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Climate change: its impacts on microbial communities and mental health

DISSERTATION

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for the degree of

DOCTOR OF PHILOSOPHY

in Biological Sciences

by

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DEDICATION

To

My husband, Jay, for his undying patience, love, and support

And my parents, Robert and Diane, for always believing in me.

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Lovero, K.G. and Treseder, K.K. 2021. Trade-offs between growth rate and other fungal traits. *Frontiers in Forests and Global Change* 4:756650. doi: 10.3389/ffgc.2021.756650

Treseder, K., Alster, C. Cat, L.A., Gorris, M., Kuhn, A., **Lovero, K.G.** et al. 2021. Nutrient and stress tolerance traits linked to fungal responses to global change: Four case studies. *Elementa: Science of the Anthropocene* 9(1): 144. https://doi.org/10.1525/elementa.2020.00144

Pratt, J., **Lovero, K.** 2020. Confronting eco-grief and eco-anxiety in the conservation classroom. North American Congress for Conservation Biology. Virtual Conference. (Interactive Session)

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ABSTRACT OF THE DISSERTATION

Climate change: its impacts on microbial communities and mental health

By

Karissa Gallego Lovero

Doctor of Philosophy in Biological Sciences

University of California, Irvine, 2022

Professor Kathleen K. Treseder, Chair

Climate change is occurring at an unprecedented rate. According to the 2021 report by the Intergovernmental Panel on Climate Change, the past five years have been the hottest on record since 1850. As a result, organisms inhabiting Earth must respond and adapt. This includes the smallest of microorganisms to human beings. To ensure that our planet remains a hospitable environment, we must improve our predictions of the impacts of climate change by furthering our knowledge of the biological processes currently in play. In turn, this information can inform policy makers of rules and regulations that must be set in place. All the while, we must address the mental health impacts that can result from studying this grim information so that we can continue to enact positive change. These topics are the focus of this dissertation.

As antibiotic resistance is expected to worsen with increasing temperatures, my first chapter elucidates the role of native aquatic bacteria in harboring and disseminating antibiotic resistance genes via mobile genetic elements. This was accomplished by collecting water from an urban environment and isolating a multidrug-resistant strain of *Aeromonas veronii*. Through plasmid DNA extraction and sequencing, I identified the extended-spectrum β-lactamase gene *bla*$_{\text{VEB-3}}$ carried on a broad-host-range and conjugative IncA/C plasmid. This highlights the

potential of aquatic *Aeromonas* in transferring antibiotic resistance genes of clinical concern to other species of bacteria.

In my second chapter, I examined trade-offs among fungal traits to improve predictions of fungal community responses to climate change. Using the Biolog Filamentous Fungi data, I calculated maximum growth rates of 37 fungal species and compared them to fungal traits from the fun^{fun} database. As a result, I identified Fast Growth, Resource Capture, and Blended life history strategies. Ultimately, this information can be incorporated into trait-based ecosystem models to link shifts in fungal communities under climate change to ecosystem feedbacks.

My third chapter investigates the mental health impacts that can result when studying climate change topics, known as eco-grief or climate anxiety. I asked how educators may confront feelings of eco-grief in the classroom to foster pro-environmental actions in students. In various biology undergraduate courses at UC Irvine, a pre-survey, eco-grief lesson, and post-survey were administered. While levels of eco-grief did not improve because of the lesson, students did report developing better coping mechanisms. In addition, attitudes and values and environmental behaviors increased from the pre- to post-survey, indicating that feelings of eco-grief did not interfere with students' ability to take action. Collectively, my dissertation addresses the impacts of climate change at various levels, provides insight towards predicting future changes, and examines methods for educating the next generation of environmentally motivated individuals.

INTRODUCTION

Due to human activity, the world as we know it is changing, and in most ways for the worse. Current global crises of concern include the development of antibiotic resistant bacteria through the misuse of antibiotics and climate change due to greenhouse gas emissions from the burning of fossil fuels. These global crises can have an impact on all living organisms, from the smallest of microorganisms to the very humans responsible for these changes, which is the focus of this dissertation.

A product of climate change is increasing temperatures, which has shown to be intimately linked to antibiotic resistance (Burnham, 2021). For example, both horizontal gene transfer and bacterial growth rates increase with increasing temperatures (Pietikäinen et al., 2005). In addition, there is evidence that bacterial infection rates are associated with increases in temperature (Eber et al., 2011; Fisman et al., 2014). The over prescription and misuse of antibiotics has led to the development of antibiotic resistance genes (ARG) in bacteria, reducing the effectiveness of available antibiotics on treating human pathogens. Furthermore, the overuse of antibiotics has increased their prevalence in freshwater environments through different sources such as agricultural runoffs, leaching from farms, and sewage discharges. This is of growing global concern, as bodies of water can serve as reservoirs selecting for the development and transmission of ARGs (Nnadozie & Odume, 2019).

In Chapter 1, I conducted a systemic screening of aquatic environments to assess the presence of ARGs and their association with mobile genetic elements that may facilitate their dissemination. The focus was on *Aeromonas* bacteria as they are ubiquitous in aquatic environments (Janda & Abbott, 2010), but less often studied as most strains are non-pathogenic. However, these bacteria can serve as reservoirs of ARGs that can be horizontally transferred to

pathogenic bacteria of clinical concern (Cattoir et al., 2008; Rhodes et al., 2000). In this study, I isolated an antibiotic-resistant strain of *Aeromonas veronii* from a freshwater suburban lake in California carrying the extended-spectrum beta-lactamase gene $bla_{\text{VEB-3}}$. To better understand the role of aquatic *Aeromonas* in harboring and dispersing ARGs, I extracted and sequenced the plasmid DNA of this strain. I then analyzed the association of mobile genetic elements with $bla_{\text{VEB-3}}$ as these may be responsible for the dissemination of this gene. Importantly, this study highlights that native bacteria such as *Aeromonas* may harbor and disperse ARGs of clinical concern to other species of bacteria with the aid of mobile genetic elements.

In Chapter 2, I investigated relationships among fungal traits to improve the understanding of how fungal communities will shift with a changing climate. Fungi perform essential ecosystem processes, such as nutrient cycling and decomposition (Dighton, 2003). To improve predictions of carbon dynamics under global change, we can link fungal traits to ecosystem processes by identifying principle microbial life history strategies, each composed of distinct functional traits (Malik et al., 2020). Specifically, I examined trade-offs among fungal traits, in which the allocation of finite resources toward one trait reduces the investment in others. I hypothesized that trade-offs among fungal traits relating to rapid growth, resource capture, and stress tolerance sort fungal species into discrete life history strategies. To test this, I compared growth rate measurements from the Biolog Filamentous Fungi database with traits from fun$^{\text{fun}}$, a fungal functional traits database. Through this, I identified trade-offs between fungal traits relating to fast growth and resource capture, sorting traits into distinct fungal life history strategies. Trait linkages such as these can be incorporated into trait-based ecosystem models to improve predictions of ecosystem function resulting from shifts in microbial communities with a changing environment (Allison, 2012; Allison & Goulden, 2017).

Furthermore, these global crises can impact both human physical and mental health (Crimmins et al., 2016; Fritze et al., 2008). Chapter 3 focuses on the phenomenon of eco-grief or eco-anxiety, defined as the psychological response associated with current and anticipated environmental losses as a result of climate change (Cunsolo et al., 2020). While grief is a rational and adaptive response, it may also present as paralyzing forms of anxiety (Pihkala, 2020). Therefore, to educate and empower the next generation of environmentally conscious students, educators must confront these feelings in the environmental classroom and foster coping mechanisms. I developed a survey tool to assess the levels of eco-grief in undergraduate students enrolled in various courses in the School of Biological Sciences at UC Irvine. I used this survey to assess how student levels of eco-grief changed after the implementation of an eco-grief classroom module. While eco-grief levels did not significantly improve after the lesson, students reported a positive shift in motivation and an increase in attitudes and values and partaking in sustainable behaviors. As climate impacts are only expected to worsen, my hope is that these results will help guide educators on how to integrate emotions into the classroom to build resilience in their students.

The world is in the midst of an anthropogenic climate crisis that both humans and microbes must respond and adapt to. Collectively, my dissertation aims to improve predictions of microbial responses under a changing climate. In addition, I provide insight on confronting the negative emotions that may result from studying climate change to ensure that future generations may continue to enact positive change.

REFERENCES

Allison, S. D. (2012). A trait-based approach for modelling microbial litter decomposition. *Ecology Letters*, *15*(9), 1058–1070. https://doi.org/10.1111/j.1461-0248.2012.01807.x

Allison, S. D., & Goulden, M. L. (2017). Consequences of drought tolerance traits for microbial decomposition in the DEMENT model. *Soil Biology and Biochemistry*, *107*, 104–113. https://doi.org/10.1016/j.soilbio.2017.01.001

Burnham, J. P. (2021). Climate change and antibiotic resistance: a deadly combination. *Therapeutic Advances in Infectious Disease*, *8*. https://doi.org/10.1177/2049936121991374

Cattoir, V., Poirel, L., Aubert, C., Soussy, C.-J., & Nordmann, P. (2008). Unexpected occurrence of plasmid-mediated quinolone resistance determinants in environmental Aeromonas spp. *Emerging Infectious Diseases*, *14*(2), 231–237. https://doi.org/10.3201/eid1402.070677

Crimmins, A., Balbus, J., Gamble, J. L., Beard, C. B., Bell, J. E., Dodgen, D., Eisen, R. J., Fann, N., Hawkins, M., Herring, S. C., Jantarasami, L., Mills, D. M., Saha, S., Sarofim, M. C., Trtanj, J., & Ziska, L. (2016). The Impacts of Climate Change on Human Health in the United States: A Scientific Assessment. *U.S. Global Change Research Program*, 312. http://dx.doi.org/doi:10.7930/J00P0WXS

Cunsolo, A., Harper, S. L., Minor, K., Hayes, K., Williams, K. G., & Howard, C. (2020). Ecological grief and anxiety: the start of a healthy response to climate change? *The Lancet. Planetary Health*, *4*(7), e261–e263. https://doi.org/10.1016/S2542-5196(20)30144-3

Dighton, J. (2003). Fungi in Ecosystem Processes. In *Fungi in ecosystem processes* (1st ed., Vol. 17). Marcel Dekker. https://doi.org/10.1201/9780824742447

Eber, M. R., Shardell, M., Schweizer, M. L., Laxminarayan, R., & Perencevich, E. N. (2011). Seasonal and temperature-associated increases in gram-negative bacterial bloodstream infections among hospitalized patients. *PLoS ONE*, *6*(9). https://doi.org/10.1371/journal.pone.0025298

Fisman, D., Patrozou, E., Carmeli, Y., Perencevich, E., Tuite, A. R., Mermel, L. A., Quirós, R. E., Vilches, V., Korman, T. M., Miyakis, S., Boutlis, C. S., Reid, A. B., Gales, A. C., Schandert, L., Affini, R., Oliveira, A. M., Marra, A. R., Camargo, L. F. A., Edmond, M. B., … Russo, A. J. (2014). Geographical variability in the likelihood of bloodstream infections due to gram-negative bacteria: Correlation with proximity to the equator and health care expenditure. *PLoS ONE*, *9*(12). https://doi.org/10.1371/journal.pone.0114548

Fritze, J. C., Blashki, G. A., Burke, S., & Wiseman, J. (2008). Hope, despair and transformation: Climate change and the promotion of mental health and wellbeing. *International Journal of Mental Health Systems*, *2*. https://doi.org/10.1186/1752-4458-2-13

Janda, J. M., & Abbott, S. L. (2010). The genus Aeromonas: taxonomy, pathogenicity, and infection. *Clinical Microbiology Reviews*, *23*(1), 35–73. https://doi.org/10.1128/CMR.00039-09

Malik, A. A., Martiny, J. B. H., Brodie, E. L., Martiny, A. C., Treseder, K. K., & Allison, S. D. (2020). Defining trait-based microbial strategies with consequences for soil carbon cycling under climate change. *ISME Journal*, *14*(1). https://doi.org/10.1038/s41396-019-0510-0

Nnadozie, C. F., & Odume, O. N. (2019). Freshwater environments as reservoirs of antibiotic resistant bacteria and their role in the dissemination of antibiotic resistance genes. In *Environmental Pollution* (Vol. 254). https://doi.org/10.1016/j.envpol.2019.113067

Pietikäinen, J., Pettersson, M., & Bååth, E. (2005). Comparison of temperature effects on soil respiration and bacterial and fungal growth rates. *FEMS Microbiology Ecology*.

https://doi.org/10.1016/j.femsec.2004.10.002
Pihkala, P. (2020). Eco-anxiety and environmental education. *Sustainability (Switzerland)*, *12*(23). https://doi.org/10.3390/su122310149
Rhodes, G., Huys, G., Swings, J., McGann, P., Hiney, M., Smith, P., & Pickup, R. W. (2000). Distribution of oxytetracycline resistance plasmids between aeromonads in hospital and aquaculture environments: implication of Tn1721 in dissemination of the tetracycline resistance determinant tet A. *Applied and Environmental Microbiology*, *66*(9), 3883–3890.

CHAPTER 1

Conjugative IncA/C plasmid carrying an extended-spectrum β–lactamase gene *bla*$_{VEB-3}$ found in an environmental *Aeromonas veronii* from California, United States

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ABSTRACT

Bodies of water harbor bacteria containing antibiotic resistance genes (ARGs) often associated with mobile genetic elements (MGEs). *Aeromonas* bacteria, in particular, are common in aquatic environments and may serve as a reservoir of clinically important ARGs. We isolated a multidrug-resistant strain of *Aeromonas veronii* from a freshwater suburban lake in California and found that it carried a conjugative and broad-host-range IncA/C plasmid. The plasmid was fully sequenced and assembled using short reads (Illumina) and long reads (Oxford Nanopore). This plasmid carried the extended-spectrum β-lactamase (ESBL) gene *bla*$_{VEB-3}$, which confers resistance to extended-spectrum cephalosporins. To our knowledge, this is the first report of *bla*$_{VEB-3}$ in the United States. The IncA/C plasmid transferred to a recipient *E. coli* strain, J53, via conjugation at a frequency of $3x10^{-2}$. Other ARGs found in the plasmid were *aadA2*, *sul1*, *tetD*, *dfrA12*, *mphA*, and *strB*. We performed antimicrobial susceptibility testing on the transconjugant and verified that the plasmid was multidrug-resistant. DNA sequencing indicated that the *bla*$_{VEB-3}$ gene was flanked by MGEs: IS*6100*, IS*26*, and a TnpF-like integrase. We also noted that signatures of a complex class 1 integron were present based on identification of conserved core and recombination regions. Furthermore, the average GC-content of the *bla*$_{VEB-3}$ and TnpF-like integrase region was 32.8%, in contrast to the 51.8% GC-content of the plasmid. This integrase was first identified from a clinical *bla*$_{VEB-3}$-carrying *Acinetobacter pittii* in Taiwan. As low GC-content is characteristic of *Acinetobacter*, this region in *Aeromonas veronii* may have originated

from *Acinetobacter*. We found further evidence that these regions were mobilized when we performed a BLASTn search of $bla_{\text{VEB-3}}$ from our isolate. The sequence matched those of environmental and clinical isolates with the same flanking regions from throughout the world. Native bacteria such as *A. veronii* may serve as reservoirs of broad-host-range multidrug-resistant plasmids that can disperse to pathogenic bacteria with a high conjugation rate.

INTRODUCTION

Antibiotic-resistant bacteria are present in the environment and serve as reservoirs of antibiotic resistance genes (ARGs; Baquero et al., 2008; Duarte et al., 2019; Kümmerer, 2009). Bacteria within the genus *Aeromonas* inhabit a wide variety of environments, but are most commonly present in aquatic ecosystems (Janda & Abbott, 2010). One of the most prevalent plasmid incompatibility types within this genus is IncA/C (Piotrowska & Popowska, 2015). These plasmids are conjugative and can associate with a broad range of hosts, facilitating the transfer between distantly-related bacteria (Piotrowska & Popowska, 2015).

Although few *Aeromonas* species serve as opportunistic pathogens, this genus is still of concern, as their resistance plasmids may transfer to bacteria of clinical significance and vice versa (Cattoir et al., 2008; Rhodes et al., 2000; Schmidt et al., 2001). Furthermore, ARGs within *Aeromonas* plasmids may be associated with mobile genetic elements (MGEs), such as transposons, insertion sequences, and integrons (Piotrowska & Popowska, 2015). MGEs play a significant role in the movement of resistance genes to other bacteria. In this way, aquatic bacteria like *Aeromonas* can spread multidrug resistance from the environment to the clinic (Lupo et al., 2012; Marti et al., 2014).

We focused on plasmid-borne genes that confer resistance to the clinically important β-lactam antibiotics, since they are of considerable health concern. Third-generation

cephalosporins were introduced in the 1980s to combat the wide spread issue of β-lactamase mediated resistance. Soon after, resistant bacterial strains with extended-spectrum β-lactamases (ESBLs) were discovered. ESBLs hydrolyze extended-spectrum cephalosporins, in addition to other antibiotics including monobactams and penicillins (Bradford, 2001; Paterson & Bonomo, 2005). The molecular class A Vietnamese ESBL (VEB) is increasing in prevalence. It was first identified in an *Escherichia coli* infecting a Vietnamese patient, and it has important functional similarities to other ESBLs commonly found in *Enterobacteriaceae* (Poirel et al., 1999). The VEB-3 variant was subsequently identified in 2003 from an *Enterobacter cloacae* isolated from a hospital patient in China and found to be located within an integron (Jiang et al., 2005).

Through a systematic screening of aquatic environments to assess the presence of antibiotic-resistant bacteria, we identified an IncA/C plasmid carrying the VEB-3 ESBL in an *Aeromonas veronii* isolate collected from a freshwater lake in Chino Hills, California. Non-pathogenic *Aeromonas* are less frequently studied, yet are still important as they may serve as reservoirs of ARGs. Accordingly, we asked whether the association of MGEs with ARGs in aquatic *Aeromonas* may facilitate the spread of resistance genes to bacteria of clinical concern. To address this question, first we described at the molecular level the fully sequenced IncA/C plasmid. Next, we examined the association of $bla_{\text{VEB-3}}$ with nearby integrons and insertion sequences by analyzing the genetic neighborhood and comparing it to previously sequenced bacteria in the NCBI GenBank. The presence of this ESBL on a broad-host-range and conjugative plasmid highlights the potential for environmental *Aeromonas* to harbor and disseminate ARGs, expediting the development of resistance and worsening the antibiotic resistance crisis.

MATERIALS AND METHODS

Collection and identification. We collected water samples from a freshwater lake in English Springs Park of Chino Hills (33°59'42.4"N 117°45'20.8"W), California on June 30th, 2015. To select for ESBL-carrying bacteria, 300 mL of water was filtered using a vacuum manifold system, with 0.45 um filter (GN-6 Pall, Michigan) placed on CHROMagar Orientation media (CHROMagar, Paris, France) containing 4 ug/mL of cefotaxime (Sigma-Aldrich), a 3rd generation cephalosporin, and supplemented with 5 g/L bile salts (Sigma-Aldrich, St. Louis, MO) and 10 g/L α-lactose monohydrate (Sigma-Aldrich). A purple isolate, labeled SW3814, was identified using matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry (Bruker, Billerica, MA).

Antimicrobial susceptibility testing. Antibiotic resistance and ESBL screening was performed for SW3814, transconjugants, and J53 using the disk diffusion test following the Clinical and Laboratory Standards Institute (CLSI) guidelines (Patel et al., 2014). We then determined minimum inhibitory concentrations (MIC) by the broth microdilution method for SW3814, transconjugants, and J53 following the CLSI guidelines (CLSI, 2012). ESBL determination was performed according to the CLSI phenotypic confirmatory test using the broth microdilution method (Patel et al., 2014). All interpretations were based on the 2014 CLSI breakpoints using *E. coli* ATCC25922 as a quality control (Patel et al., 2014).

Conjugation. To determine whether SW3814 carried a plasmid with functional conjugative machinery, we performed mating experiments between SW3814 and a recipient strain J53 (F− *met pro* Azi^{r}), an *E. coli* K-12 derivative (Jacoby & Han, 1996). Briefly, SW3814 and J53 cells were pelleted and mixed on a surface of tryptic soy agar (Becton, Dickinson, and Co., Franklin Lakes, NJ) and incubated for five hours at 35°C. The cells were resuspended in 0.9% saline solution and spread onto Mueller Hinton II Agar (Becton, Dickinson, and Co., Franklin Lakes,

NJ) containing 200 ug/mL of sodium azide (Fisher Scientific, Waltham, MA) with 20 ug/mL trimethoprim (MP Biomedicals). Trimethoprim was chosen based on the antibiotic resistance profile of SW3814, with the resistance gene presumably carried on the plasmid. Putative transconjugants were labeled as SW3814-J53-TMP. The frequency of conjugation was calculated as the CFU/mL of transconjugants divided by the CFU/mL of J53.

To verify whether transconjugants obtained were in fact J53 carrying a plasmid from SW3814 and not SW3814 mutated to become sodium azide resistant, the following steps were taken. First, putative transconjugants were streaked onto M9 minimal media, with a lack of growth indicating the presence of J53, as these are known auxotrophs while SW3814 is not. Second, putative transconjugants were screened through PCR for the insertion sequence IS*5* within the gene *orf*264, which is unique to K-12 derivative cells. The primers K12IS-F (CGCGATGGAAGATGCTCTGTA)/K12-R (ATCCTGCGCACCAATCAACAA) and K12-F (TTCCCACGGACATGAAGACTACA)/K12-R were used, giving amplicons of 969 and 1687 bps respectively, indicating the presence of a K-12 derivative. Lastly, transconjugants were streaked on CHROMagar Orientation media, with pink colonies indicating the presence of J53 *E. coli* and purple colonies indicative of *A. veronii.*

Sequencing, assembly, and annotation. Plasmids of SW3814 were isolated using the QIAGEN Plasmid Midi Kit. Plasmid DNA was visualized by gel electrophoresis using a 0.8% agarose gel run at 130V without ethidium bromide for five hours, followed by staining in ethidium bromide for 15 minutes. Plasmid DNA was submitted to the Center for Computational and Integrative Biology DNA Core (Massachusetts General Hospital) for Illumina next generation sequencing. Long reads were obtained using MinION and a MIN107 cell (Oxford Nanopore, UK). The plasmid was assembled using the software Geneious 11.1.5. The assembled plasmid had a mean

Illumina coverage of 235X. Initial annotations were gathered using the Rapid Annotation using Subsystem Technology (RAST) service (Aziz et al., 2008). Annotation of MGEs was accomplished using the BLAST program on the ISfinder website (Siguier, 2005). The Center for Genomic Epidemiology was used to annotate ARGs using ResFinder-2.1 (Zankari et al., 2012) and to determine plasmid incompatibility type using PlasmidFinder-2.1 (Carattoli et al., 2014). Manual curation and validation of annotations was done using the BLASTp and BLASTn tools.

Comparison to NCBI GenBank. To gain insight into the association of *bla*$_{\text{VEB-3}}$ with flanking MGEs, a BLASTn search was performed against the NCBI GenBank using the nucleotide sequence of *bla*$_{\text{VEB-3}}$ with and without the IS*6100* transposase. The transposase sequence was included as it may be responsible for the movement of the ESBL.

Nucleotide sequence accession number. The nucleotide sequence data for the IncA/C plasmid carrying *bla*$_{\text{VEB-3}}$ was submitted to the GenBank nucleotide database under the accession number MN900957.

RESULTS

The isolate SW3814 was identified as *A. culicicola*, synonymous with *A. veronii* (Huys et al., 2005) by MALDI-TOF mass spectrometry with a score of 2.319. SW3814 displayed resistance to seven classes of antibiotics: aminocyclitols, aminoglycosides, β-lactams, fluoroquinolones, sulfonamides, tetracyclines, and trimethoprim (Table 1.1). The presence of an ESBL was detected by the ESBL phenotypic confirmatory test. Gel electrophoresis indicated that SW3814 harbored 3 plasmids of 158, 3.3, and 2.6 kb, but only the 158 kb plasmid transferred to J53 (frequency: $3x10^{-2}$). Accordingly, we focused on the 158 kb plasmid.

First, we determined the resistance profile of the 158 kb plasmid by analyzing the transconjugant. SW3814-J53-TMP displayed resistance to the same classes of antibiotics as

SW3814, excluding the aminoglycosides and fluoroquinolones (Table 1.1). However, SW3814 displayed susceptibility to aztreonam, while the transconjugant was resistant. The plasmid revealed the presence of the ARGs *aadA2*, *bla*$_{\text{VEB-3}}$, *sul1*, *tetD*, *dfrA12*, *mphA*, and *strB* (Fig. 1.1). In addition, the plasmid was classified as the incompatibility type IncA/C.

Second, we identified an association between *bla*$_{\text{VEB-3}}$ and nearby MGEs. The insertion sequences IS*6100* and IS*26* were present immediately downstream of the ESBL (Fig. 1.2C). A TnpF-like integrase gene was upstream, followed by *sul1* encoding resistance to sulfonamides. This structure is indicative of an integron. Other conserved features of an integron were identified, including the core and inverse core sequences flanking *bla*$_{\text{VEB-3}}$ and a 33bp recombination crossover site (Fluit & Schmitz, 1999). A steady drop in the GC-content occurred at the 25 kb location containing *bla*$_{\text{VEB-3}}$ and the TnpF-like integrase gene (Fig. 1.1). Specifically, the GC-content was 32.8% in that location compared to 51.8% for the entire plasmid.

Next, we performed a nucleotide BLAST search with the nucleotide sequence of *bla*$_{\text{VEB-3}}$. We found matches to bacteria isolated from around the world carrying the VEB ESBL with a similar genetic neighborhood to that seen in SW3814 (Fig. 1.2). All isolates contained the core and inverse core sites flanking VEB, which are associated with 59-base elements (59-be's) (Stokes et al., 1997). This was followed by the recombination crossover site associated with common region 1 (CR1; Rodriguez-Martinez et al., 2006). Isolates A through F each contain the TnpF-like integrase immediately upstream of VEB. This integrase was first identified from isolate A, an *Acinetobacter pittii* collected from a blood sample in a Taiwanese hospital (Huang et al., 2010). The mobile element IS*6100* was immediately downstream of VEB in *A. pittii*. IS*6100* was also identified in isolates A through G. However, in some cases this gene was truncated by the presence of IS*26*, a mobile element also belonging to the IS*6* family (Mahillon

& Chandler, 1998). Either full or partial class 1 integrons were present in isolates A through C, but with differing ARGs in the cassette region. D, F, and G all correspond to aeromonads collected from the Seine River in Paris, France during an environmental survey to identify whether *Aeromonas* spp. may serve as a reservoir of ESBLs (Girlich et al., 2011). These isolates all carried VEB-1a, a variant that differs in one amino acid from VEB-3.

The *bla*$_{\text{VEB}}$-carrying bacteria were isolated from Asia, Europe, and America (Table 1.2, Fig. 1.2). In addition, the VEB ESBL was identified from diverse species collected from the environment as well as the clinic. Furthermore, the gene was found on both chromosomal and plasmid genetic locations; for example, although isolate B has highly similar regions to SW3814, the locations of these genes are on the chromosome.

DISCUSSION

Of the many studies that have examined environmentally-derived antibiotic-resistant bacteria, most have focused on phenotypes or simply identifying resistance genes, rather than a comprehensive analysis of their genetic location and environment (Kindle et al., 2019; Fernandes et al., 2019; Godinho et al., 2019). We therefore analyzed the complete sequence of a plasmid of an aquatic *A. veronii*. We asked whether the ARGs were associated with MGEs, as MGEs play a significant role in the horizontal transfer to pathogenic or opportunistic bacteria.

Specifically, we analyzed an IncA/C conjugative plasmid carrying the VEB-3 ESBL in an *A. veronii* isolate collected from a freshwater suburban lake. We found that the resistance genes *aadA2, bla*$_{\text{VEB-3}}$, *sul1*, *tetD*, and *dfrA12* were functional for both the wildtype SW3814 and transconjugant SW3814-J53-TMP. Therefore, we classified the plasmid as multidrug-resistant, following the definition given by the European Centre for Disease Prevention and Control (ECDC) and the Centers for Disease Control and Prevention (CDC; Magiorakos et al., 2012).

Although the plasmid possessed the complete nucleotide sequence of *strB,* it did not confer resistance to streptomycin. This is likely due to the absence of *strA*. Notably, *strA* and *strB* are commonly found in conjunction, and deletion of either gene dramatically decreases streptomycin resistance (Chiou & Jones, 1995; Scholz et al., 1989). Furthermore, the resistance gene *aadA2* can confer resistance to spectinomycin, and to a lesser extent, streptomycin. Both the position of this gene within the gene cassette of an integron and the gene copy number can affect streptomycin resistance levels, which may also explain the susceptibility of the transconjugant to this antibiotic (Collis & Hall, 1995).

In addition, the transconjugant SW3814-J53-TMP displayed resistance to aztreonam, while SW3814 did not (Table 1.1). In most cases, *bla*$_{\text{VEB}}$ displays the representative ESBL phenotype, conferring resistance to expanded-spectrum cephalosporins and the monobactam aztreonam (Chanawong et al., 2001; Naas et al., 1999; Poirel et al., 1999). In contrast, the only other published case of *Aeromonas* carrying *bla*$_{\text{VEB}}$ reports susceptibility to aztreonam for all species tested (Girlich et al., 2011). The genetic background in which *bla*$_{\text{VEB-3}}$ is present—within the *E. coli* transconjugant or the *A. veronii* wildtype—seems to affect the expression of the gene.

Next, we examined whether *bla*$_{\text{VEB-3}}$ was associated with any nearby MGEs. Integrons are a genetic element that can capture and incorporate ARGs into gene cassettes. Class 1 integrons consist of two conserved regions: an integrase gene *intI1* at the 5' end, and *qacE*Δ*1* and *sul1* genes at the 3'end (Collis & Hall, 1995). Cassette-associated recombination sites, referred to as 59-be's, are located downstream of a resistance gene within a cassette and are recognized by integrase (Collis & Hall, 1992; Hall et al., 1991). Although 59-be's vary in length, they contain a highly conserved 7-bp inverse core and core site at their left and right end, respectively (Hall et al., 1991). The first identification of a VEB-type ESBL was in 1996 from a 4-month-old

Vietnamese hospital patient in France (Naas et al., 2001; Poirel et al., 1999). The gene was plasmid-borne from an *E. coli* isolate (Naas et al., 2001; Poirel et al., 1999). Moreover, it was carried within the cassette region of an integron, with the 59-be, inverse core, and core regions present (Naas et al., 2001; Poirel et al., 1999). The *bla*$_{\text{VEB-3}}$ gene identified in this study also appears to be located within the cassette region of an integron, based on the presence of both an inverse core and core site. Nevertheless, a 59-be could not be identified downstream of the gene. These same features are also present in other isolates (Fig. 1.2).

Furthermore, complex class 1 integrons tend to possess a common region (CR) just downstream of the 3' conserved end (Mazel, 2006). This CR consists of *orf513* and a 33bp recombination crossover site (Mazel, 2006). In the isolates we examined, the inverse core site overlapped with the 33bp sequence corresponding to the right-hand boundary of the *CR*1 element (Fig. 1.2). This feature was also present in the first identification of *bla*$_{\text{VEB-3}}$ (Jiang et al., 2005; Rodriguez-Martinez et al., 2006). The gene IS*6100* is directly adjacent to *CR1*, leading to the hypothesis that *bla*$_{\text{VEB}}$ was initially mobilized by *CR*1 and then truncated by the insertion of IS*6100* (Toleman et al., 2006). The identification of a CR element is of interest. CRs are unique from most insertion sequences—they can transpose adjacent DNA with only a single copy of the element (Tavakoli et al., 2000). They also can mobilize any ARG, including ESBLs such as VEB (Tavakoli et al., 2000).

In addition, isolates A through F possess a Tnp-F like integrase, which is an enzyme that captures and incorporates ARGs within integrons (Fig. 1.2). This integrase was first identified from the plasmid of an *Acinetobacter pittii* isolated from a Taiwanese hospital (Fig. 1.2A; Deng et al., 2015; Huang et al., 2010). In SW3814, the percent GC-content of the region containing the TnpF-like integrase and *bla*$_{\text{VEB-3}}$ was 32.6%, which is much lower than the average of the

plasmid at 61.6%. Since *Acinetobacter* spp. can host plasmids with low GC-content, the *bla*$_{VEB-3}$ gene and surrounding genetic neighborhood on SW3814 may have originated from an isolate related to the *Acinetobacter pittii* mentioned above (Vallenet et al., 2008). *Acinetobacter* spp. are not only found in the clinic, but also widely distributed in the environment. The TnpF-like integrase may facilitate the movement of the VEB ESBL between environmental and clinical isolates (Doughari et al., 2011).

To our knowledge, ours is the first report of *bla*$_{VEB-3}$ in the United States (Huang et al., 2010; Jiang et al., 2005). Currently, the NCBI GenBank contains only four identifications of VEB-3. The gene occurs in *Acinetobacter pittii* (GQ926879) from Taiwan, and *Enterobacter cloaecae* (AY536519.1), *Klebsiella pneumonia* (CP006657), and *Aeromonas aquatica* (CP018201.1) from China. Although *bla*$_{VEB}$ is more rare than other clinical ESBLs, its prevalence is increasing (Naas et al., 2008). Its distribution is expanding, both in terms of geography and host species (Naas et al., 2008). Furthermore, the presence of this gene on either the plasmid or chromosome of an isolate, while still retaining features of an integron via the inverse core and core regions, displays high promiscuity and mobilization of the VEB-encoding region.

In conclusion, we identified the clinically relevant ESBL *bla*$_{VEB-3}$ in an aquatic *A. veronii*. The presence of this ESBL on a broad-host-range and conjugative plasmid highlights the potential for environmental *Aeromonas* to harbor and disseminate ARGs. Furthermore, *bla*$_{VEB-3}$ was associated with MGEs sharing homologous regions with pathogenic bacteria from throughout the world. Therefore, native aquatic bacteria such as *Aeromonas* may expedite the development of antibiotic resistance.

Table 1.1 Resistance profile of wild type SW3814, transconjugant SW3814-J53-TMP, and plasmid-free recipient J53.

Antimicrobial agent	Minimum Inhibitory Concentration (μg/ml)[a]		
	A. veronii SW3814	*E. coli* SW3814-J53	*E. coli* J53
Ampicillin	64 (R)	256 (R)	8 (R)
Cefazolin	32 (R)	32 (R)	2 (S)
Cefoxitin	0.25 (S)	4 (S)	4 (S)
Cefotaxime	16 (R)	16 (R)	≤0.032 (S)
Cefotaxime + clavulanic acid[b]	≤0.032	≤0.032	≤0.032
Cefepime	2 (S)	0.5 (S)	≤0.032 (S)
Aztreonam	1 (S)	16 (R)	≤0.032 (S)
Non-β-lactams to which isolate was resistant	streptomycin[c], spectinomycin[d], tetracycline, nalidixic acid, sulfamethoxazole, trimethoprim	spectinomycin, tetracycline, sulfamethoxazole, trimethoprim	

[a](R) resistant; (S) susceptible

[b] Clavulanic acid concentration was held constant at 4 ug/ml.

[c] As there are no MIC interpretive standards for streptomycin, resistance was determined by the disk diffusion test.

[d]CLSI interpretive standards for *Neisseria gonorrhoeae* were used for spectinomycin.

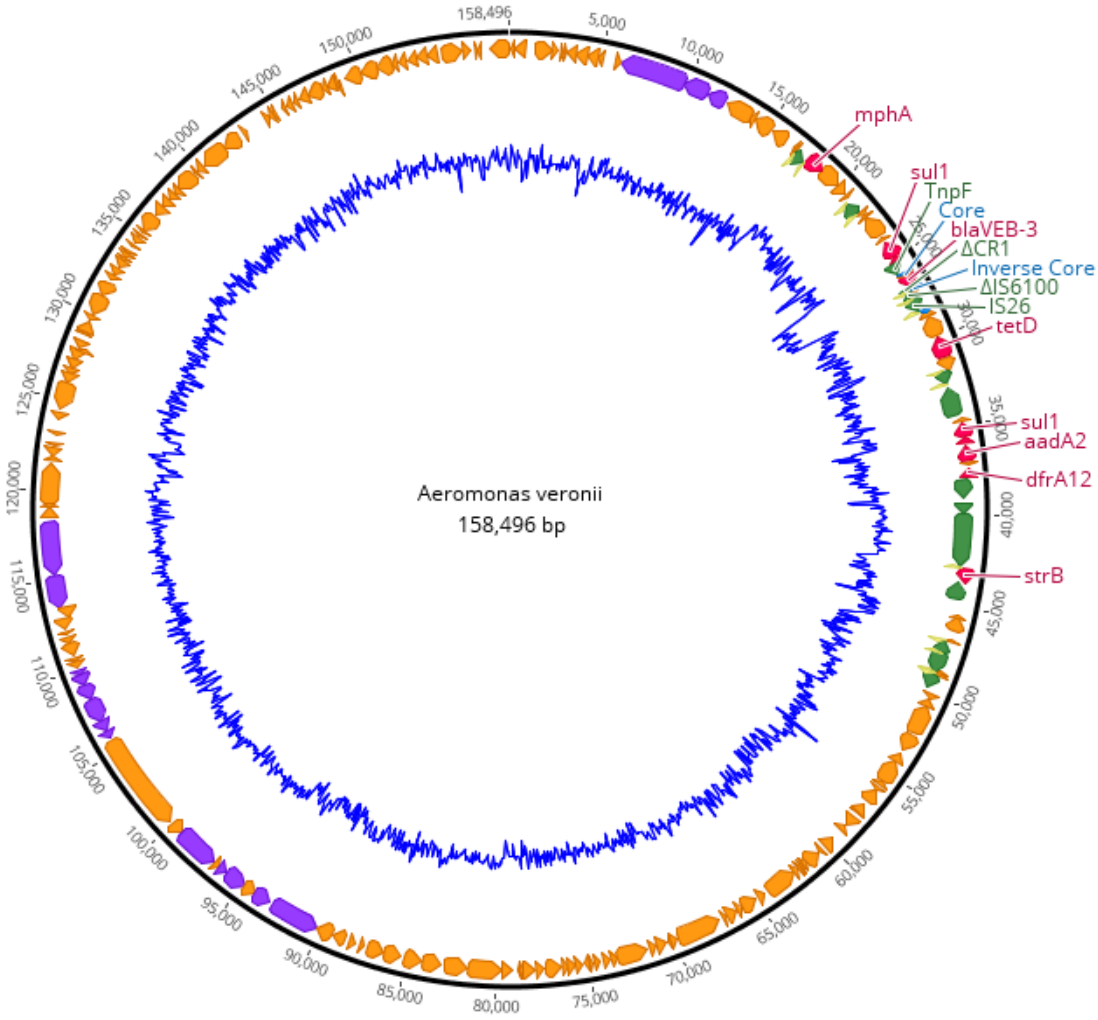

Figure 1.1. Genetic map of the158 kb plasmid of SW3814, displayed in the outermost ring. Open reading frames are represented by arrows in the direction of transcription. They are color-coded according to their putative functions: purple, conjugation machinery; red, antibiotic resistance genes; green, mobile elements; yellow, inverted repeats; orange, all other coding sequences. The inner blue ring displays the percent GC-content of the plasmid. Genes of interest are labeled. This figure was created using Geneious 11.1.5 (https://www.geneious.com).

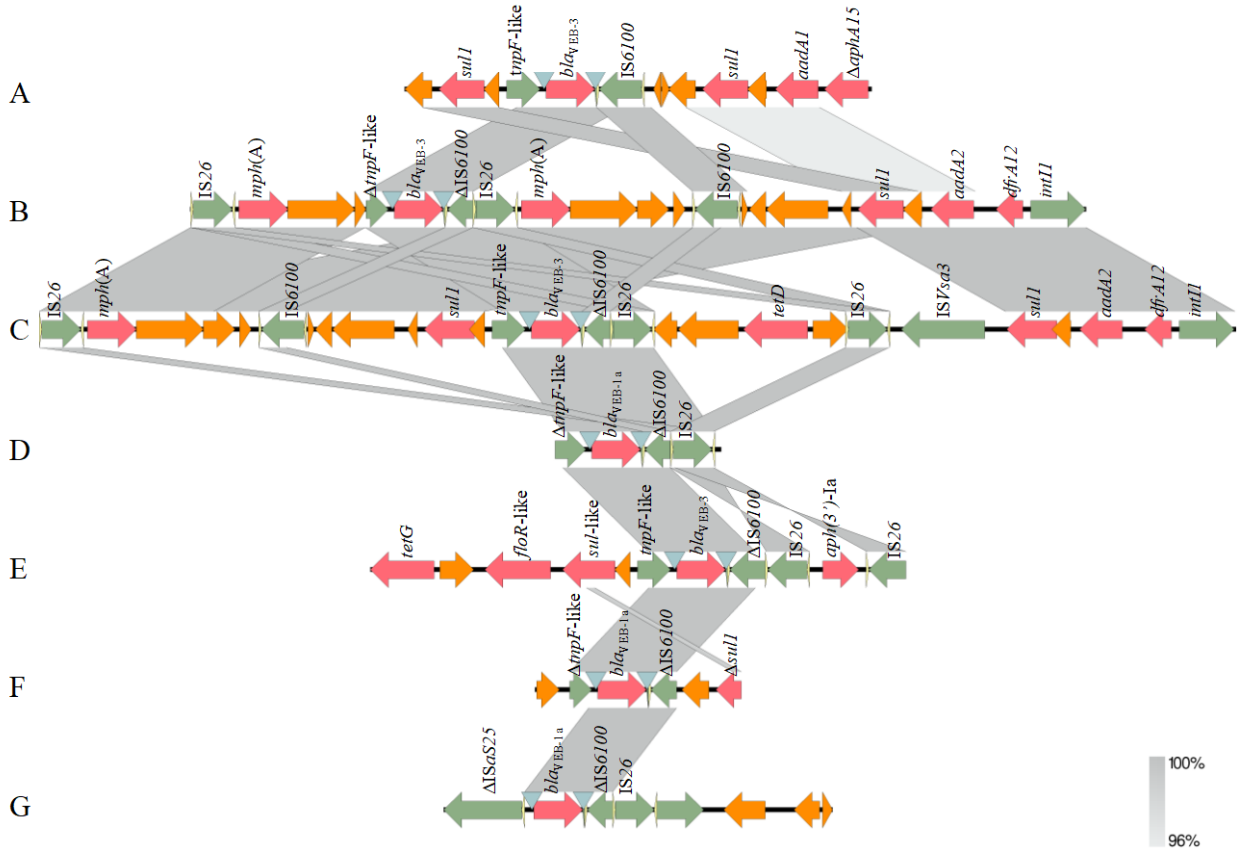

Figure 1.2. Schematic representation of isolates from the NCBI GenBank containing the *bla*$_{VEB}$ gene with regions homologous to those found in p3814 (C). Open reading frames are represented by arrows in the direction of transcription. They are color-coded according to their putative functions: red, antibiotic resistance genes; green, mobile elements; yellow, inverted repeats; orange, all other coding sequences. Blue triangles represent the recombination core and inverse core sites. The percent identity between adjacent sequences is shown as gray shading. Δ indicates the truncation of a gene. Descriptions of isolates A through G are listed in Table 1.2. This figure was created using EasyFig.

Table 1.2. Description of isolates listed in Figure 1.2.

	Accession Number	Species	Country	Isolation Source	Collection Date	Genetic Location
A	GQ926879.1	*Acinetobacter pittii*	Taiwan	Blood of hospital patient	1999-2007[a]	Plasmid
B	CP018201.1	*Aeromonas aquatica*	China	Water	2012	Chromosome
C	XXX	*Aeromonas veronii*	USA	Lake in recreational park	2015	Plasmid
D	HM370390.1	*Aeromonas caviae*	France	Seine river	2009	Chromosome
E	CP006657.1	*Klebsiella pneumonia*	China	Blood of hospital patient	2010	Plasmid
F	HM370392.1	*Aeromonas allosaccharophila*	France	Seine river	2009	Plasmid
G	HM370391.1	*Aeromonas veronii*	France	Seine river	2009	Plasmid

[a]Clinical isolates were collected from three hospitals in Taiwan from 1999 to 2007

REFERENCES

Aziz, R. K., Bartels, D., Best, A., DeJongh, M., Disz, T., Edwards, R. A., … Zagnitko, O. (2008). The RAST Server: Rapid annotations using subsystems technology. *BMC Genomics*. https://doi.org/10.1186/1471-2164-9-75

Baquero, F., Martínez, J.-L., & Cantón, R. (2008). Antibiotics and antibiotic resistance in water environments. *Current Opinion in Biotechnology*, *19*(3), 260–265. https://doi.org/10.1016/J.COPBIO.2008.05.006

Bradford, P. A. (2001). Extended-spectrum beta-lactamases in the 21st century: characterization, epidemiology, and detection of this important resistance threat. *Clinical Microbiology Reviews*, *14*(4), 933–51, table of contents. https://doi.org/10.1128/CMR.14.4.933-951.2001

Carattoli, A., Zankari, E., Garciá-Fernández, A., Larsen, M. V., Lund, O., Villa, L., … Hasman, H. (2014). In Silico detection and typing of plasmids using plasmidfinder and plasmid multilocus sequence typing. *Antimicrobial Agents and Chemotherapy*. https://doi.org/10.1128/AAC.02412-14

Cattoir, V., Poirel, L., Aubert, C., Soussy, C.-J., & Nordmann, P. (2008). Unexpected occurrence of plasmid-mediated quinolone resistance determinants in environmental *Aeromonas* spp. *Emerging Infectious Diseases*, *14*(2), 231–237. https://doi.org/10.3201/eid1402.070677

Chanawong, A., M'Zali, F. H., Heritage, J., Lulitanond, A., & Hawkey, P. M. (2001). SHV-12, SHV-5, SHV-2a and VEB-1 extended-spectrum beta-lactamases in Gram-negative bacteria isolated in a university hospital in Thailand. *The Journal of Antimicrobial Chemotherapy*, *48*(6), 839–852.

Chiou, C. S., & Jones, A. L. (1995). Expression and identification of the strA-strB gene pair from streptomycin-resistant *Erwinia amylovora*. *Gene*, *152*(1), 47–51.

CLSI. (2012). Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; Approved standard - Ninth Edition. https://doi.org/10.4103/0976-237X.91790

Collis, C. M., & Hall, R. M. (1992). Site-specific deletion and rearrangement of integron insert genes catalyzed by the integron DNA integrase. *Journal of Bacteriology*, *174*(5), 1574–1585.

Collis, C. M., & Hall, R. M. (1995). Expression of antibiotic resistance genes in the integrated cassettes of integrons. *Antimicrobial Agents and Chemotherapy*, *39*(1), 155–162.

Deng, Y., Bao, X., Ji, L., Chen, L., Liu, J., Miao, J., … Yu, G. (2015). Resistance integrons: class 1, 2 and 3 integrons. *Annals of Clinical Microbiology and Antimicrobials*, *14*, 45. https://doi.org/10.1186/s12941-015-0100-6

Doughari, H. J., Ndakidemi, P. A., Human, I. S., & Benade, S. (2011). The ecology, biology and pathogenesis of *Acinetobacter* spp.: an overview. *Microbes and Environments*, *26*(2), 101–112.

Duarte, D. J., Oldenkamp, R., & Ragas, A. M. J. (2019). Modelling environmental antibiotic-resistance gene abundance: A meta-analysis. *Science of The Total Environment*, *659*, 335–341. https://doi.org/10.1016/J.SCITOTENV.2018.12.233

Fernandes, T., Vaz-Moreira, I., & Manaia, C. M. (2019). Neighbor urban wastewater treatment plants display distinct profiles of bacterial community and antibiotic resistance genes. *Environmental Science and Pollution Research*. https://doi.org/10.1007/s11356-019-04546-y

Fluit, A. C., & Schmitz, F. J. (1999). Class 1 integrons, gene cassettes, mobility, and epidemiology. *European Journal of Clinical Microbiology & Infectious Diseases : Official*

Publication of the European Society of Clinical Microbiology, *18*(11), 761–770.
Girlich, D., Poirel, L., & Nordmann, P. (2011). Diversity of clavulanic acid-inhibited extended-spectrum beta-lactamases in *Aeromonas* spp. from the Seine River, Paris, France. *Antimicrobial Agents and Chemotherapy*, *55*(3), 1256–1261. https://doi.org/10.1128/AAC.00921-10
Godinho, O., Calisto, R., Øvreås, L., Quinteira, S., & Lage, O. M. (2019). Antibiotic susceptibility of marine Planctomycetes. *Antonie van Leeuwenhoek*. https://doi.org/10.1007/s10482-019-01259-7
Hall, R. M., Brookes, D. E., & Stokes, H. W. (1991). Site-specific insertion of genes into integrons: role of the 59-base element and determination of the recombination cross-over point. *Molecular Microbiology*, *5*(8), 1941–1959.
Huang, L.-Y., Lu, P.-L., Chen, T.-L., Chang, F.-Y., Fung, C.-P., & Siu, L. K. (2010). Molecular characterization of beta-lactamase genes and their genetic structures in *Acinetobacter* genospecies 3 isolates in Taiwan. *Antimicrobial Agents and Chemotherapy*, *54*(6), 2699–2703. https://doi.org/10.1128/AAC.01624-09
Huys, G., Cnockaert, M., & Swings, J. (2005). *Aeromonas culicicola* Pidiyar et al. 2002 is a later subjective synonym of *Aeromonas veronii* Hickman-Brenner et al. 1987. *Systematic and Applied Microbiology*, *28*(7), 604–609. https://doi.org/10.1016/j.syapm.2005.03.012
Jacoby, G. A., & Han, P. (1996). Detection of extended-spectrum beta-lactamases in clinical isolates of *Klebsiella pneumoniae* and *Escherichia coli*. *Journal of Clinical Microbiology*, *34*(4), 908–911.
Janda, J. M., & Abbott, S. L. (2010). The genus *Aeromonas*: taxonomy, pathogenicity, and infection. *Clinical Microbiology Reviews*, *23*(1), 35–73. https://doi.org/10.1128/CMR.00039-09
Jiang, X., Ni, Y., Jiang, Y., Yuan, F., Han, L., Li, M., … Lu, Y. (2005). Outbreak of infection caused by *Enterobacter cloacae* producing the novel VEB-3 beta-lactamase in China. *Journal of Clinical Microbiology*, *43*(2), 826–831. https://doi.org/10.1128/JCM.43.2.826-831.2005
Kindle, P., Zurfluh, K., Nuesch-Inderbinen, M., von Ah, S., Sidler, X., Stephan, R., & Kummerlen, D. (2019). Phenotypic and genotypic characteristics of *Escherichia coli* with non-susceptibility to quinolones isolated from environmental samples on pig farms. *Porcine Health Management*, 5, 9. https://doi.org/10.1186/s40813-019-0116-y
Kümmerer, K. (2009). Antibiotics in the aquatic environment – A review – Part II. *Chemosphere*, *75*(4), 435–441. https://doi.org/10.1016/J.CHEMOSPHERE.2008.12.006
Lupo, A., Coyne, S., & Berendonk, T. U. (2012). Origin and evolution of antibiotic resistance: the common mechanisms of emergence and spread in water bodies. *Frontiers in Microbiology*, *3*, 18. https://doi.org/10.3389/fmicb.2012.00018
Magiorakos, A.-P., Srinivasan, A., Carey, R. B., Carmeli, Y., Falagas, M. E., Giske, C. G., … Monnet, D. L. (2012). Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. *Clinical Microbiology and Infection : The Official Publication of the European Society of Clinical Microbiology and Infectious Diseases*, *18*(3), 268–281. https://doi.org/10.1111/j.1469-0691.2011.03570.x
Mahillon, J., & Chandler, M. (1998). Insertion sequences. *Microbiology and Molecular Biology Reviews : MMBR*, *62*(3), 725–774.
Marti, E., Variatza, E., & Balcazar, J. L. (2014). The role of aquatic ecosystems as reservoirs of

antibiotic resistance. *Trends in Microbiology*, *22*(1), 36–41. https://doi.org/10.1016/J.TIM.2013.11.001

Mazel, D. (2006). Integrons: agents of bacterial evolution. *Nature Reviews. Microbiology*, *4*(8), 608–620. https://doi.org/10.1038/nrmicro1462

Naas, T., Mikami, Y., Imai, T., Poirel, L., & Nordmann, P. (2001). Characterization of In53, a class 1 plasmid- and composite transposon-located integron of *Escherichia coli* which carries an unusual array of gene cassettes. *Journal of Bacteriology*, *183*(1), 235–249. https://doi.org/10.1128/JB.183.1.235-249.2001

Naas, T., Poirel, L., Karim, A., & Nordmann, P. (1999). Molecular characterization of In50, a class 1 integron encoding the gene for the extended-spectrum beta-lactamase VEB-1 in *Pseudomonas aeruginosa*. *FEMS Microbiology Letters*, *176*(2), 411–419.

Naas, T., Poirel, L., & Nordmann, P. (2008). Minor extended-spectrum beta-lactamases. *Clinical Microbiology and Infection: The Official Publication of the European Society of Clinical Microbiology and Infectious Diseases*, *14 Suppl 1*, 42–52. https://doi.org/10.1111/j.1469-0691.2007.01861.x

Patel, J. B., Cockerill, F., Alder, J., Bradford, P., Eliopoulos, G., Hardy, D., … Swenson, J. (2014). *Performance Standards for Antimicrobial Susceptibility Testing; Twenty-Fourth Informational Supplement*. *Clinical Laboratory Standards Institute*.

Paterson, D. L., & Bonomo, R. A. (2005). Extended-spectrum beta-lactamases: a clinical update. *Clinical Microbiology Reviews*, *18*(4), 657–686. https://doi.org/10.1128/CMR.18.4.657-686.2005

Piotrowska, M., & Popowska, M. (2015). Insight into the mobilome of *Aeromonas* strains. *Frontiers in Microbiology*, *6*, 494. https://doi.org/10.3389/fmicb.2015.00494

Poirel, L., Naas, T., Guibert, M., Chaibi, E. B., Labia, R., & Nordmann, P. (1999). Molecular and biochemical characterization of VEB-1, a novel class A extended-spectrum beta-lactamase encoded by an *Escherichia coli* integron gene. *Antimicrobial Agents and Chemotherapy*, *43*(3), 573–581.

Rhodes, G., Huys, G., Swings, J., McGann, P., Hiney, M., Smith, P., & Pickup, R. W. (2000). Distribution of oxytetracycline resistance plasmids between aeromonads in hospital and aquaculture environments: implication of Tn1721 in dissemination of the tetracycline resistance determinant tet A. *Applied and Environmental Microbiology*, *66*(9), 3883–3890.

Rodriguez-Martinez, J.-M., Poirel, L., Canton, R., & Nordmann, P. (2006). Common region CR1 for expression of antibiotic resistance genes. *Antimicrobial Agents and Chemotherapy*, *50*(7), 2544–2546. https://doi.org/10.1128/AAC.00609-05

Schmidt, A. S., Bruun, M. S., Dalsgaard, I., & Larsen, J. L. (2001). Incidence, distribution, and spread of tetracycline resistance determinants and tntegron-associated antibiotic resistance genes among motile Aeromonads from a fish farming environment. *Applied and Environmental Microbiology*, *67*(12), 5675 LP-5682. Retrieved from http://aem.asm.org/content/67/12/5675.abstract

Scholz, P., Haring, V., Wittmann-Liebold, B., Ashman, K., Bagdasarian, M., & Scherzinger, E. (1989). Complete nucleotide sequence and gene organization of the broad-host-range plasmid RSF1010. *Gene*, *75*(2), 271–288.

Siguier, P. (2005). ISfinder: the reference centre for bacterial insertion sequences. *Nucleic Acids Research*. https://doi.org/10.1093/nar/gkj014

Stokes, H. W., O'Gorman, D. B., Recchia, G. D., Parsekhian, M., & Hall, R. M. (1997). Structure and function of 59-base element recombination sites associated with mobile gene

cassettes. *Molecular Microbiology*. https://doi.org/10.1046/j.1365-2958.1997.6091980.x
Sullivan, M. J., Petty, N. K., & Beatson, S. A. (2011). Easyfig: a genome comparison visualizer. *Bioinformatics (Oxford, England)*, *27*(7), 1009-10.
Tavakoli, N., Comanducci, A., Dodd, H. M., Lett, M. C., Albiger, B., & Bennett, P. (2000). IS1294, a DNA element that transposes by RC transposition. *Plasmid*, *44*(1), 66–84. https://doi.org/10.1006/plas.1999.1460
Toleman, M. A., Bennett, P. M., & Walsh, T. R. (2006). ISCR elements: novel gene-capturing systems of the 21st century? *Microbiology and Molecular Biology Reviews : MMBR*, *70*(2), 296–316. https://doi.org/10.1128/MMBR.00048-05
Vallenet, D., Nordmann, P., Barbe, V., Poirel, L., Mangenot, S., Bataille, E., … Cruveiller, S. (2008). Comparative analysis of Acinetobacters: three genomes for three lifestyles. *PLOS ONE*, *3*(3), e1805. Retrieved from https://doi.org/10.1371/journal.pone.0001805
Zankari, E., Hasman, H., Cosentino, S., Vestergaard, M., Rasmussen, S., Lund, O., … Larsen, M. V. (2012). Identification of acquired antimicrobial resistance genes. *Journal of Antimicrobial Chemotherapy*. https://doi.org/10.1093/jac/dks261

CHAPTER 2

Trade-offs between growth rate and other fungal traits

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ABSTRACT

If we better understand how fungal responses to global change are governed by their traits, we can improve predictions of fungal community composition and ecosystem function. Specifically, we can examine trade-offs among traits, in which the allocation of finite resources toward one trait reduces the investment in others. We hypothesized that trade-offs among fungal traits relating to rapid growth, resource capture, and stress tolerance sort fungal species into discrete life history strategies. We used the Biolog Filamentous Fungi database to calculate maximum growth rates of 37 fungal species and then compared them to their functional traits from the fun^{fun} database. In partial support of our hypothesis, maximum growth rate displayed a negative relationship with traits related to resource capture. Moreover, maximum growth rate displayed a positive relationship with amino acid permease, forming a putative Fast Growth life history strategy. A second putative life history strategy is characterized by a positive relationship between extracellular enzymes, including cellobiohydrolase 6, cellobiohydrolase 7, crystalline cellulase AA9, and lignin peroxidase. These extracellular enzymes were negatively related to chitosanase 8, an enzyme that can break down a derivative of chitin. Chitosanase 8 displayed a positive relationship with many traits that were hypothesized to cluster separately, forming a putative Blended life history strategy characterized by certain resource capture, fast growth, and stress tolerance traits. These trait relationships complement previously explored microbial trait frameworks, such as the Competitor-Stress Tolerator-Ruderal and the Yield-Resource Acquisition-Stress Tolerance schemes.

INTRODUCTION

Fungi perform essential ecosystem processes such as decomposition and nutrient cycling (Dighton, 2003). However, it is challenging to predict how microbial communities will respond to global change and how ecosystem function will be affected (Bradford et al., 2016). For example, shifts in microbial community composition in response to a changing environment can alter carbon cycling (Allison & Martiny, 2008; Allison et al., 2013). A better understanding of relationships among fungal traits may allow us to improve predictions of carbon dynamics under global change (Allison, 2012). For example, frequently disturbed soil can provide new resources for exploitation by fast-growing fungi (Pugh & Boddy, 1988). If fast-growing fungi tend to be poor decomposers, then decomposition rates in frequently disturbed soils may be slower than otherwise expected.

Examining trade-offs among fungal traits is one approach to link community composition to ecosystem processes (Wallenstein & Hall, 2012; Crowther et al., 2014). Trade-offs occur when an allocation of finite resources toward one trait reduces investment in others. For example, fungi maximizing investment in melanin production to withstand desiccation may in turn display a decreased growth rate (Siletti et al., 2017). Yet, trade-offs between fungal growth rate and other traits are under-investigated. Here we examine trade-offs between fungal growth rate and other fungal traits related to resource capture and stress tolerance. In addition, we determine how these trade-offs structure fungal traits into various life history strategies.

The first challenge in predicting fungal responses to climate change is identifying which fungal functional traits are important for ecosystem functions (Krause et al., 2014). Functional traits are defined as measurable properties that are of physiological, morphological, or genetic origin (Crowther et al., 2014), that influence an organism's fitness, and that provide a link

between fundamental biological processes and community dynamics (McGill et al., 2006). Maximum potential growth rate is a defining feature of organisms and is thought to be an emergent feature of genomic traits. For example, an increased number of rRNA copies allows for an increase in protein synthesis, supporting rapid growth (Cox, 2003; Scott et al., 2014). Ecologists have used growth rates to predict nutrient cycling in ecosystems (Elser et al., 1996). However, predictions have primarily focused upon macroscopic organisms (Caraco et al., 1997; Allison & Vitousek, 2004; Lovett et al., 2006). Here, we quantified fungal growth rates for 653 fungal species and determined their relationships with other functional traits where possible.

To link fungal traits to ecosystem processes, it can be useful to identify principle microbial life history strategies, each composed of distinct functional traits (Malik et al., 2020). Microbial strategies or lifestyles are characterized by suites of traits which may be simultaneously selected under specific environmental conditions. While originally devised for plants, Grime's well-known Competitor-Stress Tolerator-Ruderal (CSR) scheme has been applied to mycelial fungi (Grime, 1977; Pugh, 1980; Treseder & Lennon, 2015). Here, three life history strategies exist based on the occurrence of environmental stress or disturbance (Grime, 1977). *Ruderal* organisms are often the first to colonize an area after a disturbance and do so with a rapid growth rate (Pugh, 1980). As RNA and protein synthesis is needed to support rapid growth, traits relating to the uptake of phosphate, amino acids, and nitrogen are expected to coincide with a ruderal life history strategy (Versaw & Metzenberg, 1995; Elser et al., 1996; Slot et al., 2007; Cappellazzo et al., 2008). *Stress tolerating* organisms can persist in environments that may be particularly hot, cold, or dry (Pugh, 1980). The production of osmolytes, β 1,3-glucan, and melanin can reduce desiccation during drought conditions (Acharya et al., 2004; Bowman & Free, 2006; Warren, 2014). *Competing* organisms are predicted to persist in low

stress and low disturbance environments (Pugh, 1980). They outcompete other fungi via superior resource capture (Boddy, 2000). One method is through the production of extracellular enzymes such as lignin peroxidases and cellulases, which degrade complex forms of carbon (Lynd et al., 2002; Martínez et al., 2009). Although recent studies have taken a trait-based approach toward describing bacterial communities, further research is needed to apply these schemes to fungal traits as well (Ho et al., 2013; Krause et al., 2014; Fierer, 2017; Wood et al., 2018; Ramin & Allison, 2019).

In this study, we asked (1) if there are trade-offs among fungal traits relating to fast growth, resource capture, and stress tolerance and (2) if trade-offs sort fungal traits into life history strategies, such as CSR. We hypothesized that trade-offs would occur between traits related to fungal growth rate, resource capture, and stress tolerance. Accordingly, we predicted negative relationships between these traits. To test this, we compared growth rate measurements from the Biolog Filamentous Fungi database with traits from fun^{fun}, a fungal functional traits database.

MATERIALS AND METHODS

Fungal Growth Rates

To calculate fungal growth rates, we used the Biolog Filamentous Fungi (FF) database (BIOLOG, Inc., Hayward, CA, United States; Biolog, 2017). This database includes optical density (OD) readings for various fungal species growing in a specified carbon source. Biolog measured OD at 750 nm with readings recorded at 24, 48, 72, 96, and 168 h of incubation. These OD readings indicate turbidity of the liquid culture, which is positively correlated with fungal abundance (Langvad, 1999; Meletiadis et al., 2001; Biolog, 2017). These measurements were scaled from 0 to 100, with 100 representing the maximum possible OD value. Of the various

carbon sources within the database, we chose the simple sugar α-D-glucose to calculate maximum growth rate, as not all fungi possess the capacity to breakdown more complex forms of carbon, such as cellulose (Ganesan & Nellaiappan, 2014; Hu et al., 2016). To quantify growth rate for each species, we determined the change in OD between sequential incubation time points and divided by the incubation period (hours). We then chose the maximum growth rate on α-D-glucose for each species for further analysis. For species represented by multiple strains, we averaged the maximum growth rate across strains. In total, we recorded maximum growth rates for 653 fungal strains, including clinically or environmentally important fungi, plant pathogens, and indoor or food-borne fungi (Table 2.S1).

Fungal Trait Database

We used fun[fun,] a functional trait database for fungi, to identify continuous fungal traits that may be associated with resource capture, stress tolerance, or growth rate (Table 2.1; Flores-Moreno et al., 2018). Specifically, we used gene frequencies from the fungal genomics program of the Joint Genome Institute of the Department of Energy and the 1,000 Fungal Genomes Project that were present in the database (Grigoriev et al., 2014). Genome sizes vary widely in fungi, so gene frequency was calculated as the count of a given gene per 10,000 genes in each genome. While the possession of a gene does not guarantee that it will be expressed, it indicates the genetic potential for a given trait and is useful toward identifying associations among traits (Wilmes & Bond, 2006; Myrold et al., 2014). We then merged the fungal trait data from fun[fun] with growth rate data from Biolog for all overlapping species (Tables 2.S2, 2.S3; Flores-Moreno et al., 2018). This resulted in 37 fungal species from Ascomycota, Basidiomycota, and Mucoromycota to be further analyzed (Table 2.2). The growth forms of these species included 24 filamentous fungi

and 13 yeasts. Furthermore, classification of their trophic modes resulted in 12 pathotrophs, 11 saprotrophs, 1 symbiotroph, and 13 species with mixed ecologies.

Statistics

To examine relationships between each pairwise combination of traits, we used phylogenetic independent contrasts (PIC). PICs were appropriate here because they accounted for the phylogenetic relatedness of the taxa (Webb et al., 2002, 2008). We used PICs instead of standard analyses like Pearson correlation, which require statistical independence of samples (Ricklefs & Starck, 1996).

We used the fungal phylogeny from Choi and Kim (2017), which is generated from whole genome sequences. We downloaded this phylogeny from Choi (2020, accessed 12/3/2020). Of the 37 species we analyzed, 26 were represented in the phylogeny (Table 2.S2 and Figure 2.S1). For each of the remaining species, we used the nearest taxon in the phylogeny. (Sequences were not available for these remaining taxa, so we could not construct a new phylogeny that included them.) Seven of the species were assigned to a taxon from the same genus or family (Table 2.S2). We pruned the tree to remove any taxa not represented among the 37 species (Figure 2.S1).

For PIC, we used the *aotf* function in Phylocom v 4.2 (Webb et al., 2008). The aotf function calculated the difference ("contrast") in the values of a given trait between daughter clades of each node in the phylogeny. It then generated a series of correlations of the contrasts between each pairwise combination of traits. More recently diverged clades carried more weight in the correlations, which was the default setting for *aotf*. Trait data were ranked to avoid outliers. Since this study was exploratory and aimed to be as comprehensive as possible, we did

not adjust for multiple comparisons and instead present unadjusted *P*-values (Rothman, 1990; Perneger, 1998; Feise, 2002; Althouse, 2016). A negative correlation suggests a trait trade-off.

To determine relationships among all fungal traits, we performed a non-metric multidimensional scaling (NMS) analysis, with the PIC r coefficients as the distance metric (Table 2.3). We used the monotonic multidimensional scaling function with the Kruskal method in SPSS version 13.2 (SPSS, 2017). This analysis generated coordinates for two dimensions (Table 2.S4). We visualized these data as a scatterplot, with fungal traits connected according to their PIC r coefficients using the software Polinode. We assigned traits to life history strategies based on their relatedness to one another, where traits were proposed to share a life history strategy if their PIC r coefficients were greater than 0.35. A PIC r coefficient of 0.325 or greater indicated a significant relationship ($P \leq 0.05$) before correcting for multiple comparisons. The value of 0.35 was chosen to simplify the number of relationships shown on Figure 2.2 and highlight the proposed life history strategies. This exercise generated three life history strategies, which we named the "Resource Capture," "Fast Growth," and "Blended" life history strategies. One trait, α-glucosidase 31, was associated with two life history strategies (Resource Capture and Blended) based on this metric. Since the PIC r coefficient linking α-glucosidase 31 to the Blended life history strategy was larger, we assigned it to this life history strategy.

To test if these life history strategies were statistically supported, we conducted a permutational multivariate analyses of variance (perMANOVAs). We used the *adonis* function in the *vegan* package (Oksanen et al., 2020) in R version 3.5.1, set to 10,000 permutations (R Core Team, 2020). The dependent variables were the pairwise PIC r coefficients (Table 2.3), and the independent variable was trait life history strategy. We also conducted a second perMANOVA in which traits had been *a priori* assigned to C, S, or R life history strategies.

RESULTS

Trade-Offs Between Growth Rate and Other Fungal Traits

The first question of this study was whether fungal growth rate displayed a trade-off with other fungal traits. We predicted that a negative relationship would occur between maximum growth rate and traits related to resource capture or stress tolerance, due to physiological or evolutionary trade-offs. We found that maximum growth rate was negatively related to three resource capture traits: cellobiohydrolase 7 ($P = 0.025$), crystalline cellulase AA9 ($P = 0.008$), and endoglucanase 12 ($P = 0.008$; Figure 2.1 and Table 2.3, *P*-values listed in Table 2.S4).

Identification of Fungal Trait Life History Strategies

Our second question was whether relationships among fungal traits have created discrete life history strategies, such as CSR. For these purposes, a suite of traits that tend to co-occur within fungal taxa can represent a life history strategy. Moreover, if a given suite of traits were negatively related to another suite of traits, we would consider those suites to represent distinct life history strategies.

Resource Capture Life History Strategy

Numerous resource capture traits were positively correlated with each other (Table 2.3), and they clustered in the NMS plot (Figure 2.2). Extracellular enzymes involved in the breakdown of carbon compounds clustered tightly, including β-xylosidase 43, endoglucanase 12, crystalline cellulase AA9, cellobiohydrolase 6, cellobiohydrolase 7, and lignin peroxidase. Other traits that clustered within this life history strategy include nitrate transporter and the carbon-targeting enzymes β-glucosidase 1, glycoprotein synthesis 92, and amylase 88. In addition, cellobiohydrolase 7, crystalline cellulase AA9, and endoglucanase 12 displayed negative relationships to maximum growth rate ($P < 0.05$; Table 2.3). Also, nitrate transporter, crystalline

cellulase AA9, cellobiohydrolase 6, cellobiohydrolase 7, and lignin peroxidase displayed negative relationships to chitosanase 8 ($P < 0.05$; Table 2.3). Hereafter, we refer to this life history strategy as the "Resource Capture" life history strategy.

Fast Growth Life History Strategy

Maximum growth rate was positively correlated with amino acid permease ($P = 0.010$; Figure 2.2 and Table 2.3). We call this cluster of traits the "Fast Growth" life history strategy. Furthermore, maximum growth rate was negatively related to various traits in the Resource Capture life history strategy, such as cellobiohydrolase 7, crystalline cellulase AA9, and endoglucanase 12 ($P < 0.05$; Table 2.3). In addition, maximum growth rate was negatively related to heat shock protein (see below), a trait within the Blended life history strategy ($P = 0.004$). Together, this suggests that the Fast Growth life history strategy is distinct from both the Resource Capture and Blended life history strategies.

Blended Life History Strategy

The "Blended" life history strategy consists of traits related to resource capture, stress tolerance, and fast growth (Figure 2.2). We observed a clustering of traits related to resource capture, including α-glucosidase 15, α-glucosidase 31, chitinase, chitosanase 8, endoglucanase 9, and glucosidase 81. In addition, the stress tolerance traits heat shock protein, trehalase, and cold shock protein clustered within this life history strategy. Also, ammonium transporter and acid phosphatase, traits hypothesized to support fast growth, were present within the Blended life history strategy. Furthermore, negative relationships were observed between chitosanase 8 and multiple traits of the Resource Capture life history strategy. Last, both acid phosphatase and chitosanase 8 displayed negative relationships to the stress tolerance trait β 1,3-glucan synthase.

Life History Strategy Sorting

Trait relationships varied significantly among the proposed Resource Capture, Fast Growth, and Blended life history strategies ($r^2 = 0.179$, $P < 0.001$). This result provided statistical support for this life history framework. In contrast, we did not find statistical support for the CSR framework ($r^2 = 0.084$, $P = 0.128$).

DISCUSSION

In this study, fast-growing fungal species tended to display less genetic capacity for resource capture (Figure 2.2 and Table 2.3). These results support our hypothesis that trade-offs occur among traits associated with growth rate versus resource capture. On the other hand, the lack of negative relationships between growth rate and stress tolerance traits was not consistent with this hypothesis. With respect to life history strategies, we observed clusters of traits that formed putative Fast Growth (maximum growth rate and amino acid permease) and Resource Capture (extracellular enzymes and nutrient transporters) life history strategies (Figure 2.2). In addition, we identified a Blended life history strategy consisting of traits related to resource capture, stress tolerance, and fast growth. This analysis comparing fungal growth rates to other fungal traits was possible because hundreds of fungal species were grown under common laboratory conditions to produce the Biolog database.

Microbial Trait Frameworks

Our findings share similarities with other publications exploring microbial trait frameworks. The Fast Growth life history strategy in this study was similar to the ruderal (R) strategy in Grime's CSR framework (Grime, 1977). More recently proposed is the YAS framework, a revised life history theory specifically for microbes (Malik et al., 2020). The competitor (C) strategy in CSR is analogous to the resource acquisition strategy (A) in YAS, which includes traits such as extracellular enzymes and uptake transporters (Malik et al., 2020).

The Resource Capture life history strategy in this study aligned with these C and A strategies (Grime, 1977; Malik et al., 2020). Both frameworks share the stress tolerator (S) strategy, which was not evident in this study.

Resource Capture Life History Strategy

Extracellular enzymes, such as endoglucanase, cellobiohydrolase, and β-glucosidase, clustered tightly together within the Resource Capture life history strategy (Figure 2.2). This result may be explained by the nature of cellulose breakdown by fungi as follows. Cellulose, a major component of the plant cell wall, is a significant source of energy for fungi (Klemm et al., 2005). The extracellular enzymes identified in this life history strategy can break down cellulose into simpler compounds, such as cellobiose and glucose, which may then be taken up and metabolized (Lynd et al., 2002). Cellulases act in a coordinated manner to digest cellulose (Teeri, 1997; Lynd et al., 2002). First, endoglucanases cleave cellulose chains at random internal sites, creating oligosaccharides that vary in length (Lynd et al., 2002). Then, cellobiohydrolases release cellobiose and glucose from the ends of the chains (Lynd et al., 2002). Lastly, β-glucosidases hydrolyze cellobiose to glucose for cellular uptake by fungi (Lynd et al., 2002; Payne et al., 2015). In addition, fungi produce lignin peroxidase to oxidize lignin, thereby gaining access to cellulose, nitrogen, and other nutrients (Tien & Kent Kirk, 1983). Lignin peroxidase was also present in this cluster. It therefore follows that these enzymes would be simultaneously selected to target cellulose and lignin breakdown and constitute a Resource Capture life history strategy.

Unexpectedly, certain traits that were hypothesized to support fast growth, such as nitrate and phosphate transporters, either clustered within the Resource Capture life history strategy or were positively related to traits within the Resource Capture life history strategy (Figure 2.2). Nitrate and phosphate transporters can acquire nitrogen and phosphorus, respectively, from the

environment (Versaw & Metzenberg, 1995; Slot et al., 2007). Nitrogen and phosphorus are essential to support the transcription and protein synthesis involved in extracellular enzyme production (Glenn, 1976), which might explain their affiliation with the Resource Capture life history strategy. Alternately, these genes could encode proteins that "moonlight" by exhibiting other functions in addition to nutrient uptake (Jeffery, 2018).

Fast Growth Life History Strategy

Within the Fast Growth life history strategy, maximum growth rate was positively related to amino acid permease, a transporter involved in the uptake of amino acids from the environment (Figure 2.2; Nehls et al., 1999). As amino acids are crucial for protein production, this trait may support rapid growth. Since maximum growth rate was negatively related to the resource capture traits cellobiohydrolase 7, crystalline cellular AA9, and endoglucanase 12, the Fast Growth life history strategy seemed separate from the Resource Capture life history strategy (Table 2.3). Recently, Ramin and Allison (2019) identified a negative correlation between maximum growth rate and total activity of extracellular enzymes in 49 bacterial strains isolated from leaf litter. They proposed a resource acquisition strategy characterized by bacteria investing heavily in extracellular enzyme production, versus a growth strategy characterized by high growth rates (Ramin & Allison, 2019). These strategies are consistent with our findings of a negative relationship between growth rate and resource capture traits in fungi.

Blended Life History Strategy

Surprisingly, the Blended life history strategy displayed positive relationships between traits associated with resource capture, stress tolerance, and fast growth. This contrasted with our hypothesis that trade-offs would occur between these traits. However, we observed negative relationships indicative of tradeoffs between Blended life history strategy traits and other traits.

For example, acid phosphatase and chitosanase 8 in the Blended life history strategy displayed negative relationships to β 1,3-glucan synthase, which improves stress tolerance by strengthening cell walls (Shima et al., 2008). Possession of β 1,3- glucan synthase may have been costly for fungi in the Blended life history strategy, possibly explaining the observed trade-off. Or, perhaps this trait was not beneficial for survival of fungi in this life history strategy, as they already possessed other stress tolerance traits, such as trehalase, heat shock protein, and cold shock protein.

Fungal taxa possessing resource capture traits that clustered in the Blended life history strategy may have the capacity to degrade various forms of carbon compounds to support their metabolism. The enzymes chitinase and chitosanase are responsible for the hydrolysis of chitin and chitosan, respectively (Hartl et al., 2012). Chitin is the second most abundant polysaccharide found in nature and is a structural polymer of most fungal cell walls (Bowman & Free, 2006). While these enzymes are involved in the degradation and remodeling of fungal cell walls, they also play a role in exogenous chitin decomposition (Hartl et al., 2012). Interestingly, we observed a negative relationship between chitosanase 8 and four enzymes in the Resource Capture life history strategy involved in cellulose and lignin degradation. In addition, the enzymes α-glucosidase 15 and α-glucosidase 31 hydrolyze starch, while endoglucanase 9 hydrolyzes cellulose. Last, glucosidase 81 is an enzyme responsible for catalyzing the breakdown of β 1,3-glucan, a major component of the fungal cell wall (Reese & Mandels, 1959; Chesters & Bull, 1963). This enzyme can serve a survival function by breaking down and reusing stored β-glucans during periods of carbon limitation (Pitson et al., 1993, Pitson et al., 1997). Therefore, fungi in the Blended life history strategy may have possessed the ability to degrade various sources of carbon including chitin, chitosan, and β-glucans, whereas fungi in the

Resource Capture life history strategy possessed enzymes more specialized to target cellulose and lignin to support their carbon metabolism.

As the positive relationships between traits associated with resource capture, stress tolerance, and fast growth in the Blended life history strategy did not support our hypothesis of tradeoffs, perhaps investment in these specific traits were not as costly as predicted. Similarly, Alster et al. (2021) did not observe trade-offs between traits associated with growth yield, resource acquisition, and stress tolerance in fungi from a Southern California grassland. A possible explanation may be that fungal taxa within this Blended life history strategy required equal investment in these traits for survival, which is why trade-offs were not observed. As high temperatures can lead to protein misfolding or denaturation, heat shock proteins can facilitate the survival of fungi by assisting with protein folding and stability (Tiwari et al., 2015). Since resource capture enzymes in this life history strategy, such as glucosidases and chitosanase must assume their proper three-dimensional structures to become functional, heat shock proteins may have been particularly advantageous for fungi within the Blended life history strategy. In contrast, it is also possible that resource capture and fast growth traits in this life history strategy were used to support investment in stress tolerance as suggested by Alster et al. (2021). For example, carbon gained through resource capture enzymes and nutrients gained through acid phosphatase and ammonium transporter could be invested toward trehalose production, thereby providing protection from desiccation, freeze damage, or heat shock (Singer & Lindquist, 1998; Schimel et al., 2007). While fungi within the Resource Capture or Fast Growth life history strategies can be thought of as "specialists," fungi in the Blended life history strategy may be more successful by possessing traits that support varying functions and may therefore be more of "generalists."

Stress Tolerance Traits

We found little evidence for a Stress Tolerator life history strategy as the stress tolerance traits did not cluster together as hypothesized. It was surprising that other stress tolerance traits were not positively related to one another, as has been observed in other studies (Treseder & Lennon, 2015; Maynard et al., 2019). This might have been due to the differences in fungal species analyzed in this study compared to those prior. For example, the species in this study were essentially a subset of the species analyzed in Treseder and Lennon (2015), while Maynard et al. (2019) included more Basidiomycetes. We were also constrained by which fungal species were present both in the fun[fun] and Biolog databases. It is possible that if we had more species to analyze, stress tolerance traits may have separated from others. In addition, this observation could be due to growth rates being measured under optimal conditions versus low-resource conditions as explained further below. Furthermore, some stress tolerance traits may provide additional functions. For example, while trehalase regulates levels of trehalose that protect against environmental stressors, it can also function to hydrolyze exogenous trehalose as a carbon source (Jorge et al., 1997). Last, genomic markers for stress tolerance in fungi remain largely unclassified. It is therefore possible that we have not targeted the relevant genes for stress tolerance, which may explain why a Stress Tolerator life history strategy was not identified.

Overall

While it can be argued that growth rate in the laboratory may not reflect growth rate in nature, recording growth rate under controlled conditions allows for standardized trait measurements and can improve our understanding of fungal ecology (Aguilar-Trigueros et al., 2015). For example, relative growth rate of plants measured under controlled environmental conditions has proven useful for predicting productivity in the field (Vile et al., 2006).

Furthermore, measurements of maximum growth rate in this study occurred under high-resource conditions. Tradeoffs may strengthen under low-resource conditions, as fewer resources are available to support multiple traits. Ultimately, collecting fungal trait data from as many contexts as possible will help determine trait plasticity and further our understanding of fungal communities. In addition, we acknowledge that this study examined a relatively small fungal collection of 37 species that are mostly limited to the phylum Ascomycota. We were constrained by (1) fungi that can be cultured in the laboratory and have measured growth rates and (2) the fungal species that have been sequenced and uploaded to the fun^{fun} database. As more fungal species are uploaded to the fun^{fun} database, we hope to incorporate their gene frequencies with our growth rate data from 653 species to have greater fungal diversity to be analyzed.

CONCLUSION

We identified trade-offs between fungal traits relating to fast growth and resource capture. These trade-offs led to the identification of three fungal life history strategies: a Fast Growth life history strategy characterized by rapid growth and the amino acid permease gene, a Resource Capture life history strategy encompassed by extracellular enzymes and nutrient transporters, and a Blended life history strategy consisting of traits related to rapid growth, resource capture, and stress tolerance. These relationships between fungal traits may help us predict changes in nutrient cycling under global change (Schimel & Schaeffer, 2012). For example, the frequency of wildfires is expected to increase in response to climate change (An et al., 2015). This frequent disturbance may select for fungi with fast growth rates. These fast-growing fungi might possess a lower capacity for decomposition due to trade-offs. In turn, breakdown of complex carbon compounds may slow, leading to more carbon storage than we might otherwise expect (Holden et al., 2013). Trait-based ecosystem models can incorporate trait

linkages like this to improve predictions of ecosystem feedbacks resulting from shifts in microbial communities to a changing environment (Allison, 2012; Allison & Goulden, 2017).

Table 2.1. Potential fungal traits associated with each life history strategy within the Competitor-Stress Tolerator-Ruderal scheme.

Proposed life history strategy	Fungal trait(s)	fun[fun] trait name	Function(s)
Resource capture life history strategy (a.k.a. competitor or resource acquisition)	Invertase	Invertase 32	Breakdown of sucrose (Boddy et al., 1993)
	α-glucosidase	α-glucosidase 15; α-glucosidase 31	Breakdown of starch (Nakamura et al., 1997)
	Amylase	Amylase 88	Breakdown of starch (Korman et al., 1990)
	β-xylosidase	β -xylosidase 43	Breakdown of hemicellulose (Semenova et al., 2009)
	β-glucosidases	β-glucosidase 1	Breakdown of cellulose (Znameroski et al., 2012)
	Cellobiohydrolases	Cellobiohydrolase 6; Cellobiohydrolase 7	Breakdown of cellulose (Ilmén et al., 1997)
	Endoglucanases	Endoglucanase 9; Endoglucanase 12	Breakdown of cellulose (Rungrattanakasin et al., 2018)
	α-mannosidase	Glycoprotein synthesis 92; α-mannanase 76	Breakdown of mannose (Maruyama et al., 1994; Nakajima et al., 1976)
	Crystalline cellulase	Crystalline cellulase AA9	Breakdown of cellulose (Langston et al., 2011)
	Lignin peroxidase	Lignin peroxidase	Breakdown of lignin (Ruiz-Dueñas et al., 2009)
	Chitosanase	Chitosanase 8	Breakdown of chitosan (Shimosaka et al., 1993)
	Chitinase	Chitinase	Breakdown of chitin (Seidl et al., 2005)
	Endo-β-D-1,3-glucanase	Glucosidase 81	Breakdown of β 1,3-glucan (Reese & Mandels, 1959)
	Endo-β-N-acetylglucosaminidase	Glycopeptidase 85	Breakdown of glycoproteins (Huang et al., 2018)
Stress tolerator life history strategy	β 1,3-glucan synthase	β-glucan synthase	Strengthens cell wall, reduces water loss (Mazur et al., 1995)
	Trehalase	Trehalase	Protection from desiccation, freeze damage, thermotolerance (Singer & Lindquist, 1998)
	Cold shock protein	Cold shock protein	Protection from cold stress (Homma et al., 2003; Murata et al., 2006)

	Heat shock protein	Heat shock protein	Protection from temperature, osmotic, pH stress (Lamoth et al., 2012)
Fast growth life history strategy (a.k.a. ruderal)	Fast growth		Allows rapid colonization (Siletti et al., 2017)
	Amino acid permease; Phosphate, nitrate, and ammonium transporters	Amino acid permease; Phosphate transporter; Nitrate transporter; Ammonium transporter	Augments uptake of N and P to support growth (Cappellazzo et al., 2008; Mitsuzawa, 2006; Nehls et al., 1999; Sengottaiyan et al., 2013; Slot et al., 2007; Wipf et al., 2002)
	Acid phosphatase	Acid phosphatase	P mineralization (Han et al., 1987; Nelson et al., 1976)

Table 2.2 List of fungal species analyzed in this study.

Fungal species
Aspergillus clavatus
Aspergillus fischeri
Aspergillus flavus
Aspergillus fumigatus
Aspergillus niger
Aspergillus ruber
Aspergillus terreus
Aureobasidium pullulans
Beauveria bassiana
Botrytis cinerea
Chaetomium globosum
Debaryomyces hansenii
Dekkera bruxellensis
Fusarium fujikuroi
Fusarium graminearum
Fusarium oxysporum
Fusarium solani
Kluyveromyces lactis
Komagataella pastoris
Neurospora crassa
Penicillium chrysogenum
Penicillium digitatum
Penicillium oxalicum
Pesotum piceae
Phanerodontia chrysosporium
Rhizopus arrhizus
Rhizopus microsporus
Saccharomyces cerevisiae
Schizosaccharomyces octosporus
Schizosaccharomyces pombe
Torulaspora delbrueckii
Trichoderma parceramosum
Trichoderma reesei
Trichoderma virens
Ustilago maydis
Yarrowia lipolytica
Zygosaccharomyces rouxii

Table 2.3 Phylogenetic independent contrast r coefficients for all fungal traits used for non-metric multidimensional scaling analysis.

Please see Table 2.3 in the published article at the following link:
https://www.frontiersin.org/articles/10.3389/ffgc.2021.756650/full

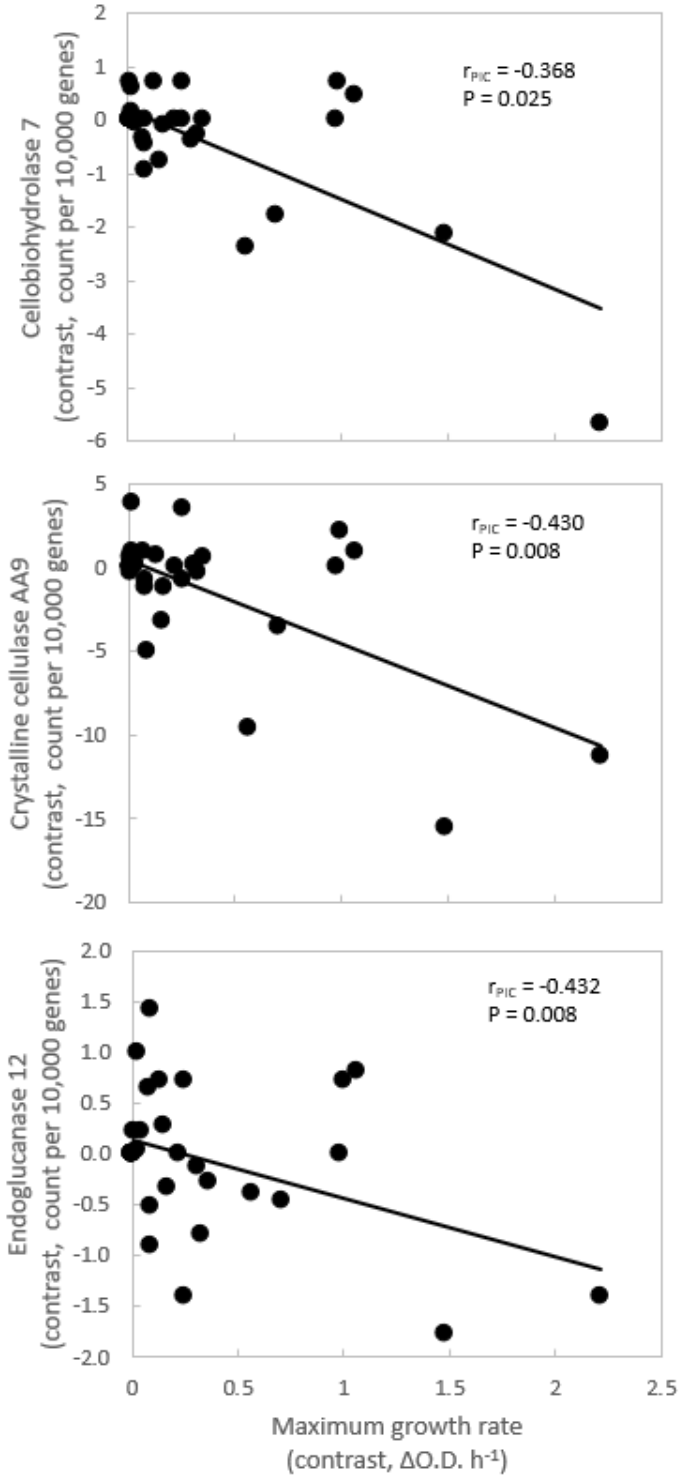

Figure 2.1. Negative relationships between maximum growth rate and other traits. Each symbol represents an internal node within the phylogeny (Fig. 2.S1). Each node's contrast value is the difference in average trait values between its daughter clades. This approach accounts for phylogenetic relatedness of taxa. Lines are best fit. *P*-values are unadjusted for multiple comparisons. Gene frequency data are from MycoCosm (Grigoriev et al., 2014). ΔO.D. = Increase in optical density at 750 nm.

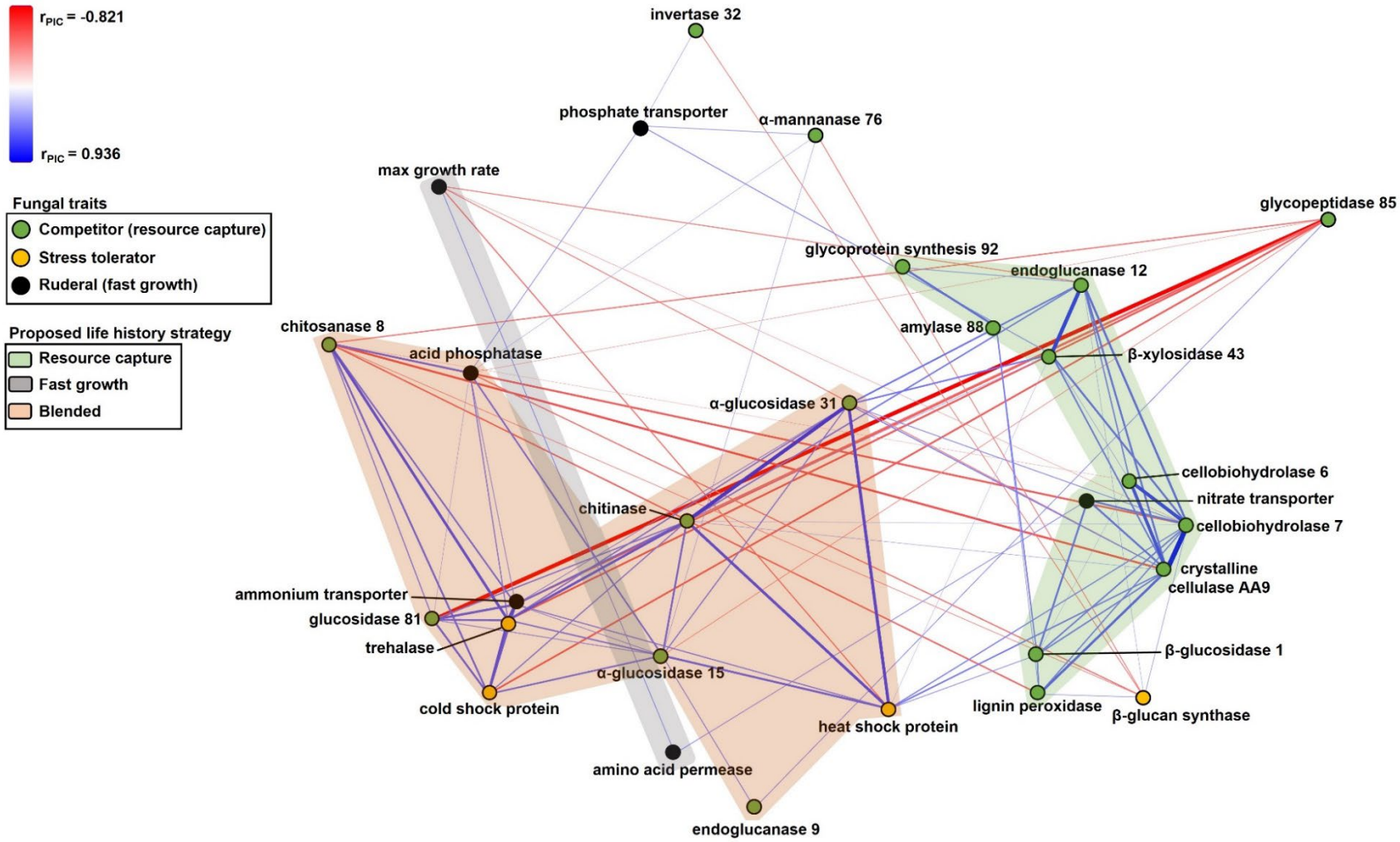

Figure 2.2 Non-metric multidimensional scaling scatterplot of fungal trait relationships calculated using PIC r coefficients between traits (n = 37). Symbols are traits; traits closer together tended to be more positively related to one another. Positive and negative relationships between traits are denoted by blue or red lines, respectively, with color intensity and thickness of lines proportional to the size of the PIC r coefficient. Trait symbols are colored according to their predicted life history strategies listed in Table 2.1. Lines corresponding to PIC r coefficients in the range of –0.35 to 0.35 have been excluded for simplicity.

Table 2.S1. Maximum growth rates of Biolog Filamentous Fungi.

Table 2.S2 List of fungal species and their corresponding traits analyzed in this study.

Table 2.S3. Units and sources of fungal traits.

Table 2.S4. *P*-values for phylogenetic independent contrast r coefficients for all fungal traits and NMS coordinates.

The Supplementary tables for this published article can be found at the following link: https://www.frontiersin.org/articles/10.3389/ffgc.2021.756650/full#supplementary-material

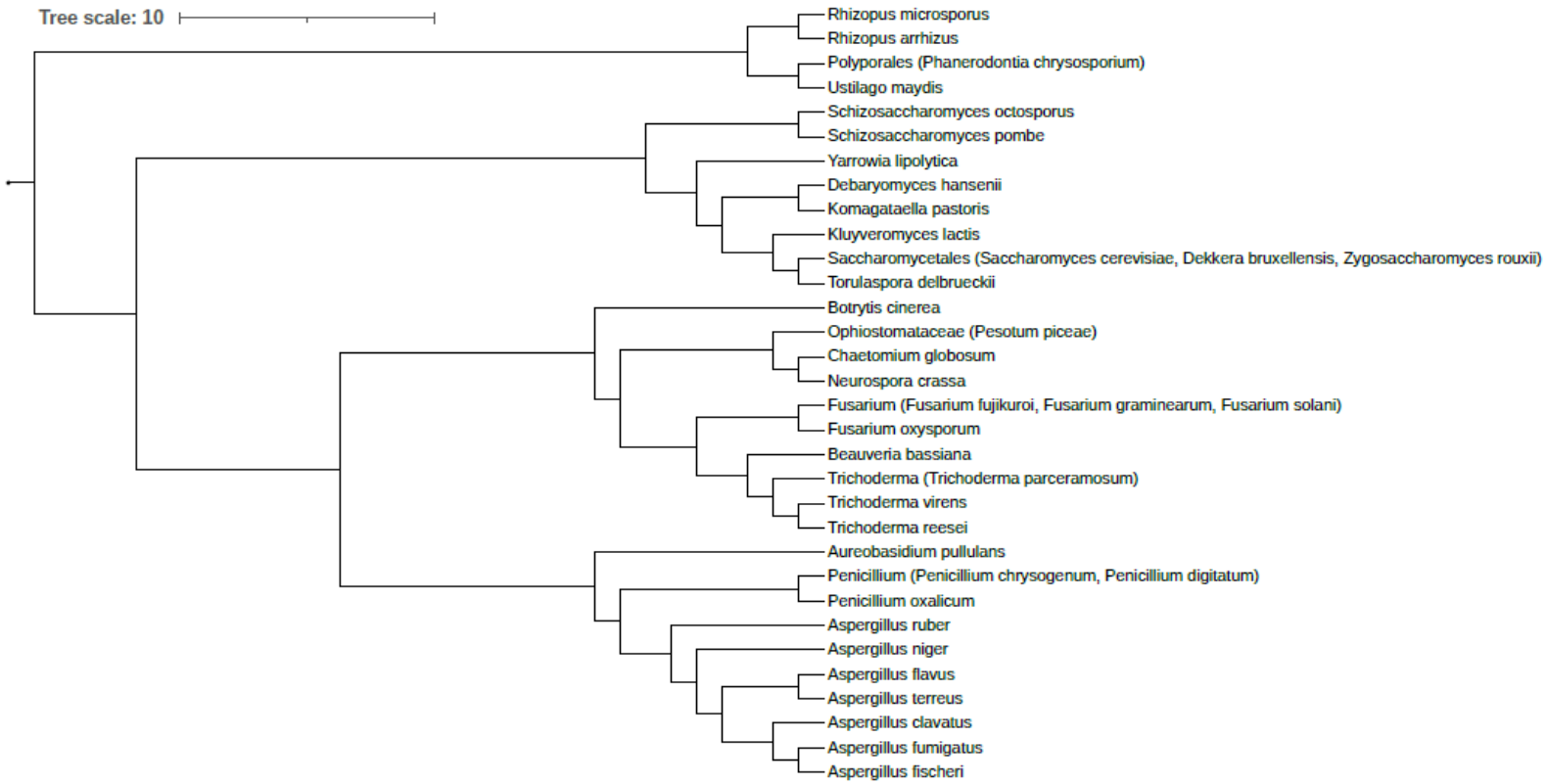

Figure 2.S1. Phylogenetic tree for fungal species analyzed in this study. If the species of interest could not be identified, the name of the order, family, or genus for the species of interest is listed with the next closest relative in parentheses. Letunic and Bork (2019) Nucleic Acids Res doi: 10.1093/nar/gkz23.

REFERENCES

Acharya, K., Samui, K., Rai, M., Dutta, B. B., and Acharya, R. (2004). Antioxidant and nitric oxide synthase activation properties of *Auricularia auricula*. *Indian J. Exp. Biol.* 42, 538–540. doi: 10.1146/annurev.py.24.090186.002211

Aguilar-Trigueros, C. A., Hempel, S., Powell, J. R., Anderson, I. C., Antonovics, J., and Bergmann, J. (2015). Branching out: towards a trait-based understanding of fungal ecology. *Fungal Biol. Rev.* 29, 34–41. doi: 10.1016/j.fbr.2015.03.001

Allison, S. D. (2012). A trait-based approach for modelling microbial litter decomposition. *Ecol. Lett.* 15, 1058–1070. doi: 10.1111/j.1461-0248.2012.01807.x

Allison, S. D., and Goulden, M. L. (2017). Consequences of drought tolerance traits for microbial decomposition in the DEMENT model. *Soil Biol. Biochem.* 107, 104–113. doi: 10.1016/j.soilbio.2017.01.001

Allison, S. D., Lu, Y., Weihe, C., Goulden, M. L., Martiny, A. C., Treseder, K. K., et al. (2013). Microbial abundance and composition influence litter decomposition response to environmental change. *Ecology* 94, 714–725. doi: 10.1890/12-1243.1

Allison, S. D., and Martiny, J. B. H. (2008). Resistance, resilience, and redundancy in microbial communities. *Proc. Natl. Acad. Sci. U. S. A.* 105, 11512–11519. doi: 10.1073/pnas.0801925105

Allison, S. D., and Vitousek, P. M. (2004). Rapid nutrient cycling in leaf litter from invasive plants in Hawai'i. *Oecologia* 141, 612–619. doi: 10.1007/s00442-004-1679-z

Alster, C. J., Allison, S. D., Glassman, S. I., Martiny, A. C., and Treseder, K. K. (2021). Exploring trait trade-offs for fungal decomposers in a Southern California grassland. *Front. Microbiol.* 12:665. doi: 10.3389/fmicb.2021.655987

Althouse, A. D. (2016). Adjust for multiple comparisons? It's not that simple. *Ann. Thorac. Surg.* 101, 1644–1645. doi: 10.1016/j.athoracsur.2015.11.024

An, H., Gan, J., and Cho, S. J. (2015). Assessing climate change impacts on wildfire risk in the United States. *Forests* 6, 3197–3211. doi: 10.3390/f6093197

Biolog. (2017). *MicroStation™ System/MicroLog User's Guide (Version 5.2.01).*

Boddy, L. (2000). Interspecific combative interactions between wood-decaying basidiomycetes. *FEMS Microbiol. Ecol.* 31, 185–194. doi: 10.1016/S0168-6496(99)00093-8

Boddy, L. M., Bergès, T., Barreau, C., Vainstein, M. H., Dobson, M. J., Ballance, D. J., et al. (1993). Purification and characterisation of an *Aspergillus niger* invertase and its DNA sequence. *Curr. Genet.* 24, 60–66. doi: 10.1007/BF00324666

Bowman, S. M., and Free, S. J. (2006). The structure and synthesis of the fungal cell wall. *BioEssays* 28, 799–808. doi: 10.1002/bies.20441

Bradford, M. A., Wieder, W. R., Bonan, G. B., Fierer, N., Raymond, P. A., and Crowther, T. W. (2016). Managing uncertainty in soil carbon feedbacks to climate change. *Nat. Clim. Chang.* 6, 751–758. doi: 10.1038/nclimate3071

Cappellazzo, G., Lanfranco, L., Fitz, M., Wipf, D., and Bonfante, P. (2008). Characterization of an amino acid permease from the endomycorrhizal fungus *Glomus mosseae*. *Plant Physiol.* 147, 429–437. doi: 10.1104/pp.108.117820

Caraco, N. F., Cole, J. J., Raymond, P. A., Strayer, D. L., Pace, M. L., Findlay, S. E. G., et al. (1997). Zebra mussel invasion in a large, turbid river: phytoplankton response to

increased grazing. *Ecology* 78, 588–602. doi: 10.1890/0012-96581997078[0588:ZMIIAL]2.0.CO;2
Chesters, C. G., and Bull, A. T. (1963). The enzymic degradation of laminarin. 1. The distribution of laminarinase among micro-organisms. *Biochem. J.* 86, 28–31. doi: 10.1042/bj0860028
Choi, J. (2020). *Feature Frequency Profile (FFP).* Available online at: https://github.com/jaejinchoi/FFP (accessed December 3, 2020).
Choi, J. J., and Kim, S.-H. (2017). A genome tree of life for the fungi kingdom. *Proc. Natl. Acad. Sci. U. S. A.* 114, 9391–9396. doi: 10.1073/pnas.1711939114
Cox, R. A. (2003). Correlation of the rate of protein synthesis and the third power of the RNA: protein ratio in *Escherichia coli* and *Mycobacterium tuberculosis*. *Microbiology* 149, 729–737. doi: 10.1099/mic.0.25645-0
Crowther, T. W., Maynard, D. S., Crowther, T. R., Peccia, J., Smith, J. R., and Bradford, M. A. (2014). Untangling the fungal niche: the trait-based approach. *Front. Microbiol.* 5:579. doi: 10.3389/fmicb.2014.00579
Dighton, J. (2003). *Fungi in Ecosystem Processes. In Fungi in ecosystem processes (1st ed.).* New York: Marcel Dekker. doi: 10.1201/9780824742447
Elser, J. J., Dobberfuhl, D. R., MacKay, N. A., and Schampel, J. H. (1996). Organism size, life-history, and N:P stoichiometry - Toward a unified view of cellular and ecosystem processes. *Bioscience* 46, 674–684. doi: 10.2307/1312897
Feise, R. J. (2002). Do multiple outcome measures require p-value adjustment? *BMC Med. Res. Methodol.* 2:8. doi: 10.1186/1471-2288-2-8
Fierer, N. (2017). Embracing the unknown: disentangling the complexities of the soil microbiome. *Nat. Rev. Microbiol.* 15, 579–590. doi: 10.1038/nrmicro.2017.87
Flores-Moreno, H., Treseder, K. K., Cornwell, W. K., Maynard, D. S., Milo, A. M., Abarenkov, K., et al. (2018). *Fungaltraits aka funfun: a dynamic functional trait database for the world's fungi.* Available online at: https://github.com/traitecoevo/fungaltraits (accessed January 25, 2019).
Ganesan, S., and Nellaiappan, O. G. (2014). Evaluation and Selection of native fungal isolates for cellulase enzyme production. *Res. J. Biotechnol.* 9, 22–29.
Glenn, A. R. (1976). Production of extracellular proteins by bacteria. *Annu. Rev. Microbiol.* 30, 41–62. doi: 10.1146/annurev.mi.30.100176.000353
Grigoriev, I. V., Nikitin, R., Haridas, S., Kuo, A., Ohm, R., Otillar, R., et al. (2014). MycoCosm portal: gearing up for 1000 fungal genomes. *Nucleic Acids Res.* 42, D669–D704. doi: 10.1093/nar/gkt1183
Grime, J. P. (1977). Evidence for the existence of three primary strategies in plants and its relevance to ecological and evolutionary theory. *Am. Nat.* 111, 1169–1194. doi: 10.1086/283244
Han, S. W., Nahas, E., and Rossi, A. (1987). Regulation of synthesis and secretion of acid and alkaline phosphatases in *Neurospora crassa*. *Curr. Genet.* 11, 521–527. doi: 10.1007/BF00384615
Hartl, L., Zach, S., and Seidl-Seiboth, V. (2012). Fungal chitinases: diversity, mechanistic properties and biotechnological potential. *Appl. Microbiol. Biotechnol.* 93, 533–543. doi: 10.1007/s00253-011-3723-3

Ho, A., Kerckhof, F. M., Luke, C., Reim, A., Krause, S., Boon, N., et al. (2013). Conceptualizing functional traits and ecological characteristics of methane-oxidizing bacteria as life strategies. *Environ. Microbiol. Rep.* 5, 335–345. doi: 10.1111/j.1758-2229.2012.00370.x

Holden, S. R., Gutierrez, A., and Treseder, K. K. (2013). Changes in soil fungal communities, extracellular enzyme activities, and litter decomposition across a fire chronosequence in Alaskan boreal forests. *Ecosystems* 16, 34–46. doi: 10.1007/s10021-012-9594-3

Homma, T., Iwahashi, H., and Komatsu, Y. (2003). Yeast gene expression during growth at low temperature. *Cryobiology* 46, 230–237. doi: 10.1016/S0011-2240(03)00028-2

Hu, M. L., Zha, J., He, L. W., Lv, Y. J., Shen, M. H., Zhong, C., et al. (2016). Enhanced bioconversion of cellobiose by industrial *Saccharomyces cerevisiae* used for cellulose utilization. *Front. Microbiol.* 7:241. doi: 10.3389/fmicb.2016.00241

Huang, Y., Higuchi, Y., Kinoshita, T., Mitani, A., Eshima, Y., and Takegawa, K. (2018). Characterization of novel endo-β-N-acetylglucosaminidases from *Sphingobacterium* species, *Beauveria bassiana* and *Cordyceps militaris* that specifically hydrolyze fucose-containing oligosaccharides and human IgG. *Sci. Rep.* 8:246. doi: 10.1038/s41598-017-17467-y

Ilmén, M., Saloheimo, A., Onnela, M. L., and Penttilä, M. E. (1997). Regulation of cellulase gene expression in the filamentous fungus *Trichoderma reesei*. *Appl.Environ. Microbiol.* 63, 1298–1306. doi: 10.1128/aem.63.4.1298-1306.1997

Jeffery, C. J. (2018). Protein moonlighting: what is it, and why is it important? *Philos. Trans. R. Soc. B Biol. Sci.* 373:1738. doi: 10.1098/rstb.2016.0523

Jorge, J. A., Polizeli, M. L., Thevelein, J. M., and Terenzi, H. F. (1997). Trehalases and trehalose hydrolysis in fungi. *FEMS Microbiol. Lett.* 154, 165–171. doi: 10.1016/S0378-1097(97)00332-7

Klemm, D., Heublein, B., Fink, H. P., and Bohn, A. (2005). Cellulose: fascinating biopolymer and sustainable raw material. *Angew. Chem. Int. Ed.* 44, 3358–3393. doi: 10.1002/anie.200460587

Korman, D. R., Bayliss, F. T., Barnett, C. C., Carmona, C. L., Kodama, K. H., Royer, T. J., et al. (1990). Cloning, characterization, and expression of two α-amylase genes from *Aspergillus niger* var. *awamori*. *Curr. Genet.* 17, 203–212. doi: 10.1007/BF00312611

Krause, S., Le Roux, X., Niklaus, P. A., van Bodegom, P. M., Lennon, T. J. T., Bertilsson, S., et al. (2014). Trait-based approaches for understanding microbial biodiversity and ecosystem functioning. *Front. Microbiol.* 5:251. doi: 10.3389/fmicb.2014.00251

Lamoth, F., Juvvadi, P. R., Fortwendel, J. R., and Steinbach, W. J. (2012). Heat shock protein 90 is required for conidiation and cell wall integrity in *Aspergillus fumigatus*. *Eukaryot. Cell* 11, 1324–1332. doi: 10.1128/EC.00032-12

Langston, J. A., Shaghasi, T., Abbate, E., Xu, F., Vlasenko, E., and Sweeney, M. D. (2011). Oxidoreductive cellulose depolymerization by the enzymes cellobiose dehydrogenase and glycoside hydrolase 61. *Appl. Environ. Microbiol.* 77, 7007–7015. doi: 10.1128/AEM.05815- 11

Langvad, F. (1999). A rapid and efficient method for growth measurement of filamentous fungi. *J. Microbiol. Methods* 37, 97–100. doi: 10.1016/S0167-7012(99)00053-6

Lovero KG and Treseder KK (2021) Trade-Offs Between Growth Rate and Other Fungal Traits. *Front. For. Glob. Change* 4:756650. doi: 10.3389/ffgc.2021.756650

Lovett, G. M., Canham, C. D., Arthur, M. A., Weathers, K. C., and Fitzhugh, R. D. (2006). Forest ecosystem responses to exotic pests and pathogens in eastern North

America. *Bioscience* 56, 395–405. doi: 10.1641/0006-3568(2006)056[0395:FERTEP]2.0.CO;2

Lynd, L. R., Weimer, P. J., van Zyl, W. H., and Pretorius, I. S. (2002). Microbial cellulose utilization: fundamentals and biotechnology. *Microbiol. Mol. Biol. Rev.* 66, 506–577. doi: 10.1128/mmbr.66.3.506-577.2002

Malik, A. A., Martiny, J. B. H., Brodie, E. L., Martiny, A. C., Treseder, K. K., and Allison, S. D. (2020). Defining trait-based microbial strategies with consequences for soil carbon cycling under climate change. *ISME J.* 14, 1–9. doi: 10.1038/s41396-019-0510-0

Martínez, Á. T., Ruiz-Dueñas, F. J., Martínez, M. J., del Río, J. C., and Gutiérrez, A. (2009). Enzymatic delignification of plant cell wall: from nature to mill. *Curr. Opin. Biotechnol.* 20, 348–357. doi: 10.1016/j.copbio.2009.05.002

Maruyama, Y., Nakajima, T., and Ichishima, E. (1994). A 1,2-α-d-mannosidase from a bacillus sp.: purification, characterization, and mode of action. *Carbohydr. Res.* 251, 89–98. doi: 10.1016/0008-6215(94)84278-7]

Maynard, D. S., Bradford, M. A., Covey, K. R., Lindner, D., Glaeser, J., Talbert, D. A., et al. (2019). Consistent trade-offs in fungal trait expression across broad spatial scales. *Nat. Microbiol.* 4, 846–853. doi: 10.1038/s41564-019-0361-5

Mazur, P., Morin, N., Baginsky, W., el-Sherbeini, M., Clemas, J. A., Nielsen, J. B., et al. (1995). Differential expression and function of two homologous subunits of yeast 1,3-beta-D-glucan synthase. *Mol. Cell. Biol.* 15, 5671–5681. doi: 10.1128/mcb.15.10.5671

McGill, B. J., Enquist, B. J., Weiher, E., and Westoby, M. (2006). Rebuilding community ecology from functional traits. *Trends Ecol. Evol.* 21, 178–185. doi: 10.1016/j.tree.2006.02.002

Meletiadis, J., Meis, J. F. G. M., Mouton, J. W., and Verweij, P. E. (2001). Analysis of growth characteristics of filamentous fungi in different nutrient media. *J. Clin. Microbiol.* 39, 478–484. doi: 10.1128/JCM.39.2.478-484.2001

Mitsuzawa, H. (2006). Ammonium transporter genes in the fission yeast *Schizosaccharomyces pombe*: role in ammonium uptake and a morphological transition. *Genes Cells* 11, 1183–1195. doi: 10.1111/j.1365-2443.2006.01014.x

Murata, Y., Homma, T., Kitagawa, E., Momose, Y., Sato, M. S., Odani, M., et al. (2006). Genome-wide expression analysis of yeast response during exposure to 4°C. *Extremophiles* 10, 117– 128. doi: 10.1007/s00792-005-0480-1

Myrold, D. D., Zeglin, L. H., and Jansson, J. K. (2014). The potential of metagenomic approaches for understanding soil microbial processes. *Soil Sci. Soc. Am. J.* 78, 3–10. doi: 10.2136/sssaj2013.07.0287dgs

Nakajima, T., Maitra, S. K., and Ballou, C. E. (1976). An endo α1 → 6 D mannanase from a soil bacterium. Purification, properties, and mode of action. *J. Biol. Chem.* 251, 174–181. doi: 10.1016/S0021-9258(17)33942-X

Nakamura, A., Nishimura, I., Yokoyama, A., Lee, D. G., Hidaka, M., Masaki, H., et al. (1997). Cloning and sequencing of an α-glucosidase gene from *Aspergillus niger* and its expression in *A. nidulans*. *J. Biotechnol.* 53, 75–84. doi: 10.1016/S0168-1656(97)01664-7

Nehls, U., Kleber, R., Wiese, J., and Hampp, R. (1999). Isolation and characterization of a general amino acid permease from the ectomycorrhizal fungus *Amanita muscaria*. *New Phytol.* 144, 343–349. doi: 10.1046/j.1469-8137.1999.00513.x

Nelson, R. E., Lehman, J. F., and Metzenberg, R. L. (1976). Regulation of phosphate metabolism in *Neurospora crassa*: identification of the structural gene for repressible acid phosphatase. *Genetics* 84, 183–192.

Oksanen, J., Blanchet, F. G., Friendly, M., Kindt, R., Legendre, P., McGlinn, D., et al. (2020). *Vegan Community Ecology Package Version 2.5-7.* Available online at: https://cran.r- project.org/web/packages/vegan/index.html (accessed October 15, 2021).

Payne, C. M., Knott, B. C., Mayes, H. B., Hansson, H., Himmel, M. E., Sandgren, M., et al. (2015). Fungal cellulases. *Chem. Rev.* 115, 1308–1448. doi: 10.1021/cr500351c

Perneger, T. V. (1998). What's wrong with Bonferroni adjustments. *Br. Med. J.* 316, 1236–1238. doi: 10.1136/bmj.316.7139.1236

Pitson, S. M., Seviour, R. J., and McDougall, B. M. (1993). Noncellulolytic fungal β-glucanases: their physiology and regulation. *Enzyme Microb. Technol.* 15, 178–192. doi: 10.1016/0141- 0229(93)90136-P

Pitson, S. M., Seviour, R. J., and McDougall, B. M. (1997). Effect of carbon source on extracellular and (1→3)- and (1→6)-β- glucanase production by *Acremonium persicinum*. *Can. J. Microbiol.* 43, 432–439. doi: 10.1139/m97-061

Pugh, G. J. F. (1980). Strategies in fungal ecology. *Trans. Br. Mycol. Soc.* 75, IN1–IN14. doi: 10.1016/s0007-1536(80)80188-4

Pugh, G. J. F., and Boddy, L. (1988). A view of disturbance and life strategies in fungi. *Proc. R. Soc. Edinb. B. Biol. Sci.* 94, 3–11. doi: 10.1017/s0269727000007053

Ramin, K. I., and Allison, S. D. (2019). Bacterial tradeoffs in growth rate and extracellular enzymes. *Front. Microbiol.* 10:2956. doi: 10.3389/fmicb.2019.02956

R Core Team (2020). *R: A language and environment for statistical computing.* Vienna: R Foundation for Statistical Computing.

Reese, E. T., and Mandels, M. (1959). Beta-D-1, 3 glucanases in fungi. *Can. J. Microbiol.* 5, 173–185. doi: 10.1139/m59-022

Ricklefs, R. E., and Starck, J. M. (1996). Applications of phylogenetically independent contrasts: a mixed progress report. *Oikos* 77:167. doi: 10.2307/3545598

Rothman, K. J. (1990). No adjustments are needed for multiple comparisons. *Epidemiology* 1, 43–46. doi: 10.1097/00001648-199001000-00010

Ruiz-Dueñas, F. J., Morales, M., García, E., Miki, Y., Martínez, M. J., and Martínez, A. T. (2009). Substrate oxidation sites in versatile peroxidase and other basidiomycete peroxidases. *J. Exp. Bot.* 60, 441–452. doi: 10.1093/jxb/ern261

Rungrattanakasin, B., Premjet, S., Thanonkeo, S., Klanrit, P., and Thanonkeo, P. (2018). Cloning and expression of an endoglucanase gene from the thermotolerant fungus *Aspergillus fumigatus* DBiNU-1 in *Kluyveromyces lactis*. *Braz. J. Microbiol.* 49, 647–655. doi: 10.1016/j.bjm.2017.10.001

Schimel, J., Balser, T. C., and Wallenstein, M. (2007). Microbial stress-response physiology and its implications for ecosystem function. *Ecology* 88, 1386–1394. doi: 10.1890/06-0219

Schimel, J. P., and Schaeffer, S. M. (2012). Microbial control over carbon cycling in soil. *Front. Microbiol.* 3:348. doi: 10.3389/fmicb.2012.00348

Scott, M., Klumpp, S., Mateescu, E. M., and Hwa, T. (2014). Emergence of robust growth laws from optimal regulation of ribosome synthesis. *Mol. Syst. Biol.* 10:747. doi: 10.15252/msb.20145379

Seidl, V., Huemer, B., Seiboth, B., and Kubicek, C. P. (2005). A complete survey of *Trichoderma chitinases* reveals three distinct subgroups of family 18 chitinases. *FEBS J.* 272, 5923–5939. doi: 10.1111/j.1742-4658.2005.04994.x

Semenova, M. V., Drachevskaya, M. I., Sinitsyna, O. A., Gusakov, A. V., and Sinitsyn, A. P. (2009). Isolation and properties of extracellular β-xylosidases from fungi *Aspergillus japonicus* and *Trichoderma reesei*. *Biochemistry* 74, 1002–1008. doi: 10.1134/S0006297909090089

Sengottaiyan, P., Ruiz-Pavõn, L., and Persson, B. L. (2013). Functional expression, purification and reconstitution of the recombinant phosphate transporter Pho89 of *Saccharomyces cerevisiae*. *FEBS J.* 280, 965–975. doi: 10.1111/febs.12090

Shima, J., Ando, A., and Takagi, H. (2008). Possible roles of vacuolar H+-ATPase and mitochondrial function in tolerance to air-drying stress revealed by genome-wide screening of *Saccharomyces cerevisiae* deletion strains. *Yeast* 25, 179–190. doi: 10.1002/yea.1577

Shimosaka, M., Nogawa, M., Ohno, Y., and Okazaki, M. (1993). Chitosanase from the plant pathogenic fungus, *Fusarium solani* f. sp. *phaseoli*—Purification and some properties. *Biosci. Biotechnol. Biochem.* 57, 231–235. doi: 10.1271/bbb.57.231

Siletti, C. E., Zeiner, C. A., and Bhatnagar, J. M. (2017). Distributions of fungal melanin across species and soils. *Soil Biol. Biochem.* 113, 285–293. doi: 10.1016/j.soilbio.2017.05.030

Singer, M. A., and Lindquist, S. (1998). Thermotolerance in *Saccharomyces cerevisiae*: the Yin and Yang of trehalose. *Trends Biotechnol.* 16, 460–468. doi: 10.1016/S0167-7799(98)01251-7

Slot, J. C., Hallstrom, K. N., Matheny, P. B., and Hibbett, D. S. (2007). Diversification of NRT2 and the origin of its fungal homolog. *Mol. Biol. Evol.* 24, 1731–1743. doi: 10.1093/molbev/msm098

SPSS (2017). *Systat (13.2).* San Jose, SA: Systat Software, Inc.

Teeri, T. T. (1997). Crystalline cellulose degradation: new insight into the function of cellobiohydrolases. *Trends. Biotechnol.* 15, 160–167. doi: 10.1016/S0167-7799(97)01032-9

Tien, M., and Kent Kirk, T. (1983). Lignin-degrading enzyme from the hymenomycete *Phanerochaete chrysasporium* burds. *Science* 221, 661–663. doi: 10.1126/science.221.4611.661

Tiwari, S., Thakur, R., and Shankar, J. (2015). Role of heat-shock proteins in cellular function and in the biology of fungi. *Biotechnol. Res. Int.* 2015:132635. doi: 10.1155/2015/132635

Treseder, K. K., and Lennon, J. T. (2015). Fungal traits that drive ecosystem dynamics on land. *Microbiol. Mol. Biol. Rev.* 79, 243–262. doi: 10.1128/mmbr.00001-15

Versaw, W. K., and Metzenberg, R. L. (1995). Repressible cation-phosphate symporters in *Neurospora crassa*. *Proc. Natl. Acad. Sci. U. S. A.* 92, 3884–3887. doi: 10.1073/pnas.92.9.3884

Vile, D., Shipley, B., and Garnier, E. (2006). Ecosystem productivity can be predicted from potential relative growth rate and species abundance. *Ecol. Lett.* 9, 1061–1067. doi: 10.1111/j.1461-0248.2006.00958.x

Wallenstein, M. D., and Hall, E. K. (2012). A trait-based framework for predicting when and where microbial adaptation to climate change will affect ecosystem functioning. *Biogeochemistry* 109, 35–47. doi: 10.1007/s10533-011-9641-8

Warren, C. R. (2014). Response of osmolytes in soil to drying and rewetting. *Soil Biol. Biochem.* 70, 22–32. doi: 10.1016/j.soilbio.2013.12.008

Webb, C. O., Ackerly, D. D., and Kembel, S. W. (2008). Phylocom: software for the analysis of phylogenetic community structure and trait evolution. *Bioinformatics* 24, 2098–2100. doi: 10.1093/bioinformatics/btn358

Webb, C. O., Ackerly, D. D., McPeek, M. A., and Donoghue, M. J. (2002). Phylogenies and community ecology. *Annu. Rev. Ecol. Syst.* 33, 475–505. doi: 10.1146/annurev.ecolsys.33.010802.150448

Wilmes, P., and Bond, P. L. (2006). Metaproteomics: studying functional gene expression in microbial ecosystems. *Trends Microbiol.* 14, 92–97. doi: 10.1016/j.tim.2005.12.006

Wipf, D., Benjdia, M., Tegeder, M., and Frommer, W. B. (2002). Characterization of a general amino acid permease from *Hebeloma cylindrosporum*. *FEBS Lett.* 528, 119–124. doi: 10.1016/S0014-5793(02)03271-4

Wood, J. L., Tang, C., and Franks, A. E. (2018). Competitive traits are more important than stress-tolerance traits in a cadmium-contaminated rhizosphere: a role for trait theory in microbial ecology. *Front. Microbiol.* 9:121. doi: 10.3389/fmicb.2018.00121

Znameroski, E. A., Coradetti, S. T., Roche, C. M., Tsai, J. C., Iavarone, A. T., Cate, J. H. D., et al. (2012). Induction of lignocellulose-degrading enzymes in *Neurospora crassa* by cellodextrins. *Proc. Natl. Acad. Sci. U. S. A.* 109, 6012–6017. doi: 10.1073/pnas.1118440109

CHAPTER 3

Addressing eco-grief and climate anxiety in the undergraduate classroom

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ABSTRACT

Teaching and studying climate change and other environmental topics can consist of a barrage of dire news resulting in feelings of anxiety, helplessness, and despair in both students and instructors. This emotional response has been coined as eco-grief or climate anxiety. To not confront these emotions in the classroom would be a disservice to students, as anxiety not only impedes cognition, but may also manifest in maladaptive and paralyzing forms. An overarching goal of most environmental classrooms is to foster pro-environmental actions in students, but it is unknown how feelings of eco-grief may affect the knowledge-to-action transformation in the classroom. Understanding how educators may effectively confront feelings of eco-grief in the classroom to foster coping mechanisms and build resilience that results in action is of growing interest to environmental educators. We conducted a two-part study at the University of California, Irvine to address two main objectives: 1) to investigate the relationships between environmental knowledge, attitudes and values, environmental behaviors, and eco-grief in undergraduate students and 2) to examine the effectiveness and impact of an eco-grief course module taught in three undergraduate courses. In support of previous studies, we found positive relationships between environmental knowledge with attitudes and values, and between attitudes and values with behaviors. However, we also found that attitudes and values predicted levels of eco-grief and modified the relationship between environmental knowledge and eco-grief. After discussing eco-grief in the classroom, we found that overall eco-grief levels did not decrease, but the subcategory of psychological impacts worsened, while optimism and hope improved. In

addition, both environmental attitudes and values and behaviors increased. Last, students' motivation toward the environment shifted to be more intrinsic and self-motivated as a result of the eco-grief module. The increase in behaviors may be due to the rise in optimism and resilience elicited by the eco-grief lesson and the shift in motivation toward the environment. We hope that these findings encourage educators to create space for confronting eco-grief and climate anxiety in the classroom.

INTRODUCTION

Globally, we face numerous environmental and socio-ecological challenges, not the least of which is climate change and its many impacts on people and the planet. According to the most recent report by the Intergovernmental Panel on Climate Change (IPCC), the past five years have been the hottest on record since 1850 (2021). This warming has caused changes to our planetary support systems, many of which are irreversible over large time scales: oceans will continue to warm and acidify; polar glaciers will continue to melt; sea levels will continue to rise leading to flooding and coastal erosion; and there will be an increasing occurrence of extreme events across the globe, including heatwaves, wildfires, drought, and heavy precipitation (IPCC, 2021). We will continue to move closer to Earth's tipping point unless large reductions in carbon dioxide and other greenhouse gas emissions occur. Furthermore, human suffering from the impacts of climate change is not equally distributed, a topic also known as climate justice (Schlosberg & Collins, 2014). This presents just a glimpse into the dire information and material that environmental researchers, educators, and students are exposed to on a daily basis.

Climate change impacts span beyond the environment and also affect both physical and mental health (Clayton & Manning, 2018; Crimmins et al., 2016; Obradovich et al., 2018; Watts et al., 2015). Researchers and practitioners who work to address these great environmental

challenges face psychological stressors and high mental health risks (Clayton, 2018; Fraser et al., 2013). Likewise, environmental educators and students spending significant time exposed to topics of climate change and other planetary crises may experience emotional distress and anxiety due to the learning of these "wicked problems" (Dillon & Krasny, 2016; Fraser et al., 2013; Kelly, 2017; Pihkala, 2019; Wallace et al., 2020). Being immersed in negative information with constant awareness of how great these challenges are and how much it will take to solve them can lead to feelings of apathy, anxiety, grief, anger, depression, and despair (Clayton, 2018). Recently, this emotional response to environmental decline has been coined "eco-grief" (Cunsolo et al., 2020), "eco-anxiety" (Usher et al., 2019), or "climate anxiety" (Clayton, 2020). While anxiety can be broadly defined as a feeling of unease due to future-related uncertainty (Grupe & Nitschke, 2013), eco-grief can be characterized into three areas: 1) grief associated with physical ecological losses, 2) grief associated with loss of environmental knowledge systems, and 3) grief associated with anticipated future ecological and cultural losses (Cunsolo & Ellis, 2018). While grief is a rational and adaptive response to climate-related ecological losses, it can also present as more problematic paralyzing forms depending on its manifestation (Pihkala, 2020b). In addition, to not explicitly address anxiety and despair in the environmental classroom could actually increase the likelihood of these emotions being experienced (Wallace et al., 2020), and worse is that anxiety can impede cognition (Maloney et al., 2014). Therefore, the question remains of how educators may confront eco-grief feelings with students to turn grief into pro-environmental action and retain students in environmental fields, as climate impacts are only expected to worsen.

While eco-grief is not fully understood, research is emerging on how to cope with these difficult feelings. A 2017 report by the American Psychological Association (APA) on the

impacts of climate change on mental health called for increased attention to the relationship between the psychological impacts of environmental crises and one's sense of being able to positively contribute or act on climate solutions (Clayton et al., 2017). In addition, Ives et al. states that to best address these great *external* global challenges, *internal* work needs to be done by addressing and channeling emotions, thoughts, identities, and beliefs in productive ways (2020). It is therefore crucial that educators acknowledge, validate, and discuss climate-related emotions to reduce any sense of isolation that students might be feeling (Wallace et al., 2020). Educators can start by sharing information about how to cope with climate anxiety, while creating safe spaces for students to discuss and confront their emotions. Some psychologists also suggest that "the cure for climate anxiety is the same as the cure for climate change – action" (Taylor & Murray, 2020). Therefore, it is important to also provide opportunities for action, empowerment, and meaningful change, however small they may be (Pihkala, 2020b). And since solutions to global problems are larger than one person's capacity to enact change, often the best way to act is collectively through joint action. Fostering a sense of community and working toward a shared goal can create feelings of hope, support, validation, and empowerment reminding students that they are not alone in the fight against climate change (Clayton, 2018). Through classroom interventions, students can come to terms with the reality of climate change and their emotions around this, developing psychological adaptation and emotional resilience so that they may still partake in environmentally-friendly behaviors (Davenport, 2017; J. P. Reser et al., 2012). Therefore, to effectively address eco-grief and climate-anxiety in students and empower action, educators must not just simply teach the discipline, but also educate for critical hope, foster optimism, cultivate coping mechanisms, and promote relational learning and connectedness to community (Ray, 2020).

Environmental educators have traditionally focused on achieving learning objectives related to environmental knowledge, and secondarily on bringing about transformative actions in students which benefit the environment (Ardoin et al., 2020). While knowledge is a prerequisite to action, it has become increasingly accepted that knowledge alone is not enough to result in pro-environmental behaviors (Coppola, 1999; Frisk & Larson, 2011; Van Kerkhoff & Lebel, 2006). Other factors, such as environmental attitudes and values or motivation toward the environment can affect a student's propensity toward engaging in environmentally-friendly behaviors (Gifford & Sussman, 2012). In addition, Carmi et al. found that the effect of knowledge on behavior is fully mediated by emotions, showing the importance of emotions in the learning process (2015). However, little is known about how feelings of eco-grief may influence this knowledge-to-action transformation. This is of interest to environmental educators, as eco-grief may impact both learning and student engagement. Therefore, environmental educators must effectively integrate emotions into the classroom to ensure that we are teaching the whole student, fostering purpose, and building resilience (Schoem et al., 2017). Using a survey tool and eco-grief lesson module, we included the topic of eco-grief in various undergraduate biology courses at the University of California, Irvine (UCI). We asked 1) what are the relationships between environmental knowledge, attitudes and values, environmental behaviors, and eco-grief in undergraduate students, 2) which of these factors best predict eco-grief levels and student engagement in pro-environmental behaviors, and 3) whether factors such as attitudes and values, behaviors, motivation, or eco-grief, might change after the eco-grief lesson module.

METHODS

Study Overview

We conducted a two-part study in the 2019-2020 academic year by surveying the following four undergraduate courses in the School of Biological Sciences at UCI. From Organisms to Ecosystems (Bio Sci 94) is a core lower-division course in the sequence required for undergraduate biology students. Ecosystem Ecology (Bio Sci E118) is an upper-division course covering ecosystem processes and the role of ecosystems in environmental change. Global Change Biology (Bio Sci 9K) is a non-majors field course exploring ways in which humans are altering the global environment. Last, Global Sustainability (Bio Sci 191) constitutes a year-long senior capstone sequence divided into 3 quarters for students working to complete the Global Sustainability Minor. For Part 1 of this study, students from Bio Sci 94 only completed a pre-survey. For Part 2 of this study, students from Bio Sci E118, 9K, and 191 completed a pre-survey, eco-grief lesson module, and a post-survey.

Eco-grief Lesson Module

We directly addressed the topic of eco-grief with students through various classroom activities. Specifically, Pratt developed a module to address this phenomenon in a way that fosters a sense of purpose and builds resilience in students – both of which are essential to turn eco-grief into action and for retention in this field. This lesson was developed for an 80-minute class period and originally designed and taught in Bio Sci 191 in 2018. It included pre-class reading with a reflective journal writing prompt; in-class presentation on the phenomenon of eco-grief; a case study of youth-led environmental action (Sunrise Movement); a structured small group discussion on the topic of eco-grief; and a call to action via the completion of a personal change plan done by each student. This lesson module is described in detail in Pratt *in press*.

Two-Part Study Description

Part 1 - Relationships between environmental knowledge, attitudes & values, behavior, and eco-grief (Pre-survey only)

As mentioned, we conducted this study in two parts. In Part 1, only the pre-survey was given to students in Bio Sci 94 to assess the relationships between the various categories of the survey: *environmental knowledge, attitudes & values, behavior,* and *eco-grief*. In addition, we asked which of these factors best determine a student's level of engaging in environmental behaviors, as this is often an overarching objective of environmental and sustainability courses. Last, we asked which factors predict the degree of eco-grief that a student may be experiencing and if eco-grief may influence whether a student partakes in pro-environmental behaviors. To test this, we surveyed 363 students from Bio Sci 94 in the winter quarter of 2020. Bio Sci 94 was selected as the focal course for Part 1 of this study because it provided us with a large sample size among a broad population of biology students with varying levels of knowledge and interest in science.

Part 2 - Impacts of Eco-grief lesson module (Pre-survey, eco-grief lesson module, post-survey)

In Part 2 of the study, the courses Bio Sci E118, 9K, and 191 completed the pre-survey, eco-grief lesson module, and post-survey to assess effectiveness and impacts of the eco-grief lesson module. Students enrolled in these environmental science and sustainability courses often have a deep interest in the environment and may be more likely to suffer from eco-grief and climate-anxiety. Therefore, these courses were ideal to survey before and after our eco-grief lesson to assess whether the module was effective in alleviating their levels of eco-grief. In addition, an overarching goal of environmental courses is to elicit action and environmental behaviors in students. We also asked whether confronting eco-grief through our lesson module would influence students' frequency of partaking in environmental behaviors. Last, because

instruction was held in a remote format in the spring quarter of 2020, the eco-grief lesson was held online and was taught by Pratt in all three of the courses (i.e. as a guest lecturer in Bio Sci E118 and 9K). After the lesson, students met through video chat to reflect on and discuss the lesson.

Pre- and Post-Survey Design

The pre-survey consisted of the following seven sections: *environmental knowledge, environmental attitudes and values, environmental behaviors, motivation toward the environment, eco-grief, and demographics*. The pre-survey was distributed to a given course after environmental science content had been covered, as we only wanted to assess levels of eco-grief after students had been exposed to information about the environment. The environmental science content covered in each course was course specific. The post-survey also consisted of the same seven sections, with the addition of a section with statements asking students how effective and impactful they felt the lesson was. The post-survey was given to students after submission of a Personal Change Plan Worksheet used in the module (Gass, 2015).

The *environmental knowledge* section consisted of 22 multiple choice questions adapted from previously published resources and reports (Natoli, 2016; O'Brien, 2007). These questions assessed student knowledge on topics such as global energy use, global pollution, climate change, biodiversity, and social and environmental justice issues. The *environmental attitudes and values* section consisted of 34 statements for students to rate on a 5-point Likert scale. These statements related to topics such as ecocentrism versus anthropocentrism, enjoyment of nature, perceived importance of environmental education, and value in economic growth versus protecting the environment. These statements were adapted from previously published surveys assessing environmental attitudes and values (Dunlap et al., 2000; La Trobe & Acott, 2000;

Milfont & Duckitt, 2010; O'Brien, 2007; Vega & Melchor, 2004). A higher score in this section indicates pro-environmental beliefs and attitudes. Students were then asked to indicate how often they partake in 11 sustainable and *environmental behaviors* in their day-to-day life. These behaviors were adapted from previously published surveys and included recycling, conserving electricity, conserving water, and participating in outdoor activities (NEETF, 2002; Vega & Melchor, 2004). Responses ranged from 1 indicating a student never partook in the behavior, to 5 indicating a student almost always partook in the behavior. The *motivation s*ection was directly adapted from the Motivation Toward the Environment Scale by Pelletier et al. (1998). Using a 5-point Likert scale, this section consisted of 24 statements regarding student motivation for adopting sustainable or eco-friendly behaviors. Based on a student's score, they were assigned to a form of motivation: intrinsic, integrated, identified, introjected, externally regulated, or amotivated. A student was assigned multiple forms of motivation if to their score was tied across categories. Next, we developed a series of 19 statements for students to rate on a 5-point Likert scale to assess their level of *eco-grief*. These statements were created to reflect the various emotional and mental health impacts linked to climate change described in the APA report, including feelings of anxiety, hopelessness, and despair (Clayton et al., 2017). Last, students were asked a series of *demographic* questions such as age, the type of environment in which they have lived the longest, their political orientation, and their current major. However, the demographic data was not used in our analyses.

Data Analysis

For Part 1 of our study, we examined single-survey data from an introductory biology course (n = 363 students) for relationships between environmental knowledge, environmental attitudes and values, environmental behaviors, motivation toward the environment, and eco-grief.

Surveys with unanswered questions or inconsistent responses for similar but reversed statements were removed, for a final sample size of n = 354 students. We established the internal consistency of our eco-grief survey questionnaire using Cronbach's alpha in the *psych* package ($\alpha = 0.78$; Revelle, 2021). In addition, we identified five subcategories of our eco-grief survey questionnaire using explanatory factor analysis in the *psych* package using the fa.parallel & fa functions (Revelle, 2021).

Environmental knowledge had a left-skewed distribution, as most scores clustered around ~70%. To transform this data for normality, we squared the proportion of correct answers. For attitudes and values, behaviors, and eco-grief, we used the per-student mean Likert score (1-5) across all questions in a category (with appropriate scores reversed for negatively phrased questions). Motivation toward the environment was analyzed as a score out of 20 separately for each motivation category. We fit simple and multiple linear regression models to test for main effects and interactions between variables in asking the following questions:

Does environmental knowledge predict attitudes and values, behaviors, or eco-grief?
Does attitudes and values predict behaviors or eco-grief?
Does eco-grief predict behaviors?
What factors predict the degree of eco-grief that students experience?
What are the subcategories of eco-grief?
What factors influence the amount of pro-environmental behaviors that students report?

For Part 2 of our study, we conducted pre- vs. post-survey comparisons to determine if and how the various categories of our survey changed after implementation of our eco-grief

lesson module. We used linear mixed effects models (*lme4* package, *lmer* function; Bates et al., 2015) to test the influence of the lesson module on changes in environmental attitudes and values, environmental behaviors, total eco-grief, and the five eco-grief subcategories in 89 students from the three classes, specifying survey (pre or post) as a fixed effect and individual as a random effect. We also nested individual within class (Bio Sci E118, 9K, 191) as a random effect, but excluded it in models which did not converge (total eco-grief, eco-grief factor 5). To test whether the eco-grief lesson influenced motivation, we tested whether students' scores for each of the six motivation categories changed after the lesson module. Since motivation score is bounded from 4-20 and skewed in all categories, we used six separate paired Wilcoxon signed-rank tests and adjusted for multiple comparisons using a Bonferroni-correction (alpha = 0.0083). All statistical analyses were performed using the R computing language (version 4.1.2, R Core Team, 2021).

RESULTS

Part 1 - Relationships between environmental knowledge, attitudes & values, behavior, and eco-grief (Pre-survey only)

Environmental Knowledge

Students in Bio Sci 94 exhibited a moderate level of environmental knowledge (Table 3.1). Environmental knowledge was positively correlated with environmental attitudes and values (Figure 3.1a; $R^2 = 0.28$, $F_{1,352} = 139.9$, $p << 0.001$). Environmental knowledge was not predictive of environmental behaviors ($F_{1,352} = 1.76$, $p = 0.19$) or eco-grief ($F_{1,352} = 0.98$, $p = 0.32$).

Environmental Attitudes & Values

Environmental attitudes and values correlated with student frequency of engaging in environmental and sustainable behaviors (Figure 3.1b; $R^2 = 0.10$, $F_{1,352} = 40.24$, $p \ll 0.001$). Students with more pro-environmental attitudes and values reported engaging more frequently in environmental behaviors. In addition, environmental attitudes and values marginally correlated with eco-grief (Figure 3.1c; $R^2 = 0.027$, $F_{1,352} = 10.07$, $p < 0.05$). Students with greater pro-environmental attitudes and values displayed higher levels of eco-grief.

What factors predict the degree of Eco-grief that students experience?

Together, knowledge (KNO) and environmental attitudes and values (EAV) were vaguely predictive of levels of eco-grief; a model including both variables outperformed models using each individually (vs. EAV, $\Delta AICc = 3.44$; vs. KNO, $\Delta AICc = 12.44$). While environmental attitudes and values weakly predictive on its own ($R^2 = 0.028$), it modified the relationship between knowledge and eco-grief ($p < 0.01$). For students with strong environmental attitudes and values, as knowledge increases so does eco-grief (Figure 3.2; $F_{3,350} = 5.923$, $p < 0.001$, $p_{EAV} = 0.08$, $p_{KNO} < 0.01$, $p_{EAV:KNO} < 0.01$, $R^2 = 0.05$).

What are the subcategories of eco-grief?

Exploratory factor analysis (fa.parallel & fa functions, psych R package) identified five factors (Table 3.2) to sub-classify the eco-grief survey questions, which fit the following general themes: (1) Emotional responses, (2) Psychological impacts, (3) Optimism and hope, (4) Responsibility, and (5) Isolation and depression. Five factors gave the best metrics and the fewest double-loaded questions (RMSR = 0.2, RMSEA = 0.045, Tucker-Lewis Index = 0.947, two cross-loaded items).

What factors influence engagement in environmental behaviors?

We found that eco-grief marginally correlated with the frequency of engaging in environmental behaviors (Figure 3.1d; $R^2 = 0.021$, $F_{1,352} = 7.532$, p-value < 0.01). Students with higher levels of eco-grief reported engaging more frequently in environmental and sustainable behaviors.

The best-fit multiple linear regression model (identified with stepwise regression, ΔAICc = 100.74 from null, ΔAICc = 14.26 from next best model) for predicting behavior had the formula: Behavior ~ EAV + Grief Factor 3 + Grief Factor 2. Together these factors explain 25% of the variance in behavior ($F_{3,350} = 41.12$, $p \ll 0.001$, adjusted $R^2 = 0.25$). Higher environmental attitudes and values ($\beta = 0.75$, $p \ll 0.001$) and higher levels of Grief Factor 2 (Psychological impacts; $\beta = 0.16$, $p \ll 0.001$) were related to higher Behavior scores, while Grief Factor 3 (Optimism, reversed; $\beta = -0.25$, $p \ll 0.001$) negatively varied with behavior; that is, less optimistic (higher score) students reported more pro-environmental behaviors. This model substantially outperformed the best fit model ignoring Grief Factors (using only Total Grief; formula: Behavior ~ EAV + Grief, adjusted $R^2 = 0.12$, $F_{3,350} = 17.73$, $p \ll 0.001$).

Part 2 - Impacts of Eco-grief lesson module (Pre-survey, eco-grief lesson module, post-survey)

In Part 2 of this study, we asked 1) are students suffering from eco-grief and climate and anxiety and 2) were students' level of eco-grief alleviated by our eco-grief lesson module. We also asked how the other categories of our survey changed, such as attitudes and values, motivation toward the environment, and behaviors after the eco-grief lesson module. A total of 89 students completed the pre-survey, eco-grief lesson module, and post-survey from the following courses: Ecosystem Ecology (Bio Sci E118), Global Change Biology (Bio Sci 9K), and Global Sustainability (Bio Sci 191). The average eco-grief score from each course was quite

similar around 3.13, with Bio Sci 191 students displaying the highest average of 3.19 for the pre-survey (Table 3.3). We found no effect of the lesson module on total eco-grief. In other words, eco-grief levels did not change on average for each student after the eco-grief lesson. As overall eco-grief levels did not change, we then analyzed whether any of the five eco-grief factors changed from pre- to post-survey. We found that changes only occur in Grief factors 2 and 3 after the eco-grief lesson module. Grief factor 2 (Psychological impacts) increased post-lesson, with a moderate effect size of 0.1888 (95% CI: 0.0667, 0.311). Grief factor 3 (Optimism and hope) decreased post-lesson, with a fixed effect size of -0.16479 (95% CI: -0.3113860 - 0.0182020).

Furthermore, environmental attitudes and values increased after the eco-grief module, with a fixed effect size of 0.07213 (95% CI: 0.02178118 0.1224885). Likewise, environmental behaviors also increased after the lesson module, with a fixed effect size of 0.15831 (Figure 3.3; 95% CI: 0.07865366 0.2379756). These models each indicate a small but real difference of means (adjusted for random effects) between the factors pre- and post-survey; a fixed effect of 0.15 indicates an increase of that size in the mean Likert score for that category (e.g., from 3.0 to 3.15).

Last, we asked whether students' motivation toward the environment might change after the eco-grief lesson. We found that there was an increase in the Identified, Integrated, and Intrinsic motivation categories after the eco-grief lesson module (Figure 3.4; Table 3.4), which aligns with a greater sense of personal motivation or self-efficacy following this lesson.

DISCUSSION

Part 1 - Relationships between environmental knowledge, attitudes & values, behavior, and eco-grief (Pre-survey only)

The first objective of this study was to determine the relationships between environmental knowledge, attitudes and values, behavior, and eco-grief in undergraduate students. While examining these relationships, we hoped to gain insight into how teachers may best educate students to foster resilience so that knowledge in the classroom may be transformed into meaning environmental action. Furthermore, we asked what role eco-grief may play in this knowledge to action transfer

First, we found that student's level of environmental *knowledge* did not correlate with engagement in environmental *behaviors*. This result was unsurprising as it supports findings from previous studies showing that purely knowledge-based education approaches do not result in environmental behaviors and action (Finger, 1994; Nolet, 2009; Stern, 2000). In addition, measured environmental *knowledge* did not predict *eco-grief* levels. This result was somewhat surprising. We expected that students with higher environmental knowledge, and therefore a better understanding of the long-term challenges posed by climate change, may be more likely to experience high levels of emotional distress and anxiety (Fritze et al., 2008). However, just because a student understands concepts of climate change does not mean that they are necessarily concerned or even care about the state of the environment. This brings us to the next category of our survey, attitudes and values.

A person possesses pro-environmental attitudes and values if they see intrinsic value in nature and promote the holistic conservation and protection of all aspects of the environment, not just those that can be exploited (La Trobe & Acott, 2000). We found that environmental *knowledge* determined environmental *attitudes and values* (Figure 3.1a). In other words, students who were more knowledgeable about the environment also possessed greater attitudes and values toward the environment. This finding was also consistent with previous studies that have

reported a positive relationship between knowledge and pro-environmental attitudes (Arcury, 2008; Bradley et al., 1999; Ramsey & Rickson, 1976). Furthermore, we found that *attitudes and values* predicted student frequency of engaging in environmental and sustainable *behaviors* (Figure 3.1b). Once more, previous studies have also demonstrated a strong link between environmental attitudes with behaviors (Heberlein & Black, 1981; Iversen & Rundmo, 2002; Kuhlemeier et al., 1999; Tarrant & Cordell, 1997). Therefore, to educate students toward partaking in environmental action, we must elicit pro-environmental attitudes and values in the classroom (Gifford & Sussman, 2012). In other words, increasing student levels of environmental knowledge alone is not enough to elicit action (Frisk & Larson, 2011).

In contrast, other prior studies have failed to identify a link between attitudes and values with behaviors (O'Riordan, 1976; Scott & Willits, 1994), indicating that that factors other than attitudes and values may be playing a role in determining behavior. We then asked whether feelings of eco-grief might relate to attitudes and values or behaviors. We identified a positive relationship between *attitudes and values* with *eco-grief* (Figure 3.1c). Students with greater pro-environmental values reported experiencing higher levels of eco-grief. Similar findings have been reported for both climate scientists (Clayton, 2018) and the general population (Searle & Gow, 2010) where people with pro-environmental orientations experienced more climate-related distress. This makes sense, as threats to the environment are experienced as direct threats to something that is highly valued, including personal identity and sense of self (Clayton, 2018).

In addition, we found that *eco-grief* levels determined the frequency of environmental *behaviors* (Figure 3.1d). While it should be mentioned that eco-grief levels were not particularly high in the student population of Bio94 (mean of 3.05), this is an important result as it emphasizes that eco-grief feelings had not reached a paralyzing state (Grose, 2020; Weber 2020)

in students to inhibit them from partaking in pro-environmental behaviors. We expand on this relationship between eco-grief and behaviors below when identifying which factors of the survey best predict engagement in environmental behaviors.

What factors predict the degree of Eco-grief that students experience?

To have a better understanding of eco-grief, we then asked which of the factors assessed in our survey best predict the degree of eco-grief that students experience. As mentioned, environmental knowledge was not predictive of eco-grief levels, while attitudes and values marginally correlated with eco-grief (Figure 3.1c). Here, we found that the best model to predict eco-grief included both environmental knowledge and attitudes and values. Specifically, environmental attitudes and values was found to modify the relationship between environmental knowledge and eco-grief (Figure 3.2). For students with greater pro-environmental attitudes and values, eco-grief levels displayed a positive relationship with environmental knowledge. Therefore, a student possessing pro-environmental attitudes and values may have a stronger emotional response to climate change material the more that they learn about this topic. In other words, for these students, their reactions to climate change may be mediated by their beliefs, values, identities, and experiences (Doherty & Clayton, 2011) all of which are encompassed in the attitudes and values assessment.

What factors influence engagement in environmental behaviors?

To further investigate our question of how to educate students in such a way to foster pro-environmental behaviors, we asked which factors of our survey best determined whether a student engaged in a high frequency of pro-environmental behaviors. We found that greater pro-environmental attitudes and values, higher levels of psychological impacts (Grief Factor 2) and lower levels of optimism (Grief Factor 3) best determined student engagement in pro-

environmental behaviors. As mentioned earlier, previous studies have reported similar findings of positive relationships between attitudes and values with environmental behaviors. However, the relationships between psychological impacts (Grief Factor 2) and optimism (Grief Factor 3) with behaviors is a novel finding.

Psychological impacts (Grief Factor 2) encompassed some of the more extreme psychological responses to climate change, such as experiencing sleep difficulties and personal relationships being affected (Table 3.2). This factor also includes statements related to feeling hyper-aware of behaviors that impact the environment, feelings of defeat toward climate change, and that mental health is negatively affected with increasing knowledge of the environment. Furthermore, high scores of Grief Factor 3 encompassed themes of low optimism and trust in community members and policy makers to protect the environment. Because of this, it is interesting that students with higher psychological impacts and lower optimism are engaging more in environmental behaviors.

It should be emphasized that this model predicting behavior was generated using the pre-survey data from Bio Sci 94. These students were simply introduced to climate change and related topics in their course and then asked to complete the pre-survey. There was no eco-grief lesson module to confront potential eco-grief feelings. Student did not develop coping mechanisms, which may explain the intense emotional and psychological responses, captured in Grief Factor 2. Furthermore, there were no opportunities to foster a sense of community or empowerment by working toward a shared goal. This may explain the lack of optimism and hope in the students who were engaged in pro-environmental behaviors. As a result, it appears that students who are taking action in Bio Sci 94 are likely motivated to do so through feelings of stress, anxiety, and lack of trust in others. Although students are experiencing these intense

emotions, this may simply be a reflection of the connection between the motivational aspects of climate anxiety (anxiety, concern) and the drive to take action to protect something that is valued (Pihkala, 2020b).

Anxiety is not always a negative emotion as it has an adaptive response to possible future threats (Blanchard et al., 2001). Likewise, climate anxiety is an adaptive response to future threats posed by climate change, and it has been shown to motivate pro-environmental actions (Clayton & Karazsia, 2020; Verplanken & Roy, 2013), explaining the above relationships. Importantly, pro-environmental behaviors are more likely to endure in the long term if they are driven by significant and meaningful experiences (Maiteny, 2002). This is akin to having pro-environmental attitudes and values. However, Maiteny also notes that if behavior is motivated by anxiety, it is much more likely to be a temporary habit (2002). Furthermore, these feelings of anxiety and despair can lead to a sense of isolation (Booth, 1997), reflected in the lack of optimism and trust in community members. This is in conflict with the underlying goals of environmental science courses, which strive to create a sense of collective agency where students feel empowered to tackle grand challenges together (Passmore & Howell, 2014; Wallace & Clark, 2018). Considering this, it would be interesting to survey these Bio Sci 94 students again later in their academic career to see whether their anxiety-fueled motivation to take action was short lasting and simply due to the fact that climate change content was just covered in their course. If so, this emphasizes the importance of addressing the emotional component of this content in the classroom.

Part 2 - Impacts of Eco-grief lesson module (Pre-survey, eco-grief lesson module, post-survey)

In part 2 of our study, we asked if students were experiencing eco-grief as they learned about environmental topics in any of the three courses we surveyed. If so, we asked whether our eco-grief lesson was effective at alleviating levels of eco-grief. Furthermore, we asked whether other factors assessed by our survey such as attitudes and values, behaviors, and motivation might change after the eco-grief lesson module. We found that there was not a significant decrease in eco-grief levels in any of the three courses surveyed after the eco-grief lesson module. There may be a few explanations for this observation. First, student reporting of eco-grief could vary from one day to the next. The surveys and eco-grief lesson module were conducted at the same time as the initial lockdown due to Covid-19. It is possible that emotional distress due to the pandemic impacted student responses to the post-survey. Second, eco-grief feelings may not just "disappear." Ecological grief has been contextualized in the field of bereavement (Comtesse et al., 2021). As the mourning of a lost loved one endures, the mourning of the loss of ecosystems and species may be the same. While students may be learning how to confront negative feelings and manage them in a healthier way, it does not mean that the negative feelings ever fully dissipate.

Despite this, the purpose of the eco-grief lesson was not to eliminate grief, but to bring an awareness to it and help students develop coping mechanisms to better live with eco-grief and alleviate its more debilitating forms. Based on student feedback, we feel this was achieved. 58% of students either agreed or strongly agree that they had developed better mechanisms to cope with their emotional responses towards global issues, such as climate change after the eco-grief lesson. In addition, 67% of students agreed or strongly agreed that they now feel better equipped to turn their emotions into action. Last, 78% of students agreed that discussing the topic of eco-

grief with their peers as it validated feelings they were currently experiencing towards global issues, while 89% agreed that this was an effective and impactful lesson.

While total eco-grief levels did not improve after the lesson module, we then analyzed whether any of the five eco-grief subcategories changed after the lesson. We found that Psychological Impacts (Grief Factor 2) worsened from pre- to post-survey. In other words, students reported experiencing more severe psychological responses to climate change material after the eco-grief lesson. One explanation could be that students now had a greater awareness of the grief that they were previously experiencing and its effect on their wellbeing. In addition, we found that optimism and hope (Grief Factor 3) decreased after the lesson module. Because climate anxiety-related feelings decreased, this means that student levels of trust, optimism, and hope increased. Therefore, by creating space to discuss eco-grief, validate emotions, and collectively engage in action, students developed increased emotional resilience and self-efficacy. This is also reflected in our next set of findings.

We found that both environmental attitudes and values and behaviors increased after the eco-grief lesson for the three courses surveyed. Therefore, as students developed coping mechanisms to better live with anxiety, they also learned how to harness the adaptive potential of these difficult emotions toward taking action (Cunsolo et al., 2020; Pihkala, 2020a). Our finding of an increase in psychological impacts (Grief Factor 2) after the lesson module aligns with previous studies on psychological adaptation to climate change. These studies reported a positive relationship between psychological distress in response to environmental destruction and engagement in pro-environmental behaviors (Helm et al., 2018; J. Reser et al., 2014). As mentioned previously, “the cure for climate anxiety is the same as the cure for climate change – action” (Taylor & Murray, 2020). We helped to facilitate a sense of collective action by

introducing students to youth-led environmental movements, creating space for small group discussions on eco-grief, and having students complete a personal change plan. Engaging in pro-environmental behaviors may serve as both a coping mechanism and an outlet for students to express their emotions regarding climate change.

Last, we observed a significant change in students' motivation toward the environment. This section of the survey assessed student levels of self-determined (intrinsic vs. extrinsic) motivation for engaging in environmental behaviors (Pelletier et al., 1998). We observed a shift in motivation toward the environment from the pre- to post-survey for the categories of Identified, Integrated, and Intrinsic motivation, indicating a greater sense of self-efficacy in environmental behaviors after the lesson module (Figure 3.4; Table 3.4). This indicated that students were more self-motivated to engage in the behaviors and choices they made in regard to the environment, as opposed to making decisions based on fear of potential consequences. As pro-environmental attitudes and values may be needed to bridge the gap between knowledge and behaviors, motivation may be similar (Pelletier, 2002).

In conducting this study, we realize that many educators may feel that it is not within their job description to be addressing student mental health in the classroom, and that this is better reserved for wellness or counseling centers on campus. Teachers are not trained on how to confront psychological distress among their students, let alone support them through it. Some might even find little encouragement for bringing emotions into the classroom. Despite this lack of training, students need support. The Center for Collegiate Mental Health reported that more than 61% of undergraduate students are experiencing stress and anxiety (CCMH 2019), which can be worsened by lack of support from faculty (Jones et al., 2018). Furthermore, college wellness centers are often understaffed and overbooked (CCMH 2019). Findings from a recent

survey of faculty members from 12 colleges and universities across the United States showed that almost 80% of faculty have had conversations with students regarding mental health in the past 12 months, while 73% welcome additional training on responding to student mental health with 61% believing such training should be mandatory (Lipson, 2021). So, while educators may not feel qualified, they play a much larger role in their student's mental health than they may realize, emphasizing the importance of integrating student wellbeing into classroom curriculum especially when there is evidence that the course curriculum itself may have negative psychological impacts on students.

In summary, climate change-induced distress is likely to become more frequent in the coming years. We found that together, environmental knowledge and environmental attitudes and values may determine whether a student experiences eco-grief. It would be expected that students enrolled in sustainability and environmental science courses hold close relationships to the natural environment. This highlights the importance of creating space in the classroom to confront the potential emotional responses that students may have as they learn about climate change and related losses. While our lesson did not eliminate eco-grief, students reported an increase in feelings of trust, optimism, hope, and resilience. In addition, motivation toward the environment shifted after the lesson module in such a way where motivation was fueled by intrinsic factors and self-efficacy. Furthermore, both environmental attitudes and values and the frequency of partaking in environmental behaviors increased. This increase may be due to the increase in optimism and resilience elicited by the eco-grief lesson and the shift in motivation toward the environment. Furthermore, taking action may be serving as an adaptive response for students, and indicates that their climate anxiety is not at debilitating levels (E. Gifford & Gifford, 2016). To not address and validate the negative emotions associated with the learning of

this dire information represents a missed opportunity that can disadvantage students. In closing, eco-grief should not be regarded as a problem, but rather an indication that our students care a great deal about the state of the planet (Clayton, 2020; Clayton & Karazsia, 2020). With these findings, we hope that educators feel more encouraged to take a holistic approach by explicitly addressing eco-grief and climate anxiety in the classroom.

Table 3.1 Descriptive summary statistics across different survey components for n = 354 students in Bio Sci 94. Statements were rated on a 5-point Likert scale. A score of 5 indicates a high level of that metric, while a score of 1 indicates a low level of that metric.

Metric	***Mean***	***Std.Dev.***	***Median***
Environmental Attitudes and Values	3.73	0.43	3.76
Behaviors	3.43	0.62	3.45
Eco-Grief	3.05	0.41	3.00
Environmental Knowledge	13.74	3.16	14.00

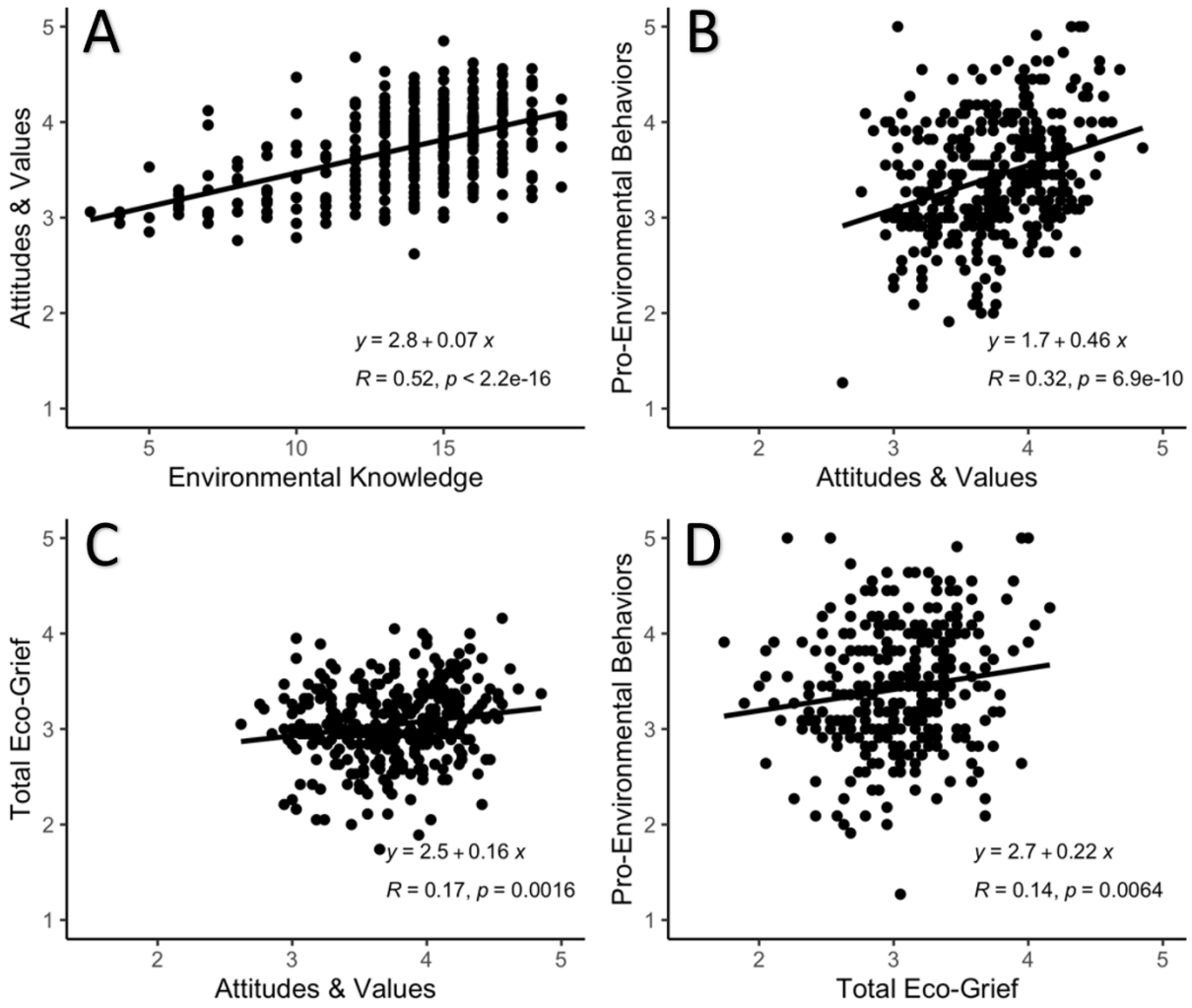

Figure 3.1. Relationships between environmental knowledge, attitudes & values, pro-environmental behaviors, and eco-grief for Bio Sci 94 students. *R* represents the correlation coefficient. A) Environmental knowledge is positively correlated with environmental attitudes and values ($R^2 = 0.28$, $F_{1,352} = 139.9$). Students who are more knowledgeable about environmental issues tend to have higher environmental attitudes and values. B) Environmental attitudes and values are positively correlated with student frequency of engaging in environmental behaviors ($R^2 = 0.10$, $F_{1,352} = 40.24$). Students with greater pro-environmental attitudes and values reported engaging more frequently in environmental behaviors. C) Environmental attitudes and values are positively correlated to eco-grief ($R^2 = 0.028$, $F_{1,352} = 10.07$). Students with greater pro-environmental attitudes and values had higher levels of eco-grief. D) Eco-grief is positively correlated with frequency of engaging in environmental behaviors ($R^2 = 0.021$, $F_{1,352} = 7.532$). Students with higher levels of eco-grief reported engaging more frequently in environmental behaviors.

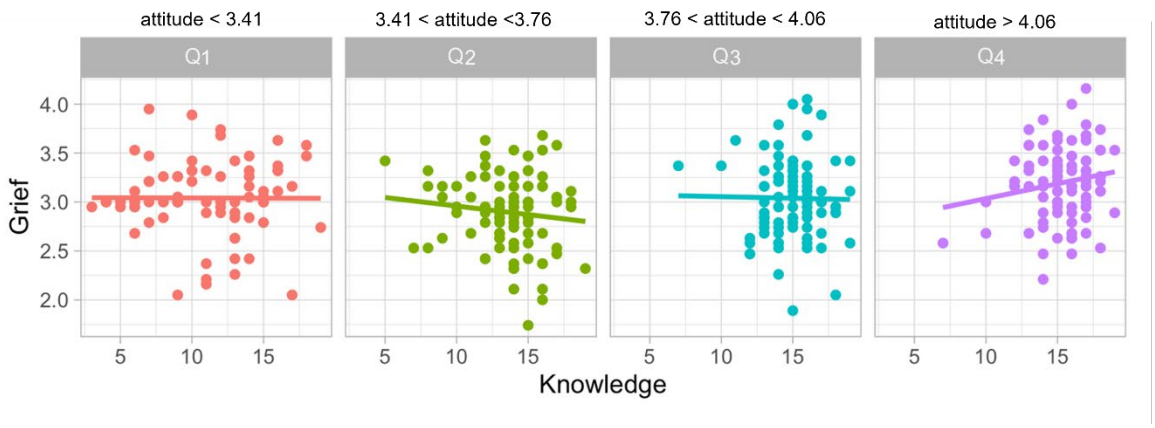

Figure 3.2. Relationship between environmental knowledge and eco-grief depending on environmental attitude and values quantile. Students with the highest scores for EAV (purple; EAV > 4.06) have a positive relationship between greater knowledge and higher levels of grief.

Table 3.2. Eco-grief factors identified with exploratory factor analysis. Five factors gave the best metrics and the fewest double-loaded questions (RMSR = 0.2, RMSEA = 0.045, Tucker-Lewis Index = 0.947, two cross-loaded items are signified in italics, meaning that a statement was placed into two different factors two different times). "*" indicates a question with a reversed expected response. Cronbach's alpha for each factor is reported in the Description column.

Factor	*Description*	*Survey Questions*
1	Emotional responses ($\alpha = 0.84$)	• I experience feelings of guilt when partaking in non-sustainable behaviors (ex: using single-use plastics, leaving the lights on, not recycling, traveling in an airplane, etc). • I feel overwhelmed when thinking of all the issues surrounding climate change. • *I feel that my overall mental health is negatively affected the more I learn about climate change.* • I feel anxious when thinking about the potential impacts of climate change. • I often feel fearful for the future of our planet and future generations. • I feel a sense of despair when picturing the world that future generations might live in if climate change continues as its current rate.
2	Behavioral effects, emotional, psychological response ($\alpha = 0.74$)	• *I feel that my overall mental health is negatively affected the more I learn about climate change.* • I feel like so much damage to our environment has already occurred that there is no point in trying to make things better. • *I feel hyper-aware of the impact that my actions have on the environment.* • I experience difficulties sleeping as I am worried about the impacts of climate change. • I feel that some of my personal relationships have been affected due to my response to climate change impacts.
3	trust, optimism, community, hope ($\alpha = 0.64$)	• *I feel that many people are doing their part to reduce their carbon footprint. • *I trust that policy makers will make the right decisions to protect our planet. • *I feel that humans are resilient creatures and we will recover from whatever the impacts of climate change will be.
4	Action-orientation, personal responsibility ($\alpha = 0.63$)	• I feel that certain populations, often those that contribute the least to the problem, are disproportionately affected by climate change. • I experience feelings of guilt when partaking in non-sustainable behaviors (ex: using single-use plastics, leaving the lights on, not recycling, traveling in an airplane, etc). • *The more I learn about environmental issues, the more motivated I am to take action. • *I feel hopeful when reading or hearing of new solutions to combat climate change. • *I feel hyper-aware of the impact that my actions have on the environment.*
5	Isolation, depression, hopelessness, fatalism ($\alpha = 0.69$)	• I feel alone in my worries about climate change. • Nothing will help with my negative feelings in regards to climate change; it is just something I need to come to terms with.

Table 3.3. Pre-survey vs. Post-survey total grief scores for the three courses assessed: Ecosystem Ecology (Bio Sci E118), Global Change Biology (Bio Sci 9K), and Global Sustainability (Bio Sci 191). Statements were rated on a 5-point Likert scale. A score of 5 indicates a high level of eco-grief, while a score of 1 indicates a low level of eco-grief.

Class	***N***	***Mean Grief (±SD)***	
		Pre	Post
Bio9K	19	3.17 ± 0.30	3.09 ± 0.45
E118	51	3.04 ± 0.43	3.09 ± 0.44
GS	19	3.19 ± 0.47	3.20 ± 0.43

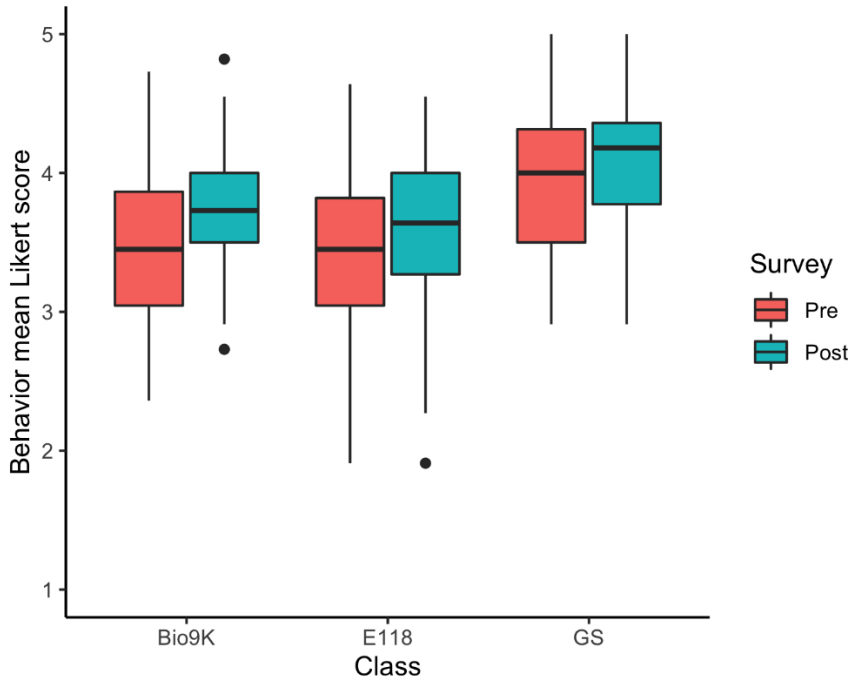

Figure 3.3. Student frequency in partaking in environmental behaviors increased after the eco-grief module. Boxes represent interquartile range and whiskers are 1.5x the interquartile range. Points represent outliers beyond the 1.5x interquartile range. The middle line of each box represents the median.

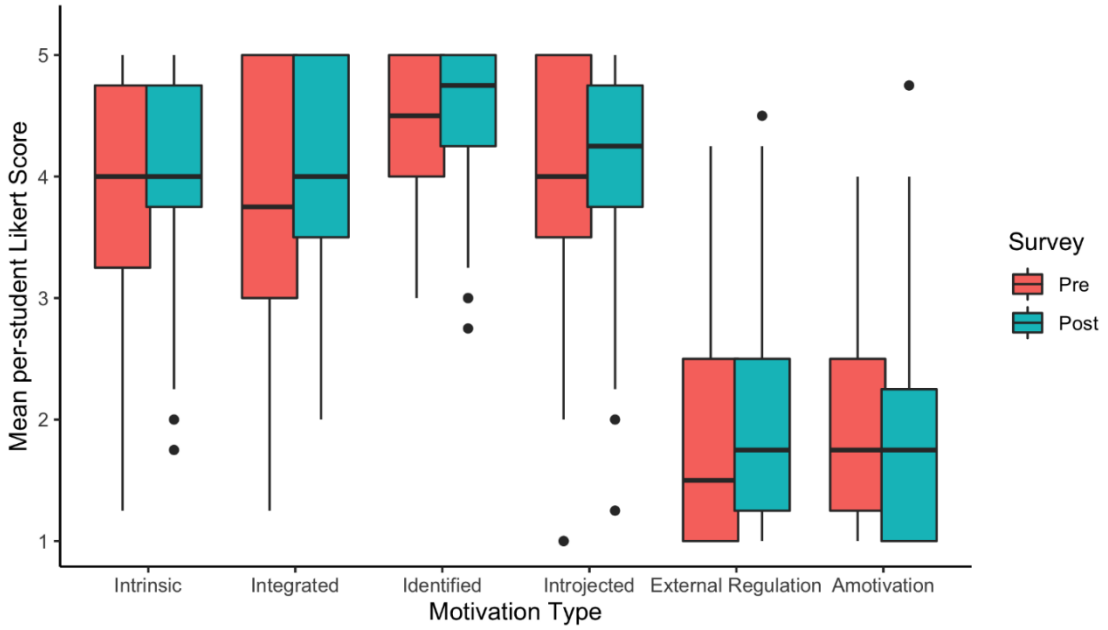

Figure 3.4. There is significant change after the eco-grief module in the identified, integrated, and intrinsic motivation categories. Motivations are ordered on the x-axis from the most self-determined type of motivation to the least. Boxes represent interquartile range and whiskers are 1.5x the interquartile range. Points represent outliers beyond the 1.5x interquartile range. The middle line of each box represents the median.

Table 3.4. There is significant increase after the eco-grief module in the identified, integrated, and intrinsic motivation categories.

Motivation	W.stat	n	p.value	alpha	significance
Intrinsic	618	89	0.0072	0.0083	*
Integrated	442.5	89	0.0013	0.0083	**
Identified	180	89	0	0.0083	***
Introjected	745	89	0.2889	0.0083	NS
External Regulation	924	89	0.7125	0.0083	NS
Amotivation	1013.5	89	0.7957	0.0083	NS

REFERENCES

Arcury, T. (2008). Environmental attitude and environmental knowledge. *Human Organization*, *49*(4), 300–304. https://doi.org/10.17730/humo.49.4.y6135676n433r880

Ardoin, N. M., Bowers, A. W., & Gaillard, E. (2020). Environmental education outcomes for conservation: A systematic review. *Biological Conservation*, *241*. https://doi.org/10.1016/j.biocon.2019.108224

Bates, D., Mächler, M., Bolker, B. M., & Walker, S. C. (2015). Fitting linear mixed-effects models using lme4. *Journal of Statistical Software*, *67*(1). https://doi.org/10.18637/jss.v067.i01

Blanchard, D. C., Hynd, A. L., Minke, K. A., Minemoto, T., & Blanchard, R. J. (2001). Human defensive behaviors to threat scenarios show parallels to fear- and anxiety-related defense patterns of non-human mammals. *Neuroscience and Biobehavioral Reviews*, *25*(7–8). https://doi.org/10.1016/S0149-7634(01)00056-2

Booth, R. (1997). Existential loneliness: the other side of the void. *International Journal of Transpersonal Studies*, *16*(1).

Bradley, J. C., Waliczek, T. M., & Zajicek, J. M. (1999). Relationship between environmental knowledge and environmental attitude of high school students. *Journal of Environmental Education*, *30*(3). https://doi.org/10.1080/00958969909601873

Carmi, N., Arnon, S., & Orion, N. (2015). Transforming environmental knowledge into behavior: the mediating role of environmental emotions. *Journal of Environmental Education*, *46*(3). https://doi.org/10.1080/00958964.2015.1028517

Clayton, S. (2018). Mental health risk and resilience among climate scientists. *Nature Climate Change, 8*(4). https://doi.org/10.1038/s41558-018-0123-z

Clayton, S. (2020). Climate anxiety: Psychological responses to climate change. *Journal of Anxiety Disorders*, *74*, 102263. https://doi.org/10.1016/j.janxdis.2020.102263

Clayton, S., & Karazsia, B. T. (2020). Development and validation of a measure of climate change anxiety. *Journal of Environmental Psychology*, *69*. https://doi.org/10.1016/j.jenvp.2020.101434

Clayton, S., & Manning, C. (2018). Psychology and climate change: Human perceptions, impacts, and responses. In *Psychology and Climate Change: Human Perceptions, Impacts, and Responses*. https://doi.org/10.1016/C2016-0-04326-7

Clayton, S., Manning, C. M., Krygsman, K., & Speiser, M. (2017). Mental Health and our changing climate: Impacts, implications, and guidance. Washington, D.C.: American Psychological Association, and ecoAmerica. *American Psychological Association*, *March*, 36. http://www.apa.org/news/press/releases/2017/03/mental-health-climate.pdf

Comtesse, H., Ertl, V., Hengst, S. M. C., Rosner, R., & Smid, G. E. (2021). Ecological grief as a response to environmental change: A mental health risk or functional response? *International Journal of Environmental Research and Public Health*, *18*(2). https://doi.org/10.3390/ijerph18020734

Coppola, N. W. (1999). Greening the technological curriculum: A model for environmental literacy. *The Journal of Technology Studies*, *25*(2). https://doi.org/10.21061/jots.v25i2.a.9

Crimmins, A., Balbus, J., Gamble, J. L., Beard, C. B., Bell, J. E., Dodgen, D., Eisen, R. J., Fann, N., Hawkins, M., Herring, S. C., Jantarasami, L., Mills, D. M., Saha, S., Sarofim, M. C., Trtanj, J., & Ziska, L. (2016). The impacts of climate change on human health in the United States: A scientific assessment. *U.S. Global Change Research Program*, 312.

http://dx.doi.org/doi:10.7930/J00P0WXS
Cunsolo, A., & Ellis, N. R. (2018). Ecological grief as a mental health response to climate change-related loss. *Nature Climate Change*, *8*(4). https://doi.org/10.1038/s41558-018-0092-2
Cunsolo, A., Harper, S. L., Minor, K., Hayes, K., Williams, K. G., & Howard, C. (2020). Ecological grief and anxiety: the start of a healthy response to climate change? *The Lancet. Planetary Health*, *4*(7), e261–e263. https://doi.org/10.1016/S2542-5196(20)30144-3
Davenport, L. (2017). *Emotional resiliency in the era of climate change: A clinician's guide*. Jessica Kingsley Publishers.
Dillon, J., & Krasny, M. E. (2016). Chapter 7. The emotional life of the environmental educator: John Fraser and Carol B. Brandt. In *Trading Zones in Environmental Education*. https://doi.org/10.3726/978-1-4539-0854-9/19
Doherty, T. J., & Clayton, S. (2011). The psychological impacts of global climate change. *American Psychologist*, *66*(4). https://doi.org/10.1037/a0023141
Dunlap, R. E., Van Liere, K. D., Mertig, A. G., & Jones, R. E. (2000). Measuring endorsement of the new ecological paradigm: A revised NEP scale. *Journal of Social Issues*, *56*(3), 425–442. https://doi.org/10.1111/0022-4537.00176
Finger, M. (1994). From knowledge to action? Exploring the relationships between environmental experiences, learning, and behavior. *Journal of Social Issues*, *50*(3). https://doi.org/10.1111/j.1540-4560.1994.tb02424.x
Fraser, J., Pantesco, V., Plemons, K., Gupta, R., & Rank, S. J. (2013). Sustaining the conservationist. *Ecopsychology*, *5*(2). https://doi.org/10.1089/eco.2012.0076
Frisk, E., & Larson, K. (2011). Educating for sustainability: Competencies & practices for transformative action. *Journal of Sustainability Education*, *2*(March).
Fritze, J. C., Blashki, G. A., Burke, S., & Wiseman, J. (2008). Hope, despair and transformation: Climate change and the promotion of mental health and wellbeing. *International Journal of Mental Health Systems*, *2*. https://doi.org/10.1186/1752-4458-2-13
Gass, R. (2015). *Personal Change Plan Worksheet*. https://atctools.org/wp-content/uploads/toolkit-files/personal-change-plan.pdf
Gifford, E., & Gifford, R. (2016). The largely unacknowledged impact of climate change on mental health. *Bulletin of the Atomic Scientists*, *72*(5). https://doi.org/10.1080/00963402.2016.1216505
Gifford, R., & Sussman, R. (2012). Oxford handbooks online environmental attitudes. In *The Oxford Handbook of Environmental and Conservation Psychology* (Issue October).
Grose, A. (2020). *A guide to eco-anxiety: how to protect the planet and your mental health*. Watkins Media.
Grupe, D. W., & Nitschke, J. B. (2013). Uncertainty and anticipation in anxiety: An integrated neurobiological and psychological perspective. *Nature Reviews Neuroscience, 14(*7). https://doi.org/10.1038/nrn3524
Heberlein, T. A., & Black, J. S. (1981). Cognitive consistency and environmental action. *Environment and Behavior*, *13*(6). https://doi.org/10.1177/0013916581136005
Helm, S. V., Pollitt, A., Barnett, M. A., Curran, M. A., & Craig, Z. R. (2018). Differentiating environmental concern in the context of psychological adaption to climate change. *Global Environmental Change*, *48*. https://doi.org/10.1016/j.gloenvcha.2017.11.012
IPCC, 2021: Climate change 2021: The physical science basis. Contribution of working group I to the sixth assessment report of the intergovernmental panel on climate change

[Masson-Delmotte, V., P. Zhai, A. Pirani, S.L. Connors, C. Péan, S. Berger, N. Caud, Y. Chen, L. Goldfarb, M.I. Gomis, M. Huang, K. Leitzell, E. Lonnoy, J.B.R. Matthews, T.K. Maycock, T. Waterfield, O. Yelekçi, R. Yu, and B. Zhou (eds.)]. Cambridge University Press. In Press.

Iversen, H., & Rundmo, T. (2002). Environmental concern and environmental behaviour among the Norwegian public. *Journal of Risk Research*, *5*(3). https://doi.org/10.1080/13669870110115434

Ives, C. D., Freeth, R., & Fischer, J. (2020). Inside-out sustainability: The neglect of inner worlds. *Ambio*, *49*(1), 208–217. https://doi.org/10.1007/s13280-019-01187-w

Jones, P. J., Park, S. Y., & Lefevor, G. T. (2018). Contemporary college student anxiety: The role of academic distress, financial stress, and support. *Journal of College Counseling*, *21*(3). https://doi.org/10.1002/jocc.12107

Kelly, A. (2017). Eco-anxiety at university: Student experiences and academic perspectives on cultivating healthy emotional responses to the climate crisis. *Independent Study Project (ISP) Collection*, *Spring*.

Kuhlemeier, H., van den Bergh, H., & Lagerweij, N. (1999). Environmental knowledge, attitudes, and behavior in dutch secondary education. *Journal of Environmental Education*, *30*(2). https://doi.org/10.1080/00958969909601864

La Trobe, H. L., & Acott, T. G. (2000). A modified NEP/DSP environmental attitudes scale. *Journal of Environmental Education*, *32*(1), 12–20. https://doi.org/10.1080/00958960009598667

Lipson, S. K. (2021). *The role of faculty in student mental health*.

Maiteny, P. T. (2002). Mind in the gap: Summary of research exploring "inner" influences on pro-sustainability learning and behaviour. *Environmental Education Research*, *8*(3). https://doi.org/10.1080/13504620220145447

Maloney, E. A., Sattizahn, J. R., & Beilock, S. L. (2014). Anxiety and cognition. *Wiley Interdisciplinary Reviews: Cognitive Science*, *5*(4). https://doi.org/10.1002/wcs.1299

Milfont, T. L., & Duckitt, J. (2010). The environmental attitudes inventory: A valid and reliable measure to assess the structure of environmental attitudes. *Journal of Environmental Psychology*, *30*(1), 80–94. https://doi.org/10.1016/j.jenvp.2009.09.001

Natoli, C. (2016). *Technical guidance for assessing environmental justice in regulatory analysis*. *June*.

Nolet, V. (2009). Preparing sustainability-literate teachers. *Teachers College Record*, *111*(2). https://doi.org/10.1177/016146810911100207

O'Brien, S. R. M. (2007). Indications of environmental literacy: Using a new survey instrument to measure awareness, knowledge, and attitudes of university-aged students. *Retrospective Theses and Dissertations*, *15055*, 113. https://lib.dr.iastate.edu/rtd/15055

O'Riordan, T. (1976). Attitudes, behavior, and environmental policy issues. In *Human Behavior and Environment*. https://doi.org/10.1007/978-1-4684-2550-5_1

Obradovich, N., Migliorini, R., Paulus, M. P., & Rahwan, I. (2018). Empirical evidence of mental health risks posed by climate change. *Proceedings of the National Academy of Sciences of the United States of America*, *115*(43), 10953–10958. https://doi.org/10.1073/pnas.1801528115

Passmore, H. A., & Howell, A. J. (2014). Eco-existential positive psychology: Experiences in nature, existential anxieties, and well-being. *Humanistic Psychologist*, *42*(4). https://doi.org/10.1080/08873267.2014.920335

Pelletier, L. G. (2002). A motivational analysis of self-determination for pro-environmental behaviors. In *Handbook of Self-Determination Research*.

Pelletier, L. G., Tuson, K. M., Green-Demers, I., Noels, K., & Beaton, A. M. (1998). Why are you doing things for the environment? The Motivation Toward the Environment Scale (MTES). *Journal of Applied Social Psychology*, *28*(5), 437–468. https://doi.org/10.1111/j.1559-1816.1998.tb01714.x

Pihkala, P. (2019). The cost of bearing witness to the environmental crisis: Vicarious traumatization and dealing with secondary traumatic stress among environmental researchers. *Soc Epistemol, 34*(1):86-100. https://doi.org/10.1080/02691728.2019.1681560

Pihkala, P. (2020a). Anxiety and the ecological crisis: An analysis of eco-anxiety and climate anxiety. *Sustainability (Switzerland)*, *12*(19). https://doi.org/10.3390/SU12197836

Pihkala, P. (2020b). Eco-anxiety and environmental education. *Sustainability (Switzerland)*, *12*(23). https://doi.org/10.3390/su122310149

R Core Team (2021). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/.

Ramsey, C. E., & Rickson, R. E. (1976). Environmental knowledge and attitudes. *The Journal of Environmental Education*, *8*(1), 10–18. https://doi.org/10.1080/00958964.1976.9941552

Ray, S. J. (2020). A Field Guide to Climate Anxiety. *University of California Press*. 217 p. https://doi.org/10.2307/j.ctvw1d67m

Reser, J., Bradley, G., Glendon, I., Ellul, M., & Callaghan, R. (2014). Public risk perceptions, understandings, and responses to climate change and natural disasters in Australia and Great Britain, Gold Coast, Australia: National Climate Change Adaptation Research Facility.

Reser, J. P., Bradley, G. L., & Ellul, M. C. (2012). Coping with climate change: Bringing psychological adaptation in from the cold. In *Handbook of the Psychology of Coping: New Research*.

Revelle, W. (2021) psych: Procedures for personality and psychological research, Northwestern University, Evanston, Illinois, USA, https://CRAN.R-project.org/package=psych Version = 2.1.9,.

Schlosberg, D., & Collins, L. B. (2014). From environmental to climate justice: climate change and the discourse of environmental justice. *WIREs Climate Change*, *5*(3), 359–374. https://doi.org/https://doi.org/10.1002/wcc.275

Schoem, D. L., Modey, C., & St.John, E. P. (2017). *Teaching the whole student: engaged learning with heart, mind, and spirit* (D. L. Schoem, C. Modey, & E. P. St. John (eds.); First edition.) [Book]. Stylus Publishing, LLC.

Scott, D., & Willits, F. K. (1994). Environmental attitudes and behavior: A Pennsylvania survey. *Environment and Behavior*, *26*(2). https://doi.org/10.1177/001391659402600206

Searle, K., & Gow, K. (2010). Do concerns about climate change lead to distress? *International Journal of Climate Change Strategies and Management*, *2*(4). https://doi.org/10.1108/17568691011089891

Stern, P. (2000). New environmental theories: toward a coherent theory of environmentally significant behavior. *Journal of Social Issues*, *56*.

Tarrant, M. A., & Cordell, H. K. (1997). The effect of respondent characteristics on general environmental attitude-behavior correspondence. *Environment and Behavior*, *29*(5). https://doi.org/10.1177/0013916597295002

Taylor, M., & Murray, J. (2020). 'Overwhelming and terrifying': the rise of climate anxiety. *The Guardian*, *February*.

Americans' Low "Energy IQ:" A Risk to Our Energy Future. The National Environmental Education and Training Foundation (NEETF). (2002). In *The Tenth Annual National Report Card: Energy Knowledge, Attitudes, and Behavior*.

Usher, K., Durkin, J., & Bhullar, N. (2019). Eco-anxiety: How thinking about climate change-related environmental decline is affecting our mental health. *International Journal of Mental Health Nursing, 28*(6). https://doi.org/10.1111/inm.12673

Van Kerkhoff, L., & Lebel, L. (2006). Linking knowledge and action for sustainable development. *Annual Review of Environment and Resources*, *31*. https://doi.org/10.1146/annurev.energy.31.102405.170850

Vega, C. L. de la, & Melchor, R. (2004). Awareness, knowledge, and attitude about environmental education: Resp. *Electronic Theses and Dissertations*, *2004–2019*(178). https://stars.library.ucf.edu/etd/178

Verplanken, B., & Roy, D. (2013). "My worries are tational, climate change is not": Habitual ecological worrying is an adaptive response. *PLoS ONE*, *8*(9). https://doi.org/10.1371/journal.pone.0074708

Wallace, R. L., & Clark, S. G. (2018). Environmental studies and sciences in a time of chaos: problems, contexts, and recommendations. *Journal of Environmental Studies and Sciences*, *8*(1). https://doi.org/10.1007/s13412-018-0469-6

Wallace, R. L., Greenburg, J., & Clark, S. G. (2020). Confronting anxiety and despair in environmental studies and sciences: an analysis and guide for students and faculty. *Journal of Environmental Studies and Sciences*, *10*(2). https://doi.org/10.1007/s13412-020-00609-6

Watts, N., Adger, W. N., Agnolucci, P., Blackstock, J., Byass, P., Cai, W., Chaytor, S., Colbourn, T., Collins, M., Cooper, A., Cox, P. M., Depledge, J., Drummond, P., Ekins, P., Galaz, V., Grace, D., Graham, H., Grubb, M., Haines, A., … Costello, A. (2015). Health and climate change: policy responses to protect public health. *Lancet (London, England)*, *386*(10006), 1861–1914. https://doi.org/10.1016/S0140-6736(15)60854-6

Weber, J.A. Climate cure: Heal yourself to heal the planet; Llewellyn Publications: Woodbury, NY, USA, 2020.