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Fungal pathogen genome stability and adaptive potential depend on host immune status and pathogen ploidy

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Abstract

Fungal pathogen genome stability and adaptive potential depend on host immune status and pathogen ploidy

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Candida albicans is the predominate opportunistic fungal pathogen of humans and can cause a broad range of infection, depending on host immune function. The interaction between the host and *C. albicans* facilitates the generation of natural variation and includes changes in ploidy. This natural variation is due to the high-level of genetic plasticity, where large-scale genome perturbations, including recombination, chromosomal aneuploidy, and whole-shifts in ploidy, are frequent. While research has demonstrated that genomic perturbations in *C. albicans* are frequent and lead to phenotypic heterogeneity, less is known about how host and pathogen genetic background impacts *C. albicans* genome dynamics and evolvability. To investigate how pathogen genetic background and ploidy impacts *C. albicans* genome dynamics *in vivo*, I evaluated the genome stability of three diploid and three tetraploid *C. albicans* strains representing different backgrounds within the host environment. I found that tetraploids rapidly undergo chromosome loss within the host environment regardless of genetic background, but diploids remain approximately diploid. However, clinical diploids had a higher frequency of loss-of-heterozygosity (LOH) compared to the laboratory diploid. Together, these findings suggest that *C. albicans* ploidy and genetic background impact the rate at which genotypic heterogeneity is generated within a host environment. Next, I evaluated how host genotype, specifically immune status, impacts *C. albicans* genome stability. Host-produced reactive oxygen species (ROS) elevated genome instability regardless of pathogen genetic background, but host-produced antimicrobial peptides (AMPs) only elevated *C. albicans* genome instability in one of the strains tested. These results suggest that a specific pathogen genotype and host genotype/environment (immunity) interaction is responsible for generating *C. albicans* genome instability. Finally, I investigated how host immunity and pathogen ploidy impact *C. albicans* virulence evolution. I predicted that more genetic variation would be generated in *C. albicans* evolved in immunocompetent hosts compared to immunocompromised hosts, enabling evolution to occur faster. Both diploid and tetraploid *C. albicans* evolved virulence rapidly in immunocompetent but not immunocompromised hosts, supporting my hypothesis that host immunity generates genetic heterogeneity in *C. albicans* that allows for evolution to occur. Additionally, I observed that tetraploids evolved faster than diploids likely due to their elevated instability and ability to generate genotypic diversity quicker. Together, this dissertation emphasizes the role both host and pathogen genotype have in generating pathogen genetic variation which enables adaptation.

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Chapter I: Introduction

1.1.1: Fungi are an important, but often overlooked component of the microbiome

The human microbiome is complex and contains just as many microbial cells as human cells[1]. Many of the microbes inside the human body are important for health, development, and protection against pathogenic species. Despite this, the normally benign microbes inside our body can cause infection and are referred to as pathobionts. The current COVID-19 pandemic and other severe disease outbreaks in history have largely focused on viruses and/or bacteria. However, deadly fungi are a new emerging threat. Many recent studies have focused on the increased risk of developing fungal infection in those that have COVID-19, specifically aspergillosis or invasive candidiasis[2,3]. Additionally, mucormycosis, a deadly fungal disease caused by Mucorales fungi, is rising in India where COVID-19 cases are surging. Aside from the pandemic, approximately 300 million people are infected with fungal diseases worldwide, with 1.6 million of those infections resulting in death yearly[4]. Thus, the need to study fungal diseases is increasingly important. *Candida albicans* is a fungus that normally colonizes humans but also causes infection and is the fourth most common cause of hospital-acquired fungal bloodstream infections with 40% of these infections resulting in mortality[5]. In addition to deadly bloodstream infections, *C. albicans* causes non-lethal mucosal infections, including vaginal and oral candidiasis[5]. One of the main risk factors for *C. albicans* infection is host immune status, with immunocompromised individuals being more susceptible to infections compared to healthy individuals[5].

1.1.2: Defining Candida albicans as a pathogen according to the damage response framework

Microbial species were once considered to either be commensal, pathogenic, or opportunistic pathogens. However, these categories only take into account the ability of the microbe to inflict damage on the host or not. Recently, the field has undergone a paradigm shift

to include the damage inflicted on the host by its own immune response after encountering the microbe[6]. The damage-response framework (DRF) defines a pathogen as a microbe that is capable of causing damage to a host[6]. This framework includes six classifications of pathogens that are based on the interaction between the host and a microorganism[6]. *C. albicans*, once considered an opportunistic pathogen, was first classified under the DRF as a class II pathogen, which is a pathogen that causes damage in either hosts with weak immune responses or in the setting of normal host responses[6]. Recently, Fidel Jr. *et al* reclassified *C. albicans* infections in each of the six classifications of DRF based on the type of infection [7]. For example; oropharyngeal candidiasis (OPC) is classified as a class 1 pathogen because damage only occurs in situations of a weakened or compromised immune system[7]. *C. albicans* that causes Vulvovaginal candidiasis (VVC), commonly thought of as a superficial infection, is classified as a class 6 pathogen[7]. In VVC, symptoms occur only after robust migration of polymorphonuclear leukocytes (PMNs) into the vaginal cavity and which then leads to chronic tissue damage [8]. This demonstrates the complexities of *C. albicans* infections. Furthermore, *C. albicans* has several virulence traits that induce host damage including; hyphal formation, hypha-associated adhesion molecules, biofilm formation, secretory aspartyl proteinases (SAPs), and candidalysin production[9,10]. Together, the host immune response and *C. albicans* virulence factors contribute to the relationship between the host and *C. albicans.*

1.2.1: C. albicans interactions with the host immune system

As a component of the human microbiome, *C. albicans* encounters many stressors including microbial interactions, changes in host physiology, and the host immune system. *C. albicans* ability to undergo morphological transitions from the yeast to hyphal state facilitate *C. albicans* penetration and dissemination throughout the body. Epithelial cells act as a mechanical

barrier against invading *C. albicans*[11]. However, *C. albicans* hyphae can actively penetrate the epithelial barrier, thus triggering an immune response[12]. *C. albicans* is then able to spread to different parts of the body through the bloodstream in the yeast form. As an initial response, antimicrobial peptides (AMPs) and cytokines are induced at the site of infection leading to the recruitment of phagocytic cells[12]. Phagocytic immune cells contain pathogen recognition receptors (PRRs) that detect the specific microbial chemical signatures called pathogen-associated molecular patterns (PAMPs)[13]. The phagocytic cells contain different receptors that recognize different parts of the fungal cell wall, including β-glucan, chitin, and mannoproteins[13]. Differences in fungal cell wall makeup trigger different immune responses. For instance, β-glucan which is more easily detected in the yeast form compared to the hyphal form is primarily recognized through CR3 and Dectin-1[14]. Once recognized, immune cells work to clear *C. albicans* through phagocytosis, production of reactive oxygen species (ROS), and/or AMPs which all have distinct mechanisms of pathogen elimination[14].

1.2.2 Antimicrobial peptides mode of action against C. albicans

AMPs are small (10-100 amino acids), soluble defense molecules the host employs during infection[15]. Although initially discovered in invertebrates[16], AMPs are an important defense mechanism found in all organisms, including plants and animals[17,18]. AMPs generally kill or inhibit pathogen growth via membrane permeabilization, inhibition of protein synthesis, and RNA and DNA disruption[15]. AMPs also promote the migration of neutrophils and monocytes to the site of infection to further control pathogen proliferation and spread[19]. The major types of AMPs active against *C. albicans* include LL-37, histatins, and defensins[20]. As *Candida* species and other fungal pathogen become increasingly resistant to the few antifungal

drugs developed, AMPs represent a promising alternative to antifungal drugs to prevent or treat fungal infections[21].

1.2.3: Reactive oxygen species mode of action against C. albicans

In addition to AMPs, ROS and reactive nitrogen species (RNS) are among the first host defense responses produced to limit pathogen proliferation. Upon phagocytosis of *C. albicans*, neutrophils and macrophages produce an oxidative burst, which generates ROS[14,22]. The NADPH oxidase generates the O_2^- anion, which can then be converted to H_2O_2 by superoxide dismutase, or to OH^- and hydroxyl anions (•OH)[23]. Additionally, phagocytic cells induce nitric oxide synthase in response to cytokine stimulation[23]. The nitric oxide synthase then forms different RNS. Both the production of ROS and RNS contribute to the killing of invading pathogens[24,25].Together both RNS and ROS cause cellular toxicity through lipid peroxidation, base oxidation and deamination, and structural changes to the DNA[26] including double strand breaks (DSBs)[27]. The inability to produce ROS through the NADPH oxidase is a severe disease known as chronic granulomatous disease (CGD)[28]. Patients with CGD often experience recurrent bacterial and fungal infections and are highly susceptible to *C. albicans*[29]. Thus, the production of RNS and ROS represent a potent host defense for controlling *C. albicans* growth and infection.

1.2.4: C. albicans evasion of immune detection and elimination

Although the immune system is effective at limiting *C. albicans* infection, *C. albicans* has several mechanisms for avoiding immune detection. *C. albicans* can avoid immune detection by "hiding" its PAMPs[12]. By redistributing the components that make up the cell wall, such as shielding β-glucan through cell wall remodeling, *C. albicans* is less likely to be recognized by Dectin-1 receptors[30]. Further, *C. albicans* is also able to escape phagocytes once engulfed, by

forming hyphae that penetrate the phagocytes, allowing *C. albicans* to escape[12]. However, if *C. albicans* is detected by host immune cells and is engulfed, it has several mechanistic strategies for mitigating host-induced damage. These mechanisms include secretion of peptide effectors[31], induction of the HOG1 stress response pathway, the RAD53 DNA damage checkpoint, and upregulation of the transcription factor *CAP1*[32]. CAP1 turns on a core set of antioxidant genes including catalase (CAT1), glutathione peroxidase (GPX), and superoxide dismutase (SOD)[32]. These antioxidants are able to break-down ROS produced by the host, essential for *C. albicans* survival in ROS.

1.3 Generating genetic variation for adaptation

While many microbes maintain a stable relationship with their host, the host environment contains stressors that exert a selective pressure on the microbe. Moreover, when a microorganism transfers to a new host niche, it is likely to be less fit compared to other microorganisms present and be outcompeted[33]. Therefore, adaptation may be required for long-term survival and proliferation. Genetic variation is needed for selection to proceed. The rate at which favorable genetic variation arises in a population is critical for determining how quickly organisms can adapt to certain stressors[34]. However, the way in which this genetic variation is created varies across species. A common mechanism that prokaryotes use to generate genetic variation is horizontal gene transfer (HGT). HGT allows for the direct transfer of genetic material between two different organisms, where one organism is not the parent of the other[35–38]. This mechanism coupled with prokaryotic short generation time allows for the rapid emergence of resistance in bacterial populations. HGT is rare in eukaryotes[39], thus eukaryotes have developed their own mechanisms for creating genetic variation. In some eukaryotic organisms, mating followed by meiosis is used to generate genetic diversity in offspring,

resulting from a new combination and reshuffling of alleles during crossing over and random assortment. However, not all eukaryotes reproduce sexually or undergo meiosis [40]. *C. albicans,* does not have a meiotic program and relies on other methods for generating genetic heterogeneity within a population of cells. *C. albicans* undergoes a process known as parasex, in which diploid nuclei fuse to form a tetraploid[40,41]. However, this process is complex and requires two cells of opposite mating types to first undergo a phenotypic switch from the "white" phase to the "opaque" phase[42]. The resulting tetraploid cell then undergoes a non-meiotic reduction to return to diploid. The reduction from tetraploid to diploid is stochastic and can generate a reassortment of alleles, loss of heterozygosity (LOH), and even transient aneuploidy[43–45].

In addition to the mechanisms described above, *de novo* mutations represent a source of genetic diversity that occurs in all organisms. De novo mutations can arise for a variety of reasons including mistakes made during DNA replication[46], mistakes made during base repair,[47] or from exogenous or endogenous mutagens which include UV, ionizing radiation, and ROS[46]. Repetitive loci are especially prone to mutation including INDELs, CNVs, and translocations[48]. These loci are also more prone to replication fork collapse and environmental damaging agents like ROS thus promoting mutation[49–51]. The Major Repeat Sequence, chromosome ends, subtelomeric *TLO* genes*,* and *ALS (Agglutinin-like sequence)* genes*,* all represent hypermutable sites in *C. albicans* with a 10-100x fold higher rate of gains-of-heterozygosity (GOH) and loss-of-heterozygosity (LOH)[52].

1.4 Consequences of genetic variation across different species

Adaptation is the result of selection acting upon genetic variation. Microorganisms living within the microbiome experience both abiotic and biotic selection pressures that may facilitate

rapid adaptation in particular niches. Thus, the ability to generate small and large-scale chromosomal changes can be especially beneficial and is often observed in stressful conditions either through a direct increase in the mutation rate i.e. causing direct DNA damage, or by indirectly increasing mutation rates[53]. In the bacterial species *Escherichia coli* and *Mycobacterium tuberculosis*, exposure to antibiotics triggers the SOS response which induces elevated mutation rates by promoting error-prone DNA repair, and thus speeds the development of antibiotic resistance[54,55]. A similar phenomenon was also observed in the yeast *Saccharyomyces cerevisae*. When mismatch repair genes are mutated, greater mutation rates are observed which causes rapid adaptation[56,57]. In other yeast, such as *C. albicans*, direct exposure to specific stressors including antifungal drugs, increase mutation rate and increase antifungal resistance [58–61]. While typically diploid, *C. albicans* mates to form pseudostable tetraploids which rapidly undergo chromosome loss to generate population heterogeneity[44,45]. Recent studies found that tetraploid *C. albicans* were able to adapt faster to antifungal drug exposure compared to diploid *C. albicans*, likely due to the ability of tetraploids to rapidly and randomly generate chromosome loss[62].

1.4.2 Genetic variation and adaptation to the host environment

The host environment induces large chromosomal changes in the environmentally acquired pathogenic yeast, *Cryptococcus neoformans*, and in the commensal *C. albicans*. These genomic changes generated by the host ultimately impact the host-pathogen relationship. While in the host lung, *C. neoformans* generates highly polyploid cells called titan cells, due to endoreduplication[63,64]. These titan cells protect the pathogen from being engulfed by host phagocytes, leading to a more virulent phenotype[65]. However, titan cells rapidly produce daughter cells that vary in ploidy, including haploid, diploid and aneuploid, which show

increased survival and adaptation to new or high stress conditions compared to titan cells[66]. Similar to *C. neoformans, C. albicans*, rapidly undergoes large-scale genomic changes inside the host environment. In a murine model of oral candidiasis, growth within the host led to the accumulation of trisomy 6, which had attenuated virulence compared to the ancestral strain[67]. Similarly, another study found that chromosome 7 trisomies frequently occurred after passaging through a GI tract of a mouse model and caused attenuated virulence[52]. Further, following serial passaging in a mouse model, *C. albicans* underwent accelerated mutation, which reduced virulence [68]. This low virulence phenotype also provided hosts with protection from other pathogenic microbes. Together, these data suggest that the host rapidly generates both genotypic and phenotypic diversity in *C. albicans* and other fungal pathogens which alters infection dynamics.

Despite *C. albicans* being both a commensal and a pathogen, only one experimental evolution study has investigated how *C. albicans* evolves virulence. Following 42 passages in a macrophage model a *cph1*Δ*/efg1*Δ non-filamentous mutant *C. albicans* strain regained the ability to form hyphae, escape macrophages, and evolved high virulence[69]. Suggesting that the macrophage environment imposes a high selective pressure on *C. albicans*. This group also investigated the infection dynamics of *Candida glabrata*, the second most common cause of candidemia, in a co-culture model with macrophages and found strikingly similar results. After six months of co-culture with macrophages*, C. glabrata* formed pseudohyphae and escaped from macrophages quicker with more damage to the macrophage[70]. Additionally, evolved populations of *C. glabrata* were more virulent in a murine model, had higher fungal burdens and an increased inflammatory response compared to the ancestral strain[70]. Yet, no study has

investigated how different host environments impact the evolutionary trajectories of the same strain.

1.5 Caenorhabditis elegans is a robust model system for investigating C. albicans genome stability and virulence evolution

Several host models, including both vertebrate and invertebrate organisms, have been developed to study *C. albicans* infection. The murine model is considered the gold standard and is often used for its adaptive and innate immune system in addition to monitoring disease progression both systemically and in specific organs[71]. However, there are significant limitations to this host model, including maintenance costs, small number of offspring, and decreased power and reproducibility associated with small sample sizes[71]. Therefore, more simple model organisms such as zebrafish (*Danio rerio*), fruit fly (*Drosophila melanogaster*), wax moth (*Galleria mellonella*), and nematode (*Caenorhabditis elegans*) have been developed to study *C. albicans*-host interactions. These non-mammalian model organisms are smaller, require less laboratory maintenance and larger sample sizes allow for greater power and reproducibility compared to murine models[72]. Each of these models have specific advantages and disadvantages that need to be considered when choosing an infection model. *G. mellonella* offers the most physiologically similar environment to humans as it can be grown at 37°C and has various phagocytic cells[73]. Furthermore, this model allows for the direct injection of a specific inoculum[73]. However, there is no fully sequenced genome, and no established method of creating mutant strains. Similar to the wax moth model, the zebrafish model allows for direct injection of a specific inoculum[71,73]. It also contains both an adaptive and innate immune system[71], which none of the other invertebrate models provide, making it an ideal model to investigate the immune response to fungal infections. Compared to the other invertebrate models, zebrafish have the costliest and space-prohibitive

maintenance. *D. melanogaster* has similar advantages and disadvantages as *C. elegans.* These advantages include easy RNAi, fully sequenced genome that is easy to manipulate[73]. Disadvantages include, optimal growth lower than physiologically relevant, no adaptive immunity or cytokines, and no tissue specific infections[73]. Compared to *D. melanogaster* and the other non-mammalian models*, C. elegans* have the most rapid life cycle, can self-fertilize to generate genetically identical offspring, and are the most amenable to large-scale screens[72–74]. The wide range of *C. elegans* mutants available and ability to store and revive host populations from cold storage make *C. elegans* an attractive model host to study *C. albicans* infection.

There are several conserved immune pathways between humans and *C. elegans* which makes it an ideal model for investigating the interactions between the immune system and pathogens. While *C. elegans* does not have an adaptive immune system or specialized innate immune cells including cytokines, it has immune pathways that are highly conserved with mammals. Similar to mammals, *C. elegans* have a mitogen-activated protein kinase (MAPK) signaling cascade[75,76]. In *C. elegans,* this signaling cascade, which includes PMK-1 *(*p38, MAPK), is responsible for producing antimicrobial peptides (AMPs) and coordinates the induction of multiple immune effectors[77]. Another parallel between mammals and *C. elegans* is the production of ROS in response to infection. *C. elegans* contains a dual-oxidase (DUOX) known as BLI-3 that has both a NADPH domain and a peroxidase domain that produces hydrogen peroxide which bears striking similarity to the human DUOX1 (Figure 1.1)[78]. Additionally, *C. elegans* also has a conserved transcription factor known as SKN-1, and ortholog of the mammalian Nrf proteins, that are responsible for protecting and detoxifying the reactive oxygen species produced to defend itself against pathogens[77]. Thus, using *C. elegans* as a model of infection can give key insight into host immune responses following infection.

C. elegans also represents a great model organism for experimental evolution. *C. elegans* have a natural relationship with many different bacterial species that are recruited from the environment it lives in[79]. Additionally, several fungal species including *Drechmeria coniospora*[80] and *microsporidia*[81] are natural pathogens of *C. elegans*, indicating that *C. elegans* are capable of interacting with diverse types of microbes. Unlike other model organisms, *C. elegans* has the ability to reproduce in a span of less than 3 days and has an average lifespan of less than one month, making it particular amenable to long-term evolution experiments. As such, many studies have utilized *C. elegans* as a powerful model to evolve virulence in several different microbial pathogens, including *Serratia marcescens* [82,83], *Enterococcus faecalis*[84] and *Bacillus thuringiensis*[85].

Figure 1

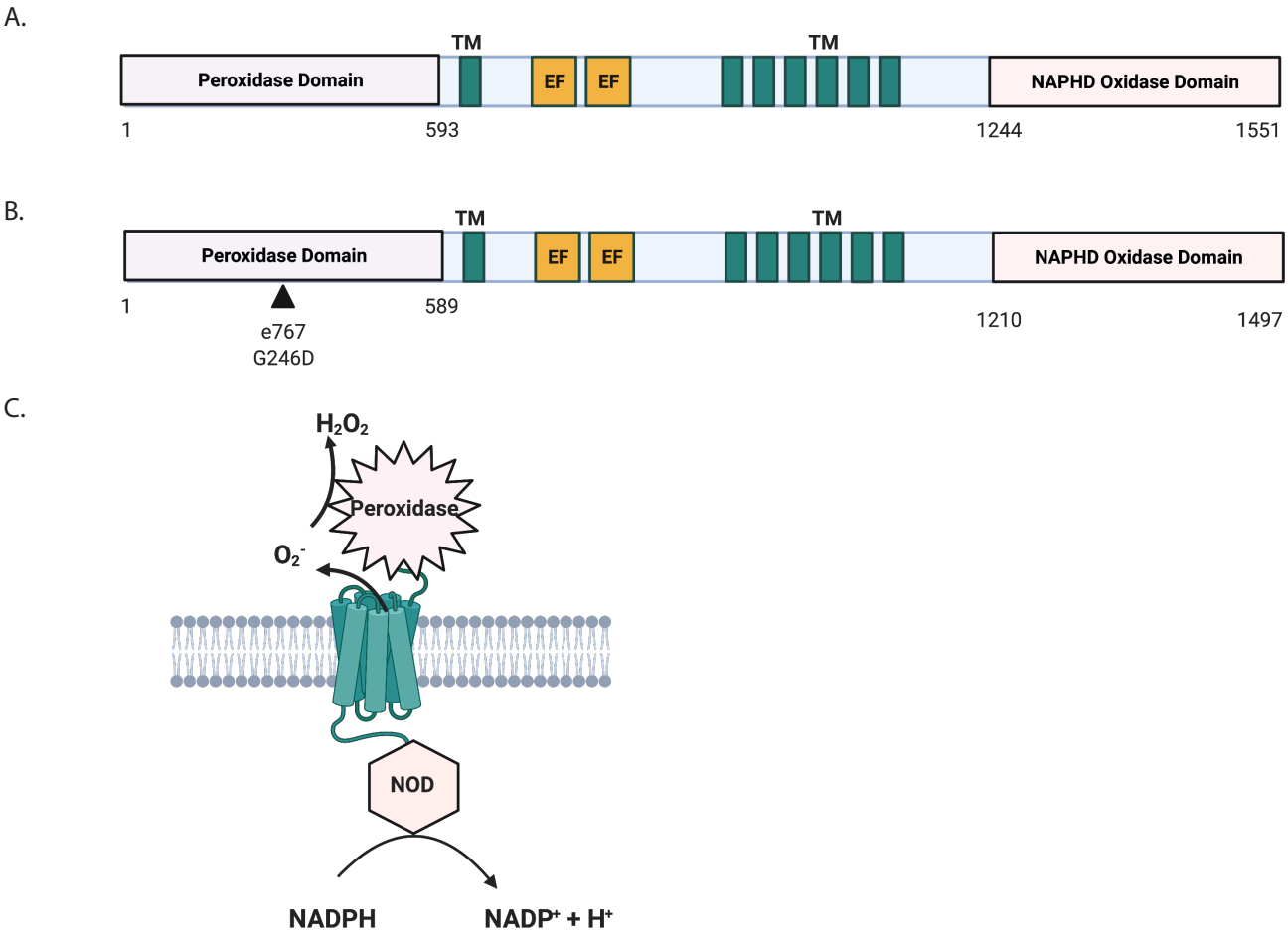

Figure 1: *C. elegans* dual oxidase conservation with human dual oxidase
Adapted from[78]. **A)** Schematic representing the human Duox1 **B)** Schematic representing *C. elegans* BLI-3/Duox. e767 represents the mutation in *bli-3* mutant worms used in this study. **C)** Topology model for *C. elegans* BLI-3. TM = transmembrane domain, EF = EF- hand, NOD = NADPH oxidase domain.

1.6 Summary

Host-microbe interactions are complex. *C. albicans*, while normally a commensal fungal component of the microbiome, can cause both minor and severe infections. While *C. albicans* infections most commonly impact immunocompromised individuals, healthy individuals also experience infection. While many *C. albicans* virulence factors have been identified and contribute to changes in *C. albicans* virulence, *C. albicans* impeccable ability to generate large-scale and small-scale genomic changes may also facilitate changes in *C. albicans* pathogenicity within the host environment. In chapter 2, I describe the impact the host environment (*C. elegans*) has on *C. albicans* genome stability across different pathogen backgrounds and ploidies. In chapter 2, I will also investigate the short-term impacts of genomic changes generated inside the host environment on *C. albicans* virulence and fitness. In chapter 3, I will pinpoint the specific host factors that drive genome instability in both diploid and tetraploid *C. albicans* by systematically using *C. elegans* backgrounds with mutations in key immune pathways. Finally, in chapter 4, I will discuss the long-term impacts of host generated genome instability on *C. albicans* virulence evolution. This chapter will specifically highlight the differences between evolution in diploid and tetraploid *C. albicans* as well as the differences in virulence evolution in healthy and immunocompromised hosts. Together, my work will demonstrate that host immunity generates genome instability in *C. albicans* which enables adaptation, but the degree to which genome instability is generated is dependent on *C. albicans* ploidy and genotype. This provides a framework for understanding how the relationship between host genotype and pathogen genotype can alter infection dynamics over time.

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Chapter II

Host-induced Genome Instability Rapidly Generates Phenotypic Variation across Candida albicans Strains and Ploidy States

Reprinted Material from: Smith AC and MA Hickman. Host-induced Genome Instability Rapidly Generates Phenotypic Variation across Candida albicans Strains and Ploidy States. mSphere 5: e00433-20.

2.1 Abstract:

Candida albicans is an opportunistic fungal pathogen of humans that is typically diploid yet, has a highly labile genome tolerant of large-scale perturbations including chromosomal aneuploidy and loss-of-heterozygosity events. The ability to rapidly generate genetic variation is crucial for *C. albicans* to adapt to changing or stressful environments, like those encountered in the host. Genetic variation occurs via stress-induced mutagenesis or can be generated through its parasexual cycle, in which tetraploids arise via diploid mating or stress-induced mitotic defects and undergo non-meiotic ploidy reduction. However, it remains largely unknown how genetic background contributes to *C. albicans* genome instability *in vitro* or in the host environment. Here, we tested how genetic background, ploidy, and host environment impact *C. albicans* genome stability. We found that host association induced both loss-of-heterozygosity events and genome size changes, regardless of genetic background or ploidy. However, the magnitude and types of genome changes varied across *C. albicans* strain background and ploidy state. We then assessed if host-induced genomic changes resulted in fitness consequences on growth rate and non-lethal virulence phenotypes and found that many host-derived isolates significantly changed relative to their parental strain. Interestingly, diploid derivatives host-associated *C. albicans* predominantly decreased host reproductive fitness, whereas tetraploid derivatives host-associated *C. albicans* increased host reproductive fitness. Together, these results are important for

understanding how host-induced genomic changes in *C. albicans* alter its relationship with the host.

2.2 Importance:

Candida albicans is an opportunistic fungal pathogen of humans. The ability to generate genetic variation is essential for adaptation and a strategy *C. albicans* and other fungal pathogens use is to change their genome size. Stressful environments, including the host, induce *C. albicans* genome instability. Here, we investigated how *C. albicans* genetic background and ploidy state impact genome instability, both *in vitro* and in a host environment. We show that the host environment induces genome instability, but the magnitude depends on *C. albicans* genetic background. Furthermore, we show that tetraploid *C. albicans* are highly unstable in host environments and rapidly reduce in genome size. These reductions in genome size often resulted in reduced virulence. In contrast, diploid *C. albicans* displayed modest host-induced genome size changes, yet these frequently resulted in increased virulence. Such studies are essential for understanding how opportunistic pathogens respond and potentially adapt to the host environment.

2.3 Introduction:

Host-pathogen interactions are multi-faceted. As a fungal opportunistic pathogen of humans, *Candida albicans* has many different relationships with the host. Typically, *C. albicans* is commensal, residing in many niches in the human body, including the gastrointestinal and urogenital tracts, oral cavity, and the skin [1]. However, *C. albicans* can be pathogenic and cause superficial mucosal infections and deadly bloodstream infections [2,3]. Much of the research

regarding *C. albicans* has focused on its virulence factors, which include filamentation, biofilm formation, secretory aspartyl proteinases (SAPs), and candidalysin production [4,5]. Host immune cells control *C. albicans* infection by recognizing fungal cells and producing antimicrobial peptides (AMPs) [6] and reactive oxygen species (ROS) which inhibit growth and cause DNA damage [7,8] However, how host-induced genome alterations in *C. albicans* impact its relationship with the host is not well understood.

Genomic alterations in *C. albicans* have important consequences in other clinical contexts, including the acquisition of resistance to the drugs used to treat fungal infections. Analysis of clinical isolates and laboratory studies show that chromosomal aneuploidy and homozygosis of hyper-active resistance alleles are associated with increased antifungal drug resistance [9–14]. The ability to generate genetic variation and tolerate genomic perturbations is a strategy that *C. albicans* leverages to adapt to changing environments [15]. As a highly heterozygous diploid [16], the *C. albicans* genome is highly labile and undergoes large-scale genome rearrangements like loss-of-heterozygosity (LOH) events and aneuploidy more frequently than small-scale DNA mutations. Furthermore, exposure to physiologically relevant stress conditions, including high temperature, oxidative stress, and antifungal drugs, increases LOH rates even further [17,18].

Another mechanism *C. albicans* uses to generate genetic variation is parasex, which involves diploid-diploid mating to produce tetraploids [19,20]. Tetraploids undergo stochastic chromosome loss to return to diploid. This process is termed ‘concerted chromosome loss’ and results in reassortment of alleles, loss of heterozygosity (LOH), and aneuploidy [21–23]. The tetraploid LOH rate is substantially higher than in diploids [23]. In the context of antifungal drugs, large-scale mutation rates are disproportionately elevated in tetraploids compared to

diploids [18]. Tetraploids, which undergo more genomic changes than diploids, have high potential to produce phenotypic changes with fitness consequences [23,24]. In fact, diploids will produce transient tetraploids via mitotic errors when exposed to high doses of the antifungal drug fluconazole [25]. Therefore, tetraploidy may be a useful evolutionary strategy to produce genetic variation.

Clinical *C. albicans* tetraploids have been isolated from human hosts [24,26–28], although whether they arise via parasex or stress-induced mitotic defects is not known. Furthermore, clinical diploid isolates also carry genomic changes such as aneuploidy and LOH [29]. A small number of experimental studies have investigated *C. albicans* genome stability within host environments and found that host-association elevates mutation rates compared to *in vitro* [30–32]. Host-induced genetic variation increases phenotypic variation including colony morphology, hyphal formation, and virulence [30,32–34]. For example, chromosome 6 and chromosome 7 trisomies arise in *C. albicans* associated with murine hosts and display attenuated virulence [31,33]. While many studies use murine models to measure virulence (reviewed in [35]), we previously developed a novel *C. elegans* infection model. Our host-pathogen system not only can assess host survival, but also host reproduction [36], an often under-looked aspect of virulence [37]. Host fecundity offers a non-lethal phenotype, which can be important when studying virulence in opportunistic pathogens that cause a wide-range of infections [37]

Here we investigated how host-association impacts *C. albicans* genome instability across multiple genetic backgrounds and ploidies. We used three diploid-tetraploid pairs of *C. albicans* strains from distinct genetic backgrounds to infect *C. elegans* hosts and subsequently measured LOH frequency and genome size changes in *C. albicans*. We found that host-association increased genome instability for all *C. albican*s strains, but the degree to which it was elevated

depended on strain background. Furthermore, host-associated diploids had minor, but significant genome size changes, whereas host-associated tetraploids rapidly underwent major reductions in genome size. We assessed how these genomic changes altered strain fitness and virulence. Most diploid isolates were more virulent following host-association, but many tetraploid isolates did not change virulence or were less virulent, despite undergoing massive genome size changes. Taken together, our results show that host-association induced genetic variation in diploid and tetraploid *C. albicans* of diverse genetic backgrounds which impacted virulence phenotypes.

2.4: Materials and Methods:

Strains and Maintenance

We used six *C. albicans* strains for this study that varied in their ploidy and their genetic background (Table 2.1). Each strain was initially struck out from glycerol stocks stored at -80°C onto a YPD [yeast peptone dextrose (1% yeast extract, 2% bactopeptone, 2% glucose, 1.5% agar, 0.004% adenine, 0.008% uridine)] plate. After 48 hours at 30°C, a single colony was arbitrarily chosen as the "parental strain".

To construct diploid clinical strains heterozygous for the *GAL1* locus, we transformed one copy of the *GAL1* open reading frame in FH1 and PN2 with the dominant drug-resistant *NAT* gene marker by lithium acetate transformation. *NAT* was amplified from plasmid pMG2120 by PCR (1. 94°C - 5 m, 2. 94°C - 30 s 3. 55°C - 45 s 4. 72°C - 4 m 5. Cycle to (2) 29x 6. 72°C - 10 min) using primers oMH112 and oMH113 listed in (Supplemental Table S2.1). Transformants were selected on YPD containing 50 μg/mL nourseothricin. PCR (1. 94°C - 3 m. 2. 94°C for 30 s. 3. 55°C - 30 s. 4. 68°C - 2 m. 5. Cycle to (2) for 35x 6. 68°C -10 m.) was performed to verify

that *NAT* was properly integrated using primers oMH106, oMH5, and oMH104 (Supplemental Table S2.1). All strains were stored at -80°C and maintained on YPD at 30°C.

C. elegans N2 bristol (wildtype) were used for fecundity and host-associated genome stability assays. *C. elegans* populations were maintained on plates containing nematode growth media (NGM) with *E. coli* (OP50) for a food source. *C. elegans* were transferred to a new plate containing freshly seeded *E. coli* every three to four days. For genome stability assays, treatment plates were seeded with both *C. albicans* and *E. coli* and supplemented with 0.2 g/L streptomycin to inhibit overgrowth of *E. coli*. For fecundity and genome stability assays, NGM was supplemented with 0.08g/L of uridine and 0.08g/L of histidine to facilitate growth of auxotrophic *C. albicans* strains.

Seeding NGM plates for genome stability assays

Single colonies of *C. albicans* were inoculated into 3 mL of YPD and incubated at 30°C overnight. *C. albicans* cultures were diluted to a final volume of 3.0 OD_{600} per mL in ddH_2O. Additionally, *E. coli* was inoculated into 50 mL of LB and incubated at 30°C for 24-48 hours. Subsequently, *E. coli* was pelleted and washed twice with 1 mL of ddH_2O. The washed pellet was then weighed and diluted to final density of 200 mg/mL. *In vitro* treatment plates contained 250 µL of diluted *C. albicans* plated and spread onto an NGM + streptomycin agar plate and incubated overnight at 30°C. *In vivo* treatment plates had 6.25 µL of *C. albicans*, 31.25 µL of *E. coli*, and brought to a final volume of 250 µL with ddH_2O. This mixture was plated and spread onto an NGM + strep agar plate and incubated overnight at 30°C.

Seeding NGM plates for fecundity assays:

Seeding NGM plates and synchronizing *C. elegans* populations for fecundity assays were performed as previously described [36]. Briefly, *C. albicans* cultures were inoculated into 3 mL of YPD and incubated at 30°C overnight. *C. albicans* cultures were diluted to a final volume of 3.0 OD_{600} per mL. Additionally, *E. coli* was inoculated into 50 mL of LB and incubated at 30°C for 24-48 hours. Subsequently, *E. coli* was pelleted and washed twice with 1 mL of ddH_2O. The washed pellet was then weighed and diluted to a final density of 200 mg/mL. Day 0 uninfected treatment plates contained 6.25 µL of *E. coli* and brought to a final volume of 50 µL with ddH_2O. Day 0 *C. albicans* treatment plates had 1.25 µL of *C. albicans*, 6.25 µL of *E. coli*, and brought to a final volume of 50 µL. The entire 50 µL was spotted onto the center of a 35-mm-diameter NGM + streptomycin agar plate, followed by incubation at room temperature overnight before the addition of eggs or transferring nematodes. For days 2-7 of the experiment, *C. albicans* treatment plates contained 0.25 µL of *C. albicans*, 1.25 µL of *E. coli* and 8.5 µL of ddH_2O. For uninfected treatments, 1.25 µL of *E. coli* was mixed with 8.75 µL of ddH_2O. The entire 10 µL was spotted onto a 35-mm-diameter NGM + streptomycin agar plate, followed by incubation at room temperature overnight before the transfer of nematodes.

Egg preparation and synchronization for genome stability and fecundity assays

To synchronize *C. elegans* populations, nematodes and eggs were washed off the plate with an M9 buffer and transferred to a 15 mL conical and pelleted at 1200 rpm for 2 minutes. The pellet was resuspended in a 25% bleach solution, inverted for 2 minutes and subsequently centrifuged for 2 minutes at 1200 rpm. The pellet was washed twice with 3 mL of ddH_2O and resuspended in 1 mL of ddH_2O. To determine the concentration of eggs, 10 µL was pipetted onto

a concave slide, the eggs were counted under a microscope, and the egg suspension was diluted with M9 to a concentration of ~100 eggs per 100 µL.

Host-associated yeast extractions

C. elegans colonized with *C. albicans* were washed off the plate with 3 mL of M9 worm buffer. This suspension was centrifuged for two minutes at 2,000 rpm to pellet the worms. The supernatant was removed and 1 mL of 3% bleach was added, transferred to a microcentrifuge tube and incubated for three minutes. The worm suspension was centrifuged for one minute at 12,000 rpm. The supernatant was removed and washed with 1 mL of M9 and centrifuged for one minute at 12,000 rpm. The wash was repeated two more times to ensure all bleach was removed. 100 ul aliquots of nematode suspension were transferred to 0.6 mL clear microtubes for manual disruption with a motorized pestle. After one minute of manual disruption, the worm intestine solution was then diluted accordingly with an M9 buffer and plated on YPD + 0.034mg/L chloramphenicol to select prevent any bacterial colonies from arising.

GAL1 Loss of Heterozygosity assay:

In vitro: Single colonies of *C. albicans* were inoculated in 3 mL YPD, grown overnight at 30°C and subsequently diluted to 3 OD in ddH_2O. 250 µL was plated and spread onto NGM + streptomycin plates and incubated overnight at 30°C and transferred to 20°C for four days. On day four, yeast cells were washed off with ddH_2O, harvested by centrifugation, washed once with ddH_2O, resuspended in 1 mL of ddH_2O and serially diluted for single colony growth. To determine the total cell viability, 100 µL of 10^{-6} dilution was plated onto YPD and grown for 48 hours at 30°C. To identify cells that lost *GAL1*, 100 µL of 10^{-2} and 10^{-3} dilution was plated onto

2-deoxygalactose (2-DOG; 0.17% yeast nitrogen base without amino acids 0.5% ammonium sulfate, 0.0004% uridine, 0.0004% histidine, 0.1% 2-deoxygalacose, 3% glycerol) and colony forming units (CFUs) counted following 72 hours incubation at 30°C.

In vivo: The approach was very similar as the *in vitro* LOH assay described above, with the following changes. A population of ~100 nematodes were plated on each treatment plate containing both *C. albicans* and *E. coli.* On day four, yeast were extracted as described in the previous section. A dilution of 10^{-1} and 10^{-2} was plated on YPD + chloramphenicol to enumerate total growth and undiluted cells were plated on 2-DOG to select for the loss of *GAL1.* Three technical replicates were used for each *C. albicans* strain for both *in vitro* and *in vivo* experiments. At least three biological replicates were used for each genome stability assay.

In vitro passaging

Serial passaging experiments were performed as previously described [23]. Briefly, we inoculated 36 single colony isolates for each *C. albicans* strain into 500 ul liquid YPD in 96-deep well culture blocks and incubated them at 30° with shaking. Every 24 hours, 5 µL of culture was diluted in 495 mL fresh YPD (1:100 dilution) and incubated at 30°C with shaking. On days 4, 7, 14 and 28 cultures were simultaneously prepared for flow cytometry or for long-term storage in 50% glycerol at -80°C. Glycerol stocks were also prepared on days 10, 17, 21 and 24.

Flow cytometry for genome size determination:

Single yeast colonies extracted from *C. elegans* were inoculated in YPD and incubated overnight at 30°C. Samples were sub-cultured into wells with 495 µL of fresh YPD and grown at 30°C for an additional 6 hours. Cells were subsequently collected by centrifugation (1000 rpm,

five minutes) and the supernatant removed and resuspended in 50:50 TE (50 mM Tris, pH 8 and 50 mM EDTA). Cells were fixed by adding 180 ul of 95% ethanol and stored overnight at 4°C. Following fixation, cells were collected by centrifugation, washed with 50:50 TE and treated with 50ul of RNase A [1mg/mL] for one hour at 37°C with shaking. Following RNase treatment, cells were collected by centrifugation, RNAase solution was removed and cells were resuspended with 50ul proteinase K [5 mg/mL] and incubated for 30 minutes at 37°C. Following proteinase K treatment, cells were collected by centrifugation and washed once with 50:50 TE and resuspended in 50ul SybrGreen (1:50 dilution with 50:50 TE; Lonza, CAT#12001-798, 10,000X) and incubated overnight at 4°C. Following SybrGreen straining, cells were collected by centrifugation, SybrGreen was removed and cells were resuspended in 50 µL 50:50 TE. Samples were sonicated to disrupt any cell clumping and subsequently run on a LSRII flow cytometer. To calibrate the LSRII and serve as internal controls, the reference diploid (SC5314) and tetraploid strains were used.

Flow cytometry data was analyzed using FlowJo, by plotting the FITC-A signal against the cell count. Two peaks were observed, the first representing the G1 mean and the second peak representing the G2 mean, which has double the genome content of the G1 peak and therefore twice the fluorescence. Genome size values were calculated using the G1 mean and compared to standard diploid and tetraploid control strains.

Host fecundity assays:

Approximately 50 host eggs were added to each control and treatment plates. After 48 hours of growth at 20°C, a single L4 nematode (x10 per treatment) was randomly selected and transferred to an uninfected or *C. albicans* treatment plate and incubated at 20°C. Each nematode

was transferred to a new plate every 24 hours for five consecutive days. Any eggs laid for each 24-hour interval were incubated for 24 hours at 20°C and the number of viable progenies produced per worm was enumerated.

Growth Rate Assays:

12 random colonies for each parental strain and 7 colonies for each host-associated isolate were grown overnight in 450 µL with shaking of YPD in a 96-well block at 30°C with shaking. Cultures were diluted 10-fold with ddH_2O. 15 µL of diluted culture was inoculated into 135 µL of YPD in a sterile round-bottom 96-well plate and placed on the Biotek ELx808 absorbance microplate reader. Optical density (OD) was measured every 15 minutes for 24 hours at 30°C with shaking. Growth rate was determined using a custom R script that calculates the maximal growth rate in each well as the spline with the highest slope from a loess fit through log-transformed optical density data that reflects the rate of cell doubling (developed by Richard Fitzjohn, as in [38]).

Statistical Analysis:

Statistical analysis was performed using GraphPad Prism 8 software. Data sets were tested for normality using the D'Agnostino & Pearson omnibus normality test. Student's t-tests were used to test for differences between LOH frequencies for host and no host treatments. Deviations from initial genome size were determined by first pooling the Day 0 data for all the diploid or tetraploid strains to calculate the mean and standard deviation (SD). Replicate lines ±1SD from the Day 0 mean were considered deviation. *In vivo* genome size deviations were calculated similarly, however the mean and standard deviations was calculated for each strain

rather than being pooled. Unpaired, Mann-Whitney U-test were used to test for differences in growth rate and host fecundity between host-associated isolates and their respective parental strains.

2.5 Results:

Host-association elevates LOH events in laboratory and clinical C. albicans isolates

The laboratory strain of *C. albicans* shows elevated genome instability in murine host environments compared to *in vitro* [30]. Here, we wanted to determine how *C. albicans* genetic background impacted *in vitro* and host-associated genome instability. Using a *C. elegans* infection model, we compared the frequency of loss-of-heterozygosity (LOH) at the *GAL1* locus [17] of a laboratory and two clinical diploid strains *in vivo* to the *in vitro* LOH frequency (Figure 2.1A). If the host environment elevated *C. albicans* genome instability, then the host-associated LOH frequency should increase relative to *in vitro*. For the laboratory and the oral clinical strains, we found that host-associated LOH is approximately ten-fold higher than *in vitro* (Figure 1B; teal and purple). However, the bloodstream clinical strain had no significant difference in LOH frequency between the *in vitro* and host-associated treatments, likely due to the high *in vitro* LOH frequency (Figure 2.1B; orange). Indeed, we found significant differences among the strain backgrounds for in the *in vitro* ($p<0.0001$, Kruskal-Wallis test) and host-associated ($p=0.0008$, Kruskal-Wallis test) treatments, with the bloodstream strain displaying higher instability than the other two strains (Supplemental Table S2.2). Our results are consistent with data from murine models [30–33] and support the hypothesis that host environments increase *C. albicans* genome instability and demonstrates that *C. albicans* genetic background influences genome instability.

Figure 2.1

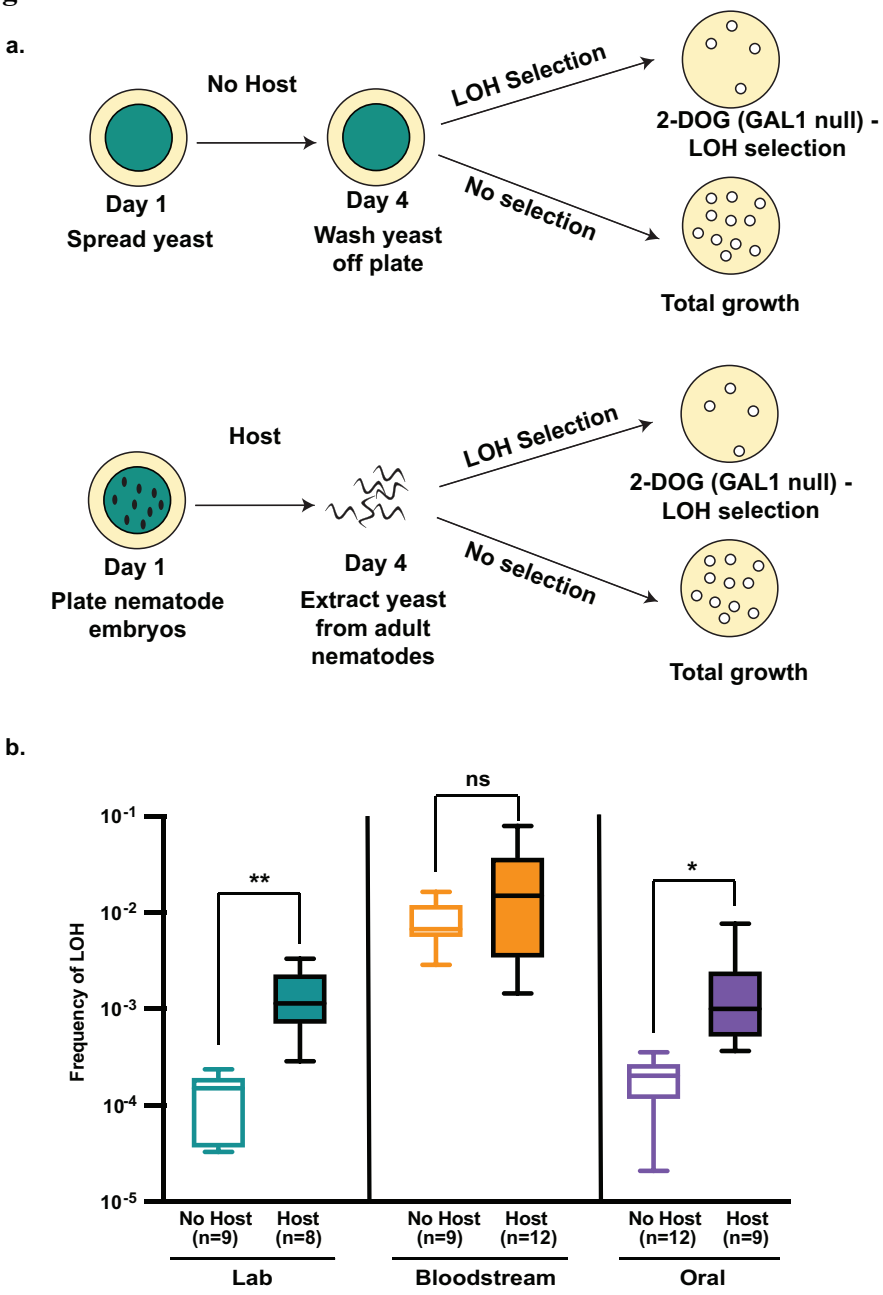

Figure 2.1: Host-association induces LOH

A) Experimental schematic. *C. albicans* was plated either in the presence or absence of hosts and subsequently extracted or washed from the treatment plates and plated on media that selected for LOH events (2-DOG) or rich media (YPD) to determine total viable growth.

B) No host- and host-association *GAL1* LOH frequencies for laboratory (green), bloodstream (orange), and oral (purple) diploid *C. albicans* strains. Boxes represent the 25th and 75th quartile with the whiskers representing the total data range. Asterisks indicate significant differences between the no-host and host treatment groups for each strain background (* $p < 0.05$, ** $p < 0.01$, ns = not significant; Unpaired Student's T-test).

Host-association induces genome size changes in clinical diploid strains

Since strain background impacts LOH frequency (Figure 2.1), we next wanted to examine if it also affects genome size changes over time. We have previously shown that clinical strains are more unstable than the laboratory strain (24), however we did not investigate changes over the course of serial passaging. To identify *in vitro* genome size changes over time, we passaged 60 replicate lines for each genetic background in nutrient-rich liquid media for 28 days and periodically measured genome size via flow cytometry for every replicate line. To assess whether replicate lines deviated in genome size changes over time, we plotted the genome size for each replicate population as the fraction of its initial genome size. Most replicate lines maintained their initial genome size throughout the 28-day experiment, regardless of strain background. However, the number of replicate lines that deviated from diploidy was higher for the clinical genetic backgrounds compared to the laboratory strain (Figure 2.2A 'deviation'). For example, on Day 14, the clinical bloodstream and oral strains had 69% and 72% of their replicate lines with deviations from their initial diploid state. In contrast, only 33% of laboratory replicate lines deviated from diploidy by that same time point. Remarkably, the bloodstream strain had deviations with both major gains (i.e. 1.5x) and losses (i.e. 0.5x) in genome size that suggest that haploidy and triploidy can arise in this strain background (Figure 2.2A and Supplemental Figure S2.1A). For the oral strain, the majority of the deviations in diploidy were minor losses in genome size (Supplemental Figure S2.1A). In addition to minor losses in genome size, in the laboratory strain multiple G1 peaks were observed during flow cytometry for several replicate lines on Day 4, indicating mixed populations. However, by Day 28, all of the laboratory replicate lines resolved back to a diploid state (Figure 2.2A and Supplemental figure S2.1A). Together,

these data demonstrate that the two clinical strains are more likely to generate genome size changes than the laboratory strain during *in vitro* passaging.

Next, we wanted to assess how the host impacts genome size stability for each of the three strains. Similar to the LOH assay, we exposed hosts to *C. albicans* for four days and subsequently extracted the yeast on day four. Colonies were picked at random and flow cytometry was performed to assess genome size (Figure 2.2B). As an *in vitro* control, we plated an equivalent lawn of *C. elegans* in the absence of nematode hosts and we calculated the relative genome size for the host-associated isolates as a fraction of the genome size of the *in vitro* control not exposed to the host. The laboratory strain had the least number of isolates that deviated from diploid (Figure 2.2C, teal). Of the 34% of isolates that were no longer diploid, all had minor losses in genome size (Figure 2.2C and Supplemental figure S2.1B). In the oral diploid strain, 69% of host-associated isolates were no longer diploid. The deviations from diploid in this strain were both minor gains and losses (Figure 2.2C, purple and Supplemental figure S2.1B). Shockingly, 93% of the bloodstream host-associated isolates were no longer diploid, after exposure to the host for four days. The majority of deviations from diploidy in this strain background were both major and minor gains in genome size (Figure 2.2C, Orange and Supplemental figure S2.1B). These results are consistent with the laboratory strain being more stable than the clinical strains, both *in vitro* and *in vivo*. Furthermore, our results indicate that the host environment increases genome instability, both in terms of LOH and genome size changes, yet the amount and direction of these genomic changes depends on strain background.

Figure 2.2

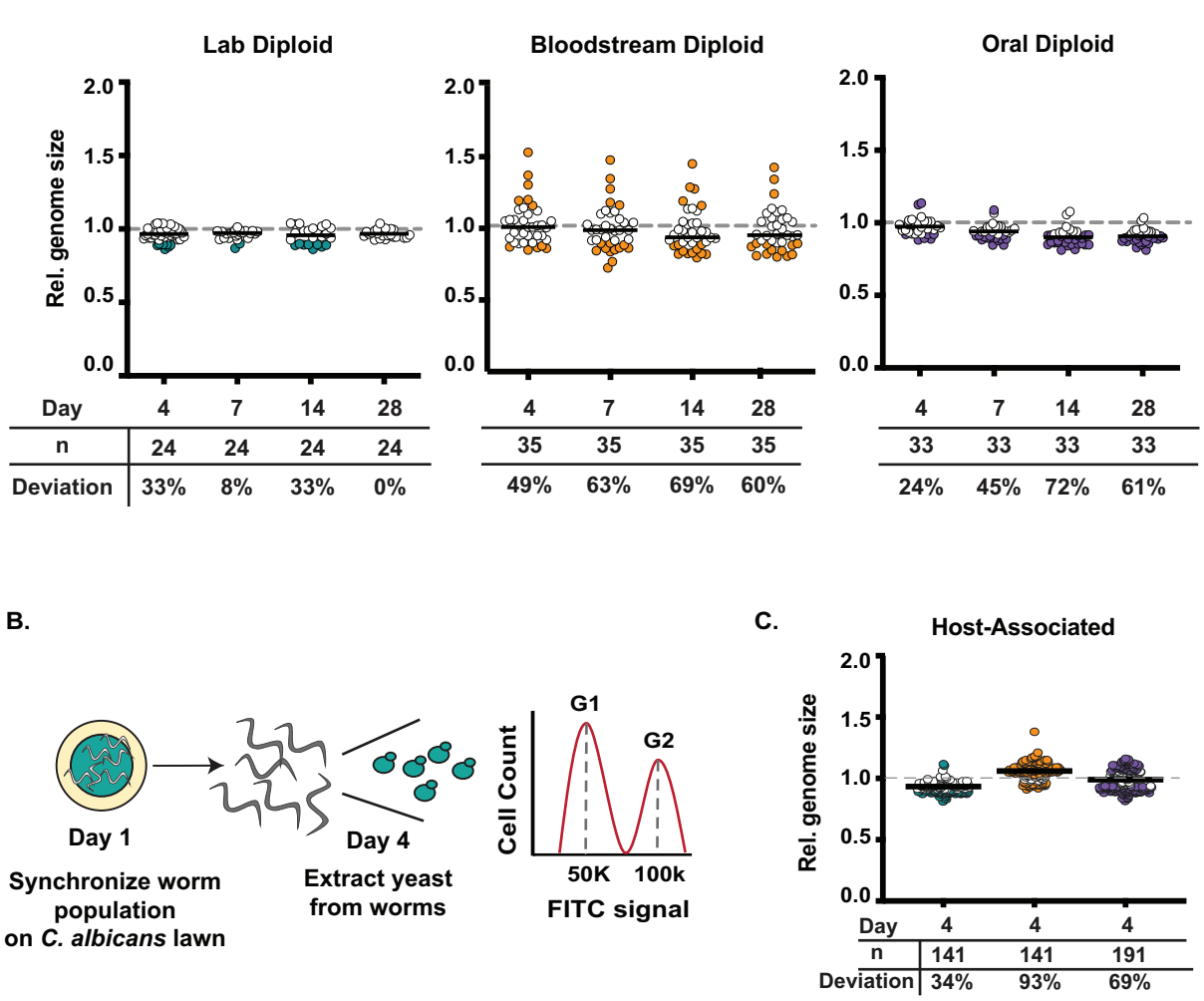

Figure 2.2 Diploid genome size stability *in vitro* and *in vivo*

A) Relative genome size of laboratory, bloodstream, and oral diploid replicate lines passaged for 28 days in rich media. Symbols represent individual replicate lines and the median indicated by the solid black line. Filled symbols indicate replicate lines more than one standard deviation away from the Day 0 mean and reflect deviations from the initial diploid state.

B) Experimental schematic for assessing host-associated genome changes.

C) Relative genome size of laboratory, bloodstream, and oral host-associated isolates. were extracted from the gut and genome size was measured via flow cytometry. Symbols represent individual *C. albicans* colonies extracted from the host and the median indicated by the solid black line. Filled symbols indicate replicate lines more than one standard deviation away from the 'No Host' mean and reflect deviations from the initial diploid state.

Tetraploids undergo rapid genome size reduction in the host environment

Our previous work demonstrated that tetraploid strains do not maintain tetraploidy during long-term passaging [23,24]. However, the dynamics of tetraploid instability for clinical strains was not captured. Therefore, we wanted to analyze the genome size stability of diverse tetraploid strains over time. We used three tetraploid strains; the laboratory tetraploid strain, a mating product between two laboratory diploids [21], a bloodstream clinical tetraploid recovered from a marrow transplant patient [27,28], and a vaginal clinical strain recovered from a vaginal infection [24]. To measure genome size changes over time *in vitro*, we passaged 60 replicate lines for each tetraploid genetic background in nutrient-rich liquid media for 28 days and periodically measured genome size via flow cytometry for each replicate line. To assess whether replicate lines deviated in genome size changes over time, we plotted the genome size for each replicate population as the fraction of its initial genome size. Unlike diploids, most tetraploid replicate lines did not maintain their initial genome size, instead most underwent genome reduction to about half their initial genome content (i.e. approximately diploid after passaging), regardless of strain background. By Day 28, 100% of the vaginal and bloodstream replicate lines were no longer tetraploid, and 97% of laboratory replicate lines were not tetraploid (Figure 2.3A and Supplemental figure S2.1C). However, we detect genome size changes at earlier timepoints for the clinical strains compared to the laboratory strain. By Day 7, more clinical replicate lines (97% and 74% for bloodstream and vaginal, respectively) had a reduction in genome size compared to the laboratory strain in which only 53% of replicate lines had a reduction in genome size. Similar to our previous work [24], we demonstrated that tetraploids reduce to diploidy after serial passaging. Additionally, we demonstrated that the clinical tetraploids undergo a more rapid reduction in genome size compared to the laboratory strain during *in vitro* passaging.

Since the host environment induces genome size changes in *C. albicans* diploid strains (26–28) we next wanted to assess how the host impacts tetraploids. To assess host-associated tetraploid stability, we infected a population of *C. elegans* with each one of the three tetraploid strains mentioned previously. After four days of exposure to *C. albicans*, we extracted *C. albicans* from the *C. elegans* gut via manual grinding and subsequently measured the genome size of the host-associated isolates via flow cytometry. We then calculated the relative genome size compared to the *in vitro* controls for each strain background. After only four days of host-association, 100% of all host-associated single colony isolates were no longer tetraploid, regardless of strain background. Furthermore, the vast majority of these tetraploid-derived isolates were approximately one-half of their initial genome content and are likely diploid (Figure 2.3B). While we anticipated elevated tetraploid instability associated with the host environment, this result was surprising, given the short time frame of host-association and the strain-independent nature. When we compare the host-associated results to the *in vitro* 28-day passaging, we observed some replicate lines could maintain tetraploidy *in vitro*, but not *in vivo*. Furthermore, while the clinical tetraploid strains showed more genome size variation than the laboratory strain during *in vitro* passaging, these size changes were more modest compared the host-associated genome size changes. Together these data suggest that the host-environment rapidly reduces the genome size of tetraploids.

Figure 2.3

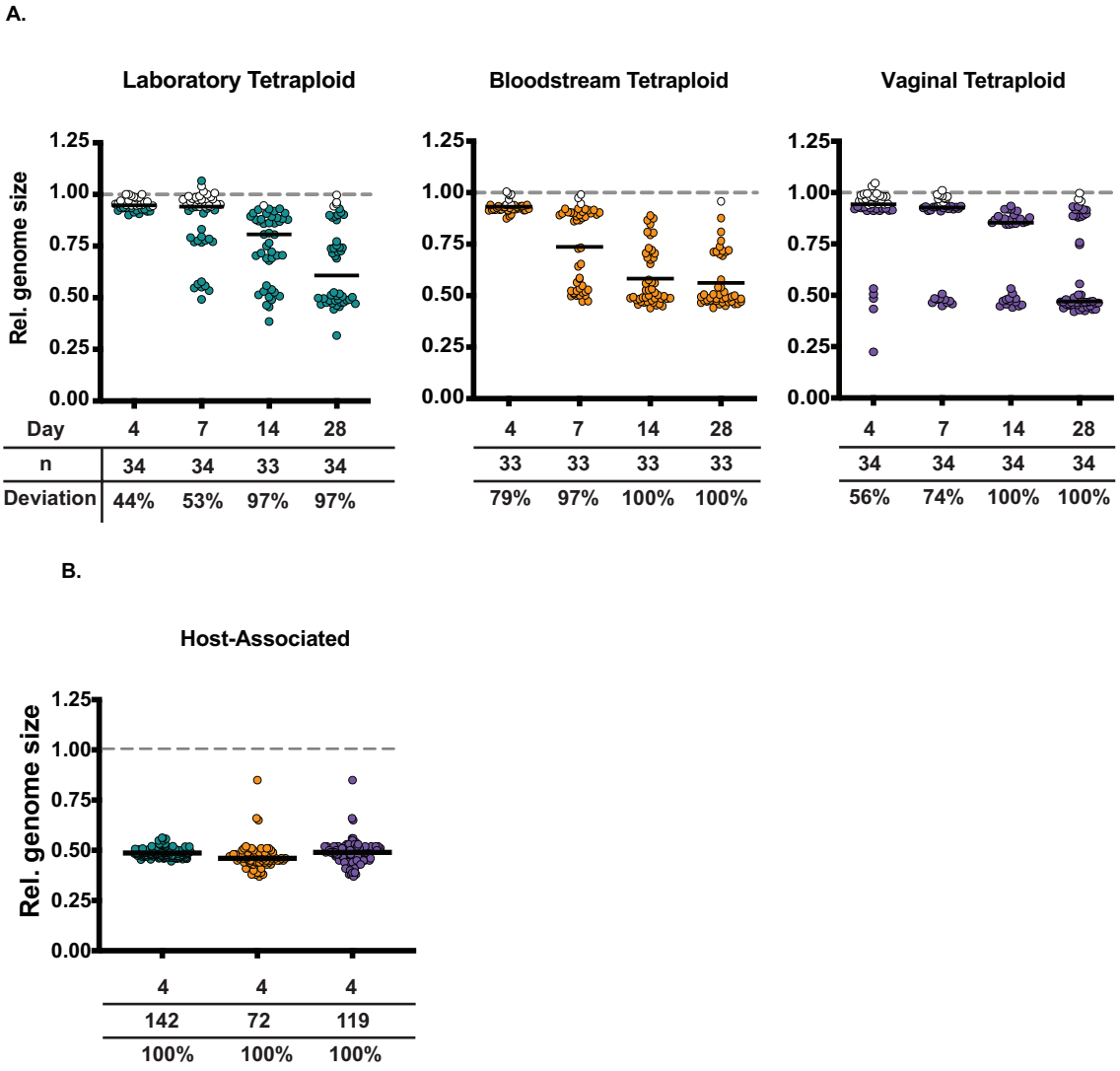

Figure 2.3 Tetraploid genome size stability *in vitro* and *in vivo*.

A) Relative genome size of laboratory, bloodstream, and vaginal tetraploid replicate lines passaged for 28 days in rich media. Symbols represent individual replicate lines and the median indicated by the solid black line. Filled symbols indicate replicate lines more than one standard deviation away from the Day 0 mean and reflect deviations from the initial tetraploid state.

B) Relative genome size of laboratory, bloodstream, and vaginal host-associated isolates. were extracted from the gut and genome size was measured via flow cytometry. Symbols represent individual *C. albicans* colonies extracted from the host and the median indicated by the solid black line. Filled symbols indicate replicate lines more than one standard deviation away from the 'No Host' mean and reflect deviations from the initial tetraploid state.

Host-induced genome size changes impact C. albicans growth and virulence

Since host-association induced genomic changes in *C. albicans*, we next wanted to assess if these changes had any fitness consequences on growth rate. We focused on host-associated derivatives with genome size changes, since genome size changes were not biased by LOH marker selection, and we did not have host-associated LOH isolates from our tetraploid strains. For each strain, we selected twelve host-associated isolates (Supplemental Table S2.3) and measured their growth rate in nutrient rich media and compared that to the growth rate of their respective parental strain (Figures 2.4A & B). It is important to note that there were some differences between genetic background and ploidy state in the parental strains. For each genetic background, the diploid growth rate was significantly different from its corresponding tetraploid growth rate (Table 2.2). For the laboratory and bloodstream ploidy pairs, the tetraploid grew more slowly than the diploid, but for the oral/vaginal genetic background the opposite was observed. Furthermore, within each ploidy class, the laboratory and bloodstream strains were significantly different from the oral/vaginal strains (Table 2.2). When we compare the host-associated isolates to their respective parental strain, we find collectively that derivatives from all strain backgrounds except the oral diploid and bloodstream tetraploid were significantly different (Figures 2.4A & B). Furthermore, the diploid host-derivatives had decreased growth rates and the tetraploid genetic backgrounds increased growth rates. When we compare the host-derived isolates as individuals to their parental strain, we detect some minor, yet significant changes in growth for all strain backgrounds and ploidies (Figures 2.4 A & B, filled symbols).

Decreases in virulence or changes to commensal-like phenotypes have been previously shown following short- and long-term association with host environments [33,34]. We have previously shown that *C. albicans* virulence can be assessed in *C. elegans* as lethal (i.e. host

survival) and non-lethal (i.e. fecundity) phenotypes that reduce host fitness [36]. To assess whether host-induced genome size changes altered virulence phenotypes, we measured the reproductive success (i.e. fecundity) of *C. elegans* hosts infected individually with each parental strain and their twelve host-derived isolates and calculated the relative fecundity by dividing the brood size of infected hosts by the brood size of uninfected hosts (Figures 2.4C & D). It should be noted that there were some differences between genetic background and ploidy state in the parental strains. The diploid-infected host fecundity was significantly higher than its corresponding tetraploid-infected host fecundity for the laboratory and oral/vaginal genetic backgrounds, indicating that tetraploids are generally more virulent than diploids (Table 2.3). We also found that genetic background impacted host fecundity for diploids, but not tetraploids (Table 2.3).

When we compare the host-associated isolates to their respective parental strain, we find collectively that derivatives from the laboratory diploid and tetraploid, and the bloodstream tetraploid were significantly different (Figures 2.4C & D). When we compare the host-derived isolates as individuals to their respective parental strain, there are significant changes to host fecundity for all strain backgrounds and ploidies (Figures 2.4C & D, filled symbols). In general, many of the diploid host-derived isolates were more virulent (i.e. reduced brood sizes) than their respective parental strain, with the exception of a small number of the host-derived isolates of the vaginal strain background that displayed reduced virulence. All of the tetraploid host-associated isolates that significantly differed showed reductions in virulence (i.e. larger brood sizes). However, there was no direct correlation between genome size changes and relative fecundity or growth (Supplemental figure S2.2). Taken together, our results indicate that even short periods of host association induce genomic changes that have direct impacts on virulence phenotypes.

Figure 2.4

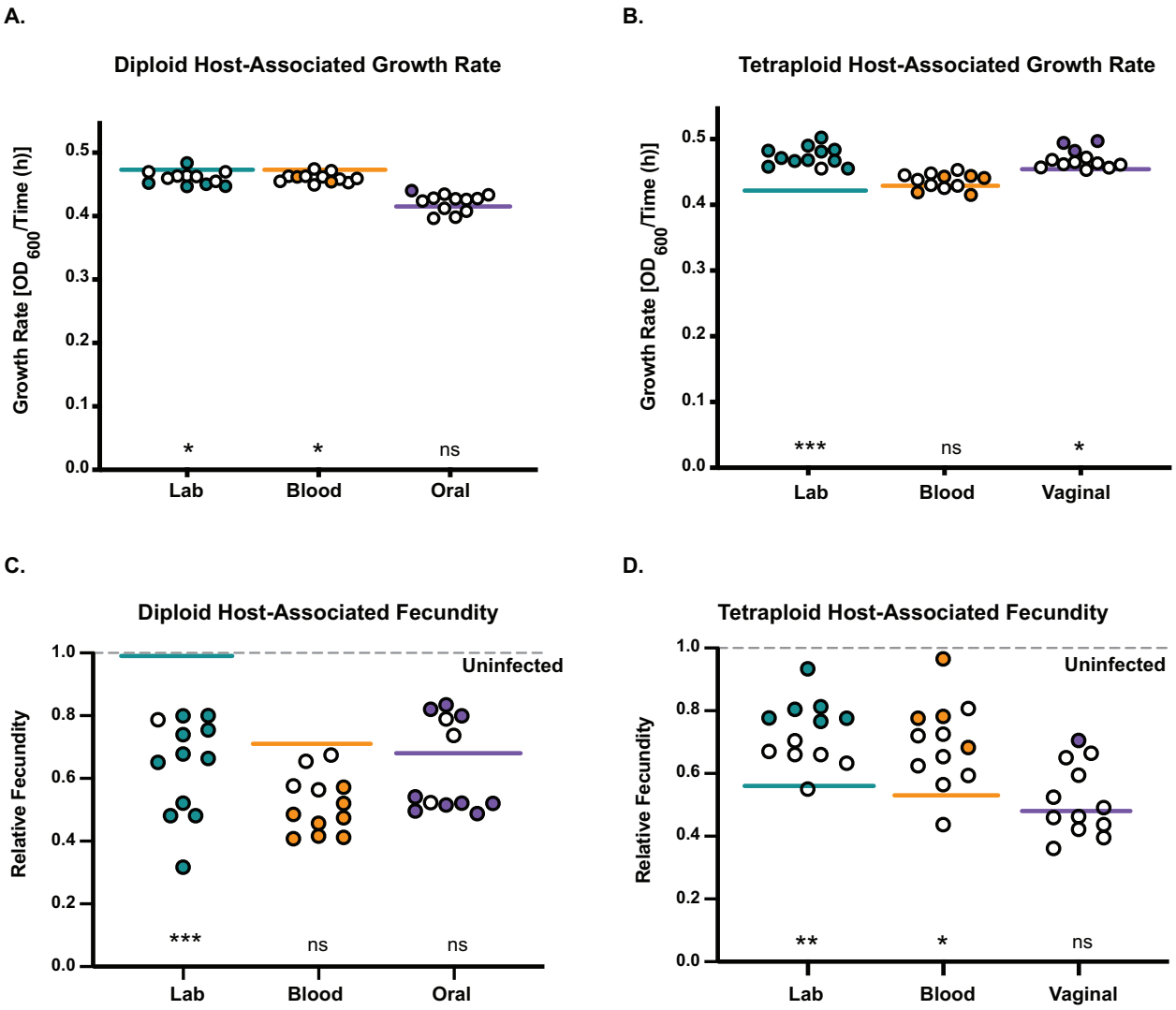

Figure 2.4: Growth rate and virulence phenotypes associated with host-induced genomic changes

A) Growth rate of the laboratory (green), bloodstream (orange), and oral (purple) host-associated isolates. Each solid line represents the mean growth rate of the parental strain. The filled in circles represent host-associated growth rates that are significantly different from the mean growth rate of the parental strain (Mann-Whitney U).

B) Same analysis as A) with tetraploid host-associated isolates

C) Relative fecundity of host infected with laboratory (green), bloodstream (orange), and oral (purple) host-associated isolates. Relative fecundity was calculated by dividing the total brood size of infected hosts by the total brood size of uninfected hosts. Solid lines represent the mean relative fecundity for each parental strain. Filled in circles are significantly different from the parental strain, open circles are not (Mann-Whitney U).

D) Same analysis as C) with tetraploid host-associated isolates. We tested for differences between the host-associated isolates and their respective parents using a Mann-Whitney U test (*** $p < 0.0001$, ** $p < 0.01$ * $p < 0.05$, ns = not significant)

2.6 Discussion:

Here, we investigated how diverse genetic backgrounds and ploidy states of *C. albicans* impact genome stability both inside and outside of the host environment. Previous work has shown that differences in genetic background give rise to phenotypic differences [29] , and also impact long-term genome dynamics [24]. Furthermore, earlier studies have shown that the host environment increases genome instability [30–33], but these studies do not address the roles genetic background and ploidy play in host-associated genome dynamics. By using three diploid-tetraploid pairs of *C. albicans* strains with distinct genetic backgrounds we were able to compare strain differences in genome stability both *in vitro* and *in vivo*. Furthermore, we analyzed how host-induced genetic changes impacted *C. albicans* growth and non-lethal host fitness phenotypes. We found that host-association increased genome instability relative to *in vitro* for all strain backgrounds (Figure 2.1). However, the magnitude by which the host elevated genome instability was dependent on strain background. We observed that diploids had minor genome size changes in the host environment (Figure 2.2), whereas our three tetraploid strains underwent rapid and large genome reductions in the host environment (Figure 2.3). Finally, when assessing whether host-induced genome size changes impacted host reproductive fitness, we found diploid derivatives generally increased in virulence and tetraploids generally decreased in virulence (Figure 2.4).

We were surprised to detect significant virulence changes in isolates derived from diploid-infected hosts, since we only detected modest genome size changes relative to tetraploids. However, 28 out of the 36 host-associated isolates had significant changes in virulence compared to their parental strain (Figure 2.4C). Host reproduction generally decreased when infected with diploid derivatives. Interestingly, 25% (3 of 12) diploid derivatives from the oral strain increased host brood sizes, indicating reduced virulence. Genetic background was also

important for diploid genome stability. For example, host-induced LOH was ~10-fold elevated in the laboratory and oral strain backgrounds, but only 2-fold elevated in the bloodstream strain. This is most likely due to the very high *in vitro* LOH frequency of the bloodstream strain. Our findings are consistent with whole-genome sequencing studies that show naturally occurring LOH events in clinical isolates [29,39–41]. These sequencing studies also detected chromosomal aneuploidy in a small number of clinical strains [28,29,40]. In our work, we observed that clinical diploid strains undergo minor genome size changes more frequently over the course of *in vitro* passaging and host-association compared to the laboratory strain (Figure 2.2). This study builds upon previous experimental studies which found that host environments elevate genome instability in *C. albicans* [30–32] and we extend this by explicitly testing for differences in multiple genetic backgrounds as well as in tetraploids.

Tetraploids underwent massive genome reductions when exposed to the host environment regardless of genetic background, and in contrast to diploids, which had significant but modest genome size changes. We propose that the host environment is inherently stressful and drives genome instability in *C. albicans* similar to stress-induced mutagenesis. There are several physiological relevant stressors that elevate LOH rates *in vitro*, including high temperature and oxidative stress [17,42]. Reactive oxygen species (ROS) production is an innate immune defense used to defend the host against invading pathogens [7,8], which inhibits growth by inducing DNA damage [43]. Our results indicate that host-induced genome instability could result from host ROS production. Given that *C. elegans* has a conserved innate immune system that includes producing ROS to defend against pathogens [44–46], it would be interesting to investigate how immune function contributes to pathogen genome instability.

The extreme genome instability observed in tetraploids, is partly due to their intrinsic highly labile nature. It has been well established that tetraploid *C. albicans* has higher levels of genome instability compared to diploids [18,22–24], and this phenomenon is also observed in related yeast species [47–50]. However, how genetic background impacts tetraploid genome stability has been extremely limited to date [24]. We have previously shown strain dependent differences in tetraploids following long-term *in vitro* serial passaging [24]. In this current work, we detected early differences across genetic backgrounds in tetraploid genome reduction during *in vitro* serial passaging (Figure 2.3A). From our collective *in vitro* results, we anticipated that host-association would likely induce genome size changes to some degree in tetraploids. However, we were surprised to observe all tetraploid host-derivatives with genome reductions, most of which were close to diploid in content (Figure 2.3B), after only four days of host association. While tetraploid *C. albicans* have been isolated in clinical settings [26–28], they are rare in comparison to diploid clinical isolates. Our results showing rapid host-induced tetraploid genome reduction may help explain the rarity of clinical tetraploids.

Host-induced genome changes resulted in subsequent changes in non-lethal virulence phenotypes for all strains (Figures 2.4C & D). We did not anticipate much phenotypic variation because host-induced genetic changes arose in the absence of selection, so it was striking that ~78% and ~31% of diploid and tetraploid host-associated derivatives changed virulence relative to their parental strains. Not only were virulence changes more frequent in the diploid derivatives, nearly all were increases in virulence (i.e. decreased host reproduction). In contrast, the few significantly different tetraploid host-derivatives had reductions in virulence. Differences in baseline virulence between the parental diploid and tetraploid strains (Table 2.3) may partially explain this result, since tetraploids were generally more virulent than diploids. It should be

noted that baseline differences in virulence between diploid and tetraploids have been observed previously, but in the opposite direction of our findings [51]. However, these differences may stem from our choice of model system (nematode vs. mouse), and virulence phenotype (lethal vs. non-lethal).

Finally, we were surprised that so few of the host-derived tetraploids changed in virulence, given the frequency and magnitude of genome size changes induced in the host environment. There was no correlation between changes in genome size and changes in virulence (Figure S2.2), similar to previous work demonstrating that *in vitro* adaptation to nutrient depletion is not correlated to genome size changes [24]. Tetraploid genome reduction generates massive karyotypic heterogeneity in cell populations through its parasexual cycle [22,23] by re-assorting chromosomes into new allelic combinations, that has been proposed to facilitate rapid adaptation [15]. Others have demonstrated that decreased heterozygosity correlates with decreased in pathogen fitness and virulence [29,40,52]. In our study, we measured genome size changes, but haven't yet characterized the allelic composition in our host-derived isolates. Thus, it is possible that after undergoing massive host-associated genome size reductions, tetraploid derivatives contained fewer heterozygous chromosomes. When we consider the large genomic but small phenotypic changes in tetraploids, coupled with the small genomic but large phenotypic changes in diploids, we propose that specific allelic combinations and potential *de novo* mutations foster virulence changes.

2.7 Acknowledgements:

I would like to thank Judy Dinh for her technical support, Ognenka Avramovska and Dr. Levi Morran for critical reading of the manuscript, and the Emory University School of Medicine

Flow Cytometry Core. This research is supported by NSF DEB-1943415 (MAH) and Emory University startup funds (MAH).

2.8 Tables & Supplementary Material: Chapter II

Table 2.1

Strain	Alias	Ploidy	Genetic Background	Source
YJB11700 ↟	Laboratory	Diploid (euploid 2N)	SC5314 reference strain; *GAL1/gal1Δ::NAT*	Gillum et al. (1984); Hickman et al 2013
RBY18	Laboratory	Tetraploid (euploid 4N)	Mating product between two SC5314-derived strains	Bennett and Johnson (2003)
FH1 ↟	Bloodstream	Diploid (euploid 2N)	Initial clinical isolate from marrow transplant patient (same as FH6); *GAL1/gal1Δ::NAT*	Hull et al. Marr et al. (1997); Abbey et al. (2014)
FH6 ↟	Bloodstream	~Tetraploid: 4xChr1, Chr2, Chr3, Chr7, ChrR; 3xChr4, Chr5, Chr6; 2x isochromosome (5L)	Mid-infection, post antifungal treatment, clinical isolate recovered from marrow transplant patient (same as FH1)	Hull et al. Marr et al. (1997); Abbey et al. (2014)
PN2	Oral	~Diploid: 2xChr1, Chr2, Chr3, Chr4, Chr5, Chr7, ChrR; 3xChr6	Clinical isolate recovered from the oral cavity from the same patient as PN1; *GAL1/gal1Δ::NAT*	Gerstein et al. (2017)
PN1	Vaginal	Tetraploid (euploid 4N)	Clinical isolate recovered from vaginal infection post antifungal treatment from the same patient as PN2	Gerstein et al. (2017)

Table 2.1: Strains used in this study
↟ Indicates strains with genome sequences available

Table 2.2

Strain		Diploid			Tetraploid		
		Laboratory	**Bloodstream**	**Oral**	**Laboratory**	**Bloodstream**	**Vaginal**
Mean Growth Rate ±*SD*		0.47 *0.02* n=12	0.47 0.01 n=12	0.42 0.01 n=12	0.42 *0.02* n=12	0.44 0.01 n=12	0.45 *0.03* n=12
Diploid	**Laboratory**						
	Bloodstream	ns					
	Oral	****	****				
Tetraploid	**Laboratory**	****	****	ns			
	Bloodstream	****	****	ns	ns		
	Vaginal	*	*	**	*	**	

Table 2.2: Parental Strain Growth Rates. Mean growth rate, standard deviation (SD) and number of biological replicates (n) are indicated for each treatment. Differences between treatments were tested in all pairwise combinations using the Mann-Whitney U test and significance indicated (ns – not significant; * $p<0.05$; ** $p>0.01$; *** $p>0.001$; **** $p>0.0001$).

Table 2.3

Strain		Diploid			Tetraploid		
		Laboratory	Bloodstream	Oral	Laboratory	Bloodstream	Vaginal
Mean Rel. Fedundity ±SD		0.91 *0.11* n=27	0.71 0.1 n=15	0.68 0.14 n=18	0.56 *0.1* n=10	0.52 0.12 n=6	0.48 *0.13* n=7
Diploid	Laboratory						
	Bloodstream	*					
	Oral	****	ns				
Tetraploid	Laboratory	****	*	*			
	Bloodstream	****	ns	*	ns		
	Vaginal	****	*	*	ns	ns	

Table 2.3: Parental Strain Relative Host Infected Fecundity. Relative fecundity (infected brood size/uninfected brood size), standard deviation (SD) and number of biological replicates (n) are indicated for each treatment. Differences between treatments were tested in all pairwise combinations using the Mann-Whitney U test and significance indicated (ns – not significant; * $p<0.05$; ** $p>0.01$; *** $p>0.001$; **** $p>0.0001$).

Supplemental Figure 2.1

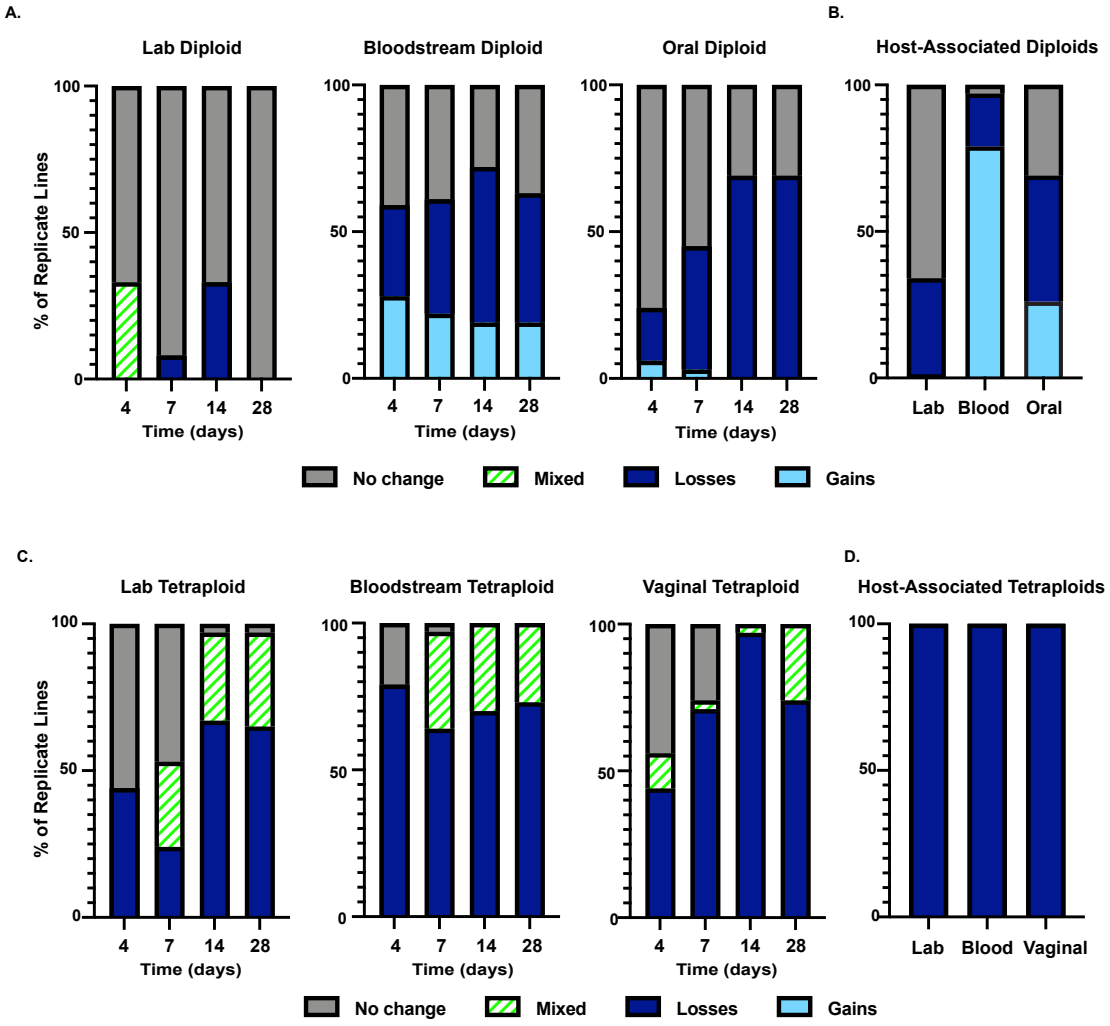

Figure S2.1: Types of genomic changes observed in diploids and tetraploids

A) The percent of genome size gains and losses in diploid strains. Flow cytometry was used to determine genome size. Gains in genome size (light blue) represent replicate lines greater than 1 SD from the Day 0 mean and losses in genome size (dark blue) are less than 1 SD from the Day 0 mean. Replicate lines with more than one G1 flow cytometry peak were considered mixed populations (green and white).

B) Same analysis as A, but for host-associated diploids.

C) Same analysis as A, but for each of the tetraploids *in vitro*.

D) Same analysis as A, but for host-associated tetraploids.

Figure S2.2

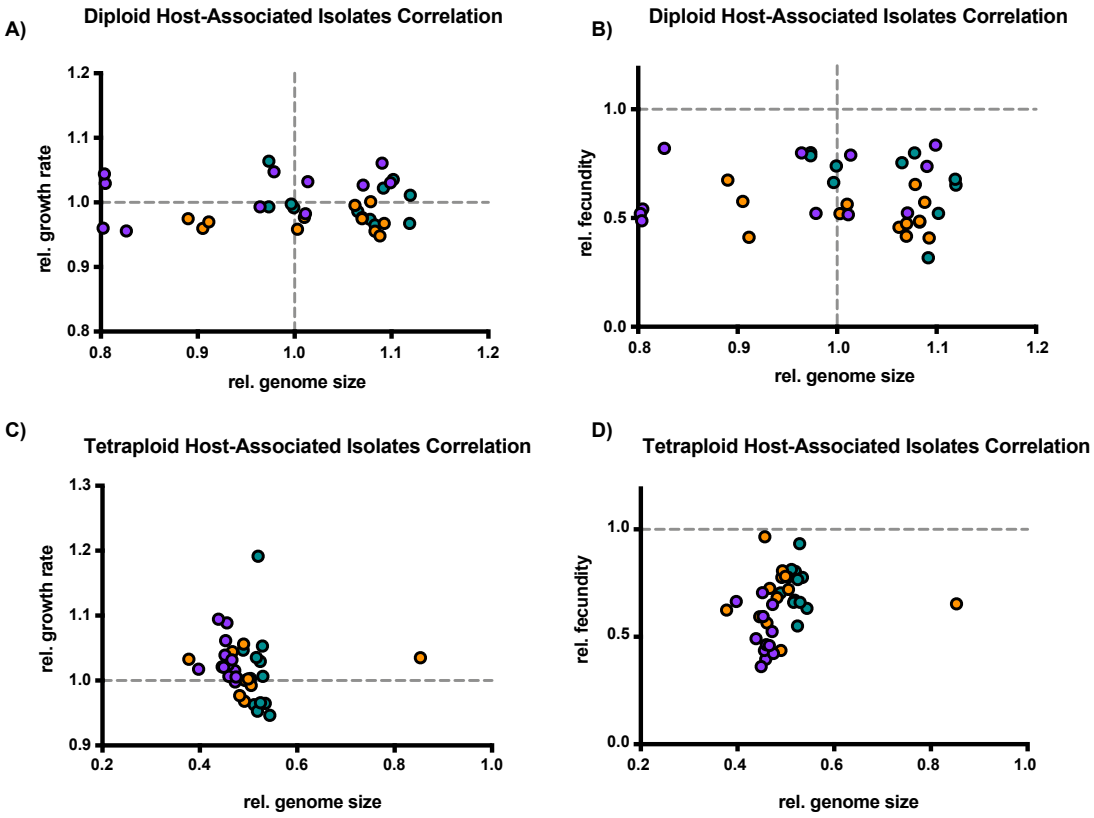

Figure S2.2 Correlation between genome size changes and fitness or virulence in both tetraploid and diploid *C. albicans* host-associated isolates.

A) Relationship between relative growth rate and relative genome size for host-associated laboratory (green), bloodstream (orange), and oral (purple) diploid isolates. Pearson correlation; laboratory: r = -0.08, p = 0.805; bloodstream: r = 0.18, p = 0.57; oral: r = 0.38, p = 0.22.

B) Relationship between the relative fecundity and the relative genome size for host-associated laboratory (green), bloodstream (orange), and oral (purple) diploid isolates. Pearson correlation; laboratory: r = -0.57, p = 0.051; bloodstream: r = -0.31, p = 0.33; oral: r = 0.42, p = 0.18).

C) Relationship between relative growth rate and relative genome size for host-associated laboratory (green), bloodstream (orange), and oral (purple) tetraploid isolates. Pearson correlation; laboratory: r = -0.24, p = 0.45; bloodstream: r = 0.08, p = 0.81; vaginal: r = -0.23, p = 0.47.

D) Relationship between the relative fecundity and the relative genome size for host-associated laboratory (green), bloodstream (orange), and oral (purple) tetraploid isolates. Pearson correlation; laboratory: r = -0.09, p = 0.78; bloodstream: r = -0.04, p = 0.91; vaginal: r = -0.33, p = 0.29.

Table S2.1

Primer name	Sequence	Direction	Purpose
oMH112	5'-CGGTTTCCGGGGGTTGGTGTTTAGTTTTCGTTTTTCGTTTTTTTTGGAAAGAATGTTTTAGCTCATTGACTGGATGGCGGCGTTAGTATCG-3'	Forward	Tagging primer to replace *GAL1* with *NAT* using pMH10
oMH113	5'-ATTTGTTTTGACCATATGGTAGTTGCGATATTCGTCGCCCTATCTATTTTTGCAACAGACTAATACCGTAAAACGACGGCCAGTGAATTC-3'	Reverse	Tagging primer to replace *GAL1* with *NAT* using pMH10
oMH104	5'-CTGGTTGGGGTGGTTCAATTG-3'	Forward	Verification of *GAL1*
oMH106	5'-GGTAGTTGCGATATTCGTCGC-3'	Reverse	Verification of *GAL1/NAT*
oMH5	5'-GGTGGATCAACTGGAACTTCTC-3'	Forward	Verification of *NAT*

Table S2.1: Oligos used in this study to generate *gal1::NAT/GAL1* heterozygous loci

Table S2.2

Treatment		*in vitro*			Host associated		
		Laboratory	**Bloodstream**	**Oral**	**Laboratory**	**Bloodstream**	**Oral**
Mean LOH ±*SD*		0.00013 *0.000079* n=9	0.0085 0.0043 n=9	0.0002 0.000095 n=12	0.0015 0.001 n=8	0.022 0.025 n=12	0.0019 *0.0023* n=9
in vitro	**Laboratory**						
	Bloodstream	****					
	Oral	ns	****				
Host associated	**Laboratory**	****	***	****			
	Bloodstream	****	ns	****	***		
	Oral	****	**	****	ns	***	

Table S2.2: Diploid LOH frequency. Mean LOH frequency, standard deviation (SD) and number of biological replicates (n) are indicated for each treatment. Differences between treatments were tested in all pairwise combinations using the Mann Whitney U test and significance is indicated (ns – not significant; * $p<0.05$; ** $p>0.01$; *** $p>0.001$; **** $p>0.0001$).

Table S2.3

		Diploids				Tetraploids			
	Strain	Genome size	Growth Rate ±1SD (n)	Rel. Fecundity ±1SD (n)		Strain	Genome size	Growth Rate ±1SD (n)	Rel. Fecundity ±1SD (n)
Laboratory	Parent	45,915	0.47±0.02 (12)	.99±.12 (27)		Parent	96,000	0.42 ± .02 (12)	.56 ± .10 (10)
	Host-associated derivatives 1	45,882	0.47±0.01 (3)	.74±.20 (8)		Host-associated derivatives 1	49,911	0.50 ± .005 (3)	.80 ± .07 (7)
	Host-associated derivatives 2	48,906	0.46±0.01(3)	.75±.14 (9)		Host-associated derivatives 2	49,128	0.48 ± 0.02 (3)	.81 ± .19 (8)
	Host-associated derivatives 3	45,755	0.46±0.01(3)	.66±.09 (8)		Host-associated derivatives 3	51,312	0.47 ± 0.03 (3)	.78 ±.10 (8)
	Host-associated derivatives 4	49,496	0.45±0.01 (3)	.80±.15 (7)		Host-associated derivatives 4	48,403	0.47 ± 0.02 (3)	.78 ± .14 (8)
	Host-associated derivatives 5	44,699	0.45±0.01 (3)	.80±.08 (10)		Host-associated derivatives 5	46,948	0.49 ± 0.02 (3)	.70 ± 22 (8)
	Host-associated derivatives 6	50,593	0.46±0.01 (3)	.52±.16 (7)		Host-associated derivatives 6	49,753	0.47 ± 0.02 (3)	.67 ± .17 (5)
	Host-associated derivatives 7	49,721	0.45±0.01 (3)	.48±.11 (8)		Host-associated derivatives 7	50,343	0.48 ± 0.02 (3)	.55 ± .06 (6)
	Host-associated derivatives 8	51,403	0.45±0.02 (3)	.65±.11 (8)		Host-associated derivatives 8	52,191	0.46 ± 0.04 (3)	.63 ± .11 (8)
	Host-associated derivatives 9	56,532	0.46±0.003 (3)	.48±.10 (5)		Host-associated derivatives 9	49,562	0.47 ± 0.03 (3)	.66± .19 (9)
	Host-associated derivatives 10	50,120	0.47±0.002 (3)	.32±.04 (3)		Host-associated derivatives 10	50,347	0.46 ± 0.03 (3)	.77 ± .14 (7)
	Host-associated derivatives 11	51,364	0.45±0.01 (3)	.68±.20 (7)		Host-associated derivatives 11	50,851	0.46 ± 0.01 (3)	.66± .16 (5)
	Host-associated derivatives 12	44,692	0.48±0.02 (3)	.79±.24 (6)		Host-associated derivatives 12	50,756	0.48 ± 0.01 (3)	.93 ± .17 (7)
Bloodstream	Parent	49,040	.47 ± .02 (12)	.71± .10 (15)		Parent	102,918	.43 ± 0.01 (12)	.53 ± .12 (6)
	Host-associated derivatives 1	52,094	0.47±0.05 (7)	.46±.04 (5)		Host-associated derivatives 1	45,892	0.44 ± 0.03 (7)	.59 ± .09 (8)
	Host-associated derivatives 2	52,470	0.46±0.03 (7)	.47±.09 (5)		Host-associated derivatives 2	47,018	0.44 ± 0.01 (7)	.96 ± .16 (10)
	Host-associated derivatives 3	44,389	0.45±0.03 (7)	.57±.09 (5)		Host-associated derivatives 3	50,442	0.45 ± 0.01 (7)	.43 ± .12 (7)
	Host-associated derivatives 4	53,108	0.45±0.02 (7)	.49±.03 (6)		Host-associated derivatives 4	47,452	0.44 ± 0.01 (7)	.57 ± .14 (8)
	Host-associated derivatives 5	44,699	0.46±0.04 (7)	.41±.08 (8)		Host-associated derivatives 5	50,599	0.42 ± 0.02 (7)	.77± .22 (5)
	Host-associated derivatives 6	52,456	0.46±0.02 (7)	.42±.06 (5)		Host-associated derivatives 6	50,743	0.43 ± 0.02 (7)	.81 ± .30 (4)
	Host-associated derivatives 7	53,578	0.46±0.03 (7)	.41±.11 (7)		Host-associated derivatives 7	52,037	0.43 ± 0.02 (7)	.72 ± .16 (4)
	Host-associated derivatives 8	53,358	0.45±0.03 (7)	.57±.11 (8)		Host-associated derivatives 8	48,062	0.45 ± 0.03 (7)	.73 ± .18 (6)
	Host-associated derivatives 9	43,653	0.46±0.02 (7)	.67± .00 (2)		Host-associated derivatives 9	51,368	0.43 ± 0.02 (7)	.78 ± .15 (5)
	Host-associated derivatives 10	49,532	0.46±0.01 (7)	.56±.09 (6)		Host-associated derivatives 10	49,621	0.42 ± 0.01 (7)	.68 ± .11 (5)
	Host-associated derivatives 11	49,182	0.45±0.01 (7)	.52±.13 (7)		Host-associated derivatives 11	38,843	0.44 ± 0.03 (7)	.62 ± .11 (10)
	Host-associated derivatives 12	52,892	0.47±0.02 (7)	.65±.14(9)		Host-associated derivatives 12	87,795	0.44 ± 0.01 (7)	.65 ± .13 (6)
Oral/Vaginal	Parent	50,680	0.38±0.03 (12)	.68±.14 (18)		Parent	98,243	.45 ± 0.03 (12)	.48±.08 (7)
	Host-associated derivatives 1	54,272	0.42±0.01 (7)	.52±.13 (5)		Host-associated derivatives 1	46,350	0.46 ± 0.02 (7)	.52 ± .09 (4)
	Host-associated derivatives 2	51,373	0.43±0.01 (7)	.97±.16 (6)		Host-associated derivatives 2	45,041	0.47 ± 0.02 (7)	.39 ± .01 (2)
	Host-associated derivatives 3	49,609	0.43±0.01 (7)	.52±.07 (7)		Host-associated derivatives 3	44,761	0.49 ± 0.02 (7)	.44 ± .08 (5)
	Host-associated derivatives 4	40,777	0.43±0.03 (7)	.54±.17 (7)		Host-associated derivatives 4	43,069	0.49 ± 0.05 (7)	.49 ± .12 (5)
	Host-associated derivatives 5	55,695	0.43±0.02 (7)	.84±.08 (5)		Host-associated derivatives 5	46,420	0.45 ± 0.02 (7)	.65 ± .09 (5)
	Host-associated derivatives 6	48,867	0.41±0.01 (7)	.80±.15 (9)		Host-associated derivatives 6	44,373	0.47 ± 0.03 (7)	.71 ± .09 (5)
	Host-associated derivatives 7	55,257	0.44±0.01 (7)	.74±.17 (6)		Host-associated derivatives 7	44,528	0.48 ± 0.02 (7)	.59 ± .10 (6)
	Host-associated derivatives 8	41,878	0.40±0.02 (7)	.82±.05 (8)		Host-associated derivatives 8	39,051	0.46 ± 0..01 (7)	.66 ± .10 (4)
	Host-associated derivatives 9	39,722	0.42±0.04 (7)	.49±.10 (9)		Host-associated derivatives 9	45,178	0.46 ± .0.02 (7)	.46 ± .15 (6)
	Host-associated derivatives 10	51,250	0.41±0.02 (7)	.52±.11 (10)		Host-associated derivatives 10	44,115	0.46 ± 0.03 (7)	.36 ± .13 (4)
	Host-associated derivatives 11	40,667	0.40 ± .01 (7)	.52±.10 (10)		Host-associated derivatives 11	46,617	0.46 ± 0.02 (7)	.42± .07 (3)
	Host-associated derivatives 12	40,727	0.43 ± .01 (7)	.49 ± .08(10)		Host-associated derivatives 12	45,786	0.47 ± 0.03 (7)	.46± .03 (4)

Table S2.3 Characteristics of host-associated isolates.
For each genetic background and ploidy, the genome size (measured as mean G1 FITC signal), growth rate, and infected host fecundity values are indicated.

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Chapter III

Host defense mechanisms induce genome instability in an opportunistic fungal pathogen

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3.1 Abstract:

The ability to generate genetic variation facilitates rapid adaptation in stressful environments. The opportunistic fungal pathogen *Candida albicans* frequently undergoes large-scale genomic changes, including aneuploidy and loss-of heterozygosity (LOH), following exposure to physiological stressors and host environments. However, the specific host factors that induce *C. albicans* genome instability remains largely unknown. Here, we leveraged genetically-tractable nematode hosts to specifically investigate the innate immune components driving host-associated *C. albicans* genome instability, which include host production of antimicrobial peptides (AMPs) and reactive oxygen species (ROS). *C. albicans* associated with wildtype, immunocompetent hosts carried multiple large-scale genomic changes including LOH, whole chromosome, and segmental aneuploidies. In contrast, *C. albicans* associated with immunocompromised hosts deficient in AMPs or ROS production had reduced LOH frequencies and fewer, if any, additional genomic changes. We also found that *C. albicans cap1*Δ/Δ strains deficient in ROS detoxification, were more susceptible to host-produced ROS genome instability compared to wildtype *C. albicans*. Further, genomic perturbations resulting from host-produced ROS are mitigated by the addition of antioxidants. Taken together, this work suggests that host-produced ROS and AMPs induces genotypic plasticity in *C. albicans* which may facilitate rapid adaptation and lead to phenotypic changes.

3.2 Introduction:

The opportunistic fungal pathogen *Candida albicans* is typically commensal and a component of the human microbiome[1], yet is a leading cause of fungal bloodstream infections and 40% of these infections result in mortality[2]. In addition to bloodstream infections, *C. albicans* causes non-lethal mucosal infections, including vaginal and oral candidiasis[2]. *C. albicans* infection is highly dependent on the host context, including high estrogen levels[3], chronic stress[4], antibiotic use[5–7], uncontrolled diabetes[8,9], and immunosuppression[10,11]. In the absence of proper immune recognition, fungal proliferation is uncontrolled, leading to infection. However, healthy individuals, despite having a fully functioning immune system, also experience *C. albicans* infections resulting from the commensal isolates becoming pathogenic rather than an infection acquired from outside sources[12]. The transition from commensal to pathogenic organism may be facilitated by *C. albicans* phenotypic and genotypic heterogeneity. *C. albicans* genetic diversity within and among individuals is very high and often include numerous single nucleotide polymorphisms (SNPs) and loss of heterozygosity (LOH) events [13]. This genetic variation may be a direct consequence of the stressors *C. albicans* encounters in the host which include immune stressors and other microbes. Recent work has demonstrated the host environment elevates *C. albicans* genome instability similar to *in vitro* stressors [14–18]. However, the specific host components that generate this instability is largely unknown.

Genomic variation is quite common in clinical *C. albicans* isolates [13, 19] and includes polymorphisms, copy number variation, LOH, and partial or whole chromosomal aneuploidies [19]. This genomic variation indicates that host environments either induce or maintain genetic variation in *C. albicans*. Murine infection models have found that when exposed to different host

niches, *C. albicans* has a greater than 10-fold increase in LOH and frequent aneuploidy compared to *in vitro* [14–16]. Following host exposure, genomic changes generated in the murine environment often resulted in a more commensal-like phenotype and higher fitness inside the host [15,16,18,20]. However, the long-term genotypic and phenotypic consequences have not yet been extensively studied [20]. *Caenorhabditis elegans* has also been used as a host model to assess *C. albicans* genome stability and host-associated genome instability is detected across multiple *C. albicans* strains and that even short-term host association resulted in changes to virulence [17]. Together, murine and *C. elegans* models clearly demonstrate that host environments drive genetic diversity in *C. albicans* which cause phenotypic changes, however, it is remains unclear what specific host components contribute to *C. albicans* genome instability.

C. albicans encounters many different stressors within the host environment, including the immune system, which is designed to control and remove pathogens. The immune system recognizes *C. albicans* and other pathogens through pathogen recognition receptors (PRRs) that detect the specific microbial chemical signatures called pathogen-associated molecular patterns (PAMPs) [21]. Recognition of pathogens like *C. albicans* trigger a specific cellular response. Similar to humans, *C. elegans* produce reactive oxygen species (ROS) and antimicrobial peptides (AMPs) in response to pathogens. AMPs inhibit microbial growth through a variety of methods, including disrupting the cell membrane, and halting DNA, RNA, and protein synthesis [22]. *C. elegans* AMP production is activated through a mitogen-activated protein kinase (MAPK) signaling cascade [23,24] and includes SEK-1 (MAPKK), which is homologous to the mammalian MKK3/6 and MKK4 MAPKKs [25]. Mutations in *C. elegans* SEK-1 increase host susceptibility to *C. albicans* and other microbial pathogens [26,27]. Another conserved host defense mechanism is ROS production. Mammals produce ROS via five NADPH oxidases and

two dual oxidases [32,33] while *C. elegans* ROS production is mediated by a single dual oxidase BLI-3 in response to bacterial and fungal pathogens [26–29]. BLI-3 mutant hosts are more susceptible to *C. albicans* infection [30]. ROS causes cellular toxicity through structural changes to the DNA [31] and generates double strand breaks in *C. albicans* [32]. Together, host-produced AMPs and ROS act in various ways in order to inhibit *C. albicans* growth.

Here we investigated if host-produced AMPs and ROS induce *C. albicans* genome instability using the model host *C. elegans*. We infected immunocompetent and two different immunocompromised hosts with mutations in either *sek-1* (AMP production) or *bli-3* (ROS production) with *C. albicans* and measured the frequency and types of genome changes. Immunocompetent hosts elevate genome instability and generate greater genetic diversity in *C. albicans* compared to immunocompromised hosts. Host-produced ROS elevated *C. albicans* LOH regardless of strain background. However, host-produced AMPs only elevated *C. albicans* genome instability in a strain dependent manner. Taken together, our results suggest that host innate immune pathways are a source of genome instability in *C. albicans*.

3.3 Materials and Methods:

Strains and Maintenance

C. albicans and *C. elegans* strains for this study are listed in Table 1. Yeast strains were stored at -80°C and maintained on YPD (yeast peptone dextrose; 1% yeast extract, 2% bactopeptone, 2% glucose, 0.004% adenine, 0.008% uridine) at 30°C. Strains were initially struck onto YPD agar plates from frozen glycerol stocks and incubated at 30°C for 48 hours and single colonies used as the “parental strain” in subsequent in vivo experiments. Nematode populations were maintained on plates containing nematode growth media (NGM) with *E. coli*

(OP50) for a food source. *C. elegans* were transferred to a new plate containing freshly seeded *E. coli* every three to four days. For genome stability assays, treatment plates were seeded with both *C. albicans* and *E. coli* and supplemented with 0.2 g/L streptomycin to inhibit overgrowth of *E. coli*. For fecundity and genome stability assays, NGM was supplemented with 0.08g/L of uridine and 0.08g/L of histidine to facilitate growth of auxotrophic *C. albicans* strains.

Host-associated C. albicans genome stability

Host preparation: NGM plates are seeded with a mixture of *E. coli* and *C. albicans* 24 h prior to host preparation. To seed plates, single colonies of *C. albicans* were inoculated into 3 mL YPD and incubated overnight at 30°C. Cultures were adjusted with ddH_2O to a final concentration of 3.0 OD_{600} per mL. Simultaneously, a single colony of *E. coli* was inoculated into 50 mL LB and incubated for 24-48 h at 30°C. The *E. coli* culture was pelleted and washed twice with 1 mL ddH_2O. The pellet was weighed and diluted to final concentration of 200 mg/mL. For *in vitro* treatments, 250 µL *C. albicans* was spread onto NGM + streptomycin agar plates and incubated overnight at 30°C. For *in vivo* treatment plates, 6.25 µL *C. albicans*, 31.25 µL *E. coli*, and brought to a final volume of 250 µL with ddH_2O, was spread onto NGM + streptomycin agar plates and incubated overnight at 30°C. For experimental evolution experiments, *C. albicans* treatment plates had 1.25 µL of *C. albicans* and 6.25 µL of *E. coli* and were brought to a final volume of 50 µL. The entire 50 µL was spotted onto the center of a 35-mm-diameter NGM plus streptomycin agar plate, followed by incubation at room temperature overnight before the addition of eggs or transferring nematodes.

To synchronize *C. elegans* populations, nematodes and eggs were washed off plates with M9 buffer, transferred to 15 mL conical tubes, and pelleted via centrifugation (2 min at 1200

rpm). The pellet was resuspended in 2 mL of 25% bleach, mixed via inversion for 2 min, and centrifuged for 2 min at 1200 rpm. The pellet was washed twice with 3 mL ddH_2O and resuspended with 1 mL ddH_2O. To determine the concentration of eggs, 10 µL was pipetted onto a concave slide, eggs were visualized microscopely and counted, and the suspension was adjusted to a concentration of ~100 eggs per 100 µL with M9.

Host-associated yeast extractions: Yeast extractions were performed as described previously [17]. Hosts exposed to *C. albicans* were washed from plates with 3 mL M9 and pelleted via centrifugation (2 min at 2,000 RPM). The supernatant was removed and the pellet was resuspended with 1 mL 3% bleach, transferred to a microcentrifuge tube, and incubated for three minutes and subsequently centrifuged for 1 min at 12,000 rpm. The supernatant was removed and washed with 1 mL of M9 and centrifuged for one minute at 12,000 rpm. The wash was repeated two more times to ensure all bleach was removed. 100 µl aliquots of nematode suspension were transferred to 0.6 mL clear microtubes for manual disruption with a motorized pestle. After one minute of manual disruption, the worm intestine solution was then diluted accordingly with an M9 buffer and plated on YPD + 0.034mg/L chloramphenicol to select prevent any bacterial colonies from arising.

GAL1 Loss of Heterozygosity assay

In vitro: Single colonies of *C. albicans* were inoculated in 3 mL YPD grown overnight at 30°C and subsequently diluted to 3 OD in ddH_2O. 250 µL was plated and spread onto NGM + streptomycin plates and incubated overnight at 30°C and transferred to 20°C for four days. On day four, yeast cells were washed off with ddH_2O, harvested by centrifugation, washed once with ddH_2O, resuspended in 1 mL of ddH_2O and serially diluted for single colony growth. To determine the total cell viability, 100 µL of 10^{-6} dilution was plated onto YPD and grown for 48

h at 30°C. To identify cells that lost *GAL1*, 100 µL of 10^{-2} and 10^{-3} dilution was plated onto 2-deoxygalactose (2-DOG; 0.17% yeast nitrogen base without amino acids 0.5% ammonium sulfate, 0.0004% uridine, 0.0004% histidine, 0.1% 2-deoxygalacose, 3% glycerol) and colony forming units (CFUs) counted following 72 hours incubation at 30°C. LOH assays performed with α-Lipoic acid were performed as described above but α-Lipoic acid (Sigma-Aldrich #1077-28-7) was dissolved in 100% ethanol and added to NGM media containing 0.2 g/L streptomycin sulfate to a final concentration of 25 µM.

In vivo: The approach was very similar as the *in vitro* LOH assay described above, with the following changes. A population of ~100 nematodes were plated on each treatment plate containing both *C. albicans* and *E. coli.* On day four, yeast were extracted as described in the previous section. A dilution of 10^{-1} and 10^{-2} was plated on YPD + chloramphenicol to enumerate total growth and undiluted cells were plated on 2-DOG to select for the loss of *GAL1.* Three technical replicates were used for each *C. albicans* strain for both *in vitro* and *in vivo* experiments. At least three biological replicates were used for each genome stability assay.

Hydrogen Peroxide Exposure and Genome Stability:

Six single colonies of *C. albicans* were inoculated in either 2 ml of YPD or in 2 mL of YPD containing 5 mM H_2O_2, grown for 20 h at 30°C. Cultures were centrifuged at 2000 rpm for 2 minutes. The supernatant was removed, and the pellet was washed once with 1 mL of ddH_2O. Cultures were serially diluted for single colony growth. Loss-of-heterozygosity assays were performed to determine the frequency of LOH.

Whole genome sequencing and analysis

Genomic DNA was isolated with phenol chloroform as described previously[50]. Whole genome sequencing was performed through the Microbial Genome Sequencing Center using a single library preparation method based on the Illumina Nextera kit. Libraries were sequencing using paired end (2 x 150 bp) reads on the NextSeq 550 platform. Adaptor sequences and low-quality reads were trimmed using Trimmomatic (v0.39 LEADING:3 TRAILING: 3 SLIDINGWINDOW: 4:15 MINLEN: 36 TOPHRED33)[51]. All reads were mapped to the phased *C. albicans* reference genome[52] using the haplotypo python script 'mapping.py'. This tool uses the Burrows-Wheeler Aligner MEM (BWA v0.1.19) algorithm to align the sequencing reads to the reference genome followed by Samtools (v0.1.19) to sort, mark duplicates, and create a BAM file. Variant files were created using the haplotypo python script 'var_calling.py' using the BCFtools calling method. Identification of aneuploidy, CNVs, and LOH were conducted using whole genome sequencing data and the Yeast Mapping Analysis Pipeline (YMAP). BAM files were uploaded to YMAP and plotted using the *Candida albicans* reference genome (A21-s02smo8-r09) with corrections for chromosome end bias and GC content[53]. Identification of SNPs, INDELs and Ts/Tv: Variant files were analyzed using BCFtools 'stats' followed by plot-vcfstats to generate a visual representation of the proportion of each type of variant.

Statistical analysis:

Statistical analysis was performed using GraphPad Prism 8 software. Data sets were tested for normality using the D'Agnostino & Pearson omnibus normality test.

3.4 Results:

Host defense pathways elevate C. albicans genome instability

We and others have shown that nematode and murine host environments induce *C. albicans* genome instability compared to *in vitro* [14–18], yet the specific host attributes driving genome instability have not been elucidated. Here, we tested whether components of host innate immune function drove host-associated genome instability by comparing *C. albicans* LOH between yeast extracted from immunocompetent and immunocompromised hosts (Fig. 3.1A). We used two different immunocompromised host genotypes, one carried a *sek-1* deletion and does not produce AMPs [33], the second host genotype carried a *bli*-3 deletion and could not produce ROS [27,29]. *C. albicans* LOH frequency was significantly reduced when extracted from *sek-1* and *bli-3* hosts compared to wildtype (N2) hosts (Figs. 3.1B, S3.1A&B) and implicates both *sek-1* and *bli-3* immune pathways as a source of pathogen genome instability.

Since *C. albicans* genetic background impacts mutation rates [15,17] we also measured host-induced LOH frequency in a clinical *C. albicans* strain (Marr 1997). Similar to the laboratory *C. albicans* strain, the clinical strain had significantly reduced LOH frequency when extracted from *bli-3* hosts compared to wildtype hosts (Fig. 3.1C), yet the clinical *C. albicans* LOH frequency extracted from *sek-1* and wildtype hosts was not statistically different. Together, these data suggest that the *bli-3* pathway induces diploid *C. albicans* genome instability regardless of pathogen genetic background, and the *sek-1* pathway may only contribute to genome instability in a strain-dependent manner.

While, tetraploid *C. albicans* are isolated from patients, they are more unstable than diploids and rapidly lose chromosomes within the host environment. Therefore, I wanted to uncover if host immune components were responsible for the genome size reduction observed in tetraploid *C. albicans* exposed to the host environment. In this case, I measured genome size changes with flow cytometry of three different tetraploid *C. albicans* strains following exposure

to healthy, *sek-1*, and *bli-3* hosts. After exposure to either *sek-1* or *bli-3* hosts a majority of *C. albicans* isolates regardless of strain background, maintained ploidy (Fig. S3.2). This is similar to what we observed *in vitro*, but in contrast to *C. albicans* extracted from wildtype hosts which had were mostly diploid. Across all *C. albicans* strains, less than 5% of isolates extracted from either *sek-1* or *bli-3* immunocompromised hosts had genome size reductions to approximately diploid. Together, this suggests that both host-produced AMPs and ROS play a role in generating genome stability in tetraploid *C. albicans*.

Figure 3.1

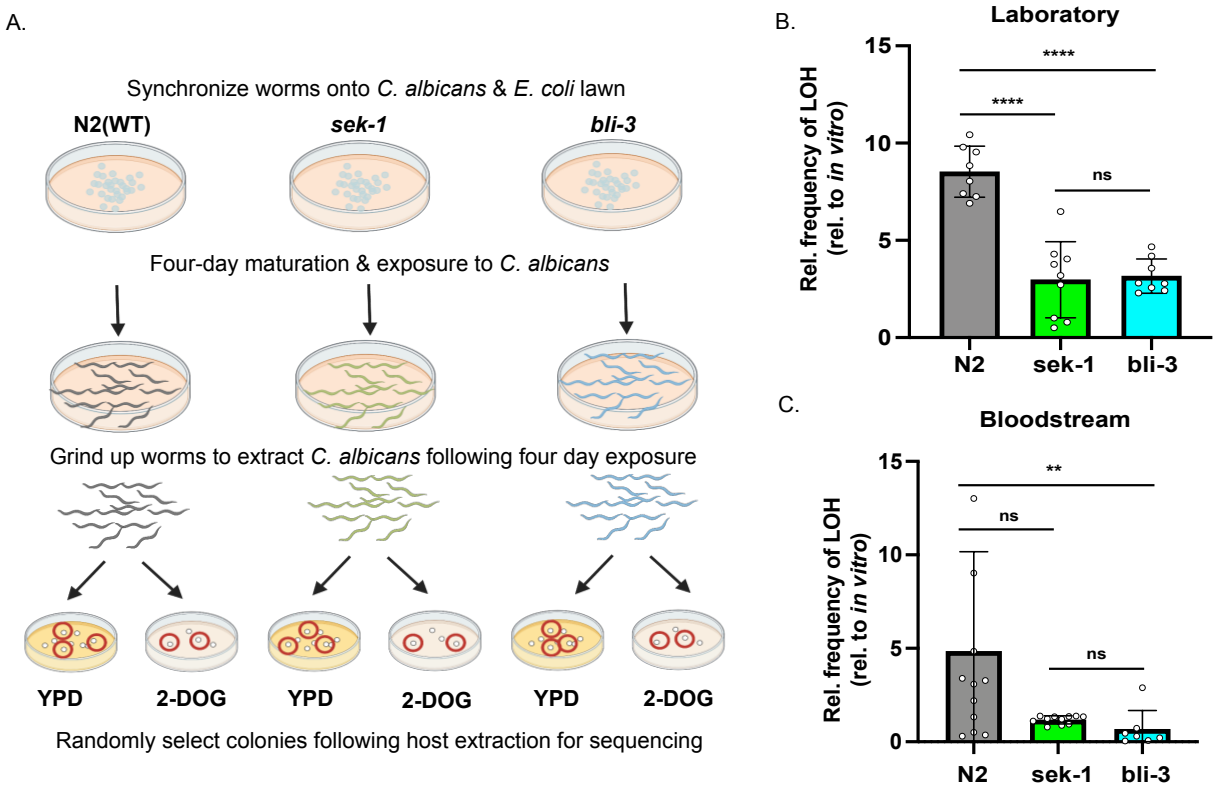

Figure 3.1: Host immunity impacts *C. albicans* genome stability. A) Experimental overview of in vivo experiments. B) Laboratory *C. albicans* LOH frequency following host association relative to the LOH frequency of *C. albicans* no host control for N2 (grey, n = 8), *sek-1* (green, n = 9), and *bli-3 (*blue, n = 8). C) Bloodstream *C. albicans* LOH frequency following host association relative to the LOH frequency of *C. albicans* no host control for N2 (grey, n = 11), *sek-1* (green, n = 11), and *bli-3 (*blue, n = 8). Plotted are the means and SD. Asterisks indicate significant differences (**** $p < 0.0001$, ** $p < 0.01$ ns = not significant; Kruskal-Wallis with post-hoc Dunn's multiple test).

In vitro and in vivo ROS elevate genome instability in cap1Δ/Δ C. albicans

In response to pathogens bacterial and fungal pathogens, *C. elegans* produces ROS via the BLI-3 dual oxidase [30]. However, *C. albicans* has several mechanisms to combat ROS, including activation of antioxidant genes that detoxify ROS, whose expression is regulated by the Cap1p transcription factor [34,35]. To directly test whether *CAP1* protects *C. albicans* from ROS-induced genome instability, we compared *in vitro* LOH frequencies of wildtype and *cap1*Δ/Δ *C. albicans* strains exposed to 5 mM H_2O_2. While exposure to H_2O_2 elevated the LOH frequency in both wildtype and *cap1*Δ/Δ *C. albicans* strains, the increase in LOH was significantly higher in *cap1*Δ/Δ that had a 40-fold increase compared to wildtype that had a 15-fold increase (Fig. 3.2A & S3.3A). To assess whether *CAP1* mitigates *C. albicans* genome instability from host-produced ROS, we compared LOH frequencies between wildtype and *cap1*Δ/Δ *C. albicans* associated with immunocompetent and immunocompromised hosts. If host-produced ROS induces *C. albicans* genome instability, then *cap1*Δ/Δ *C. albicans* will have increased LOH in N2 and *sek*-1 hosts that are capable of producing ROS, but not in *bli-3* hosts that cannot. Compared to *in vitro*, all host environments increased *cap1*Δ/Δ LOH (Fig. S3.3B), and host-extracted *cap1*Δ/Δ LOH frequencies were higher compared to wildtype *C. albicans* for both immunocompetent and *sek-1* hosts, but not from *bli-3* hosts (Fig. 3.2B). Together, these data demonstrate that host-produced ROS elevated *C. albicans* genome instability and that Cap1p-mediated ROS detoxification is important for mitigating ROS-induced *C. albicans* genome instability both *in vitro* and *in vivo*.

Endogenous and exogenous antioxidants break down ROS through a variety of mechanisms, thus we next wanted to determine whether antioxidants mitigate *C. albicans* genome instability resulting from host-produced ROS. We compared host-associated *C. albicans*

LOH in the presence or absence of 25 μM of lipoic acid, an antioxidant involved in the breakdown of ROS [29]. In the presence of lipoic acid, host-associated *C. albicans* LOH was significantly reduced when extracted from immunocompetent and *sek-1* hosts (Figs. 3.2C & S3.3C). In contrast, lipoic acid did not decrease *C. albicans* LOH when extracted from *bli-3* hosts (Figs. 3.2C & S3.3C). This suggests that antioxidants are effective in reducing *C. albicans* genome instability in ROS-producing hosts. Together, these data suggest that both *in vitro* and *in vivo* ROS elevates genome instability in a *cap1*Δ/Δ mutant, and that *C. albicans* genome instability caused by host-produced ROS can be alleviated with the addition of antioxidants.

Figure 3.2

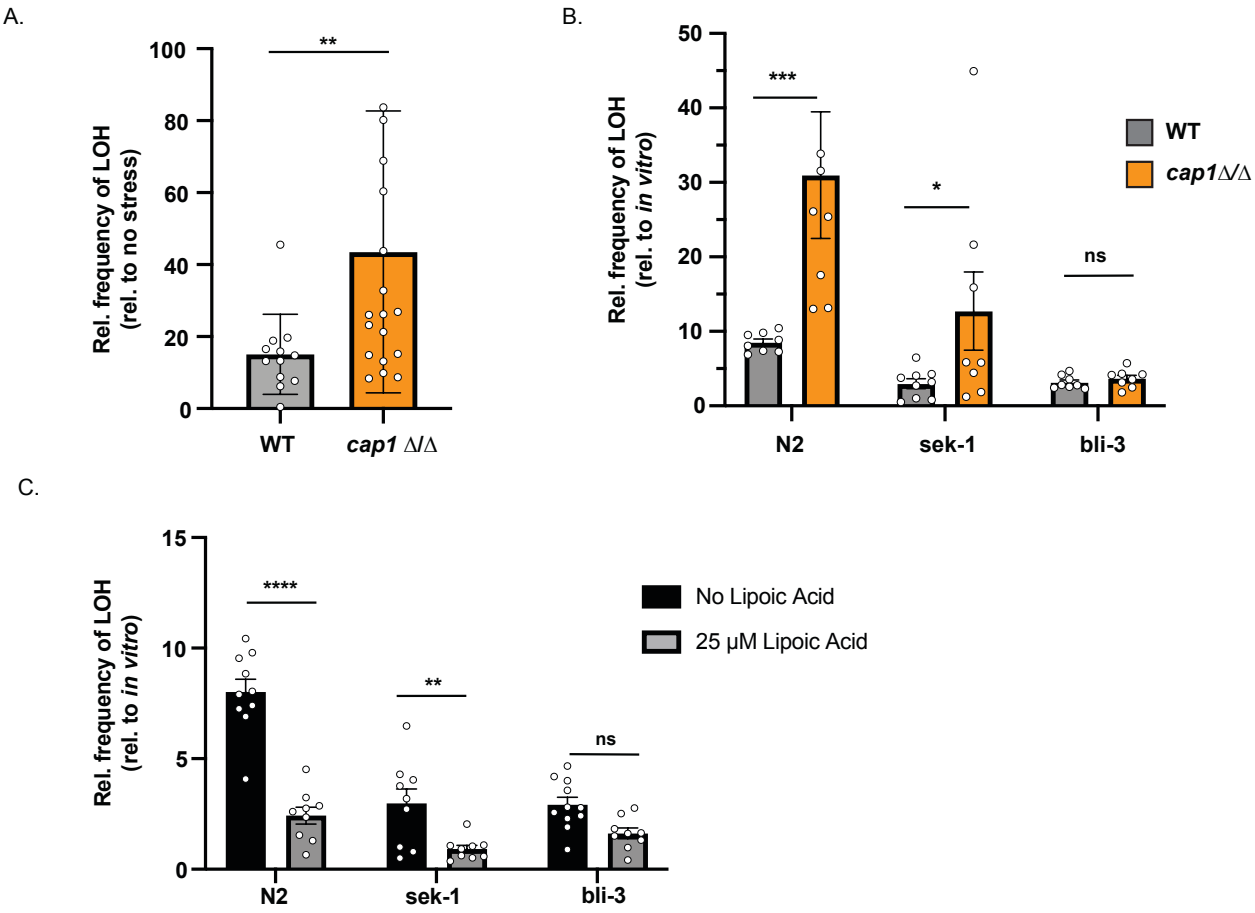

Figure 3.2: *C. albicans cap1* Δ/Δ strain is more susceptible to *in vitro* and *in vivo* ROS
A) Plotted are the LOH frequencies of C. albicans exposed to 5mM H_2O_2 for 24 h relative to the frequencies of LOH of C. albicans without stress exposure. Plotted are the means and SD for both wildtype (WT) (grey, n = 12) and cap1Δ/Δ (orange, n = 19). Each data point represents an individual measurement. B) Plotted are the LOH frequencies of C. albicans exposed to each host environment relative to the no host frequency of LOH. Plotted are the means and SD. C) C. albicans LOH frequency following host exposure relative to the no host LOH frequency with 25 μM of alpha-lipoic acid (grey, N2: n = 9, sek-1: n = 9, bli-3: n = 9) and without 25 μM of alpha-lipoic acid (black, N2: n = 16, sek-1: n = 9, bli-3: n = 19). Each data point represents an individual measurement. Plotted are the means and SD. Asterisks indicate significant differences (**** $p < 0.0001$, *** $p < 0.005$, ** $p < 0.01$, * $p < 0.05$, ns = not significant; Mann-Whitney U test).

Host-produced AMPs and ROS cause aneuploidy and abundant LOH events in C. albicans

LOH assays are an easy, useful and established way to measure *C. albicans* genome instability [36,37], however, they are limited to measuring a heterozygous marker at a single genomic location. To characterize whether host immunity drives other genomic alterations, we whole-genome sequenced single colony isolates associated with wildtype, *sek-1* and *bli-3* hosts that were isolated either on media that selected for LOH events (2-DOG; Fig. 3.3A) or media that did not select for LOH events (YPD; Fig. S3.4). Among LOH-selected isolates extracted from immunocompetent hosts, *GAL1* LOH on Chr1 was mediated via break-induced recombination (4/6) or via whole chromosome loss and reduplication (2/6). For the break-induced recombination LOH events, there was no evidence for a common breakpoint or recombination hotspot mediating host-induced *GAL1* LOH. Additional large-tract LOH events, as well as whole-chromosomal and segmental aneuploidies were identified in all LOH-selected isolates from N2 immunocompetent hosts, but not from immunocompromised host backgrounds (Fig. 3.3A). Four isolates carried a long-tract LOH or segmental aneuploidy on Chr2 with breakpoints ranging between positions 1,795,723 and 2,155,170; four carried a 170 kb LOH tract or segmental aneuploidy on Chr6, whose breakpoint corresponds with position 861,044, the beginning of a major repeat sequence (MRS). One isolate was trisomic for Chr7 and tetrasomic for Chr6. Even among non-LOH selected isolates associated with immunocompetent hosts, 50% had undergone whole-chromosome loss and reduplication on Chr2 (Fig. S3.4), but no large-scale genomic changes were detected in non-LOH selected isolates associated with immunocompromised hosts (Fig. S3.4A). Thus, immune components in immunocompetent hosts induced large-scale genomic changes in *C. albicans*.

We also investigated whether there were differences in smaller-scale genetic changes in *C. albicans* extracted from immunocompetent or immunocompromised hosts. Given to the

mutagenic nature of ROS, we anticipated more SNPs, specifically GC→TA and GC→CG transversions [38], in ROS-producing hosts, but did not detect any significant differences among host backgrounds (Fig. 3.3B). Further, there were no significant differences in the proportion of C→A and C→G substitutions. We did detect a significantly lower number of INDELs in isolates extracted from *bli-3* hosts compared to *sek-1* hosts (Fig. 3.3B), indicating that host-produced ROS may generate INDELs in *C. albicans*. Despite incurring roughly the same number of mutations regardless of host background, larger chromosomal events including aneuploidy and large-scale LOH are primarily observed in isolates from immunocompetent hosts and not immunocompromised hosts.

Figure 3.3

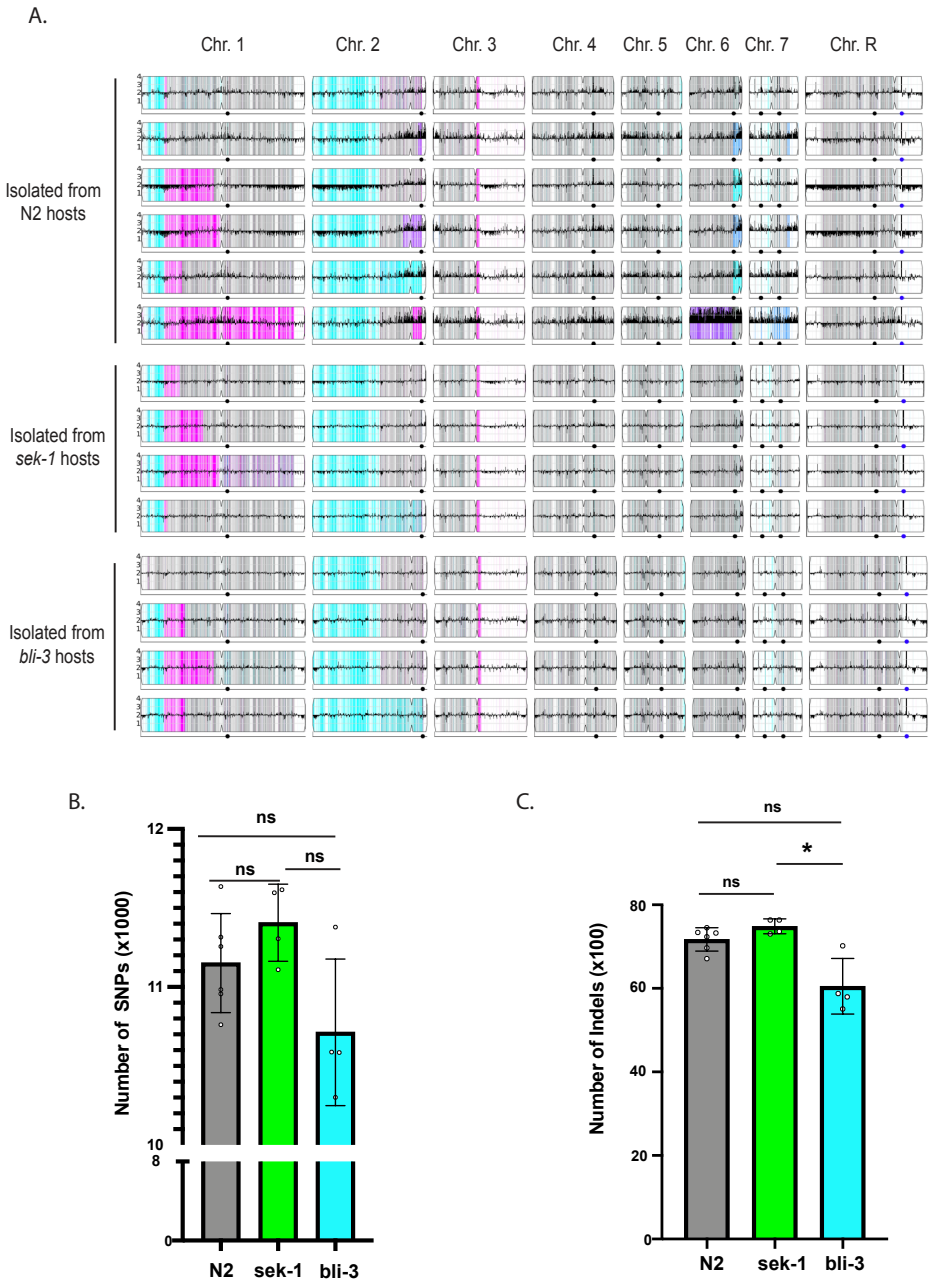

Figure 3.3: Genome-wide changes following host association. A) YMAPs showing the copy number and allelic ratio for each GAL$^-$ isolate following host exposure. B) Number of genome-wide SNPs (x1000) in *C. albicans* following exposure to N2 (wildtype, grey, n = 6), *sek-1* (green, n = 4), and *bli-3* (blue, n = 4) hosts. Plotted are the mean and SD. Individual data points represent the number of SNPs in one isolate. C) Number of genome-wide INDELs (x100) in *C. albicans* following exposure to N2 (wildtype, grey, n = 6), *sek-1* (green, n = 4), and *bli-3* (blue, n = 4) hosts. Plotted are the mean and SD. Individual data points represent the number of INDELs in one isolate. Asterisks indicate significant differences (* $p < 0.05$, ns = not significant; ANOVA with post-hoc Tukey's multiple test).

3.5 Discussion:

We previously reported that healthy, immunocompetent hosts induce *C. albicans* genome instability [17] and we followed this up by investigating whether host innate immune pathways drive pathogen genome instability. By using wildtype and two immunocompromised hosts deficient for AMP production (*sek-1*) and ROS production (*bli-3*), we compared the differences in host-associated *C. albicans* genome stability and mutational landscape. *C. albicans* associated with either immunocompromised host had reduced relative LOH frequencies compared to those associated with immunocompetent hosts (Figure 3.1). Similar to several other host passaging experiments and whole-genome sequencing results from clinical isolates [14,15,19], many of our isolates extracted from wildtype hosts contained large-scale genomic changes including whole and segmental chromosomal aneuploidy and/or additional LOH events (Figure 3.3). We detected the presence of an extra copies of Chr6 following host exposure, consistent with previous observations of host-induced genomic alterations that used a murine OPC infection model [14]. That study suggested Chr6 aneuploidy produces more commensal-like phenotypes inside the host environment. Whether this also occured in our isolated associated with a nematode host context has yet to be investigated. We also detected an extra copy of Chr7 in one isolate following immunocompetent host exposure. In a gastrointestinal murine model of candidiasis, Chr7 trisomy results in higher *C. albicans* fitness within the gastrointestinal tract compared to the euploid strain [15] which suggests that generating specific aneuploidies may enable host adaptation. However, immunocompromised host-associated *C. albicans* isolates did not carry any detectable aneuploidy and only a small number (2/8) had an LOH event that was not selected for (Figure 3.2). This suggests that both AMPs and ROS act as stressors on *C. albicans* that enable the generation of genetic variation which might lead to phenotypic changes that create a more synergistic host–pathogen relationship.

The host has a variety of mechanisms in order to control microbial growth. Because *C. albicans* is normally a commensal and resides in many niches in the human body, it must strike a delicate balance with the host to evade detection. Overgrowth of *C. albicans* initiates an immune response that initially includes the production of AMPs and the recruitment of phagocytes to the site of infection which produce ROS [39]. We found that the removal of host-produced AMPs and ROS decrease LOH frequency in *C. albicans* and overall genomic changes compared to *C. albicans* extracted from wildtype hosts (Figures 3.1 & 3.3). However, we only found a significant decrease in the relative LOH frequency in both the laboratory and bloodstream *C. albicans* extracted from *bli-3* hosts compared to wildtype hosts, whereas we did not detect a significant decrease the relative LOH frequency in the bloodstream strain extracted from *sek-1* hosts (Figs 3.1B&C). This suggests that host-produced ROS generates genome instability regardless of pathogen background, but the *sek-1* pathway may only produce AMPs in response to certain strains of *C. albicans*. We propose that the differences observed in genome instability between different strains of *C. albicans* is potentially due to recognition of different PAMPs by host PRR which can trigger different immune responses [40].

Because host-produced ROS represented a conserved source of genome instability across two different strains of *C. albicans*, we further investigated the role of host-produced ROS in generating genome instability. *C. albicans* has several mechanisms for detoxify ROS, one of which is regulated by the transcription factor Cap1p. *Cap1*Δ/Δ mutants are more susceptible to *in vitro* ROS killing [34,41]. Here we show that *cap1*Δ/Δ *C. albicans* genome instability was increased compared to wildtype *C. albicans* in hosts capable of producing ROS (N2 and *sek-1*) but not in *bli-3* hosts incapable of producing ROS (Fig. 3.2B). Which suggests that host-produced ROS through the bli-3 dual-oxidase induces genome instability in *C. albicans* and that

Cap1p is important for detoxification of host-produced ROS. Recent work in *E. coli*, demonstrated that when exposed to low levels of ROS, *E. coli* exhibits a priming response in which evolution in ROS occurs faster and cells develop greater resistance as opposed to non-primed cells [42]. Our results suggest that host-produced ROS might be priming *C. albicans* allowing for tolerance of greater stress.

Although counterintuitive, LOH increases genetic diversity by unmasking recessive alleles, leading to phenotypic changes [43]. For example, LOH of drug-resistant alleles of *ERG11*, *TAC1* or *MRR1* increases antifungal drug resistance [44–46]. Similarly, aneuploidy offers a short-term solution that organisms use during adaptation [47–49] and have been shown to be advantages under certain conditions including the host environment[14–16]. Because we detected large-scale LOH events and aneuploidy in isolates exposed to immunocompetent hosts, we suggest that these genetic changes facilitate rapid adaptation to the host. Our work identifies AMPs and ROS as important conserved innate immune responses that generate genome instability in the fungal pathogen, *C. albicans*. We propose that the generation of genetic variation in response to host-produce ROS and AMPs represents a way in which *C. albicans* can quickly respond to host stressors thus further tolerating these stressors or avoiding further immune attack. Our work has important implications for opportunistic pathogens that are often commensal in immunocompetent hosts but more frequently cause infection in immunocompromised hosts.

3.6 Acknowledgements

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3.7 Supplementary Materials and Tables:

Table S3.1

***C. albicans* strains**		
Strain	**Genetic Background**	**Source**
YJB11700	SC5314 reference strain, *GAL1/galΔ::NAT*	Gillum et. al. 1984; Hickman et al 2013
cap1Δ/Δ	his1Δ/his1Δ, leu2Δ::C.dubliniensis HIS1/leu2Δ::C.maltosa LEU2, arg4Δ /arg4Δ, URA3/ura3Δ::imm434, IRO1/iro1Δ::imm434 orf19.1623Δ::C.dubliniensisHIS1/orf19.1623Δ::C.maltosaLEU2	Nobel et al 2010
***C. elegans* strains**		
Alias	**Genotype**	
WT	N2 (bristol)	
bli-3	bli-3(e767)	
sek-1	sek-1(km4)	

Table S3.1 List of strains used in this study

Figure S3.1

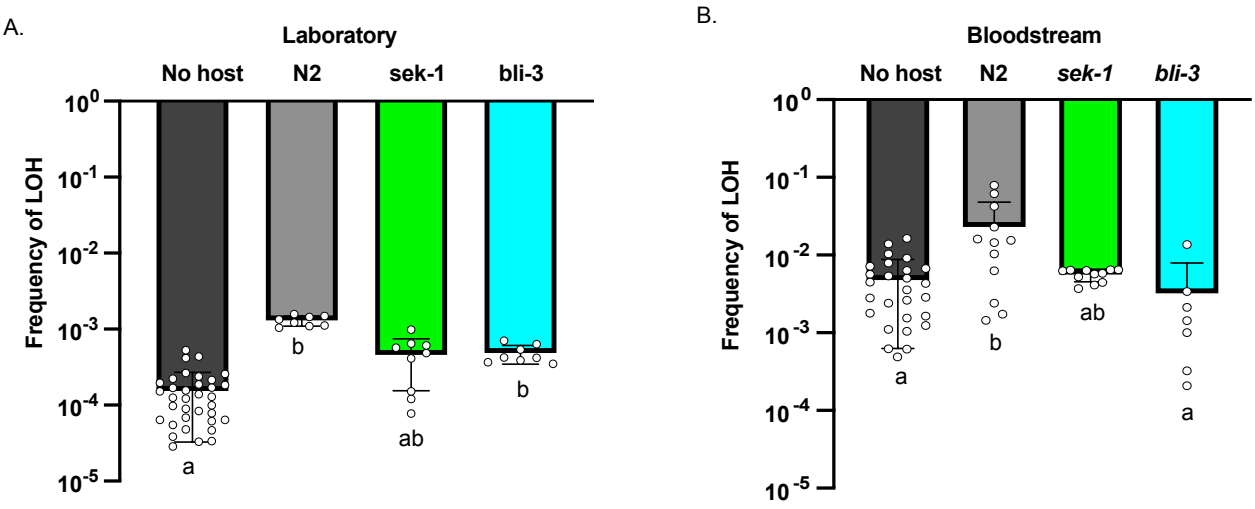

Figure S3.1: A) No host (dark grey, n = 35) and host-associated *GAL1* LOH frequencies for laboratory *C. albicans* extracted from wildtype (N2) (grey, n = 8), *sek-1* (green, n = 9), and *bli-3* hosts (blue, n = 8). Plotted are the mean and standard deviation. Symbols represent individual measurements. B) No host (dark grey, n = 27) and host-associated *GAL1* LOH frequencies for a clinical bloodstream strain of *C. albicans* extracted from wildtype (N2) (grey, n = 12), *sek-1* (green, n = 11), and *bli-3* hosts (blue, n = 7). Plotted are the mean and standard deviation. Symbols represent individual measurements. Treatments that share letters are not significantly different, whereas treatments with different letters are statistically different according to a Kruskal-Wallis test with post hoc Dunn's multiple comparisons ($p < 0.05$).

Figure S3.2

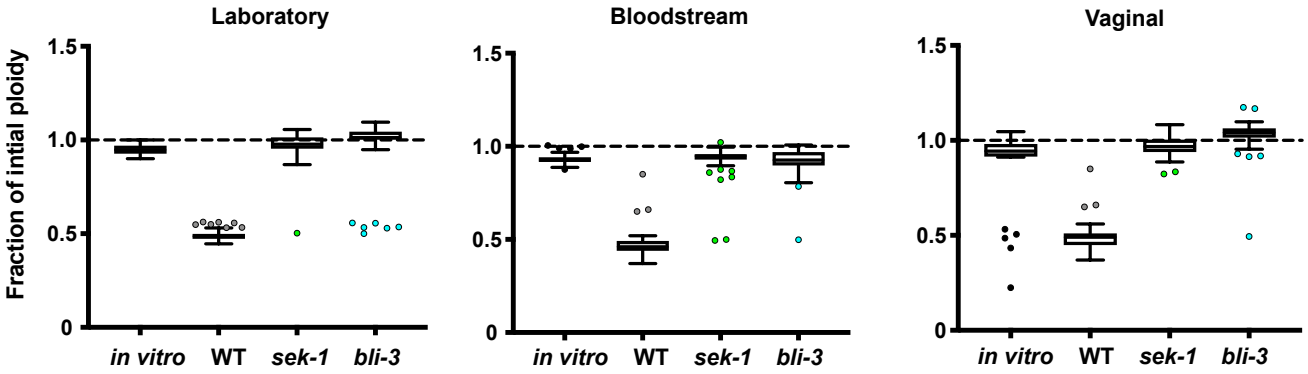

Figure S3.2: Host immunity generates genome instability in tetraploid *C. albicans*.
Fraction of initial ploidy for laboratory tetraploids following four days in vitro exposure (black), wildtype host exposure (grey), sek-1 host exposure (green) and bli-3 host exposure (blue)
The relative genome size was calculated by dividing the genome size of each isolate by the mean genome size of the C. albicans strain not exposed to the host. The tukey method was used for plotting whiskers and outliers are represented as single dot points.

Figure S3.3

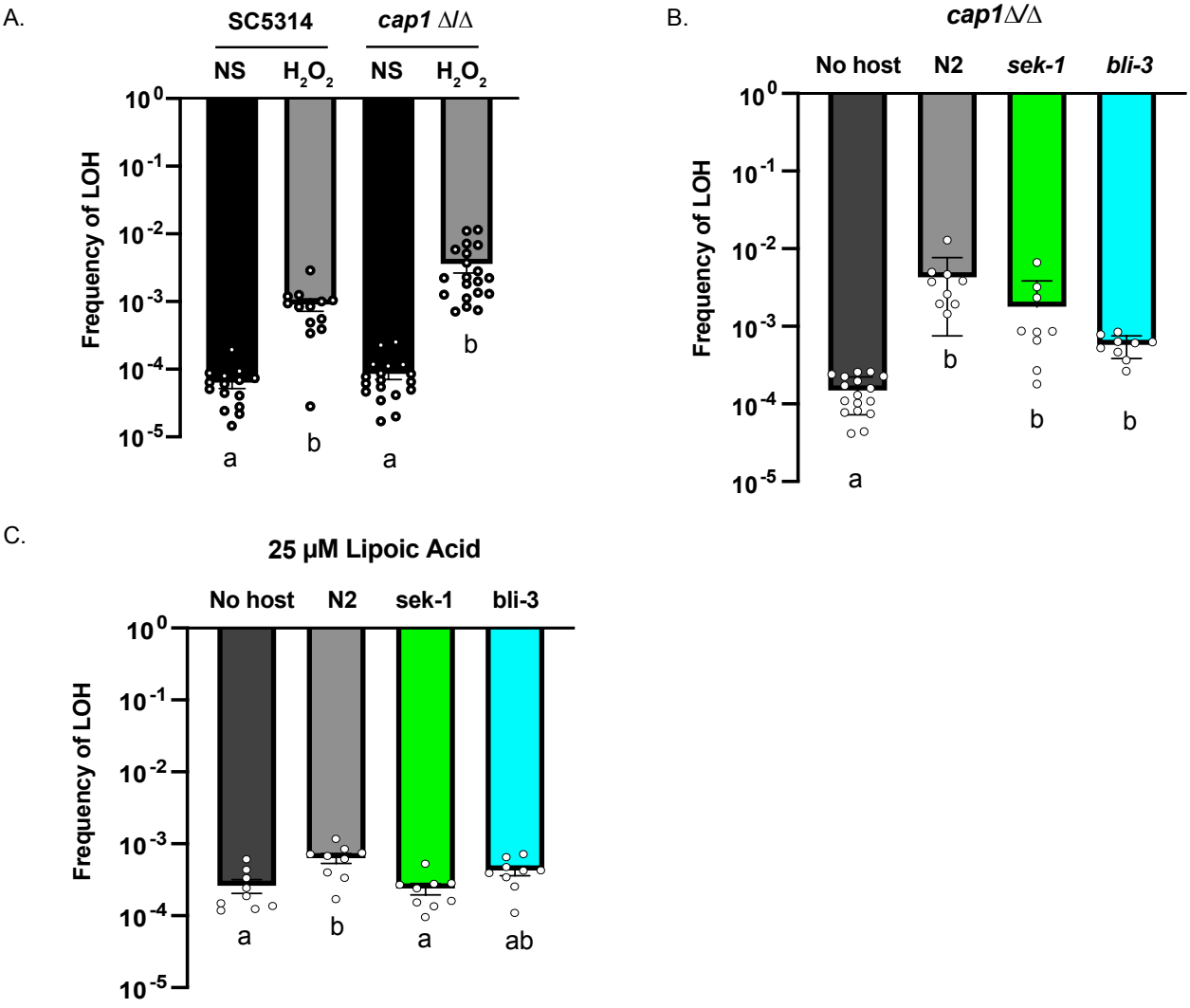

Figure S3.3: A) A) Plotted are the LOH frequencies *C. albicans* exposed to 5mM H_2O_2 or no stress for 24 h. Plotted are the means and SD for both SC5314 (no stress: black, n = 15; H_2O_2: grey, n = 13) and *cap1*Δ/Δ (no stress: black, n = 18; H_2O_2: grey, n = 20). Each data point represents an individual measurement. B) No host (dark grey, n = 17) and host-associated *GAL1* LOH frequencies for *C. albicans* extracted from wildtype (N2) (grey, n = 9), sek-1 (green, n = 9), and bli-3 hosts (blue, n = 9). Plotted are the mean and standard deviation. Symbols represent individual measurements. **B)** No host (dark grey, n = 9) and host-associated *GAL1* LOH frequencies for a *C. albicans* extracted from wildtype (N2) (grey, n = 9), sek-1 (green, n = 9), and bli-3 hosts (blue, n = 9) with 25 µM of alpha-lipoic acid. Plotted are the mean and SD. Symbols represent individual measurements. Treatments that share letters are not significantly different, whereas treatments with different letters are statistically different according to a Kruskal-Wallis test with post hoc Dunn's multiple comparisons ($p < 0.05$).

Figure S3.4

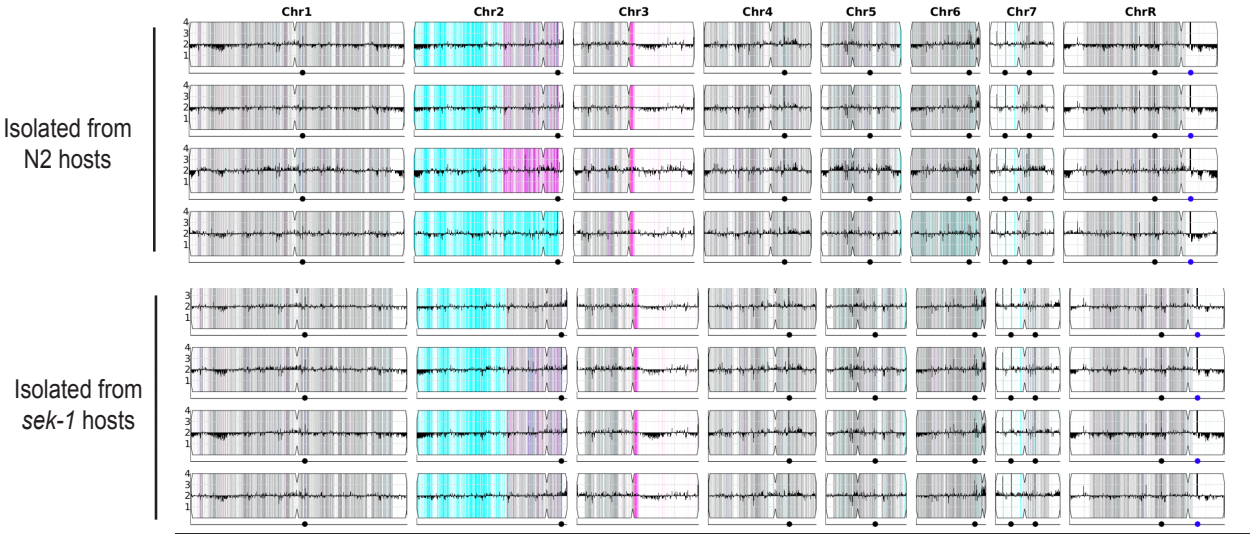

Figure S3.4: A) Following exposure to N2 (wildtype) and *sek-1 C. albicans* was extracted and colonies were chosen at random for DNA extraction and whole-genome sequencing. YMAPs showing the copy number and allelic ratio for each isolate following host exposure.

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Chapter IV

Host immunity and pathogen ploidy determine virulence evolution trajectories

4.1 Abstract:

Virulence is a result of the interaction between a microbe and a susceptible host. Immunocompromised and immunosuppressed individuals are more likely to get infections compared to healthy individuals and pathogen genotype impacts virulence dynamics. Yet, no study has investigated the impact of host immunity and pathogen genotype on virulence evolution of a pathobiont. Here, I leveraged the genetically-tractable host *Caenorhabditis elegans* to test how host immunity and pathogen ploidy impacts virulence evolution of the fungal pathobiont *Candida albicans*. Using experimental evolution with selection for increased virulence, I infected immunocompetent and immunocompromised hosts with either diploid or tetraploid *C. albicans* and monitored the change in host survival for each passage. Both diploid and tetraploid *C. albicans* evolved virulence quickly in immunocompetent hosts, but not at all in immunocompromised host. Further, tetraploids evolved virulence faster than diploids, but it was not fully maintained throughout the experiment. Flow cytometry was performed on *C. albicans* populations following each passage to determine if genomic changes were responsible for virulence changes. Diploids remained primarily diploid regardless of host genotype, but tetraploids underwent a massive genome size reduction corresponding to significant changes in virulence. Together this suggests that tetraploids have an advantage over diploids during adaptation likely due to the ability to stochastically lose chromosomes generating population heterogeneity. Yet, the response to selection in diploids was potentially more stable than in the tetraploid populations. Additionally, my work suggests that the host immune system exerts a strong selective pressure on *C. albicans* which allows for rapid adaptation.

4.2 Introduction:

C. albicans is a fungal pathobiont of the human microbiota[1]. While often commensal, it causes benign mucosal infections, as well as deadly bloodstream infections[2]. These infections are more common in immunocompromised individuals[2]. *C. albicans* often maintains a stable relationship with the host, but the host contains multiple stressors, including the immune system, that exert selective pressures on the microbe to adapt to the host environment[3,4]. Beyond exerting selective pressure, I demonstrated in chapter 3 that the host immune system also induces genome instability in *C. albicans*. *C. albicans* is normally a diploid organism, has a highly labile genome that rapidly undergoes large-scale genomic changes including loss-of-heterozygosity (LOH), aneuploidy and whole ploidy shifts in presence of physiological stressors[5], including the host environment [6–9]. Yet, little is known regarding the impact of host generated genetic variation on the long-term evolution of *C. albicans*.

Genetic variation is needed for evolution by natural selection. The rate at which beneficial mutations arise in a population is critical for determining how quickly organisms can adapt[10]. However, the way in which this genetic variation is created varies across species. Meiosis and sexual reproduction are commonly used to generate genetic variation in eukaryotic organisms. However, some eukaryotes, including the fungus *C. albicans*, do not reproduce sexually and must rely on other means of generating genetic variation in order to adapt to environmental changes. *C. albicans* is normally diploid but exists on a spectrum from haploid to highly polyploid[11]. *C. albicans* forms tetraploids via a process known as parasex, in which diploid nuclei fuse to form a tetraploid[12,13]. This cell then undergoes a non-meiotic reduction to return to diploid. The reduction from tetraploid to diploid is stochastic and causes reassortment of alleles, LOH, and even transient aneuploidy in a cell population[14–16]. *C. albicans* ability to

rapidly undergo substantial genomic changes may allow for rapid adaptation during environmental changes.

Ploidy, the number of sets of chromosomes, impacts the rate of adaptation. Despite having a higher mutational target, haploids adapt faster than diploid yeast population in a range of environments due to a higher probability for fixation of beneficial alleles[17]. Similar to diploids, tetraploids have the ability to buffer deleterious alleles, yet they have orders of magnitude higher mutation rates[18]. With higher mutation rates and greater fitness effects, tetraploid populations adapt faster than both diploids and haploids[19]. While this has been extensively studied *in vitro* to different environmental stressors, the impact *C. albicans* ploidy has on adaptation to a host environment has yet to be investigated. My results in chapter 3, demonstrate that tetraploids are more unstable than diploids within the host, and that host immune status impacts the level of *C. albicans* genome instability. Therefore, I predict that tetraploids evolved within an immunocompetent host will evolve faster than a diploid population of *C. albicans*.

To date, only four studies performed long-term serial passaging experiments with *C. albicans*, which were all in murine model systems. *Tso* et al., investigated how the gut environment shaped the evolution of *C. albicans* by passaging *C. albicans* for ten generations without selecting for virulence and found that the mouse gut selects for hyphal defective variants that are avirulent[20]. Similarly, Cheng *et al.,* serially passaged *C. albicans* in mouse spleens and on the fifth passage found an isolated with delayed filamentation initiation, more resistant to phagocytes, and had attenuated virulence[21]. However, neither of these studies were selecting for virulence. Only one study in *C. albicans* has directly selected for virulence, but it was

performed *in vitro,* and they started with an avirulent strain of *C. albicans*[3]. Therefore, it is still unknown how virulence evolves in different host contexts.

Here, I used a *C. elegans* model to investigate how the host environment impacts the evolutionary trajectories of both diploid and tetraploid *C. albicans* populations under selection for increased virulence. I used experimental evolution to select for virulence while passaging *C. albicans* through either immunocompetent or immunocompromised *C. elegans.* I predicted virulence evolution to occur the quickest with tetraploid *C. albicans* in immunocompetent hosts due to the high selective pressure of the immune system and higher genome instability in tetraploids compared to diploids. Both diploids and tetraploids evolved higher virulence in immunocompetent hosts, but tetraploids evolved faster. Neither diploids nor tetraploid *C. albicans* evolved changes in virulence in immunocompromised hosts despite the same selection for increased virulence. Suggesting that the immune system exerts a selective pressure on *C. albicans*. To understand if differences in genome dynamics contributed to why tetraploids evolved quicker, I measured genome size following each passage in both diploids and tetraploids. While diploid *C. albicans* remained diploid in both immunocompetent and immunocompromised hosts, I found that all tetraploid replicate populations dropped to approximately diploid by passage 3 in immunocompetent hosts, but only 1/3 of replicate populations dropped to diploid by passage 6 in immunocompromised hosts. Suggesting that reduction in tetraploid genome size facilitated rapid adaptation. Together this work suggests that host immunity and ploidy changes have the capacity to alter the evolutionary trajectories of *C. albicans* populations.

4.3 Materials and Methods:

Experimental Evolution

Six replicate populations of 50 *C. elegans glp-4* (bn2) and AU37 (glp-4 (bn2); sek-1 (km-4)) were infected with a mixture of *C. albicans* strain and OP50 (*Escherichia coli*). We measured virulence as infection-induced host mortality rate, and imposed selection for increased virulence by passaging MH128 (tetraploid) or MH88 (diploid) only from the first 50% of the population that died. *C. elegans* populations were enumerated daily and transferred to new plates with the same seed culture every 48 hr. Dead nematodes were collected daily by picking into in .5 mL M9 buffer in a microcentrifuge tube until 50% of the population died. Following 50% death, the dead worm solution was centrifuged for 30 seconds at maximum speed. The supernatant was removed and 500 uL of 3% bleach was added to the pellet to kill any bacteria or yeast on the surface of the nematodes. After two minutes of bleach treatment, the microcentrifuge tube was centrifuged for 30 seconds at max speed. The supernatant was removed and 500 uL of M9 was added to the pellet and centrifuged for 30 seconds at max speed. This step was repeated two additional times to ensure the bleach was removed. The worm pellet was then exposed to manual disruption with a motorized pestle. After one minute of manual disruption, all the intestinal extracts were inoculated into 2 mL of YPD + 0.034mg/L chloramphenicol to prevent any bacterial growth. After 24 h of growth at 30°C, extracted *C. albicans* was used to seed for the subsequent generation with fresh OP50. New, synchronized populations of *C. elegans* were used for each passage. To ensure changes in virulence were due to host exposure, *C. albicans* with OP50 was passaged on NGM + streptomycin in the absence of hosts.

Flow Cytometry

C. albicans extracted from *C. elegans* at the end of each passage were stored in glycerol stocks at -80 °C. *C. albicans* from glycerol stocks were inoculated in YPD and incubated overnight at 30°C. Following logarithmic growth, cells were fixed with 95% ethanol, treated with 50 µl of RNase A (1 mg/ml) for 1 h at 37°C with shaking, then 50 µl proteinase K (5 mg/ml) and incubated for 30 min at 37°C, and resuspended in 50 µl Sybr green (1:50 dilution with 50:50 TE; Lonza, catalog no. 12001-798, 10,000×). Samples were sonicated to disrupt any cell clumping and subsequently run on an LSRII flow cytometer. To calibrate the LSRII and serve as internal controls, the reference diploid (SC5314) and tetraploid strains were used.

Flow cytometry data were analyzed using FlowJo, by plotting the fluorescein isothiocyanate A (FITC-A) signal against the cell count. Two peaks were observed, the first representing the G1 mean and the second peak representing the G2 mean, which has double the genome content of the G1 peak and therefore twice the fluorescence. Genome size values were calculated using the G1 mean and compared to standard diploid and tetraploid control strains.

Egg preparation and nematode synchronization

To synchronize *C. elegans* populations, nematodes and eggs were washed off the plate with 5 mL of M9 buffer, transferred to a 15-ml conical tube, and pelleted at 1,200 rpm for 2 min. The pellet was resuspended in a 25% bleach solution, inverted for 2 min, and subsequently centrifuged for 2 min at 1,200 rpm. The pellet was washed twice with 3 ml of ddH2O and resuspended in 1 ml of ddH2O. To determine the concentration of eggs, 10 µL was pipetted onto a concave slide, the eggs were counted under a microscope, and the egg suspension was diluted

with M9 to a concentration of 50-100 eggs per 10 µL. 10 µL or 50-100 eggs were pipetted onto each seeded plate.

Seeding:

Briefly, *C. albicans* cultures were inoculated into 3 ml of YPD and incubated at 30°C overnight. *C. albicans* cultures were diluted to a final volume of 3.0 OD per ml. Additionally, *E. coli* was inoculated into 50 ml of LB and incubated at 30°C for 24 to 48 h. Subsequently, *E. coli* was pelleted and washed twice with 1 ml of ddH2O. The washed pellet was then weighed and diluted to a final density of 200 mg/ml. *C. albicans* treatment plates had 1.25 ul of *C. albicans* and 6.25 ul of E. coli and were brought to a final volume of 50 ul. The entire 50 ul was spotted onto the center of a 35-mm-diameter NGM plus streptomycin agar plate, followed by incubation at room temperature overnight before the addition of eggs or transferring nematodes. For the first passage, the parental *C. albicans* strain was used for seeding.

Statistical analysis:

Significant differences in the average time to 50% death for each passage relative to the initial passage was calculated using a one-way Kruskal-Wallis followed by post-hoc Dunn's multiple comparisons test.

4.4 Results:

C. albicans evolves rapidly in immunocompetent, but not immunocompromised hosts

My results described in the previous chapter, support a model that innate immunity generates *C. albicans* genetic variation by inducing genome instability. As genetic variation is a fundamental requirement for evolution to occur, I hypothesized that *C. albicans* would evolve

more rapidly in immunocompetent hosts, compared to immunocompromised hosts. To test this hypothesis, I performed *in vivo* experimental evolution utilizing immunocompetent and immunocompromised hosts and selected for increased *C. albicans* virulence. I infected six parallel populations of 50 hosts and when a host population reached 50% death, I extracted *C. albicans* from the dead hosts to infect a new host population, thus selecting for *C. albicans* associated with early host death (Fig. 4.1A). I evolved *C. albicans* in this manner for a total of ten passages (Fig. 4.1A). After five passages in immunocompetent hosts, the average time to 50% death was ~8 days which was significantly lower than the initial passage with an average of 12 days (Fig. 4.1B), indicating increased virulence in *C. albicans*. This increased virulence was subsequently maintained throughout the remainder of the evolution experiment (Fig. 4.1B). In contrast, there was no change in the average time to 50% death in immunocompromised hosts throughout ten passages, despite being initially more susceptible to *C. albicans* infection compared to immunocompetent hosts, (Fig. 4.1C). Therefore, *C. albicans* did not respond to selection for increased virulence in the immunocompromised hosts within ten passages. Together, these results support our hypothesis that host immunity-induced genomic changes generated in *C. albicans* populations facilitated a strong response to this selection regime.

To evaluate whether evolved virulence was specific to the immunocompetent host environment that *C. albicans* was evolved in, I infected immunocompromised hosts with *C. albicans* evolved in immunocompetent hosts and vice versa. We found that immunocompromised hosts infected with immunocompetent-host evolved *C. albicans* reached 50% death two days faster compared to immunocompromised hosts infected with immunocompromised host-evolved *C. albicans.* In contrast, immunocompetent hosts infected with immunocompromised host-evolved *C. albicans* took on average 2.5 days longer to reach

50% death compared to immunocompetent hosts infected with immunocompetent host-evolved *C. albicans* (Figs. 4.1D). Taken together, this result indicates that evolved virulence is not specific to host context and the genomic changes generating a virulent phenotype in one host context still give rise to a virulent phenotype in another host context. To validate that increased virulence observed from immunocompetent hosts was a direct result of our selection pressure and not the passaging itself, we passaged *C. albicans* in parallel experimental conditions but in the absence of hosts. We then infected immunocompetent and immunocompromised hosts to the no-host evolved *C. albicans* and found that no-host evolved *C. albicans* did not change their virulence relative to the ancestral (P0) state, regardless of host immune status (Fig. 4.1E). Thus, selection for virulence was responsible for the increase in virulence in *C. albicans* evolved in immunocompetent hosts.

Figure 4.1

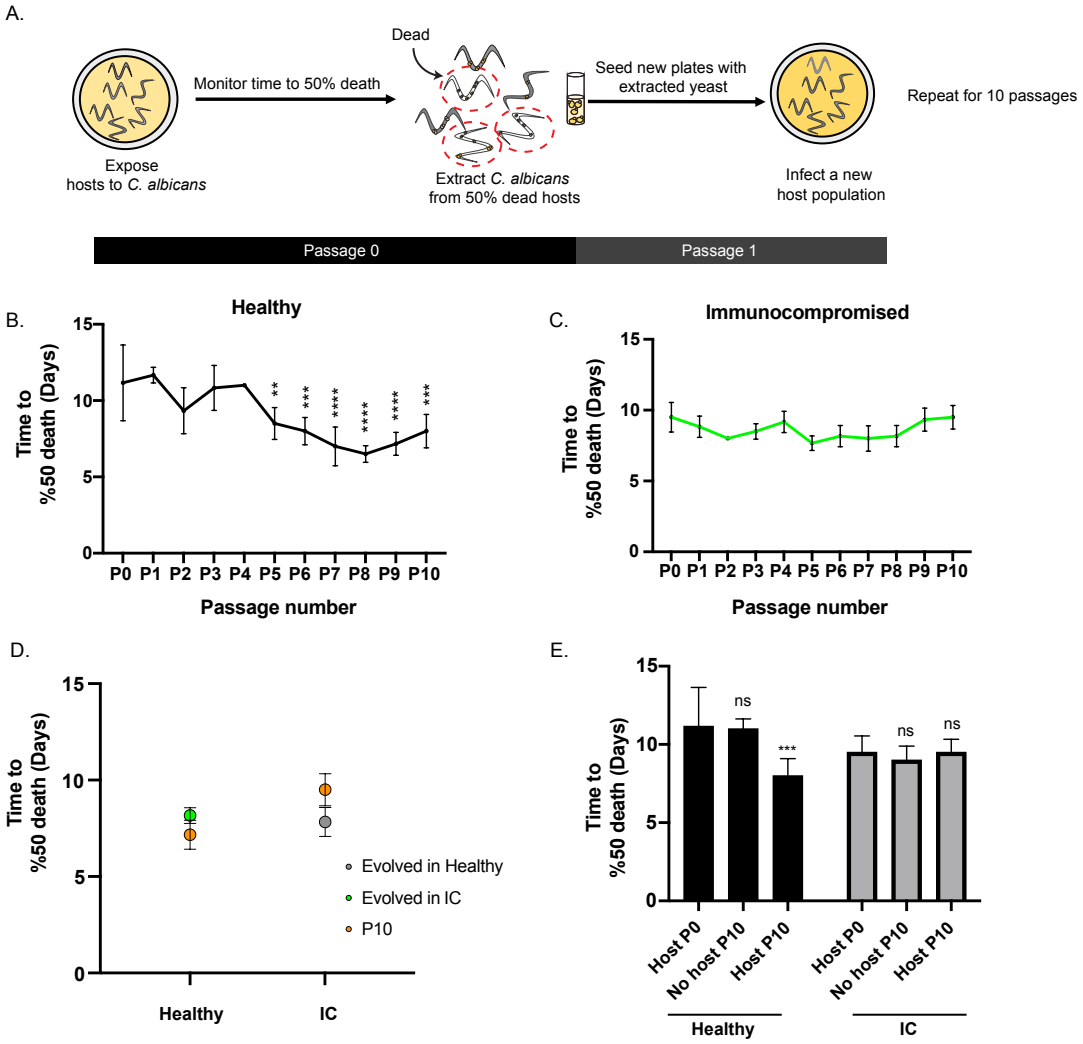

Figure 4.1 *C. albicans* evolves rapidly in healthy hosts

A) Experimental schematic **B)** Time to 50% death for ten passages in healthy, immunocompetent (*glp-4)* hosts. Plotted are the mean (n=6) and SD for each passage. **C)** Time to 50% death for ten passages in immunocompromised (*glp-4, sek-1*) hosts. Plotted are the mean (n=6) and SD for each passage. Asterisks represent significant differences compared to the initial hoc Dunn's multiple test). **D)** Time to 50% death in healthy and immunocompromised hosts. Plotted is the average time to 50% death for the final (P10) time point and hosts exposed to *C. albicans* evolved in the other host environment. Plotted are the means (n = 6) and SD. Asterisks indicate significant differences (* $p < 0.05$, ns = not significant, Mann-Whitney U test). **E)** Time to 50% death for immunocompetent (black) and immunocompromised hosts (grey) infected with *C. albicans* for the initial passage (Host P0), *C. albicans* evolved for ten generations in the absence of hosts (No host P10), and C. albicans evolved in that host environment for ten generations (Host P10). Asterisks represent significant differences compared to the initial (P0) time point (*** $p < 0.005$, ns = not significant, Kruskal-Wallis with post-hoc Dunn's multiple test). (P0) time point (**** $p < 0.0001$, *** $p < 0.005$, ** $p < 0.01$, * $p < 0.05$ Kruskal-Wallis with post-hoc Dunn's multiple testing.

Tetraploids undergo rapid evolution

Tetraploids are more unstable than diploid *C. albicans*[16] and thus rapidly generate genetic variation that allows for more rapid adaptation under *in vitro* stressors[22]. Yet, this idea has not been investigated in a host environment. Therefore, I wanted to investigate whether tetraploids evolve more rapidly than diploids *in vivo*. Immunocompetent *C. elegans* infected with tetraploid *C. albicans* on passage 2 had a significantly lower average time to 50% population death compared to the initial passage, indicating increased *C. albicans* virulence (Fig. 4.2A). In contrast, it took five passages in diploid *C. albicans* to observe any significant changes in virulence, suggesting that tetraploids have an initial advantage. Interestingly, on passage 4, immunocompetent hosts survived just as long as the initial passage, with an average time to 50% death of 11 days (Fig. 4.2A). I observed no significant changes in survival for immunocompetent hosts in the subsequent four passages. Together this suggests that the virulent populations in passage two were maybe less fit and not maintained throughout subsequent passages. Despite virulence selection in immunocompromised hosts, we did not observe any significant changes in the average time to 50% death compared to the initial passage (Fig. 4.2B). We observed the same trend for immunocompromised hosts infected with diploid *C. albicans*. Therefore, selection in immunocompetent hosts resulted in an increase and subsequent decrease in *C. albicans* virulence, while selection in immunocompromised hosts did not alter *C. albicans* virulence over the course of experimental evolution. Together, these data demonstrate that the *C. elegans* immune system can alter the evolutionary trajectories of tetraploid *C. albicans* populations and that tetraploid populations evolve faster than diploid *C. albicans*.

Figure 4.2

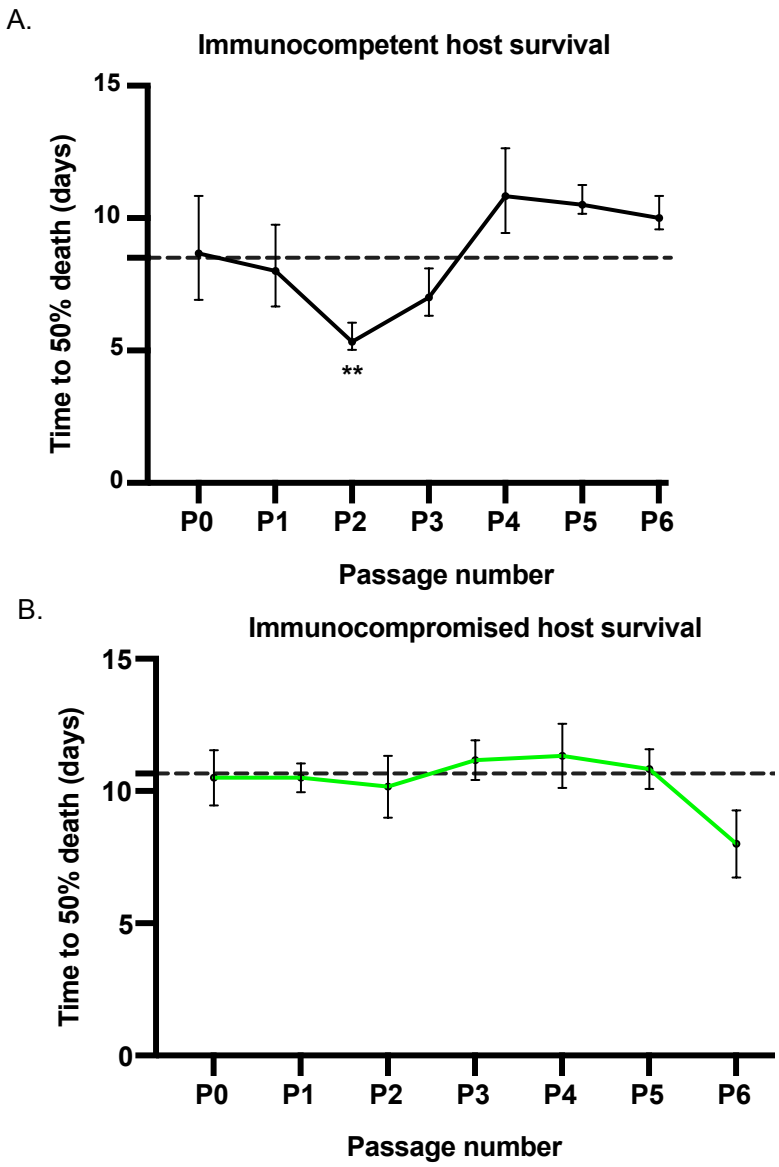

Figure 4.2 Tetraploids evolve rapidly in immunocompetent hosts
A) Time to 50% death for six passages in healthy, immunocompetent (*glp-4)* hosts. Plotted are the mean (n=6) and SD for each passage. **B)** Time to 50% death for six passages in immunocompromised (*glp-4, sek-1*) hosts. Plotted are the mean (n=6) and SD for each passage. Asterisks represent significant differences compared to the initial (P0) time point (** $p < 0.01$ Kruskal-Wallis with post-hoc Dunn's multiple test).

Ploidy changes induce rapid adaptation

Recent studies have demonstrated that the murine host environment causes large-scale genomic changes in diploid *C. albicans* including LOH, aneuploidy and whole changes in ploidy which generate a more commensal-like phenotype[7,8,23]. Similarly, in chapter 3 I demonstrated that *C. elegans* induces genetic changes in both diploid and tetraploid *C. albicans*. However, my study extended upon the previous literature and found innate immune components reactive oxygen species (ROS) and antimicrobial peptides (AMPs) generate large-scale genomic changes in *C. albicans*. Therefore, I wanted to determine if the virulence changes in both diploid and tetraploid *C. albicans* passaged in immunocompetent hosts were due to genomic changes. Following each passage, I measured genome size for each replicate population with flow cytometry. I observed diploid populations passaged through either immunocompetent or immunocompromised hosts mostly maintained ploidy (Fig. 4.3A&B). Yet, it is unclear from flow cytometry alone if aneuploidy and LOH events were present. Similar to what I observed in chapter 3, a majority of the tetraploid replicate lines were approximately diploid following passage 2 within immunocompetent hosts (Fig. 4.3C), which corresponds to the significant increase in virulence (Fig. 4.2). Diploidy was maintained in all six of the replicate lines throughout the subsequent passages in immunocompetent hosts (Fig. 4.3C). I did not observe any large changes in *C. albicans* genome size until passage 4 in immunocompromised hosts and only 1/3 of the replicate populations had a reduction in genome size (Fig. 4.3D). However, this reduction in genome size in a subset of replicate populations did not result in any changes in virulence. Together these data suggest that host innate immunity causes genome size reduction in tetraploid *C. albicans* which increases the rate at which virulence evolution occurs, likely due to

allelic reassortment during chromosome loss. However, genome size reduction is not required for virulence evolution, because diploids were able to evolve within immunocompetent hosts.

Figure 4.3

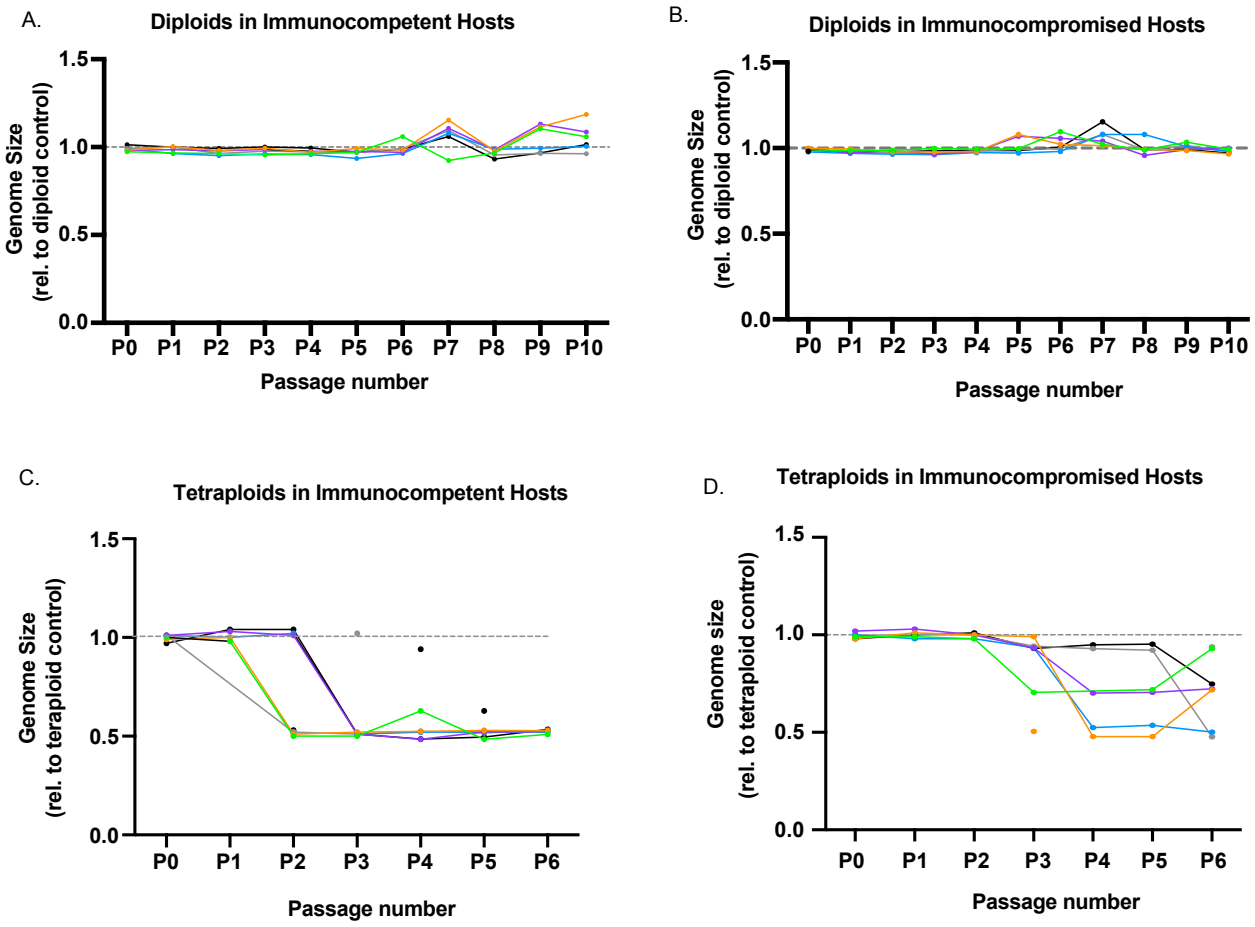

Figure 4.3: Genome size changes are abundant in tetraploids following immunocompetent host passage

A) Diploid genome size relative to diploid control for ten passages in immunocompetent hosts. B) Diploid genome size relative to diploid control for ten passages in immunocompromised hosts. C) Tetraploid genome size relative to tetraploid control for six passages in immunocompetent hosts. D)Tetraploid genome size relative to tetraploid control for six passages in immunocompromised hosts. Each colored line corresponds to the same replicate population throughout each passage (i.e. population A = black, B = grey).

4.5 Discussion:

Evolving increased virulence in both diploid and tetraploid *C. albicans* demonstrates that host immunity can propel rapid adaptation under selection favoring virulence (Figs. 4.1 & 4.2). Additionally, my study demonstrates that genome size reduction occurs rapidly in tetraploid *C. albicans* under selection in immunocompetent hosts (*glp-4*), but not immunocompromised (*glp-4;sek-1*) hosts or in diploid *C. albicans* (Fig. 4.3). Together, these results indicated that host immunity coupled with virulence selection generated genome instability allowing for rapid virulence evolution in *C. albicans*.

C. albicans has a highly labile genome, which frequently carries aneuploid chromosomes following host exposure[7,8,24], drug stress[25], and following the stochastic process of concerted chromosome loss in tetraploids[14,16,26]. Here, I demonstrated that tetraploid *C. albicans* are highly unstable in an immunocompetent host environment. Following only two passages in immunocompetent host under selection, all of the replicate lines were approximately diploid (Fig. 4.3). Tetraploid reduction corresponded to reduced host survival, i.e. an increase in *C. albicans* virulence (Fig. 4.2). Suggesting that the reduction in ploidy accelerated adaptation. However, despite constant selection pressure and lack of ploidy changes following the initial drop in ploidy, immunocompetent host survival increased back to baseline levels by passage 4 (Fig. 4.2). We hypothesize that although more virulent, the initial diploid population in passage 2 in immunocompetent hosts were not as fit in the host environment and were thus replaced by a more fit and less virulent population of cells, a trade-off often observed with increased virulence[27–31].

We observed a significant increase in tetraploid *C. albicans* virulence in immunocompetent hosts by passage two (Fig. 4.2). For comparison, diploid *C. albicans* evolved virulence in

immunocompetent hosts by passage five (Fig. 4.1). Together, this suggests that tetraploidy facilitates rapid adaptation, a result that is consistent with other recent evolution studies. For example, tetraploid *C. albicans* evolved faster than diploid *C. albicans* under antifungal drug selection[22] and tetraploid *Saccharomyces cerevisiae* adapted faster to raffinose selection compared to diploids[19]. This accelerated adaptation is likely due to the higher mutation rates in tetraploids and frequent chromosome loss generating new favorable allelic ratios[15,16].

Although counterintuitive, LOH increases genetic diversity by unmasking recessive alleles, leading to phenotypic changes[32]. For example, LOH of drug-resistant alleles of *ERG11*, *TAC1* or *MRR1* increases antifungal drug resistance [33–35]. We did not detect any major changes in genome size in diploids evolved in either immunocompetent or immunocompromised hosts (Fig. 4.3). Yet, diploids in immunocompetent hosts likely had multiple LOH events, a phenomenon I demonstrated in the previous chapter and others have previously demonstrated[6–8]. Therefore, changes in virulence may result from LOH events, SNPs, and INDELs rather than genomic size changes.

Aneuploidy represents a short-term and transient solution that allows for rapid adaptation in stressful conditions [36–38], and our results demonstrate that tetraploid *C. albicans* populations rapidly undergo chromosome loss in immunocompetent hosts under selection, which corresponded to an increase in virulence. However, this trend was not observed in *C. albicans* evolved in immunocompromised hosts. Despite a lack of genome size changes, diploids also evolved virulence in immunocompetent hosts, albeit at a slower rate. Together this suggests that host immunity coupled with ploidy reduction cause rapid evolution in the opportunistic pathogen, *C. albicans*. This research suggests that host immunity forces rapid adaptation in *C. albicans* which enables it to switch from a commensal to a pathogen and vice versa. In

immunocompromised individuals however, *C. albicans* is able to proliferate and continue to cause infection because the immune system isn't providing as strong of a selection pressure. Therefore, I predict that long-term persistence in the host is more causative of pathogen virulence than rapid adaptation. For *C. albicans* populations within immunocompromised hosts, persisting, while adapting slowly might allow for *C. albicans* to grow to larger population sizes without triggering an immune response, thus allowing *C. albicans* to exploit these hosts to a greater degree causing a more severe infection. Future investigations should evaluate the connection between *C. albicans* populations size and overall virulence in both healthy and immunocompromised hosts.

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Chapter V: Discussion

Dissertation Discussion:

In this section, I summarize my questions and major findings, briefly describe how these findings fit into the current literature, address any outstanding questions and/or limitations of my study and finally highlight the implications of my work and future directions.

5.1 Summary of results & contributions to the current literature:

C. albicans is considered a pathobiont, normally harmless but can become pathogenic in the right context[1]. The transition from harmless to pathogen is induced by a variety of factors including both abiotic and biotic factors within the host[2]. *C. albicans* has a highly labile genome[3] and grows very rapidly, which I predict enables the generation of genetic variation and fast adaptation in the presence of host stressors. However, the impact of the host environment on *C. albicans* genome stability, virulence and evolution has largely been ignored. In this dissertation I investigated 1) how the host environment impacts genome dynamics in *C. albicans* of differing genetic backgrounds and ploidy, 2) the specific host components that drive genome instability in *C. albicans*, and 3) the long-term impacts of host-generated genome instability on *C. albicans* virulence evolution.

C. albicans, although typically diploid, has a highly plastic genome which gives rise to genetic heterogeneity[3–7]. Genetically distinct, but closely related *C. albicans* isolates exist within a single individual [8]. But *C. albicans* populations are even more distinct between different host individuals[8]. Yet, *C. albicans* infections often result from an existing population rather than an outside source[9]. Therefore, it is important to understand how distinct *C. albicans* populations give rise to genetic heterogeneity to promote phenotypic changes. To investigate

how pathogen genetic background impacts genome stability and phenotypic outcomes following host association, I infected *C. elegans* hosts with *C. albicans* from six different genetic backgrounds representing two ploidies from three different host backgrounds. Similar to previous literature, I found that host-association indeed increased *C. albicans* genome instability[10–12]. However, I found that the magnitude of genome instability was dependent on both genetic background and ploidy. Tetraploids underwent rapid genome size reductions in the host environment, while diploids mostly maintained ploidy. Furthermore, clinical isolates were generally more unstable than the laboratory strain. I next evaluated the phenotypic consequences of genome instability by measuring pathogen fitness and virulence. Many of the isolates following host association exhibited changes in fitness and virulence, the direction of these changes was dependent on both ploidy and genetic background. Yet, I found that there was no overall correlation between changes in genome size and the magnitude of phenotypic change. Which suggests that allelic composition rather than ploidy is a better predictor of phenotypic outcome. Together, my data supports a model that pathogen genetic background is important for understanding how quickly genetic variation is generated, which ultimately impacts pathogen fitness and virulence.

In Chapter 3, I expanded on my first aim and uncovered the specific host immune factors that generated *C. albicans* genome instability. I found that both host-produced reactive oxygen species (ROS) and antimicrobial peptides (AMPs) generated large-scale genomic changes in *C. albicans* including whole and partial chromosomal aneuploidy as well as large-scale LOH events. However, I found that ROS consistently elevates genome instability across all strains and ploidies, but AMPs only elevated genome instability in some *C. albicans* strains. Together this suggests that there is an interaction between the host environment/genotype and the pathogen

genotype. I hypothesize that the same host genotype induced different levels of genome instability for different strains of *C. albicans* due to a difference in the recognition of pathogen associated molecular patterns (PAMPs). Different cell wall components are recognized by host pathogen recognition receptors (PRRs), which trigger different immune responses[13]. However, this has not been fully investigated in *C. elegans*. This work provides an understanding of how both pathogen genotype and host genotype interact to generate genetic variation which can facilitate evolution.

Virulence is a result of the interaction between a microbe and a susceptible host[14]. Immunocompromised and immunosuppressed individuals are more likely to have *C. albicans* infections compared to healthy individuals. Yet, no study had previously investigated the impact of host environment on *C. albicans* virulence evolution. In chapter 4, I evaluated the impact of host genetic background (immunocompetent v immunocompromised) on *C. albicans* virulence evolution and the impact pathogen ploidy (diploid v tetraploid) had on the rate of adaptation. Under selection for increased virulence, both diploid and tetraploid *C. albicans* evolved greater virulence in immunocompetent, but not immunocompromised hosts. However, tetraploids were able to evolve changes in virulence more rapidly than diploid populations. Further, this change in virulence within tetraploid populations corresponded with a reduction in genome size to approximately diploid. Thus, the tetraploids were highly unstable and underwent a stochastic process of concerted chromosome loss which generated a genetically heterogenous population. This generation of genetic heterogeneity likely facilitated rapid adaptation, and may also explain the later loss of virulence in these populations. Together my work suggests that immunocompetent hosts provide a greater selective pressure which generates genetic variation in *C. albicans* allowing for rapid adaption. In immunocompromised hosts, *C. albicans* is likely able

to proliferate to higher levels uncontrolled by the lack of part of the immune system, thus there is less selective pressure imposed by the host and a lack of host immune stress to generate genome instability for selection to act upon.

5.2 Outstanding Questions & Limitations:

1. Do fungal phenotypes within worms correlate to phenotypes within humans? Does it matter?

It is assumed that all humans carry *C. albicans* within their gastrointestinal tract[15]. *C. elegans* do not form a natural relationship with *C. albicans* but can easily be infected by ingestion. The most common infections caused by *C. albicans* is mucosal infections[1] which likely represents the type of infection we induce in *C. elegans*. Furthermore, we find that in addition to causing lethal infections, *C. albicans* more often induces non-lethal infections in *C. elegans* including a reduction in fecundity[16]. While this is not a commonly investigated phenotype, several studies suggest that *C. albicans* impacts reproduction by reducing sperm viability[17,18]. Importantly, many genes that have been important for virulence in murine models, are also critical for virulence in *C. elegans*[19], suggesting a conservation in *C. albicans* virulence across models, despite *C. elegans* lacking a complex organ system. Therefore, despite the inability to study tissue-specific infections or bloodstream infections, *C. elegans* represents a promising model to study *C. albicans* infections and virulence.

2. How well do the host effects of worms correlate to humans? What can we learn from these immune mutant host strains? What factors contribute to virulence that are missed?

Similar to humans, *C. elegans* encounters and ingests many different types of pathogens. To protect itself against invaders, *C. elegans* also has an innate immune system that recognizes different PAMPs. The *C. elegans* innate immune system is comprised of four highly conserved pathways within the intestinal lumen which also provide protection against pathogens in mammals [20–22]. The *sek-1* pathway, which I discussed throughout Chapters 3 & 4 is a MAPK signaling pathway [21] that leads to the production of antimicrobial peptides responsible for defense against pathogens including *C. albicans*[16]. This pathway is comprised of NSY-1, SEK-1 and PMK-1 which are the *C. elegans* homologs of human ASK-1, MKK-3 and P38 which lead to the production of pro-inflammatory cytokines[19]. Furthermore, *C. elegans* also produce ROS in response to pathogens through the dual oxidase bli-3, which we also investigated and is highly conserved in humans[23–25]. Although humans have five dual oxidases while *C. elegans* only have one. Several studies have demonstrated that bli-3 mutant *C. elegans* are more susceptible to *C. albicans*[25]. Similarly, humans with mutations in the NADPH peroxidase, a disease known as chronic granulomatous disease (CGD) are more susceptible to many different fungal infections, including *C. albicans*[26]. Together, this demonstrates that *C. elegans* is an effective model for understanding the host effects on *C. albicans*. However, we have yet to evaluate the impact of adaptive immune responses on *C. albicans* genome stability and the long and short-term consequences for the host-pathogen relationships. Therefore, it would be useful to investigate these questions in a more complex host model with an adaptive immune response. Furthermore, one element that was not thoroughly investigated in this dissertation is the interaction between *C. albicans* and other components of the microbiome. Because *C. albicans* has both synergistic and antagonistic relationships with other microbes in the host, this may also

be a source of genome instability observed in a more complex system, such as the murine host or by using the newly developed CeMBio kit for investigating the microbiome in *C. elegans*[27].

3. Is experimental evolution in *C. elegans* predictive of *C. albicans* evolution in humans? What can we learn from experimental evolution in *C. albicans*-*C. elegans* system?

In Chapter 4, I evaluated the long-term consequences of host-induced genome instability in immunocompromised and immunocompetent hosts and found changes in *C. albicans* virulence only in immunocompetent hosts. Evolution in nature takes a long time, studying evolution using experimental evolution within the laboratory allows us to understand how different conditions, i.e., the host environment impacts evolution. Additionally, host-pathogen interactions are an area that is often studied theoretically and notoriously difficult to investigate from a microbial perspective. However, the ease of infectivity and the rapid generation time of *C. elegans* make them amenable for performing large-scale experimental evolution studies to investigate host-pathogen interactions. As such, several studies have used *C. elegans* to investigate host pathogen interactions, including pathogen resistance[28–30], resistance trade-offs[28,31], and local adaptation[32,33]. Yet, there are a few caveats including the limited population size and the population bottlenecks *C. albicans* goes through when passaging from one host population to the next. Furthermore, because I am inducing *C. albicans* to a more simplified environment compared to humans and I imposed strong selection, I likely selected for alleles with different patterns of pleiotropy than would happen naturally[34]. However, by using an experimental evolution setup, I can control the specific selection imposed. By picking two host genotypes with distinct immune components, I investigated how immunity impacts the rate

of adaptation in *C. albicans* by minimizing additional confounding factors. I found that virulence evolution occurs rapidly in immunocompetent, but not immunocompromised hosts, which suggests that the selection pressure within the immunocompromised hosts is not as strong. Together, this research can provide an understanding of how normally commensal pathobionts, such as *C. albicans* evolve quickly to become pathogenic.

5.3 Future directions:

I demonstrated that the host environment coupled with genomic changes contribute to virulence evolution. Yet, to link genotypic changes to the phenotypic outcomes, analysis of what is happening at the allelic level rather than just genome size changes is necessary. To potentially uncover new mutations that increase *C. albicans* virulence, whole-genome sequencing on isolates throughout the passaging experiment could be performed. A comparison between the ancestral population and the populations that had increased virulence, might shed some light on how *C. albicans* increased virulence in immunocompetent hosts.

Virulence is a result of not only the microbe but a susceptible host[14]. My research highlights the importance of understanding the relationship between the pathogen genotype and the host environment. My study demonstrates pathogen genetic background and ploidy determine how quickly genetic variation is generated, which is needed for evolution to occur. In addition, I found that host genotype, more specifically the host immunity is a source of genome instability in *C. albicans* that drives rapid virulence evolution. However, we often see that virulence arises in immunocompromised or immunosuppressed individuals[1]. In my study, I selected for *C. albicans* populations that killed the hosts the fastest. In humans, it is in the best interest for a pathobiont such as *C. albicans* to maintain a commensal relationship in order to evade triggering an immune response that would cause more harm to the pathogen. Which is

why studies investigating short-term and long-term impacts of host association without any selection have found that *C. albicans* is often more commensal following host association[12,29,30]. In immunocompromised individuals *C. albicans* is able to proliferate without triggering a strong immune response and thus it can cause infection, and although there might be less selective pressure in immunocompromised people, there are still functional immune components and other microbes that could be a source of *C. albicans* genome instability. As such, investigation into why immunocompromised hosts experience more deadly infections compared to immunocompetent hosts, despite having a lesser selective pressure should be pursued.

5.4 Final thoughts and implications:

Mycotic diseases are common in other organisms including reptiles, amphibians, and plants. However, they are quite rare in humans. Part of what differentiates humans from these other species that are often infected by fungi is our warm body temperatures and our adaptive immune system. The barrier for fungi to colonize and infect us is quite high and thus there are only a small number of fungal species that can actually cause infection in humans. Despite this, researchers predict that human fungal infections are only becoming more common. Thus, investigating fungal pathogens and pathobionts is of upmost importance. The research discussed above has important implications understanding how other pathobionts switch from being a commensal to a pathogen. Each individual harbors unique microbes, which interact with each other and the human body including the immune system. Determining how quickly the microbial communities within our body can mutate and adapt will be crucial for preventing infection. Therefore, continued investigation into the effects the host environment has on genotypic and

phenotypic outcomes of microorganisms within the body is needed to improve not only fungal infection disease outcome, but also other microbial diseases caused by residents of the microbiome.

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