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# Phenotypic plasticity in the productions of virulence factors within and among serotypes in the *Cryptococcus neoformans* species complex

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# Abstract

The *Cryptococcus neoformans* species complex (CNSC) is a common opportunistic human fungal pathogen and the most frequent cause of fungal meningitis. There are three major serotypes in CNSC: A, D, and their hybrids AD, and they have different geographic distributions and medical significance. Melanin pigment and a polysaccharide capsule are the two major virulence factors in CNSC. However, the relationships between serotype and virulence factor production and how environmental factors might impact their relationships are not known. This study investigated the expressions of melanin and capsular polysaccharide in a genetically diverse group of CNSC strains and how their phenotypic expressions were influenced by oxidative and nitrosative stress levels. We found significant differences in melanin and capsular polysaccharide productions among serotypes and across stress conditions. Under oxidative stress, the laboratory hybrids exhibited the highest phenotypic plasticity for melanin production while serotype A showed the highest for capsular polysaccharide production. In contrast, serotype D exhibited the highest phenotypic plasticity for capsular polysaccharide production and clinical serotype AD the highest phenotypic plasticity for melanin production under nitrosative stress. These results demonstrated that different serotypes have different environmental condition-specific mechanisms to modulate the expression of virulence factors.


# Introduction

*Cryptococcus neoformans* species complex (CNSC) is a major human fungal pathogen that causes hundreds of thousands of deaths each year [1, 2]. The CNSC is comprised of two main evolutionary divergent lineages that have been variably called serotypes (A and D), genotype groups (VNI, VNII, VNB, and VNIV), varieties (var. *grubii* and var. *neoformans*), and species (*C. neoformans* and *C. deneoformans*). The strains of serotype A correspond to genotype groups VNI, VNII, VNB; variety *grubii*; and species *C. neoformans*. In contrast, the strains of serotype D correspond to genotype group VNIV; var. *neoformans*; and species *C. deneoformans*. Within CNSC, serotype AD hybrids (genotype group VNIII) are also commonly found in certain geographic regions [1]. In this study, we will use the term serotype to refer to the genetically divergent groups of strains within CNSC. The serotypes differ in their geographic distributions and in medical significance. In clinics, the dominant serotype infecting patients is serotype A. At present, the reason(s) for the high prevalence of serotype A strains in patients remains largely unknown.

The primary mode of transmission for CNSC into a host is through the respiratory system. Inhalation of spores or aerosolized vegetative cells will initially lead to the development of a pulmonary burden in both immunocompetent and immunocompromised individuals [1]. For immunocompromised individuals, invasive cryptococcosis will likely develop, leading to meningoencephalitis [2–4]. Within the host, invading CNSC pathogens will first encounter alveolar macrophages as the primary phagocytic and host defense cells at the time of pulmonary infection [1, 5–8]. As part of the innate immune response, these macrophages will phagocytose the CNSC cells and attempt to destroy them using internally released reactive oxygen species (ROS) and reactive nitrogen species (RNS) [9, 10]. In particular, the generalized

RNS-based inhibition of CNSC cells within macrophages has been shown to be predominantly activated by interferon-gamma (IFN-γ) following the initial establishment of fungal burden within the lungs [11–14]. However, despite the oxidizing environment created by the release of ROS and RNS, CNSC cells have been observed to persist within macrophages and resist the primary defensive attacks from the host [7, 10, 15, 16]. This persistence has been shown to be related to the particular serotype of the invading pathogen with serotype A strains being more resistant to host defenses than serotype D strains [17, 18]. However, it is not known whether the differential survival in vivo between serotypes A and D strains is related to their differential virulence factor production abilities.

Within CNSC, melanin and capsule productions are two major virulence factors [19, 20]. Previous studies have shown that infection of CNSC cells coincides with their melanin pigment production, potentially enabling the pathogen cells to neutralize harmful oxygen and nitrogen radicals inside macrophages [21–24]. Indeed, melanin-lacking CNSC strains due to mutation in the laccase gene have shown decreased virulence and reduced capacity to neutralize oxidants as compared to wild-type strains [19, 23, 25–27]. Furthermore, reintroducing the wildtype laccase gene into laccase-deficient cells prevented the killing of CNSC cells via ROS within an *in vivo* murine alveolar macrophage model [24]. Similarly, previous studies have also shown growth of the CNSC capsules in oxidative environments following macrophage phagocytosis [10, 20]. The subsequent enlargement of the polysaccharide capsule was associated with inhibition of macrophage phagocytic action [7, 10, 15, 16, 28–30]. Indeed, the necessity of the capsule for pathogenicity and virulence has been extensively demonstrated, with acapsular mutant strains displaying attenuated virulence in both *in vitro* and *in vivo* models [7, 26, 31–33]. Together, these studies have demonstrated that both melanin and capsule productions are important virulence factors in CNSC.

While the productions of both melanin and capsule seem to be influenced by *in vivo* factors during CNSC infection and pathogenesis, the potential quantitative responses of different serotypes to different stressors in their melanin and capsule productions are not known. During their evolutionary divergence, serotypes A and D may have evolved different mechanisms to respond to oxidative and nitrosative stresses. The differences in phenotypic expression of specific traits under different environmental conditions are commonly referred to as “phenotypic plasticity” [34]. Given the changing environments associated with host defense systems, with the release of ROS and RNS creating chemically dangerous environments for the pathogen, it is clear that having the ability to adapt and modulate a response to these factors is beneficial for the pathogen. However, though they have been commonly observed, the extent of phenotypic plasticity in virulence factor production in CNSC have yet to be determined [35].

The objectives of this study are to fill in the gaps of knowledge with regard to the patterns of variation in melanin and capsular polysaccharide productions in CNSC. Specifically, we are interested in addressing the following questions. First, do strains of serotypes A and D in CNSC produce different amounts of melanin and capsular polysaccharides? Second, how do oxidative and nitrosative stresses influence variations in melanin and capsular polysaccharide productions? We will use clinically relevant oxidative and nitrosative stress conditions that have been used previously to investigate their influences in these two traits. Third, do strains of serotypes A and D respond to oxidative and nitrosative stresses differently?

While the focus of our study is on comparing natural strains of the two evolutionarily divergent serotypes A and D, we are also interested in how natural hybrids and a population of laboratory hybrids would perform under similar test conditions. Specifically, we are interested in the amount of variation in both melanin and capsule productions and whether these hybrids will show intermediate patterns of virulence factor productions and responses to stresses as the serotype A and serotype D strains?

# Materials And Methods

### Strains of the *Cryptococcus neoformans* species complex

A total of 50 strains of the CNSC were analyzed in this study, comprising 14 strains of serotypes A, 14 strains of serotype D, and 22 strains of serotype AD (Table 1). These strains were derived from a variety of sources. Twelve serotype A, five serotype D, and eight serotype AD strains were randomly selected from the samples obtained in the 1992–1994 US cryptococcosis surveillance program [36–38]. The remaining two serotype A isolates were KN99a and KN99α, isogenic strains derived from repeated backcrosses between two serotype A strains H99 and 125.91 [39, 40]. KN99a and KN99α differ from each other at the mating type locus but are otherwise assumed identical to each other. Of the remaining nine serotype D isolates, seven were natural isolates with two from Germany, three from Greece, one each from Belgium and Italy [37]. The remaining two serotype D isolates JEC20 and JEC21 are model laboratory strains, differ from each other primarily at the mating type locus [41]. Among the 22 serotype AD strains, 14 were progeny from a previously described cross between a serotype D strain JEC20 and a serotype A strain CDC15, with the remaining eight from patients in San Francisco, USA [42, 43]. These strains represent a variety of genotypes assayed by multilocus enzyme electrophoresis, multilocus sequence typing, multilocus PCR-RFLP genotyping and/or whole-genome sequencing [37, 41, 44–46]. The details of all strains are presented in Table 1.

Table 1
Strains of *C. neoformans* species complex analyzed in this study

| Strain Code | Serotype | Ecological Niche | Geographic Origin | Reference |
|---|---|---|---|---|
| RKI 13–0490 | D | Environmental | Germany | 39 |
| RKI 13–0491 | D | Environmental | Germany | 39 |
| CDC27 | D | Clinical | Southern USA | 38 |
| CDC32 | D | Clinical | Southern USA | 38 |
| CDC76 | D | Clinical | Southern USA | 38 |
| CDC77 | D | Clinical | Southern USA | 38 |
| Y494-91 | D | Environmental | Atlanta, USA | 38 |
| GRAKI13HO1-1 | D | Environmental | Greece | 39 |
| GRACP30BK1-1 | D | Environmental | Greece | 39 |
| GRAKI28HO1-1 | D | Environmental | Greece | 39 |
| IUM 01-4786 | D | Environmental | Belgium | 39 |
| IUM 93-1656 | D | Clinical | Italy | 39 |
| JEC20 | D | Laboratory | NIAID, NIH | 42 |
| JEC21 | D | Laboratory | NIAID, NIH | 42 |
| H99 | A | Clinical | Southern USA | 40 |
| KN99a | A | Laboratory | Duke University | 39 |
| KN99α | A | Laboratory | Duke University | 39 |
| M92-0064 | A | Clinical | Southern USA | 44 |
| M92-0074 | A | Clinical | Southern USA | 44 |
| M92-0113 | A | Clinical | Southern USA | 44 |
| M92-0123 | A | Clinical | Southern USA | 44 |
| M92-0131 | A | Clinical | Southern USA | 44 |
| M92-0161 | A | Clinical | Southern USA | 44 |
| M92-0168 | A | Clinical | Southern USA | 44 |
| M92-0178 | A | Clinical | Southern USA | 44 |
| M92-0192 | A | Clinical | Southern USA | 44 |
| M92-0219 | A | Clinical | Southern USA | 44 |

| Strain Code | Serotype | Ecological Niche | Geographic Origin | Reference |
| --- | --- | --- | --- | --- |
| M92-0223 | A | Clinical | Southern USA | 44 |
| 14 strains | AD | Laboratory hybrid progeny | McMaster University | 45 |
| M92-0203 | AD | Clinical | San Francisco, USA | 44 |
| M92-0086 | AD | Clinical | San Francisco, USA | 44 |
| M94-0118 | AD | Clinical | San Francisco, USA | 44 |
| M93-0695 | AD | Clinical | San Francisco, USA | 44 |
| M93-0610 | AD | Clinical | San Francisco, USA | 44 |
| M94-0018 | AD | Clinical | San Francisco, USA | 44 |
| M94-0241 | AD | Clinical | San Francisco, USA | 44 |
| M94-0793 | AD | Clinical | San Francisco, USA | 44 |

**Quantification of melanin production:**

To assay their ability to produce melanin pigments, all *C. neoformans* strains were retrieved from − 80°C freezer stock and warmed to room temperature. A 10 µL sample of each strain was spotted onto a standard solid yeast extract peptone dextrose (YEPD) growth medium and incubated at 30°C for 72 hours. The actively growing cells were then transferred into a 1 mL sterile $dH_2O$ and diluted to a $10^6$ cells/mL density. Afterwards, 5 µl of the $10^6$ cells/mL suspension was spotted onto a solid caffeic acid media plate. Four spots were created for each strain under each treatment condition. The plates contained three concentrations of hydrogen peroxide or sodium nitrite: low (0.25 mM), intermediate (0.5 mM), and high (1 mM). A 0 mM no stressor condition functioned as the negative control. Stressors were created separately from stock solutions and added to the autoclaved caffeic acid agar medium, mixed gently with a stir bar, and then poured onto petri-plates. The plates were placed in a 30°C incubator for 7 days. Following this growth period on the caffeic acid medium, cells were exposed to a transilluminator to measure melanin pigmentation using spot densitometry [37]. Individual caffeic acid plates were placed in a white-light transilluminator with an exposure time of 150 milli seconds [37]. Captured images were then processed using spot densitometry using ImageJ. Melanin pigment density values were determined for each colony, with relative melanin production calculated as the ratio between colony pigmentation and background pigmentation of agar medium. Four replicates were performed for each isolate under each of the treatment conditions.

**Quantification of capsular polysaccharide production:**

To assay their ability to produce capsular polysaccharides, all *C. neoformans* strains were retrieved from − 80°C freezer stock and warmed to room temperature. A 10 µL sample of each strain was spotted onto a standard solid YEPD growth medium and incubated at 30°C for 72 hours. The actively growing cells were

then transferred into a 1 mL sterile $dH_2O$ and diluted to a $10^6$ cells/mL density. To assay their capsular polysaccharide production, 5 µL of the cell suspensions were transferred to a liquid Sabouraud Dextrose (SD) broth containing 5% MOPS and incubated at 30°C for 48 hours to elicit capsule production. After this point, cell suspensions were transferred to a 10% SD broth containing various concentrations of oxidative and nitrosative stressors and incubated at 30°C for 72 hours. Specifically, hydrogen peroxide was used to elicit oxidative stress and sodium nitrite was used to create nitrosative stress. Three concentration levels were used for each stressor type: low (0.25 mM), intermediate (0.5 mM), and high (1 mM). A 0 mM no stressor condition was used as the negative control. Stressors were created separately from stock solutions and added directly to the 10% SD broth growth phase.

After stressor treatments, the capsular polysaccharides were extracted using DMSO exposure and centrifugation [47]. Briefly, after the 72 hours growth period, cells were diluted to a 1 mL volume of $10^6$ cells/mL density with sterile $dH_2O$. For each tube, 75 µL of dimethyl sulfoxide (DMSO) was added in twice at 30 minutes apart to initiate capsule removal [47]. After DMSO addition, the samples were centrifuged at ~ 13000 rpm for 10 min to pellet the removed capsular polysaccharides. The capsular polysaccharides were then treated with phenol-sulphuric acid colorimetry to measure polysaccharide concentration [48]. Three replicates were performed for each isolate under each treatment condition.

**Data Analysis:**

All statistical analyses were conducted using the R-Studio software and associated packages. Analysis of Variance (ANOVA) and Tukey's Honest Significant post-hoc testing were conducted using the stats v3.6.2 R package. Relative melanin production and capsular polysaccharide production of each tested CNSC serotype and strain were compared among each other in individual environments. In order to control for normality of residuals and homoscedasticity, relative melanin production data was logit transformed and capsule polysaccharide production data was log transformed. Below we describe the specific tests to determine the statistical significance of the phenotypic differences between serotypes and between stress conditions.

**Partitioning of phenotypic variations**

To determine the relative contributions and statistical significance of the various factors to the overall melanin and capsular polysaccharide productions, we performed the following tests. First, we partitioned the variance associated with melanin and capsular polysaccharide productions within each of the two types of stressors (oxidative and nitrosative stress) using a three-way ANOVA with the three factors being strain, serotype, and the concentrations of stressors, including their interaction effects. Second, we conducted a Tukey's Honest Significant Post-hoc test to determine differences in virulence trait production between pairs of environment conditions and pairs of serotypes. Lastly, the Pearson's correlation coefficient between capsular polysaccharide concentration and relative melanin production of the tested CNSC strains and in the seven environmental conditions was calculated to determine any association between the two virulence factors.

**Phenotypic plasticity within serotypes**: In addition to estimating the contributions of various factors to the total observed variances of the two traits, we also investigated phenotypic plasticity of the individual serotypes. To calculate phenotypic plasticity, a simplified relative distance plasticity index (RDPI) model for capsular polysaccharide production and relative melanin production according to each serotype group and environmental stressor combination was calculated and compared using the formula described in Valladares et al. (2006). In short, RDPI was calculated by separating individual CNSC serotypes as three separate groups, with all strains within each group considered as separate subjects. Each virulence factor trait (either capsular polysaccharide production or relative melanin production) was considered separately for each of the seven treatments. Pairwise differences in the average virulence factor measurement between pairs of strains at each treatment were calculated and used to scale the change in production from a value between 0 and 1, representing the individual RDPI value [49]. RDPI values for each strain within each serotype group in each environment stressor type (oxidative or nitrosative stress) were then averaged to derive the mean and standard deviation of serotype RDPI value for each environmental stress condition. A two-way ANOVA was then used to compare differences in the capsular polysaccharide concentration and relative melanin production RDPI values among CNSC serotypes for each environmental stressor type. Within each treatment condition, a Tukey's Honest Significant Post-hoc test was used to compare pairwise differences between environment type and serotype. The null hypotheses for these tests were: (i) within each of the seven treatment conditions, the phenotypic plasticity of relative melanin production and capsular polysaccharide concentrations will not differ among CNSC serotypes; and (ii) within each serotype, the phenotypic plasticity of relative melanin production and capsular polysaccharide concentrations will not differ among the seven treatment conditions.

# Results

## Variation in Melanin Production

A total of 50 strains from the CNSC were analyzed in this study. The mean melanin production level for each strain under each environmental condition is shown in Supplementary Table S1. Significant variations in relative melanin production were observed among these strains across the seven tested environmental conditions (Fig. 1). Overall, we observed serotype-specific differences in melanin production at all investigated oxidative and nitrosative stress levels (Fig. 1A & 1B). Furthermore, different environmental stressor levels showed differential contributions to the variances associated with melanin production, and there were significant interaction effects between environmental conditions with CNSC strains and serotypes (Fig. 1C). Finally, though the phenotypic plasticity within individual CNSC serotypes was relatively limited (~ 0.1), significant differences were observed between serotypes A and D in their relative phenotypic plasticity indices in both oxidative and nitrosative stress environments (Fig. 1D). Below we describe the observed variations in detail.

## Effects of oxidative stress on CNSC melanin production

Our analyses showed that the three serotypes contributed significantly to variations in melanin production (Fig. 1A; $F_{3,796} = 92.85$, $P < 0.001$). Similarly, we observed that oxidative stress levels contributed to differences in melanin production ($F_{3,796} = 13.57$, $P < 0.001$). Furthermore, there were significant interaction effects between serotype and oxidative stress levels ($F_{9,784} = 16.53$, $P < 0.001$). Within serotype D, the highest melanin production was observed under intermediate oxidative stress, significantly higher than that under all other oxidative stress levels ($F_{3,220} = 11.95$, $P < 0.001$). In contrast, serotype A strains produced more melanin under no oxidative stress than that under all three oxidative stressor concentrations ($F_{3,220} = 20.66$, $P < 0.001$), with the three oxidative stress conditions showing no statistically significant difference among each other in melanin production. Interestingly, the laboratory serotype AD group showed a pattern similar to that of serotype D strains, with melanin production under intermediate oxidative stress being significantly higher than that under all other conditions except the no stressor condition ($F_{3,220} = 17.20$, $P < 0.001$). Lastly, the clinical serotype AD hybrids group showed a different pattern as the laboratory hybrids whereby melanin production of the clinical serotype AD hybrids under no stressor condition was significantly lower than that under all other oxidative stress levels ($F_{3,124} = 30.28$, $P < 0.001$).

Comparisons between the serotypes showed that serotype D strains produced significantly lower melanin than both serotypes A and laboratory serotype AD under all oxidative stress conditions: (i) no stressor ( $F_{1,110} = 262.30$, $P < 0.001$; $F_{1,110} = 28.66$, $P < 0.001$, values respectively when compared with serotype A and laboratory serotype AD), (ii) low stressor ($F_{1,110} = 50.97$, $P < 0.001$; $F_{1,110} = 42.88$, $P < 0.001$), (iii) intermediate stressor ($F_{1,110} = 63.09$, $P < 0.001$; $F_{1,110} = 84.09$, $P < 0.001$), and (iv) high stressor ($F_{1,110} = 97.59$, $P < 0.001$; $F_{1,110} = 5.449$, $P < 0.05$) conditions. Interestingly, serotype D strains produced significantly lower melanin than clinical serotype AD strains under only the low ($F_{1,86} = 29.55$, $P < 0.001$) and high stressor ($F_{1,86} = 46.2$, $P < 0.001$) conditions, but produced significantly higher melanin than clinical serotype AD strains under the no stressor condition ($F_{1,86} = 15.5$, $P < 0.001$). Furthermore, serotype A strains were shown to have significantly higher melanin production than laboratory serotype AD strains under only the no stressor ($F_{1,110} = 14.31$, $P < 0.001$) and the high oxidative stress condition ($F_{1,110} = 29.38$, $P < 0.001$). No other significant pairwise difference was observed between serotype A and laboratory serotype AD strains. Lastly, clinical serotype AD strains were shown to have overall significantly lower melanin production than both serotypes A and laboratory serotype AD strains under the no stressor ($F_{1,86} = 279$, $P < 0.001$; $F_{1,86} = 36.98$, $P < 0.001$), intermediate stress ($F_{1,86} = 40.89$, $P < 0.001$; $F_{1,86} = 52.72$, $P < 0.001$), and high stress conditions ($F_{1,86} = 11.17$; $F_{1,86} = 4.714$, $P < 0.001$).

We further compared the relative differences in melanin production between serotypes in response to oxidative stress (Fig. 1B). Overall, we found that the relative difference in melanin production of serotype A strains to be significantly lower than those of serotypes D, laboratory serotype AD, and clinical serotype AD strains under the low ($F_{1,110} = 117.7$, $P < 0.001$; $F_{1,110} = 82.15$, $P < 0.001$; $F_{1,86} = 158.9$, $P < 0.001$) and intermediate ($F_{1,110} = 116.1$, $P < 0.001$; $F_{1,110} = 42.30$, $P < 0.001$; $F_{1,86} = 159.1$, $P < 0.001$) oxidative stress conditions. In addition, the relative change in melanin production of serotype D strains was significantly

higher than those of serotypes A and laboratory AD strains under the high oxidative stress condition ($F_{1,110}$ = 45.46, P < 0.001; $F_{1,110}$ = 35.20, P < 0.001). Furthermore, serotype D strains demonstrated significantly lower relative changes in melanin production as compared to clinical serotype AD strains under low and intermediate oxidative stress ($F_{1,86}$ = 59.53, P < 0.001; $F_{1,86}$ = 22.88, P < 0.001), with laboratory serotype AD strains also being observed to have significantly lower changes in melanin production under low oxidative stress as compared to their clinically isolated counterparts ($F_{1,86}$ = 50.58, P < 0.001). Lastly, clinical serotype AD strains were shown to have significantly higher relative changes in melanin production as compared to serotypes A, D and laboratory serotype AD under the high oxidative stress condition ($F_{1,86}$ = 179.7, P < 0.001; $F_{1,86}$ = 108.5, P < 0.001; $F_{1,86}$ = 153.5, P < 0.001).

We partitioned the variance associated with CNSC relative melanin production under oxidative stress conditions to determine the primary contributing variables based on the three-way ANOVA used (Fig. 1C). We observed that strain-specific differences in our tested CNSC population created the largest amount of variation in relative melanin production in response to oxidative stress, at 38.28%. Interestingly, serotype-environment interactions only accounted 10.07% of associated melanin production variance, whereas 27.35% of variance was associated with serotype-specific differences.

We quantified phenotypic plasticity for each serotype using the relative distance plasticity indices under oxidative stress (Fig. 1D). In response to oxidative stress, we observed that all three serotypes had relatively minor phenotypic plasticity. However, significant serotype-specific differences were observed. Overall, laboratory serotype AD demonstrated the highest RDPI value under oxidative stress, but it was only significantly higher than serotype D (*$F_{1,26}$ = 15.74, P < 0.001*). Serotype A also exhibited significantly higher RDPI value than serotype D (*$F_{1,26}$ = 25.06, P < 0.001*). The RDPI values of serotypes AD and A were statistically not significantly different from each other. Lastly, clinical serotype AD strains displayed RDPI that were only significantly higher than that of Serotype D (*$F_{1,20}$ = 8.842, P < 0.01*). While clinical serotype AD RDPIs were lower than those of laboratory serotype AD and serotype A, they were not significantly different.

## Impact of nitrosative stress on CNSC relative melanin production

The use of nitrosative stress similarly created differences in the relative melanin production of the tested CNSC population. Similar to that observed in oxidative stress conditions, we found serotype-specific differences influenced melanin production (Fig. 1A; $F_{3,796}$ = 67.71, P < 0.001). The presence of nitrosative stressor also significantly influenced the extent of melanin production in the tested CNSC population ($F_{3,796}$ = 9.25, P < 0.001). Lastly, we observed significant serotype and nitrosative stress level interactions influencing melanin production ($F_{9,784}$ = 20.41, P < 0.001). Serotype D strains had significantly lower melanin production in the no stressor condition as compared to all other nitrosative stress levels ($F_{3,220}$ = 23.13, P < 0.001). Serotype A strains showed significantly lower melanin production in the intermediate nitrosative stress condition than those in all other conditions ($F_{3,220}$ = 19.29, P < 0.001). In contrast, laboratory serotype AD strains had significantly higher melanin production under intermediate nitrosative

stress than that under other nitrosative stressor conditions ($F_{3,220} = 9.929$, $P < 0.01$). Lastly, clinical serotype AD strains demonstrated significantly higher melanin production under the high nitrosative stress condition than those in all other conditions ($F_{3,124} = 68.13$, $P < 0.001$).

Among the three serotypes, serotype A showed higher melanin production than both serotypes D, laboratory serotype AD, and clinical serotype AD under the low ( $F_{1,110} = 18.45$, $P < 0.001$; $F_{1,110} = 31.35$, $P < 0.001$, $F_{1,86} = 144$, $P < 0.001$) and high ($F_{1,110} = 15.27$, $P < 0.001$; $F_{1,110} = 33.38$, $P < 0.001$, $F_{1,86} = 38.98$, $P < 0.001$) nitrosative stress conditions. Interestingly, laboratory serotype AD had significantly higher melanin production than serotypes D, A and clinical AD in the intermediate nitrosative stress condition ($F_{1,110} = 9.985$, $P < 0.001$; $F_{1,110} = 18.74$, $P < 0.001$; $F_{1,86} = 74.29$, $P < 0.001$). Though serotype D was observed to have slightly higher melanin production under the low and high nitrosative stress conditions when compared to serotype AD, these differences were not significant. Next, clinical serotype AD strains demonstrated significantly lower melanin production than both serotype D and laboratory serotype AD under the low ($F_{1,86} = 35.67$, $P < 0.001$; $F_{1,86} = 144$, $P < 0.001$), and intermediate ($F_{1,86} = 58.11$, $P < 0.001$; $F_{1,86} = 79.22$, $P < 0.001$) nitrosative stressors. Lastly, clinical serotype AD strains demonstrated significantly lower melanin production as compared to serotype A strains under intermediate nitrosative stress ($F_{1,86} = 79.22$, $P < 0.001$).

We compared the relative change in melanin production between serotypes among nitrosative stress levels (Fig. 1B). Compared to the no stress condition, the relative change in melanin production of serotype D was significantly higher than those of serotypes A, laboratory serotype AD, and clinical serotype AD strains under low ($F_{1,110} = 169.2$, $P < 0.001$; $F_{1,110} = 45.85$, $P < 0.001$; $F_{1,86} = 15.5$, $P < 0.001$), and intermediate ($F_{1,110} = 348.1$, $P < 0.001$; $F_{1,110} = 6.371$, $P < 0.05$; $F_{1,86} = 21.27$, $P < 0.001$) nitrosative stress conditions. Under high nitrosative stress conditions serotype D strains were shown to have significantly higher relative changes in melanin production as compared to serotype A and laboratory serotype AD strains ($F_{1,110} = 128.1$, $P < 0.001$; $F_{1,110} = 38.55$, $P < 0.001$) but significantly lower relative changes as compared to clinical serotype AD strains ($F_{1,86} = 9.77$, $P < 0.01$). Serotypes A and AD showed no significant differences in relative melanin production under low and high nitrosative stress levels. However, serotype AD was recorded as having significantly higher relative changes in melanin production as compared to serotype A under intermediate nitrosative stress ($F_{1,110} = 68.43$, $P < 0.001$). Lastly, clinical serotype AD strains demonstrated significantly higher relative changes in melanin production as compared to both serotype A and laboratory AD strains under the low ($F_{1,86} = 74.93$, $P < 0.001$; $F_{1,86} = 12.49$, $P < 0.001$), and high nitrosative stress conditions ($F_{1,86} = 254.3$, $P < 0.001$; $F_{1,86} = 51.02$, $P < 0.001$), but was only significantly higher as compared to serotype A under intermediate nitrosative stress ($F_{1,86} = 182.9$, $P < 0.001$).

Overall, similar to those under oxidative stress, these results highlighted the differential responses among serotypes to various nitrosative stress levels in their melanin production (Fig. 1C). Within individual serotypes, we also observed that strain-specific effects contributed the highest percentage (at 38.84%) to the total variance of the melanin production under nitrosative stresses. Interestingly, serotype level

interactions accounted for the second highest contribution at 21.04% and serotype-stress level interactions contributed 15.78%, both of which were statistically significant.

We further analyzed the phenotypic plasticity of individual serotypes based on RDPI to specifically quantify the extent of melanin production in response to nitrosative stress. Minimal phenotypic plasticity was observed within each of the three serotypes following application of the nitrosative stress treatment (Fig. 1D). However, serotype-specific differences were recorded. Clinical serotype AD had the highest RDPI value in response to nitrosative stress but was only significantly different from serotype A ($F_{1,20} = 19.05, P < 0.001$) and not laboratory serotype AD. Serotype D also displayed significantly higher RDPI values as compared to only serotype A ($F_{1,26} = 42.66, P < 0.001$). The RDPI value of laboratory serotype AD was also significantly higher than that in serotype A ($F_{1,26} = 6.657, P < 0.05$).

### Variation in capsular polysaccharide production

The same CNSC strains examined above were used to measure capsular polysaccharide production (CPP) under the same stress treatments as described above. The mean CPP (and standard deviation) for each strain under each environmental condition is shown in Supplementary Table S2. Figure 2 illustrates serotype-specific effects in CPP in both sets of environmental stressors. Furthermore, there were significant interaction effects between serotype and stress levels as well as between strain and stress levels. Lastly, CPP phenotypic plasticity indices showed serotype-dependent effects among the nitrosative stress levels, despite the relatively low phenotypic plasticity of CPP within individual serotypes.

### Effect of oxidative stress on CNSC capsular polysaccharide concentration

Figure 2A illustrates the serotype-specific and oxidative stress level-dependent effects on CPP. Under oxidative stress, we observed a major difference among serotypes in their CPP ($F_{3,796} = 131.3, P < 0.001$). In addition, the oxidative stress levels also contributed significantly to variations in CPP values ($F_{3,796} = 11.61, P < 0.001$). Finally, we observed serotype and environment interactions in their combined effect on CPP under oxidative stress ($F_{9,784} = 31.29, P < 0.001$). Consistently across all oxidative stressor conditions and the no stressor condition, clinical serotype AD demonstrated the highest CPP values. Similar to the trends seen for melanin production, serotype A strains were shown to produce significantly lower CPP under the intermediate oxidative stress condition as compared to all other concentration levels ($F_{3,220} = 51.81, P < 0.001$). Serotype D CPP levels were observed to be similar under the intermediate and high oxidative stress conditions, with both being significantly higher than the no and low oxidative stressor levels ($F_{3,220} = 32.05, P < 0.001$). Next, laboratory serotype AD CPP under the no stressor condition was significantly lower than those in the presence of oxidative stresses (all three oxidative stress levels), but the three oxidative stresses didn't differ from each other in their effects on CPP ($F_{3,220} = 7.823, P < 0.001$). Lastly, clinical serotype AD CPP under the no stressor condition was significantly higher than that in all three oxidative stress levels, with CPP under the intermediate stressor being significantly higher than that in low and high stress conditions ($F_{3,124} = 41.68, P < 0.001$).

Among the serotypes, serotype A strains showed higher CPP than both serotype D and serotype AD under the no stressor and high oxidative stress conditions. However, these differences were only statistically significant under the no stressor condition ($F_{1,110} = 11.57, P < 0.001$; $F_{1,110} = 8.096, P < 0.01$). Contrastingly, serotype AD strains were observed to have significantly higher CPP as compared to both serotypes A and D under the low oxidative stress condition ($F_{1,110} = 18.46, P < 0.001$; $F_{1,110} = 9.134, P < 0.01$). Interestingly, under the intermediate oxidative stress condition, both serotype D ($F_{1,110} = 97.32, P < 0.001$) and serotype AD ($F_{1,110} = 44.62, P < 0.001$) had significantly higher CPP than serotype A. Furthermore, serotype D was also shown to have significantly higher CPP than serotype AD under the same condition ($F_{1,110} = 12.52, P < 0.001$). Clinical serotype AD strains showed significantly higher CPP values as compared to serotypes A, D and laboratory AD under all oxidative stress conditions: (i) no stressor ($F_{1,86} = 359.5, P < 0.001$; $F_{1,86} = 360.3, P < 0.001; F_{1,86} = 1116, P < 0.001$ values respectively when compared with serotype A, serotype D, and laboratory serotype AD), (ii) low stressor ($F_{1,86} = 105.9, P < 0.001$; $F_{1,86} = 108.3, P < 0.001; F_{1,86} = 29.59, P < 0.001$), (iii) intermediate stressor ($F_{1,86} = 289.2, P < 0.001$; $F_{1,86} = 52.43, P < 0.001; F_{1,86} = 130.5, P < 0.001$), and (iv) high stressor ($F_{1,86} = 11.95, P < 0.001$; $F_{1,86} = 18.01, P < 0.001; F_{1,86} = 20.86, P < 0.001$) conditions.

Similar to results in melanin production, we compared differences in the relative change in CPP between serotypes under oxidative stress (Fig. 2B). Compared to the no stress condition, serotype A strains showed significantly lower relative changes in CPP as compared to both serotypes D and laboratory AD strains under the low ($F_{1,110} = 19.30, P < 0.001$; $F_{1,110} = 28.10, P < 0.001$), and intermediate oxidative stress levels ($F_{1,110} = 121.5, P < 0.001$; $F_{1,110} = 29.42, P < 0.001$). Furthermore, serotype D strains showed significantly higher relative changes in CPP as compared to laboratory serotype AD strains under both the intermediate and the high oxidative stress conditions ($F_{1,110} = 16.83, P < 0.001$; $F_{1,110} = 21.35, P < 0.001$). Interestingly, serotype A strains were also observed to have significantly higher relative changes in CPP as compared to laboratory serotype AD strains under the high oxidative stress condition ($F_{1,110} = 9.268, P < 0.01$) and yet significantly lower relative changes in CPP as compared to serotype D strains under the same concentration level ($F_{1,110} = 4.969, P < 0.05$). Clinical serotype AD strains demonstrated significantly lower changes in CPP as compared to serotypes D, A, and laboratory AD strains under the low ($F_{1,86} = 64.58, P < 0.001$; $F_{1,86} = 16.52, P < 0.001$; $F_{1,86} = 64.08, P < 0.001$) and high oxidative stress conditions ($F_{1,86} = 68.91, P < 0.001$; $F_{1,86} = 83.72, P < 0.001$; $F_{1,86} = 39.56, P < 0.001$). Finally, under intermediate oxidative stress clinical serotype AD strain relative changes in CPP were significantly lower than that of serotype D and laboratory serotype AD ($F_{1,86} = 116.1, P < 0.001$; $F_{1,86} = 7.71, P < 0.01$) but significantly higher than that of serotype A ($F_{1,86} = 8.456, P < 0.01$). Together, these results demonstrated serotype-specific responses in CPP to oxidative stresses, with clinical serotype AD hybrids showing the lowest relative change in CPP in the low and high oxidative stress conditions, while serotype D had the highest in these conditions.

Our three-factor ANOVA showed differential contributions of oxidative stress levels, strains, serotypes, and their interactions to the total CPP variations (Fig. 2C). Here, we noted that the serotype level

interactions explained the greatest proportion of CPP variance (43.17%), followed by serotype-oxidative stress effects (17.53%), strain-oxidative stress level interaction effect (16.15%), with the strain-specific effect accounting for only 8.25% of CPP variance due to oxidative stress.

We quantified CPP phenotypic plasticity in response to oxidative stress using the RDPI measure for each serotype (Fig. 2D). Despite the overall low level of phenotypic plasticity, we identified serotype-specific response effects. Specifically, serotype A maintained the highest RDPI value, followed by serotype D, laboratory serotype AD and then clinical serotype AD. The comparisons between serotype A and laboratory serotype AD ($F_{1,26} = 8.166, P < 0.01$), serotype D and clinical serotype AD ($F_{1,20} = 9.323, P < 0.01$), and serotype A and clinical serotype AD ($F_{1,20} = 8.768, P < 0.01$) were significantly different. In contrast, the serotype D RDPI value was not significantly different to either serotype A or laboratory serotype AD.

### Impact of nitrosative stress on CNSC capsular polysaccharide concentration

Similar to our analyses concerning serotype CPP under oxidative stress, we also observed the presence of serotype-specific effects on CPP values under nitrosative stress ($F_{3,796} = 162.4, P < 0.001$). Within individual serotypes, there was a significant nitrosative stress level effect ($F_{3,796} = 0.519, P < 0.001$). In addition, serotype and nitrosative stress level interactions also significantly impacted CPP values ($F_{9,784} = 62.496, P < 0.001$). Under high nitrosative stress, serotype D CPP was significantly higher than that under no, low, and intermediate nitrosative stress levels ($F_{3,220} = 80.39, P < 0.001$). Contrastingly, serotype AD CPP values under high oxidative stress were shown to be significantly lower than that under all other stress levels ($F_{3,220} = 30.53, P < 0.001$). Interestingly, serotype A strain CPP values did not differ significantly across the four nitrosative stress levels. Clinical serotype AD strain CPP values were shown to be significantly higher under the low nitrosative stress condition as compared to the intermediate and high stressor levels ($F_{3,124} = 111.4, P < 0.001$).

Similar to that under oxidative stresses, the difference in CPP among serotypes was highly dependent on the nitrosative stress levels (Fig. 2A). Under no nitrosative stress, the serotypes A, D and the laboratory AD strains produced similar CPPs values, being quite distinct from that produced by clinical serotype AD strains. Under nitrosative stress, serotype D strains had significantly higher CPP than both serotypes A and AD at the low ($F_{1,110} = 23.14, P < 0.001$; $F_{1,110} = 94.53, P < 0.001$), intermediate ($F_{1,110} = 27.13, P < 0.001$; $F_{1,110} = 103.1, P < 0.001$) and high ($F_{1,110} = 192.1, P < 0.001$; $F_{1,110} = 601.9, P < 0.001$) levels. Furthermore, serotype AD CPP was shown to be significantly lower than that of serotype A at the low ($F_{1,110} = 14.79, P < 0.001$), intermediate ($F_{1,110} = 8.284, P < 0.01$) and high nitrosative stress levels ($F_{1,110} = 117.7, P < 0.001$). Clinical serotype AD strains showed significantly higher CPP values as compared to serotype D, A and laboratory serotype AD strains at the low nitrosative stress level ($F_{1,86} = 44.93, P < 0.001$; $F_{1,86} = 126.9, P < 0.001; F_{1,86} = 410, P < 0.001$). Furthermore, while clinical serotype AD strain CPP values were shown to be significantly higher than laboratory AD strains at the intermediate ($F_{1,86} = 48.11,$

$P < 0.001$) and high stressor level ($F_{1,86} = 184.6, P < 0.001$), they were significantly lower than serotype D at these same stress levels ($F_{1,86} = 5.979, P < 0.001; F_{1,86} = 71.96, P < 0.001$).

We investigated the impact of each nitrosative stress level on the relative changes in CPP (over CPP in the absence of nitrosative stress) among serotypes (Fig. 2B). Our analyses showed that serotype D had significantly higher relative changes in CPP as compared to serotypes A and AD under the low ($F_{1,110} = 37.59, P < 0.001$; $F_{1,110} = 66.78, P < 0.01$), intermediate ($F_{1,110} = 32.50, P < 0.001$; $F_{1,110} = 44.75, P < 0.01$), and high ($F_{1,110} = 169.5, P < 0.001$; $F_{1,110} = 347.2, P < 0.01$) nitrosative stress conditions. Furthermore, we observed that the relative change in CPP for serotype A was significantly higher than that of serotype AD under high nitrosative stress ($F_{1,110} = 40.42, P < 0.001$). Lastly, clinical serotype AD strains demonstrated significantly lower relative changes in CPP than serotype D, A and laboratory serotype AD strains under low ($F_{1,86} = 75.34, P < 0.001$; $F_{1,86} = 7.107, P < 0.01$; $F_{1,86} = 9.359, P < 0.01$), intermediate ($F_{1,86} = 143.6, P < 0.001$; $F_{1,86} = 106.9, P < 0.001$; $F_{1,86} = 213.6, P < 0.001$) and high nitrosative stress conditions ($F_{1,86} = 458.3, P < 0.001$; $F_{1,86} = 166.4, P < 0.001$; $F_{1,86} = 56.26, P < 0.001$).

The three-factor ANOVA demonstrated that among the factors, serotype contributed the highest to CPP variance under nitrosative stress conditions, at 38.46% (Fig. 2C). This was followed by significant serotype-nitrosative stress interaction (32.85% of the total variance), and strain- nitrosative stress interaction (14.41%). Interestingly, the strain-specific effects only explained 4.95% of the total variance associated with CPP due to nitrosative stressors.

Differences in RDPI values in response to nitrosative stress additionally indicated the presence of serotype-specific effects (Fig. 2D). Serotype D was shown to have significantly higher RDPI values as compared to both serotype A and laboratory serotype AD ($F_{1,26} = 59.11, P < 0.001; F_{1,26} = 36.44, P < 0.001$), but not clinical serotype AD. Though serotype A RDPI values were slightly higher than that of laboratory serotype AD, the difference was statistically not significant. Lastly, clinical serotype AD RDPI values were shown to be significantly higher than that of serotype A and laboratory serotype AD ($F_{1,20} = 19.43, P < 0.001; F_{1,20} = 42.29, P < 0.001$).

## Relationship between melanin production and CPP under oxidative and nitrosative stresses

Our above analyses showed that both oxidative and nitrosative stresses impacted melanin production and CPP to different extents for different serotypes and/or strains. Here, we were interested in how CPP and relative melanin production might be correlated under various stress conditions. To address this objective, we calculated the Pearson correlation coefficients between CPP and relative melanin production under each of the seven environmental stress conditions. Here, to ensure homoscedasticity and normality of residuals, the relative melanin productions were logit transformed and CPP data were log transformed prior to analysis (similar to those described above in the analyses of melanin and capsular polysaccharide productions). Our analyses showed that under low oxidative stress, CPP and relative melanin productions were weakly but significantly negatively correlated (Fig. 3; $r = -0.08, P < 0.05$). In contrast, under high nitrosative stress condition, CPP and relative melanin productions were weakly but

significantly positively correlated (Fig. 3; r = 0.06, P < 0.05). Under the remaining five conditions (no stressor, intermediate and high oxidative stresses, and low and intermediate nitrosative stresses), CPP and relative melanin productions were not significantly correlated (Supplementary Fig. 1).

# Discussions

Melanin pigment and a polysaccharide capsule are two major virulence factors of the CNSC. Elimination of either melanin formation or capsule biosynthesis renders CNSC strains avirulent. However, little is known about the patterns of quantitative variation in virulence factor production. Here we quantified the variations in melanin and capsular polysaccharide productions of strains in the three serotypes within CNSC under seven culture conditions and investigated their patterns of variation. Our results showed extensive variation in both traits in the analyzed population and identified that serotype, strain, stress levels, and their interactions can all contribute significantly to the observed variations. Furthermore, we were able to provide, for the first time, a quantification of the phenotypic plasticity associated with these virulence factors within and among the three CNSC serotypes. Interestingly, the clinical and laboratory serotype AD hybrids showed notable differences in their virulence factor productions and responses to oxidative and nitrosative stresses. Below we discuss the potential mechanisms for the observed variations and the relevance of our *in vitro* data to virulence in CNSC.

### Variation in melanin production

Our results indicate that the two divergent serotypes A and D in our sample of CNSC differed significantly in melanin production. In addition, environmental conditions play a significant role in the relative differences between these two serotypes. In general, serotype A showed a higher melanin production level than serotype D, consistent with the general observation of higher virulence of serotype A strains than serotype D strains. As expected, the laboratory hybrid progeny from a single cross showed an intermediate melanin level in-between serotypes A and D under most conditions except at intermediate oxidative and intermediate nitrosative stresses where they produced more melanin than both the serotypes A and D natural strains. In comparison, clinical serotype AD strains demonstrated significantly more variation in melanin production than laboratory serotype AD hybrids. Interestingly, despite having overall low melanin production levels, serotype D showed the largest relative changes (increase) in melanin production in response to nitrosative stresses. The phenotypic plasticity analyses largely complemented these observations as the rank order of RDPI values among individual serotypes depends on the type of environments. Together, these observations suggest that the production and regulation in melanin synthesis are impacted not only by genetic factors accumulated through evolutionary divergence but also by how the genetic factors interact with environmental factors. Our results suggest different serotypes respond differently to different oxidative and nitrosative stressors. At present, the difference in mechanism(s) by which the different serotypes regulating their melanin productions in response to oxidative and nitrosative stresses is not known. Regardless, the extensive phenotypic variations in melanin production among hybrid progeny from a single cross, as demonstrated both within an

environment and among the tested environments, indicate their significant potential in adaptation and virulence trait evolution in CNSC.

### Variation in capsular polysaccharide production

Different from melanin production, the three serotypes produce similar amounts of capsular polysaccharides under no, low and high oxidative stress conditions. The exception was the clinical serotype AD strains that demonstrated among the highest levels of capsular polysaccharides under oxidative and nitrosative stresses. In addition, under intermediate oxidative stress and all three nitrosative stress conditions, serotype D strains produced the second most capsular polysaccharides. Furthermore, serotype D strains upregulated their CPP more prominently than the other two serotypes under both oxidative and nitrosative stress conditions.

Previous studies have noted changes in colony morphology in response to environmental conditions [50, 51]. For example, Guerrero et al. (2010) and Fries et al. (1999) both noted cases of phenotypic switching from smooth colony morphologies to mucoid morphologies among CNSC serotype A, D and C strains. The increased mucoidy was due to upregulation of capsular polysaccharide productions and such phenotypic differences can impact the level of immune activation. Specifically, T-cell activation was more pronounced by cells from mucoid colonies than by cells from smooth colony morphologies [50]. In addition, CNSC cells displaying wrinkled cell morphologies elicit smaller antibody and inflammatory responses as compared to those with mucoid phenotypes [52]. These observations suggest that quantitative variations in CPP plays a role in the virulence of CNSC strains.

The smaller amount of CPP in the more virulent serotype A strains compared to serotype D strains seems to counter the argument about the importance of CPP in virulence. However, it should be pointed out that aside from the quantity of CPP, the type of capsular polysaccharides may also impact host immune response and pathogen virulence. Fries et al. (2001) demonstrated that CNSC cells with mucoid phenotypes had an increased phagocytic inhibitory activity but such a capacity was dependent on glucuronoxylomannan (GXM) specific to their cell type. Indeed, serotypes A and D strains differ in their GXM compositions[10, 51–54]. In addition, the *in vivo* environment may offer other potential signals to allow serotype A strains to produce more capsular polysaccharides to a level comparable to that of serotype D strains.

Our observed variation in CPP among CNSC serotypes likely includes both the types and quantities of GXMs. A previous study by McFadden et al. (2007) reported antigenic differences during various growth phases of serotypes C and D strains. While the potential influence of different environmental conditions on CPP were not considered in their study, the capsules of serotypes C and D changed noticeably in antigenic compositions throughout their growth phase, as indicated by NMR spectroscopy [55]. Thus, it is highly possible that the presence of oxidative and nitrosative stressors could impact the serotypes A, D, and AD differently in their types of capsular polysaccharides. Additional research is needed in order to investigate this possibility.

### Relationships between capsule and melanin production

Depending on environmental conditions, the relationships between CPP and melanin productions were shown to be highly variable, from weakly positive to neutral and weakly negative correlations. The negative correlation between CPP and melanin production under low oxidative stress condition suggested a potential tradeoff between the two virulence traits in this environment. The capsular polysaccharides and melanin pigments play some similar/overlapping roles in pathogen protection against host immune system attacks, including phagocytosis and oxidative and nitrosative damages by hosts. As the productions of both traits are likely energetically and materially expensive, the upregulation of one could lead to the downregulation of the other, causing the observed negative correlation. However, under high nitrosative stress condition, CPP and melanin productions were weakly positively correlated, suggesting the potential co-regulation of these two traits in this environment. Interestingly, other types of correlations have also been observed in previous studies between these two virulence traits and other traits in CNSC. For example, though CPP was not analyzed, a previous study indicated a positive association between high fungal burden and increased melanization due to enhanced laccase activity [56]. However, in a different study, an *in vitro* analysis conducted by Zaragoza et al. (2008) confirmed that high fungal uptake strains manifest smaller capsule sizes. Furthermore, increased intracranial pressure has been associated with high cerebrospinal fluid fungal burdens which showed highly heterogenous capsule-forming CNSC populations [57]. The statistically significant correlations observed here between melanin and capsular polysaccharide productions at specific oxidative and nitrosative stress environments suggest that the tradeoffs and coregulations were especially important for the fungal pathogens in these environments. The genetic basis underlying such tradeoffs remains to be examined.

## Hybrid serotype AD

In this study, we chose two serotype AD populations for analyses: eight clinical serotype AD strains and 14 hybrids from a single cross between strains of serotypes A and D. The patterns of phenotypic variation between these two serotype AD populations were compared with each other and with those of serotypes A and D natural strains. Our results showed that in some environments, the laboratory hybrid strains exhibited intermediate melanin production and CPP between serotypes A and D strains. However, in other environments, e.g., intermediate oxidative and nitrosative stresses, the laboratory hybrids showed superior melanin production over both serotypes A and D strains. Still in other environments such as under nitrosative stresses, the laboratory hybrids showed the least amounts of capsular polysaccharides and did not show upregulation in their capsular polysaccharide productions. Interestingly, hybrid strains from clinical sources demonstrated different patterns of melanin and capsular polysaccharide production as the laboratory serotype AD hybrids (as well as the serotype A and D samples). By and large, across the oxidative and nitrosative environments, melanin production of clinical serotype AD strains was marginally elicited in comparison to other serotype groups, however capsular polysaccharides of these strains were the highest recorded. Furthermore, melanin production in the clinical serotype AD strains demonstrated greater upregulation in contrast to a general downregulation of capsular polysaccharides when exposed to both oxidative and nitrosative environments. Two factors may have contributed to the observed differences between clinical and laboratory serotype AD hybrids. In the first, the strain sources were different, with all the laboratory hybrids from a single cross while the clinical hybrids were from

multiple hybridization events [58]. Secondly, aside from being able to grow on rich artificial media, there was no other selection pressure for the laboratory hybrids. In contrast, the clinical serotype AD strains had likely gone through a variety of environmental and host selective pressure to become the causal agents of cryptococcal meningitis. Regardless of the potential reasons, the observed diversity of phenotypes under different environmental conditions of both groups of hybrids suggests a potentially high capacity of hybrid strains to regulate their virulence factor expressions in vivo. Indeed, previously studies have shown hybrid progeny derived from crossing serotypes A and D strains often show transgressive phenotypes in high temperature growth and in resistance to antifungal drugs [59]. Furthermore, the hybrid strains are capable of rapid adaptation through loss of heterozygosity to generate aneuploid mitotic recombinants [60]. Our results here extend previous studies by demonstrating the capacity of serotype AD strains to regulate virulence factor production under different environmental conditions. Such a capacity indicates their significant evolutionary potential that could pose an increasingly important threat to human health. Better understanding of the generation, spread, and virulence production in hybrids are needed in order to develop effective approaches to better control these hybrids.

**Limitations of our study and future perspectives**

As indicated in the Materials section, the strains analyzed in this study were obtained from a diversity of sources, with multiple genetic backgrounds, to explore the broad patterns of virulence trait production and phenotypic plasticity. While these strains were randomly picked from our collections, we would like to stress that our strains are unlikely representative of the whole natural and clinical populations of CNSC. However, despite the small sample size and limited geographic regions represented here, we found large phenotypic variations and plasticity within and between each of the serotype groups. For example, despite from the same geographic region, serotypes A and D strains from southern US showed significantly different melanin pigmentation and CPP under the seven environmental conditions (Supplementary Tables 3 and 4; $p < 0.05$). The difference in melanin production was much greater than that for capsular polysaccharides. Furthermore, in the case of the serotype D strains, a comparison between natural (both clinical and environmental) strains from the southern US and those from Europe also showed significant differences in both melanin production and CPP in the seven test conditions (Supplementary Tables 5 and 6; $p < 0.05$). This result suggests geography could be a significant factor in virulence trait expression. Whether the patterns observed here also apply to other geographic populations requires further investigation.

In our study, we compared melanin production and CPP among seven environmental conditions. These conditions were chosen based on previous studies and/or their clinical relevance. Specifically, the three concentrations of hydrogen peroxide and sodium nitrite have been used to examine stress responses of CNSC cells in both *in vivo* murine models and/or *in vitro* assays [61–65]. Such a concentration gradient (0, 0.25mM, 0.5mM, and 1.0mM) also allowed us to explore the response of CNSC strains to changes in stress levels, which in turn provided insights into potential regulatory mechanisms of virulence factor production. Indeed, testing additional stressors in the future could provide further insights into the regulation and adaptation of CNSC.

A number of methods have been used to quantify melanin production and CPP. For CPP, a common method is Indian ink staining and measuring the size of capsules of individual cells under a microscope. However, using this method, large variations are commonly observed among cells of the same strain under the same incubation condition, making inter-strain and inter-serotype comparisons often meaningless. In our method, we extracted capsular polysaccharides from populations of cells for each strain under each condition and quantified them using colorimetry. Our analyses revealed high reproducibility among repeats of the same strain under the same environmental condition. Such a high repeatability allowed us to critically analyze the contributions of strains, environmental factors, and their interactions to the overall CPP variation. Similarly, for melanin production, a common method involves the generation of “melanin ghosts” through treatments using corrosive chemicals such as sulfuric acid, followed by quantification using spectrometry [64]. However, the process is time consuming. The method used here relies on transilluminator-based spot densitometry to determine relative melanin production. This method is fast and high reproducible, requires no chemical treatment [37]. Indeed, this method has led to the identification of genetic loci contributing to melanin production among natural strains, including those by natural single nucleotide polymorphisms at the lac1 gene, the essential gene for melanin biosynthesis in CNSC [37, 46]. However, as noted above, the relationship between our *in vitro* results to those of *in vivo* observations needs to be critically evaluated to determine the clinical significance of the observed phenotypic variation in our in vitro study.

# Conclusions

In summary, our study indicates that both melanin and capsular polysaccharide productions in CNSC are significantly influenced by serotypes, strains, environmental stressors, and interactions among these factors. We demonstrated that among the three serotypes in CNSC, environmental stressors play a major role in the phenotypic plasticity associated with these two virulence factors. Interestingly, we found an overall negative correlation between melanin production and CPP but that the strength of the relationship depends on specific environmental factors. Together, these results suggest complex regulatory networks controlling the expressions of these two virulence traits, with both genetic and environmental factors playing major roles. Even though we chose the clinically relevant oxidative and nitrosative stress levels in our in vitro investigations, their relevance of our observations to the *in vivo* host environments awaits further investigation.

# Declarations

### Data availability statement

All data described in the study are presented in the manuscript and as supplementary files.

### Ethical statement

We confirm that all methods in this study were carried out in accordance with relevant guidelines and regulations. In addition, all experimental protocols were approved by McMaster University. No human nor animal was used as subject in this research.

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**Competing Interests Statement**

The authors declare that we have no competing interests or other interests that might be perceived to influence the results and/or discussion reported in this manuscript.

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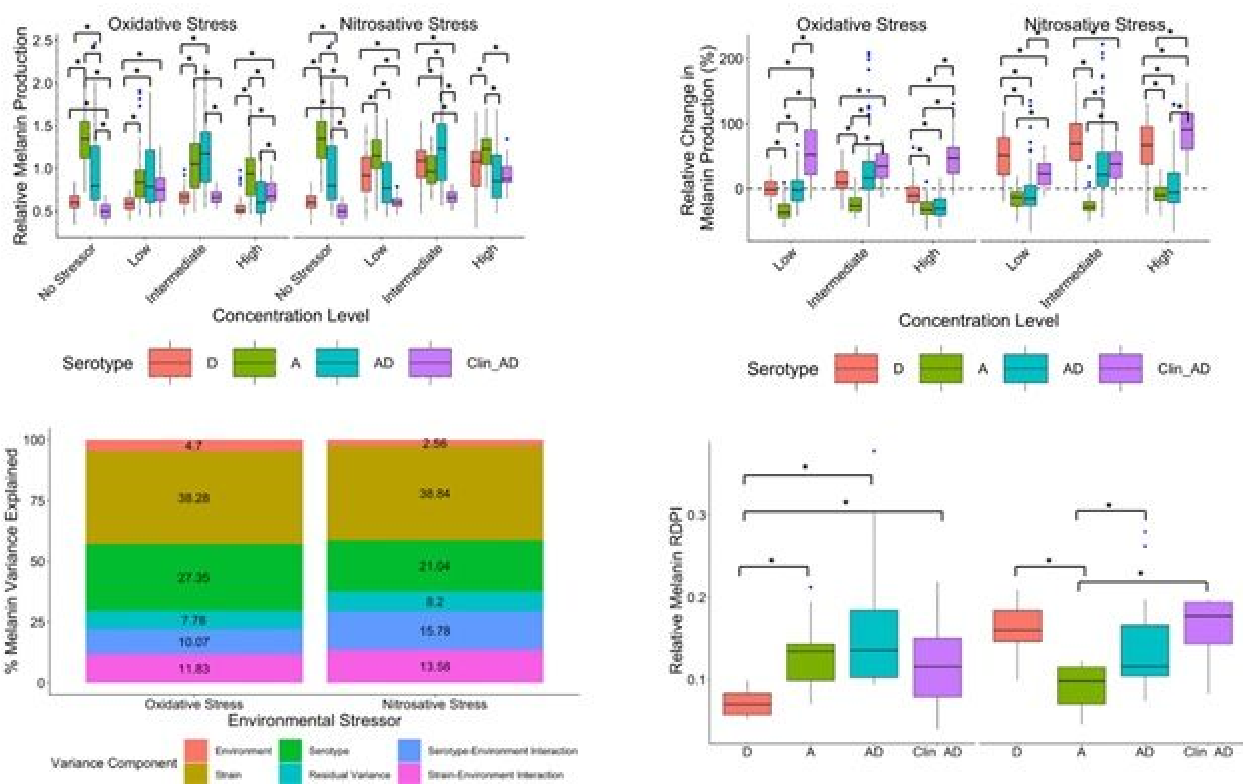
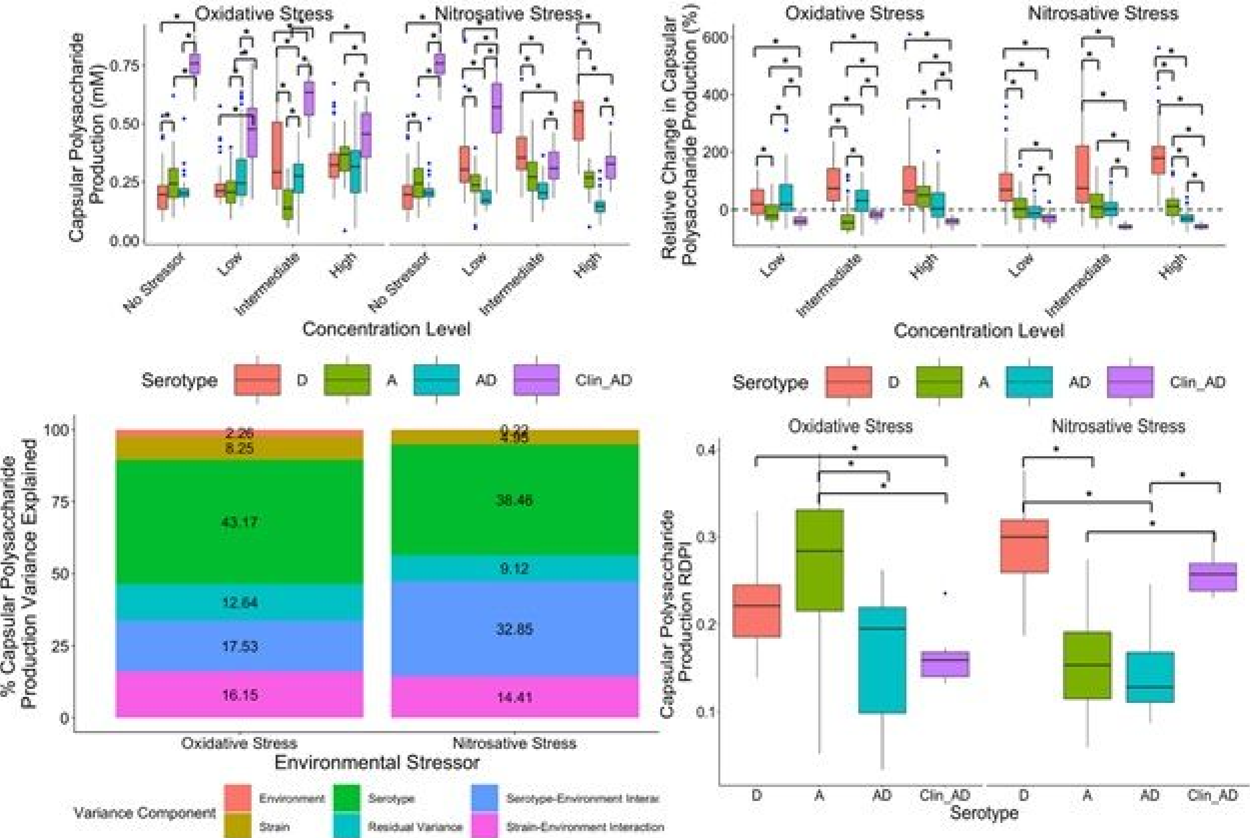
# Figures




## Figure 1

Environmental stressors induce serotype-specific effects in melanin production. Relative melanin production of Cryptococcus neoformans species complex (CNSC) samples consisting of serotypes A, D, and two samples of serotype AD. One serotype AD sub-sample was a hybrid progeny population derived from a cross between a serotype A and a serotype D strain, and the other was clinical serotype AD hybrids. These strains were exposed to seven types of growth conditions (A). Relative change in melanin production of a Cryptococcus neoformans species complex (CNSC) samples consisting of serotypes A, D, and two samples of serotype AD. One serotype AD sub-sample was a hybrid progeny population derived from a cross between a serotype A and a serotype D strain, and the other was clinical serotype AD hybrids. These strains were grown in six types of conditions and the relative changes were calculated by comparing with the negative control (B). Contribution of environment, serotype, strain and associated interactions to % melanin variance (C). Relative distance plasticity indices (RDPI) for melanin production of a Cryptococcus neoformans species complex (CNSC) population consisting of serotypes A, D, and two

samples of serotype AD. One serotype AD sub-sample was a hybrid progeny population derived from a cross between a serotype A and a serotype D strain, and the other was clinical serotype AD hybrids. The RDPI values were generalized to oxidative and nitrosative stress conditions (D). Presence of * indicates significant difference between groups at the p<0.05 significance level.




## Figure 2

Environmental stressors induce serotype-specific effects in terms of capsular polysaccharide production. Relative capsular polysaccharide production of Cryptococcus neoformans species complex (CNSC) samples consisting of serotypes A, D, and two samples of serotype AD. One serotype AD sub-sample was a hybrid progeny population derived from a cross between a serotype A and a serotype D strain, and the other was clinical serotype AD hybrids. These strains were exposed to seven types of growth conditions (A). Relative change in capsular polysaccharide production of a Cryptococcus neoformans species complex (CNSC) samples consisting of serotypes A, D, and two samples of serotype AD. One serotype AD sub-sample was a hybrid progeny population derived from a cross between a serotype A and a serotype D strain, and the other was clinical serotype AD hybrids. These strains were grown in six types of conditions and the relative changes were calculated by comparing with the negative control (B). Contribution of environment, serotype, strain, and associated interactions to % capsular polysaccharide variance (C). Relative distance plasticity indices (RDPI) for capsular polysaccharide production of a Cryptococcus neoformans species complex (CNSC) population consisting of serotypes A, D, and two samples of serotype AD. One serotype AD sub-sample was a hybrid progeny population derived from a cross between a serotype A and a serotype D strain, and the other was clinical serotype AD hybrids. The RDPI values

were generalized to oxidative and nitrosative stress conditions (D). Presence of * indicates significant difference between groups at the p<0.05 significance level.

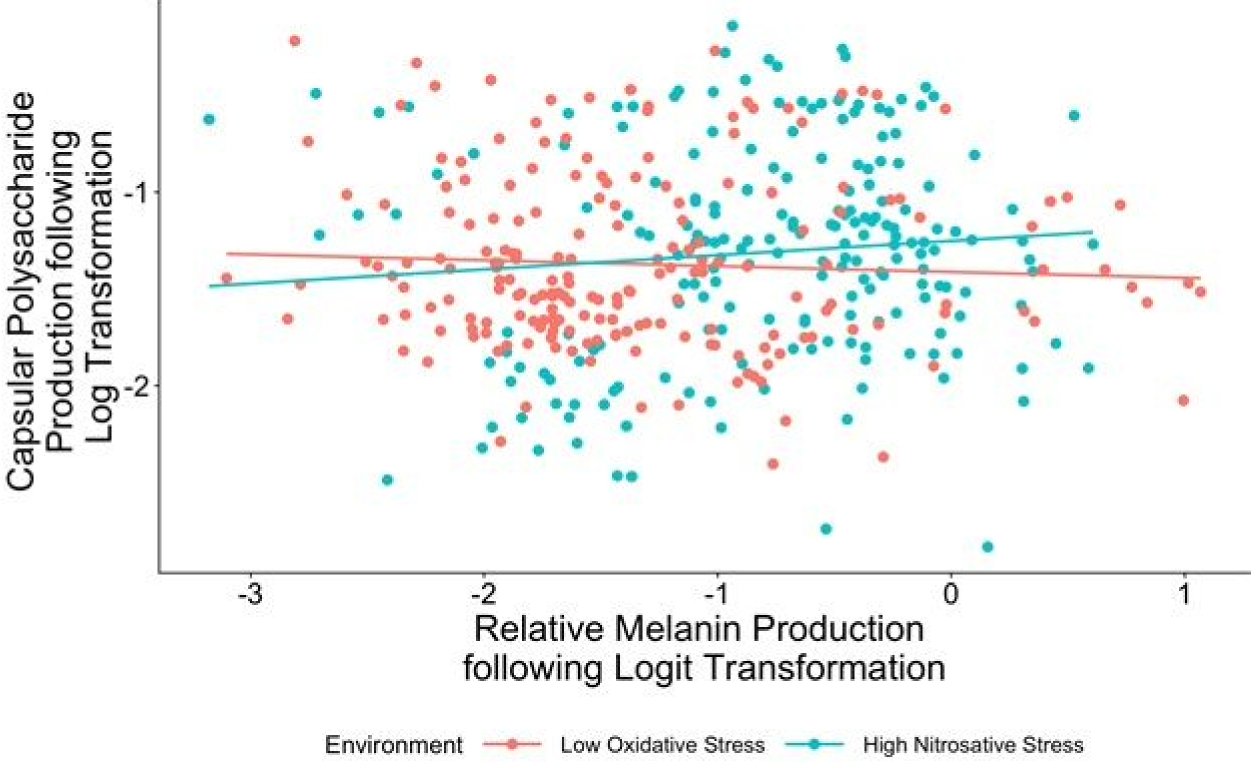



**Figure 3**

Capsular polysaccharide concentration and relative melanin production demonstrate environment-dependent correlations. Correlation between capsular polysaccharide production and relative melanin production of a Cryptococcus neoformans species complex (CNSC) population consisting of serotypes A, D, and two samples of serotype AD. One serotype AD sub-sample was a hybrid progeny population derived from a cross between a serotype A and a serotype D strain, and the other was clinical serotype AD hybrids. Correlation under seven experimental conditions were separated estimated. Capsular polysaccharide production and relative melanin production under low oxidative stress showed a negative correlation ($r = -0.08$; $p < 0.005$). However, capsular polysaccharide production and relative melanin production under high nitrosative stress showed a positive correlation ($r = 0.06$; $p < 0.005$). Correlations under other five experimental conditions were statistically not significant.

# Supplementary Files

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