An evolutionary consideration of stress response, biodiversity, and anthropogenic context

by

Sam Lipson Sharpe

B.A., Carleton College, 2014

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Division of Biology
College of Arts and Sciences

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2024

Abstract

Variation is inherent in nature and underlies all evolutionary processes, with organismal diversity representing both a response to and a prerequisite for these processes. This dissertation considers fundamental concepts of evolutionary biology, including biodiversity, categorization of similarity and difference, and environmental heterogeneity, against the backdrop of profound threats to ecological diversity and marginalized human populations due to anthropogenic activities. In this way, evolutionary considerations of biodiversity in the Anthropocene epoch occur at the interface of nature and culture and can be understood through Donna Haraway's term *natureculture*. My research uses a naturecultural framework to investigate stress response across populations of a wild plant species and explore strategies for improving representation and inclusion of sex, gender, and sexuality diversity in biology classrooms. To assess how local adaptation can impact physiological and transcriptomic responses to anthropogenically mediated sources of abiotic stress, I utilized climatically diverse populations of the wild foxtail millet, *Setaria viridis*. This species has many advantageous qualities as a model system, including widespread populations across a range of habitats, high genetic diversity, short generation times, small size, high rates of self-pollination, copious seed production, and a relatively small, sequenced diploid genome. Using 9 populations of *S. viridis* from geographically and climatically diverse home ranges, I compared photosynthetic response to drought stress in a greenhouse dry down experiment. With a subset of these populations, I assessed root surface area, aboveground biomass, and sensitivity to drought stress across plants of different ages. I found significant differences in drought tolerance by population which was not correlated with climate of origin but may be partially explained by differences in total plant size and ratio of root surface area to aboveground biomass. For *S. viridis* plants within the same population, plant age

at the start of drought impacted both overall drought sensitivity and total production of inflorescences. To further address questions of local adaptation and abiotic stress response, I selected 2 *S. viridis* populations that demonstrated divergent physiological responses to drought stress and compared transcriptomic response across drought, chilling, and salinity stress conditions. I found substantial variability in the number and function of genes significantly up or down regulated between populations and treatment conditions, with only a few genes and gene functions showing similar response patterns across both populations or multiple stress conditions. There was, however, notable alignment of differently expressed genes from both *S. viridis* populations with a database of nearly 200 molecularly characterized drought tolerance genes. In an additional consideration of diversity, interface between science and culture, and anthropogenically mediated stress and resilience, I explored current issues in biology education that exclude or mischaracterize sex, gender, and sexuality diversity, and discussed emerging alternate pedagogical strategies for increasing inclusivity. I drew parallels between the oversimplified presentations of sex across taxa as a universal male/female binary in many biology classrooms, the ongoing social, legal and medical harms faced by queer, transgender, and intersex people, and the use of appeals to just such a universal sex binary to justify and perpetuate these harms. Importantly, biology education also offers many powerful opportunities to challenge such harmful misconceptions through providing more comprehensive, nuanced, and inclusive conceptualizations of sex, gender, and sexuality diversity across the tree of life. Through the integration of evolutionary, ecological, genomic, pedagogical, and naturecultural approaches, the research presented in this dissertation has important implications for understanding biodiversity, stress response, and resilience to anthropogenic contexts for human and non-human organisms.

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Major Professor
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Land Acknowledgement

"As the first land-grant institution established under the 1862 Morrill Act, we acknowledge that the state of Kansas is historically home to many Native nations, including the Kaw, Osage, and Pawnee, among others. Furthermore, Kansas is the current home to four federally recognized Native nations: The Prairie Band Potawatomi, the Kickapoo Tribe of Kansas, the Iowa Tribe of Kansas and Nebraska, and Sac and Fox Nation of Missouri in Kansas and Nebraska. Many Native nations utilized the western plains of Kansas as their hunting grounds, and others--such as the Delaware--were moved through this region during Indian removal efforts to make way for White settlers. It's important to acknowledge this, since the land that serves as the foundation for this institution was, and still is, stolen land. We remember these truths because K-State's status as a land-grant institution is a story that exists within ongoing settler-colonialism, and rests on the dispossession of Indigenous peoples and nations from their lands. These truths are often invisible to many. The recognition that K-State's history begins and continues through Indigenous contexts is essential" (K-State's Indigenous Faculty and Staff Alliance 2020).

Parts of this dissertation have also been written during my time living in Mni Sota Makoce on the ancestral homeland of the Dakota people who were forcibly exiled from this land because of aggressive and persistent settler colonialism. In making these land acknowledgements, we recognize the continued significance of these lands for many Native nations, and we pay our respects to the ancestors as well as past, present, and emerging Native leaders. In acknowledging the Indigenous contexts through which K-State's history (and the history of all settler colonial states and institutions) begins and continues on stolen land, we remember that "settler colonialism must be understood as a multi-fronted project of making the First Peoples of a place extinct; it is a relentless structure, not contained in a period of time" (Arvin et al. 2013).

And in making these acknowledgements, we make visible the ways in which the work presented in this dissertation has not only been conducted on stolen land but is also situated within settler colonial structures. As a white settler with Ashkenazi and Eastern European ancestry, I have had access to freedom of movement to travel nationally and internationally for

academic conferences and research collaborations during my graduate program, a freedom that settler colonial states often have denied and continue to deny the Indigenous peoples they have colonized. My research on plant response to environmental stress must be understood through the context of increasingly frequent, severe, and stochastic extreme weather events, soil salinization, and habitat loss due to anthropogenic climate change and ecological degradation. Colonialism and imperialism are major drivers of climate change, environmental contamination, and habitat destruction, and Indigenous peoples are disproportionately impacted by the consequences of these processes while simultaneously safeguarding 80% of the world's remaining biodiversity ("Climate Stories | Indigenous Peoples" 2023). As I prepare to submit my revised dissertation in August of 2024, the genocide being carried out in Gaza by the occupying settler colonial state of Israel is in its 10th month, the blockade on goods and movement in and out of Gaza is in its 17th year, and the occupation of Palestine is in its 76th year (Haddad 2022). In 2023, during the first 2 months of this escalated violence towards Palestinians living under decades-long settler colonial occupation in Gaza, the state of Israel's aerial bombardment and ground invasion generated over 281,000 metric tons of carbon dioxide, the equivalent of burning more than 150,000 tons of coal (Lakhani 2024). While it is not currently possible to calculate the total human and environmental impacts of this ongoing colonial military besiegement (which is heavily financially supported by US institutions and US taxpayer dollars), the ecological devastation and genocide of Indigenous people are intertwined, long-lasting, and catastrophic (Murray and Bishara 2024; Salvatori 2024).

The fourth chapter of this dissertation is a response to the complicity of biology and biology education in upholding colonial and white supremacist ideologies that position sex and gender as intrinsically binary. Settler colonial violence against Indigenous peoples has been both justified by and escalated towards Indigenous cultural conceptualizations of gender beyond a man/woman binary (Miranda 2010). Indigenous queer, transgender, 2 Spirit, and gender diverse individuals continue to face escalated and intersectional forms of oppression (CRIB 2021), including state-sanctioned and state-condoned violence. In 2023, Tortuguita, an Indigenous queer and nonbinary forest defender, was killed by Georgia State Troopers while protesting the creation of a police training center within Atlanta's Weelaunee Forest (Goldberg 2023; Riley and Hershkind 2023). Just over a year later, Nex Benedict, an Indigenous trans and nonbinary high school student, died the day after being harassed and physically assaulted by

3 other students in a girl's bathroom at his high school in Oklahoma, a state which legally requires students to use gendered bathroom facilities corresponding to their sex designated at birth (Logan 2024).

Just as settler colonialism is an ongoing and relentless structure (Arvin et al. 2013), so must be the work for those of us like myself who are settlers to continue learning these often intentionally suppressed truths, to interrogate the histories we were taught and the structures and institutions we exist within, and to support and be in solidarity with the liberation and sovereignty of Indigenous peoples. As a place to start, I direct those reading this who are also settlers to visit the K-State Indigenous Faculty and Staff Alliance's website if they are K-State affiliated, and to use the tools at https://native-land.ca/ to learn more about whose lands they are on.

In acknowledging the connections between academic activities and institutions, settler colonialism, land theft and occupation, ecological destruction, and the resulting inequitable life chances for Indigenous peoples, it is critical to address our multifaceted complicity in these systems and engage in ongoing and material work, beyond just acknowledgement, to dismantle colonial structures and oppressions.

Personal Acknowledgements

The completion of this dissertation has been made possible through the support, wisdom, and encouragement of an entire ecosystem of people, and this section represents only a partial list. I would first like to thank my advisor, Mark Ungerer, for his incredible support and guidance as I have navigated my doctoral research and a variety of other life challenges. I am profoundly grateful for his kindness, patience, expertise, and creativity throughout this process. He has been extraordinarily flexible and generous in encouraging some of the more unconventional turns that my path through graduate school has taken, including designing my own interdisciplinary course, taking science communication and critical theory graduate classes in other departments, and writing one of my chapters on a topic outside of his lab's typical study systems. I would not have been able to complete this program without the phenomenal support he provided me, both as a scientist and a person.

I thank my other committee members Michi Tobler, Christopher Toomajian, and Jesse Nippert for their guidance and advise in developing my research and scholarship. Jesse Nippert

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Weaver for letting me take their graduate courses as a biology student, helping me design and teach my own interdisciplinary course, and serving as unofficial mentors to me as a trans person navigating academia. They allowed me the possibility to find a wholeness I had never imagined in my relationship with science, and without which this dissertation and my previous publications would not have been achievable.

Before coming to K-State, I had no idea how I would navigate life in Kansas as a queer and trans person, and I thank the many incredible members of the Manhattan and broader Kansas queer community for doing such essential work and providing incredible friendships. Thank you to the SAGA and Gender Collective leaders who have taught me so much and modeled so much fierce love and resistance over the years, including Seth, Riley, Lain, Jakki, Ash, Sophie P., Colton, Rachel H., Milo, Alex, Ray, Roxie, Ryn, Delia, Jarred, Anna, Julia, Courage, and Jamie P. Thank you to the other queer Kansas community members who I have had the privilege of meeting and organizing with, including Kevin S., An, Trish, Elise, Steph, Carol, Brandi, Emerson, Courtney H., Graham S., and Katie B. Thank you to CJ Janovy for the work you are doing to tell the stories of LGBTQIA+ people in Kansas and for including me in your documentary. Thank you to all the amazing drag performers I've had the honor of sharing both physical and virtual stages with. I thank Rexy, Phoenix, Katie R., and Stephanie Mott for the opportunity to know them and for everything they were as activists and trans people. May their memories be for a blessing and a revolution.

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Dedication

As Dr. Robin Wall Kimmerer writes in her book *Braiding Sweetgrass*, “plants can be eloquent in their physical responses and behaviors. Plants answer questions by the way they live, by their responses to change; you just need to learn how to ask.” This dissertation is dedicated to them.

Chapter 1 - Introduction

> ***We learn about worlds when they do not accommodate us. Not being accommodated can be pedagogy. We generate ideas through the struggles we have to be in the world; we come to question worlds when we are in question.*** **-Sarah Ahmed, An Affinity of Hammers**

Variation is inherent and myriad across biological life on earth, serving as both a progenitor and consequence of evolution. Through the scale of evolutionary time and the setting of a non-homogenous biosphere, natural selection has produced a multitude of bidirectional transitions between difference and sameness. Single species partition available niches or disperse and adapt to disparate climates, ancestrally distinct species converge on analogous phenotypes to accommodate a shared environment, and assemblages of species form coevolutionary dynamics in which each one drives the other's adaptive trajectory through reciprocation or antagonism. Across these processes of sameness and difference, convergence and radiation, and biological or spatial barriers that lead to reproductive isolation, evolutionary biologists have applied taxonomies and phylogenies, names and categories and nodes of genetic divergence. Through these methods of systematics, the field has sought to quantify the precise moments in phylogenetic time when ancestral sameness becomes evolutionary difference, an imperfect and repeatedly revised process.

Evolutionary histories themselves are not intentional nor are they inevitable. Phenotypic and abiotic constraints, environmental heterogeneity, and genetic drift contribute substantially to the history of life on earth and the composition of extant biodiversity. Chance, context, and diversity are integral components of these histories. Natural selection cannot occur without the

presence of heritable variation, and evolutionary fitness always exists relative to a specific climatic, geographic, and ecosystemic context. No single phenotype or trait is universally optimal as these contexts are highly variable across place and time, and each presents particular challenges that render certain organismal characteristics more advantageous than others. Constitutive and plastic traits which confer higher tolerance to the stressors and challenges of a given ecological context are critical contributors to differential outcomes of competition, survival, and reproductive capacity that ultimately determine whether a species will persist there.

Since the Industrial Revolution, the effect of human activities on the Earth's ecosystems has been so great that some believe we have created an Anthropocene epoch, substantially shaping the contexts driving current and future patterns of evolution across taxa (Harden et al. 2014). Consequently, scientific inquiry into the predicted evolutionary trajectories of wild populations must consider the impact of both historical and human contexts. Species with highly specialized niches, limited opportunity for migration, acute sensitivity to environmental change, and vulnerable habitats are likely to be more threatened by the impacts of climate change and other anthropogenic effects than those with more generalist habits. At the ecosystem level, biodiversity has been shown to predict greater resilience and functionality (Naeem et al. 2012; Oliver et al. 2015; Dainese et al. 2019).

These evolutionary principals of convergence and divergence, heterogeneity, context, and resilience are prominent both in ecological genomics, the already interdisciplinary field in which my doctoral research was initiated, and in the self-reflective process of engaging in scientific inquiry itself. This perspective on evolutionary processes and knowledge creation spans the human and biological worlds, sometimes in unexpected ways, resulting in what Donna Haraway and Banu Sumbramaniam describe as a *natureculture*. (Haraway and Goodeve 2013;

Subramaniam and Bartlett 2023). The concept of a natureculture includes the many reciprocal ways in which biodiversity, scientific inquiry, and human culture shape and intersect with one another. For example, an unusually severe drought in Kansas during the summer of 2018 provided the opportunity for several of my classmates to study the responses of various fishes to the unprecedented fragmentation of a perennial reach of a headwater prairie stream within the context of predicted increases in frequency and severity of drought under anthropogenic climate change (Hopper et al. 2020). Another group of classmates utilized data collected on grassland songbirds during the 2018 drought to contrast their behavior during this anomalous year to other years in a longer term study, enabling unexpected and potentially predictive insights into the impacts of a more arid future (Smith et al. 2023) And while the global impacts of the COVID-19 pandemic in 2020 were profoundly disruptive for many field biologists, others were able to pivot towards previously unexplored areas of research, such as Caleb Krueger, who, unable to collect the anticipated data for his master's research, instead analyzed 50 years of turtle sex ratio data and developed the Reptilian Offspring Sex and Incubation Environment Database (ROSIE) (Krueger and Janzen 2022).

Similarly, the naturecultural context in which the work of my dissertation took place has also played a fundamental role in shaping its content. CJ Janovy's 2018 book and the 2022 documentary it inspired, both titled *No Place Like Home*, explore the experiences of queer and trans people who come to or stay in Kansas, which "In the American imagination…is a place LGBT people leave" (Janovy 2018). To survive in a context with such strong selective pressures, "Activism is a constant necessity for LGBTQ+ people in Kansas" (Janovy qtd in Dulin 2023).

I came to Kansas in 2015 to study the ecological genomics of plant adaptation. A key feature of plant biology is that, as sessile organisms, plants must adjust their development,

remodel their morphology, and reconfigure their biochemistry to survive inhospitable or changing environments. In doing so, they can be extraordinarily resilient to a variety of contexts. I saw analogous forms of resilience, adaptability, and fierce self-preservation in queer communities that persist perennially in a place many would expect them to leave. Over time, to survive in these contexts, I have also been compelled to develop similar adaptations, and this process has had indelible impact on my work as a biologist.

Sarah Ahmed writes, "we come to question worlds when we are in question" (pg. 1., Ahmed 2016). As a queer and visibly gender-non-conforming person in Kansas, my existence, my civil rights, and the validity of my own organismal biological have been constantly called into question through unaccommodating social and political contexts. This has impacted my life and research in daily and material ways. During the course of my graduate studies at Kansas State University, I encountered reminders that I was unwelcome and in question when insults were shouted at me as I rode my bike to campus, when I had to go to a different floor or sometimes a different building to locate a bathroom in which I would not receive denigratory stares or comments, and when I struggled to find health care providers that would provide me with competent medical treatment rather than fixating on my unconventional body and appearance.

As Ahmed predicted, these experiences, while distracting and distressing, have compelled me to questions and pedagogies and areas of inquiry that I may otherwise have ignored. I learned that the buildings in which I couldn't find a bathroom, the doctors who had never been trained to treat a patient like me, and the empowerment of strangers to shout slurs at me were all connected through specific naturecultural trajectories that were entrenched in the history of biology yet not taught to biology students. I saw the eerie parallels between the

political appeals to the supposed biological validity of sex binaries, gender essentialism, and conscripted heterosexuality that were increasingly being used to invalidate my existence and the universalizing language about sex and reproduction in the undergraduate curriculum I was being asked to teach. The personal and communal threats posed by these weaponized misconceptions of human biology, the disconnect between understandings of diversity in biology research and in workforce diversity and inclusion initiatives, and the presentation of a ubiquitous biological sex binary in contrast to the acceptance of profound variability in other phenotypic traits became unignorable to me. I questioned whether there could not be a better way to approach teaching, support for human diversity, and conceptualizations of sex variation, an approach that could counteract these anthropogenic threats to resilient, biodiverse, and sustainable cultural and scientific communities.

These questions have led me to other scholars and other disciplines seeking similar answers, and ultimately to the final research chapter of my dissertation. It is this area of inquiry that has led me to understand evolutionary biology as a natureculture that encompasses my own lived experience of gender diversity, the endeavor of my doctoral research, and the context of escalating pseudoscientific political attacks on LGBTQIA+ community members. It took an interdisciplinary approach to understand the biological sciences as itself a context, one with a specific, naturecultural history. From reading critical theory, I developed an understanding of disciplinary systems of knowledge production that legitimate some ways of knowing while rendering others illegible (Kimmerer 2013). Through collaboration with feminist studies scholars, I learned that the idea of a discrete sex binary did not originate from validated biological inquiry but from cultural prejudices validated by eugenics and subsequently enshrined into a self-perpetuating ideology (Somerville 1994; Subramaniam and Bartlett 2023). Gaining

access to these intellectual frameworks for understanding context, categorization, and anthropogenic effects on biodiversity has been an essential part of my own biological education that I passionately believe all students should be able to access. Therefore, one of my chapters is devoted to an examination of these topics and approaches for incorporating them into biology classrooms and curricula.

While my dissertation is unconventional in including both plant ecological genomics research and considerations of strategies for queer inclusive biology education, the concepts of diversity and variation, imperfect categorization, relationship to context, anthropogenic impacts, and resilience to stress and change are prominent throughout all chapters. Chapters 2 and 3 consider the physiological and transcriptomic responses of a wild panicoid grass to environmental stress, assessing differences in stress tolerance and adaptive mechanisms across climatically diverse populations. Chapter 4 is an exploration of existing limitations and promising approaches for creating more LGBTQIA+ inclusive curricula and pedagogy within biology classrooms.

Chapter 2- Variations in drought tolerance across climatically diverse populations of the wild foxtail millet, *Setaria viridis*

Local adaptation is a putative driver of differences in abiotic stress tolerance between populations within a species. Drought is a potent source of abiotic stress, particularly for sessile organisms, which is predicted to increase in severity and frequency as climate change progresses. Susceptibility to the effects of drought stress can also vary throughout an organism's life cycle. Domesticated crop species can experience genetic bottlenecks which reduce diversity, and wild relatives can be an important source of genetic diversity which confers favorable traits such as

stress tolerance. The wild foxtail millet *Setaria viridis* is a close relative to the economically important domestical foxtail millet *Setaria italica*, and *S. viridis* possess many favorable characteristics for a plant model system, including small size, short life cycle, high fecundity, a sequenced, diploid genome, self-fertilization, and considerable genetic diversity across populations. We measured the physiological impact of drought stress on 9 populations of *S. viridis* from climatically diverse home ranges, as well as the susceptibility of *S. viridis* plants of different ages to the effects of drought stress.

Chapter 3- Comparisons of transcriptomic response to drought, chilling, and salinity stress in two *Setaria viridis* populations

As sessile organisms, plants cannot flee from stress and therefore must rely on developmental and physiological strategies to withstand the impacts of stressful or changing environments. Regulation of gene expression in response to abiotic stress is a key mediator of these stress response mechanisms. Changes in expression of functional genes and transcription factor genes in response to stress are integral to these transcriptomic responses, and both functional and transcription factor gene families may be conserved across response pathways to different forms of abiotic stress. Some common stress responsive genes and pathways have been identified both across species and across populations within a species. We assessed the transcriptomic response of 2 populations of *S. viridis* to drought, salinity, and chilling stress through comparing the numbers, fold change, and ontology of differently expressed genes across population and treatment condition. We also blasted differently expressed genes identified in our experiment against the DroughtDB, a database of nearly 200 molecularly characterized genes involved in plant drought response (Alter et al. 2015).

Chapter 4- Creating LGBTQIA+ inclusive classrooms and curricula

Considerations of biodiversity and human diversity within the biological sciences are frequently siloed, and issues of human diversity are typically excluded from the core content of research and instruction. However, these mindsets and practices ignore the co-constitutive nature of science and society as it impacts marginalized populations who have been historically excluded from participation in the production of biological knowledge and historically exploited or neglected by its practices. Biology education plays a key role in maintaining this harmful status quo and presents a critical and promising opportunity to better account for naturecultural histories of science and society and reduce barriers to advancement and participation for marginalized demographics. We explore the current barriers preventing biology education from adequately serving and representing the LGBTQIA+ community and propose alternate approaches for curricular representation and inclusive pedagogy.

Chapter 5- Synthesis and Implications

Attentiveness to context, ongoing processes of change, interrogation of categories, and variation along spectrums are key principles of evolutionary biology across scales and contexts. In Chapter 2, we found significant variation in drought response (as measured by declines in photosynthetic rate) across *S. viridis* populations. In contrast to our predictions, there were no significant correlations between population drought tolerance and either root surface area or climatic variables characterizing each population's home range. We noted a tendency of plants which flowered earlier to have improved drought tolerance, which aligns with existing evidence that a shorter life cycle and earlier reproduction can improve the chances of successful

reproduction for plants living in stressful environments. Plants exposed to drought stress at 4 weeks old were also shown to produce more inflorescences than plants exposed to drought stress at either 3 or 5 weeks old, indicating variability in the impacts of drought stress across development.

In Chapter 3, overall patterns of gene expression regulation likely indicated an increase in the number of up and downregulated genes for the population more susceptible to each form of stress. We found that transcriptomic responses were largely distinct at the gene level, with minimal overlap in differently expressed genes and overrepresented gene ontology terms across population or treatment condition. However, there was notable overlap in the DroughtDB genes to which these differently expressed genes from each population and treatment condition aligned. The broadly divergent responses to environmental stress across populations and treatment conditions may be indicative of the high heterogeneity previously observed between populations of *S. viridis,* though the overlap in alignment with the DroughtDB genes across populations may point to some shared expression patterns for genes with similar functions.

In Chapter 4, we discussed the potential harms of taking a status quo approach to teaching sex, gender, and sexuality diversity within biology classrooms, including limiting exposure to the full range of organismic diversity, alienating LGBTQIA+ students, and fomenting prejudicial attitudes about queerness as unnatural or "un-biological." We offered alternate strategies for discussing sex and reproductive diversity across taxa without anthropomorphizing non-human organisms or projecting human gender stereotypes onto them. In approaching human sex, gender, and sexuality diversity, we provided methods for contextualizing these traits as naturecultures whose conceptualization has been shaped by

intertwined biological and cultural trajectories. Finally, we suggested teaching approaches to support student learning around these topics in an inclusive and validating learning environment.

Overall, these areas of inquiry collectively engage with key evolutionary principals and further a unique approach to biological research and education within both proximate and broader disciplinary contexts.

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Chapter 2 - Variations in drought tolerance across climatically diverse populations of the wild foxtail millet, *Setaria viridis*

Abstract

Local adaption, in which different populations of the same species develop traits that increase fitness within the local environment, is an important process in driving intraspecies diversity. Location specific adaptations to commonly encountered sources of abiotic stress are especially important for plants, as they are sessile organisms unable to relocate when subjected to stressors such as drought, which can cause substantially deleterious physiological effects. We investigated local adaptation to drought stress in 9 climatically diverse populations of the wild foxtail millet, *Setaria viridis*, through a greenhouse dry down experiment in which changes in photosynthetic rate over time were measured to assess drought response. We also assessed differences in drought response for plants of different ages from 2 *S. viridis* populations with variable drought response. Our analyses indicated significant differences in drought response across the 9 populations as well as significant differences in drought response between plants of different ages within the same population. We did not find significant relationships between population drought response and root surface area or any of 19 bioclimatic variables related to home range climate. Plant biomass appeared to be a driver of drought response in both experiments, with populations that had more vegetative tissue showing greater sensitivity to drought stress than smaller populations and older (and bigger) plants succumbing more quickly to drought stress than younger plants from the same population. We also noted that some populations produced more inflorescences than others, and plants that were 4 weeks old at the start of the dry down experiment produced more total inflorescences than plants that were either 3 or 5 weeks old.

Overall, our results indicate that populations of *S. viridis* from climatically diverse home ranges differ in their response to drought stress, that plant size influences drought tolerance, and that reproductive output is differentially affected by drought stress based on plant age at the start of drought. These findings may aid in identifying favorable adaptive traits present in some *S. viridis* populations that can be utilized to improve abiotic stress tolerance in its agriculturally important relative, *Setaria italica*.

Introduction

Plants vary substantially in their ability to withstand abiotic stress (Sharma et al. 2020). Adaptation to different types of stress allows plants to occupy a variety of climatic and ecosystem niches across the planet (Kawakami et al. 2011). Significant differences in abiotic stress tolerance exist not only between species, but also between populations within a species (Kooyers 2015). The primary process that can lead to the development of differences in tolerance of environmental stress between populations of the same species is local adaptation (Sork 2017). Local adaptation that differentiates populations within a species commonly arises in response to widespread geographic distribution and differences in selective pressures between locations (Kawakami et al. 2011; Farkas et al. 2015). However, divergent selective pressures can also drive local adaption among individuals in close geographical proximity, especially when environments are highly heterogeneous (Selby and Willis 2018; Tobler et al. 2019) . Additionally, variations in stress tolerance adaptation can exist not only between populations within a species, but also between different parts of a single organism's life cycle (Zhang et al. 2011; Motamedi et al. 2019).

Drought is a significant source of abiotic stress in many parts of the world and is predicted to increase in frequency and severity as climate change progresses (Dai 2013; Fracasso et al. 2016). Shifting and intensifying patterns of drought can significantly impact plants' ability to survive and reproduce optimally within a particular geographic range (Sharma et al. 2020). These effects are predicted to be substantial for both wild plants and domesticated crop species.

To explore questions of drought tolerance and local adaptation, we focused on *Setaria viridis*, the wild foxtail millet. *Setaria viridis* (L.) P. Beauv. is a grass species in the Poaceae family and the subfamily Panicoideae (Kellog 2017 (book chapter)). It is closely related to the domesticated foxtail millet, *Setaria italica*, which has been cultivated in China for over 8,000 years, nearly as long as the common millet *Panicum miliaceu* (Lu et al. 2009). *Setaria italica* remains an agriculturally important crop in China and India and is cultivated in other parts of the world for birdseed and fodder (Li and Brutnell 2011).

Domesticated crop species like *S. italica* can experience genetic bottlenecks, and introducing genetic material from wild relatives can be an effective way to enhance traits like drought tolerance. Diversifying and increasing drought tolerance of staple crops is increasingly important as droughts are predicted to increase in severity and frequency across much of the world due to anthropogenic climate change (Peng et al. 2010; Lata et al. 2011; Feldman et al. 2018; Li et al. 2021). *Setaria italica* has been shown to have only 55% of the genetic diversity of *S. viridis*, and the wild species has many more unique polymorphisms compared to its domesticated relative (Hu et al. 2018). Notably, *S. italica* is quite drought tolerant relative to many cereal crops (Peng and Zhang 2021) and is cross compatible with *S. viridis* (Li and Brutnell 2011), providing a useful opportunity to increase diversity and abiotic stress tolerance of this domesticated crop species through interbreeding with its wild relative.

Setaria viridis possesses many qualities that make it an excellent plant model system. It is a short-lived annual with C_4 photosynthesis (Brutnell et al. 2010), high rates of self-fertilization (Defelice 2002), relatively small size, copious seed production (Li and Brutnell 2011), and a small (ca. 500 Mb) sequenced diploid genome (Mamidi et al. 2020). Originating in Eurasia, *S. viridis* is now found in many temperate regions worldwide in both native and introduced locations, and demonstrates considerable morphological variation (Defelice 2002), providing ample opportunity for studies of local adaptation.

In the current study, we explored whether populations of *S. viridis* from diverse locations, selected based on variation in precipitation regimes, differed in their drought response utilizing a common garden greenhouse dry-down experiment. In subsequent stages of the experiment, we assessed the combined effects of plant age and population on drought tolerance in this species. We used photosynthetic rate as our primary physiological measurement of drought tolerance and drought response. Photosynthesis is a universally important plant process (Sharma et al. 2020) and one of the most sensitive to drought stress (Sun et al. 2013). Measuring changes in photosynthetic rate allowed us to precisely capture the progressing effects of drought stress on our experimental plants. Reproductive output of plants was assessed under drought conditions, as were other traits including root surface area and aboveground biomass.

Methods

Setaria viridis populations

Seeds were obtained from the United States Department of Agriculture's Germplasm Resources Information Network (USDA GRIN) (**https://www.ars-grin.gov**) for 9 populations of *S. viridis* from geographically diverse parts of the world with different levels of average annual

precipitation (Table 2.1, Figure 2.1). Average annual precipitation values for each location of origin were extracted from the WorldClim database (Fick and Hijmans 2017). Populations were selected to represent a diverse variety of home-range precipitation levels, with average annual precipitation for the 9 populations varying from 158 to 1279 mm per year (Table 2.1, Fick and Hijmans 2017).

Germination procedure

Seeds were stored in a 4°C refrigerator in sealed bags with desiccant beads prior to planting. Germination was performed in an indoor growth room with a 12-hour photo period and 22°C temperature . Seeds were planted in MetroMix 360 in 6 cm-deep open trays. Soil was fully hydrated and allowed to rest for 10 minutes before seeds were added and pressed slightly below the soil surface. Trays were covered with clear plastic covers for the first 3 nights to increase humidity. Seedlings began germinating after 5-7 days. Approximately 10-14 days after planting, trays were moved to the greenhouse where they received natural light and were watered as necessary.

After 2-3 weeks, seedlings were transferred to individual 8.9 cm diameter plastic pots. Pots were filled with a 6:3:1 mixture of MetroMix360, Quikrete Commercial Grade Medium Sand, and local prairie soil from which all large roots, rocks, and insects were removed. Pots were filled and weighed to standardize the amount of soil, then watered to saturation before seedlings were transplanted.

Dry down 1: population comparison

Seeds from 9 populations representing a diverse range of precipitation regimes were planted in May 2018, as previously described. Plants were transferred to individual pots 16 days after planting and regular watering continued until the dry down experiment commenced.

Thirty-four days after the seeds were planted, an experimental dry down treatment was implemented in which water was withheld from all plants. Six replicates from each of the 9 populations were used. One day prior to the start of the drought treatment, plants were arranged in 6 spatial blocks on the greenhouse tables. One plant from each of the 9 population was placed in each block and the positions within blocks were randomly assigned.

On day 1 of the dry down, the soil surface of each pot was covered with 2 layers of white cotton cloth and secured to the pot using a rubber band (Figure A.1). This manipulation was intended to reduce the effects of water evaporation from the soil surface during the experimental drought treatment. Pot weight was recorded daily and used as a proxy for transpiration. Chlorophyll fluorescence (effective photochemical quantum yields of Photosystem II) was measured daily with a MiniPam fluorometer (Walz, Bremen, Germany) as an indicator of photosynthetic rate (Figure A.2). Photosynthetic rate was measured using a Li Cor 6400 (Li Cor, Inc., Lincoln, NE) every 3 days until chlorophyll fluorescence began to decline, at which point photosynthetic rate was measured daily with the Li Cor. Fluorometer measurements provide an alternative indicator of photosynthetic function (Baker 2008), providing insight into the light reactions of photosynthesis while being less time consuming and causing less stress to the plant from each measurement. Each plant remained in the dry down experiment until it registered a measurement of photosynthetic rate of less than 1 $\mu mol\ m^{-2}sec^{-1}$ at flow meter settings of both

200 and 500 µmol sec^{-1}. The fabric covering was then removed from the pot and the plant was rewatered. Two days later, photosynthetic rate, pot weight, and fluorescence were remeasured.

On the first day of the dry down experiment, when plants were 35 days of age, it was observed that in some populations, every individual had already produced at least 1 inflorescence. For other populations, some or all individuals had not yet produced their first inflorescence. For all plants that had not yet produced an inflorescence at day 35, the day on which an inflorescence was first observed was recorded. The number of inflorescences was monitored until all plants had fully matured and produced inflorescences.

Data were analyzed in R 4.3.2 (R Core Team 2023) using the RStudio integrated development environment (Posit Team 2023). One-way ANOVAs were used to compare means (using the 'anova' function in RStudio). Data was assessed using Levene's and Shapiro-Wilks tests. When the assumptions of the tests were violated, ANOVA was still used as sample sizes were equal and ANOVA test is known to be robust to non-normal data (Blanca et al. 2017). Tukey's HSD posthoc tests were used to calculate differences between means across populations. The cor.test function with the Kendall correlation coefficient in RStudio was used to look for associations between population, days to photosynthetic rate of 0 µmol $m^{-2}sec^{-1}$, and pot weight. Kendall's tau was selected due to its robustness to non-normal data and outliers (Chok 2010). Figures were created in RStudio using the 'ggplot2' package (Wickham 2016).

Comparison with environmental variables

For each of the 9 populations, 19 environmental variables were extracted from the WorldClim database (Fick and Hijmans 2017). The cor.test function with the Kendall correlation coefficient in RStudio was used to look for association between each variable and population

averages for days to photosynthetic rate of 0 μmol $m^{-2}sec^{-1}$ (representing drought response). Kendall's tau was selected due to its robustness to non-normal data and outliers (Chok 2010). Map showing the global variation for average annual precipitation based on WorldClim data were generated in RStudio. Correlation figures were created in RStudio using the 'ggplot2' package (Wickham 2016).

Root surface area comparison

Four of the 9 populations from the initial drought experiment were selected to assess comparative root surface area. Seeds were germinated from 2 drought tolerant populations (China and Portugal) and 2 drought sensitive populations (Russia and Afghanistan). Seeds were germinated and transplanted as previously specified. At 33 days after planting, plant height was measured, and plants were harvested. Aboveground biomass was harvested, dried in a 65°C oven for 4 days, and weighed.

Plants were removed from pots and roots were separated from soil. Roots were thoroughly washed to remove the maximum amount of soil and debris. To assess root surface area, roots were floated in water in a single layer on a clear plexiglass tray and adjusted to maximize extension and separation. Trays were scanned using EpsonScan and surface area for each plant was calculated using WinRhizo (Regent Instruments Inc.), with manual adjustments made as needed to ensure the accuracy of the measurements.

One-way ANOVAs were used to compare population means (using the 'anova' function in RStudio). Data was assessed using Levene's and Shapiro-Wilks tests. When the assumptions of the tests were violated, ANOVA was still used as sample sizes were equal and ANOVA test is known to be robust to non-normal data (Blanca et al. 2017). Tukey's HSD posthoc tests were

used to calculated differences between means across groups. The cor.test function with the Kendall correlation coefficient in RStudio was used to look for associations between root surface area, aboveground biomass, the ratio of root surface area to aboveground biomass, and population drought tolerance as assessed in the first dry down experiment. Kendall's tau was selected due to its robustness to non-normal data and outliers (Chok 2010). Figures were created in RStudio using the 'ggplot2' package (Wickham 2016).

Dry down 2: plant age and developmental stage comparison

Two of the 9 populations from the first dry down experiment were selected for a second dry down experiment comparing the effects of both population and plant age on drought response. Populations utilized for this subsequent experiment were selected based on drought sensitivity differences observed in the first experiment and consisted of populations from China (more drought tolerant) and Russia (less drought tolerant). Seeds were germinated as previously described. Seeds were planted on August 6th, 13th, and 20th in 2018. The experimental dry down period was started on September 12th, 2018, with plants that were 3, 4, and 5 weeks old for a total of 18 plants (2 populations x 3 age groups x 3 replicates). Plants were arranged in spatial blocks on the greenhouse benches. One plant from each age/population/treatment group was placed in each block and the positions within blocks were randomly assigned.

The soil surface of each pot was covered with fabric as in the previous drought stress experiment. Plants were weighed daily as a proxy for water lost through transpiration. Photosynthesis was measured every 3 days until fluorescence began to decline, at which point it was measured daily. Plants were rewatered when they reached a photosynthetic rate of less than 4 μmol $m^{-2}sec^{-1}$ at flow meter settings of both 200 and 500 μmol s^{-1}. The photosynthetic rate

criterion was adjusted from below 1 to below 4 μmol $m^{-2}sec^{-1}$ in this dry down experiment as it was conducted in the fall when temperatures were lower and photoperiod was shorter, causing plants to decline in photosynthetic rate due to drought stress more slowly. When plants reached a photosynthetic rate of less than 4 μmol $m^{-2}sec^{-1}$, the fabric was removed from the soil surface and plants were rewatered. Photosynthesis, fluorescence, and pot weight were remeasured 2 days after rewatering.

One-way and two-way ANOVAs were used to compare populations means (using the 'anova' function in RStudio). Data was assessed using Levene's and Shapiro-Wilks tests. When the assumptions of the tests were violated, ANOVA was still used as sample sizes were equal and ANOVA test is known to be robust to non-normal data (Blanca et al. 2017). Tukey's HSD posthoc tests were used to calculated differences between means across population and age groups. Figures were created in RStudio using the 'ggplot2' package (Wickham 2016).

Results

Dry down 1: population comparison

A one-way ANOVA was used to compare population responses to the dry down treatment (Figure 2.2). The number of days required for plants to reach a photosynthetic rate of 0 μ mol m^{-2} s^{-1} differed substantially by population (one-way ANOVA: $F = 31.6$, $Df = 8$, $p = 5.11*10^{-16}$). A Tukey's HSD posthoc test at $\alpha=0.05$ was used to assess differences between populations. The populations from Afghanistan and Russia had the lowest mean number of days between initiation of the dry down and reaching a photosynthetic rate of 0 μ mol m^{-2} s^{-1}, while the China, Portugal, and Michigan 2 populations had the highest mean number of days.

The relationship between population drought tolerance and average population pot weight at photosynthetic rate of 0 μ mol m^{-2} s^{-1} was nonsignificant (Kendall's Rank Correlation: T= 12, p-value= 0.260, tau = -0.333), as was the relationship between population drought tolerance and average rate of pot weight loss by population (Kendall's Rank Correlation: T=9, p= 0.275, tau= -0.357).

At the start of the dry down experiment (plants 35 days of age) it was noted that some plants had produced at least 1 inflorescence and others had not. A general trend was noted in which populations that flowered by day 35 were more drought tolerant, though the correlation was not significant (Kendall's Rank Correlation: Z= -0.560, p= 0.575, tau= -0.160). However, whether or not plants flowered by day 35 differed significantly by population (Figure 2.3, one-way ANOVA: F = 29.4, Df =8, p= $1.97*10^{-15}$). Notably, the population from Afghanistan flowered later than all other populations, with 0 of the 6 plants flowering before day 48.

The total number of inflorescences that each plant produced differed significantly by population (Figure 2.4, one-way ANOVA: F=64.0, Df =8, p=$2*10^{-16}$) but the relationship between average total inflorescence number by population and population drought tolerance was not significant (Kendall's Rank Correlation: T=20, p=0.761, tau = 0.111).

Bioclim variables and drought response

After performing all correlations between the 19 Bioclim variables and our experimental measurement of drought tolerance at the population level using Kendall's Rank Coefficient, none of the Bioclim variables were found to be significant predictors of drought tolerance (Table 2.2). The lowest p-value was for Bioclim variable 12 (Figure 2.5), average annual precipitation, which

showed a positive relationship with drought tolerance (Kendall's Rank Coefficient: z = 1.57, p-value = 0.116, tau= 0.423).

Root surface area comparison

Four representative populations (2 drought-tolerant and 2 drought-sensitive) from the 9 populations included in the dry down were selected for a follow-up root surface area assessment. The ratio of root surface area to aboveground biomass differed significantly by population (Figure 2.6, one-way ANOVA: F=6.24, Df =3, p=0.0036), with the population from Portugal having a significantly higher root surface area to aboveground biomass ratio than the other 3 populations. Root surface area also differed significantly by population (Figure A.3, one-way ANOVA: F=80.85, Df =3, $p=2.35 *10^{-11}$), with the population from Afghanistan having substantially more root surface area than the other populations. Plants from the Afghanistan population also had significantly more aboveground biomass than the plants from other populations (Figure A.4, one-way ANOVA: F= 114, Df =3, p= 9.44e-13). Root surface area was not significantly correlated with drought tolerance at the population level (Kendall's Rank Correlation: T = 1, p = 0.333, tau= -0.667) but it was marginally significantly correlated with aboveground biomass (Figure A.5a, Kendall's Rank Correlation: T = 6, p-value = 0.0833, tau=1). Although the population from Afghanistan represented an influential point, removing it from the correlation did not change the direction of the relationship (Figure A.5b, Kendall's Rank Correlation: T = 3, p-value = 0.333, tau =1).

Dry down 2: plant age and developmental stage comparison

A second dry down experiment was conducted on one of the more drought-tolerant populations (China) and one of the more drought-sensitive populations (Russia) that had similar flowering times during the initial dry down. For each of the 2 populations, 3 different ages of plants were used in the experiment. A two-way ANOVA was used to compare the effects of population and plant age on the number of days until plants reached a photosynthetic rate of 4 μmol $m^{-2}sec^{-1}$. Both population and plant age significantly influenced the average number of days to photosynthetic rate of 4 μmol $m^{-2}sec^{-1}$ at alpha =0.05 (two-way ANOVA: Population F= 5.37, p=0.039, Df =1; Age F=42.1, p < 0.001, Df =2), though the interaction was not significant (F=2.45, p=0.128, Df =2). When the interaction was removed from the model, age remained significant at alpha=0.05 (F=34.9, Df =2, p < 0.001), while population significance decreased to p=0.053 (F= 4.446, Df =1).

One-way ANOVAs were performed to compare differences in drought response by population within each age group (Figure 2.9a). A posthoc Tukey test indicated significant differences in drought response between the youngest plants from the China and Russia populations (diff= -8, p= 0.00133), though the differences in drought response for the other age groups were not significant (4 weeks: diff= -2, p= 0.647, 5 weeks: diff= -0.333, p= 0.845).

Separate one-way ANOVAs were performed for the effect of plant age on days to photosynthetic rate of 4 μmol $m^{-2}sec^{-1}$ for each population (Figure 2.9b). Larger, older plants generally declined more quickly than smaller, younger plants. Three out of the four pairwise comparisons performed across plant age within population were significant at α=0.05. For the population from China, age significantly influenced days to photosynthetic rate of 4 μmol $m^{-2}sec^{-1}$ (one-way ANOVA: F= 31.9, Df =2, p < 0.001). A posthoc Tukey test indicated

significant differences between all 3 age groups (4 week - 5 week: diff=11.3, p=0.0104; 3 week – 5 week: diff=20.3, p < 0.001; 3 week - 4 week: diff=9, p=0.0288). For the population from Russia, age significantly influenced days to photosynthetic rate of 4 μmol $m^{-2}sec^{-1}$ for each population (one-way ANOVA: F= 13.0, Df =2, p=0.00659). A posthoc Tukey test indicated significant differences between 4 and 5 weeks and between 3 and 5 weeks but not between 3 and 4 weeks, though the trend was in the same direction (4 week - 5 week: diff=9.667, p=0.0229; 3 week – 5 week: diff=12.667, p=0.00663; 3 week - 4 week: diff=3, p=0.519).

The number of inflorescences on plants differed significantly by plant age, though not by population (two-way ANOVA: Age: F= 5.62 Df =2 p= 0.0162, Population: F= 1.31, Df =1 p= 0.271). When population was removed from the model, age remained significant (one-way ANOVA: F= 5.5, Df =2, p= 0.0161). A posthoc Tukey test found significant differences between the number of inflorescences produced by 4 and 5-week-old plants (Figure 2.10, 4 week-5 week: diff= 6.67, p= 0.0135), though other pairwise comparisons were not significant.

Discussion

With anthropogenic climate change predicted to increase the frequency and severity of droughts in many parts of the world, identifying and developing drought tolerant crop species is a priority (Peng et al. 2010; Lata et al. 2011; Feldman et al. 2018; Li et al. 2021). As a known water-use-efficient cereal crop (Peng and Zheng 2021), *S. italica* is an important genetic resource for further development of drought-resistant crops (Feldman et al. 2018), and its interfertility with the more diverse wild relative *S. viridis* (Li and Brutnell 2011) offers potential for additional improvement and discovery of tolerance traits. Our experiment explores variation in drought resistance of *S. viridis* across both population and plant age.

In the first dry down comparing 9 different populations of *S. viridis*, populations were found to differ significantly in how quickly they succumbed to drought stress and reached a photosynthetic rate of less than 1 μmol $m^{-2}sec^{-1}$. Based on these measurements, the populations from Afghanistan and Russia were found to be the most drought sensitive while the China, Portugal, and Michigan 2 populations were found to be the most drought resistant.

Overall, the differences we measured in drought response across populations of *S. viridis* align with previous research on geographically widespread populations of *S. viridis* across Eurasia and North America, which also found significant differences in drought response by population (Saha et al. 2016; Duarte et al. 2019; Travassos-Lins et al. 2021). Saha et al. 2016 compared physiological, molecular, and biochemical responses to drought stress in 6 populations from different areas of the world and found significant differences between populations. Duarte et al. 2019 and Travassos-Lins et al. 2021 both utilized 2 of the populations assessed by Saha et al. 2016 in their experiments, also finding significant differences in drought response. Saha et al. 2016 included 2 of the same populations assessed in our experiment, Iran and Azerbaijan, and found them to be in the less drought tolerant group. Similarly, we found these 2 populations to have similar levels of drought tolerance compared to each other and intermediate drought tolerance (neither the highest nor the lowest) in comparison to the other 7 populations that we assessed. None of these previous studies quantitatively assessed potential connections between climate of origin and drought response as evaluated in a common garden experiment.

Seemingly paradoxically, the 2 Michigan populations were collected at the same longitude and latitude based on their GRIN passport source histories, but they showed a statistically significant difference in drought tolerance. This unexpected result may be related to the history of *S. viridis'* introduction to North America from Eurasian ancestral lineages.

Previous work on genetic and geographic diversity of *S. viridis* populations in North America indicate multiple separate introductions of this species from different Eurasian lineages, which have not become either fully admixed or genetically differentiated from Eurasian populations following introduction (Huang et al. 2014; Layton and Kellog 2014; Schröder et al. 2017). A study which created majority-rule Bayesian trees from both chloroplast and nuclear genes for 7 populations of *S. viridis* and several relatives within the *Setaria* genus found that the 5 populations collected in North America did not cluster in a single distinct lineage apart from the 2 populations collected in China, with some North American populations clustering more closely with the populations from China than with each other (Layton and Kellog 2014). Another study, which surveyed the genetic diversity of a worldwide sample of over 200 *S. viridis* populations and 21 *S. italica* populations, found evidence of strong population structure with 3 distinct groups (2 in *S. viridis* and one resembling *S. italica*) and many admixed individuals (Huang et al. 2014). All 3 of the identified genetic groups included individuals in both Eurasia and North America, with a clear geographic pattern in group distribution in Central North America (Huang et al. 2014). Generally, Group 1 individuals were observed north of 43.5° N and Group 2 to the south, with some admixed individuals clustered in between (Wang et al 1995). Another study that assessed population structure based on simple sequence repeat markers in 232 *S. viridis* populations also found two US subpopulation clusters, one of which tended to occur above 46° N Latitude and the other below 44° N Latitude (Schröder et al. 2017). Huang et al. 2014 included 3 populations from Michigan (collected between 44° and 46° N Latitude), 1 in Group 1 and 2 that were admixed between Group 1 and Group 2. Notably, Michigan spans roughly 41° to 49° N Latitude, and the Michigan populations included in our study were collected at 45.109° N, close to the latitudinal divide between Group 1 and Group 2.

Based on this evidence of genetic and phylogenetic diversity in North American populations of *S. viridis*, specifically in Michigan, it is possible that genetically distinct populations are growing in sympatry within the collection site area from which the 2 Michigan populations we used in this experiment were collected. Although local adaptation to a shared environment can result in populations that show similar responses to environmental stressors like drought, local adaptation is not the only type of evolutionary force that can impact genotypic and phenotypic diversity, and the strong population structure detected in previous research on *S. viridis* populations in North America may be playing a substantial role in the differences we observed between these two populations.

During this dry down, differences in flowering time by population were observed, with populations that flowered earlier tending to be more drought tolerant than those that flowered later. This result is supported by previous work which has associated earlier flowering time with enhanced drought escape (Kooyers et al. 2015). Plants that can reproduce quickly, shorten their growing season, and escape drought stress can attain a selective advantage over later flowering plants, which may have the potential to grow larger and produce more seeds, but risk succumbing to lethal drought stress before maturation (Franks et al. 2007). Plants from the Afghanistan population decreased in photosynthetic rate most rapidly on average, produced more vegetative biomass, and flowered later than the other populations, indicating a potential difference in life history. Notably, the Afghanistan population was sourced from a cultivated germplasm and was observed to have seed abscission more similar to *S. italica* than wildtype *S. viridis* (USDA 2023), which may account for some of these observed differences. The larger size and more prolonged life cycle of this population may indicate polyploidy (Corneillie et al. 2019). While *S. viridis* individuals have been consistently found to be diploid, polyploidy has been

identified in other closely related species across the *Setaria* lineage, including allopolyploids for which *S. viridis* has supplied one of the genomes (Layton and Kellog 2014), indicating the possibility of this GRIN sample representing a polyploid hybrid lineage. Additionally, all populations were grown in pots of the same size with the same amount of soil, and the lower drought tolerance observed in the larger Afghanistan plants may have been influenced by the ratio of soil to plant size causing the larger plants to deplete the available water more quickly during the dry down (Ray and Sinclair 1998; Poorter et al. 2012).

Our analysis of root surface area found that 1 of the 2 more drought tolerant populations assessed, Portugal, had a significantly higher root surface area to aboveground biomass ratio than the other three populations. Previous research has found that increased root surface area can contribute to increased drought tolerance in some plant populations compared to less drought tolerant populations or genotypes of the same species (Idrissi et al. 2015; Abenavoli et al. 2016; Ding et al. 2016; Manju et al. 2018). Root surface area has been shown to play an important role in helping plants absorb water from the soil, retain water, and maintain cell turgor during drought stress (Manju et al. 2018). Given the overall size differences in the plants from these four populations and the potential for allometric influence of plant size on root surface area (especially with the Afghanistan population), the root surface area to aboveground biomass ratio is likely a more accurate indicator of relative surface area than the surface area measurement alone. The experimental finding that the population from Portugal had both increased drought tolerance and a higher root surface area to biomass ratio indicates that the previously established role of root surface area in enhancing drought tolerance may explain some of the variability in drought tolerance observed in our experiments. However, as the root surface area to biomass ratio for the drought tolerant China population did not significantly differ from that of the less

drought tolerant populations, differences in relative root surface area cannot fully account for the variability in drought tolerance between populations that we observed.

The Afghanistan population had substantially more aboveground biomass and root surface area than the other populations assessed and also had low drought tolerance, suggesting that overall plant size may serve as an explanatory variable impacting both total (but not relative) root surface and drought tolerance. One of the populations used by Saha et al. 2016, Duarte et al. 2019, and Travassos-Lins et al. 2021 (Ast-1) also produced much more vegetative tissue, attained a larger size at the same age, and was visually observed to have more dense root architecture compared to the other population utilized in these experiments (A10.1). Saha et al. 2016 and Travassos-Lins et al. 2021 both found A10.1 to be more drought tolerant than Ast-1, which supports the trend we observed of populations with larger plants proving more sensitive to drought stress. In contrast, Duarte et al. 2019 described Ast-1 as more drought tolerant than A10.1, though this experiment only assessed response to water withholding for 45 hours. A separate study that assessed water use efficiency in a *S. viridis* x *S. italica* recombinant inbred line also found a strong correlation between plant size and water use, with larger plants using more water than smaller plants (Feldmen et al. 2018).

The 19 environmental variables from the Bioclim database were used to test for associations between experimental drought response and features of the climate of origin for each population. Average annual precipitation and precipitation seasonality (how evenly total annual precipitation is spread across the months of the year) were of particular interest. While no correlations were found to be significant, trends were observed between some Bioclim variables and drought tolerance, especially average annual precipitation. The direction of the relationship indicated that as average annual precipitation tends to increase, drought resistance also tends to

increase. However, as with all the relationships between Bioclim variables and drought tolerance, this observed trend was not statistically significant, likely because of the small number of populations included in the experiment, which was constrained by the time involved in obtaining photosynthetic rate measurements. Measuring photosynthetic rate to assess physiological response to drought stress provided us with a precise assessment of differences in drought response between *S. viridis* populations. Although the time intensive nature of photosynthetic measurements limited the sample size of our experiment, it provided a highly sensitive (Sun et al. 2013) comparison of drought response between populations.

Measurements of average annual precipitation are limited in their ability to determine the actual environmental conditions experienced by a short-lived annual plant. With a 2-to-3-month life span, *S. viridis* may be able to avoid drought conditions even in very arid locations by growing during the wettest time of year. And in areas with higher levels of precipitation per year, it is not known how much of that precipitation is available to *S. viridis* plants during their growing season. The Bioclim variables provide an estimate of local environment, but do not give a precise picture of the growing conditions experience by *S. viridis* in each location during their lifetime. A 2020 study by Akman et al. similarly found a positive correlation between survival under drought and average annual precipitation when comparing 8 wild populations of the common sugar bush in South Africa. The authors also found that plants from wetter sites had higher shoot starch content, leading them to propose that greater carbohydrate reserves may have contributed to better survival through drought (Ackman et al. 2020).

As a short-lived annual plant, *S. viridis* may rely on a drought escape strategy in addition to drought tolerance mechanisms. Plants that escape drought do so by developing and reproducing rapidly prior to the onset of drought (Kooyers 2015; Kooyers 2019). This strategy is

most typical in annual plants (Bouzid et al. 2019; Monroe et al. 2019) and could provide an explanation for why we did not see significantly increased drought tolerance in populations from locations that receive less average annual precipitation. It is also possible that inherent tradeoffs between growth and drought tolerance (Bouzid et al. 2009) mean *S. viridis* populations from more water limited regions prioritize rapid growth to facilitate drought escape over investing in drought tolerance mechanisms. Drought escape strategies can be both constitutive and plastic, and plasticity in flowering time in response to photoperiod and water availability can help to facilitate an adaptive drought escape response (Kooypers 2019). *S. viridis* is known to show plasticity in flowering time in response to photoperiod (Swanton et al. 1999; Li and Yang 2008), which we also observed experimentally.

As previously noted, earlier studies that compared drought tolerance across geographically widespread populations of *S. viridis* did not investigate potential correlations between physiological response to drought and climatic of origin. Huang et al. 2014 surveyed the genetic diversity of over 200 *S. viridis* populations from across the world, though they did not assess experimental phenotype. As part of their analysis, they performed a genome-wide association study of the 19 Bioclim variables to detect potential genomic regions responding to climatic conditions. Positive associations of bioclimatic variables with certain genomic regions would be potentially indicative of local adaptation to one or more of these variables. While climatic and genetic distances (calculated through neighbor-joining) were shown to be strongly correlated, association mapping did not show significant association of genomic regions and bioclimatic variables (Huang et al. 2014). The authors noted that this may be attributable to strong population genetic structure (with the surveyed populations clustering into 3 genetic groups), which may overwhelm signals of local adaptation (Huang et al. 2014).

Our second dry down compared drought response of *S. viridi*s not only across populations, but also at different ages. In this dry down, we were able to confirm that the population from China was more drought tolerant than the Russia population for at least 1 age group (plants that were 3 weeks old at the start of the dry down), replicating what we had seen in the first dry down. While the 4- and 5-week-old age groups did not show statistically significant differences in drought tolerance by population, the apparent trends were in the same direction, whereby the population from China trended as more tolerant, though more notably for the 4-week age group. For both populations, there was a trend towards smaller, younger plants lasting longer before succumbing to drought stress, as might be expected based on plant size to soil volume ratios (Ray and Sinclair 1998; Poorter et al. 2012). For the China population, there were significant differences in drought tolerance between all 3 age groups, while for the Russia population, only the difference between the 5-week-old plants and the other 2 age groups was significant.

For both populations, the 4-week-old plants produced the greatest number of inflorescences by the time of senescence. Variations in drought sensitivity between different phases of *Setaria* life cycle have been previously documented, with greater drought sensitivity observed in the reproductive stage in both *S. italica* (Lata and Shivhare 2017) and *S. viridis* (Rodrigues et al. 2020). Rodrigues et al. 2020 additionally noted differences in sensitivity between the grain filling and maturation phases of the reproductive organs. However, due to the timing of this dry down experiment, all or most individuals from all 3 age groups had begun producing reproductive tissue by the start of the experiment, so we were not able to compare the effects of drought stress on vegetative vs reproductive life stages of *S. viridis*.

The intermediate age group (4-weeks-old at the start of the dry down) may have been at a size and phase of development which made reproductive output least vulnerable to the effects of drought stress compared to either the 3-or 5-week-old plants. Previous research has demonstrated that *S. viridis* plants produced maximum reproductive output when they were able to delay flowering and establish more vegetative biomass before inflorescence production (Li and Yang 2008), that larger plants had higher water usage (Feldmen et al. 2018), and that *S. viridis* plants slow their growth rate when experiencing osmotic stress (Valença et al. 2020). In our dry down, the 5-week-old plants may have been more compromised by the experimental drought stress than the 4-week-old plants due to their larger size, as discussed previously, and the 3-week-old plants may not have produced sufficient biomass by the start of the dry down to achieve the same level of fecundity as the 4-week-old-plants, despite being less drought sensitive.

This finding may have important implications for maximizing crop yields under drought conditions, especially in closely related cultivated species, such as *S. italica*. Total inflorescence number is often used as an indicator of yield and overall reproductive output (Walker 2022) and evidence that yield is maximized when these plants encounter drought conditions at a stage where they have been able to produce sufficient vegetative biomass to increase fecundity but not so much as to further increase drought susceptibility could help inform timing for planting crops. Further research including field-based experiments comparing growth, survival, and reproductive output of plants with staggered planting dates across the growing season is needed to assess how these results might translate to agricultural contexts.

Compared to the plants in the first dry down, which was performed in June, we noted that plants in the second dry down, which was performed in the fall, flowered at a younger age and smaller size. This is likely due to seasonal differences in photoperiod, which are known to

influence the rate of phenological development in *S. viridis* (Swanton et. al 1999; Li and Yang 2008). Previous studies of *S. viridis* have found that individuals planted earlier in the growing season have a longer interval between germination and flowering, and that life history patterns and reproductive allocation are highly plastic (Li and Yang 2008) and can be influenced climatic conditions (Schröder et al. 2017). Seasonal differences also affected ambient temperature and light between the first and second dry downs. In spite of these seasonal variations, the relationship between the drought tolerance of the China and Russia populations remained the same, indicating that it is robust to differences in temperature and photoperiod.

Conclusions

In our exploration of drought response across populations and age in *S. viridis*, we found significant differences in drought sensitivity across both populations and age groups, as well as variation in root surface area and aboveground biomass across populations. These experiments explored a gap in the existing literature about drought response across populations of *S. viridis* by comparing experimental phenotype with bioclimatic data from each population's climate of origin. We did not find drought response to be significantly correlated with any of these bioclimatic variables, indicating that variation in drought tolerance across geographically diverse populations of *S. viridis* is not explainable solely by local adaptation to annual precipitation regimes in their home ranges. There are also important limitations on the ability of bioclimatic data from a single point to represent the conditions a plant may actually encounter within a larger and potentially more heterogeneous landscape.

Measuring physiological changes provided valuable insight into variation in drought sensitivity across populations of *S. viridis*, though the molecular changes that underlie these

physical differences, which could potentially be used to inform crop improvement strategies, have not been fully elucidated.

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Figures

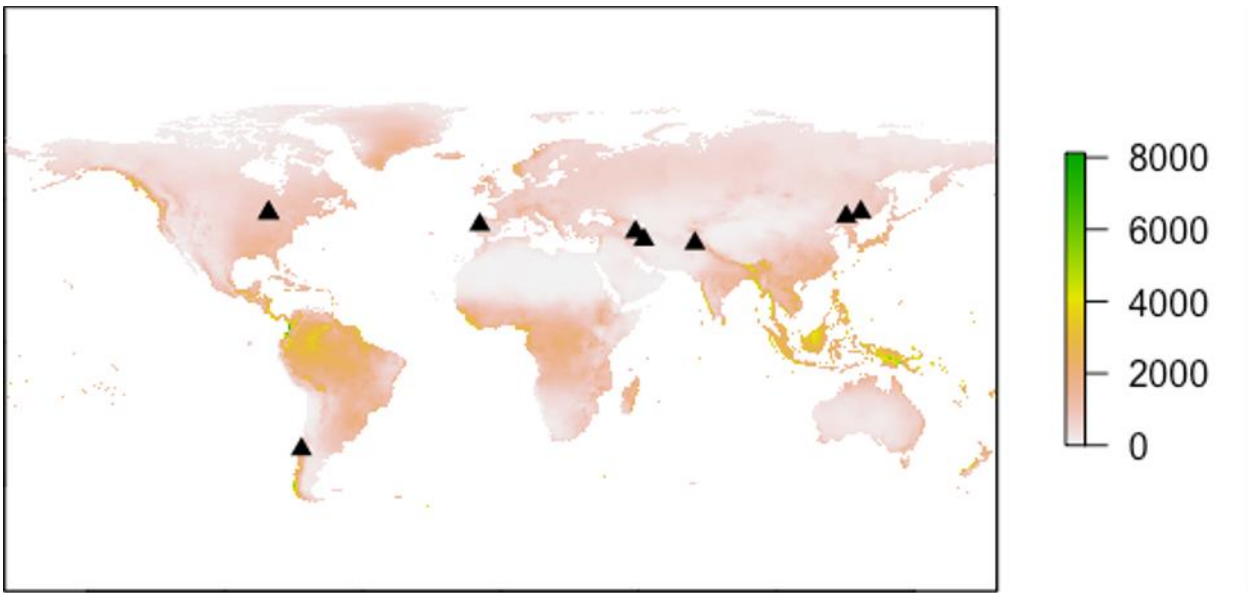

Figure 2.1 : Range of global average annual precipitation in millimeters, ranging from close to 8000 mm in the tropics to close to 0 in drier areas. Black triangles mark the locations from which seeds for experimental *Setaria viridis* populations were collected. Although the differences in average annual precipitation between these locations do not appear striking on the scale of the map, they range from 167mm per year to 1264 mm per year. Map generated from Bioclim dataset.

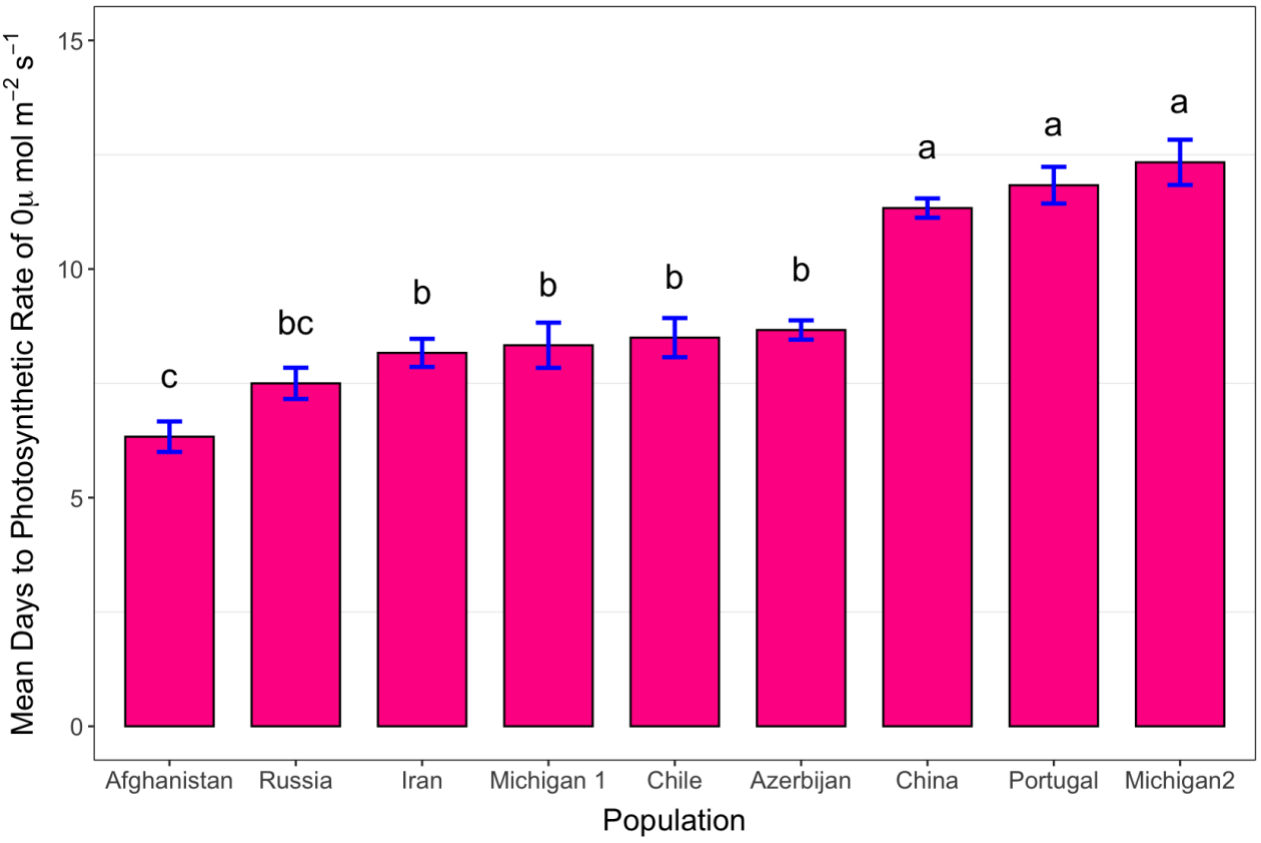

Figure 2.2 : Comparison of drought response across 9 populations of *Setaria viridis* during greenhouse dry down experiment. Error bars represent one standard error. Means sharing a letter are not significantly different.

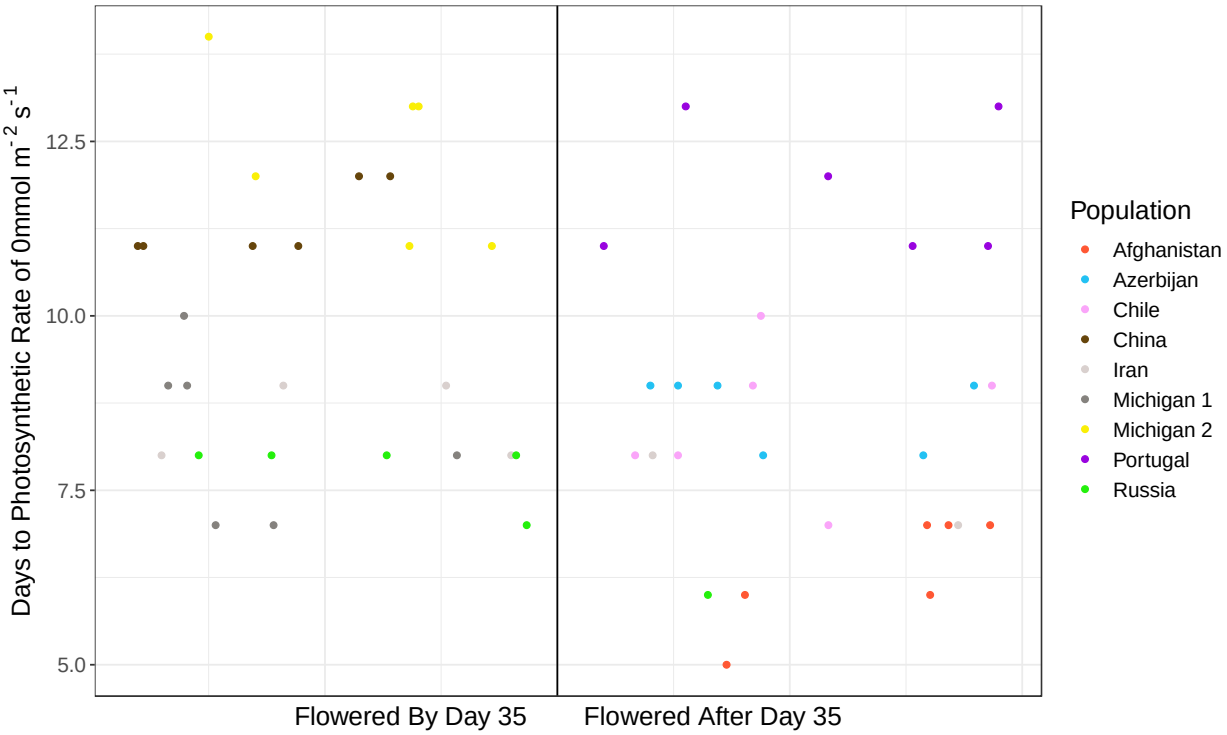

Figure 2.3 : Flowering time across population. Each plant was assessed for the emergence of at least 1 inflorescence by day 35. Points were artificially jittered horizontally within each category (flowered by day 35 and flowered after day 35) so that all observations would be visible.

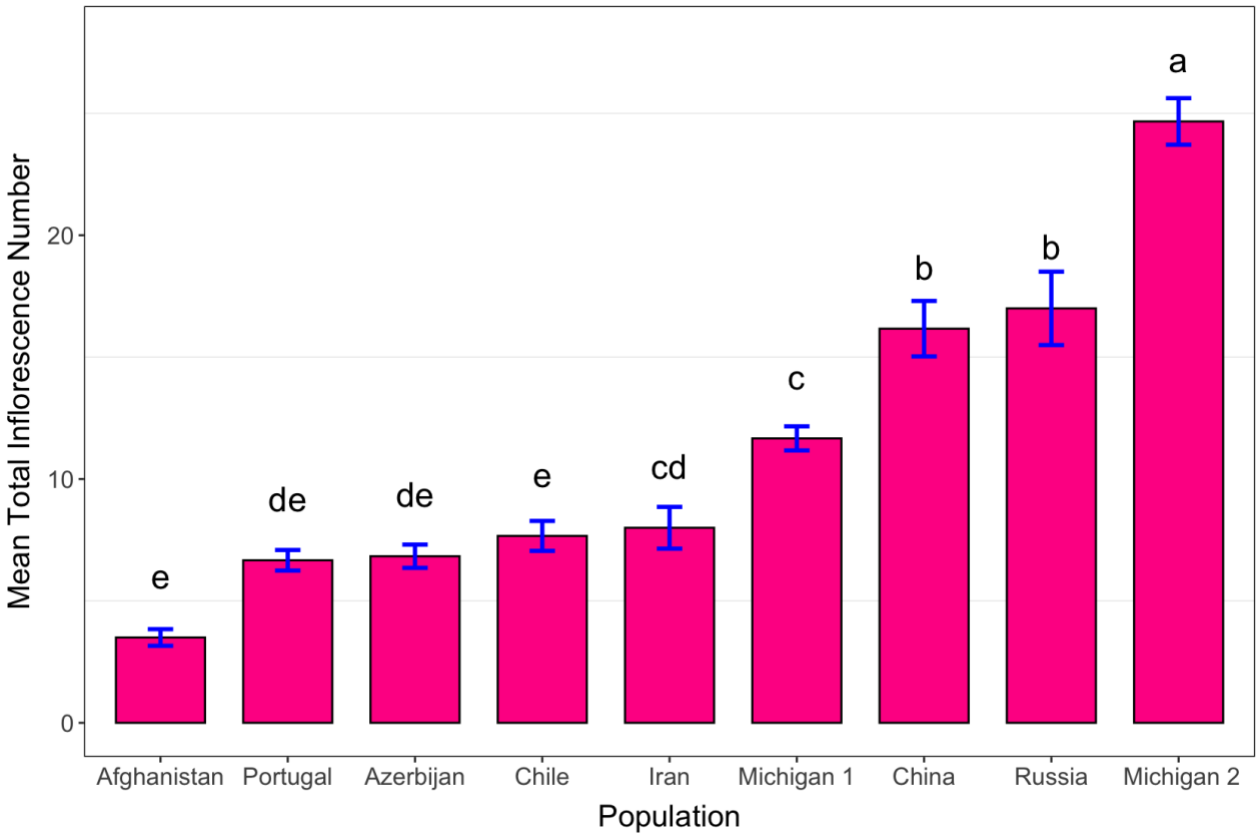

Figure 2.4 : Comparison of total inflorescence number across 9 populations of *Setaria viridis* observed during a greenhouse dry down experiment. Error bars represent one standard error. Means sharing a letter are not significantly different.

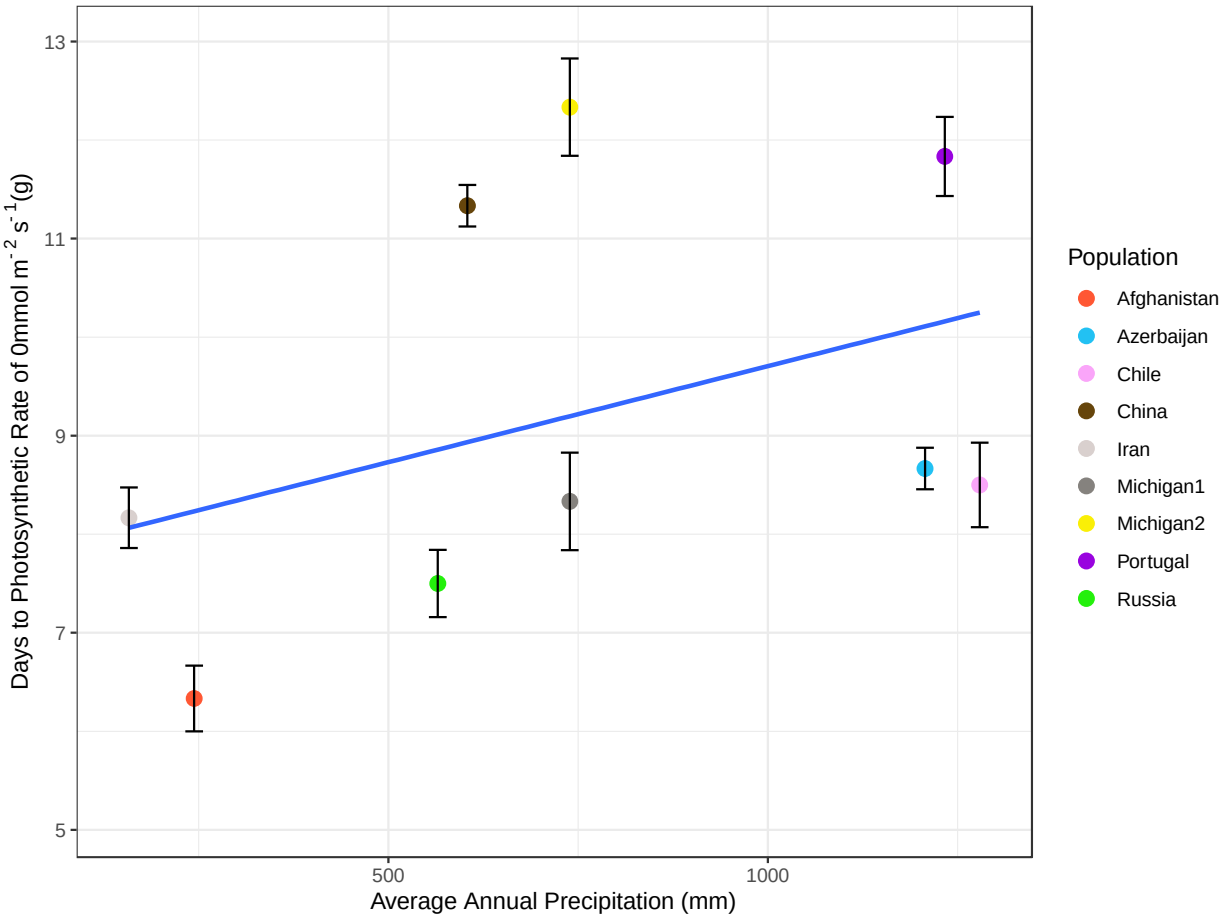

Figure 2.5 : Comparison between average annual precipitation and population average drought response of 9 *Setaria viridis* populations. Error bars represent one standard error.

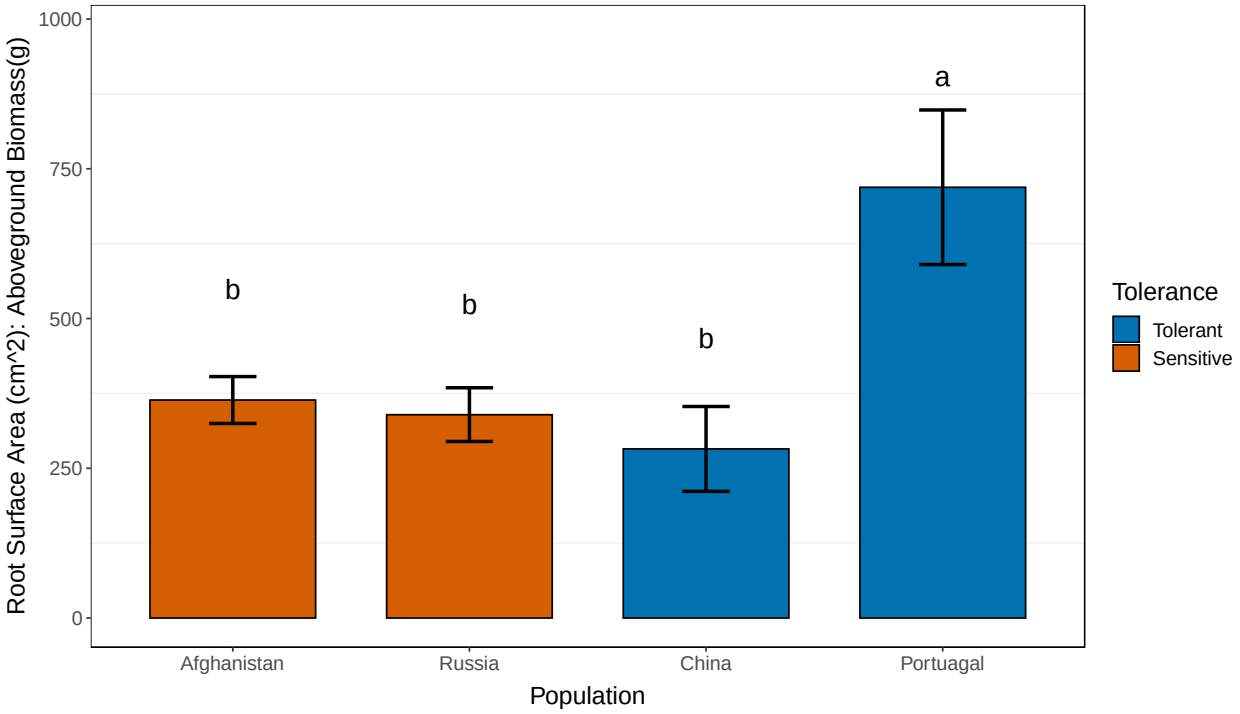

Figure 2.6 : Comparison of root surface area to aboveground biomass across 4 *Setaria viridis* populations. Error bars represent one standard error.

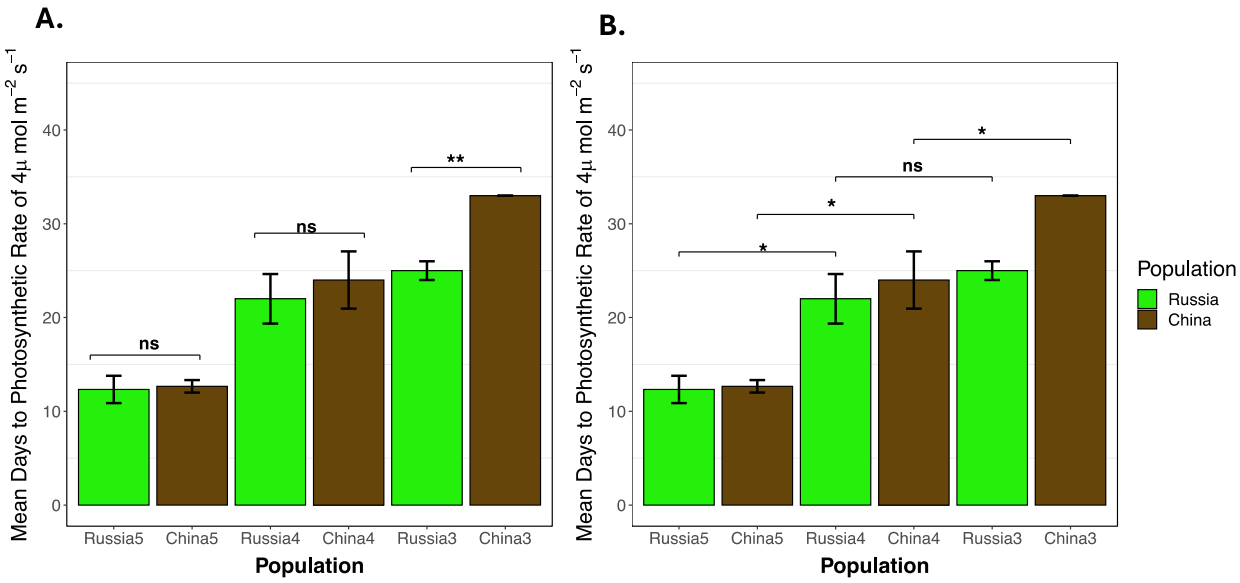

Figure 2.7 : Comparison of drought response across age and population during greenhouse dry down experiment. Error bars represent standard error. A. Results of a pairwise comparison of population within each age category. B. Results of a pairwise comparison of age within each population. ns = nonsignificant ($p > 0.05$), * = $0.05 \geq p > 0.01$,
** = $0.01 \geq p > 0.001$.

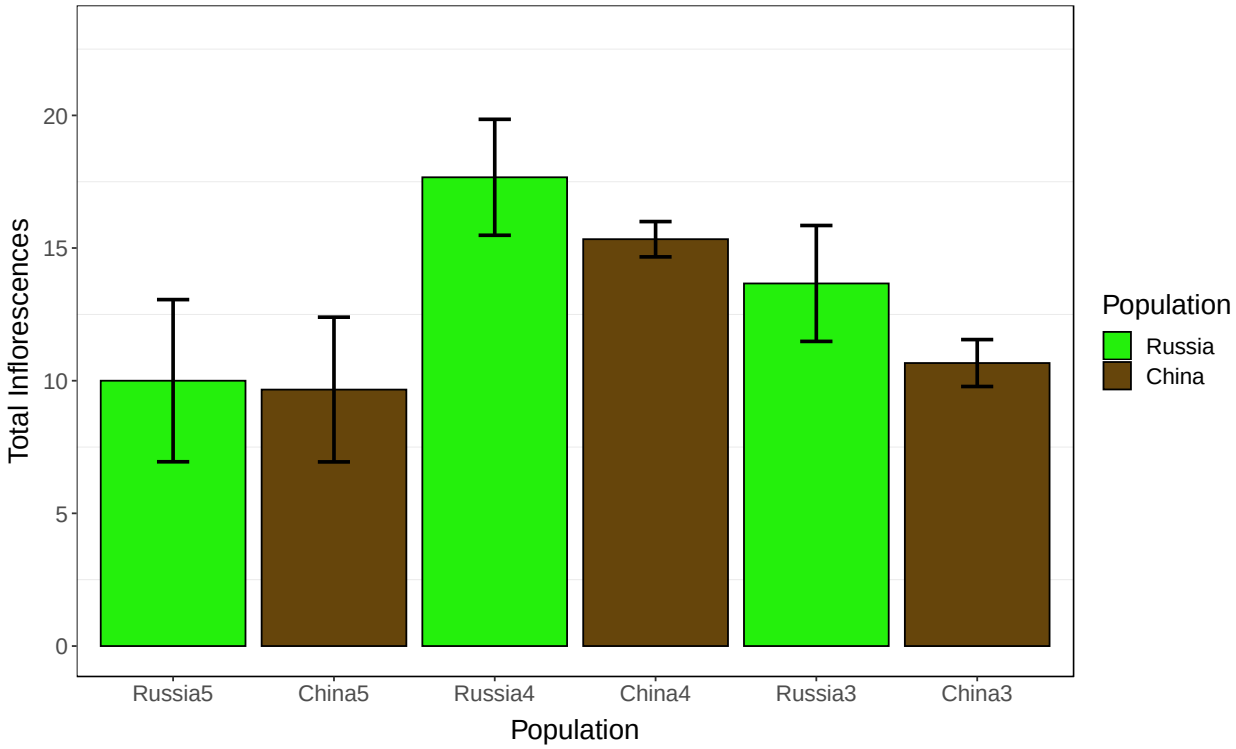

Figure 2.8 : Total inflorescence number by population and age group. Error bars represent one standard error.

Tables

Table 2.1 : *Setaria viridis* populations used in this chapter.

Plant ID	**Origin**	**Longitude**	**Latitude**	**Average Annual Precipitation in mm (WorldClim)**
PI 230135	Iran	51.965 E	35.762 N	158
PI 212625	Afghanistan	70.467 E	34.433 N	244
PI 677118	Russian Federation	130.628 E	45.404 N	565
PI 408810	China	125.307 E	43.848 N	604
Ames 32291	United States (Michigan 1)	-84.166 W	45.109 N	739
Ames 32292	United States (Michigan 2)	-84.166 W	45.109 N	739
PI 223677	Azerbaijan	48.879 E	38.456 N	1207
PI 677122	Portugal	-7.592 W	40.967 N	1233
PI 202407	Chile	-72.247 W	-37.438 S	1279

Table 2.2 : Output of correlations between Bioclimatic variables from WorldClim (Fick and Hijmans 2017) from each population's location of origin and average drought tolerance by population. Correlation tests were conducted using the corr.test functionality in RStudio with the Kendall correlation coefficient for non-parametric statistics. None produced p-values that were significant at alpha=0.05.

WorldClim Variable	Z-value	p-value	R (tau)
BIO1 = Annual Mean Temperature	-0.315	0.753	-0.0845
BIO2 = Mean Diurnal Range (Mean of monthly (max temp - min temp))	-1.36	0.173	-0.366
BIO3 = Isothermality (BIO2/BIO7) (* 100)	-0.314	0.753	-0.0845
BIO4 = Temperature Seasonality (standard deviation *100)	-0.104	0.917	-0.0282
BIO5 = Max Temperature of Warmest Month	-1.15	0.249	-0.310
BIO6 = Min Temperature of Coldest Month	-0.105	0.917	-0.0282
BIO7 = Temperature Annual Range (BIO5-BIO6)	-0.943	0.345	-0.254
BIO8 = Mean Temperature of Wettest Quarter	0.314	0.753	0.0845
BIO9 = Mean Temperature of Driest Quarter	-0.734	0.463	-0.197
BIO10 = Mean Temperature of Warmest Quarter	-1.15	0.249	-0.310
BIO11 = Mean Temperature of Coldest Quarter	-0.524	0.600	-0.141
BIO12 = Annual Precipitation	1.57	0.116	0.423
BIO13 = Precipitation of Wettest Month	0.943	0.345	0.254
BIO14 = Precipitation of Driest Month	1.476	0.14	0.400
BIO15 = Precipitation Seasonality (Coefficient of Variation)	-1.363	0.173	-0.366
BIO16 = Precipitation of Wettest Quarter	0.943	0.345	0.253
BIO17 = Precipitation of Driest Quarter	1.15	0.249	0.310
BIO18 = Precipitation of Warmest Quarter	0.734	0.463	0.197
BIO19 = Precipitation of Coldest Quarter	0.943	0.345	0.254

Chapter 3 - Comparisons of transcriptomic response to drought, chilling, and salinity stress in two *Setaria viridis* populations

Abstract

Abiotic stressors such as drought, chilling, and salinity pose an increasing challenge for both wild and domesticated plants due to the intensification of these stressors under anthropogenic climate change. Changes in gene regulation constitute a critical component of plant response to environmental stress, involving both functional genes and transcription factor genes. Some types of transcriptomic stress response patterns have been found to be conserved across different populations and different sources of stress, especially those which have similar impacts on plants at the cellular level. In this experiment, we analyzed the transcriptomic response of 2 populations of the wild foxtail millet, *Setaria viridis*, to drought, chilling, and salinity stress. While several individual genes and overrepresented gene ontology terms were shared across both populations or multiple stressors, overall, transcriptomic responses differed substantially between populations and treatment conditions . Blasting the differentially expressed genes from this experiment with genes from the DroughtDB, an established database of genes known to be involved in plant drought response, resulted in substantial alignment for both populations. This may indicate that there are shared gene functions and genes families involved in stress response across population and treatment combinations, although patterns of up or downregulation for specific genes in these families may differ. Overall, these results affirm the high interpopulation diversity of *S. viridis* and provide additional insight into shared and unique responses to multiple forms of stress across two wild populations of this promising model species.

Introduction

As sessile organisms, plants cannot flee from external sources of stress and instead must rely on metabolic, developmental, morphological, physiological, and biochemical strategies to respond to the deleterious effects of these stressors (Essamine et al. 2020). Drought, salinity stress, and cold stress that does not result in ice formation (subsequently referred to as chilling stress) are some of the most damaging environmental stressors for plants and have substantial negative impacts on crop plant productivity worldwide (Duarte et al. 2019). All of these stressors are predicted to increase in frequency and stochasticity as anthropogenic environmental disruption and climate change progress (Dai 2013; van Zelm et al. 2020; Augspurger 2013). Droughts threaten plant survival and productivity (Sharma et al. 2020) and are predicted to become more severe and frequent across many parts of the world (Dai 2013; Fracasso et al. 2016). Salinity stress produces significantly negative impacts on plants, especially salt-intolerant glycophytes (Essamine et al. 2020), and soil salinity is increasing worldwide due to increased evaporation during droughts associated with climate change (van Zelm et al. 2020) and inappropriate irrigation management on cultivated lands (Valeriano et al. 2021). Chilling can impair plant growth and reproduction (Sun et al. 2022), and the frequency, intensity, and duration of extreme low-temperature events has been increasing worldwide, even with the overall trend of climatic warming (Augspurger 2013; Shi et al. 2022).

Drought, chilling, and salinity stress can all disrupt plant water status, resulting in the experience of low water potential, cellular dehydration, and osmotic stress (Verslues et al. 2006; Benny et al. 2020). They also impact ion transport, membrane integrity, and production of reactive oxygen species (ROS) (Sharma et al. 2020; Valeriano et al. 2021). Excessive ROS production without sufficient detoxification by antioxidant systems is extremely damaging to

plants at the cellular level and can result in the degradation of cell membranes, enzymes, proteins, DNA, and other important macromolecules (Hussain et al. 2018).

While many of the negative effects of drought, chilling, and salinity stress on plants anatomy and physiology are shared, each stressor can also produce unique deleterious effects. Drought stress can increase leaf and canopy temperature, trigger early maturation, and reduce chlorophyll content (Hussain et al. 2018). Chilling stress can result in reduced root surface area, decreased cell cycle production, and delayed development (Hussain et al. 2018). Salinity stress can produce toxic effects due to excess sodium ions that disrupt cellular function and signaling (Chan et al. 2012; Ariño-Estrada et al. 2019).

Plants have developed complex adaptive responses to cope with these impacts of environmental stress (Essamine et al. 2020), and changes in gene expression are key to facilitating these response mechanisms. Stress response genes have been broadly categorized as either functional genes, which code for enzymes and metabolic proteins that directly function to protect plant cells from stress, or regulatory genes, which code for proteins that function as transcription factors (TFs), protein kinases, and protein phosphatases (Wang et al. 2016). Functional genes involved in stress response include detoxification enzymes, ion transporters, water channel proteins, chaperonins, flavonoid synthesis-related genes, and other protective proteins (Wang et al. 2016).

Studies of stress response gene regulation have increasingly focused on the expression and effects of TFs, as they can regulate a wide spectrum of downstream stress-responsive genes that may be responsive to multiple forms of stress and provide more effective potential targets for gene modification in economically important species (Wang et al. 2016). TFs are proteins which bind to *cis*-acting elements in order to activate expression of the gene (Li et al. 2019). In

this way, TFs can regulate transcription of functional genes, phytohormones (which can also impact the regulation of TFs), other TFs, or even themselves through autoregulation and cross-regulation of other TFs in the same family (Li et al. 2019; Myers et al. 2023). There are six main classes of TFs involved in plant response to stress: AP2/ERF (APETALA2/ethylene responsive factor), bHLH (basic helix-loop-helix), MYB (myeloblastosis related), NAC (no apical meristem (NAM), *Arabidopsis* transcription activation factor (ATAF1/2), and cup-shaped cotyledon (CUC2)), WRKY, and bZIP (basic leucine zipper) (Ng et al. 2018).

Many of the functional and TF gene families integral to these responses are conserved across different forms of stress, in part due to the similarities in how they are experienced by the plant at the molecular and cellular level (Verslues et al. 2006) and in part due to crosstalk between TF mediated regulatory pathways (Meyers et al. 2023). The PIP2 aquaporin gene *MaPIP2-7* in banana was found to be upregulated following cold, drought, and salinity treatments, and overexpression of this gene enhanced plant tolerance to all three stressors (Xu et al. 2020). In a 2020 metanalysis of drought and salinity stress in fruit tree crops, 39 differently expressed genes with shared regulation patterns for both stressors were identified, including TFs, genes involved in hormone signaling, signal transduction related kinases (Benny et al. 2020). Some stress response mechanisms may also be unique, especially in plants that are adapted to one form of environmental stress but not others, to mitigate stress-specific functional impacts, or when different genes within the same family differ in the magnitude or direction of regulation change in response to different forms of stress (Hussain et al. 2018; Duarte et al. 2019). A comparison of gene expression changes in maize subjected to cold and heat stress found that genes for calcium signaling and ROS scavenging enzymes had conserved responses across both stressors while genes involved in hormone signaling, heat shock proteins, and several TF

families, such as ERF, demonstrated different responses (Li et al. 2020). In an analysis of transcriptomic response to drought, heat, and light stress in two species of Portuguese grapevine, a higher number of differently expressed genes were unique to one stressor than shared between all three stressors (Rocheta et al. 2016).

In the context of escalating and diverse consequences of climate change throughout the world, many plants are likely to endure escalating forms of abiotic stress. Understanding how shared and unique transcriptomic changes enable plants to respond to the effects of different types of abiotic stress is important for better predicting and mitigating impacts on plant survival and productivity (Wang et al. 2016). The escalation of various environmental stressors due to climate change and anthropomorphic habitat degradation will extend to both wild plants and agriculturally cultivated crops, and some researchers have proposed taking a transgenic approach to modifying multi-stress response regulatory genes in economically important cultivated species to most effectively mitigate a world of more extreme and stochastic abiotic stressors (Ng et al. 2018; Raza et al. 2021).

The annual C_4 grass *Setaria viridis* has emerged as a potential model genetic system for panicoid grasses in the Poaceae family due to its sequenced, diploid genome, short life cycle, high rate of self-fertilization, abundant seed production, and small size (Carvalho et al. 2022). Its wide geographic range, high phenotypic and genetic diversity, and cross-compatibility with the agriculturally important domestical millet *Setaria italica* also make *S. viridis* a relevant candidate for exploring conserved and variable transcriptomic responses to different forms of environmental stress (Defelice 2002; Li and Brutnell 2011; Xiong et al. 2022).

In this experiment, we build upon our previous work comparing the physiological responses of nine geographically diverse populations of *S. viridis* to drought stress and perform

an assessment of the transcriptomic response of two of these populations to multiple forms of abiotic stress. Existing research has found *S. viridis* to exhibit plasticity in physiological, transcriptomic, and biochemical responses to different types of environmental stress (Saha et al. 2016; Duarte et al. 2019). However, comparisons of stress tolerance across accessions of *S. viridis* have frequently been limited to the Ast-1 and A10.1 accessions (Duarte et al. 2019, Ariño-Estrada et al. 2019; Travassos-Lins et al. 2021; Carvhalo et al. 2022). While these accessions have been shown to differ in their abiotic stress tolerance, their response to variable edaphic and climatic factors is not necessarily representative of stress tolerance throughout the species. Ast-1 is from Azerbaijan, which has a fairly temperate climate, and A101.1 is an inbred accession propagated by single seed descended from germplasm collected in Canada but believed to have originated in China (Jia 2017; Mamadi et al. 2020), limiting meaningful determination of home range climate. Variability and adaptation in abiotic stress response across the full range of climatic conditions in which wild populations of *S. viridis* grow has not been fully explored. Our experiment addresses some of these gaps in knowledge by comparing transcriptomic response to drought, salinity, and chilling stress in two wild populations of *S. viridis* from climatically distinct home ranges with previously observed differences in drought tolerance.

Methods

Germination procedure

Seeds were stored in a 4° C fridge in sealed bags with desiccant beads prior to the beginning of the experiment. Germination was performed in an indoor grow room with a 12-hour photo period. Seeds were planted in MetroMix360 in open trays. Soil was fully hydrated and

allowed to rest for 10 minutes before seeds were added and pressed slightly below the soil surface. Trays were covered with plastic covers for the first three nights to increase humidity. Seedlings began germinating after 5-7 days. Approximately 10-14 days after planting, trays were moved to the greenhouse where they received natural light and were watered as necessary.

After 2-3 weeks, seedlings were transferred to individual 3.5-inch (8.9 cm) diameter plastic pots. Pots were filled with a 6:3:1 mixture of MetroMix360, Quikrete Commercial Grade Medium Sand, and local prairie soil from which all large roots, rocks, and insects were removed. Pots were filled and weighed to standardize the amount of soil, then watered to saturation before seedlings were transplanted.

Two populations were used: Chile (PI 202407) and China (PI 408810; Table 3.1). Populations had been shown to differ in a previous drought tolerance experiment in which China was shown to be relatively more drought tolerant than Chile.

Drought and cold stress experiments

Seeds were planted in May of 2019. Six plants were randomly assigned to each of the 4 groups: dry down, dry down control, chilling stress, and chilling stress control. Plants were arranged in 6 spatial blocks on the greenhouse bench and randomly assigned by group to positions within the blocks. Experimental interventions began when plants were 38 days old.

The dry down was performed in the same way as the dry downs described in Chapter 2 of this dissertation. RNA from control and droughted plants was harvested when droughted plants reached 50% of initial photosynthetic rate as measured by a LiCor 6400. Two 3-4 cm pieces of leaf were sampled from each plant and immediately placed in a 25mL Fisherbrand centrifuge tube filled with liquid nitrogen. (Leaf tissue was used to enable comparison of transcriptomic

response to stress with physiological responses to stress measured through decline in leaf photosynthetic rate in Chapter 2.) After the samples were flash-frozen, tubes were drained of liquid nitrogen and capped. Samples were either placed in a dewar of liquid Nitrogen or in a cooler of dry ice for transport back to the lab, where they were placed in a -80°C freezer. This process was repeated for each plant producing 2 sample tubes per plant.

Chilling stressed plants were placed in a 4°C cold room with a 12-hour photo period for 1 week. Controls for the cold room were kept in a growth room with an equivalent photo period at 22°C. Plants were randomly assigned positions within the cold room and growth room by group. All plants were watered as needed when the soil surface became dry.

RNA was harvested from control and chilling stressed plants immediately after chilling stressed plants were removed from the cold room. Two 3-4 cm pieces of leaf were sampled from each plant and immediately placed in a 25mL Fisherbrand centrifuge tube filled with liquid nitrogen. After the sample was flash-frozen, tubes were drained of liquid nitrogen and capped. Samples were immediately placed in a -80°C freezer. This process was repeated for each plant producing 2 sample tubes per plant.

Salinity stress experiment

Seeds were planted in June 2019. Germination was carried out as previously described. Eight plants from each population (Chile PI 202407 and China PI 408810) were randomly assigned to control or salinity stress categories. Plants were arranged in 6 spatial blocks on the greenhouse bench and randomly assigned by group to positions within the block. Plants were 42 days old at the start of the experiment. During the experiment, they were watered each day with 100mL of either distilled water or 200 mmol/L NaCl solution. RNA was harvested either when

experimental plants reached approximately 50% of initial photosynthetic rate as measured by a LiCor 6400 or after 12 days of salt treatment, whichever was earlier.

Two 3-4 cm pieces of leaf were sampled from each plant and immediately placed in a 25mL Fisherbrand tube filled with liquid nitrogen. After the sample was flash-frozen, tubes were drained of liquid nitrogen and capped. Samples were either placed in a dewar of liquid Nitrogen or in a cooler of dry ice for transport back to the lab, where they were placed in a -80° C freezer. This process was repeated for each plant producing 2 sample tubes per plant.

RNA extraction

Three biological replicates were selected for each treatment and control group from each population for a total of 36 samples (2 populations x 6 treatments x 3 replicates).

A Qiagen RNeasy Plant Mini Kit (Qiagen 2016) was used to perform extractions. Leaf tissue was thoroughly ground using a mortar and pestle chilled with liquid Nitrogen. The remainder of the extraction procedure was carried out according to the Qiagen RNeasy Plant Mini Kit (Qiagen 2016), utilizing the suggested DNAse Max protocol (Qiagen 2019).

A NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE) was used to asses RNA quality and quantity. Samples that did not meet concentration and purity standards of 1.9 260/280 ratio and 25 nmol/ul were re-eluted. Samples that still did not meet this threshold after re-elution were discarded and replaced with other samples from the same populations and treatment groups.

Sequencing

Library preparation and sequencing were performed at the Integrated Genomics Facility of the Throckmorton Plant Sciences Center at Kansas State University. Libraries were

prepared with TruSeq stranded mRNA kit (Illumina). Sample quality was checked using with Agilent Bioanalyzer before and after library preparation.

Single end sequencing was carried out through an Illumina NextSeq500 Sequencing run using with NextSeq Hi Output 75 cycles v2.5 kit. TruSeq 6bp single indexes were used to barcode the samples. 20-40 million reads were generated per sample.

Sequence assembly and differential gene expression

Raw sequences were quality assessed with FASTQC (Andrews 2010), then trimmed and filtered using Trimmomatic V0.39 (Bolger et al. 2014) according to the following criteria: (1) adapters and barcodes removed, (2) reads < 30 bases removed, (3) bases trimmed from read ends if quality < 30, and (4) read ends trimmed while mean quality < 30 in a 5 bp sliding window.

Trimmed sequences were then re-assessed with FASTQC. Technical replicates were concatenated before Bowtie2 v.2.4.1 (Langmead and Salzberg 2012) was used for aligning reads to the *Setaria viridis* reference genome v. 2.0 (*Setaria viridis* v2.1, DOE-JGI, http://phytozome.jgi.doe.gov/) in conjunction with RSEM v1.3.3 (Li and Dewey 2011), which was used to calculate expression and generate a count matrix. Separate matrices were generated to compare gene expression in each population/treatment to the level of gene expression in its matched control.

EBSeq was used for differential expression analyses of read counts arising from RNA-Seq (Leng and Kendziorski 2020). For each population/treatment combination, the corresponding control plants were used as baseline and set as condition 1. Lists of Differently Expressed Genes (DEGs) were generated for each population and treatment at 0.01 False Discovery Rate cutoff. FDR was calculated from PPDE >= 1 – FDR, where PPDE represents the posterior probability that a gene/transcript is differentially expressed. R (R Core Team 2023)

with an RStudio integrated development environment (Posit Team 2023) was used to assess overlapping DEGs between populations and treatments. The 'ggVennDiagram' function of the ggplot2 package was used to create Venn Diagrams displaying overlapping DEGs between populations and treatment conditions (Wickham 2016). The 'geom_col_pattern' function of the ggplot2 package was used to create Figure 3.1 (Wickham 2016).

Fold Change analysis

RealFC (Fold Change) values were generated from EBseq. This value is the normalized (within condition 1 mean count) / (normalized within condition 2 mean count). A fold change > 1 indicates that condition 1 has higher expression than condition 2, while a fold change <1 indicates that condition 2 has a higher expression than condition 1. As a result, the RealFC values generated from EBseq were less than 1 for upregulated genes and greater than 1 for downregulated genes. The inverse (1/x) RealFC values for DEG fold change were calculated to enable a more intuitive analysis of regulation difference relative to the gene expression values in the control condition (set as 1). The $\log_2$ of the inverse RealFC values were then calculated to create a more easily interpretable symmetric distribution of fold change values around zero, in which downregulated values are negative and upregulated values are positive. We will therefore use $\log_2$(1/RealFC) values to assess fold change throughout the paper. RFC outliers were calculated as genes with RFC values greater than Quartile 3 + (1.5 * Interquartile Range) or lesser than Quartile 1-(1.5 * Interquartile Range) with Interquartile Range calculated as Quartile 3-Quartile 1. Figures were created in RStudio using the 'ggVennDiagram' and 'geom_boxplot" function of the ggplot2 package (Wickham et al. 2016).

Gene Ontology analysis

Ensembl Genomes Protein IDs were extracted from the local IDs for all DEGs generated at FDR cutoff 0.01 (all references to "DEGs" from here on out refer to those generated at FDR 0.01 cutoff). These Ensembl Genomes Protein IDs were converted to UniProtKB IDs using the Retrieve/ID mapping tool (UniProt 2021).

Gene ontology (GO) analysis was performed using the statistical overrepresentation test from PANTHER Classification System (Mi et al. 2019, Thomas et al. 2021). The representation of GO terms in each of our 12 lists of DEGs (up and down regulated DEGs for each of 2 populations x 3 treatments) was compared to the representation of GO terms in the 38,120 genes from the *Setaria viridis* (SETVI) reference proteome genome in PANTHER. We conducted statistical overrepresentation tests for three functional categories (PANTHER GO-Slim Biological Process, PANTHER GO-Slim Molecular Function, and PANTHER GO-Slim Cellular Component), using a Fischer's exact test with FDR $P < 0.05$ to evaluate overrepresentation of GO terms in our DEG lists compared to the reference proteome genome for each functional category. After performing these statistical overrepresentation tests in PANTHER, we removed any GO terms found to be statistically overrepresented at FDR $P < 0.05$ but not FDR $P < 0.01$ to maintain consistency with the FDR cutoff of 0.01 used in our calculation of DEGs. For some of the DEG lists, the GO term "Unclassified" was found to be overrepresented compared to the reference, and this was also removed from further analyses as it did not provide additional information about gene functions overrepresented in the DEG lists. We then compared the remaining lists of statistically overrepresented GO terms to assess shared terms between populations and treatment conditions for up and down regulated DEGs.

DroughtDB comparison

To help contextualize the gene expression changes observed in our experiment within the existing literature on stress response genes, we performed a local blast search comparing our DEGs to the DroughtDB (DDB), a database of known plant drought stress genes and their homologues (Alter et al. 2015). The database contains a total of 199 genes from 38 plant species, with unique fasta sequences available for 190 genes.

Using the NCBI blast command line tools (NCBI), we created a local database from the 190 DDB genes and blasted each set of population and treatment specific DEGs against it. We then assessed how many of DEGs for each population and treatment combination for up and down regulated genes were found to have significant alignment with genes from the DroughtDB, and how many DDB genes they aligned with. The 'ggVennDiagram' function of the ggplot2 package was used to create Venn Diagrams displaying overlap of DDB blast alignments with DEGs (Wickham 2016).

Results

Differentially Expressed Genes

Out of the 52,459 protein-coding transcripts in the *S. viridis* reference genome v2.1, between 40,335 and 42,127 protein-coding transcripts aligned to the reference for each of our six population and treatment combinations (Table 3.2). Upregulated and downregulated DEG lists for each population and treatment condition were generated from EBSEQ at FDR 0.01. The number of upregulated and downregulated DEGs varied widely across population and treatment combinations (Figure 3.1, Table 3.2). The highest number of total DEGs were from the China population under chilling stress, with 6880 genes upregulated and 7343 genes downregulated for

a total of 14,223 DEGs, 33.9% of the total aligned sequences for this condition (Figure 3.1, Table 3.2). The smallest total number of DEGs were in the drought stress condition for the China population, with 181 genes upregulated and 207 genes downregulated for a total of 388 DEGs, 1% of the total aligned sequences for this condition (Figure 3.1, Table 3.2). Differences were found in the number of DEGs for each population in response to each of the treatment conditions. The Chile population had approximately 1.5x more DEGs than the China population in the drought stress condition. The China population had approximately 14x more DEGs in the chilling stress condition and approximately 1.8x more DEGs in the salinity stress condition compared to the Chile population. The largest difference in the number of upregulated and downregulated DEGs for one treatment condition was observed for the Chile population in the chilling stress condition, in which there were 893 upregulated DEGs and only 71 downregulated DEGs. For both populations, the chilling stress condition had the largest number of DEGs and the drought stress condition had the smallest number of DEGs. There were more upregulated than downregulated DEGs in four of six experimental conditions, with the exceptions being the China population under chilling stress and the Chile population under salinity stress, in which there were more downregulated than upregulated DEGs.

Very few DEGs (zero for the Chile downregulated DEGs and one each for the others) were found to overlap between all three treatments for each population, with a greater number of genes overlapping between 2 of 3 treatment conditions (Figure 3.2). The individual genes upregulated across all 3 treatment conditions for each population were found in a gene ontology analysis to both have functionalities related to ATPase activity, peptidase activity, proteolysis, and mitochondrion (Table 3.3). The single gene that was downregulated across all 3 treatment conditions for the China population was found in a gene ontology analysis to have functionalities

related to protein serine/threonine kinase activity, protein phosphorylation, defense response to bacterium, and plasma membrane. Comparisons of DEGs across population within treatment condition (Figure 3.3a-c) found overlaps between populations for all conditions, with the largest number of overlapping DEGs for genes upregulated under chilling stress (Figure 3.3a) and the smallest number of overlapping DEGs for genes upregulated under drought stress (Figure 3.3b). However, with the exception of DEGs upregulated under chilling stress (for which the majority of the genes upregulated in the Chile population were also upregulated in the China population, Figure 3.3a), the majority of DEGs were not shared between populations within treatment condition.

Fold Change

Real Fold Change (RFC) values were generated from EBseq and converted to $\log_2(1/RFC)$ for a more interpretable format. Summary statistics for RFC distributions for each population and treatment condition are shown in Table 3.4. For 11 of the 12 populations and treatment conditions, the absolute value of the mean was greater than the absolute value of the median (Table 3.4), indicating a right skewed distribution in which many genes have an RFC value close to zero and a relatively small number of genes have RFC absolute values much greater than the median. This is further evinced by plotting the RFC distribution (Figure 3.4), which also shows the presence of multiple outliers for 5 of the 12 populations and treatment conditions. RFC outliers were identified in upregulated DEGs for both populations under chilling stress, in downregulated DEGs for both populations under salinity stress, and in downregulated DEGs for the China population under chilling stress. The greatest number of outliers were in the upregulated DEGs from the China population under chilling stress.

In a comparison of upregulated and downregulated RFC outlier DEGs across populations and treatment conditions (Figure 3.5), 8 DEGs were identified as RFC outliers, which were upregulated for both populations under chilling stress and a total of 10 DEGs were identified as RFC outliers for 2 of the 3 downregulated populations and treatment conditions (Figure 3.5), with zero DEGs identified as outliers across all three population-treatment combinations. Most genes thus were identified as RFC outliers for only one population and treatment condition. Of the 18 total genes identified as RFC outliers for 2 populations and treatment conditions, 4 were found to correspond to GO term entries in PANTHER, with functions including ubiquitin protein transferase activity, protein serine/threonine kinase activity, protein autophosphorylation, carbohydrate kinase activity, phosphotransferase activity, alcohol as a group acceptor, and plasma membrane (Table 3.5.) A0A4U6VFD5 was both an RFC outlier for China population downregulated DEGs under chilling stress and salinity stress, and the only gene that was significantly downregulated in the China population across all three stressors (Table 3.3).

Functional classification

A statistical overrepresentation analysis was performed using PANTHER to determine which GO terms were overrepresented in up and down regulated DEGs compared to the *S. viridis* reference genome. In total, 518 GO terms were found to be overrepresented in DEGs from the China population and 25 GO terms were found to be overrepresented in DEGs from the Chile population (Table B.1). There were no overrepresented GO terms in DEGs from the China population under drought stress, the Chile population under drought stress, or the Chile population under chilling stress. The majority of overrepresented GO terms were in DEGs from the China population under chilling stress (504) and the majority of those (422) were in upregulated DEGs (Appendix Table B.1). Twelve GO terms were overrepresented in two

conditions (either in DEGs from both populations or in DEGs from more than one treatment condition within the same population), including seven in the Biological Function category, three in the Molecular Function category, and two in the Cellular Component category (Table 3.6).

Out of the 543 significantly overrepresented GO term findings across both populations, we assessed the top 1% most overrepresented as measured by the magnitude of the fold enrichment. The top 5 highest fold enrichments values from our analysis were from four GO terms (as 2 of them were the same GO term found to be overrepresented in both populations). All were from DEGs upregulated under salinity stress, were in the Biological Process functional category, and showed an enrichment of 10-fold or greater compared to the expectations based on the reference genome (Table 3.7). Response to cold (GO:0009409) was overrepresented over 10-fold in both the China and Chile populations under salinity stress, response to salt stress (GO:0009651) was overrepresented over 10-fold in the China population under salinity stress, response to reactive oxygen species (GO:0000302) was upregulated over 10-fold in the China population under salinity stress and also over 2-fold in this population under chilling stress, and response to osmotic stress (GO:0006970) was upregulated over 10-fold in the China population under Chilling stress.

DroughtDB comparison

To assess potential overlap between the DEGs identified in our current study with previously identified genes responsive to drought stress, we performed a BLAST search of the DEGs from our abiotic stress experiments against a local database of the genes included in the DDB (Alter et al. 2015). This database includes 199 genes from 38 plant species which have previously been identified and molecularly characterized as related to drought stress. Within the

database, these genes are categorized based on their involvement in physiological and molecular adaptation related pathways, as well as more specific functions within each of these pathways (Alter et al. 2015).

Unique fasta sequences were available for 190 of the 199 DDB genes, and we used these sequences to create a local database against which we blasted each of our 12 sets of DEGs (up and down regulated DEGs from each population and treatment condition). At least some of the genes from all 12 of the upregulated and downregulated DEG lists were found to align significantly with genes from the DDB at threshold $1e^{-20}$ (Table 3.8). The smallest fraction of DEG genes aligned to DDB genes was from the Chile salinity upregulated DEG list (0.072) and the largest fraction of DEG genes aligned to DDB genes was from the Chile salinity downregulated DEG list (0.231). The DEG list that aligned with the smallest fraction of the DDB genes was the Chile salinity downregulated DEG list (0.063) and the DEG list which aligned with the largest fraction of DDB genes was the China chilling downregulated list (0.700).

There was notable overlap in which DDB genes DEGs aligned with when comparing the DEGs across treatment within population (Figure 3.6) and across population within treatment (Figure 3.7). For both populations, there were multiple DDB genes to which DEGs from all treatment conditions aligned (Figure 3.6). We identified the DDB genes to which only upregulated DEGs from all treatment conditions aligned for each population (meaning that any DDB genes that also aligned with downregulated genes for all treatment conditions within the population were removed) and their biological functions (Table B.2). Functions include calcium-dependent protein kinases, transcription factors, hormone transporters, and serine/threonine specific protein phosphatases.

From our original lists of DDB genes to which DEGs aligned, we eliminated all DDB genes that aligned with both up and down regulated DEGs from the same population and treatment condition to identify DDB genes that were uniquely aligned with upregulated DEGs for each population and treatment condition (Table 3.9). For DDB genes to which upregulated DEGs uniquely aligned, the largest fraction of alignment was in the Chile chilling DEG list (0.308) and the smallest fraction of genes aligned to DDB genes was from the China drought DEG list (0.087).

Finally, using the DDB Pathway Browser, which incorporates 169 of the DDB genes, (Alter et al. 2015), we identified the pathway nodes of the DDB genes to which upregulated DEGs for each population and treatment condition uniquely aligned to upregulated DEGs (Figure 3.9, Table B.3). Upregulated DEGs from the Chile population aligned with 35/79 DDB genes in the Physiological Adaptation category and 78/158 DDB genes in the Molecular Adaptation category, while upregulated DEGs from the China population aligned with 28/79 DDB genes in the Physiological Adaptation category and 60/158 DDB genes in the Molecular Adaptation category. Upregulated DEGs from the Chile population aligned with a greater number of DDB genes in the pathway nodes for ion and osmotic homeostasis, ion channels, water channels, protein kinases, and ABA degradation. Upregulated DEGs from the China population aligned with a greater number of DDB genes in the pathway nodes for osmolyte production, osmolyte biosynthesis, detoxification, protein phosphatases, ABA biosynthesis, and ABA signaling. There was an equal number of DEGs upregulated from each population with DDB genes in the pathway nodes for removal of ROS, detoxification signaling, LEA proteins, proteases, ABA importer, ABA exporter, and ethylene biosynthesis (Table B.3). Of the 62 TFs in the DDB Pathway Browser, upregulated DEGs from the Chile population aligned to 33 of them

and upregulated DEGs from the China population aligned to 29 of them. Upregulated genes from the Chile population aligned with a greater number of MYB, MYC, NAC and WRKY TFs, upregulated genes from the China population aligned with a greater number of DREB, bZIP, AP-2 Domain, and ERF TFs, and alignment of both populations with Zinc Finger TFs was equal.

Discussion

Environmental stressors, including drought stress, chilling stress, and salinity stress, are predicted to increase in severity, frequency, and stochasticity in many parts of the world due to anthropogenically mediated climate change (Dai 2013; van Zelm et al. 2020; Augspurger 2013). Understanding how plants respond to these changes is important for predicting the impacts of these shifting climatic and edaphic trends, especially for agriculturally and economically important species. As a continuation of our exploration of local adaptation and response to environmental stress in *S. viridis*, a close relative of the cereal crop *S. italica*, this transcriptomic experiment sought to provide additional information about the biological mechanisms facilitating observed stress responses within and between populations from different climates of origin.

Due to the overlap in adverse impacts experienced by plants subjected to drought, chilling, and salinity stress, including low water potential, increased production of ROS, membrane damage, and ionic stress (Verslues et al. 2006; Benny et al. 2020; Sharma et al. 2020; Valeriano et al. 2021), we had anticipated potential overlaps in DEGs responsive to each of our experimental stress conditions. As other studies have found that some stress response genes may be commonly modulated across different plant species (Benny et al. 2020) we expected that stress response pathways would be relatively conserved between our two *S. viridis* populations , but with potential differences in the fold change of key stress response genes, especially given that the China population was shown in one of our previous experiments to have superior

drought tolerance. While plants navigate many adaptive trade-offs in vegetative, reproductive, and stress-responsive traits, some prior studies have shown certain populations or genotypes to possess superior tolerance to multiple stressors, potentially due to simultaneous selection for forms of abiotic stress that may co-occur in their home ranges and the overlap in various stress response pathways (Saha et al. 2016; Xu et al. 2020; Valeriano et al. 2021).

Analysis of DEGs and RFC

Transcriptomic response was assessed through the number of up and downregulated genes in the environmental stress treatment conditions relative to the control condition, and through the gene expression fold change for these differently expressed genes. Both of these metrics are important indicators of gene expression and regulation changes in response to environmental stress. Alterations in gene expression are an important part of plant response to stress as sessile organisms who cannot relocate when environmental conditions present challenges to their survival. Elevated levels of transcriptomic change can potentially be indicative of adaptive (facilitating fitness-promoting traits conferring stress resistance or modulating genetic perturbation) or non-adaptive (reflecting response to injury) responses to stress (Chan et al. 2012).

In several previous studies, plants that were more tolerant of specific environmental stressors were found to have fewer DEGs under stress than less tolerant plants, due either to better constitutive adaptation or more precisely adaptive induced responses (Essamine et al. 2020). Increased numbers of upregulated genes can indicate greater physiologic disruption (Essamine et al. 2020), while increased numbers of downregulated genes may indicated decreased homeostatic maintenance and reduced allocation to non-stress responsive functions (Chan et al. 2012; van Zelm et al. 2020). Increased expression levels in a specific subset of genes

that support adaptive stress responses can indicate superior stress tolerance (Fracasso et al. 2016). Therefore, both the number DEGs and the function of those DEGs can be important to evaluating a plant's tolerance to a particular source of stress.

In our previous experiments, the China population was shown to be more tolerant of drought stress than the Chile population (Table 3.1), and in our transcriptomic analysis, the China population had fewer DEGs under drought stress than the Chile population. Our experiment utilized a single timepoint in the transcriptomic response to stress for each population and treatment combination. Previous research has shown that transcriptomic responses may vary based on stress intensity (Li et al. 2020), time of day (Sun et al. 2022), and duration (Lata et al. 2014) and that patterns of DEGs shift across different stages of stress response, with expression of some genes increasing early on in the response and expression of others increasing later due to either acclimation to or increasing decline from the stress over time. The greater level of gene expression changes occurring in the Chile population while experiencing drought conditions may indicate higher levels of stress relative to the China population at the time of sampling (Essamine et al. 2020). The mean and maximum RFC values for up and downregulated genes across populations experiencing drought stress were very similar, which may mean that the number of genes up and down regulated under drought conditions is more indicative of experienced drought stress than the average or maximum expression fold change of those genes.

The average annual precipitation in the home range of the Chile population (1279 mm/year) is much greater than that of the China population (604 mm/year; Table 3.1). In addition to the differences in average annual precipitation between the two population's home ranges, based on data from WorldClim, the home range of the China population also had a higher precipitation seasonality coefficient of variation (Fick and Hijmans 2017). This measurement is

the ratio of the standard deviation of the precipitation for each month to the mean precipitation per month, so a higher value indicates greater variability of precipitation throughout the year (O'Donnell and Ignizio 2012). It is possible that the observed differences in drought response between these populations are due in part to different levels of selective pressure to endure water limited conditions and inconsistent precipitation availability within their home ranges, especially as a short-lived, annual species. While there was not found to be an overall significant relationship between average annual precipitation in the home range and observed drought tolerance across 9 *S. viridis* populations (see Chapter 2), that does not eliminate the possible influence of this environmental variable on drought response for any individual population.

For the chilling stress condition, the population from China had many more DEGs, more RFC outliers, and greater mean absolute value RFCs for both up and down regulated genes as compared to the Chile population. The Chile population however had higher maximum RFC values for upregulated DEGs and a lower minimum RFC values for downregulated DEGs. The China population had more downregulated than upregulated DEGs, whereas the Chile population had more upregulated than downregulated DEGs for this condition (Figure 3.1). The difference in the number of chilling stress DEGs between populations is striking, which may demonstrate much higher levels of stress for the China population experiencing chilling relative to both the Chile population and the other types of stress treatments. The average annual mean temperature for the China (5.1ºC) population's home range is cooler than that of the Chile population (13.1ºC), however this may not indicate the actual climatic conditions experienced by a short-lived annual plant during its growing season. For example, if both the Chile and China populations typically grow during the wettest quarter in each of their native ranges, the wettest quarter for the Chile population has a mean temperature of 8.4 ºC with 695mm precipitation and

the wettest quarter for the China population has a mean temperature of 21.8 ºC with 408mm precipitation. It is therefore possible that the China population may be less likely to encounter cooler temperatures in its native range and growing season and would experience relatively less selective pressure to develop greater tolerance/reduced stress when experiencing chilling.

A recent study evaluating two accessions of *S. viridis* for chilling stress found both to be impacted by but capable of recovering from the impacts of chilling stress at temperatures as low as 0 ºC once returned to warmer temperatures (Carvalho et al. 2022). During chilling stress, photosynthetic rate decreased, likely due to increased stomatal closure and impaired functionality of photosynthetic enzyme complexes below 10 ºC (Carvalho et al. 2022). Plants kept at lower chilling temperatures for longer durations did not recover to baseline photosynthesis or chlorophyll fluorescence levels following chilling stress, and plants in the reproductive phase were more susceptible to damage from chilling stress than vegetative plants, with a somewhat greater susceptibility shown in the A10.1 accession than the Ast-1 accession (Carvalho et al. 2022). Another study assessing transcriptomic response to chilling stress over time in an unspecified accession of *S. viridis* identified 7911 total DEGs in response to chilling stress with increasing levels of gene expression change over time (Sun et al. 2022). While this is still only about half the number of total DEGs observed in the China population under chilling stress, there is some evidence that the need to synthesize substances that facilitate cold resistance, such as purines and pyrimidines, coupled with the inhibition of enzymatic activity, growth, and many basic biological processes under chilling stress can produce substantial gene expression changes (Sun et al. 2022).

Under the salinity stress condition, there were more up and down regulated DEGs in the China population than in the Chile population, though the difference in these DEG counts was

much less substantial than for the chilling stress condition. The China population had greater mean and maximum RFC values for upregulated DEGs and a lower minimum RFC value for downregulated DEGs while the Chile population had a lower mean RFC value for downregulated DEGs. Only the China population had RFC outliers for downregulated DEGs. A study comparing transcriptomic response of *S. viridis* under salinity stress to a highly salt tolerant halophyte found that *S. viridis* showed substantial changes in gene expression, both up and down regulation, in comparison to the more salt tolerant species (Essemine et al. 2020). In keeping with this trend, the increased number and fold change of differently expressed genes in the China population may indicate an overall more elevated stress response to salinity in this population compared to the Chile population.

Previous research has also observed differences in salinity tolerance between populations of *S. viridis*. One study comparing the A10.1 accession to the Ast-1 accession noted different patterns of upregulation in ABA signaling related genes between the two accessions in the 48 hours following the addition of a 200mM NaCl solution (Duarte et al. 2019). Another study comparing the salt spray tolerance of coastal populations of *S. viridis* (*Setaria viridis* var. pachystachys (Franch et Savat.)) to inland populations found significantly higher salt spray tolerance in the coastal population, as well as evidence that this salt tolerance trait was highly heritable (Itoh et al. 2021; Itoh et al. 2024). Based on an accompanying common garden experiment, which found that the inland plants were quickly eliminated in coastal regions by the strong winds and salt spray, the researchers suggested that the salt-spray tolerant coastal ecotype may have evolved rapidly due to the strong selective pressure of this harsh environment and the high rates of self-pollination in *S. viridis*, though potential candidate genes conferring this tolerance have not been identified (Itoh et al. 2024). While this type of extreme ecotypic

difference in salinity tolerance was not present in our experiment, these results indicate that natural selection on salt tolerance in *S. viridis* is a putative driver of divergence in this trait, which may be observable within more moderate differences between populations. There are many other potential causes of mild salinity stress that may drive selection for slight variations in constitutive salinity tolerance or increased plasticity in response to salinity stress, including soil salinization that develops from evapotranspiration in arid and semi-arid environments, irrigation, deforestation, and anthropogenic industrial chemicals (Yadav et al. 2011).

Comparisons of co-tolerance to multiple stressors across different accessions of *S. viridis* have shown mixed results. A comparison of six *S. viridis* accessions found that three of them were more tolerant to both water-deficit and heat stress, while the other three were less tolerant to both (Saha et. al 2016). Interestingly, different comparisons of the relative stress tolerance of the Ast-1 and A10.1 accessions have produced conflicting results. Two studies found the A10.1 accession to be more tolerant of water-deficit than Ast-1 (Saha et al. 2016; Travassos-Lins et al. 2021), while another study found the opposite (Duarte et al. 2019). Studies of comparative NaCl tolerance have produced results indicating that Ast-1 may be more salt tolerant (Ariño-Estrada et al. 2019), less salt tolerant (Valeriano et al. 2021), or equally salt tolerant (Duarte et al. 2019) compared to A10.1. Two studies failed to find substantial differences in chilling tolerance between the two accessions (Duarte et al. 2019; Carvalho et al. 2022), but one indicated that A10.1 may be somewhat more sensitive to chilling stress than Ast-1 only during its reproductive stage (Carvalho et al. 2022). These inconsistent observations may be driven by differences in experimental conditions, including intensity, duration, and method of stress application as well as which markers of stress were assessed. It is also possible that these accessions utilize different primary response mechanisms to tolerate these sources of stress which may be more effective

with some methods of experimentally induced stress than others (ex: grown in soil and irrigation with NaCl solution in Duarte et al. 2019 versus incubation in a tube of solution with ^{22}Na Ariño-Estrada et al. 2019 versus grown in hydroponic NaCl solutions in Valeriano et al. 2021).

Differences in gene expression between different *S. viridis* accessions exposed to stress have also been observed. While many previous studies have assessed a specific subset of genes, rather than the entire transcriptome, in concordance with our results, other researchers observed significant variability between the expression profiles of different *S. viridis* accessions when exposed to drought and salinity stress (Duarte et al. 2019), drought and heat stress (Saha et al. 2016) and drought stress (Travassoss-Lins et al. 2021). A study assessing expression of aquaporin genes in the roots of two *S. viridis* accessions under non-stressed conditions also found differences in gene regulation (Gal et al. 2023), indicating both variability in both constitutive and plastic mechanisms of stress tolerance across accessions. This divergence in stress tolerance and transcriptomic response to stress between *S. viridis* accessions aligns with the characterization of *S. viridis* as a species with "unusually low intrapopulation genetic diversity, and unusually high genetic diversity between populations compared with an average plant species" (Dekker 2003, p. 641).

Functional and GO Analysis

Although drought, chilling, and salinity stress all create the experience of low water potential for the plant (Verslues et al. 2006), we found few genes which were upregulated across all three conditions for each population, though there were somewhat more genes upregulated either across two treatments for a population, or in both populations for the same treatment. We further investigated functions of the small number of genes which were identified as DEGs across all 3 stressors, or which were found to be RFC outliers for at least 2 stressors.

For upregulated DEGs, there was a single gene upregulated across all 3 treatment conditions for the China and Chile population each and 8 RFC outliers from upregulated DEGs across both the China and Chile populations under chilling stress (only 1 of these 8 genes was found to have functional category entries in GO term analysis using PANTHER). The shared upregulated DEG across stressors for the Chile population (Gene ID: lcl|CM016555.1_cds_TKW20581.1_20284, Mapped ID: A0A4U6UV8) was associated with the following GO terms: ATPase activity, peptidase activity, proteolysis, mitochondrion, and chloroplast envelope. The shared upregulated DEG across stressors for the China population (Gene ID: lcl|CM016554.1_cds_TKW26389.1_15288, Mapped ID: A0A4U6T5Z1) was associated with a very similar set of GO terms: ATPase activity, peptidase activity, proteolysis, and mitochondrion. Breakdown of damaged proteins and cellular components during abiotic stress is important for the preservation of cellular integrity, and upregulation of peptidases and proteolysis activity may be related to this stress response pathway (Flick and Kaiser 2012). The upregulation of ATPase and mitochondrion may be related to the multifaceted functionalities of mitochondria in stress response, including signaling, regulation of redox in the context of elevated ROS generation, and provision of cellular energy needed to carry out all components of the stress response (Aken 2021).

The shared upregulated RFC outlier between the Chile and China populations undergoing chilling stress was associated with the following GO terms: ubiquitin-protein transferase activity, ubiquitin protein ligase binding, protein ubiquitination, and cullin-RING ubiquitin ligase complex. Ubiquitination is an important cellular process that is essential to maintaining homeostasis by removing degraded proteins, and the upregulation of this process, as well as genes for proteolysis and peptidase related activates, may indicate an increased need to remove

proteins which have been damaged due to chilling stress, as noted above (Flick and Kaiser 2012).

For downregulated DEGs, there was a single gene downregulated across all 3 treatment conditions of the China population and a total of 10 RFC outliers from downregulated DEGs shared by 2 populations and treatment conditions (3 of which were found to have functional category entries in GO term analysis using PANTHER). The shared downregulated DEG across stressors for the China population (Gene ID: lcl|CM016557.1_cds_TKW09159.1_31771, Mapped ID: A0A4U6U6Y4) was associated with the following GO terms: protein serine/threonine kinase activity, protein phosphorylation, defense response to bacterium, and plasma membrane. This gene was also 1 of the 2 shared RFC outliers under chilling and salinity stress for the China population; the other 1 was associated with the following GO terms: carbohydrate kinase activity, phosphotransferase activity, alcohol group as acceptor, hexose metabolic process, and cytosol. The other RFC outlier with PANTHER GO term entries was shared between the China and Chile populations under salinity stress and was associated with the following GO terms: protein serine/threonine kinase activity, protein autophosphorylation, and plasma membrane. These specific and overlapping functions indicate a reduction in phosphate transfer, and as phosphorylation is a major regulator of cellular processes and metabolism (Cheng et al. 2011), the downregulation of these genes may be related to an overall decrease in metabolic rate in plants experiencing these forms of stress. Protein kinases and protein phosphatases can function in signaling pathways which trigger morphological changes to protect the integrity of the cellular membrane and macromolecules in plants experiencing drought stress (Benny et al. 2020). The downregulation of defense response to bacterium may also represent a prioritization of defense response to abiotic stress over biotic stressors.

In addition to investigating the specific functions of the small number of overlapping genes between populations and stressors, we performed a statistical overrepresentation test in PANTHER to identify which GO terms were overrepresented in identified DEGs compared to their representation in the *S. viridis* reference genome. Statistically overrepresented GO terms were not evenly distributed across the 12 DEG lists, with no GO terms overrepresented in DEGs under drought stress for either population and no GO terms overrepresented in the DEGs under chilling stress in the Chile population. The lack of identified overrepresented GO terms in these conditions does not mean that certain gene functions did not play a critical or conserved role in stress response, just that the number of genes associated with each functions was not greater than what would be expected by random selection.

Many more overrepresented GO terms were identified in DEGs under chilling stress in the China population than any other population treatment combination, including GO terms from all three functional categories. This may indicate that the large number of DEGs in this population when subjected to chilling represent a multifaceted response (though not necessarily an adaptive one) to this stressor. Notably, although the greatest number of overrepresented GO terms for the China population were from DEGs under chilling stress, the GO terms with the highest fold enrichment were from DEGs under salinity stress. The only GO terms with a fold enrichment of over 10 in the China population were from salinity stress DEGs, while in comparison, the highest fold enrichment for any chilling stress DEGs was 6.26. The higher fold enrichment of this smaller number of GO terms may demonstrate a targeted functional response to salinity stress, especially as their functions are all directly related to abiotic stress response (response to cold, salt stress, ROS, and osmotic stress).

Of the 4 GO terms with the highest fold enrichment from the China population upregulated salinity stress DEGs, one (response to cold) was also highly overrepresented in the Chile population salinity stress DEGs, and another (response to ROS) was also overrepresented in the China population chilling stress DEGs. Although the only GO term overrepresented in the Chile population upregulated DEGs was response to cold, it had the highest fold enrichment of any GO term in our analysis (21.6). Other GO terms overrepresented in upregulated DEGs for more than one population and treatment condition were response to heat stress, response to temperature stimulus, and unfolded protein binding. Though the overrepresentation of GO terms related to heat stress response in DEGs from plants exposed to chilling stress may seem paradoxical, both hot and cold temperatures outside of a plant's optimum temperature range can result in dehydration and osmotic stress (Li et al. 2020). The shared overrepresentation and high fold enrichment of these abiotic-stress-related GO terms may reflect their role in mounting a functional response to address the similar biological challenges plants can face under temperature and salinity stress, including the excessive ROS production, low water potential, and cellular dehydration (Verslues et al. 2006; Hussain et al. 2018, Benney et al. 2020). Previous research on *A. thaliana* also identified over-expression of genes related to unfolded protein binding, as they play an important role in repairing stress induced protein damage to maintain protein structure and function (Chaddad et al. 2023).

We identified 7 GO terms overrepresented in downregulated DEGs from more than one population and treatment combination with the following functions: cell surface receptor signaling pathway, kinase activity, transferase activity, transferring phosphorus-containing groups, phosphate-containing compound metabolic process, phosphorus metabolic process, membrane, and plasma membrane. These functions are quite similar to those identified for the

single downregulated DEG shared across all three stressors for the China population (Table 3.3) and the shared RFC outliers (Table 3.5). Downregulation of these functions can be related to stress-induced reduction of metabolic rate and cellular processes and changes in signaling pathways (Cheng et al. 2011, Benny et al. 2020), indicating that these may be some of the most commonly and significantly downregulated functions for plants in both populations across treatment conditions.

DroughtDB

To help situate our exploration of *S. viridis* transcriptomic response to 3 different environmental stressors within existing abiotic stress research, we performed a local BLAST and pathway comparison with the DDB (Alter et al. 2015). This database contains 199 genes known to be involved in plant drought response from 38 plant species as well as a "Pathway Browser" which presents these genes in increasingly more detailed categories within the pathways involved in plant adaptation to drought stress, including the roles of specific gene families (Alter et al. 2015) . After blasting our DEGs against a local database made up of 190 genes from the DDB that had unique fasta sequences available, we found that DEGs from all 12 of our DEG lists (1 list each for upregulated and downregulated genes from each population and treatment combination) aligned with DDB genes.

To better assess the alignment of DEGs upregulated under stress with the DDB genes, we identified the sets of DDB genes which aligned only with upregulated DEGs for each population and treatment condition (removing any DDB genes which aligned with both up and down regulated DEGs for the same population and treatment condition). Using these lists of genes, we recreated the Pathway Browser diagram from the DDB based on the representation of uniquely upregulated DEGs from the China and Chile populations. There was substantial overlap in the

pathway alignment of the China and Chile DEGs, but differences were also apparent. This may reflect the presence of conserved and divergent stress response mechanisms across these populations, as well as potential differences in strategies towards and degree of stress tolerance.

Both populations had upregulated DEGs align with DDB genes in the pathway nodes for removal of ROS, detoxification signaling, LEA proteins, proteases, ABA importer, ABA exporter, and ethylene biosynthesis. This may reflect a shared utilization of detoxification processes, ABA regulation, and AP2/ERF family TFs in stress response across both populations. Detoxification signaling is a crucial component of withstanding abiotic stress, especially salinity stress (Chan et al. 2012; Ariño-Estrada et al. 2019). An important component of this is managing rates of ROS production and detoxification in order to maintain normal cell function and prevent oxidative stress (Hussain et al. 2018). Increased accumulation of ROS during stress can substantially damage cellular machinery, degrade DNA, and inhibit photosynthesis, which may be fatal for the plant (Chen et al. 2021). The AP2/ERF family includes ethylene-responsive TFs which are known to be involved in many abiotic stress response pathways such as chilling, salinity, and drought (Chan et al. 2012; Wang et al. 2016; Benny et al. 2020). Abscisic Acid (ABA) is known to play an important role in physiologic responses to biotic and abiotic sources of stress (Bouzid et al. 2019; Benny et al. 2020), with ABA levels rising in response to multiple abiotic stressors (Duarte et al. 2019).

We found upregulated DEGs from the Chile population to align with a greater number of genes for ion and osmotic homeostasis, ion channels, water channels, protein kinases, and ABA degradation in the DDB, whereas upregulated DEGs from the China population aligned with a greater number of osmolyte production, osmolyte biosynthesis, detoxification, protein phosphatases, ABA biosynthesis, and ABA signaling genes. There were also differences in the

number of DEGs from each population aligned to the different types of TFs in the DDB. These differences may indicate disparate key strategies in each population's response to abiotic stressors.

The alignment of the China population DEGs with ABA biosynthesis and signaling genes may reflect the important role of ABA in drought response, including through ABA responsive transcription factors such as DREBs and bZIPs (Wang et al. 2016; Huang et al. 2016; Trono and Pecchioni 2022). The increased alignment with osmolyte production-related genes could point to an important role of osmolytes in this population's drought response mechanism through providing a driving gradient for water uptake into the cell to allow the maintenance of turgor without producing toxicity (Trono and Pecchioni 2022).

Upregulated genes from the Chile population aligned with substantially more MYB TF genes and with slightly more MYC, NAC and WRKY TF genes, upregulated genes from the China population aligned with substantially more DREB and AP-2 Domain TF genes and with slightly more bZIP and ERF TF genes, and alignment of both populations with Zinc Finger TFs was equal. The combination of upregulated gene alignment in the Chile population points to a possibly increased emphasis on mitigating stress through utilization of water and ion channels, such as aquaporins, integral membrane proteins that regulate water flow into or out of cells, and which are known to play an important role in regulating water movement during drought stress (Alexandersson et al. 2010; Tang et al. 2017; Benny et al. 2020). The increased upregulation of these genes the Chile population but not the China population may be part of the overall observed trend of decreased transcriptomic changes in the population more tolerant to a give stress and could also indicate a greater utilization of other drought response mechanisms by the China population or greater constitutive expression of these proteins.

The alignment of upregulated Chile DEGs with ABA degradation genes (in contrast to the alignment with ABA biosynthesis and ABA signaling genes in the China population) and protein kinases (some of which can be activated by abiotic stress directly in an ABA-independent manner) may be indicative of increase importance of ABA-independent stress response pathways for this population (van Zelm et al. 2020). There is some indication that chilling stress response pathways are less ABA dependent than drought stress response pathways (Hussain et al. 2018), and an enhanced tolerance of chilling stress relative to drought stress in this population is plausible if the pattern of decreased transcriptomic change indicating better tolerance holds true. Similarly, the Chile population also had fewer DEGs total under salinity stress than the China population, and WRKY TFs have also been shown to confer increased salinity tolerance across a range of species (Wang et al. 2016).

While we identified some instances of shared DEGs and DEG functions between the Chile and China populations when subjected to 3 forms of abiotic stress, we also found many substantial differences in the number of DEGs, the RFC of DEGs, and the GO terms overrepresented among DEGs when comparing these populations. Although some existing research has shown that aspects of transcriptomic response to stress can be conserved between different plant populations and species (Benny et al. 2020), the level of divergence we found between these two *S. viridis* populations is in keeping with the unusually low intrapopulation genetic diversity and unusually high genetic diversity between populations characteristic of this species (likely related to its high rates of self-fertilization; Dekker 2003). This trend was also reflected in previous studies on interpopulation differences in transcriptomic response to abiotic stress in *S. viridis* (Saha et al. 2016; Travassoss-Lins et al. 2021; Gal et al. 2023). These variable responses may be due to morphological differences between the accessions that they utilized as

well as differing adaptations to stress developed through local adaptation to distinct home environments (Saha et al. 2016; Duarte et al. 2019). As previously noted, the China and Chile populations that we utilized are morphologically similar in terms of overall plant size and inflorescence production, but they originate from climatically different home ranges, providing the opportunity for divergent adaptive response to the local environment to occur.

Limitations

It is important to note that assessing transcriptomes of plants under abiotic stress at a single time point presents a snapshot, rather than a comprehensive census of the complete process of how gene expression is altered in response to that type of stress. Studies of transcriptomic response to abiotic stress that have sampled at multiple time points have indicated changes over time in the identity and fold change of DEGs (Lata et al. 2014; Sun et al. 2022). Our experiment sampled leaf tissue, and stress response, signaling, and metabolite production may also differ between different plant organs (Lata et al. 2014; Muthamilarasan et al. 2015; Guedes et al. 2023), especially in the case of drought and salinity stress, which are typically first encountered by the roots and subsequently by aboveground structures through signaling and transport.

While we used photosynthetic rate measurements to increase standardization in stress intensity at time of sampling for the drought and salinity stress conditions, it is not possible to sample plants experiencing an identical level of stress from different stressors. In particular, the plants subjected to chilling stress may have been experiencing more acute or cumulative stress at the time of sampling, as more upregulated DEGs were identified in both populations under chilling stress as compared to drought and salinity stress. It is also possible that *S. viridis* is less

adapted to chilling stress, though this is not supported by previous research (Duarte et al. 2019; Carvalho et al. 2022) .

Conclusions and Future Directions

This study represents a multifaceted examination of adaptation and response to environmental stress across 2 different populations of *S. viridis* from different home ranges subjected to drought, salinity, and chilling stress. We compared the quantity and magnitude of gene expression changes, the functions of differently expressed genes, and the alignment of these genes to a known set of drought responsive genes. This provided some insight into potential biological mechanisms by which the previously established enhanced drought tolerance of the China population relative to the Chile population may be achieved. We also observed substantial changes in gene expression occurring in the China population when subjected to chilling stress that were nearly 10 times greater than for any other population and treatment combination. In spite of the commonalities in how plants generally are known to experience drought, chilling, and salinity stress, our analysis found minimal overlap in DEGs across the 2 populations and 3 stressors, though some similarities in gene ontology and alignment with DDB genes indicate that some overall response mechanisms, if not individual genes, may show similar response patterns. This pattern of variable DEGs across both populations and stressors is in concordance with existing literature comparing transcriptomic responses of *S. viridis* accessions to different sources of environmental stress. As this species has a broad range and elevated heterogeneity between wild populations, a variety of strategies for surviving environmental stress may be utilized across populations and types of stress. This genetic and functional variability may

provide an enhanced diversity of favorable genetic traits available to increase the stress tolerance. of *S. italica* varieties.

The broad geographical and climatic range across which *S. viridis* populations occur provides an excellent opportunity for further transcriptomic exploration of local adaptation in response to environmental stress. As we determined in our previous experiment, exposure to stress at different points in the life cycle can result in different outcomes, including those which may have important implications for agricultural species, making comparisons of transcriptomic response to stress at different time especially relevant. Abiotic stressors such as drought, chilling, and salinity stress are predicted to become more severe along with other multifaceted and stochastic environmental stressors. These increasingly complex, challenging, and unpredictable growing environments will be experienced simultaneously by wild plants and agricultural crops, underscoring the importance of developing proactive strategies to maintain food security and ecosystem resilience.

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Figures

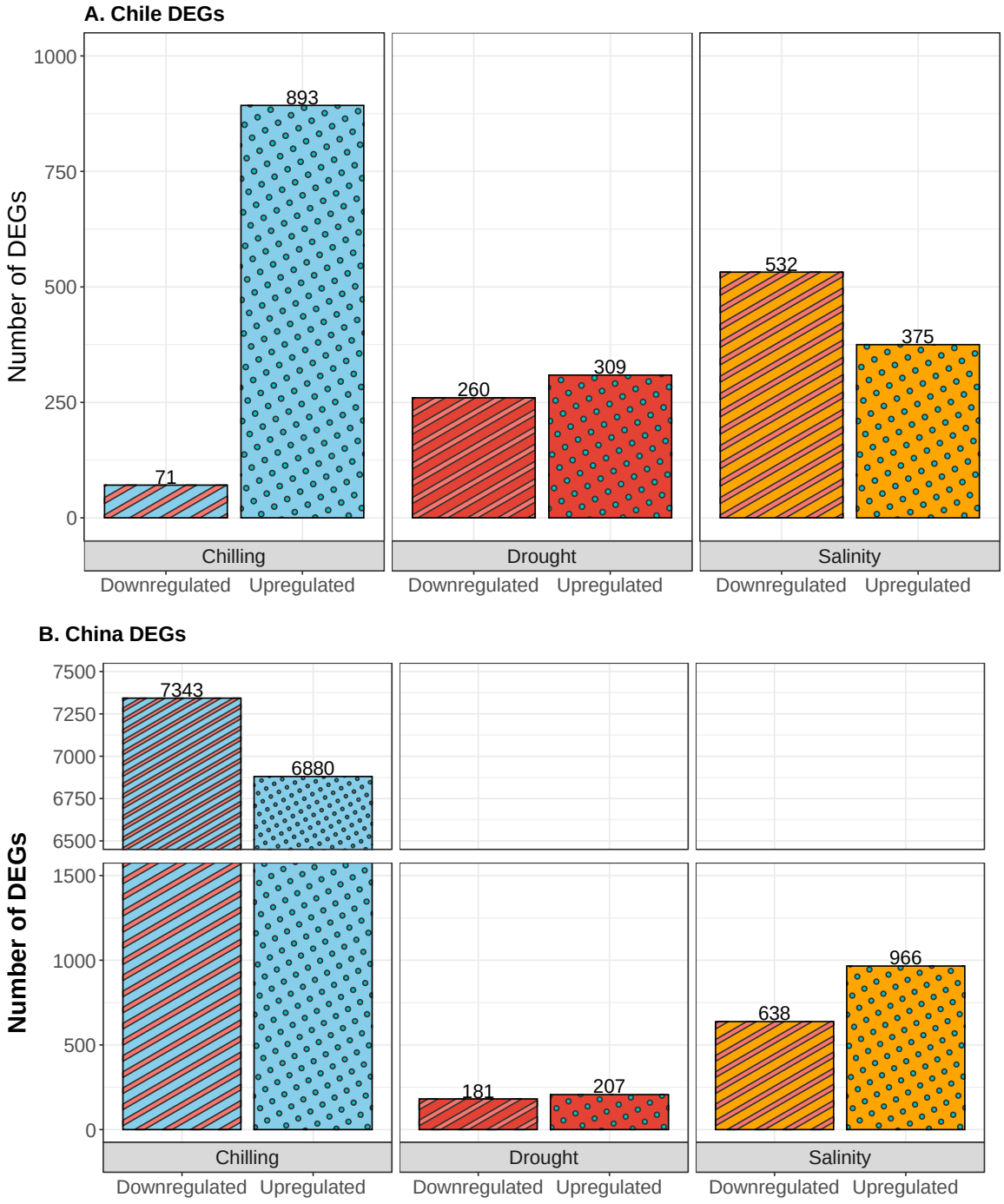


Figure 3.1 : Number of Differently Expressed Genes across populations and treatment conditions. A. DEGs in plants from the Chile population. B. DEGs in plants from the China population. The scale of the Y-axis differs between panels A and B.

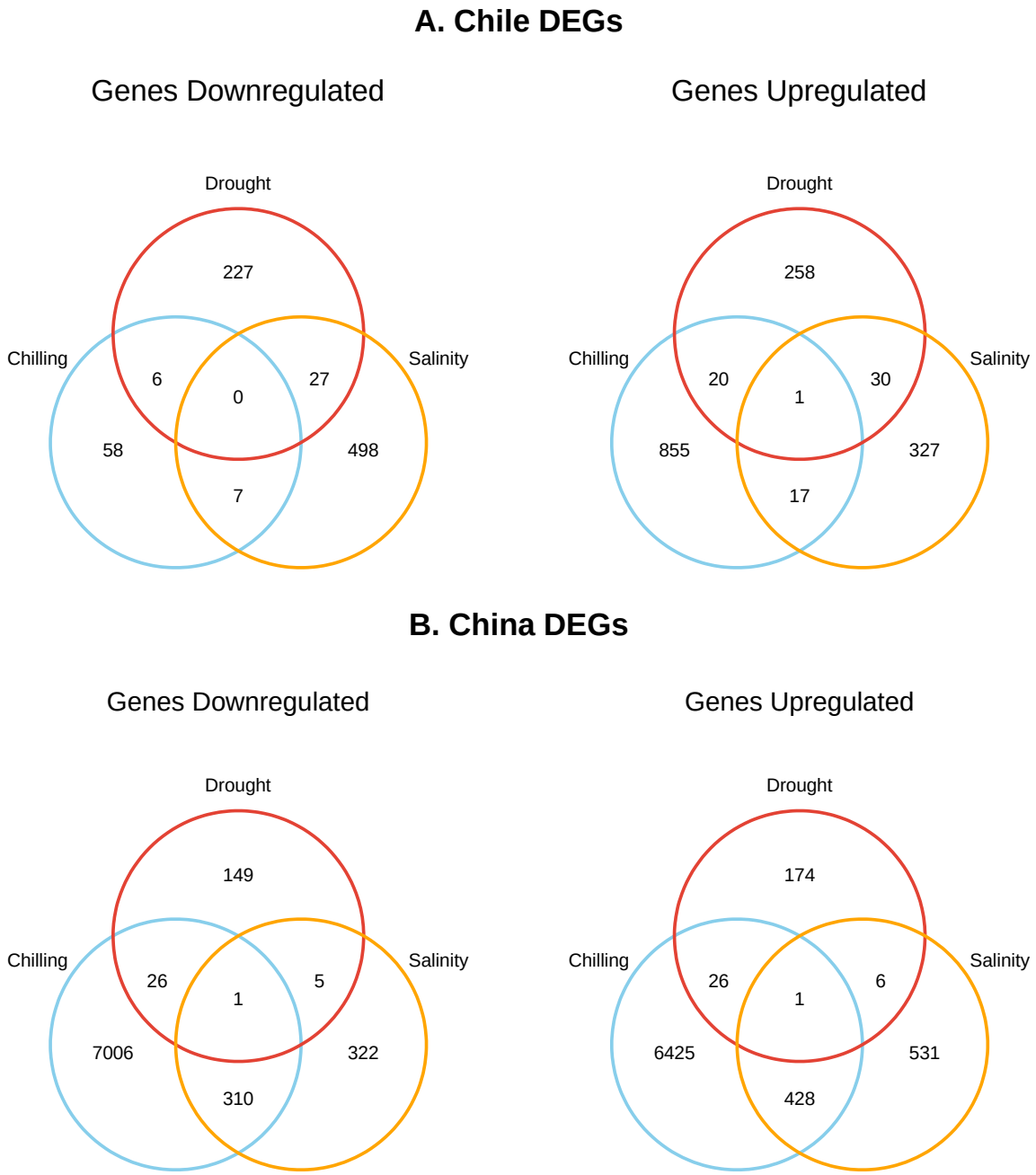
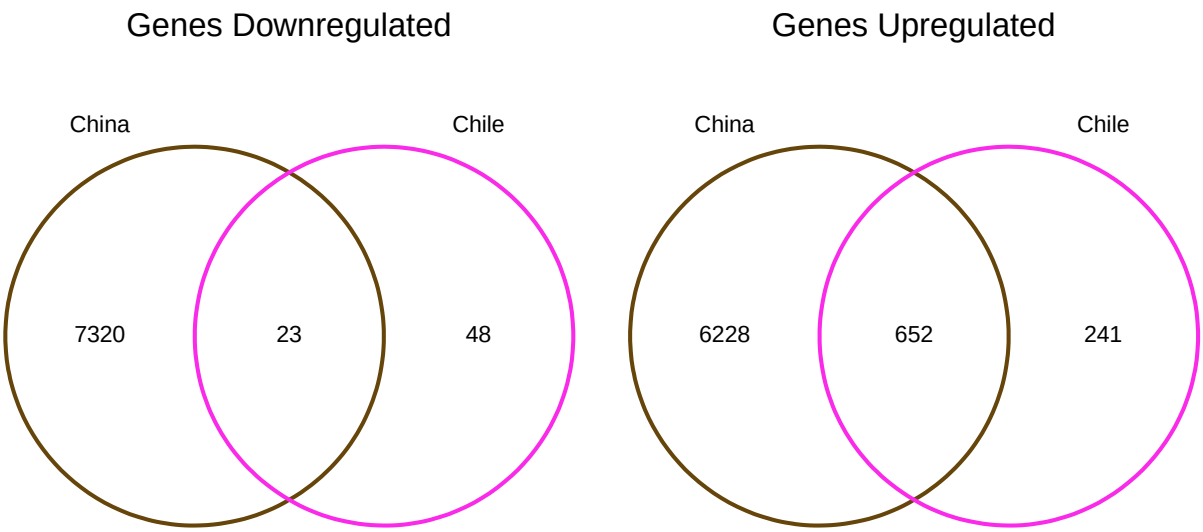
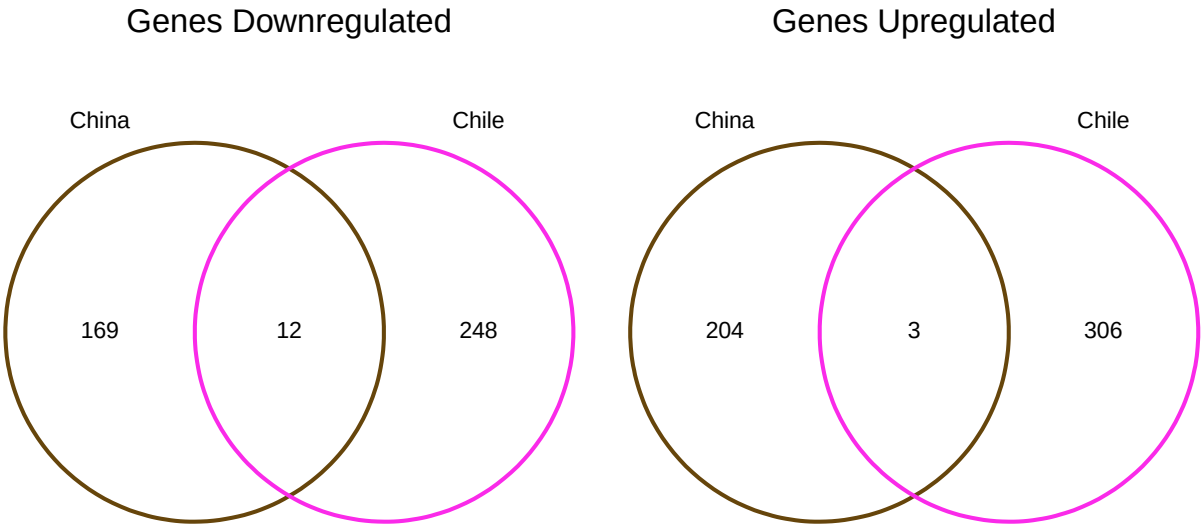


Figure 3.2 : Overlapping DEGs across treatment within each population. A. Chile population DEGs. B. China population DEGs.

A. Chilling Stress DEGs



B. Drought Stress DEGs



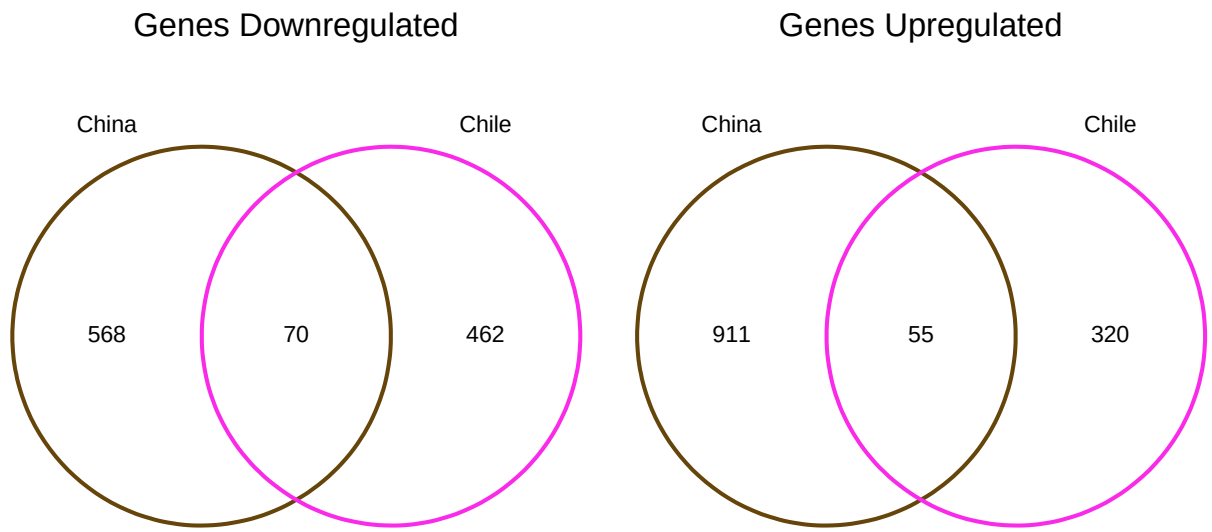


Figure 3.3 : Overlapping DEGs across population within treatment. A. Chilling stress DEGs. B. Drought stress DEGs. C. Salinity stress DEGs.

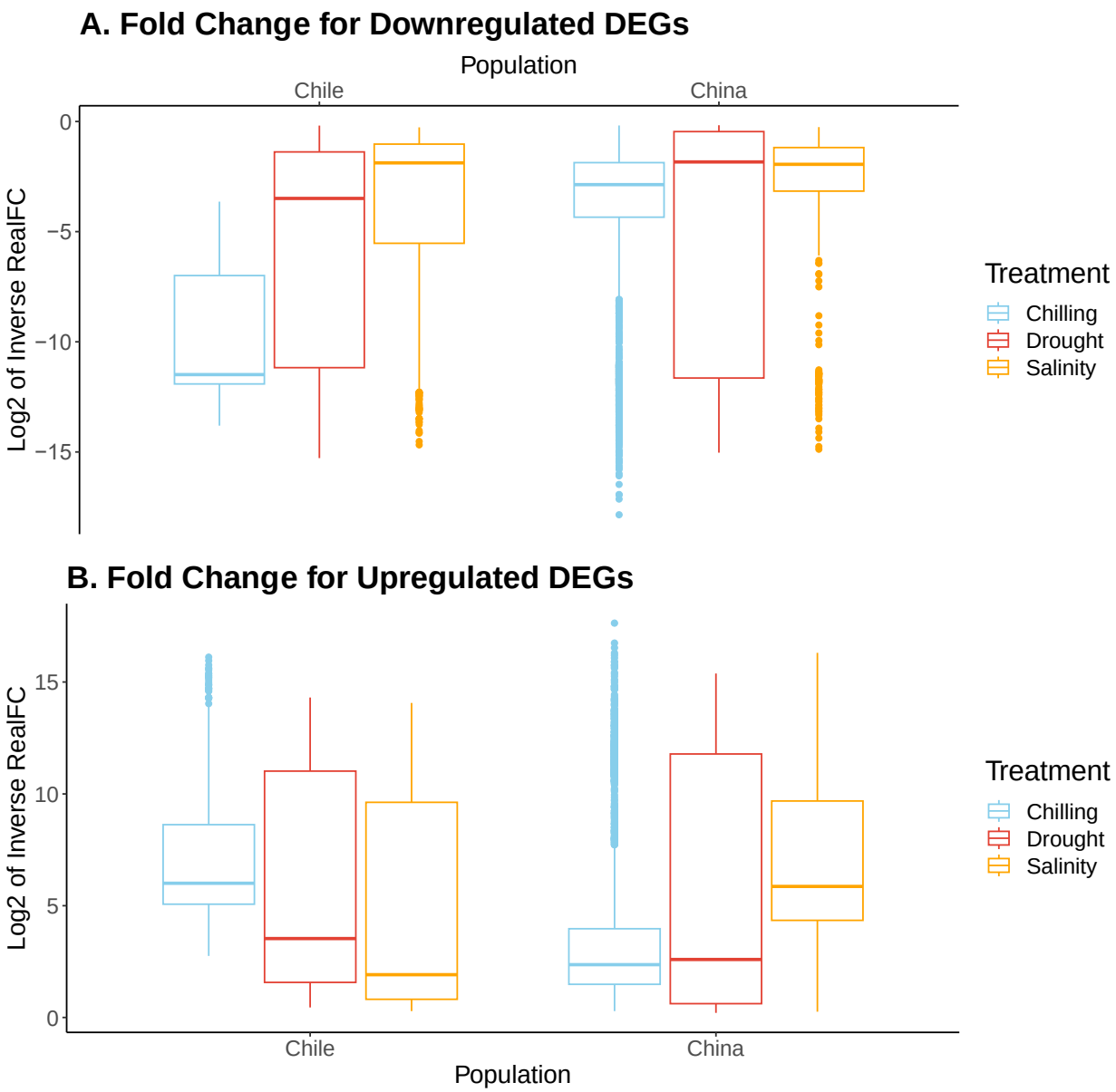


Figure 3.4 : Box and whisker plots for the $\log_2$ of the inverse RFC for DEGs for each population and treatment condition. A. Downregulated. B. Upregulated.

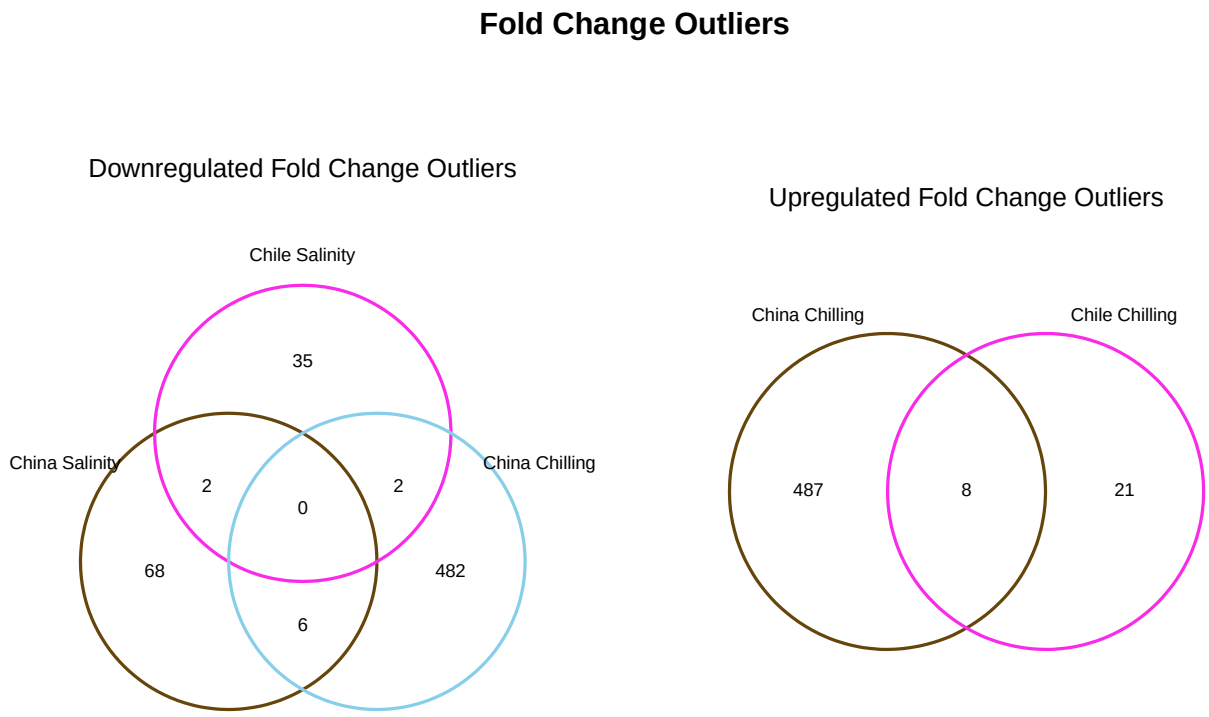


Figure 3.5 : Overlapping fold change outliers for upregulated and downregulated DEGs. Outliers were calculated based on the upper and lower bounds of the $\log_2$ inverse of RFC values.

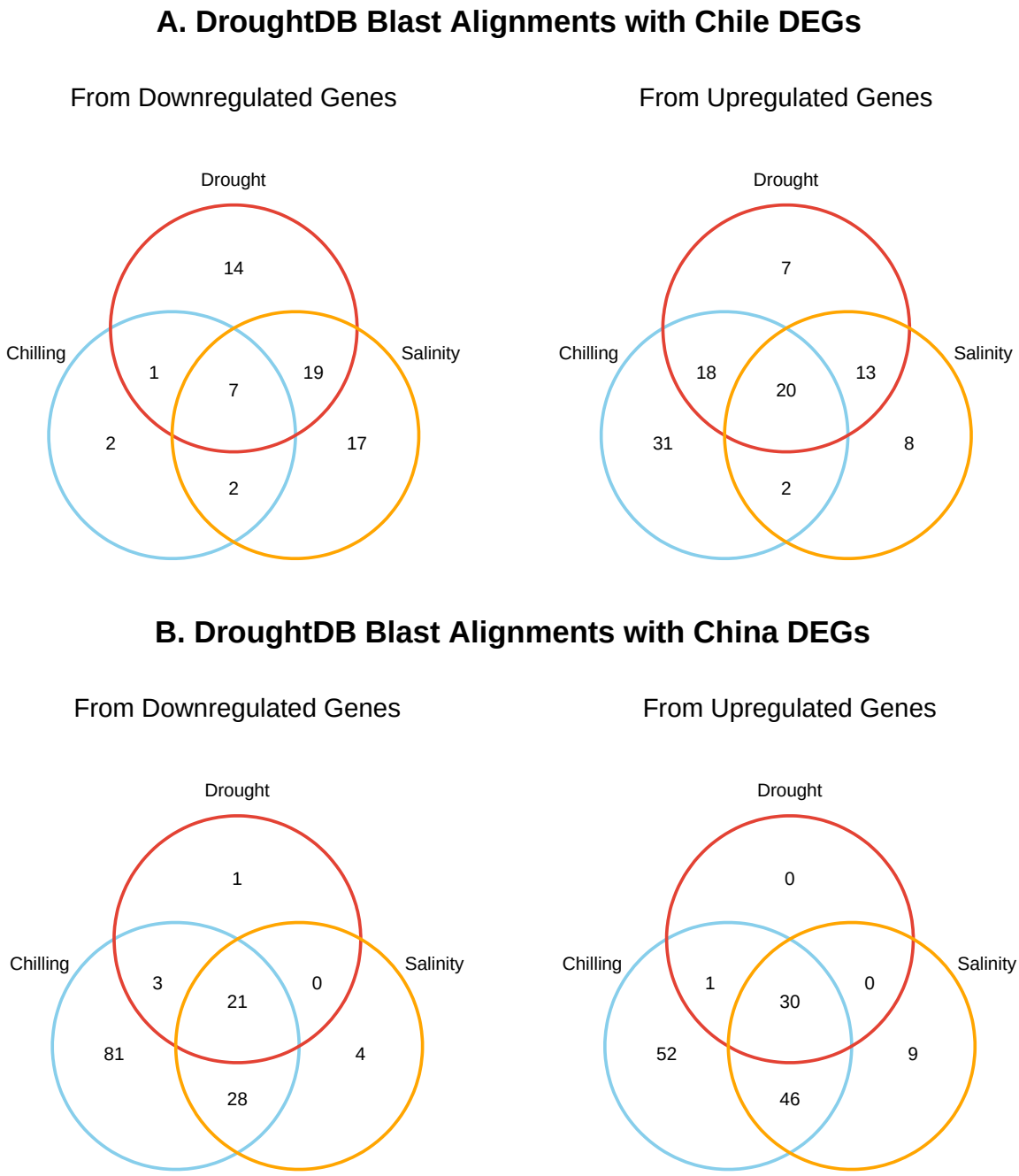
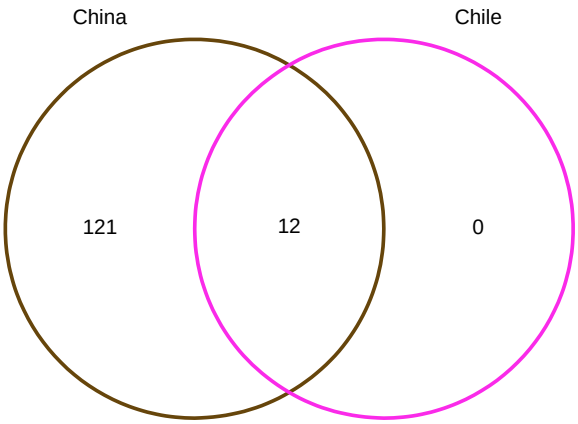


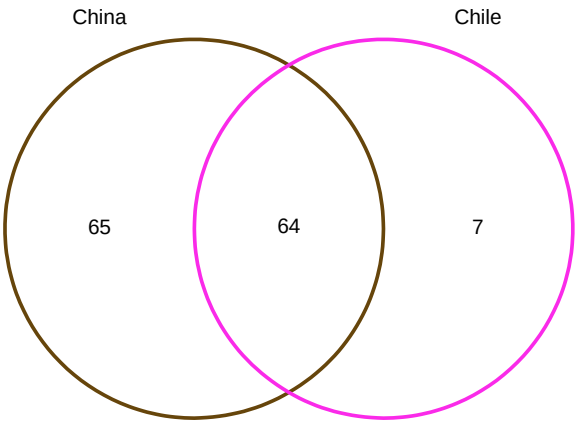
Figure 3.6 : Comparison of genes from the DroughtDB that produced significant alignments with DEGs across each treatment within each population. A. DEGs from the Chile population. B. DEGs from the China population.

A. DroughtDB Blast Alignments with Chilling Stress DEGs

From Downregulated Genes

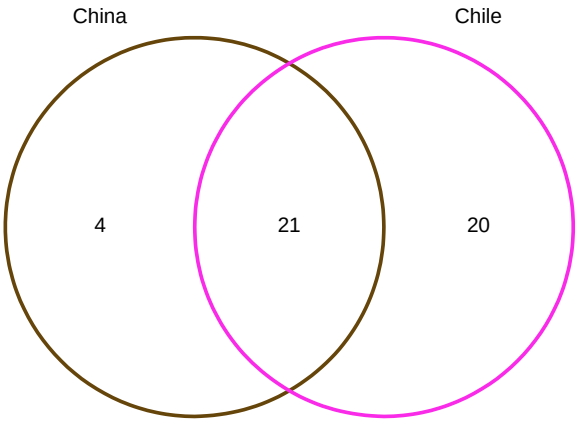


From Upregulated Genes

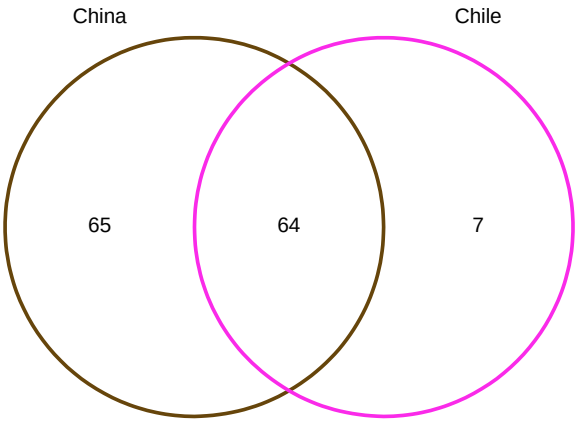


B. DroughtDB Blast Alignments with Drought Stress DEGs

From Downregulated Genes



From Upregulated Genes



C. DroughtDB Blast Alignments with Salinity Stress DEGs

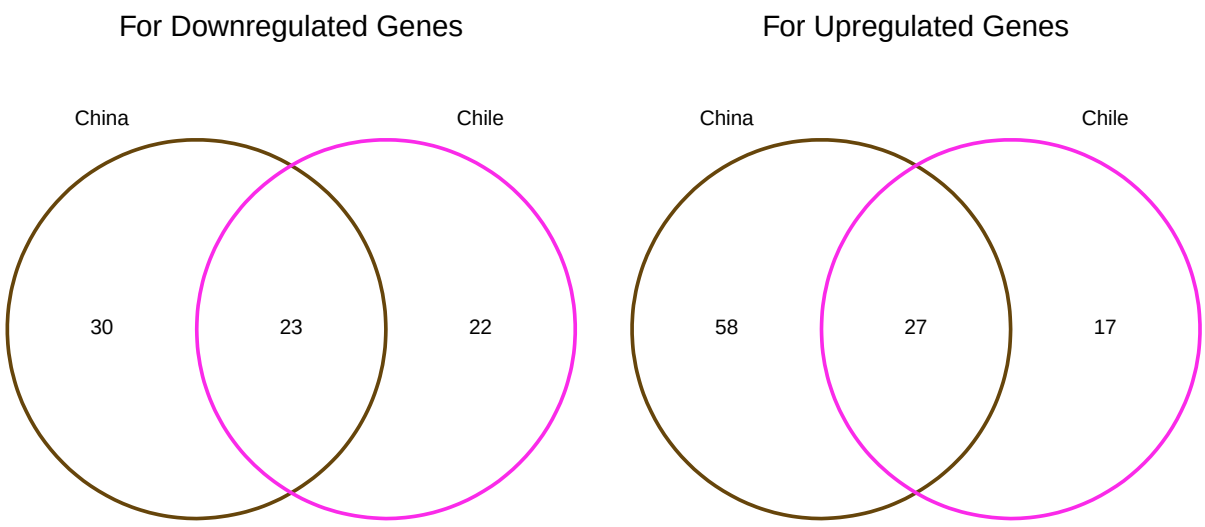


Figure 3.7 : Comparison of genes from the DroughtDB that produced significant alignments with DEGs between each population within each treatment. A. DEGs from the Chilling Stress treatment. B. DEGs at 0.01 FDR from the Drought Stress treatment. C. DEGs at 0.01 FDR from the Salinity Stress treatment.

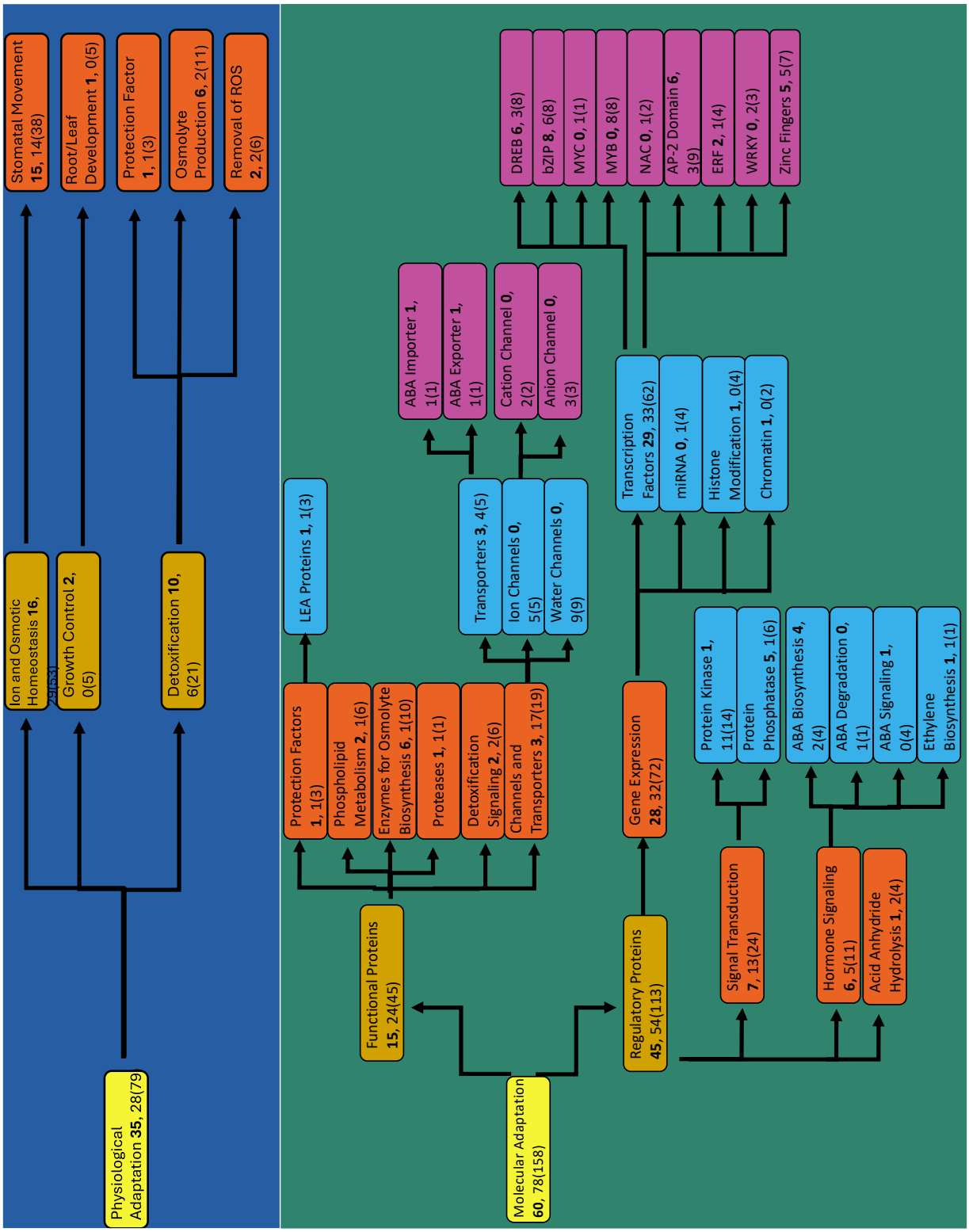


Figure 3.8 : DroughtDB pathway adapted to reflect representation of *Setaria viridis* DEGs uniquely upregulated under stress conditions. The number of DDB genes for each category is indicated in parenthesis and the numbers preceding parenthesis in indicate the number of genes from the China population (bold) and the Chile population (non-bold) with at least 1 BLAST hit

(E-value cutoff: 10^{-20}) based on BLAST analyses of DEGs upregulated at FDR 0.01 from both populations and all 3 treatment conditions. DDB genes that matched with both up and down regulated DEGs for the same population and treatment condition were not included. Figure redrawn with modification based on Figure 4 in Qui et al. 2021 and Figure 1 in Alter et al. 2015.

Tables

Table 3.1 : *Setaria viridis* accessions. The 2 right most columns summarize results from Chapter 2 of this dissertation. The rows corresponding to the 2 populations used for transcriptomic analyses in Chapter 3 are in bold.

Plant ID	Origin	Longitude	Latitude	Average Annual Precipitation mm (WorldClim)	Average days of drought stress for plants to reach a photosynthetic rate of 0 µ mol m^{-2} s^{-1}	Tukey's HSD posthoc test compact letter display category for drought response calculated with
Ames 32292	United States (Michigan 2)	-84.166 W	45.109 N	739	12.333	a
PI 677122	Portugal	-7.592 W	40.967 N	1233	11.833	a
PI 408810	**China**	**125.307 E**	**43.848 N**	**604**	**11.333**	**a**
PI 223677	Azerbaijan	48.879 E	38.456 N	1207	8.667	b
PI 202407	**Chile**	**-72.247 W**	**-37.438 S**	**1279**	**8.500**	**b**
Ames 32291	United States (Michigan 1)	-84.166 W	45.109 N	739	8.333	b
PI 230135	Iran	51.965 E	35.762 N	158	8.167	b
PI 677118	Russian Federation	130.628 E	45.404 N	565	7.500	bc
PI 212625	Afghanistan	70.467 E	34.433 N	244	6.333	c

Table 3.2 : Number of Differently Expressed Genes for each population and treatment condition at FDR 0.01.

Population	Treatment	# Upregulated DEGs	# Downregulated DEGs	# Total DEGs	# Total Aligned Transcripts	#Total DEGs/ #Total Aligned Transcripts
Chile	Chilling	893	71	964	41392	0.023
Chile	Salinity	375	532	907	40522	0.022
Chile	Drought	309	260	569	40335	0.014
China	Chilling	6880	7343	14223	41949	0.339
China	Salinity	966	638	1604	42127	0.038
China	Drought	207	181	388	40639	0.010

Table 3.3 : DEGs upregulated across all three stress conditions within a population. Molecular Function, Biological Process, and Cellular Component gene ontology information was derived from PANTHER.

Population	Regulation Change	Gene ID	Mapped ID	Chilling Stress Log2(InverseRealFC)	Drought Stress Log2(InverseRealFC)	Salinity Stress Log2(InverseRealFC)	Molecular Function	Biological Process	Cellular Component
Chile	Up	lcl|CM016555.1_cds_TKW20581.1_20284	A0A4U6UV81	5.528	5.051	4.711	ATPase activity; peptidase activity	proteolysis	Mitochondrion ; chloroplast envelope
Chile	Down	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
China	Up	lcl|CM016554.1_cds_TKW26389.1_15288	A0A4U6T5Z1	1.875	12.329	12.177	ATPase activity; peptidase activity	proteolysis	Mitochondrion
China	Down	lcl|CM016557.1_cds_TKW09159.1_31771	A0A4U6U6Y4	-11.333	-13.121	-11.704	protein serine/threonine kinase activity	protein phosphorylation; defense response to bacterium	plasma membrane

Table 3.4 : Summary statistics and outliers for the $\log_2$ of the inverse RFC values for up and downregulated DEGs for each population and treatment condition.

Population	Treatment	Regulation Change	Min	1st Quartile	Median	Mean	Third Quartile	Max	Outliers
Chile	Drought	Up	0.448	1.570	3.527	5.389	11.018	14.312	0
Chile	Drought	Down	-15.281	-11.174	-3.497	-5.749	-1.384	-0.188	0
Chile	Salinity	Up	0.283	0.815	1.911	4.501	9.624	14.067	0
Chile	Salinity	Down	-14.678	-5.533	-1.885	-4.065	-1.029	-0.270	39
Chile	Chilling	Up	2.752	5.067	6.000	7.329	8.625	16.115	29
Chile	Chilling	Down	-13.808	-11.917	-11.489	-9.804	-6.995	-3.638	0
China	Drought	Up	0.206	0.617	2.595	5.679	11.786	15.383	0
China	Drought	Down	-15.033	-11.645	-1.841	-5.070	-0.461	-0.175	0
China	Salinity	Up	0.258	4.345	5.858	6.747	9.681	16.310	0
China	Salinity	Down	-14.875	-3.162	-1.948	-3.119	-1.183	-0.261	76
China	Chilling	Up	0.284	1.484	2.361	3.263	3.970	17.636	495
China	Chilling	Down	-17.850	-4.345	-2.870	-3.598	-1.870	-0.183	490

Table 3.5 : DEGs identified as RFC outliers for at least two treatment conditions.

Gene ID	Mapping ID	Condition 1	Condition 1 Log2(InverseRFC)	Condition 2	Condition 2 Log2(InverseRFC)	Molecular Function	Biological Process	Cellular Component
lcl|CM016556.1_cds_TKW14432.1_25917	A0A4U6UGG0	ChinaChilling Up	9.570	ChileChilling Up	15.765	ubiquitin-protein transferase activity; ubiquitin protein ligase binding	protein ubiquitination	cullin-RING ubiquitin ligase complex
lcl|CM016559.1_cds_TKW00302.1_41155	A0A4U6TFH6	ChinaSalinity Down	-12.398	ChileSalinity Down	-13.642	protein serine/threonine kinase activity	protein autophosphorylation	plasma membrane
lcl|CM016557.1_cds_TKW09159.1_31771	A0A4U6U6Y4	ChinaSalinity Down	-11.704	ChinaChilling Down	-11.333	protein serine/threonine kinase activity	protein phosphorylation; defense response to bacterium	plasma membrane
lcl|CM016555.1_cds_TKW22827.1_22530	A0A4U6VFD5	ChinaSalinity Down	-6.438	ChinaChilling Down	-8.382	carbohydrate kinase activity; phosphotransferase activity, alcohol group as acceptor	hexose metabolic process	cytosol

Table 3.6 : GO terms overrepresented in two conditions based on a PANTHER statistical overrepresentation analysis of all 12 DEG lists with FCR P < 0.01.

GO Terms	Functional Category	Condition 1	Fold Enrichment	raw p-value	FDR	Condition 2	Fold Enrichment	raw p-value	FDR
Upregulated DEGs									
response to cold (GO:0009409)	Biological Process	China Salinity	11.81	1.36E-06	0.000226	Chile Salinity	21.6	3.02E-06	0.00225
response to heat (GO:0009408)	Biological Process	China Chilling	2.33	1.80E-06	2.16E-05	China Salinity	6.37	1.22E-07	0.0000259
response to reactive oxygen species (GO:0000302)	Biological Process	China Chilling	2.35	8.12E-04	5.50E-03	China Salinity	10.54	2.38E-08	0.00000710
response to temperature stimulus (GO:0009266)	Biological Process	China Chilling	2.13	7.87E-06	8.03E-05	China Salinity	8.43	2.31E-13	0.00000000
unfolded protein binding (GO:0051082)	Molecular Function	China Chilling	2.66	1.54E-08	4.00E-07	China Salinity	5.27	1.97E-05	0.00974
Downregulated DEGs									
cell surface receptor signaling pathway (GO:0007166)	Biological Process	China Salinity	6.78	7.44E-08	1.11E-04	Chile Salinity	9.75	1.05E-11	3.93E-09
kinase activity (GO:0016301)	Molecular Function	China Chilling	1.28	3.52E-04	7.91E-03	Chile Salinity	3.33	7.55E-10	0.00000009
membrane (GO:0016020)	Cellular Component	China Chilling	1.15	9.74E-05	1.07E-03	China Salinity	1.69	5.95E-06	2.36E-03
phosphate-containing compound metabolic process (GO:0006796)	Biological Process	China Chilling	1.31	6.65E-06	0.000276	Chile Salinity	3.22	7.76E-12	3.85E-09
phosphorus metabolic process (GO:0006793)	Biological Process	China Chilling	1.31	4.29E-06	0.000194	Chile Salinity	3.18	1.22E-11	3.63E-09
plasma membrane (GO:0005886)	Cellular Component	China Salinity	2.06	2.66E-05	3.52E-03	Chile Salinity	2.53	6.95E-08	9.20E-06
transferase activity, transferring	Molecular Function	China Chilling	1.27	1.57E-04	4.31E-03	Chile Salinity	2.94	4.47E-09	0.00000044

phosphorus-containing groups (GO:0016772)									

Table 3.7 : Top 1% of overrepresented GO term findings from PANTHER statistical overrepresentation test by fold enrichment. Fold enrichment values are shaded in gray. A complete list of all overrepresented GO terms can be found in Appendix Table B.1.

Population	**Treatment**	**Regulation Change**	**Functional Category**	**GO Term**	**Number genes from *Setaria viridis* (SETVI) - REFLIST (38120)**	**Actual Number**	**Expected Number**	**Fold Enrichment**	**raw P-value**	**FDR**
Chile	Salinity	Up	Biological Process	response to cold (GO:0009409)	25	5	0.23	21.6	3.02E-06	0.00225000
China	Salinity	Up	Biological Process	response to salt stress (GO:0009651)	30	9	0.71	12.65	2.09E-08	0.00000778
China	Salinity	Up	Biological Process	response to cold (GO:0009409)	25	7	0.59	11.81	1.36E-06	0.00022600
China	Salinity	Up	Biological Process	response to reactive oxygen species (GO:0000302)	40	10	0.95	10.54	2.38E-08	0.00000710
China	Salinity	Up	Biological Process	response to osmotic stress (GO:0006970)	36	9	0.85	10.54	1.21E-07	0.00003000

Table 3.8 : Results of blasting each DEG list against the local database of genes from the Drought Database (DDB) at threshold $1e^{-20}$. The number of DEGs (column 4) that aligned with genes from the DDB (column 6) are listed. Column 5 contains the percentage of DEGs from each list with aligned to genes in the DDB. Column 7 contains the percentage of genes from the DDB which aligned with DEGs from the given list.

Population	Treatment	Regulation Change	#DEGs aligned with DDB genes	Aligned DEG/total DEG	#DDB genes that DEGs aligned with	Aligned DDB genes/total DDB genes
Chile	Drought	Up	47	0.152	58	0.305
Chile	Drought	Down	56	0.215	41	0.216
Chile	Salinity	Up	27	0.072	43	0.226
Chile	Salinity	Down	123	0.231	45	0.237
Chile	Chilling	Up	112	0.125	71	0.374
Chile	Chilling	Down	14	0.197	12	0.063
China	Drought	Up	23	0.111	31	0.163
China	Drought	Down	23	0.088	25	0.132
China	Salinity	Up	106	0.110	85	0.447
China	Salinity	Down	114	0.179	53	0.279
China	Chilling	Up	603	0.088	129	0.679
China	Chilling	Down	740	0.101	133	0.700

Table 3.9 : Number of DEGs from each population and treatment condition producing significant alignment when blasting DEG lists against the DroughtDB at threshold $1e^{-20}$. Only DDB genes that aligned uniquely with upregulated DEGs for each treatment are included.

Population	Treatment	#DDB genes that upregulated genes aligned with	#DDB genes that only upregulated genes aligned with	DDB genes aligned only with upregulated DEGs/total DDB genes
Chile	Drought	58	31	0.159
Chile	Salinity	43	25	0.128
Chile	Chilling	71	60	0.308
China	Drought	31	17	0.087
China	Salinity	85	52	0.267
China	Chilling	129	26	0.133

Chapter 4 - Creating LGBTQIA+ inclusive classrooms and curricula

Abstract

Biology courses are unique in that they often involve topics related to human diversity, including those that reflect students' life experiences, within a STEM context. However, academic biology and by extension biology education has historically treated human diversity (particularly the experiences of marginalized populations) as separate from scientific considerations of biodiversity, producing textbooks and curricula that decline to engage with many relevant intersections of science and culture. We discuss the limitations and problems with this dominant approach to biology education, and instead propose using a naturecultural framework for incorporating concepts of sex, gender, and sexuality diversity in biology classes. Teaching about the wide array of sex traits and reproduction strategies across taxa without incorporating gendered human stereotypes can help elucidate these topics and give students an increased appreciation of the truly vast diversity of life on earth. Including discussions of queer sexuality, intersex variation, and gender diversity as they are relevant to different subjects within biology can help affirm students who hold these identities and challenge the common and insidious idea that only heteronormative and binary understandings of human biology are scientifically legitimized. Utilizing inclusive language and addressing prejudicial or inadequate representation of LGBTQIA+ identities in biology textbooks can create a more supportive classroom environment and teach students to understand and engage with the interactive and ongoing process of producing scientific knowledge. Taking steps such as these to increase LGBTQIA+ affirmation and representation in biology classrooms stands to provide a variety of academic and

interpersonal benefits for all students and challenge the use of appeals to widely held biological misconceptions as justification for anti-LGBTQIA+ beliefs and policies.

Part 1: Introduction

Variation is an integral component of biology at every level from the genetic diversity that provides a population with resilience to environmental change to the variety of convergent strategies that enable the completion of the same essential life functions through different pathways. Evolutionary biology in particular is deeply rooted in exploring questions of diversity and difference, as natural selection is made possible by the presence of trait variation and has resulted in the rich distinctiveness between organisms that have adapted to inhabit different biomes and occupy different niches. Although diversity in biological life on earth is considered imperative for maintaining ecosystem function and producing ecosystem services (Naeem et al., 2012; Oliver et al. 2015; Dainese et al., 2019), it is often treated as categorically separate from the conversation surrounding human diversity in science and the academy. At universities, offices of diversity and inclusion are housed in separate departments from science labs and lectures, at conferences, these topics are discussed in separate sessions, and in grant proposals, tie-ins to outreach and equity work with historically underrepresented groups are often confined to the broader impacts section.

Despite these attempts at siloing them into separate buildings and conversations, human diversity and its cultural implications are not separate from biological science. Biological research and knowledge production are co-constitutive with the societies in which they take places, with culture influencing science and science influencing culture. As Banu Subramaniam writes in *Ghost Stories for Darwin: The Science of Variation and the Politics of Diversity*, "…science has been central in the invention of biological categories such as sex, gender, race,

class, nation, and sexuality, and subsequently reifying social prejudices in the inferiority of women, people of color, colonized, poor, or homosexuals" (p. 4, Subramaniam 2014). The production of these categories through scientific practices and institutions and the fomenting of these social prejudices creates a self-perpetuating cycle in which individuals designated socially and biologically inferior are frequently denied the education and access to participate in the research and academic discourse that produce these designations. Describing this ongoing coevolution, Subramaniam continues "In coining the term *natureculture*, Donna Haraway challenges us to reject the binaries of nature and culture and attend to the constant traffic of discourses, information, and theories between the worlds of natures and cultures. There is no nature or culture, only natureculture (Haraway and Goodeve 2013; p. x, Subramaniam 2014)."

The consequences of these naturecultural interactions are critical. Subramaniam continues "Questions of genetic variation in human and nonhuman organisms are deeply linked to questions of diversity and difference in human populations steeped in tortured histories of slavery, colonialism, and genocide. *A naturecultural analysis reveals that the question of variation is fundamentally about power-the politics of life and death*" (p. 7, Subramaniam 2014). There are myriad examples of how naturecultural construction of human difference continue to affect access to power, autonomy, and life chances of marginalized populations. Black patients with melanoma have a 2-3-fold greater risk of mortality than white patients, due at least in part to underrepresentation of people of color in dermatological professions and research and the lack of proficiency at detecting these cancers early on darker skin tones (Nelson 2020). In 12 US states and 1 territory, transgender individuals are required to undergo sex reassignment surgery, which may include sterilization, in order to legally alter the sex marker on their birth certificates, potentially forcing them to choose between fertility and accurate legal identification (MAP

2024). Recent research attempting to use CRISPR 2.0 gene editing technology to reverse certain forms of hereditary deafness is viewed by some deaf and disability rights activists as a form of eugenics, which ignores the social barriers that cause deafness itself to be seen as a deficit, rather than a natural form of human difference that hearing culture fails to accommodate (Katz 2020).

As is evinced by the previous examples, the work done by the quantitative and categorical tools of biology is not value neutral or divorced from human history and culture (Subramaniam and Bartlett 2023). These historical, cultural, and value-based aspects of science influence both how research is conducted and how science is taught. Pedagogy plays a profound role both in influencing how students come to understand human diversity from a biological perspective and in shaping which students have the support, role models, and access to become biologists themselves (Hughes 2018; Cooper et al. 2020).

As biology instructors, we have a responsibility to interrogate bias, engage with the naturecultural construction of demographic categories, and counteract histories of prejudice and violence in our content and classrooms. This chapter will specifically focus on practices that increase the inclusiveness of biology education for students who are gay, lesbian, bisexual, transgender, queer, intersex, asexual or otherwise not heterosexual, cisgender, and or/endosex (See Glossary for more detailed definitions). We will use the acronym LGBTQIA+ (lesbian, gay, bisexual, transgender, queer/questioning, intersex, and asexual/aromantic) to describe this population (see Glossary for more detailed definitions). The "+" indicates other identities and experiences not explicitly represented by the acronym that are impacted by the issues discussed. This includes but is not limited to nonbinary individuals who do not identify as transgender, gender nonconforming cisgender individuals, and those with culturally specific sexualities and gender identities. LGBTQIA+ individuals exist at every intersection of human diversity,

including race, class, religion, disability status, and geographic location, and their experiences of identity and oppression are not homogenous (Crenshaw 1989; Leyva et al. 2022). Other acronyms may be used in this chapter to accurately reflect the specific populations considered in cited literature.

Several recent studies indicate that more of the current college population in the US identifies as LGBTQIA+ than in previous generations. A 2015 national survey found that at least 7% of Americans ages 18-35 identify as lesbian, gay, bisexual, or transgender (Jones and Cox 2015), while a 2017 study put that number at 20% (GLAAD 2017). A recent survey conducted in Arizona estimated that the percentage of students in undergraduate biology classrooms who are LGBTQ+ is between 9 and 16% (Cooper et al. 2020). Although the presence of students with diverse sexualities and gender identities on college campuses is increasing, the culture of many of these campuses remains hostile towards them. According to the 2015 US Trans Survey, nearly a quarter of transgender college students reported being verbally, physically, or sexually harassed by classmates, staff, and faculty (James et al. 2016). Sixteen percent of college and vocational students who were openly transgender experienced such significant harassment and transphobia that they left school (James et al. 2016).

According to a 2020 study, more than 1 in 3 LGBTQIA+ Americans experienced discrimination in the past year, while 3 in 5 transgender individuals reported discrimination (Mahowald et al. 2020). Both systemic and interpersonal prejudice against LGBTQIA+ individuals are often justified by the supposed biological immutability of mandatory heterosexuality, the "scientific fact" of sex and gender binary, and the belief that cisgender life histories are naturally superior (Sun 2019). Given the pervasiveness of such prejudice, LGBTQIA+ students may avoid or mistrust biology courses, assuming their sexuality, gender

identity, and/or intersex status will be erased or pathologized by both instructors and course content (Cooper and Brownell 2016; Cooper et al. 2020; Fitzgerald-Russell and Kowalske 2022).

Positive representations of LGBTQIA+ individuals in biology are important for creating an inclusive classroom environment but are often lacking (Cooper et al. 2020). Most states do not require high school and middle school students to receive comprehensive sex education, and even sex ed curricula that goes beyond an abstinence-only perspective often do not mention the developmental and sexual needs and experiences of LGBTQIA+ individuals (Valens 2017). This lack of inclusive and useful information can alienate LGBTQIA+ students and impeded their ability to learn at school, access resources, and make safe decisions.

Heterosexual, cisgender students are also negatively influenced by curricula that pathologizes or excludes LGBTQIA+ identities (Casper et al. 2022). Many students in college biology classes plan to go into health care professions, where they will increasingly, reflecting current demographics, be called upon to meet the needs of people whose bodies, genders, and sexualities do not conform to what is often assumed to be a heterosexual, endosex, cisgender default. For non-science majors, an introductory college biology class may be their last exposure to formal science instruction. A curriculum that fails to portray sex, sexuality, and gender diversity as an inherent and unignorable aspect of human biological variation will do a great disservice to their understanding of their own species.

As Snyder and Broadway conclude in their review of high school biology textbooks, "The heteronormative treatment of sexuality amplified the silence of missed opportunities to discuss the full range of sexuality in nature and society" (pg. 632, Snyder and Broadway 2004). Biology courses, especially those taught at the college level, have both the opportunity and the responsibility to do justice to the full scope of human diversity and the identities of all students

by creating inclusive classrooms and curricula.

Part 2: Teaching sex and reproductive diversity across species

The language that we choose to use and the examples that we provide in describing non-human organisms are an important part of emphasizing the inherent diversity of life and critical for avoiding heteronormative, binary, or anthropocentric biases. Reproductive strategies, sex determination systems, and courtship behaviors vary widely across species (p. 5, Roughgarden 2013), and it is important to treat these behaviors as natural variation, rather than projecting stereotypical gender roles (which are based in context-specific, human cultural formulations and expectations) or values judgements onto them. This is both scientifically sound and can help equip students to combat stigmas against non-straight, non-cisgender, and non-monogamous individuals, who are often stigmatized by claims that such behaviors are "against nature" or in denial of biology.

Applying human gender stereotypes animals, plants, or fungi can contribute to anthropomorphization, the conflation of sex and gender (Cooper et al. 2020), and critical misunderstandings of plant biology, in which sex and reproduction differ substantially from human life cycles (Casper et al. 2022; Subramaniam and Bartlett 2023). Survey approaches to biological diversity may summarize biological sex across taxa as a consistent, binary, and always determined the same way (pp. 62-63, Urry et al. 2017). However, specifically defining what sex means and how sex works in different organisms can provide students with an understanding of the true and fascinating diversity of sex determination, sex differentiation, and mating strategies across taxa (p.181, Roughgarden 2013; Zemenick et al. 2022). Not all species have life cycles that include sexual reproduction or distinct sex categories. Some animals, including multiple types of invertebrates and some vertebrates (primarily whiptail lizards) only have one sex and

rely solely on asexual reproduction through parthenogenesis (Cosentino et al. 2019). Not all species with sexes have only two sexes, such as some basidiomycete fungi, which have thousands of mating types (Coelho et al. 2017). Not all species have separate sexes, as many plants and some animals can produce two different types of gametes from a single individual (Sumbramaniam and Bartlett 2023). Not all species have individuals whose sex stays consistent throughout their lifetime, given that some fish, like clownfish, can undergo sequential hermaphroditism (Casas et al. 2016).

There are many plants and animals that regularly change sex throughout their lives. Some perennial plants can produce either a staminate or carpellate sporophyte in a given year based on bulb size in spring. A variety of animals (including fishes, amphibians, birds, and reptiles) change from a fertile adult of one sex to a fertile adult of another sex during their lifecycles, either once they attain a specific age, or in response to the sex ratio of their proximate conspecifics (p. 31, Roughgarden 2013). Much of the genetic pathway that enables this change in gonadal tissue type in fishes is conserved and present in other vertebrates (Matson et al. 2011). Even animals that do not regularly undergo gonadal sex reversal, including mice and humans, rely on the expression of transcription factors such as *Dmrt1*, *Foxl2*, and *Sox9* to maintain adult gonadal tissue and prevent ovary cells from converting to testicular cells and vice versa (Uhlenhaut et al. 2009; Matson et al. 2011). Blocking the *Foxl2* gene in adult mice resulted in the transdifferentiation of ovaries to testis at the cellular level (Uhlenhaut et al. 2009) and blocking *Dmrt1* in adult mice similarly caused testicular cells to transdifferentiate into ovary cells (Matson et al. 2011). While these transcription factors have been shown to play a role in some human intersex variations (Matson et al. 2011) and are important for maintaining gonadal cell type in adults, the experimental strategies used to induce transdifferentiation in mice cannot be

translated to human medicine.

For anisogamic organisms, sex determination can occur through multiple strategies, including sex chromosomes, egg incubation temperature, or social cues (Li and Gui 2018). Among species that utilize chromosomal systems of sex determination, there is considerable variation in the number of sex chromosomes, how they are inherited, and whether different sexes have the same number of sex chromosomes (Figure 4.1). ZW/ZZ sex determination is widespread in birds, while XX/XY sex determination is common in mammals, though some species have lost the Y chromosome and others have multiple sets of X and Y chromosomes (Li and Gui 2018). Even attempts to demonstrate variation in chromosomal systems of sex determination may still perpetuate the misconception that all species produce dyadic male and female individuals (Figure 4.1A). Such examples also may neglect to mention that intersex variation also occurs in species with chromosome-based sex determination. A more comprehensive and accurate understanding of sex variation can be achieved by including these variations, as well as discussing how environmental sex determination, based on temperature, photoperiod, social factors, and other variables, occurs across reptiles, amphibians, and fishes (Li and Gui 2018). Insects also show substantial variation in sex determination systems, including ZW/ZZ, XX/XY, and haplodiploidy (Blackmon et al. 2017).

Another way to challenge overgeneralizations and stereotypes about binary sex categories is to explain what sex operationally means in different systems and how it is practically identified by researchers. Generally, categorical sex has been conceptualized in relation to anisogamy, and an individual's sex is determined by which size of gametes they produce (p. 22, Roughgarden 2013). While gamete production is evolutionarily extremely important, the extrapolation of the existence of anisogamy to the conclusion a universal sex binary (especially

among animals) is not supported by empirical data across organisms (McLaughlin et al. 2023). Across anisogamic, dioecious and gonochoristic species, sex traits (i.e. those related directly or indirectly to reproduction) do not manifest as contiguous phenotypic suites universal to all male or all female individuals (McLaughlin et al. 2023). In some species, different sexes are nearly identical except for differences in gonadal tissue type, while others show dramatic dimorphism in size, appearance, and behavior.

It is possible and practical to reject universalizing language equating anisogamy with a holistic sex binary (i.e. the assumption that if a species has 2 types of gametes, then all sex-related traits manifest as a consistently binary based on gamete production), and to instead provide students with a multi-faceted, species specific understanding of sex variation (Subramaniam and Bartlett 2023). It can be explained to students that in anisogamy, gamete sizes differ and some gametes are retained in the location where they are produced by meiosis while others are dispersed without projecting gender stereotypes onto the gametes or their interactions (Hales 2020, Subramaniam and Bartlett 2023). Gamete size can be significant because, in many cases, ova production requires a greater investment than sperm production. Ova usually are the vehicle of inheritance for not only genomic DNA but also mitochondria, chloroplasts, cytoplasm, and other organelles, as well as sometimes a yolk to nourish the developing embryo. However, this is not universally true. The investment may be greater for the sperm contributor in animals where sperm is delivered alongside or as part of a "nuptial gift" that is given to the partner immediately before or during copulation (Prokop and Okrouhlík 2021). These gifts benefit the ova producing parent by providing extra nutrients and sometimes protection from predation and may increase the likelihood of successful mating and contributions to parentage for the gift-giver (Prokop and Okrouhlík 2021). In seahorses and pipefish, the sperm

producing parent also gestates the fertilized eggs and gives birth to live young (Wilson et al. 2003). In other species, including many birds, the sperm producing partner devotes relatively few resources to gamete production but invests in energy-intensive physical ornaments and/or behavioral mating displays (Fusasni et al. 2014). Such examples are a useful way to highlight that there is nothing inevitable or biological about pervasive Western gender roles which cast women and by extension all female organisms as more decorative, performative, and nurturing; indeed, this is the reverse of patterns in many birds, fishes, and other animals. Division of parental care also varies widely across animals and is not always or only a solely maternal duty. In some birds, both parents take turns incubating eggs, in some fishes, both parents attend to their young, and may species across taxa show biparental or no parental care.

Defining sexual reproduction itself in direct, biological terms can help students see it apart from gendered and anthropomorphic connotations. In simplified terms, sexual reproduction is the recombination of genetic material from the genomes of 2 different individuals (p. 22, Roughgarden 2013). Because sex simply requires recombination of DNA, in multicellular organisms, some monoecious species can perform sexual reproduction within a single organism.

Sex and mating in dioecious/gonochoristic organisms are often described in heavily anthropomorphized terms (p. 100, Roughgarden 2013), which may include assigning differential levels of morality, value, or sophistication to certain types of mating strategies. This misrepresents the process of evolution by suggesting a hierarchy of organisms with (dimorphic, heterosexual, monogamous) humans at the top. However, all extant organisms are equally "evolved," and, as previously noted, the unique adaptations present throughout the vast diversity of life on earth crucially contribute to a stable and productive biosphere (Naeem et al. 2012; Oliver et al. 2015; Dainese et al. 2019). This diversity includes different systems of mating and

reproduction, and there are interesting consequences for anatomy, physiology, and behavior based on mating patterns, which may be relevant to explore in detail. Monogamous species may be either facultative or obligate. Polygamous species may demonstrate polyandry (one male mating with many females), polygyny (one female mating with many males), or polygynandry (both males and females mate with multiple partners). Homosexual behavior is not unique to humans and has been documented in hundreds of animal species across taxa (p. 128, Roughgarden 2013). Asexual forms of reproduction also occur across taxa (p. 16, Roughgarden, 2013). Including diverse examples such as these provides an important lesson that, in nature, sex is not always used for reproduction and reproduction can occur without sex.

Part 3: Teaching human sex, gender, and sexuality diversity

Biology is uniquely situated as a STEM discipline that directly relates to aspects of human diversity and identity (including those which may be visibly or invisibly represented by students), providing a crucial opportunity for instructors to challenge or foment forms of social prejudice often justified by claims of biological immutability (Lewis and Sharpe 2023). And while all topics within the field of biological sciences are to some extent naturecultural (Subramaniam 2014), discussions and studies of human biology are particularly intertwined with social, legal, and political discourses (Casper et al. 2022).

In introductory biology classes, as well as in many advanced topics, human sex is often described as a binary: two sexes, male and female, with sex determined at conception by the presence or absence of the Y chromosome (Urry et al. 2017). Conception itself is often described in stereotypically gendered language that portrays the sperm as courageous, enduring, and forceful, and the ova as either passive and sedentary or coercive and devouring (Martin 1991). As an additional complexity, many textbooks, academic articles, and other resources use the

terms male/female and man/woman interchangeably or to refer to both sex and gender.

This narrative of human sex (and, as many therefore assume, also gender) as binary is biologically inaccurate and has emerged from a specific culturally and racially mediated history. Conceptualizations of sexual dimorphism in humans developed co-constitutively with ideas of race and racial hierarchy through specific interplay between science and society during the 19th century (Henderson 2020). In "The Sex That Dare Not Speak Its Name", Emily Nussbaum writes, "…only since 1800 have male and female bodies come to be viewed as utterly distinct" (1999). Prior to that time, men were seen as "the amped-up, deluxe model, and women the budget version, with genitals identical to males' but inverted for lack of the creative heat to pop them out" (Nussbaum 1999). As scientists learned of the existence of different gonads, ovaries and testes, they "increasingly viewed men and women as opposing types rather than points on a continuum" (Nussbaum 1999). The publication of Darwin's evolutionary theories in the mid-1800s provided substantial evidence against polygenesis (Somerville 1994). Within a context of increasing fears among white communities in the US and Europe about losing power and increasing popularity of eugenics, aspects of evolutionary theory were misappropriated to provide support for new pseudoscientific justifications of racism.

Rather than the claim of polygenesis (that different racial groups constituted different species), this new model of human racial hierarchy was undergirded by the fabricated assertion that human sex dimorphism was both a marker of superiority and a racial trait (Somerville 1994; Markowitz 2001; Henderson 2020). According to this model, the extent of human sex dimorphism varies by race (including genital size and shape, pelvic width, secondary sex characteristics, temperament, and "female beauty"), with Europeans having the greatest amount of sex dimorphism and other racial groups, which they argued were more "primitive" and

"savage," demonstrating less dimorphism (Somerville 1994; Markowitz 2001; Henderson 2020). Sexologists in the late 19th century used pseudoscientific biometrics and comparative anatomy to claim that these purported physiological differences between racial groups evinced their belief in the superior power, intelligence, and virility of European men and the complementarily superior beauty, fertility, and tenderness of European women, which placed them as sufficiently inferior to European men, yet superior as compared to non-Europeans (Markowitz 2001; Henderson 2020). Biometrics was also used to claim that decreased dimorphism was exhibited by "degenerate" Europeans, particularly women, including lesbians and sex workers, rendering them less evolved and more physically and hierarchically similar to non-European women than to their heterosexual European counterparts (Somerville 1994; Henderson 2020). These claims about the supposedly racialized science of biological sex were used in service of eugenic projects (Somerville 1994; Henderson 2020) and have continued to impact the cultural understanding and medical treatment of people deemed inferior by or seen as in violation of the racialized sex binary, including Black and Indigenous women, transgender individuals, and intersex people (Lewis and Sharpe 2023).

In contrast to the claims made by 19th century sexologists, eugenicists, and comparative anatomists, even at the moment of conception, human sex exists along a spectrum, not as a binary (Ainsworth 2015). The terms sex and gender are frequently conflated in biology, as well as legally and colloquially (California Law Review 1995), and when differentiated, gender is often viewed as something scaffolded onto and less legitimate than sex. Sex in humans is a multifaceted and non-contiguous phenotype that includes of a collection of variable traits, including primary sex characteristics (such as gonads, chromosomes, external reproductive structures, internal reproductive structures, sex hormone production, and sex hormone response)

and secondary sex characteristics, which are directly or indirectly related to reproduction (Serano 2017; Lindahl 2021). Socially and legally, sex is designated at birth, either male or female, usually based primarily on the appearance of external genitals (Serano 2017). The designation of sex at birth also represents an expectation of gender, which is socially, legally, and in some cases medically enforced.

The enforcement of designated sex and gender is particularly precipitous for intersex individuals who are born with variations in their sex traits or reproductive anatomy (Lewis and Sharpe 2023). Determining the exact percentage of intersex people in the population is an imprecise process because some people do not realize they are intersex until they reach puberty or try to have children, some variations are never identified, and others may be lied to about their medical history throughout their lives. Current medical interpretations of sex trait variations are designed to minimize their prevalence due to medical stigma and dominant cultural narratives enforcing the belief in sex and gender as binary (Costello 2012; Costello 2013). A single precise and comprehensive definition of intersex may not exist, due to the inherent diversity of sex and the variability in phenotypic presentation of some intersex traits which may mean they are never detected. Because "sex" is comprised of so many different traits, all of which vary along spectrums, attempting to sort all human bodies into 3 discrete categories of male, female, and intersex is a subjective and imperfect process (Figure 4.2). The estimate that 0.5-1.7 % of the population has intersex traits is frequently referenced (Blackless et al. 2000), but is considered by many to be an underestimation, with updated calculations including 5% or more of the human population (Maria Marsden, personal communication, October 19, 2021). A substantial contributor to the difference between these estimates is the inclusion of Polycystic Ovary Syndrome (Lindahl 2023), an endocrine condition that affects an estimated 5-20% of people with

ovaries worldwide and frequently hormonal and reproductive sex trait variation (Ntumy et al. 2019). Although most historical estimates of the prevalence of intersex variation in human populations have not included PCOS, this condition can result in symptoms which are clinically indistinguishable from other intersex variations such as non-classic Congenital Adrenal Hyperplasia (Yesiladali et al. 2022) and can be met with similar types of social and medical stigma as other types of sex trait variations that do not conform to binary expectations (Lindahl 2023).

Acknowledging intersex development whenever human sex and human sex determination is mentioned is a simple and important way to disrupt the misconception that human sex is binary. Non-intersex people can be referred to as endosex to avoid the tendency towards designations of "intersex people and normal people". Depending on the course and topic, it is not always necessary to provide detailed information about specific intersex variations. However, whenever the XX/XY system of sex determination is discussed, it should be acknowledged that XXY, XYY, XO, and other combinations also occur, and that some people with XX or XY chromosomes are also intersex (Table 4.1). This provides an opportunity to point out the difference between genotype and phenotype in sex determination (an XX genotype usually results in an individual with ovaries and a vulva, but interactions with hormones and genes on other chromosomes can result in different phenotypes). Many textbooks and course materials will present a simplified view of sex hormones that labels testosterone the "male" sex hormone and estrogen as the "female" sex hormone (p. 215, Roughgarden 2013). Stating that this is inaccurate and ignores the functions of estrogens, androgens, progestogens, and many other hormones in all sexes can increase accuracy and inclusion, even in settings where providing a detailed explanation of hormonal activity is impractical (Figure 4.3).

Including more in-depth discussions of intersex topics in Genetics, Anatomy and Physiology, Animal Development (Figure 4.4), and other relevant courses provides an important opportunity to address misconceptions and increase awareness (Sharpe et al. 2023). Students may have never heard of intersex humans before, be confused about the difference between transgender and intersex, and have misconceptions about individual intersex people being able to produce both types of gametes or having "both" sets of external genitals. There may be intersex students present in these classes, students who are unaware of their own intersex status due to being undiagnosed or deceived by parents and doctors, as well as students who believe they have never met an intersex person. Given that 5+% of the population may have variations of sex characteristics, the widespread lack of awareness about intersex is a result of how this aspect of human diversity has been culturally and medically erased, especially over the past 70 years.

The dominant cultural belief in a sex and gender binary impact on intersex people's lives and bodies in profound and often damaging ways and can only be fully understood through a naturecultural framework. Although intersex people have existed across time, culture, and geography, current mainstream medical protocols function to surgically and socially force intersex children into a binary sex category without their knowledge or consent (Chase 1998). The surgical technologies that enabled this paradigm were largely developed by urologist Hugh Hampton Young in the 1920s and 1930s at John Hopkins, and their widespread use was initiated in the 1950s by psychologist and sex researcher John Money (Colapinto 1997; Chase 1998). Money believed that gender was completely malleably through social factors and that intersex children could be made "formerly intersex" if they were operated on in infancy, subjected to aggressive gender-typical socialization, and never told the truth of their birth status (Hendricks 1993; Colapinto 1997). In trying to eradicate intersex bodies and silence intersex experiences

through medical and social repression, this protocol of early and aggressive intervention has contributed substantially to the extensive medical trauma many intersex people have experienced (Chase 1998) and the widespread erasure and lack of representation of intersex variation in education, media, and public life.

Forty years after Money's paradigm of early surgery and repressive gender socialization was initiated, intersex adults began gathering, organizing, and sharing their stories about the physical and psychological harm these protocols caused (Chase 1998; Nussbaum 1999), beginning the modern intersex rights movement. Substantial progress has been made in building community and raising awareness, and both intersex activists and some medical professionals are now calling for parents and doctors to forgo non-medically emergent genital surgeries on intersex infants, providing intersex people with bodily autonomy and the opportunity to decide for themselves if they want to these types of surgeries or not old enough to decide for themselves (Caplan-Bricker 2017). However, in spite of decades of activist efforts to end these non-emergent, non-consensual surgeries on intersex infants, many hospitals still perform them (interACT).

How is this a natureculture? Sex diversity is an inherent part of human biodiversity. However, the process of determining which bodies are classified as intersex, whether these variations are seen as neutral or pathological, and if medical and social tools are used to suppress intersex variation are all culturally mediated processes. Especially in the case of intersex people who are subjected to nonconsensual hormone replacement therapy and surgeries as children or adolescents, cultural adherence to an artificial sex and gender binary very literally alters the nature of their bodies and biology. Including thoughtful and actively-moderated discussions of these issues in Biology classes can provide intersex students (who may or may not disclose their

intersex status to a professor or other students) to see themselves represented as part of the human biological experience, rather than being further erased or enfreaked, and provides important and possibly unprecedented knowledge to endosex students, some of whom may later become doctors who treat intersex patients or parents of intersex children (Sharpe et al. 2023).

Although some biology instructors may be reluctant to include discussions of gender in their curricula, not doing so can leave unchallenged the misconception that Biology only legitimates binary and bioessentialist conceptualizations of gender (Bazzul and Sykes 2010; Junkala et al 2021; Avielo et al. 2022). As Harris et al. 2024 writes, "When instructors try to remain neutral by not engaging with issues of sex, gender, and sexuality diversity in their curricula and pedagogy, their attempts at neutrality or non-engagement tacitly enforce a 'hidden curriculum' that implicitly affirms heterosexual, cisgender, and endosex bodies and lives as the only ones that are natural, normal, and "biological" (pg. 2, Harris et al. 2024). Providing a gender-affirming perspective does not require expertise or extensive deviations from course content. Clarifying that human sex and gender both show considerable variation, that gender is not determined by binary sex phenotypes, and that demeaning gender stereotypes and denial of gender diversity are not supported by biological evidence can go a long way.

Having a foundational understanding of current affirming gender terminology can also be helpful to creating a supportive classroom environment and addressing any questions arise about sex, gender, and biology. The term transgender refers to a person whose self-knowledge of gender differs from the sex/gender they were designated at birth (Cooper and Brownell 2016). This includes transgender men and women, whose gender identity corresponds to a culturally recognized gender category different from the one they were designated at birth, nonbinary, agender, genderqueer individuals whose gender falls outside mainstream cultural understandings

of binary men and women, and people with culturally specific genders that may not be legible through hegemonic systems of gender norms. The term transgender is itself an adjective (transgender man, transgender woman, transgender children). It is not an adverb (avoid "transgendered") or a noun (avoid "a transgender") (Kapitan 2021). Non-transgender individuals can be referred to as cisgender, which prevents the juxtaposition of transgender vs "normal" people. Being transgender is not a pathological condition, and the term "Gender Identity Disorder" has been removed from the DSM V and does not reflect current understandings of human gender diversity (APA 2022).

Terms such as "biologically male," "biologically female," "genetically male," "genetically female," "born a man," "born a woman" don't accurately describe a person's gender or what they "really" are (Kapitan 2021). Importantly, legal sex assigned to a baby at birth is usually based exclusively or primarily on external physical structures, which are only one of component of sex-specific development, and, as in the case of intersex people, may not align as expected with internal sex organs, chromosomes, and hormone levels (Chase 1998). As previously discussed, biological sex in humans is not only a multifaceted and highly variable set of traits, but it can also be altered by various medical interventions. Transgender individuals may undergo hormone replacement or gender affirming surgeries that change some aspects of their biological sex characteristics, making it inaccurate to describe a medically transitioning transgender individual as having identical biological sex traits to a cisgender person of the same assigned sex. Although transgender people use a variety of terms to describe their own experiences of dissonance between the sex/gender they were assigned and the gender they understand themselves to be, defaulting to "designated gender" for the former and "affirmed gender" for the latter respects the individual identities and self-knowledge of transgender people

(Kapitan 2021). Most often, it is not necessary to refer to a transgender individual's designated gender at all.

One of the insidious impacts of the sex and gender binary is that transgender and intersex people are frequently subject to medical gatekeeping and denied autonomy to make affirming decisions about their own bodies and sex characteristics (Chase 1998; Henderson 2020). Transgender individuals are increasingly facing legislative bans on accessing gender affirming hormones and surgeries (Lewis and Sharpe 2023) or may be required to obtain considerable paperwork and documentation to access them. However, these same prescriptions and procedures are readily given to cisgender adults who request them and are often forced on non-consenting intersex infants (Costello 2016). Many cisgender individuals choose to alter their biological sex characteristics through surgeries (i.e. cisgender women who breast implants) and hormones (i.e. cisgender men who take exogenous testosterone and cisgender women who use hormonal birth control), however, these same procedures are seen as unnatural and suspect when pursued by transgender individuals (Henderson 2020). When relevant to course materials, it may be useful to discuss how current medical technologies enable a variety of alterations to be made to primary and secondary human sex characteristics, which consenting individuals should be able to access regardless of their gender identity as part of their right to bodily autonomy. This further illustrates the naturecultural impacts of social beliefs, medical policies, and legislation on human biology.

Another area in which sex and gender inclusive language is especially important is when describing puberty and sexual development in humans. These topics are often presented according to a set of assumptions that physical development, chromosomal sex, and gender identity will always align in a cisgender, endosex manner. However, puberty is a complicated,

sometimes unpredictable, and potentially traumatic experience for many people, including transgender individuals who may feel that their body is developing in ways that contradict their sense of self, and intersex people who may not discover that they have an intersex condition until secondary sex characteristics begin (or do not begin) to develop. Implementing non-gendered language around puberty may not feel intuitive or efficient, but it pushes both instructors and students to explore the variation in development in people of all sexes and genders. This is especially critical when working with middle and high school students, whose bodies may still be changing or about to change.

Removing gender essentialist terms and assumptions also creates a more inclusive environment for students who are not transgender or intersex. Teenagers of all genders and sexes whose bodies look different or change at a different rate than most of their peers are often subject to bullying or made to feel self-conscious. Using "many" and "some" rather than "every" or "all" language to describe physical changes (i.e. "many girls, some boys, and some people of other genders have a menstrual cycle") reduces stigma against those who diverge from expected patterns. For example, describing facial hair growth as a normally or exclusively male trait stigmatizes not only transgender and intersex people but also cisgender men who have extremely light or spare facial hair and cisgender women who may grow noticeable facial hair. Many developmental processes and healthcare needs can be described using anatomy rather than gender ("penises grow, chests/breasts develop, and facial hair grows") or by referring to people or bodies with specific needs and characteristics, rather than assuming identity ("people who have a cervix should get regular PAP tests" or "people with testicles should check them regularly for lumps") (SextEd 2017).

Part 4: Inclusive pedagogical strategies

To provide an inclusive biology learning environment for students of all genders and sexualities, effective pedagogical strategies are important as well as course content. Like students in other marginalized groups, LGBTQIA+ students frequently encounter a "hidden curriculum" of expectations regarding gender, sexuality, and gender expression in college courses in all subject areas (Bazzul & Sykes 2010; Zook 2017, Junkala et al 2021; Avielo et al. 2022). Removing this "hidden curriculum" from classrooms and syllabi is an ongoing process of self-education, challenging implicit biases, and inviting authentic feedback from students and colleagues. For instructors who are straight and cisgender, it can be easy to unintentionally write case studies, examples, and questions featuring only cisgender, endosex individuals and heterosexual relationships. Incorporating examples of real or theoretical people representing diverse gender identities and sexual orientations in a respectful, normalizing way can make LGBTQIA+ students feel more included and challenge the default assumptions of other students.

In confronting their implicit biases, and coming to terms with knowledge gaps, instructors may be put in the position of learning from their students. It is important to balance acknowledging students' expertise about their own identities and avoiding positioning students to speak for the experiences or views of an entire identity group or to disclose personal information they are not comfortable with. Deferring to student's knowledge can be challenging and humbling, but it is an important way to establish mutual respect and create an authentic environment in which to grow and move towards a more inclusive paradigm. This also does not give license to assume that openly LGBTQIA+ students should become the de facto instructor on topics related to their identities, or that it is permissible for the instructor or other students to ask personal or invasive questions about their bodies, identities, or behaviors.

Creating a culture of respect and inclusion for people of all gender can be established at the beginning of the semester by inviting, affirming, and using each student's correct name and pronouns. This is especially important in classes that involve active learning, group work, or discussions, as being consistently misgendered by groupmates can decrease learning, participation, and attendance for LGBTQIA+ students (Cooper and Brownell 2016). To avoid putting students on the spot, it may be easier to do this through email or by collecting names and pronouns with other relevant written information on notecards during the first day of class. If a student indicates a preferred name that differs from their legal name, use the preferred name and pronouns exclusively in both oral and written communication, and consistently correct other students it they fail to do so. When working with another instructor or if a substitute teacher will be teaching the class, make sure they are informed about which names to use. In some cases, a student may express that they want to use the name and pronouns that they find affirming in class but are not able to be out to their family or within all spaces in the school. If a student requests that their given name and pronouns be used outside the classroom, it is important for their privacy and safety to respect this request. Even if there are not any openly transgender or gender non-conforming students in a particular classroom, modeling an environment in which other people's gender is respected and not assumed sets a positive example for all students, and is a good practice for supporting gender diversity.

Depending on the institution, subject area, and limitations of the course, inclusive textbooks and other teaching materials may not be available or convenient to use. Most standard resources describe human anatomy based on the assumptions of cisgender, endosex bodies. Several articles have reviewed the presentation of human sex, gender, and sexuality in Biology textbooks and found queer representation to typically be minimal or stigmatizing. Snyder and

Broadway found that, in many Biology textbooks, the primary or only mention of sexualities beyond heterosexual will be in the context of HIV and AIDS (Snyder and Broadway 2004). This can bolster the impression that reproductively focused heterosexuality is the only natural/normal/positive form of human sexuality, while queerness is nonbiological except as it is associated with pathology. Bazzul and Sykes found no references to any non-straight sexualities in their review of a Canadian Biology textbook and 11 instances in the text in which implicitly or explicitly promoted heteronormative and/or binary conceptualizations of human biology (Bazzul and Sykes 2011). Junkala et al. reviewed 5 Swedish biology textbooks for grades 7-9, and while they noted many more examples of sexuality and gender diversity in these textbooks than the other authors, they still found explicit and implicit examples in which only binary sex/gender and heterosexuality were shown to be grounded in human biology (Junkala et al. 2021). Avielo et al. compared a variety of Finnish biology textbooks from the 20th and 21st century, finding that recent texts presented fewer gender stereotypes but still emphasized biological causes of sex and gender differences, additionally noting that "overtly fixed ideas of human sex/gender can make it more difficult to understand the reproductive modes of other organisms" (pg. 309, Avielo et al. 2022).

When possible, it may be beneficial to look for resources outside of or in addition to these types of standard textbooks when covering topics related to human sex, gender, and sexuality. Even if course texts use non-affirming language, providing students with a clear guide and consistent modeling of inclusive, anatomy-focused, rather than gender-focused language, to be used in classroom dialogue and in class assignments can help to create an inclusive environment. Involving students in the process of searching for and challenging examples of the "hidden curriculum" in textbooks and other materials can be a useful exercise. There is also an

important opportunity to challenge the view of textbooks as biological doctrine or a "final science" presenting irreducible facts, rather than existing in relationship to science as a process that continues to progress, self-interrogate, and be revised (Avielo et al. 2022). Pointing out binary and heteronormative thinking in Biology textbooks is another way to demonstrate the naturecultural quality of biology. In the example of HIV/AIDS, students can learn about how a cultural of homophobia and political disregard for queer life shaped the biological trajectory of a virus, facilitated the spread of misinformation and increased bigotry, and restricted the ability of science and research to save lives. This can also be connected to current disparities in access to newer medications to treat and prevent the transmission of HIV within marginalized communities. A related public health issue that students can explore is sex and gender bias in clinical trials used to test drugs and medical procedures. Historically, many drugs and procedures have only been tested on white, cisgender, endosex men before being approved for use, although their effects on women, transgender people, and intersex people may be quite different and potentially harmful (Chen et al. 2018; Brenna 2021; Zemenick et al. 2022). And even when cisgender, endosex women are included in models of disease and treatment response, transgender and intersex individuals are usually not considered (Zemenick et al. 2022).

Part 5: Conclusions

The guidelines suggested here are general and meant to evolve and adapt to different courses, institutions, and student populations. Our understanding of sexuality and gender diversity continues to change as language and dialogue becomes more nuanced, inclusive, and comprehensive. Operating based on a flexible and inclusive framework will help to accommodate these changes as inevitable and welcome. Understanding human diversity from a naturecultural lens provides a framework for including a diversity of experiences as part of our

species' biological truth while neither essentializing cultural categories as stemming from physiological determinism or discounting their impact on physical and social realities. Sharing with students the vast ranges of sex and reproductive variation across taxa provides critical context that nature is wildly queer and creative, and that sex binaries and compulsory heterosexuality do not hold exclusive claim to biological truth. Incorporating these principals into biology education at every level is an important step towards allowing all students to feel included and affirmed by Biology, to support increased representation of LGBTQIA+ people and perspectives in the field, and to dismantle the oppressive power structures that continue to weaponize misconceptions about biology to enforce bigotry and violence.

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Glossary of Terms

Agender: umbrella term for individuals who do not identify with any gender category or do not relate to conceptualizations of gender.

Anisogamy: sexual reproduction through the fusion of 2 different gamete types (often small sperm and large ova). It is common in multicellular organisms.

Aromantic: a term used to describe individuals who experience little or no romantic attraction.

Asexual: umbrella term used to describe individuals who experiences little or no sexual and/or physical attraction to other people.

Bisexual: a term used to describe individuals who experience attraction to people of more than one gender. Some people use bisexual and pansexual interchangeably, though pansexual may also specifically refers to attraction to people regardless of sex or gender.

Dioecious: reproductive strategy in which sperm and ova are produced by separate individuals. Usually used to refer to plants but can also be used more broadly.

Endosex: Used to describe humans whose are born with bodies that conform to typical medical expectations of male or female anatomy (non-intersex).

Gay: a term used to describe experiencing attraction to people of the same gender.

Gender: cultural system of categorization that applies social norms, expectations, and constraints to individuals perceived to belong to culturally recognized gender categories. Different cultures have conceptualized gender in different ways throughout history, and not all cultures have a strong emphasis on gender as a social organizing principle.

Genotype: combination of alleles an individual inherits. May be used to refer to the whole genome collectively or the gene(s) related to a specific trait.

Gonochoristic: reproductive strategy in which sperm and ova are produced by separate individuals. Typically used to describe animals.

Haplodiploidy: System of sex determination in which females develop from fertilized eggs and are diploid (have the 2N number of chromosomes) and males develop from unfertilized eggs and are haploid (have the 1N number of chromosomes).

Hermaphrodite: reproductive strategy in which a single individual can produce both sperm and ova. In plants, it refers to an individual that produces both carpellate (ova-producing) and staminate (pollen-producing) organs on the same flower. This term has historically been used to describe intersex humans, though this usage is inaccurate as no intersex humans can produce both functional ova and functional sperm. It also regarded as a slur when used to refer to transgender or intersex people. Due to the stigma and misunderstanding surrounding this term, we recommend being intentional about its usage in the classroom and defining appropriate and inappropriate usage very specifically.

Intersex: an umbrella term for people who are born with, or later develop naturally, variations in their sex traits or reproductive anatomy.

Isogamy: a form of sexual reproduction that involves fusion of gametes of similar size and form. This strategy is often found in unicellular organisms which have two or more mating types.

Lesbian: a term for women (including transgender, cisgender, and nonbinary women) who experience attraction to other women (including transgender, cisgender, and nonbinary women).

Monoecious: reproductive strategy in which a single individual can produce both ova and sperm. In plants, it refers to individuals that that grow both pistillate (ova producing) and staminate (pollen producing) flowers on the same plant.

Natureculture: term coined by Donna Haraway to provide a framework for interrogating the ongoing interactions between nature and culture and the ways in which they are continuously making and remaking each other.

Nonbinary: a term that refers to individuals who do not identify (entirely or at all) with the binary genders man and woman. This is also used as an umbrella term for a variety of gender

identities outside the binary. Nonbinary identities are part of the transgender umbrella, though some individual nonbinary people do not identify as transgender.

Phenotype: observable traits of an individual that result from the interactions of their genotype with the environment. As with genotype, it can refer to a single trait or include all developmental, morphological, biochemical, and behavioral characteristics.

Queer: umbrella term for non-heterosexual and/or non-cisgender individuals. This term has historically been used as a slur, and some individuals in the community find it hurtful, though others, (including the author) have reclaimed it.

Questioning: refers to individuals in the process of exploring their LGBTQA+ identity.

Sex: collection of variable traits related directly or indirectly to sexual reproduction. In anisogamous organisms, sex is often divided into male and female categories based on gamete size. In humans, definitions of sex based solely on gamete production are limited in their utility to encompass both muti-trait variations in sex phenotypes and the many legal, medical, and social dimensions of sex. Sex and gender are frequently culturally conflated as female/woman and male/man. However, sex does not determine gender, sex and gender do not align for all individuals, and neither sex nor gender is binary.

Sex and gender binary: A prevalent set of connected cultural beliefs, including 1) all humans have 1 of 2 contiguous sex trait phenotypes (male or female) 2) all humans have 1 of 2 gender identities (man or woman) that correspond to and are seen as a natural extension of their sex traits 3) Through a combination of sex traits and culturally normative gender expression (masculine or feminine), it is always immediately and clearly apparent what someone's sex and gender are. These beliefs do not reflect the reality of human sex and gender diversity but are legally, medically, and socially enforced in many cultural contexts and significantly impact LGBTQIA+ people's lives and experiences of oppression.

Transgender/trans: umbrella term for individuals whose self-understanding of gender does not align with the sex category which they were designated at birth. This includes individuals with agender and nonbinary gender identities as well as transgender men and transgender women. Transgender individuals may socially and/or medically transition. Gender diversity beyond a man/woman binary has existed throughout human cultures across time and geography, and many of these variations cannot be fully understood through the modern, culturally specific framework of transgender.

Figures

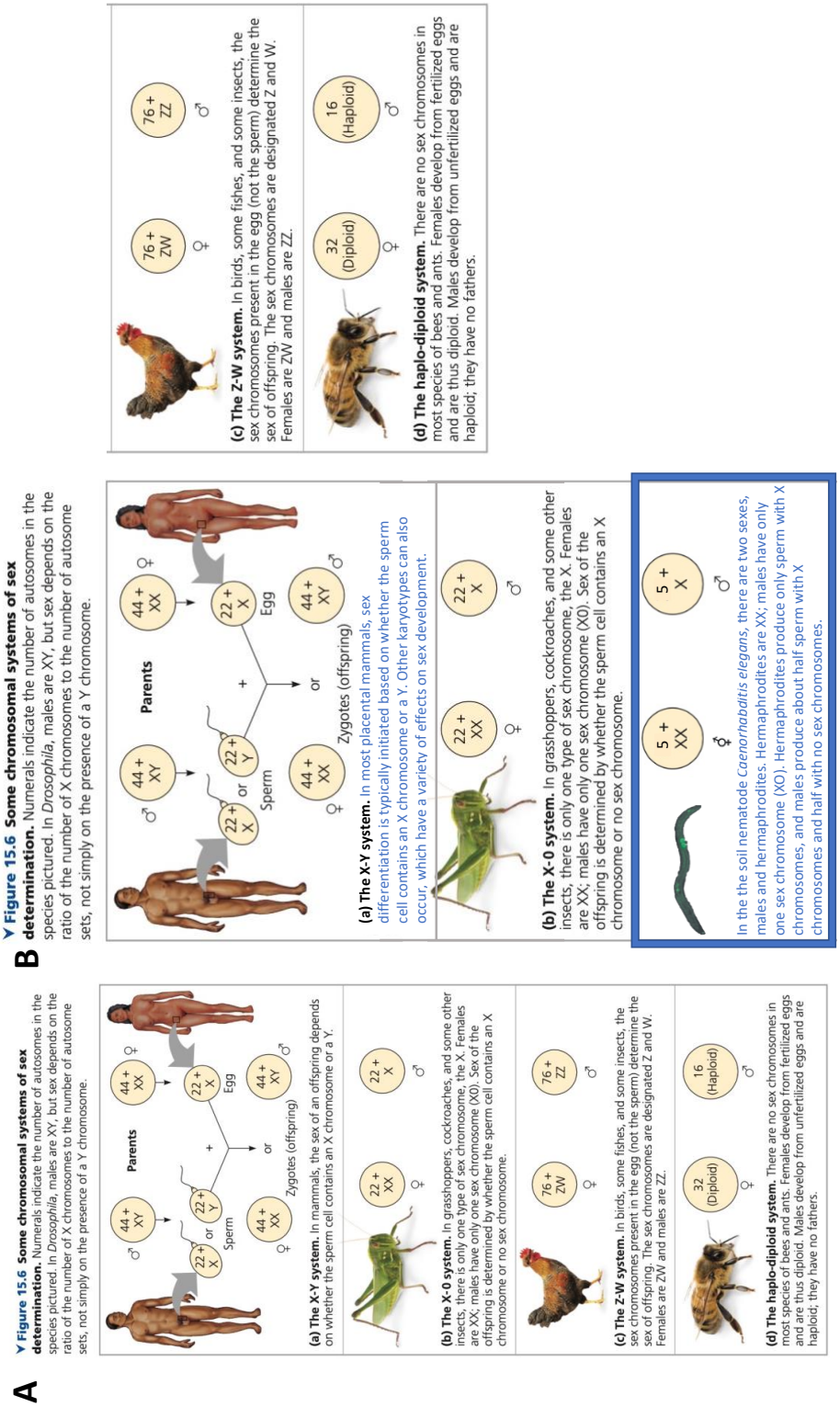

Figure 4.1 : A. Examples of chromosomal sex determination strategies presented in Campbell Biology 11th Edition, Figure 15.6 (p. 298, Urry et al. 2017). B. Figure with suggested

modifications shown in blue. Panel (a) has been adjusted to the presence of intersex development in humans within the XX/XY chromosome system, and a panel has been added to show an example of a self-fertile species with multiple sexes that also utilizes sex chromosomes.

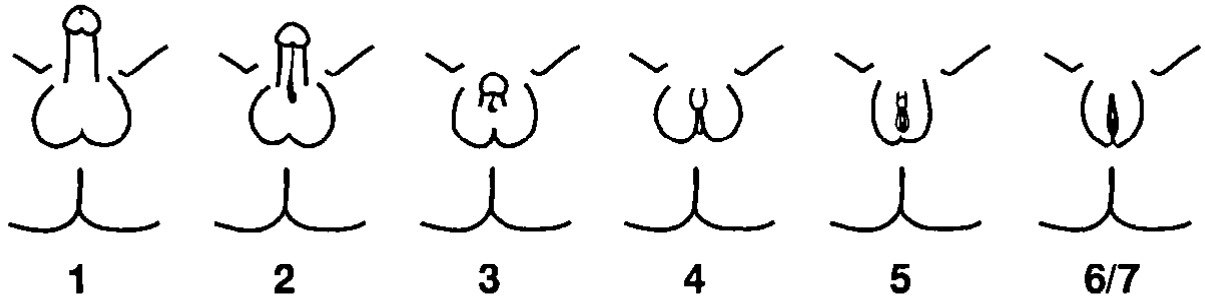

Figure 4.2 : The Quigley Scale. This scale was developed to evaluate external genital phenotype in children diagnosed with Androgen Insensitivity Syndrome but is also a highly useful visualization of the external genital structure diversity as a spectrum, rather than a binary. Students may be unfamiliar with the existence of intersex variation or may have misconceptions about intersex people having "both" sets of genitals or reproductive organisms, and providing specific examples of sex diversity can introduce new perspectives. Image is adapted from Figure 8 in Quigley et al. 1995.

A

The Chemical Groups Most Important in the Processes of Life

Consider the differences between estradiol (a type of estrogen) and testosterone. These compounds are female and male sex hormones, respectively, in humans and other vertebrates. Both are steroids, organic molecules with a common carbon skeleton in the form of four fused rings. They differ only in the chemical groups attached to the rings (shown here in abbreviated form); the distinctions in molecular architecture are shaded in blue:

The different actions of these two molecules on many targets throughout the body are the basis of gender, producing the contrasting features of male and female vertebrates. In this case, the chemical groups are important because they affect molecular shape, contributing to function.

B

Messages in current text	Suggested adjustments
There are separate and unique male and female sex hormones.	Ratios of different sex hormones affect how sex characteristics develop
Testosterone and estrogens are the only sex hormones. Only male individuals have testosterone and only female individuals have estrogens.	There are a variety of hormones that contribute to sex development in vertebrates, including androgens, estrogens, and progestogens.
Sex and gender are conflated and used interchangeably.	Sex is a multi-faceted phenotype that varies across vertebrates and is not synonymous with cultural understandings of gender.
Male and female sex differences produce the basis of gender.	Sex characteristics vary along spectrums, both in humans and in other vertebrates, and people experience a variety of different relationships between their sex traits and gender identity.
Male and female vertebrates (including humans) have contrasting sex characteristics.	Sex development differs considerably between different vertebrates, and some species have significant sexual dimorphism while others have fewer differences between sexes.

Figure 4.3 : A. Excerpt from Campbell Biology 11th Edition, a popular textbook used for AP Biology. Terminology that presents sex as a binary and conflates sex and gender have been added (p. 62, Urry et al. 2017). B. Discussion of how the current text explicitly and implicitly delivers misinformation about sex and gender diversity, with suggestions for a more inclusive approach.

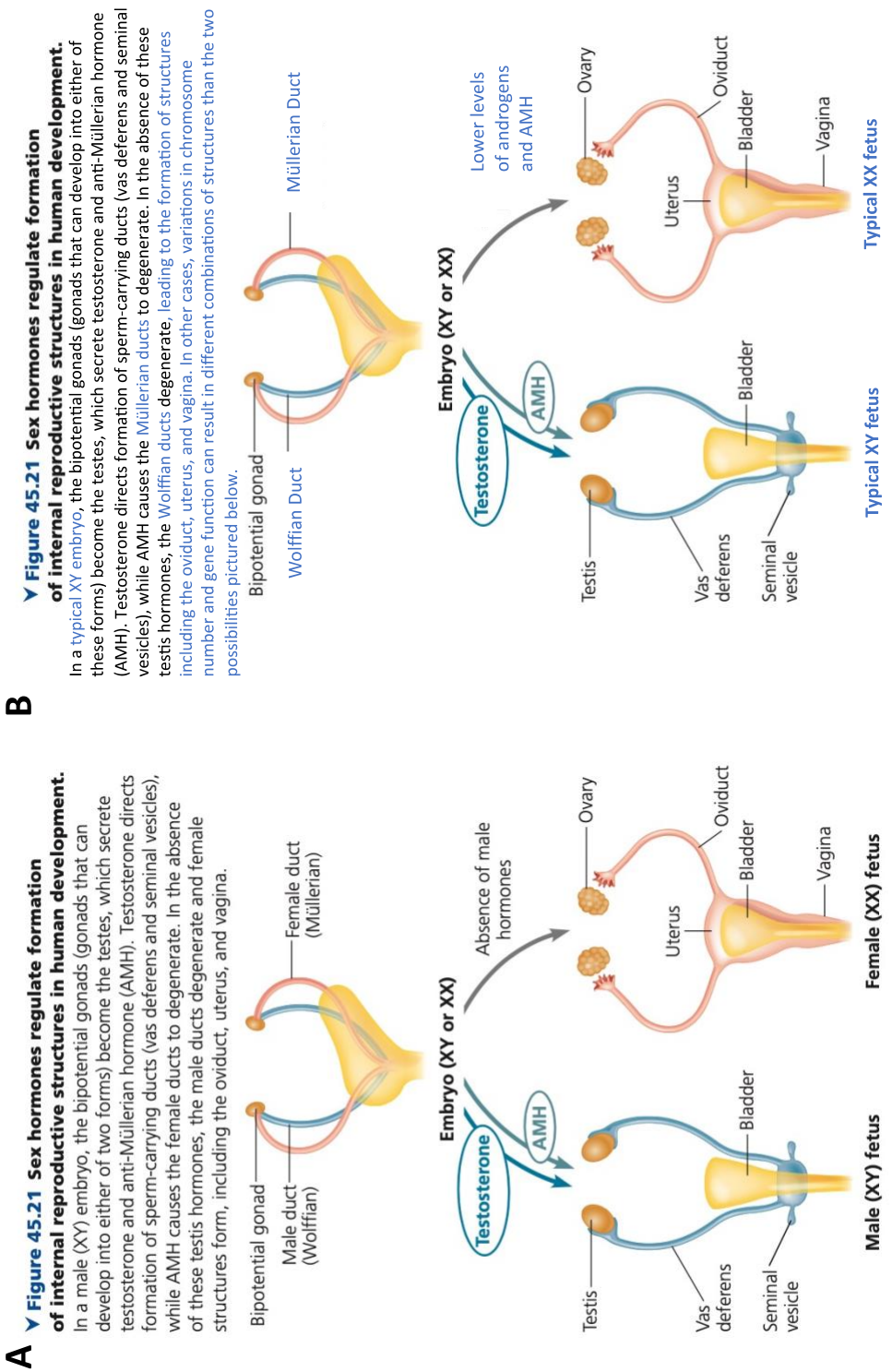

Figure 4.4 : A. Diagram of human reproductive structures during embryonic development adapted from Campbell Biology 11th Edition (p. 1013, Urry et al., 2017). B. Figure with suggested modifications shown in blue. Language which is gendered or enforces the idea of a sex binary has been replaced with structure-specific language and reference to alternative developmental pathways that can lead to intersex development have been included.

Tables

Table 4.1 : Examples of genotype and possible phenotypes for 6 of the most common intersex variations. Over 40 named intersex variations exist, as well as those which have not been named. Table adapted from the interACT website (interACT).

Medical Term	Chromosomes	External	Internal	Puberty
Complete Androgen Insensitivity	XY	Vulva, clitoris	Testes, no uterus, sometimes partial vagina, or complete vagina	If testes are left alone, body goes through puberty via converting testosterone into estrogen
Partial Androgen Insensitivity	XY	Vulva and visibly large clitoris, or other differences	Testes, no uterus, varies	If testes are left alone, body has varying levels of response to testosterone
Congenital Adrenal Hyperplasia	XX	Vulva (labia may be fused), often visibly large clitoris	Ovaries, uterus, sometimes partial vagina or complete vagina	May be early, higher testosterone can lead to features such as facial hair, changed fat distribution
Swyer's	XY	Vulva, clitoris	Streak gonads, uterus, sometimes partial vagina or complete vagina	No puberty because streak gonads do not produce any hormones
Klinefelter's	XXY	Penis, small testicles	May have low sperm count	Low T may cause breast development or other atypical features, may be very tall
Hypospadias	Varies by cause (often XY)	Penis (with urethral opening somewhere other than tip) and testicles; or small penis (with urethra near base or perineum) and open labioscrotal folds; or other differences		Varies by cause (often typical testosterone puberty)

Chapter 5 - Synthesis and Implications

"when we are not at home, when we are asked where we are from or who we are, or even what we are, we experience a chip, chip, chip, a hammering away at our being. To experience that hammering is to be given a hammer, a tool through which we, too, can chip away at the surfaces of what is, or who is, including the very categories through which personhood is made meaningful" **-Sarah Ahmed, An Affinity of Hammers**

Evolution is a central concept within biology, and evolutionary biology encompasses a wide variety of approaches to inquiry about the origins of life on earth and the ways in which living organisms change over time in relation to their environments and each other. My dissertation chapters utilize different methods for exploring evolutionary questions (physiological comparisons, transcriptomics, and inclusive pedagogy) but all incorporate many of the same key principles of biodiversity, adaptation, classification, and resilience against the backdrop of increasingly stochastic anthropomorphic contexts.

Chapters 2 and 3 are focused on questions of local adaptation in *Setaria viridis*, the wild foxtail millet and seek to contribute to the growing knowledge base about the evolutionary ecology and genomics of this emerging model species. These chapters utilized the substantial diversity of *S. viridis* populations across a wide species range and the previously sequenced *S. viridis* genome assembly to build upon existing research on local adaptation and physiological and transcriptomic responses to stress.

Our findings from Chapter 2 add new knowledge about the extent of variability between populations in their responses to drought stress, the differences in drought sensitivity between plants of different ages, and potential impacts of root surface area to aboveground biomass ratios

and flowering time on drought tolerance which invites further exploration through future research. We also generated further evidence to support previous findings about the impact of photoperiod on morphology and timing of reproduction in *S. viridis*.

The findings from Chapter 3 contribute to an increasing number of studies characterizing transcriptomic responses to environmental stress in *S. viridis*. To our knowledge, ours is the first study to assess full leaf transcriptomic responses to multiple forms of abiotic stress in wild *S. viridis* populations from both China and Chile. Other researchers have analyzed the response of various putative stress response genes across a range of forms of abiotic stress in several different populations of *S. viridis*, and our results fill some existing gaps in this body of literature while also corroborating some previous findings about overall variability in stress response between populations.

Chapter 4 also contributed to an emerging area of research and innovation in imagining and developing novel approaches to teaching biology in a way that is responsive to and supportive of human diversity in relation to sex, gender, and sexuality. An increasing number of papers have presented research findings, proposed guidelines, and provided community input surrounding this topic (Cooper et al. 2016, Cooper et al. 2020, Hales 2020, Caspar et al. 2022, Leyva et al. 2022, Zemenick et al. 2022, Lewis and Sharpe 2023, Sharpe et al. 2023), representing some of the many considerations and approaches needed to improve inclusion and discussions of LGBTQIA+ diversity in biology classrooms and curricula. The ideas, approaches, and strategies covered in this chapter are responsive to and built upon existing literature on addressing the short comings of status quo teaching approaches which ignore or minimize these forms of biodiversity.

Collectively, this research represents a multi-faceted consideration of naturecultures, a term that was "coined by Haraway and Goodeve (2013) and insists on an interdisciplinary approach to studying the world. Refusing binary worlds of nature and culture, naturecultures allows us to understand the entangled worlds of bacteria, viruses, fungi, plants, animals (and human) as more-than- human worlds" (pg. 950, Subramaniam and Bartlett 2023). This concept of naturecultures is present throughout the contexts that have brought about my research questions, the variables impacting their interpretation, and broader implications of this work.

Setaria viridis is a member of Poaceae, the grass family, which has been described as "the modern world's most successful angiosperm family," in part because grasses, like humans, have both adapted to ecosystemic contexts across the globe and have altered the ecosystems they colonize, in effect creating their own eco-evolutionary contexts (Linder et al. 2018). Poaceae's evolutionary success also extends to its coevolutionary relationship with humans, wherein 60% of human energy intake globally is now derived from 3 grass family crops, and human activities have facilitated many recent grass dispersal and invasion events (Linder et al. 2018). Several indicators of this coevolutionary context between humans and grasses became apparent through the analysis of the data we generated in Chapter 2 and 3, as well as related themes of unstable categorization and tensions between sameness and difference.

In Chapter 2, we assessed variation in drought response across 9 populations of *S. viridis*, as well as the relationship between drought tolerance and 19 bioclimatic variables from the location of origin for each population. While we did not find any significant relationships between population drought tolerance and these variables, difference in drought tolerance by population were significant, including in some unexpected ways. Two of the populations in the dry down experiment were, per the GRIN database (USDA), collected at the same Latitude and

Longitude in Michigan, USA, but showed statistically significant differences in their response to drought stress. This result is in opposition to what would be predicted through local adaptation, but local adaptation is not the only contributor to evolution. Population genetic analyses of *S. viridis* populations across the globe have identified multiple, likely human mediated introductions of this species in North America from different Eurasian populations (Huang et al. 2014). Several researchers all identified 43.5°- 46° N Latitude as the transition point between the more northern population cluster and the more southern population cluster of *S. viridis* in central North America (Wang et al. 1995, Huang et al. 2014, Schröder et al. 2017). Huang et al. observed that this remaining, strong population genetic structure may overwhelm signals of local adaptation in North American populations of *S. viridis* (Huang et al. 2014). The 2 Michigan populations we assessed were both collected within this latitudinal range, suggesting that drought tolerance in these populations may be more attributable to divergent human-mediated dispersal histories than to contemporary sympatry.

The *S. viridis* population that showed the most susceptibility to drought stress in the dry down experiment was from Afghanistan, which had the second lowest average annual precipitation of all the populations assessed. This result was also in contrast to what would be predicted based on local adaptation, but upon further investigation, this population's GRIN Passport noted that it was obtained from at a market in Jalalabad where it was being sold for birdseed and described it as "Taxonomically ambiguous as determined by David Brenner, November, 2012. It has the thin multiple stems, and un-lobed head of wild-type *viridis*; but the seed abscission of cultivated *S. italica*." (USDA). Despite being listed under *S. viridis* in the GRIN database, this population's anthropogenically-mediated history likely resulted in a phenotype that could not be definitively categorized by existing taxonomic categories, and its

cultivation process may have occurred through artificial selection for seed traits that disregarded any evolutionary tradeoffs reducing drought tolerance.

The analysis of transcriptomic responses to drought, chilling, and salinity stress in Chapter 3 is also interpretable through these themes. The increasing threat posed to both wild and domesticated plants by these 3 environmental stressors through the multifaceted and extensive impacts of climate change illustrates the extent to which the Anthropocene epoch is creating new, unpredictable, and increasingly inhospitable evolutionary contexts. In our transcriptomic assessments, at the level of individual differently expressed genes, we observed considerable difference between populations and treatment conditions. But in the context of shared alignment with a database of 190 molecularly characterized plant drought response genes (Alter et al. 2015), we observed considerably more similarity across both populations and all treatment conditions. This may indicate some level of functionally similar responses to stress across the 2 populations and 3 stressors that are accomplished through different genes with related functions.

In situating these experiments within the existing literature on stress tolerance between populations of *S. viridis*, we found strikingly inconsistent results across several researcher's comparisons of stress tolerance in the Ast-1 and A10.1 populations (Saha et al. 2016, Ariño-Estrada et al. 2019, Duarte et al. 2019, Travassos-Lins et al. 2021, Valeriano et al. 2021, Carvalho et al. 2022). Notably, each of these previous experiments involved slightly different methods and durations of stress exposure, which may have led to biologically relevant differences in how these stressors were experienced by the plants at the cellular and organismic levels. This potential explanation is supported by other studies which have shown that plants, as sessile organisms which experience evolutionary pressure to be acutely responsive to a variety of

abiotic threats from which they cannot escape, may demonstrate highly specific and variable patterns of gene regulation depending on the duration and severity of stress (Sun et al. 2022).

Chapter 4 of this dissertation elucidates several key examples of how specific naturecultures have impacted LGBTQIA+ populations and interrogates the broader natureculture of knowledge production about the natural world situated within human cultural contexts. LGBTQIA+ students face complex forms of systemic oppression which can result in both general challenges in navigating educational contexts and completing degree programs as well as specific forms of invalidation and harm related to binary and heteronormative conceptualizations of biology that are often present in biology textbooks and curricula. Addressing these sources of harm, inequity, and under consideration of human sex, gender, and sexuality diversity is relevant to not only JEDI (justice, equity, diversity, and inclusion) workforce initiatives but also to how biology itself is taught and conceptualized. A full appreciation of diversity across taxa includes the variety of evolutionary approaches to sex traits and reproductive behaviors beyond anthropomorphic analogies that postulate a universal sameness through a shared sex binary in spite of evidence of considerable forms of difference (McLaughlin et al. 2023; Subramaniam and Bartlett 2023). Categorization of human sex and gender as a fundamental binary has and continues to endanger transgender and intersex communities in a variety of ways, including the ongoing legislative and cultural threats against transgender individuals and the ongoing medical harms and erasure that intersex people face. Biology instructors are powerfully situated to challenge these deleterious paradigms through including these topics as part of their curricula and taking intentional steps to create inclusive classrooms for all students.

My interest in and approach to these topics over the course of my graduate program has been shaped by questions about context, diversity, categorization, sameness and difference, and

the constant co-construction of science and culture. These questions have been inescapable for me, due in part to the overwhelming experiences of what Ahmed describes as "a chip, chip, chip, a hammering away" at my own queer biology and personhood (pg. 1., Ahmed 2016). Ultimately, the research presented in these chapters provides explorations of some of these questions, a contribution to the field of evolutionary biology, a response to some ways I have been hammered away at, and an invitation for further questions, continued areas of research, and the ongoing importance of chipping away at the surfaces of what is.

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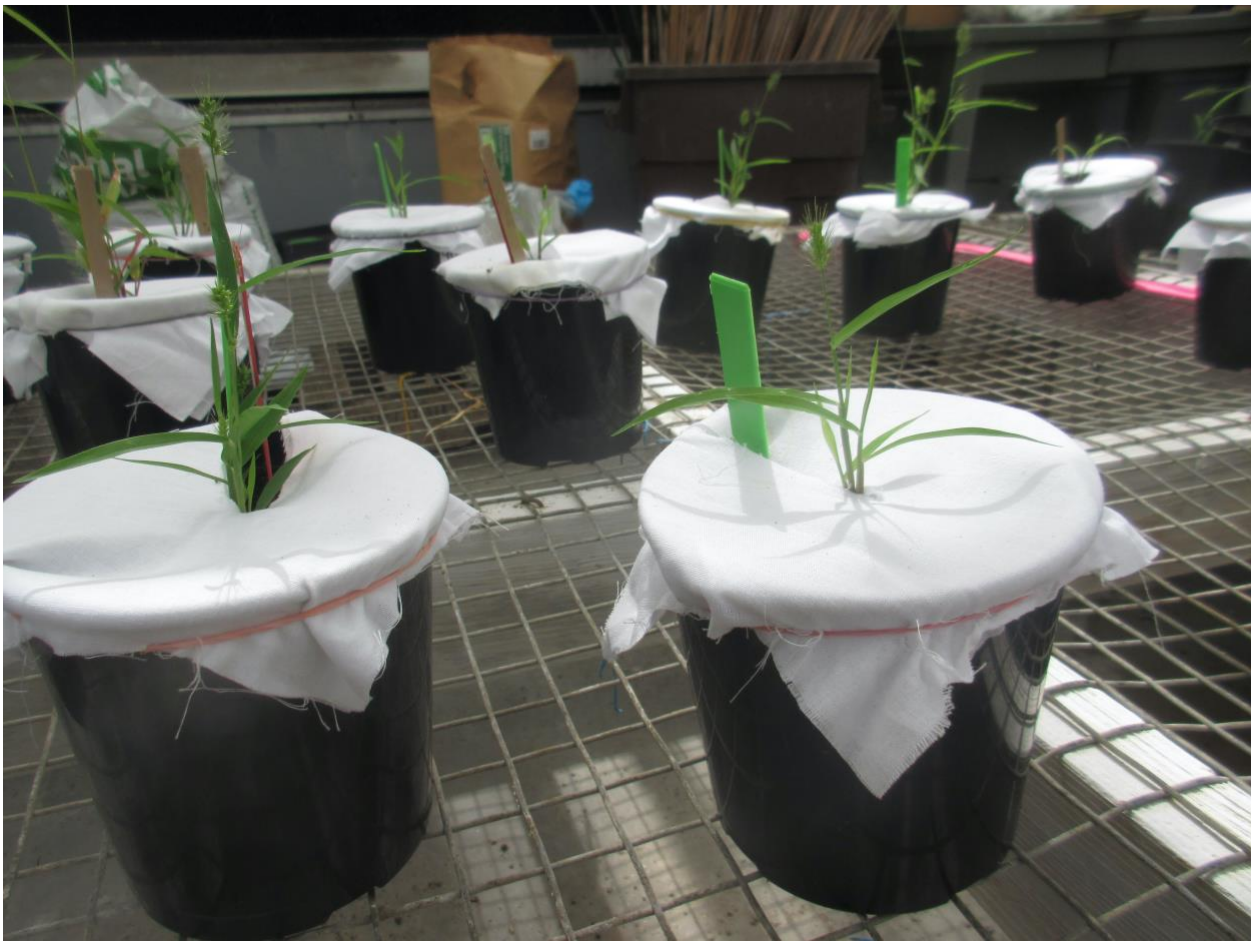
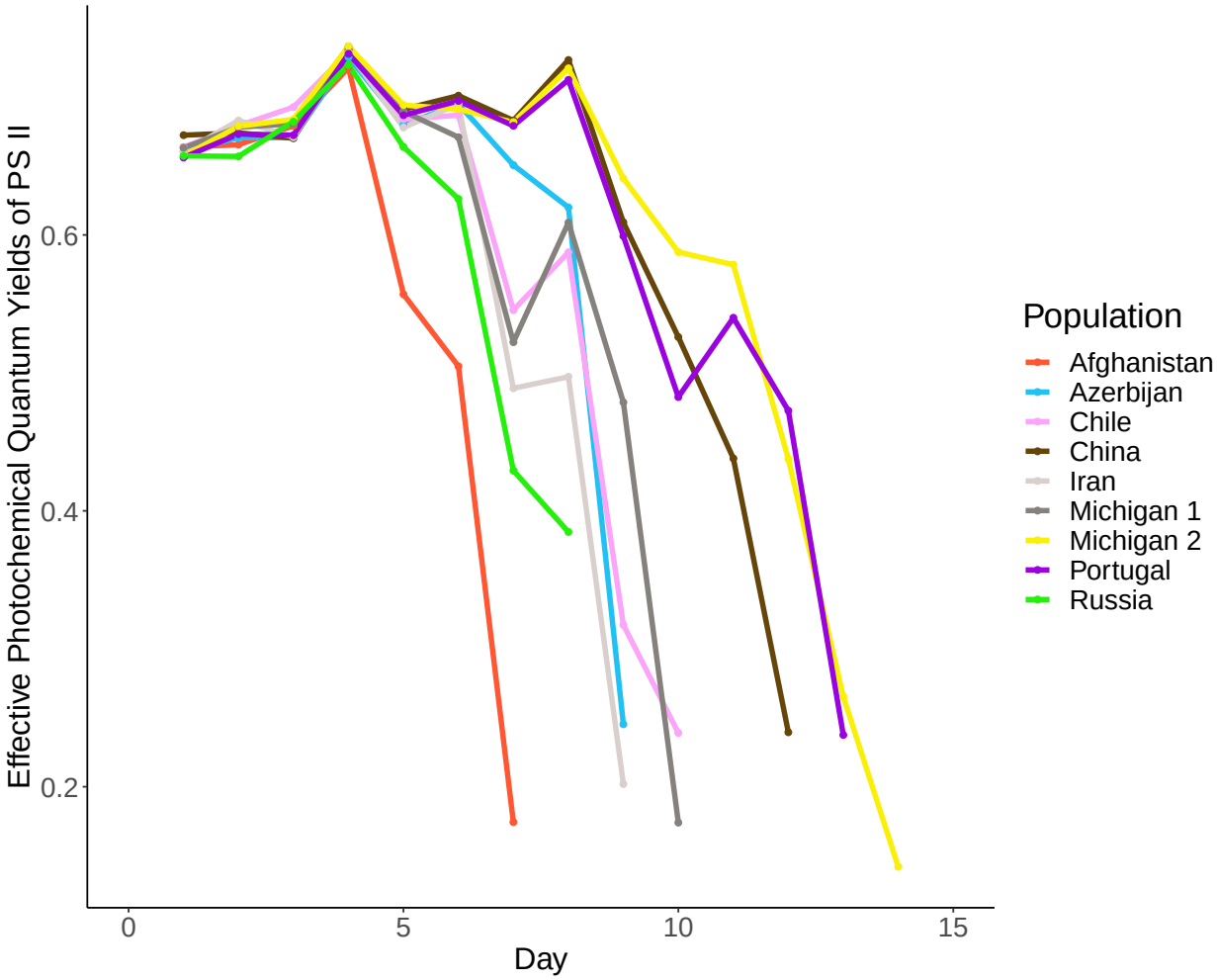
Appendix A - Supplementary materials from Chapter 2

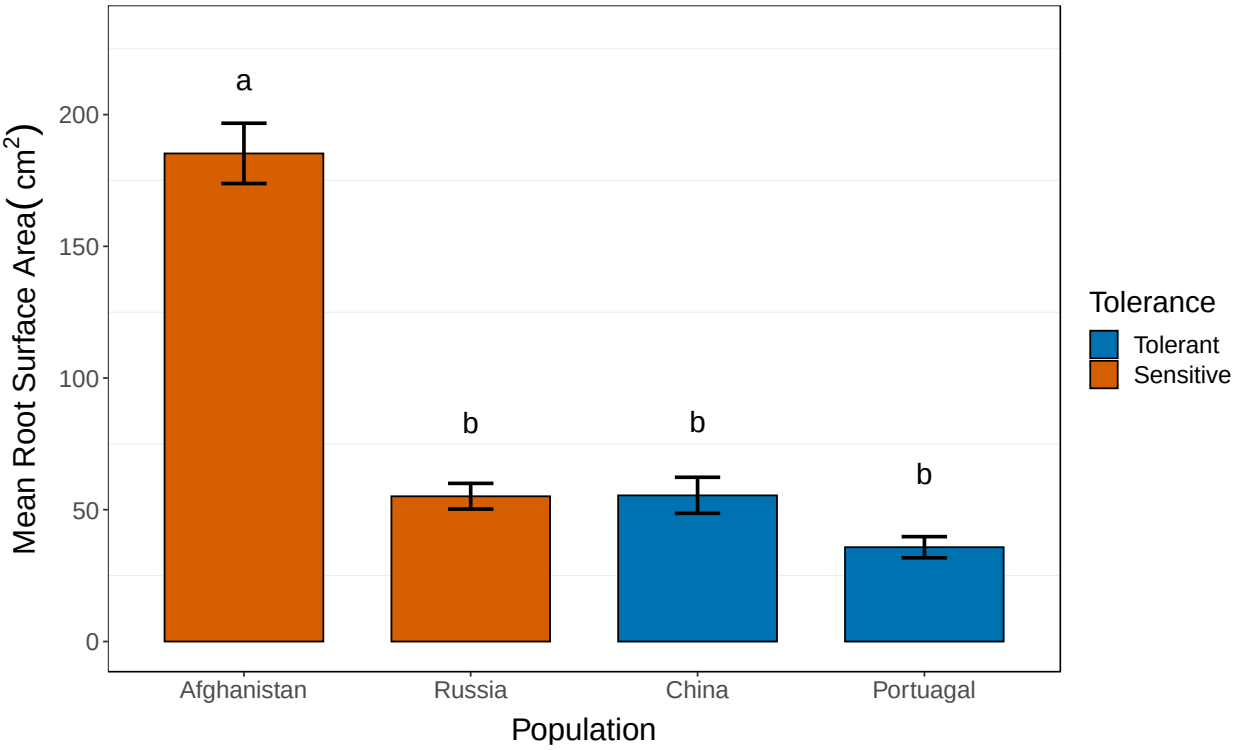
Supplementary Figures



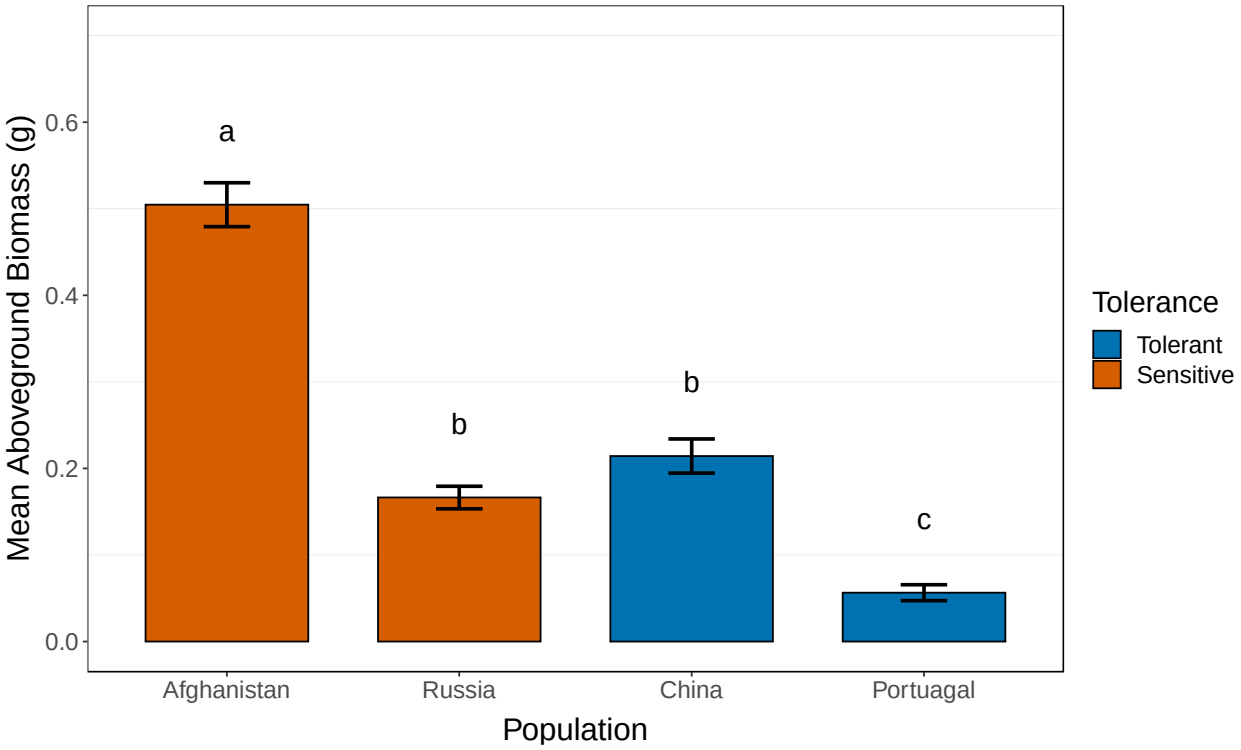
Appendix Figure A.1 : Pot set-up for dry down experiments. Two layers of white cotton fabric were used to cover the soil surface of experimental plants to reduce water evaporation off the soil surface.



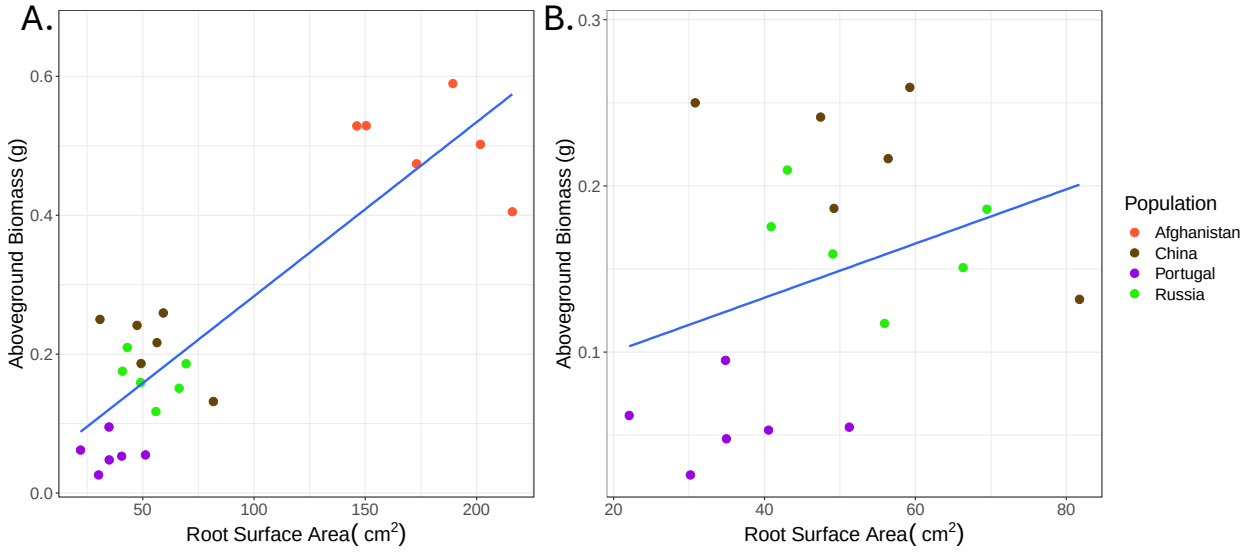
Appendix Figure A.2 : Effective photochemical quantum yields of Photosystem II compared across 9 populations of *Setaria viridis*. Points for each population for each day represent the average of all plants from that population which were still in the dry-down experiment (had not yet reached photosynthetic rate of less than 1 $\mu mol\ m^{-2}sec^{-1}$).



Appendix Figure A.3 : Comparison of root surface area between 4 *Setaria viridis* populations. Error bars represent one standard error.



Appendix Figure A.4 : Comparison of aboveground biomass between 4 *Setaria viridis* populations. Error bars represent one standard error.



Appendix Figure A.5 : Comparison between aboveground biomass and root surface area. Statistics were calculated from population averages while the figure shows individual observations. A. Comparison across all 4 *Setaria viridis* populations. B. Comparison across 3 *Setaria viridis* populations with the Afghanistan population removed. (Note that the x- and y-axes scales differ between the panels).

Appendix B - Supplementary materials for Chapter 3

Supplementary Tables

Appendix Table B.1: Complete list of overrepresented GO terms for all DEG lists based on a PANTHER statistical overrepresentation test. This table has been uploaded as a supplementary document due to its size.

Appendix Table B.2 : Biological function of DroughtDB genes uniquely aligned to upregulated DEGs across treatment conditions based on the BLAST analyses of DEGs upregulated at FDR 0.01. DDB genes that aligned with both up and downregulated DEGs across all three treatment conditions within a population were not included. Biological functions were obtained from the DDB.

Population	Treatment Condition	Regulation Change	Gene	ID	Biological Function
Chile	Chilling, Drought, Salinity	Up	CDPK7	LOC_Os03g03660	calcium-dependent protein kinase
Chile	Chilling, Drought, Salinity	Up	CIPK03	LOC_Os07g48760	calcineurin B-like protein-interacting protein kinase
Chile	Chilling, Drought, Salinity	Up	CIPK15	LOC_Os11g02240	calcineurin B-like protein-interacting protein kinase
Chile	Chilling, Drought, Salinity	Up	CPK21	AT4G04720	calcium-dependent protein kinase
Chile	Chilling, Drought, Salinity	Up	CPK23	AT4G04740	calcium-dependent protein kinase
Chile	Chilling, Drought, Salinity	Up	MRP4	AT2G47800	multidrug resistance-associated protein, ABC transporter
Chile	Chilling, Drought, Salinity	Up	MYB44	AT5G67300	MYB type TF

Chile	Chilling, Drought, Salinity	Up	NADP-ME	GRMZM2G085019	NADP-malic enzyme
Chile	Chilling, Drought, Salinity	Up	NPK1	Solyc01g079750	MAPKKK from tobacco; stress tolerance in tobacco
Chile	Chilling, Drought, Salinity	Up	OsMYB3R-2	LOC_Os01g62410	MYB TF
Chile	Chilling, Drought, Salinity	Up	OST1/SRK2E	AT4G33950	Kinase-like (open stomata 1), activated by ABA, activates SLAC1
Chile	Chilling, Drought, Salinity	Up	OST2	AT2G18960	plasma membrane proton ATPase
Chile	Chilling, Drought, Salinity	Up	SRK2C	AT1G78290	SNF1-related protein kinase 2, osmotic-stress-activated protein kinase
Chile	Chilling, Drought, Salinity	Up	ZmCPK4	GRMZM2G321239	Calcium-dependent protein kinases
China	Chilling, Drought, Salinity	Up	ABCG22	AT5G06530	ABC-transporter
China	Chilling, Drought, Salinity	Up	ABCG25	AT1G71960	ABC-transporter, ABA export from cells
China	Chilling, Drought, Salinity	Up	ABCG40	AT1G15520	ABC-transporter, ABA import
China	Chilling, Drought, Salinity	Up	ABF3	AT4G34000	ABA responsive element (ABRE) binding bZIP factor
China	Chilling, Drought, Salinity	Up	ABF4	AT3G19290	ABA responsive element (ABRE) binding bZIP factor
China	Chilling, Drought, Salinity	Up	ABI1	AT4G26080	PP2C

China	Chilling, Drought, Salinity	Up	ABI2	AT5G57050	PP2C
China	Chilling, Drought, Salinity	Up	AREB1	AT1G45249	bZIP TF, ABRE binding
China	Chilling, Drought, Salinity	Up	AtBG1	AT1G52400	beta-glucosidase; hydrolyzes glucose-conjugated, inactive ABA to active ABA
China	Chilling, Drought, Salinity	Up	FAR1	AT4G15090	FAR-RED IMPAIRED RESPONSE1, key positive transcription factors in the phytochrome A pathway
China	Chilling, Drought, Salinity	Up	FHY3	AT3G22170	FAR-RED ELONGATED HYPOCOTYL3, key positive transcription factors in the phytochrome A pathway
China	Chilling, Drought, Salinity	Up	HAB1	AT1G72770	PP2C
China	Chilling, Drought, Salinity	Up	OsbZIP23	LOC_Os02g52780	bZIP transcription factor
China	Chilling, Drought, Salinity	Up	OsbZIP72	LOC_Os09g28310	bZIP transcription factor
China	Chilling, Drought, Salinity	Up	PLD	Solyc06g068090	Phospholipase D, lipid degrading enzyme
China	Chilling, Drought, Salinity	Up	PP2C	GRMZM2G166035	PP2C

Appendix Table B.3 : Uniquely upregulated DEGs aligned with DDB genes from each pathway node in the DroughtDB (Alter et al. 2015). DroughtDB genes that matched with both up and down regulated DEGs for the same population and treatment condition were not included in the counts for each population. The remaining upregulated genes that had at least 1 BLAST hit (E-value cutoff: 10^{-20}) based on BLAST analyses of DEGs upregulated at FDR 0.01 in at least one of the three treatment conditions were counted for each pathway node.

Pathway Node	# China aligned, uniquely upregulated DEGs	# Chile aligned uniquely upregulated DEGs	Total DDB genes per node
Physiological Adaptation	28	35	79
Ion and Osmotic Homeostasis	16	29	53
Stomatal Movement	15	14	38
Growth Control	2	0	5
Root/Leaf Development	1	0	5
Detoxification	10	6	21
Osmolyte Production	6	2	11
Protection Factors	1	1	3
Removal of ROS	2	2	6
Molecular Adaptation	60	78	158
Functional Proteins	15	24	45
Protection Factors	1	1	3
LEA Proteins	1	1	3
Phospholipid Metabolism	2	1	6
Enzymes for Osmolyte Biosynthesis	6	1	10
Proteases	1	1	1
Detoxification Signaling	2	2	6
Channels and Transporters	3	17	19
Ion Channels	0	5	5
Anion Channel	0	3	3
Cation Channel	0	2	2
Transporters	3	4	5
ABA Importer	1	1	1
ABA Exporter	1	1	1
Water Channels	0	9	9
Regulatory Proteins	45	54	113
Gene Expression	28	32	72

Transcription Factors	29	33	62
AP2-Domain	6	3	9
bZIP	8	6	8
DREB	6	3	8
ERF	2	1	4
MYB	0	8	8
MYC	0	1	1
NAC	0	1	2
NF-Y	0	0	3
WRKY	0	2	3
Zinc Fingers	5	5	7
miRNA	0	1	4
Histone Modification	1	0	4
Chromatin	1	0	2
Post-translational Modification	0	0	3
Ubiquitin Ligases	0	0	2
Signal Transduction	7	13	24
Protein Kinases	1	11	14
Protein Phosphatases	5	1	6
Hormone Signaling	6	5	11
ABA Biosynthesis	4	2	4
ABA Degradation	0	1	1
ABA Signaling	1	0	4
Ethylene Biosynthesis	1	1	1
Acid Anhydride Hydrolases	1	2	4
All Genes	67	82	179