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The Effects Of Whole Genome Duplication On Functional Traits And Interactions With Arbuscular Mycorrhizal Fungi

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ABSTRACT

Whole genome duplication is one of the most pervasive forces driving plant evolution, yet modelling efforts on the establishment of polyploid plants have shown that early generation polyploids, or "neopolyploids", should experience high extinction rates due to a population demographic disadvantage. A major gap in understanding why established polyploids are so prevalent despite theoretically high extinction rates is that we know so little about the ecological consequences of neopolyploidy. For example, by investigating how neopolyploidy affects plant ecophysiology, or functional trait variation across environments, we can understand the ecological contexts that can promote polyploid establishment. Thus, a major goal of this dissertation was to understand how neopolyploidy affects plant ecophysiology and organismal performance. The first chapter tests the hypothesis that neopolyploids are more nutrient limited by comparing growth and leaf functional trait responses of diploids, neotetraploids, and established tetraploids of *Heuchera cylindrica* to different levels of nutrient supply. The results support the hypothesis that neopolyploidy increases growth-liming nutrient requirements, and this pattern was consistent across multiple independent origins of neopolyploidy, suggesting that increased nutrient limitation of neopolyploids is a consistent feature of whole genome duplication. The leaf functional traits and biomass allocation patterns of neotetraploids also showed that whole genome duplication induces a shift towards a more competitive or resource-acquisitive growth strategy. Although functional traits are key components of plant performance, we also lack information on the reproductive effects of neopolyploidy. Consequently, the second chapter examines the reproductive effects of neotetraploidy in a quick-growing annual. Specifically, I investigated how neopolyploidy and nutrient supply affects lifetime fitness in *Arabidopsis thaliana.* Similar to the first chapter, I found evidence that neotetraploidy increased

nutrient requirements in *A. thaliana.* Neotetraploids had greater lifetime fitness than diploids, but only in high nutrient environments, further supporting the idea that neopolyploidy causes a shift to a more competitive growth strategy. Although increased nutrient limitation is a major ecophysiological consequence of neopolyploidy, another long-hypothesized ecophysiological effect is that neopolyploidy improves tolerance to harsh abiotic environments. As such, in the third chapter I tested the hypothesis that neopolyploidy improves stress tolerance. Using diploids and synthetic neotetraploids of *H. cylindrica* and *A. thaliana,* I found that neotetraploid functional traits were less responsive to salt and drought stress than diploids, supporting the hypothesis that neopolyploidy enhances stress tolerance. As opposed to the first two chapters that found consistent effects of neopolyploidy across multiple independent origins, in this experiment I found that the effect of neopolyploidy on stress tolerance was largely dependent on the independent polyploid origin. The results from the first three chapters show that neopolyploidy can improve stress tolerance but increase nutritional needs, and one mechanistic way that polyploids can overcome increased nutrient needs is through mutualistic species interactions. Thus, the fourth chapter tests whether diploids and established tetraploids differ in colonization rates by arbuscular mycorrhizal fungi (AMF) that generally serve as nutrient-providing mutualists. From field-sampled roots, I not only found greater total colonization of AMF on tetraploids than diploids, but that tetraploids also had a greater colonization by arbuscules, the interface of nutrient exchange between the host plant and fungus. The results from chapter four support the conclusion of chapters 1 and 2, that neopolyploidy increases nutrient limitation. Taken together, the results from this dissertation show that physiological changes in neopolyploids can lead to immediate differences in performance across resource environments, and that certain environmental contexts may promote the odds of neopolyploid establishment.

THE EFFECTS OF WHOLE GENOME DUPLICATION ON FUNCTIONAL TRAITS AND INTERACTIONS WITH ARBUSCULAR MYCORRHIZAL FUNGI

by

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Chapter 1: Neopolyploidy in *Heuchera cylindrica* increases nutrient limitation and causes a shift in plant growth strategy

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Running Title: Neopolyploidy shifts plant growth strategy

Summary:

- Whole genome duplication frequently occurs within plant lineages and can cause a myriad of physiological changes. One proposed physiological consequence of neopolyploidy is that it elicits a greater requirement for growth limiting nutrients, but this hypothesis remains untested. Furthermore, we do not know how plant growth strategies associated with nutrient acquisition are affected by neopolyploidy. To address these gaps, we provide evidence of how neopolyploidy affects functional traits associated with plant nutrient limitation.
- We synthesized neotetraploid *Heuchera cylindrica,* an herbaceous perennial, and compared the growth and functional trait responses of diploids, neotetraploids, and naturally occurring tetraploids to nitrogen and phosphorus manipulations.
- We found strong support for the hypothesis that neopolyploidy causes increased nutrient limitation, as evidenced by the reduced productivity and increased concentrations of nitrogen and phosphorus in tissues of neotetraploids as compared to diploids. We also found evidence that multiple independent origins of neotetraploidy led to differing responses to nutrient supply, but neotetraploidy generally shifted functional traits toward a faster, more resource-inefficient growth strategy.
- Taken together, the results from this study suggest that neotetraploidy can cause shifts in plant growth strategies indicative of stronger nutrient limitation.

Keywords: Nutrient limitation; nitrate; phosphate; plant economics spectrum; Saxifragaceae; synthetic polyploid; whole genome duplication

Introduction

Polyploidy has been increasingly appreciated as a key driver of plant evolution, with recent work estimating that whole genome duplication (WGD) is associated with 15% of angiosperm speciation events, and that 35% of vascular plant species are recently formed polyploid lineages (Wood et al., 2009). Although established polyploids are commonly found in nature, first-generation polyploids (hereafter "neopolyploids") must overcome ecological barriers before they can form persistent populations. In particular, when neopolyploids arise, they are expected to suffer high extinction rates due to competitive interactions with their diploid parents as well as having to overcome a severe population bottleneck (Arrigo and Barker, 2012; Levin, 2019). Thus, understanding the abundance of established polyploid taxa despite ecological barriers to establishment is a major focus of ecological research on polyploid plants.

One of the primary hypothesized mechanisms by which neopolyploids may become established is through ecological niche segregation (Thompson and Lumaret, 1992; Ramsey and Schemske, 2002). To achieve a niche shift, however, WGD must first change the physiology of neopolyploids in a way that alters how they interact with their environment. Consequently, we need studies that profile the physiological changes that immediately follow WGD and how those changes can lead to novel plant-environment interactions. Indeed, there have been calls for work on neopolyploids (Ramsey and Schemske, 2002) and studies that investigate the immediate effects of polyploidy on ecophysiology (Soltis et al. 2016). Furthermore, studies that incorporate multiple maternal origins of neopolyploidy can reveal if functionally important traits vary with independent WGD events or if these traits are universally affected by polyploidy. By investigating traits that affect the growth and fitness of multiple lineages of neopolyploids, we

can begin to understand the ecological contexts that promote or hinder the ecological establishment of nascent polyploid lineages.

Although WGD-induced physiological divergence is expected to facilitate ecological niche segregation and promote polyploid establishment in the long term (Levin, 2019), these physiological changes may pose a greater challenge to the performance and survival of neopolyploid lineages in the near term. Specifically, whole-genome duplication is expected to increase nutrient requirements (Leitch and Bennett, 2004; Leitch and Leitch, 2008; Hessen et al., 2010; Guignard et al., 2017), thus limiting where and when neopolyploids can grow (Segraves and Anneberg, 2016). For example, increasing genomic and RNA content is hypothesized to correlate negatively with growth and reproductive effort in nutrient-limited environments because nitrogen and phosphorus will instead be used to construct additional DNA and RNA (Lewis, 1985; Hessen et al., 2010). Corroborating this idea is the observation that plant growth rates are negatively correlated with genome size, which is thought to be caused by longer cell division times associated with larger genome contents (Bennett, 1972). Consequently, the ability of neopolyploids to become established may be heavily influenced by the nutritional quality of the soil. Furthermore, experimental validation of how plant growth strategies are affected by neopolyploidy and nutrient supply rates are still needed, since plants with large genomes may respond more strongly to nutrient addition than plants with small genomes.

We have some empirical evidence supporting the hypothesis that WGD alters plant growth strategies and leads to increased nutrient limitation. For example, a nutrient addition study found that neotetraploids grew more in response to nutrient addition than diploids (Walczyk and Hersch-Green, 2019), suggesting that neotetraploidy increases resource limitation. Similarly, two grassland studies have shown that established polyploids have greater productivity

in response to increased nutrient supply than co-occurring, unrelated diploid species (Šmarda et al., 2013; Guignard et al., 2016). Polyploids with a competitive growth strategy were the primary functional group that outperformed diploids (Guignard et al., 2016). Together, these results lead to an interesting question about how plant functional traits are affected by within-lineage WGD. By investigating functional traits, or characteristics that affect growth, reproduction, and survival of a plant, we can ascertain whether WGD affects plant growth strategies. To address this question, we carried out a comparison of functional trait responses between diploids and their neopolyploid descendants to variation in nutrient supply to test if WGD *per se* affects nutrient limitation. By comparing the functional trait responses of diploids and their first-generation neopolyploid progeny, we are able to minimize the effect of selection and drift that have shaped the ecophysiology of established polyploids. For instance, we know that selection acts strongly to reduce genome size of neopolyploids after they arise (Leitch and Bennett, 2004; Cavalier-Smith, 2005; Dodsworth et al., 2016), thus potentially confounding the effect of WGD on plant growth strategies. Therefore, to investigate how WGD immediately affects plant growth strategies, we need to compare the functional trait responses of diploids and their neopolyploid progeny to variation in nutrient supply.

To investigate the immediate effects of neopolyploidy on plant nutrient limitation, we used the plant economics spectrum framework (Grime, 1977; Westoby, 1998; Reich, 2014; Diaz et al., 2016), which classifies plant functional traits along a spectrum of resource-acquisitiveness versus resource-conservatism (Wright et al., 2004). For example, Reich *et al.* (2014) distinguishes resource-acquisitive plants with a high relative growth rate as having a "fast" growth strategy, and resource-conservative plants with longer lived tissues having a "slow" strategy. Since there is evidence that polyploidy increases plant nutritional needs (Šmarda et al.,

2013; Guignard et al., 2016; Walczyk and Hersch-Green, 2019), we predicted that neopolyploidy will elicit greater investment into traits associated with faster resource acquisition and this will be coupled with a concomitant decrease in allocation to traits associated with resource conservation. By extension of this prediction, we expected that neopolyploidy causes plants to shift towards a fast growth strategy.

Here we test how neopolyploidy in *Heuchera cylindrica* (Saxifragaceae) affects traits associated with resource acquisition and conservation. *Heuchera cylindrica* is an herbaceous perennial with diploid and autotetraploid populations that grow in close proximity in the Pacific Northwest of North America (Godsoe et al., 2013). The low relative growth rate of *Heuchera*, coupled with the formation of a rhizomatous storage organ, typifies this plant as a 'slow' plant under the framework of Reich (2014). This slow growth strategy of *Heuchera* allows us to not only investigate the effect of neopolyploidy on resource acquisition, but also the effect on resource storage, making it an excellent study system to examine the effect of neopolyploidy on plant nutrient limitation. In this study, we asked three questions: 1) Does neopolyploidy increase plant nutritional needs? 2) How do neopolyploid functional trait responses to nutrient supply differ from diploid and established polyploids? 3) Are the effects of neopolyploidy consistent across multiple independent origins?

Materials and Methods

Study organism and growth conditions

Seed stocks of diploids and established polyploids of *H. cylindrica* were produced from plants that had been previously collected from six field sites (Fig. 1). These field sites are

geographically adjacent to one another within a contact zone in Idaho (Table S1; See Anneberg & Segraves (2019) for a description of site characteristics). The seed stocks were generated by hand-pollinating among flowering plants collected from the same field site. The majority of the developing seeds from the diploids were allowed to fully mature, but a subset of the developing seeds were treated to induce neopolyploidy as described below.

For the experiment, seeds were germinated in a nursery tray filled with autoclaved quartz sand in a growth chamber set to a 12:12 L:D cycle, with a 14ºC daytime temperature and a 10ºC nighttime temperature. Once the seedlings had their first true leaves, we transplanted them into 311.5 cm^3 (7 cm x 7cm x 6.35cm) pots filled with autoclaved sand. We transplanted one plant per pot to avoid competitive effects. Transplants were moved to a greenhouse room set to 21–24ºC daytime and 15–18ºC nighttime with a supplemental 16:8 L:D cycle. Although the use of sand as a potting medium is disparate from natural edaphic conditions including biotic interactions such as with arbuscular mycorrhizal fungi, our reductionist approach allowed us to control the concentration of nutrient supply to plants. The seeds for the experiment were germinated in August 2019, transplanted into pots in later that month, and the experiment was harvested in late December 2019.

Induction and confirmation of neopolyploidy

To induce neotetraploidy in *H. cylindrica*, we treated the developing embryos from diploid maternal plants with nitrous oxide gas. Following a method that uses pressurized nitrous oxide gas to arrest the first mitotic division of zygotes (Ostergren, 1957; Dhooghe et al., 2011; Wongprichachan et al., 2013), we waited until just prior to the first zygotic division (~37 – 42 h post-pollination in *H. cylindrica*) before placing the plants in 620.53 kPa nitrous oxide gas for 24

hours (Dvorak et al., 1973). Plants were returned to the greenhouse to set seed. We collected putatively neotetraploid seeds from these plants, and then verified the cytotype on a small subset of the seeds from each maternal plant using the flow cytometry methods of Godsoe et al. (2013). In total, we produced seed stocks representing 12 independent origins of neotetraploidy. To confirm that nitrous oxide treatment did not confound the cytotype-specific patterns observed in this study, we included 12 seed stocks that had received nitrous oxide treatment but failed to become neotetraploid.

To avoid the confounding effect of selection after WGD, we used first-generation neotetraploid seeds, rather than second generation seed stocks with verified ploidy. As a result, the neotetraploid seed stocks contained a mixture of neotetraploid and diploid (failed induction) seeds. Therefore, to verify the cytotype of all experimental plants, we conducted a qualitative survey of four traits that are predictive of cytotype in *Heuchera.* The qualitative survey consisted of a binary scoring matrix (0 or 1) based on either having flat or curled leaves, thin or thick leaves, thin or thick leaf petioles, and having symmetrically straight or whorled lobes at the base of leaves. Traits that correlated with tetraploidy received a 1, and traits that correlated with diploidy received a 0. For each plant, we took the sum of the scores from all four traits, with possible scores ranging from zero to four (Appendix S1). We cross-validated that the survey scores correlated with plant cytotype by using flow cytometry to determine ploidy of 317 of the experimental plants one week prior to harvest. To account for the removal of material from these plants, the removed leaf tissue was weighed and later added to the estimate of biomass.

Experimental Design

We investigated how WGD and nutrient supply rate affect *H. cylindrica* performance by growing plants at three nitrogen and phosphorus fertilization levels. The control nutrient concentrations of nitrogen and phosphorus were defined by their respective average plant-available concentrations from previously sampled field sites (Anneberg and Segraves 2019). The concentration of phosphorus in the control treatment was adjusted slightly higher to attain a molar nitrogen to phosphorus ratio of 16, a value considered optimally co-limiting for terrestrial plant growth (Gusewell, 2004). We either quartered or quadrupled the control concentrations of nitrogen and phosphorus to arrive at the low and high nutrient treatment levels, respectively. We supplied fertilizers in the form of a modified Hoagland's solution with adjusted nitrogen and phosphorus concentrations (Table S2), and adjusted the pH to 5.5 – 5.75 to ensure proper nutrient availability to plants (Argo and Fisher, 2002). We watered the plants by adding 1.5 L of nutrient solution to each bottom-watering tray once per week. After a four day dry-down, the plants were watered again with 1.5 L de-ionized water.

We grew 40 maternal lines across three nutrient treatments with four replicates per treatment level, with each bottom-watering tray (20 plants each) treated as a block. Of the 40 maternal lines, we included four naturally occurring established autotetraploid maternal lines from two populations to compare the performance of neotetraploids to established tetraploids. Consequently, we have four levels within cytotype: diploids, neotetraploids, nitrous oxide treated diploids (failed inductions), and established tetraploids. Of these four cytotypes, we included 12 neotetraploid, 12 diploid, 12 nitrous oxide-treated diploid, and four established tetraploid maternal lines, for a total of 40 maternal lines. Because of uncertainty in the identity of the neotetraploids, when possible, we planted additional replicates of these plants to improve statistical power. In total, we grew 522 plants in the experiment.

Functional Trait Measurements

We estimated plant performance by measuring ten functional traits associated with resource acquisition and conservation: total biomass, percent aboveground biomass, percent fine root mass, percent rhizome mass, leaf dry matter content (LDMC), specific leaf area (SLA), leaf chlorophyll, average fine root diameter, tissue nitrogen concentration, and tissue phosphorus concentration. We chose to measure SLA since it embodies the efficiency of leaf carbon capture and correlates positively with relative growth rate (Poorter and Remkes, 1990; Reich et al., 1992), whereas LDMC measures structural carbon investment and correlates positively with leaf lifespan (Wilson et al., 1999). Thus, plants that display low SLA and high LDMC typify a resource-conservative strategy, whereas high SLA and low LDMC suggests a faster, more resource-acquisitive growth strategy. During harvest, two or three leaves per plant were collected for individual measurements of chlorophyll fluorescence, SLA, and LDMC. We measured SLA by taking digital scans of the fresh leaves, which were used to determine the area of each leaf with ImageJ (Schneider et al., 2012), and then SLA was calculated by dividing the fresh leaf area by its dry mass. To calculate LDMC, we divided the dry mass of a leaf by its fresh mass. We controlled for leaf moisture content variation by measuring fresh leaf mass within three hours of collection from a well-watered plant for all collected leaves in this study. We report the average leaf-level measure for each plant.

The aboveground dry biomass of plants was measured by adding the dry mass of harvested leaves to the remainder of the dried aboveground biomass. We additionally calculated a linear regression between leaf fresh mass and leaf dry mass for each of the 40 maternal lines and used the best fit line to estimate the dry biomass of leaves that were used during the flow

cytometry procedure. Briefly, we weighed each leaf prior to chopping for flow cytometry and estimated its dry mass by fitting the fresh weight to the best fit line equation. The estimated dry biomass from each fresh leaf that was used for flow cytometry was added to the total aboveground biomass measure.

The investment into belowground biomass and root traits was determined by separating the aboveground and belowground biomass during harvest. We ensured that all root materials were recovered from the pots at the time of harvest by gently flipping each well-watered plant in a pot upside down and removing the pot before gently lowering the entire plant and the attached sand substrate into a tank of deionized water to carefully shake off the sand. Fine root diameter was then measured on a subsample of plants by collecting two intact, first-order fine roots per plant. The root systems were scanned and measured with WinRhizo (Regen Instrument Inc., Quebec, Canada) and fine root diameters were averaged per plant. Average fine root diameter was used as an indicator of how efficiently a root may probe the soil for limiting nutrients without any biotic interactions that facilitate nutrient uptake (e.g. arbuscular mycorrhizal fungi), such that a plant with small diameter roots is more efficient at acquiring soil nutrients than thick roots. Because of time constraints, we were only able to collect root subsamples for 76 plants. These subsampled roots were included in the belowground dry biomass per plant. To discriminate between the biomass investment into storage versus fine roots, we separately weighed the dry biomass of the rhizome and fine roots of each plant.

To assess how cytotype and nutrient treatment affected the investment of nitrogen and phosphorus into tissues, we measured the concentration of both nutrients from a subsample of the plants. To do so, we milled the dried aboveground biomass and fine roots of plants with a Wiley Minimill (Thomas Scientific, Swedesboro, NJ, USA). We then measured tissue

phosphorus concentrations using a colorimetric staining approach (Ohno and Zibilske, 1991). Briefly, 30 mg of milled tissue was ashed in a muffle furnace and acidified with 6M hydrochloric acid. The acidified solutions were diluted with deionized water to 10% (v/v) HCl, and 200 µl of each sample was stained with malachite green (Rao et al., 1997). The intensity of staining was measured with a BioRad Smart Spec spectrophotometer (Thomas Scientific, Swedesboro, NJ, USA) at 630 nm. The phosphorus concentration in the stained solutions was calculated from the linear equation of standard solutions of known phosphorus concentration. To determine the concentration of nitrogen in tissues, we used a NC 2100 autoanalyzer (CE Elantech, Lakewood, NJ, USA). For samples in which we quantified carbon and nitrogen concentrations, approximately 2.5 mg of milled tissue was weighed into tins and the concentration of nitrogen was measured through an oxidation-reduction reaction coupled with a direct measurement of thermal conductivity change. In total, we measured tissue nitrogen from 224 plants, and we were only able to measure tissue phosphorus from 167 of the plants because of the more time-consuming procedure.

Statistical Analyses

We controlled for intrinsic size differences among plants when reporting on biomass allocation to different tissues (Osnas et al., 2018). Mass-based normalizations were carried out by dividing the dry mass of a tissue (i.e., aboveground, rhizome, and fine roots) by the corresponding total dry mass of the entire plant. Linear mixed models were fitted to each functional trait with nutrient treatment, cytotype, and their interaction as fixed effects and the site of maternal origin as a random effect. We first tested if nitrous oxide had an effect on the functional traits by fitting linear mixed models to the data from untreated diploids and nitrous

oxide treated diploids, and a two-way ANOVA was used to assess the statistical significance of the main effects. We then determined how neotetraploidy affected plant functional traits across nutrient environments by fitting linear mixed models to the data from diploids, neotetraploids, and established tetraploids. For each two-way ANOVA, we also tested how each group differed from one another by carrying out a Tukey's HSD post hoc analysis. We used R software (R Core Team, 2019) for all statistical analyses. The linear mixed models were constructed with the lme4 package (Bates et al., 2015) and the two-way ANOVA tests were carried out with the lmerTest package (Kuznetsova et al., 2017).

We also tested how multiple independent origins of neotetraploidy affected plant responses to nutrient treatments using linear mixed models. We used population of origin rather than each of the 12 maternal lines because our qualitative morphological survey and flow cytometry results revealed that we had unequal sample sizes or missing values for some of the neotetraploid maternal lines across nutrient treatments. We thus constructed linear mixed models as outlined above, but also incorporated the population of maternal origin as a main effect along with the main effects of nutrient treatment and cytotype. By necessity, we excluded the established tetraploids from this analysis since they were from non-overlapping populations with the diploids and neotetraploids. The diploids and neotetraploids used in this analysis comprised four independent populations of origin.

Results

Validation of plant cytotype

Flow cytometry confirmed that 86 plants were neotetraploids. All neotetraploids subjected to flow cytometry scored as either a three or four in the qualitative morphological survey. In contrast, the diploids and nitrous oxide-treated diploids had scores ranging from zero to two (Appendix S1). We used these scores to assign a cytotype to each plant that was treated with nitrous oxide, where plants with a score of 3-4 were assigned as neotetraploid, and a score of 0-2 assigned as a diploid. This resulted in 6 of 216 nitrous oxide treated 'diploid' plants being reassigned as neotetraploids, and 109 of 189 putatively neotetraploid plants being reassigned as nitrous oxide treated diploids.

Effect of nitrous oxide treatment

The comparison between untreated diploids and nitrous oxide-treated diploids revealed that there was no effect of nitrous oxide treatment on any of the traits except for total biomass (Table S3). There was a significant interaction between cytotype and nutrient treatment on total biomass production. Nitrous oxide treatment caused a slight increase in total biomass with high nutrient treatment (Fig. S1).

Biomass

In comparison to diploids and established tetraploids, the neotetraploids were less productive, but only when treated with high nutrients (Fig. 2A; Table 1). The relative biomass allocation pattern of neotetraploids also significantly differed from diploids and established tetraploids because they allocated more biomass into aboveground tissues with a concomitant reduction in rhizome tissue (Fig. 2B; Fig. 2D). However, the cytotypes did not differ in relative allocation to fine roots.

Functional traits

There was a significant cytotype by nutrient treatment interaction for SLA, with neotetraploids having significantly lower SLA than diploids and established tetraploids in the high nutrient treatment, but there was no difference in SLA among cytotypes in the control and low nutrient treatments (Table 1). Although cytotype and nutrient treatment did not interact for LDMC, neotetraploids had lower LDMC compared to diploids and established tetraploids, and LDMC decreased as nutrient treatment increased (Fig. 3A). For leaf chlorophyll content, the interaction between nutrient treatment and cytotype was driven by the established tetraploids differing from the diploids and neotetraploids (Table 1). For fine root diameters, neotetraploidy caused significantly larger diameter roots, and established tetraploids did not statistically differ from either diploids or neotetraploids in root diameter (Fig. 3B).

Nitrogen and phosphorus content

There was a positive relationship between aboveground tissue nutrient concentration and nutrient supply (Table 2). Although neotetraploids had higher nitrogen concentrations than diploids and established tetraploids (Fig. 4A), there was no effect of cytotype on aboveground phosphorus concentrations (Fig. 4B). Belowground tissue nitrogen and phosphorus concentrations increased with increasing nutrient supply, and neotetraploids had a higher concentration of both nutrients in the high nutrient treatment (Table 2; Fig. 4C; Fig. 4D).

Multiple origins of neopolyploidy

The effect of multiple origins of neotetraploidy was evident from the interaction term between cytotype and population of origin in the lme models. Population of maternal origin significantly interacted with cytotype for leaf chlorophyll contents and had a marginally significant interaction effect for SLA (Fig. 5; Table S4). There was no effect of multiple origins on the remaining traits (Table S4).

Discussion

Whole genome duplication is a frequent and recurring process in plants and has long been recognized as a driver of phenotypic differentiation (Soltis and Soltis, 1999; Ramsey and Schemske, 2002). Only a handful of studies, however, have investigated the consequences of neopolyploidy on plant ecophysiology (Maherali et al., 2009; Ramsey, 2011; Husband et al., 2016; Van Drunen and Husband, 2018; Walczyk and Hersch-Green, 2019). A key ecophysiological consequence expected in neopolyploids is increased nutrient limitation (Leitch and Leitch, 2008; Hessen et al., 2010; Guignard et al., 2017), but we have yet to assess how functional traits that are affected by resource limitation are also affected by neopolyploidy. Here we provide strong support for the hypothesis that WGD causes a greater need for growth limiting nutrients. Specifically, we show that neopolyploidy causes a reduction in total biomass production while at the same time causing an increase in tissue nutrient concentrations. We further show how leaf functional traits associated with resource use efficiency and carbon capture were simultaneously affected by polyploidy and nutrient treatment.

The strongest support for the hypothesis that WGD causes greater nutrient limitation comes from the observed increase in tissue nutrient concentrations and the associated reduction of growth in neotetraploids as compared to their diploid parents. Although neotetraploidy

increased aboveground tissue nitrogen, we did not observe a corresponding increase in aboveground phosphorus concentrations. This is unsurprising since neotetraploids displayed stronger carbon limitation phenotypes, and carbon limited plants often allocate more nitrogen aboveground to increase photosynthetic rate (Field, 1983). The lack of effect of neotetraploidy on aboveground phosphorus content, however, is probably due to the strong plasticity of plant nutrient concentrations in leaves and stems (Sterner and Elser, 2002). An alternative and non-mutually exclusive hypothesis is that aboveground tissues of neotetraploids did not have greater phosphorus content than diploids because they may have plastically reduced RNA concentrations in tissues, since RNA is a chief constituent of plant phosphorus pools and is positively correlated with growth rates (Sterner and Elser, 2002). Although plants have a high degree of plasticity over tissue nutrient concentrations, fine roots have one of the highest degrees of homeostatic control over nitrogen and phosphorus contents (Sterner and Elser, 2002) and are therefore a good indicator of how neotetraploidy affects the nutrient construction cost of tissues. Because neotetraploidy caused fine roots to have higher nitrogen and phosphorus concentrations and caused a corresponding decrease in total biomass production, we conclude that neotetraploidy led to increased nitrogen and phosphorus limitation.

Across the functional traits measured, we found multiple lines of evidence that neotetraploidy increased plant carbon limitation. First, despite low nitrogen and phosphorus supply, neotetraploids consistently allocated a greater proportion of biomass to aboveground tissues and less into rhizome storage than either diploids or established tetraploids (Fig. 2), demonstrating that neotetraploidy caused a diminished carbon budget. Second, neotetraploids had lower LDMC than diploids and established tetraploids, and since LDMC correlates positively with leaf lifespan (Wilson et al., 1999), the decreased LDMC of neotetraploids

suggests that WGD causes a more carbon-inefficient and fast growth strategy. Lastly, high nutrient supply increased SLA for diploids and established tetraploids, but not for neotetraploids. Within the SLA measurement, neotetraploid leaf area positively correlated with nutrient supply, but the correspondingly greater increase in neotetraploid leaf dry mass than the relative gain in leaf area caused neotetraploid leaves to have lower SLA than diploid leaves. Therefore, the greater biomass investment to construct neotetraploid leaves further shows that neotetraploid tissues are inherently more costly to build and reduces the total return on leaf carbon investment.

Along with stronger nutrient limitation in neotetraploids, we also observed that neotetraploidy in *H. cylindrica* led to changes in functional traits, indicating a shift in growth strategy. Using the fast and slow economics spectrum put forth by Reich (2014), we generally found that neotetraploidy caused functional traits to shift towards a faster growth strategy, meaning that functional traits became less conservative of resources. The observation that neotetraploidy led to a more carbon-limited growth strategy is apparent from their increased allocation to aboveground biomass, lower SLA, and lower LDMC. A faster growth strategy may be maladaptive for *H. cylindrica,* since it is a perennial plant that must overwinter and generate early spring tissues by drawing on stored carbon reserves from the rhizome (Soltis, 2007). One of the greatest challenges of shifting to a faster growth strategy for neotetraploid *H. cylindrica*, then, may be that the increased biomass allocation into aboveground growth comes at the cost of investing biomass into storage (Fig. 2). In addition to a reduced capacity to invest in storage, there were other physiologically costly features of neotetraploidy. Specifically, neotetraploidy increased the concentrations of growth-limiting nutrients in tissues. Since neotetraploidy caused a shift towards a more nutrient-acquisitive phenotype, this was apparently insufficient to overcome the added cost of allocating more nitrogen and phosphorus into tissues because

neotetraploidy also resulted in reduced total biomass production. Furthermore, neotetraploidy caused fine roots to become thicker (Fig. 3B), suggesting they are less effective at probing fine pore spaces in the soil for limiting mineral nutrients (Eissenstat, 1992). The finding that neotetraploid roots are less able to acquire nutrients than diploids comes with the caveat that we did not test how biotic interactions can affect nutrient acquisition abilities. Future studies that inoculate diploids and neotetraploids with belowground nutritional mutualists such as arbuscular mycorrhizal fungi will reveal how biotic interactions that facilitate nutrient uptake are affected by neopolyploidy. Together, these results suggest that neotetraploidy causes functional traits to become less efficient and shift plants towards a more resource-intensive growth strategy.

In addition to finding that cytotype and nutrient supply affected functional traits, there was also marked trait variation associated with multiple independent origins of neotetraploidy. We found that the site of neotetraploid origin had a significant effect on SLA and leaf chlorophyll content (Fig. 5; Fig. S2). Plant responses to neotetraploidy for leaf chlorophyll contents were contingent on the population of maternal origin, where neotetraploids from Palouse had increased chlorophyll content compared to their diploid parents, but there was no difference between diploids and their neotetraploid offspring from the other three sites. This pattern was perhaps driven by site-specific environmental variation, since the Palouse site is more exposed than the other field sites (TJA, personal observation). Therefore, the diploids from Palouse may have experienced differential selection pressure than the other sites of maternal origin for leaf chlorophyll content. The fact that there was a strong effect of independent origin on the neotetraploid trait responses *H. cylindrica* underscores how multiple origins can bolster the standing phenotypic variation of nascent neotetraploid populations and thereby improve the odds of establishment.

Since site of origin did not affect total biomass production, relative biomass allocation strategy, LDMC, and tissue nutrient concentrations (Fig. 5), neotetraploidy likely has a universal effect on these traits. Across repeated origins, the majority of the measured traits responded the same way to WGD, suggesting that a shift to a more resource-intensive growth strategy is a feature of neopolyploidy in *H. cylindrica*. Indeed, studies have provided similar evidence of traits that are invariant across repeated maternal origins of neopolyploidy (Soltis and Soltis, 1999; Pacey et al., 2020; Wei et al., 2020). In this study, we observed that multiple origins of neotetraploidy caused a consistent reduction in growth rate as well as increased nutrient content of tissues. For perennial plants such as *H. cylindrica,* growth rate and survival are the most deterministic traits correlated with fitness (Silvertown et al. 1993), and thus the increased nutrient limitation caused by neopolyploidy would likely drive a higher extinction risk for neopolyploid populations. The consistently reduced growth and more nutritionally intensive tissues across all of the sites of origin of *H. cylindrica* suggests that increased nutrient limitation is a direct consequence of WGD.

Although we used multiple synthetic origins of neotetraploidy to show the direct effects of WGD on nutrient limitation, our neotetraploids may not resemble natural neotetraploids for two reasons. First, the nitrous oxide gas treatment may directly affect the phenotype; however, this seems unlikely because the nitrous oxide-treated diploids consistently resembled the untreated diploids across nutrient treatments. The only effect of nitrous oxide treatment detected was that it caused greater total biomass in the high nutrient treatment. Because the nitrous oxide-treated plants were obtained primarily from genetically distinct maternal diploids, the effect of nitrous oxide may be a feature of the experimental design rather than an intrinsic treatment effect. Either way, because neotetraploids responded in the opposite direction to nitrous oxide-

treated diploids indicates that our estimate of the effect of neotetraploidy on biomass is conservative. Second, natural autopolyploids are expected to have increased heterozygosity due to having four possible alleles per genetic locus (Levin, 2002), but our synthetic neotetraploids have only two alleles possible per locus since WGD was induced post-fertilization. Because of this, we expect that these neotetraploids represent a conservative estimate of the effect of WGD on plant responses to nutrient supply.

In conclusion, we show that neopolyploidy can cause an abrupt shift in the growth strategy of a species, yielding a better understanding of the ecological contexts that may drive neopolyploid establishment. A shift to a more resource intensive growth strategy after WGD may be disadvantageous for plants that are preadapted to stressful habitats that select for slow growth (Grime, 1977; Reich, 2014), whereas a faster growth strategy may be selectively beneficial for neopolyploids growing in competitive environments, such as grasslands (Šmarda et al., 2013; Guignard et al., 2016). This study has uniquely shown that increased nutrient limitation due to neopolyploidy is followed by an immediate shift in functional traits associated with resource acquisition and storage, indicating that the nutrient environment in which diploids undergo WGD can have a dramatic effect on the performance of their neopolyploid progeny.

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Author Contribution:

TJA and KAS conceptualized and designed the experiment. TJA performed the experiment and analyzed the data. TJA and KAS drafted the manuscript.

Tables and Figures

Table 1: ANOVA table summarizing the linear mixed models of biomass traits of diploids, neotetraploids and established tetraploids of *Heuchera cylindrica*. Statistical significance is denoted with p-values in bold.

Trait	Fixed Effect	df (treatment, error)	F	P
Total Biomass	Treatment	2, 257	490.434	**<0.001**
	Cytotype	2, 33	17.957	**<0.001**
	Treatment x Cytotype	4, 257	3.404	**0.010**
Aboveground Biomass	Treatment	2, 253	79.714	**<0.001**
	Cytotype	2, 8	11.812	**0.004**
	Treatment x Cytotype	4, 253	2.011	0.093
Rhizome Biomass	Treatment	2, 252	46.512	**<0.001**
	Cytotype	2, 10	28.117	**<0.001**
	Treatment x Cytotype	4, 252	2.513	**0.042**
Fine Root Biomass	Treatment	2, 253	31.776	**<0.001**
	Cytotype	2, 15	5.201	**0.0192**
	Treatment x Cytotype	4, 253	1.680	0.155
Fine Root Diameter	Treatment	2, 34	4.540	**0.018**
	Cytotype	2, 5	32.587	**0.002**
	Treatment x Cytotype	4, 34	2.506	0.060
Average Chlorophyll	Treatment	2, 243	68.592	**<0.001**
	Cytotype	2, 6	0.355	0.714
	Treatment x Cytotype	4, 243	4.455	**0.002**
SLA	Treatment	2, 247	23.529	**<0.001**
	Cytotype	2, 6	9.236	**0.013**
	Treatment x Cytotype	4, 247	3.572	**0.007**

LDMC	Treatment	2, 248	133.275	**<0.001**
	Cytotype	2, 10	15.332	**0.001**
	Treatment x Cytotype	4, 248	1.809	0.128

Table 2: ANOVA table summarizing the linear mixed models associated with tissue nitrogen and phosphorus concentrations in diploids, neotetraploids and established tetraploids of *Heuchera cylindrica*. Statistical significance is denoted with p-values in bold.

Trait	Fixed Effect	df (treatment, error)	F	P
Aboveground N	Treatment	2, 113	40.749	**<0.001**
	Cytotype	2, 15	5.520	**0.017**
	Treatment x Cytotype	4, 113	2.364	0.057
Aboveground P	Treatment	2, 75	122.237	**<0.001**
	Cytotype	2, 20	0.258	0.775
	Treatment x Cytotype	4, 75	1.535	0.201
Belowground N	Treatment	2, 124	78.356	**<0.001**
	Cytotype	2, 124	7.191	**0.001**
	Treatment x Cytotype	4, 124	2.372	0.056
Belowground P	Treatment	2, 56	81.174	**<0.001**
	Cytotype	2, 56	4.793	**0.012**
	Treatment x Cytotype	4, 56	2.218	0.079

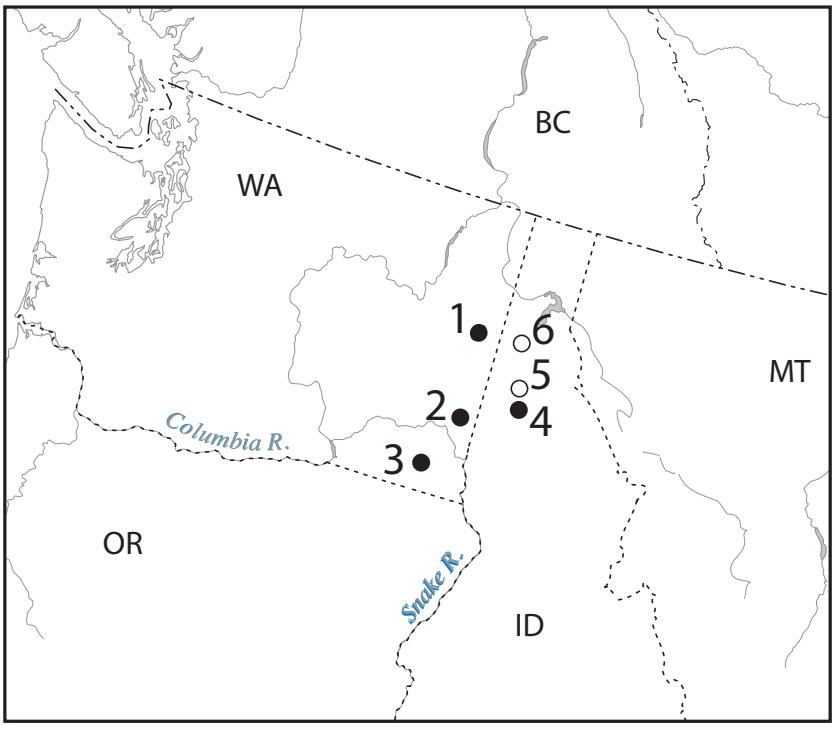

Figure 1: Map of collection sites for maternal diploid and established tetraploid seed stock of *Heuchera cylindrica* used in this study. Closed circles are diploid sites and open circles are established tetraploid sites. Numbers associated with each field site are associated with corresponding site info on Table S1.

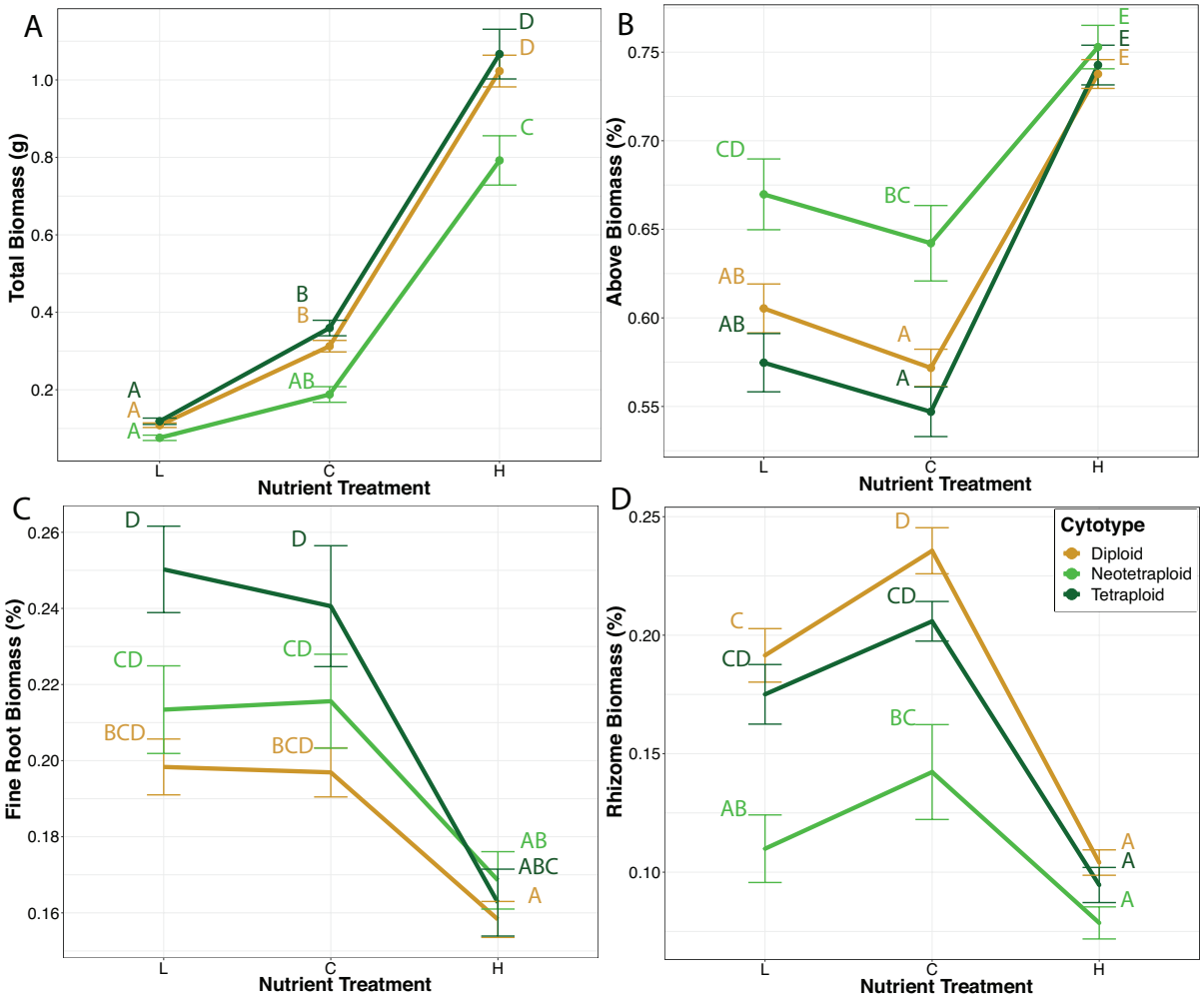

Figure 2: Mean and standard errors for biomass traits of diploids, neotetraploids, and established tetraploids to nutrient manipulation in *Heuchera cylindrica*. A) the mean total biomass response, B) the proportion of biomass allocated to aboveground tissues, C) the proportion of biomass allocated to fine roots, and D) the proportion of biomass allocated as storage rhizome. “L”, “C”, and “H” represent the low, control, and high nutrient treatments, respectively. Letters denote significance of post hoc tests comparing all means.

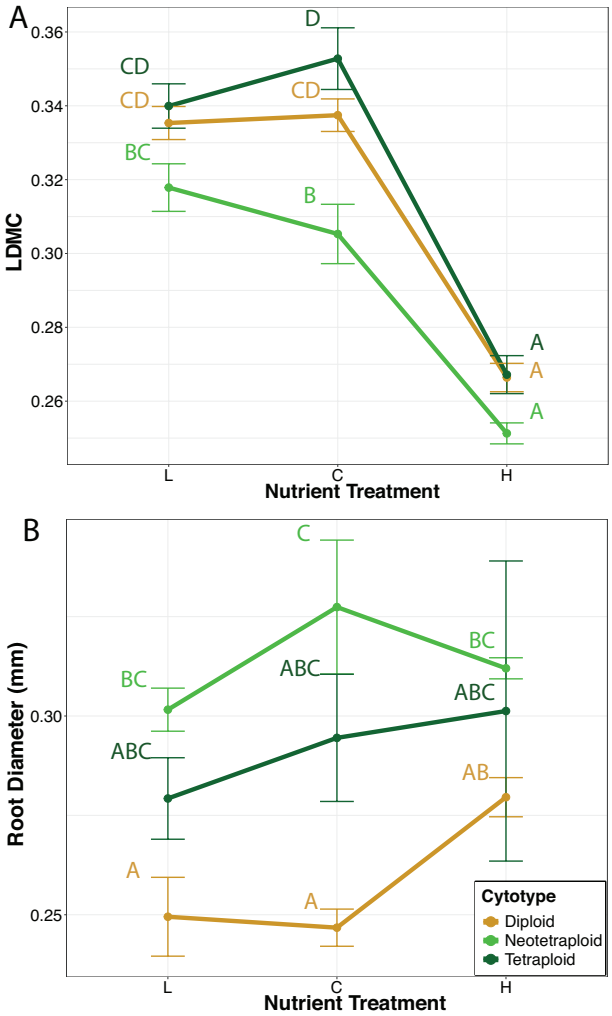

Figure 3: Mean and standard errors of functional trait responses to nutrient manipulation of diploid, neotetraploid, and established tetraploid *Heuchera cylindrica.* A) the average leaf dry matter content (LDMC) trait response of plants, and B) The average root diameter of plants. "L", "C", and "H" represent the low, control, and high nutrient treatments, respectively. Letters denote significance of post hoc tests comparing all means.

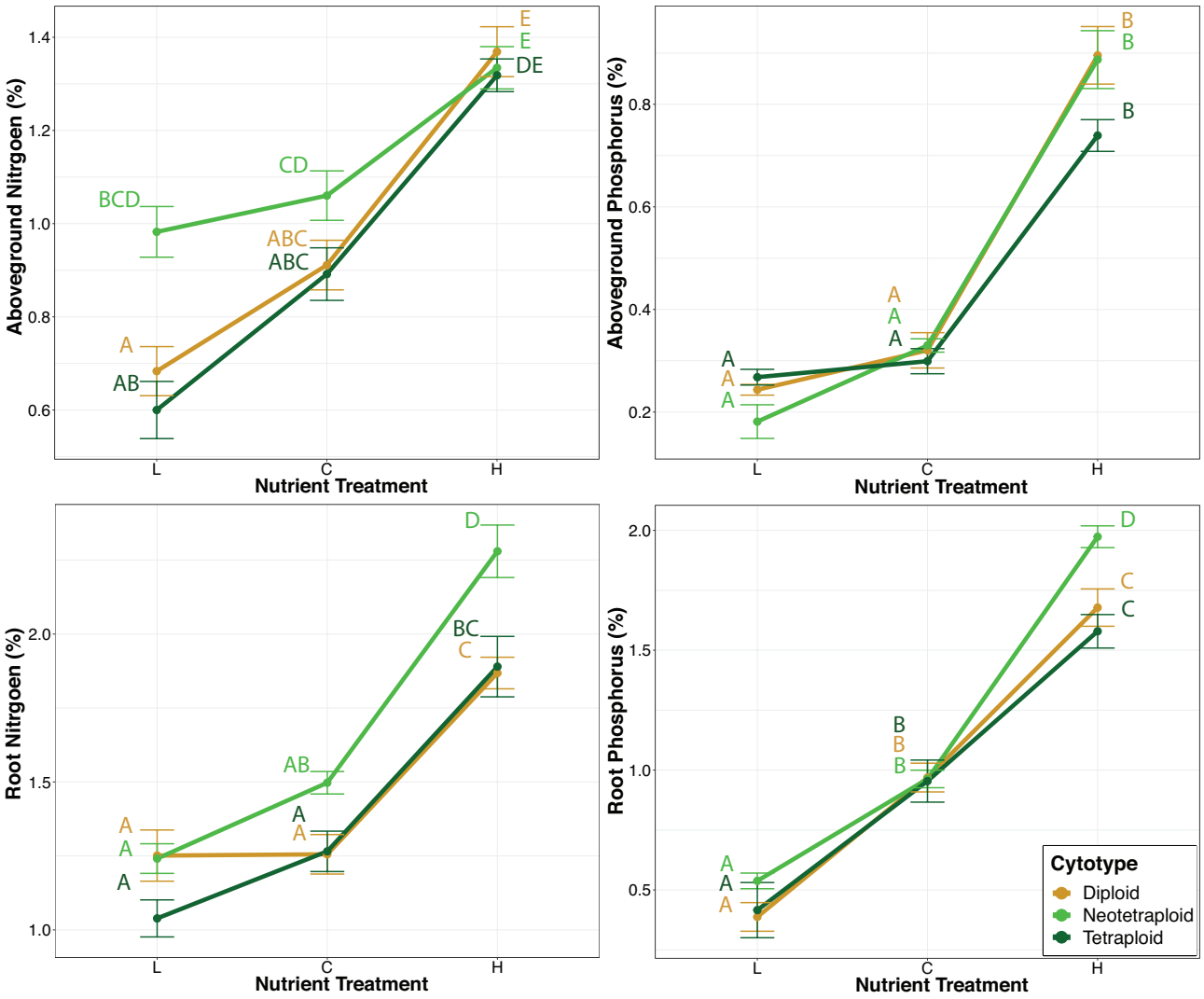

Figure 4: Mean and standard errors of tissue nutrient concentration responses to nutrient manipulation of diploid, neotetraploid, and established tetraploid *Heuchera cylindrica*. A) aboveground tissue nitrogen concentrations, B) aboveground tissue phosphorus concentrations, C) belowground tissue nitrogen concentrations, and D) belowground tissue phosphorus concentrations. "L", "C", and "H" represent the low, control, and high nutrient treatments, respectively. Letters denote significance of post hoc tests comparing all means.

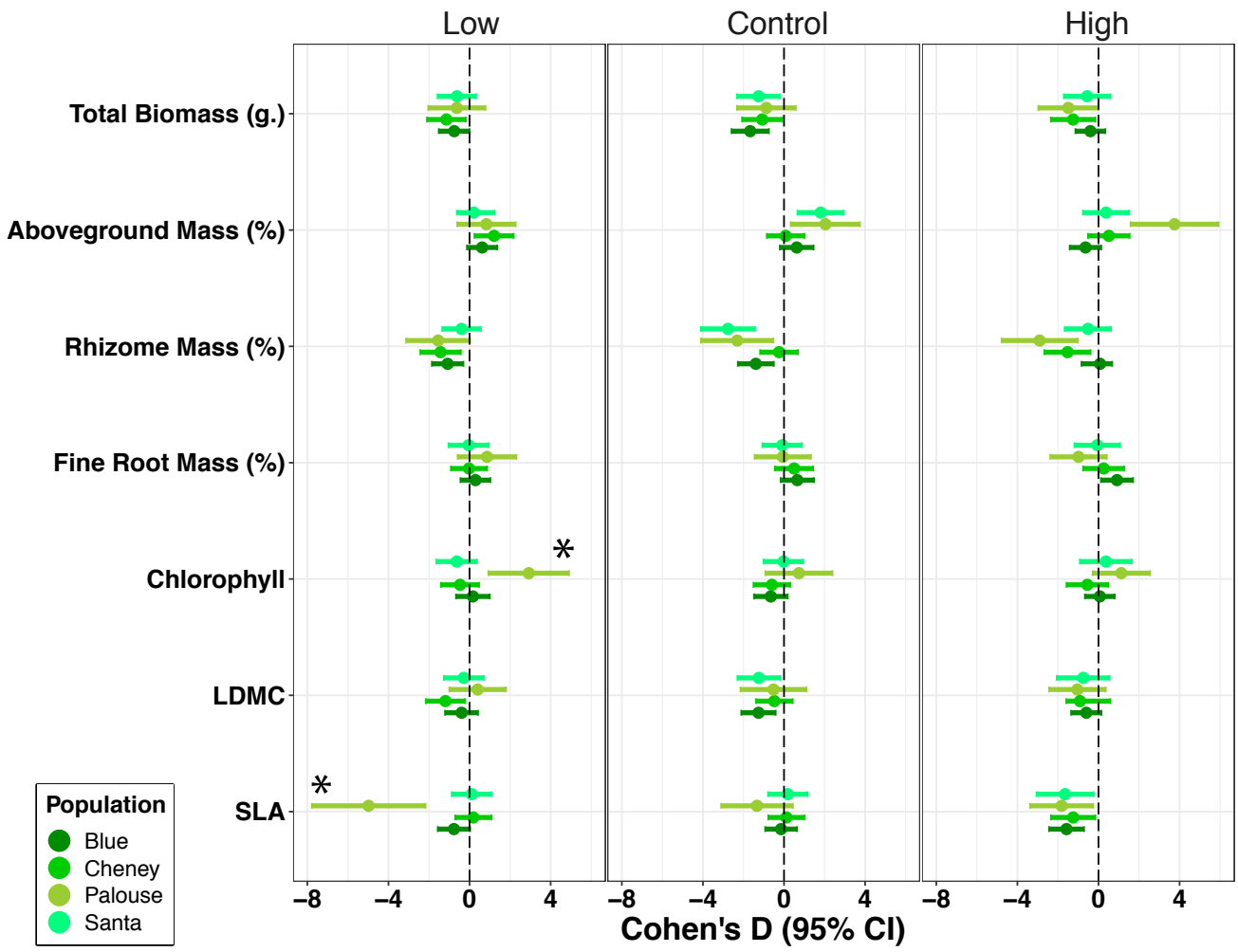

Figure 5: Cohen's D measurements and 95% confidence intervals of the effect of multiple origins of neotetraploidy. Cohen's D was determined as the difference in trait measurements between diploids and their neopolyploid progeny for each population (origin) by nutrient treatment level. The low, control, and high panels correspond to the respective nutrient treatments. Significant effect of multiple origins is indicated with asterisks.

Table S1: Site information of the maternal diploid and established tetraploids of *Heuchera cylindrica* used in this study.

Map Number	Site	Coordinates	Population Cytotype
1	Cheney, WA	47.481677 N, 117.567133 W	Diploid
2	Palouse, WA	46.910055 N, 117.055666 W	Diploid
3	Dayton, WA	46.199712 N, 117.766992 W	Diploid
4	Santa, ID	47.166822 N, 116.483605 W	Diploid
5	Benewah, ID	47.337876 N, 116.827284 W	Tetraploid
6	Coeur d'Alene, ID	47.618344 N, 116.662353 W	Tetraploid

Table S2: Description of the modified Hoagland's fertilizer solution treatments and the associated micronutrients that were applied with each nutrient treatment.

Treatment	Nutrient Name	Molarity
Control	NH_4NO_3	1.33 mM
	KH_2PO_4	0.051 mM
Low	NH_4NO_3	0.33 mM
	KH_2PO_4	0.013 mM
High	NH_4NO_3	5.31 mM
	KH_2PO_4	0.203 mM
Micronutrients	K_2SO_4	0.75 mM
	$MgSO_4$	0.65 mM
	$MnSO_4$	1 μM
	$CuSO_4$	0.1 μM
	$ZnSO_4$	1 μM
	Na_2MoO_4	0.035 μM
	H_3BO_3	0.01 mM
	Fe-EDTA	0.1 mM
	$CaCl_2$	2 mM

Table S3: ANOVA table of linear mixed models, comparing untreated diploids of *Heuchera cylindrica* to nitrous oxide – treated diploids. Statistical significance is denoted with p-values in bold.

Trait	Fixed Effect	df	F	P
Total Biomass	Treatment	2, 375	940.073	**<0.001**
	Cytotype	1, 378	6.647	**0.010**
	Treatment x Cytotype	2, 375	4.273	**0.015**
Normalized Above Biomass	Treatment	2, 373	169.180	**<0.001**
	Cytotype	1, 376	0.048	0.828
	Treatment x Cytotype	2, 373	0.255	0.775
Normalized Rhizome Biomass	Treatment	2, 372	149.337	**<0.001**
	Cytotype	1, 375	0.031	0.862
	Treatment x Cytotype	2, 372	0.642	0.527
Normalized Fine Roots Biomass	Treatment	2, 372	32.964	**<0.001**
	Cytotype	1, 375	0.283	0.595
	Treatment x Cytotype	2, 372	0.047	0.954
Average Chlorophyll	Treatment	2, 370	109.948	**<0.001**
	Cytotype	1, 371	0.657	0.418
	Treatment x Cytotype	2, 370	0.055	0.946

Average SLA	Treatment	2, 367	64.555	**<0.001**
	Cytotype	1, 368	0.344	0.558
	Treatment x Cytotype	2, 367	0.204	0.816
Average DMC	Treatment	2, 369	217.631	**<0.001**
	Cytotype	1, 371	1.315	0.252
	Treatment x Cytotype	2, 369	0.429	0.652
Aboveground Phosphorus	Treatment	2, 49	88.463	**<0.001**
	Cytotype	1, 50	0.0405	0.841
	Treatment x Cytotype	2, 49	2.589	0.085
Aboveground Nitrogen	Treatment	2, 78	33.175	**<0.001**
	Cytotype	1, 79	0.699	0.406
	Treatment x Cytotype	2, 78	2.275	0.110
Belowground Phosphorus	Treatment	2, 40	84.266	**<0.001**
	Cytotype	1, 40	0.105	0.748
	Treatment x Cytotype	2, 40	1.192	0.314
Belowground Nitrogen	Treatment	2, 90	48.872	**<0.001**
	Cytotype	1, 90	0.112	0.739

	Treatment x Cytotype	2, 90	1.513	0.226

Table S4: ANOVA table summarizing the linear mixed models of biomass and leaf functional traits of *Heuchera cylindrica*. Statistical significance is denoted with p-values in bold.

Trait	Fixed Effect	df	F	P
Total Biomass	Treatment	2, 25	114.64	**<0.001**
	Cytotype	1, 188	24.13	**<0.001**
	Population	3, 181	1.78	0.15
	Treatment x Cytotype	2, 188	5.16	**<0.007**
	Treatment x Population	6, 181	2.63	**0.018**
	Cytotype x Population	3, 178	0.58	0.631
	Treatment x Cytotype x Population	6, 178	1.41	0.211
Normalized Above Biomass	Treatment	2, 28	25.43	**<0.001**
	Cytotype	1, 191	26.79	**<0.001**
	Population	3, 183	3.82	**0.011**
	Treatment x Cytotype	2, 191	1.10	0.335
	Treatment x Population	6, 183	2.17	**0.048**
	Cytotype x Population	3, 179	1.86	0.138
	Treatment x Cytotype x Population	6, 179	2.03	0.064
Normalized Rhizome Biomass	Treatment	2, 28	15.88	**<0.001**
	Cytotype	1, 191	58.28	**<0.001**
	Population	3, 183	2.89	**0.037**

	Treatment x Cytotype	2, 191	3.63	**0.028**
	Treatment x Population	6, 182	2.01	0.066
	Cytotype x Population	3, 179	1.47	0.223
	Treatment x Cytotype x Population	6, 179	1.90	0.084
Normalized Fine Roots Biomass	Treatment	2, 29	11.59	**<0.001**
	Cytotype	1, 192	2.13	0.146
	Population	3, 183	2.56	0.056
	Treatment x Cytotype	2, 192	0.54	0.581
	Treatment x Population	6, 183	0.97	0.450
	Cytotype x Population	3, 179	1.13	0.338
	Treatment x Cytotype x Population	6, 179	0.87	0.522
Average Chlorophyll	Treatment	2, 28	20.58	**<0.001**
	Cytotype	1, 183	1.30	0.255
	Population	3, 174	10.77	**<0.001**
	Treatment x Cytotype	2, 182	1.14	0.321
	Treatment x Population	6, 174	0.90	0.499
	Cytotype x Population	3, 170	6.14	**<0.001**
	Treatment x Cytotype x Population	6, 170	0.59	0.740
Average SLA	Treatment	2, 33	12.73	**<0.001**

	Cytotype	1, 189	17.95	**<0.001**
	Population	3, 178	7.89	**<0.001**
	Treatment x Cytotype	2, 188	4.09	**0.0183**
	Treatment x Population	6, 178	0.82	0.559
	Cytotype x Population	3, 176	2.37	0.072
	Treatment x Cytotype x Population	6, 176	0.50	0.805
Average DMC	Treatment	2, 28	40.65	**<0.001**
	Cytotype	1, 183	18.96	**<0.001**
	Population	3, 176	7.35	**0.001**
	Treatment x Cytotype	2, 182	1.45	0.236
	Treatment x Population	6, 175	0.64	0.702
	Cytotype x Population	3, 171	0.81	0.492
	Treatment x Cytotype x Population	6, 171	1.42	0.211

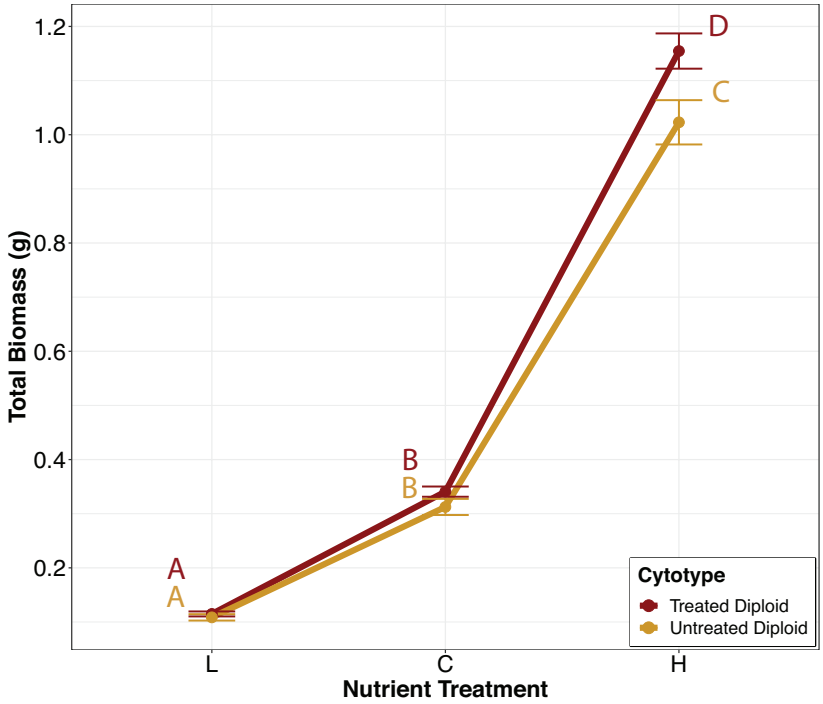

Figure S1: Mean and standard errors for total biomass of treated and untreated diploids to nutrient manipulation in *Heuchera cylindrica*. Diploid reaction norms are in gold and nitrous oxide-treated diploids are in red. “L”, “C”, and “H” represents the low, control, and high nutrient treatments. Letters denote groupings of a Tukey’s post hoc test, where groups with different letters are significantly different.

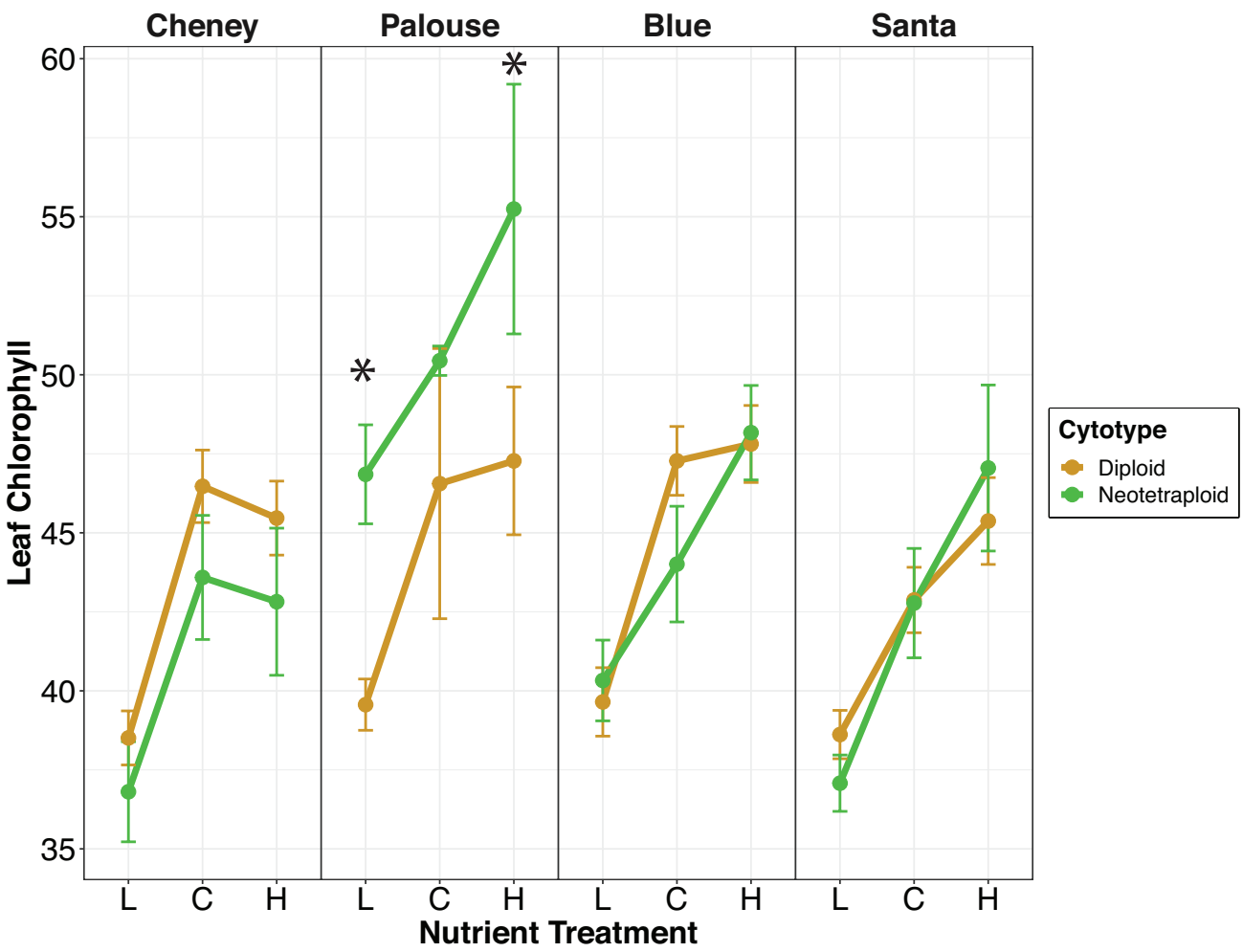

Figure S2: Mean and standard error for leaf chlorophyll content of untreated diploids and neotetraploids to nutrient manipulation in *Heuchera cylindrica*. Each of the four panels represents an independently derived neopolyploid origin. "L", "C", and "H" represent the low, control, and high nutrient treatments. Asterisks denote significant differences between diploids and neotetraploids within a treatment group.

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Chapter 2: Nutrient enrichment and neopolyploidy interact to increase lifetime fitness of *Arabidopsis thaliana*

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Abstract

Aims:

Nascent polyploids, or neopolyploids, frequently arise within diploid plant lineages and are expected to experience increased requirements for growth-limiting nutrients because of building a larger genome. Because this may have important consequences for the ecology of neopolyploids, we need studies that track the lifetime fitness effects of whole genome duplication. Here we investigated how multiple origins of neopolyploidy and nutrient supply rate affected fitness-related traits of *Arabidopsis thaliana*.

Methods:

We investigated the interaction between cytotype, independent neopolyploid origins, and soil fertility by conducting a greenhouse experiment with five nutrient treatments that varied nitrogen and phosphorus supply. We compared biomass, flowering phenology, fecundity, average mass per seed, and offspring germination rates of diploids and their descendent neotetraploids from four independent origins.

Results:

The results supported the hypothesis that neopolyploidy increases nutrient limitation. Diploids outpaced their neotetraploid descendants in growth and composite fitness in all nutrient treatments except with high supply of nitrogen and phosphorus, where neotetraploid growth and composite fitness exceeded diploids. In contrast, we did not detect an interaction between cytotype and nutrient treatment for flowering phenology, but neotetraploids flowered later, and

low nutrient supply caused earlier flowering. We additionally found that the trait responses of neotetraploids were strongly contingent on their independent, maternal origin.

Conclusions:

Polyploidy has myriad effects on plant physiology, but few studies have tested how neopolyploid-induced physiological changes can affect plant environmental interactions. By showing that neopolyploid fitness is more responsive to nutrient supply, we conclude that neotetraploidy increases nutrient limitation in *A. thaliana*.

Introduction

Whole genome duplication (WGD) is one of the most pervasive evolutionary forces in the plant kingdom, where multiple independent origins of first-generation polyploids, or "neopolyploids", arise from their progenitor diploids as frequently as the somatic mutation rate (Ramsey and Schemske 1998). When neopolyploids first arise they must immediately overcome a demographic mating disadvantage from their diploid parents in order to become established or else they are expected to quickly go extinct (Arrigo and Barker 2012; Mayrose et al. 2015). Although models indicate that neopolyploid populations should be ephemeral (Fowler and Levin 2016; Rodriguez 1996), we often observe established polyploids at rates that are higher than expected (Levin 2019). Although there may be no simple mechanism that fully explains the abundance of polyploids in nature, recent efforts have hypothesized that the high incidence of established polyploid populations may be a result of both ecological opportunity and the apparent ability of extant polyploids to tolerate harsh conditions (Levin 2019; Levin and Soltis 2018). As a result, there has been a call for work characterizing how neopolyploidy can alter plant physiology and how those alterations affect performance under environmentally challenging conditions.

One of the major assumptions about the physiological effects of polyploidy is that it increases nutrient requirements (Guignard et al. 2017; Leitch and Leitch 2008). This assumption is rooted in the idea that WGD causes a greater need for nitrogen and phosphorus because polyploids synthesize more nucleic acids (Lewis 1985). Furthermore, polyploids may be more nutritionally constrained by a need to produce additional proteins as compared to diploids, since they must maintain gene balance in their genetic networks (Birchler and Veitia 2010; Osborn et al. 2003). Plant protein production may not linearly scale with changes in genome size because

rRNA production requires more phosphorus to facilitate growth (Hessen et al. 2010). Thus, WGD may shift the ratio of required nitrogen to phosphorus. For example, if WGD causes a doubling in nucleic acid production but not protein production, it may result in altered stoichiometric requirements for growth limiting nutrients such as nitrogen and phosphorus. Because we expect altered nitrogen and phosphorus stoichiometry at the cellular level, we also expect an increase in the total requirements of these nutrients at the tissue or whole-organism level, since WGD is strongly associated with increased tissue size at the macroscopic scale, otherwise referred to as the 'gigas' effect (Muntzing et al. 1936; Stebbins 1971). Although the gigas effect results in lower cell densities that may compensate for elevated nutrient needs, the net effect of polyploidy is predicted to result in a more nutrient limited plant because building overall larger tissues will incur a greater need for growth limiting nutrients. We thus expect that WGD will cause neopolyploids to be intrinsically more limited in their ability to grow and reproduce under nutrient-limited environments than their diploid parents.

Although our understanding of how neopolyploidy affects plant nutrient requirements is limited, we do have some evidence suggesting that polyploidy elicits a greater need for nutrients. For instance, two long-term fertilization studies in grasslands found that productivity in established polyploid species increased more with nutrient enrichment than co-occurring diploid species (Guignard et al. 2016; Šmarda et al. 2013). These findings highlight how polyploids that have become established and evolved over many generations can outperform diploid species, but the immediate effects of neopolyploidy on plant nutritional requirements remains unclear. Thus, to investigate the direct effects of WGD on plant nutrient needs, we need studies that examine early generation polyploids that have not had substantial time for evolutionary processes to act. Furthermore, we also need direct comparisons between diploids and their neopolyploid progeny

in order to control for phylogenetic relatedness between diploids and polyploids. In doing so, we can directly test the immediate effects of WGD on plant nutrient limitation.

Indeed, there has been one study thus far to consider the effect of neopolyploidy on plant performance under variable nutrient supply rates. Neotetraploid *Chamerion angustifolium* grew more than diploids in response to nutrient enrichment, but these neotetraploids always produced fewer flowers than their diploid progenitors (Walczyk and Hersch-Green 2019), suggesting a tradeoff between productivity and reproductive output in polyploids. Indeed, a recent meta-analysis showed that WGD typically reduced the reproductive output of neopolyploid plants and increased the size of reproductive tissues, suggesting a negative relationship between fecundity and reproductive biomass investment (Porturas et al. 2019). Although neopolyploidy can negatively affect reproductive rates, we do not know how other complex fitness-related traits such as individual seed mass or progeny germination rates are affected by WGD. Since there is a negative relationship between genome size and seed mass (Beaulieu et al. 2007), we expect a negative relationship between reproductive output and the allocation to individual seeds. There may thus be a compensatory effect of neopolyploidy on plant fitness; if plant fecundity is reduced by WGD, but neopolyploids invest more into seeds, then the lifetime fitness of neopolyploids may exceed that of their diploid parents. By taking fecundity, seed mass investment, and progeny germination rate into account, we will be able to track the complex lifetime fitness response of plants to neopolyploidy and nutrient supply rate.

Although there is evidence that neopolyploidy will cause plant reproductive traits to be more constrained by the nutrient environment, we do not know how WGD and nutrient supply rates also affect other fitness-related traits such as ontogeny. Polyploids often grow more slowly than related diploids (Levin 1983), and we expect this slower growth to result in delayed

reproductive phenology of neopolyploids, which can indirectly affect plant lifetime fitness (Munguia-Rosas et al. 2011). There is some evidence that established polyploids have a delayed phenology compared to diploids (Simon-Porcar et al. 2017), yet there are other instances in which established polyploids have earlier flowering phenology than their diploid ancestors (Bretagnolle and Thompson 1996; Petit et al. 1997). These mixed results may be due to a genotype by environment interaction, that can differentially shape the flowering phenology of diploids versus polyploids. For example, a study found that tetraploids flowered earlier in a mixed-ploidy population, but the opposite pattern was observed in a common garden environment, suggesting that the environment has a strong effect on polyploidy-derived phenology differences (Segraves and Thompson 1999). Therefore, the effect of environmental nutrient supply may strongly interact with neopolyploidy on flowering phenology. Since WGD causes cells to divide more slowly and neopolyploids are expected to be more growth limited by the soil nutrient environment, we predict that neopolyploidy and soil fertility will interact to affect flowering phenology.

Here we use *Arabidopsis thaliana* (Brassicaceae) to test how nutrient limitation differs between diploids and their neopolyploid offspring. *Arabidopsis thaliana* is a good model for testing how neopolyploidy affects plant responses to nutrient addition because it is a fast-growing annual, making it possible to assess lifetime fitness. Furthermore, multiple, independent autotetraploid lineages have recently been synthesized by Luca Comai and were made publicly available by (Solhaug et al. 2016). By inducing autopolyploidy, an intraspecific WGD event, it allows us to discern the direct effect of WGD, and thus avoid the confounding effect of interspecific hybridization. These *A. thaliana* lines also represent a broad geographic sampling, providing a unique opportunity to assess how multiple independent origins of neopolyploidy

affect the ecophysiological responses of plants to nutrient supply. The fact that these independent neotetraploid origins were synthesized from geographically disparate diploid maternal lineages also allows us to avoid confounding effects of local adaptation, and thus we can draw conclusions about whether the effects of polyploidy are consistent among origins. In the present study, we addressed three questions: 1) Are neotetraploid *A. thaliana* more nutrient limited than their diploid parents? 2) Are fitness related traits and flowering phenology differentially affected by plant cytotype and nutrient supply rates? 3) Are the fitness responses of plants consistent across multiple origins of neotetraploidy?

Materials and Methods

Study organism and growth conditions

We used *A. thaliana* to study how neotetraploidy affects plant responses to nitrogen and phosphorus manipulation. We acquired eight accessions, comprised of four diploid ecotypes and their corresponding neotetraploid descendants that were synthesized with colchicine. Thus, these four ecotypes represent four independent origins of neotetraploidy. Seed stocks were sourced from the Arabidopsis Biological Resource Center (ABRC, Columbus, Ohio; Table S5). Although three of the four seed stocks used in this study are from Germany, they were acquired from broadly distributed sites and represent independent ecological backgrounds.

To encourage proper germination, we stratified a subset of the seeds from each maternal line for four days before planting into 108 cubic cm pots filled with autoclaved quartz sand. The plants were grown in a climate-controlled greenhouse maintained at 21-24ºC daytime and 18-21ºC nighttime, under ambient light conditions. We watered plants by placing the pots into

nursery trays and bottom watering with deionized water. Approximately two weeks after the seeds had germinated and true leaves had begun to emerge, we thinned to one plant per pot to avoid competitive effects. The plants were allowed to become established for two weeks before treatments were applied, and during this time, we supplied the nursery trays with a modified Hoagland's fertilizer solution that is considered optimal for growth of *A. thaliana* (Cai et al. 2017).

Experimental Design

To investigate how neotetraploidy affects the growth and fitness of *A. thaliana* under different nutrient supply rates, we conducted a factorial nutrient manipulation experiment. This experiment was conducted from late May 2019 to early September 2019. During this time, the eight accessions were grown under five nutrient treatment levels. We planted 60 pots of each of the eight accessions, for a total of 480 pots. The plants were distributed evenly among 20 nursery trays, such that each tray contained three pots of each of the eight accessions. We avoided artifacts driven by microclimatic variation within the greenhouse by rotating the nursery trays twice per week. Tray rotations were carried out by systematically moving the trays on the greenhouse benches while also rotating each tray 180 degrees, so that each tray occupied every possible tray location during the course of the experiment.

We investigated how cytotype and nutrient supply affected *A. thaliana* performance by varying both the nitrogen and phosphorus of nutrient treatments (Table 4). By varying both the concentration and stoichiometry of nitrogen and phosphorus in the nutrient treatments, it allowed us to discern how these nutrients differentially affected growth and reproduction. Following Cai et al. (2017), the control nutrient treatment was a modified Hoagland fertilizer solution with a

molar N:P ratio of 16, a value considered to be optimally colimiting for growth of terrestrial plants (Koerselman and Meuleman 1996). In addition to the control, we applied four experimental nutrient treatment levels: low N & P, high N & P, low N:P, and high N:P. The concentrations of each nutrient treatment were determined by either quartering (low N & P) or quadrupling (high N & P) the concentration of the control level of nitrogen and phosphorus. We also investigated how altered stoichiometric supply ratios of nitrogen to phosphorus affected diploid versus neotetraploid growth by either supplying plants with a molar N:P ratio of 256 for the high N:P treatment level, or a molar N:P ratio of 1 for the low N:P treatment level. The molar ratio of the high N:P treatment was calculated by quadrupling the concentration of nitrogen and quartering the concentration of phosphorus relative to control, whereas the low N:P treatment molar ratio was calculated by quartering the concentration of nitrogen and quadrupling the concentration of phosphorus relative to control. The high N:P treatment is well within the theoretical zone of phosphorus limitation, and the low N:P treatment is considered a strongly nitrogen limited environment (Gusewell 2004; Koerselman and Meuleman 1996). All nutrient solutions were adjusted to a pH of 5.5-5.75 to ensure nutrient availability to plants.

We applied the nutrient treatments by placing the nutrient solution into bottom-watering trays and allowing it to sit for five days per week. To avoid differential accumulation of micronutrients in the watering trays, each week we replaced the nutrient solution with deionized water for two days. In this way, we supplied the same volume to each bottom-watering tray for five days per week during the experiment; the only variation in nutrient supply was based on the relative concentration of nitrogen and phosphorus in the nutrient treatment solutions.

Fitness – Related Trait Measurements

We investigated how neotetraploidy and soil nutrient treatments interact by measuring a suite of fitness-related traits in *A thaliana*. First, we determined how key flowering phenology events in diploids and their neotetraploid progeny were affected by altered nitrogen and phosphorus supply rates. We recorded the days to bolting, days to first flowering, and the latency between bolting and first flowering. For a subset of the plants (~4 plants per treatment level: ecotype x cytotype x nutrient treatment), we also collected three ripe, intact siliques to assess seed traits. From the siliques, we determined average seed set per silique and seed weight. A subset of these seeds were used to assess germination rate by placing approximately 20 stratified seeds on an autoclaved filter paper moistened with deionized water. The seeds were given two weeks to germinate and then germination success was scored using a dissecting microscope.

Plants were harvested after a majority of the tissues had senesced in order to assess productivity and fitness responses. The harvested aboveground tissues were dried in a 60˚C drying oven for three days before the dry weights were determined. At harvest, we also recorded the total number of siliques per plant. From the counts of the total number of siliques per plant, we multiplied by the average number of seeds per silique to estimate the plant fecundity, or the total number of seeds produced per plant. Rather than relying on fecundity alone as our estimate of plant fitness, we created a composite fitness metric by incorporating average seed mass and germination rate (Campbell 1991). Thus, our final estimate of plant fitness was the multiplicative product of fecundity, average mass per seed, and average seed germination rate. All data in this study are included in this article and its supplementary information files.

Statistical Analyses

We assessed the effects of cytotype, nutrient supply rate, and independent origin of neotetraploidy by fitting individual linear mixed effect (lme) models on phenology measurements, fitness-related traits, and biomass. We used a three-way ANOVA to analyze the interaction between the main effects of cytotype, nutrient treatment, and ecotype (independent origins of neotetraploidy). By incorporating the ecotype of the plants as a main effect in our models, we were able to determine if there was an effect of independent origins of neotetraploidy on trait responses. In all our models, we included bottom watering tray as a random effect. Since we were also interested in the direction and magnitude of the differences when comparing diploids and neotetraploids, we used Tukey's HSD post hoc tests. We dropped 55 plants from the analyses because they had lost a large fraction of their potting medium due to the flushing of nutrient solutions and water during the experiment and we wanted to avoid potential artifacts caused by this stressful disturbance. To build our statistical models, we used the lme4 package (Bates et al. 2015) for lme models in R (R Core Team 2019).

Results

Phenological effects of neotetraploidy and nutrient treatment

Although there was no interaction between cytotype and nutrient treatment for the three measures of flowering phenology (Table 5), we did observe that neotetraploids generally had delayed flowering phenology compared to their diploid parents (Fig. 6). Additionally, plants in the high N & P and high N:P treatments took longer to bolt than the low N:P treatment level, whereas the control and low N & P treatment level were not statistically different from any other group. The effect of nutrient treatment on the days to first flowering was driven by delayed flowering with high N:P and high N & P treatment levels (Fig. 6; Table 5). We also observed a

three-way interaction between cytotype, nutrient treatment, and ecotype on the latency between bolting and first flowering, in which there were idiosyncratic responses by the ecotypes to variation in nutrient supply and cytotype (Table 5; Fig. 7).

Reproductive and biomass effects of neotetraploidy and nutrient treatment

We found that the number of seeds per silique and average weight per seed were not responsive to nutrient treatment; however, neotetraploids had consistently fewer and heavier seeds than diploids (Table 6; Fig. 8). Although neotetraploids produced heavier and fewer seeds than diploids, the cytotype by ecotype interaction on both average weight per seed and seeds per silique was driven by variation in the magnitude of ecotype-specific responses to neotetraploidy for average weight per seed and seeds per silique, respectively. Overall, there appeared to be a negative relationship between average mass per seed and the average number of seeds per silique (Fig. 8).

There was a cytotype by ecotype interaction for fecundity (Table 6), where the C24 and Landsberg neotetraploid origins caused fecundity to be generally similar between diploids and neotetraploids, but neotetraploids from Aua and Niederzenz had lower fecundity than diploids. The main effect of neotetraploidy caused a significant reduction in plant fecundity, whereas high N & P and the control nutrient treatment increased fecundity compared to the other nutrient treatments. The germination success of these seeds was also increased by neotetraploidy, and there was variation among ecotypes in germination rate. We also compiled all three of the independent reproductive measures of fitness by multiplying fecundity, average mass per seed, and average germination rate of seeds as a composite measure of fitness and found a significant interaction between cytotype and nutrient treatment as well as between cytotype and ecotype

(Fig. 9; Table 6). The cytotype by nutrient treatment interaction was driven by an increase in neotetraploid composite fitness with high N & P nutrient treatment, but was not different in the other nutrient treatments (Fig. 9).

Lastly, we assessed the total aboveground biomass of plants as another fitness-related measure. There were significant interaction effects between nutrient treatment and cytotype (Fig. 10; Table 6) where neotetraploids had greater biomass production with high N & P treatment compared to no differences in biomass between diploids and neotetraploids in the other nutrient treatments. The high N & P treatment also caused greater biomass production for both diploids and neotetraploids, but there was no difference among the other nutrient treatments in biomass production.

The effect of independent origins of neotetraploidy on trait responses

We addressed how multiple independent origins of neotetraploidy affected the cytotype-specific responses of plants to nutrient treatment. We found only one instance of a three-way interaction between ecotype or "multiple origins", cytotype, and nutrient treatment for the latency from date of bolting to first flowering (Table 5; Fig. 7). This three-way interaction was driven by the Landsburg and C24 neotetraploid origins not differing between diploids and neotetraploids in bolting to flowering time, the Aua origin of neotetraploidy had delayed bolting to flowering phenology, whereas the Niederzenz origin of neotetraploidy had delayed phenology only with high N:P treatment. For all other traits that were measured, the ecotype of neotetraploid origin and plant cytotype significantly interacted (Table 5, Table 6; Fig. 11), indicating that multiple origins of neotetraploidy has a strong effect on trait responses of *A. thaliana.*

Discussion

Polyploidy is predicted to increase the nutrient requirements of plants and thus constrain their ability to grow and reproduce in resource-limited environments. Although a few studies have shown that polyploids can be more productive with high nutrient supply rates (Guignard et al. 2016; Walczyk and Hersch-Green 2019; Šmarda et al. 2013), we do not know how other corresponding traits associated with lifetime fitness are affected. In this study, we specifically tested how neotetraploidy affects plant nutrient limitation by varying nitrogen and phosphorus supply rates and comparing the responsiveness of fitness-related traits of neotetraploids to their diploids parents. The results were consistent with the prediction that WGD causes greater nutrient requirements in plants. Nutrient limitation is often defined by the responsiveness of a plant to an increasing supply of the nutrients of interest (Vitousek et al. 2010). In the present study, we observed that growth and key fitness-related traits of neotetraploid *A. thaliana* responded more positively to a high supply of nitrogen and phosphorus than their diploid parents (Fig. 9, 5), indicating that there is strong evidence that neopolyploidy increases nutrient limitation in *A. thaliana*. Similar to previous studies that have observed that polyploids are more responsive to nutrient environment than diploids (Guignard et al. 2016; Šmarda et al. 2013), we found that the reproductive output of neotetraploids was more responsive to the nutrient content of the soil. Interestingly, with low and control nutrient supply, diploids and neotetraploids had equivalent performance (Table 6). The comparatively stronger response of neotetraploid composite fitness to the high nutrient treatment suggests that neotetraploids were more plastic to nutrient environments (Figs. 4-5). This result supports the prediction that WGD causes an

increase in phenotypic plasticity (Parisod et al. 2010). The enhanced performance of neotetraploids when given a high supply of nitrogen and phosphorus suggests that high resource soil environments may be more favorable for the establishment and persistence of neopolyploids.

Although we expected that neotetraploid *A. thaliana* would respond differently than their diploid parents to an altered ratio of nitrogen to phosphorus, we saw no interaction between cytotype and altered N:P stoichiometry. In fact, when we compared the growth and fitness responses of plants between all nutrient treatments, we observed that diploids and neotetraploids responded similarly to altered N:P stoichiometry as they did to low nitrogen and phosphorus. This observation was likely due to the fact that both nitrogen and phosphorus are essential nutrients for plant growth, and neither resource is substitutable for the other when a plant uses them for physiological processes (Sperfeld et al. 2016). Thus, variation in the quantity of non-substitutable nutrients supplied to plants, rather than their molar ratio of supply, is the only situation that is expected to result in substantial variation of trait responses, indicating that they are co-limiting resources (Sperfeld et al. 2016). Indeed, when we simultaneously supplied both nitrogen and phosphorus along a molar N:P ratio of 16, a value often considered to be co-limiting for many terrestrial plants, we observed an appreciable increase in growth and fitness in the plants. Consequently, we conclude that neotetraploidy does not affect the sensitivity of *A. thaliana* to nitrogen and phosphorus stoichiometry, rather, WGD affects plant sensitivity to the concentration of these co-limiting nutrients.

Similar to the measure of composite fitness, we found that neotetraploid *A. thaliana* produced significantly more aboveground biomass than their diploid progenitors, but only when supplied with a balanced and high supply of nitrogen and phosphorus. This enhancement in productivity of neotetraploids in response to nutrient enrichment corroborates long-term

fertilization studies in grasslands that show that nutrient enrichment causes established polyploid plants to have a greater response in biomass productivity than co-occurring, unrelated diploid species (Guignard et al. 2016; Šmarda et al. 2013). The greater responsiveness of neotetraploids to increased nutrient supply in this study was likely driven by an increase in the nutritional cost of building inherently larger polyploid tissues, where a low nutrient supply allowed for only minimal growth of both cytotypes, but the neotetraploids achieved greater maximum growth with high nutrient supply. Although some mechanisms have been proposed to explain the greater responsiveness of polyploids to high nutrient supply, such as greater nutrient uptake efficiency, the examples we have are mostly species dependent (Cacco et al. 1976). As more studies that compare diploids and their neopolyploid progeny emerge from a broad phylogenetic sampling, we will be able to disentangle the species-specific effects from the universal effects of WGD that affect neopolyploid responses to increased nutrient supplies.

In addition to finding that neotetraploidy promoted the biomass production of *A. thaliana,* we also observed a strong interaction between cytotype and nutrient supply on the lifetime fitness of plants. The measure of lifetime fitness of plants in this study was an emergent property of multiple fitness-related traits. For instance, when we considered fecundity alone, we did not detect an interaction between plant cytotype and nutrient environment, but when we factored in the average weight per seed and the germination rates of those seeds, we saw a strong interaction between cytotype and nutrient treatment. The disparity between the results for fecundity versus composite fitness was caused by diploids outpacing their neotetraploid progeny in seed production, but neotetraploids made heavier seeds that germinated at higher rates (Fig. 8). This suggests that fecundity is the most labile reproductive effect of neopolyploidy and thus serves as a compensatory mechanism to overcome the inherently greater costs of building larger

neopolyploid seeds. Similar to the negative effect of neotetraploidy on fecundity that we observed, (Walczyk and Hersch-Green 2019) also observed that neotetraploidy in *C. angustifolium* caused a reduction in the number of flowers per plant, suggesting that reduced fecundity occurs in neotetraploid *C. angustifolium*. Given that numerous studies have found a strong positive correlation between seed mass and genome size (Caceres et al. 1998; Chung et al. 1998; Richardson et al. 2015), increased nutrient supplies may commonly promote the lifetime fitness of neopolyploid plants more than their diploid parents when we take into account the greater investment into individual seeds.

Despite finding a strong interaction between cytotype and nutrient supply on growth and fitness related traits in *A. thaliana,* we did not observe a similar effect on plant flowering phenology. Instead, we observed that both the main effect of nutrient treatment and cytotype independently explained a significant amount the variation in flowering phenology. Specifically, neotetraploids had delayed flowering phenology compared to their diploid ancestors at all nutrient treatment levels, but the high N & P treatment also caused both diploids and neotetraploids to flower later. This suggests that temporal separation may segregate diploids and their neopolyploid descendants. The divergence of flowering phenology between potentially competing cytotypes has been viewed as a mechanism that can promote the odds of neopolyploid establishment (Fowler and Levin 2016; Husband and Sabara 2004; Oswald and Nuismer 2011; Rodriguez 1996; Segraves and Thompson 1999). Although the pattern of delayed flowering in neotetraploids was consistent across nutrient treatments, we do not know if our findings from the greenhouse are directly relatable to field settings. Specifically, the greenhouse growth conditions used in this study likely deviate from natural *A. thaliana* growing conditions in terms of light and temperature. However, because the greenhouse conditions were common to all plants in the

experiment, we consider the differences in performance observed between plants to be the result of treatments, rather than the climate controls. Furthermore, we were unable to conclude if the differences in flowering phenology between diploids and neotetraploids are adaptive, since *A. thaliana* is primarily a selfing annual, although rare outcrossing events do occur (Abbott and Gomes 1989; Hoffmann et al. 2003; Platt et al. 2010). Future experimental work that investigates the evolutionary lability of flowering phenology in synthetic neopolyploids will help to resolve whether temporal isolation affects neopolyploid persistence.

An additional interesting finding was that the trait responses of *A. thaliana* neotetraploids often interacted with the ecotype of origin, supporting the hypothesis that variation in neopolyploid phenotypes is increased by the repeated genesis of independent neopolyploid lineages. Although the only instance in which we observed a three-way interaction between nutrient treatment, cytotype, and ecotype of neotetraploid origin was for the latency between date of bolting to first flower, we observed a significant interaction between cytotype and ecotype on individual traits in all other cases (Table 6). Numerous studies have highlighted the idea that multiple origins of neopolyploidy within a species can support the odds of polyploid establishment by integrating more of the standing genetic variation present in diploids into nascent polyploid populations (Soltis and Soltis 1999). Other recent studies have also supported this idea through comparisons of independent origins neopolyploidy, where they observed that trait responses of neopolyploids varied with maternal origin (Husband et al. 2016; Pacey et al. 2020; Wei et al. 2020). Although the four ecotypes of origin were chosen based on their availability at the time, we recognize that having only four ecotypes constrains our ability to make conclusions about the universal effects of polyploidy on *A. thaliana*. Despite this

constraint, we found evidence that the trait responses of cytotypes of *A. thaliana* to nutrient supply were contingent on their independent ecotype of origin.

We expect that the three independent origins of synthetic neotetraploidy in *A. thaliana* used in this study will more closely resemble naturally derived neotetraploids since outcrossing in *A. thaliana* is rare (Abbott and Gomes 1989; Hoffmann et al. 2003; Platt et al. 2010). In contrast, colchicine-induced synthetic neopolyploids of outcrossing species may not be representative of naturally occurring neopolyploids, since colchicine is typically applied to zygotes, meaning that there are only two alleles possible per locus in the resulting neopolyploid (Dhooghe et al. 2011). Natural neopolyploids are expected to experience higher allelic diversity, since WGD primarily occurs through the union of unreduced gametes, leading to a maximum of four unique alleles per locus in neotetraploids (Otto and Whitton 2000). Additionally, we recognize that the application of mitotic inhibitors, such as colchicine, can have trans-generational effects on plant physiology, but a colchicine treatment effect is improbable in the neotetraploid maternal lines that were used here. This is because other studies have shown that the effects of colchicine effect dissipate after the first generation (Husband et al. 2016), and the neotetraploid seed stocks used here have had a few generations to remove this treatment effect.

The results show that neotetraploidy causes *A. thaliana* to be more responsive to nitrogen and phosphorus addition, supporting the hypothesis that WGD causes greater nutrient limitation in plants, and also that multiple origins of neopolyploidy can affect trait responses to nutrient supply. This study, along with other recent examples, underscores the need to investigate first-generation polyploids to capture the immediate consequences of WGD on plant ecophysiology. Studies that incorporate multiple independent genetic origins of neopolyploidy will reveal how WGD affects plant ecological interactions. In addition to highlighting the need to incorporate

multiple genetic origins of neopolyploidy, this study has uniquely shown that neopolyploidy promotes the complex lifetime fitness response of *A. thaliana* to increased nutrient supply.

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Conflict of Interest Statement

The authors declare that they have no conflict of interest.

Table Captions

Table 4: Description of nitrogen and phosphorus concentrations for the nutrient treatments as well as the concentrations of micronutrients that were added to each treatment.

Treatment	Nutrient Name	Molarity
Control	NH_4NO_3	4 mM
	KH_2PO_4	0.25 mM
Low Both	NH_4NO_3	1 mM
	KH_2PO_4	0.0625 mM
High Both	NH_4NO_3	16 mM
	KH_2PO_4	1 mM
Low N:P	NH_4NO_3	1 mM
	KH_2PO_4	1 mM
High N:P	NH_4NO_3	16 mM
	KH_2PO_4	0.3402 mM
Micronutrients	K_2SO_4	0.75 mM
	$MgSO_4$	0.65 mM
	$MnSO_4$	1 µM
	$CuSO_4$	0.1 µM
	$ZnSO_4$	1 µM
	Na_2MoO_4	0.035 µM
	H_3BO_3	0.01 mM
	Fe-EDTA	0.1 mM
	$CaCl_2$	2 mM

Table 5: Summary of linear mixed effect models for three phenology traits.

Measurement	Fixed Effect	df (treatment, error)	F	P
Days to Bolt	Treatment	4, 343	7.12	**<0.001**
	Cytotype	1, 343	8.13	**0.005**
	Ecotype	3, 343	122.96	**<0.001**
	Treatment x Cytotype	4, 343	1.65	0.161
	Treatment x Ecotype	12, 343	1.95	**0.028**
	Cytotype x Ecotype	3, 343	5.30	**0.001**
	Treatment x Cytotype x Ecotype	12, 343	0.94	0.512
Days to Flower	Treatment	4, 331	8.40	**<0.001**
	Cytotype	1, 331	10.72	**0.001**
	Ecotype	3, 331	131.75	**<0.001**
	Treatment x Cytotype	4, 331	1.65	0.163
	Treatment x Ecotype	12, 331	2.01	**0.023**
	Cytotype x Ecotype	3, 331	5.49	**0.001**
	Treatment x Cytotype x Ecotype	12, 331	1.12	0.341
Bolt to Flower	Treatment	4, 331	2.60	**0.036**
	Cytotype	1, 331	18.71	**<0.001**
	Ecotype	3, 331	12.68	**<0.001**
	Treatment x Cytotype	4, 331	0.86	0.491
	Treatment x Ecotype	12, 331	2.33	**0.007**
	Cytotype x Ecotype	3, 331	6.02	**<0.001**
	Treatment x Cytotype x Ecotype	12, 331	1.85	**0.040**

Table 6: Summary of linear mixed effect models of biomass and fitness-related traits. Significant results are bolded.

Measurement	Fixed Effect	df (treatment, error)	F	P
Avg. Weight per Seed	Treatment	4, 14	0.64	0.642
	Cytotype	1, 111	309.89	**<0.001**
	Ecotype	3, 109	14.19	**<0.001**
	Treatment x Cytotype	4, 111	1.70	0.155
	Treatment x Ecotype	12, 109	0.99	0.462
	Cytotype x Ecotype	3, 111	3.87	**0.011**
	Treatment x Cytotype x Ecotype	12, 110	0.95	0.500
Seeds per Silique	Treatment	4, 13	3.01	0.060
	Cytotype	1, 120	129.05	**<0.001**
	Ecotype	3, 117	18.21	**<0.001**
	Treatment x Cytotype	4, 117	0.59	0.673
	Treatment x Ecotype	12, 116	1.46	0.148
	Cytotype x Ecotype	3, 119	4.25	**0.007**
	Treatment x Cytotype x Ecotype	12, 117	1.02	0.435
Fecundity	Treatment	4, 17	9.67	**<0.001**
	Cytotype	1, 301	22.92	**<0.001**
	Ecotype	3, 302	7.09	**<0.001**
	Treatment x Cytotype	4, 302	0.39	0.813
	Treatment x Ecotype	12, 301	1.23	0.261
	Cytotype x Ecotype	3, 302	7.31	**<0.001**
	Treatment x Cytotype x Ecotype	12, 301	1.27	0.234
Progeny Germination Rate	Treatment	4, 13	0.10	0.980
	Cytotype	1, 112	9.79	**0.002**
	Ecotype	3, 110	3.16	**0.028**
	Treatment x Cytotype	4, 112	1.28	0.283
	Treatment x Ecotype	12, 110	1.25	0.257
	Cytotype x Ecotype	3, 111	0.58	0.630
	Treatment x Cytotype x Ecotype	12, 111	1.01	0.449
Composite Fitness	Treatment	4, 17	14.52	**<0.001**

	Cytotype	1, 302	0.07	0.795
	Ecotype	3, 302	8.40	**<0.001**
	Treatment x Cytotype	4, 302	4.50	**0.002**
	Treatment x Ecotype	12, 302	1.76	0.053
	Cytotype x Ecotype	3, 302	5.76	**<0.001**
	Treatment x Cytotype x Ecotype	12, 301	1.40	0.165
Biomass	Treatment	4, 14	38.77	**<0.001**
	Cytotype	1, 320	0.59	0.445
	Ecotype	3, 320	9.00	<**0.001**
	Treatment x Cytotype	4, 319	4.06	**0.003**
	Treatment x Ecotype	12, 318	2.26	**0.009**
	Cytotype x Ecotype	3, 320	3.87	**0.001**
	Treatment x Cytotype x Ecotype	12, 319	1.08	0.375

Figure legends

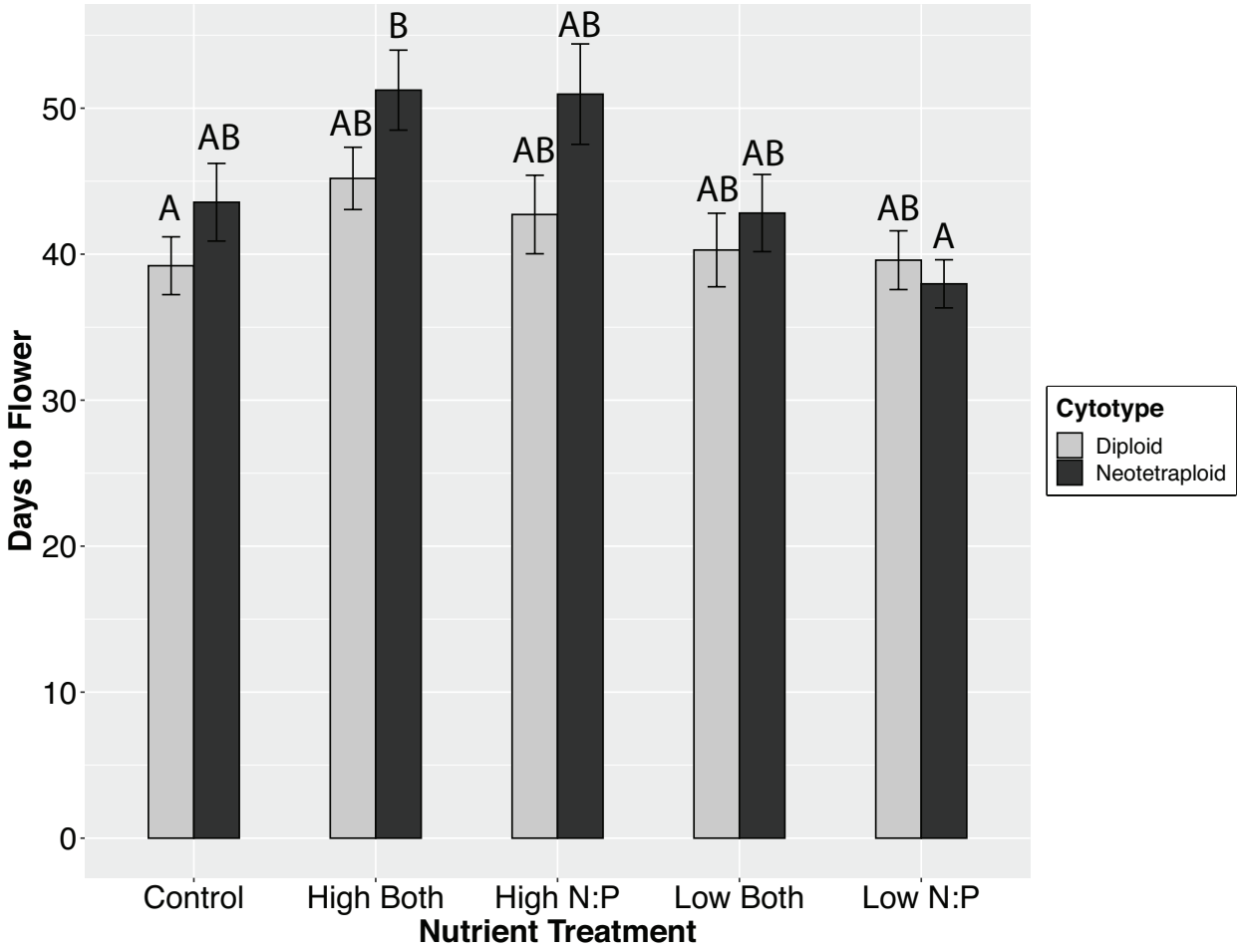

Figure 6 Days to first flowering for diploid and neotetraploid *Arabidopsis thaliana.* Grey bars represent diploids, and black bars correspond to neotetraploids (± standard error). Letters denote groupings of Tukey's post hoc tests of the nutrient supply by cytotype interaction, where groups that do not significantly differ at p=0.05 are marked with the same letter

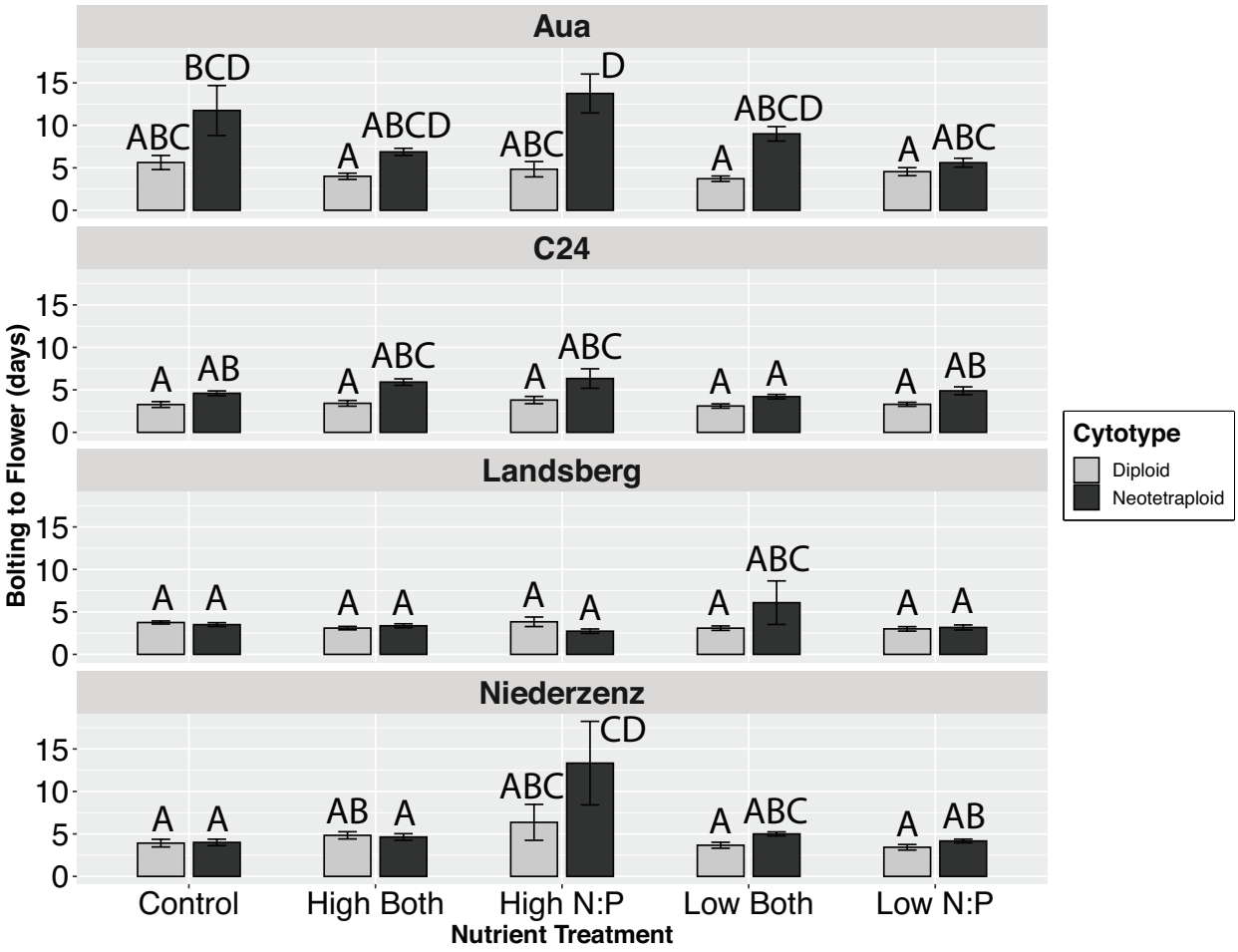

Figure 7 The latency in days between time of first bolting and first flowering. Each subpanel represents an ecotype or "independent origin" of neotetraploidy, with grey bars representing the diploid ancestor and black bars representing the neotetraploid offspring lineage (± standard error). Letters denote groupings of Tukey's post hoc tests of the ecotype by nutrient supply by cytotype interaction, where groups that do not significantly differ at $p \geq 0.05$ are marked with the same letter

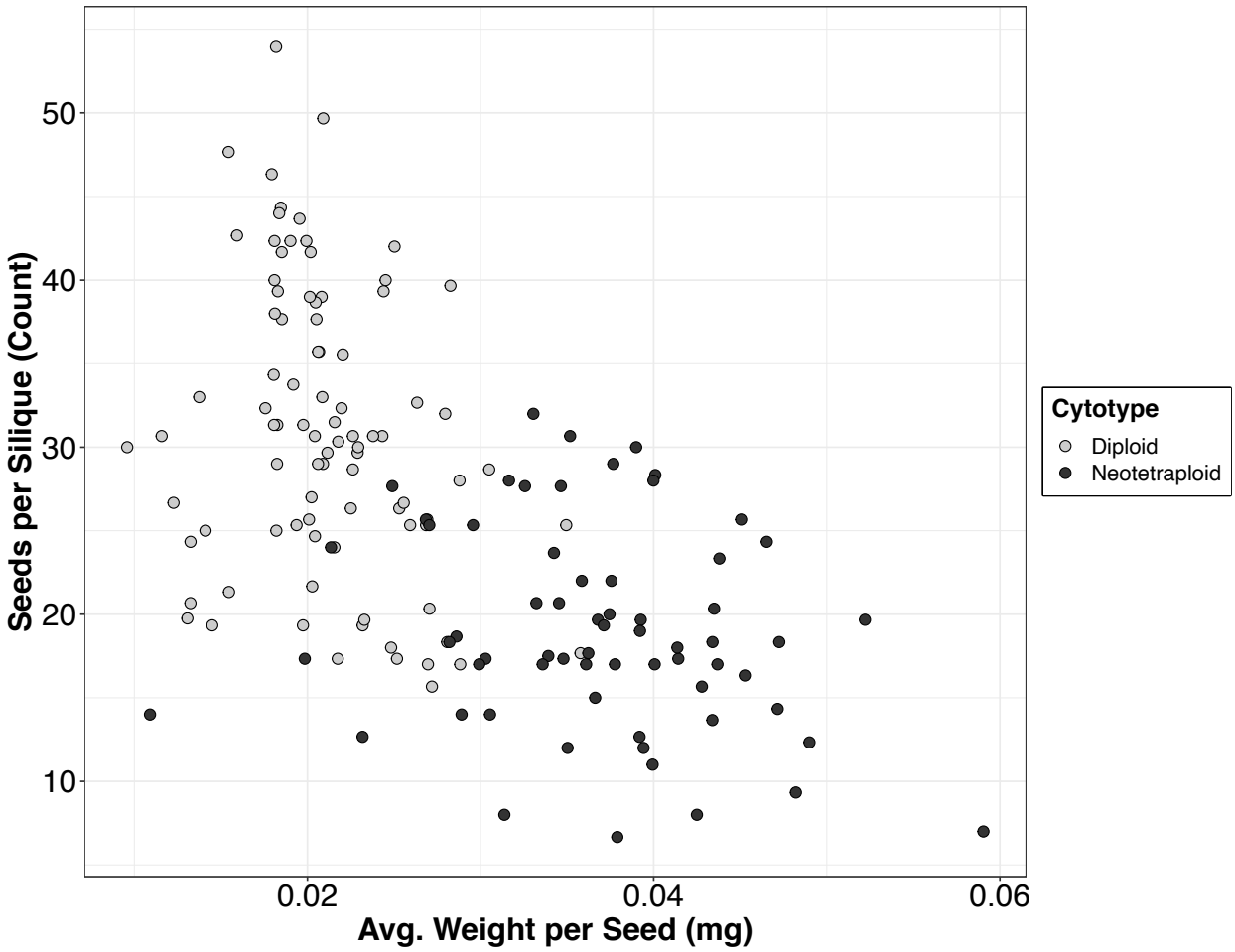

Figure 8 Scatterplot showing the effect of cytotype on the relationship between the average weight per seed and the number of seeds per silique. Grey dots indicate diploids and black dots neotetraploids.

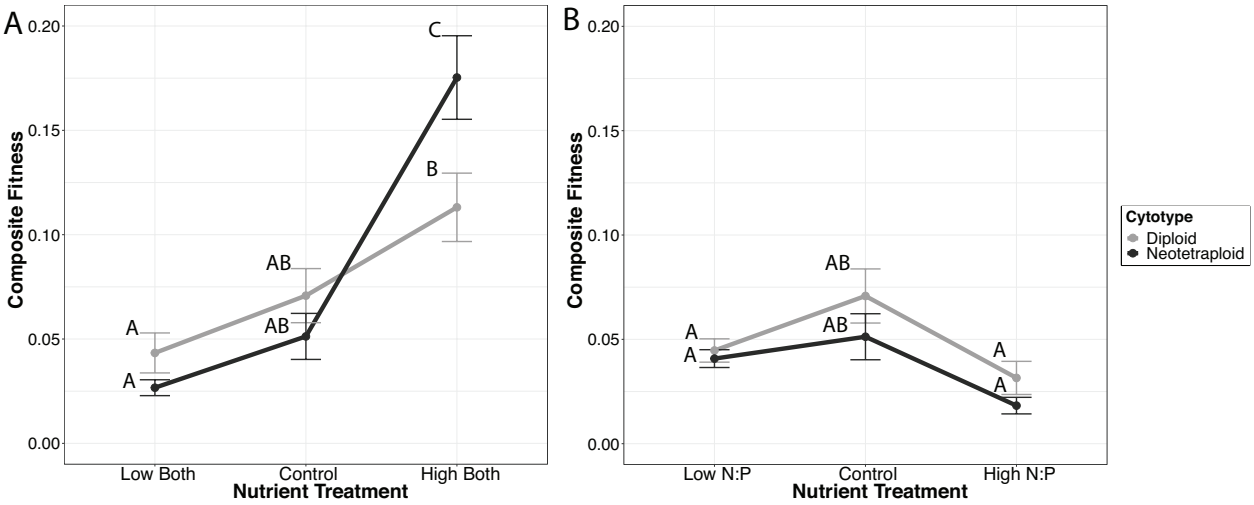

Figure 9 Reaction norm plots of composite fitness variation in response to nutrient supply. Balanced nutrient treatments (A) and adjusted stoichiometry treatments (B). Grey lines are diploids, and black lines are neotetraploids (± standard error). Letters denote groupings of Tukey's post hoc tests of the nutrient supply by cytotype interaction, where groups that are significant have different letters

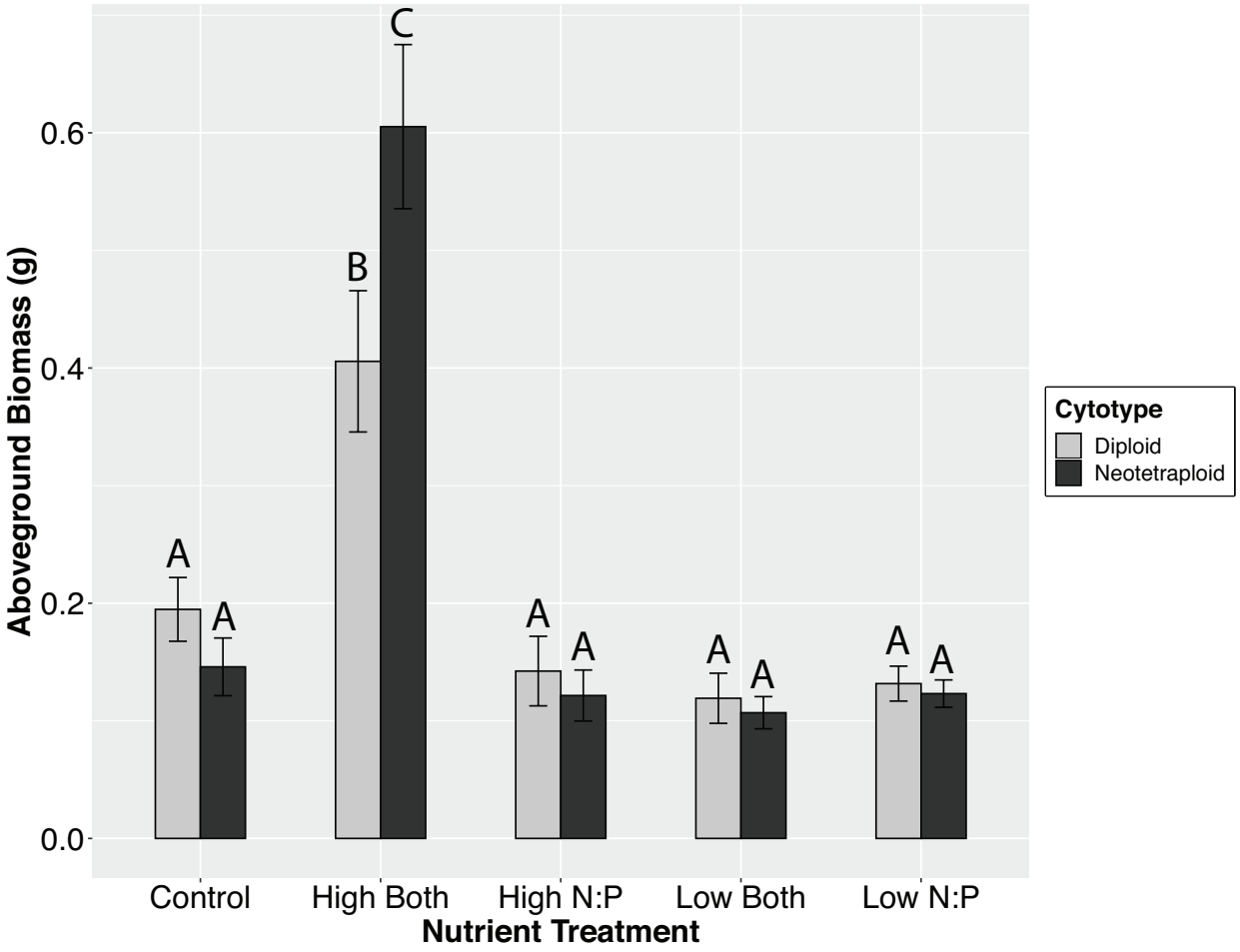

Figure 10 Aboveground biomass production of *Arabidopsis thaliana* diploids and neotetraploids across nutrient treatments. Grey bars indicate diploids and black bars neotetraploids (± standard error). Letters denote groupings of Tukey's post hoc tests of the nutrient supply by cytotype interaction, where groups that are significant have different letters

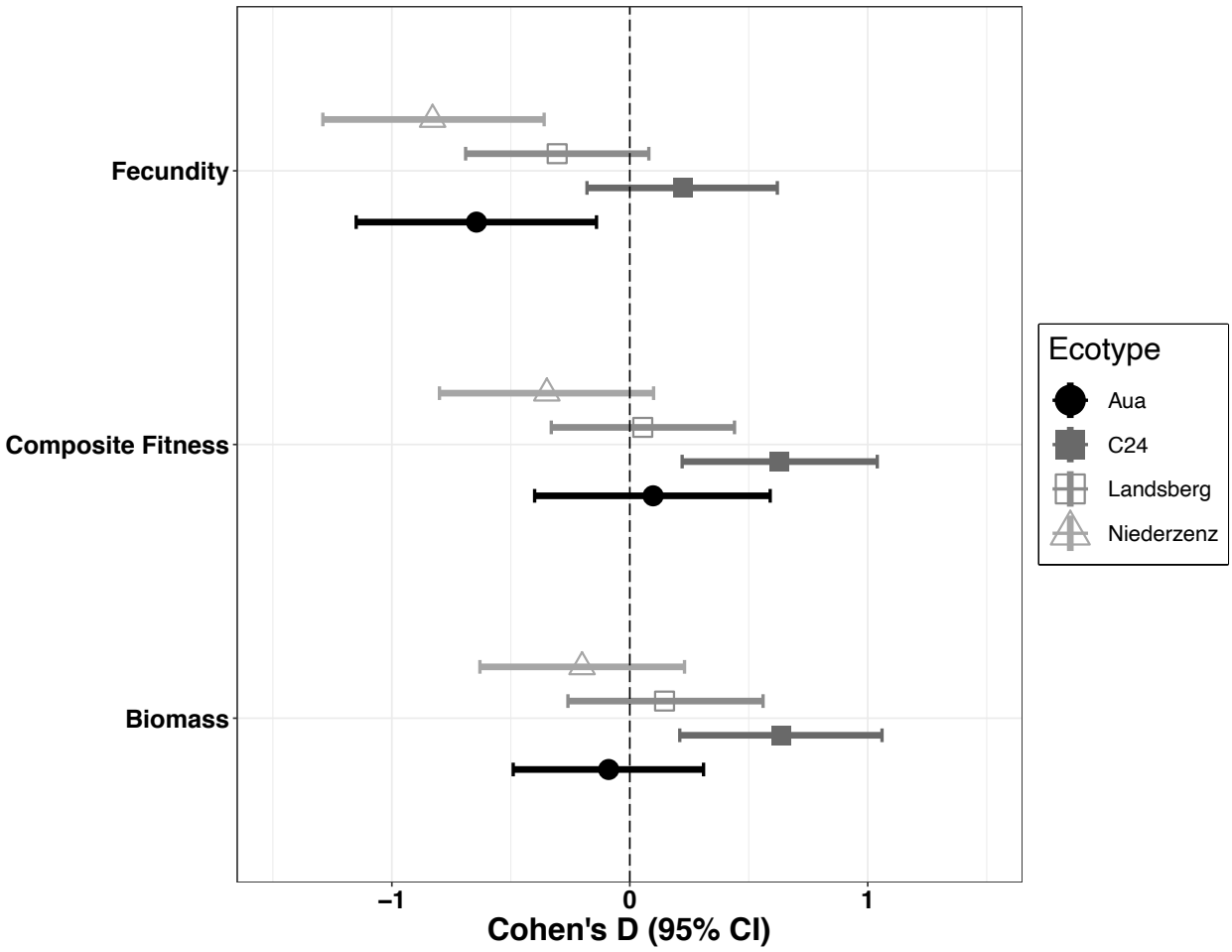

Figure 11. Effect of multiple origins of neotetraploidy. Cohen's D was determined as the difference between diploids and neotetraploids for each ecotype (origin). Dots represent the average Cohen's D measurement and bars denote the 95% confidence interval.

Table S5: Accession information for each *Arabidopsis thaliana* ecotype pair of diploids and neotetraploids acquired.

Ecotype	Background	Cytotype	notes	Germplasm Identifier	link
Niederzenz	Niederzenz, Germany	Diploid		CS69109	https://www.arabidopsis.org/servlets/TairObject?type=stock&id=4502158719
Niederzenz	Niederzenz, Germany	Neotetraploid	Synthetic Neotetraploid	CS69110	https://www.arabidopsis.org/servlets/TairObject?type=stock&id=4502158720
Landsberg	Landsberg, Germany	Diploid		CS69111	https://www.arabidopsis.org/servlets/TairObject?type=stock&id=4502158721
Landsberg	Landsberg, Germany	Neotetraploid	Synthetic Neotetraploid	CS69112	https://www.arabidopsis.org/servlets/TairObject?type=stock&id=4502158722
C24	Coimbra, Portugal	Diploid		CS69115	https://www.arabidopsis.org/servlets/TairObject?type=stock&id=4502158725
C24	Coimbra, Portugal	Neotetraploid	Synthetic Neotetraploid	CS69116	https://www.arabidopsis.org/servlets/TairObject?type=stock&id=4502158726
Aua	Rhon, Germany	Diploid		CS69121	https://www.arabidopsis.org/servlets/TairObject?type=stock&id=4502158731
Aua	Rhon, Germany	Neotetraploid	Synthetic Neotetraploid	CS69122	https://www.arabidopsis.org/servlets/TairObject?type=stock&id=4502158732

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Chapter 3: Neotetraploids are more tolerant to stress than their diploid progenitors

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Running Head: Neopolyploidy affects performance under stress

Abstract

Aims:

Early generation polyploids, or neopolyploids, are expected to be more tolerant to stressful growing conditions than their diploid parents, yet we have little evidence of how neopolyploidy and stressful environments affect plant functional traits or reproduction. In this study, we investigated how neotetraploidy, drought stress, and salt stress affects the growth of a stress tolerant perennial *Heuchera cylindrica* and reproduction in the ruderal annual *Arabidopsis thaliana*. We additionally tested if multiple origins of neopolyploidy affects variation in stress response.

Methods:

In a greenhouse experiment, we tested how neotetraploidy, independent origins of neotetraploidy, and stress affect performance. We used four independently derived neotetraploid *H. cylindrica* populations to assess how whole genome duplication impacts biomass allocation strategy and leaf functional trait variation since growth is a key measure of perennial plant performance. Similarly, we used four independently derived neotetraploid *A. thaliana* ecotypes to test how reproductive traits are affected by stress since reproductive effort is a strong determinant of performance in annual plants.

Results:

Although diploids outperformed their neotetraploid descendants under control conditions, diploids and their neotetraploid descendants often performed similarly when grown under drought or salt stress. Additionally, independently derived origins of neotetraploidy caused

significant variation in reproductive traits, whereas there was little variation in growth, biomass allocation, and leaf functional traits.

Conclusions:

We show that neotetraploidy causes *H. cylindrica* and *A. thaliana* to be more tolerant to drought and salt stress than their diploid progenitors. We also show that independently derived neopolyploid origins cause variation in the fitness responses of plants to stressful environments and that the effects of whole genome duplication may universally impact neopolyploids in terms of biomass allocation strategy and leaf functional traits. Together, these results suggest that stressful habitats may provide more favorable odds of establishment for neopolyploids in natural populations.

Keywords

Arabidopsis; Brassicaceae; drought stress; *Heuchera*; polyploidy; salt stress; Saxifragaceae; whole genome duplication

INTRODUCTION

Polyploidy, or the retention of additional chromosome sets from the previous parental generation, has long been recognized as an evolutionary mechanism that induces phenotypic novelty in plants (Ramsey and Schemske, 2002; Soltis et al., 2014). These phenotypic novelties are thought to facilitate shifts in niche space that may promote the otherwise poor odds of polyploid establishment in natural populations (Soltis et al., 2016). Indeed, numerous efforts have modeled polyploid establishment (Levin, 1975; Rodriguez, 1996; Fowler and Levin, 2016), and a recent study highlighted that a reduction in niche overlap between nascent polyploids, or "neopolyploids" and their diploid parents can substantially improve the probability of neopolyploid persistence through evolutionary time (Fowler and Levin, 2016). As a result, understanding how neopolyploidy affects plant trait responses to different environments, or their ecophysiology, can help us discern the ecological contexts that facilitate polyploid establishment.

One of the most widely cited effects of polyploidy on plant ecophysiology is that whole genome duplication improves the tolerance of plants to harsh abiotic conditions (Madlung, 2013). Most of the evidence supporting the idea that polyploidy improves stress tolerance comes from biogeographic studies on established polyploids. This work has shown that polyploids occur more frequently in harsh environments including more arid, colder, or disturbed habitats (Rothera and Davy, 1986; Lumaret et al., 1987; Brochmann et al., 2004; Manzaneda et al., 2012; Hao et al., 2013; Castro et al., 2020; Decanter et al., 2020). Another piece of evidence is that the timing of whole genome duplication events of established polyploids is correlated with periods of mass extinction, suggesting that whole genome duplication enhances stress tolerance (Fawcett et al., 2009; Levin and Soltis, 2018). Although we have good evidence that polyploidy promotes plant tolerance to stressful conditions, there remains the possibility that the established polyploid

ranges we observe today are the byproduct of subsequent evolution acting on polyploid lineages, rather than the direct effect of whole genome duplication. Rather than whole genome duplication itself improving stress tolerance during cataclysmic times, empty niche space filling due to mass extinction or directional selection following whole genome duplication may instead be driving the pattern of polyploid associations to stressful conditions. What is needed to reconcile these competing explanations are studies on early generation polyploids, or neopolyploids. Doing so would allow us to compare the performance of diploids and their neopolyploid progeny in stressful abiotic growing conditions and reveal if polyploidy *per se* affects the stress responses of plants.

Although few, there are several studies of neopolyploids that support the view that polyploidy increases plant tolerance to environmentally harsh conditions. For example, a transplant study found that neohexaploids of *Achillea borealis* derived from mesic tetraploid sites had higher survivorship than tetraploids when they were transplanted into the xeric range of established hexaploids (Ramsey, 2011). Since *A. borealis* survivorship was improved by neohexaploidy in the established hexaploid range, this suggests that the direct effect of polyploidy increased the tolerance of *A. borealis* to xeric conditions. Conversely, a drought assay on *Chamerion angustifolium* revealed that neotetraploidy did not alter drought tolerance as compared to diploids; however, established tetraploids took significantly longer to wilt, suggesting that drought tolerance evolved after whole genome duplication (Maherali et al., 2009). The contrasting results between *A. borealis* and *C. angustifolium* on polyploid-induced drought tolerance is likely driven by the fact that the two studies investigated inherently different traits (i.e., survival versus days to wilt). To resolve the effects of whole genome duplication on

plant stress response will require integrating more traits that contribute to lifetime fitness and growth.

In addition to water limitation studies on neopolyploids, we also have evidence that neopolyploidy enhances salt stress tolerance (Meng et al., 2011; Chao et al., 2013; Zhu et al., 2018). In an experiment on *Arabidopsis thaliana*, neotetraploids were able to survive longer and produce more seeds than diploids when grown with added sodium chloride, but the diploids had a greater reproductive effort than neotetraploids under control conditions (Chao et al., 2013). Similarly, *Brassica rapa* neotetraploids grew more than their diploid progenitors under salt stress (Meng et al., 2011). Taken together, there is compelling evidence that polyploidy promotes plant tolerance to salt stress, but it is unclear how salt stress affects the complex lifetime fitness of plants, given their relative life history strategy. That is, for annual plants in the above examples, the most meaningful performance measures for plants with this life history strategy are reproductive effort, whereas the performance of perennial plants is determined by growth, biomass allocation, and survival (Kozlowski, 1992).

Another often overlooked factor that may confound our understanding of the effect of polyploidy on plant stress response is the repeated formation of independent neopolyploid lineages. We know from previous work that multiple origins of neopolyploidy can strongly influence phenotypic variation in nascent polyploid populations (Soltis et al., 2004). For example, a recent study using multiple independent origins of neotetraploidy in *Fragaria vesca* found that trait responses to neotetraploidy were strongly dependent on polyploid origin (Wei et al., 2020). This suggests that the repeated formation of independent neopolyploid lineages can meaningfully affect trait variation in response to different environments. However, we do not know if multiple origins of polyploidy cause increased variation in the stress responses of

neopolyploid plants, or if neopolyploidy affects stress responses of plants in a universal way across independent origins of polyploidy.

To test how neopolyploidy and multiple origins affect plant stress responses, we used two plant species that differ strongly in life history traits: *Arabidopsis thaliana* (Brassicaceae) and *Heuchera cylindrica* (Saxifragaceae). The annual life cycle of *A. thaliana* makes it an ideal model species for assessing how reproductive traits are affected by neopolyploidy and stress. In contrast, the slow-growing perennial habit of *H. cylindrica* provides a complementary system in which to assess how growth, leaf functional traits and biomass allocation strategies are affected by neotetraploidy. These species are phylogenetically distant and therefore improves the likelihood that the commonalities observed between these diploid-neopolyploid species represent more general features of whole-genome duplication. Additionally, synthetic neotetraploids for both species were readily available (Solhaug et al., 2016; Anneberg and Segraves, 2020), alleviating the laborious effort of synthesizing neotetraploids *de novo.* Furthermore, multiple independent origins of neotetraploidy were available for both *A. thaliana* and *H. cylindrica*, allowing us to understand how the repeated formation of neotetraploidy affects trait variation in response to stressful growing conditions.

Here, we test how neotetraploidy in *H. cylindrica* and *A. thaliana* affects plant performance under drought and high salinity stress. We asked the following questions: 1) Does neotetraploidy improve growth and biomass allocation of plants under stressful conditions? 2) How are lifetime fitness-related reproductive traits affected by neotetraploidy and abiotic stress? 3) How do multiple independent origins of neotetraploidy affect the responses of plants to stressful conditions? To address these questions, we used *H. cylindrica* to assess how

neotetraploidy affects growth and biomass allocation patterns of plants and *A. thaliana* to track reproductive traits that strongly influence plant fitness.

MATERIALS AND METHODS

Study organisms and growth conditions

To assess how neopolyploidy affects plant performance under stressful environments, we conducted separate, parallel experiments on *H. cylindrica* and *A. thaliana*. Seed stocks of *H. cylindrica* were the same as those used in Anneberg and Segraves (2020), consisting of 12 untreated diploid, 12 putative neotetraploid, 12 treated diploid (failed neotetraploid induction), and 4 established tetraploid seed stocks. Seeds of diploid and established tetraploid plants were collected from the Pacific Northwest of the United States (Fig. S1). The 'treated diploid' and 'putative neotetraploid' *H. cylindrica* seeds were produced by treating hand-pollinated flowers with pressurized nitrous oxide for approximately 32 hours (Anneberg and Segraves, 2020). This arrests the first division of the zygote, creating a subset of neotetraploid seeds.

For *A. thaliana,* the seeds originated from four ecotypes that each represent a diploid maternal lineage and their corresponding neotetraploid descendants that were synthesized using colchicine as described by Solhaug et al. (2016). We acquired these seeds from the Arabidopsis Biological Resource Center (ABRC, Columbus, Ohio: Table S1). Although we acknowledge that including colchicine-treated diploids as well as established tetraploids of *A. thaliana* would enhance the experimental design, we were unable to acquire treated diploids, and chose not to include established tetraploids because they were geographically disparate from the diploids. Geographic isolation may confound any observable patterns in the established tetraploid cytotype.

We germinated *H. cylindrica* seeds by planting them in nursery trays filled with autoclaved quartz sand. We misted the seeds daily, until the seedlings began to form their first true leaves. At this point, we transplanted the seedlings into individual 311.5 cm^3 pots filled with autoclaved quartz sand, such that there was one plant per pot. The pots were randomly placed within nursery trays that held either 20 or 21 pots per tray, and these trays were rotated twice per week to avoid microclimatic effects within the greenhouse. Throughout the course of the experiment, each tray was rotated such that they occupied all possible positions in the greenhouse at least once. We encouraged seedling establishment by supplying a modified Hoagland's fertilizer solution to the nursery trays for two weeks after transplantation. Plants were grown in a greenhouse with a 21–24ºC daytime and 15–18ºC nighttime and a 16:8 L:D cycle. The plants were transplanted in late August 2019 and harvested in December 2019.

Seeds of *A. thaliana* were directly sowed into individual 108 cm^3 pots filled with autoclaved quartz sand and placed in nursery trays, with 24 pots per nursery tray. The volume of the pots is suitable for A. thaliana (Pacey et al., 2020) and allowed us to maximize our sample size in the greenhouse. To encourage plant establishment, a modified Hoagland's fertilizer solution was supplied to each nursery tray for two weeks prior to thinning the plants. We thinned the germinated *A. thaliana* seedlings to one plant per pot when the first true leaves began to emerge, and we could apply treatments. We conducted this experiment in a greenhouse with 21–24ºC daytime and 18–21ºC nighttime, under ambient light conditions. The *A. thaliana* were planted in late May 2019, and the plants were harvested in early September 2019.

Heuchera cylindrica **stress assay**

We investigated how neotetraploidy affected performance of *H. cylindrica* under stress by applying drought and salt treatments to plants in a greenhouse growth assay. As a control, we supplied 1.25 L of a Hoagland's fertilizer solution (Table S2) to nursery trays on a weekly basis. Salt stress treatments were applied to plants by including 50 mM sodium chloride in the control fertilizer solution that was supplied to the nursery trays. After fertilizer solutions were supplied to the nursery trays, we waited for a five-day dry down period before emptying the trays and adding 1.25 L of deionized water to the control and salinity stress treatment plants. Plants in the drought treatment were not provided with deionized water, such that they only received the fertilizer solution each week.

In total, we grew 428 plants in 21 nursery trays (20-21 plants per tray). Of the 21 nursery trays, 9 trays had plants that were controls, 6 trays had plants that received drought, and 6 trays had plants that received salt treatment. We assessed biomass allocation patterns and functional traits of all plants at harvest by measuring total biomass, aboveground and belowground biomass allocation, specific leaf area (SLA), leaf dry matter content (LDMC), and leaf chlorophyll content. At the time of harvest, we separately measured the biomass of aboveground tissues, fine roots, and rhizomes to assess the relative biomass allocation patterns of plants. We measured SLA as the fresh leaf area divided by its corresponding dry biomass, and LDMC was measured as leaf fresh mass divided by its corresponding dry mass. Chlorophyll content of leaves was measured by taking the average of three leaf chlorophyll readings from sections of an individual leaf that were free of major leaf veins.

Because we used first-generation, putatively neotetraploid seed stocks of *H. cylindrica,* we assigned a confirmed cytotype to each plant using a qualitative morphological survey. This survey consisted of a binary scoring matrix of four leaf and stem traits that were previously

found to strongly correlate with neotetraploidy in this species (Anneberg and Segraves, 2020). The traits that correlated with neotetraploidy were scored as a "1" and were: having a thick leaf, increased length of lobes at the leaf base, having a thick stem, and leaf curling. Diploid traits were scored as "0" and corresponded with thin leaves, reduced lobes at the leaf base, thin stems, and no leaf curling. We summed the binary scores and any putatively neotetraploid plant that received a total score of 3 or 4 was assigned as neotetraploid. Plants with a score of 0-2 were considered failed neotetraploid inductions, or "treated diploid" (Appendix S1).

Arabidopsis thaliana stress assay

We used *A. thaliana* to assess how reproductive traits were affected by both neotetraploidy and stressful abiotic conditions. We supplied each nursery tray with 1.5 L of a Hoagland's fertilizer solution (Table S2) on a weekly basis and grew the plants under constant irrigation. We avoided the harmful accumulation of micronutrients in the trays by emptying their contents every four days and refilling the trays with deionized water for three days before adding more fertilizer. We applied salinity stress to 96 plants by including 50 mM of sodium chloride in the fertilizer solution. The drought treatment was applied as an intense, one-week episodic drought, in which we waited until a majority of the plants began to bolt before applying treatment. Drought was applied by removing the fertilizer solution and leaving the pots to dry for one week. With the exception of the one-week drought, we treated the plants that received drought treatment identical to the controls.

In total, we grew 288 plants in 12 trays (24 pots per tray), comprising 96 plants per stress treatment with four trays of plants per treatment. At the time of harvest, three ripe and intact siliques were harvested from a subset of the plants so that we could estimate the average number

of seeds produced per silique as well as the average mass per seed from plants at each treatment level (cytotype, stress treatment, and ecotype). In total, we collected siliques from 100 plants across treatment levels. The siliques were dried, and the seeds were counted, weighed, and averaged per silique. From the average number of seeds per silique for each treatment level, we estimated the total fecundity of plants by counting the total number of ripe siliques produced per plant and multiplying that count by the corresponding average seeds per silique. The average germination rate of progeny was also assessed in a subset of ~30 seeds per plant. Seeds were surface sterilized with ethanol and transferred to sterile filter paper moistened with deionized water, and germination rates were assessed after two weeks. We estimated a composite lifetime fitness measure for the plants following Campbell (1991) that was the multiplicative product of fecundity, average mass per seed, and the average germination rate of progeny.

Statistical Analysis

The data from the *H. cylindrica* and *A. thaliana* experiments were analyzed separately. For traits with continuous numeric measurements, we constructed full factorial linear mixed effect (lme) models with cytotype, stress treatment, maternal origin, and the interaction terms as main effects, and tray as a random effect. Models were constructed using the lme4 package (Bates et al., 2015) with R software (R Core Team, 2019). To discern the relative differences among treatment groups, we carried out Tukey HSD post hoc analyses. For *A. thaliana,* we considered the four ecotypes as independent origins, whereas the four populations of *H. cylindrica* were considered independent origins. Because the established tetraploids of *H. cylindrica* were collected from disparate populations from the diploids, we excluded them from the models that incorporated the main effect of 'population' to test multiple origins. The treated

diploids that failed to become neotetraploid in the *H. cylindrica* dataset were only used to confirm that there were no trans-generational effects of nitrous oxide on the plant traits that we measured, and these data were otherwise excluded from the analysis.

RESULTS

Confirmation of neotetraploidy and effect of nitrous oxide on *H. cylindrica*

The qualitative morphological survey revealed that there were 102 confirmed neotetraploids, with 29 of those plants in the control treatment, 39 treated with drought, and 34 treated with high salinity (Appendix S1). The remaining 64 putative neotetraploids that scored a 2 or less were reassigned as treated diploids. We did not detect an effect of nitrous oxide gas on any of the *H. cylindrica* traits (Table S3), and therefore conducted the remaining analyses without corrections.

Effect of stress and neotetraploidy on *H. cylindrica*

The stress treatments had a more negative effect on diploids and established tetraploids than neotetraploids (Table 7; Fig. 12). When we considered the distribution of total biomass investment into aboveground tissues, we did not detect an interaction between stress treatment and cytotype on aboveground tissue allocation patterns (Table 7); however, neotetraploids allocated significantly more biomass into aboveground structures than diploids. Stress treatments caused diploids and established tetraploids to reduce their relative biomass allocation to rhizomes, but stress did not affect neotetraploid rhizome biomass allocation (Table 7; Fig. 12). For biomass allocation to fine roots, salt treatment caused a significant increase in diploid and

established tetraploid fine root allocation but did not affect neotetraploid fine root production (Fig. 12).

In comparison to the drastic effect of cytotype and stress treatment on *H. cylindrica* biomass allocation, leaf functional traits were only responsive to stress treatment (Table 8). Although we observed a significant interaction between cytotype and stress treatment on LDMC (Table 8), this pattern was driven by neotetraploids having slightly lower LDMC values in control conditions compared to no differences among cytotypes in stress treatments. For leaf chlorophyll and SLA, only stress treatment had a significant effect on variation in those traits (Table 8), where drought and salt stress caused an increase in SLA, and a decrease in leaf chlorophyll content.

Effect of neotetraploidy and stress on *A. thaliana*

Neotetraploidy, stress, and independent neopolyploid origins together had strong effects on many reproductive traits. For instance, we found that neotetraploidy caused plants to produce significantly heavier seeds, whereas salt treatment caused a reduction in seed mass (Fig. 13). Although we did not detect an effect of stress treatment on the average number of seeds per silique, we did observe that neotetraploids produced significantly fewer seeds per silique than diploids (Fig. 13) and also that diploids from the C24 independent neopolyploid origin produced fewer seeds per silique than diploids from the other three origins (Table 9). There was no effect of cytotype or stress treatment on germination rates (Table 9).

When considering fecundity and composite fitness, we identified a three-way interaction between cytotype, stress, and ecotype of origin (Table 9). This three-way interaction was driven by neotetraploids producing fewer seeds than diploids in the Aua ecotype when drought

treatment was applied (Fig. S2). The interaction between cytotype, stress, and ecotype of origin on composite fitness was caused by neotetraploids from the Aua and C24 neopolyploid origins having greater composite fitness under control conditions, but neotetraploids from the Landsburg and Niederzenz neopolyploid origins having lower composite fitness than diploids under control conditions (Fig. 14). Similarly, independent neopolyploid origins defined the response of *A. thaliana* neotetraploids to salt treatment, where salt stress caused neotetraploid composite fitness to be lower than diploids for the C24 ecotype of origin, whereas neotetraploid composite fitness was greater than diploids with salt stress in the Landsberg ecotype (Fig. 14).

Effect of multiple neotetraploid origins on stress responses

For *A. thaliana,* ecotype of origin, cytotype, and stress treatment interacted on fecundity and composite fitness (Table 9), in which the tolerance of an ecotype to stress treatment was improved by neotetraploidy in two origins and became worse or not different for the other two ecotypes (Fig. 14). Additionally, cytotype and ecotype of origin often interacted on all the other reproductive traits except for germination rate of *A. thaliana* (Table 9). Although the reproductive traits of the four ecotypes generally responded to neotetraploidy in the same direction, there was substantial variation among the ecotypes in their relative magnitude of increase or decrease in a trait with neotetraploidy. In contrast to *A. thaliana,* independent neopolyploid origins in *H. cylindrica* were only significantly different for aboveground biomass allocation (Table S4). For all other leaf functional traits and biomass-related traits there was a consistent response to neotetraploidy and stress treatment.

DISCUSSION

Polyploidy is expected to improve the tolerance of plants to harsh abiotic conditions, but the majority of evidence that we have supporting this idea stems from studies on established polyploids. The few studies that have compared the stress responses of neopolyploids to their diploid ancestors have shown that neopolyploidy can either improve stress tolerance (Meng et al., 2011; Ramsey, 2011; Chao et al., 2013; Zhu et al., 2018) or has no affect (Maherali et al., 2009). In order to understand how stress affects the odds of neopolyploid establishment, we need studies that assess how traits that are associated with complex lifetime fitness respond to harsh abiotic conditions. To address this, we investigated how neotetraploidy, drought, and salt stress affects the growth, biomass allocation, and leaf functional traits responses of *H. cylindrica* and lifetime fitness traits of *A. thaliana*. We additionally incorporated multiple independent origins of neotetraploidy in order to understand if whole genome duplication has universal effects on traits or if there is variability based on maternal origin.

We observed that neotetraploids were more tolerant to drought and salt stress than their diploid progenitors. Specifically, when we compared the responses of diploids and neotetraploids to drought or salt stress, the neotetraploids were less negatively affected by stress treatments. For example, the growth of diploids and established tetraploids of *H. cylindrica* outperformed the neotetraploids under control conditions, but when either salt or drought stress was applied, the diploids and established tetraploids experienced a strong reduction in total biomass production whereas the neotetraploids had similar measures as control plants (Fig. 12). We found similar patterns in *A. thaliana*, in which the composite fitness of diploids was often more labile to salt or drought stress than their neotetraploid descendants, although this pattern varied among independent neopolyploid origins (Fig. 14). Together, the results indicate that neotetraploids were generally less susceptible to stressful growing conditions than their diploid progenitors,

suggesting that neopolyploid establishment may be more favored in marginal habitats with harsh abiotic conditions.

Despite previous work suggesting that neotetraploidy could cause *H. cylindrica* to be more susceptible to stress (Anneberg and Segraves, 2020), we found that neotetraploidy promoted the tolerance and ability of *H. cylindrica* to avoid stressful conditions. A previous study found that neotetraploidy causes *H. cylindrica* to shift towards a more resource-acquisitive growth strategy (Anneberg and Segraves 2020), suggesting that neotetraploids should be more sensitive to abiotic stress (Grime, 1977; Reich, 2014). In contrast, we observed that neotetraploid performance was less affected by drought and salt stress than were diploids and established tetraploids (Fig. 12). This unexpected result may be explained by neotetraploids allocating less biomass to fine root production than the other two cytotypes, regardless of stress treatment (Fig. S3). Thus, the belowground drought and salt stresses that were applied may have affected neotetraploids differently than diploids. Since neotetraploids innately allocated more of their biomass aboveground (Fig. S3) – as indicated by their growth under control conditions – this trait may have allowed neotetraploid *H. cylindrica* to avoid salt stress, because they had less fine root material in physical contact with the saline solutions. Furthermore, salt and drought stress have previously been found to increase fine root proliferation in the soil as a means of plastically overcoming a water or mineral nutrient deficiency (Ali et al., 1999; Arif et al., 2019), but this may further exacerbate the negative consequences of salt or drought by having a greater proportion of the total biomass in direct contact with the belowground stressor. In *H. cylindrica,* we observed a pattern of increased allocation to fine root production in response to drought and salt treatment, but neotetraploid fine root production did not increase as much as that observed in diploids and established tetraploids (Fig. 12). We therefore conclude that the increased

aboveground biomass allocation and reduced fine root allocation pattern caused by neotetraploidy can serve as an avoidance strategy to salt stress, since the neotetraploids performed similarly under control and stress conditions.

Another surprising result observed in *H. cylindrica* was that there was a general lack of leaf functional trait responses to neotetraploidy in this species. Despite our expectation that neotetraploidy and stress treatment would interact, we only detected an effect of cytotype on one of the three leaf functional traits (Table 8). The reason why neotetraploidy did not affect leaf chlorophyll and SLA in *H. cylindrica* may be due in part to the control nutrient solution used in this experiment. A previous study assessing the responses of these leaf functional traits to nutrient addition and neotetraploidy found that they were primarily only responsive to high nutrient addition (Anneberg and Segraves 2020). The control fertilizer solution that was supplied to *H. cylindrica* in this study, then, may not have been sufficiently enriched to elicit a strong response to neotetraploidy across stress treatments. However, the control fertilizer solution we used was based on plant-available nitrogen and phosphorus concentrations from field sites in the spring, so the lack of effect of neotetraploidy on the stress responses of leaf chlorophyll and SLA would likely hold in natural settings. The only leaf functional trait that cytotype and stress treatment interacted on was LDMC, where drought caused equivalent LDMC responses between diploids and neotetraploids, compared to neopolyploids having lower LDMC under control conditions (Table 8; Fig. S2). Since LDMC correlates positively with leaf lifespan and the ability of plants to conserve resources (Grime et al., 1997), the equalizing force that drought had on diploid versus neotetraploid suggests that droughted environments can improve the odds of neotetraploids to become established.

The reproductive traits of *A. thaliana* responded to neotetraploidy similar to *H. cylindrica* growth, where they both became more tolerant, but the apparent improvement of *A. thaliana* reproductive stress tolerance may have been driven by the increase in individual seed mass. Although the mass of individual seeds did not respond to stress treatment, neotetraploidy caused individual seeds of *A. thaliana* to become significantly heavier than their diploid progenitors (Fig. 13). The neotetraploid-induced increase in seed mass likely improves their odds of becoming established in stressful habitats, since an increased biomass investment into individual seeds is often viewed as adaptive due to improved offspring vigor (Dalling and Hubbell, 2002; Moles and Westoby, 2004). In addition to having a greater mass per seed, neotetraploidy also caused plants of *A. thaliana* to produce fewer seeds per silique and total seed set (Table 9). The apparently greater reproductive effort of diploids is indicative of a greater colonization ability, although the heavier seeds of neotetraploids suggests that the subsequent polyploid seedlings may be more robust to stress (Muller-Landau, 2010). We find modest support for this idea, since the composite fitness of diploids and neotetraploids generally became equivalent when drought or salt stress was applied.

Although both *H. cylindrica* and *A. thaliana* generally became more tolerant to drought and salt stress with neotetraploidy, we also detected strong variation in the response of *A. thaliana* traits among the independently derived origins of neotetraploidy. Specifically, with the exception of progeny germination rates, we found that all of the reproductive trait responses to neotetraploidy varied based on their independent origin (ecotype). The strong contingency of the composite fitness response of *A. thaliana* on the independent ecotype of origin to stress and neotetraploidy complicates the patterns we observed, since neotetraploidy either caused an increased or decreased tolerance to drought and salt stress, depending on the ecotype of origin

(Fig. 14). In fact, the composite fitness of neotetraploids from the Aua and C24 ecotypes were marginally greater than diploids when grown under control conditions but became worse or not different from diploids when stress was applied. The dependency of ecotype of origin on *A. thaliana* fitness responses to neotetraploidy and stress treatment suggests that the investment into reproductive effort may be particularly labile to the genetic background of the maternal plant. These results are in stark contrast to those for *H. cylindrica,* which did not have any effect of independent origin on biomass traits. The contrasting effect of independent origin of neotetraploidy on *A. thaliana* versus *H. cylindrica* may be due to differences in evolutionary forces between these species. However, another possibility that explains the discrepancy of the effect of independent origin on *A. thaliana* versus *H. cylindrica* is that biomass-related traits may be universally affected by neotetraploidy compared to the more labile reproductive traits. A future multi-year experiment that measures the reproductive effort of the slow-growing perennial *H. cylindrica* would reveal if a similar effect of multiple origins on reproduction would hold true.

CONCLUSIONS

The results from this study show that neopolyploidy can promote the tolerance of plants to stressful abiotic conditions, as indicated by the more tolerant growth and fitness responses of neotetraploids across stress treatments. Thus, we find broad support for the hypothesis that stressful habitats would represent environments more favorable to neopolyploid establishment. We additionally show that multiple independent origins can affect the reproductive responses of neopolyploid plants to salt and drought stress. However, growth and belowground biomass allocation traits were consistent across multiple independent origins, suggesting that

neotetraploidy has universal effects on these traits. Taken together, reproductive traits are likely to be more labile to multiple origins of neopolyploidy, and the repeated formation of neopolyploid lineages may thus allow for selection to act more strongly across heterogeneous environments for reproductive effort. This study underscores the need to assess how multiple independent origins affect trait variation and plant ecophysiological responses to neopolyploidy.

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AUTHOR CONTRIBUTIONS

TJA and KAS designed the experiment. TJA planted and carried out the experiment. TJA wrote the first draft of the manuscript, and TJA and KAS edited subsequent drafts.

DATA AVAILABILITY

All associated data files of this manuscript will be made publicly available in an online data repository such as Dryad or ResearchGate.

Tables and Figure Legends

Table 7: Summary of linear mixed effect models of growth and biomass allocation traits in *Heuchera cylindrica*. Significant results are bolded.

Measurement	Fixed Effect	df (treatment, error)	F	P
Total Biomass	Stress	2, 25	37.32	**<0.001**
	Cytotype	2, 205	7.89	**<0.001**
	Stress x Cytotype	4, 205	5.73	**<0.001**
Percent Aboveground Mass	Stress	2, 22	1.86	0.180
	Cytotype	2, 204	12.86	**<0.001**
	Stress x Cytotype	4, 204	2.07	0.085
Percent Rhizome Mass	Stress	2, 23	24.89	**<0.001**
	Cytotype	2, 206	5.29	**0.006**
	Stress x Cytotype	4, 206	5.82	**<0.001**
Percent Fine Root Mass	Stress	2, 22	17.88	**<0.001**
	Cytotype	2, 204	6.95	**0.001**
	Stress x Cytotype	4, 204	2.30	0.060

Table 8: Summary of linear mixed effect models of leaf functional traits in *Heuchera cylindrica*. Significant results are bolded.

Measurement	Fixed Effect	df (treatment, error)	F	P
Chlorophyll Content	Stress	2, 28	33.15	**<0.001**
	Cytotype	2, 217	0.88	0.414
	Stress x Cytotype	4, 216	0.96	0.433
LDMC	Stress	2, 28	7.43	**0.003**
	Cytotype	2, 211	1.95	0.144
	Stress x Cytotype	4, 211	2.56	**0.0395**
SLA	Stress	2, 27	8.31	**0.002**
	Cytotype	2, 213	0.38	0.688
	Stress x Cytotype	4, 213	0.29	0.885

Table 9: Summary of linear mixed effect models of leaf reproductive traits in *Arabidopsis thaliana*. Significant results are bolded.

Measurement	Fixed Effect	df (treatment, error)	F	P
Weight per Seed	Stress	2, 7	4.84	**0.046**
	Cytotype	1, 72	144.67	**<0.001**
	Ecotype	3, 71	10.02	**<0.001**
	Stress x Cytotype	2, 72	0.46	0.631
	Stress x Ecotype	6, 71	1.76	0.120
	Cytotype x Ecotype	3, 69	5.00	**0.003**
	Stress x Cytotype x Ecotype	6, 69	0.46	0.832
Seeds per Silique	Stress	2, 6	0.77	0.505
	Cytotype	1, 71	80.18	**<0.001**
	Ecotype	3, 69	6.83	**<0.001**
	Stress x Cytotype	2, 71	1.29	0.282
	Stress x Ecotype	6, 69	2.60	**0.025**
	Cytotype x Ecotype	3, 68	3.98	**0.011**
	Stress x Cytotype x Ecotype	6, 68	1.24	0.299
Progeny Germination Rate	Stress	2, 7	1.81	0.231
	Cytotype	1, 70	2.55	0.115
	Ecotype	3, 69	2.86	**0.043**
	Stress x Cytotype	2, 70	0.46	0.635
	Stress x Ecotype	6, 69	0.31	0.931
	Cytotype x Ecotype	3, 68	0.66	0.582
	Stress x Cytotype x Ecotype	6, 68	0.65	0.688
Fecundity	Stress	2, 9	3.13	0.093
	Cytotype	1, 192	23.15	**<0.001**
	Ecotype	3, 191	4.89	**0.003**
	Stress x Cytotype	2, 192	0.33	0.721
	Stress x Ecotype	6, 191	0.85	0.531
	Cytotype x Ecotype	3, 191	2.25	0.084
	Stress x Cytotype x Ecotype	6, 191	2.65	**0.017**
Composite Fitness	Stress	2, 9	3.18	0.090
	Cytotype	1, 191	4.66	**0.032**

	Ecotype	3, 191	13.83	**<0.001**
	Stress x Cytotype	2, 191	0.57	0.569
	Stress x Ecotype	6, 191	1.06	0.386
	Cytotype x Ecotype	3, 191	1.14	0.334
	Stress x Cytotype x Ecotype	6, 191	2.43	**0.027**

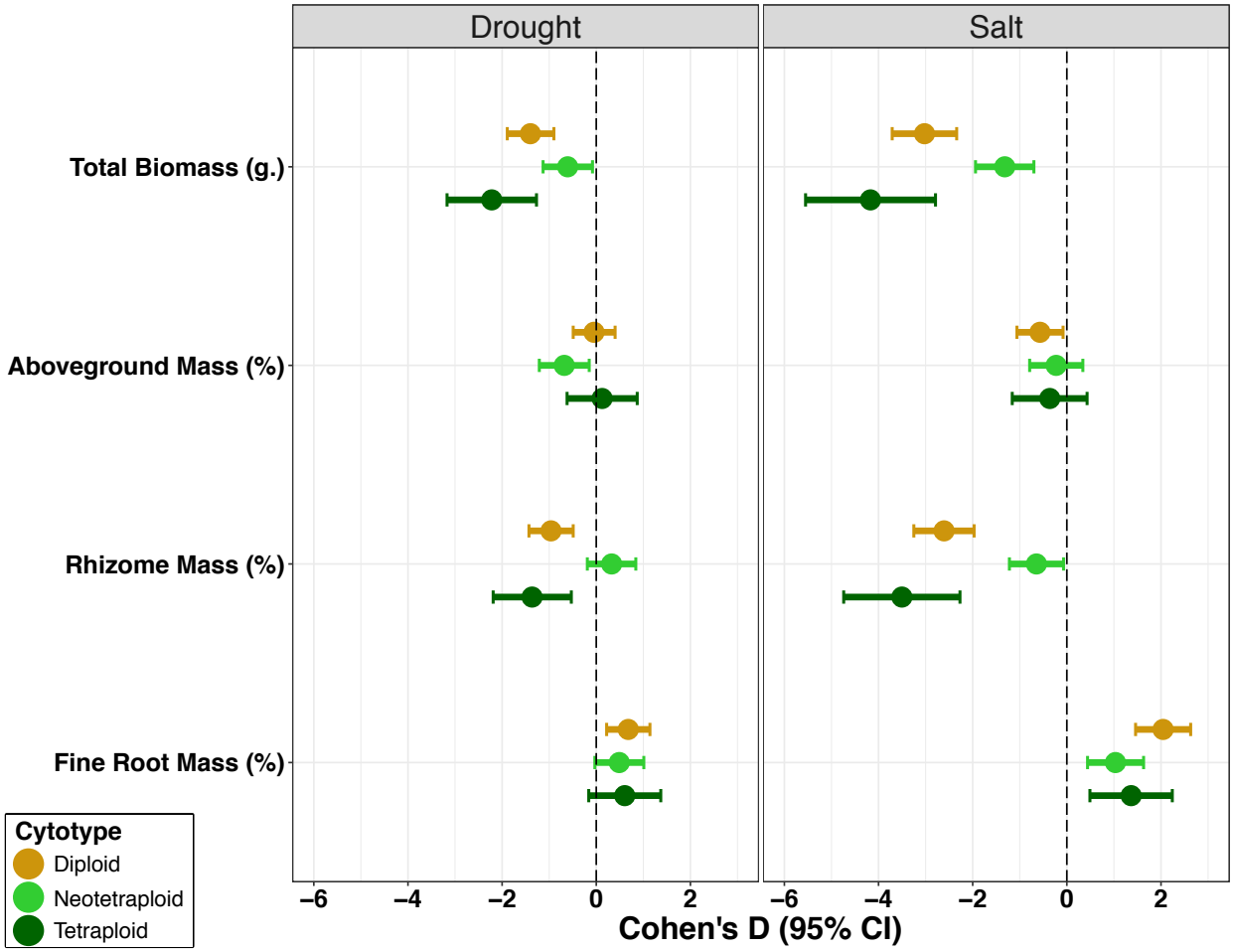

Figure 12. The effect of cytotype and stress on total biomass and allocation responses in *Heuchera cylindrica*. Cohen's D was calculated as the difference of each cytotype response to either drought or salt stress relative to control conditions. The dots are the average Cohen's D measurement, and the bars are 95% confidence intervals.

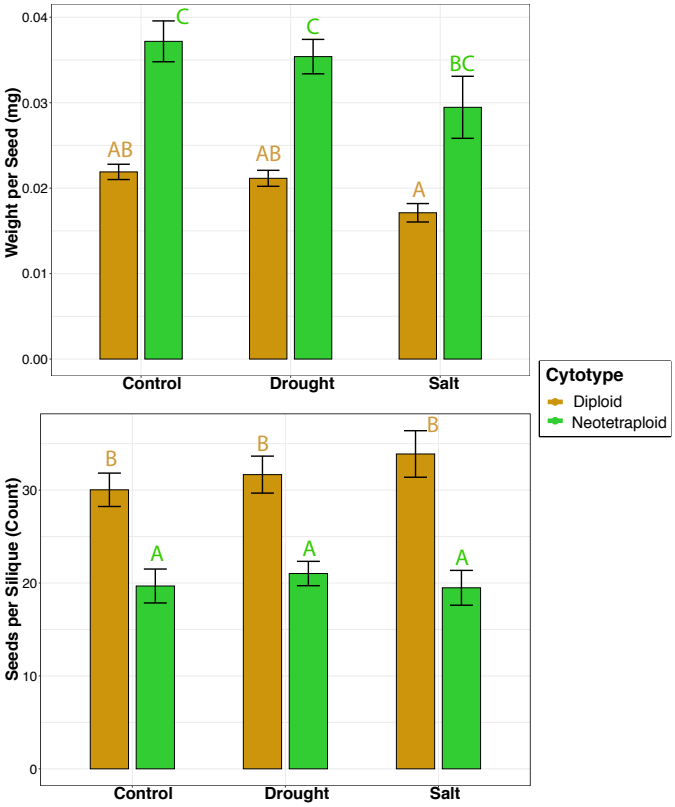

Figure 13. Seed traits of *Arabidopsis thaliana* diploids and neotetraploids in response to stress. Gold bars are diploids and green bars are neotetraploids, representing the average composite fitness response ± standard error. Letters denote groupings of Tukey's post hoc tests of the ecotype by nutrient supply by cytotype interaction, where groups that do not significantly differ at $p \geq 0.05$ are marked with the same letter.

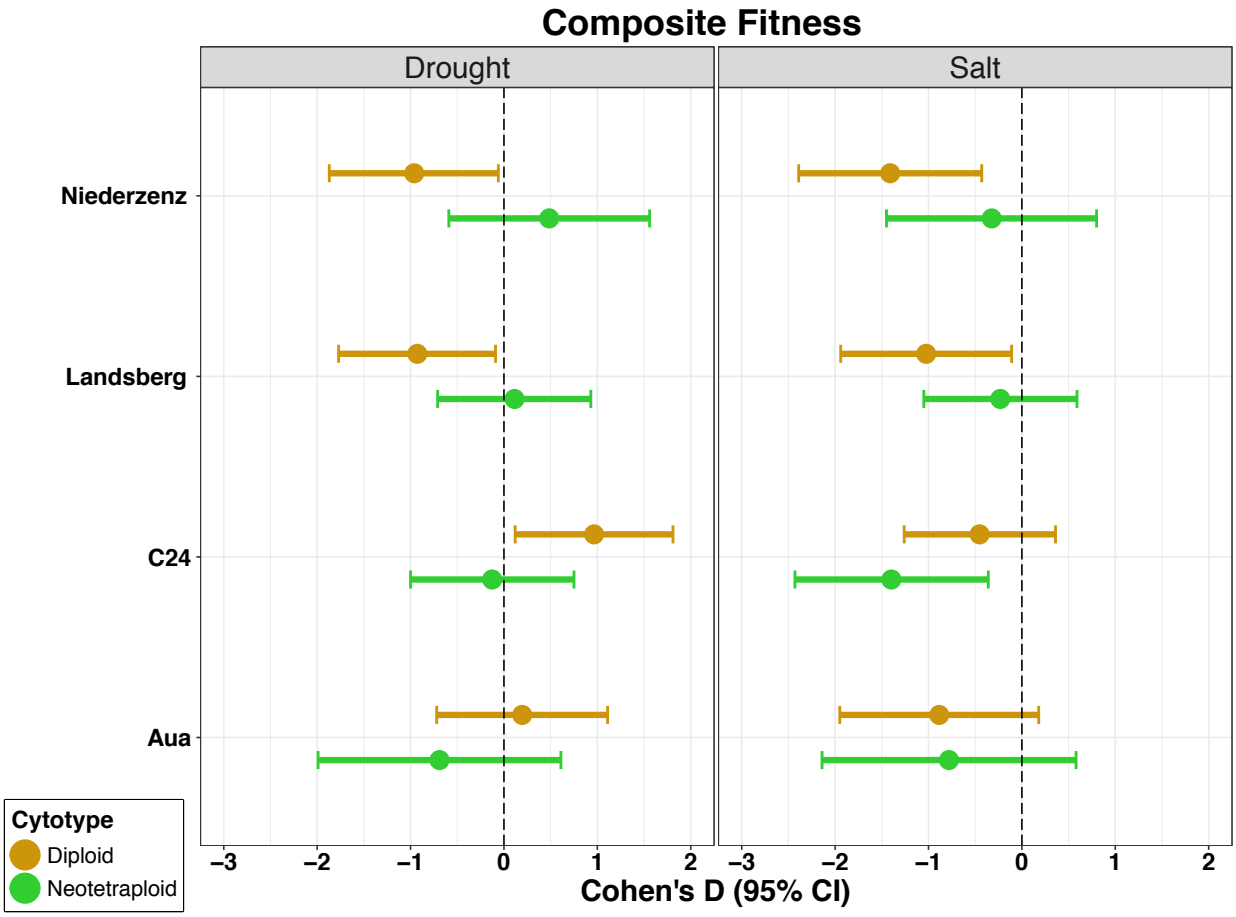

Figure 14. The effect of neotetraploidy, multiple origins (Ecotype), and stress on the composite fitness (mass per seed x fecundity x percent seed germination) of *Arabidopsis thaliana*. Cohen's D was calculated as the difference of each cytotype by ecotype level response to either drought or salt stress relative to control conditions. The dots are the average Cohen's D measurement, and the bars are 95% confidence intervals.

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Chapter 4: Intraspecific polyploidy correlates with colonization by arbuscular mycorrhizal fungi in *Heuchera cylindrica* (Saxifragaceae)

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RUNNING HEAD: Polyploidy and mycorrhizal colonization

ABSTRACT

- Premise of the study: Polyploidy is known to cause physiological changes in plants which, in turn, can affect species interactions. One major physiological change predicted in polyploid plants is a heightened demand for growth-limiting nutrients. Consequently, we expect polyploidy to cause an increased reliance on the belowground mutualists that supply these growth-limiting nutrients. An important first step in investigating how polyploidy affects nutritional mutualisms in plants, then, is to characterize differences in the rate at which diploids and polyploids interact with belowground mutualists.
- Methods: We used *Heuchera cylindrica* (Saxifragaceae) to test how polyploidy influences interactions with arbuscular mycorrhizal fungi (AMF). AMF are key mutualists of plants that supply growth limiting nutrients to their hosts in exchange for carbon. Here we first confirmed the presence of AMF in *H. cylindrica*, and then we used field collected specimens to quantify and compare the presence of AMF structures while controlling for site-specific variation.
- Key results: Tetraploids had higher colonization rates as measured by total, hyphal, and nutritional exchange structures; however, we found that diploids and tetraploids did not differ in vesicle colonization rates.
- Conclusions: The results suggest that polyploidy may alter belowground nutritional mutualisms with plants. Because colonization by nutritional exchange structures was higher in polyploids but vesicle colonization was not, polyploids might form stronger associations with their AMF partners. Controlled experiments are necessary to test whether this pattern is driven by a change in host use of AMF or by shifts in the identity of the fungi.

KEYWORDS

arbuscular mycorrhizae; polyploidy; Saxifragaceae; belowground species interactions; colonization

INTRODUCTION

Polyploidy, or whole-genome duplication (WGD) is exceptionally common in the plant kingdom, with an estimated 35% of vascular plant species being polyploid (Wood et al., 2009). Although polyploids are clearly abundant among plants, theory predicts that polyploids should often go locally extinct shortly after they arise due to competition with their diploid ancestors (Fowler and Levin, 2016). Indeed, there is evidence that although polyploidy occurs frequently within plant lineages, they suffer from higher extinction rates than their diploid ancestors (Levin, 2018). Reconciling this apparent contradiction has thus been a major focus of contemporary research on polyploidy.

A key mechanism that can explain why polyploids are so common despite theoretical predictions, is that WGD often induces changes in plant physiology that can promote persistence by shifting ecological interactions (Madlung, 2013). Although we have accumulated evidence that WGD-induced shifts in physiology can greatly affect the way that plants interact with their abiotic environment (Maherali et al., 2009; Ramsey, 2011), we have only recently begun to appreciate the consequences for the biotic interactions of plants, and most of these studies have focused on aboveground interactions (Ramsey and Ramsey, 2014; Segraves and Anneberg, 2016). This is surprising, given that many belowground interactions involve nutritional mutualisms that are crucial to the growth of the host plant (Vandenkoornhuyse et al., 2015), and one of the major expected physiological consequences of polyploidy is an increased need for nutrients from the soil (Leitch and Bennett, 2004; Guignard et al., 2016; Segraves and Anneberg, 2016). Consequently, we expect that polyploids will be more dependent on the belowground mutualists that provision plants with limiting nutrients. Despite these proposed linkages between polyploidy and belowground mutualisms, it remains unclear how polyploidy affects these

interactions.

An attractive model for testing how polyploidy affects belowground species interactions is the association between plants and arbuscular mycorrhizal fungi (AMF). AMF are a group of near-ubiquitous belowground mutualists that provision their host plant with growth-limiting nutrients in exchange for carbohydrates (Smith and Read, 2008). Because colonization rates of AMF on their host are primarily driven by the nutritional needs of the plant and the nutritional quality of the soil, we expect that polyploids will have increased colonization rates by AMF. From a plant-centric perspective, we can examine how polyploidy influences the quality of the mutualism by quantifying the percent of colonization by arbuscules, the nutrient exchange structures formed by AMF. Greater colonization rates by arbuscules implies that the plants experience a greater benefit from their AMF symbionts (Johnson et al., 2003). From a myco-centric point of view, fungi that have increased colonization by vesicles, the structures that AMF form to store carbon acquired from their hosts, implies that the fungi receive more benefits from the mycorrhizal interaction (Johnson, 1993; Smith and Read, 2008). As a result, simultaneous assessment of colonization by arbuscules and vesicles can offer a way to examine how polyploidy impacts the placement of the plant-AMF interaction along the mutualism-antagonism continuum (Johnson and Graham, 2013). For example, if more arbuscules are observed in polyploids than their diploid ancestors, but vesicle formation by AMF is unaffected, this would imply that polyploids have a lower cost to benefit ratio than diploids, since maintenance costs of the interaction (i.e., vesicle formation rates) are static while benefits (i.e., arbuscules) are greater.

One of the first steps in determining how WGD affects the plant interaction with AMF is to compare colonization rates by AMF on diploid and polyploid host plants. To date, only five studies have investigated how within lineage WGD affects the interaction with AMF, and none

have found differences in total AMF colonization on host roots (Jun and Allen, 1991; Sudová et al., 2010; Doubkova et al., 2012; Sudová et al., 2014; Sudová et al., 2018). To better understand the contextual nature of how polyploidy influences plant – AMF interactions, we need studies that separately consider colonization by arbuscules, vesicles, and hyphae. By comparing the colonization rates of these structures individually, rather than reporting on total colonization alone, we may be better able to frame the consequences of polyploidy on this belowground interaction.

A good system for investigating how polyploidy affects the mycorrhizal interaction is *Heuchera cylindrica*. *Heuchera* has been used extensively to test ideas about how species interactions differ between diploids and polyploids (e.g., Thompson et al., 2004). This species has naturally occurring diploid and autotetraploid populations (Godsoe et al., 2013) that formed via the union of unreduced, intraspecific gametes (Wolf et al., 1989). This is in contrast to allopolyploidy, where WGD occurs following an interspecific hybridization event. As a result, *H. cylindrica* is a good model for studying the effect of polyploidy on species interactions because it allows us to focus on the effects of WGD without the complexity of interspecific hybridization. Furthermore, sampling efforts have shown that diploid and tetraploid populations grow adjacent to one another in overlapping climatic environments (Godsoe et al., 2013), mitigating the possibility that other ecological factors will confound differences between cytotypes. Although the Saxifragaceae are thought to be a non-mycorrhizal family (Maherali et al., 2016; Werner et al., 2018), there is evidence showing that some members of this group can form mycorrhizal associations (Peters et al., 2011; Oehl and Korner, 2014). Here we present evidence that *Heuchera cylindrica* is indeed able to associate with mycorrhizal fungi.

In the present study, we first confirmed that *H. cylindrica* engages in the mycorrhizal interaction by quantifying percent AMF colonization on field collected plants. Then we tested whether the rate of AMF colonization on *H. cylindrica* differed between ploidy levels. Specifically, we examined whether the total percent colonization by AMF, colonization by nutrient exchange structures, vesicle colonization, or hyphal colonization rates differed between cytotypes, as an estimator of the quality of the mutualism.

MATERIALS AND METHODS

Study organism and field sampling — *Heuchera cylindrica* is a semi-evergreen perennial herb, that occurs in the western United States. To quantify root colonization by AMF in a natural setting, we collected roots from three diploid and two tetraploid field sites in May 2008, and then from six diploid and six tetraploid populations in early June 2018 (Table 10). In our 2018 collection, all populations were sampled at the time of peak flowering, since the reproductive period is when plants are the most phosphorus limited (Gusewell, 2004), and would thus be more likely reliant on their AMF partners. Ploidy levels of plants from nine of the twelve populations had previously been determined via flow cytometry (Godsoe et al., 2013), and we determined the ploidy levels of the remaining three uncharacterized populations using the same methods.

We controlled for site-specific differences in soil quality by characterizing five edaphic factors (pH, electrical conductivity, Kjeldahl N, Olsen P, and molar N: P) and AMF spore densities, all of which are known to affect AMF colonization rates. We collected soil samples from each site at depths of 0 – 20cm from directly beneath *H. cylindrica* individuals. Soil samples were stored in plastic bags and transported to Syracuse University where we assessed

their chemical profile. We first air dried a half liter subsample of each soil sample and then passed the dried soil samples through a 2mm sieve. We determined the pH and electrical conductivity of soils in a 1:1 soil:de-ionized water mixture. We measured soil pH with a Mettler Toledo SevenGo™ SG2 pH meter, and the electrical conductivity of soils was measured with an Orion™ conductivity meter (Model 122). We estimated the quantity of plant-available nitrogen with a 1M KCl extraction and subsequent colorimetric analysis (EPA, 1978) at the Cornell Nutrient Analysis Laboratory. We then extracted plant-available phosphorus as Olsen phosphorus (Olsen, 1954) and stained with malachite green to estimate the concentration of plant-available phosphorus from each site (Rao et al., 1997). Although mycorrhizal fungi and plants may differ in their ability to acquire nitrogen and phosphorus from the soil, the plant-available estimation of nutrient availability is a significant driver of plant-mycorrhizal colonization rates (Smith and Read 2008). From the estimated nitrogen and phosphorus data, we calculated the molar nitrogen to phosphorus ratio available in each soil sample. We also controlled for potential differences in AMF propagule density across sites by estimating AMF spore densities via the sucrose flotation method (Allen et al., 1979). The total number of AMF spores per site was estimated by counting the number of spores on one-quarter of a petri dish and multiplying by four. We expressed our site-specific estimates as the total number of spores per 5g of soil.

Confirmation and quantification of AMF colonization — We confirmed *H. cylindrica* is a mycorrhizal species and quantified differences in colonization rates of AMF on roots from our pilot data from 2008 and our 2018 dataset (Table 10). To do so, we took subsamples of fine roots from an average of four individuals per site (ranging from 3 – 7 individuals). We preserved

roots in 70% ethanol and kept them at 4ºC. Roots were cleared and stained for fungal structures within 14 days after collection. Roots were cut into 2cm segments and placed in 10 % KOH (w/v) and cleared of cellular content via autoclaving at 121°C for 15 minutes. Roots were then washed with de-ionized water and stained with 0.03% Chlorazol Black E (w/v) during a second autoclave cycle at 121˚C for 15 minutes. We then mounted the stained roots on glass slides with a 1:1:1 lactic acid:glycerol:de-ionized water solution (v/v/v) (Brundrett et al., 1984). Lactic acid was included in the mounting solution to improve the quality of the stained roots, since lactic acid reduces the intensity of staining in plant tissues, while preserving the stain in fungal structures. We first confirmed that *H. cylindrica* is a mycorrhizal species by identifying AMF structures (i.e., aseptate hyphae, arbuscules, and vesicles) colonized on stained roots of diploids and tetraploids. We then observed individual root sections with a differential interference contrast microscope set to 200x total magnification and counted the number of times that arbuscules, vesicles, and hyphae intersected with the graticule of the ocular, for a total of 50 views per root sample. We then calculated total percent AMF colonization as the proportion of views containing at least one AMF structure to the total number of views for that root sample (Brundrett et al., 1984; McGonigle et al., 1990). Our root sampling and staining procedure from 2008 was carried out according to the methods as described above with the exceptions that only total colonization was determined and the colonization data were collected by another individual using a compound light microscope. In total, we quantified root colonization on 30 individuals in 2008 and 53 individuals in 2018.

Statistical analysis — We used our measures of the percent colonization by arbuscules + hyphal coils as an estimator of the benefit received by the plant, and we used vesicle colonization

as an estimator of the benefit the fungus received from the plant (Johnson, 1993; Johnson et al., 2003). We partitioned our 2018 dataset to separately quantify the percent colonization by nutrient exchange structures (arbuscules and coils), vesicles, and hyphae. The colonization rates of individual structures will not necessarily sum to the total colonization rate because multiple structures were often found together in the same view.

To address the question of how polyploidy in *H. cylindrica* affects colonization rates by AMF, we controlled for geographic variation in edaphic factors and AMF spore density. To accomplish this, we first summarized the site-specific data using a principal components analysis of the five edaphic factors and AMF spore density. We saved the loadings of the first three axes which explained >90% of the variation and used them as covariates in a subsequent ANCOVA. This allowed us to compare the main effect of host plant cytotype while accounting for site-specific soil quality co-variation. We calculated a separate ANCOVA for total AMF colonization, arbuscule + vesicle colonization, vesicle colonization, and hyphal colonization rates, respectively. For our 2008 dataset, we calculated the total percent AMF colonization and used an ANOVA to compare the main effect of host plant ploidy level on total AMF colonization rates.

RESULTS

The 2008 dataset revealed that diploid and tetraploid *H. cylindrica* significantly differed in total colonization by AMF ($F_{1,4} = 8.63$, $P < 0.05$), with diploids hosting a mean of 28.0% total colonization as compared to 43.4% in tetraploids. In our 2018 dataset all root segments of *H. cylindrica* had greater than 50% colonization by AMF. After controlling for site-specific edaphic factors and AMF spore density, the ANCOVA comparing total colonization rates of plants

showed that diploids and tetraploids were significantly different ($F_{1,11} = 6.67$, $P < 0.04$), with diploids hosting an average of 58.9% total AMF colonization and tetraploids hosting 69.3% (Fig. 15).

To separately quantify the percent colonization of nutrient exchange structures, vesicles, and hyphae, we partitioned the total colonization data of each individual root sample into three categories: arbuscules plus hyphal coils, vesicles, and hyphae. After controlling for site specific differences in the soil, we found that nutrient exchange structure colonization significantly differed between diploids and tetraploids ($F_{1,11} = 69.63$, $P < 0.0001$). Diploids hosted an average of 21.4% colonization by nutrient exchange structures as compared to 36.4% in tetraploids (Fig. 15). We found that the frequency of vesicles in plant roots did not statistically differ between diploids and tetraploids ($F_{1,11} = 0.87$, $P > 0.38$); however, hyphal colonization rates differed significantly between diploids and tetraploids ($F_{1,11} = 5.96$, $P < 0.05$), with diploids hosting an average of 54.9% hyphal colonization compared to an average of 67.2% in tetraploids.

DISCUSSION

Polyploidy can cause strongly divergent physiologies in plants and, in turn, is expected to result in altered species interactions between diploids and polyploids in their belowground nutritional mutualisms. The handful of studies that have investigated the effect of polyploidy on plant-AMF interactions, however, have found no differences in total colonization by AMF. We argue that working at a finer scale by comparing the colonization rates of arbuscules, vesicles, and hyphae separately, may reveal differences in the way that diploids and polyploids interact with their AMF mutualists. We were thus interested in characterizing how polyploidy affects the

belowground nutritional mutualism with AMF by comparing the rates of colonization by different AMF structures on roots of diploid and polyploid plants from the field.

In contrast with previous work in other systems, we found that polyploidy strongly correlates with the rate of AMF colonization. After controlling for site-specific differences, we found that diploid and tetraploid *H. cylindrica* differed in the total rates of AMF colonization and that this was consistent for the two sampling periods ten years apart. We found a similar, but stronger pattern in nutrient exchange structure colonization, specifically that tetraploids were colonized by more nutrient exchange structures than diploids. This finding is intriguing since we observed that vesicle colonization did not differ between diploid and tetraploid *H. cylindrica*. Thus, our results suggest that polyploids of *H. cylindrica* benefit more from the interaction with AMF, because nutrient exchange structure colonization increased while carbon storage structures of the fungi did not. We are cautious in our interpretation of these results, however, since we did not profile the AMF communities colonized on plants. Some AMF species do not form vesicles, so it is possible that the patterns may have been driven by differences in the taxonomic profile of the AMF communities on diploids and tetraploids. Nevertheless, observing differences in colonization between diploids and tetraploids suggests that the dynamics of belowground mutualisms may shift.

Nutrient exchange structure colonization by AMF may have been greater on tetraploids because polyploidy is expected to increase the nutritional needs of plants (Leitch and Bennett, 2004; Segraves and Anneberg, 2016; Guignard et al., 2017). Indeed, comparative studies suggest that polyploids are more nutrient limited than diploids. For example, polyploids have been shown to produce less biomass and are less abundant than phylogenetically unrelated diploids in experimental plots with low nutrients as compared to nutrient enrichment plots (Šmarda et al.,

2013; Guignard et al., 2016). By participating in nutrient exchange mutualisms, perhaps polyploids are able to overcome an increased need for growth-limiting nutrients. Although we found that polyploids of *H. cylindrica* had significantly greater nutrient exchange structure colonization of AMF than diploids, our results may have differed from previous studies since we did not control for the taxonomy of the potential AMF mutualists available to plants. For example, Sudová et al. (2014) conducted a pot study in which they inoculated diploids and polyploids of *Aster amellus* with *Rhizophagus* isolates of AMF from field sites and found no differences in total AMF colonization or in extra-radical hyphal lengths, AMF structures that forage for mineralized nutrients in the soil. They also included a non-sterile soil treatment in their inoculation experiment, however, and found that the AMF that colonized hexaploids of *A. amellus* produced significantly longer extra-radical hyphae than diploids. Together, the results of Sudová et al. (2014) and the results from the present study suggest that polyploids invest more into the AMF mutualism than their diploid ancestors. To better understand how WGD affects host plant investment into the AMF mutualism, we require inoculation experiments that provide AMF partner options to their host plants (Kiers et al. 2011). This would allow us to disentangle the effect of AMF taxonomy from the effect of divergent polyploid physiologies on the plant and AMF mutualism.

The fact that nutrient exchange structure colonization rates increased while vesicle colonization rates were static between tetraploids and their diploid ancestors raises the question of how polyploidy affects carbon allocation strategies of plants. That is, we do not know if polyploids invest carbon into the plant – AMF interaction differently than their diploid progenitors. For example, we know from other studies that AMF can act as sinks for a significant amount of plant carbon (Kaschuk et al., 2009), so the question remains whether polyploids

exchange more carbon for growth-limiting nutrients. The answer to this may be complex, since there is evidence of a negative correlation between polyploidy and maximum photosynthetic rates (Knight et al., 2005). Since polyploids may have a diminished capacity to photosynthesize at the same rate as their diploid ancestors, it may then limit their ability to invest as much carbon into the interaction with AMF. If polyploidy does result in reduced pools of carbon for plants to allocate towards belowground interactions, then polyploids may preferentially associate with AMF partners that offer the highest carbon to nutrient exchange rate. The next step in addressing this problem is to quantify and compare the rates at which diploids and their polyploid offspring acquire carbon and how much of that carbon is allocated to AMF partners in exchange for growth limiting nutrients. Furthermore, the quantification of carbon allocation to AMF partners should account for more than just vesicle structure colonization, and instead account for all intraradical and extraradical AMF structures. Even though vesicles are enriched in carbohydrates, other AMF structures may represent substantial pools of carbon that were derived from the host plant. By taking a more comprehensive estimation of AMF biomass production, we can more precisely quantify the total carbon investment by host plants into their mycorrhizal partners.

Our results suggest that polyploids may interact with their AMF partners differently than their diploid ancestors, however, this result may be confounded by the evolutionary history of the polyploid plants used in this study. Specifically, naturally occurring polyploids of *H. cylindrica* underwent WGD hundreds, if not thousands, of generations ago. The observed differences in colonization rates, then, may have been caused either by polyploidy, drift, selection that favored polyploid lineages to form stronger mycorrhizal associations, or by a product of any of these processes. Consequently, the phenotypes of the extant polyploids of today may not necessarily

match those of first-generation polyploids. This may explain why our results contrast those of previous efforts (Jun and Allen, 1991; Sudová et al., 2010; Doubkova et al., 2012; Sudová et al., 2014; Sudová et al., 2018). In our future efforts, we will make use of first-generation polyploids, or neopolyploids, in order to understand the direct effect of polyploidy on nutritional limitation, and ultimately, on species interactions.

In addition to examining AMF colonization patterns between diploids and polyploids, we also confirmed the mycorrhizal status of *H. cylindrica.* Historically, the Saxifragaceae have been considered non-mycorrhizal or only weakly mycorrhizal (Maherali et al., 2016; Werner et al., 2018), and up to this point, efforts have solely focused on the genus *Saxifraga*. For example, three studies have shown that species of *Saxifraga* are not colonized by AMF in the field (Treu et al., 1996; Ruotsalainen et al., 2004; Brown and Jumpponen, 2014); in contrast, Peters et al. (2011) sampled four species of *Saxifraga* and found an average total AMF colonization rate upwards of 39%. Oehl and Korner (2014) sampled roots of *Saxifraga oppositifolia* from the Swiss Alps and found that plants hosted five different morphotypes of AMF, and although colonization rates were not quantified, they reported on the presence of intra-radical AMF structures. Although the genus *Saxifraga* clearly includes species that can host AMF, until the present study, we were missing colonization information from the second major lineage within the Saxifragaceae.

Together, our results show that diploids and polyploids differ in how they interact with their AMF mutualists. We hypothesize that this pattern is driven by increased nutritional needs of plants as a result of polyploidy; however, we require further studies that control for the identity of the AMF partners to advance our understanding of how polyploidy affects the plant – AMF association. Doing so will help us discern if polyploidy affects preferential carbon

allocation of a host plant to root segments with superior AMF mutualists. Furthermore, we must make use of early-generation polyploids in future efforts, this will allow us to disentangle the effects of selection and drift acting on polyploids following WGD, and thereby understand the immediate effect of WGD on species interactions.

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Tables

Table 10. Sampling locations and their site-specific soil qualities.

Site	Coordinates	2008 Sample Size	2018 Sample Size	Ploidy	pH	Electrical Conductivity	Olsen P (mg/Kg)	Kjeldahl N (mg/Kg)	Molar N:P	Spore Count (count/5g)
Dayton, WA	46.199712 N, 117.766992 W	-	7	Diploid	6.19	18.7	1.44	3.97	6.09	1641
Santa, ID	47.166822 N, 116.483605 W	-	3	Diploid	6.57	21.7	1.80	3.03	3.73	1004
Cheney, WA	47.481677 N, 117.567133 W	-	5	Diploid	6.51	20.3	2.27	4.74	4.63	1035
Boise, ID	44.314285 N, 116.070428 W	-	4	Diploid	6.39	27.7	4.67	4.35	2.06	1186
Orofino, ID	46.489156 N, 116.732209 W	5	6	Diploid	7.48	42.1	0.73	3.19	9.63	766
Albion, WA	46.839574 N, 117.280947 W	5	3	Diploid	6.35	20.85	2.08	4.01	4.28	457
Benewah, ID	47.337876 N, 116.827284 W	-	5	Tetraploid	6.43	16.1	0.91	4.08	9.86	520
Calder, ID	47.279133 N, 116.215752 W	-	3	Tetraploid	6.05	12.6	1.21	2.84	5.21	410

Coeur d'Alene, ID	47.618344 N, 116.662353 W	-	6	Tetraploid	5.95	18.2	1.91	4.51	5.22	1242
St. Joe, ID	47.315246 N, 116.270866 W	-	3	Tetraploid	6.91	52.8	0.93	9.31	22.13	1121
Ashley, MT	48.120512 N, 114.576281 W	-	4	Tetraploid	7.21	36.9	3.05	7.38	5.34	757
St. Regis, MT	47.229085 N, 115.255366 W	-	4	Tetraploid	6.89	43.8	0.47	6.62	31.30	1857
Spalding, ID	46.57972 N, 116.7847 W	5	-	Diploid	-	-	-	-	-	-
Thompson		5	-	Tetraploid	-	-	-	-	-	-
Beaver Creek, MT	47.71667 N, 115.6925 W	5	-	Tetraploid	-	-	-	-	-	-
Calder (Big Creek), ID	47.48111 N, 116.2231 W	5	-	Tetraploid	-	-	-	-	-	-

Figure Legends

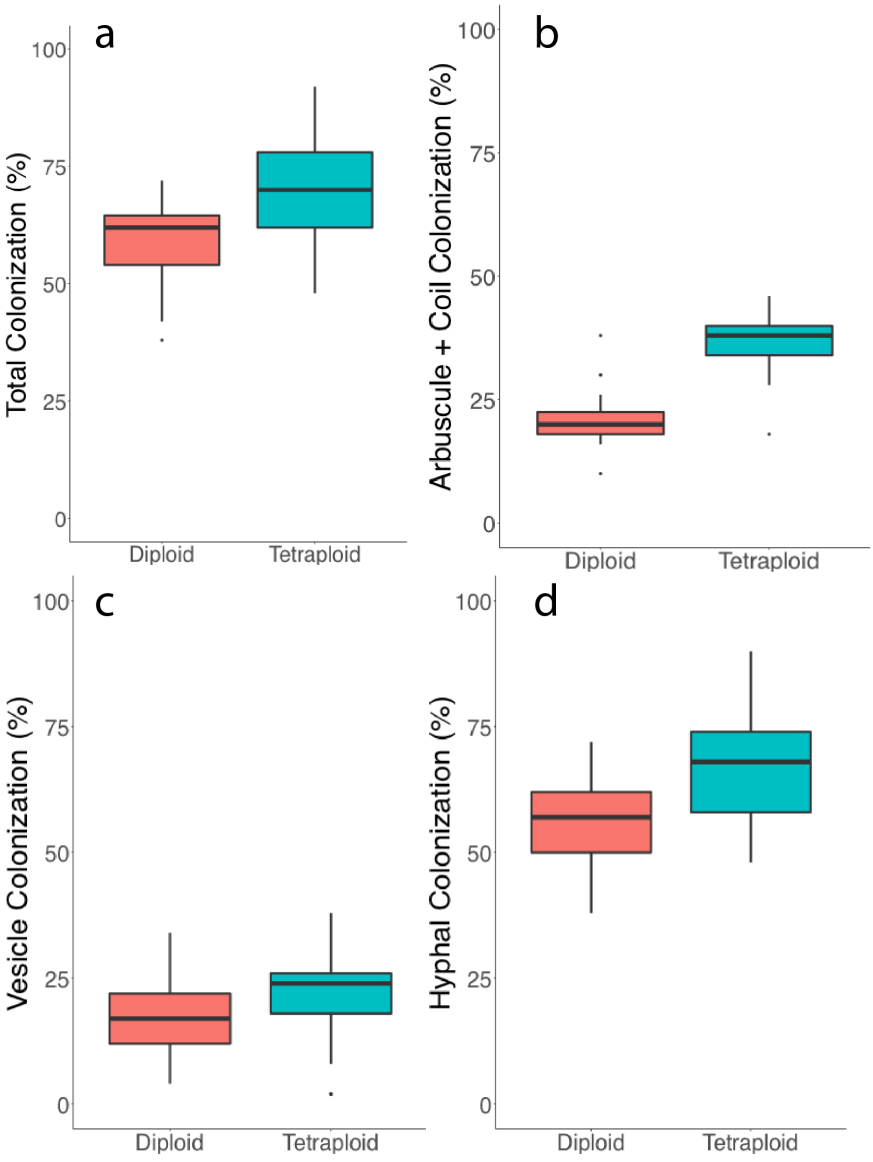

Figure 15. Colonization by arbuscular mycorrhizal fungi on *Heuchera cylindrica* roots collected in 2018. (a) Total colonization. (b) Nutritional exchange structures (arbuscules + coils). (c) Vesicles. (d) Hyphae.

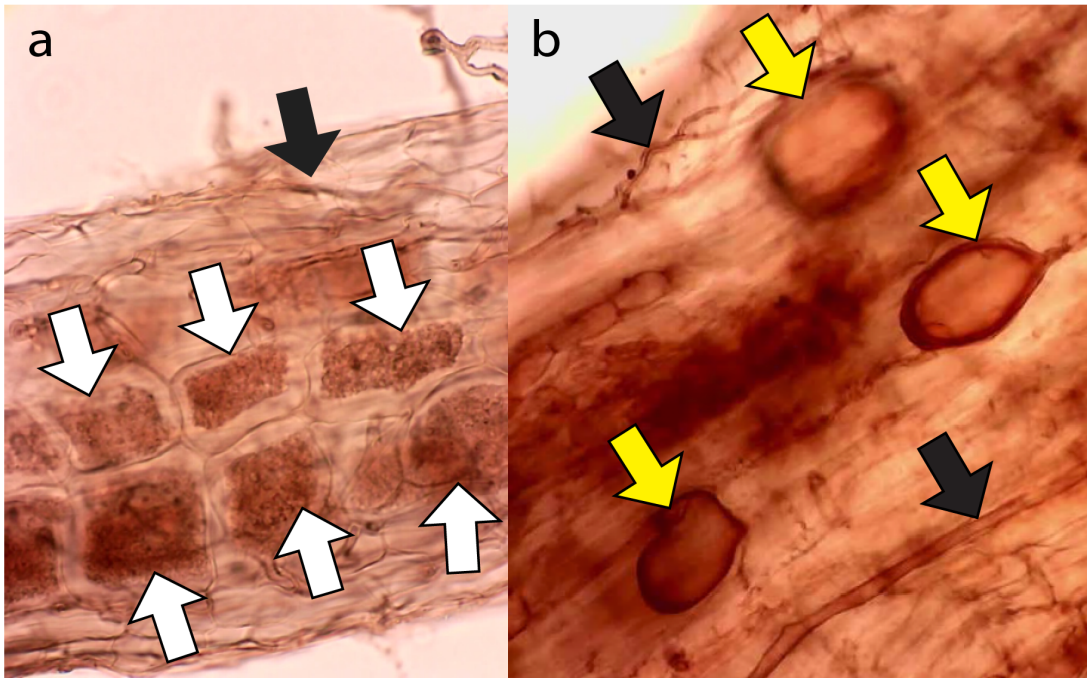

Figure 16. Photographs of *Heuchera cylindrica* roots colonized by (a) arbuscules and hyphae and (b) vesicles and hyphae. 200x magnification. Black arrows indicate hyphae, white arrows indicate arbuscules, and yellow arrows indicate vesicles

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EDUCATION

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2011 – 2015 B.S. University of Kansas, 2015. Focus in Organismal Biology

GRANTS AND FELLOWSHIPS

2019 Syracuse University Graduate School Travel Grant

2018 Sigma Xi Grant in Aid of Research

2015 Syracuse University Graduate Research Fellowship

2014 University of Kansas Howieson Opportunity Fund Recipient

2014 Ecological Genomics Institute Travel Fellowship

2013 University of Kansas Undergraduate Research Award

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PUBLICATIONS

Anneberg T.J. and K.A. Segraves (2020). Neotetraploids are more tolerant to stress than their diploid progenitors. Manuscript in prep.

Anneberg T.J. and K.A. Segraves (2020). Neopolyploidy causes increased nutrient limitation phenotypes of functional traits in *Heuchera cylindrica.* Manuscript in review.

Anneberg T.J. and K.A. Segraves (2020). Neopolyploidy improves the lifetime fitness response of *Arabidopsis thaliana* to nutrient enrichment. *Plant and Soil* 456: 439-453.

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Segraves K.A., **T.J. Anneberg** (2016). Species interactions and plant polyploidy. *American Journal of Botany* 103 (10): 1-10.

PRESENTATIONS

Anneberg T.J and K.A. Segraves (2020, July). Neopolyploidy in *Heuchera cylindrica* causes increased nutrient limitation phenotypes of functional traits. Invited talk at the virtual Botanical Society of America conference.

Anneberg T.J and K.A. Segraves (2020, May). Neopolyploid *Heuchera cylindrica* are more nutrient limited. Contributed talk to the Polyploid Webinar series by Michael Barker.

Anneberg T.J., S. Wang, D.M. Althoff, K.A. Segraves (2019, July). Plant polyploidy promotes resilience to stress. Contributed poster at the Botanical Society of America conference in Tucson, AZ.

Anneberg T.J., S. Wang, D.M. Althoff, K.A. Segraves (2019, June). Plant polyploidy promotes resilience to stress. Contributed talk at the Society for the Study of Evolution conference in Providence, RI.

Anneberg T.J. and J. Gleason (2014, June). I got you babe: Analyzing the courtship duet of *Drosophila americana*. Contributed poster at the University of Kansas Undergraduate Research Symposium, Lawrence, KS.

Anneberg T.J. and J. Gleason (2014, March). Resolving the subspecies status of *Drosophila americana americana* and *Drosophila americana texana*. Contributed poster at the University of Kansas Undergraduate Research Symposium, Lawrence, KS.

Anneberg T.J and J. Gleason (2014, October). Are the chromosomally distinct subspecies of Drosophila americana reproductively isolated? Contributed poster at the Ecological Genomics Symposium, Kansas City, MO.

RESEARCH and PROFESSIONAL EXPERIENCE

Spring 2020 Research Assistant, Syracuse University, Laboratory of Kari Segraves

Synthesized putative neotetraploids of Pennycress via pressurized nitrous oxide gas; worked on progress towards dissertation

2014 – 2015 Undergraduate Research Ambassador, University of Kansas

Counseled fellow undergraduates on engaging with independent research on campus

2013 – 2015 Volunteer Undergraduate Research Assistant, University of Kansas, Laboratory of Jennifer Gleason

Investigated the courtship duet in Drosophila americana and the subspecies status of D. a. americana and D. a. texana

2014 – 2015 Volunteer Undergraduate Research Assistant, University of Kansas, Laboratory of Benjamin Sikes

Assisted a PhD student in extracting wild yeast DNA for an experiment investigating fungal diversity at different depths of aquatic environment

2011 – 2015 Database Analyst, CivicPlus, Manhattan, KS

Managed a large cloud database of local government data, performing mass imports and exports which often entailed custom Microsoft Excel formatting. Also organized and wrote submissions for government website awards on behalf of clientele.

2011 Agronomy Intern, Kansas State University Department of Agriculture, Manhattan, KS

Collected infrared readings from soybean leaves from experimental plots in eastern Kansas; estimated phytic acid concentrations of seeds; removed weeds from plots; field-collected and counted soybean pollen on agar plates daily

TEACHING

2020 & 2018 Technology Inspired by Nature teaching assistant, Biology Department, Syracuse University

In addition to leading weekly discussion sections, graded exams and written assignments for a lecture course of approximately 100 students. I also gave a guest lecture on perennial agriculture.

2019 Ecology and Evolution teaching assistant, Biology Department, Syracuse University

Coordinated weekly reviews and quizzes of course content. Helped write short essay exam questions in addition to exam grading.

2018 Field Biology Laboratory teaching assistant, Biology Department, Syracuse University

I helped teach a field biology course that included a half-semester of lectures and culminated in a week of student-led research projects at Archbold Research Station in Venus, FL. I helped drive the van and chaperoned the students at numerous destinations that showcased neotropical habitats of Florida. I mentored students and helped them develop their independent research questions. I also gave a guest lecture on estuary habitats.

2017 Integrative Biology teaching assistant, Biology Department, Syracuse University

Helped teach a lab section that encompassed topics such as biostatistics, genetics, population level selection, and reviewing primary literature.

2016 Introduction to Biology teaching assistant, Biology Department, Syracuse University

Taught a multiple laboratory sections of approximately 25 students each, covering basic concepts in biology.

PROFESSIONAL MEMBERSHIPS

2016 – Present Mycological Society of America

2019 – Present Botanical Society of America

2019 – Present Society for the Study of Evolution

TECHNICAL SKILLS

Analysis and Programming

- R Statistics
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- WinRHIZO
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