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Functional Traits 2.0: The Power of Metabolomics for Plant Ecology

Metabolic responses of two pioneer wood decay fungi to diurnally cycling temperature

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Abstract

1. Decomposition of lignin-rich wood by fungi drives nutrient recycling in woodland ecosystems. Fluctuating abiotic conditions are known to promote the functioning of ecological communities and ecosystems. In the context of wood decay, fluctuating temperature increases decomposition rates. Metabolomics, in tandem with other 'omics tools, can highlight the metabolic processes affected by experimental treatments, even in the absence of genome sequences and annotations. Globally, natural wood decay communities are dominated by the phylum Basidiomycota. We examined the metabolic responses of *Mucidula mucida*, a dominant constituent of pioneer communities in beech branches in British woodlands, and *Exidia glandulosa*, a stress-selected constituent of the same communities, in response to constant and diurnally cycling temperature.
2. We applied untargeted metabolomics and proteomics to beech wood blocks, colonised by *M. mucida* or *E. glandulosa* and exposed to either diurnally cycling (mean $15 \pm 10^\circ\text{C}$) or constant (15°C) temperature, in a fully factorial design.
3. Metabolites and proteins linked to lignin breakdown, the citric acid cycle, pentose phosphate pathway, carbohydrate metabolism, fatty acid metabolism and protein biosynthesis and turnover were under-enriched in fluctuating, compared to stable temperatures, in the generalist *M. mucida*. Conversely, *E. glandulosa* showed little differential response to the experimental treatments.
4. *Synthesis*. By demonstrating temperature-dependant metabolic signatures related to nutrient acquisition in a generalist wood decay fungus, we provide new insights into how abiotic conditions can affect community-mediated decomposition and carbon turnover in forests. We show that mechanisms underpinning important biogeochemical processes can be highlighted using untargeted metabolomics and proteomics in the absence of well-annotated genomes.

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KEYWORDS

diurnal cycles, ligninolytic fungi, metabolomics, proteomics, temperature, wood decay

1 | INTRODUCTION

The decomposition of lignin-rich wood, a process which is largely mediated almost entirely by a polyphyletic group of fungi—ligninolytic basidiomycete and xylariaceous ascomycetes—drives key ecosystem processes like nutrient cycling and the sequestration and release of carbon in forest systems (Bradford et al., 2014; Harmon et al., 1986; Tláskal et al., 2021). The nutrient profile of dead and decaying wood is characterised by abundant carbon, limiting levels of nitrogen, high concentrations of polyphenolic substances to which nitrogenous compounds bind and low pH (Bending & Read, 1996; Read, 1991). Up to 20% of the carbon sequestered in organic matter globally is stored in dead wood (Delaney et al., 1998). In un-decayed wood, the C/N ratio can be as high as 1250/1 and both the carbon and nitrogen present are insoluble, protected within the polymeric cellulose and lignin (Rayner & Boddy, 1988). Decomposition of these recalcitrant polymers in angiosperm wood relies on a suite of oxidases predominantly produced by white-rot basidiomycete fungi (Baldrian, 2008; Buswell et al., 1987; Krah et al., 2018). After decomposing the lignocellulose, ligninolytic fungi store assimilable nitrogen in their mycelia, which is then available to other organisms (Lindahl et al., 2002; Watkinson et al., 2006). In modulating the availability of nutrient resources and ecological niches for several other organisms in these ways, ligninolytic fungi are essential ecosystem engineers in wooded biomes and contribute directly to their biodiversity and productivity (Lonsdale et al., 2008).

Temperature plays a pivotal role in determining the rate of decomposition in wooded biomes by influencing rates of fungal growth, enzyme production and fungal–fungal interaction outcomes (Boddy, 1983; Cartwright & Findlay, 1958; Hiscox et al., 2016; Magan, 2008). Fungal decay rates increase with temperature (A'Bear et al., 2012; Boddy, 1986; Venugopal et al., 2016), due in part to the thermodynamics of enzymatic reactions (Laidler, 1984). Intricate thermally driven changes in the proteomic profile of ligninolytic fungi have been demonstrated, with the diversity of cellulose- and hemicellulose-metabolising enzymes increasing under warmer experimental conditions indicating that increased temperature might enhance access to wood-bound nutrients (Moody et al., 2018).

During the decomposition of woody resources in nature, ligninolytic fungi are exposed to fluctuations in the full range of abiotic variables, including temperature (Boddy, 1984; Magan, 2008). Several authors have shown that temperature is an important driver of fungal respiration, and therefore wood decay rate (Boddy, 1983a, 1983b; Bond-Lamberty et al., 2002; Jomura et al., 2008; Liu et al., 2013; Mackensen et al., 2003). Responses to changes in environmental variables such as temperature can be highly species-specific (A'Bear et al., 2012; Venugopal et al., 2016). To our knowledge, only one study has investigated fluctuating temperature in wood decay communities, showing experimentally that fluctuations

increase decomposition rates by supporting greater species richness (Toljander et al., 2006). Thus, temperature fluctuations are likely to be an important driver of function in this system; however, most experimental studies of ligninolysis have been undertaken at constant temperatures. Where the effect of temperature on wood decay has not been the primary aim, constant temperatures known to be optimum for mycelium growth rates are routinely used, but wood decay rate does not always track with mycelial extension rate (Wells & Boddy, 1995). In natural wood decay communities, pioneer species are restricted to attached dead branches which are not buffered by the woodland floor microclimate (Boddy et al., 2017; Unterseher & Tal, 2006), suggesting they may be particularly prone and adapted to fluctuating temperatures.

As chemo-heterotrophs, fungi interact with their environment and sequester nutrients through the secretion of enzymes, other proteins and low molecular weight compounds into their substrate (Brakhage, 2013; Keller et al., 2005). These products are utilised by ligninolytic fungi in the invasion and degradation of the wood (Higuchi, 1997; Lynd et al., 2002) and in mediating other interactions with their external environment (Baldrian, 2004; Boddy, 2000; Brakhage & Schroeckh, 2011; Criquet et al., 2000; D'Annibale et al., 2005; Hiscox & Boddy, 2017; Moody et al., 2018; Yim et al., 2007). Understanding how these processes are mediated requires tools to characterise fungal biochemical responses to changing stimuli.

Metabolomics and proteomics are powerful tools that improve our understanding of how organisms and ecosystems function. Profiling the full range of proteins and metabolites an organism produces when interacting with its environment can give an instantaneous snapshot of the functional end-points of complex biological networks, and accurately describe how cellular responses change in the face of stress factors such as non-natural abiotic conditions. Metabolomics is particularly powerful given that putative identifications can be assigned to compounds in the absence of well-annotated genomes, allowing for the interpretation of stress responses in ecologically relevant non-model organisms. Many metabolites are universal between large groups of polyphyletic organisms; others may be novel with interesting applications in technology or medicine. It is therefore important to look for 'metabolomic traits', that is, products of metabolism which may be modulated under differing conditions and therefore distinguish the responses of different species to environmental change (Peters et al., 2018).

Here, we report on a simple wood-block experiment to examine proteomic and metabolomic responses of two basidiomycete fungi, *Mucidula mucida* and *Exidia glandulosa*, to both stable and diurnally cycling temperature. Both organisms are common constituents of naturally occurring pioneer communities in beech *Fagus sylvatica* tree branches in South Wales, UK (Rawlings, 2018). *E. glandulosa* is tolerant of desiccation and often found in sun-baked or other highly desiccated wood (Boddy, 2001; Boddy & Rayner, 1983;

Heilmann-Clausen, 2001) and as such we consider it to have a stress-selected strategy. On the other hand, *M. mucida* was the most commonly isolated species, associated with less desiccated beech branches in the same study (South Wales, UK; Rawlings, 2018) and we therefore consider it to be more of a generalist. We anticipated that variable temperature would elicit a different biochemical response compared with stable temperature and that this difference may be less marked in *E. glandulosa* due to its stress-selected nature.

2 | MATERIALS AND METHODS

The two fungal strains *M. mucida* and *E. glandulosa* were isolated from dead branches attached to standing beech (*F. sylvatica*) trees at Clyne Valley Woodlands, a minimally managed woodland in Swansea, South Wales, UK (Lat 51.6063, Long -4.0068). Isolates were collected from different but closely positioned trees within the same woodland stand.

2.1 | Experimental design and microcosm conditions

F. sylvatica blocks (John Harrison, Wrexham, UK) with dimensions 2 cm³ were autoclaved three times and then pre-colonised by placing them on 0.5% malt-extract agar (MEA; 0.5% malt, 1.5% agar w/v; Sigma-Aldrich) cultures of the appropriate fungal strain and incubated in the dark at 20°C. Following 8-week pre-colonisation, experimental treatments consisting of 2 × 2 block matrices colonised with one of the two fungal strains per microcosm. Microcosms were subjected to a diurnally cycling temperature sequence of 5, 15, 25 and 15°C per 24 hr so that temperature changed by 10°C every 6 hr with a mean of 15 ± 10°C. Temperatures were chosen to represent the minima and maxima canopy-dwelling ligninolytic fungi encounter during the growing season in a temperate woodland ecosystem. Control microcosms were kept at a constant temperature of 15°C for the duration of the experiment. These methods were also chosen to be comparable with those of Toljander et al. (2006). All microcosms were incubated in the dark for 8 weeks with three replicates per species per treatment ($n = 12$).

At the end of the experiment, each block was split into three segments from top to bottom using a sterile chisel. The top and middle sections were quenched in liquid nitrogen, lyophilised and stored at -20°C. From the bottom third of each block, four wood chips (~0.5 mm²) were extracted, one from each quadrant, and placed onto 2% (w/v) MEA. Cultures were incubated at 20°C for 7–10 days and mycelial morphology confirmed that no woodblocks had become contaminated during the experiment.

2.2 | Direct shotgun LC-MS/MS proteomics

One-third of the frozen woodblock sections from each treatment group was manually chipped and the weight of wood standardised to

the lowest weight across samples. As such, 0.87 g of each sample was added to 50 ml of cold phosphate buffer (50 mM potassium phosphate pH 7.5, 1 µg/ml Pepstatin A, 1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA) and agitated for 40 hr (4°C). The sample suspension was filtered and filtrate centrifuged at 4°C and 25,000 g for 30 min. The supernatant was brought to 15% (v/v) trichloroacetic acid and agitated overnight (4°C). Protein precipitate was centrifuged at 1,700 g for 45 min (4°C) and pellets were washed with tris-buffered acetone (20% (v/v) 50 mM tris-base and acetone) three times, followed by an acetone wash. Dried-protein pellets were re-suspended in 6 M urea, 2 M thiourea and 100 mM Tris-HCl, pH 8.0. Protein concentrations were normalised following protein quantification by Bradford assay. Urea concentration was adjusted to 1 M by addition of 50 mM ammonium bicarbonate. Trypsin digestion of re-suspended proteins was carried out as described in Owens et al. (2015). Proteins were prepared for LC-MS/MS analysis as in Dolan et al. (2014) and Moloney et al. (2016).

For LC-MS/MS analysis, 1 µg of peptide mixtures was eluted onto a Q-Exactive quadrupole MS coupled to a Dionex RSLCnano (ThermoFisher Scientific). LC gradients from 3% to 45% were run over 65 min and data collected using a Top15 method for MS/MS scans. Spectra were analysed using the predicted protein databases of *E. glandulosa* HHB12029 (Nagy et al., 2016) and *M. mucida* CBS55879 (Ruiz-Dueñas et al., 2020) using MaxQuant (version 1.6.2.3) with integrated Andromeda for database searching (Cox & Mann, 2008). MaxQuant parameters are as described in Owens et al. (2015). The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository (Vizcaino et al., 2016) with the dataset identifier to be provided. Removal of irrelevant protein groups and log₂ transformation of LFQ intensities were performed in Perseus (version 1.4.1.3; Tyanova et al., 2016).

2.3 | Functional annotation and differential analysis of fungal proteomes

Functional analyses were undertaken in Blast2GO Pro 5.2.4 (B2G; Conesa et al., 2005; Gotz et al., 2008). Peptide sequences were subjected to the complete Gene Ontology (GO) pipeline and annotated using both the NCBI *nr* fungi database using BLASTp [BLAST expectation (*E*) value 1.0⁻³] and the EMBL-EBI InterPro database (Jones et al., 2014). Results were mapped to the GO Association and UniProt ID-Mapping service (GO annotation database version: 2018.09; 57,58). NCBI *nr* and InterPro results were merged using the Annotation module (Gotz et al., 2008), run with the parameters: annotation cut-off 55; GO weight 5; BLAST *E*-value filter 1.0⁻⁶; HSP-Hit coverage cut-off 0 and hit filter 500.

2.4 | Untargeted GC-MS metabolomics

One-third of the frozen woodblock sections from each treatment group was each weighed and manually chipped, added to ≤10 ml

acetonitrile:methanol:H₂O (2:2:1) and shaken at 180 rpm at room temperature for 1 hr. Extracts were filtered through glass wool and Whatman filter paper (no. 1) and centrifuged at 3,500 g for 30 min. The supernatant was dried in vacuo (Eppendorf) overnight.

Dried samples were derivatised by addition of 30 µl methoxylamine hydrochloride (15 mg/ml in pyridine; Sigma) followed by incubation at 60°C for 90 min. Subsequently, 50 µl MSTFA+1% (v/v) TMCS (Thermo Scientific) was added to the sample and incubated at 40°C for 60 min. Derivatised samples were transferred to autosampler vials and 10 µl tetracosane (5 mg/ml in heptane; Sigma-Aldrich) added as an internal standard.

Samples (1 µl) were injected onto a 5,975 gas chromatographer (GC) with inert MS detector, 5% Phenyl Methyl Siloxane column (30 m × 250 µm × 0.25 µm) and a helium carrier gas flow rate of 1 ml/min (Agilent). The injector was operated in splitless mode at 250°C and the column oven temperature was held at 60°C for 1 min before being increased at a rate of 10°C per min until 180°C and then at 4°C until reaching 300°C. The mass spectrometer utilised an electron emission of 70 eV and scanned from 50 to 650 amu per scan.

GC-MS data were initially produced using MSD ChemStation software and chromatograms imported into AMDIS for deconvolution and manual comparison of deconvoluted spectra with the NIST 2011 (NIST, 2017) and the GOLM Metabolome Database (GMD) mass spectral reference libraries (41; Downloaded 13th September 2018). Putative identifications were based on matches of mass spectra (≥80% similarity) and visual inspection of matched spectra in NIST MS Search 2.0 in line with Level 2 of the Metabolomics Standards Initiative (Sumner et al., 2007).

Data were further pre-processed using SpectConnect (Styczynski et al., 2007) with elution threshold set to 1 min and support threshold 50%. These conditions were chosen as the most conservative level able to align the tetracosane peaks across chromatograms. The integrated signal of each peak was normalised to that of tetracosane and to the initial weight of the wood block chips. Common contaminants of GC-MS datasets (e.g. disiloxanes) were removed from the datasets along with mass spectra that comprised <6 ion peaks.

2.5 | Statistical analysis of fungal proteomes and metabolomes

Data were analysed using MetaboAnalyst 4.0 following normalisation by median, g-log transformation and mean centring. Where multiple derivatisation products or peptides resulted in several peaks or sequences being detected for the same metabolite or protein, intensity values were combined additively for each sample to give total molecule concentration. Partial least squares discrimination analysis (PLS-DA) was used to visualise whether temperature treatments influenced proteomes and metabolomes within acceptable confidence limits (95%). The predictive accuracy of the model was assessed by cross-validation and inspection of Q^2 values, with values approaching 1 being taken as an indication of strong predictive accuracy and negative values as an indication of over-fitted models, indicating a

high probability that separation of samples in the two-dimensional PLS-DA scores plot arose by chance.

Compounds that contributed most to the separation of samples into experimental treatment groups were identified by variable importance in projection (VIP) analysis. All compounds with VIP >1.0 were deemed 'important' compounds and, therefore, were considered to differ in concentration across the three treatment conditions.

Data matrices were subsequently split into two experiments—one comprising *M. mucida*-colonised samples and the other comprising *E. glandulosa*-colonised samples—and the effect of variable versus stable temperature on the organism's metabolome and proteome was assessed using two-factor between-subjects ANOVA with a 5% Benjamini-Hochberg false discovery rate (FDR) correction for multiple comparisons.

3 | RESULTS

3.1 | Cycling temperature drives variation in the metabolism of two wood decaying fungi

We observed greater variation in both the metabolome and proteome of *E. glandulosa* under cycling temperature than under stable temperature. *M. mucida* produced a more variable proteome under cycling temperature but a less varied metabolome (Figure 1).

In the metabolomics study, a total of 540 GC-MS peaks conserved in at least two replicates under each treatment were detected across the two experiments (*M. mucida* and *E. glandulosa*). Of these we were able to assign putative identifications to 55 metabolites linked to carbohydrate metabolism, decomposition of wood and wood products, fatty acid metabolism, amino acid metabolism and secondary metabolic processes (Tables S1 and S2). In the proteomics study, we identified the sequences of 447 conserved proteins present in at least two replicates under each treatment condition (Table S3).

Metabolomics samples could be separated into temperature treatments by PLS-DA analysis based on the variation in the metabolome of both species (Figure 1). The primary axis, which in PLS-DA describes between-group variation, showed separation of samples into their temperature treatment groups and explained 50.8% of the metabolic variation. Component 2, which describes within-treatment variation, explained a further 7.4% of the total observed variation (Full model = 3 components; $R^2 = 1.0$; $Q^2 = 0.90$; Accuracy = 0.75; Figure 1).

The identity and concentration of 178 metabolites contributed to the separation of samples into their treatment groups. In the top 50 were the putatively identified metabolites xylobiose, sorbitol, erythritol, erythrose, benzaldehyde, heneicosylic acid, ethyl galate, glucuronolactone and malonic acid (VIP score >1.0; Table S4). The greatest degree of variation in the metabolome was explained by variation in the concentration of unidentified compound T98 but xylobiose, sorbitol and erythritol also ranked very highly (Table S3). The *E. glandulosa* metabolome was subject to greater within-treatment

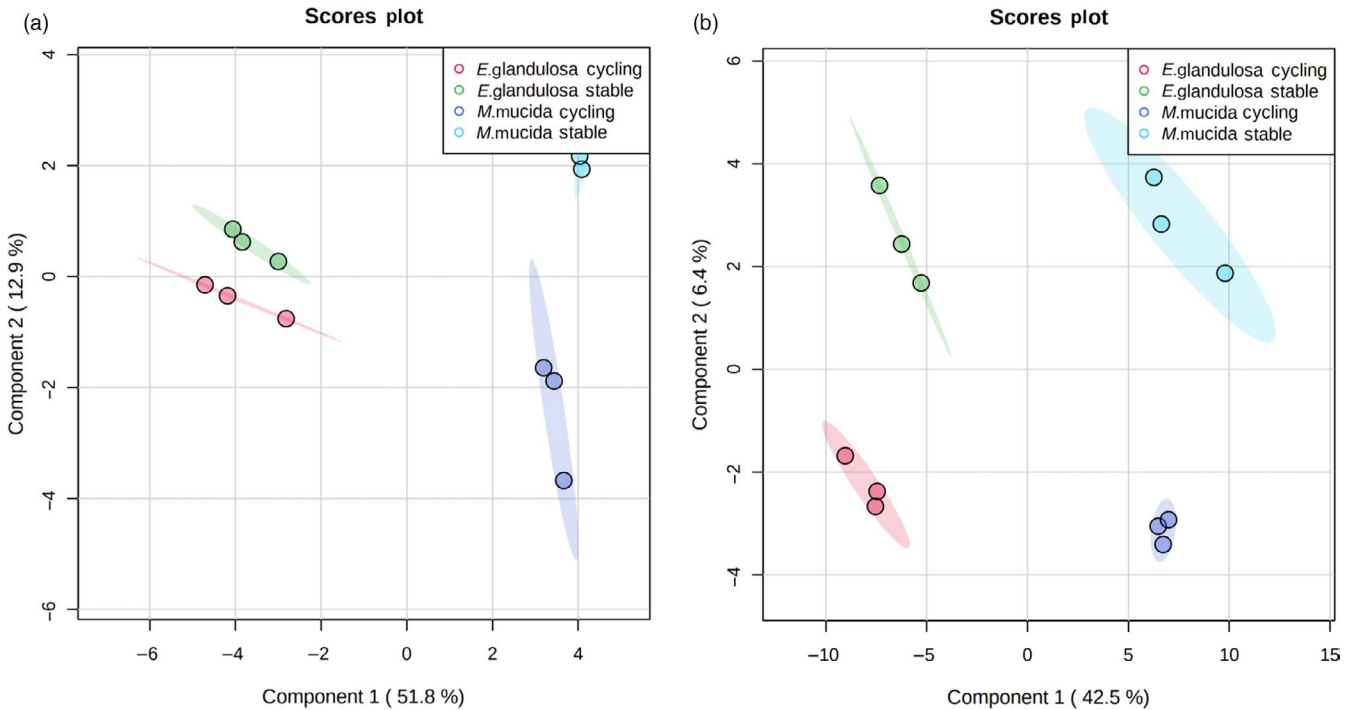


FIGURE 1 Partial least squares-discrimination analysis (PLS-DA) comparing the full quantified (a) proteome and (b) metabolome. Metabolomes of *Exidia glandulosa* under stable (red) and variable (green) and *Oudemansiella mucida* under stable (dark blue) and variable (light blue) temperature. Clouds represent 95% confidence intervals

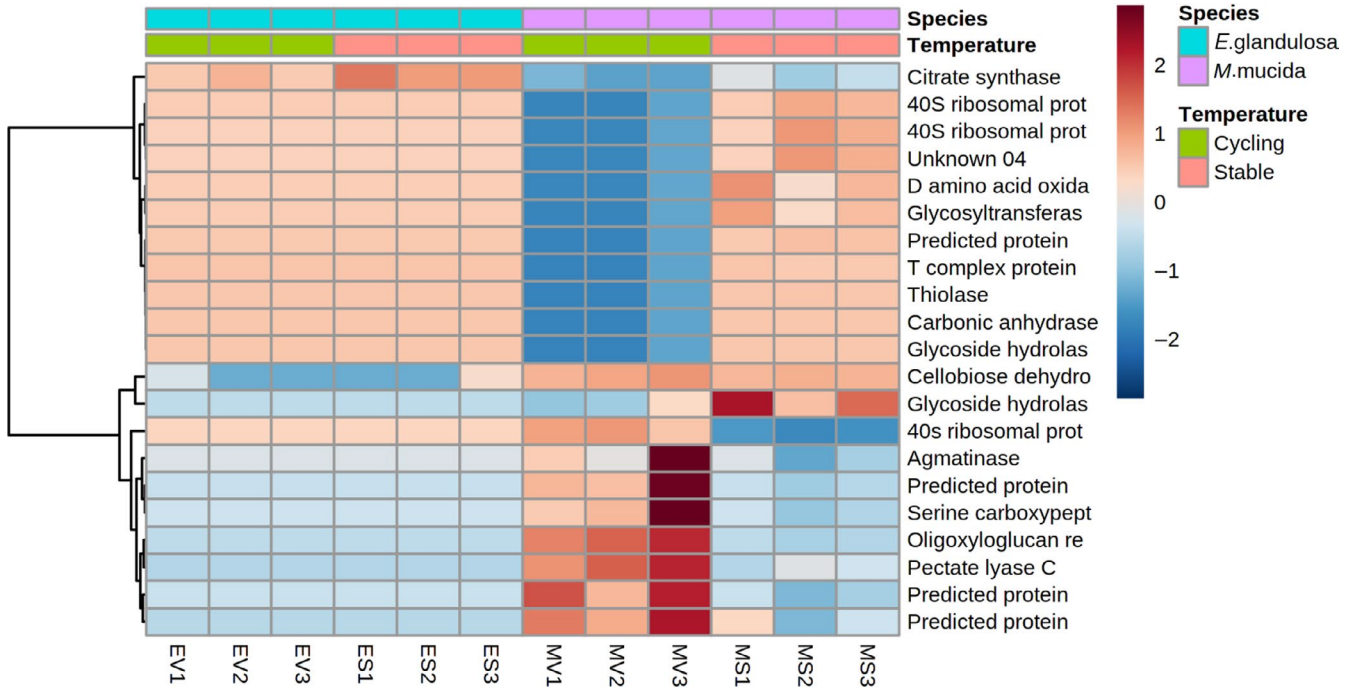


FIGURE 2 Heatmap of proteins produced by *Exidia glandulosa* and *Oudemansiella mucida* found to have been significantly altered by cycling temperature of two-way between-subjects ANOVA

variation compared with that of *M. mucida* under both temperature treatments, as shown by tighter clustering along the secondary PLS-DA axis.

Proteomics samples were separated into treatment groups based on the identity and abundance of proteins produced by *M.*

mucida and *E. glandulosa* in the experiment (Figure 1). The most accurate model comprised two components ($R^2 = 0.98$; $Q^2 = 0.74$; Accuracy = 0.50), with between-treatment differences explaining 72.7% of variation and within-treatment variation explaining a further 6.2%.

FIGURE 3 (a) Heatmap of top 50 metabolites produced by *Exidia glandulosa* and *Oudemansiella mucida* found to have been significantly altered by cycling temperature of two-way between subjects ANOVA; (b) Venn diagram showing the results of two-way between subjects ANOVA on concentration levels of all metabolites produced by *E. glandulosa* and *Oudemansiella mucida* under stable and variable temperature

VIP analysis showed that the abundance of 118 proteins contributed to the separation of samples into treatment groups in the model (Table S5). Of these, a transaldolase contributed most to sample separation, followed by an unknown protein, ketol acid reductoisomerase, methionine adenosyltransferase, a thiamine diphosphate binding protein and a heat shock cognate 70 protein. In all, 14 of the 15 most important proteins in driving samples into their treatment groups were present in higher concentration in samples colonised by *E. glandulosa* than in those colonised by *M. mucida*. Transaldolase was present in higher concentration under variable temperature in *E. glandulosa*-colonised wood and in lower concentration under variable temperature in *M. mucida*-colonised wood compared with stable temperature treatments. An aldo/keto reductase was the only protein in the top 15 most important proteins for sample clustering that was present in high concentration in wood colonised by *M. mucida* compared with *E. glandulosa*.

3.2 | Cycling temperature inhibits several major metabolic pathways in *E. glandulosa* but drives their over-expression in *M. mucida*

We employed separate two-way ANOVAs to compare the (a) proteome and (b) metabolome of our two wood decaying fungi under stable and diurnally cycling temperature treatments and observed resulting significant differences in the metabolism of both species (Figures 2 and 3; Figures S7 and S9; Tables S6 and S8). We then took the putative proteins and metabolites significantly altered by temperature in the experiments, along with those identified in VIP analysis, and calculated effect sizes (Cohen's *d*) elicited upon their abundance by cycling temperature for each species (Figure 4; Table S1). We treated the stable temperature as the reference condition and cycling temperature as the comparator in all treatment contrasts, thereby assessing whether metabolic products were present in higher or lower concentration under cycling temperature than under stable temperature. Where products were observed in higher concentrations under cycling temperature (i.e. accumulated), we suggest that this may be linked to an upregulation of the genes controlling the pathways with which they are associated. Conversely where products were observed in lower concentrations (i.e. dissipated), this may indicate a downregulation of the genes controlling their associated pathways.

We observed the concentration of 348 metabolites to differ significantly between treatment groups. Of these, 122 were altered only by the difference in species colonising the wood and we do not consider differences in abundance of these metabolites to be of interest in testing our hypothesis. The concentration of 20 metabolites was significantly altered by the effect of temperature in

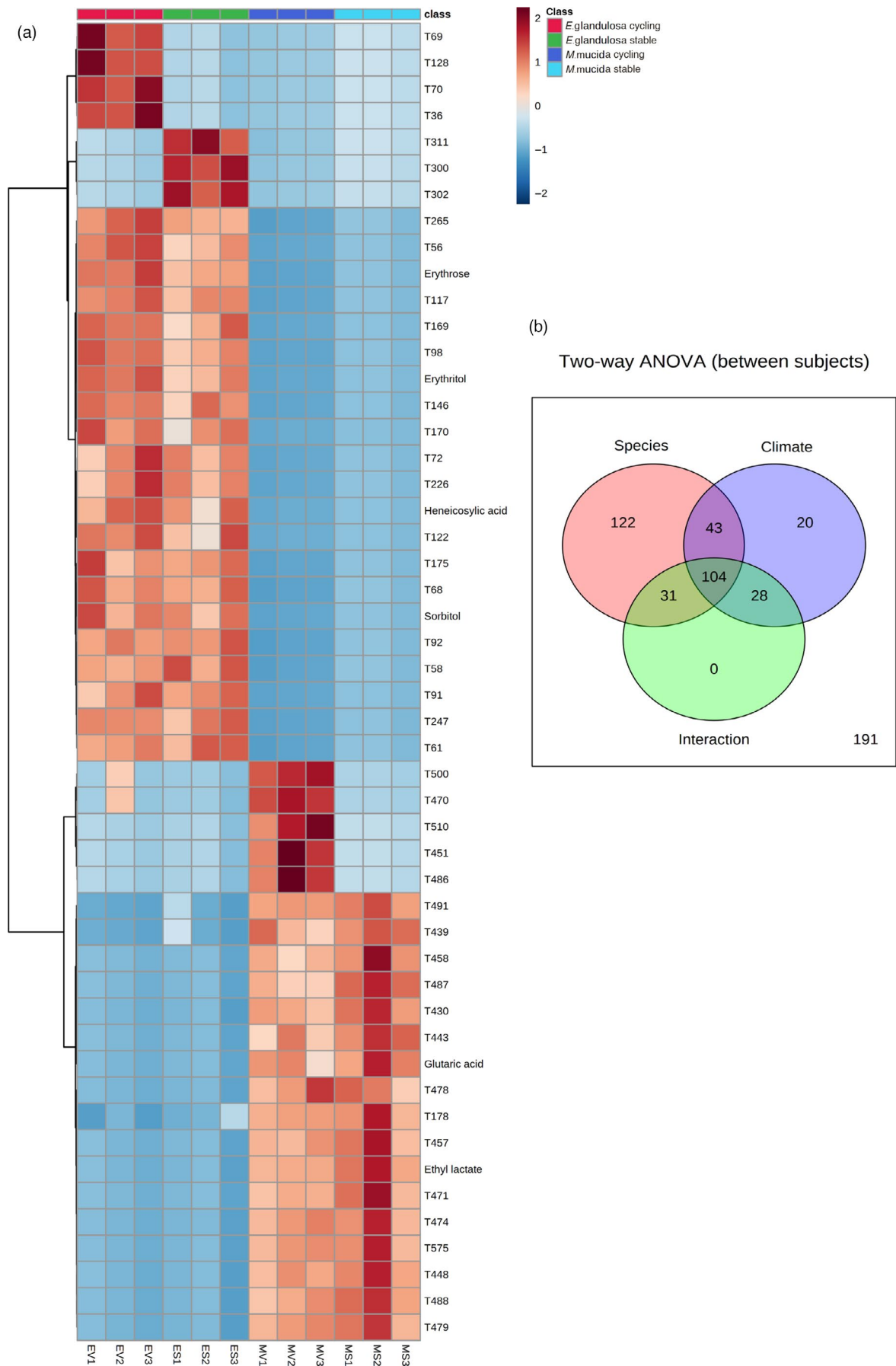
both species, including glycerol and protocatechuic acid. A further 43 metabolites were significantly altered by temperature treatment for a single species including ethyl galate, vanillic acid and xylobiose. We observed that 104 metabolites were significantly altered by species identity, temperature treatment and the interaction between the two independent variables (Table S8; Figure S8), indicating that unmeasured abiotic variables interact with temperature and species identity in driving the abundance of many metabolites. In total, 35 significantly altered metabolites were putatively identified and the majority could be linked to the citric acid cycle and glycolysis, carbohydrate metabolism, fatty acid metabolism and wood degradation (Figure 4).

We observed the abundance of 326 proteins to differ significantly between treatment groups. Of these, 302 were altered only by species identity. The abundance of two proteins, citrate synthase and an amidase signature domain-containing protein were altered by both temperature and species. In the main, protein abundance was altered by a combination of temperature, species and the interaction between the two, again indicating that unmeasured abiotic variables influence the relationship between species identity and temperature regime in determining protein abundance.

Standardised mean differences in the concentrations of metabolic products indicated that the vast majority of molecules present in *M. mucida* samples, 54 out of 65, were dissipated under cycling temperature with the remainder being accreted (Figure 4). Overall, there was net dissipation within each of the five pathways of interest in *M. mucida* samples under cycling temperature, suggesting these pathways may have been less active in this species. In *E. glandulosa* samples, cycling temperature led to accumulation of 49 molecules, dissipation of 11 molecules and no change in the remaining five. There was net-accumulation within our five pathways of interest in *E. glandulosa* samples under cycling temperature, suggesting these pathways may have been more active. In the main, cycling temperature had a greater effect on the concentration of molecules associated with these pathways than *M. mucida* and of the 65 molecules identified, 46 showed contrasting concentrations between the two species. The two species both exhibited dissipation of neotrehalose, glycerol, fumaric acid, citrate synthase, syringic acid, benzoic acid, stearic acid, pimelic acid and arachidic acid under cycling temperature. Both species accumulated pectate lyase, cellobiohydrolase, cellobiose dehydrogenase and acyl CoA dehydrogenase under cycling temperature compared with 15°C static temperature.

4 | DISCUSSION

We demonstrated that both the metabolome and proteome of two lignolytic fungi differ under a diurnally cycling temperature regime



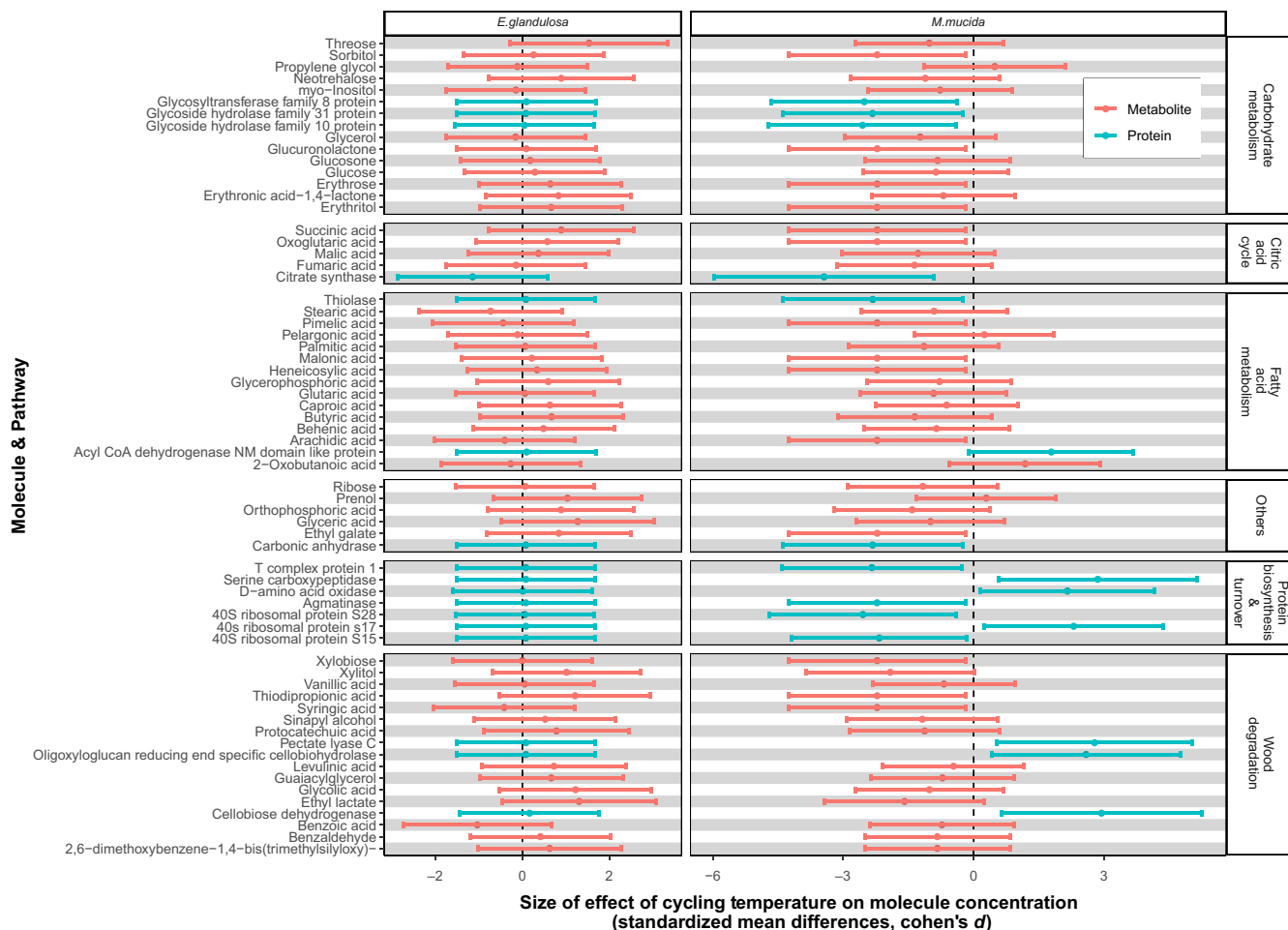


FIGURE 4 Forest plot of standardised mean differences in proteins and metabolites produced by *Mucidula mucida* and *Exidia glandulosa* found to be significantly altered by cycling temperature. Other putatively identified proteins and metabolites linked to the same pathways are also included

compared with stable conditions. In support of our hypothesis, cycling temperature elicited shifts in the profile of metabolites and proteins produced, as well as significantly altering the concentrations of a number of molecules. In our study, cycling temperature had a generally dissipating effect on metabolites and proteins that could be linked to wood decomposition, carbohydrate metabolism, the citrate cycle, fatty acid metabolism and protein turnover when produced by *M. mucida*. Conversely, cycling temperature led to the accumulation of metabolites and proteins linked to the same pathways when produced by *E. glandulosa*. Cycling temperature resulted in a more varied metabolic profile and greater effect sizes on the concentration of altered molecules for *M. mucida* than for *E. glandulosa*.

The citric acid or tricarboxylic (TCA) cycle is the most important, centrally connecting pathway for energy metabolism in living systems (Akram, 2014) which links carbohydrate, protein and lipid metabolism with energy generation in the form of adenosine triphosphate (ATP; Meléndez-Hevia et al., 1996). In aerobic organisms, ATP is usually formed through the processing of glucose via either glycolysis or the pentose phosphate pathway (Fleck et al., 2011). In addition to fuelling cellular processes through the production of ATP, upregulation of the citric acid cycle allows for the over production

and accumulation of citric acid cycle intermediaries by filamentous fungi. These organic acids are thought to contribute to a competitive advantage for filamentous fungi over other micro-organisms in the environment by lowering environmental pH (Liaud et al., 2014) as well as to their ability to buffer against environmental change (Sazanova et al., 2014). For ligninolytic fungi, organic acid production can be linked to increased productivity due to the acid-catalysed nature of the hydrolases they employ in the degradation of cellulose and hemicellulose (Shimada et al., 1997).

In our study, *M. mucida* showed depletion of glucose along with intermediates of the pentose phosphate pathway (ribose and glyceric acid) and the citric acid cycle (oxoglutaric acid, malic acid, fumaric acid, succinic acid) and one of the major enzymes regulating the citric acid cycle, citrate synthase (Akram, 2014). *M. mucida* produced each of these metabolites and proteins in lower concentrations under variable temperature than *E. glandulosa* even though, under stable temperature, the fungi did not differ at least in their production of intermediary organic acids of the citric acid cycle. *E. glandulosa* exhibited an accretion of glucose, the pentose phosphate pathway-linked compounds ribose and glyceric acid and the citric acid cycle-linked compounds oxoglutaric acid and succinic acid

under variable temperature. However, *E. glandulosa* also showed a depletion of malic acid, fumaric acid and citrate synthase under variable temperature. *E. glandulosa* produced each of these metabolites and proteins in higher concentrations than *M. mucida* under variable temperature suggesting higher levels of cellular activity and lignocellulose decomposition by *E. glandulosa* in response to diurnal temperature cycles.

Linked to the citric acid cycle are various pathways involved in the metabolism of carbohydrates, fatty acids and amino acids both for the production of glucose to fuel ATP production but also for the biosynthesis of nucleic acids, lipids and amino acids. In our study, proteins and metabolites linked to carbohydrate metabolism were depleted for *M. mucida* by variable temperature as were metabolites linked specifically to lignocellulose degradation. Similarly, with the exception of 2-oxobutonic acid, all proteins and metabolites linked to fatty acid metabolism were depleted under variable temperature for *M. mucida*. Although we observed an accretion of proteins linked to lignocellulose decomposition (an oligoxyloglucan reducing cellobiohydrolase, a pectate lyase and a cellobiose hydrolase) and of some proteins linked to protein biosynthesis and turnover (an agmatinase, 40S ribosomal protein S17 and a serine carboxypeptidase), our observations suggest that, in the main, the major metabolic pathways were downregulated for *M. mucida* as a consequence of diurnal temperature cycles.

Fatty acid biosynthesis and degradation pathways are essential for vegetative growth and development in filamentous fungi due to the high lipid content of cell membranes (Hynes et al., 2006). Lipids may also contribute to adaptation to temperature stress in basidiomycetes (Dart, 1976). The levels of structural ribosomal proteins as well as proteins involved in protein assembly, folding and proteolysis are also linked to cellular activity, growth and the ability to respond to a changing environment (An & Harper, 2020; Blazewicz et al., 2013; Efeyan et al., 2015; Saxton & Sabatini, 2017). It is possible therefore that our observations related to reduced and arrested growth rates in *M. mucida* and *E. glandulosa*, respectively. Mechanisms of 'self-restraint' that result in slower growth rates in multi-species microbial communities have been demonstrated to support coexistence (Niehaus et al., 2019). Previous work found that under variable temperature greater community richness was supported but that under these same conditions vegetative biomass was diminished (Toljander et al., 2006) and our results may go some way towards supporting these findings.

Taken together our results indicate that, for the two species examined, cycling temperature resulted in alterations to carbon processing indicating a potential linkage between fluctuating temperatures and carbon flux in wood decay systems. Each 24-hr temperature cycle consisted of 6-hr blocks where fungi were held at 5°C (25% of the time), 15°C (50% of the time) and 25°C (25% of the time) while controls were held at 15°C 100% of the time. With each 10°C increase in temperature, microbial growth rate increases due to increased enzyme activity (Laidler, 1984). We cannot rule out the possibility that the presence of higher temperatures in our cycling temperature treatment drives the responses we observed given that

we did not contrast cycling temperature with the minima and maxima. However, we would expect the equivalent ratio of time held at 5°C to counterbalance any effect of the maxima.

We observed differences in the remaining concentration of wood products such as sinapyl alcohol, one of the three lignin monolignols, suggesting differences in rate of wood decomposition. Under cycling temperature, both species exhibited accretion of several proteins utilised for degradation of pectins and cellobiose, although effect sizes were smaller for *E. glandulosa*. The concentration of various wood products and phytochemicals was present in higher concentration under cycling temperature for *E. glandulosa* and depleted for *M. mucida*. These results suggest that cycling temperature may have facilitated greater decomposition of the wood by *M. mucida* and lesser decay by *E. glandulosa*. In the woodland, wood decay rates are known to rely partially on species identity, with temperature driving the outcomes of competitive interactions between species (Hiscox et al., 2016; Wells & Boddy, 1995). Our study provides insight into some potential metabolic traits that may underpin these relationships and species' adaptations to a changing environment, which may inform future hypothesis testing.

Stable temperatures are not the norm in natural communities which are subject to diurnal and seasonal fluctuations. However, stable temperatures are often maintained under experimental conditions when the objective is to examine the impact of temperature on interaction outcomes and metabolic responses (Hiscox et al., 2016; Moody et al., 2018; O'Leary, 2018). Our study indicates that inferring real-world processes from the behaviours of experimental systems under stable laboratory conditions may not always be appropriate as those artificial conditions may produce misleading interaction outcomes and metabolic responses. Inconsistencies may be introduced by the very conditions designed to examine the mechanisms and drivers of ecosystem function in nature.

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AUTHORS' CONTRIBUTIONS

A.R., S.C.M., L.B. and D.C.E. conceived the ideas and developed the experimental design; A.R., S.C.M. and E.O. developed and applied the laboratory methodologies for data acquisition; A.R., E.O. and E.D. produced the mass spectral data; A.R. and M.S.F. analysed the data; A.R. led the writing of the manuscript. D.C.E., M.S.F., L.B., S.C.M., E.D., S.D., D.F. and E.O. contributed critically to the drafts and gave final approval for publication.

PEER REVIEW

The peer review history for this article is available at <https://publons.com/publon/10.1111/1365-2745.13716>.

DATA AVAILABILITY STATEMENT

Pre-processed proteomics and metabolomics data have been deposited to Dryad with DOI <https://doi.org/10.5061/dryad.v15dv41w3> (Rawlings et al., 2021). The mass spectrometry proteomics have been deposited to the ProteomeXchange Consortium (<http://proteomecentral.proteomexchange.org>) via the PRIDE partner repository (Vizcaino et al., 2016) with dataset identifier: PXD026003. Unprocessed mass spectrometry metabolomics data are available at the NIH Common Fund's National Metabolomics Data Repository (NMDR) website, the Metabolomics Workbench (Sud et al., 2016), <https://www.metabolomicsworkbench.org> with Study ID: ST001805 (<https://doi.org/10.21228/M8BQ3C>).

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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