A NEW QUORUM SENSING MOLECULE IN *C. NEOFORMANS*, GIBBERELLIC ACID, INCREASES MELANIZATION IN THE PRESENCE OF TESTOSTERONE

by

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ABSTRACT

C. neoformans, a spore-producing pathogenic yeast, affects immunocompromised individuals causing meningoencephalitis. Once *C. neoformans* is introduced via the respiratory tract, it is engulfed by macrophages and other phagocytes. One of *C. neoformans's* primary virulence factors is the pigment, melanin, which is produced in the cell wall and protects the yeast against UV radiation and oxidizing agents produced by macrophages during phagocytosis. To better understand the observed sex bias (3:1; male: female) in cryptococcosis infections, I determined the phenotype of various virulence factors in the presence of exogenous sex hormones. The data show a difference in melanization of *C. neoformans* cells in the presence of exogenous testosterone and estrogen, where *C. neoformans* melanizes faster in testosterone. Using a combination of RNA sequencing analysis and ELISA results, I have identified a new quorum sensing molecule, gibberellic acid (GA) in *C. neoformans*. These data suggest that this melanization difference in testosterone is due to increased production of GA, as GA is highly upregulated in the presence of testosterone. Thus, this may help explain the sex bias observed in *C. neoformans* infections.

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L-DOPA- L-3,4-dihydroxyphenylalanine

QSM(s)- Quorum Sensing Molecule(s)

QS- Quorum Sensing

PA- Pantothenic Acid

GA- Gibberellic Acid

RNAseq- RNA sequencing

qRT-PCR- quantitative Reverse Transcription Polymerase Chain Reaction

KO Strains- Knockout Strains

YPD- Yeast Peptone Dextrose

PBS- Phosphate Buffered Saline

Test- Testosterone

Estr- Estrogen

Eth- Ethanol

CC- Chlormequat Chloride

DS- DL-Serine

LS- L-Serine

I
INTRODUCTION

1.1. *Cryptococcus neoformans*

1.1.1. Pathogenesis of *C. neoformans*

Cryptococcus neoformans, a spore-producing pathogenic yeast, affects immunocompromised individuals causing meningoencephalitis (1). According to the CDC, there are about 200,000 cryptococcosis infections yearly, with 181,000 leading to death ("CDC," 2015). *C. neoformans* is typically found in the environment and is most commonly associated with pigeon excreta (Valiente *et al*., 1997), hollows of a variety of trees (4), and excreta-contaminated what? Soil? Missing a word here (5). A *C. neoformans* infection begins by the inhalation of airborne spores that causes pulmonary inflamation (6). This infection is typically asymptomatic (5) and in immunocompetent individuals, *C. neoformans* may stay in a latent state or be cleared by the immune response (2). However, in immunocompromised individuals the infection can develop into cryptococcal pneumonia (2), and using its many virulence factors, *C. neoformans* disseminates from the lungs, crosses the blood brain barrier, causes meningitis, and may ultimately lead to death (7).

C. neoformans infections are a grave concern for HIV/AIDS patients because of their immunosuppressed state. Sub-Saharan Africa currently has the most documented cryptococcosis infections due to the high incidence of HIV (8). Although highly active antiretroviral therapy (HAART) is available, mortality rates among HIV patients remain high in developing countries due to cost or accessibility (8). There is a known sex-bias

associated with *Cryptococcus* infections, whereby seven in ten males will develop the infection, while there is a decreased incidence of disease in females (9-10). Currently the reason for this phenomenon is unknown.

1.1.2. Virulence Factors of *C. neoformans*

C. neoformans has many virulence factors that enable it to manipulate and escape the host's immune system to survive. Its ability to grow at 37ºC makes it an immense threat to humans and aids in the yeast's virulence. One of its main virulence factors is its polysaccharide capsule which is mostly composed of glucuronoxylomannan (GXM) and glucuronoxylomannogalactan (GXMGal) (11). In the environment the capsule protects the yeast from desiccation and from predators such as amoeba (12). In the host, the capsule aids *C. neoformans* survival in a variety of ways. First, it has antiphagocytic properties that aid in evading killing by macrophages (13). This decrease in phagocytosis causes a decrease in antigen presentation which results in T-cell interference (12). The capsule also plays a role once *C. neoformans* is engulfed by exploiting the macrophage environment for replication; this contributes to its parasitism and spread throughout the host (11). The capsule also has the ability to change its structure. This allows for tissue evasion and dissemination from the lungs (11). Lastly, the capsule has immunomodulatory effects that are mediated through GXM (11). It induces the secretion of IL-10, which down regulates proinflammatory cytokines, and causes L-selectin shedding in neutrophils (12).

C. neoformans expresses many enzymes that function as virulence factors. These include: urease, which aids *C. neoformans* dissemination from the lungs into the blood

stream (13). The production of DNases provides the yeast with nucleotides by degrading neutrophilic DNA (14). Superoxide dismutase helps *C. neoformans* to evade macrophage killing and aids in replication within the phagocyte, while phospholipase B degrades the cell membrane allowing for tissue invasion (14). Lastly, *C. neoformans* produces a melanin pigment that provides protection for the yeast both environmentally and inside its host.

1.2. Melanin Production

1.2.1. Melanin Biosynthesis

Melanin synthesis was first discovered by Staib in the 1960s (15). Melanin is a hydrophobic brown/black pigment that protects many fungal species in the environment (16). Fungi utilize two pathways for melanin synthesis. Most pathogenic fungi use the pentaketide pathway (also referred to as the 1,8-dihydroxynaphthalene (DHN) pathway) (16). This pathway requires an endogenous substrate produced by yeast to polymerize DHN melanin (Pal, Gajjar & Vasavada, 2015). Melanin can also be synthesized by the L-3,4-dihydroxyphenylalanine (L-DOPA) pathway, which requires an exogenous substrate (such as host catecholamines), that is catalyzed by laccase to form melanin (16). Very few pathogenic fungal organisms synthesize melanin using this pathway (19). However, *C. neoformans* can only synthesize melanin via the L-DOPA pathway (20). This pathway is similar to mammalian melanin synthesis, which makes *C. neoformans* an ideal organism to study melanin synthesis (16).

1.2.2. Melanin as a Virulence Factor in Pathogenic Yeast

In many pathogenic fungi, melanin production is used as a barrier to protect the yeast from harsh environmental conditions and UV radiation, which aids in survival (21). Within a host however, melanin production is considered a virulence factor because it is used for pathogenesis in a variety of ways depending on the organism. In rice and cucumber plant parasitism, the pathogenic fungi *Magnaporthe grisea* (22) and *Colletotrichum lindemuthianum* (23) both use melanin to create turgor pressure, which allows for tissue invasion (24). Unlike other fungi, *Aspergillus* species produce a blue/green melanin pigment through the DHN pathway that also contributes to virulence (25). *Aspergillus niger* uses melanin as a redox buffer (24), while *Aspergillus fumigatus* uses it to prevent phagocytosis by monocytes (25). Some fungal species, such as *Sporothrix schenckii,* have the ability to produce two types of melanin using both the DHN and L-DOPA pathways; which allows for the expression of different virulence phenotypes depending on the type of melanin that is produced (26).

1.2.3. Melanin as a Virulence Factor in *C. neoformans*

Melanin production is regulated by laccase genes and located in the cell wall of *C. neoformans* (27). Studies have shown that when *LAC1*, the laccase gene, is removed melanization does not occur and the strain is less virulent (28). This indicates that melanin is important to the virulence of *C. neoformans* inside the host. As in other pathogenic fungi, melanin production aids *C. neoformans* survival in the environment against harsh environmental conditions and UV radiation (15). However, inside the host, melanization benefits *C. neoformans* in multiple ways. Melanin production has been

associated with the reduction of tumor necrosis factor-α and lymphoproliferation, which allows *C. neoformans* to avoid phagocytosis (29). If phagocytosed, melanin can also protect the yeast from oxidizing agents produced by macrophages, which allows *C. neoformans* to avoid oxidative damage, and allowing for replication inside the macrophage (21). Lastly, melanin also plays a role in redox buffering because it has a high affinity for metal ions, such as copper and iron (29).

1.3. Testosterone Affects *C. neoformans* Virulence

Sex hormones are naturally secreted in humans and given the observed sex bias in cryptococcosis infections (30), could influence *C. neoformans* virulence in the host. Previous studies have shown that testosterone increases extracellular release of the polysaccharide capsule (31), which is a key virulence factor with immunomodulator activity in the host, suggesting that a testosterone-rich environment could be contributing to an increase in virulence. Previous studies revealed that sex hormones could also affect melanization, with *C. neoformans* cultures grown in the presence of testosterone producing more melanin than those grown in the presence of estrogen (Dr. Tiffany Guess, personal communication). However, the explanation for this observation was undetermined. Melanization is known to be a quorum sensing mediated phenotype, thus I hypothesized that this difference was occurring because testosterone, a known transcriptional regulator, was affecting growth rate through transcriptional regulation.

The adaptation for increased melanization in the presence of testosterone could have possibly evolved from hormones secreted into the environment. Steroid hormones are naturally released into the extracellular environment by both humans and animals (32)

and are stable insoluble lipids that are not broken down when excreted (32). The amount of hormone secreted depends on a variety of factors (diet, pregnancy, pharmaceutical consumption, species, etc.) (32). Regardless of the source, these excreted hormones may have potentially aided the adaptation of *C. neoformans* to testosterone, which could, in part, explain the sex-bias observed in cryptococcosis infections.

1.4. Melanization is Quorum Sensing Mediated

1.4.1. Quorum Sensing Mechanism

Quorum sensing is a mechanism by which microbes release chemical signals called quorum sensing molecules (QSMs) that increase in concentration as a function of cell density (33). Once QSMs exceed a threshold concentration, they stimulate changes in transcription to produce a specific phenotype, such as a melanin pigment, in response to the current environmental condition (34). This mechanism was first identified in the late 1960s in the bioluminescent species *Vibrio fischeri* (35). This bacterium forms a symbiotic relationship with other species and bioluminates when cell density is relatively high (36). This cell density-dependent mechanism is described in other fungal species. *Candida albicans* uses a quorum sensing mechanism for biofilm production and chlamydospore formation (37), while the *Saccharomyces cerevisiae* QSM farnesol, stimulates the reduction of reactive oxygen species levels (38).

1.4.2. Pantothenic Acid Acts as a QSM in *C. neoformans*

The discovery of quorum sensing in *C. neoformans* is fairly recent. A study conducted by Albuquerque *et al* (2013) identified the QSM pantothenic acid (PA) (39). This molecule is thought to be secreted and affects the melanization of *C. neoformans* (39). PA was first discovered in 1933 as a growth factor for microorganisms (40). It is a precursor to coenzyme A (41) and is known to stimulate growth in *Aspergillus niger* (40-41). In other yeast, such as *Saccharomyces cerevisiae,* exogenous PA is a requirement for growth (41), suggesting that the yeast does not make all the required enzymes in the PA pathway for biosynthesis (41). However, in *C. neoformans*, NMR results indicate that PA is naturally secreted by the yeast (39). Since PA acts as a growth factor, it is likely increasing the growth of *C. neoformans* and the increase in cell density results in increased melanization.

1.4.3. Gibberellic Acid is a Potential QSM in *C. neoformans*

Gibberellic Acid (GA) is a growth hormone most commonly found in plants, and regulates the growth rate of seedlings (44). This acid, first identified in the pathogenic fungi *Gibberella fujikuroi,* functions in cell growth (45) and, has since been found in a number of other fungi (46). Although identified in fungi, the ability to produce this metabolite is thought to have evolved from plants through a symbiotic relationship (46). *Gibberella fujikuroi* is commonly associated with rice plants (47). When the fungi secretes GA there is an increase in the rice plant growth by controlling and promoting germination (47).

To our knowledge GA production has never been identified in *C. neoformans*. However, transcriptome analysis revealed multiple genes in the gibberellic biosynthesis pathway that were upregulated when *C. neoformans* melanized in the presence of testosterone. These data lead to the hypothesis that GA production is upregulated in the presence of testosterone and affects growth rate, which increases the rate of melanization, thus acting as a QSM in *C. neoformans*.

II

MATERIALS AND METHODS

2.1. *C. neoformans* Strains

All experiments were conducted using the wildtype *C. neoformans* strain, H99S (serotype A) (48-49), unless otherwise stated. Clinical isolates obtained from patients in Botswana (50) were used in preliminary experiments to determine if melanization varied from the wildtype. *C. neoformans* knock out strains of genes identified from the RNA sequencing data were obtained from Dr. Hiten Madhani (UCSF).

Table 1: List of strains used in experiments.

Strains	Strain Information
H99S	*C. neoformans* wildtype (serotype A) (48-49)
H99S-GFP	Fluorescently labeled *C. neoformans* wildtype
B68	Clinical isolate of *C. neoformans* (serotype A) (50)
B62	Clinical isolate of *C. neoformans* (serotype A) (50)
B58	Clinical isolate of *C. neoformans* (serotype A) (50)
B30	Clinical isolate of *C. neoformans* (serotype A) (50)
ΔLAC1	Mutant strain derived from wildtype (serotype A)
CNAG_03340	Flavonol synthase knockout strain (a kind gift form Dr. Hiten Madhani, UCSF)
CNAG_03857	Hypothetical protein knockout strain (a kind gift form Dr. Hiten Madhani, UCSF)
CNAG_05356	Hypothetical protein knockout strain (a kind gift form Dr. Hiten Madhani, UCSF)
CNAG_04029	Cytochrome P450 knockout strain (a kind gift form Dr. Hiten Madhani, UCSF)
CNAG_02841	Cytochrome P450 monooxygenase pc-2 knockout strain (a kind gift form Dr. Hiten Madhani, UCSF)

2.2. Thawing and Preparing *C. neoformans* Cell Stocks and Hormone Preparation

C. neoformans was stored at -80°C to prevent microevolution. All yeast cultures were prepared in a Biosafety Level II cabinet to prevent contamination. Yeast Peptone Dextrose (YPD) broth in varying volumes was inoculated in flasks using frozen stocks . The flask was rotated in a 37°C orbital incubator at 150 RPM for 24-36 h to achieve log phase. After incubation, the culture was centrifuged for 5 minutes at 700 x *g* to pellet the yeast. The culture was washed three times and suspended in phosphate buffered saline (PBS). Using a hemocytometer, cells were counted and adjusted to a final concentration of 1×10^6 cells/mL for all experiments unless otherwise stated.

All experiments with exogenous steroid hormones contained physiological concentrations of exogenous steroid hormones, unless otherwise stated. Testosterone was

diluted to a concentration of 10 ng/mL (51) and 17β-estradiol (estrogen) was diluted to a concentration of 400 pg/mL (51). Both hormone stocks were diluted using ethanol; therefore, an ethanol treatment was utilized in experiments as a vehicle control.

2.3. Cultivating *C. neoformans* on Agar Plates

H99S and clinical isolates (50) (Table 1) were prepared as stated in section 2.2. On three L-DOPA plates, supplemented with testosterone, estrogen, or ethanol, 25 µL of *C. neoformans* were streaked. To test if melanization could occur on various substrates, 25 µL of *C. neoformans* was plated on four plates containing L-DOPA, testosterone, estrogen or ethanol. All plates were protected from light and grown in a 37°C incubator for seven days. On days, three, five, and seven, images were taken using a digital camera to qualitatively document the degree of melanization.

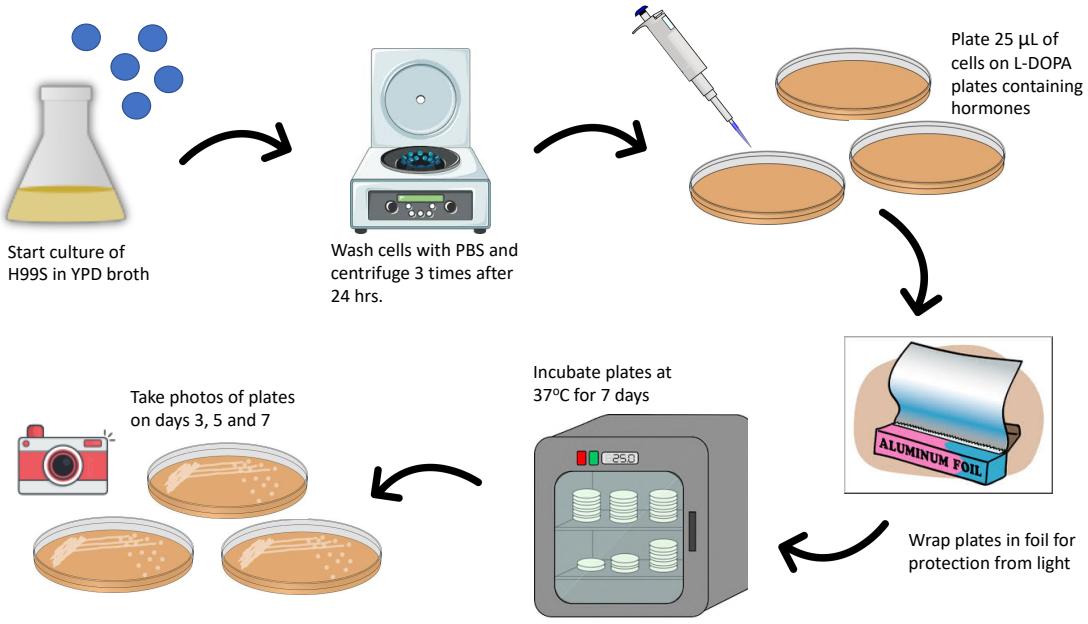


Figure 1: Schematic showing the method for plating *C. neoformans* on L-DOPA plates to observe melanization.

2.4. Cultivating *C. neoformans* Wildtype and Knockout Strains in L-DOPA Liquid Medium

H99S and knockout (KO) strains (Table 1; Hiten Madhani, UCSF) were prepared as stated in section 2.2. Three flasks were treated with testosterone, estrogen, or ethanol, respectively for H99S. Flasks treated with and without testosterone were prepared for KO strains. Cells, hormones and L-DOPA liquid media were all added to the flasks at a final volume of 10 mL. The flasks were protected from light and placed in a 37°C orbital shaker for five days. Every 24 h, 1 mL was removed for an absorbance reading at 400 nm and 600 nm.

2.5. Testosterone Dose Response During Melanization

H99S was prepared as stated in section 2.2. Five concentrations of exogenous testosterone, 0 ng/mL, 0.5 ng/mL, 2 ng/mL, 5 ng/mL, and 10 ng/mL were added to the cells and L-DOPA media respectively. The flasks were protected from light and incubated for five days in a 37°C orbital shaker at 150 RPM. Every 24 h, 1 mL was removed for an absorbance measurement of 400 nm and 600 nm.

2.6. RNA Isolation

H99S was prepared as stated in section 2.2. Three flasks were treated with testosterone, estrogen, or ethanol, respectively. Cells, hormones and L-DOPA liquid media were all added to the flasks at a final volume of 10 mL. The flasks were protected from light and placed in a 37°C orbital shaker for three days. After incubation, cells were spun at 700 x *g* for 5 minutes, counted and diluted to a final concentration of 2×10^8 cells for RNA isolation using a RNeasy Midi Kit (Qiagen). Genomic DNA contamination was

removed from the total RNA using a Message Clean Kit (GeneHunter) and the RNA concentration was measured using a NanoDrop 2000 Spectrophotometer (Thermo-Scientific).

2.7. Quantitative Reverse Transcription Polymerase Chain Reaction

Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was performed using isolated RNA from *C. neoformans* at day three of melanization. cDNA was prepared using the QuantiNova™ Reverse Transcription kit (Qiagen). cDNA, primers, and SYBR green were added to a 96 well PCR plate. Each cDNA treatment was prefrormed in quadruplicate and normalized using the geometric mean of the housekeeping genes *UBC6* or *TFC1*. The data was analyzed using the CFX96 Real-time PCR Detection System (BioRad). Fold changes were determined using the $2^{-\Delta\Delta C'_T}$ method as by KJ Livak and TD Schmittgen (53).

2.8. cDNA Preparation for RNA Sequencing

cDNA libraries for RNA sequencing (RNAseq) were prepared from 1 µg of isolated RNA using the NEBNext® Ultra™ Directional RNA Library Prep Kit for Illumina® (New England BioLabs), the NEBNext Multiplex Oligos for Illumina® Index Primers and the NEBNext Poly(A) Magnetic Isolation Module (New England BioLabs). Sequencing was completed by HudsonAlpha Genomic Sequencing Lab (Huntsville, AL) using the HiSeq 2500 system.

2.9. RNA Sequencing Analysis (Bioinformatics)

RNAseq analysis (Figure 2) was completed using two analytical platforms, CyVerse (https://de.cyverse.org) and Galaxy (https://usegalaxy.org). Quality check was completed on each paired end read using FASTQC (version 0.11.5 multi-file). Due to issues with STAR (version 2.5.3a-index-align), the paired end reads were aligned using HISAT2 (version 2.1-index-align) to a *C. neoformans* reference genome (H99S) (https://useast.ensembl.org).

Bam files produced from the HISAT2 (version 2.1-index-align) alignment were utilized in two ways. In Galaxy the bam files were used to quantify the number of reads using a genome annotation file (https://useast.ensembl.org) of *C. neoformans* (featureCounts). MultiJoin (also in Galaxy) was used to join the output files of featureCounts into a single tabular file. This file was utilized in R studio using the edgeR differential expression package. To determine the similarities among replicates in the treatment groups (testosterone, estrogen and ethanol) and to ensure any outliers would be removed, multidimensional scale plots (MDS plot) and a cluster dendrogram were utilized.

Once cluster analysis was complete, the reads were aligned into any potential transcripts using StringTie and the genome annotation (version 1.3.3) (CyVerse). Transcripts for all replicates in the treatment groups were then merged into a single gtf file using StringTie-Merge (version 1.3.3). Lastly, CuffDiff2 (version 16-way max) was utilized to compare the genome alignments (bam files) and the transcript alignments (gtf files) for gene expression changes that were significantly different. This output file was

used to determine any genes that were upregulated when *C. neoformans* was in the presence of testosterone compared to the other treatment groups.

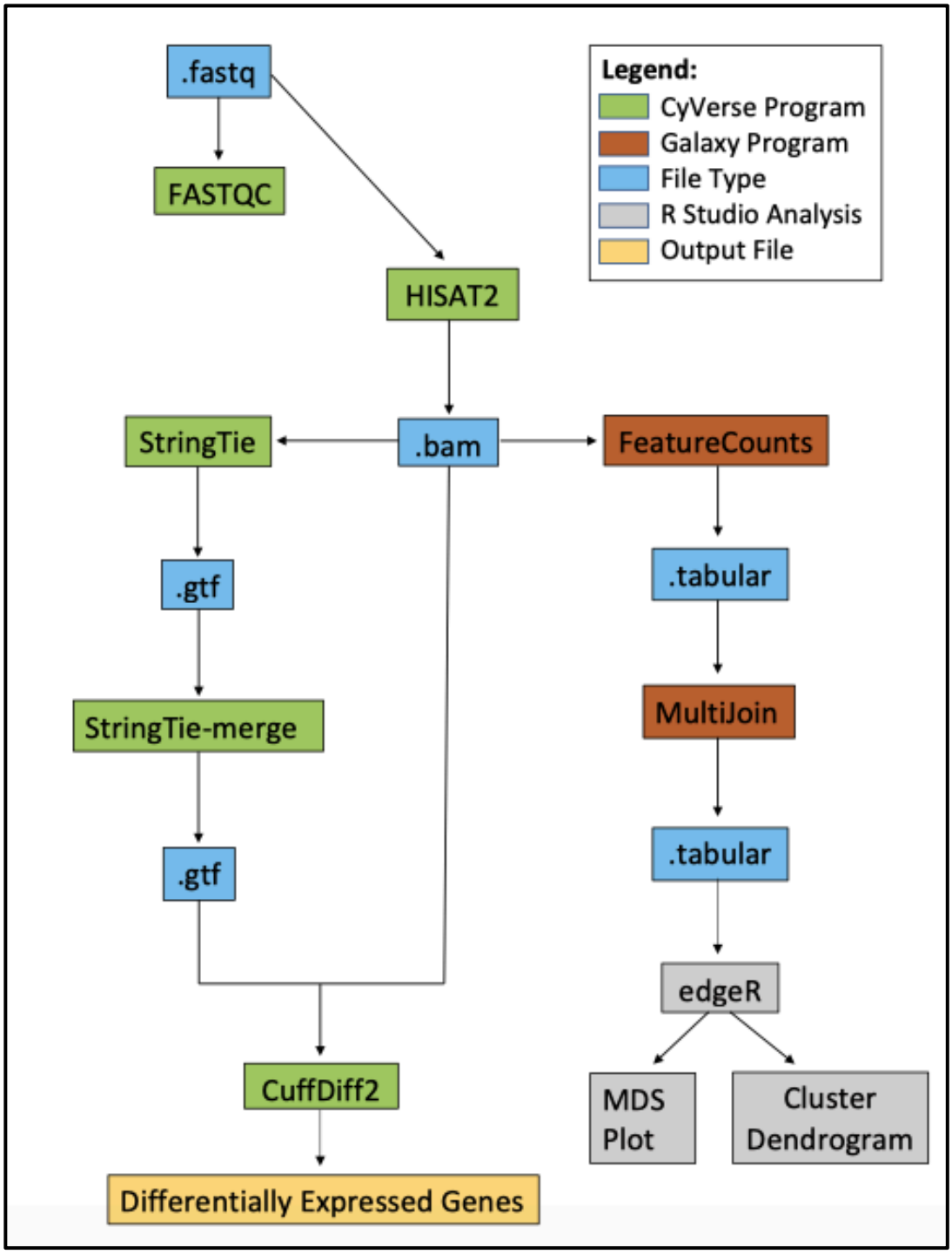


Figure 2: Schematic of the pipeline for RNA sequencing analyses

2.10. Serial Dilutions of *C. neoformans*

H99S was prepared as stated in section 2.2 with the exception that cells were counted and diluted to a starting concentration of $1x10^6$ cells/mL in a total volume of 200 µL. Three 10-fold dilutions (10^5, 10^4, 10^3) were also prepared from the starting concentration in a total volume of 100 µL. On L-DOPA plates, treated with testosterone, estrogen, or ethanol, 100 µL of each cell concentration were plated in spots. The plates were protected from light and grown in a 37°C incubator for seven days. On days, three, five and seven, images were taken using a digital camera to document the amount of melanization.

For quantitative serial dilution data, liquid L-DOPA media containing testosterone, estrogen, or ethanol was added to polypropylene round bottom snap-cap tubes (Falcon®), along with each of the cell concentrations. The tubes were protected from light and grown in a 37°C orbital incubator at 150 RPM for five days. Every 24 h, 1 mL was removed for an absorbance reading at 400 nm and 600 nm.

2.11. Cultivating *C. neoformans* Wildtype and Knockout Strains in Conditioned Media

H99S and KO strains (Table 1, Hiten Madhani, UCSF) were prepared as stated in section 2.2. For H99S, flasks were treated with testosterone, estrogen, or ethanol, respectively. For KO strains, flasks were treated with and without testosterone only. Cells, hormones and L-DOPA liquid media were all added to the flasks at a final volume of 10 mL. The flasks were protected from light and placed in a 37°C shaker for seven days. On day six, new H99S and KO cultures were grown for 24 h in YPD. On day seven, the melanized cultures were centrifuged at 700 x *g* for 5 minutes to pellet the

yeast. The new H99S and KO cultures were prepared as stated in section 2.2. In three flasks treated with testosterone, estrogen, or ethanol, respectively, new L-DOPA media and the supernatants of the melanized cultures were added in a 1:1 ratio along with fresh *C. neoformans* cells for a total volume of 10 mL. The flasks were protected from light and incubated in an orbital shaker. An absorbance reading was measured at 400 nm and 600 nm at 16, 24 and 32 h time points. Flasks were imaged using a digital camera at the same time points.

2.12. Cultivating *C. neoformans* in Conditioned Media with Exogenous Quorum Sensing Molecules and Quorum Sensing Molecule Inhibitors (Figure 3)

H99S was prepared as stated in section 2.2. Cells and L-DOPA liquid media were added to the flasks at a final volume of 10 mL. The flasks were protected from light and placed in a 37^{o}C shaker for seven days. On day six, a new H99S culture was grown for 24 h in YPD. On day seven, the melanized cultures were centrifuged at 2000 RPM (700 x *g*) for 5 minutes to pellet the yeast. The new H99S culture was prepared as stated in section 2.2. Using polypropylene round bottom snap-cap tubes (Falcon®), 3 µg/mL of GA (Sigma), 2×10^{-6} M of chlormequat chloride (GA inhibitor, Sigma), 10 µM of PA (MP Biomedicals), 1 mg/mL of DL-Serine (PA inhibitor, Alfa Aesar) and 2 mg/mL of L-Serine (Honeywell Fluka™) were added, to respective tubes with or without exogenous testosterone along with the new *C. neoformans* cells. Two tubes contained H99S (plus/minus testosterone) without additives added for comparison. New L-DOPA media and the supernatants of the melanized cultures were added in a 1:1 ratio. The tubes were

protected from light and incubated at 37ºC in an orbital shaker at 150 RPM. An absorbance reading was measured at 400 nm at the 16, 24, and 32 h time points.

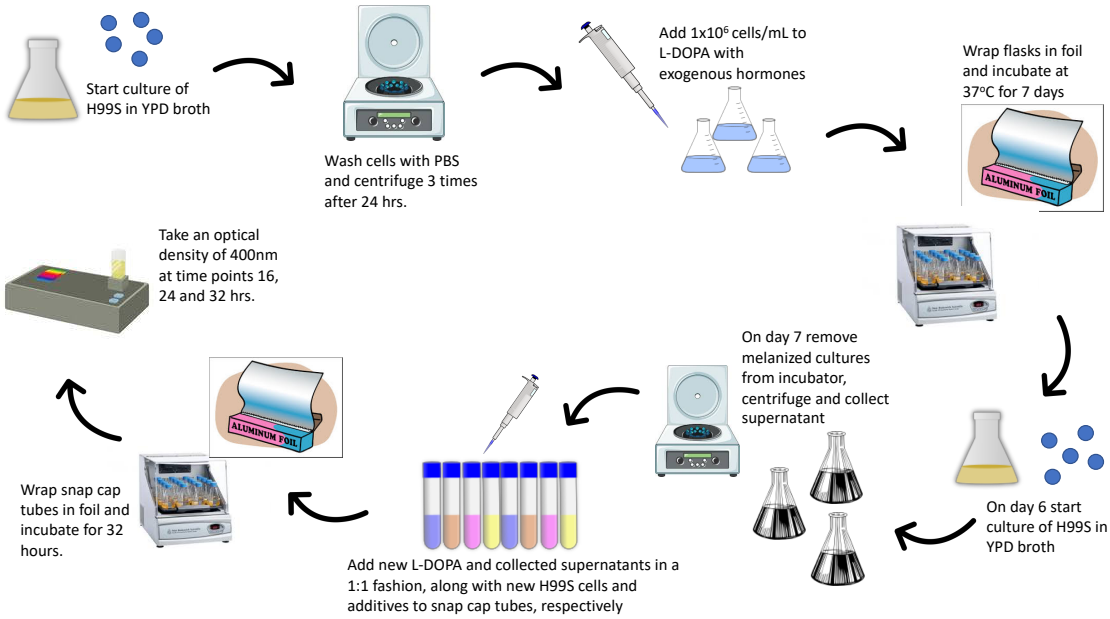


Figure 3: Schematic showing the method for cultivating *C. neoformans* in conditioned media with additives to observe melanization.

2.13. ELISAs

GA and PA concentrations in *C. neoformans* supernatants during melanization were determined using ELISAs. A standard curve for both QSMs were utilized using known standard values (Gokani & Thaker, 2002 ;Gonthier *et al*, 1998). A primary rabbit polyclonal antibody (Lifespan Biosciences) for both GA and PA was added to the samples and they were incubated in a 37ºC water bath for 1 h. After incubation the samples were transferred to a 96 well F-bottom microplate (Greiner Bio-One) in duplicate and incubated again at 37ºC for 1 h. Using a microplate washer (BioTek), the wells were washed with PBS + 0.5% Tween 20 wash buffer. A secondary donkey anti-

rabbit horseradish peroxidase polyclonal antibody (Southern Biotech) was added to each well and the plate was incubated for another hour at 37ºC. After incubation, the plate was washed using PBS + 0.5% Tween 20. A TMB substrate (Thermo Fisher Scientific) was added to each well and the plate was incubated for 30 minutes at room temperature. After incubation the stop solution, 2N sulfuric acid, was added to each well. The absorbance of each sample was measured using the CLARIOstar Plus Microplate Reader (BMG LABTECH) at an 450 nm.

2.14. Tissue Culture Techniques- Passaging, Thawing and Freezing

J774A.1 (ATCC® TIB-67™) murine macrophages were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (HyClone™) containing 4.5 g/L D-glucose with 4 mM L-glutamine, supplemented with 10% of heat inactivated fetal bovine serum (FBS) (CORNING Inc.), 1% nonessential amino acids (CORNING Inc.), 1% penicillin, and 10% 1X NCTC-109 (Thermo Fisher) in a 100 mm tissue culture petri dish (Falcon®). The cells were maintained in a 37°C incubator with 5% CO_2 humidified atmosphere and grown until they were 80% confluent.

Passaging:

Once cells were confluent, growth medium was aspirated, the cells were washed with 5 mL of warm PBS and dissociated from the surface of the plate by repeated washing with 10 mL of warm DMEM (HyClone™). Once resuspended, the cells were transferred to a conical tube. The cells were reseeded by completing a 1:10 split in a new 100 mm x 20

mm tissue culture petri dish (Falcon®) containing fresh medium. Once passage twenty-five was reached, the cells were discarded and fresh cells were thawed.

Thawing:

J774A.1 macrophages were kept frozen in a liquid nitrogen cryogenic tank and thawed in a 37°C water bath. Once thawed, cells were added to 10 mL of DMEM (HyClone™) and centrifuged at 300 *x g* for 7 minutes to pellet and remove dimethyl sulfide (DMSO). The supernatant was discarded, and the pellet was resuspended in 10 mL of DMEM (HyClone™) and seeded into a 100 mm x 20 mm tissue culture petri dish (Falcon®).

Freezing:

Low passage J774A.1 macrophages were grown until they were 80% confluent. The cells were washed with 5 mL of warm PBS and dissociated from the plate by resuspending in 10 mL of warm DMEM (HyClone™). The resuspended cells were transferred to a conical tube and centrifuged at 300 *x g* for 5 minutes to pellet. The media was discarded, and the cells were resuspended in 90% cell medium and 10% DMSO in a total volume of 1 mL. The cells were then transferred to a labeled 2 mL screw cap (Greiner Bio-One) tube and stored in a liquid nitrogen tank.

2.15. Fungal burden of J774A.1 macrophages

H99S was prepared as stated in section 2.2. Three flasks were treated with testosterone, estrogen, or ethanol, respectively. Cells, hormones and L-DOPA liquid

media were added to the flasks at a final volume of 10 mL. The flasks were incubated for seven days at 37 °C in an orbital shaker.

Seeding:

On day six, J774A.1 macrophages were diluted and counted using trypan blue (Sigma). Macrophages were seeded in a tissue culture 96 well flat bottom plate with a low evaporation lid (Falcon®) at a concentration of 2 x 10^5 cells/mL by adding macrophages, 200 units/mL IFN-γ, and DMEM (HyClone™). Two hundred µl of the macrophage mix was added to each well (28 wells). A new culture of H99S was also grown in YPD for 24 h.

Infecting:

The 24 h H99S culture was washed three times and suspended in PBS. The cells were diluted and counted to a final concentration of 3.6 X 10^6 cells/mL. H99S cultures that melanized for seven days were centrifuged and suspended in PBS, diluted and counted to a final concentration of 3.6 X 10^6 cells/mL for a 2:1 infection ratio with macrophages. *C. neoformans* cells were opsonized for 30 minutes in a 37°C water bath by adding 1 µg/mL LPS, 10 µg/mL anti-GXM IgG 18B7 (a kind gift of Dr. Arturo Casadevall), 200 U/mL IFN-γ, and DMEM (HyClone™) for a total volume of 1 mL; hormones were added to the unmelanized *C. neoformans* cells as well. Media was removed from the 96 well plate and 100 µl of each opsonized *C. neoformans* mix was added to the appropriate well. An additional 100 microliters of DMEM (HyClone™) was added to each well and the plate was centrifuged at 300 *x g* for 1 minute to bring the floating *C. neoformans* cells down to

contact the macrophages to ensure phagocytosis. The plate was incubated at 37 °C and 5% $CO_2$for 1 hour. After incubation, the media was removed, and each well was washed with 300 µl of PBS. Two hundred microliters of DMEM (HyClone™) was added to each well and the plate was incubated for 18 hours at 37 °C and 5% CO_2.

Inoculum Plating:

A 1:200 dilution was made using PBS and the inoculum. Ten microliters of that dilution was added to 350 µl of PBS and 100 µl was plated on two labeled YPD plates for each inoculum. The plates were incubated inverted on the lab bench at ambient temperature for three days.

Lysing:

After the 18-hour incubation, 100 µl 0.5% sodium dodecyl sulfate (SDS) was added to each well. The plate was incubated for 5 minutes at room temperature to lyse macrophages. After 5 minutes, all contents in the wells were transferred to individual microcentrifuge tubes. All wells were then washed with 100 µl of PBS and added to the same individual microcentrifuge tubes.

Plating:

For each strain, one well was counted to determine the appropriate dilution for plating. For each tube, 100 µl was plated on two labeled YPD plates. All plates were incubated inverted for two days in a 37 °C incubator. After the two-day incubation (three-day incubation for inoculum plates), the plates were counted and the CFU's were determined.

2.16. Phagocytosis of J774A.1 macrophages

H99S-GFP was prepared as stated in section 2.2. Flasks were treated with exogenous hormones. Cells, hormones and L-DOPA liquid media were all added to the flasks at a final volume of 10 mL. The flasks were incubated for three days in an orbital shaker. On day two a new culture of *C. neoformans* (H99S-GFP, a kind gift of Dr. Robin May) was grown in YPD and J774A.1 macrophages were counted, diluted and seeded in a 29 mm glass dish with a 14 mm bottom well (Cellvis) at a concentration of 5 x 10^5 cells/mL for 24 h. On the third day, all *C. neoformans* cultures were centrifuged (700 x *g*) and suspended in PBS, diluted, counted and adjusted to a final concentration of 3.6 X 10^6 cells/mL for a 2:1 infection ratio with macrophages. *C. neoformans* cells were opsonized for 30 minutes in a 37°C water bath by adding 1 μg/mL LPS, 10 μg/mL anti-GXM IgG 18B7, 200 U/mL IFN-γ, and DMEM (HyClone™) for a total volume of 1 mL; hormones were added to the unmelanized *C. neoformans* cells as well. Media was removed from the dishes and opsonized *C. neoformans* along with new DMEM (HyClone™) was added for a total volume of 1 mL. The plate was centrifuged at 300 *x g* for 1 minute to bring the floating *C. neoformans* cells down to contact the macrophages to ensure phagocytosis and incubated at 37 °C and 5% CO_2 for 1 hour. After incubation the media was removed, and cells were suspended in 1 mL of PBS containing 5 μg/mL of calcofluor white (Sigma-Aldrich) and 10 μg/mL propidium iodide (Invitrogen). Macrophages were imaged using the Nikon Elements Software and a Nikon Eclipse Ti-E widefield confocal microscope. To calculate percentages of phagocytosis, one-hundred macrophages with and without *C. neoformans* cells were counted.

2.17. Statistical Analysis

Melanization experments in liquid L-DOPA media over 5 days were analyzed using linear regression analysis. Conditioned media experiments were analyzed using MANOVA with simple contrasts. ELISA data were analyzed using Tukey Kramers Honest Significant Difference for multiple comparisons. qRT-PCR data were analyzed using the $2^{-\Delta\Delta C'_T}$ method (Rao *et al*., 2013) for relative gene expression analysis. RNAseq data were analyzed using edgeR differential expression analysis using R studio (Boston, MA). Statistical analysis was conducted using JMP, version 14 (SAS Institute, Cary, NC). A p-value < 0.05 was considered significant.

III

RESULTS

3.1. *C. neoformans* Exhibits Melanization Differences When Grown on L-DOPA Agar Plates and Liquid Medium Supplemented with Steroid Hormones

Previous studies completed by Dr. Tiffany Guess suggested that steroid hormones could influence the melanization rate of *C. neoformans.* To verify this, a number of *C. neoformans* clinical isolates and the wildtype strain, H99S, were grown on L-DOPA agar plates containing steroid hormones for seven days to determine if there was any variation in melanization (Figure 4A). On days three, five, and seven, images were taken of plates using a digital camera to document the amount of melanization. Visually, there was a clear difference in melanization when *C. neoformans* was in the presence of testosterone; unfortunately, this data was only qualitative. In order to derive quantitative data, H99S was grown in liquid L-DOPA medium for five days (Figure 4B and 4C). Each day an absorbance reading at 400 nm and 600 nm was measured. By day three, an increase in melanization when *C. neoformans* was in the presence of testosterone became apparent. This trend continued throughout day five. The growth rate of *C. neoformans* also increased more in the presence of testosterone (Figure 4C). These data suggest that this variation in *C. neoformans* melanization was cell density dependent and the growth rate was affected by testosterone.

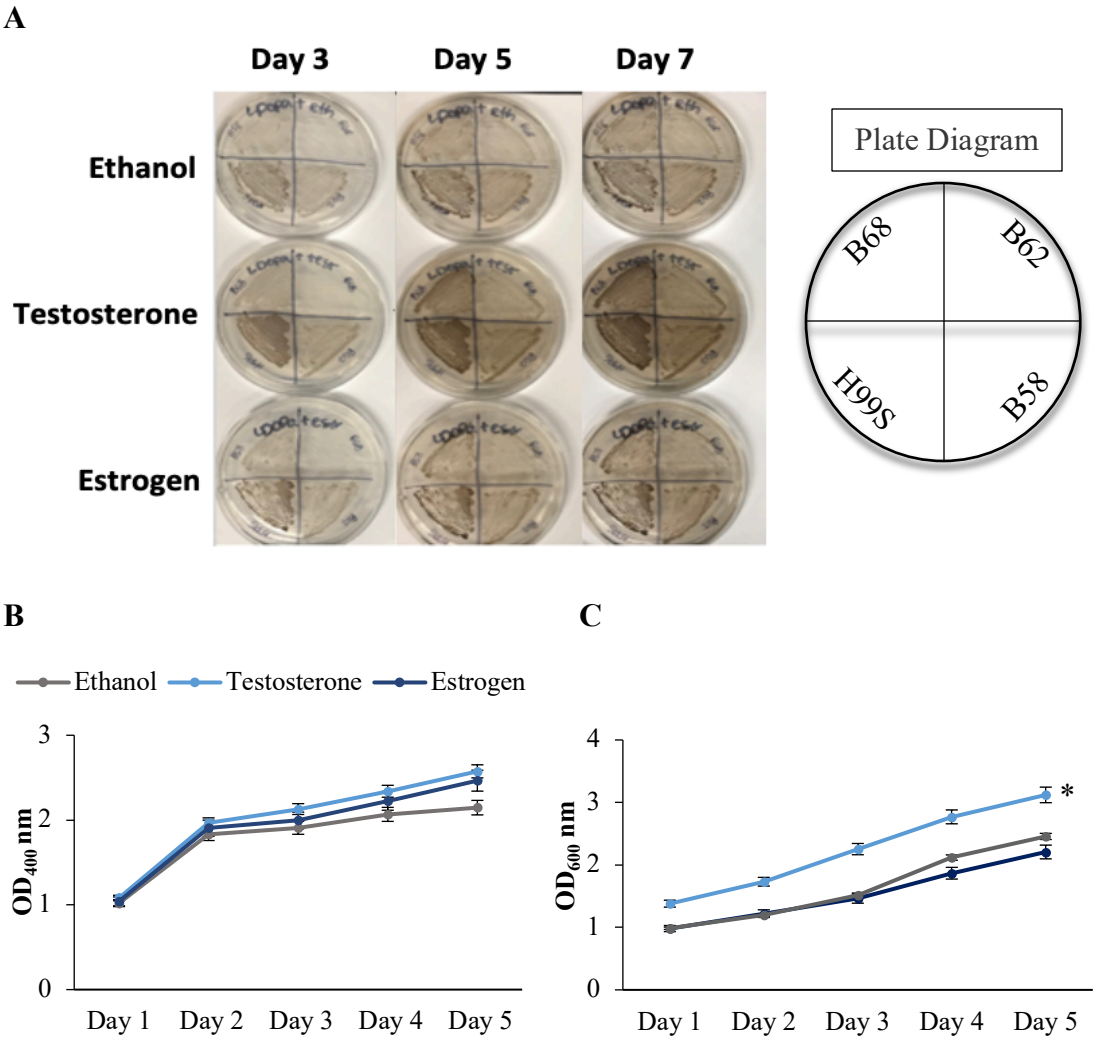


Figure 4: Melanization of *C. neoformans* in the presence of steroid hormones. A: The wildtype strain, H99S and clinical isolates: B62, B68 and B58 grown on L-DOPA agar plates for seven days. Pictures were taken using a digital camera on days 3, 5 and 7. **B:** The wildtype strain, H99S, was grown in liquid L-DOPA media for five days, and read at an absorbance of 400 nm to detect melanization differences. Error bars represent the standard error of the mean. Data is representative of three independent experiments, N=3. **C:** The wildtype strain, H99S, was grown in liquid L-DOPA media for five days, and read at an absorbance of 600 nm to detect growth rate during melanization. Error bars represent the standard error of the mean. Data are representative of three independent experiments. Statistical significance is represented as follows: $p < 0.01$ using linear regression analysis comparing testosterone to estrogen, N=3.

3.2. Confirmation That Testosterone is Not a Substrate Utilized for Melanization in *C. neoformans*

Due to an increase in growth rate and melanization when *C. neoformans* was in the presence of testosterone, the initial hypothesis was that *C. neoformans* was using testosterone as a substrate to melanize. To test this, *C. neoformans* was grown on four plates containing different substrates, testosterone, estrogen, ethanol, and L-DOPA, for seven days (Figure 5). From previous experiments and the literature, we expected melanization to occur on the plate containing L-DOPA, so this served as the positive control in the experiment. However, by day seven *C. neoformans* had grown on all the plates and while melanization was evident on plates supplemented with L-DOPA, no melanization had occurred on the steroid hormone plates. This indicated that our original hypothesis was incorrect, and that testosterone was affecting melanization of *C. neoformans* another way.

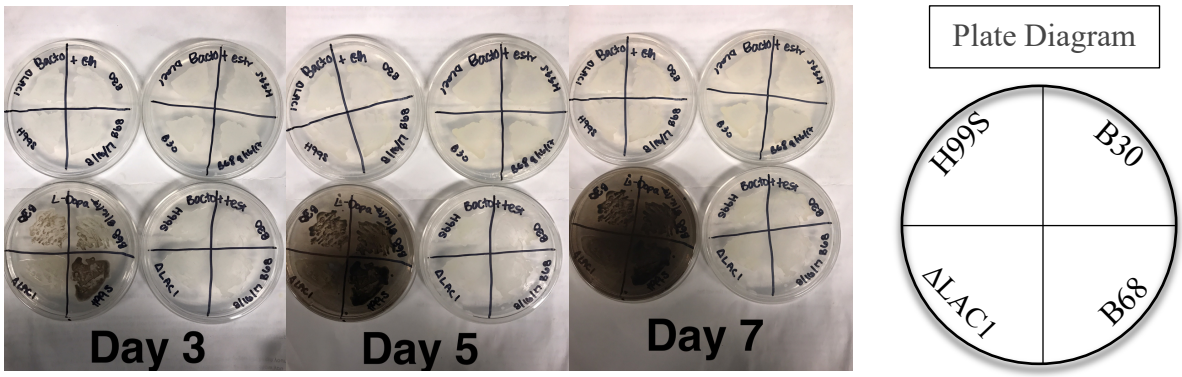


Figure 5: Melanization of *C. neoformans* using steroid hormones as a substrate compared to L-DOPA on days three, five, and seven. Plates were treated with testosterone, estrogen, ethanol, or L-DOPA, respectively and incubated at 37°C for seven days.

3.3. Various Concentrations of Testosterone Affect Melanization

With the knowledge that testosterone was not being used as a substrate for *C. neoformans* to melanize, I decided to look at additional aspects of testosterone that could have an effect on melanization. One factor that was controlled throughout the experiments was the amount of testosterone added. In every experiment, a dose of 10 ng/mL of testosterone was added to represent physiological levels. However, all males do not produce the same amount of testosterone (Kelsey *et al*., 2014), and some males receive additional testosterone through therapeutic shots for various reasons. This led to the hypothesis that the amount of testosterone present could have an effect on melanization. A dose response experiment was preformed to test this hypothesis using the following concentrations of testosterone; 0 ng/mL, 0.5 ng/mL, 2 ng/mL, 5 ng/mL and 10 ng/mL (Figure 6). Testing this range is beneficial because it reflects physiological levels of testosterone in males (2-10 ng/mL) (52) and females (0.15-0.7 ng/mL) (59). Over five days there was a direct relationship between the concentration of testosterone and melanization (Figure 6A). The data also revealed that once a certain threshold was met (between 2 ng/mL and 5 ng/mL), melanization increased exponentially. We also saw this same threshold and proportional relationship in *C. neoformans* growth rate as testosterone concentration increased, further validating that melanization was cell density dependent (Figure 6B). This data could potentially aid in determining if cryptococcosis infections in males are virulent depending on the concentration of testosterone present.

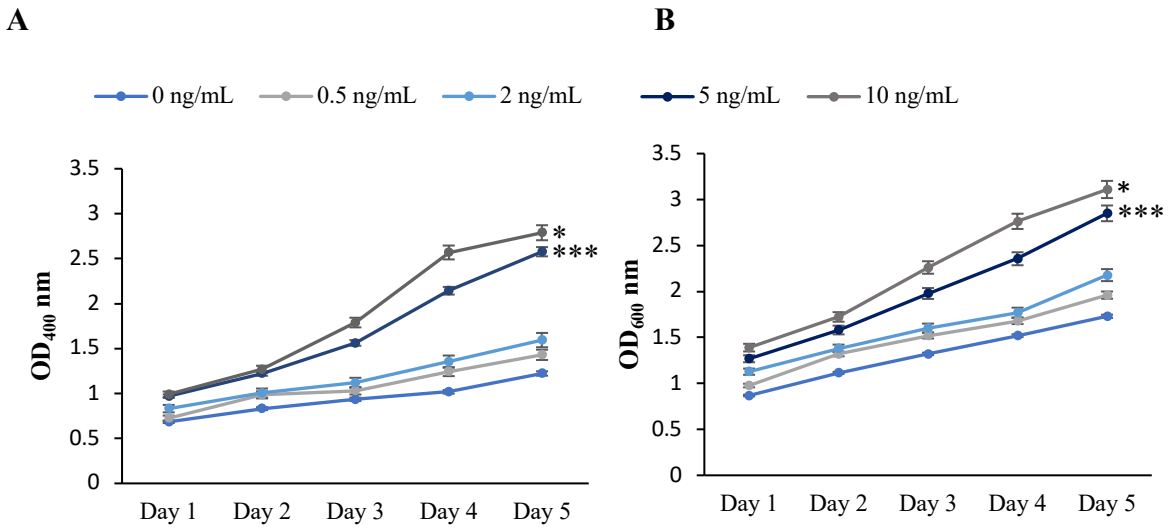


Figure 6: Dose response of testosterone during the melanization of *C. neoformans*. **A:** The wildtype strain, H99S, was grown in liquid L-DOPA media with varying concentrations of testosterone; 0 ng/mL, 0.5 ng/mL, 2 ng/mL, 5 ng/mL and 10 ng/mL. *C. neoformans* was grown for five days and read at an absorbance of 400 nm to detect melanization differences. Error bars represent the standard error of the mean. Data is representative of three independent experiments. Statistical significance is represented as follows: $p = 0.036$ comparing 10 ng/mL to 5 ng/ mL, $p < 0.0001$ comparing 5 ng/mL to 2 ng/mL using linear regression analysis, N=3**. B:** The wildtype strain, H99S, was grown in liquid L-DOPA media with varying concentrations of testosterone; 0 ng/mL, 0.5 ng/mL, 2 ng/mL, 5 ng/mL and 10 ng/mL. *C. neoformans* was grown for five days and read at an absorbance 600 nm to detect growth rate during melanization. Error bars represent the standard error of the mean. Data are representative of three independent experiments. Statistical significance is represented as follows: $p = 0.02$ comparing 10 ng/mL to 5 ng/ mL, $p < 0.0001$ comparing 5 ng/mL to 2 ng/mL using linear regression analysis, N=3.

3.4. Initial qRT-PCR Screen Identified Genes Upregulated When *C. neoformans* is in the Presence of Testosterone

Due to the known effects of steroid hormones on gene regulation (Ing, 2005; Parker, 1988), I hypothesized that testosterones effects were at the transcriptional level, leading me to utilize qRT-PCR on genes identified in the literature that were known to be involved in *C. neoformans* melanization or quorum sensing (Figure 7). Tup1 is a general regulator of a variety of genes and thought to be involved in the quorum sensing

mechanism observed in *C. neoformans* (39). qRT-PCR data shows it was upregulated when *C. neoformans* was grown in the presence of testosterone (Figure 7A). This suggested that testosterone was possibly affecting melanization by regulating quorum sensing genes. The gene CNAG_03922 was identified in a microarray study completed by Eisenmann *et al, 2011* and encodes a signal peptide upregulated during the melanization of *C. neoformans* (28). This gene was chosen to determine if it was upregulated more when *C. neoformans* was cultured in the presence of testosterone than in estrogen during melanization. qRT-PCR data confirmed that this was true (Figure 7B). The genes *RAS1* and *CYR1* are known to be involved in the *C. albicans* farnesol quorum sensing pathway. *C. neoformans* has homologues (CNAG_01672 and CNAG_03202) for these genes and qRT-PCR results revealed that they were also upregulated when *C. neoformans* was cultured in the presence of testosterone (Figures 7C + 7D). These data suggest that these genes could also be a part of a quorum sensing pathway in *C. neoformans* as well, which is consistent with the idea that testosterone could be affecting melanization by influencing the expression of quorum sensing genes. Due to the difference in gene expression seen in these four genes when *C. neoformans* was in the presence of testosterone compared to estrogen, RNAseq was performed to determine if any other genes were significantly affected.

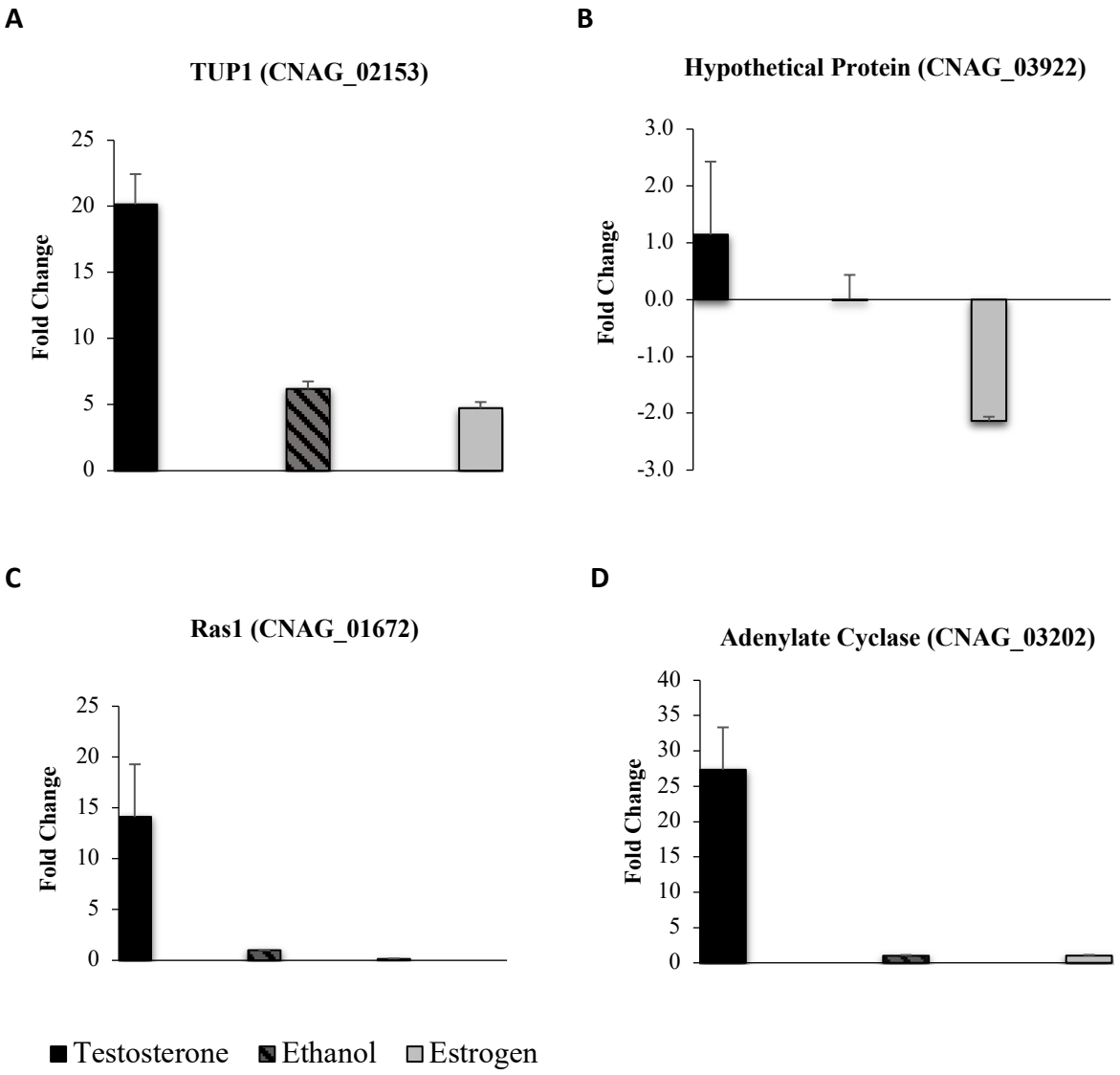


Figure 7: qRT-PCR analysis of genes associated with quorum sensing after exposure of *C. neoformans* to steroid hormones. A: qRT-PCR results of *TUP1*, a gene thought to be involved in quorum sensing in *C. neoformans*. **B:** qRT-PCR results of a hypothetical protein, a gene upregulated during melanization of *C. neoformans* without steroid hormones. **C:** qRT-PCR results of *RAS1*, a gene involved in the *C. albicans* farnesol quorum sensing pathway. **D:** qRT-PCR results of adenylate cyclase, a gene involved in the *C. albicans* farnesol quorum sensing pathway. Error bars represent the standard error of the mean. Fold change was calculated using the geometric mean of two housekeeping genes. Data are representative of three independent experiments.

3.5. Transcriptome Analysis of *C. neoformans* Cultured in the Presence of Steroid Hormones Reveals Genes Significantly Regulated in the Presence of Testosterone

RNA sequencing analysis comparing *C. neoformans* in the presence of steroid hormones was used to identify gene expression changes during melanization. We hypothesized that the increase in melanization when testosterone was present was due to changes in gene expression occurring at day three, due to visually observing (Figure 4) *C. neoformans* starting to melanize. RNA was extracted from *C. neoformans* on day three and cDNA libraries were prepared and sequenced. The data revealed four genes (flavonol synthase, a hypothetical RNA and two hypothetical proteins) that showed significant upregulation in *C. neoformans* in the presence of testosterone compared to estrogen (Table 2). CNAG_03340, a flavonol synthase, is a transmembrane protein that is part of the gibberellic biosynthesis pathway (FungiDB, CNAG_03340). Given this finding, we looked for other genes in the gibberellic biosynthesis pathway in the RNAseq data and identified four additional genes that were also upregulated in the presence of testosterone (but not significantly): cytochrome p450 monooxygenase pc-2 (CNAG_02841), cytochrome p450 (CNAG_05842 and CNAG_04029), and sphingolipid delta-4 desaturase (CNAG_00644). The gibberellic biosynthesis pathway produces GA, which typically increases growth rates in other organisms (44, 63), although this pathway has not been identified in *C. neoformans*.

Table 2: RNA sequencing data of genes upregulated during the melanization of *C. neoformans* in the presence of steroid hormones. Genes positioned above the table break are significantly up regulated (q-value < 0.05). Genes positioned below the table break are part of a gibberellic biosynthesis pathway that are not significantly up regulated (q-value > 0.05) but were validated with qRT-PCR (qRT-PCR data located in Appendix A)

Locus	Putative Function	RNA Seq. Fold Change	Real-Time PCR Fold Change	Validated	Significant (RNAseq)
CNAG_03340	Flavonol Synthase	17.88	75.04	YES	**YES**
CNAG_05356	Hypothetical Protein	45.84	23.82	YES	**YES**
CNAG_03857	Hypothetical Protein	18.41	25.55	YES	**YES**
CNAG_13056	Hypothetical RNA (ncRNA)	88.47	57.31	YES	**YES**
CNAG_03465	Laccase	1.6	5.72	YES	**NO**
CNAG_02841	Cytochrome P450 Monooxygenase	1.59	11.68	YES	**NO**
CNAG_05842	Cytochrome P450	1.36	8.08	YES	**NO**
CNAG_04029	Cytochrome P450	3.19	19.54	YES	**NO**
CNAG_00644	Sphingolipid Delta-4 Desaturase	1.34	6.42	YES	**NO**

3.6. Serial Dilutions of *C. neoformans* Validates that Melanization is Cell-Density Dependent

To validate that melanization was cell-density dependent in *C. neoformans*, serial dilutions were made in liquid L-DOPA media and ELISAs were completed to determine GA and PA concentrations. Serial dilutions revealed concentrations of 10^5 cells had melanized more compared to lower concentrations of *C. neoformans* (Figure 8). This

indicated that an increase in cell-density resulted in faster melanization, as expected for a phenotype controlled by quorum sensing. When H99S (all concentrations) was in the presence of testosterone, there was increased GA production in comparison to estrogen and ethanol (Table 3). This suggested that testosterone stimulates GA production, and this could be responsible for the variation in melanization seen when *C. neoformans* is cultured in the presence of testosterone.

Table 3: Concentrations of GA and PA in serial dilutions from conditioned media samples (32h) using ELISA. GA in all cell concentrations in testosterone is significantly higher than GA in all cell concentrations in estrogen or ethanol ($p < 0.05$, Tukey-Kramer HSD). PA is similarly affected by both testosterone and estrogen at 10^5 and 10^4 cell concentrations but is significantly increased with higher cell concentrations ($p < 0.05$, Tukey-Kramer HSD). Fold changes were determined by comparing both testosterone and estrogen concentrations to ethanol (control) concentrations, respectively. Data are representative of three independent experiments, N=3.

Treatment (Dilution)	GA Concentration (ng/mL)	PA Concentration (ng/mL)	Fold Change of GA secretion compared to ethanol (control)	Fold Change of PA secretion compared to ethanol (control)
H99S Alone	21.905	35.647		
Test 10^5	462.325	114.302	+23.404*	+3.78*
Test 10^4	328.183	86.994	+19.648*	+3.368*
Test 10^3	201.482	65.532	+15.825*	+3.659
Test 10^2	187.928	31.752	+15.709*	+3.515
Estr 10^5	20.864	112.367	+1.05	+3.72*
Estr 10^4	15.631	75.835	-0.935	+2.936
Estr 10^3	12.995	37.126	+1.020	+2.073
Estr 10^2	12.822	28.619	+1.071	+3.168
Eth 10^5	19.754	30.194	1.0	1.0
Eth 10^4	16.703	25.831	1.0	1.0
Eth 10^3	12.732	17.907	1.0	1.0
Eth 10^2	11.963	9.032	1.0	1.0

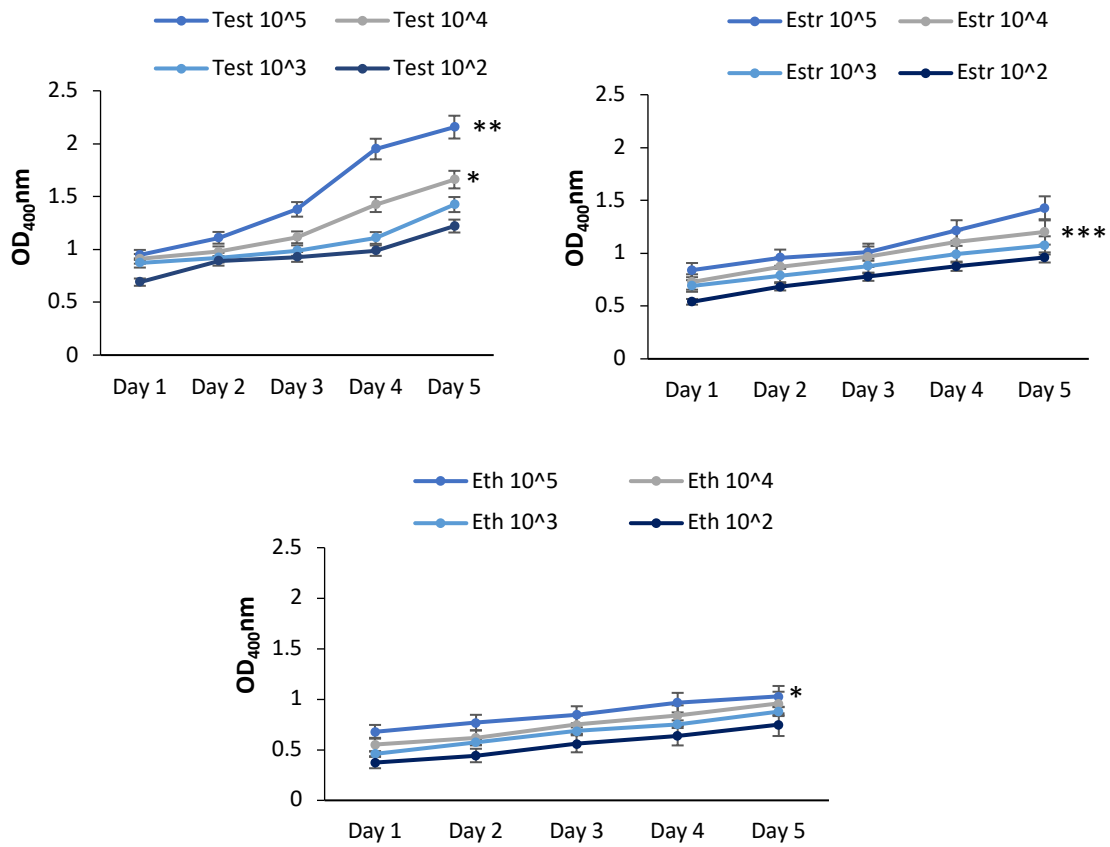


Figure 8: Serial dilutions of *C. neoformans* to observe melanization. The wildtype strain, H99S, was grown in liquid L-DOPA media containing hormone treatments for five days, and read at an absorbance of 400 nm to detect melanization differences. Statistical significance is represented as follows: $^{**}p = 0.0003$ comparing Test 10^5 to Test 10^4, $^{*}p = 0.019$ comparing Test 10^4 to Test 10^3, $^{***}p < 0.0001$ comparing Estr 10^4 to Estr 10^3, and $^{*}p = 0.011$ comparing Eth 10^5 to Eth 10^4 using linear regression analysis, N=3. Error bars represent the standard error of the mean. Data are representative of three independent experiments. OD600 data located in Appendix B.

3.7. Conditioned Media Increases Melanization in *C. neoformans*

The regulation of gene expression in response to population density is a phenomenon known as quorum sensing (64). To determine if *C. neoformans* was producing QSMs, the wild type strain, H99S, was grown in conditioned media for 32 h (Figure 9). An

absorbance reading at 400 and 600 nm was taken at three time points, 16, 24, and 32 h. In previous experiments the variation in melanization occurred at day three; however, when conditioned media was used, this variation was seen at 16 h (Figure 9B), suggesting *C. neoformans* was producing a QSM that was affecting melanization. There was also a significant increase in melanization ($p < 0.002$) when *C. neoformans* was cultured in the presence of testosterone compared to estrogen (Figure 9A). These data supports the hypothesis that *C. neoformans* produced higher levels of one or more QSMs when it was cultured with testosterone.

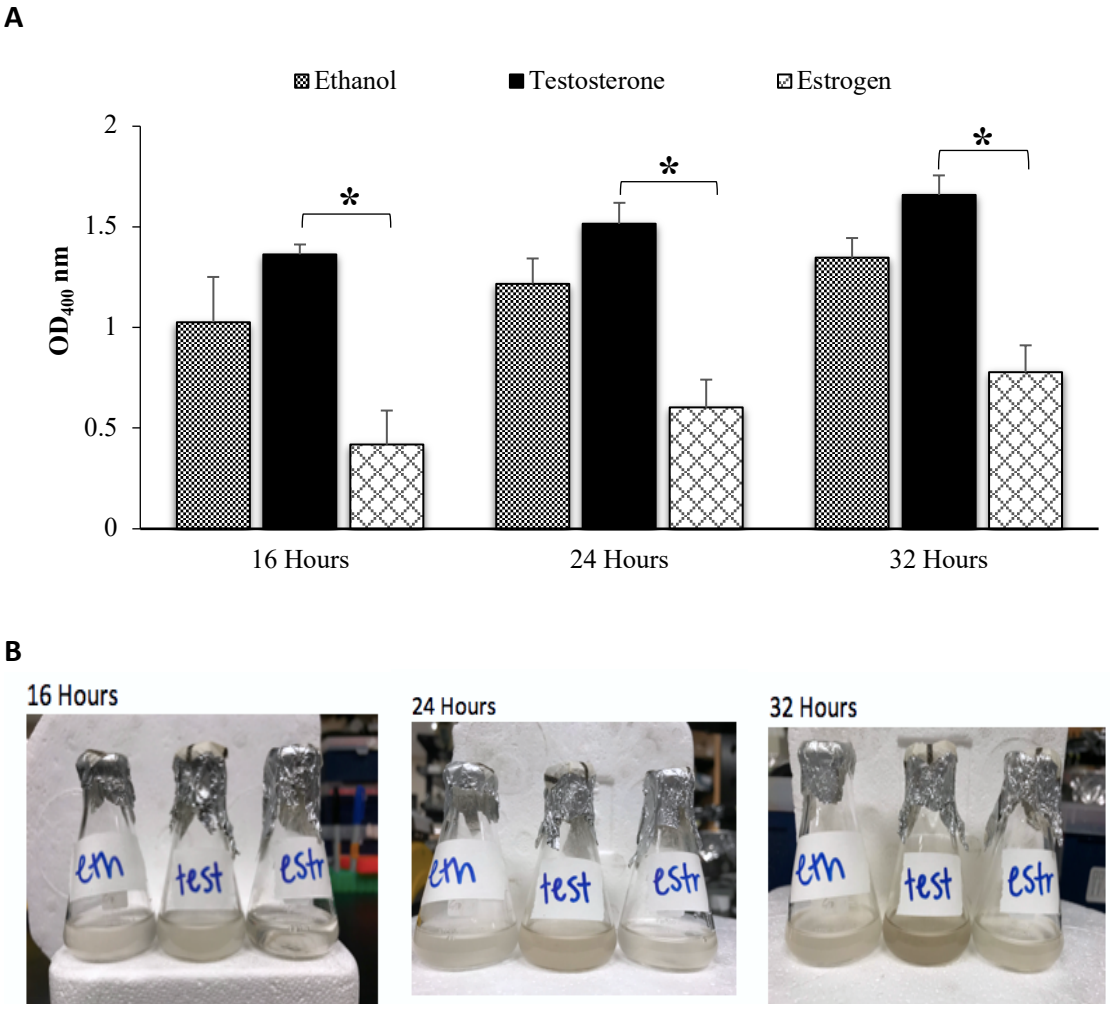


Figure 9: Melanization of *C. neoformans* in the presence of steroid hormones grown in conditioned media. A: Melanization of the wildtype strain, H99S, grown in conditioned media treated with steroid hormones with absorbances taken at three timepoints; 16 h, 24 h, and 32 h. Statistical significance is represented as follows: $p < 0.002$ using MANOVA with simple contrasts, N=3. Error bars represent the standard error of the mean. Data are representative of three independent experiments. **B:** Pictures were taken using a digital camera of melanized H99S in conditioned media treated with steroid hormones at three time points; 16 h, 24 h, and 32 h.

3.8. Cultivating *C. neoformans* in Conditioned Media with Exogenous Quorum Sensing Molecules and Quorum Sensing Molecule Inhibitors Reveals GA has an Effect on Melanization

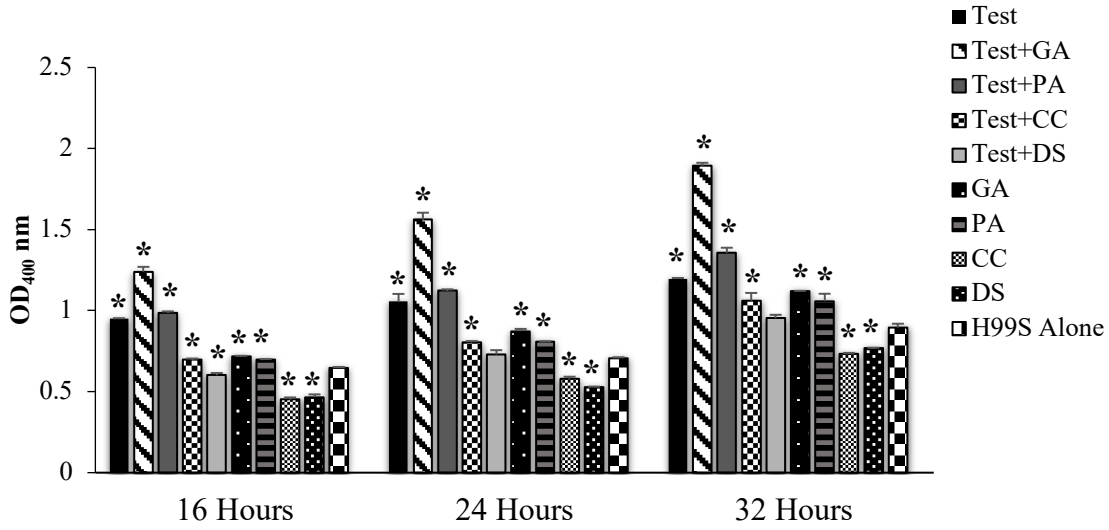
RNAseq analysis revealed multiple genes that were part of a gibberellic biosynthesis pathway that were upregulated during melanization. To determine the role of GA in melanization in the presence of testosterone, another conditioned media experiment was conducted. All treatments of H99S contained GA, chlormequat chloride (a GA inhibitor), PA, or DL-Serine (a PA inhibitor) plus/minus the presence of testosterone. For comparison, H99S was used in the presence or absence of exogenous testosterone without the addition of QSMs or their inhibitors (Figure 10A + 10C). When exogenous GA and testosterone were added to *C. neoformans*, there was a significant increase in melanization compared to *C. neoformans* in the presence of exogenous PA and testosterone or *C. neoformans* and testosterone without additives. These data suggest that GA affects melanization in *C. neoformans.* Chlormequat chloride and DL-Serine are known to inhibit 50% of GA and PA production, respectively, in other organisms (65). This experiment demonstrated a significant decrease in melanization when chlormequat chloride or DL-Serine was present compared to when *C. neoformans* melanized in testosterone without additives, suggesting that *C. neoformans* is producing both GA and PA molecules, and their production is being inhibited; thereby, decreasing melanization in *C. neoformans*.

Interestingly, at 24 and 32 h, there was no difference in melanization when DL-Serine and testosterone were present compared to H99S without additives (without testosterone), suggesting that DL-Serine may be abrogating the increased melanization seen in the

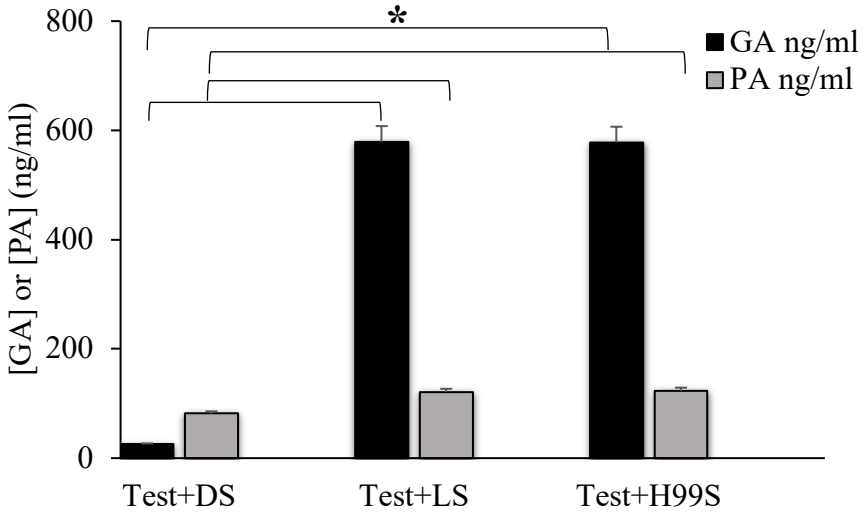
presence of testosterone. To determine if DL-Serine was also inhibiting GA, ELISAs were conducted to determine the concentration of GA and PA after 32 h of melanization on cell supernatants from conditioned media with DL-Serine + testosterone. These data suggest that DL-Serine significantly inhibits PA by 50%, as seen in Figure 10A, and also inhibits GA by 100% (Figure 10B).

I also used L-Serine as an inhibitor to determine if it had the same effects on GA production as DL-Serine (Figure 11). Conditioned media experiments (Figure 11A) revealed that when H99S was grown in the presence of L-Serine (without testosterone) compared to H99S without additives, there was no change in melanization. This data also revealed that when H99S was cultured in the presence of L-Serine plus testosterone compared to H99S plus testosterone, the amount of melanization was the same. These data suggest that L-Serine had no effect on melanization. ELISAs were also preformed to determine if GA or PA production was altered in the presence of L-Serine and the data suggests that L-Serine has little effect on GA and PA production compared to DL-Serine (Figure 11B).

A


Test
Test+GA
Test+PA
Test+CC
Test+DS
GA
PA
CC
DS
H99S Alone
OD400 nm
2.5
2
1.5
1
0.5
0
16 Hours
24 Hours
32 Hours

B


GA ng/ml
PA ng/ml
[GA] or [PA] (ng/ml)
800
600
400
200
0
Test+DS
Test+LS
Test+H99S

C

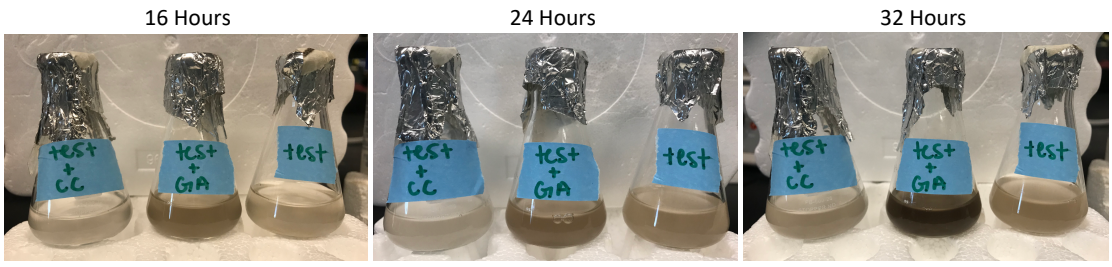
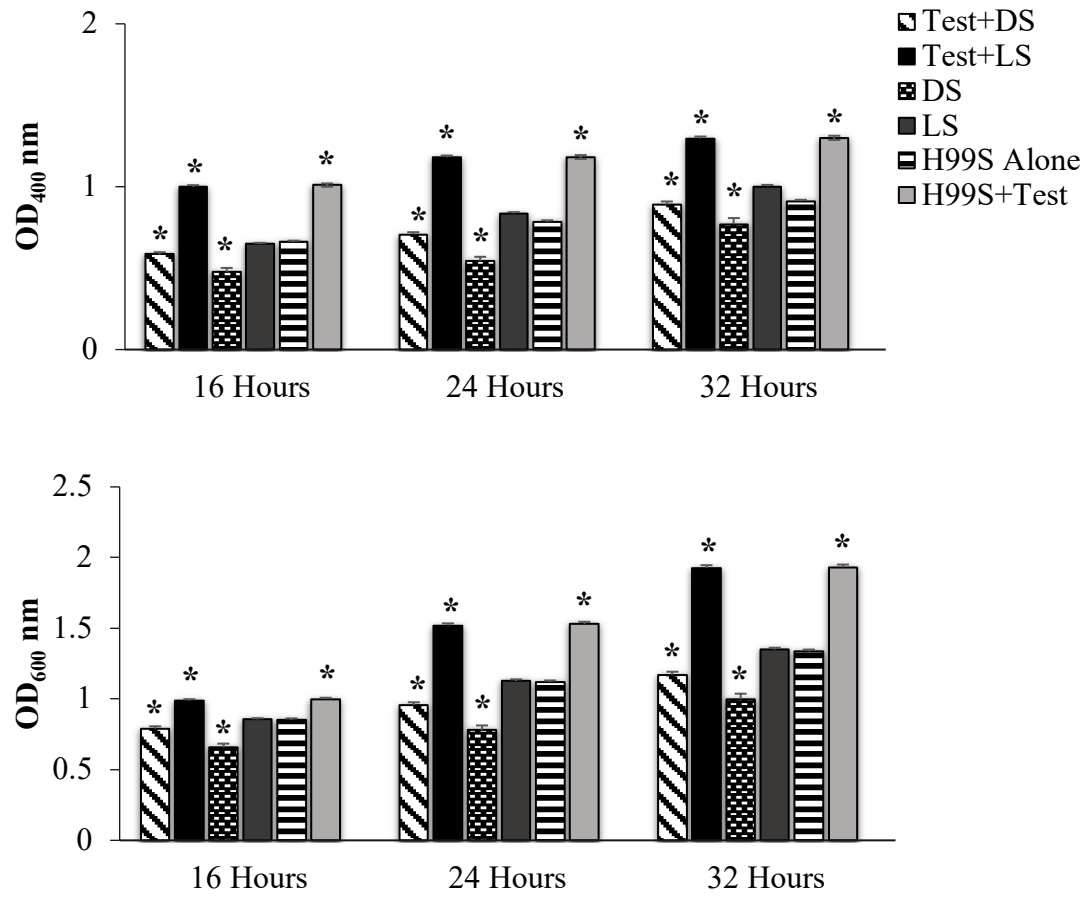

16 Hours
24 Hours
32 Hours
test + CC
test + GA
test

Figure 10: Melanization of *C. neoformans* grown in conditioned media with additives. A: Melanization of the wildtype strain, H99S, grown in conditioned media with additives and absorbances taken at three timepoints; 16 h, 24 h, and 32 h. Data are representative of three independent experiments. Error bars represent the standard error of the mean. Statistical significance is represented as follows: $p < 0.001$ compared to H99S without additives using MANOVA with simple contrasts, N=3. **B:** Concentrations of GA and PA when exogenous DL-Serine was added to conditioned media. Confirming DL-Serine also inhibited GA production. Data are the average of three independent experiments. Error bars represent the standard deviation of the mean. Statistical significance is represented as follows: $p < 0.05$ using Tukey-Kramer HSD for multiple comparisons, N=3. **C:** Pictures were taken using a digital camera of H99S in conditioned media when exogenous GA and chlormequat chloride were added at three time points; 16 h, 24 h, and 32 h.

A


Test+DS
Test+LS
DS
LS
H99S Alone
H99S+Test
OD400 nm
2
1
0
16 Hours
24 Hours
32 Hours
OD600 nm
2.5
2
1.5
1
0.5
0
16 Hours
24 Hours
32 Hours

B

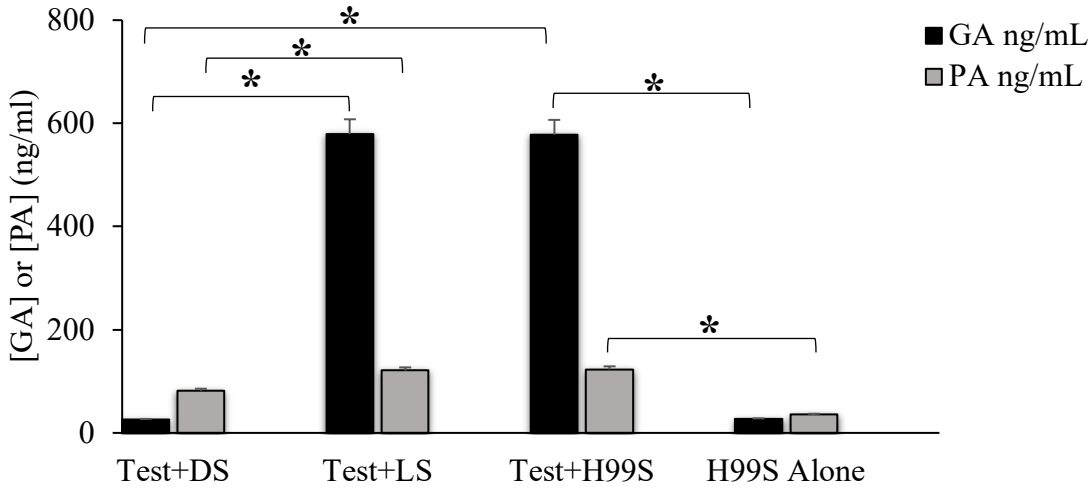

GA ng/mL
PA ng/mL
[GA] or [PA] (ng/ml)
800
600
400
200
0
Test+DS
Test+LS
Test+H99S
H99S Alone

Figure 11: Melanization of *C. neoformans* grown in conditioned media with additives. A: Melanization of the wildtype strain, H99S, grown in conditioned media with additives and absorbances taken at three timepoints; 16 h, 24 h, and 32 h. Absorbances were taken at 400 and 600 nm. Data are representative of three independent experiments. Error bars represent the standard error of the mean. Statistical significance is represented as follows: $p < 0.0001$ compared to H99S Alone using MANOVA with simple contrasts, N=3. **B:** Concentrations of GA and PA when exogenous DL-Serine or L-Serine was added to conditioned media using ELISA. Confirming that DL-Serine inhibits GA production while L-Serine has no effect. Data is representative of three independent experiments. Error bars represent the standard error of the mean. Statistical significance is represented as follows: $p < 0.001$ using Tukey-Kramer HSD for multiple comparisons, N=3. Full statistics can be found in Appendix C.

3.9.Phenotypic Testing of Knockout Strains in L-DOPA Liquid Medium and Conditioned Media Reveals a Decrease in Melanization

KO strains of genes identified in the RNAseq analysis, including genes involved in the GA biosynthesis pathway, were tested for amounts of melanization in both L-DOPA media (over 5 days) and in conditioned media (over 32 h) compared to H99S in the presence or absence of testosterone. All of the KO strains tested in liquid medium showed decreased melanization over five days and over 32 h in conditioned media (Figure 12) when compared to H99S in the presence or absence of testosterone. These data suggest that these genes (cytochrome p450 monooxygenase pc-2, flavonol synthase, the hypothetical proteins, and cytochrome p450), all involved in the gibberellic biosynthesis pathway, play a role in *C. neoformans* melanization in testosterone.

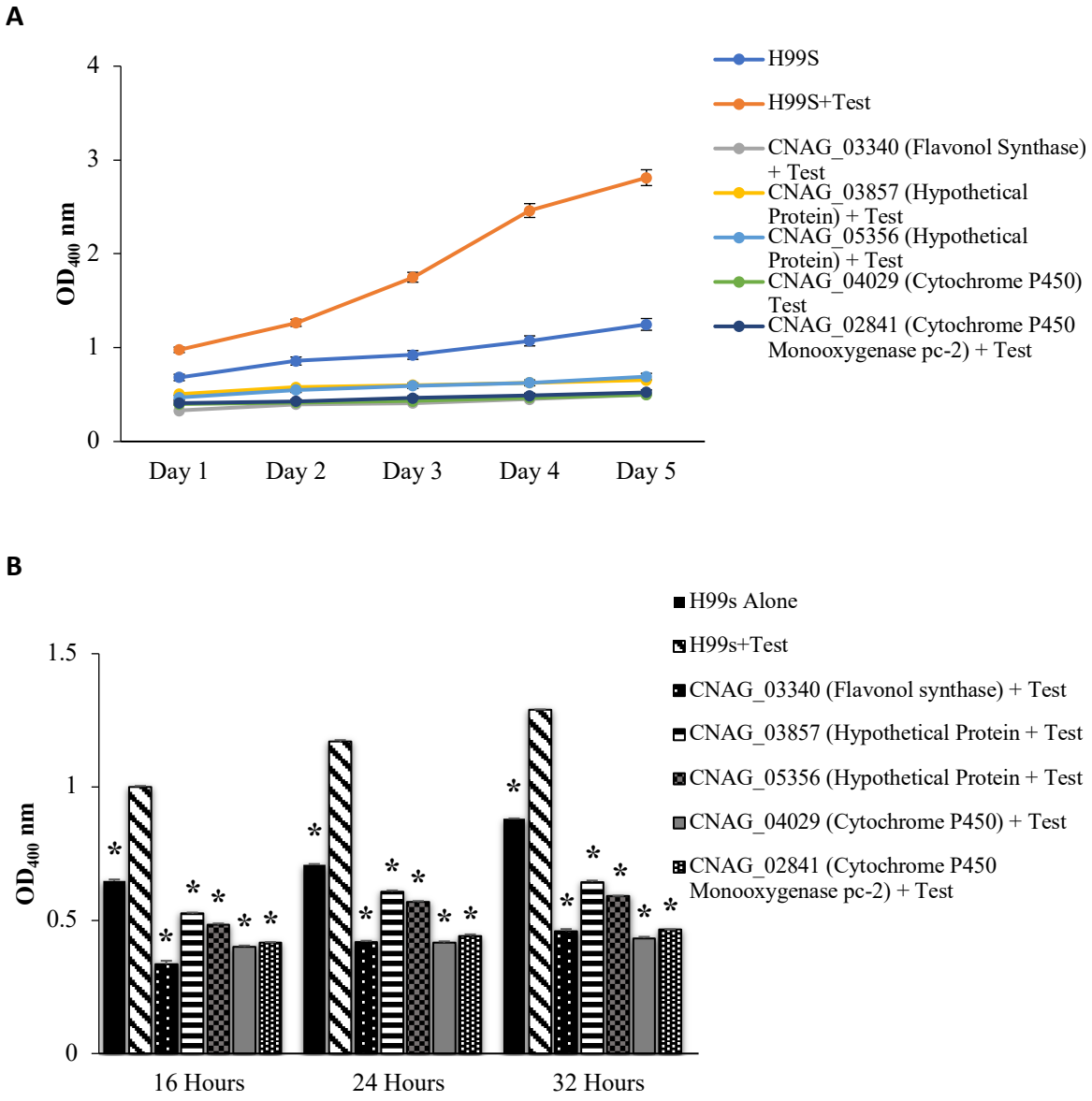


Figure 12: Phenotypic testing of KO strains in liquid L-DOPA media. A: KO strains and the wildtype strain, H99S, were grown in L-DOPA media for five days to determine any changes in melanization. Error bars represent the standard error of the mean. Data are the average of three independent experiments. Error bars represent the standard error of the mean. H99S is significantly different from all other strains tested ($p < 0.0001$, linear regression analysis, N=3). **B:** KO strains and H99S were grown in conditioned media at three time points; 16 h, 24 h, 32 h. All treatments compared to H99S plus testosterone using MANOVA with simple contrasts, $p < 0.0001$, N=3. Data are representative of three independent experiments. Error bars represent the standard error of the mean.

3.10. ELISAs Confirm Both GA and PA Concentrations in *C. neoformans* KO Strains

ELISAs were conducted to determine the concentration of GA and PA after 32 h of growth in conditioned media (Figure 10). PA concentrations in the KO strains grown in the presence of testosterone ranged from 30-90 ng/mL (Table 4). These concentrations were all lower than the concentration of PA in H99S in the presence of testosterone (about 140 ng/mL), suggesting that when *C. neoformans* melanized in testosterone, it produced more PA than the KO strains and that these genes are likely involved in PA production.

GA concentrations varied widely when the KO strains were compared to H99S (Table 4). In the KO strains, concentrations ranged from 10-20 ng/mL compared to 568 ng/mL in H99S plus testosterone. These data suggest that the genes that were knocked out in these strains affected GA production, which in turn affected melanization. The flavonol synthase (CNAG_03340), cytochrome p450 (CNAG_04029), and cytochrome p450 monooxygenase pc-2 (CNAG_02841) genes all had a 2-fold lower production of GA compared to H99S without testosterone, suggesting these genes are involved in GA-induced melanization. This data also indicates that *C. neoformans* produced more GA when H99S melanized in testosterone compared to without testosterone (about 22 ng/mL, a 29.5-fold change).

GA and PA concentrations were also measured when *C. neoformans* was cultured in the presence of estrogen in comparison to the presence of testosterone (Figure 13). These data revealed a 1-fold difference in PA concentration when comparing *C. neoformans* in the presence of testosterone to estrogen (Table 5). This suggested that both hormones

were regulating PA synthesis in similar ways. However, when *C. neoformans* was in the presence of testosterone it secreted 20-fold more GA compared to when it was in the presence of estrogen (Table 5). This is similar to when H99S was not in the presence of hormones, suggesting that testosterone was regulating GA production, which was acting as a quorum sensing molecule and increasing melanization.

Table 4: GA and PA concentrations using ELISA when KO strains and the wildtype strain, H99S were in the presence of testosterone compared to when testosterone was absent in H99S. Data are the average of three independent experiments. All knockout strains had significantly less GA & PA than H99S plus testosterone ($p <$ 0.0001, Tukey Kramer HSD, N=3).

Strain	GA (ng/mL)	PA (ng/mL)	Fold Change of GA Compared to H99S + Testosterone	Fold Change of PA Compared to H99S + Testosterone
H99S	25.845	35.688	-22.479	-3.45
H99S + Testosterone	580.956	123.222	1.0	1.0
Flavonol Synthase (CNAG_03340) + Testosterone	10.231	42.101	-56.781	-2.926
Hypothetical Protein (CNAG_03857) + Testosterone	15.6120	56.129	-37.212	-2.1953
Hypothetical Protein (CNAG_05356) + Testosterone	28.817	52.115	-20.160	-2.364
Cytochrome p450 (CNAG_04029) + Testosterone	10.395	38.165	-55.886	-3.229
Cytochrome p450 monooygenase pc-2 CNAG_02841 + Testosterone	17.292	36.436	-33.596	-3.381

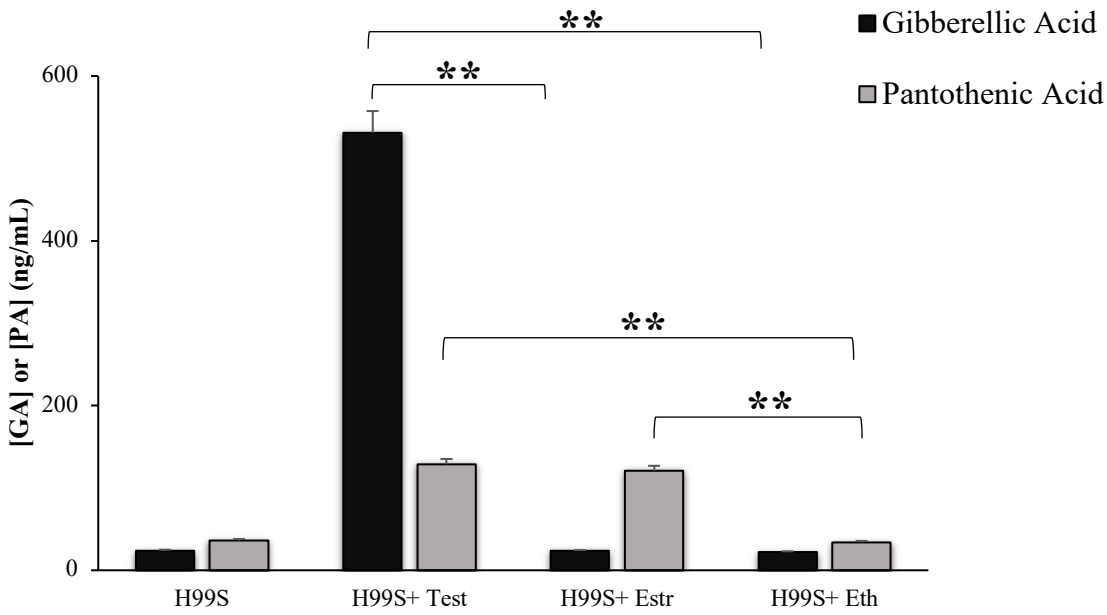


Figure 13: GA and PA concentrations using ELISA of the the wildtype strain, H99S, in the presence of estrogen and ethanol compared to H99S plus testosterone. H99S without steroid hormones present was used as a control. Data are the average of three independent experiments. Error bars represent the standard deviation of the mean. Tukey-Kramer HSD for multiple comparisons, $p < 0.001$, N=3. Full statistics can be found in Appendix D.

Table 5: GA and PA concentrations using ELISA of the the wildtype strain, H99S in the presence of estrogen and ethanol compared to H99S plus testosterone. H99S without steroid hormones present was used as a control. H99S plus testosterone had significantly more GA & PA than all other treatments ($p < 0.001$, Tukey Kramer HSD, N=3).

Strain	GA (ng/mL)	PA (ng/mL)	Fold Change of GA compared to H99S + Testosterone	Fold Change of PA compared to H99S + Testosterone
H99S	23.769	36.175	-22.338	-3.557
H99S + Testosterone	530.952	128.679	1.0	1.0
H99S + Estrogen	23.356	120.806	-22.733	-1.065
H99S + Ethanol	21.965	33.962	-24.173	-3.789

3.11. Fungal Burden using J774A.1 Macrophages Shows Inconsistency with the Literature

Since melanin is a major virulence factor in *C. neoformans*, if there is a significant variation in melanization in the presence of steroid hormones, there could be a difference in virulence as well. To test this, J774A.1 macrophages were infected with melanized and unmelanized *C. neoformans* in the presence of steroid hormones to determine fungal burden (Figure 14). These data suggest that unmelanized *C. neoformans* had a higher fungal burden than melanized *C. neoformans*. This was surprising as I would expect to see the opposite result, because melanin protects *C. neoformans* against the respiratory burst created by macrophages (1). It has previously been shown that *C. neoformans* fungal burden experiments in model organisms also suggests that melanized *C. neoformans* should have a higher fungal burden (66). I expected to see the same results using macrophage cell lines. Although these data suggested melanized *C. neoformans* cells show less fungal burden, I hypothesized the efficiency of phagocytosis may have been a factor that could be altering the fungal burden results. This led me to pursue a phagocytosis assay with melanized *C. neoformans* cells and a revised hypothesis that when *C. neoformans* was melanized and in the presence of testosterone there was less fungal burden compared to other hormone treatments due to a decrease in phagocytosis.

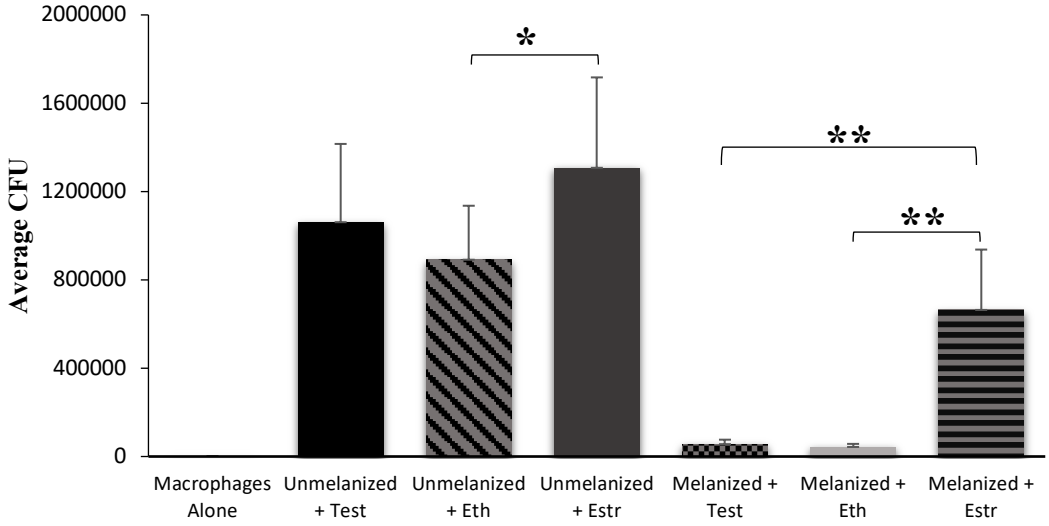


Figure 14: Fungal Burden of melanized and unmelanized *C. neoformans* in the presence of steroid hormones during J774A.1 macrophage infections. Data are the average of two independent experiments. Error bars represent the standard deviation of the mean. Statistical significance is represented as follows: $**p < 0.001$ and $*p < 0.05$ using Tukey-Kramer HSD for multiple comparisons, N=2. Full statistics can be found in Appendix E.

3.12. Phagocytosis Assay using J774A.1 Macrophages Reveals Melanization Prevents Efficient Phagocytosis

The efficiency of phagocytosis was determined by a phagocytosis assay using J774A.1 macrophages infected with melanized and unmelanized *C. neoformans* cells (Figure 15). The assay results revealed that there was about a 3-fold decrease in phagocytosis of melanized *C. neoformans* cells compared to unmelanized cells. These data confirm the previous results in the fungal burden experiment. Due to the difference in phagocytosis efficiency of unmelanized and melanized cells, it was not feasible to compare the two for a reliable outcome. This assay also revealed that there was not a difference in phagocytosis among melanized cells in different hormone treatments. This

suggests that steroid hormones do not have an effect on the number of cells that are phagocytosed by macrophages.

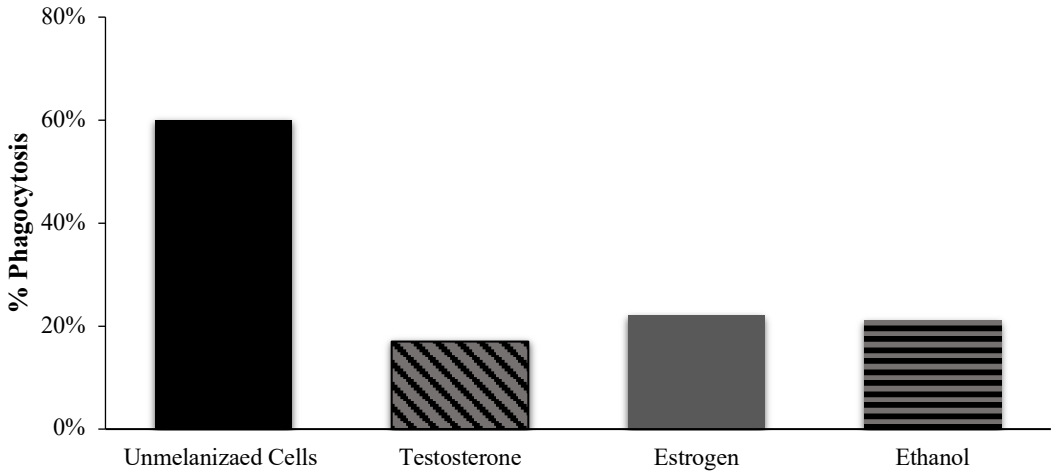


Figure 15: J774A.1 phagocytosis percentage of melanized *C. neoformans* in the presence of steroid hormones. Percentages were determined using a using the Nikon Elements Software and a Nikon Eclipse Ti-E widefield confocal microscope to take photos of 100 *C. neoformans* cells. Data are representative of one experiment.

IV

DISCUSSION

The sex-bias observed during *C. neoformans* infections has been a topic of debate as early as the 1960s (30). Although research on the effects of steroid hormones on *C. neoformans* melanin production is fairly new, this phenomenon has also been documented in animals (44). By characterizing the effects of testosterone and estrogen on *C. neoformans* virulence factors, I hoped to unearth the cause of this difference.

Initial data (Figure 4) suggested that melanization differed in the presence of steroid hormones. These data led to the hypothesis that *C. neoformans* could be using testosterone as a substrate to melanize and this could explain the variation seen between hormones during melanization. However, when this hypothesis was tested (Figure 5) we found that neither testosterone nor estrogen were being used as a substrate to melanize, leading me to utilize other experiments to determine why this difference in melanization was occurring.

One factor that could play a role in this increased melanization is the concentration of testosterone present. Males are known to produce various amounts of testosterone (51), and this could be advantageous to pathogenic organisms such as *C. neoformans*. According to a study completed by Furman *et al* (2013) higher levels of testosterone have been linked to immunosuppression, whereas estrogen and lower testosterone levels have not (68). This study parallels the data obtained from the dose response experiment using various concentrations of testosterone (Figure 6). When

testosterone concentrations are high, there is an increase in melanization, suggesting that increased testosterone concentrations can cause an increase in virulence.

A modified conditioned media experiment (28) (Figure 9) was also preformed because *C. neoformans* has a known QSM, PA, that is thought to effect melanization. This led to a new hypothesis that when *C. neoformans* is in the presence of testosterone it secretes more PA, thus causing an increase in melanization. This experiment suggested that when conditioned *C. neoformans* culture media with L-DOPA was added to a new culture, melanization occurred at a faster rate. This was similar to the findings of Eisenman *et al* (2011) in which *C. neoformans* was grown for seven days in minimal medium, after which L-DOPA was added. With the late addition of L-DOPA to the old culture, the investigators observed an increase in melanization compared to a *C. neoformans* culture that was initially grown in L-DOPA (13). Although Eisenman *et al* did not test the effects of steroid hormones on melanization, both studies suggest that *C. neoformans* was secreting a molecule(s) that increased melanization, a common quorum sensing phenomenon. Additionally, our conditioned media experiment with steroid hormones revealed that when *C. neoformans* melanized in testosterone, melanization was increased compared to *C. neoformans* melanizing in estrogen. These data are consistent with my hypothesis that when *C. neoformans* melanized in the presence of testosterone, there was an increase in the secretion of these molecules, suggesting a quorum sensing-like mechanism.

Microarray data by Eisenman *et al* (2013). revealed genes that were upregulated when *C. neoformans* was melanized. A few of these genes were also shown to be upregulated by qRT-PCR (Figure 7) when *C. neoformans* was melanized and in the

presence of testosterone compared to estrogen. However, results of RNA sequencing (Table 2) indicated several other genes were upregulated when *C. neoformans* was melanized in testosterone. One of these genes is involved in the GA biosynthesis pathway, providing the first indication that GA could be involved in melanization Thus, I hypothesized that the increase in expression of these genes in the presence of testosterone contributed to the observed difference in melanization.

Melanization is a phenotype controlled by quorum sensing, a cell density dependent mechanism. Serial dilutions completed using liquid L-DOPA media revealed that as cell-density increases, melanization occurs at a faster rate (Figure 8). A study completed by Albuquerque *et al* (2013) found increased melanization when they added exogenous PA to samples, but not as much of an increase was observed in their conditioned media samples (39). This study suggested that *C. neoformans* was secreting additional molecules that were contributing to the increased melanization. RNA sequencing analysis, (Table 2) along with ELISA data, (Table 4) suggest that at least one of these molecules was GA. Serial dilution ELISA (Table 2) also revealed that as cell density increased, the concentration of GA and PA also increased, further suggesting that GA and PA could both be acting as QSMs. GA is secreted by many other fungi and is thought to aid survival in harsh environments and increase pathogenesis, but has not yet been described as a QSM (46). ELISA data completed on *C. neoformans* in the presence of both hormones revealed that when *C. neoformans* was in the presence of estrogen there was only a 1-fold decrease in PA production compared to when testosterone was present (Figure 13 + Table 5). This suggests that PA production was not affected by the presence of either hormone. However, there was a 22-fold decrease in GA production in

the presence of estrogen compared to testosterone, which is similar to GA production when hormones were absent. This further indicated that testosterone was regulating GA production. Thus, we hypothesize that GA is acting as a QSM in *C. neoformans,* and secretion is increased when *C. neoformans* is in the presence of testosterone, thus increasing melanization.

Another recent study by Tian *et al* (2018) identified a quorum sensing peptide, QSP1, in *C. neoformans* that controls sexual reproduction (69). *C. neoformans,* like other fungi, has two sexual cycles (bi and unisexual). The Tian study suggests that QSP1 is a signaling molecule being utilized in both cycles by affecting cell density and paracrine regulation (69). This further suggests that *C. neoformans* is likely secreting other QSMs.

By inhibiting the individual QSMs that *C. neoformans* secretes, I was able to determine how melanization varies when either PA or GA were absent (Figure 10A). However, DL-Serine (plus testosterone), an inhibitor of PA, did not show a significant decrease in melanization compared to H99S (minus testosterone). Using ELISAs (Figure 10B), I found that DL-Serine also inhibits GA production, thus blocking the effects of testosterone on melanization. Unfortunately, DL-Serine could not serve as a potential therapeutic for men with cryptococcosis because it is highly toxic and causes kidney necrosis (70). DL-Serine's isomer, L-Serine was tested to determine if it could also inhibit GA production and serve as an alternative therapeutic. However, ELISA data (Figure 11B) revealed that L-Serine has no effect on GA or PA production, indicating that future studies to identify the mechanism by which DL-serine inhibits GA are needed to determine potential therapeutics.

Due to QSM secretion, there was an increase in melanization over a shorter amount of time when using conditioned media (Figure 9) compared to L-DOPA media (Figure 4B). To determine if QSM secretion and melanization could be altered, KO strains of genes involved in the gibberellic biosynthesis pathway were also grown in conditioned media. These strains all showed a decrease in melanization when compared to H99S (Figure 11). Interestingly, examination of the data (Figure 11A) revealed that the flavonol synthase (CNAG_03340), cytochrome p450 (CNAG_04029), and cytochrome p450 monooxygenase pc-2 (CNAG_02841) genes all appear to cluster together. These genes are known to be a part of the GA biosynthesis pathway in other organisms (63); suggesting that they are also a part of the same pathway in *C. neoformans*. The two hypothetical proteins (CNAG_03857 and CNAG_05356) also cluster together and both contain transmembrane domains (71- 72). ELISA data (Table 4) suggest these genes produce the same amount of PA as H99S without testosterone, which suggest they are not involved in the PA pathway or its production. However, there was a different effect in the GA ELISA data, where the hypothetical protein KO strain, CNAG_03857, produced less GA compared to H99S without testosterone. I hypothesize that testosterone may be binding to this protein, subsequently signaling the GA pathway. Future studies will test this hypothesis.

As stated before, *C. neoformans* melanin production benefits the yeast in a variety of ways. A phagocytosis assay (Figure 15) confirmed one of these benefits, that melanization aids *C. neoformans* to avoid phagocytosis. However, with these results I would expect to see an increase in fungal burden of melanized cells compared to unmelanized cells, but instead the data revealed the opposite result (Figure 14). I

hypothesized that this is due to a lack in phagocytosis efficiency or non-lytic exocytosis. In future studies a technique to achieve equal phagocytosis of both melanized and unmelanized *C. neoformans* cells should be completed before an accurate fungal burden comparison can be made.

In conclusion, our data indicate that GA is a newly identified QSM in *C. neoformans*, whose production is stimulated by testosterone, which may contribute to increased disease in males. As melanin production is known to increase virulence and contribute to antifungal resistance in *C. neoformans*, disrupting both the PA and GA pathways could cause a significant reduction of melaninizaion. Once the mechnism by which testosterone interacts with *C. neoformans* is known, potential drug targets can be identified in future research.

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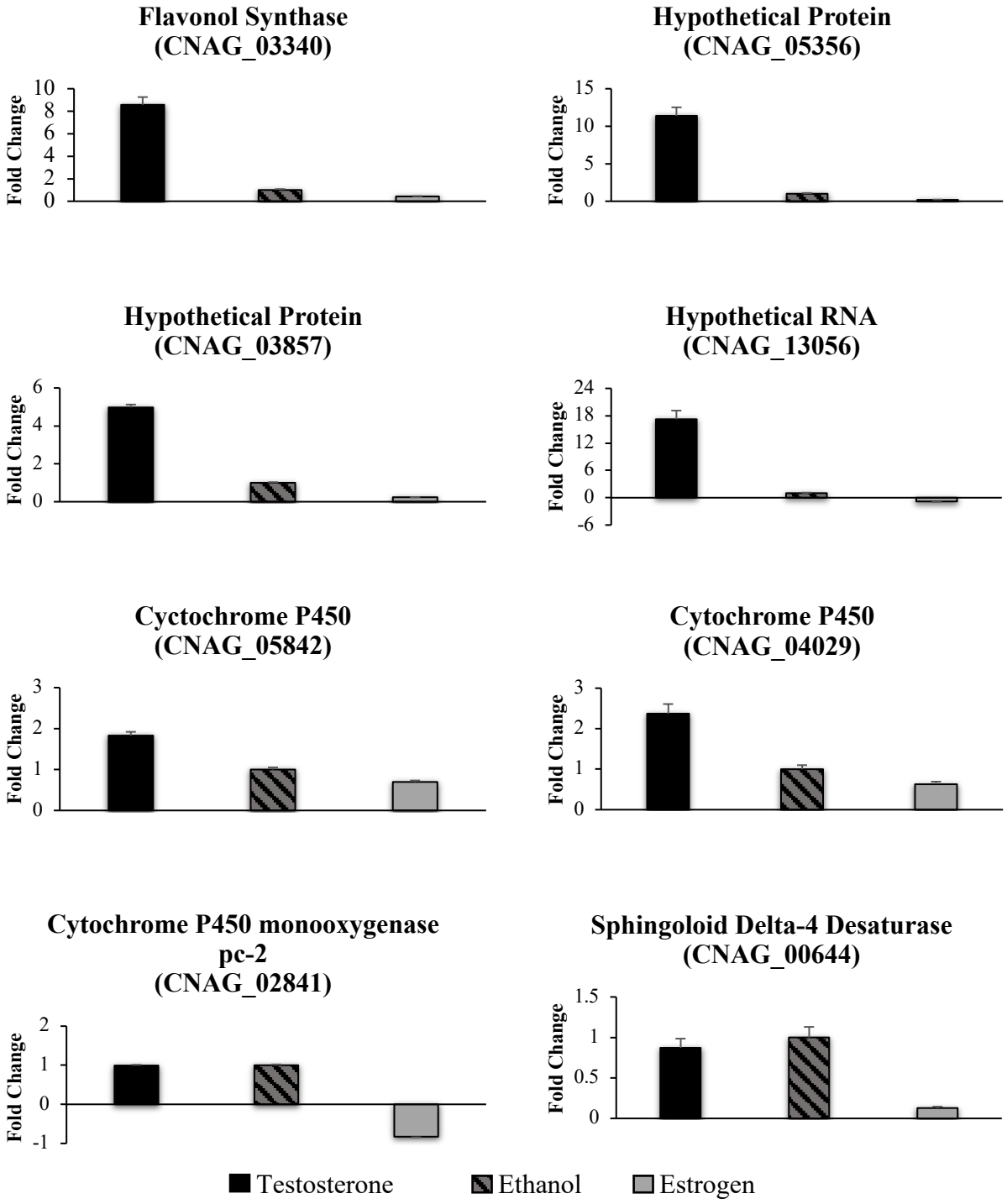
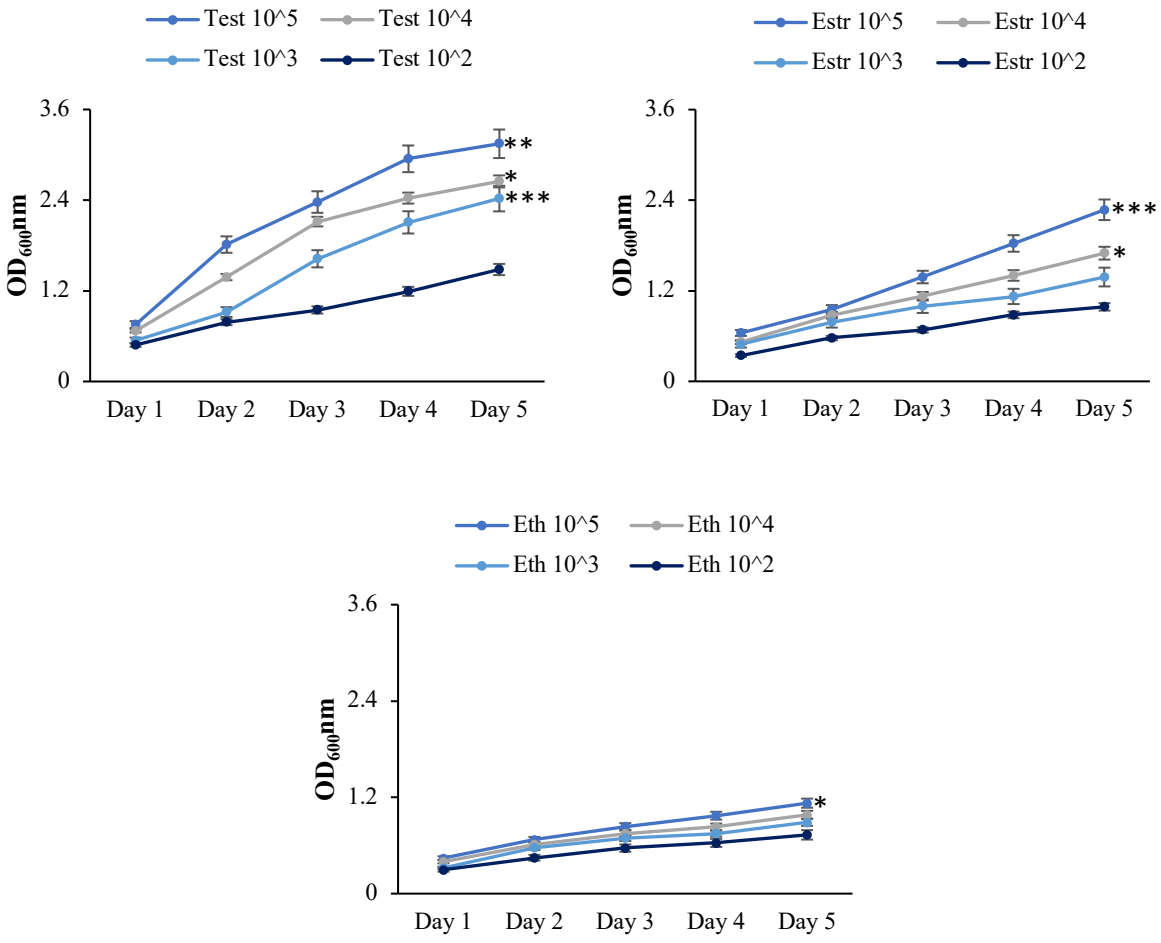
APPENDICES

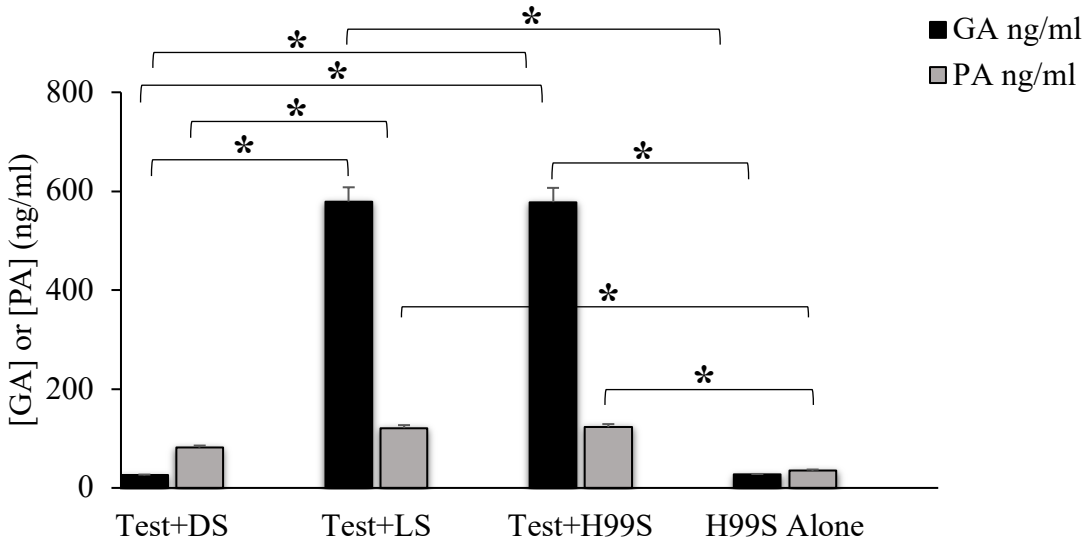
APPENDIX A. qRT-PCR analysis validating genes identified in the RNAseq data. Error bars represent the standard error of the mean. Data are representative of two independent experiments. Fold change was calculated using the geometric mean of two housekeeping genes.



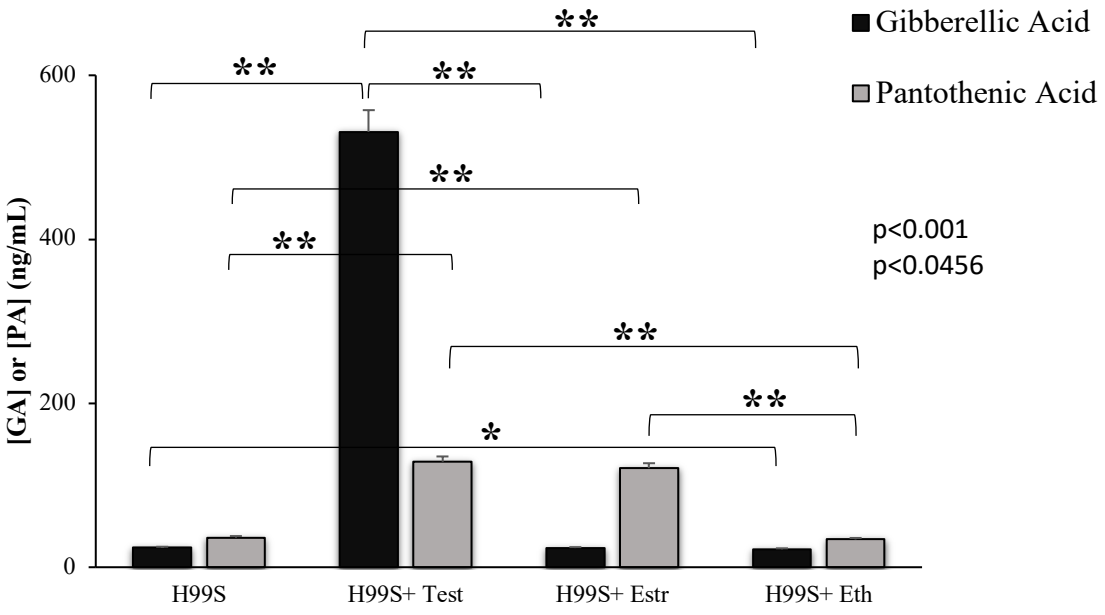
APPENDIX B. Serial dilutions of *C. neoformans* to observe growth rate. The wildtype strain, H99S, was grown in liquid L-DOPA media containing hormone treatments for five days, and read at an absorbance of 600 nm to detect growth rate differences. Statistical significance is represented as follows: $**p < 0.001$ comparing Test 10^5 to Test 10^4, $*p < 0.05$ comparing Test 10^4 to Test 10^3, $***p < 0.0001$ comparing Test 10^3 to Test 10^2, $***p < 0.0001$ comparing Estr 10^5 to Estr 10^4, and $*p < 0.05$ comparing Estr 10^4 to Estr 10^3, and $*p < 0.05$ comparing Eth 10^5 to Eth 10^4 using linear regression analysis, N=2. Error bars represent the standard error of the mean. Data is representative of two independent experiments.



APPENDIX C. Full statistics from Figure 11B. Concentrations of GA and PA when exogenous DL-Serine or L-Serine was added to conditioned media using ELISA. Confirming that DL-Serine inhibits GA production while L-Serine has no effect. Data are representative of three independent experiments. Error bars represent the standard error of the mean. Statistical significance is represented as follows: $p < 0.001$ using Tukey-Kramer HSD for multiple comparisons, N=3.



APPENDIX D. Full statistics from Figure 13. GA and PA concentrations using ELISA of the the wildtype strain, H99S, grown in the presence of estrogen and ethanol compared to H99S plus testosterone. H99S without steroid hormones present was used as a control. Data are the average of three independent experiments. Error bars represent the standard deviation of the mean. Tukey-Kramer HSD for multiple comparisons, $^{**}p < 0.001$, with the exception of H99S vs. H99S+Eth, $^{*}p < 0.0456$, N=3.



APPENDIX E. Full statistics from Figure 14. Fungal burden of melanized and unmelanized *C. neoformans* in the presence of steroid hormones during J774A.1 macrophage infections. Data are the average of two independent experiments. Error bars represent the standard deviation of the mean. Statistical significance is represented as follows: *** $p < 0.0001$, ** $p < 0.001$ and * $p < 0.05$ using Tukey-Kramer HSD for multiple comparisons, N=2.