Analysis of Sex Bias in *Cryptococcus neoformans* Infections

by

Tiffany Guess

A Dissertation Submitted in Partial Fulfillment of the Requirements for the Degree of

Doctorate of Philosophy in Molecular Biosciences

Middle Tennessee State University

August 2018

Thesis Committee:

Dr. Erin E. McClelland, Advisor

Dr. Anthony Farone

Dr. Sebastian Joyce

Dr. David Nelson

Dr. Rebecca Seipelt-Thiemann, Chair

I dedicate this research to my parents, my late mother Bertha Guess and my father Oscar Paul Guess III, without whose love, guidance, and emphasis on education, I would be lost.

ACKNOWLEDGEMENTS

I would like to gratefully acknowledge the numerous people who have made this dissertation possible. I came to the MTSU Biology Department as an undergraduate in 2000 and have been fortunate to work with brilliant, caring, dedicated scientists that have had an outsized effect on my life and career. Drs. Mary and Tony Farone were instrumental in shaping my love for biology. They guided me, allowed me to make mistakes, expected me to remedy those mistakes, and in this, I gained confidence in myself as a student and as a researcher. I would like to thank my advisor, Dr. Erin McClelland for her support and guidance in this project as well serving as a role model for myself and numerous other female scientists. Additionally, I extend my gratitude to my committee: Dr. Anthony Farone, Dr. Sebastian Joyce, Dr. David Nelson, and Dr. Rebecca Seipelt-Theimann for all of the expertise, support, and encouragement they have generously given over these last years. I would like to offer a special thanks to Dr. David Nelson who has served as a collaborator on several projects, as an excellent editor, and whose high standards push me further than I would go on my own. I am grateful for the opportunity afforded to me by MOBI, and particularly its director, Dr. Jason Jessen, who has consistently offered advice and encouragement along the way. Finally, I would be remiss if I didn't thank my sweet husband, Jarrod Miller, and my close friends and family for their support and counsel through this journey. I am deeply indebted to you all.

ABSTRACT

Cryptococcus neoformans, a pathogenic yeast and the causative agent of cryptococcosis, is responsible for an estimated quarter million new cases of the disease resulting in more than 180,000 deaths each year worldwide. For decades, researchers have noticed that the prevalence of cryptococcosis is skewed between males and females. Numerous studies show sex-specific differences in *C. neoformans* infection rates, with males having a higher incidence of disease and death (~7M:3F). Sexual dimorphism in infection is not uncommon. However, the cause of these differences, in *C. neoformans*, has not been well elucidated. The aim of this project was to gain a deeper understanding of the causes underlying the sex bias observed in cryptococcosis. To accomplish this, a comprehensive approach was designed to examine the effect of sex-defining hormones on the pathogen itself as well as experiments to detect differences in the host-pathogen interface of males and females. There were three major components to this study. First, to establish whether sex hormones were taken up by *C. neoformans*, fluorescently tagged 17β-estradiol was incubated with wild type and mutant strains, and the results imaged on a confocal microscope. Second, to assess any changes in virulence factors in the presence of sex hormones, *C. neoformans* clinical isolates were grown in an environment designed to mimic the central nervous system (CNS) and a series of *in vitro* biochemical experiments were conducted to examine each of the virulence factors individually in the presence of sex hormones. Third, the response of healthy human male and female immune cells were evaluated during infection with *C. neoformans*. The results show that estrogen is, in fact, capable of entering *C. neoformans* at normal

physiological levels seen in females. Additionally, a number of virulence factors are changed in the presence of estrogen and testosterone at normal physiological levels. Finally, the percentages of T cells differ between males and females during a *C. neoformans* infection, which may suggest differences in the adaptive immune response to this pathogen. Taken together, these results indicate that the sex bias observed for many years has a biologic basis and cannot simply be explained by environmental factors. Further, the sexual dimorphism stems from both differences in the pathogen and host-pathogen interface in the presence of a male and female environment.

TABLE OF CONTENTS

Page

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

7AAD	7-Aminoactinomycin D
AIDS	Acquired immunodeficiency syndrome
ANOVA	Analysis of variance
AMI	Antibody-mediated immunity
AmpB	Amphotericin B
APC	Allophycocyanin
A.U.	Arbitrary units
BLAST	Basic local alignment search tool
BPS	Bathophenanthrolinedisulfonic acid
$CaCl_2$	Calcium chloride
CD	Cluster of differentiation
Cdr1	*Candida* multidrug resistance
CFW	Calcofluor white
$C_4H_6O_4$	Succinic acid
Cir1	*Cryptococcu*s iron regulator
CMI	Cell-mediated immunity
CNAG	*Cryptococcus neoformans* assigned gene number
CO_2	Carbon dioxide
CR	Congo Red
CTR	*Cryptococcus* copper transport regulator
DMEM	Dulbecco's Modified Eagle medium

EBP	Estrogen-binding protein
Ebp1p	Estrogen-binding protein 1
EG	Estradiol Glow
FITC	Fluorescein isothiocyanate
GM_CSF	Granulocyte-macrophage colony-stimulating factor
g	Gravity
GalXM	Galactoxylomannan
GXM	Glucuronoxylomannan
H_2O_2	Hydrogen peroxide
HSP	Heat shock protein
HIV	Human immunodeficiency virus
HOG	High-osmolarity glycerol
F	Female
FBS	Fetal bovine serum
HK	Heat-killed
H	Hours
IFN-γ	Interferon-gamma
Ig	Immunoglobulin
IL	Interleukin
IRB	Institutional Review Board
KCl	Potassium chloride
KO	Knockout

LPS	Lipopolysaccharide
M	Male
MEM	Minimal essential media
Min	Minutes
MPs	Mannoproteins
MANOVA	Multivariate analysis of variance
MAPK	Mitogen-activated protein kinase
MOI	Multiplicity of infection
mRNA	Messenger ribonucleic acid
NaCl	Sodium chloride
NADPH	Nicotinamide adenine dinucleotide phosphate
$NaNO_2$	Sodium nitrite
NF-κB	Nuclear factor-kappa B
NIH	National Institute of Health
NK	Natural killer
OD	Optical density
OYE	Old yellow enzyme
PBS	Phosphate buffered saline
PBS2	Polymyxin B sensitivity
PBMCs	Peripheral blood mononuclear cells
PCM	Paracoccidioidmycosis
PE	Phycoerythrin

PE-Cy7	Phycoerythrin-Cyanine 7
PI	Propidium iodide
PLB	Phospholipase B
ROI	Region of interest
RPM	Revolutions per minute
RPMI	Roswell Park Memorial Institute medium
SE	Standard error
SD	Standard deviation
SDS	Sodium dodecyl sulfate
Th	T-helper
TNF-α	Tumor necrosis factor-alpha
YPD	Yeast peptone dextrose

CHAPTER 1

INTRODUCTION

1.1 General Introduction

The opportunistic pathogen, *Cryptococcus neoformans* causes disease in a quarter of a million people every year, resulting in more than 180,000 deaths (Rajasingham et al., 2017). Hardest hit are HIV^+ patients in developing countries that do not have ready access to antiretroviral therapy (ART), making them acutely susceptible to the illness (reviewed in(Coelho et al., 2014a, b; Maduro et al., 2015)). Data from the Centers for Disease Control (CDC) indicate that in sub-Saharan Africa*, C. neoformans* kills more people than tuberculosis annually and more every month than any Ebola outbreak on record (Rajasingham et al., 2017). Without treatment, the disease is 100% fatal and mortality rates are approximately 20-50% with treatment (Chiapello et al., 2004; Coelho et al., 2014b; McClelland et al., 2013). Furthermore, the anti-fungal drugs used to treat *C. neoformans,* where available, are not well tolerated due to their inherent toxicity and require long-term use (Coelho and Casadevall, 2016). Flucytosine and Amphotericin B (AmpB), typically in conjunction with each other, are the most frequently prescribed anti-fungals in cryptococcal meningitis cases (Perfect et al., 2010). Flucytosine has been shown to suppress bone marrow and cause anaphylaxis, whereas AmpB can cause acute kidney and cardiac failure (Perfect et al., 2010).

1.2 Pathogenesis

Cryptococcosis, an infection of *C. neoformans,* results most often from inhaling desiccated spores that are commonplace in the environment, occurring naturally in soil

and bird droppings (Alvarez et al., 2009). Serological tests indicate that upwards of 90% of the population have been exposed to *C. neoformans* (Abadi and Pirofski, 1999; Rothstein, 1971). In healthy individuals, the immune system mounts an effective response and the pulmonary infection is self-resolving and asymptomatic (Giles et al., 2009). In immunocompromised people, however, the yeast can cause pneumonia and if able to disseminate from the lungs to the central nervous system (CNS), progresses to meningitis, which is often fatal (Eisenman et al., 2007).

1.3 Virulence of *C. neoformans*

Upon entering the lungs, there are several virulence factors that contribute to the success of *C. neoformans* within infected individuals. The polysaccharide capsule is considered one of the primary virulence factors, as acapsular strains are not virulent (Eisenman et al., 2007; Kwon-Chung and Rhodes, 1986). The capsule is made of three key components: glucuronoxylomannan (GXM), galactoxylomannan (GalXM), and mannoproteins (Fig 1.1). GXM and GalXM are polysaccharides and make up the bulk of the capsule. Mannoproteins (MPs) are located in the cell wall and are involved in T cell stimulation (Huang et al., 2002). The capsule is strongly anti-phagocytic and when shed into the intracellular space becomes a major immunomodulator of the host response in a number of ways. Specifically, GXM inhibits the production of pro-inflammatory cytokines TNF-α and IFN-γ and increases the production of anti-inflammatory cytokines, such as IL-4 and IL-5 (Casadevall and Pirofski, 2005; Schop, 2007). In macrophages, GXM inhibits NF-κB signaling, which in turn regulates TNF-α expression (Hayes et al., 2016)

Additionally, GXM interferes with antigen-presenting cells (APCs), which, in turn, inhibits T cell proliferation (Eisenman et al., 2007; Garcia-Rodas et al., 2014).

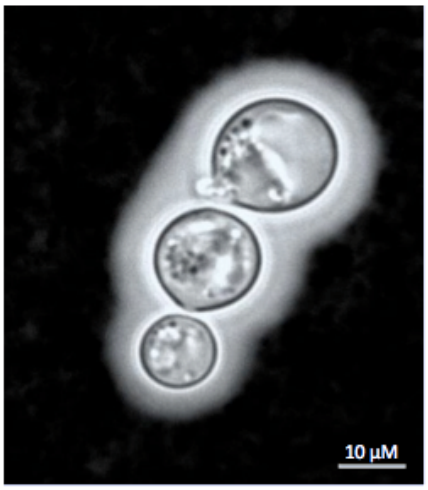


Figure 1.1: An Image of *C. neoformans* Stained with India Ink.

Melanin is another important virulence factor. Like capsule, melanin aids *C. neoformans* pathogenesis on multiple fronts. Despite being located beneath the capsule in the inner portion of the cell wall, this brown-black pigment appears to "cloak" the yeast from recognition by host cells by preventing opsonization and, therefore, reduces the likelihood of both phagocytosis and a protective T cell-mediated immune response (Jacobson and Emery, 1991). In the event that melanized *C. neoformans* are phagocytosed, the virulence factor acts as an antioxidant, neutralizing oxidative bursts from host macrophages (Wang and Casadevall, 1994). Melaninized *C. neoformans* cells are resistant to the most commonly prescribed drug used to treat cryptococcal meningitis, Amphotericin B, which works by binding to ergosterol and creating holes in the membrane of *C. neoformans* resulting in cell death as well as causing oxidative stress within the cell (Baginski and Czub, 2009; Wang et al., 1995).

A third virulence factor, phospholipase B (PLB) is a secreted protein and is necessary for initial infection as it enhances *C. neoformans* adherence to lung epithelial cells (Ganendren et al., 2006). Additionally, it is required for dissemination out of the lungs to cause systemic disease, and has the ability to damage host tissues through the alteration of lipid signaling molecules (Eisenman et al., 2007; Noverr et al., 2003). Urease, a fourth virulence factor, is produced in large quantities in *C. neoformans* and increases the pH in the phagolysosome assisting in immune system evasion as well as inducing ammonia toxicity in host cells, which aids the pathogen in crossing the blood-brain barrier (Fu et al., 2018). Further, urease has been shown to elicit a non-protective Th2 response from the host rather than the more effective, fungicidal Th1 immune response during a *C. neoformans* infection (Eisenman et al., 2007; Olszewski et al., 2004; Rutherford, 2014).

In addition to the virulence factors listed above, there are other aspects that allow *C. neoformans* to thrive once it is inside the host. The pathogen has the ability to withstand oxidative and nitrosative stress, conditions common in the phagolysosomes of macrophages (Missall et al., 2004a; Missall et al., 2004b). *C. neoformans* can survive and replicate inside host macrophages, therefore, these are conditions that the yeast encounters *in vivo*. The rigidity of the cell wall provides strength and integrity to *C. neoformans* (McClelland, 2007). Additionally, iron is essential for the expression of the aforementioned virulence factors and is regulated by *Cryptococcus* iron regulator (*Cir1*), which also controls the expression of all known virulence factors (Jung et al., 2006). *Cryptococcus* copper transporters (CTR) 1 and 4 work in tandem to maintain

homeostasis of copper encountered in the host, a process necessary for the successful migration of *C. neoformans* from the lungs (high copper environment) to the brain (low copper environment) (Festa et al., 2014; Li et al., 2015a; Sun et al., 2014; Zaragoza and Casadevall, 2004). A basidiomycete, *C. neoformans* is typically a haploid-budding yeast (Garcia-Rodas et al., 2014). However, it can also undergo sexual reproduction and produce spores (Kwon-Chung, 1976; McClelland, 2007). This can be seen *in vitro* under specific conditions and involves complex regulatory mechanisms and morphological transitions (McClelland, 2007; Sia et al., 2000). Some of the factors that control mating also contribute to virulence such as MAPK signaling (Fox and Heitman, 2005). Another aspect that makes mating factors of particular interest is the generation of virulent strains through genetic recombination. Sequence analysis of progeny of two *C. neoformans* strains revealed at least twenty genes thought to contribute to virulence (Mitchell, 2003). Lastly, a fundamental characteristic of a fungal pathogen, the ability of *C. neoformans* to grow and thrive at 37^{o}C makes it particularly dangerous to humans (Casadevall, 1998).

1.4 The Immune Response to *C. neoformans*

The immune response to *C. neoformans* is also quite complex. Phagocytosis by alveolar macrophages is a primary method for clearance, but in those instances where *C. neoformans* is able to establish a successful infection, the human host is typically immunocompromised and unable to upregulate the necessary, pro-inflammatory cytokines for an effective cell-mediated immune response (Schop, 2007). When macrophage activation is triggered it is often via the alternative rather than the classical

pathway (Schop, 2007). Classically activated macrophages produce reactive nitrogen and oxygen species that can kill *C. neoformans* as well as present antigens to T cells to stimulate an adaptive immune response. In contrast, alternatively activated macrophages do not kill *C. neoformans* and stimulate an anti-inflammatory, wound-healing reaction (Leopold Wager and Wormley, 2014). In addition to phagocytic killing of *C. neoformans*, a strong B and T cell response is required for successful defense against the pathogen. The polysaccharide capsule often induces T cell-independent antibodies and therefore triggers a Th2 response rather than a Th1 response that will enable clearing of *C. neoformans* from the body (McClelland, 2007). While not linked directly to decreasing fungal burden, IgG and IgM producing B cells play an important role in down-regulating inflammation and lessening damage to the host during a *C. neoformans* infection (Rivera et al., 2005).

1.5 Sexual Dimorphism in Infection

Sexual dimorphism in the immune response is observed as early as fetal life. Multiple studies show that the differences in the specific and non-specific immune response are carried forward as we age with females generally faring better during infection (Dromer et al., 1996; Durrani et al., 2012; Shah et al., 2015; Yang et al., 2014). There are no differences in reference ranges of lymphocytes in healthy males and females, suggesting that these differences arise when the immune system is challenged (Valiathan et al., 2014). There appear to be two primary sources that account for the differences seen during infection, sex chromosomes and sex hormones. Numerous genes and microRNAs related to immune cell function are located on the X chromosome

(Reardon, 2016; van Lunzen and Altfeld, 2014). While the majority of the second X chromosome is inactivated in females, inactivation is incomplete (Reardon, 2016). Given this, researchers hypothesize that women often have access to "backup" copies of these important immune cell regulators, where men, with only one X chromosome, do not (Reardon, 2016; Syrett et al., 2017; Wang et al., 2016).

Like sex chromosomes, sex hormones generally provide protection to females during infection. Estrogen has pro-inflammatory properties and has been shown to activate immune cells during an anti-microbial response, whereas testosterone suppresses inflammation, which favors continuing infection (Grossman, 1989; Reardon, 2016). The ability of sex hormones to affect the immune response is further supported by the fact that the female immune privilege is common until menopause where immune responses between the sexes equilibrate (Giefing-Kroll et al., 2015). Furthermore, women on estrogen replacement therapy typically see their immune systems buoyed to pre-menopausal levels (Colton et al., 2005). In sum, there appears to be no difference in the absence of an immune challenge, but when presented with a stimulus, the immune response between the sexes is quite different.

Both estrogen and androgen receptors are present in circulating monocytes and lymphocytes suggesting involvement of sex hormones in regulating the development and function of the immune system (Lai et al., 2012; Scariano et al., 2008). In general, estrogen appears to confer protection during an immune challenge whereas testosterone has been shown both to suppress certain components of the immune system and, interestingly, cause an increase in pro-inflammatory cytokines, typically

leading to a less successful outcome during infection and periods of physiological stress (Candore et al., 2006; McClelland, 2011; Solianik et al., 2014). Females tend to have a Th1 cytokine response to most infections, which is often helpful in viral and parasitic infections, as well as *C. neoformans* infections (Ashman and Papadimitriou, 1995; Hepworth and Grencis, 2009; Zivkovic et al., 2015), and could explain the more effective immune response (with exceptions) in females. Females typically also have higher, longer-lasting antibody titers after vaccination (Muenchhoff and Goulder, 2014). Additionally, estrogen seems to allow for increased somatic hypermutation and isotype switching and therefore a higher affinity of Ig-producing cells (Giefing-Kroll et al., 2015). Simply put, estrogen appears to foster a more robust immune reaction when responding to pathogens.

1.6 Sex Bias in *C. neoformans* Infections

Sexual dimorphism in infections is not uncommon. Sex biases are observed in the rate and outcomes of infection in tuberculosis (Agaba et al., 2011; de Faria Gomes et al., 2015; Nhamoyebonde and Leslie, 2014; van Lunzen and Altfeld, 2014), HIV (Abioye et al., 2015; Boulle et al., 2015; de Faria Gomes et al., 2015; DeSilva et al., 2009), Leishmaniasis (Murback et al., 2011; Travi et al., 2002), and *Candida albicans* (Alves et al., 2014; Javed et al., 2009; Loster et al., 2016) to name a few. However, the reasons for these differences, particularly as it pertains to *C. neoformans*, have not been well elucidated. Despite the gap between rates of males and females with cryptococcosis, to date there are only seven primary research articles published that focus on these differences (Table 1).

Interestingly, despite almost identical rates of exposure to *C. neoformans* in males and females, males have a higher incidence of disease and death from cryptococcosis (Carroll et al., 2008; Lortholary et al., 2002). This is true in both HIV^+ (~5M:1F) and HIV^- (~4M:1F) populations (Bava and Negroni, 1992; Escandon et al., 2018; Maduro et al., 2015). A phenomenon that has been documented for more than a half a century, one of the first reports of sex bias in *C. neoformans* infections was in 1966 by a researcher noting that 82% of patients with cryptococcal pneumonia were male (Campbell, 1966). A separate study published in 1970, detailed a patient population where 68% of cryptococcal meningitis patients were male (Edwards et al., 1970). These findings have been echoed by multiple other studies, but the onset of the AIDS epidemic in the 1990s drastically highlighted the increased incidence of males with cryptococcosis. With a new immunocompromised population, the AIDS era gave rise to numerous case studies and reports from around the world citing higher percentages of males suffering and dying from cryptococcal infections compared to their female counterparts (Dromer et al., 2004; Dromer et al., 1996; Escandon et al., 2018; Shaheen et al., 2017).

A trio of papers published in the 1970s are considered the basis for sex bias studies in *C. neoformans (Mohr et al., 1972; Mohr et al., 1974; Mohr et al., 1973).* Prior to the HIV crisis, researchers noted the increased frequency of cryptococcosis in males despite evidence indicating males and females were exposed at similar rates (Rothstein, 1971). In an attempt to determine the cause of this gap, Mohr et al. tested the effects of hormones on seven human *C. neoformans* isolates (Mohr et al., 1972). Incubation with

diethystillbesterol, a non-steroidal, synthetic estrogen, completely inhibited growth in all seven clinical strains at six days of incubation (Mohr et al., 1972). The concentration of estrogen used in this study (1µg/mL) was higher than the concentration occurring in human females (100 pg/mL). Growth in three of seven isolates was completely inhibited in the presence of estrogen, and the remaining four showed markedly inhibited growth (Mohr et al., 1972). There was no growth inhibition observed in samples incubated with progesterone, testosterone, or dorethynodrel, a synthetic progestin (Mohr et al., 1972). The authors note that inhibitory effects of estrogens, but not testosterone, are likely not solely responsible for the lower levels of cryptococcosis in females but may partially explain the differences in incidence between the sexes (Mohr et al., 1972).

In a follow-up study using the same isolates, Mohr tested inhibition of *C. neoformans* when incubated with AmpB and hormones (Mohr et al., 1973). Results showed complete inhibition of all isolates incubated with diethylstillbesterol and AmpB (Mohr et al., 1973). Estradiol and AmpB completely suppressed the growth of four isolates (Mohr et al., 1973). Either compound alone failed to suppress the growth completely (Mohr et al., 1973). Progesterone plus AmpB showed slight inhibition, and there were no inhibitory effects with testosterone (Mohr et al., 1973). The effective concentration of estradiol was much lower than that of diethylstilbesterol or AmpB alone, providing direct evidence of the inhibitory effects of estrogen on *C. neoformans*.

To further discern the efficacy of estrogens in conjunction with AmpB, the same researchers in the aforementioned studies collected blood samples from eleven cryptococcal meningitis patients before, during, and after treatment of AmpB and

before, during, and after administration of 5 mg diethylstilbesterol (Mohr et al., 1974). The samples were infected with a non-encapsulated strain of *C. neoformans* and phagocytosis recorded. Phagocytic activity of all patient samples were markedly depressed both before and during treatment of AmpB (Mohr et al., 1974). In patients receiving diethylstilbesterol, however, phagocytic activity increased significantly (Mohr et al., 1974). Further, antigen titers decreased in all patients administered the synthetic estrogen (Mohr et al., 1974). When administration of the estrogen was stopped in one patient, antigen titers increased, and there was an abrupt decline in the percent phagocytosis of the patient's blood samples (Mohr et al., 1974). The results of these studies indicate that estrogens play a dual role in *C. neoformans* infections by both inhibiting growth of the pathogen as well as increasing phagocytic activity of host immune cells.

After Mohr's work in the 1970s, there was a gap of nearly three decades before another primary research paper was published focusing on the male/female inequality of *C. neoformans* infections, despite the sex bias of cryptococcosis growing larger during the AIDs epidemic of the 1980s and 1990s. In a manuscript published in 2002, researchers suggested that female outbred mice fared better than males because they had higher levels of the cytokines TNF-α and IFN-γ in the blood and spleen during *C. neoformans* infection, suggesting a more protective Th1 immune response in females (Lortholary et al., 2002).

In 2007, a five-year observational study from France reported that male sex, along with positive HIV status were major determinants of presentation and outcome of

cryptococcosis (Dromer et al., 2007). Enrolled patients were either HIV^+ or HIV^- that had at least one positive *C. neoformans* culture from urine, blood, or CSF. Of the 230 patients enrolled in the study, 78% were male and 62% were both male and HIV^+ (Dromer et al., 2007). Of the HIV^+ population, males had a greater incidence of fungaemia (fungal sepsis), positive urine cultures, and more disseminated infections (Dromer et al., 2007). $CD4^+$ T cells were approximately the same in males and females. In the HIV- population, the only difference seen between sexes was higher CSF antigen titers in males (Dromer et al., 2007). Based on the patients in this study, cryptococcosis was more severe in men than women, and those differences were more pronounced in the HIV^+ population.

A group of experiments published in 2013 focused on both host and pathogen features as they pertain to sex. Using a cryptococcal $meningitis^+/HIV^+$ patient cohort from Botswana the researchers found that males had higher mortality rates than females despite having increased numbers of $CD4^+$ T cells at the time of hospitalization (Bisson et al., 2008; McClelland et al., 2013). *C. neoformans* strains isolated from the cerebral spinal fluid (CSF) of those patients showed strains isolated from females released more capsular glucuronoxylomannan (GXM) and had longer doubling times than strains isolated from males (McClelland et al., 2013). When incubated with exogenous testosterone, however, strains showed increased GXM release suggesting that exposure to a male environment may increase the virulence of a *C. neoformans* infection (McClelland et al., 2013). Looking at the innate immune response in healthy donors to *C. neoformans* infection, they found macrophages isolated from females

phagocytosed higher numbers of *C. neoformans* than macrophages isolated from males, yet male macrophages had a higher fungal burden and were killed at increased rates compared to female macrophages (McClelland et al., 2013). Additionally, after a chronic cryptococcal infection, male Balb/c mice had a significantly higher splenic fungal burden than did female mice (McClelland et al., 2013). This data suggests the reciprocal interplay between pathogen and the sex-specific host environment contributes to a differential immune response and results in an increased prevalence of cryptococcal meningitis in males.

Table 1: A Summary of Major Findings From Studies Investigating Sexual Dimorphism in the Response to *C. neoformans* Over the Past 45 Years.

Published	Author	Organism Studied	Major Findings
1972	Mohr et al.	*C. neoformans*	Growth of clinical isolates was inhibited when incubated with either a synthetic or natural human estrogen.
1973	Mohr et al.	*C. neoformans*	Estrogens, when combined with AmpB, markedly inhibited *C. neoformans* growth *in vitro.*
1974	Mohr et al.	Humans	Phagocytic activity increased and antigen titers decreased in cryptococcal meningitis patients administered synthetic estrogen.
2002	Lortholary et al.	Mice	Females had increased levels of the helpful Th1 cytokines TNF-α and IFN-γ in blood and spleen during *C. neoformans* infection.
2006	van den Berg et al.	*C. elegans*	Males were found to be more resistant to *C. neoformans*. This resistance was linked to increased activity of the DAF-16 stress-response transcription factor.
2007	Dromer et al.	Humans	Male gender was a major determinant of outcome during *C. neoformans* infection. Cryptococcosis was more severe in men.
2013	McClelland et al.	Mice, Humans	Spleens of male mice showed higher fungal burden than female mice after chronic cryptococcosis infection. Human males had higher $CD4^+$ T cells yet had higher mortality rates. Macrophages isolated from females were more effective during a *C. neoformans* infection than male macrophages.

1.7 Other Fungi Exhibiting Sexual Dimorphism in Infection

There are other fungi that exhibit sexual dimorphism in infection. Similar to *C. neoformans*, *Paracoccidioides brasilienis,* the causative agent of paracoccidioidmycosis (PCM), exhibits gender susceptibility during infection with males more likely to suffer overt disease than females (11-30M:1F) (Restrepo et al., 2008; Shankar et al., 2011a; Shankar et al., 2011b). Also echoing *C. neoformans, P. brasilienis* is found frequently in soil and most often afflicts agricultural workers. Researchers initially hypothesized that males suffered disease in greater numbers due to increased exposure. However, skin test results showing equal rates of infection indicate that is not the case (Restrepo et al., 1968). Although the mechanism of action is still unknown, multiple studies point to sex hormones, particularly estrogen, playing a key role in the differences of PCM seen in males and females. In one cohort, 70% of the women diagnosed with PCM were menopausal, which is characterized by many symptoms including decreased estrogen production (Shikanai-Yasuda et al., 2006). Further, the sex bias does not exist in children suffering from PCM, with males and females suffering at similar rates. The sex differences are only observed in patients around the age of puberty, thirteen, and upward (Fonseca et al., 1999; Goncalves et al., 1998).

Microarray analysis revealed incubation of *P. brasilienis* with 17β-estradiol results in the up or down regulation of over 500 genes (Monteiro et al., 2009), and binding studies suggest a hormone-binding protein is present in the cytosol of this yeast (Loose et al., 1983). Increased levels of estrogen clearly appear to confer protection among people

exposed to *P. brasilienis,* but more research needs to be done to understand the mechanism.

In contrast to *C. neoformans* and *P. brasilienis*, *C. albicans* infections occur with greater frequency and severity in females (1M:3-5F) (Javed et al., 2009; Kali et al., 2013; Loster et al., 2016; Rubaihayo et al., 2016). Considered typical gut and mouth flora, *C. albicans* can act as an opportunistic pathogen and overgrow to the point of infection. It is the main cause of vaginal candidiasis but can also infect the mouth, throat, and bloodstream (Alves et al., 2014; Kurakado et al., 2017; Rapp, 2004). The reversal of the sex bias in *C. albicans*, compared to *C. neoformans* and *P. brasilienis* may be explained in a few ways. First, the female anatomy puts women at a greater risk for genital candidiasis (Goncalves et al., 2016). Second, while estrogens are known immunostimulators, there is a noted exception – the female reproductive tract. In fact, estrogen decreases expression of NF-κB, a transcriptional regulator, which in turn down regulates the production of several cytokines in the uterine and vaginal epithelium, suggesting that the hormone may be a key factor in weakening female host defenses in the face of opportunistic microflora such as *C. albicans* (Wagner and Johnson, 2012; Wira et al., 2010). Increased levels of estrogen during pregnancy, the use of oral contraceptives, and hormone replacement therapy have all been positively associated with increased *C. albicans* infection (Cheng et al., 2006; Hamad, 2014). Mechanistic studies show that an estrogen binding protein (Ebp1) located in the cytosol of *C. albicans* binds host estrogen, specifically 17β-estradiol, with a high affinity (Skowronski and Feldman, 1989). Further, *C. albicans* cells treated with estrogen survive better than

untreated cells at higher temperatures (48°C) by upregulating a heat-stress protein (*hsp90*) (Zhang et al., 2000). In addition, increased levels of *Candida* multidrug resistance (cdr1) mRNA occur in cells treated with estrogen compared to control cells (Zhang et al., 2000). Similar to *C. neoformans* and *P. brasilienis*, *C. albicans* is influenced by the host hormonal environment. A greater understanding of how hormone responses differ during candidiasis may provide insights to uncover mechanisms in other fungi with sex biases during infection.

1.8 Project Objectives and Summary

Three key findings are evident from this limited body of work. First, females consistently have more favorable outcomes during cryptococcosis compared to their male counterparts. Second, sex hormones appear to be a significant source of the differences observed between males and females during an infection. Lastly, the published work largely focuses on sex hormones and how they modulate the host response. Very little is known about the effect of the hormonal environment on the pathogen itself. Taking the above into account, this project aimed to address both host and pathogen components of sexual dimorphism in infection to help determine what roles biological sex and sex hormones play in the virulence of and immune response to *C. neoformans*.

Since sex susceptibility to infection is primarily about the interaction between host and pathogen, with both contributing to disease, it is necessary to determine how sex hormones affect both the host and the pathogen. The first aim of this project was to determine whether estrogen was able to enter the *C. neoformans* cell. Fluorescently

tagged 17β-estradiol was incubated with multiple *C. neoformans* strains in varying concentrations, and the relative amount of 17β-estradiol accumulating was determined by laser scanning confocal imaging. In fact, estrogen was able to permeate *C. neoformans* cells at normal physiological levels. Second, the effect of sex hormones on the expression of virulence factors of this pathogen was characterized. Using a group of clinical *C. neoformans* strains isolated from HIV^{+}/cryptococcal $meningitis^{+}$ patients, a series of biochemical experiments were performed to establish what effects exogenously added testosterone and estrogen had on virulence. The results of virulence testing in the presence of sex hormones indicate that melanin production was increased in the presence of testosterone and growth of the yeast was inhibited in the presence of estrogen coupled with oxidative stress. These results provide direct evidence that the hormonal environment affects *C. neoformans.* The third aim of this project investigated whether aspects of the healthy male and female immune response were different in the presence and absence of exogenous sex hormones. Peripheral blood was collected from 40 volunteers (20 male, 20 female) and their PBMCs isolated and infected with *C. neoformans*. B and T cell proliferation was measured via multi-color flow cytometry. Males had a significantly decreased T cell percentages compared to females during infection. Additionally, total B cell percentages in males dropped during infection whereas female B cell populations remained the same in both uninfected and infected cell populations. These results suggest an inherent deficit in the healthy male immune response during a *C. neoformans* infection.

Considering both the sexual dimorphism observed and the impact of *C. neoformans* on public health, especially in the developing world, it is striking that so little is known about the sex susceptibility aspect of this pathogen. This research was designed as a novel, comprehensive approach to investigating the sexual dimorphism occurring during *C. neoformans* infections. We expect this work to contribute in meaningful ways both to the general understanding of sexual dimorphism of infection and specifically, to the sex bias in cryptococcosis. Up until this point, no one has tested the impact of sex hormones on the virulence of *C. neoformans*. That testing coupled with our investigation on the effect of biological sex on the immune response to *C. neoformans* deepens our understanding of what is currently a very poorly understood phenomenon.

CHAPTER 2
MATERIALS AND METHODS

2.1 *C. neoformans* Strains and Maintenance

All *C. neoformans* strains were cultured from frozen stock to prevent microevolution and grown in yeast peptone dextrose (YPD) broth (BD Difco) at 37 ^{o}C in a 150 RPM shaking incubator (Table 2).

Table 2: *C. neoformans* Strains Used in These Experiments.

Strain Name	Description	Source
H99S	Wild type, serotype A	John Perfect
CNAG_00544 Δ	*NADPH dehydrogenase* Δ, H99S, serotype A	Hiten Madhani
CNAG_01523 Δ	*hog1*Δ, H99S, serotype A	Hiten Madhani
B5	Male-derived clinical isolate	Gregory Bisson
B10	Male-derived clinical isolate	Gregory Bisson
B12	Male-derived clinical isolate	Gregory Bisson
B14	Male-derived clinical isolate	Gregory Bisson
B15	Male-derived clinical isolate	Gregory Bisson
B18	Female-derived clinical isolate	Gregory Bisson
B20	Female-derived clinical isolate	Gregory Bisson
B24	Male-derived clinical isolate	Gregory Bisson
B26	Male-derived clinical isolate	Gregory Bisson
B27	Female-derived clinical isolate	Gregory Bisson
B30	Female-derived clinical isolate	Gregory Bisson
B33	Female-derived clinical isolate	Gregory Bisson
B40	Male-derived clinical isolate	Gregory Bisson
B43	Female-derived clinical isolate	Gregory Bisson
B45	Male-derived clinical isolate	Gregory Bisson
B52	Female-derived clinical isolate	Gregory Bisson
B55	Female-derived clinical isolate	Gregory Bisson
B58	Male-derived clinical isolate	Gregory Bisson
B62	Female-derived clinical isolate	Gregory Bisson
B65	Male-derived clinical isolate	Gregory Bisson
B68	Female-derived clinical isolate	Gregory Bisson
B74	Female-derived clinical isolate	Gregory Bisson

Table 2 *C. neoformans* Strains Used in These Experiments. (Contd.)

Strain Name	Description	Source
B75	Female-derived clinical isolate	Gregory Bisson
B77	Female-derived clinical isolate	Gregory Bisson
B88	Female-derived clinical isolate	Gregory Bisson
B93	Female-derived clinical isolate	Gregory Bisson
B99	Female-derived clinical isolate	Gregory Bisson
B106	Male-derived clinical isolate	Gregory Bisson

2.2 Mammalian Cell Culture

The BALB/c murine macrophage cell line, J774.16, were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 4.5 g/L glucose and 0.11g/L sodium pyruvate supplemented with 10% FBS, 2 mM L-glutamine, 1% penicillin/streptomycin antibiotic, 10% NCTC-109 medium, and 1X MEM non-essential amino acid solution from Thermo Fisher Scientific. The cells were cultured in an incubator at 37 ^{o}C and 5% CO_2. Cells were cultured in 75 cm^2 tissue culture flasks until ~80% confluent. The cells were washed with warm phosphate buffered saline (PBS) and removed by gently scraping the walls of the flask. Two mL of cells were transferred to a new 75 cm^2 tissue culture flask and 20 mL fresh J774 media and stored for later use.

2.3 Estrogen Permeability Studies

2.3.1 Estradiol Glow Experiments

C. neoformans strains were grown in a liquid culture of YPD at 37^{o}c and 150 RPM until cells reached log phase (typically 18-48 hours). The cells were pelleted by centrifugation at 870 x *g* for 5 minutes (min) and washed three times with PBS to remove residual YPD. Cells were resuspended in PBS, counted on a hemocytometer and a concentration of 5 x 10^8 cells/mL in 200 µL was incubated with Estradiol Glow (EG) (Jena Bioscience) at physiologically relevant levels of estrogen (400 pg/mL), 10x, or 100x concentrations in the dark at room temperature for two hours (h) or 24h. EG has spectroscopic properties of λ_{exc} 467 nm and λ_{em} 618 nm. After incubation, 4 µL of cell suspension was placed on a slide, a cover slip was added, and sealed with nail polish. One hundred cells/condition were imaged using a LSM 700 laser scanning confocal

microscope equipped with a Plan-Apochromat 63X NA 1.4 oil immersion DIC M27 objective using 63X magnification. EG was excited using the 488 nm laser. AF488 fluorescence was excited through a 465-495 nm excitation filter, and emitted light can be detected through a 515-555 nm barrier filter reflected from a 505-nm dichroic mirror.

2.3.2 Heat Killing Experiments

To assess whether estrogen uptake differs in dead compared to live *C. neoformans* cells, the wild type strain, H99S was heat-killed (HK) by incubating cells in a 65 ^{o}C water bath for 40 min. After incubation, the cell culture was divided. Half of the cells were stained with Estradiol Glow at 100X concentration and imaged as described in 2.3.1. To confirm cell death, the other half of the culture was stained with the dead cell stain, propidium iodide (PI) (1 µg/mL) for 20 min and imaged on a confocal microscope. Unstained H99 both live and heat-killed were used as negative controls.

2.3.3. Capsule Induction

To determine whether varying sizes of capsule interfered with the ability of estrogen to permeate *C. neoformans*, capsule inductions were performed as described in (Guess et al., 2018). Briefly, *C. neoformans* strains were grown in YPD liquid culture at 37^{o}C and 150 RPM until log phase and washed three times to remove residual YPD. The cells were counted and 1 x 10^{5} cells/2 mL were seeded into a 6-well plate containing DMEM. Cells were incubated for 18 h at 37^{o} C and 5% CO_2. The combination of minimal nutrients and CO_2 causes *C. neoformans* to induce capsule growth. After 18 h, the cells

were collected and centrifuged at 870 x *g* for 5 minutes in a microcentrifuge. The supernatant was carefully aspirated and the cells were resuspended in 10 µL PBS.

2.3.4 Staining with Estradiol Glow and Calcofluor White

To determine whether estrogen was taken up into the cell body, some cultures stained with Estradiol Glow were co-stained with Calcofluor White (CFW) (Sigma Aldrich), which stains the cell wall of *C. neoformans.* This was done by adding CFW (1:1, cell suspension: CFW) to the cell suspension at the 1.5 h incubation period of Estradiol Glow. Incubation continued as described in 2.3.1. After 30 min of incubation with either (single-stained controls) or both stains, the cell suspensions were pelleted at 670 x *g* for five min. The supernatant was discarded and, 4 µL of cell suspension was placed on a slide, a cover slip was added, and sealed with nail polish.

2.3.5 Fluorescence Analysis

Images acquired from experiments described in 2.2 were analyzed using FIJI, version 1 (ImageJ) (Schindelin et al., 2012). Cells were identified and measured using the Region of Interest (ROI) tool placed in the cytosol of each cell. Background fluorescence, determined using extracellular signal, was subtracted from total fluorescence during analysis. Capsule diameter was measured manually using Axiovision software (Zeiss) and the equation: ([total diameter] – [cell body diameter]).

2.3.6 Capsule and Cell Body Size Analysis

In an effort to determine whether estrogen permeability was correlated with surface area of *C. neoformans*, cells were stained with India ink and imaged using a Zeiss A1 Axio light microscope with a Plan - NEOFLUAR 100X oil immersion NA 1.30 objective

at 100X. One hundred cells per strain were imaged and measured. Capsule diameter was measured manually using Axiovision software (Zeiss) and the equation: (total diameter – cell body diameter).

2.4 Virulence Factor Testing

For these studies, 28 unique clinical *C. neoformans* isolates, listed in table 2.1, were used. These strains were cultured from frozen stock and were collected from patients with HIV/AIDS and cryptococcal meningitis in Botswana (Bisson et al., 2008). Sixteen isolates were collected from females and 12 isolates were collected from males. Using a series of biochemical methods (see sections 2.4.1 – 2.4.5), the virulence factors of *C. neoformans* were tested with and without sex hormones and the subsequent changes measured. Isolates were cultured as described in 2.1. The cells were then centrifuged and washed once with PBS. After washing, isolates were resuspended in asparagine medium + 0.3% glucose, and the cells from each strain were divided into three sterile flasks containing asparagine medium and physiological levels of testosterone (10 ng/mL, Sigma), estrogen (400 pg/mL, Sigma), or ethanol (0.001%) as a negative control. Asparagine medium is a low nutrient medium designed to mimic the environment of the CNS (Jacobson and Compton, 1996). These cultures were incubated in a 37 ^{o}C shaking incubator until each was in log phase. The wash steps were repeated three times with PBS and each culture was resuspended in asparagine media without glucose and corresponding hormones and incubated for 24 h at 37 ^{o}C in a shaking incubator. After 24 h, the cells were pelleted and washed three times with PBS, and

counted with a hemocytometer. From this point, each isolate was tested using previously validated, optimal parameters for each experiment.

2.4.1 Capsule Size

After growing each strain as described above (2.4), cell suspensions of 1 x 10^5 cells/2 mL were seeded into a 6-well plate containing DMEM and testosterone, at physiological concentrations or a negative control and incubated for 18 h at 37^o C + 5% CO_2. After 18 h, cells were subsequently centrifuged, and washed 3 times with PBS. Slides for each condition were made using a 1:1 ratio of cell suspension:India ink and imaged by light microscopy. Fifty cells per condition were imaged and analyzed.

2.4.2 Melanin

This protocol was adapted from (McClelland et al., 2005). To test for melanin production, clinical isolates were grown as stated in 2.3 and after incubating in asparagine medium with hormones, 25 µL of each isolate were plated on corresponding L-dopa agar plates containing testosterone, estrogen, or ethanol. The plates were incubated at 37 ^{o}C for 7 days in the dark. At days 3, 5, and 7, the plates were photographed and examined for melanin production and given a qualitative score (0 = no visible melanin production, 1 = some visible melanization, 2 = partial melanization, 3 =significant but not complete melanization, 4 = complete melanization) based on the amount of color produced by the colonies.

2.4.3 Urease

This protocol was adapted from (Kwon-Chung et al., 1987). To measure urease activity, each isolate was grown under the conditions described in 2.3. Next, 5 x 10^7 cells

in 2 mL of 2X Robert's urease broth were transferred to two sets of 15 mL tubes (2 h and 4 h tubes) and incubated at 37^{o} C/150 RPM for 2 and 4 h. At each time point appropriate cell samples were centrifuged, and 1 mL of the supernatant was transferred to cuvettes. The optical density (OD) was measured at 560 nm and recorded. A higher OD indicates higher urease production.

2.4.4 Resistance to Cell Stressors

To test for resistance to cell stressors, clinical isolates were grown as described in 2.3 and a protocol was adapted from (Gerik et al., 2005), by plating them on cell stressor plates that correspond with each condition (e.g. an isolate that is grown in medium containing testosterone will also be plated on rich medium plates containing both the inhibitor to be tested and testosterone) (Fig. 2.1). Specifically, 100 µL of 1 x 10^{6} cells/mL of each isolate was used as the first of five 1:10 serial dilutions made in PBS. Five µL of each dilution was spotted on plates containing rich medium, corresponding hormones at physiological levels, and one of ten cell stressors. The stressors included: 2 mM hydrogen peroxide (H_2O_2) or 1 mM sodium nitrite ($NaNO_2$) and 25 mM succinic acid ($C_4H_6O_4$) (oxidative and nitrosative stressors), 0.03% sodium dodecyl sulphate (SDS), 0.5% Congo Red (CR), 1.5 µg/ml Calcofluor white (CFW), 1 M potassium chloride (KCl), 1 M sodium chloride (NaCl), 400 mM calcium chloride ($CaCl_2$) or 1 M sorbitol (cell wall stressors), and 100 µM bathophenanthrolinedisulfonic acid (BPS) (iron stressor). The plates were stored at 37 ^{o}C and checked for growth and given a qualitative score (0 = no colonies, 1 = 1-10 colonies, 2 = 11-50 colonies/partial lawn, 3 = >50 colonies/full lawn) at 3, 5, and 7 days. The scores were recorded and images were taken of each plate.

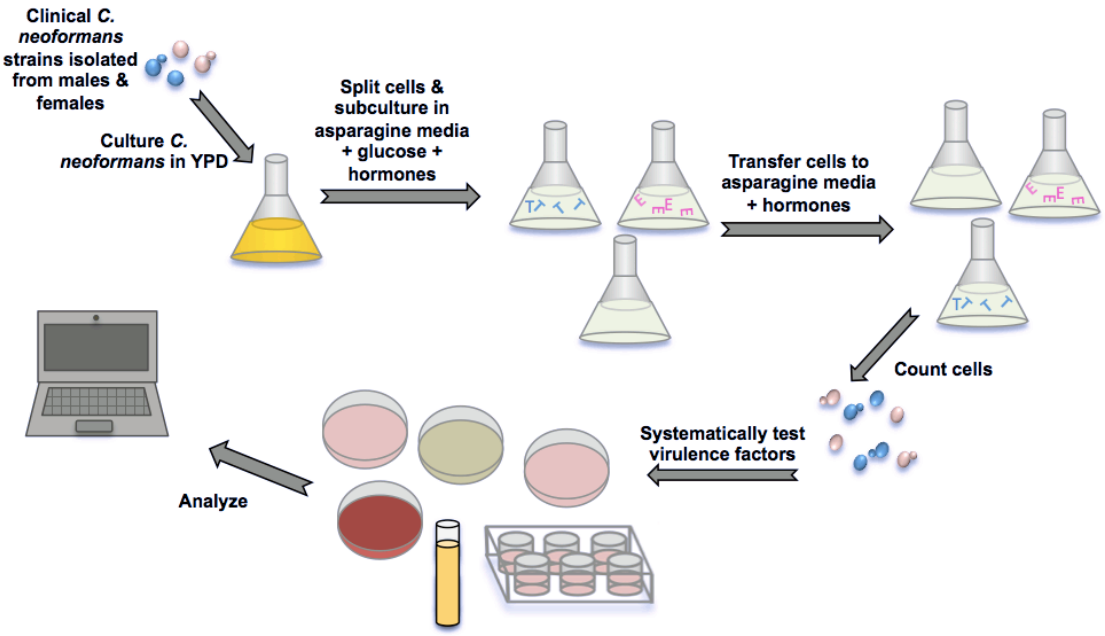


Figure 2.1: Workflow Schematic of Virulence Factor Testing.

Clinical *C. neoformans* isolates were first cultured in YPD then subsequently transferred to asparagine media containing testosterone, estrogen, or a negative control. The cells from each culture were counted and the virulence factors of *C. neoformans* were systematically tested using a variety of biochemical methods. The data was collected and analyzed.

2.4.5 Fungal Burden

This protocol utilized the murine macrophage cell-line J774.16. The macrophages were seeded on day one of the experiment. When cells were 70-80%

confluent they were washed with warm PBS, resuspended in 10 mL warm J774 medium (described in 2.2), and removed from the flask using a cell scraper. Cells were counted using Trypan Blue viability stain and adjusted to 5 x 10^5 viable cells/mL in 2.5 mL total volume. Then, 20 units of IFN-ϒ was added to the cell suspension and 200 µL/well was aliquoted into a 96-well plate. Seeded macrophages were incubated overnight in a 37 ^{o}C incubator with 5% CO_2. On day two, *C. neoformans* in log phase cultured in YPD was washed three times with PBS and counted. *C. neoformans* cells were adjusted to 1 x 10^6 cells/mL (2:1 MOI) and LPS (1 µg/mL) and IFN- ϒ (200 Units) was added. *C. neoformans* cells were opsonized for 30-60 min at 37 ^{o}C. Medium was then removed from seeded macrophages and infected with 100 µL opsonized *C. neoformans*/well. An additional 100 µL of warm J774 medium was added to all wells. The 96-well plate was centrifuged at 500 x *g* for five min and the infected macrophages were incubated for one h at 37 ^{o}C with 5% CO_2. The macrophages were examined under a dissecting microscope to confirm phagocytosis. The media and any extracellular *C. neoformans* were removed. Each well was washed twice with warm PBS. The cells were resuspended in 200 µL warm J774 medium/well and incubated for 24 h at 37 ^{o}C with 5% CO_2. Following this incubation period, 100 µL of 0.5% SDS was added to every well to lyse the macrophages. After five min of SDS incubation, the liquid from all wells was transferred to centrifuge tubes and the wells were washed two times with 100 µL PBS. To retrieve residual *C. neformans* from wells, these washes were added to the corresponding centrifuge tubes. The *C. neoformans* cells in these tubes were counted to determine correction dilutions for plating. Cells from each condition were plated on YPD plates (100 cell suspension µL

/plate, two plates/condition). The inoculated plates were incubated at room temperature, and colonies were counted and recorded after four days incubation.

2.5 Immunology Study

2.5.1 Blood Draws

This study was carried out in the Biology Department of Middle Tennessee State University in Murfreesboro, TN under an approved IRB protocol (#15-301). Written consent was obtained from forty-three healthy volunteers aged ≥ 18 years (21M:22F), who were prospectively enrolled in the study. Of those 43, 40 (20M:20F) samples were utilized for this study.

2.5.2 Peripheral Blood Mononuclear Cell (PBMC) Isolation

Peripheral blood samples were obtained from volunteers by a licensed phlebotomist, Dr. Erin Park, by venipuncture. Approximately 25 mL of blood per person was collected in sodium heparin collection tubes and labeled with a unique identifier. Heparanized blood was overlayed onto room temperature Ficoll in a 1:1 ratio in a sterile 50 mL conical tube. The samples were centrifuged at 400 x *g* for 30 min at room temperature with the brake off to ensure that deceleration did not disrupt the density gradient. Two mL of sera from each patient sample was stored at -80 ^{o}C for subsequent antibody-reactivity testing. The buffy coat, containing the mononuclear cell layer, was extracted using a transfer pipette and washed three times with lipopolysaccharide (LPS) -free phosphate buffered saline (PBS). The cells were resuspended in RPMI 1640 medium (Sigma, USA) plus 10% human serum (50:50 male:female) and counted on a hemocytometer using a 1:1 Trypan Blue exclusion analysis (0.4% solution). Aliquots of 2

x 10^8 viable cells/2 mL were cryopreserved using a method previously outlined (Nazarpour et al., 2012) to be infected at a later date.

2.5.3 MOI Determination

To establish the appropriate multiplicity of infection (MOI), preliminary experiments were conducted using PBMCs infected with *C. neoformans* using a variety of MOIs: 1:1, 1:10, 1:50, 1:100, 1:1,000 (*C. neoformans*:PBMCs). The cells were incubated in a 37 ^{o}C CO_2 incubator and assessed via flow cytometry after seven days. The 1:100 MOI was determined to be the most beneficial ratio as it stimulated the immune response of both B and T cells but did not cause significant death of the PBMCs.

2.5.4 PBMC Infection and Flow Analysis

Cryopreserved PBMCs were removed from liquid nitrogen and thawed in a 37 ^{o}C water bath. After thawing, cells were added to 10 mL warm RPMI 1640 medium (Sigma, USA) plus 10% human serum (50:50 male:female) and centrifuged at 400 x *g* to pellet PBMCs. Cells were resuspended in 500 µL warm RPMI and counted on a hemocytometer using a 1:1 ratio for Trypan Blue exclusion analysis. One hundred µL of 5 x 10^6 viable cells/mL PBMCs were seeded in 96-well round-bottomed plates and infected with the *C. neoformans* strain, H99S, at a concentration of 5 x 10^4 cells/mL in 100 µL RPMI (1 *C. neoformans*:100 PBMC MOI) and allowed to incubate for seven days in a 37 ^{o}C CO_2 incubator, with 100 µl new medium added on day three of incubation

Physiological levels of testosterone (10 ng/mL, Sigma) or 17β-estradiol (400 pg/mL, Sigma) were added to subsets of infected and uninfected PBMCs on day one of incubation. Separate PBMC and *C. neoformans* negative controls were incubated with

RPMI only. All samples were done in triplicate. One hundred µL warm RPMI was added to each well on day four of incubation. See figure 2.1 for an illustration of experimental set up. PBMCs were resuspended in LPS-free PBS and incubated with fluorescent antibodies as indicated in Table 2.2 for 20 min at approximately 21 ^{o}C (lab temperature), in the dark. See Figure 2.1.

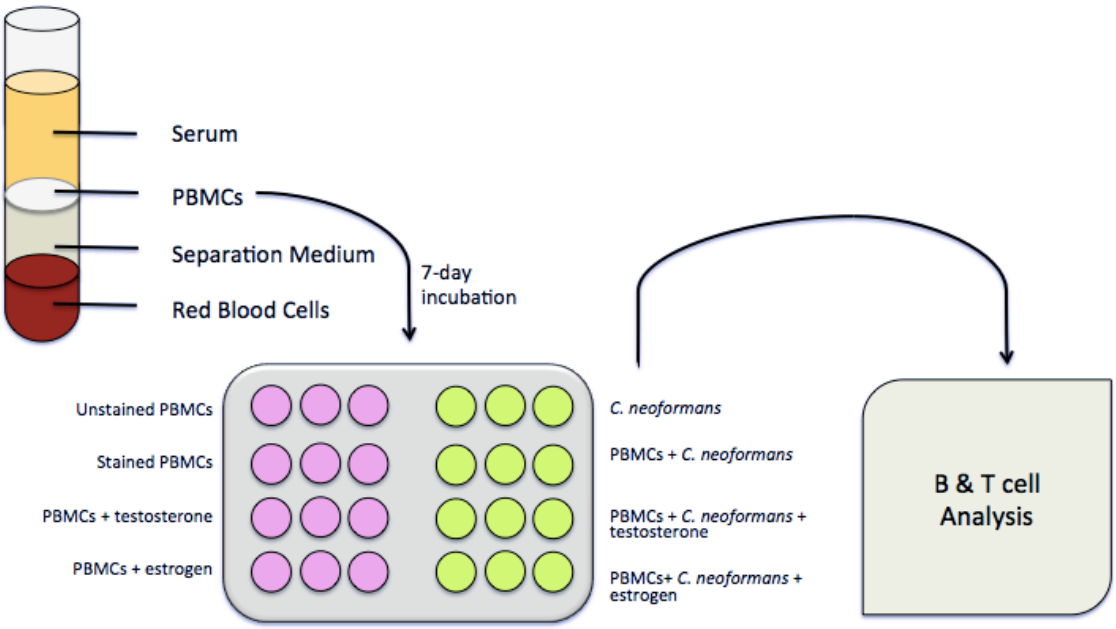


Figure 2.2: Workflow Schematic of PBMC Infection and Analysis.

Whole blood was extracted by venipuncture and separated using a density gradient. Isolated PBMCs were incubated for 7 days either alone or in the presence of *C. neoformans*. Subsets of both uninfected and infected groups were incubated with testosterone or estrogen. All samples were setup in triplicate and the B and T cell response was determined via flow cytometry.

Table 3: Antibodies Used in Flow Cytometry.

Instrument: Guava easyCyte 8HT

Antibody/Probe	Fluorochrome	Excitation/Emission Spectra (nm)	Vendor/Cat. No.
Anti-human CD3	APC	650/660	BD Biosciences/555342
Anti-human CD3	PE	496/578	BD Biosciences/555347
Anti-human CD3	FITC	494/520	BD Biosciences/557085
Anti-human CD3	PC7	496/785	Beckman Coulter/IM3628U
Live-dead discriminator	7AAD	546/647	ThermoFishcer/A1310

A Guava® easyCyte 8HT was used for flow cytometry (Millipore). A forward scatter threshold of 800 was used to avoid most debris in counting; 30,000 events/well were collected in all wells with the exception of *C. neoformans* only controls where 5,000 events/well were collected. Compensation was performed using single-color controls of uninfected PBMCs. Gating of flow cytometry data was analyzed using InCyte version 3.1 (Millipore). For multi-color analysis, lymphocytes were identified in the same manner, and standard elliptic gates were applied to all samples. Within those gates, the T cell population was identified as cells expressing the surface markers CD3, CD4, or CD8. The B cell population was identified as cells expressing the surface marker CD19. *C. neoformans* controls were used to inform gating strategies to ensure the least possible amount of *C. neoformans* was included in the lymphocyte gate during analysis as illustrated in Figure 2.3.

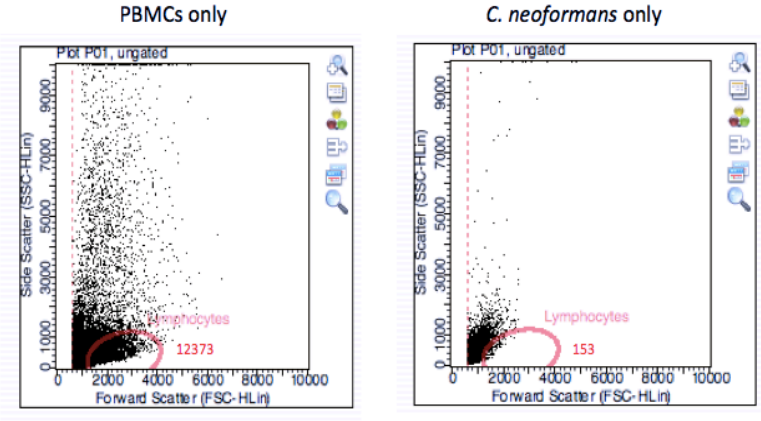


Figure 2.3: Representative Images of Lymphocyte Gating.

The image on the left is a forward vs. side scatter plot of PBMCs only with an elliptic gate drawn around the lymphocyte population. 12,373 of 30,000 events collected fell into the lymphocyte gate (41.24%). The image on the right is a forward vs. side scatter plot of *C. neoformans* only with the same elliptic gate. 153 of 5,000 events collected (3.06%) are included in the lymphocyte gate.

2.6 Statistical Analysis

Sample sizes and number of experiments performed are noted in each figure legend. The type of statistical test that was performed for each experiment is indicated in each figure legend. All statistical analysis was derived using JMP, version 13 (SAS Institute). Graphs were created using Excel, version 14 (Microsoft) or GraphPad, version 7.0 (Prism). All error bars presented in this dissertation represent standard error. Statistical significance was defined as p value of ≤ 0.05.

Chapter 3

THE HORMONAL MILIEU: ESTROGEN PERMEATES *C. neoformans*

3.1 Introduction

Produced by all vertebrates with the exception of bony fish, sex hormones are steroids synthesized by the gonads and adrenal glands, reviewed by (Bentley, 2001). In humans, the most important sex hormones are testosterone and estrogen (Marino et al., 2006). Responsible for key roles in numerous physiological functions, these fat-soluble molecules travel throughout the body and enter cells freely, where, in target cells, they bind to specific hormone receptors in the nucleus and trigger a cascade of events resulting in protein production by the target cell (Gruber et al., 2002). The closest analogue to sex steroids in fungi is ergosterol (Fig 3.1) (Dupont et al., 2012). A sterol located in cell membranes of fungi and functioning much the way cholesterol does in humans, ergosterol is essential for multiple cell processes in yeast, including *C. neoformans* (Kim et al., 1975). A key role of ergosterol is to regulate both membrane fluidity and permeability by interacting with phospholipids and other components of the cell membrane (Mitchell and Perfect, 1995).

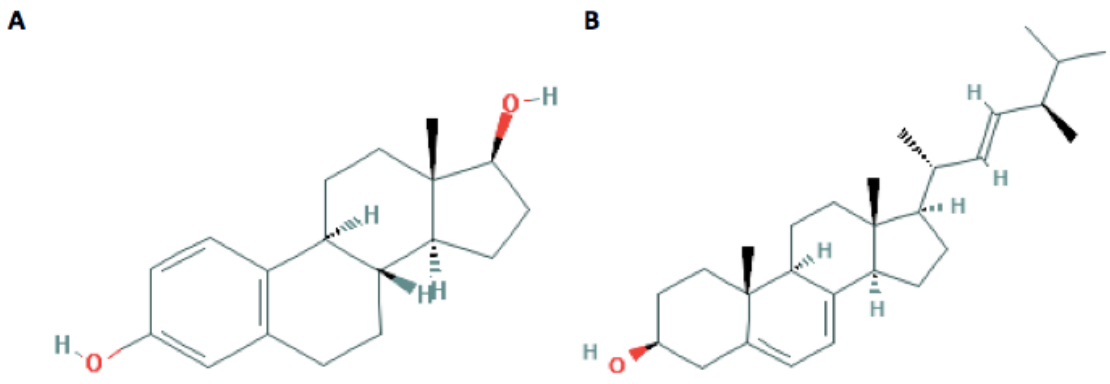


Figure 3.1: Chemical Structure of (A) 17-β Estradiol and (B) Ergosterol (Source: PubChem).

Heretofore, it has not been shown that estrogen is capable of entering *C. neoformans*. A logical first step in investigating the effect estrogen has on *C. neoformans* directly is to determine whether or not the hormone is able to permeate the cell and enter into the cell body and whether this occurs at concentrations encountered *in vivo*. Prior research has shown that estrogen enters *Saccharomyces cerevisiae* and *Candida albicans,* but the mechanism in which the hormone enters these cells has not been discerned (Burshell et al., 1984; Skowronski and Feldman, 1989). The hormone may permeate by either diffusion or by engaging cell surface receptors. Diffusion is the most likely mechanism since, to date, no hormone-like cellular receptors have been identified on the cell surface of *C. neoformans*.

In the 1980s, researchers isolated and characterized an estrogen-binding protein (EBP) located in the cytosol of *S. cerevisiae* (Burshell et al., 1984). While it has shown no sequence homology to the human estrogen receptor, the EBP appears to be functionally conserved among eukaryotes to such an extent that the human estrogen receptor is able to reconstitute estrogen signaling within *S. cerevisiae* (Paumi et al., 2009). Very similar to *S. cerevisiae*, an estrogen receptor was located and isolated in the cytosol of *C. albicans* (Skowronski and Feldman, 1989). Termed estrogen-binding protein 1 (Ebp1p), it binds 17β-estradiol with a high affinity and is analogous to a gene in *S. cerevisiae* that encodes old yellow enzyme (OYE), an oxidoreductase (Skowronski and Feldman, 1989). Characterization of Ebp1p shows that it, like the EBP in *S. cerevisiae,* has no sequence homology to the mammalian steroid receptor family (Madani et al., 1994). To date, an EBP1p orthologue or other EPB has not been located in *C. neoformans*, nor are there any published reports of researchers searching for and failing to find one.

A BLAST search did not identify, in *C. neoformans*, a sequence similar to the EBPs in either *C. albicans* or *S. cerevisiae*. However, *C. neoformans* contains a single NADPH dehydrogenase, of which its importance in cell virulence and viability has not been characterized. Given that the EBP is a NADPH dehydrogenase in *C. albicans*, we hypothesized that a knockout (KO) strain that does not express this protein would show decreased uptake of fluorescently-tagged estrogen when compared to wild type (Skowronski and Feldman, 1989).

Highly conserved, the high-osmolarity glycerol (HOG) pathway is a complex mitogen-activated protein kinase (MAPK) pathway containing multiple signaling

components that regulate the stress response in *C. neoformans.* The HOG pathway acts most commonly as it relates to the adaptation to hyperosmotic stress but it can also be activated as a response to numerous environmental stresses, including: oxidation, acid, heavy metal, or temperature stress, anti-fungal drug response, and breaches of the cell wall (Bahn et al., 2005). The pathway can be activated by two different mechanisms. One mechanism leads to the activation of PBS2 and involves a "two-component phospho-relay signaling system", which results in the activation of Hog1 (Bahn, 2008). Hog1 is located in both the cytosol and the nucleus during unstressed conditions but rapidly dephosphorylates and concentrates in the nucleus during environmental stress where it controls osmotic regulation and the transcription of target genes (Bahn, 2008; Bahn et al., 2005). Additionally, Hog1 regulates a number of genes implicated in the virulence of *C. neoformans,* such as *CAP59*, *ERG11*, *LAC1*, and *SCH9* (Jung and Bahn, 2009). Serotype A *Hog1Δ* mutants are attenuated for virulence, which underscores the significance of Hog1 in the pathogenesis of cryptococcosis (Bahn et al., 2005). Given the importance of Hog1 in both cell function and virulence, we hypothesized that estrogen uptake may be increased in a *Hog1Δ* mutant compared to wildtype strains

The aim of these experiments was threefold: 1. To definitively show that estrogen was able to enter into *C. neoformans* cells and at physiological concentrations. 2. To decipher to what extent capsule and cell membrane integrity effect hormone uptake. 3. Investigate the differences in estrogen uptake in wild type *C. neoformans* compared to *NADPH dehydrogenaseΔ* and *Hog1Δ* mutant strains in *C. neoformans.*

To accomplish these aims, *C. neoformans* was incubated with a fluorescently labeled 17-β-estradiol and imaged using fluorescent microscopy. Once it was established that estrogen does penetrate *C. neoformans,* the next step was to examine how changing the cellular structure would affect the penetration of the hormone, if at all. The first obstacle molecules encounter when coming into contact with *C. neoformans* is its complex capsule. To this end, experiments were conducted that manipulated the capsule and cell membrane in the presence of fluorescently tagged estrogen and the differences in uptake were measured. Finally, *Hog1Δ* and *NADPH dehydrogenase Δ* mutants were incubated with fluorescent estrogen and the differences in uptake were measured.

3.2 Results

3.2.1 Estrogen Permeates *C. neoformans* at Physiologically Relevant Concentrations

Given that females fare better during a *C. neoformans* infection, the aim was to determine whether the primary female sex hormone, 17β-estradiol, enters the *C. neoformans* cell. To accomplish this, a fluorescently tagged estrogen, Estradiol Glow (EG), was incubated with the wild type *C. neoformans* strain, H99S, along with the cell wall stain, calcofluor white (CFW) (Figure 3.1). Once it was determined that estrogen was able to enter *C. neoformans*, the next set of experiments was conducted to establish at what concentrations EG could be detected in the cell. Physiologically relevant concentrations (400 pg/mL), 10X, and 100X concentrations of EG were incubated with *C. neoformans.* Cells were imaged on a confocal microscope and analyzed using the method outlined in 2.3.5. Fluorescent images showed that estrogen, at normal physiological levels seen in females, was taken up by *C. neoformans.* When compared to background fluorescence, there was a significant difference at 1X EG concentrations ($p < 0.001$). There was no difference between 1X and 10X concentrations, but there was a difference between 1X and 100X concentrations ($p < 0.0001$, $n = 3$) (Figure 3.1B and 3.1C). It should be noted that background fluorescence is primarily seen in budding cells. To ensure that the difference in fluorescence between the negative control and increasing EG concentrations was real and not an artifact, all budding cells were excluded from the analysis.

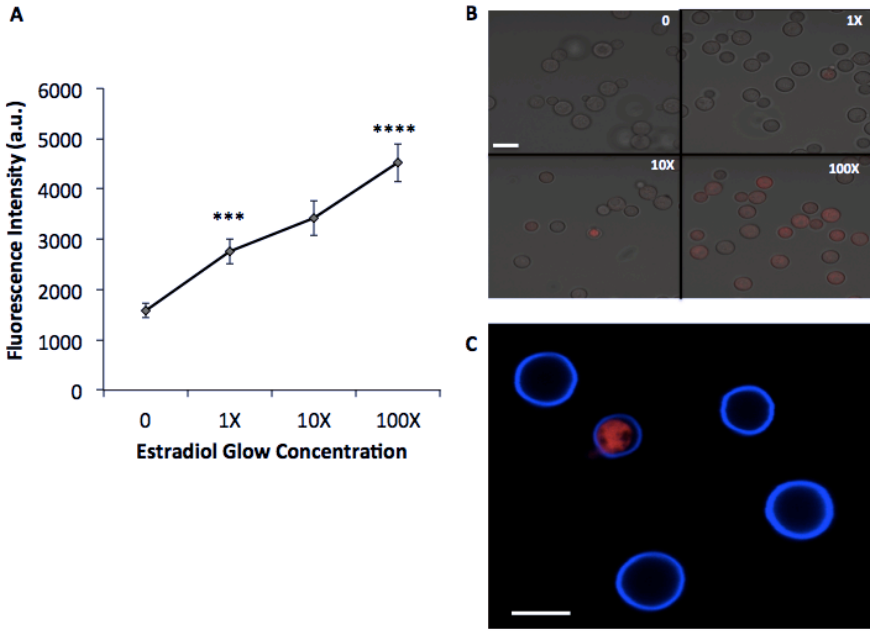


Figure 3.2: Estrogen Permeates *C. neoformans* at Physiologically Relevant Concentrations.

(A) The results are of a representative experiment illustrating the fluorescence intensity as measured in arbitrary units (a.u.) for *C. neoformans* when incubated at increasing levels of EG concentration. These experiments were performed as three discrete biological repeats. One hundred cells per condition were imaged. Data was analyzed using ANOVA with simple contrasts. Error bars are SE. Statistical significance is indicated as follows: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; and ****, $p < 0.0001$ **(B)** Clockwise from top left: *C. neoformans* without staining, stained with EG at 1X (400 pg/ml), 10X (0.004 µg/ml), and 100X (0.04 µg/ml) concentrations at 2.5 h post-treatment. **(C)** *C. neoformans* stained with EG (1X; red) and CFW (1:1, cell suspension: CFW; blue) and imaged at 2.5 h incubation. Scale bars indicate 10 µm.

3.2.2 *C. neoformans* Capsule and Cell Structure Affect Estrogen Uptake

3.2.2.1 Estrogen Uptake is Increased in the Presence of Enlarged Capsule

Given that estrogen from the surrounding environment could permeate *C. neoformans,* the next step in this study was to investigate how estrogen was entering the cell and what methods may block or facilitate its entrance. To that end, a series of experiments was undertaken that would either increase the size of the capsule or damage the cell membrane, both in the presence of EG, and the differences in estradiol uptake were measured. The proprietary fluorophore used in EG is naturally dim. Once it was established that estrogen enters the cell at levels seen in humans, it was decided to use a 100X concentration (0.04 μg/mL) in all other experiments to better visualize the fluorophore. The capsule of H99S was induced to grow as described in 2.3.3. The cells were then incubated with 100X EG and imaged on a confocal microscope. The estradiol uptake was measured using ROI analysis and compared with uninduced cells of the same strain. Results show significantly increased uptake of estradiol in cells with induced capsule ($p < 0.001$) (Figure 3.2). Additionally, a different staining pattern was observed. In uninduced cells, EG tends to distribute equally throughout the cell, whereas in cells with induced capsule, EG is concentrated in a ring around the cell wall or cell membrane and is more diffuse through the body of the cell (Fig 3.3D).

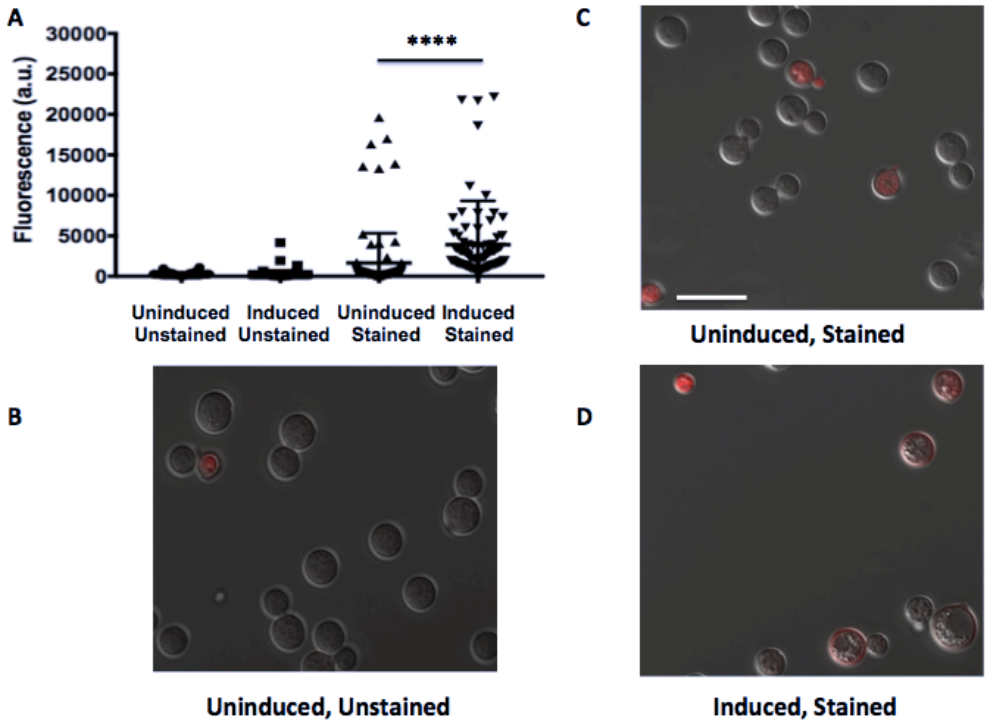


Figure 3.3: Estrogen Uptake is Increased in the Presence of Enlarged Capsule.

(A) The results are of a representative experiment illustrating the fluorescence intensity as measured in arbitrary units (a.u.) of *C. neoformans* when incubated with EG in cells with uninduced and induced capsule. These experiments were performed as three discrete biological repeats. One hundred cells per condition were imaged. Data was analyzed using ANOVA with simple contrasts. Error bars are SE. Statistical significance is indicated as follows: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; and ****, $p < 0.0001$. **(B)** Uninduced *C. neoformans* unstained **(C)** Uninduced *C. neoformans* stained with 100x (0.04 µg/mL) EG imaged at 2.5 h incubation. **(D)** Induced *C. neoformans* stained with 100x (0.04 µg/mL) EG and imaged at 2.5 h incubation. Scale bar indicates 10 µm.

3.2.2.2 Estrogen Uptake is Dramatically Increased in Heat-Killed Cells

Like other yeast, *C. neoformans* cannot survive temperatures exceeding 60 ^{o}C for extended periods of time (Maxwell and Magwene, 2017). Death occurs quickly at these temperatures, primarily due to the irreversible denaturation of proteins that are required for the life of the cell (Leuenberger et al., 2017). Both the polysaccharide capsule and the cell wall contain small amounts of mannoproteins (Kollar et al., 1997). The cell membrane, situated just inside the cell wall, is composed primarily of phospholipids and proteins (Takeo et al., 1973). To establish how much environmental estrogen enters *C. neoformans* when the integrity of the cell membrane is compromised, the wild type strain, H99S, was killed using heat as described in 2.3.2 and incubated with 100X EG. Cells were imaged on a confocal microscope and EG fluorescence intensity in the yeast cell body was quantified using FIJI as described in 2.3.5. Results indicate dramatically increased uptake of estrogen in heat-killed H99S ($p < 0.0001$) (Figure 3.3). To confirm death, a subset of heat-killed cells were stained with PI, a membrane-impermeable DNA stain that only labels dead cells, and imaged. All cells were dead as they all contained PI (data not shown).

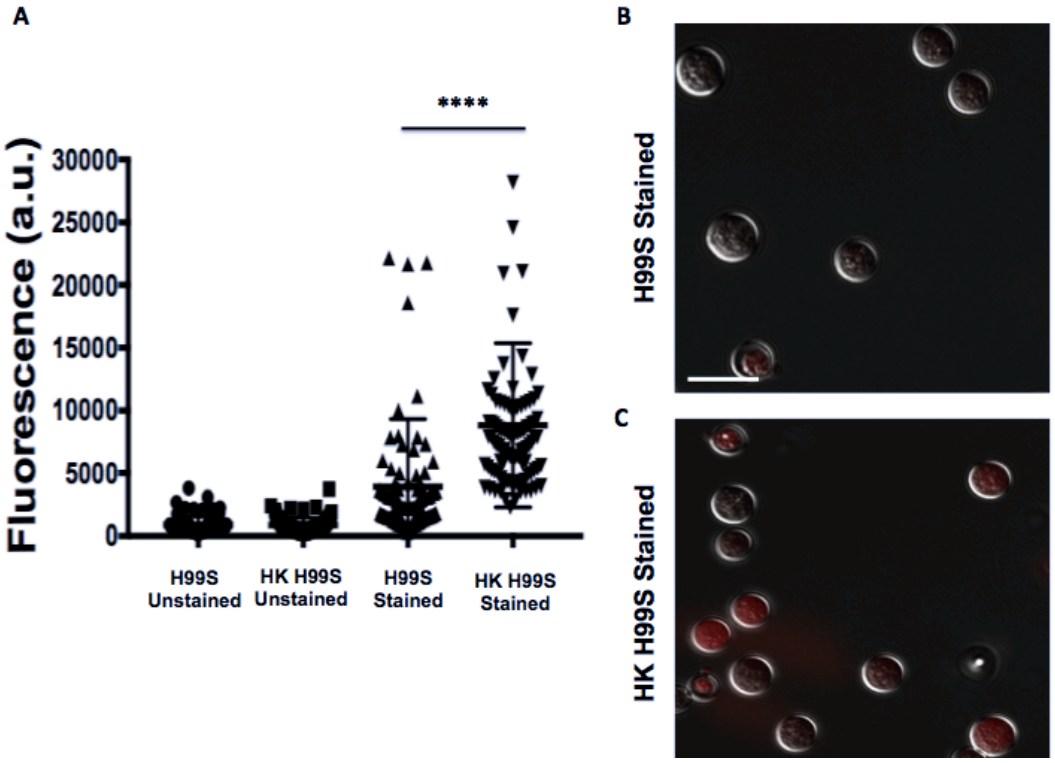


Figure 3.4: Estrogen Uptake is Dramatically Increased in Heat-Killed Cells.

(A) The results are of a representative experiment illustrating the fluorescence intensity as measured in arbitrary units (a.u.) of *C. neoformans* when incubated with EG in live and heat-killed cells. These experiments were performed as two discrete biological repeats. One hundred cells per condition were imaged. Data was analyzed using ANOVA with simple contrasts. Error bars are SE. Statistical significance is indicated as follows: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; and ****, $p < 0.0001$. **(B)** Live *C. neoformans* stained with 100x (0.04 µg/mL) EG imaged at 2.5 h incubation. **(C)** Heat-killed *C. neoformans* stained with 100x (0.04 µg/mL) EG and imaged at 2.5 h incubation. Scale bar indicates 10 µm.

3.2.3 Estrogen Uptake Changes with Strain and Cell Size

3.2.3.1 *C. neoformans* Uptake of Estrogen is Strain Dependent

To further characterize the ability of estrogen to permeate *C. neoformans* and what conditions influence its uptake, two mutant strains and wild type H99S were incubated with 100X EG and cell images were taken on a confocal microscope.

Hormonal uptake was measured and compared between the strains using ROI analysis. Compared to wild type H99S, the *HOG1Δ* mutant (CNAG_01523) showed significantly decreased uptake of estrogen ($p < 0.0001$). Conversely, the *NADPH dehydrogenaseΔ* mutant (CNAG_05444) showed significantly increased uptake of estrogen when compared to wild type H99S ($p < 0.0001$) (Figure 3.4).

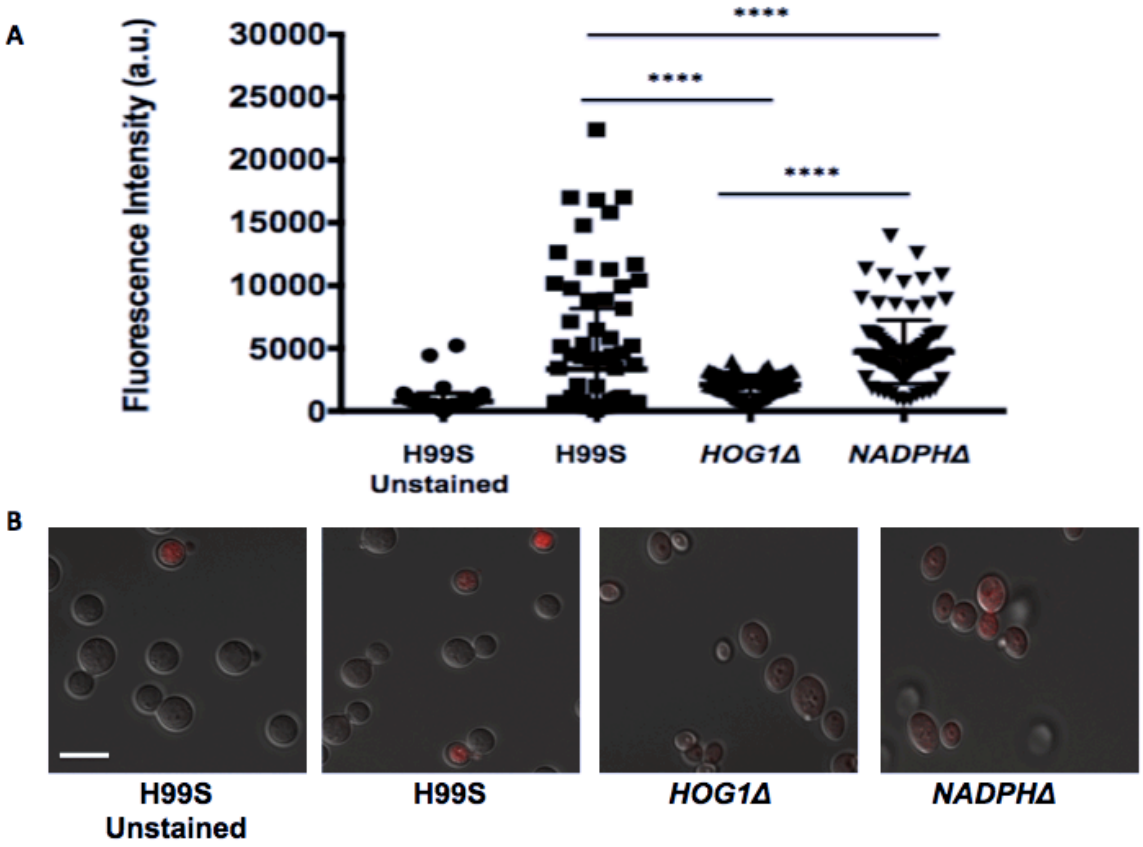


Figure 3.5 *C. neoformans* Uptake of Estrogen is Strain Dependent.

(A) The results of a representative experiment illustrating the fluorescence intensity as measured in arbitrary units (a.u.) of the wild type strain H99S and two mutant strains, *HOG1Δ* and *NADPHΔ.* These experiments were performed as three discrete biological repeats. One hundred cells per strain were imaged. Data was analyzed using ANOVA with simple contrasts. Error bars are SE. Statistical significance is indicated as follows: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; and ****, $p < 0.0001$. **(B)** Images of H99S unstained, H99S, *HOG1Δ* and *NADPHΔ,* respectively stained with 100X (0.04 µg/mL) EG imaged at 2.5 h incubation. Scale bar indicates 10 µm.

3.2.3.2 Differences in Estrogen Uptake by Strain are Positively Correlated by Cell Size

Given the results of both the capsule induction experiments and the differential levels of estrogen uptake in the three strains tested, it was important to understand if those differences were due to the surface area of the cells or the genetic make-up of the strain. To accomplish this, cells of all three strains were stained with India ink, imaged, and measured as described in 2.3.6. Results showed significantly different cell sizes between strains with *HOG1Δ* having the smallest mean cell size, H99S having an intermediate size, and *NADPHΔ* having the largest cell size ($p < 0.05$) (Figure 3.5). These results positively correlate with the amounts of hormone uptake seen in 3.2.3.1.

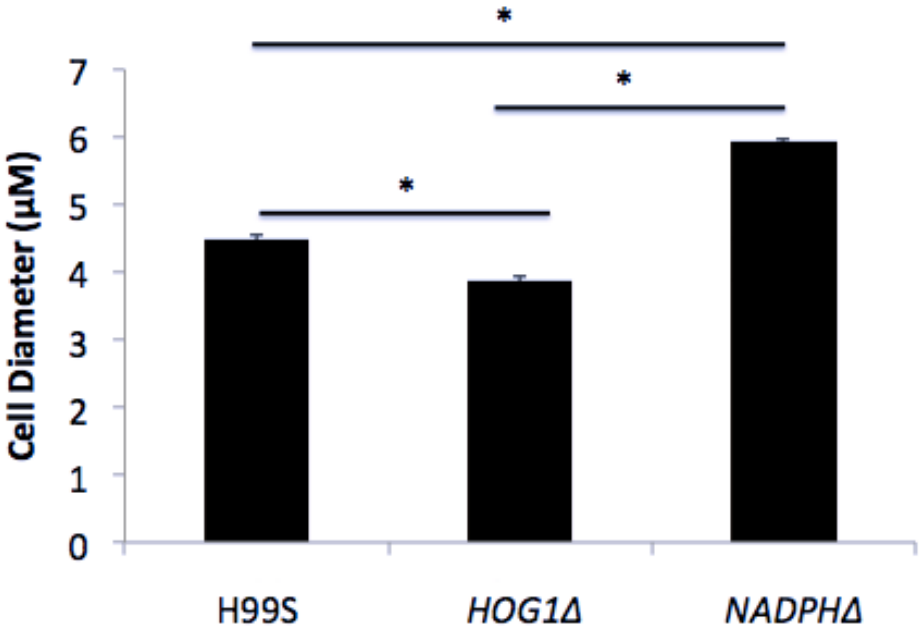


Figure 3.6: Differences in Estrogen Uptake by Strain are Positively Correlated with Cell Size.

The results of a representative experiment illustrating mean cell diameter as measured in µm of the wild type strain H99S and two mutant strains, *HOG1Δ* and *NADPHΔ*. These experiments were performed as three discrete biological repeats. One hundred cells per strain were imaged. Data was analyzed using ANOVA with simple contrasts. Error bars are SE. Statistical significance is indicated as follows: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; and ****, $p < 0.0001$.

3.3 Discussion

The primary aim of these experiments was to determine, definitively, whether estrogen has the ability to permeate the *C. neoformans* cell and whether this occurs at physiological concentrations. We are the first to show that a fluorescently tagged version of estradiol in the surrounding environment does penetrate the capsule and cell wall of *C. neoformans* and was taken up by the cell. Importantly, it does so at normal physiological levels seen in humans. Implications of this may be far-reaching. The sex bias in cryptococcosis infections is stark but not well understood. Establishing that the hormonal milieu of *C. neoformans* has the ability to enter the yeast provides an important initial understanding of the complex interplay of the hormonal host-pathogen interaction.

In addition to showing that estrogen in the environment penetrates *C. neoformans*, further experiments showed that the hormone levels entering the cell vary, depending on capsule and cell structure. Both inducing capsule growth and killing the cell by heat treatment allow for increased uptake of estrogen steroid. Hormones enter into mammalian cells freely via diffusion; however, we considered it possible that the capsule of *C. neoformans* would block estrogen from entering the cell. The capsule of *C. neoformans* is a dynamic, semi-permeable structure that protects the pathogen, however, results indicated that increasing the size of *C. neoforman's* "shield", the capsule, makes it more vulnerable to penetration of environmental estrogen, possibly due to increased capsule permeability post-induction (Jalsenjak and Kondo, 1981).

Furthermore, the images of induced *C. neoformans* reveal a concentrated ring of estrogen around the cell wall and cell membrane of *C. neoformans* rather than the more uniform distribution observed in cells that have not had the capsule induced. Given that ergosterol is located in the cell membrane, and the similar structures of ergosterol and estrogen, it is possible that the yeast is incorporating estrogen into the cell membrane in addition to, or instead of, ergosterol during the process of capsule (and possibly cell body) growth. Ergosterol inhibits growth of human breast cancer cells in the presence of 17β-estradiol (Subbiah and Abplanalp, 2003). Further, that inhibition is greater in the estrogen receptor (+) breast cancer cell MCF-7 compared to the estrogen receptor (-) breast cancer cell line MDA-231 (Subbiah and Abplanalp, 2003). This could be indicative of estrogenic activity in ergosterol and, therefore, ergosterol-like activity in estrogen. In the future, measuring ergosterol levels in induced verses uninduced *C. neoformans* in the presence of estrogen may shed light on the mechanism of action the cell is using to incorporate the hormone inside the cell, which is possibly affecting the virulence of *C. neoformans*. (Subbiah and Abplanalp, 2003)

Likewise, HK cells allowed in statistically significantly greater levels of estrogen compared to living cells. During the heat-killing process, proteins are denatured subsequently causing damage to the structure of *C. neoformans* (Mody and Syme, 1993). This damage occurs primarily to the cell membrane but also occurs in the capsule and cell wall (Leuenberger et al., 2017). The results of these experiments illustrate that hormone uptake is significantly affected by changes in capsule/cell body size, and cell membrane integrity. In the future, incubating EG stained *C. neoformans*

with increasing doses of lysozyme would allow for more precise measurements of estradiol uptake of cells with varying levels of cell wall damage.

Lastly, the differences in estrogen uptake compared to *NADPH dehydrogenaseΔ* and *Hog1Δ* were investigated. The *NADPH dehydrogenaseΔ* mutant was chosen because a NADPH dehydrogenase in *C. albicans* was found to serve as an EBP. Although the two proteins in *C. neoformans* and *C. albicans* do not share sequence homology, we hypothesized that if the NADPH dehydrogenase was acting as an EBP in *C. neoformans*, the *NADPH dehydrogenaseΔ* mutant would show little to no estrogen uptake. Interestingly, however, it showed the opposite: increased estrogen uptake compared to wild type H99S, which suggests that this protein does not serve as an EBP in *C. neoformans*.

HOG1 plays a vital role in the HOG pathway and stress response of *C. neoformans*. It is upstream of numerous genes that contribute to virulence in this yeast. Hog1 is activated during environmental stress. Assuming that estrogen in the surrounding environment is stressful to *C. neoformans*, the *HOG1Δ* mutant may lack the ability to regulate estrogen uptake and show increased estrogen uptake, however this strain took in significantly less of the hormone than wild type, suggesting that the presence of estrogen alone is not enough to activate the HOG pathway.

Since the levels of estrogen in our KO strains showed the opposite of what was expected when considering their mutations, the diameter of the cells was measured for each strain. Analysis revealed a positive correlation with strain cell body size and environmental estrogen uptake. While the surface area increases in larger cells, the

surface area-to-volume ratio decreases as cells grow in size. Simply stated, if the method of entry were solely diffusion, we would expect a negative correlation of strain size and estrogen uptake – smaller cells should yield higher concentrations of estrogen. Since this is not the case, we are forced to consider the possibility that while simple diffusion is very likely the primary method of entry; other differences in genetics between strains may also play a role in estrogen's ability to permeate *C. neoformans*. Future studies examining the relationship between size and EG uptake should be conducted using different sized cells within a single strain. This would allow for a more accurate comparison by ruling out potential compounding factors such as genetic differences between strains. Additionally, the shape of the cells could influence the size to volume ratio and should be considered when making comparisons between different strains.

In conclusion, this group of experiments reveals a great deal about *C. neoformans* and estrogen. We now know that estrogen permeates the cell at levels seen in healthy human females. Further, in cells with enlarged capsule, estrogen is able to enter into *C. neoformans* in higher concentrations and with a different pattern. Estrogen levels in HK cells are dramatically increased compared to living *C. neoformans*. Finally, estrogen uptake varies by strain, and that variation is positively correlated with cell size. Much of the research on sex bias in infections is focused on the host immune response and neglects to consider the environment that the pathogen encounters inside the host. This work is an important step to showing that the relationship between the host hormonal environment and *C. neoformans* is a dynamic rather than static one. The

next question is what effect do sex hormones in the environment have on the virulence of *C. neoformans in vitro?*

CHAPTER 4

THE EFFECTS OF SEX HORMONES ON THE VIRULENCE OF *C. NEOFORMANS*

4.1 Introduction

Differences in susceptibility to infections in males and females are not uncommon. However, research on the sexual dimorphism of infections is primarily focused on the different immune responses to these pathogens. Little is known about how the hormonal milieu affects pathogens directly. Experiments in the 1970's showed that incubation of estrogen and synthetic estrogen with *C. neoformans* inhibited growth (Mohr et al., 1972; Mohr et al., 1973). Work done by McClelland *et al* (2013) detailed differences in doubling times and GXM capsule shedding in clinical isolates derived from male and female cryptococcosis patients treated with exogenous testosterone or estrogen, with female isolates having increased doubling times and strains incubated with testosterone showing increased GXM shedding. Despite the significant difference in the number of men and women suffering from cryptococcosis, the above studies comprise the entirety of the published research on the effect of the hormonal milieu on *C. neoformans.* The effects of sex hormones on the virulence factors of *C. neoformans* may have a significant impact on disease severity and may partially explain the sex bias seen in these infections. The aim of these experiments was to begin to unravel the complexity of sex-specific, host-pathogen interactions by examining the effect of sex hormones on *C. neoformans* and its virulence factors. The hypothesis was that *C. neoformans* is capable of responding to mammalian sex hormones, particularly

testosterone, as a molecular signal to up-regulate the expression of virulence factors and that estrogen may down regulate the expression of virulence factors.

Few studies using other fungi have examined differences in virulence in the presence of sex hormones. In *C. albicans*, where the sex bias is the opposite of *C. neoformans* infections, with females suffering in disproportionate numbers compared to males, strains grown in the presence of 17β-estradiol exhibited both increased growth and enhanced survival at higher temperatures than did controls (Zhang et al., 2000). The researchers of this study determined that the heat shock protein (hsp)90 was up-regulated in the presence of estrogen. Additionally, kinetic studies revealed estrogen increased the amount of *Candida* multidrug resistance (*cdr1*) mRNA compared to control cells. Furthermore, growth of *C. albicans* in the presence of estrogen was not uniform among all strains, suggesting a quantitative, genetically-based variation of response to the hormone (Gujjar et al., 1997). This variation could also be due to differential uptake of estrogen as described in chapter three (Fig. 3.5).

In the case of *P. brasiliensis*, a pathogenic fungus similar to *C. neoformans* in its environmental ubiquity and sexual dimorphism in infection, estrogen has been shown to mitigate virulence. Estrogen was shown to inhibit the mycelium-to-yeast transformation, a step that must occur during the establishment of an infection *in vivo* (Shankar et al., 2011a). Analysis of microarray data found a correlation between estradiol exposure and differential expression of genes associated with cell wall remodeling, energy metabolism, and cell signaling during the mycelium-to-yeast transition (Camacho and Nino-Vega, 2017). This could begin to explain the ~30M:1F sex

bias seen in these infections, however exact mechanisms remain unclear. Additionally, and in contrast to *C. alibicans*, HSP genes are down-regulated in *P. brasiliensis* when incubated with estrogen or human female serum *in vitro* (Shankar et al., 2011b).

Previous researchers have reported changes in virulence in both *C. neoformans* and other pathogenic fungi in the presence of sex hormones, albeit the studies are limited in their scope. Given this, the goal of these experiments was to determine, in a comprehensive manner, how the hormonal environment affects the virulence factors of *C. neoformans*.

4.2 Results

To assess any changes in virulence factors in the presence of sex hormones, *C. neoformans* clinical isolates from males and females were cultured in rich medium then transferred to an environment designed to mimic the CNS and used to conduct a series of *in vitro* biochemical experiments that examined the virulence factors individually in the presence of sex hormones as described in 2.4. The results are listed below in Table 4.1. Where a difference was seen, the data is illustrated and described. Negative data is shown in the appendices.

Table 4 Virulence factors tested using *C. neoformans* strains isolated from males and females.

Virulence Factor	Method of Testing	Results
Capsule Size	Capsule Induction	Increased size in the presence of sex hormones
Melanin Production	L-Dopa Plating	Increased in the presence of testosterone
Urease Production	Urease Assay	No difference
Cell Wall Stress	SDS, CR, CFW, KCl, NaCl, CaCl2, $C_4H_6O_4$ Plating	No difference
Oxidative Stress	H_2O_2 Plating	Inhibited growth in female strains in the presence of estrogen
Nitrosative Stress	$NaNO_2$ Plating	No difference
Iron Stress	BPS Plating	No difference
In vitro growth	Fungal Burden Assays	No difference

4.2.1 Capsule Size Changes in the Presence of Sex Hormones

In times of nutrition deficiency, the capsule of *C. neoformans* grows in size to protect the yeast (Granger et al., 1985). Capsule size varies greatly between strains, and capsule size has been positively correlated with virulence (McClelland, 2007). Given this, capsule inductions were performed on all clinical strains in the presence of sex hormones to determine what, if any, differences occur. When exposed to sex hormones, isolates produced capsule that was significantly larger in diameter than the

negative control (ethanol) but with no significant difference between capsule sizes for the two hormone groups ($p < 0.001$, $n = 1$) (Figure 4.1)

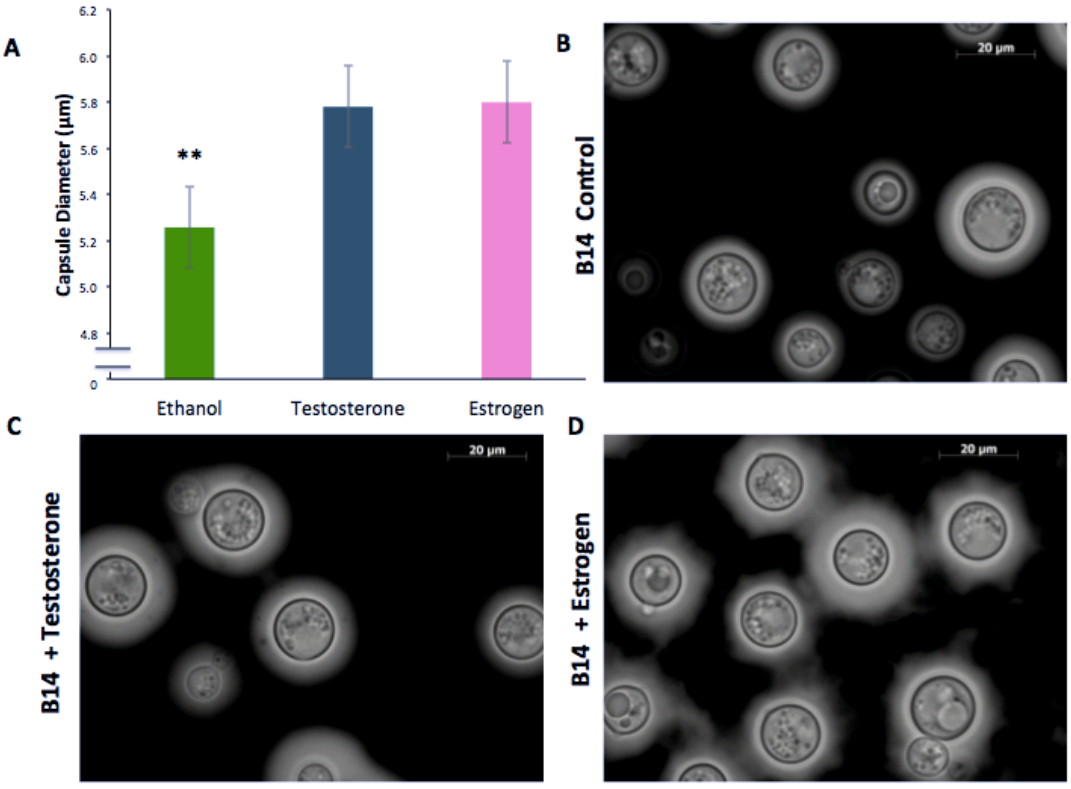


Figure 4.1 Induced Capsule Diameter is Increased in the Presence of Sex Hormones. Mean capsule diameter (µM) of all 28 clinical *C. neoformans* isolates in the presence of ethanol (0.001%) (negative control), testosterone (10 ng/ml), or estrogen (400 pg/ml). Fifty cells of each condition from each strain were imaged and analyzed measured manually using Axiovision software. This experiment was done once. Data was analyzed using ANOVA with simple contrasts. Error bars are SE. Statistical significance is indicated as follows: *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$. **(B)** Images of strain B14 incubated in ethanol (0.001%)(negative control), **(C)** testosterone (10 ng/ml), and **(D)** estrogen (400 pg/ml) post-capsule induction.

4.2.2 Melanin Production Increases in the Presence of Testosterone.

Considered another primary virulence factor, melanin is a dark pigment that is formed by oxidizing L-DOPA and protects *C. neoformans* from a variety of host and environmental assaults (Jacobson and Tinnell, 1993). Without melanin, *C. neoformans* is unable to disseminate outside of the lungs (Nosanchuk et al., 2015). Further, melanin helps the pathogen evade killing by acting as an antioxidant for *C. neoformans* as well as inhibiting opsonization by specific antibodies thereby helping to evade phagocytosis (Nosanchuk et al., 2015). Understanding the extent to which changes occur in this virulence factor in a male vs. female environment is valuable in helping us understand the larger effect of sex hormones during a *C. neoformans* infection. Results showed a significant increase in melanin production when strains were exposed to testosterone at 3, 5, and 7 days ($p < 0.05$) (Figure 4.2).

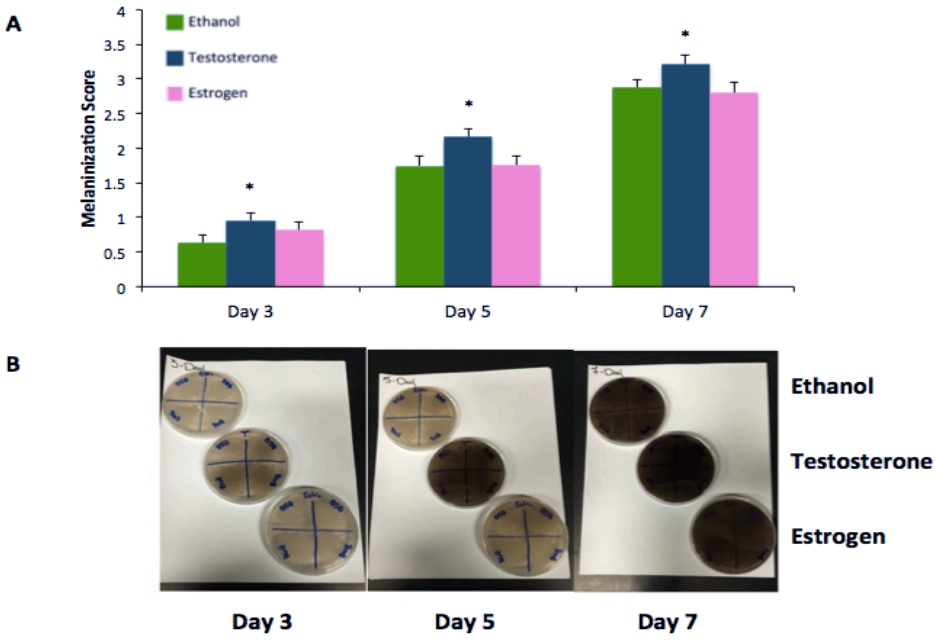


Figure 4.2 Melaninization is Increased in the Presence of Testosterone.

(A) Mean qualitative melanization scores of all 28 clinical *C. neoformans* isolates in the presence of ethanol (negative control), testosterone, or estrogen. Cells were plated on L-Dopa plates containing corresponding hormones and incubated in the dark at 37 ^{o}C. Plates were imaged at days 3, 5, and 7. These experiments were performed as three discrete biological repeats. Data was analyzed using ANOVA with simple contrasts. Error bars are SE. Statistical significance is indicated as follows: *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$. **(B)** Images of strains B52, B58, B62, and B68 cultured in medium containing ethanol (0.001%) (negative control), testosterone (10 ng/ml), or estrogen (400 pg/ml), and grown on L-Dopa plates containing corresponding hormones at days 3, 5, and 7.

4.2.3 Estrogen inhibits growth of *C. neoformans* strains isolated from females in the presence of oxidative stress.

During the first stages of infection inside the host, *C. neoformans* is phagocytosed by aveolar macrophages in an attempt to control the pathogen and avoid dissemination outside of the lungs (Yasuoka et al., 1994). These macrophages produce respiratory bursts of hydrogen peroxide as a means of weakening and killing *C. neoformans* (Brown et al., 2007). Resistance to this oxidative stress is necessary for successful colonization in mammalian hosts. In order to test whether the presence of sex hormones would alter the ability of *C. neoformans* to overcome this stressor *in vitro*, clinical isolates were cultured in the presence of sex hormones and plated on YPD plates containing H_2O_2. Growth was significantly inhibited in female-derived (but not male) strains at days 3 and 5 *in vitro* ($p < 0.05$, $n = 3$) (Figure 4.3).

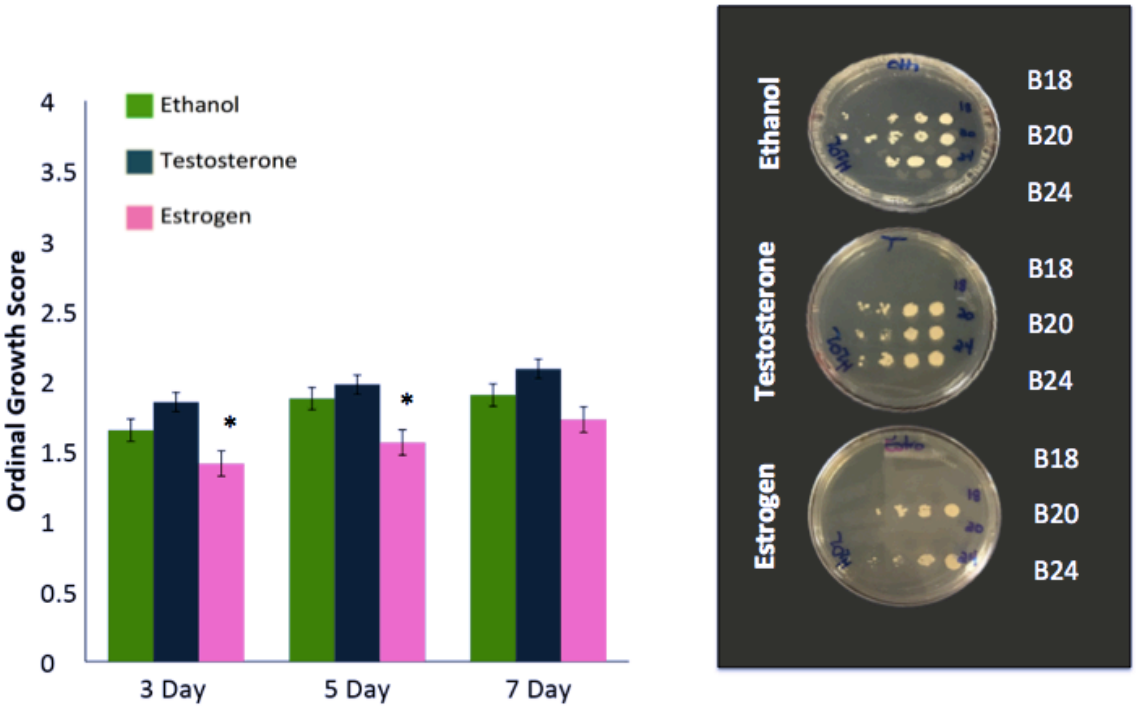


Figure 4.3 Growth of Female-Derived Isolates is Inhibited in the Presence of Estrogen and Hydrogen Peroxide.

(A) Mean ordinal growth scores of all 16 female-derived clinical *C. neoformans* isolates in the presence of ethanol (negative control), testosterone, or estrogen. Cells were plated on YPD plates containing corresponding hormones and H_2O_2 and incubated at 37 ^{o}C. Plates were imaged at days 3, 5, and 7. These experiments were done three times. Data was analyzed using ANOVA with simple contrasts. Error bars are SE. Statistical significance is indicated as follows: *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$. **(B)** Images of strains B18, B20, and B24 cultured in medium containing ethanol, estrogen, and grown on YPD plates containing corresponding hormones and H_2O_2 at day 3.

4.3 Discussion

The results of this screen indicate significant changes in some virulence factors in the presence of exogenous sex hormones in strains isolated from both males and females. However, several virulence factors showed no difference when exposed to testosterone or estrogen. Urease experiments were conducted on all isolates and showed no significant difference between treatment groups or between strains isolated from males and females (data not shown). As mentioned previously, urease aids *C. neoformans* in crossing the blood-brain barrier, which occurs several days post-infection (Olszewski et al., 2004). It is possible, but not experimentally tested, that the sexual dimorphism observed in *C. neoformans* infections is due largely to the hormonal environment during the acute stage of the disease. Further investigation is needed, but if accurate, offers some explanation as to why there is no change in urease activity between treatment groups.

Additionally, no significant difference in *C. neoformans* growth was observed in response to cell wall, osmotic, nitrosative, or iron cell stressors between treatment groups, and, surprisingly, there was no difference in *in vitro* fungal burden testing (data not shown). Given previous research documenting that greater numbers of *C. neoformans* are phagocytosed and killed by female-derived macrophages, we expected to see similar results in murine macrophages treated with estrogen (McClelland et al., 2013). It should be noted, however, that unlike the rest of the virulence factor screen, fungal burden experiments were conducted using the wild type lab strain, H99S. Clinical strains were isolated from male and female humans, where they were exposed to their

host's hormonal environment and could have adapted to that environment unlike the lab strain that has been passaged numerous times in medium not containing hormones.

Exposure to sex hormones led to increased capsule diameter compared to the negative control, which suggests that *C. neoformans* responds to its hormonal milieu and, in turn, produces more capsule, which is protective. Given that the difference observed was between treatment groups and the negative control and not between testosterone and estrogen exposure, this set of experiments was only conducted once, and therefore these results should be interpreted with caution.

The increase of melanin production in the presence of testosterone is of particular interest given that while *C. neoformans* researchers have shown various inhibitory effects of estrogen (McClelland et al., 2013; Mohr et al., 1972; Mohr et al., 1974), an increase in overall virulence or virulence factors when strains are exposed to testosterone has not been shown. Melanin production is vital for a successful infection aiding the pathogen in both dissemination to the CNS and evasion of the host immune response. These results are the first to show that exposure to a male hormonal environment leads to an increase in one of the primary virulence factors, which may explain, at least partially, the greater symptom severity and higher likelihood of mortality of males suffering from cryptococcosis.

In addition to the differences observed in the primary *C. neoformans* virulence factors when in different hormonal environments, strains isolated from females exposed to exogenous, physiological levels of estrogen showed inhibited growth when also exposed to increased levels of H_2O_2, an oxidative stressor and a condition found

inside host macrophage phagolysosomes. This is important given the role of macrophages as a primary, first-line of defense during a *C. neoformans* infection and suggests that when inside a female host, *C. neoformans* may be at a greater disadvantage to establish a successful infection.

The data from the virulence factor and cell stressor testing indicate that *C. neoformans* strains behave differently in the presence of male and female sex hormones. Not only might these results aid researchers in understanding the sex gap seen in lethal *C. neoformans* infections, but they also shed a bright light on the often-ignored effects that the host hormonal environment directly has on *C. neoformans*.

CHAPTER 5

An Inherent Immune Deficit in Healthy Males to *C. neoformans* Infection May Begin to Explain the Sex Susceptibility in Incidence of Cryptococcosis

5.1 Introduction

Another variable in cryptococcosis is the host immune response, which can vary widely between individuals. Protective host responses and increased survival occur when host macrophages are classically (M1) rather than alternatively (M2) activated, which leads to robust cell-mediated immunity (CMI) (Rohatgi and Pirofski, 2015; Voelz and May, 2010). This, in turn, causes strong antibody-mediated immunity (AMI), as indicated by the activation of B and T cells. The B cell response has been linked to both resistance to cryptococcosis and control of pulmonary inflammation in mice infected with *C. neoformans* (Aguirre and Johnson, 1997; Rivera et al., 2005). Historically, $CD4^+$ T cells have been shown to mediate fungal clearance and offer protection to the host, but recent studies describe a more complex picture implicating these T cells in advanced disease severity and higher mortality rates in both mice and HIV^+/$cryptococcosis^+$ patients (Neal et al., 2017; Panackal et al., 2015). Th1 immunity is another necessary, pro-inflammatory response, which is characterized by the secretion of interleukin (IL)-2, IL-6, IL-12, interferon-gamma (IFN-ϒ), and tumor necrosis factor-alpha (TNF-α), rather than an anti-inflammatory Th2 cytokine response of IL-4, IL-5, IL-10, and IL-13 secretion (Eschke et al., 2015; Li et al., 2015b; Mora et al., 2017; Mora et al., 2015; Schop, 2007). $CD8^+$ T cells mediate killing of *C. neoformans* and limit the pathogen growth and survival inside macrophages by producing IFN-γ, independent of $CD4^+$ T cells (Lindell et al., 2005;

Rohatgi and Pirofski, 2015; Schop, 2007). Due to the combined nature of *C. neoformans* as a potent immune-modulator and its ability to cause illness in the immuno-compromised population, robust immune responses are not commonly seen (Vecchiarelli et al., 2011).

In an effort to better understand the early normal immune response of both males and females to a *C. neoformans* infection, this study utilized peripheral blood mononuclear cells (PBMCs) from healthy donors to evaluate the relationship of host sex with the immune response to a *C. neoformans* infection, providing insight into how it may affect outcome. B ($CD19^+$) and T ($CD4^+/CD3^+$, and $CD8^+/CD3^+$) cell proliferation were measured in both uninfected and infected PBMCs. Subsets of both cell populations from all donors were incubated with testosterone or 17β-estradiol to determine if steroid hormones acutely affected the outcome. When infected with *C. neoformans*, T cell percentages in both sexes and B cell percentages in males decreased significantly compared to uninfected cell populations. Also, males had lower T cell percentages than females during infection. Cells incubated with exogenous sex hormones showed no difference in immune responses during our analysis. This study is the first to investigate the adaptive immune response of healthy individuals to *C. neoformans*, and the results show that males have an inherent deficit in their immune response to *C. neoformans.* These findings are an important first step in explaining the sex-based incidence of disease and mortality from cryptococcosis.

5.2 Results

5.2.1 T Cell Response

The aim of this study was to determine whether features of the adaptive immune response to *C. neoformans* differ between healthy males and females, given that males have a higher incidence of both disease and death from cryptococcosis (Bisson et al., 2008; Jimenez-Mejias et al., 1991; Manfredi et al., 1995; McClelland et al., 2013; Millogo et al., 2004; Zheng et al., 2016). First, PBMCs from healthy donors were isolated and incubated for seven days with or without *C. neoformans* (1:100 MOI). Subsets of both of these groups were also treated with physiologically relevant levels of testosterone or estrogen. Multi-color flow cytometry was utilized to determine percentages of B and T cell populations as well as the percentage of dead cells at 7 days of culture. In the uninfected control group, males had a lower percentage of cells expressing CD3, a surface marker expressed on all T cells, compared to females (61% vs. 68%, $p < 0.001$) (Fig. 5.1). Amounts of $CD4^+$ helper T cell percentages and $CD8^+$ cytotoxic T cell percentages were approximately the same in control cultures from males and females. PBMCs infected with *C. neoformans* showed significantly decreased percentages of all three measured T cell markers in both males (CD3: 61% vs. 34%, CD4: 42% vs. 22%, CD8: 22% vs. 11%, $p < 0.0001$ for CD3 and CD4, $p= 0.0004$ for CD8) and females (CD3: 68% vs. 47%, CD4: 45% vs. 30%, and CD8: 25% vs. 18%, $p < 0.0001$ for CD3 and CD4, $p = 0.0167$ for CD8) compared to the uninfected group. However, males had strikingly lower percentages of T cells when compared to females in the infected group (CD3: 33% vs. 47%, CD4: 22% vs. 30%, CD8: 11% vs. 18%, $p < 0.0001$ for all markers). The

difference observed in percentages of $CD3^+$ cells between males and females in uninfected cells grew significantly during infection (34% vs. 47%, $p < 0.001$) with males having much fewer cells than females. Additionally, males had significantly less $CD4^+$ and $CD8^+$ cell percentages than females in the infected group (22% vs. 30% and 11% vs. 18%, $p < 0.0001$ for both markers).

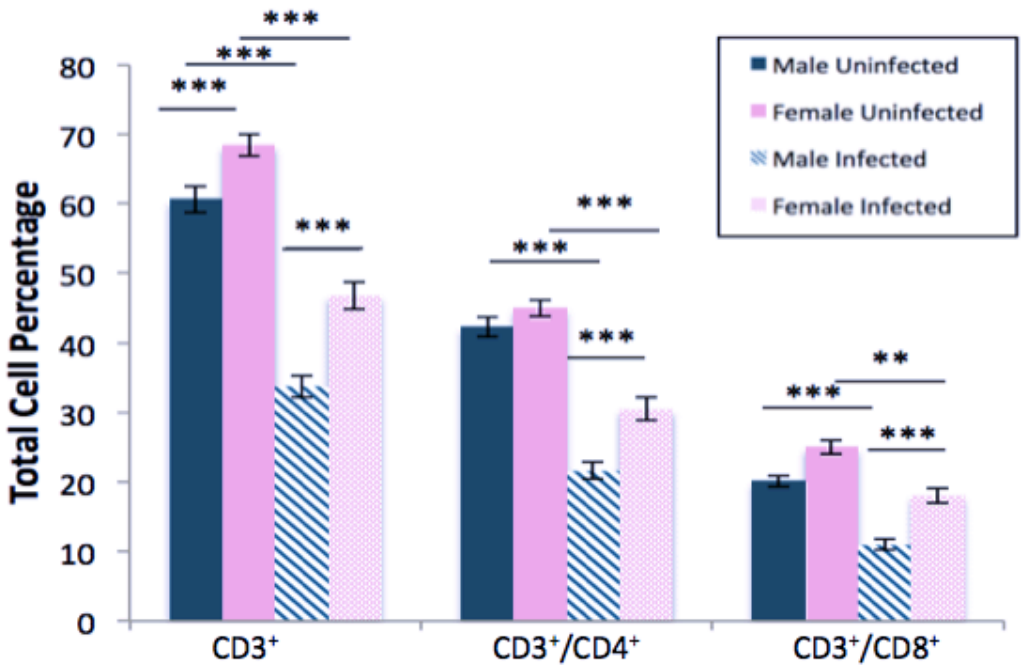


Figure 5.1: T Cell Response of Males and Females in Uninfected and Infected Groups. Mean cell percentages for T cell markers ($CD3^+$/APC, $CD3^+/CD4^+$ APC/PE, $CD3^+/CD8^+$ APC/FITC) in male and female groups of both cells only (uninfected) and in the presence of *C. neoformans* (infected) based on flow cytometric analysis of PBMCs in suspension. The total percentage given for each marker represents the total percentage of cells expressing the antigen indicated. N=40 (20 males & 20 females). This experiment was done in triplicate. Data was analyzed using MANOVA with simple contrasts. Error bars are SE. Statistical significance is indicated as follows: *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$.

5.2.2. B Cell Response

While T cells represent one arm of the adaptive immune response, B cells represent the other. To investigate whether B cell percentages were different between males and females in uninfected and *C. neoformans* infected cultures, flow cytometry was again utilized with a broad B cell marker, CD19. Males and females did not differ in B cell percentage in the uninfected control group. There was no change in B cell percentage in females when infected with *C. neoformans*, but the cell percentage did decrease significantly during infection in males during infection (17% vs. 10%, $p = 0.0034$; Fig. 5.2). There was no difference in dead cells, as measured by 7-Aminoactinomycin D (7AAD) staining for male, female, uninfected, or infected groups (Fig. 5.2). There was also no difference in cell percentages treated with testosterone or estrogen and cells without hormones (data not shown).

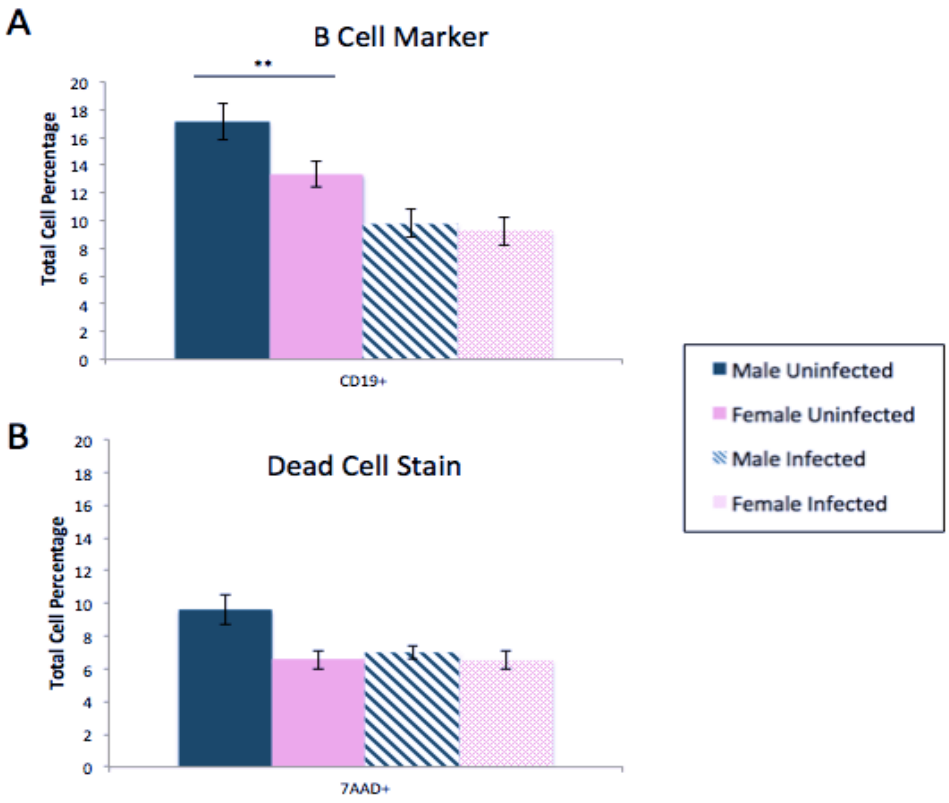


Figure 5.2: B Cell Response and Dead Cells of Males and Females in Uninfected and Infected Groups.

Mean cell percentages for **(A)** B cell markers ($CD19^{+}$/PC7), and (**B)** dead cell marker (7AAD) in male and female groups of both cells only (uninfected) and in the presence of *C. neoformans* (infected) based on flow cytometric analysis of PBMCs in suspension. The total percentage given for each marker represents the total percentage of cells expressing the antigen indicated. N=40 (20 males & 20 females). This experiment was done in triplicate. Data was analyzed using MANOVA with simple contrasts. Error bars are SE. Statistical significance is indicated as follows: *, $p < 0.05$; **, $p < 0.01$; and

***, $p < 0.001$. Representative dot plots for a male and female donor are illustrated in figures 5.3 and 5.4, respectively.

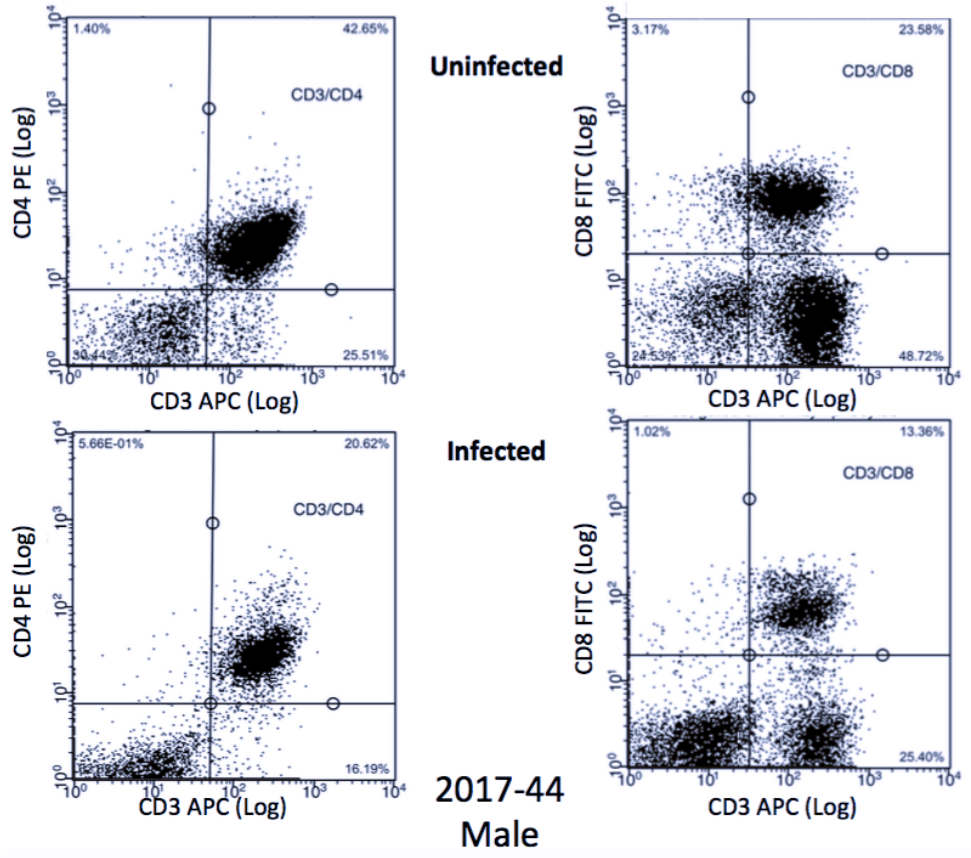


Figure 5.3: Male T Cell Dot Plots.

Dot plots from a representative male donor (2017-44) of CD3+/CD4+ and CD3+/CD8+ T cells in both uninfected and infected treatment groups.

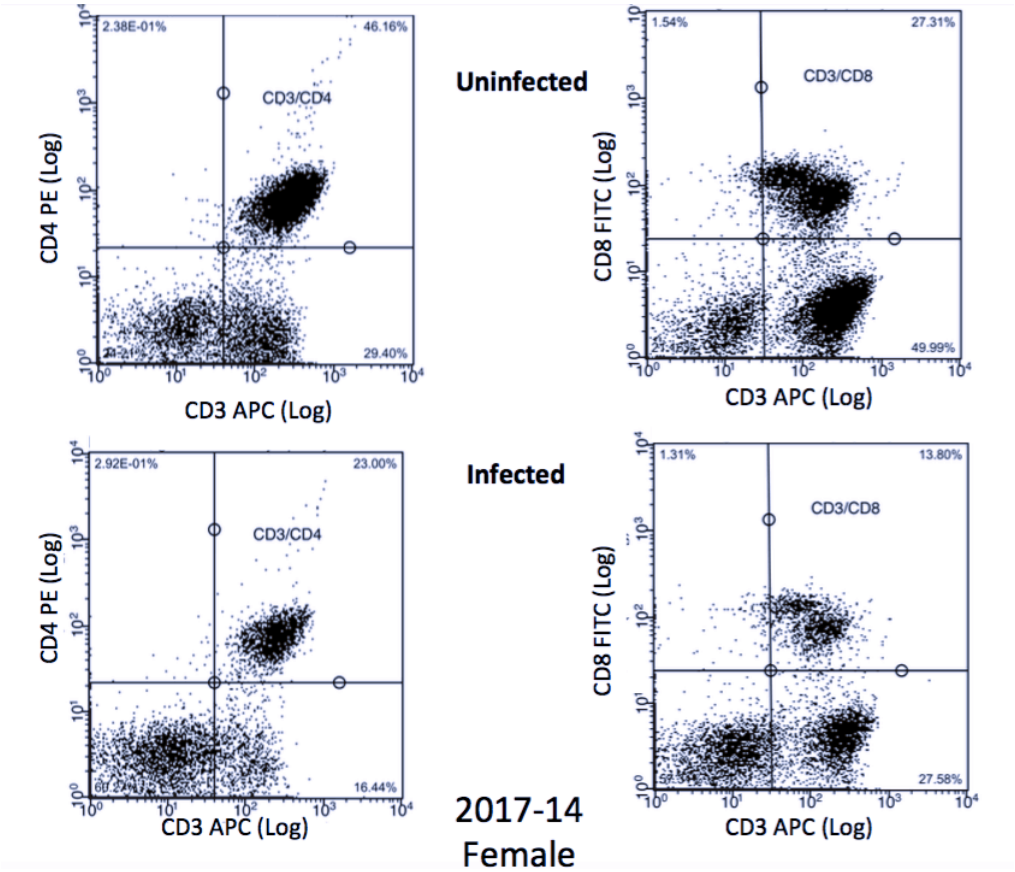


Figure 5.4: Female T Cell Dot Plots.

Dot plots from a representative female donor (2017-14) of CD3+/CD4+ and CD3+/CD8+ T cells in both uninfected and infected treatment groups

5.3 Discussion

This is the first study to investigate the adaptive immune responses of healthy male and female PBMCs when exposed to *C. neoformans* in the absence or presence of testosterone or estrogen. Our goal was to begin to understand the differences in the host immune response between males and females to determine if those differences may explain the observed differences in cryptococccosis between the sexes (7M:3F). To do this, the B and T cell percentages of infected and uninfected PBMCs from males and females were compared. Subsets of both were treated with exogenous sex hormones.

Our cohort of subjects is unique, in that, they are healthy and not typical candidates for cryptococcal disease. Given the nature of cryptococcosis, it is accepted that patients suffering from the disease have multiple comorbidities, or at the very least, a compromised immune system making it difficult for researchers to discern how *C. neoformans* acts on the host devoid of other variables. Keeping this in mind, these experiments were designed with the aim of understanding the differences that may be masked or accentuated when studying typical *C. neoformans* patients. Reference ranges for healthy adults in the U.S. show that $CD3^+$ and $CD4^+$ counts are typically higher in females (Reichert et al., 1991; Valiathan et al., 2014). Uninfected PBMCs served as the negative control in this study and reflected those differences in the $CD3^+$ population. While $CD4^+$ cell percentages were also higher in females in our control group, the differences were not statistically significant nor were any other markers in the control group. During infection, T cell percentages in both males and females dropped significantly. This could be a result of *C. neoformans* known immunomodulatory effects

depressing the immune response, *C. neoformans* cells contaminating the analysis, or, most likely, a combination of both (Casadevall and Pirofski, 2005; Ellerbroek et al., 2004; Mednick et al., 2005; Vecchiarelli et al., 2011). However, percentages of all measured T cell markers were consistently and significantly lower in males than females in infected groups. The gap in $CD3^+$ cell percentages that was observed between uninfected male and female PBMCs grew significantly during infection, suggesting a weakened T cell response in males in the presence of *C. neoformans*.

During infection, B cell (CD19-expressing) percentages remained the same in females (compared to uninfected cells) but dropped significantly in males. This, like the decline in T cell percentages, could be indicative of a less robust immune response in males during a *C. neoformans* infection. The role B cells play in a *C. neoformans* infection have not been as well elucidated as those of T cells, but multiple studies report the necessity of B cells, often serving as a first line of defense during *C. neoformans* infections and a correlation between increased levels of B cells (IgM^+) and a decreased likelihood of immunocompromised patients developing cryptococcosis (Rivera et al., 2005; Rohatgi and Pirofski, 2012; Subramaniam et al., 2009). It should be noted, however, that most of these studies have taken place with HIV^+ subjects that already have T cell deficiencies and therefore may rely more heavily on an effective B cell response.

A robust cytotoxic T cell ($CD8^+$) response is needed to successfully mediate the immune response to a cryptococcal infection and has been shown to directly inhibit the growth of *C. neoformans* (Huffnagle et al., 1991; Levitz et al., 1994). The helper T cell

($CD4^+$) response is more complex. Despite their ability to decrease fungal burden in the host, $CD4^+$ cells have recently been linked to advanced clinical symptoms and mortality (Neal et al., 2017; Panackal et al., 2015). One such study examined HIV^+/cryptococcal $meningitis^+$ patients and found that despite having higher $CD4^+$ T cell counts; males had higher risk of death (McClelland et al., 2013). In HIV^+ patients, T cell depletion is the primary predisposing cause for development of cryptococcal meningitis (Jarvis et al., 2013). Given this, our results suggest an inherit deficit in the male immune response when confronted with *C. neoformans.* This may begin to explain why males experience higher rates of disease and death from cryptococcosis.

Cell percentages of $CD3^+$, $CD4^+$ and $CD8^+$ T cell markers in males and females and the $CD19^+$ B cell marker in males decreased during infection, even though the same numbers of events were collected. The question that naturally follows is, "Did any cell percentages increase and if so, what were they?" Due to limitations with our flow cytometer, we were limited on what we could measure. Our design utilized cell percentages rather than total cell numbers. The flow analysis was done using a gating strategy to isolate lymphocytes excluding the vast majority of *C. neoformans* (Fig 2.3), however, we recognize now that the amount *C. neoformans* in each sample could vary considerably. Given this, we acknowledge the possibility that the differences seen in immune cell percentages between males and females could be a less robust response in male immune cells as we first concluded, or, just as interestingly, increased proliferation of *C. neoformans* in male verses female samples during the week-long incubation period.

That said, given the results of the cytokine analysis (described below), specifically increased levels of IFN-γ and IL-2 during infection, it is feasible that natural killer (NK) cells make up some of the "other" category (Kawakami et al., 2001; Rohatgi and Pirofski, 2015). There were likely some monocytes in this population as well. Monocytes regularly overlap with lymphocyte gates and show up as background, especially since Ficoll separation was used for PBMC isolation (Thompson et al., 1985). Finally, despite our best efforts, it was impossible to gate out all of the yeast. On average, about 3% of the total cell population within the lymphocyte gate is *C. neoformans*.

To further characterize the immune response to *C. neoformans* in healthy cell populations, another graduate researcher in a collaborating lab, Natalia Castro-Lopez, conducted cytokine analysis on infected cell supernatants from each donor in this study. Interestingly, uninfected cell supernatants from males showed lower levels of both the Th1 cytokines IL-2, IFN-γ, TNF-α and the Th2 cytokines IL-4, and IL-6 compared to uninfected female supernatants. During infection, however, the differences in cytokine expression between males and females subsided. This differs from a previous mouse study *in vivo* that described higher levels of IFN-γ, and TNF-α in female outbred mice with a cryptococcal infection (Lortholary et al., 2002). There were increases in IL-2, IL-4, GM-CSF, IFN-γ, and TNF-α in infected supernatants as a whole compared to uninfected samples, which is indicative of a healthy response consisting of mostly Th-1 cytokines (Mora et al., 2017; Scriven et al., 2016; Van Dyke et al., 2017). There was no significant increase of IL-10, a cytokine linked, at higher levels, with low survival rates (Mora et al.,

2015; Scriven et al., 2016). Initially called T cell growth factor (TCGF), IL-2 is synthesized by activated T cells (Zhang et al., 2018). Given this, it is perplexing that during infection, IL-2 levels were the same between sexes yet males had lower percentages of all T cell markers measured. It is possible that male cell populations included higher numbers of NK cells, which are activated in the presence of IL-2 and have limited anti-cryptococcal properties (Lehmann et al., 2001; Levitz et al., 1994). The lower levels of cytokine expression in uninfected male supernatants, but the ability to overcome those deficits during infection paints a complex picture and does not point to a definitive cytokine profile between the sexes. This is a healthy cohort, however, and may be more suggestive of an ideal immune response to *C. neoformans* than anything else.

Prior to the HIV epidemic, it was postulated that males had a higher incidence of cryptococcosis because of higher exposure rates, particularly in rural areas (Hajjeh et al., 1999; Mohr et al., 1972). More recent studies show that not to be the case with similar exposure rates for males and females, suggesting biological reasons for the sex bias in *C. neoformans* infections (Abadi and Pirofski, 1999; Davis et al., 2007). To gain a deeper insight into the donor population for this study, an undergraduate researcher, Joseph Rosen, used donor sera to determine serological status. Of the 40 subjects in this trial, 39 (97.5%) demonstrated immune reactivity to cryptococcal antigens indicating previous exposure, 20 males and 19 females. This study was performed in Murfreesboro, TN, a city with approximately 130,000 residents (Census, 2016). PBMCs from the single serological negative donor showed higher levels of CD3+/CD4+ T cell percentages compared to PBMCs from serologically positive donors, possibly due to the immune-

suppressive effects of a previous *C. neoformans* exposure. However, since there was only one negative donor, these data should be interpreted with caution.

Many factors are thought to influence the differences between male and female immune responses. Environmental stimuli, microRNAs, and X-linked immunoregulatory genes are all evidenced to contribute to what is commonly considered the female immune advantage (Angele et al., 2014; Candore et al., 2006; Imahara et al., 2005). The majority of research in sexual dimorphism between males and females during an immune challenge, however, is focused on sex hormones (Angele et al., 2014; Colton et al., 2005; Giefing-Kroll et al., 2015; Guess and McClelland, 2017; Kim-Fine et al., 2012; Roved et al., 2017). Testosterone has been shown to have anti-inflammatory properties whereas estrogen exhibits pro-inflammatory properties, often a much needed response during an infection (Colton et al., 2005; Giefing-Kroll et al., 2015; Roved et al., 2017). Therefore, when designing this study, we hypothesized that overlaying exogenous levels of sex hormones onto PBMCs would exaggerate the immune response (i.e. added levels of estrogen would increase B and T cell proliferation, and added levels of testosterone would show a corresponding decrease). There was, however, no change in B and T cell percentages in the presence of added sex hormones in both the infected and uninfected groups. This is suggestive of a few possibilities: 1. The differences we see in the male and female immune response are due to something other than sex hormones. The strain used for these experiments, H99S, may need much longer than the 7-day incubation to “adapt” to a changed hormone environment. 3. The immune response in males and females is static enough that it requires more than the addition of normal

physiological levels of hormones to overcome its typical response. We would posit the latter two possibilities are the most likely. Previous research suggests variation in cryptococcal strains can influence the human immune response and mortality outcome (Wiesner et al., 2012). Isolated after passaging a mixture of frozen stock through a rabbit model, H99S was derived from the *C. neoformans* var. *grubbii* type strain H99 that was originally isolated in 1978 from a male patient with Hodgkin's disease and kept on plates at 4^{o}C until 1994 when it was frozen (Janbon et al., 2014). Thus, it is reasonable to expect a clinical strain that has been in the host's hormonal environment and with a different genotype may be more susceptible to hormone changes than a common laboratory strain that has likely lost any hormonal adaption. Further, the range of sex hormone levels considered normal is vast. For healthy males, age 20-29, testosterone levels vary between 0.8 -11 ng/mL, and in females, estradiol falls between 20-430 pg/mL with the levels fluctuating widely throughout the menstrual cycle (2015; Sofronescu, 2015). For the purposes of this study, we chose normal/high-range hormone concentrations to overlay onto PBMCs. Yet, given the scope of what is considered normal, it is not altogether surprising that these levels did not alter the immune response in systems that have adapted over millions of years to weather such variations.

The immune response of healthy individuals reveals differences that may otherwise be overlooked or attributed to other factors and are suggestive of a biological immune deficit present in males when infected with *C. neoformans*. On its face, male-derived PBMCs exhibited a less robust immune response during a *C. neoformans*

infection compared to female-derived PBMCs. All percentages of measured T cell markers were significantly lower, and B cell percentages declined in males during infection. That said, failing to label either *C. neoformans* or mammalian cells with a fluorescent antibody, thereby not being able to completely exclude *C. neoformans* from total cell numbers cast a shadow on these results, and they should be interpreted with caution. To remedy this, we anticipate isolating PBMCs from additional healthy donors, labeling *C. neoformans* during flow analysis, and assuming proliferation of the yeast is consistent, extrapolate that data to confirm the results outlined in this chapter.

At present it is not clear what, if any, direct roles testosterone and estrogen play in the immune response to *C. neoformans.* In total, these results indicate that the difference in outcomes of cryptococcosis between men and women may well be biologic and can be at least partially explained by the adaptive immune response, and cannot simply bedue to differences in exposure rates. These experiments are an important first step in elucidating the biological sex differences seen in response to a *C. neoformans* infection and hopefully lay the groundwork for future research that uncovers the mechanisms behind these differences.

CHAPTER SIX

SEX MATTERS: CONCLUSION AND FINAL THOUGHTS

6.1 Outcomes and Discussion

Causing the death of nearly 200,000 people annually, *C. neoformans* is a lethal, yet often overlooked disease (Rajasingham et al., 2017). Even more overlooked and understudied, despite decades of evidence, is the sex bias in cryptococcosis. Males are at a distinct disadvantage, however, little time and research effort has been dedicated to this area of study. This body of work was designed to begin to understand the biologic reasons for the sex bias in *C. neoformans* infections. Taking a comprehensive approach by examining both the pathogen and host-pathogen interface, we have learned a great deal about the relationship of sex and *C. neoformans*.

We were the first to show that estrogen permeates *C. neoformans*, very likely by diffusion, and that mammalian sex hormones have an effect on several of the pathogen's virulence factors. The implications of these experiments have the potential to reframe the discussion of sex bias in cryptococcosis infections. Understanding that the hormonal milieu that the pathogen is exposed to plays a role in the expression of virulence factors is a novel concept. To date, no one had examined the effects of testosterone and estrogen on *C. neoformans* virulence. While testosterone has been linked to increased melanin production in several species at the organismal level, the correlation has never been made connecting testosterone in the pathogen's surrounding environment to increased melaninization of the pathogen as used for virulence (Beziers et al., 2017; Bischitz and Snell, 1959; Fargallo et al., 2007), which we

detail in chapter four. Further, our experiments showing that the combination of estrogen and hydrogen peroxide, a condition found inside the phagosomes of macrophages, inhibits the growth of female-derived strains of *C. neoformans,* confirming the findings reported in McClelland et al. (2013) that describe enhanced killing of the yeast by female-derived macrophages. To be sure, there remain many unanswered questions, namely the mechanism by which sex hormones are acting on *C. neoformans* to cause enhanced virulence, in the case of testosterone and inhibited growth, in the case of estrogen. However, I feel our work demonstrating estrogen's ability to permeate *C. neoformans* taken together with the outcome of the virulence factor screen constitute another step towards better understanding the complex host-fungi interplay.

By examining not only the effect of sex on the pathogen itself, but on the host-pathogen interaction, the work described in this dissertation also sheds light on how the immune response to *C. neoformans* may differ between healthy men and women. Most exciting were our findings that during infection the percentages of male T cells were significantly lower than the percentages of T cells from their female counterparts, suggesting a less robust immune response in men during an acute cryptococcosis infection. However, given that we did not label *C. neoformans* allowing it to be completely excluded from analysis, this study suffers from a serious oversight. We are actively working to remedy this, but at present there remain many unanswered questions. Similar to ours, a recent study used blood from healthy volunteers to examine the macrophage response to *C. neoformans* by quantifying intracellular fungal

proliferation and vomocytosis (non-lytic expulsion) over an 18 h period and found a large variation in both metrics between individuals but no correlation in that variation between sexes (Garelnabi et al., 2018). If male and female macrophages are not behaving differently during a *C. neoformans* infection, one must imagine that it is indeed the adaptive immune response, B and T cells, which are affecting the differences in outcomes in males and females. We hope to resolve the issues of this study quickly in order to offer a definitive answer to this question.

Over the last several years, the scientific community has come to recognize the importance of improving the balance of biological sex represented in research. In 2014, the National Institute of Health (NIH) announced a proposal to help remedy the over reliance of male cells and animals in pre-clinical research (Clayton and Collins, 2014). This came almost 20 years after the NIH Revitalization Act mandating the inclusion of women in clinical studies (Congress, 1993). These changes reflect the findings of scientists over the decades, findings that illustrate differences in the male and female immune response to pathogens, differences in neurology and cardiovascular health, drug susceptibility, symptom presentation, etc (Angele et al., 2014; Atabani et al., 2000; Auer et al., 2018; Beierle et al., 1999; de Faria Gomes et al., 2015; Klein and Pekosz, 2014; van Lunzen and Altfeld, 2014). Simply put, sex matters. We see this specifically in the data described in this dissertation and in a broader context throughout biology. This research helps contribute to our understanding of the importance of the differences between the male and female environment and immune response to *C. neoformans*, a very deadly pathogen.

REFERENCES

(2015). Labatory Reference Ranges. In Endocrine Self-Assessment Program (Endocrine Society: Center for Learning).

Abadi, J., and Pirofski, L. (1999). Antibodies reactive with the cryptococcal capsular polysaccharide glucuronoxylomannan are present in sera from children with and without human immunodeficiency virus infection. The Journal of infectious diseases *180*, 915-919.

Abioye, A.I., Soipe, A.I., Salako, A.A., Odesanya, M.O., Okuneye, T.A., Abioye, A.I., Ismail, K.A., and Omotayo, M.O. (2015). Are there differences in disease progression and mortality among male and female HIV patients on antiretroviral therapy? A meta-analysis of observational cohorts. AIDS Care *27*, 1468-1486.

Agaba, P.A., Digin, E., Makai, R., Apena, L., Agbaji, O.O., Idoko, J.A., Murphy, R., and Kanki, P. (2011). Clinical characteristics and predictors of mortality in hospitalized HIV-infected Nigerians. J Infect Dev Ctries *5*, 377-382.

Aguirre, K.M., and Johnson, L.L. (1997). A role for B cells in resistance to Cryptococcus neoformans in mice. Infect Immun *65*, 525-530.

Alvarez, M., Burn, T., Luo, Y., Pirofski, L.A., and Casadevall, A. (2009). The outcome of Cryptococcus neoformans intracellular pathogenesis in human monocytes. BMC Microbiol *9*, 51.

Alves, C.T., Silva, S., Pereira, L., Williams, D.W., Azeredo, J., and Henriques, M. (2014). Effect of progesterone on Candida albicans vaginal pathogenicity. Int J Med Microbiol *304*, 1011-1017.

Angele, M.K., Pratschke, S., Hubbard, W.J., and Chaudry, I.H. (2014). Gender differences in sepsis: cardiovascular and immunological aspects. Virulence *5*, 12-19.

Ashman, R.B., and Papadimitriou, J.M. (1995). Production and function of cytokines in natural and acquired immunity to Candida albicans infection. Microbiological reviews *59*, 646-672.

Atabani, S., Landucci, G., Steward, M.W., Whittle, H., Tilles, J.G., and Forthal, D.N. (2000). Sex-associated differences in the antibody-dependent cellular cytotoxicity antibody response to measles vaccines. Clin Diagn Lab Immunol *7*, 111-113.

Auer, M., Frauscher, B., Hochleitner, M., and Hogl, B. (2018). Gender-Specific Differences in Access to Polysomnography and Prevalence of Sleep Disorders. J Womens Health (Larchmt) *27*, 525-530.

Baginski, M., and Czub, J. (2009). Amphotericin B and its new derivatives - mode of action. Curr Drug Metab *10*, 459-469.

Bahn, Y.S. (2008). Master and commander in fungal pathogens: the two-component system and the HOG signaling pathway. Eukaryotic cell *7*, 2017-2036.

Bahn, Y.S., Kojima, K., Cox, G.M., and Heitman, J. (2005). Specialization of the HOG pathway and its impact on differentiation and virulence of Cryptococcus neoformans. Mol Biol Cell *16*, 2285-2300.

Bava, A.J., and Negroni, R. (1992). [The epidemiological characteristics of 105 cases of cryptococcosis diagnosed in the Republic of Argentina between 1981-1990]. Rev Inst Med Trop Sao Paulo *34*, 335-340.

Beierle, I., Meibohm, B., and Derendorf, H. (1999). Gender differences in pharmacokinetics and pharmacodynamics. Int J Clin Pharmacol Ther *37*, 529-547.
Bentley, P. (2001). Sex Hormones in Vertebrates (eLS).

Beziers, P., Ducrest, A.L., Simon, C., and Roulin, A. (2017). Circulating testosterone and feather-gene expression of receptors and metabolic enzymes in relation to melanin-based colouration in the barn owl. Gen Comp Endocrinol *250*, 36-45.

Bischitz, P.G., and Snell, R.S. (1959). The effect of testosterone on the melanocytes and melanin in the skin of the intact and orchidectomised male guinea-pig. J Invest Dermatol *33*, 299-306.

Bisson, G.P., Nthobatsong, R., Thakur, R., Lesetedi, G., Vinekar, K., Tebas, P., Bennett, J.E., Gluckman, S., Gaolathe, T., and MacGregor, R.R. (2008). The use of HAART is associated with decreased risk of death during initial treatment of cryptococcal meningitis in adults in Botswana. J Acquir Immune Defic Syndr *49*, 227-229.

Boulle, C., Kouanfack, C., Laborde-Balen, G., Boyer, S., Aghokeng, A.F., Carrieri, M.P., Kaze, S., Dontsop, M., Mben, J.M., Koulla-Shiro, S.*, et al.* (2015). Gender Differences in Adherence and Response to Antiretroviral Treatment in the Stratall Trial in Rural District Hospitals in Cameroon. J Acquir Immune Defic Syndr *69*, 355-364.

Brown, S.M., Campbell, L.T., and Lodge, J.K. (2007). Cryptococcus neoformans, a fungus under stress. Curr Opin Microbiol *10*, 320-325.

Burshell, A., Stathis, P.A., Do, Y., Miller, S.C., and Feldman, D. (1984). Characterization of an estrogen-binding protein in the yeast Saccharomyces cerevisiae. J Biol Chem *259*, 3450-3456.

Camacho, E., and Nino-Vega, G.A. (2017). Paracoccidioides Spp.: Virulence Factors and Immune-Evasion Strategies. Mediators Inflamm *2017*, 5313691.

Campbell, G.D. (1966). Primary pulmonary cryptococcosis. Am Rev Respir Dis *94*, 236-243.

Candore, G., Balistreri, C.R., Listi, F., Grimaldi, M.P., Vasto, S., Colonna-Romano, G., Franceschi, C., Lio, D., Caselli, G., and Caruso, C. (2006). Immunogenetics, gender, and longevity. Annals of the New York Academy of Sciences *1089*, 516-537.

Carroll, S.F., Loredo Osti, J.C., Guillot, L., Morgan, K., and Qureshi, S.T. (2008). Sex differences in the genetic architecture of susceptibility to Cryptococcus neoformans pulmonary infection. Genes and immunity *9*, 536-545.

Casadevall, A., and Pirofski, L. (2005). Insights into mechanisms of antibody-mediated immunity from studies with Cryptococcus neoformans. Curr Mol Med *5*, 421-433.

Casadevall, A.a.P., J.R. (1998). Cryptococcus neoformans. Journal of Antimicrobial Chemotherapy *44*, 139.

Census, U.S. (2016). Murfreesboro, TN (Retrieved from: https://www.census.gov/quickfacts/fact/table/murfreesborocitytennessee/PST045216).

Cheng, G., Yeater, K.M., and Hoyer, L.L. (2006). Cellular and molecular biology of Candida albicans estrogen response. Eukaryot Cell *5*, 180-191.

Chiapello, L.S., Baronetti, J.L., Aoki, M.P., Gea, S., Rubinstein, H., and Masih, D.T. (2004). Immunosuppression, interleukin-10 synthesis and apoptosis are induced in rats inoculated with Cryptococcus neoformans glucuronoxylomannan. Immunology *113*, 392-400.

Clayton, J.A., and Collins, F.S. (2014). Policy: NIH to balance sex in cell and animal studies. Nature *509*, 282-283.

Coelho, C., Bocca, A.L., and Casadevall, A. (2014a). The intracellular life of Cryptococcus neoformans. Annual review of pathology *9*, 219-238.

Coelho, C., Bocca, A.L., and Casadevall, A. (2014b). The tools for virulence of Cryptococcus neoformans. Adv Appl Microbiol *87*, 1-41.

Coelho, C., and Casadevall, A. (2016). Cryptococcal therapies and drug targets:the old, the new and the promising. Cellular microbiology.

Colton, C.A., Brown, C.M., and Vitek, M.P. (2005). Sex steroids, APOE genotype and the innate immune system. Neurobiology of aging *26*, 363-372.

Congress, r. (1993). NIH Policy and Guidelines on The Inclusion of Women and Minorities as Subjects in Clinical Research, NIH, ed. (Washington DC).

Davis, J., Zheng, W.Y., Glatman-Freedman, A., Ng, J.A., Pagcatipunan, M.R., Lessin, H., Casadevall, A., and Goldman, D.L. (2007). Serologic evidence for regional differences in pediatric cryptococcal infection. Pediatr Infect Dis J *26*, 549-551.

de Faria Gomes, N.M., da Mota Bastos, M.C., Marins, R.M., Barbosa, A.A., Soares, L.C., de Oliveira Wilken de Abreu, A.M., and Souto Filho, J.T. (2015). Differences between Risk Factors Associated with Tuberculosis Treatment Abandonment and Mortality. Pulmonary medicine *2015*, 546106.

DeSilva, M.B., Merry, S.P., Fischer, P.R., Rohrer, J.E., Isichei, C.O., and Cha, S.S. (2009). Youth, unemployment, and male gender predict mortality in AIDS patients started on HAART in Nigeria. AIDS Care *21*, 70-77.

Dromer, F., Mathoulin-Pelissier, S., Fontanet, A., Ronin, O., Dupont, B., Lortholary, O., and French Cryptococcosis Study, G. (2004). Epidemiology of HIV-associated cryptococcosis in France (1985-2001): comparison of the pre- and post-HAART eras. AIDS *18*, 555-562.

Dromer, F., Mathoulin-Pelissier, S., Launay, O., Lortholary, O., and French Cryptococcosis Study, G. (2007). Determinants of disease presentation and outcome during cryptococcosis: the CryptoA/D study. PLoS Med *4*, e21.

Dromer, F., Mathoulin, S., Dupont, B., Letenneur, L., and Ronin, O. (1996). Individual and environmental factors associated with infection due to Cryptococcus neoformans serotype D. French Cryptococcosis Study Group. Clin Infect Dis *23*, 91-96.

Dupont, S., Lemetais, G., Ferreira, T., Cayot, P., Gervais, P., and Beney, L. (2012). Ergosterol biosynthesis: a fungal pathway for life on land? Evolution *66*, 2961-2968.
Durrani, F., Phelps, D.S., Weisz, J., Silveyra, P., Hu, S., Mikerov, A.N., and Floros, J. (2012). Gonadal hormones and oxidative stress interaction differentially affects survival of male and female mice after lung Klebsiella pneumoniae infection. Experimental lung research *38*, 165-172.

Edwards, V.E., Sutherland, J.M., and Tyrer, J.H. (1970). Cryptococcosis of the central nervous system. Epidemiological, clinical, and therapeutic features. J Neurol Neurosurg Psychiatry *33*, 415-425.

Eisenman, H.C., Casadevall, A., and McClelland, E.E. (2007). New insights on the pathogenesis of invasive Cryptococcus neoformans infection. Curr Infect Dis Rep *9*, 457-464.

Ellerbroek, P.M., Walenkamp, A.M., Hoepelman, A.I., and Coenjaerts, F.E. (2004). Effects of the capsular polysaccharides of Cryptococcus neoformans on phagocyte migration and inflammatory mediators. Curr Med Chem *11*, 253-266.

Escandon, P., Lizarazo, J., Agudelo, C.I., and Castaneda, E. (2018). Cryptococcosis in Colombia: Compilation and Analysis of Data from Laboratory-Based Surveillance. J Fungi (Basel) *4*.

Eschke, M., Piehler, D., Schulze, B., Richter, T., Grahnert, A., Protschka, M., Muller, U., Kohler, G., Hofling, C., Rossner, S.*, et al.* (2015). A novel experimental model of Cryptococcus neoformans-related immune reconstitution inflammatory syndrome (IRIS) provides insights into pathogenesis. Eur J Immunol *45*, 3339-3350.

Fargallo, J.A., Martinez-Padilla, J., Toledano-Diaz, A., Santiago-Moreno, J., and Davila, J.A. (2007). Sex and testosterone effects on growth, immunity and melanin coloration of nestling Eurasian kestrels. J Anim Ecol *76*, 201-209.

Festa, R.A., Helsel, M.E., Franz, K.J., and Thiele, D.J. (2014). Exploiting innate immune cell activation of a copper-dependent antimicrobial agent during infection. Chemistry & biology *21*, 977-987.

Fonseca, E.R., Pardal, P.P., and Severo, L.C. (1999). [Paracoccidioidomycosis in children in Belem, Para]. Rev Soc Bras Med Trop *32*, 31-33.

Fox, D.S., and Heitman, J. (2005). Calcineurin-binding protein Cbp1 directs the specificity of calcineurin-dependent hyphal elongation during mating in Cryptococcus neoformans. Eukaryot Cell *4*, 1526-1538.

Fu, M.S., Coelho, C., De Leon-Rodriguez, C.M., Rossi, D.C.P., Camacho, E., Jung, E.H., Kulkarni, M., and Casadevall, A. (2018). Cryptococcus neoformans urease affects the outcome of intracellular pathogenesis by modulating phagolysosomal pH. PLoS Pathog *14*, e1007144.

Ganendren, R., Carter, E., Sorrell, T., Widmer, F., and Wright, L. (2006). Phospholipase B activity enhances adhesion of Cryptococcus neoformans to a human lung epithelial cell line. Microbes Infect *8*, 1006-1015.

Garcia-Rodas, R., Cordero, R.J., Trevijano-Contador, N., Janbon, G., Moyrand, F., Casadevall, A., and Zaragoza, O. (2014). Capsule growth in Cryptococcus neoformans is coordinated with cell cycle progression. mBio *5*, e00945-00914.

Garelnabi, M., Taylor-Smith, L.M., Bielska, E., Hall, R.A., Stones, D., and May, R.C. (2018). Quantifying donor-to-donor variation in macrophage responses to the human fungal pathogen Cryptococcus neoformans. PloS one *13*, e0194615.

Gerik, K.J., Donlin, M.J., Soto, C.E., Banks, A.M., Banks, I.R., Maligie, M.A., Selitrennikoff, C.P., and Lodge, J.K. (2005). Cell wall integrity is dependent on the PKC1 signal transduction pathway in Cryptococcus neoformans. Molecular microbiology *58*, 393-408.

Giefing-Kroll, C., Berger, P., Lepperdinger, G., and Grubeck-Loebenstein, B. (2015). How sex and age affect immune responses, susceptibility to infections, and response to vaccination. Aging cell *14*, 309-321.

Giles, S.S., Dagenais, T.R., Botts, M.R., Keller, N.P., and Hull, C.M. (2009). Elucidating the pathogenesis of spores from the human fungal pathogen Cryptococcus neoformans. Infection and immunity *77*, 3491-3500.

Goncalves, A.J., Londero, A.T., Terra, G.M., Rozenbaum, R., Abreu, T.F., and Nogueira, S.A. (1998). Paracoccidioidomycosis in children in the state of Rio de Janeiro (Brazil). Geographic distribution and the study of a "reservarea". Rev Inst Med Trop Sao Paulo *40*, 11-13.

Goncalves, B., Ferreira, C., Alves, C.T., Henriques, M., Azeredo, J., and Silva, S. (2016). Vulvovaginal candidiasis: Epidemiology, microbiology and risk factors. Crit Rev Microbiol *42*, 905-927.

Granger, D.L., Perfect, J.R., and Durack, D.T. (1985). Virulence of Cryptococcus neoformans. Regulation of capsule synthesis by carbon dioxide. J Clin Invest *76*, 508-516.

Grossman, C. (1989). Possible underlying mechanisms of sexual dimorphism in the immune response, fact and hypothesis. J Steroid Biochem *34*, 241-251.

Gruber, C.J., Tschugguel, W., Schneeberger, C., and Huber, J.C. (2002). Production and actions of estrogens. N Engl J Med *346*, 340-352.

Guess, T., and McClelland, E. (2017). An Updated Overview of the Gender-Specific Response to Infection. In Principles of Gender-Specific Medicine (Cambridge: Elsevier, Inc.), pp. 417-428.

Guess, T.E., Rosen, J.A., and McClelland, E.E. (2018). An Overview of Sex Bias in C. neoformans Infections. J Fungi (Basel) *4*.

Gujjar, P.R., Finucane, M., and Larsen, B. (1997). The effect of estradiol on Candida albicans growth. Ann Clin Lab Sci *27*, 151-156.

Hajjeh, R.A., Conn, L.A., Stephens, D.S., Baughman, W., Hamill, R., Graviss, E., Pappas, P.G., Thomas, C., Reingold, A., Rothrock, G.*, et al.* (1999). Cryptococcosis: population-based multistate active surveillance and risk factors in human immunodeficiency virus-infected persons. Cryptococcal Active Surveillance Group. J Infect Dis *179*, 449-454.

Hamad, M. (2014). Estrogen treatment predisposes to severe and persistent vaginal candidiasis in diabetic mice. J Diabetes Metab Disord *13*, 15.

Hayes, J.B., Sircy, L.M., Heusinkveld, L.E., Ding, W., Leander, R.N., McClelland, E.E., and Nelson, D.E. (2016). Modulation of Macrophage Inflammatory Nuclear Factor kappaB (NF-kappaB) Signaling by Intracellular Cryptococcus neoformans. J Biol Chem *291*, 15614-15627.

Hepworth, M.R., and Grencis, R.K. (2009). Disruption of Th2 immunity results in a gender-specific expansion of IL-13 producing accessory NK cells during helminth infection. J Immunol *183*, 3906-3914.

Huang, C., Nong, S.H., Mansour, M.K., Specht, C.A., and Levitz, S.M. (2002). Purification and characterization of a second immunoreactive mannoprotein from Cryptococcus neoformans that stimulates T-Cell responses. Infect Immun *70*, 5485-5493.

Huffnagle, G.B., Yates, J.L., and Lipscomb, M.F. (1991). Immunity to a pulmonary Cryptococcus neoformans infection requires both CD4+ and CD8+ T cells. J Exp Med *173*, 793-800.

Imahara, S.D., Jelacic, S., Junker, C.E., and O'Keefe, G.E. (2005). The influence of gender on human innate immunity. Surgery *138*, 275-282.

Jacobson, E.S., and Compton, G.M. (1996). Discordant regulation of phenoloxidase and capsular polysaccharide in Cryptococcus neoformans. J Med Vet Mycol *34*, 289-291.

Jacobson, E.S., and Emery, H.S. (1991). Temperature regulation of the cryptococcal phenoloxidase. J Med Vet Mycol *29*, 121-124.

Jacobson, E.S., and Tinnell, S.B. (1993). Antioxidant function of fungal melanin. J Bacteriol *175*, 7102-7104.

Jalsenjak, I., and Kondo, T. (1981). Effect of capsule size on permeability of gelatin-acacia microcapsules toward sodium chloride. J Pharm Sci *70*, 456-457.

Janbon, G., Ormerod, K.L., Paulet, D., Byrnes, E.J., 3rd, Yadav, V., Chatterjee, G., Mullapudi, N., Hon, C.C., Billmyre, R.B., Brunel, F.*, et al.* (2014). Analysis of the genome and transcriptome of Cryptococcus neoformans var. grubii reveals complex RNA expression and microevolution leading to virulence attenuation. PLoS genetics *10*, e1004261.

Jarvis, J.N., Casazza, J.P., Stone, H.H., Meintjes, G., Lawn, S.D., Levitz, S.M., Harrison, T.S., and Koup, R.A. (2013). The phenotype of the Cryptococcus-specific CD4+ memory T-cell response is associated with disease severity and outcome in HIV-associated cryptococcal meningitis. The Journal of infectious diseases *207*, 1817-1828.

Javed, F., Klingspor, L., Sundin, U., Altamash, M., Klinge, B., and Engstrom, P.E. (2009). Periodontal conditions, oral Candida albicans and salivary proteins in type 2 diabetic subjects with emphasis on gender. BMC oral health *9*, 12.

Jimenez-Mejias, M.E., Fernandez, A., Alfaro, E., Regordan, C., and Pachon Diaz, J. (1991). [Cryptococcosis of the central nervous system. Clinical and diagnostic characteristics]. Med Clin (Barc) *97*, 604-608.

Jung, K.W., and Bahn, Y.S. (2009). The Stress-Activated Signaling (SAS) Pathways of a Human Fungal Pathogen, Cryptococcus neoformans. Mycobiology *37*, 161-170.

Jung, W.H., Sham, A., White, R., and Kronstad, J.W. (2006). Iron regulation of the major virulence factors in the AIDS-associated pathogen Cryptococcus neoformans. PLoS biology *4*, e410.

Kali, A., Charles, M.P., Noyal, M.J., Sivaraman, U., Kumar, S., and Easow, J.M. (2013). Prevalence of Candida co-infection in patients with pulmonary tuberculosis. The Australasian medical journal *6*, 387-391.

Kawakami, K., Kinjo, Y., Yara, S., Koguchi, Y., Uezu, K., Nakayama, T., Taniguchi, M., and Saito, A. (2001). Activation of Valpha14(+) natural killer T cells by alpha-galactosylceramide results in development of Th1 response and local host resistance in mice infected with Cryptococcus neoformans. Infect Immun *69*, 213-220.

Kim-Fine, S., Regnault, T.R., Lee, J.S., Gimbel, S.A., Greenspoon, J.A., Fairbairn, J., Summers, K., and de Vrijer, B. (2012). Male gender promotes an increased inflammatory response to lipopolysaccharide in umbilical vein blood. The journal of maternal-fetal & neonatal medicine : the official journal of the European Association

of Perinatal Medicine, the Federation of Asia and Oceania Perinatal Societies, the International Society of Perinatal Obstet *25*, 2470-2474.

Kim, S.J., Kwon-Chung, J., Milne, G.W., Hill, W.B., and Patterson, G. (1975). Relationship between polyene resistance and sterol compositions in Cryptococcus neoformans. Antimicrob Agents Chemother *7*, 99-106.

Klein, S.L., and Pekosz, A. (2014). Sex-based biology and the rational design of influenza vaccination strategies. The Journal of infectious diseases *209 Suppl 3*, S114-119.

Kollar, R., Reinhold, B.B., Petrakova, E., Yeh, H.J., Ashwell, G., Drgonova, J., Kapteyn, J.C., Klis, F.M., and Cabib, E. (1997). Architecture of the yeast cell wall. Beta(1-->6)-glucan interconnects mannoprotein, beta(1-->)3-glucan, and chitin. J Biol Chem *272*, 17762-17775.

Kurakado, S., Kurogane, R., and Sugita, T. (2017). 17beta-Estradiol inhibits estrogen binding protein-mediated hypha formation in Candida albicans. Microb Pathog *109*, 151-155.

Kwon-Chung, K.J. (1976). Morphogenesis of Filobasidiella neoformans, the sexual state of Cryptococcus neoformans. Mycologia *68*, 821-833.

Kwon-Chung, K.J., and Rhodes, J.C. (1986). Encapsulation and melanin formation as indicators of virulence in Cryptococcus neoformans. Infect Immun *51*, 218-223.
Kwon-Chung, K.J., Wickes, B.L., Booth, J.L., Vishniac, H.S., and Bennett, J.E. (1987). Urease inhibition by EDTA in the two varieties of Cryptococcus neoformans. Infect Immun *55*, 1751-1754.

Lai, J.J., Lai, K.P., Zeng, W., Chuang, K.H., Altuwaijri, S., and Chang, C. (2012). Androgen receptor influences on body defense system via modulation of innate and adaptive immune systems: lessons from conditional AR knockout mice. Am J Pathol *181*, 1504-1512.

Lehmann, C., Zeis, M., and Uharek, L. (2001). Activation of natural killer cells with interleukin 2 (IL-2) and IL-12 increases perforin binding and subsequent lysis of tumour cells. Br J Haematol *114*, 660-665.

Leopold Wager, C.M., and Wormley, F.L., Jr. (2014). Classical versus alternative macrophage activation: the Ying and the Yang in host defense against pulmonary fungal infections. Mucosal Immunol *7*, 1023-1035.

Leuenberger, P., Ganscha, S., Kahraman, A., Cappelletti, V., Boersema, P.J., von Mering, C., Claassen, M., and Picotti, P. (2017). Cell-wide analysis of protein thermal unfolding reveals determinants of thermostability. Science *355*.

Levitz, S.M., Dupont, M.P., and Smail, E.H. (1994). Direct activity of human T lymphocytes and natural killer cells against Cryptococcus neoformans. Infection and immunity *62*, 194-202.

Li, J., Chang, Y.C., Wu, C.H., Liu, J., Kwon-Chung, K.J., Huang, S.H., Shimada, H., Fante, R., Fu, X., and Jong, A. (2015a). The 14-3-3 gene function of Cryptococcus neoformans is required for its growth and virulence. Journal of microbiology and biotechnology.

Li, X., Liu, G., Ma, J., Zhou, L., Zhang, Q., and Gao, L. (2015b). Lack of IL-6 increases blood-brain barrier permeability in fungal meningitis. J Biosci *40*, 7-12.

Lindell, D.M., Moore, T.A., McDonald, R.A., Toews, G.B., and Huffnagle, G.B. (2005). Generation of antifungal effector CD8+ T cells in the absence of CD4+ T cells during Cryptococcus neoformans infection. J Immunol *174*, 7920-7928.

Loose, D.S., Stover, E.P., Restrepo, A., Stevens, D.A., and Feldman, D. (1983). Estradiol binds to a receptor-like cytosol binding protein and initiates a biological response in Paracoccidioides brasiliensis. Proc Natl Acad Sci U S A *80*, 7659-7663.

Lortholary, O., Improvisi, L., Fitting, C., Cavaillon, J.M., and Dromer, F. (2002). Influence of gender and age on course of infection and cytokine responses in mice with disseminated Cryptococcus neoformans infection. Clin Microbiol Infect *8*, 31-37.

Loster, J.E., Wieczorek, A., and Loster, B.W. (2016). Correlation between age and gender in Candida species infections of complete denture wearers: a retrospective analysis. Clin Interv Aging *11*, 1707-1714.

Madani, N.D., Malloy, P.J., Rodriguez-Pombo, P., Krishnan, A.V., and Feldman, D. (1994). Candida albicans estrogen-binding protein gene encodes an oxidoreductase that is inhibited by estradiol. Proc Natl Acad Sci U S A *91*, 922-926.

Maduro, A.P., Goncalves, L., Inacio, J., Faria, N.C., Martins, M.L., and Teles, F.R. (2015). HIV/AIDS-Associated Cryptococcosis in Portugal Spanning the Pre- to Post-HAART Era: A Retrospective Assessment at the Genotypic Level Based on URA5-RFLP. Current microbiology *71*, 449-457.

Manfredi, R., Rezza, G., Coronado, V.G., Cozzi Lepri, A., Coronado, O.V., Mastroianni, A., and Chiodo, F. (1995). Is AIDS-related cryptococcosis more frequent among men? Aids *9*, 397-398.

Marino, M., Galluzzo, P., and Ascenzi, P. (2006). Estrogen signaling multiple pathways to impact gene transcription. Curr Genomics *7*, 497-508.

Maxwell, C.S., and Magwene, P.M. (2017). The quick and the dead: microbial demography at the yeast thermal limit. Mol Ecol *26*, 1631-1640.

McClelland, E.a.S., Jennifer (2011). Gender specific differences in the immune response to infection. Archivum Immunologiae et Therapiae Experimentalis *59*, 10.

McClelland, E.E., Hobbs, L.M., Rivera, J., Casadevall, A., Potts, W.K., Smith, J.M., and Ory, J.J. (2013). The role of host gender in the pathogenesis of Cryptococcus neoformans infections. PLoS One *8*, e63632.

McClelland, E.E., Perrine, W.T., Potts, W.K., and Casadevall, A. (2005). Relationship of virulence factor expression to evolved virulence in mouse-passaged Cryptococcus neoformans lines. Infection and immunity *73*, 7047-7050.

McClelland, E.E.C., A.; Eisenmann, H. (2007). Pathogenesis of Cryptococcus neoformans. In New Insights in Medical Mycology (Springer Netherlands), pp. 131-157.

Mednick, A.J., Nosanchuk, J.D., and Casadevall, A. (2005). Melanization of Cryptococcus neoformans affects lung inflammatory responses during cryptococcal infection. Infection and immunity *73*, 2012-2019.

Millogo, A., Ki-Zerbo, G.A., Andonaba, J.B., Lankoande, D., Sawadogo, A., Yameogo, I., and Sawadogo, A.B. (2004). [Cryptococcal meningitis in HIV-infected patients at Bobo-Dioulasso hospital (Burkina Faso)]. Bulletin de la Societe de pathologie exotique (1990) *97*, 119-121.

Missall, T.A., Lodge, J.K., and McEwen, J.E. (2004a). Mechanisms of resistance to oxidative and nitrosative stress: implications for fungal survival in mammalian hosts. Eukaryotic cell *3*, 835-846.

Missall, T.A., Pusateri, M.E., and Lodge, J.K. (2004b). Thiol peroxidase is critical for virulence and resistance to nitric oxide and peroxide in the fungal pathogen, Cryptococcus neoformans. Mol Microbiol *51*, 1447-1458.

Mitchell, A.P. (2003). Updated view of Cryptococcus neoformans mating type and virulence. Infection and immunity *71*, 4829-4830.

Mitchell, T.G., and Perfect, J.R. (1995). Cryptococcosis in the era of AIDS--100 years after the discovery of Cryptococcus neoformans. Clin Microbiol Rev *8*, 515-548.

Mody, C.H., and Syme, R.M. (1993). Effect of polysaccharide capsule and methods of preparation on human lymphocyte proliferation in response to Cryptococcus neoformans. Infect Immun *61*, 464-469.

Mohr, J.A., Long, H., McKown, B.A., and Muchmore, H.G. (1972). In vitro susceptibility of Cryptococcus neoformans to steroids. Sabouraudia *10*, 171-172.

Mohr, J.A., Muchmore, H.G., and Tacker, R. (1974). Stimulation of phagocytosis of Cryptococcus neoformans in human cryptococcal meningitis. J Reticuloendothel Soc *15*, 149-154.

Mohr, J.A., Tatem, B.A., Long, H., Muchmore, H.G., and Felton, F.G. (1973). Increased susceptibility of Cryptococcus neoformans to amphotericin B in the presence of steroids. Sabouraudia *11*, 140-142.

Monteiro, J.P., Clemons, K.V., Mirels, L.F., Coller, J.A., Jr., Wu, T.D., Shankar, J., Lopes, C.R., and Stevens, D.A. (2009). Genomic DNA microarray comparison of gene expression patterns in Paracoccidioides brasiliensis mycelia and yeasts in vitro. Microbiology *155*, 2795-2808.

Mora, D.J., Ferreira-Paim, K., Andrade-Silva, L.E., Bragine, T., Rocha, I.H., Ribeiro, B.M., Machado, G.H., Rodrigues Junior, V., Silva-Teixeira, D.N., Meyer, W.*, et al.* (2017). Cytokine patterns in a prospective cohort of HIV-infected patients with cryptococcal meningitis following initiation of antifungal and antiretroviral therapy. PLoS One *12*, e0176304.

Mora, D.J., Fortunato, L.R., Andrade-Silva, L.E., Ferreira-Paim, K., Rocha, I.H., Vasconcelos, R.R., Silva-Teixeira, D.N., Nascentes, G.A., and Silva-Vergara, M.L. (2015). Cytokine profiles at admission can be related to outcome in AIDS patients with cryptococcal meningitis. PloS one *10*, e0120297.

Muenchhoff, M., and Goulder, P.J. (2014). Sex differences in pediatric infectious diseases. The Journal of infectious diseases *209 Suppl 3*, S120-126.

Murback, N.D., Hans Filho, G., Nascimento, R.A., Nakazato, K.R., and Dorval, M.E. (2011). American cutaneous leishmaniasis: clinical, epidemiological and laboratory studies conducted at a university teaching hospital in Campo Grande, Mato Grosso do Sul, Brazil. An Bras Dermatol *86*, 55-63.

Nazarpour, R., Zabihi, E., Alijanpour, E., Abedian, Z., Mehdizadeh, H., and Rahimi, F. (2012). Optimization of Human Peripheral Blood Mononuclear Cells (PBMCs) Cryopreservation. Int J Mol Cell Med *1*, 88-93.

Neal, L.M., Xing, E., Xu, J., Kolbe, J.L., Osterholzer, J.J., Segal, B.M., Williamson, P.R., and Olszewski, M.A. (2017). CD4(+) T Cells Orchestrate Lethal Immune Pathology despite Fungal Clearance during Cryptococcus neoformans Meningoencephalitis. MBio *8*.

Nhamoyebonde, S., and Leslie, A. (2014). Biological differences between the sexes and susceptibility to tuberculosis. The Journal of infectious diseases *209 Suppl 3*, S100-106.

Nosanchuk, J.D., Stark, R.E., and Casadevall, A. (2015). Fungal Melanin: What do We Know About Structure? Frontiers in microbiology *6*, 1463.

Noverr, M.C., Cox, G.M., Perfect, J.R., and Huffnagle, G.B. (2003). Role of PLB1 in pulmonary inflammation and cryptococcal eicosanoid production. Infect Immun *71*, 1538-1547.

Olszewski, M.A., Noverr, M.C., Chen, G.H., Toews, G.B., Cox, G.M., Perfect, J.R., and Huffnagle, G.B. (2004). Urease expression by Cryptococcus neoformans promotes microvascular sequestration, thereby enhancing central nervous system invasion. Am J Pathol *164*, 1761-1771.

Panackal, A.A., Wuest, S.C., Lin, Y.C., Wu, T., Zhang, N., Kosa, P., Komori, M., Blake, A., Browne, S.K., Rosen, L.B.*, et al.* (2015). Paradoxical Immune Responses in Non-HIV Cryptococcal Meningitis. PLoS Pathog *11*, e1004884.

Paumi, C.M., Chuk, M., Snider, J., Stagljar, I., and Michaelis, S. (2009). ABC transporters in Saccharomyces cerevisiae and their interactors: new technology advances the biology of the ABCC (MRP) subfamily. Microbiol Mol Biol Rev *73*, 577-593.

Perfect, J.R., Dismukes, W.E., Dromer, F., Goldman, D.L., Graybill, J.R., Hamill, R.J., Harrison, T.S., Larsen, R.A., Lortholary, O., Nguyen, M.H.*, et al.* (2010). Clinical practice guidelines for the management of cryptococcal disease: 2010 update by the infectious diseases society of america. Clin Infect Dis *50*, 291-322.

Rajasingham, R., Smith, R.M., Park, B.J., Jarvis, J.N., Govender, N.P., Chiller, T.M., Denning, D.W., Loyse, A., and Boulware, D.R. (2017). Global burden of disease of HIV-associated cryptococcal meningitis: an updated analysis. Lancet Infect Dis *17*, 873-881.

Rapp, R.P. (2004). Changing strategies for the management of invasive fungal infections. Pharmacotherapy *24*, 4S-28S; quiz 29S-32S.

Reardon, S. (2016). Infections reveal inequality between the sexes. Nature *534*, 447.

Reichert, T., DeBruyere, M., Deneys, V., Totterman, T., Lydyard, P., Yuksel, F., Chapel, H., Jewell, D., Van Hove, L., Linden, J.*, et al.* (1991). Lymphocyte subset reference ranges in adult Caucasians. Clin Immunol Immunopathol *60*, 190-208.

Restrepo, A., Benard, G., de Castro, C.C., Agudelo, C.A., and Tobon, A.M. (2008). Pulmonary paracoccidioidomycosis. Semin Respir Crit Care Med *29*, 182-197.

Restrepo, A., Robledo, M., Ospina, S., Restrepo, M., and Correa, A. (1968). Distribution of paracoccidioidin sensitivity in Colombia. Am J Trop Med Hyg *17*, 25-37.

Rivera, J., Zaragoza, O., and Casadevall, A. (2005). Antibody-mediated protection against Cryptococcus neoformans pulmonary infection is dependent on B cells. Infect Immun *73*, 1141-1150.

Rohatgi, S., and Pirofski, L.A. (2012). Molecular characterization of the early B cell response to pulmonary Cryptococcus neoformans infection. J Immunol *189*, 5820-5830.

Rohatgi, S., and Pirofski, L.A. (2015). Host immunity to Cryptococcus neoformans. Future Microbiol *10*, 565-581.

Rothstein, E. (1971). The Thirtieth Veterans Administration - Armed Forces Pulmonary Disease Research Conference. The American Review of Respiratory Disease *103*, 11.

Roved, J., Westerdahl, H., and Hasselquist, D. (2017). Sex differences in immune responses: Hormonal effects, antagonistic selection, and evolutionary consequences. Horm Behav *88*, 95-105.

Rubaihayo, J., Tumwesigye, N.M., Konde-Lule, J., Wamani, H., Nakku-Joloba, E., and Makumbi, F. (2016). Frequency and distribution patterns of opportunistic infections associated with HIV/AIDS in Uganda. BMC Res Notes *9*, 501.

Rutherford, J.C. (2014). The emerging role of urease as a general microbial virulence factor. PLoS Pathog *10*, e1004062.

Scariano, J.K., Emery-Cohen, A.J., Pickett, G.G., Morgan, M., Simons, P.C., and Alba, F. (2008). Estrogen receptors alpha (ESR1) and beta (ESR2) are expressed in circulating human lymphocytes. J Recept Signal Transduct Res *28*, 285-293.

Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B.*, et al.* (2012). Fiji: an open-source platform for biological-image analysis. Nat Methods *9*, 676-682.

Schop, J. (2007). Protective immunity against cryptococcus neoformans infection. Mcgill J Med *10*, 35-43.

Scriven, J.E., Graham, L.M., Schutz, C., Scriba, T.J., Wilkinson, K.A., Wilkinson, R.J., Boulware, D.R., Urban, B.C., Lalloo, D.G., and Meintjes, G. (2016). A Glucuronoxylomannan-Associated Immune Signature, Characterized by Monocyte Deactivation and an Increased Interleukin 10 Level, Is a Predictor of Death in Cryptococcal Meningitis. J Infect Dis *213*, 1725-1734.

Shah, N.S., Greenberg, J.A., McNulty, M.C., Gregg, K.S., Riddell, J., Mangino, J.E., Weber, D.M., Hebert, C.L., Marzec, N.S., Barron, M.A.*, et al.* (2015). Severe Influenza in 33 US Hospitals, 2013-2014: Complications and Risk Factors for Death in 507 Patients. Infect Control Hosp Epidemiol *36*, 1251-1260.

Shaheen, A.A., Somayaji, R., Myers, R., and Mody, C.H. (2017). Epidemiology and trends of cryptococcosis in the United States from 2000 to 2007: A population-based study. Int J STD AIDS, 956462417732649.

Shankar, J., Restrepo, A., Clemons, K.V., and Stevens, D.A. (2011a). Hormones and the resistance of women to paracoccidioidomycosis. Clinical microbiology reviews *24*, 296-313.

Shankar, J., Wu, T.D., Clemons, K.V., Monteiro, J.P., Mirels, L.F., and Stevens, D.A. (2011b). Influence of 17beta-estradiol on gene expression of Paracoccidioides during mycelia-to-yeast transition. PloS one *6*, e28402.

Shikanai-Yasuda, M.A., Telles Filho Fde, Q., Mendes, R.P., Colombo, A.L., and Moretti, M.L. (2006). [Guidelines in paracoccidioidomycosis]. Rev Soc Bras Med Trop *39*, 297-310.

Sia, R.A., Lengeler, K.B., and Heitman, J. (2000). Diploid strains of the pathogenic basidiomycete Cryptococcus neoformans are thermally dimorphic. Fungal Genet Biol *29*, 153-163.

Skowronski, R., and Feldman, D. (1989). Characterization of an estrogen-binding protein in the yeast Candida albicans. Endocrinology *124*, 1965-1972.

Sofronescu, A. (2015). Estradiol Reference Range, E. Staros, ed. (MedScape).

Solianik, R., Skurvydas, A., Vitkauskiene, A., and Brazaitis, M. (2014). Gender-specific cold responses induce a similar body-cooling rate but different neuroendocrine and immune responses. Cryobiology *69*, 26-33.

Subbiah, M.T., and Abplanalp, W. (2003). Ergosterol (major sterol of baker's and brewer's yeast extracts) inhibits the growth of human breast cancer cells in vitro and the potential role of its oxidation products. Int J Vitam Nutr Res *73*, 19-23.

Subramaniam, K., Metzger, B., Hanau, L.H., Guh, A., Rucker, L., Badri, S., and Pirofski, L.A. (2009). IgM(+) memory B cell expression predicts HIV-associated cryptococcosis status. J Infect Dis *200*, 244-251.

Sun, T.S., Ju, X., Gao, H.L., Wang, T., Thiele, D.J., Li, J.Y., Wang, Z.Y., and Ding, C. (2014). Reciprocal functions of Cryptococcus neoformans copper homeostasis machinery during pulmonary infection and meningoencephalitis. Nat Commun *5*, 5550.

Syrett, C.M., Sindhava, V., Hodawadekar, S., Myles, A., Liang, G., Zhang, Y., Nandi, S., Cancro, M., Atchison, M., and Anguera, M.C. (2017). Loss of Xist RNA from the inactive X during B cell development is restored in a dynamic YY1-dependent two-step process in activated B cells. PLoS Genet *13*, e1007050.

Takeo, K., Uesaka, I., Uehira, K., and Nishiura, M. (1973). Fine structure of Cryptococcus neoformans grown in vitro as observed by freeze-etching. J Bacteriol *113*, 1442-1448.

Thompson, J.M., Gralow, J.R., Levy, R., and Miller, R.A. (1985). The optimal application of forward and ninety-degree light scatter in flow cytometry for the gating of mononuclear cells. Cytometry *6*, 401-406.

Travi, B.L., Osorio, Y., Melby, P.C., Chandrasekar, B., Arteaga, L., and Saravia, N.G. (2002). Gender is a major determinant of the clinical evolution and immune response in hamsters infected with Leishmania spp. Infect Immun *70*, 2288-2296.

Valiathan, R., Deeb, K., Diamante, M., Ashman, M., Sachdeva, N., and Asthana, D. (2014). Reference ranges of lymphocyte subsets in healthy adults and adolescents with special mention of T cell maturation subsets in adults of South Florida. Immunobiology *219*, 487-496.

Van Dyke, M.C.C., Chaturvedi, A.K., Hardison, S.E., Leopold Wager, C.M., Castro-Lopez, N., Hole, C.R., Wozniak, K.L., and Wormley, F.L., Jr. (2017). Induction of Broad-Spectrum Protective Immunity against Disparate Cryptococcus Serotypes. Front Immunol *8*, 1359.

van Lunzen, J., and Altfeld, M. (2014). Sex differences in infectious diseases-common but neglected. The Journal of infectious diseases *209 Suppl 3*, S79-80.

Vecchiarelli, A., Pericolini, E., Gabrielli, E., Chow, S.K., Bistoni, F., Cenci, E., and Casadevall, A. (2011). Cryptococcus neoformans galactoxylomannan is a potent negative immunomodulator, inspiring new approaches in anti-inflammatory immunotherapy. Immunotherapy *3*, 997-1005.

Voelz, K., and May, R.C. (2010). Cryptococcal interactions with the host immune system. Eukaryot Cell *9*, 835-846.

Wagner, R.D., and Johnson, S.J. (2012). Probiotic lactobacillus and estrogen effects on vaginal epithelial gene expression responses to Candida albicans. J Biomed Sci *19*, 58.

Wang, J., Syrett, C.M., Kramer, M.C., Basu, A., Atchison, M.L., and Anguera, M.C. (2016). Unusual maintenance of X chromosome inactivation predisposes female lymphocytes for increased expression from the inactive X. Proceedings of the National Academy of Sciences of the United States of America *113*, E2029-2038.
Wang, Y., Aisen, P., and Casadevall, A. (1995). Cryptococcus neoformans melanin and virulence: mechanism of action. Infect Immun *63*, 3131-3136.

Wang, Y., and Casadevall, A. (1994). Susceptibility of melanized and nonmelanized Cryptococcus neoformans to nitrogen- and oxygen-derived oxidants. Infection and immunity *62*, 3004-3007.

Wiesner, D.L., Moskalenko, O., Corcoran, J.M., McDonald, T., Rolfes, M.A., Meya, D.B., Kajumbula, H., Kambugu, A., Bohjanen, P.R., Knight, J.F.*, et al.* (2012). Cryptococcal genotype influences immunologic response and human clinical outcome after meningitis. MBio *3*.

Wira, C.R., Fahey, J.V., Ghosh, M., Patel, M.V., Hickey, D.K., and Ochiel, D.O. (2010). Sex hormone regulation of innate immunity in the female reproductive tract: the role of epithelial cells in balancing reproductive potential with protection against sexually transmitted pathogens. American journal of reproductive immunology (New York, NY : 1989) *63*, 544-565.

Yang, Z., Huang, Y.C., Koziel, H., de Crom, R., Ruetten, H., Wohlfart, P., Thomsen, R.W., Kahlert, J.A., Sorensen, H.T., Jozefowski, S.*, et al.* (2014). Female resistance to pneumonia identifies lung macrophage nitric oxide synthase-3 as a therapeutic target. eLife *3*.

Yasuoka, A., Kohno, S., Yamada, H., Kaku, M., and Koga, H. (1994). Influence of molecular sizes of Cryptococcus neoformans capsular polysaccharide on phagocytosis. Microbiol Immunol *38*, 851-856.

Zaragoza, O., and Casadevall, A. (2004). Experimental modulation of capsule size in Cryptococcus neoformans. Biol Proced Online *6*, 10-15.

Zhang, X., Essmann, M., Burt, E.T., and Larsen, B. (2000). Estrogen effects on Candida albicans: a potential virulence-regulating mechanism. J Infect Dis *181*, 1441-1446.

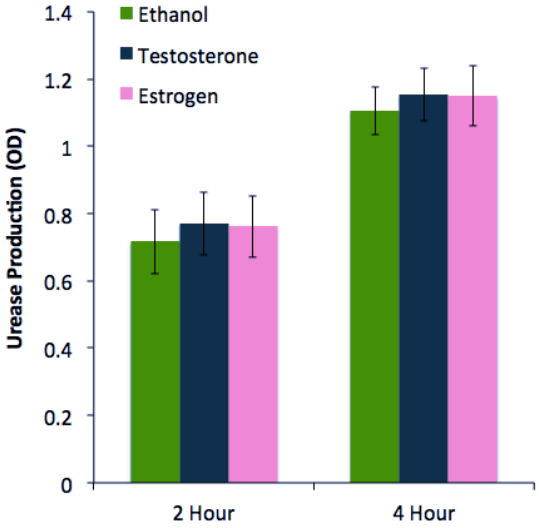
Zhang, X., Lv, X., and Song, Y. (2018). Short-term culture with IL-2 is beneficial for potent memory chimeric antigen receptor T cell production. Biochem Biophys Res Commun *495*, 1833-1838.

Zheng, H., Li, M., Wang, D., ling Yang, J., Chen, Q., Zhang, X., Man, Y., Lao, J., Chen, N., and Pan, S. (2016). Gender-specific contributing risk factors and outcome of female cryptococcal meningoencephalitis patients. BMC Infect Dis *16*, 22.

Zivkovic, I., Bufan, B., Petrusic, V., Minic, R., Arsenovic-Ranin, N., Petrovic, R., and Leposavic, G. (2015). Sexual diergism in antibody response to whole virus trivalent inactivated influenza vaccine in outbred mice. Vaccine *33*, 5546-5552.
**

APPENDICES

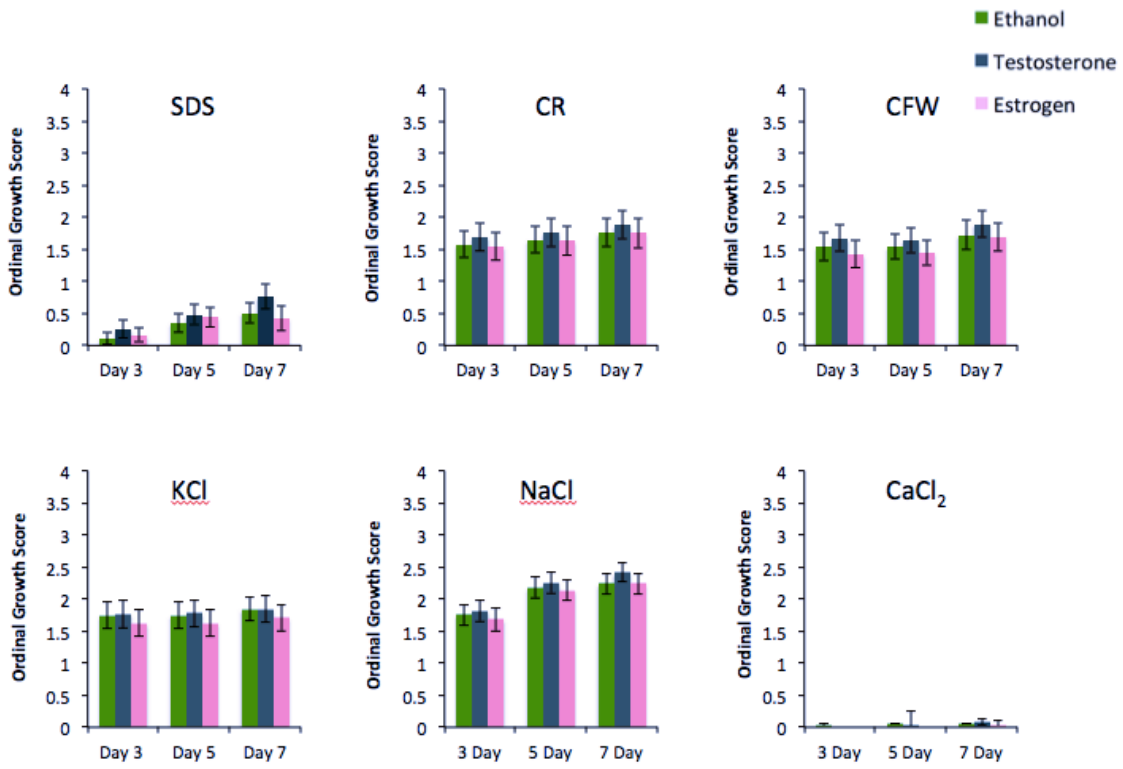
APPENDIX A: UREASE PRODUCTION



Supplemental Figure 1. Urease Production in the Presence of Sex Hormones.

Mean optical density of all 28 clinical *C. neoformans* isolates in the presence of ethanol (negative control), testosterone, or estrogen. Cells were incubated in Robert's Urea Broth at 37 ^{o}C for two or four hours. Data was analyzed using ANOVA with simple contrasts. Error bars are SE.

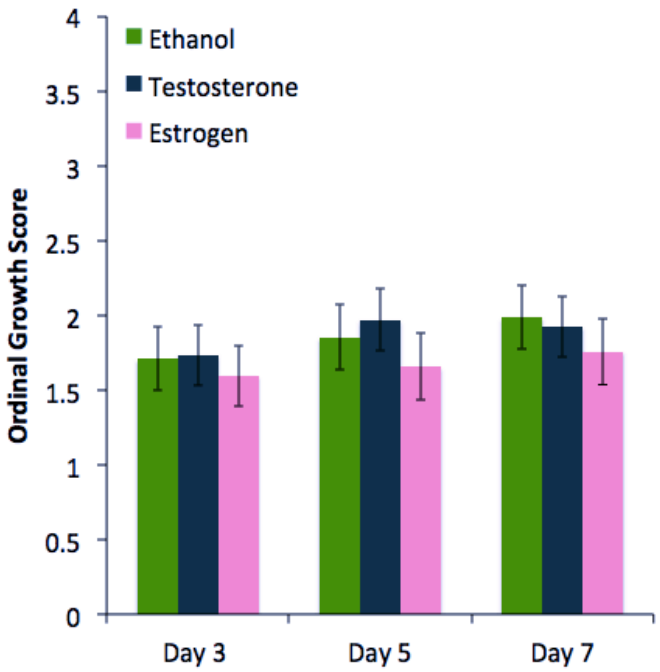
Appendix B: Cell Wall Stress



Supplemental Figure 2. *C. neoformans* Growth on Cell Stressor Plates in the Presence of Sex Hormones.

Mean ordinal growth score of all 28 clinical *C. neoformans* isolates in the presence of ethanol (negative control), testosterone, or estrogen. Cells were plated on YPD plates containing SDS, CR, CFW, KCl, NaCl, or $CaCl_2$ and incubated at 37°C. Plates were imaged at days 3, 5, and 7. Data was analyzed using ANOVA with simple contrasts. Error bars are SE.

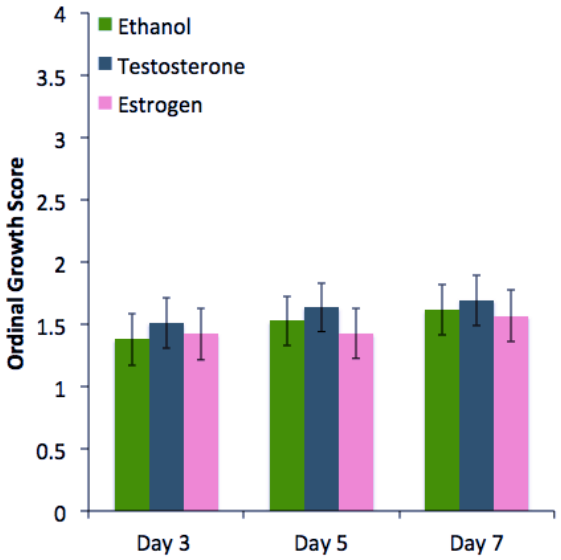
Appendix C: Nitrosative Stress



Supplemental Figure 3. *C. neoformans* Growth on Nitrosative Stressor Plates in the Presence of Sex Hormones.

Mean ordinal growth score of all 28 clinical *C. neoformans* isolates in the presence of ethanol (negative control), testosterone, or estrogen. Cells were plated on YPD plates containing $NaNO_3$ and succinic acid and incubated at 37°C. Plates were imaged at days 3, 5, and 7. Data was analyzed using ANOVA with simple contrasts. Error bars are SE.

Appendix D: Iron Stress



Supplemental Figure 4. *C. neoformans* Growth on Iron Stressor Plates in the Presence of Sex Hormones.

Mean ordinal growth score of all 28 clinical *C. neoformans* isolates in the presence of ethanol (negative control), testosterone, or estrogen. Cells were plated on YPD plates containing $NaNO_3$ and succinic acid and incubated at 37°C. Plates were imaged at days 3, 5, and 7. Data was analyzed using ANOVA with simple contrasts. Error bars are SE.