ml of caspofungin. Statistical significance was determined by using Log-rank (Mantel-Cox) test, p-values comparing survival rates of worms infected with susceptible and resistant *C. albicans* are included. In the presence of caspofungin, worms infected with susceptible or resistant *C. albicans* were not significantly different than the uninfected ($p>0.05$).

B) Survival curves for *C. elegans* populations in the absence of antifungal. Treatment groups and statistical comparison are the same as 'A'. *C. elegans* infected with susceptible and resistant *C. albicans* had significantly lower survival rates than the uninfected *C. elegans* ($p<0.05$).

C) Box and whiskers plot of the of viable population size of F1 and F2 progeny from a single host after 7 days. Uninfected are represented in grey (n=8), host infected with caspofungin susceptible in light blue (n=11), and host infected with caspofungin susceptible in dark blue (n=7). Whiskers go from the minimum to the maximum and the box represents the 25th to 75th quartiles. Statistical comparison is between uninfected and infected hosts and represents a Mann-Whitney U-test (*** or **, $p<0.0005$, $p<0.005$). Difference between lineage growth of host infected with caspofungin susceptible and resistant *C. albicans* was not significant ($p> 0.05$).

Discussion:

In this study, I explore if there is a cost to being caspofungin resistant in the opportunistic fungal pathogen *C. albicans* by investigating if drug-resistant strains have attenuated growth rates, virulence and ability to adapt to other antifungals. In the absence of caspofungin, resistant *C. albicans* do not have attenuated *in vitro* growth or virulence in a *C. elegans* host compared to susceptible *C. albicans*. Therefore, our results demonstrate that in *C. albic*ans there is not a trade-off between caspofungin resistance and growth rate or virulence. Surprisingly, caspofungin evolved tetraploids but not diploids were more resistant to fluconazole than their ancestors. Altogether, these results that demonstrate that trade-offs due to antimicrobial resistance are often complex and should not be always assumed.

In many bacterial species, antibiotic resistance had been demonstrated to come at a cost to growth rate (Ferenci, 2016; Phan & Ferenci, 2013; Schulz Zur Wiesch et al., 2010). However, recent work suggests that the negative relationship between growth rate and resistance is often dependent on the drug and the abundance of nutrients in the environment (Basra et al., 2018). Therefore, in this paper, I attempted to specifically investigate if there are growth trade-offs to caspofungin resistant *C. albicans* in the absence of antifungal. In contrast to findings by other researchers, caspofungin resistant *C. albicans* did not demonstrate attenuated growth rates in the absence of caspofungin (Ben-Ami et al., 2011). The lack of growth trade-off due to antifungal resistance may be attributed to the loss of deleterious mutations or the accumulation of compensatory mutations during evolution. Compensatory mutations may counteract the costs associated with antimicrobial resistance. For example, treatment with caspofungin can cause a compensatory increase in chitin in the fungal cell in *C. albicans* (Walker & Munro, 2020).

Further investigation could determine if there are compensatory mutations in genes related to growth in the resistant lines.

Given the mutagenic nature of caspofungin, strains evolved in this antifungal may also have adapted to other antifungals. For diploid evolved lines, I observe reduced fluconazole MIC compared to their ancestors, suggesting that there are costs from prolonged exposure to caspofungin. However, an unexpected result was that evolved tetraploids were more resistant to fluconazole than ancestral tetraploids (Fig. 3b) In addition, there was no association between resistance to caspofungin and resistance to fluconazole in diploids or tetraploids that resistance to caspofungin did not confer resistance to fluconazole. Resistance to fluconazole may occur in evolved tetraploids but not diploids due to exposure to antifungals disproportionately increasing genome instability in tetraploids compared to diploids (Avramovska & Hickman, 2019). Therefore, the increased genomic instability caused by evolution in caspofungin may allow for adaptation to other antifungals due to mutations in genes that control fluconazole resistance. Resistance to fluconazole has been demonstrated to occur through mutations in different genes such as *ERG11* which is involved in ergosterol synthesis or *CDR1* which encodes an efflux pump (Arendrup & Patterson, 2017).

Some studies have demonstrated that caspofungin resistance in *Candida* species leads to attenuated virulence in a range of different hosts (Ben-Ami et al., 2011). However, in our study, I found no difference between the virulence of caspofungin susceptible and resistant *C. albicans* in a *C. elegans* host. The absence of a cost to virulence due to antifungal resistance again may be attributed to the loss of deleterious mutations or the accumulation of compensatory mutations during evolution. In other organisms, antimicrobial resistance and hypovirulent have been

demonstrated to rapidly evolve compensatory mutations and restore virulence (Bjorkman et al., 1998). Further investigations in if there are compensatory mutations in important *C. albicans* virulence pathways would help illuminate why there is no observed trade-off.

In conclusion, our results demonstrate that a cost to antimicrobial resistance is not universal. Trade-offs due to antimicrobial resistant are dependent on a multitude of factors including the drug, organismal properties and what functions are impacted by evolved resistance. Our findings complement recent work that suggest that antimicrobial resistance may not necessarily come at a cost to growth rate and virulence (Basra et al., 2018; Roux et al., 2015). Though ploidy does not appear affect trade-offs of caspofungin resistance, it may play a role in adaptation to antifungals in eukaryotic pathogens such as *C. albicans*. Therefore, this study may serve as an important reminder of the perils of applying broad concepts like a universal cost of resistance to complex and dynamic microbes like *C. albicans*.

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