Pseudogymnoascus destructans transcriptome changes during white-nose syndrome infections

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ABSTRACT
White nose syndrome (WNS) is caused by the psychrophilic fungus Pseudogymnoascus destructans that can grow in the environment saprotrophically or parasitically by infecting hibernating bats. Infections are pathological in many species of North American bats, disrupting hibernation and causing mortality. To determine what fungal pathways are involved in infection of living tissue, we examined fungal gene expression using RNA-Seq. We compared P. destructans gene expression when grown in culture to that during infection of a North American bat species, Myotis lucifugus, that shows high WNS mortality. Cultured P. destructans was grown at 10 to 14 °C and P. destructans growing in vivo was presumably exposed to temperatures ranging from 4 to 8 °C during torpor and up to 37 °C during periodic arousals. We found that when P. destructans is causing WNS, the most significant differentially expressed genes were involved in heat shock responses, cell wall remodeling, and micronutrient acquisition. These results indicate that this fungal pathogen responds to host-pathogen interactions by regulating gene expression in ways that may contribute to evasion of host responses. Alterations in fungal cell wall structures could allow P. destructans to avoid detection by host pattern recognition receptors and antibody responses. This study has also identified several fungal pathways upregulated during WNS infection that may be candidates for mitigating infection pathology. By identifying host-specific pathogen responses, these observations have important implications for host-pathogen evolutionary relationships in WNS and other fungal diseases.

Introduction
Fungal pathogens have emerged as major threats to biodiversity and human health. The diversity of these infectious eukaryotes and their hosts present new challenges in characterizing the interactions between host, pathogen, and the environment that lead to pathogenesis. One successful approach is to use systems biology to compare whole-transcriptome changes in gene expression between the pathogen infecting the host, the host without the pathogen, and the pathogen without the host. This dual RNA-Seq approach can be used to identify genetic factors from the pathogen that contribute to host colonization and manipulation of host-pathogen interactions.

Among fungal emerging infectious diseases, white-nose syndrome (WNS) in bats has spread from Eurasia, where it is endemic, to North America, where it is decimating several species of hibernating bats. Susceptible species, such as the little brown myotis (Myotis lucifugus) have shown population declines up to 90% in affected hibernacula. WNS is caused by Pseudogymnoascus destructans, a psychrophilic fungus that grows in cold hibernacula and causes cutaneous infections in bats while they hibernate. During WNS, P. destructans invades the skin tissue, forming subcutaneous lesions identified as cupping erosions by histopathology. The infection disrupts the hibernation behavior of susceptible bats and leads to more frequent arousals from torpor, premature energy depletion, electrolyte imbalance, and death.

WNS does not affect all species of bats equally. Many, but not all, North American species are being severely affected, while most European bats can host P. destructans infections, but have low mortality from WNS. Coevolution of P. destructans and Eurasian bats, such as Daubenton’s myotis (M. daubentoni), appears to have adapted these populations to a commensal or parasitic relationship with lower pathology. North American bats, on the other hand, have yet to...
benefit from such selection against extirpation of the host species\textsuperscript{23} and some species face the possibility of regional extinctions.\textsuperscript{11,18,24} The virulence of the \textit{P. destructans} infection is controlled by a combination of the environment (i.e., temperature and humidity of the hibernaculum), the host (and the host’s response to infection), and the pathogen (and the pathogen’s response to the host).\textsuperscript{25} In this study, we focus on the third component of this epidemiological triangle by dissecting the genetic components that allow \textit{P. destructans} to infect hosts and become a virulent pathogen.

Whether \textit{P. destructans} remains a commensal parasite or becomes pathogenic is determined by host-pathogen interactions.\textsuperscript{8,26} We have previously examined the host response of the WNS-susceptible \textit{M. lucifugus} to \textit{P. destructans} infection in the wing membrane and found robust gene expression changes in the host during hibernation.\textsuperscript{27} We now shift our focus to characterize previously hypothesized virulence attributes of the fungus by measuring in any sample, the cultured samples expressed 324 genes were expressed at a minimum count of 1, representing 63% of all \textit{P. destructans} genes (Table S1). These samples expressed 13,512 ± 357 \textit{M. lucifugus} genes, representing 52% of all bat genes. Using a minimum of 1 count in any sample, the cultured samples expressed 8825 genes and the wing samples expressed 7264 genes of 9575 known \textit{P. destructans} genes (Table S1). These results indicate that sufficient read depth was obtained in this data set to measure \textit{P. destructans} gene expression, at least for the majority of genes.

\textbf{Comparison of infected and uninfected bats}

Prior to comparing the expression of \textit{P. destructans} genes during host infection to those in culture, we confirmed that infection levels in host tissues were sufficient to measure pathogen gene expression by quantifying the number of RNA-Seq reads that mapped to the \textit{P. destructans} transcriptome (Table S1). Compared to a group of samples from \textit{M. lucifugus} not infected with \textit{P. destructans} (Figure S1), the samples from the infected bats from Kentucky showed significantly higher levels of \textit{P. destructans} transcripts ($t = 8.84$, $p < 0.00001$). In the wing samples from infected bats, we found that $5990 \pm 324 \textit{P. destructans}$ genes were expressed at a minimum count of 1, representing 63% of all \textit{P. destructans} genes (Table S1). These samples expressed 13,512 ± 357 \textit{M. lucifugus} genes, representing 52% of all bat genes. Using a minimum of 1 count in any sample, the cultured samples expressed 8825 genes and the wing samples expressed 7264 genes of 9575 known \textit{P. destructans} genes (Table S1). These results indicate that sufficient read depth was obtained in this data set to measure \textit{P. destructans} gene expression, at least for the majority of genes.

\textbf{Comparison of \textit{P. destructans} gene expression during WNS and culture}

Using both hierarchical clustering (Fig. 1A) and principal component analysis (Fig. 1B), we found that the patterns of \textit{P. destructans} gene expression were similar in each group of samples (cultured or WNS). We observed a small batch effect between the cultured samples that were grown at different times and sequenced differently (Table 1). We also found that samples from bats KY19 and KY23, which came from a different cave in the same county as bats KY06, KY07, and KY11,\textsuperscript{27} clustered separately from these samples and from sample KY39, which came from a different county. These results suggest that some of the differences in gene expression that we observe within the 2 groups could be due to variations in the environmental conditions or genetic differences between the \textit{P. destructans} isolated growing in different hibernacula. However, the largest differences appear to be due to the different growth conditions between culture and growth on bats.

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|c|c|c|}
\hline
\textbf{Group} & \textbf{Sample} & \textbf{SRA Accession} & \textbf{Sequencing} & \textbf{Reads post-trim} & \textbf{\textit{P. destructans} counts} & \textbf{\textit{M. lucifugus} counts} & \textbf{Percent \textit{Pd}} \\
\hline
\textit{MyLu} & KYMYLU06W & SRR1916825 & PE 101 bp & 19,289,825 & 99,055 & 5,190,125 & 1.9\% \\
& KYMYLU07W & SRR1916826 & PE 101 bp & 18,862,520 & 121,838 & 5,379,370 & 2.2\% \\
& KYMYLU11W & SRR1916827 & PE 101 bp & 19,302,516 & 98,878 & 5,034,500 & 1.9\% \\
& KYMYLU19W & SRR1916842 & PE 101 bp & 17,642,460 & 123,139 & 4,535,156 & 2.6\% \\
& KYMYLU23W & SRR1916830 & PE 101 bp & 14,997,956 & 85,249 & 3,599,787 & 2.3\% \\
& KYMYLU39W & SRR1916832 & PE 101 bp & 17,609,994 & 59,888 & 4,252,402 & 1.4\% \\
\hline
\textit{Culture} & SRR1270408 & PE 50 bp & 22,792,423 & 13,820,072 & & & \\
& SRR1270408 & PE 50 bp & 24,400,308 & 15,080,834 & & & \\
& SRR1270412 & PE 101 bp & 107,250,955 & 63,836,991 & & & \\
& SRR1270417 & SE 51 bp & 27,402,575 & 18,301,930 & & & \\
\hline
\end{tabular}
\caption{RNA-Seq data sets used for analysis and RSEM expected counts.}
\end{table}
We then compared *P. destructans* gene expression during WNS infection of *M. lucifugus* to the 20631–21 strain of *P. destructans* grown in culture (Fig. 3D, Table S2). Using our Trinotate annotation, we identified 39 genes that showed significant changes in expression during WNS whose putative functions could contribute to virulence by affecting tissue invasion, the heat shock response, nutrient acquisition, immune evasion, and other pathways (Table 2).

We specifically examined the expression levels of secreted proteases, because they have been implicated in the pathogenesis of WNS.30,31 Protease genes were identified by homology and by PFAM analysis32 and the expression of these genes was compared in the 5 culture samples and 6 *M. lucifugus* WNS samples (Table S2). Table 3 lists selected protease genes and demonstrates that the genes for subtilase-family proteases are more highly expressed during culture than during tissue invasion. Other proteases are highly expressed during host infection, such as VC83_01361, the *P. destructans* homolog of the *Aspergillus fumigatus* major allergen Aspf2, show lower gene expression when *P. destructans* is growing in culture.

To further explore the functional pathways that regulate infection, gene ontology enrichment analysis was performed using the genes identified by edgeR at a maximum FDR of 0.05 and minimum fold-change of 2. We examined the annotated functions of *P. destructans* genes upregulated in either *M. lucifugus* infections or in culture (Table 4). This analysis determined that several pathways involved in peptide and nitrogen metabolism are significantly enriched in *P. destructans* during infection (FDR < 0.05). While growing in culture, *P. destructans* showed enrichment of oxidation-reduction and transport pathways (FDR < 0.001) and depletion of other metabolic pathways (FDR < 0.05).

**Discussion**

We determined how parasitism affects the expression patterns of *P. destructans* genes by comparing expression levels between the fungus in culture and during host infection. We used dual RNA-Seq data and an approach that simultaneously mapped the reads to both host and pathogen transcriptomes followed by the removal of reads that mapped to host transcripts. This approach allowed for the estimation of expression levels of *P. destructans* genes with high levels of confidence by using RSEM to control for the uncertainty of multi-mapped
reads. We compared gene expression changes of the cultured 20631–21 North American strain of *P. destructans* to infection of a naïve North American species. Although the data set had limited read depth for *P. destructans* genes in the *M. lucifugus* samples, we observed significant differential gene expression in 211 genes, or 2.2% of the 9575 known *P. destructans* genes. This initial study has validated this approach to identifying changing patterns of pathogen gene expression. Future studies will be needed to overcome some of the limitations of the currently available data sets by using greater read depth for the dual RNA-Seq data, better matching environmental conditions *in vitro* to those in hibernacula, and using the identical isolate of *P. destructans* for both data sets.

**Figure 2.** Expression levels of differentially expressed *P. destructans* genes. Heatmaps show the expression level in counts per million (CPM) of (a) the 94 *P. destructans* genes upregulated in the MyLu samples compared with the Culture samples or (b) the 117 genes upregulated in the Culture samples compared with the MyLu samples. Genes were identified as differentially expressed (FDR < 0.001) by both edgeR and DESeq2 and expressed (CPM > 0) in at least 2 of the MyLu samples. The scale is log10 CPM with a maximum of 4.5 (a) or 4.1 (b).
Future work could also compare changes in *P. destructans* gene expression during infection of North American or European bat species that show more resistance to WNS mortality than *M. lucifugus*.8,19,20,33,34

As expected, we found that the transition from abiotic to parasitic growth was accompanied by many changes in *P. destructans* gene expression. Differences in temperature and humidity could also contribute to the differences in gene expression that we observed. Some of the gene expression changes are also presumably due to alterations in nutrient availability, such as the increased expression of lipase (VC83_00616) *in vivo* due to the high lipid content of mammalian skin. Although the cultured *P. destructans* was not grown on the same substrate that it would find in the environment, many of the gene expression changes that we observed appear consistent with adaptation to the host environment, rather than changes due to nutrient sources. For example, the increased expression of heat shock genes is consistent with the response to arousal from torpor to euthermic
body temperatures that occurred 60 to 120 minutes before collecting the *M. lucifugus* samples. Correspondingly, a single sample from a bat that was allowed to become euthermic only briefly did not show upregulation of *P. destructans* heat shock genes (unpublished results). Thermal stress caused by a febrile response in the human host has been shown to activate a heat shock response in *Candida albicans*, preventing deleterious protein unfolding and aggregation. This heat shock response could be important for fungal survival in our system, as bats arouse to euthermic temperatures several times throughout hibernation (thus several times throughout *P. destructans* infection), and susceptible populations arouse from torpor more frequently during WNS.  
Consistent with a response of the pathogen to evade host immune recognition, we also found large increases in expression of genes involved in fungal cell wall structures (Table 2). The fungal cell wall is composed of an inner layer of chitin, a middle layer of glucans and mannan, and an outer layer of glucuronoxylomannans, chitin, mannan, and glucans. The fungal cell wall is composed of an inner layer of chitin, a middle layer of glucans and mannan, and an outer layer of glucuronoxylomannans, chitin, mannan, and glucans. The fungal cell wall structure is dynamic, and its components can be modified in response to environmental stress. The fungal cell wall is composed of an inner layer of chitin, a middle layer of glucans and mannan, and an outer layer of glucuronoxylomannans, chitin, mannan, and glucans. The fungal cell wall structure is dynamic, and its components can be modified in response to environmental stress. The fungal cell wall structure is dynamic, and its components can be modified in response to environmental stress. The fungal cell wall structure is dynamic, and its components can be modified in response to environmental stress. The fungal cell wall structure is dynamic, and its components can be modified in response to environmental stress.

### Table 2. Selected *P. destructans* genes differentially expressed between culture and WNS-affected *M. lucifugus* that have putative functions implicated in fungal virulence.

| Genea | Full Name | BLASTXb | edgeRc | DESeq2d |
|-------|-----------|---------|--------|---------|
| VCB3_01361 | Major allergen Asp f 2 | ALL2_ASFU | 36.0 | 1.11E-09 |
| VCB3_00616 | Lipase 1 | LIP1_GEOCN | 9.1 | 1.84E-05 |
| VCB3_02553 | 30 kDa heat shock protein | HSP30_NEUCR | 29.4 | 9.69E-07 |
| VCB3_07843 | Hsp70 nucleotide exchange factor FES1 | FES1_NEUCR | 19.0 | 8.14E-08 |
| VCB3_00970 | Heat shock protein 78, mitochondrial | HSP78_SCHPO | 12.4 | 4.17E-06 |
| VCB3_00522 | Protein pm1 | PS1_SCHPO | 9.9 | 5.21E-06 |
| VCB3_01964 | Heat shock protein hsp88 | HSP88_NEUCR | 9.5 | 2.15E-05 |
| VCB3_08137 | Heat shock protein hsp98 | HSP98_NEUCR | 9.0 | 7.52E-05 |
| VCB3_01046 | Heat shock 70 kDa protein 2 | HSP72_PARBA | 7.3 | 2.04E-04 |
| VCB3_02466 | Uncharacterized protein C1711.08 | YNY8_SCHPO | 5.3 | 1.41E-04 |
| VCB3_08167 | Heat shock protein 82 | HSP82_AJCEA | 4.3 | 1.91E-03 |
| VCB3_09034 | Unchar. J domain-containing protein C63.13 | YCJD_SCHPO | 4.3 | 3.20E-03 |
| VCB3_06435 | Heat shock protein still homolog | STI1_SCHPO | 4.1 | 2.03E-03 |
| VCB3_01360 | Zinc-regulated transporter 1 | ZRT1_YEAST | 18.6 | 5.62E-08 |
| VCB3_07026 | Calcium-transporting ATPase 3 | ATC3_SCHPO | 11.7 | 2.23E-04 |
| VCB3_00191 | Putative Copper transporter protein (PFAM) | PMD1_SCHPO | 10.3 | 2.37E-05 |
| VCB3_06862 | Calcium-transporting ATPase 3 | ATC3_SCHPO | 6.2 | 4.74E-05 |
| VCB3_01014 | Calcium-transporting ATPase 2 | ATC2_SCHPO | 3.4 | 2.64E-03 |
| VCB3_04094 | Metal homeostasis factor | ATX1_YEAST | 7.5 | 4.90E-03 |
| VCB3_00261 | Mannan endo-1,6-glucosidase | EXG1_COCCA | 6.5 | 1.93E-03 |
| VCB3_00736 | Na(Ca) antiporter 1 | NAH1_ZYGRO | 11.6 | 2.07E-04 |
| VCB3_03500 | Spherulin-1A | SR1A_PHYPO | 22.2 | 9.41E-05 |
| VCB3_07867 | Uncharacterized protein AFUA_6G02800 | YAP200_ASPFU | 21.2 | 8.98E-07 |
| VCB3_00788 | Endochitinase 1 | CH1_APHAL | 11.6 | 2.07E-04 |
| VCB3_07327 | Probable glucan endo-1,3-β-glucosidase egiC | EGLC_NEOFI | 6.5 | 1.93E-03 |
| VCB3_04729 | Endochitinase 1 | CH1_COCCM | 6.3 | 8.24E-05 |
| VCB3_07145 | Mannan endo-1,6-α-mannosidase DCW1 | DCW1_YEAST | 5.9 | 2.51E-05 |
| VCB3_05104 | Chitin synthase 4 | CH54_NEUCR | -3.4 | 8.74E-04 |
| VCB3_09076 | Glucan 1,3-β-glucosidase | EXG1_COCCA | -3.5 | 1.99E-04 |
| VCB3_00261 | Mannan endo-1,6-α-mannosidase DFG5 | DFG5_CANAL | -3.8 | 3.97E-03 |
| VCB3_08448 | Protein SUR7 | SUR7_CANAL | -10.1 | 1.43E-03 |
| VCB3_05292 | Cell wall mannoprotein C53 | C53_YEAST | -14.8 | 3.30E-05 |
| VCB3_01650 | Mannan endo-1,6-α-mannosidase DCW1 | DCW1_YEAST | -15.4 | 1.18E-06 |
| VCB3_06309 | Putative heme-binding peroxidase | CCR2_ASFU | 7.5 | 4.90E-03 |
| VCB3_00225 | Putative cytochrome DASH, mitochondrial | CRD6_NEUCR | 7.5 | 2.15E-05 |
| VCB3_06307 | Squalene monoxygenase | ERG1_CANAL | 4.5 | 2.51E-04 |
| VCB3_03222 | Probable GTP cyclohydrolase-2 | RIB1_SCHPO | 4.1 | 7.67E-04 |
| VCB3_01624 | Leptomycin B resistance protein | PMD1_SCHPO | 3.8 | 3.55E-03 |
| VCB3_06509 | Thioredoxin reductase | TRX5_NEUCR | 3.1 | 2.03E-03 |
| VCB3_08771 | Probable transporter MCH5 | MCH5_YEAST | -4.2 | 2.18E-04 |

Notes.  
aDESeq2 gene (Drees et al. 2016).  
bBLAST hit with the lowest E-value in the Swissprot database. Only homologs with E < 1E-04 were considered.  
cFold change in gene expression of the WNS samples compared with culture samples determined by edgeR. Negative values indicate higher expression in the culture samples. Dashed lines separate genes with higher expression in WNS from genes with higher expression in culture.  
dAdjusted p-value of differential expression determined by edgeR after Benjamini-Hochberg FDR correction.  
eMean normalized expression level (TPM) in culture or WNS samples determined by DESeq2.  
fAdjusted p-value of differential expression determined by DESeq2 after Benjamini-Hochberg FDR correction.
β-glucans, and an outer layer of mannose. The cell wall provides rigidity and structure, however is also highly dynamic. The pattern recognition receptor Dectin-1 has been shown to be a receptor for fungal 1,3-β glucans and 1,6-β glucans,\textsuperscript{37,38} thus cell wall components serve to alert the mammalian immune system of a fungal pathogen. We have observed that Dectin-1 and several other C-type lectin domain family members are significantly upregulated in bat tissues infected with \textit{P. destructans}.\textsuperscript{27}

Consistent with this host observation, we detected significant alterations in \textit{P. destructans} enzymes predicted to be involved in fungal cell wall remodeling (Table 2). VC83_00788 and VC83_04729, homologs of Endochitinase 1, an enzyme which randomly cleaves and breaks down chitin, are upregulated 11.6 and 6.3-fold, respectively, while VC83_05104, a homolog of Chitin synthase.

### Table 3. Expression of selected \textit{P. destructans} protease genes.

| Gene  | Full Name                               | BLASTX\textsuperscript{b} | FC\textsuperscript{c} | FDR\textsuperscript{d} | Cult\textsuperscript{e} | WNS\textsuperscript{e} | FDR\textsuperscript{e} |
|-------|-----------------------------------------|---------------------------|-----------------------|-------------------------|-------------------------|-------------------------|-------------------------|
| VC83_09074 | Subtilisin-like protease 3 (Destructin-3) | SUB3_PSED2                 | 1.8                    | 0.66                    | 29.3                    | 51.6                    | 0.67                    |
| VC83_06062 | Subtilisin-like protease 2 (Destructin-1) | SUB2_PSED2                 | −1.3                   | 0.78                    | 502.4                   | 375.4                   | 0.80                    |
| VC83_07090 | Subtilisin-like protease Spm1             | SPM1_MAGO7                 | −1.5                   | 0.30                    | 2056.9                  | 1421.4                  | 0.12                    |
| VC83_06607 | Protease Kexin 2                         | KEK2_CANAW                 | −2.3                   | 0.05                    | 147.8                   | 66.9                    | 0.04                    |
| VC83_04892 | Subtilisin-like protease 1 (Destructin-2) | SUB1_PSED2                 | −3.0                   | 0.33                    | 5800.6                  | 1962.1                  | 1.00                    |
| VC83_02181 | Tripeptidyl-peptidase sed2               | SED2_ASPFU                 | −5.5                   | 0.0011                  | 791.6                   | 152.6                   | 0.0045                  |

\textsuperscript{37}\textsuperscript{38}DESeq2

\textsuperscript{a}P. destructans\textsuperscript{b} gene (Drees et al. 2016).

\textsuperscript{b}BLAST hit with the lowest E-value in the Swissprot database. Only proteins with E < 1E-04 were considered.

\textsuperscript{c}Fold change in gene expression of the WNS samples compared with the culture samples determined by EdgeR. Negative values indicate higher expression in the culture samples. A dashed line separate genes with higher expression in WNS from genes with higher expression in culture.

\textsuperscript{d}Adjusted p-value of differential expression determined by edgeR after Benjamini-Hochberg FDR correction.

\textsuperscript{e}Mean normalized expression level (TPM) in culture or WNS samples determined by DESeq2.

\textsuperscript{f}Adjusted p-value of differential expression determined by DESeq2 after Benjamini-Hochberg FDR correction.

### Table 4. Gene ontology analysis of \textit{P. destructans} pathways altered during WNS.

| GO Category | Biological Process | E/P\textsuperscript{1} | Ratio in study\textsuperscript{2} | p | FDR\textsuperscript{3} |
|-------------|--------------------|-------------------------|----------------------------------|---|------------------------|
| Upregulated during WNS infection | peptide metabolic process | e | 23/410 | 2.49E-08 | <0.001 |
| GO:0006518 | peptide metabolic process | e | 21/410 | 9.07E-08 | <0.001 |
| GO:0006412 | translation | e | 21/410 | 1.90E-07 | <0.001 |
| GO:0043040 | peptide biosynthetic process | e | 23/410 | 5.57E-07 | 0.002 |
| GO:0043603 | cellular amide metabolic process | e | 8/410 | 1.04E-06 | 0.002 |
| GO:0042254 | ribosome biogenesis | e | 21/410 | 1.58E-06 | 0.002 |
| GO:0043604 | amide biosynthetic process | e | 8/410 | 2.59E-06 | 0.002 |
| GO:0022613 | ribonucleoprotein complex biogenesis | e | 8/410 | 1.16E-05 | 0.008 |
| GO:0044085 | cellular component biogenesis | e | 8/410 | 2.62E-05 | 0.012 |
| GO:0034645 | cellular macromolecule biosynthetic process | e | 28/410 | 2.82E-05 | 0.012 |
| GO:1901566 | organonitrogen compound biosynthetic process | e | 31/410 | 2.82E-05 | 0.012 |
| GO:0044271 | cellular nitrogen compound biosynthetic process | e | 34/410 | 9.20E-05 | 0.028 |

| GO Category | Biological Process | E/P\textsuperscript{1} | Ratio in study\textsuperscript{2} | p | FDR\textsuperscript{3} |
|-------------|--------------------|-------------------------|----------------------------------|---|------------------------|
| Upregulated in culture | oxidation-reduction process | e | 75/846 | 2.42E-07 | <0.001 |
| GO:0055114 | oxidation-reduction process | e | 56/846 | 1.09E-06 | <0.001 |
| GO:0055085 | transmembrane transport | e | 119/846 | 8.21E-06 | 0.002 |
| GO:0044710 | single-organism metabolic process | p | 18/846 | 3.19E-05 | 0.004 |
| GO:0093034 | nucleic acid metabolic process | p | 1/846 | 3.71E-05 | 0.01 |
| GO:0006396 | RNA processing | p | 31/846 | 6.80E-05 | 0.01 |
| GO:0027230 | tricarboxylic acid metabolic process | e | 7/846 | 7.88E-05 | 0.012 |
| GO:1901360 | organic cyclic compound metabolic process | p | 33/846 | 0.00011 | 0.024 |
| GO:0006139 | nucleobase-containing compound metabolic process | p | 27/846 | 0.00013 | 0.032 |
| GO:0034641 | cellular nitrogen compound metabolic process | p | 42/846 | 0.00013 | 0.032 |
| GO:0016070 | RNA metabolic process | p | 11/846 | 0.00017 | 0.046 |

\textsuperscript{1}Enrichment (e) or purification (p) detected. Enrichment indicates that the GO category is more highly represented than expected by chance and purification indicates that the category is underrepresented.

\textsuperscript{2}Number of differentially expressed genes in this category compared with total differentially expressed genes.

\textsuperscript{3}Adjusted p-value of enrichment or purification after Benjamini-Hochberg FDR correction.
4 is downregulated 3.4-fold in *P. destructans* during infection compared with culture. Two homologs to Glucan endo-1,3-β glucosidases were differentially regulated; VC83_07327 was upregulated in *P. destructans* during infection while VC83_09076 was upregulated during culture. These enzymes presumably regulate cell wall β-glycan turnover and catabolism of β-glycans by removal of non-reducing terminal glucosyl residues from saccharides and glycosides.

Additionally, 3 Mannan endo-1,6-α mannosidases that were differentially expressed between *P. destructans* actively infecting a host and growing in culture (*Table 2*). Two were upregulated in culture (VC83_00261 and VC83_01650), and one was upregulated during WNS (VC83_07145). Mannan endo-1,6-α mannosidases are required for normal synthesis of the cell wall and alkaline pH-induced hypha formation, as well as being responsible for random hydrolysis of α-mannosidic linkages in unbranched mannosides. It is likely that the changes in Glucan endo-1,3-β glucosidase and Mannan endo-1,6-α mannosidase gene expression that we observed upon the switch from abiotic growth to host colonization lead to substantial alterations in the cell wall structures. The resulting differences in saccharide and glycoside branching patterns in the cell wall could make the pathogen less recognizable to the mammalian immune system.

Alternatively, these changes in cell wall enzyme gene expression could be due to changes in metabolic pathways that accompany the shift from abiotic to infectious niches. Different carbon sources can modulate cell wall structure and virulence in *C. albicans*. It is possible that changes in cell wall structures are caused by differences in metabolism when infecting bats, rather than direct adaptation to the host.

Alterations in cell wall structures also accompany shifts in the morphological growth type of fungi, such as a shift from yeast to hyphal phase in *C. albicans*. However, *P. destructans* grows vegetatively as hyphae on both Sabouraud’s dextrose agar medium in culture, and when forming cupping erosions in the wing tissue of the host. Thus there is no difference in morphotype between our cultured and WNS *P. destructans* samples that might explain the dramatic alterations in expression of cell wall remodeling enzymes that we observed. Consequently, we propose that changes in the β-glucan landscape on the fungal surface via cell wall remodeling are a mechanism of immune evasion for *P. destructans*, similar to other fungal pathogens.

Alterations of the cell wall during infection could explain the ineffectiveness of antibodies that recognize the cell wall of cultured *P. destructans* in providing protection from WNS. These results may also explain why immunization with either cultured *P. destructans* or a β-glucan vaccine did not affect the susceptibility of *M. lucifugus* to WNS (J. Johnson, J. McMichael, D. Reeder, and K. Field, unpublished). The antigens provided by these immunizations may not be present on the surface of *P. destructans* during infection because of changes in the cell wall structure that accompany the transition from abiotic to parasitic growth.

Because tissue invasion is a hallmark characteristic of *P. destructans* infections during WNS, we expected that expression of genes involved in degradation of the extracellular matrix would be upregulated. Unexpectedly, we found that the previously characterized subtilase-family of secreted proteases showed lower expression in *P. destructans* during infection than in culture. Instead, the homolog of the *A. fumigatus* vacuolar protease, major allergen Aspf2, showed high levels of expression during infection of *M. lucifugus* and was significantly upregulated compared with culture conditions. This suggests that other proteases may be better targets for preventing colonization than the subtilase-family proteases, although the possible role of Aspf2 in tissue invasion remains unknown. It is also plausible that subtilase-family proteases are regulated at a post-transcriptional level or are used by the fungus primarily during initial colonization. Therefore, further proteomic and expression time-course experiments may prove useful to further dissect the infection. Nevertheless, the abundant expression of Aspf2, known to be an *A. fumigatus* allergen in humans, suggests that further investigation of IgE-mediated allergic reactions during WNS may be warranted.

Infection of hosts was also associated with changes in expression for several genes involved in the transport or homeostasis of metal ions, including zinc, iron, and copper. This fungal response may be due to limited availability of some of these micronutrients in the host, which is likely sequestering metal ions as a form of nutritional immunity. Changes in micronutrient acquisition gene expression appear to be associated with host colonization, including increased expression of the zinc transporter Zrt1, the copper homeostasis factor ATX1, and a putative copper transporter, as well as the unexpected loss of siderophore import using Mirb. Homeostasis of these micronutrients is essential for normal fungal metabolism and for the ability of the pathogen to respond to oxidative stress activated by the host immune response. However, our gene ontology analysis (*Table 4*) indicates that genes involved in oxidation-reduction pathways are more highly expressed during growth in culture than host colonization. Enrichment of pathways involving peptide metabolism and translation in *P. destructans* infecting bats (*Table 4*) indicates that host colonization demands higher levels of protein expression than abiotic growth. Competition between the host and pathogen for
Micronutrients and the generation of oxidative stress likely varies over the course of infection and further study is needed to dissect this time course.

Together, these results provide a model of gene expression changes in *P. destructans* that accompany the transitions from abiotic to parasitic growth (Figure 4). This model provides a framework to understand how the pathogen responds with phenotypic plasticity to the environment and its host to adopt a virulent phenotype. Our results also suggest approaches to minimize virulence and/or colonization by targeting immune evasion, micronutrient acquisition, tissue invasion, or the heat shock response. Efforts to understand why some species are more susceptible to WNS than others will require further examination of host-pathogen interactions to determine if the pathogen responds differently in hosts that exhibit lower WNS susceptibility.

**Materials and methods**

**Sample collection**

Two different data sets were used for this study (Table 1). The samples for the first data set (MyLu) consisted of wing tissue from 6 individual *P. destructans*-infected *M. lucifugus* (little brown myotis) collected 60–120 minutes after arousal to euthermy from hibernation from caves in Kentucky, USA, as described previously.27 Hibernacula temperatures were 4–6 C at the time of collection and, based on our previous experience, we estimate that skin temperature varied between 4 and 8 C during torpor and up to 37 C during periodic arousals. The second data set was obtained from the North American 20631–21 strain of *P. destructans* growing in culture by D. Akiyoshi and A. Robbins (Department of Infectious Disease and Global Health, Cummings School of Veterinary Medicine, Tufts University). The 20631–21 strain of *P. destructans* was obtained from D. Blehert (National Wildlife Health Center, US. Geological Survey, Madison, WI, USA). The fungus was grown in culture at 10–14°C for 23 d on Sabouraud dextrose agar plates (BD Diagnostics, #221180) (Table 1). Sabouraud dextrose agar contains nutrient sources of dextrose, pancreatic digest of casein, and peptic digest of animal tissue. RNA was isolated using a Qiagen RNeasy Lipid Tissue Kit after disruption of the cells using Zymo BashingBead Lysis Tubes and a bead beater on maximum speed for 30 sec for 3 times and then 20 sec once, with cooling on ice between each.

**RNA sequencing**

RNA sequencing was performed using Illumina sequencing as summarized in Table 1. Prior to analysis all data sets were quality trimmed using Trimomatic v.0.35 with the parameters SLIDINGWINDOW:4:5 LEADING:5 TRAILING:5 MINLEN:25. For samples with paired-end sequencing, only reads with both pairs remaining after trimming were used for further analysis. Analysis of the reads using FastQC v0.11.5 and the results of STAR mapping indicate that there are no significant differences in the quality of the RNA in any of the cultured samples from the MyLu samples.

**Differential expression analysis**

The quality trimmed reads were aligned using STAR v.2.5.1b to the concatenated genomes of *M. lucifugus* and *P. destructans*. For *M. lucifugus*, we used genome assembly Myoluc2.0 and gene models from Ensembl release 84.56 For *P. destructans*, we used the genome assembly and gene models from Drees et al..57 RSEM v1.2.25 was then used to apply an expectation maximization algorithm to predict gene expression counts for each transcript. The expected count matrix for all samples is available in Data Set S1. To determine if the number of reads mapped to *P. destructans* transcripts provided sufficient statistical power to detect differential expression of these genes, we used Scotty to analyze the expected counts generated by RSEM. We determined that 65% of *P. destructans* genes expressed at a minimum of 4-fold change could be detected with a p-value cutoff.
of 0.05. Transcripts per million (TPM) was calculated by normalizing read counts for the length of each transcript and adjusting for the library size of mapped reads for each sample. The M. lucifugus transcripts were then removed from the analysis and differential expression was determined using only *P. destructans* transcripts.

Differential expression between conditions was determined using either DESeq2 v1.10.1 or edgeR v.3.12.1 after normalizing across samples using the trimmed mean of M-values (TMM) method and a minimum expression level of 2 TPM combined across all samples. False discovery rate (FDR) was used to control for multiple comparisons using the Benjamini-Hochberg procedure. Hierarchical clustering was performed using R stats package R v2.0.4–64 at an *α* level of 99% and 100 000 iterations. Genes without expression (expected count < 1) in at least 2 MyLu samples were excluded from the final analysis. Annotations for each gene were determined by using Trinotate v3.0, NCBI BLAST v2.2.29 analysis. Annotations for each gene were determined by using GOATOOLS v0.6.967 with enrichment or purification of gene ontology enrichment analysis was performed using either DESeq2 v1.10.1 or edgeR v.3.12.1 after normalizing across samples using the trimmed mean of M-values (TMM) method and a minimum expression level of 2 TPM combined across all samples. False discovery rate (FDR) was used to control for multiple comparisons using the Benjamini-Hochberg procedure. Hierarchical clustering was performed using R stats package v3.3.1 with Pearson correlation complete-linkage clustering of Euclidean distances. Clustering was confirmed by bootstrap analysis using pvclust level of 99% and 100 000 iterations.

**Disclosure of potential conflicts of interest**

No potential conflicts of interest were disclosed.

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