MELANIN PRODUCTION IN *CRYPTOCOCCUS NEOFORMANS*

# QUANTIFYING THE CONTRIBUTION OF ENVIRONMENTAL AND GENETIC FACTORS TO THE VARIATION IN MELANIN PRODUCTION AMONG STRAINS OF *CRYPTOCOCCUS NEOFORMANS*

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# Lay Abstract

The yeast, *Cryptococcus neoformans*, is naturally found worldwide in soil and pigeon droppings, and causes fatal infections in people with a weakened immune system. It produces dark melanin pigments that aid the yeast in neutralizing the immune response of the infected host. My project sought to characterize the variation in melanin levels found among natural populations of *C. neoformans* and to determine the factors that contribute to this variation. I identified genetic differences between the strains to be the biggest cause of variation in melanin levels. Changes in the *LAC1* gene which is essential for melanin synthesis, were found to shape the melanin production significantly when the yeast is exposed to stressful conditions. Interactions between genes and the environment were also revealed to be a significant contribution to the observed differences in melanin levels. These results highlight the complex interplay of factors that shape the expression of observable traits.

# Abstract

*Cryptococcus neoformans* is a basidiomycetous yeast capable of causing fatal meningoencephalitis in immunocompromised individuals. Naturally found worldwide in association with pigeon droppings, *C. neoformans* produces melanin pigments that play a protective role in both natural and physiological settings. Significant variation in the level of melanin production has been observed among natural populations of *C. neoformans*, although the factors behind this phenotypic variation remain unclear. In my project, I quantified the level of melanin production in 54 strains of *C. neoformans* and characterized the change in melanin response to three common stressors encountered by *C. neoformans* in natural and clinical environments: thermal, oxidative and nitrosative stresses. Using this data, I assessed the contribution of genetic factors, environmental factors and genotype-environmental interactions to the observed variance in melanin. The analysis revealed that over 50% of the variance in melanin was attributable to genetic differences among the strains. I identified three single nucleotide polymorphisms (SNPs) in the *LAC1* gene, which is essential for melanin synthesis, to be significantly associated with melanin production in thermal and oxidative stresses. One of the SNPs is predicted to cause an amino acid change (P182A/T) in a putative catalytic domain of the Lac1 protein. The genotype-environment interactions were also found to contribute to a significant proportion of the variance in phenotype. In the majority of tested stress conditions, environmental factors only contributed to a small amount of variance ranging from 5-15%. These results highlight the complex interplay of genetic, environmental and interaction factors that contribute to an

observable phenotype. *C. neoformans* is a model fungal pathogen whose ability to produce melanin is a well-established virulence factor. The results from this project contribute to our understanding of the evolutionary trajectory and regulation of melanin production in *C. neoformans*.

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# Chapter 1

# General Introduction

## 1.1 *Cryptococcus* species complex

### 1.1.1 Serotypes and molecular types of *Cryptococcus* sp. complex

Of the estimated 5 million fungal species on earth (Blackwell, 2011), only those in a small number of genera are capable of causing life-threatening infections in humans. The most common fungal pathogens belong to *Candida, Aspergillus* and *Cryptococcus* species complexes (Mirza et al., 2003; Pfaller & Diekema, 2004; Pfaller, Pappas, & Wingard, 2006). *Candida* and *Cryptococcus* sp. predominantly exist in the unicellular yeast form whereas *Aspergillus* sp. are moulds that present as mycelia of branching thread-like hyphae. *Candida* infections are the most common type of fungal infections in humans as *Candida* sp. are a natural component of the human flora, and changes in physiological environment can lead to their outgrowths, resulting in infections (Hube, 2004; Pfaller & Diekema, 2007; Romani, Bistoni, & Puccetti, 2003). *Aspergillus* and *Cryptococcus* sp. are opportunistic pathogens that mainly infect individuals with compromised immune systems, usually manifested as pulmonary infections that can disseminate into the central nervous system. *Cryptococcal* infections are the most common fungal infections affecting the central nervous system, clinically presenting as inflammation of the meninges leading to meningoencephalitis (Del Valle & Pina-Oviedo, 2006). In Sub-Saharan Africa where

antifungal treatments are not readily accessible, *Cryptococcal* infections are a leading cause of death among HIV/AIDS patients, even surpassing the death toll of tuberculosis (Park et al., 2009).

*Cryptococcus* sp. are basidiomycetous, encapsulated yeasts with a widespread global distribution. *Cryptococcus* sp. complex comprises two major species, *Cryptococcus gattii* and *Cryptococcus neoformans* that are further subcategorized based on molecular and genealogical differences. *Cryptococcal* strains can be classified in to four major serotypes termed A, B, C and D, based on cell surface antigen properties. In 1935, Benham (Benham, 1935) used agglutination reactions in serums derived from rabbits to recognize three distinct species, and potentially a fourth, within the genus *Cryptococcus* that exhibited unique antigenic and morphological properties. However, this study included strains that were later identified as belonging to the genera *Candida* and *Rhodotorula.* In 1949, Evans exclusively used *Cryptococcal* strains isolated from infected patients in agglutination reactions to identify three main serotypes – A, B and C (Evans, 1949). In 1968, Wilson identified a fourth serotype, D, in *Cryptococcus* complex (Ikeda, Shinoda, Fukazawa, & Kaufman, 1982; Wilson, Bennett, & Bailey, 1968). Serotype specificity is determined by the structural variations of glucuronoxylomannan ($G_A$XM), the most abundant polysaccharide in the capsule that surrounds the *Cryptococcal* cells (Bhattacharjee, Bennett, & Glaudemans, 1984).

In addition to serotypes, different molecular types within the species complex can be distinguished using PCR fingerprinting, amplified fragment length polymorphism (AFLP) and more recently multi-locus sequence typing (MLST) (Boekhout et al., 2001; Cogliati 2013; Fraser

et al., 2005; Litvintseva, Thakur, Vilgalys, & Mitchell, 2006; Meyer, Mitchell, Freedman, & Vilgalys, 1993; Viviani et al., 1997). These methods consistently reveal 8 different molecular types among *Cryptococcal* strains, called VNI, VNII, VNIII, VNIV, VGI, VGII, VGIII and VGIV. The molecular types and serotypes are strongly correlated: strains with serotypes A and D belong to molecular types VNI to VNIV, whereas serotypes B and C usually have one of the four VG molecular types. Strains of serotypes B and C and molecular types VGI, VGII, VGIII and VGIV have been classified as the species *C. gattii* (Kwon-Chung et al., 2017). Its sister species *C. neoformans* is composed of serotypes A and D which have been elevated into variety status, thus designated *C. neoformans* var. *grubii* (serotype A) and *C. neoformans* var. *neoformans* (serotype D). These include the molecular types VNI, VNII, VNIII and VNIV (Kwon-Chung et al., 2017).

### 1.1.2 Distinct characteristics of the two *Cryptococcal* species

The two sister species *C. gattii* and *C. neoformans* are markedly different in a number of aspects including geographical distribution, basidiospore morphology, virulence, level of expression of virulence factors, clinical presentation and epidemiology. Both species can be naturally found in the environment: *C. gattii* is usually isolated from decaying tree barks whereas *C. neoformans* is typically associated with pigeon excreta and soil (Lazéra, Mitchell, Nielsen, Castañeda, & Wanke, 2011). Together their geographical territories span many parts of the world. *C. gattii* was thought to be restricted to tropical and subtropical regions (Kwon-Chung & Bennett, 1984b; Sorrell, 2001) until in 2001, a major *Cryptococcal* outbreak, determined to be caused by a rare

genotype of *C. gatti* affecting healthy individuals and animals, emerged in Vancouver Island in British Columbia (Kidd et al., 2004). *C. gatti* natural isolates and clinical cases have since been reported in various countries in North America, South America, Europe and Asia, leading to the expansion of its distribution (Springer & Chaturvedi, 2010). *C. neoformans* var. *grubii* has a worldwide distribution with 99% of the cryptococcal infections in HIV patients being caused by this variety (Mitchell & Perfect, 1995). *C. neoformans* var. *neoformans* also has a global distribution but is more commonly found in temperate climates, especially in Europe where 30% of the reported cryptococcol infections are caused by this variety (Bovers, Hagen, & Boekhout, 2008; Kwon-Chung & Bennett, 1984a).

With its ability to infect apparently healthy individuals, *C. gattii* is the more virulent of the two sister species and usually results in more severe infections (Georgi et al., 2009; Kidd et al., 2004). However, *C. neoformans* infections are far more abundant at a global scale. Infections by *C. neoformans* in healthy individuals are extremely rare: it usually causes infections in individuals who have compromised immunity due to HIV/AIDS, diabetes, organ transplants, chemotherapy, etc (Speed & Dunt, 1995). Out of the two varieties, *C. neoformans* var. *grubii* tends to be more virulent with the majority of reported cases of cryptococcal infections being caused by this variety (see above).

The focus of the rest of this chapter will be on *C. neoformans* as it is the focus of this thesis and is the more prevalent of the two sister species in clinical settings.

## 1.2 Epidemiology and pathobiology of Cryptococcosis

Cryptococcal infections, collectively referred to as cryptococcosis, are clinically manifested in a variety of forms with the most common being pulmonary infections, cutaneous infections and meningoencephalitis which is the inflammation of the meninges in the central nervous system. If untreated, cryptococcosis will result in death. *C. neoformans* is an opportunistic pathogen that primarily infects immunocompromised individuals: the impact of cryptococcosis is mostly felt among the HIV/AIDS population where it is considered an AIDS-defining illness by the Centers for Disease Prevention and Control (CDC) (Mamidi, DeSimone, & Pomerantz, 2002; Wright, Schneider, & Berger, 2011). It has been estimated that 1 million new cases of HIV-related cryptococcal meningitis are diagnosed every year with 625,000 of them resulting in mortality (Park et al., 2009). The vast majority of these cases occur among the HIV/AIDS population in Sub-Saharan Africa where HIV is highly prevalent and antiretroviral and antifungal therapies are not readily accessible. In contrast, the prevalence of cryptococcosis in the United States' AIDS population has declined by almost 90% in the 1990s due to the increasingly successful use of antifungals to combat these infections (Mirza et al., 2003). However, it remains a concern as a recent survey discovered that the incidence of cryptococcosis among the AIDS patients in the United States continues to persist at 2.9% (McKenney et al., 2014).

The infectious agent of *Cryptococcus* is thought to be the basidiospores that upon inhalation germinate and establish an infection in the lungs. The cells can then potentially enter the bloodstream and disseminate throughout the rest of the body: if it crosses the blood-brain barrier

of the central nervous system, meningoencephalitis can arise. A recent body of evidence shows that, despite the long-held belief of *C. neoformans* establishing infections through extracellular growth in host tissues (Kozel & Gotschlich, 1982; Littman, 1959), it is also a facultative intracellular pathogen that can replicate and propagate inside the macrophages (Alvarez & Casadevall, 2006; Feldmesser, Kress, Novikoff, & Casadevall, 2000; Lee, Kress, Zhao, Dickson, & Casadevall, 1995). Upon detecting the invading cryptococcal cells, the host immune system, even if compromised, will mount a defence by mobilizing available macrophages to engulf the yeast cells. Once the yeast cells are internalized, macrophages release antimicrobial molecules including reactive oxygen species (ROS) and reactive nitrogen species (RNS) into the intracellular environment to damage and kill the engulfed cells (Forman & Torres, 2002; Nathan & Shiloh, 2000). ROS and RNS refer to derivatives of $O_2^{\bullet}$ and $NO^{\bullet}$ radicals respectively that can modify organic molecules (Fang, 2004). ROS and RNS can target a range of molecules in a microbial cell including thiols, metal centers, protein tyrosinases, nucleotides and lipids (Fang, 2004; Nathan & Shiloh, 2000), thus causing widespread damage to invading microbes. However, research evidence suggests that *C. neoformans* has a number of adaptive strategies to survive and persist within the human macrophages, making it a successful intracellular pathogen. Feldmesser and colleagues (2000) showed that over the course of a murine pulmonary infection, *C. neoformans* cells persisted inside macrophages accompanied by replication, residence in a membrane-bound phagosome and the accumulation of polysaccharides that are thought to protect the yeast cells from lytic activity of the macrophages (Feldmesser et al., 2000). It was recently found that *C. neoformans* cells can exit the macrophages without damaging the host cell through phagosome extrusion (Alvarez & Casadevall, 2006), and also transfer laterally from infected to uninfected macrophages through direct cell-to-cell contact (Alvarez & Casadevall, 2007; Ma,

Croudace, Lammas, & May, 2007). These findings shed light on the route of infection of *C. neoformans* and how it is able to disseminate through the blood stream and eventually cross the blood-brain barrier to establish infections in the central nervous system.

## 1.3 Virulence factors of *C. neoformans*

The three main virulence factors of *C. neoformans* are its ability to grow at $37^0$C, production of a protective polysaccharide capsule around the cell, and the production of melanin pigments. All three play crucial roles in successfully counteracting antimicrobial activities of the host to propagate and establish an infection. It is believed that, as an environmental yeast, *C. neoformans* evolved these traits in response to selection pressures exerted by environmental stressors present in its natural habitats: the presence of similar stressors inside mammalian hosts allowed it to become an opportunistic pathogen. The body of evidence in support of this was synthesized by Casadevall and colleagues (2003) to propose that *C. neoformans* is a 'ready-made' pathogen with its virulence factors playing a 'dual-role' in both environmental and clinical settings (Casadevall, Steenbergen, & Nosanchuk, 2003). *C. neoformans* cells are preyed on by amoebas such as *Acanthamoeba palestinensis*, mites and sow bugs (*Metoponorthus pruinosus*) in its natural environment (Ruiz et al., 1982). Once engulfed by *A. palestinensis* cells, *C. neoformans* employs the same strategies that it uses to survive inside human macrophages (discussed above) to replicate within amoeba cytoplasm and eventually kill the amoeba cells (Casadevall et al., 2003). It is likely that the defense mechanisms that first evolved to survive predation by amoeba enabled *C.*

*neoformans* to successfully grow within human macrophages during infections. The three main virulence factors of *C. neoformans* and their roles in survival and pathogenesis are discussed below.

### 1.3.1 Ability to grow at $37^0$C

The ability to grow at temperatures of and above $37^0$C has enabled *C. neoformans* to successfully infect humans as well as other mammals including dogs, cats, horses, sheep and even dolphins (McGill et al., 2009; Venn-Watson, Daniels, & Smith, 2012). Given its global distribution, *C. neoformans* can be exposed to high ambient temperatures in its natural habitats, especially in tropical regions. A genome-wide analysis of temperature-regulated transcriptome of *C. neoformans* revealed that when temperature increased from $25^0$C to $37^0$C, the transcription of genes encoding several heat-shock proteins and translational machinery was upregulated (Steen et al., 2002). Several other signaling cascade pathways and biosynthesis pathways have been implicated in high temperature growth of *C. neoformans*: some notable examples are RAS1 signaling cascade (Alspaugh, Cavallo, Perfect, & Heitman, 2000), calcineurin signaling pathway (Odom et al., 1997) and pyrimidine biosynthetic pathway (de Gontijo et al., 2014). A partial genome-microarray analysis discovered a transcription factor homolog Mga2, whose potential targets include fatty acid biosynthetic enzymes, to be induced at $37^0$C, suggesting that membrane remodeling is an important component of *C. neoformans'* adaptation to high temperature (Kraus et al., 2004). These findings highlight the presence of adaptive strategies in *C. neoformans* to successfully grow in high temperatures. The strains of the two varieties of *C. neoformans* tend to be slightly different in their thermotolerance: an analysis of 19 clinical strains revealed that *C.*

*neoformans* var. *neoformans* strains were more susceptible to heat killing by high temperatures ($45^0$c – $47^0$C) than *C. neoformans* var. *grubii* (Martinez, Garcia-Rivera, & Casadevall, 2001). This is likely explained by the geographical distributions of the two varieties: *C. neoformans* var. *neoformans* is mostly confined to temperate regions where it is not regularly exposed to high temperatures whereas *C. neoformans* var. *grubii* has a global distribution including tropical regions. However, it should be noted that both varieties are perfectly capable of growth at $37^0$C.

### 1.3.2 Production of the polysaccharide capsule

The production of a polysaccharide capsule is a unique trait that sets *C. neoformans* apart from other fungal pathogen. It is also essential for *C. neoformans* pathogenesis as it has been consistently shown that acapsular mutants of *C. neoformans* are unable to establish infections in mammalian hosts (Chang & Kwon-Chung, 1994; Fromtling, Shadomy, & Jacobson, 1982; Kwon-Chung & Rhodes, 1986). Furthermore, during murine infections, structure of the capsule was observed to undergo changes with organ invasion, leading investigators to believe that these structural changes enable *C. neoformans* to avoid host immune responses and cross the blood-brain barrier (Charlier et al., 2005).

The *C. neoformans* capsule, which surrounds the cell wall, is predominantly made of polysaccharides with the two most abundant being glucuronoxylomannan (GXM) and galactoxylomannan (GalXM) (Bose, Reese, Ory, Janbon, & Doering, 2003). Structural variations in GXM determine the serotype of the *C. neoformans* strains. The capsule is thought to aid the survival of *C. neoformans* by hindering the phagocytosis of yeast cells by predatory amoeba in

the natural environment and by host macrophages during infections (Kozel & Gotschlich, 1982). Macrophages can however overcome the anti-phagocytic effects of the capsule in the presence of opsonins to ingest *C. neoformans* cells (Feldmesser et al., 2000). The polysaccharide capsule seems to play a beneficial role even when the cell is engulfed as it was shown that acapsular mutants of *C. neoformans* were unable to replicate intracellularly within the macrophages (Feldmesser et al., 2000). These findings highlight the crucial role played by the polysaccharide capsule to promote survival of *C. neoformans* both in the natural and clinical environments.

### 1.3.3 Melanin production

The production of melanin pigments by *C. neoformans* is another important virulence factor and the focus of this thesis. In the presence of phenolic substrates, *C. neoformans* produces melanin pigments that turn the colonies a characteristic dark brown color. The ability to produce dark pigments when grown on bird seed agar has been historically used as an indicative characteristic of *C. neoforman* (Staib et al., 1987). These dark pigments were first identified as a type of melanin in 1995 by researchers using electron spin resonance spectroscopy (Wang, Aisen, & Casadevall, 1995). Since then, many research efforts have focused on elucidating the structure and biosynthesis of *C. neoformans* melanin, and its contribution to virulence.

Many fungal species are capable of producing melanin: the majority of them use the endogenous substrate dihydroxynaphthalene (DHN) through the pentaketide pathway to produce DHN-melanin (Eisenman & Casadevall, 2012). In contrast, *C. neoformans* utilizes a

phenoloxidase enzyme to oxidize exogenous ortho-phenolic compounds such as L-dopamine (L-DOPA) and its structural analogs to produce DOPA-melanin which is closely related to melanin pigments synthesized by mammals. *C. neoformans* is unable to produce melanin if the substrate molecules are not available for uptake in its surroundings. L-DOPA is first oxidized by the laccase enzyme, a phenoloxidase, to dopaquinone. Only this first step is believed to be enzymatically catalyzed in the L-dopa melanin synthesis pathway, which is followed by a series of spontaneous oxidation and polymerization reactions to yield the final melanin pigments. Dopaquinone is converted to cyclodopa through a nucleophilic addition reaction: cyclodopa is then oxidized to dopachrome which tautomerizes into dihydroxyindoles that eventually polymerize into melanin (Eisenman & Casadevall, 2012; Land, Ramsden, & Riley, 2004; Langfelder, Streibel, Jahn, Haase, & Brakhage, 2003a). The rate-limiting step of the melanin biosynthesis pathway is the initial oxidation of L-DOPA, catalyzed by the laccase enzyme. Two laccase enzymes were found to be present in *C. neoformans* cells, coded for by the *LAC1* and *lac2* genes respectively, which are located in tandem on chromosome 7 (Zhu & Williamson, 2004). The *LAC1* enzyme is the main producer of melanin in *C. neoformans* with mutations in *lac2* gene causing only slight delays in melanin formation (Pukkila-Worley et al., 2005b). The basal transcript level of *lac2* was also found to be much lower than that of *LAC1* (Pukkila-Worley et al., 2005b).

In a series of elegant experiments, Salas and colleagues (1996) demonstrated the relationship between *LAC1*, melanin production and virulence by deriving a melanin-mutant of *C. neoformans* that had a deletion in the 5' end of the *LAC1* gene (Salas, Bennett, Kwon-Chung, Perfect, & Williamson, 1996). The melanin production of the mutant strain and its virulence in

mice were significantly diminished: complementation of the *LAC1* deletion restored both melanin production and virulence. However, it is not clear whether the survival and pathogenic benefits of melanin biosynthesis stem from melanin pigments themselves or from the activity of the *LAC1* enzyme. Melanised cryptococcal cells have shown to be more resistant *in vitro* to oxidative stress caused by ROS, nitrosative stress caused by RNS, and to antimicrobial activity of antifungal drugs than non-melanized cells (Doering, Nosanchuk, Roberts, & Casadevall, 1999; Emery et al., 1994; Jacobson & Tinnell, 1993; Y. Wang & Casadevall, 1994b). However, it is debatable if strains produce melanin to the same extent during an infection as they do *in vitro*: *C. neoformans* cells isolated from infected mouse brains were found to contain intermediate products of the melanin synthesis pathway but no melanin pigments (Liu, Wakamatsu, Ito, & Williamson, 1999). A later study utilizing monoclonal antibodies specific to melanin showed that *C. neoformans* in fact produced melanin *in vivo* in the lung and brain tissues of infected mice (Rosas et al., 2000). Either melanin pigments themselves or the *LAC1* enzyme act as scavengers of free oxygen and nitrogen radicals, removing them from the cellular environment. This protects the yeast cells from cellular stress caused by ROS and RNS inside macrophages during an infection, and also provides protection from ultraviolet light and temperature extremes in its natural habitat (Rosas & Casadevall, 1997; Y. Wang & Casadevall, 1994a).

In addition to its role in catalyzing melanin synthesis, *LAC1* enzyme was also found to contain iron oxidase activity. Liu and colleagues (1999) showed that laccase inhibited hydroxyl radical-mediated killing of *C. neoformans* cells by oxidizing Fe(II) to Fe(III) in a cell-free system (Liu, Tewari, & Williamson, 1999). Their results suggested that iron oxidase activity of laccase might play a role in protecting *C. neoformans* from oxidative stress inside macrophages through

iron oxidation which would result in a decrease in hydroxyl radical formation. Even though it is not yet clear the exact mechanisms through which melanin biosynthesis pathway and its components render protection to *C. neoformans*, the ability to produce melanin is unarguably a major virulence factor of this pathogenic yeast.

### 1.3.4 Variation in melanin production among strains of *C. neoformans*

It has been observed that strains of *C. neoformans* can vary significantly in their level of melanin production, ranging from strains that produce no melanin at all and appear white on the substrate media, to strains that produce a large amount of melanin and turn black/dark brown in a matter of days (Vogan, Khankhet, Samarasinghe, & Xu, 2016). The genetic basis behind this observed variation in phenotype has not been fully elucidated. Vogan (2016) conducted a Quantitative Trait Loci (QTL) analysis where he quantified the melanin production of 230 hybrid strains derived from a mating event between the two varieties of *C. neoformans* (Vogan et al., 2016). His analysis identified five QTLs that together explained 49% of the observed variation in melanin production among the hybrid strains. One of the QTLS that explained 10% of the total variance was located near the genetic marker CNG01240 which lies close to the *LAC1* gene. These results indicated that variations in the *LAC1* gene was contributing to the variation in melanin production among strains of *C. neoformans*. It also remains to be investigated whether the level of melanin production has a quantitative effect on the degree of virulence of *C. neoformans* strains. The role of the *LAC1* gene in variation in melanin production and its effects on the virulence of *C.*

*neoformans* strains were explored in my Masters thesis project which is described in Chapter 2 below.

# Chapter 2

# Quantifying the Contribution of Environmental and Genetic Factors to the Variation in Melanin Production among Strains of *Cryptococcus neoformans*.

## 2.1 Preface

Even though significant variation has been observed in the level of melanin production among natural populations of *Cryptococcus neoformans*, the factors responsible for this phenotypic variation remain largely unknown. The gene that is crucial for melanin synthesis, *LAC1*, is located near one of the genetic markers recently identified as contributing to the variation in melanin production. Melanin synthesis is a well-established virulence factor of *C. neoformans* but, whether the variation in melanin levels has an association with the degree of virulence has not been investigated.

This chapter has been written as a draft manuscript with the aim to be published in the journal Molecular ecology.

I am the primary contributor of this work. Jianping Xu conceived the experiments, with the majority of the experiments conducted by me, with technical help from David Acetuno-Caicedo on *Galleria mellonella* virulence assays. . Analyses and writing of the manuscript were also predominantly completed by me, with much help and input from Jianping Xu.

# Abstract

The interplay between genetic, environmental and genotype-environment interaction effects that lead to phenotypic variation among natural populations often remain understudied due to its many layers of complexity. In the pathogenic yeast *Cryptococcus neoformans*, found worldwide in association with pigeon excreta and soil, the level of melanin production varies significantly between strains. Melanin production in *C. neoformans* is a major virulence factor that contributes to its pathogenicity. In this study, we characterized the melanin response of 54 *C. neoformans* var. *neoformans* strains spanning 3 continents and 13 countries to three common stressors encountered in the natural and human host environments: thermal, oxidative and nitrosative stresses. We also sequenced their *LAC1* gene whose protein product catalyzes melanin synthesis. Our analysis revealed that over 50% of the variance in melanin was attributable to genetic differences among the strains, with three single nucleotide polymorphisms (SNPs) in the *LAC1* gene significantly associated with melanin production in thermal and oxidative stresses. One of the SNPs is predicted to cause an amino acid change (P182A/T) in the putative catalytic domain of the Lac1 protein. Furthermore, we found significant genotype-environment interactions that contributed 28%, 29%, and 43% to the total melanin variance in thermal, nitrosative and oxidative stresses respectively. In contrast, though statistically significant, environmental stress contributed relatively little to the total variance, ranging from 5-15%. Our study underscores the importance of ecology and environmental factors for better understanding the evolution and regulation of melanin production in *C. neoformans* and other virulence traits in fungal pathogens.

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# 2.2 Introduction

Of the estimated 5 million fungal species on earth (Blackwell, 2011), only those in a small number of genera are capable of causing life-threatening infections in humans. The most common fungal pathogens belong to *Candida*, *Aspergillus* and *Cryptococcus* species complexes (Mirza et al., 2003; Pfaller & Diekema, 2004; Pfaller et al., 2006). Within these species, the virulence and the severity of resulting infections can vary significantly among strains. For example, some strains of *Cryptococcus* are highly virulent and cause fatal infections in both immunocompromised and immunocompetent humans, whereas others are completely avirulent in humans as well as in other mammalian hosts (Chen et al., 2008; Kidd et al., 2004; Ross & Taylor, 1981). The variation in virulence has been linked to the differential expression of virulence factors by the strains. In the human pathogenic *Cryptococcus* species complex, virulent strains generally show excellent survival at $37^0$C, and produce melanin pigments and polysaccharide capsules (Kwon-Chung & Rhodes, 1986; Perfect et al., 2006; Salas et al., 1996). However, differences at the genetic level that contribute to natural phenotypic variations have not been fully elucidated in many of the clinically-relevant fungal pathogens. Identifying these genetic factors can shed light on the pathogens' mechanisms of action during infection. It can also provide insights into the selection pressures encountered by human pathogens across evolutionary time.

*Cryptococcus neoformans* is a basidiomycetous yeast that causes meningoencephalitis and a range of associated infections, collectively known as

cryptococcosis, primarily in immunocompromised individuals. Each year, there is an estimated one million new cases of cryptococcosis worldwide, with ~625 000 of them resulting in mortality (Park et al., 2009). *C. neoformans* has a global distribution and is naturally found in a wide range of ecological niches including pigeon droppings, soil and tree barks (Lazéra et al., 2011). *C. neoformans* has been subcategorized into two varieties based on cell surface antigen properties, namely *C. neoformans* var. *grubii* (serotype A) and *C. neoformans* var. *neoformans* (serotype D) (Evans, 1949; Ikeda et al., 1982; Wilson et al., 1968). Strains of *C. neoformans* var. *grubii* generally tend to be more virulent and are found in a wider geographical range than *C. neoformans* var. *neoformans* (Bovers et al., 2008; K .J. Kwon-Chung & Bennett, 1984).

The ability of *C. neoformans* to produce melanin pigments using exogenous phenolic compounds is a well-documented virulence factor that contributes to its pathogenicity (Butler & Day, 1998; Salas et al., 1996). In *C. neoformans'* melanin biosynthesis pathway, a phenolic substrate, such as L-DOPA or a structural analog, is first oxidized by the laccase enzyme, a phenoloxidase, into dopaquinone. This is followed by a series of spontaneous oxidation reactions to yield dihydroxyindole which polymerizes into melanin pigments (Eisenman & Casadevall, 2012; Land et al., 2004; Langfelder, Streibel, Jahn, Haase, & Brakhage, 2003b). The laccase enzyme (*LAC1*) coded for by the *LAC1* gene is essential for this process (Salas et al., 1996). The dual-use virulence hypothesis contends that melanin pigmentation first evolved in *C. neoformans* as a protective shield against environmental stressors in its natural habitat, but the presence of similar stressors inside mammalian hosts allowed *C. neoformans* to become an

opportunistic pathogen (Casadevall et al., 2003). Previous research has established the protective role of melanin in *C. neoformans* against oxidative stress, nitrosative stress and antimicrobial activities of antifungal drugs (Doering, Nosanchuk, Roberts, & Casadevall, 1999; Emery et al., 1994; Jacobson & Tinnell, 1993; Rosas & Casadevall, 1997; Wang & Casadevall, 1994). *C. neoformans* cells are exposed to oxidative and nitrosative stresses during an infection as macrophages release bursts of reactive oxygen species (ROS) and reactive nitrogen species (RNS) to kill the invading yeast cells (Fang, 2004). Furthermore, melanized *C. neoformans* cells were found to be less susceptible to ultraviolet light and temperature extremes than non-melanized cells: these are two stressors the yeast very likely encounters in its natural habitat (Rosas & Casadevall, 1997; Wang & Casadevall, 1994).

Variation in melanin production has been observed among different strains of *C. neoformans*, but the genetic basis of this natural phenotypic variation has not been elucidated. A Quantitative Trait Loci (QTL) analysis conducted by Vogan and colleagues (2016) identified five QTLs that were contributing to the variation in melanin production among 230 hybrid strains derived from a mating event between the two varieties of *C. neoformans* (Vogan et al., 2016). One of the QTL regions explained 10% of the phenotypic variance in the study population and this QTL was located near the genetic marker CNG01240 which lies close to the *LAC1* gene. Although it has been shown that disrupting the *LAC1* gene eliminates the synthesis of melanin pigments in *C. neoformans* cells (Salas et al., 1996), the QTL analysis provided evidence for the possibility that variations within the *LAC1* gene could have a quantitative effect on the level of

pigmentation in *C. neoformans*. In addition to genetic variation, environmental factors and genotype-environmental interactions are two important contributors to phenotypic variation in natural populations. Genotype-environmental interactions of spontaneous mutations were demonstrated to have a significant effect on the vegetative fitness in *C. neoformans* (Xu, 2004). Similarly, while the protective role of melanin against various stress conditions has been well-documented, it is less clear how the level of melanin production changes in response to these environments. Indeed, the contributions of these environmental factors such as thermal, oxidative and nitrosative stresses, to variation in melanin production among natural strains of *C. neoformans* have not been quantified yet.

The objectives of this study were to (i) quantify the variation in melanin production among natural strains of *C. neoformans* var. *neoformans*; (ii) examine and quantify the potential effects of thermal, oxidative and nitrosative stresses on melanin production; (iii) investigate the relationship between DNA sequence variation at *LAC1* gene and melanin production; and (iv) assess the correlation between level of melanin production and degree of virulence using *Galleria mellonella* waxworms as a model host. To accomplish these objectives, we obtained 54 strains of *C. neoformans* var. *neoformans* from 13 countries spanning 3 continents (North America, Europe, and Asia): these included both clinical and environmental strains of varied origins. We quantified their level of melanin production *in vitro* under optimum growth conditions as well as in the presence of thermal, oxidative and nitrosative stresses. For each strain, we obtained its complete *LAC1* gene sequence, including the promoter region. Finally, the virulence of the strains was assayed using *Galleria mellonella* (greater waxworm) as the model host. These data

were used to quantify the contributions of genetic and environmental factors to melanin production and their influences on *in vivo* virulence in waxworms.

# 2.4 Materials and methods

## 2.4.1 Strains

A total of 54 strains of *Cryptococcus neoformans* var. *neoformans* (serotype D) were analyzed in this study. Among these, 52 strains were natural isolates of clinical and environmental origins, from diverse geographical regions. The detailed information about these strains are shown in **Table S1** (Supplementary Information Table 1). The common laboratory strains JEC20 and JEC21, both of serotype D, were included in the population as reference strains.

## 2.4.2 General protocol for melanin assay

Melanin production was quantified using a spot densitometry assay on caffeic acid-containing solid agar medium as described previously (Vogan et al., 2016). Caffeic acid is a structural analog of L-DOPA and C. *neoformans* uses these two substrates interchangeably to synthesize melanin. Briefly, for our assay, the stock cultures maintained at -800C were first revived on solid YEPD medium and grown for 2-3 days at 300C. Cells from freshly grown cultures were suspended in sterile water and the cell

density was adjusted to 1x106 cells/ml using the hemocytometer cell counter. 5µl of this solution was spotted onto the caffeic-acid agar (Hopfer & Blank, 1976). The plates containing the spots were then incubated in the relevant environmental conditions for 72 hours, after which measurements were taken. A transilluminator was used to expose the resulting colonies to reflective white light, and the amount of light reflected from the surface of the colonies was quantified using the spot densitometry function of the Alpha-Imager (InnovTech). The light is reflected according to the darkness of the yeast colonies: the darker colonies that produced more melanin reflect less light. For each strain at each incubation condition, at least 4 replicate spots were assayed. Each experiment was repeated three times.

### 2.4.3 Effect of temperature on melanin production

The melanin assay protocol mentioned above was performed with the plates being incubated at either $30^0$C or $37^0$C. The optimum growth temperature of *C. neoformans* is around $30^0$C. The human physiological temperature is around $37^0$C, the temperature yeast cells are exposed to during an infection. The remaining protocol for assaying melanin production was the same as that described above.

### 2.4.4 Effect of oxidative stress on melanin production

To simulate oxidative stress caused by ROS, hydrogen peroxide ($H_2O_2$) was added to the molten caffeic acid-agar before solidification in one of three concentrations – 0.25mM, 0.5mM and 1mM. The medium without any added $H_2O_2$ was used as the negative control. The remaining protocol for assaying melanin production was followed as described above.

### 2.4.5 Effects of nitrosative stress on melanin production

To simulate nitrosative stress caused by RNS, sodium nitrite ($NaNO_2$) salt was added to the molten caffeic acid agar before solidification in one of three concentrations – 0.25mM, 0.5mM and 1mM. The medium without any added $NaNO_2$ was used as the negative control. The remaining protocol for assaying melanin production was followed as described above.

### 2.4.6 Sequencing of the *LAC1* gene

To obtain the complete nucleotide sequence of the *LAC1* gene including both the coding region (2886 bps) and up to 900bp upstream of the transcription start site, we designed 6 primer pairs based on the fully sequenced genome of JEC21, available on NCBI

(Accession number NC_006692.1, range 353455..357328 - reverse complement). These primers were designed to ensure sufficient overlap between the resulting fragments for accurate alignment. The primer sequences and their PCR protocols are listed in **Table S2** in Supplementary Information. The primers were used to PCR amplify the six fragments of the *LAC1* gene in each strain and were then sequenced using the Sanger method. The software CLC Sequence Viewer 7.8.1 was used to combine the fragments to obtain the complete *LAC1* sequence for each strain. The "adegenet" package in R-studio was used to extract polymorphic sites from the aligned *LAC1* sequences of the population.

### 2.4.7 *Galleria mellonella* mortality assay

The virulence of each strain was assayed in the surrogate host *Galleria mellonella* (Greater Wax Worm) using a protocol described previously (Fuchs, O'Brien, Khoury, & Mylonakis, 2010). Briefly, *C. neoformans* strains were freshly grown on YEPD medium for 72 hours followed by the preparation of a cell suspension in Phosphate-Buffered Saline (PBS) with the concentration adjusted to $2x10^7$ cells/ml using the hemocytometer cell counter. 5ul of this solution was injected using a 26mm gauge Hamilton syringe (Fisher Scientific) into the last left proleg of the waxworms, so that each worm received an inoculum of ~$1x10^5$ cells. For each strain, 10-12 waxworms were injected. Each trial included three control groups of wax worms: the first group was left untouched, the second group was injected with sterile PBS only, and a third group was poked in the last

left proleg with the needle to stimulate the physical trauma of injections but were not injected with any liquid. The groups of waxworms were then placed in separate petri dishes supplemented with wheat-bran as food, and incubated in the dark at $30^0$C for up to 14 days. The number of dead worms was scored daily. Waxworms were declared dead when they did not respond to repeated touch stimuli. Kaplan-Meier survival curves for waxworms injected with different *C. neoformans* strains were generated using the "Survival" package in R-Studio, based on daily mortality counts.

### 2.4.8 Statistical Analysis

All statistical analyses were carried out using the R-Studio software and associated packages. First, we conducted two-way Analysis of Variance (ANOVA) tests for each of the 3 different stress conditions to partition the total population level phenotypic variation in melanin production to environmental effect, genotypic effect (i.e. broad-sense heritability) and genotype-environment interaction effects. Second, for those conditions that showed statistically significant genotypic effects, one-way ANOVA tests were used to determine which levels of stress (e.g. $30^o$C vs $37^o$C; and different concentrations of $H_2O_2$ and different concentrations of $NaNO_2$) were more conducive for melanin production. Third, for each of the 8 environmental conditions (i.e. different levels of high temperature, oxidative stress, nitrosative stress), we used one-way ANOVA to estimate the percentage of population variance in melanin explained by the *LAC1* locus. Next,

within each environmental condition, the strains were grouped into environmental and clinical categories and their melanin levels were compared using student's t-tests to determine if source of the strains was a contributing factor to variation in melanin levels. Also, the strains were grouped according to their mating type (MAT locus) into MAT-a and MAT-α groups. The average melanin production of the two groups was compared within each environmental condition using student's t-tests to determine if MAT locus was a contributing factor to the variance in melanin. Fifth, the effect of *LAC1* genotypes on melanin production within each environmental condition was tested using ANOVA tests. Finally, a mixed-linear model was constructed using the R package 'lme4' to evaluate the quantitative association between virulence, melanin production and *LAC1* genotypes. In all tests, Bonferroni correction was applied to reduce false positive signals in our results.

## 2.5 Results

Within each of the environmental conditions, we observed significant variation in melanin production among strains of *C. neoformans* var. *neoformans* (**Figure 2.1**). Furthermore, the average melanin production of the population changed significantly in response to thermal, oxidative and nitrosative stresses (**Figure 2.2**). Given the significant amount of observed variation in melanin production, we conducted Two-factor ANOVAs to determine the proportional contribution of genetic and environmental factors, as well

as that of genetic-environmental interactions, to the observed phenotypic variance. We also calculated the proportion of melanin variance explained by the *LAC1* gene in our study population of *C. neoformans* var. *neoformans*. The results from variance analyses are summarized in **Table 2.1** and

**Table 2.2**. Below we describe the individual results.

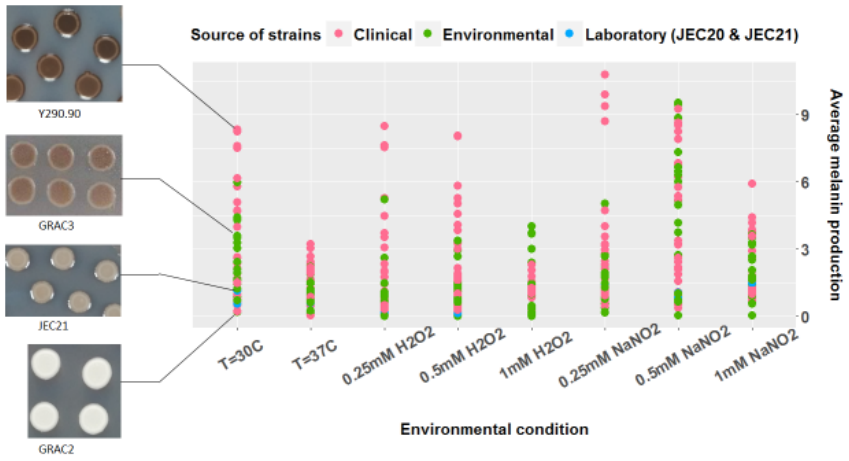


**Figure 2.1: Melanin production of individual *C. neoformans* var. *neoformans* strains.**

This figures represents the level of melanin production of each of the 54 tested strains of *C. neoformans* var. *neoformans* in 8 different environmental conditions, from left, $30^0$C (no added stressors), $37^0$C (thermal stress), low oxidative stress (0.25mM $H_2O_2$), intermediate oxidative stress (0.5mM $H_2O_2$), high oxidative stress (1mM $H_2O_2$), low nitrosative stress (0.25mM $NaNO_2$), intermediate nitrosative stress (0.5mM $NaNO_2$) and high nitrosative stress (1mM $NaNO_2$).

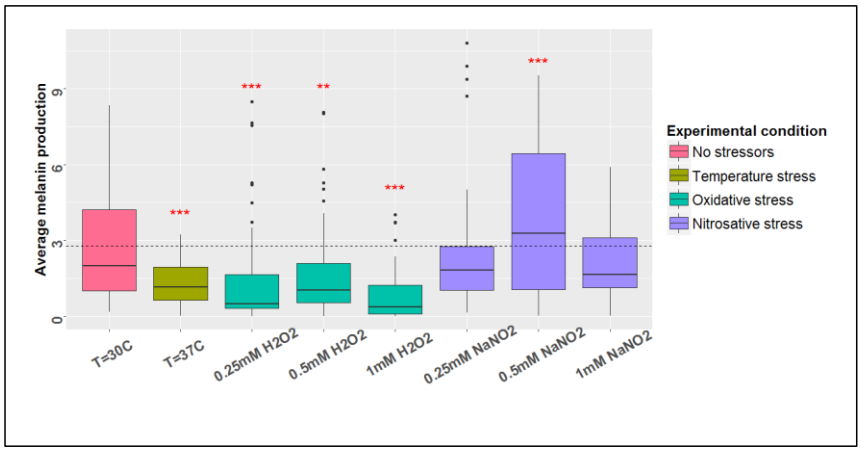


**Figure 2.2: Melanin production of *C. neoformans* in different environmental stresses.**

These boxplots show the distribution of melanin production scores of the tested *C. neoformans* var. *neoformans* population in 8 different environmental conditions, namely, from left, temperature of $30^0$C, temperature of $37^0$C, low oxidative stress (0.25mM $H_2O_2$), intermediate oxidative stress (0.5mM $H_2O_2$), high oxidative stress (1mM $H_2O_2$), low nitrosative stress (0.25mM $NaNO_2$), intermediate nitrosative stress (0.5mM $NaNO_2$) and high nitrosative stress (1mM $NaNO_2$). The dashed line represents the average melanin production of the population in the negative control (no added stressors). The red stars above the boxplots represent the degree of statistical significance to which the average melanin production of the conditions differ from that of the negative control.

### 2.5.1 Effect of temperature on melanin production

The average melanin production of the *C. neoformans* var. *neoformans* population was severely depressed at $37^0$C when compared to $30^0$C (p= 1.56E-06). However, clinical strains produced significantly more melanin on average than environmental strains at $37^0$C (p=0.011). At $30^0$C, the clinical and environmental populations did not differ in average melanin production (**Figure 2.3**). In $30^0$C, clinical strains exhibited a wider range of melanin levels (0.21 - 8.35) than the environmental population (0.16 to 6.0) whereas at $37^0$C both populations showed a more restricted range of melanin levels. In thermal stress, 55.5% of the variation in melanin between strains was attributable to genetic differences between the strains, followed by 28% due to genetic-environmental interactions and, 15% due to environmental factors alone. The *LAC1* locus explained 54% of the genetic variance and 30% of the total observed variance in melanin in thermal stress. The presence of genotype-environmental interactions was evidenced by several strains whose response to thermal stress did not conform to the average population behavior (**Panel A in Figure 2.4**). One such strain was I0156, a clinical strain isolated from an AIDS patient in Italy, whose melanin production increased almost 7-fold when exposed to thermal stress.

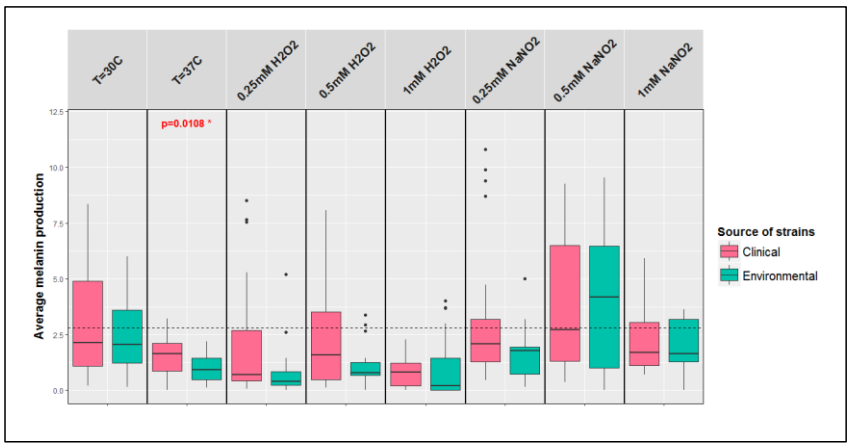


**Figure 2.3: Melanin production of *C. neoformans* by source.**

These boxplots show the average melanin production of the clinical and environmental populations of *C. neoformans* var.*neoformans* within 8 environmental conditions, depicted in panels above. The panels are, from left, temperature of $30^0$C, temperature of $37^0$C, low oxidative stress (0.25mM $H_2O_2$), intermediate oxidative stress (0.5mM $H_2O_2$), high oxidative stress (1mM $H_2O_2$), low nitrosative stress (0.25mM $NaNO_2$), intermediate nitrosative stress (0.5mM $NaNO_2$) and high nitrosative stress (1mM $NaNO_2$). The environmental and clinical populations produced similar melanin levels on average in all environmental conditions except in a temperature of 37C (thermal stress) where clinical strains produced significantly more melanin on average than the clinical strains (p=0.0188). The dashed line represents the average melanin production of the population in the negative control (no added stressors).

### 2.5.2 Effect of oxidative stress on melanin production

In all three tested levels of oxidative stress (0.25mM, 0.5mM and 1mM $H_2O_2$), the *C. neoformans* population produced significantly less melanin on average than when grown without any oxidative stress (p values 3.09E-05, 0.00459, 5.28E-11 respectively). Interestingly, the average melanin production of the population did not differ significantly between the three levels of oxidative stress. In high oxidative stress, most strains did not produce any melanin at all and appeared as white colonies. In low and intermediate oxidative stress, clinical strains produced more melanin on average than the environmental strains but these differences were not statistically significant (**Figure 2.3**). In high oxidative stress, both populations were equally repressed.

In oxidative stress, a significant proportion of the variance between strains was attributed to genotype-environment interactions at 43%. This was highlighted by the inconsistent patterns of strains' responses under different levels of oxidative stress. For example, in the environmental strain GRAC1 from Greece, melanin production was induced in response to oxidative stress with increasingly higher amounts of melanin being produced as stress level increased (**Panel B in Figure 2.4**). Similarly, a few other strains produced high levels of melanin in low and intermediate oxidative stress, leading to higher variance within the population. According to the ANOVA results, genetic factors contributed 51% to the overall variance in melanin in oxidative stress whereas variance due to environmental factors was a mere 5%.

### 2.5.3 Effect of nitrosative stress on melanin production

Unlike oxidative stress, nitrosative stress did not seem to have a significant repressive effect on melanin production. In low and high nitrosative stress, the average melanin production of the *C. neoformans* population was slightly lower compared to the negative control but these differences were not statistically significant. In the presence of intermediate nitrosative stress (0.5mM $NaNO_2$), the average melanin production of the population was unexpectedly higher, even surpassing that of optimum growth conditions at $30^0$C (p=0.00032). This was a significant increase from the average melanin production in low nitrosative stress (p= 3.09E-05) and high nitrosative stress (p= 5.75E-11). Out of the 8 tested environmental conditions, intermediate nitrosative stress also induced the highest variation in melanin levels among the population, ranging from 0.007 to 9.53. In all three levels of nitrosative stress, the environmental and clinical populations did not significantly differ from each other in average melanin production (**Figure 2.3**).

In nitrosative stress, 59% of the variance in melanin was due to genetic differences between strains. Genotype-environment interactions contributed to 29% of the variance and environmental factors explained 11% of the overall variance. Similar to thermal and oxidative stresses, the melanin response of a few strains did not follow the overall pattern of the population, signifying the effects of genotype-environment interactions on melanin production in *C. neoformans* (**Panel C in Figure 2.4**).

**Table 2.1: Statistical analyses of variation in melanin production.**

This table displays the results of the two-way ANOVA conducted to assess the contribution of genotype (all strains were assumed to have different genotypes) and environment (thermal, oxidative and nitrosative stresses) on the variation in melanin production observed among 54 strains of *C. neoformans* var. *neoformans.*

| | **Two Way ANOVA: Melanin production ~ Strain (genotype) * Stressor (environment)** | | | | | |
|---|---|---|---|---|---|---|
| **Environmental Stress** | **Factor** | **Degrees of freedom** | **Sum of Squares** | **Mean sum of squares** | **F-value** | **P- value** |
| **Thermal stress** | Strain | 53 | 718.28 | 13.552 | 311.34 | <2.2e-16 *** |
| | Stressor | 1 | 199.67 | 199.670 | 4587.05 | <2.2e-16 *** |
| | Strain: Stressor Interactions | 53 | 363.75 | 6.863 | 157.67 | <2.2e-16 *** |
| | Residuals | 274 | 1.93 | 0.044 | | |
| | **% Variance due to genetic factors** | | | | | **55.52** |
| | **% Variance due to environmental factors** | | | | | **15.43** |
| | **% Variance due to genetic x environmental interactions** | | | | | **28.12** |
| **Oxidative stress** | **Factor** | **Degrees of freedom** | **Sum of Squares** | **Mean sum of squares** | **F-value** | **P- value** |
| | Strain | 53 | 847.82 | 15.997 | 378.09 | <2.2e-16 *** |
| | Stressor | 2 | 77.91 | 38.955 | 920.75 | <2.2e-16 *** |
| | Strain: Stressor Interactions | 106 | 71.14 | 6.699 | 158.35 | <2.2e-16 *** |
| | Residuals | 462 | 19.55 | 0.042 | | |
| | **% Variance due to genetic factors** | | | | | **51.21** |
| | **% Variance due to environmental factors** | | | | | **4.71** |
| | **% Variance due to genetic x environmental interactions** | | | | | **42.9** |
| **Nitrosative stress** | **Factor** | **Degrees of freedom** | **Sum of Squares** | **Mean sum of squares** | **F-value** | **P- value** |
| | Strain | 53 | 1954.30 | 36.874 | 540.33 | <2.2e-16 *** |

| | | | | | | |
|---|---|---|---|---|---|---|
| | Stressor | 2 | 371.73 | 185.863 | 2723.55 | <2.2e-16 *** |
| | Strain: Stressor Interactions | 106 | 967.27 | 9.125 | 133.72 | <2.2e-16 *** |
| | Residuals | 412 | 28.12 | 0.068 | | |
| | **% Variance due to genetic factors** | | | | | **58.84** |
| | **% Variance due to environmental factors** | | | | | **11.19** |
| | **% Variance due to genetic x environmental interactions** | | | | | **29.12** |

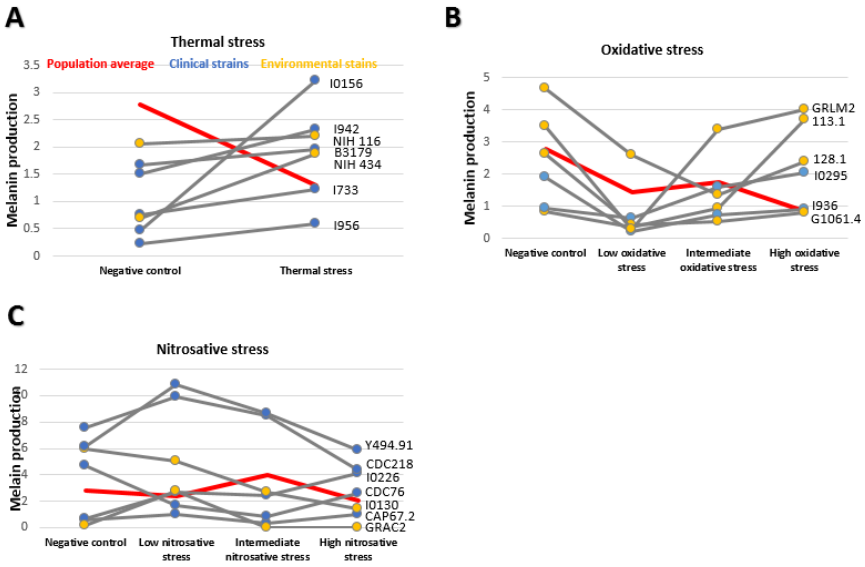


**Figure 2.4: Variable melanin responses among strains of *C. neoformans*.**

In each stress condition, we identified several strains whose melanin production varied significantly from the rest of the population, highlighting the contribution of genotype-environmental interactions to melanin production in *C. neoformans* var. *neoformans*. The red lines represent the population average. **A)** Melanin production of several strains increased in thermal stress in contrast to the rest of the population whose average melanin production decreased. **B)** Several strains exhibited increased melanin production in oxidative stress, in contrast to the decreasing trend observed in the average population. **C)** The average melanin production of the population showed a peak in melanin production at intermediate nitrosative stress, whereas a few strains peaked at low or intermediate nitrosative stress.

### 2.5.4 Melanin production by mating type

*C. neoformans* cells belong to one of two mating types – 'a' or 'α', determined by their allelic composition at the MAT locus. The test population consisted of 39 MAT-α strains and 15 MAT-a strains. The two mating types significantly differed in average melanin production in three out of the eight tested environmental conditions, namely thermal stress, low oxidative stress and high oxidative stress. In thermal and low oxidative stress conditions, the MAT-α strains produced significantly more melanin on average than MAT-a strains ($p=0.0007$ and $p=0.03$ respectively), whereas in high oxidative stress, MAT-a strains significantly out-produced MAT-α strains ($p=0.03$) (**Figure 2.5**). In all other tested conditions, the average melanin production of the MAT-α strains was higher than that of MAT-a strains, although these differences were not statistically significant. Therefore, based on the ANOVA results, MAT locus explained 11% of the total melanin variance in thermal stress, 4.4% in low oxidative stress and 4.8% in high oxidative stress.

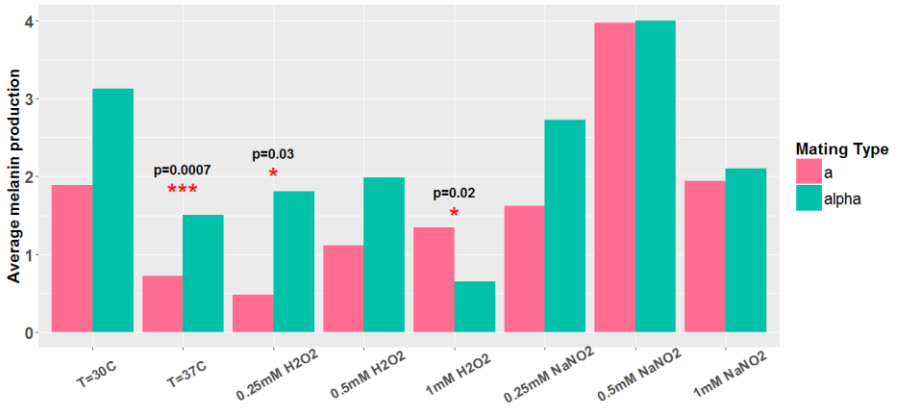


**Figure 2.5: Melanin by mating type:** The average melanin production of the *C. neoformans* var. *neoformans* strains grouped by mating type is shown here. Blue bars represent MAT-a strains and pink bars represent MAT-α strains. In $37^0$C and low oxidative stress, MAT-α strains produced significantly more melanin than MAT-a strains, whereas in high oxidative stress, MAT-a strains produced significantly more melanin than MAT-α strains.

### 2.5.5 Genetic polymorphisms at the *LAC1* locus and their contributions to melanin production

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We obtained the *LAC1* gene sequence from all 54 strains. A total of 110 SNPs were found within the combined promoter and coding regions of the *LAC1* gene, leading to 21 different *LAC1* alleles in our population of *C. neoformans* var. *neoformans*. ANOVA tests revealed that this locus accounted for 13% to 30% of the total variance in melanin production, depending on the environmental condition (

**Table 2.2**). In thermal and oxidative stress, the contribution of the *LAC1* gene sequence variation to melanin production increased with higher levels of stress. However, in nitrosative stress, the biggest contribution from *LAC1* to melanin production was found at intermediate nitrosative stress. Specifically, the *LAC1* gene explained 18% of the total variance in melanin production at 30$^{o}$C without any stress, 30% at 37$^{o}$C, 13% in low oxidative stress, 21% in intermediate oxidative stress, 23% in high oxidative stress, 23% in low nitrosative stress, 27% in intermediate nitrosative stress, and 22% in high nitrosative stress. To determine the effects of specific *LAC1* polymorphisms on melanin production, we focused on the SNPs with a minor allele frequency (MAF) of 10% or higher.

**Table 2.2: Table: Percent melanin variance explained by *LAC1* and *MAT* loci.**

The % variance in melanin explained by the *LAC1* locus in each of the tested environmental conditions is shown in this table. The results were obtained from ANOVA-based analyses.

| **% Variance explained by *LAC1* in each stress condition** | | |
|---|---|---|
| **Environmental stressor** | **% Genetic variance due to *LAC1*** | **% Total variance due to *LAC1*** |
| No stress | 31.99 | 17.76 |
| Thermal stress ($37^0$C) | 53.73 | 29.83 |
| Low oxidative stress | 25.55 | 13.08 |
| Intermediate oxidative stress | 41.25 | 21.12 |
| High oxidative stress | 45.24 | 23.17 |
| Low nitrosative stress | 38.84 | 22.85 |
| Intermediate nitrosative stress | 45.45 | 26.74 |
| High nitrosative stress | 37.59 | 22.12 |

### 2.5.6 *LAC1* promoter and 5' UTR

A total of 2232 base pairs immediately upstream of the start codon of the *LAC1* gene was analyzed for genetic polymorphisms. This included the 5' Untranslated Region (5' UTR) that spanned from -1 to -433 and the preceding region from –434 to -2232 within which the promoter was located. The nucleotides were numbered relative to the start codon ATG, with 'A' considered +1. A total of 38 SNPs were identified of which 28 were located in the promoter region and 10 in the 5' UTR. The genetic polymorphisms are approximately uniformly distributed throughout the length of the region without any obvious clustering. A significant portion of the SNPs (11/38) was due to the three environmental strains from Germany deviating from the rest of the population while remaining identical to each other.

11 out of the 38 SNPs had a MAF ≥ 10% and were thus included in the statistical analysis. A schematic of the promoter and 5' UTR of *LAC1* with the 11 significant SNPs is shown in **Figure 2.6**. One of the 11 SNPS, G-925A was located within a repressor region in the promoter previously identified as having an inhibitory effect on the transcription of the *LAC1* gene ((Zhang, Varma, & Williamson, 1999). We also identified a tandem duplication of a 322 base pair segment that spanned the promoter and 5' UTR regions (-77 to -720). Both duplicates contained a small INDEL of 8 nucleotides (CGATACAT) (-137 and -459) and two SNPs located several base pairs downstream of the INDEL. All 6 of these polymorphisms were present in the same 9/54 strains and they

surpassed our MAF threshold of 10%. Due to their identical distributions, only one was included in downstream statistical analyses to avoid redundancy.

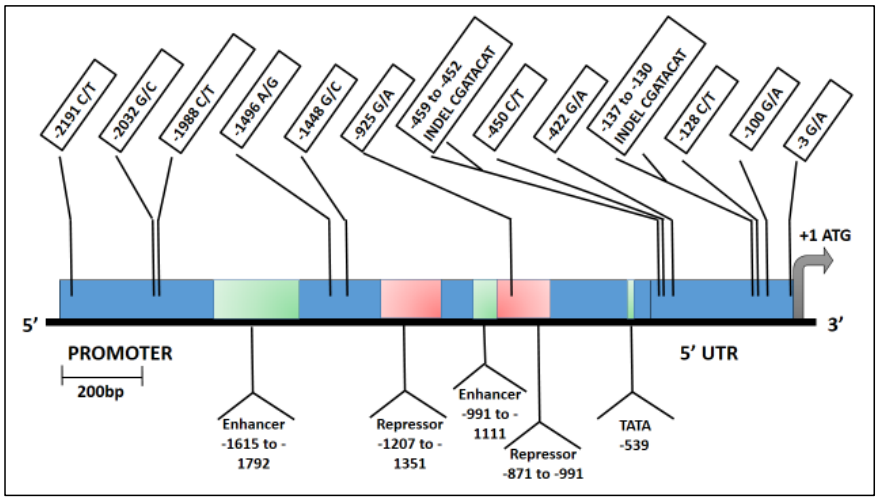


**Figure 2.6: SNPs in the promoter of *LAC1*.** This schematic shows the Single Nucleotide Polymorphisms (SNPs) and INDELs that we found within the promoter and 5' untranslated regions of the *LAC1* gene, in our study population of *C.neoformans* var. *neoformans*. Only those polymorphisms that met a minor allele frequency threshold of 10% are shown here. The green regions within the promoter represent transcriptional enhancer elements and the red regions represent the transcriptional repressor elements identified in a previous study. One SNP, -925 G/A, was located within a transcriptional repressor element.

### 2.5.7 *LAC1* coding region

The Lac1 protein is coded for by 14 exons that add up to a total length of 1872 base pairs, and are translated into a final protein product of 624 amino acid residues. Within our population of *C. neoformans* var. *neoformans* strains, we identified a total of 48 SNPs located within the exons of *LAC1*, 12 of which surpassed the MAF threshold of 10%. Only 4/12 are predicted cause non-synonymous amino acid changes. These are F159Y (MAF 44.4%), P182T and P182A (MAF 27.8%), M360I (MAF 38.9%) and R590K (MAF 25.9%). F159Y and P182T represent changes from non-polar to polar amino acids whereas M360I represents a switch within the non-polar amino acid category. All three of these SNPs are located within putative catalytic domains of the Lac1 protein, namely a copper-oxidase domain and a diphenol oxidase domain. The fourth non-synonymous SNP R590K signifies a change from one basic amino acid to a different basic amino acid and is located at the 3' end of the protein within the 14$^{th}$ exon. A schematic of the *LAC1* coding region with the SNPs identified within exons is shown in **Figure 2.7**.

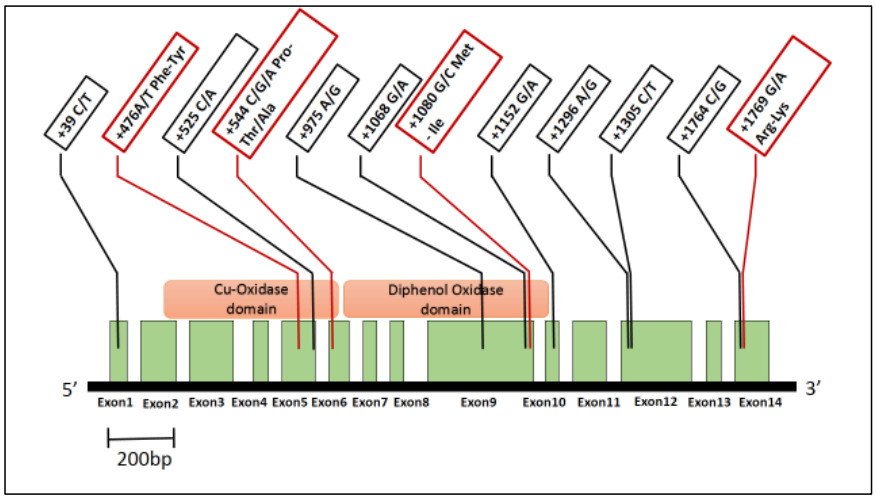


**Figure 2.7: SNPs in the coding region of *LAC1*.** This schematic shows the Single Nucleotide Polymorphisms (SNPs) that we found within the coding region of the *LAC1* gene, in our study population of *C. neoformans* var. *neoformans*. Only those polymorphisms that met a minor allele frequency threshold of 10% are shown here. The green boxes represent exons and the white boxes in between represent the introns. The four boxes in bold are the SNPs that cause a non-synonymous amino acid change whereas the rest of the SNPs are synonymous and do not lead to a change in the amino acid. Three of the four non-synonymous SNPs are located within putative catalytic domains of the Lac1 protein.

### 2.5.8 *LAC1* introns and the 3' Untranslated region

We also sequenced the 13 introns and a few nucleotides into the 3' Untranslated Region (3' UTR) of the *LAC1* gene that added up to a total length of 721 bps. Within this, we identified a total of 24 SNPs, 8 of which surpassed the MAF of 10%. The SNPs were clustered towards the 3' end of the gene with 15/24 SNPs located within the last 3 introns and the 3' UTR. Intron 12, one of the two smallest introns of the gene (49 bps each), contained the highest number of SNPS in any intron (6 SNPs), but none of them met the MAF threshold of 10%. Introns 1, 2 and 7 had zero SNPs, introns 3,5,10 and 11 contained 2 SNPS each and intron 8 contained 3 SNPs. Due to the absence of any evidence of *LAC1* introns contributing to the transcriptional regulation of the gene, SNPs located within the introns and the 3' UTR were not included in downstream statistical analyses.

### 2.5.9 Association between selected *LAC1* haplotypes and melanin production

The 11 SNPs from the promoter region and the 4 non-synonymous SNPs from the coding region with MAF $\geq$ 10% were concatenated to form strings of SNPs here on referred to as *LAC1* haplotypes. There was a total of 11 *LAC1* haplotypes with the majority of strains (42/54) belonging to 4 haplotype groups. The haplotypes were numbered 1 to 11 depending on their prevalence with 1 being the most prevalent (14/54) and 11 being the least (1/54). Strains with similar origins (clinical vs. environmental) tended to cluster within haplotypes. For example, in haplotype 1, 12 out of the 14 strains were

environmental, whereas in haplotype 2, 8 out of the total of 9 strains were of clinical origin.

The association between *LAC1* haplotypes and melanin production within each environmental condition was evaluated using ANOVA tests. *LAC1* haplotypes were only significantly associated with melanin production in thermal stress (p= 0.0138) and high oxidative stress (p= 0.00602). The average melanin production of the different haplotype groups within these two environmental conditions are shown in **Figure 2.8**. To determine which SNPs contributed the most to the observed differences in melanin production, each of the 15 SNP were individually assessed using ANOVA tests. One SNP, A-1496G, located in the *LAC1* promoter was significantly associated with melanin production in thermal stress (p= 2.86E-03). Strains with G at this position produced significantly more melanin under thermal stress than strains with A. In high oxidative stress, two SNPs, A-925G and P182T/A, were significantly associated with melanin production. The former is located within a repressor region of the *LAC1* promoter whereas the latter is located within the putative copper-oxidase domain of the Lac1 protein and causes a non-synonymous amino acid switch from proline to threonine or alanine. The results on the significant *LAC1* SNPs are summarized in **Table 2.3**.

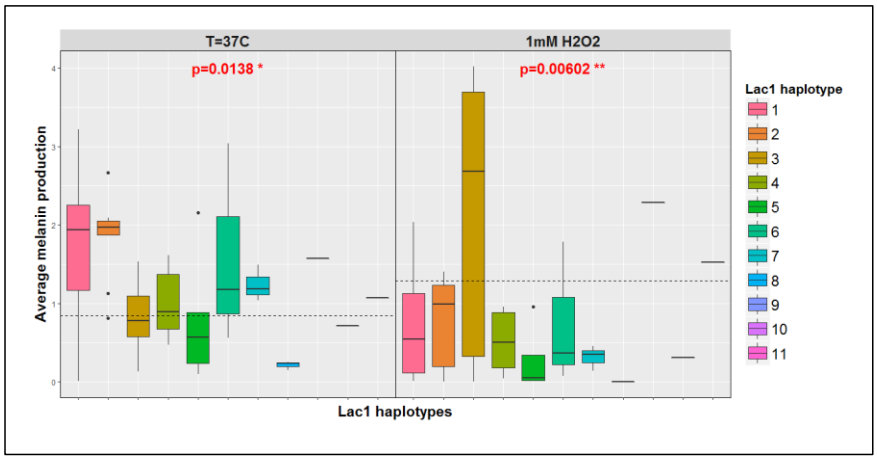


**Figure 2.8: Difference in melanin production between lac1 haplotypes.**.

The boxplots depict the melanin production levels of *C. neoformans* var. *neoformans* strains grouped by their *LAC1* haplotypes in thermal stress and high oxidative stress. The dashed line in each panel represents the average melanin production of the population in that stress condition. In thermal stress, haplotypes 2 and 8 are significantly different from each other whereas in high oxidative stress, haplotype 3 produced significantly more melanin than the rest of the population. The *LAC1* haplotypes did not significantly differ in average melanin production in other tested environmental conditions.

**Table 2.3: *LAC1* SNPs significantly associated with melanin production.**

The non-synonymous SNPs and the corresponding amino acid changes in the *LAC1* exons that were identified in the study population of *C. neoformans* var. *neoformans* are listed in this table.

| Specific SNPs/amino acid changes associated with melanin production | | | | | |
|---|---|---|---|---|---|
| **Stressor** | **SNP position** | **SNP alleles** | **Amino acid change** | **Effect on melanin production** | **Adjusted p-value** |
| Thermal stress ($37^0$C) | -1496 | A | - | ↓ melanin | 2.86E-03 |
| | | G | - | ↑ melanin | |
| High oxidative stress | -925 | A | - | ↑ melanin | 1.65E-04 |
| | | G | - | ↓ melanin | |
| | 544 | C | Proline | ↓ melanin | 1.30E-03 |
| | | G | Threonine | ↓ melanin | |
| | | A | Alanine | ↑ melanin | |

### 2.5.9 *Galleria mellonella* survival assay

We assessed the virulence of *C. neoformans* var. *neoformans* strains using the Greater waxworms (*Galleria mellonella)* to determine if virulence was quantitatively associated with the level of melanin production and the genetic polymorphisms at the *LAC1* locus. Once we obtained survival data for all the strains, we constructed a mixed-effect model to analyze the relationship between virulence and *LAC1* haplotypes. Our model failed to provide evidence of a significant association between *LAC1* haplotype and virulence in the tested population (p=0.76). Next we constructed mixed-models to include the melanin production of the strains in the eight environmental conditions. The analysis indicated that virulence of the strains was significantly associated with melanin production in high nitrosative stress (p= 0.025) in an inverse manner (correlation coefficient = -0.18). Strains that produced higher amounts of melanin in high nitrosative stress tended to be less virulent than strains that produced less melanin in this condition (**Figure 2.9**). The models failed to provide evidence of significant links between virulence and melanin production in other environmental conditions. Furthermore, we did not identify a significant difference in virulence between environmental and clinical strains of *C. neoformans* var. *neoformans.*

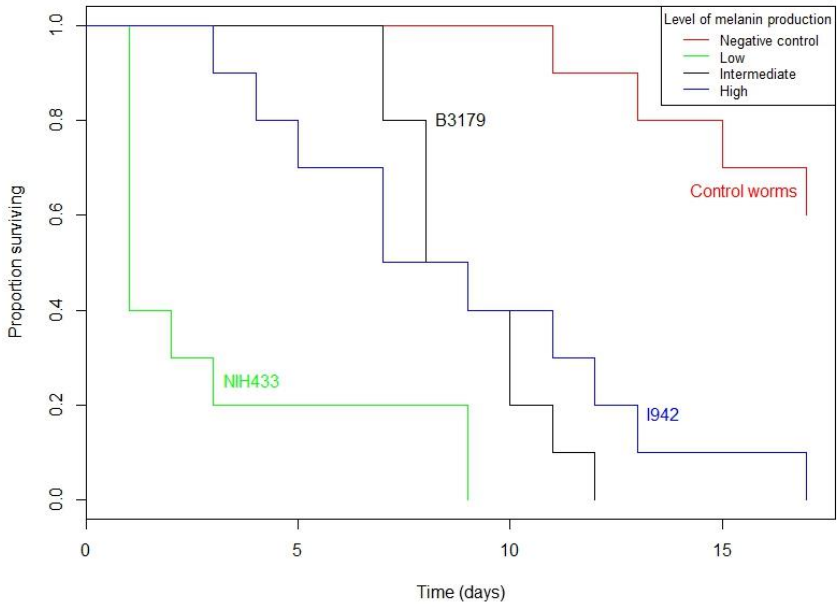


**Figure 2.9: Kaplan-Meier Survival Curves**. The Kaplan-Meier survival estimates of waxworms injected with *C. neoformans* var. *neoformans* strains that produced low, medium and high levels of melanin in high nitrosative stress. I942 (blue), a high producer killed the waxworms at a slower rate than B3179 (black), an intermediate producer and NIH433 (green), a low producer.

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# 2.6 Discussion

Our results indicate strong effects of genotypes, environmental factors, and genotype-environment interactions on the level of melanin produced in *Cryptococcus neoformans* var. *neoformans* (serotype D). With regard to environmental factors, both high temperature and high oxidative stress markedly suppressed melanin production. We identified a putative association between *LAC1* sequence variation and melanin production, and an inverse correlation between melanin production in nitrosative stress and the virulence of the strains in waxworms. Below we discuss the implications of these results.

## 2.6.1 Effect of temperature on melanin production

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At $37^0$C, both environmental and clinical strains of *C. neoformans* var. *neoformans* produced significantly less melanin compared to $30^0$C. This is consistent with previous findings that Lac1 protein is severely inhibited at $37^0$C and the catalysis of melanin synthesis is negatively affected (Jacobson & Emery, 1991). However, a study that quantified the melanin production of seven *C. neoformans* strains including 4 var. *neoformans* strains over a course of 14-days found the melanin content to be similar in cells grown in $30^0$C and $37^0$C at all days except day 10 (Wang, Aisen, & Casadevall, 1996). The apparent contradiction between this and our result could be due to the small sample size

used in the previous study that failed to capture the full extent of variation in melanin that exists in natural populations of *C. neoformans*.

During infections within mammalian hosts, *C. neoformans* cells are constantly exposed to temperatures of or above $37^0$C. It has been shown that *C. neoformans* is capable of producing melanin *in vivo* during infections (Rosas et al., 2000). In fact, clinical strains in our population of *C. neoformans* var. *neoformans* produced significantly more melanin than environmental strains at $37^0$C, implying that exposure to thermal stress within the host may have acted as a selective pressure for higher melanin production in clinical strains. The majority of the tested environmental strains were isolated from Europe and regions of Asia that are located away from the equator: thus, these strains were unlikely to have been exposed to temperatures $\geq$ 37ºC for significant amount of time in their natural habitats.

The decrease, but not complete elimination, of overall melanin production under thermal stress suggests that the presence of melanin pigments at low concentrations might be sufficient for survival within a host during an infection. The lower melanin production at $37^0$C is likely due to the decreased functionality of the Lac1 protein at $37^0$C, combined with the rerouting of cellular resources to heat response pathways. A transcriptome analysis conducted by Steen and colleagues discovered that transcript levels of heat shock proteins HSP60, HSP70 and HSP80, along with translation machinery, were elevated when temperature was increased from $25^0$C to $37^0$C in both varieties of *C. neoformans* (Steen et al., 2002). All the tested strains in our population were able to grow at $37^0$C which points to the presence of a successful heat response pathway, possibly at the cost of higher melanin production.

### 2.6.2 Effect of oxidative stress on melanin production

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$H_2O_2$ breaks down into reactive oxygen species (ROS) that are harmful to yeast cells. This is one of the mechanisms macrophages utilize to combat *C. neoformans* cells during an infection. Previous studies have shown that melanised *C. neoformans* cells are more resistant to oxidative stress than non-melanized cells *in vitro* (Wang & Casadevall, 1994). We found that the presence of $H_2O_2$ in the growth medium actually led to a significant decrease in mean melanin production of the *C. neoformans* var. *neoformans* population with both environmental and clinical strains being equally affected. At high oxidative stress (1mM $H_2O_2$), melanin production in most strains was almost completely inhibited at least up until 3 days following initial exposure. Our results indicate that melanin synthesis is unlikely to be a component of the initial response to oxidative stress in *C. neoformans*. The growth of the strains was not affected in the three tested concentrations of oxidative stress (0.25mM, 0.5mM and 1mM $H_2O_2$) which shows that *C. neoformans* has response mechanisms to successfully counteract oxidative stress, and that such a response does not require the presence of melanin pigments inside the cells. In addition to catalyzing melanin synthesis, *LAC1* enzyme contains iron oxidase activity and mediates the oxidation of Fe(II) to Fe(III) which indirectly leads to the removal of hydroxyl radicals from the environment (Liu, Tewari, et al., 1999). Redirecting the *LAC1* enzyme from melanin synthesis to iron oxidation can lead to decreased levels of melanisation in *C. neoformans* cells in the presence of oxidative stress. Our results support the notion that, given the importance of melanin synthesis pathway to virulence in *C. neoformans* (Kwon-Chung, Polacheck, &

Popkin, 1982), the *LAC1* enzyme, rather than the melanin pigments, carry out a crucial role in protecting the yeast cells from oxidative stress.

### 2.6.3 Effect of nitrosative stress on melanin production

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During an infection, macrophages release bursts of RNS along with ROS into the cytoplasm to cause stress to engulfed yeast cells. Both species are believed to cause cellular stress in a similar manner, and consequently the responses of *C. neoformans* to ROS and RNS were expected to be similar as well. However, our results show that *C. neoformans* population produced significantly more melanin on average in all three levels of nitrosative stress than in oxidative stress. The average melanin production in low and high nitrosative stress was somewhat repressed compared to baseline but increased unexpectedly in intermediate nitrosative stress. Based on our results, we propose that the production of melanin pigments is a stress response to nitrosative stress in *C. neoformans* but this response is only triggered within a small window of RNS concentrations (approximately 0.25mM to 0.5mM $NaNO_2$) in the environment. When nitrosative stress is encountered in concentrations outside of this narrow window, melanin production is suppressed in favor of other defense mechanisms. Our findings are consistent with the results of a previous study where it was found that the concentration of synthetic DOPA-melanin pigments in an acidic buffer increased four-fold in the presence of 0.5mM $NaNO_2$ whereas the presence of $H_2O_2$ in the medium did not produce the same effect (Matuszak et al., 2015). They proposed that the interaction of

DOPA-melanin with nitrite at acidic pH leads to melanin oxidation, followed by the generation of a large number of melanin radicals. To the best of our knowledge, this is the first instance of this phenomenon being observed in a cellular setting. Also, this is the first evidence of melanin synthesis being used as a response to nitrosative stress in a nitrite-concentration-dependent manner in *C. neoformans* var. *neoformans.* Both environmental and clinical strains exhibited this pattern of melanin production suggesting that nitrosative stress and the adaptive response to it is likely widespread in natural and clinical environments of *C. neoformans.*

### 2.6.4 Melanin production of mating type

Mating type, similar to melanin production, has been linked to the virulence of *C. neoformans,* with MAT-α strains clearly more virulent than MAT-a strains (K. J. Kwon-Chung, Edman, & Wickes, 1991). In fact, strains of the more virulent variety *C. neoformans* var. *grubii* almost exclusively belong to the α mating type (K J Kwon-Chung & Bennett, 1978; Liaw, Wu, & Hsueh, 2010). In our test population of 54 *C. neoformans* var. *neoformans* strains, 28 out of the 30 clinical strains belonged to α mating type whereas the environmental strains were evenly distributed between the two mating types with 10 MAT-α strains and 12 MAT-a strains.

Given the previously shown association of MAT-α strains with higher virulence, it was expected that they should express higher melanin production as well. While this was the case for the test population in thermal stress and low oxidative stress, the MAT locus

failed to be significantly associated with melanin production in nitrosative stress and intermediate oxidative stress. In fact, in high oxidative stress, MAT-a strains out-produced MAT-α strains significantly. The MAT locus spans a region of >100kb and contains over 20 genes including regulators of sexual development, pheromones and pheromone receptors, as well as other genes that have no obvious function in mating (Lengeler et al., 2002). These genes likely interact with other loci, potentially including those involved in melanin production, as well as with environmental factors, to shape the melanin response in different environments, leading to an inconsistent link between mating type and melanin production.

Furthermore, the two mating types in our test population did not significantly differ in virulence for waxworms. However, the association of α mating type with virulence is supported by the overwhelming majority of clinical strains in the study population being MAT-α. The virulence in waxworms may not have resembled human infections closely enough to detect the difference in virulence between MAT-α and MAT-a strains.

### 2.6.5 Analysis of Variance in melanin production

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Despite the crucial role of melanin in the virulence of *C. neoformans*, the current knowledge about the genetic basis of its natural quantitative variation is largely incomplete. It has been shown that melanin synthesis is eliminated in the absence of the *LAC1* gene (Salas et al., 1996). Other than that, it is not yet clear which genetic loci and

environmental factors are responsible for the significant variation in melanin levels observed among strains of *C. neoformans*. By analyzing hybrid progeny of a *C. neoformans* var. *neoformans* and a *C. neoformans* var. *grubii* strain, a recent study identified five genetic regions influencing melanin production at the $30^0$C environment without any added stresses (Vogan et al., 2016). The results from the current study are consistent with multiple loci contributing to melanin production in natural populations of *C. neoformans* var. *neoformans*. Our ANOVA-based analyses reveal that over 50% of the variance in melanin production at each of the eight tested environments was due to genetic variation between the strains. Among these, *LAC1* locus accounted for 25% to 55% of the total genetic variance, while the MAT locus contributed 8% to 20% of the genetic variance, leaving up to 65% of the genetic variance unexplained. This reveals the presence of other genetic loci that are significantly associated with melanin production. However, at present, we don't know what those genes are and where they are located in the genome. As shown in **Error! Reference source not found.Error! Reference source not found.**, the contribution of *LAC1* to variance in melanin is significantly higher in the presence of environmental stress, with the exception of low oxidative stress. This implies that *LAC1* plays a greater role in shaping the response of melanin to environmental stressors, than in melanin production in the absence of stress.

The variance in melanin production explained by *LAC1* in the absence of environmental stress is ~18% in our test population: this is slightly higher than the estimate of 10% obtained by the QTL analysis of hybrids progeny of *C. neoformans* var. *grubii* and *C. neoformans* var. *neoformans* (Vogan et al., 2016). The differences might be

attributed to a couple of factors. First, due to their historical divergence (Xu, Vilgalys, & Mitchell, 2000a), there might be more loci involved in melanin production variation between the two varieties than within a single variety, diminishing the contribution of *LAC1* to the overall variance. Second, since the hybrid progeny shared the same parents and were more related to each other than our study population, they are less likely to have been variable at *LAC1* and other genetic loci, leading to smaller proportions of variance being explained by *LAC1* itself. Our results indicate that genetic-environment interactions also contributed to a significant proportion of the variance between the strains, with them causing up to 43% of the melanin variance in oxidative stress. Our findings are consistent with melanin production in *C. neoformans* being a complex trait with many genetic, environmental and interaction factors having a role in its regulation and response to environmental stress.

### 2.6.6 Significant genetic polymorphisms in *LAC1* gene

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We identified specific SNPs located within *LAC1* that were significantly associated with melanin production in *C. neoformans* var. *neoformans*. *LAC1* haplotypes were significantly associated with melanin production in thermal and high oxidative stresses, the two conditions in which *LAC1* was estimated to contribute the most to overall variance in melanin production, at 54% and 45% respectively. The SNP significantly associated with melanin production in thermal stress was located in the promoter, but not

in regions known to have an effect on the rate of transcription (Zhang et al., 1999). This SNP likely arose in response to high temperature stress in the clinical environment: 20 out of the tested 31 clinical strains contain the allele for higher melanin production at this SNP, in contrast to only 4 environmental strains having this allele.

Melanin production in high oxidative stress was significantly associated with an amino acid switch in a putative copper-oxidase domain of the Lac1 protein. This change likely affects the 3D structure of the catalytic domain since the 3 different alleles code for amino acids with differing characteristics: specifically, proline (39/54 strains) has a unique side chain that forms a ring with the backbone of the amino acid, Alanine (8/54) has a non-polar, methyl side group, whereas threonine (7/54) has a polar, hydroxyl side group. Strains with threonine produced significantly more melanin in high oxidative stress than strains with proline or alanine: the allele with threonine was found in all tested environmental strains from Greece, one clinical strain from Italy and one clinical strain from Japan. Given this mixed distribution, this SNP likely evolved independently in *C. neoformans* var. *neoformans* on multiple occasions and confers a significant advantage in oxidative stress by elevating melanin production. The second SNP significantly associated with melanin production in high oxidative stress is located within a repressor region of the *LAC1* promoter. Variation at this position likely alters the rate of transcription of the *LAC1* gene, leading to changes in melanin production. The allele associated with higher melanin levels at this SNP is present in the same 7 strains that contain threonine at the amino acid switch, suggesting that these two positions act in concert to elevate melanin production.

### 2.6.7 *Galleria mellonella* virulence assays

Even though melanin production is a well-known virulence factor of *C. neoformans*, the quantitative effects of melanisation on *in vivo* virulence have not been investigated before. Our results from the *Galleria mellonella* virulence assays showed that strains that produced more melanin in high nitrosative stress killed the waxworms at a slower rate. This result, combined with the finding that melanin production is upregulated within a small window of nitrosative stress, led us to believe that the presence of melanin at lower concentrations is likely sufficient to gain its survival and pathogenic benefits. Continuing to produce higher levels of melanin in the presence of nitrosative stress may adversely affect the yeast's ability to establish a successful infection inside the waxworms.

The degree of melanisation in other tested stress conditions was not associated with virulence in our population of *C. neoformans*. Similarly, *LAC1* haplotypes also failed to be significantly linked to virulence of the strains in waxworms. Given the significant differences among strains in their *in vivo* virulence in waxworms, our results suggest that the waxworm virulence of *C. neoformans* are affected by multiple loci and that the effects of *LAC1* genotype on virulence could be masked by other loci. Interestingly, the environmental strains in this study were equally as virulent in the waxworm model as the clinical strains. This aligns with previous observations that found environmental strains of *C. neoformans* to be fully capable of causing infections in humans and other hosts (Da Silva et al., 2006; Dromer, Mathoulin, Dupont, Letenneur, & Ronin, 1996; Pedroso, Lavrador,

Ferreira, Candido, & Maffei, 2010). It further supports the dual-use virulence paradigm of *C. neoformans* as our results illustrate the capability of this environmental yeast to successfully transition into a deadly pathogen in animal models.

## 2.7 Conclusions

Even though the significance of melanin production to the virulence of model pathogenic yeast *C. neoformans* has been well-established, the extent of variation in melanin levels among natural isolates and populations has remained largely unknown. In this study we quantified the melanin production of 54 strains of *C. neoformans* var. *neoformans* in thermal, oxidative and nitrosative stresses. We demonstrated that melanin production in nitrosative stress varied depending on the concentration of nitrite ions in the environment, whereas thermal and oxidative stresses exerted a uniformly negative effect on melanin production. Our results indicate that genetic factors, including the *LAC1* lcous, and genotype-environment interactions are responsible for the majority of the observed melanin variance in natural populations of *C. neoformans* var. *neoformans*. We identified three SNPs in *LAC1*, one causing an amino acid switch in the Lac1 protein, that were significantly associated with melanin production. We also found a quantitative association between melanin production in intermediate nitrosative stress and degree of virulence in our test population. Our results demonstrate the complex interplay of genetic, environmental and interaction factors that result in observable phenotypic variation in

natural populations. Our results suggest novel QTLs that are specific for certain environmental conditions. Identifying such QTLs, their regulations, and how they interact with each other and with environmental factors will further enhance our understanding of the evolution and maintenance of fungal virulence traits in natural populstions. Our findings on intra-varietal variance in melanin production in *C. neoformans* var. *neoformans* also help shed light on potential mechanisms of divergence between the two varieties.

# Chapter 3

# General Conclusion

The importance of melanin production to the virulence of human fungal pathogen *C. neoformans* has been demonstrated through the creation of melanin-mutant strains that concurrently lose their ability to cause infections (Kwon-Chung et al., 1982). It was observed that there is significant variation in melanin levels among strains of natural *C. neoformans* populations (Vogan et al., 2016). It is not clear what genetic, environmental and interaction factors are involved and the extent of their contribution to the melanin variance. Furthermore the implications of this phenotypic variance to the survival and virulence of *C. neoformans* strains have not been previously investigated. In the current project, I calculated the percent melanin variance explained by genetic factors, environmental factors, and genotype-environment interactions in a natural *C. neoformans* var. *neoformans* population of 54 strains. By sequencing the *LAC1* gene which is essential for melanin synthesis in *C. neoformans*, I was able to calculate the percent melanin variance explained by the *LAC1* locus. I further identified 3 single nucleotide polymorphisms (SNPs) in the *LAC1* gene, one of which is predicted to cause an amino acid switch in a putative catalytic domain, to be significantly associated with melanin production in *C. neoformans*. The contributions of these findings to the current body of knowledge on *C. neoformans* pathogenesis and virulence are discussed below.

Evidence suggests that the two varieties of *C. neoformans* have been diverging from each other for ~18 million years (Fan, Currie, Gutell, Ragan, & Casadevall, 1994; Xu, Vilgalys, & Mitchell, 2000b). One major area of ongoing *Cryptococcus* research is the study of genetic and genealogical factors responsible for the significant difference in virulence observed between the two varieties, where *C. neoformans* var. *grubii* is generally more virulent than *C. neoformans* var. *neoformans* and is responsible for 99% of cryptococcal infections in certain geographical areas (Mitchell & Perfect, 1995). Previous work from our research group has identified multiple genetic loci contributing to the variation between the two varieties in virulence factors such as capsule production, melanin production and antifungal drug resistance (Vogan et al., 2016). The findings of this project suggest that rather than being qualitative in nature, the genetic variance behind the inter-varietal differences might act in a quantitative manner resulting in quantitative phenotypic differences. These results represent the first instance of a proven quantitative association between *LAC1* sequence variance and melanin variance in *C. neoformans*. It was observed during the project that some of the PCR primers designed based on a reference strain of *C. neoformans* var. *neoformans* to amplify *LAC1* gene fragments did not successfully amplify *C. neoformans* var. *grubii* strains. The sequence identity between the consensus *LAC1* sequence of the tested *C. neoformans* var. *neoformans* strains and the *LAC1* sequence of H99, a model clinical strain *C. neoformans* var. *grubii* was 89%, with approximately 300 SNPs scattered throughout the length of the gene. Given the findings of this thesis project, these genetic polymorphisms in the *LAC1*

gene likely contribute to the differences in virulence and melanin production between the two varieties of *C. neoformans*.

The results from this project showed that melanin production in strains of *C. neoformans* var. *neoformans* was greatly repressed in oxidative stress, but was either slightly repressed or induced below/above baseline levels in nitrosative stress depending on the concentration of RNS in the environment. A study that quantified *LAC1* transcript levels in the *C. neoformans* var. *grubii* strain H99 using real-time PCR found the opposite trend where *LAC1* was greatly induced in oxidative stress and repressed in nitrosative stress. One thing to be noted is that level of melanin produced does not necessarily reflect the level of *LAC1* expression in the cell: *LAC1* also contains iron oxidase activity that is not related to the melanin biosynthesis pathway. It is possible that *LAC1* expression is induced in *C. neoformans* var. *neoformans* when exposed to oxidative stress but the enzyme is utilized for iron oxidase activity leading to an overall decrease in melanin synthesis. Another explanation for this apparent contradiction between the two studies is that the regulation of melanin synthesis/*LAC1* expression could be different between the two varieties of *C. neoformans*. The ability to elevate *LAC1* expression in oxidative stress might be contributing to the higher virulence of *C. neoformans* var. *grubii* strains. I am currently in the process of quantifying the level of *LAC1* expression of *C. neoformans* var. *neoformans* strains in different stress conditions.

The results from this thesis confirm the polygenic nature of melanin production in *C. neoformans*, with the genetic factors contributing up to 65% of the phenotypic variance remaining unknown. *LAC1*, the only enzyme known to catalyze melanin

synthesis in *C. neoformans* only contributed 25%- 55% to the genetic variance responsible for observed variation in melanin, depending on the environmental condition. Similarly, in my test population, the *MAT* locus that determines the mating type of *C. neoformans* cells accounted for 8-20% of the total observed melanin variance. Previous studies have identified several other genes associated with melanin production including cyclic AMP pathway (Pukkila-Worley et al., 2005a), *VPH1* (Erickson et al., 2001) and *CLC1* (Zhu & Williamson, 2003) proteins that regulate proton and chloride ion movements, as well as homologs of *Ccc2* and *Atx1* (Walton, Idnurm, & Heitman, 2005) which code for proteins involved in copper transport. Sequence polymorphisms at these genetic loci potentially contribute to the variation in melanin production observed among strains of *C. neoformans.*

Given the importance of melanin production to the virulence of the opportunistic fungal pathogen *C. neoformans,* it is imperative to understand the genetic basis behind the natural variation in melanin observed among strains. The genetic loci identified as having a role in this phenotypic variance could potentially be considered as drug targets to successfully treat cryptococcal infections. No vaccines have yet been developed against infections by *C. neoformans* species. The SNPs identified within the *LAC1* gene in this study should be further investigated to determine their potential causative effects on the Lac1 protein and consequently, melanin production.

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