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The effect of genome duplication on the reproductive ecology of plants

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Abstract

Whole genome duplication, or polyploidy, is the largest genomic alteration observed in nature. Polyploidy occurs in many different taxa, but is a widely tolerated and recurrent evolutionary phenomenon in plants. Although the importance of polyploidy in plants has been touted for approximately 100 years, we have yet to fully understand the ecological consequences of whole genome duplication on plant reproductive biology. Here I investigated how whole genome duplication impacts plant reproductive ecology. Specifically, I studied the effects of whole genome duplication on flowering phenotypes and the contributions of whole genome duplication to three premating barriers. I used a combination of genomic modifications of plants to induce polyploidy in experimental populations, manipulative field experiments to test ecological hypotheses, and literature surveys to examine evolutionary trends. In the first chapter, I used meta-analytical approaches based on published studies to explore the effect of whole genome duplication on several aspects of floral morphology, phenology, and reproductive output in plants. The results suggested that across a wide variety of plant species, morphological traits increase in size (e.g., flower diameter increases), reproductive output decreases, and there were no general trends in the effect of whole genome duplication on flowering phenology. I also observed that variation in reproductive output increases after whole genome duplication, whereas variation does not increase or decrease in phenology or morphology traits. In the second chapter, I build on existing knowledge of the mechanisms involved in premating reproductive isolation of polyploid lineages by investigating the factors that are important in driving assortative mating in the generations immediately following whole genome duplication. I accomplished this by using synthetic polyploids which provide the opportunity to study polyploidy in the generations immediately following formation when reproductive isolation will be critical to establishment.

Trifolium pratense, or red clover, was used in an experimental study of diploids and newly formed polyploids to determine if the phenotypic differences caused by whole genome duplication facilitated premating isolation. The premating barriers examined included flowering phenology, self-fertilization rates, flower visitor community, and flower visitor behavior. I found that whole genome duplication increases flower size, but there were no cascading effects that facilitated premating isolation of newly formed polyploids. Together, my results suggest that polyploidy puts plants at a reproductive disadvantage and that if newly formed polyploids are found in sympatry with their diploid progenitors, rapid adaptation is likely necessary to establish and avoid extinction.

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Chapter 1

Abstract

Polyploidy, or whole genome duplication (WGD), is a phenomenon that is ubiquitous in plants; this is remarkable given that theory suggests polyploids should be evolutionarily transitory. Recently, there has been an expanding interest in the ecological aspects of polyploids that could explain their pervasiveness in nature. In particular, much research has focused on the ecological mechanisms leading to reproductive isolation from their diploid progenitors. WGD is often accompanied by changes in a number of different traits, but there are numerous conflicting examples of the phenotypic effect of WGD in the literature. Because the phenotype dictates how an organism interacts with its environment, it is critical that we first have a solid understanding of the effects of WGD on reproductive traits to understand the ecological mechanisms leading to assortative mating. In this study, we used literature surveys and meta-analysis approaches to comprehensively describe how WGD affects floral morphology, phenology, and reproductive output in plants. We focused on comparisons of newly generated polyploids and their diploid parents to mitigate the potential confounding effects of adaptation and drift that can occur when examining older, established polyploid populations. The results indicated that across a broad representation of angiosperms, floral morphological traits tend to increase in size, reproductive output tends to decrease, but phenology is unaffected by WGD. Additionally, we found that variation in reproductive output increases after WGD, whereas variation does not change for phenology or size-related traits. These results provide a more comprehensive picture of the phenotypes resulting from WGD, which can help us understand which traits may be important in shifting ecological dynamics of plants in the generations immediately following polyploidization.

Introduction

The consequences of large-scale genomic modifications can be extensive, and linking these changes to their subsequent phenotypes is important for understanding ecological and evolutionary dynamics (Otto and Whitton, 2000; Segraves and Anneberg, 2016). For instance, chromosomal rearrangements such as inversions can give rise to polytypic species with differences in life history or reproductive strategies (Lowry and Willis, 2010; Kupper et al., 2016; Tuttle et al., 2016). We also know that genome size can vary greatly within a single species and can correlate with a number of environmental variables such as elevation, altitude, and moisture levels (reviewed in Levin, 2002; Smarda and Bures, 2010), suggesting that genome size can contribute to local adaptation (Levin, 2002). Additionally, we know that whole genome duplication can cause instant reproductive isolation, setting individuals with duplicated genomes on independent evolutionary trajectories, allowing them to diverge from their ancestors (Ramsey and Schemske, 2002; Otto, 2007; Ramsey and Ramsey, 2014). Ultimately, illustrating the relationship between genomic modifications and phenotype is central to our understanding of how it will impact an organism's life history (Otto and Whitton, 2000; Segraves and Anneberg, 2016).

Perhaps the most substantial class of genomic restructuring is whole genome duplication (WGD). WGD, or polyploidy, is thought to be particularly important in the evolution of plants (Adams and Wendel, 2005; Soltis et al., 2009; Soltis et al., 2016). WGD is estimated to have given rise to 15% of speciation events in angiosperms (Wood et al., 2009), and recent evaluations suggest that nearly a quarter of extant plant taxa are polyploid (Barker et al., 2016). WGD can immediately impact gene expression (reviewed in Chen and Ni, 2006), morphology (summarized in Table 1 in Ramsey and Schemske, 2002), and also provides duplicated genetic

material that can spur the evolution of novel phenotypes ("neosubfunctionalization", reviewed in Flagel and Wendel, 2009). These immediate changes in polyploids can have cascading effects on plant ecology and evolution. Indeed, changes in ploidy level have been implicated in the ability to colonize new habitats (Leitch and Leitch, 2008; Parisod et al., 2010; te Beest et al., 2012), altering how an organism interacts with its abiotic and biotic environments (Maherali et al., 2009; Liu et al., 2011; Ramsey, 2011; Segraves and Anneberg, 2016), and driving species diversification (Soltis et al., 2009). Novel phenotypes that accompany WGD may contribute to exaptations that mitigate difficulties in establishment (Buggs and Pannel 2007), which might be linked to the pervasiveness of polyploids in nature.

The ubiquity of polyploidy in plants is intriguing because polyploids are expected to be extremely uncommon. Theory predicts that polyploids should be rare and evolutionarily ephemeral due to frequency-dependent reproductive disadvantages associated with being the minority cytotype in a population; i.e., the minority cytotype exclusion principle (Levin, 1975). Thus, the answer to why plant polyploidy is so common remains to be explained. One hypothesis is that genome duplication may confer changes in phenotype that allow new polyploids to overcome minority cytotype exclusion by becoming, at least in part, prezygotically isolated from their parental species during initial establishment (Levin, 1975; Husband, 2000). For example, if polyploidy leads to larger flowers, pollinators may be able to detect these differences and either favor or avoid polyploids, leading to assortative mating (Segraves, 2017). Additionally, if polyploids have overall larger structures, it might be expected that they would take longer to develop (Cavalier-Smith, 1978; Ramsey and Ramsey, 2014). Possessing larger flowers could impose constraints on flowering time, resulting in later flowering dates and a shift in flowering phenology. To determine if phenotypic changes associated with genome duplication could play a

role in reproductive isolation, the first step is to fully understand the consequences of genome duplication with respect to phenotypic traits related to reproduction. Decades of studies have documented the effect of WGD on many plant phenotypes including reproductive traits (reviewed by Ramsey and Schemske, 2002; Vamosi et al., 2007); nevertheless, the predictability and magnitude of phenotypic changes due to genome duplication remains unclear.

A common prediction of WGD is that phenotypic changes such as the increase in size and greater robustness that is often seen in polyploids is termed the 'gigas effect'. The gigas effect is thought to be the result of polyploids having greater quantities of DNA that causes larger cells and cascades into larger tissues and organs (Muntzing, 1936; Stebbins, 1971). However, this directional effect on plant phenotype is not the rule (Stebbins, 1950; Otto and Whitton, 2000; Vamosi et al., 2007) as there are numerous examples of polyploids having smaller or identically sized floral organs (tables 1 and 2 of Vamosi et al., 2007; Ning et al., 2009; Trojak-Goluch and Skomra, 2013). Another phenotype other than size that might be impacted by the gigas effect is flowering phenology. If larger organs require more time to develop, then the time of reproductive peak should occur at a later date. However, similar to size traits, this prediction is not always observed (Nuismer and Cunningham, 2005; Thompson and Merg, 2008; Nghiem et al., 2011). There is considerable variation among studies in the effect of WGD, and that variation is probably in part caused by examining polyploids with differing evolutionary histories. Many studies have examined natural polyploid systems that have evolved for many generations and thus have had time to ameliorate the initial phenotypic effects of WGD. Indeed, there is some evidence to suggest that phenotypes can degrade or change in subsequent generations after polyploidization (Butterfass, 1987; Oswald and Nuismer, 2011; Ramsey, 2011;

Husband et al., 2016), suggesting that if we want to understand the direct effects of WGD, that we need to study newly formed polyploids.

Therefore, the next step is to quantitatively assess how WGD impacts phenotypes immediately after WGD occurs. To understand if there are predictable, quantitative effects of WGD on plant phenotypes, results of single case studies that compare diploids with their polyploid offspring immediately after WGD need to be compiled and analyzed. In particular, we need to understand if polyploidy results in significant shifts in reproductive traits that could play a role in allowing new polyploids to escape minority cytotype exclusion. Here, we surveyed the literature and performed a meta-analysis to quantitatively assess the immediate consequences of whole genome duplication on reproductive traits. In our analysis, we included studies that contained data from newly synthesized polyploids to disentangle the effects of genome duplication from subsequent adaptation. This was done to mitigate confounding effects of adaptation and drift and because the phenotypic effects of genome duplication will be most critical in determining which traits might facilitate reproductive isolation during initial establishment immediately following WGD. Our goals were to 1) determine the impact of genome duplication on floral morphology, phenology, and reproductive output, and 2) identify the traits that are most affected by genome duplication.

Methods

Literature Search

To find relevant literature that would address our questions, we performed searches in three separate databases using Syracuse University Libraries' subscription packages. First, we used Web of Science (ISI) to search for the terms (neopoly* or *synthes* or colchicine or oryzalin or trifluralin or nitrous) and (phenoty* or morphol* or phenolo*) and (flower* or floral or pollen or petal) and (plant* or *ploid*) from 1900 to the present. This search returned 234 results. For the second search, we used the database Agricola open to all years with the same search terms as above except it excluded the precursory asterisks because Agricola does not support that search function; this search returned 339 results. Third, we searched JSTOR open to all years, with the search identical to Agricola but without the term 'synthes*' because removing it reduced the results to a feasible number to examine. This search was open to any content type and filtered by subject types 'Biological Sciences', 'Botany & Plant Science', 'Ecology & Evolutionary Biology', and 'General Science' which returned 2,805 results. This initial pool of 3,378 publications was further narrowed by including only the subset of articles that indicated in the title or abstract that traits were measured before and after polyploid induction. This narrowed the results to 130 research papers, all of which were examined and excluded from subsequent analysis if they did not meet the following conditions: 1) contained extractable quantitative data on floral phenotype or phenology of both polyploids and their progenitors, and 2) reported sample sizes, means, and either standard deviation or standard error. In instances when the publication did not include the data necessary to calculate effect sizes, the corresponding author was contacted to request those data or data were extracted from the figures using Plot Digitizer Ver. 2.6.8. In addition to data collected from database searches, we also obtained data from two

unpublished studies that were shared by the authors (Comai and Wu, unpublished data; Porturas et al., unpublished data). When a study reported data from multiple genotypes of a single species, we collapsed the genotypic data into an average for the species. If a study reported data from multiple varieties, they were treated individually because varieties of a single species often display very different floral traits (e.g., *Brassica oleracea*). In our compiled dataset, we included information on the reference, species, ploidy level, chromosome number, mode of genome duplication, selection history (e.g., artificial selection of horticultural plants), the means of polyploidy synthesis (e.g., colchicine), trait types and trait measurements. Selection history type was assigned subjectively. If the species' floral phenotype or related features such as fruit had been subject to a well-known history of strong artificial selection (e.g., maize, *Brassica oleracea*, *Chrysanthemum*) they were assigned to the agricultural/horticultural selection history type. Otherwise, the species was assigned to the natural selection history type. We collated data on three major trait categories (phenology, size, and reproductive output) that included many different trait measurement types (Table S1).

Meta-analyses

We used the R Statistical Software (R Development Core Team, 2016) to perform our meta-analyses. For all analyses, we used the log response ratio ($lnRR = ln(mean_{after\ WGD} / mean_{before\ WGD})$) as the effect size measure to compare trait differences before and after WGD. This was calculated using the 'escalc' function in the R package 'metafor' (Viechtbauer, 2010). We also estimated the coefficient of variation ratio ($lnCVR = ln(CV_{after\ WGD} / CV_{before\ WGD})$), calculated using the 'calc.lnCVR' function provided by Nakagawa et al. (2015) to compare variation in those traits before and after WGD.

We first determined whether phylogenetic history and genome size would be important covariates to account for in our models. To do this, we mapped the lnRR of size-related traits onto the plant phylogeny published by Zanne et al. (2014). Size-related traits were used for this analysis because size traits were expected to have a similar directional change and subsequent analysis verified that there were no differences in how WGD affected various size-related traits. Because many species in our dataset were not included in this phylogeny, the phylogeny was trimmed so that the tips represented genera instead of species. We used the 'drop.tip' function from the R package 'phytools' (Revell, 2012). If there was more than one representative species or lnRR measure per genus, the average lnRR was used. The generic name of one species in our database, *Dendranthema nankingense*, was not included in the phylogeny, so the name was replaced by its suggested synonym (*Chrysanthemum indicum*) according to The Plant List database (www.theplantlist.org/). We tested for phylogenetic signal in the data using Blomberg's K and Pagel's λ. Tests for non-random distribution of the effect size of WGD across the phylogeny were done using the 'phylosig' function from the R package 'phytools' (Revell, 2012), specifying both Blomberg's K and Pagel's λ as output variables. We also determined whether genome size influenced the effect of genome duplication in plants. We calculated the Pearson's correlation coefficient between C-values and average effect size (lnRR) of size-related traits for a species. C-values were obtained from the Kew Royal Botanical Gardens Plant DNA C-values Database.

Linear mixed models were used to estimate the average effect size of WGD on phenology, reproductive output, and size. Because we found no evidence of correlation between the effect size and either phylogenetic history or genome size, these variables were excluded from our models. The first model included all our calculated effect sizes that were assigned to

one of three trait categories: ‘phenology’, ‘output’, and ‘size’. Trait category was used as the fixed effect variable for this model. The random effects variables were 1) the paper reference, 2) plant species nested within paper reference, and 3) trait category nested within plant species nested within paper reference. These were the assigned random effects because some studies measured multiple traits (e.g., flower length, flower width, pollen size) on multiple species. The log response ratio was used as the response variable for the model. The mechanism of polyploid formation (allopolyploid versus autopolyploid) and selection history (horticultural/agricultural versus natural) were analyzed as interactive fixed effects. In the second model, we tested the hypothesis that WGD increases variation in traits by using the same model but substituting the coefficient of variation ratio for the response variable.

Next, we estimated the average effect size of WGD on the size of gametes, petals, flowers, and inflorescences. This model included 106 effect sizes that were grouped into four morphology trait categories: ‘gamete’, ‘petal’, ‘flower’, and ‘inflorescence’. Some size traits were excluded from this dataset because there were insufficient measurements to calculate reliable estimates. Morphology trait category was used as the fixed effects variable and the other factors were identical to the first model. Lastly, we used a similar approach to estimate the average effect size of WGD on the reproductive output of gametes, flowers, and inflorescences. This model included 29 effect sizes that were placed into three reproductive output trait categories: ‘gamete’, ‘flower’, and ‘inflorescence’. No interactions were tested because there were not enough measurements to calculate reliable estimates when parsed between the interaction categories.

Estimated average effect sizes were modeled using the ‘rma.mv’ function. For all models, we tested for significant differences among factors of the trait categories and whether there was

significant evidence of interactions (Wald-type chi-square tests, Q_M). If there was a significant interaction effect, we used Tukey's HSD post-hoc tests to determine whether there were pairwise differences between the levels of the trait categories; significant differences were detected using the function 'ghlt' from the R package 'multcomp' (Torsten et al., 2008). All null models are summarized in Table S2. We also tested for publication bias with Eggar's regression test by including variance as a moderator to our null models. If the studies included in our analysis are not impacted by publication bias, then the intercept should not significantly deviate from zero at $\alpha = 0.10$ (Egger et al., 1997).

Results

Overview

We had 185 effect size and variation size measures from 41 studies and 60 independent WGD events. In our dataset, we had representatives of 30 genera across 18 plant families. The vast majority of our measures came from diploid to tetraploid genome duplications (89.2%), and the remaining forms of WGD events were relatively rare (haploid to diploid 4.3%, triploid to hexaploid 4.3%, tetraploid to octaploid 1.6%, octaploid to hexadecaploid 0.5%). Many measures also came from WGDs that were induced using the mitotic inhibitor colchicine (72.4%), The other polyploid induction types included somaclonal variation during embryo culture (8.1%), oryzalin (4.9%), nitrous oxide gas (4.3%), protoplast fusion (1%), trifluralin (0.5%), or were unspecified (8.6%). Based on Eggar's regression test, we found evidence of publication bias in all three of our datasets as the intercepts were significantly different from zero at $\alpha = 0.10$: all trait categories ($p = 0.072$), size trait categories ($p = 0.003$), and reproductive output trait categories ($p = 0.053$).

Phylogenetic history and genome size correlations

We found no evidence of a correlation between the effect of WGD on size traits and evolutionary history (Blomberg's K: 0.297, $p = 0.111$; Pagel's lambda: 0.252, $p = 0.441$; Fig. 1). We also found no evidence of a correlation between the effect of WGD on size traits and genome size (Pearson's correlation estimate = 0.029, $p = 0.937$). Thus, subsequent analyses did not correct for phylogenetic history or genome size.

Overall effect of genome duplication on reproductive output, size, and phenology

There were significant differences in how WGD impacted reproductive output, size and phenology (Q_M = 952.318, df = 2, p < 0.0001). The estimated mean effect size for reproductive output was negative (-0.190 ± 0.078), indicating that WGD reduced the reproductive output of polyploid plants. In contrast, the estimated mean effect size of size-related traits was positive (0.195 ± 0.075), showing that the size of floral traits generally increased following WGD. The estimated mean effect size of phenology (0.010 ± 0.140) was not significantly different from zero (Fig. 2). We found no evidence for an interaction between these trait categories and the mechanism of polyploid formation (Q_M = 1.430, df = 1, p = 0.232). Reproductive output was dropped from this test for an interaction because the dataset had no allopolyploids with that measure. In addition, we did find a significant interaction between trait category and selection history (Q_M = 32.961, df = 2, p < 0.0001), but there were no significant differences in pairwise comparisons of the two selection history categories for the three traits (Fig. 3).

We were also interested in knowing whether WGD significantly increased trait variation after WGD. Indeed, we found significant differences in how WGD affected variation in reproductive output, size and phenology (Q_M = 5059.650, df = 2, p < 0.0001). There was no significant difference in the mean estimated variation in phenology and size (0.288 ± 0.476 and 0.076 ± 0.280, respectively); however, we did see an increase in variation after WGD for reproductive output (0.974 ± 0.281) (Fig. 4). Similar to the trends observed in effect size, we found no evidence of an interaction between trait category and mechanism of polyploid formation (Q_M = 1.576, df = 1, p = 0.209). Reproductive output was dropped from this test due to a lack of allopolyploids with that measure. Moreover, we did find a significant interaction between trait category and selection history (Q_M = 954.605, df = 2, p < 0.0001). Pairwise

comparisons examining differences between the two selection history categories (agricultural/horticultural versus natural) for the three traits showed that reproductive output was significantly different (Tukey's HSD post hoc test, $p < 0.0001$, Fig. 5).

Effect of genome duplication on size traits

Although we found an overall significant increase in the size-related traits after WGD (Fig. 3), we found no significant differences in the magnitude of effect size when comparing across gametes, petals, flowers, and inflorescences ($Q_M = 1.920$, df = 3, $p = 0.590$) (Fig. 6). There was also no evidence of an interaction between the size traits and mechanism of polyploid formation ($Q_M = 2.274$, df = 1, $p = 0.132$); gamete and inflorescence data were dropped from this test because the dataset lacked allopolyploids with either of those measures. Finally, we found no interaction between size and selection history category ($Q_M = 0.731$, df = 2, $p = 0.694$). We dropped inflorescence from this test because there was only one effect size measure of a natural inflorescence.

When we tested for overall changes in variation after WGD, we found no significant impact on variation in size. However, when we tested for changes in variation after WGD within the size-related traits, we found WGD impacts variation in size differently among gametes, petals, flowers, and inflorescences ($Q_M = 21.657$, df = 3, $p < 0.0001$) (Fig. 7). We also found there was no evidence of an interaction between the size traits and mode of genome duplication ($Q_M = 0.171$, df = 1, $p = 0.680$), or between size traits and selection history category ($Q_M = 0.517$, df = 2, $p = 0.772$). We dropped gamete and inflorescences from the test for interactions with mode of genome duplication because there were no allopolyploids with either of those

measures in our dataset, and for similar reasons, we also dropped inflorescence from the test for interactions with selection history category.

Effect of genome duplication on reproductive output

Although we found an overall significant decrease after WGD in reproductive output (Fig. 3), there was no significant difference in the magnitude of effect size when comparing across gametes, flowers, and inflorescences ($Q_M = 1.677$, df = 2, p = 0.432) (Fig. 8). When we tested for overall changes in variation after WGD, there was a significant increase in variation in reproductive output related traits. For just the reproductive traits, however, we found WGD did not impact variation in reproductive output differently among gamete, flowers, and inflorescences ($Q_M = 0.544$, df = 2, p = 0.762). Additionally, the significance observed in reproductive output when examining overall changes was lost in this smaller dataset when we excluded data on petals; petals were excluded because all data on petals came from a single study.

Discussion

Polyploidy is a conundrum because it is so ubiquitous among plants despite theory suggesting polyploids should be rarely able to successfully establish natural populations (Levin, 1983; Fowler and Levin, 1984; Felber, 1991; Baack, 2005; Fowler and Levin, 2016). Better understanding the phenotypes resulting from WGD can help us understand which traits might play key ecological roles during establishment in the critical generations immediately following polyploidization (Segraves and Anneberg, 2016). This study is the first to use meta-analytical approaches to assess how WGD impacts various aspects of floral traits in the generations immediately following genome duplication. Using data available in the literature, we examined how size traits, reproductive output, and phenology are impacted by WGD. This study builds on the previous work of Vamosi et al. (2007) by including a larger number of studies and estimating the effect sizes of WGD. Our goals were to quantify how genome duplication impacted floral phenotypes and phenology across a wide representation of plants, identify the traits that are more affected than others, and whether there are correlates that might help us to better predict variation after WGD.

In concert with broad expectations (Stebbins, 1971; Levin, 2002), we found that on average, WGD increased the size of floral traits. The gigas effect has long been recognized as a consequence of WGD despite some reports of WGD imparting no differences or a decrease in size. The data here support the gigas effect and suggest that the plants that experience no size increase after WGD are in the minority. We also observed a general decrease in reproductive output measures (Fig. 2). An increase in size traits coupled with a decrease in reproductive output suggest that WGD results in differences in resource allocation to reproductive structures (Segraves and Thompson, 1999). When we broke down size and reproductive output into

individual components, the data suggest that WGD had consistent effects on the components within their respective categories. For example, the magnitude of the effect that genome duplication had on the increase in size of gametes was not significantly different than the magnitude of the effect on petals, flowers, and inflorescences (Fig. 6). This is surprising because we expected the largest effect to be seen in gametes because they are single cells as opposed to the other anatomical structures which are aggregates of cells and different tissue types. These larger structures could have displayed smaller effect sizes if fewer but larger cells were used to compose those structures. Similar to size traits, the magnitude of the effect that WGD had on reproductive output was not different between pollen, flowers, and inflorescences (Fig. 8).

In contrast to changes in morphological traits, the results suggested that WGD does not result in a shift in flowering phenology. This is surprising because some of the seminal studies that investigate the effect of WGD on phenology have identified later flowering phenology in polyploids (Segraves and Thompson, 1999; Husband and Sabara, 2003; Jersáková et al., 2010; Oswald and Nuismer, 2011; Ramsey, 2011; Roccaforte et al., 2015). Additionally, one might expect longer mitotic division times of polyploid cells to translate into later or longer flowering periods (Ramsey and Schemske, 2002). Our data trend towards that expectation, however, the effect size was not significantly different from zero, and our results do not provide evidence that WGD significantly shifts flowering phenology of plants.

Another general expectation of polyploids is that they will likely exhibit greater variability in traits due to increased or fixed heterozygosity, or phenotypic and genomic instability in the generations following WGD (Soltis and Soltis, 1995; Comai et al., 2000; Otto and Whitton, 2000; Ramsey and Schemske, 2002). If this were the case, it would likely be a beneficial artifact of WGD for polyploids to exhibit a wide variety of phenotypes on which

selection can act during critical establishment periods, allowing faster evolution to the most appropriate phenotype for their environment. However, we did not find a general trend of greater variation in traits after genome duplication. The only traits that showed significantly increased variation after WGD were inflorescence size (Fig. 7) and reproductive output traits from non-horticultural/agricultural species (Fig. 5). Furthermore, tradeoffs between increased size of polyploids with decreased reproductive output may limit the ability of selection to differentiate polyploids from their diploid progenitors or increase their reproductive output over diploids. At best, reproductive output might return to the baseline output of diploids. Because so few traits seemed to display greater variation after WGD, increased variation in phenotypes may be less common and a less important consequence of polyploidy than previously expected.

In addition to investigating variation after WGD, we were also interested in determining whether phylogenetic history or genome size might reliably predict how WGD impacts floral phenotype. We expected evolutionary history to correlate with the magnitude of effect because developmental or genetic constraints on flower development could be shared within clades and create similar responses to WGD. However, there was no evidence of phylogenetic signal in the effect that WGD had on size-related traits (Fig. 1). We also predicted that genome size might correlate with the effect of genome duplication. We know there is a strong relationship between cell and genome size (Beaulieu et al., 2008), so we expected that doubling the genomic content of a plant with a large C-value would generate a stronger response than doubling the genomic content of a plant with a small C-value. Nonetheless, we did not detect a correlation between genome size and the effect of genome duplication on size-related traits. These results, in combination with a lack of effect of our other predictors, mode of genome duplication (allopolyploidy vs. autopolyploidy) and selective history category (agricultural or horticultural

vs. natural), having little to no predictive power was surprising given the large variation we see in the effect of WGD on traits. These four factors we predicted might be important in dictating the relative strength of WGD were for the most part not significant, suggesting that the processes dictating the effect that WGD has on various traits are dynamic.

One possible explanation for the lack of patterns seen from our predictors is that there simply might not have been enough data to detect a signal. We had to exclude more than 25 studies that compared plants before and after WGD because they did not report the data necessary to calculate effect sizes. Additionally, within some categories, there were too few samples to reliably calculate estimates so they were excluded from the model. This also meant that some interactions between traits and predictors such as mechanism of polyploid formation and selection history could not be tested.

Despite these caveats, our study is the most extensive to date examining the effects of genome duplication on the floral phenotype. Our results indicate that WGD has an immediate but contrasting effect on morphological traits and reproductive output. There was a general increase in the size of floral traits, but reproductive output decreased. We also found that phenotypic variation did not generally increase after WGD, suggesting that neopolyploids do not necessarily have more variation for selection to act on than diploids. If we are to understand the enigmatic ubiquity of polyploids, linking these phenotypic effects to their ecological roles in the generations following genome duplication is an important next step.

Figures and Tables

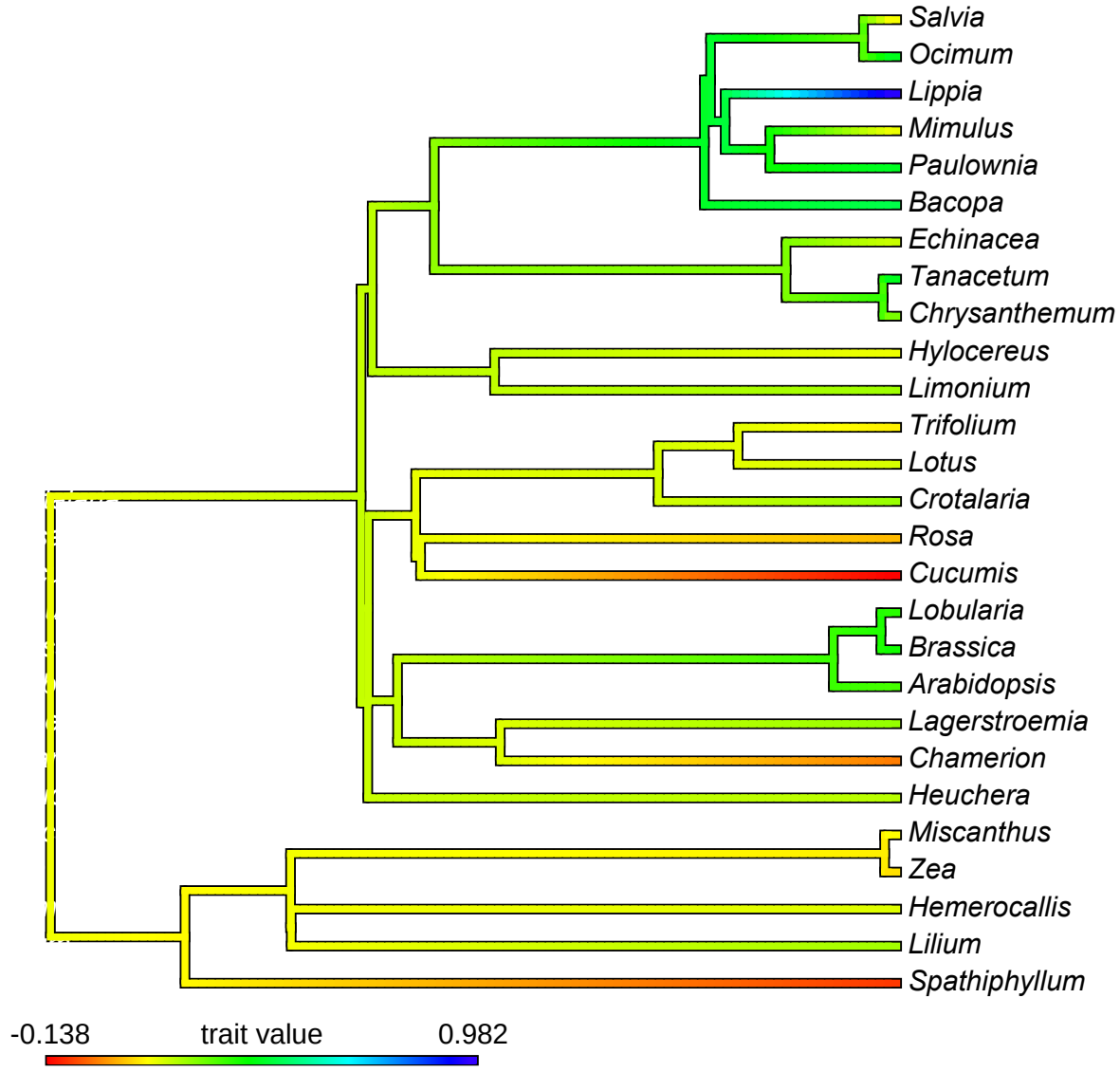

Figure 1. Phylogenetic patterns of the effect size of whole genome duplication on size-related traits across 27 genera. Trait values are the average log response ratio for each genus, where positive values correspond to an increase in size after whole genome duplication, Blomberg's K: 0.297, p = 0.111; Pagel's lambda: 0.252, p = 0.441.

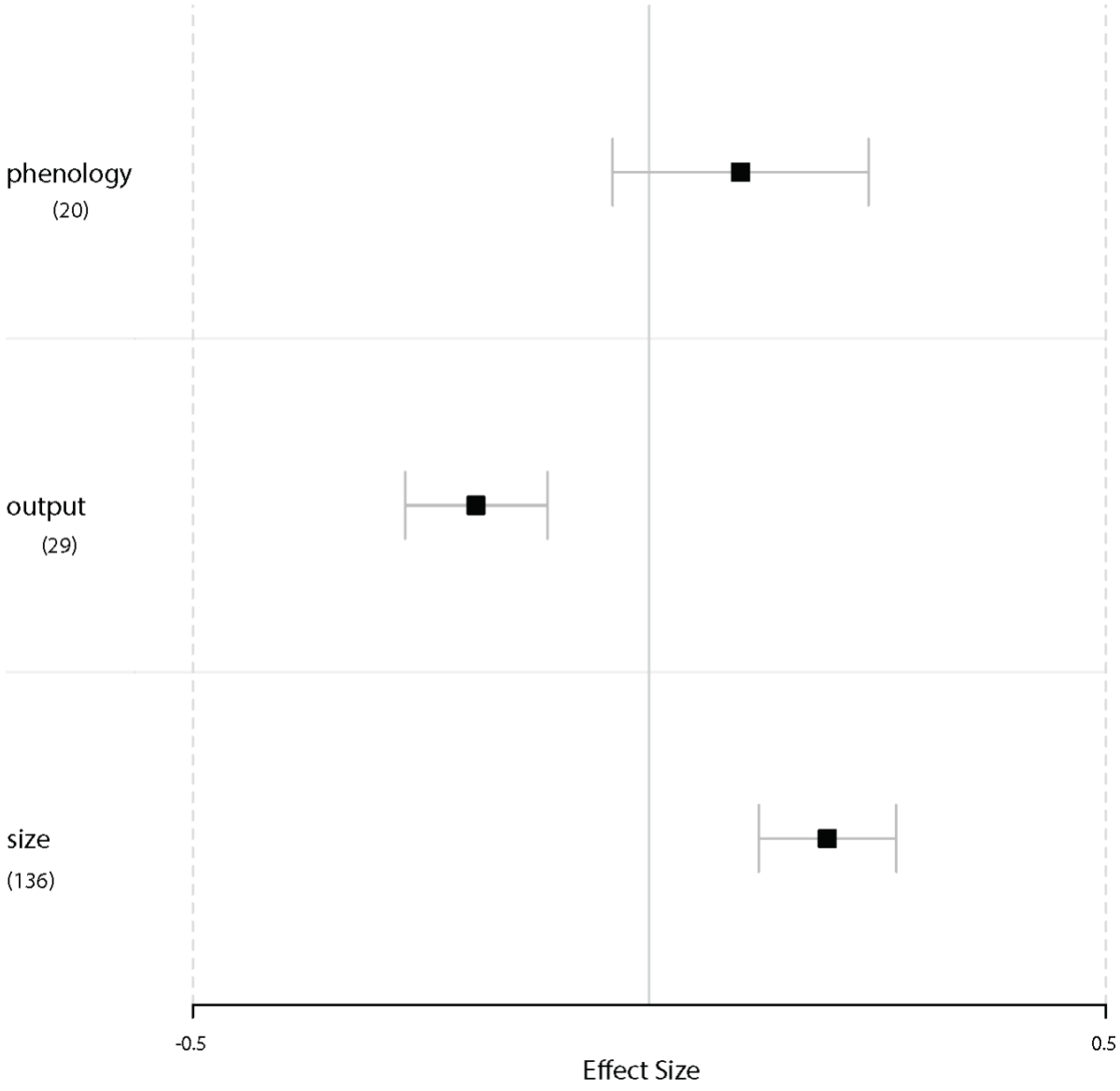

Figure 2. The average estimated effect size of whole genome duplication on phenology, reproductive output, and size-related traits. Values are coefficient estimates of log response ratios and their corresponding 95% confidence intervals. If the confidence interval includes zero, the estimate is not statistically different from zero. Number of effect size measures are in parentheses following the trait identifier.

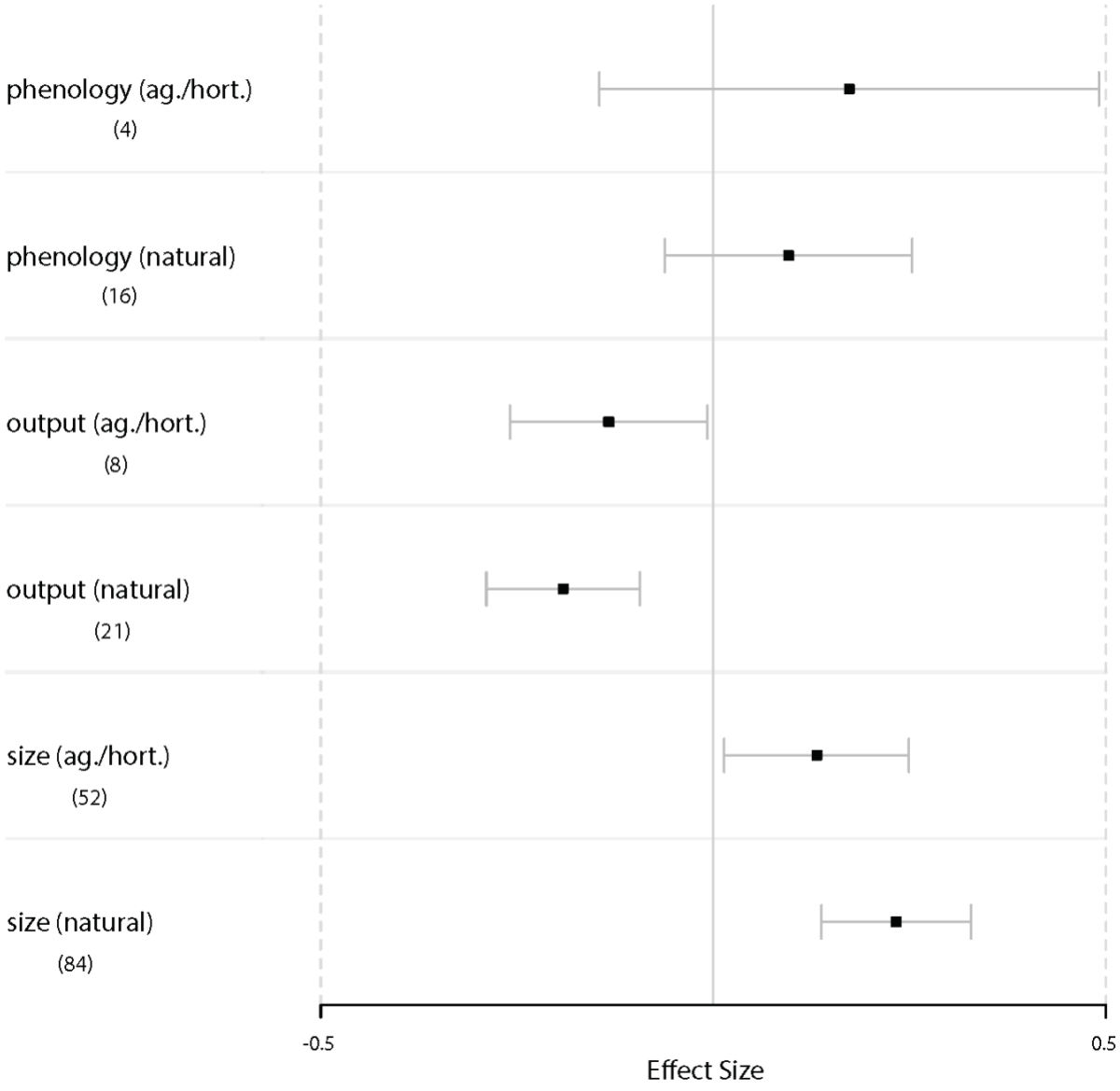

Figure 3. The average estimated effect size of whole genome duplication on phenology, reproductive output, and size-related traits by their selection history (agricultural/horticultural or natural). Values are coefficient estimates of log response ratios and their corresponding 95% confidence intervals. If the confidence interval passes through zero, the estimate is not statistically different from zero. Number of effect size measures are in parentheses following the trait identifier.

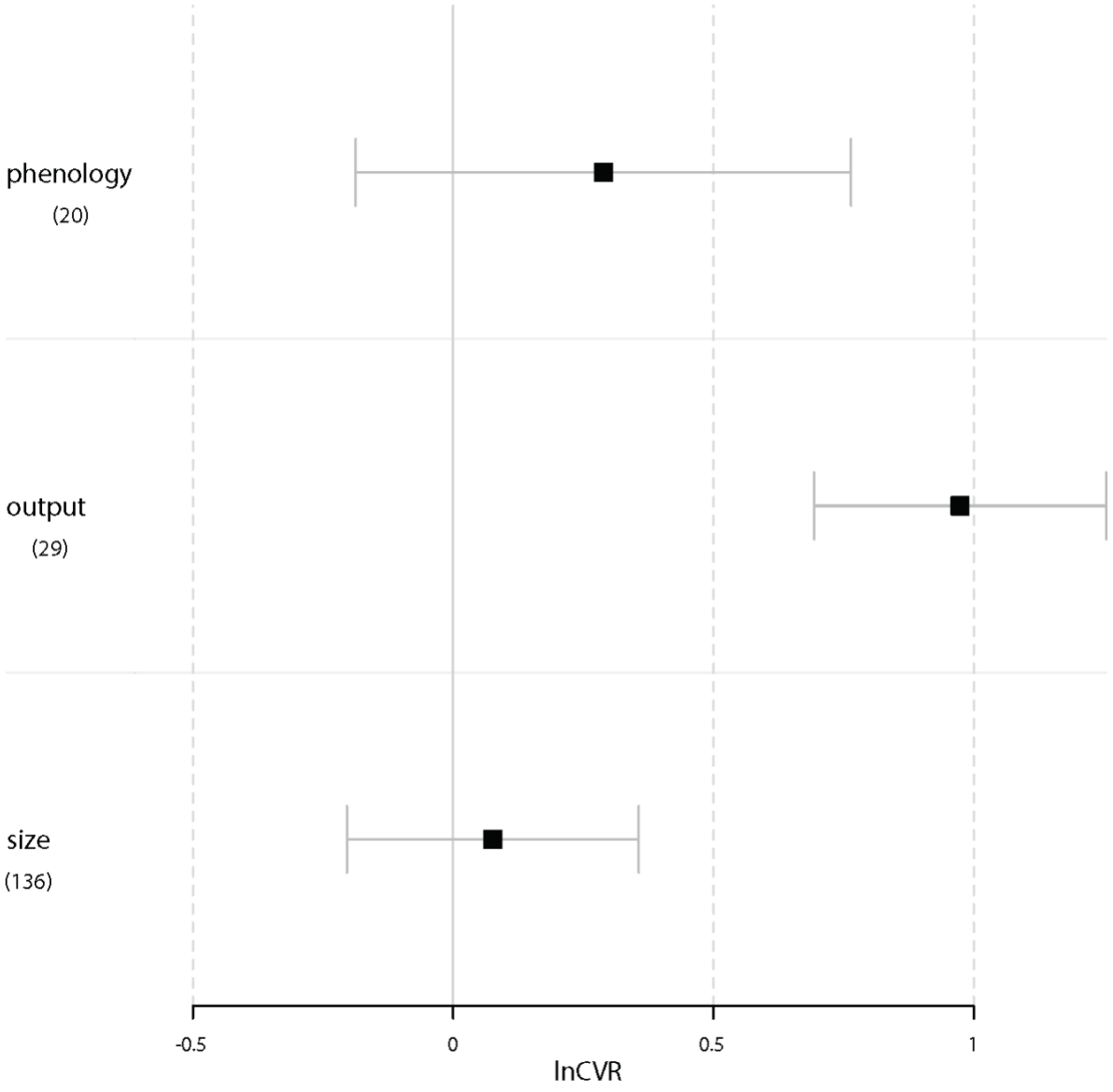

Figure 4. The average estimated effect on the amount of trait variation in response to whole genome duplication in phenology, reproductive output, and size related traits. Values are coefficient estimates of the log coefficient of variation ratios (lnCVR) and the corresponding 95% confidence intervals. If the confidence interval passes through zero, the estimate is not statistically different from zero. Number of effect size measures are in parentheses following the trait identifier.

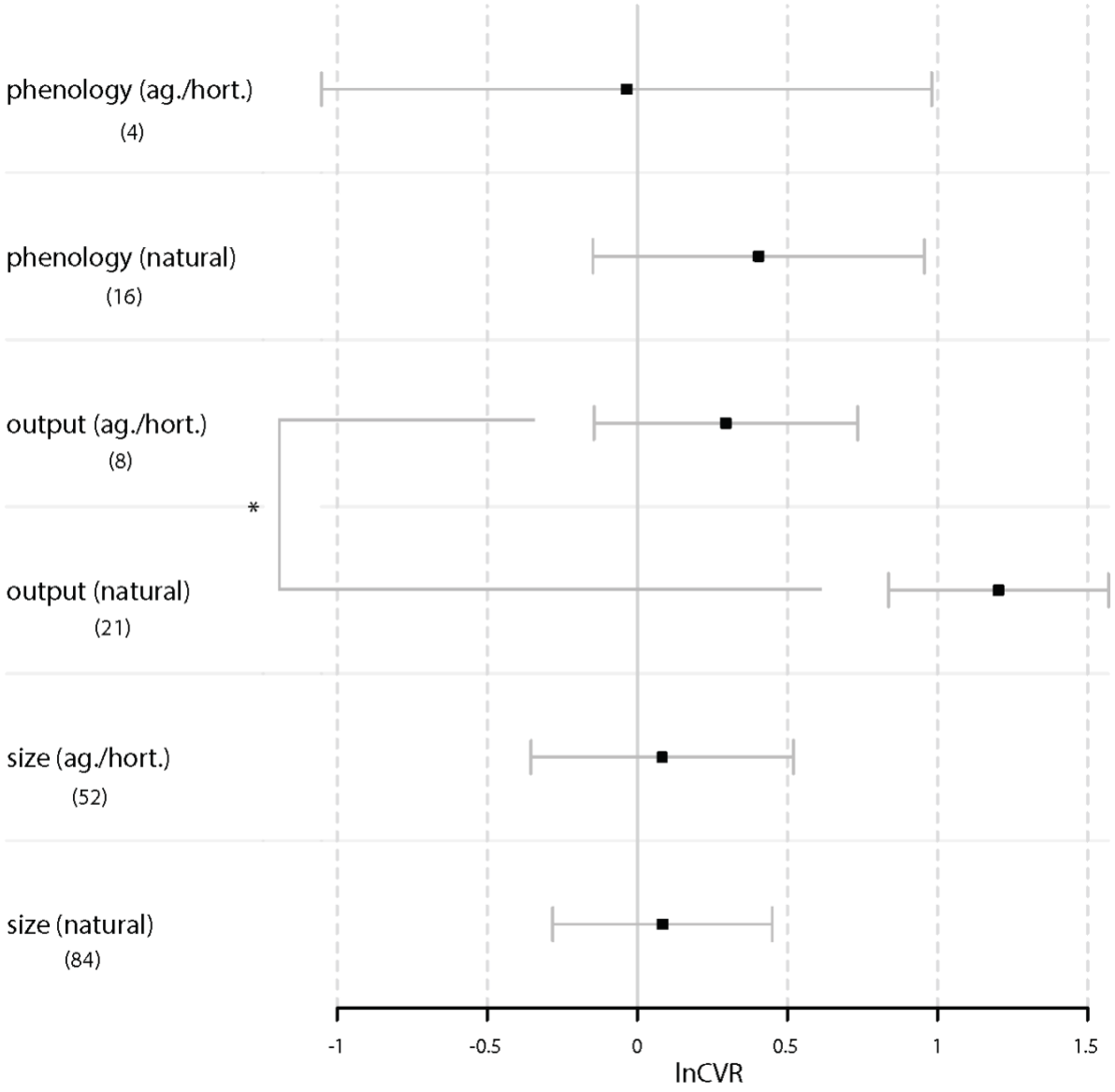

Figure 5. The average estimated effect on the amount of trait variation in response to whole genome duplication in phenology, reproductive output, and size related traits by their selection history (agricultural/horticultural or natural). Values are coefficient estimates of the log coefficient of variation ratios (lnCVR) and the corresponding 95% confidence intervals. If the confidence interval passes through zero, the estimate is not statistically different from zero. Number of coefficient of variation ratio measures are in parentheses following the trait identifier. Asterisks signify significant differences between the selection history category within that trait as determined by Tukey's HSD post hoc tests.

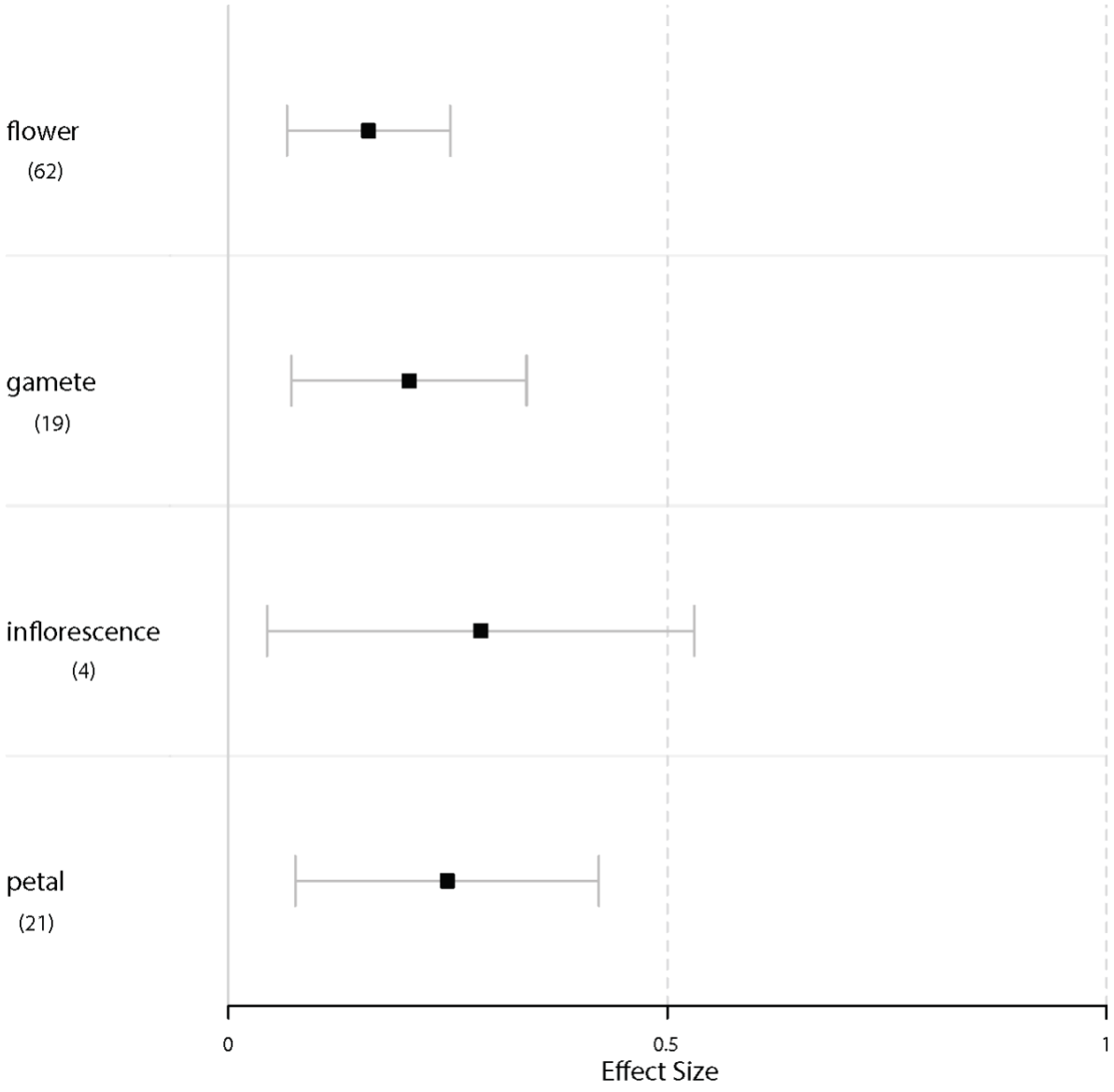

Figure 6. The average estimated effect size of whole genome duplication on size-related traits. Values are coefficient estimates of log response ratios and their corresponding 95% confidence intervals. Number of effect size measures are in parentheses following the trait identifier.

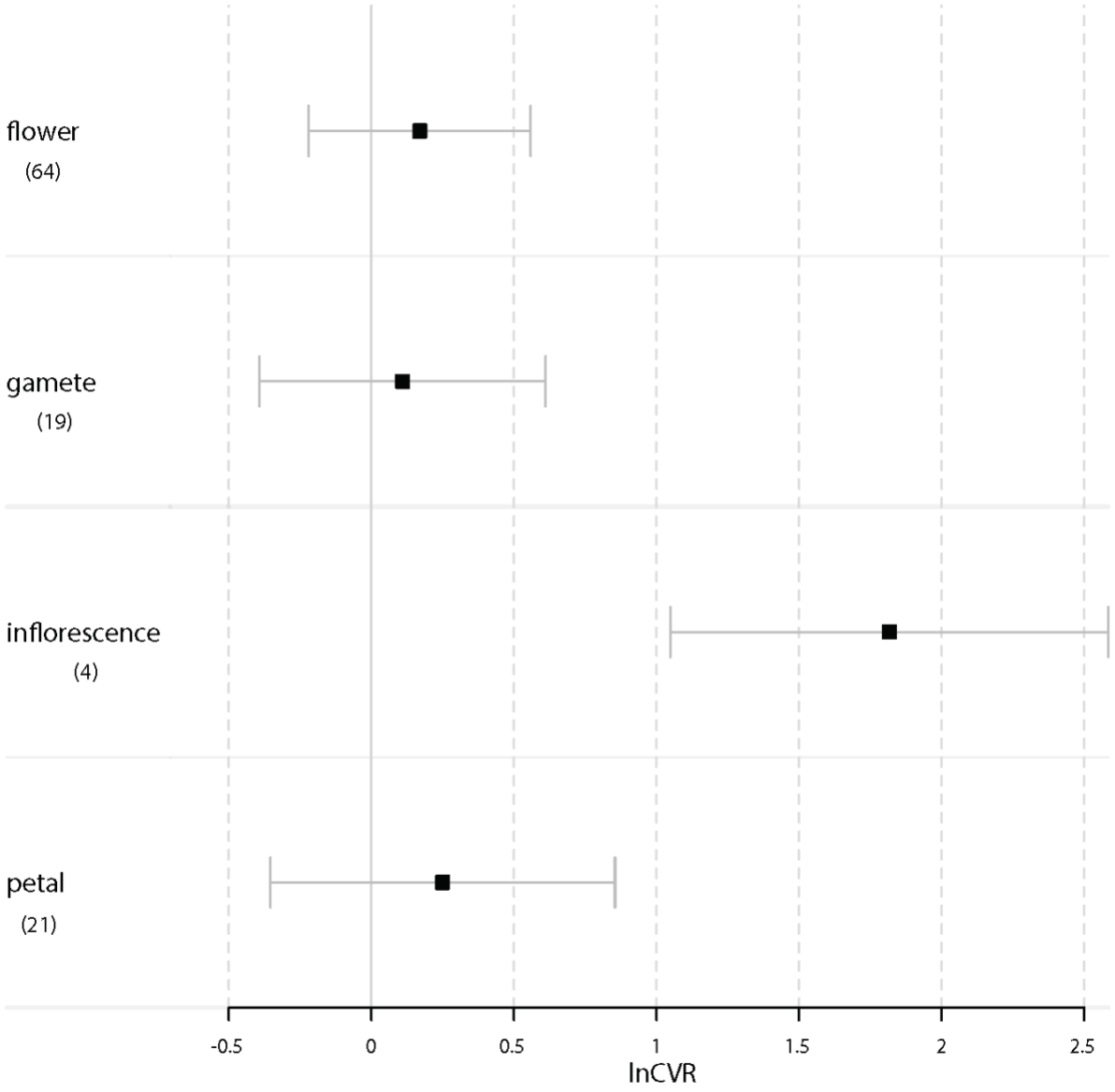

Figure 7. The average estimated effect on variation in response to whole genome duplication in size-related traits. Values are coefficient estimates of the log coefficient of variation ratios (lnCVR) and the corresponding 95% confidence intervals. If the confidence interval passes through zero, the estimate is not statistically different from zero. Number of coefficient of variation ratio measures are in parentheses following the trait identifier.

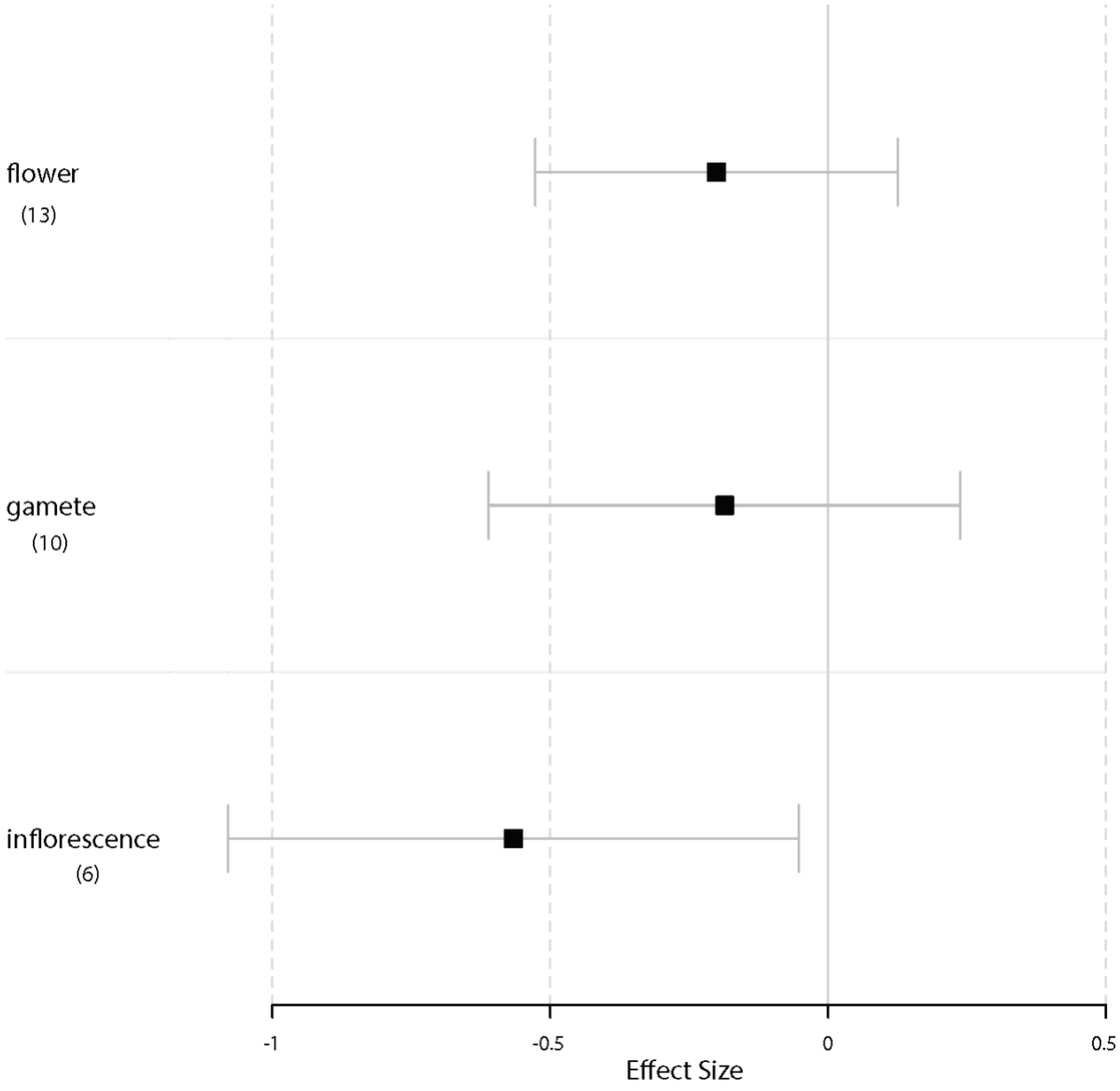

Figure 8. The average estimated effect size of whole genome duplication on the reproductive output traits. Values are coefficient estimates of log response ratios and their corresponding 95% confidence intervals. Number of effect size measures are in parentheses following the trait identifier.

Table S1. Trait table for completed dataset including all the measures collected from the literature that fell within the three main categories.

Phenology	**Size**	**Reproductive Output**
Weeks to flowering	Flower diameter	No. of inflorescences
Days to flowering	Pollen diameter	Percent pollen viability
Days to first flower	Pistillate corolla length	No. of ovules
Days to first inflorescence	Staminate corolla length	No. of ligulate florets
Days to peak flower	Staminate calyx length	No. of tubular florets
Flower date	Staminate pedicle	No. of flowers
Length of flowering	Pollen size	No. of pollens
	Disc flower diameter	No. of capitula
	Inflorescence diameter	No. of ray flowers per capitulum
	Ligulate flower dry weight	No. of flowers per inflorescence
	Tubular flower dry weight	No. of basal inflorescences
	Floral bud length	No. of lateral inflorescences
	Petal length	No. of petals
	Petal width	No. of scapes
	Style length	
	Pollen area	
	Flower length	
	Flower lip height	
	Flower lip width	
	Flower tube length	
	Pollen length	
	Pollen width	
	Ligulate flower length	
	Ligulate flower width	
	Tubular flower diameter	
	Tubular flower length	
	Flower weight	
	Calyx width	
	Flower corolla length	
	Flower corolla width	
	Pistil length	
	Stamen length	
	Ovary length	
	Spathum length/width ratio	
	Spathum thickness	
	Petal back width	
	Petal front width	
	Calyx tube length	
	Standard of corolla breadth	
	Standard of corolla width	
	Ray flower length	
	Anther length	

	Anther width	
	Calyx diameter	
	Filament length	
	Ovary diameter	
	Banner petal length	

Table S2. Table listing the null models used in the meta-analysis.

Dataset	**Response variable**	**Null models**
Entire dataset Effect types = phenology, reproductive output, and size	Effect size	lnRR = fixed(effect type) + random(paper reference + plant species nested within paper reference + trait type nested within plant species nested within paper reference)
	Coefficient of variation ratio	lnCVR = fixed(effect type) + random(paper reference + plant species nested within paper reference + trait type nested within plant species nested within paper reference)
Size dataset Trait types = Gametes, petals, flowers, inflorescences	Effect size	lnRR = fixed(trait type) + random(paper reference + plant species nested within paper reference + trait type nested within plant species nested within paper reference)
	Coefficient of variation ratio	lnCVR = fixed(trait type) + random(paper reference + plant species nested within paper reference + trait type nested within plant species nested within paper reference)
Reproductive output dataset Output types = gametes, flowers, inflorescences	Effect size	lnRR = fixed(output type) + random(paper reference + plant species nested within paper reference + trait type nested within plant species nested within paper reference)
	Coefficient of variation ratio	lnCVR = fixed(output type) + random(paper reference + plant species nested within paper reference + trait type nested within plant species nested within paper reference)

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Chapter 2

Abstract

Although polyploidy has been studied since the early 1900's, most attention has focused on the genomic consequences of polyploidy and consequently, fundamental aspects of polyploid ecology and evolution remain unexplored. In particular, surprisingly little is known about how newly formed polyploid species become demographically established. Models predict that most polyploids should go extinct within the first few generations due to reproductive disadvantages associated with being the minority in a primarily diploid population (i.e., the minority cytotype principle), yet polyploidy is extremely common. Therefore, a key goal in the study of polyploidy is to determine the mechanisms that promote polyploid establishment in nature. Because premating isolation will be critical for newly formed polyploids (neopolyploids) to avoid minority cytotype exclusion and thus help facilitate establishment, we induced polyploidy in *Trifolium pratense* and examined changes in floral morphology and three common premating barriers to determine their importance in generating reproductive isolation from diploids. These premating barriers included isolation by self-fertilization, flowering time asynchrony, and pollinator mediated isolation. We found significant differences in the morphology of diploid and neopolyploid flowers, but these results did not in turn facilitate differences in premating barriers that would generate reproductive isolation of neopolyploids from diploids. Our results indicate that none of the three common premating barriers that we tested are important in facilitating reproductive isolation of neopolyploid *Trifolium pratense.* This work adds to the current paucity of studies investigating premating isolation of neopolyploids from their diploid progenitors in the generations immediately following polyploid formation.

Introduction

Understanding the factors that drive speciation and reproductive isolation is a major focus in evolutionary ecology. One common mode of speciation in plants is polyploidy, or the duplication of an entire set of chromosomes (Reisberg and Willis, 2007; Wood et al., 2009); and although polyploidy has been studied since the early 1900's, most attention has focused on the molecular and genomic consequences of whole genome duplication (Soltis et al., 2010). Consequently, fundamental aspects of polyploid ecology and evolution remain unexplored. In particular, surprisingly little is known about how newly formed polyploid species (hereafter 'neopolyploids') become established in nature. Models predict that under many conditions, polyploids should be relatively ephemeral and go extinct within a few generations due to reproductive disadvantages associated with being the minority in a primarily diploid population (Levin, 1975; Fowler and Levin, 1984; Felber, 1991; Rodriguez, 1996; Baack, 2005; Rausch and Morgan, 2005; Fowler and Levin, 2016). Yet, polyploidy is extremely common (Barker et al., 2016, 24% in extant plant species); thus, a key goal in the study of polyploidy is to determine the mechanisms that promote neopolyploid establishment in populations.

For neopolyploids to establish and persist in a predominantly diploid population, neopolyploids must be at least partially reproductively isolated from their diploid progenitor. If no reproductive isolation exists between neopolyploids and their diploid progenitors and the two cytotypes mated freely, we would expect one of two outcomes. First, if the two cytotypes were capable of producing offspring, the offspring would be triploid which are often semi-fertile due to meiotic irregularities and a high production of gametes with an abnormal number of chromosomes (Ramsey and Schemske, 1998). The second outcome would be that no offspring could be generated, known as the "triploid block" (Marks, 1966). Both of these scenarios would

lead to reduced fecundity of neopolyploids and fewer successful pollinations relative to diploids. Because neopolyploids will be fewer in number in a population than their diploid counterparts, the disadvantage of the minority cytotype will increase with each successive generation until the neopolyploids become extinct. This is known as the minority cytotype exclusion principle (Levin, 1975). Minority exclusion can be mitigated through reproductive isolation, and although many polyploids experience instant postzygotic isolation from their diploid sister group, prezygotic barriers must also exist to facilitate assortative mating and avoid ineffective pollinations that result in wasted gametes and proportionally fewer offspring (Levin, 1975; Husband and Sabara, 2003; Husband et al., 2016).

To date, studies investigating the role of prezygotic barriers in reproductive isolation of polyploids have primarily compared systems of established polyploids and their diploid sister group (Segraves and Thompson 1999; Husband and Sabara, 2003; Schranz and Osborn 2004; Thompson and Merg 2008; Jersáková et al., 2010; Gross and Schiestl 2015; Roccaforte et al., 2015; Husband et al., 2016; Pegoraro et al., 2016). For example, Husband and Sabara (2003) estimated mechanisms of reproductive isolation in natural populations of *Chamerion angustifolium* and determined that the majority of isolation between cytotypes was due to prezygotic isolation, specifically pollinator fidelity and the spatial distribution of cytotypes within populations. Similarly, Roccaforte et al. (2015) quantified the contribution of isolating barriers between diploid *Erythronium mesochoreum* and its tetraploid sister species *Erythronium albidum.* They found that geographic isolation was driving reproductive isolation in this polyploidy complex, followed by pollinator mediated-isolation and floral phenology, with postzygotic barriers contributing the least to reproductive isolation. Polyploidization is also known to break down reproductive self-incompatibility mechanisms, be correlated with changes

in mating systems, and alter the rate of selfing (Ramsey and Schemske, 1998; Glick et al., 2016). There is evidence from phylogenetic comparative studies that polyploids generally tend to self-fertilize at higher rates than diploids and this propensity towards selfing may help neopolyploids to overcome minority cytotype exclusion (Barringer, 2007; Robertson et al., 2011). Together, these studies suggest that established polyploids and diploids are often isolated through at least one, but often a combination of prezygotic barriers, particularly when living in sympatry. Although this previous work investigating the mechanisms maintaining reproductive isolation and promoting persistence of established polyploids has been instrumental in the study of polyploid reproductive ecology, there remains a gap in our understanding of how polyploids establish given their reproductive disadvantages. Specifically, we have yet to understand which prezygotic mechanisms promote isolation and facilitate establishment in the generations immediately following polyploid speciation (Husband et al., 2016).

To the best of our knowledge, only one study to date has quantified the relative importance of various prezygotic isolating mechanisms of neopolyploids from their diploid progenitors. Husband et al. (2016) found that in *Chamerion angustifolium,* neopolyploids had some phenotypic traits that were more similar to diploids than established polyploids, and other traits that more closely resembled established polyploids and differed from diploids. Additionally, they found differences in the degree to which the various reproductive barriers contributed to reproductive isolation of neopolyploids and established polyploids from diploids. This study provides direct evidence that the mechanisms and degree of reproductive isolation experienced by established polyploids may not be the same for neopolyploids especially during the critical generations immediately following whole genome duplication. These results highlight how the phenotypes of neopolyploids can be significantly different from older generation

polyploids (Butterfass, 1987; Oswald and Nuismer, 2011) and suggest that to truly understand the pervasiveness of polyploidy, we require more studies investigating the mechanisms of prezygotic isolation of neopolyploids.

To address this deficit and build upon the foundational work of Husband et al. (2016), we induced neopolyploidy in red clover, *Trifolium pratense,* and observed changes in floral morphology and three common prezygotic barriers to determine their importance in generating reproductive isolation from diploids. This study is the first to assess multiple modes of prezygotic isolation on a single set of neopolyploids under common garden conditions. The prezygotic barriers that we examined were temporal isolation via flowering phenology, the breakdown of self-incompatibility, pollinator-mediated isolation via differences in flower visitor communities and flower visitor behavior.

Methods

Study organism

To investigate whether prezygotic isolation occurs in neopolyploids relative to their diploid parents, we used the herb *Trifolium pratense,* or common red clover. Red clover is frequently planted as fodder and although it has origins in Europe, *T. pratense* is now globally naturalized (GBIF). Red clover is an excellent species to use for studies of reproductive isolation in neopolyploids for a number of reasons. First, there are published methods for inducing polyploidy in this species (Taylor et al., 1976), diploid red clover naturally produce unreduced gametes at low frequencies (Parrott and Smith, 1986), tetraploid populations have been identified in nature (Pinar et al., 2001; Buyukkartal, 2008; Buyukkartal, 2013), the species is outcrossing and diploids are known to be strongly self-incompatible, and lastly, it reaches reproductive maturity relatively quickly (3-4 months).

Generating neopolyploids

Neopolyploid red clover seeds were generated following the methods described by Taylor et al. (1976). In brief, diploid plants were grown from seed (Dirt Works, New Haven VT, Organic Medium Red Clover) and cross pollinated by hand. Twenty-four hours after pollination, we clipped the inflorescences with fertilized flowers and placed the inflorescence stalks in 2% w/v sucrose. These were then incubated in a pressure chamber filled with nitrous oxide at 90 psi for either 24 or 36 hours, and seeds were then allowed to develop with a constant supply of sucrose solution until the inflorescence tissue was dried.

Cytological analysis

We identified the cytotype of plantlets grown from nitrous oxide treated seeds by evaluating nuclear DNA content using flow cytometry (Kron et al., 2007). Flow cytometric methods followed the protocols of Godsoe et al. (2013). In brief, plant nuclei were isolated from leaf tissues by chopping leaves in magnesium sulfate buffer ([10 mM $MgSO_4$-$7H_2O$, 50 mM KCl, 5mM Hepes, adjusted to pH 8], 6.8 mM dithiothreitol, Triton X 100 at 1mg/mL, and 1 mM PVP-40). The resulting supernatant was filtered through a 30 µm nylon filter, and samples were centrifuged and supernatant discarded. We then stained the nuclei with propidium iodide solution containing a rainbow trout red blood cell standard (Rainbow trout blood diluted with 1:11 Alsever's solution, 5mg/mL propidium iodide, and magnesium sulfate buffer). Our propidium iodide solution differed from Godsoe et al. (2013) recipe by omitting RNase from the solution. Samples were processed on a BDAccuri C6 flow cytometer at the Syracuse University Flow Core facility, and cytotype was determined by analyzing the data using Flowing Software (Version 2.5.1, Perttu Terho, Turku Centre for Biotechnology, Finland; www.flowingsoftware.com).

Plants identified as tetraploids via flow cytometry analysis were then subject to chromosome counts from root tip cells. We sampled fine roots, and soaked them in Farmer's Fixative (3:1 absolute ethanol to glacial acetic acid) for approximately 24 hours, followed by treatment with 10% HCl at 60°C for 5 minutes, and last stained the roots with acetocarmine at 60°C for approximately 1.5 hours. Four plants identified as tetraploids via flow cytometry were confirmed as tetraploids with direct counts of chromosomes. Two other tetraploids identified via flow cytometry had approximately double the number of chromosomes as determined by chromosome squashes, but small overlapping chromosomes made it difficult to provide

definitive confirmation. However, these two plants displayed similar phenotypes to the chromosome squash verified tetraploids, and did not display characteristics of the aneuploids such as stunted growth and bumps over the leaf and stem surfaces.

Seed stocks for experiments

To obtain enough tetraploid plants to do a comparative study between neopolyploids and diploids, and to ensure that our neopolyploid and diploid plants were treated identically, both nitrous oxide treated red clover and untreated diploids were grown to flowering together in a greenhouse at 14-16℃ day and 11-13℃ night temperature cycles and 15-hour daylight conditions. We cross pollinated 14 diploids to generate a stock of diploid seeds. Once nitrous oxide treated plants were confirmed as tetraploids via flow cytometry or both flow cytometry and chromosome counts, we cross pollinated these six neopolyploids to generate a stock of neopolyploid seeds.

Plant care

Diploid and neopolyploid seeds were grown in the Syracuse University greenhouse. These seeds were germinated in Miracle-Gro Potting Mix and sown in individual cells of propagation trays. We set the greenhouse room conditions at 20-22°C day and 17-19°C night temperature cycles with light conditions that mimicked natural sunrise and sunset conditions of Syracuse NY, USA. Four weeks after planting, the seedlings that germinated were transplanted to 1.89 L pots. Both diploid and neopolyploid seeds had low germination success. Therefore, in an attempt to increase germination rates, we cold treated the remaining seeds that had not yet germinated. Cold treatment lasted for two weeks at 6-8°C in a reach-in growth chamber.

Following the cold treatment, seeds were returned to the greenhouse and grown under standard growing conditions as before. Approximately four weeks after being returned to the greenhouse this second group of plants was transplanted into 1.89 L pots. For the remainder of the experiment, both groups were grown in the same greenhouse conditions, then moved to the Syracuse University experimental gardens by 'group' once they began bolting. Once transferred to the common garden, plants remained there through the end of the experiment. In total, 85 non-cold treated seeds (hereafter group 1) germinated (diploids, N = 31 and neopolyploids, N = 54) and 89 cold treated seeds (hereafter group 2) germinated (diploids, N = 39 and neopolyploids, N = 50). Once neopolyploid seedlings had at least three trifoliate leaves, they were screened via flow cytometry against the rainbow trout red blood cell standard and a diploid red clover individual to confirm cytotype.

Flower morphology

Three flowers from the top, middle, and base of an inflorescence were collected from each flowering plant. Flowers were placed on ice and transported to the lab to photograph. Flowers were photographed individually and pictures were taken using an Olympus Camedia c 7070 wide-zoom 7.1 MP camera with a Leica S8 APO dissecting microscope, including a 0.5 cm Minitool Micro-Scale ruler (BioQuip). Total length (TL), length of the banner petal (LB), distance between the tip of wing petals (WD), width of banner petal (BW), width of the tube (WT), stigma-anther separation (SA), wing length (WL), and the angle of the banner (AB) were measured (Fig. 1). All morphological traits were measured using ImageJ 1.50i software (Schneider et al., 2012). Total length was measured using the curved line tool to follow the shape of the flower on the ventral side of the tube and banner petal. The angle of the banner petal was

measured using the angle tool, and the rest of the traits were measured using the straight line tool in ImageJ 1.50i.

Floral phenology

For each plant, the date of germination was recorded and they were observed for flowering. For the plants that flowered, we tracked their flowering phenology throughout the season. We counted the total number of inflorescences in bloom per plant. Inflorescences were scored as in bloom if more than half of the flowers on the inflorescence were open. This was used as the cut-off because previous observations of local bees foraging on red clover were attracted to inflorescences with the majority of flowers in bloom. Because red clover is outcrossing, if bees are not visiting the inflorescence when only a few flowers are open, then it is effectively not reproductively active.

Self-fertilization

Each diploid and neopolyploid plant were assigned to one of two self-fertilization treatments prior to flowering: hand-pollination and autonomous self-pollination. The hand-pollination treatment was designed to determine the frequency of self-pollinating individuals while simulating the presence of pollinators, and the autonomous self-pollination treatment determined the frequency of self-pollinating individuals regardless of pollinator presence. We used both self-fertilization treatments because self-incompatibility mechanisms may break down in neopolyploids if morphology changes in a way that reduces the ability of pollen to autonomously reach the stigma such that self-pollination may only occur in the presence of pollinators. For both treatments, a single inflorescence on the plant was covered with a small

mesh bag before flowering to ensure that no pollinators would be able to visit. For the hand-pollination treatment, we temporarily removed the mesh bag and hand pollinated flowers on the selected inflorescence with pollen originating from the same inflorescence. For the autonomous self-pollination treatment, the mesh bag remained in place throughout flowering to test if floral morphology allowed for self-pollination in the absence of pollinators. Four weeks after self-fertilization treatments, the inflorescences were removed from the plant, bagged, and brought back to the lab to assess presence or absence of seeds.

Flower visitors

Flower visitor behavior was monitored to determine whether there were immediate behavioral differences in bee responses to neopolyploid plants. Depending on the number of plants in bloom on a given day, 6-12 plants were set up approximately one meter apart from one another in a rectangular checkerboard array with alternating cytotypes. Arrays were placed in various locations within one kilometer of the experimental garden. Observations of flower visitor behavior began when an insect landed on an inflorescence in the array and they were followed until leaving the array. The visitation pattern (whether landing on diploid or neopolyploid inflorescence), the number of inflorescences visited, and whether the insect actively foraged or simply visited a flower was recorded. When possible, insects were collected after visitation and were brought back to the lab for identification. If we were unable to catch the insects, a size estimate was recorded. Small bees are unlikely to be effective pollinators, as previous studies have suggested only larger bees pollinate red clover (Bender, 1999a, b). We were easily able to identify *Bombus impatiens* to species level in the field because of unique abdomen markings. Other species in the genus have variable color patterns so field identification was unreliable.

Species identified during these observations were used to generate diploid and neopolyploid bee community profiles.

Statistical analysis

To determine whether there were differences in flower morphology between diploids and neopolyploids, we performed a two-way multivariate analysis of variance (MANOVA). Our model included the eight flower morphology traits as response variables with cytotype as a fixed predictor variable and 'group' as an interacting fixed predictor variable. The group predictor variable allowed us to determine whether the cold treatment or difference in development time impacted the differences between cytotypes. Tukey's HSD post-hoc test was then used to further evaluate differences of the morphological traits between cytotypes of the individual morphological traits. We also performed a principal components analysis to visualize the differences and characterize the variation. Top, middle, and bottom flower measurements were averaged per plant, and experimental units were at the plant level.

To determine whether there were differences in phenology, we first calculated the days to first flower (first day of recorded flowering – first day of recorded germination) and days to peak flower (day of recorded max flowering – first day of recorded germination). We then used a two-way MANOVA to investigate whether there were differences in these floral phenology traits between diploids and neopolyploids. This model included the two phenology variables as response variables with cytotype as a fixed predictor variable and 'group' as an interacting fixed predictor variable to determine whether the cold treatment impacted differences between cytotypes.

To determine whether neopolyploids differed from diploids in the proportion of individuals able to self-pollinate, we used a chi-square test for equality of proportions. To determine if bees played a role in prezygotic isolation of neopolyploids by flying non-randomly between cytotypes, we used a chi-square goodness of fit test to see if flights between cytotypes differed from what would be expected by random. And lastly, to determine if bees were differentially visiting diploids and neopolyploids, we used a chi-square test of independence. All analyses were carried out using R Statistical Software (R Development Core Team, 2016).

Results

Flower morphology

In total, 318 flowers were photographed and measured, providing 2501 individual measurements from 48 diploid and 57 neopolyploid plants. A two-way MANOVA indicated that there were significant effects of cytotype and group on floral morphology ($F_{8,94} = 8.271$, $p < 0.0001$; $F_{8,94} = 2.613$, $p = 0.013$), but the interaction term was not significant ($F_{8,94} = 0.332$, $p = 0.952$). Tukey's post hoc tests indicated significant differences between cytotypes in all size traits and also the angle of the banner (Fig. 2). Although 'group' significantly affected morphology, univariate tests show that differences between groups were only present for one shape trait, the distance between wings (Fig. 3). For all size traits where diploids and neopolyploids were significantly different from one another, neopolyploids were larger and the angle of the banner petal was sharper. We also used principal components analysis to explore differences in floral morphology (Fig. 4). In this analysis, we found that size-related traits were more important in driving the differences between diploids and neopolyploids because all of the size traits along with wing distance had larger loadings on the first principal component which accounted for 53% of the total variation. Stigma-anther separation and angle of the banner petal had larger loadings on the second principal component which accounted for 14% of the total variation.

Floral phenology

We tracked floral phenology on 43 diploids and 55 neopolyploids. A two-way MANOVA examining the effect of cytotype and 'group' on floral phenology traits showed that there were significant effects of both cytotype ($F_{2,93} = 7.533$, $p < 0.001$), and 'group' ($F_{2,93} =$

15.015, $p < 0.0001$) on floral phenology traits. There was, however, no interaction between 'group' and cytotype that impacted these phenology traits ($F_{2,93} = 1.464$, $p = 0.237$). Group 1 plants that did not receive cold treatment flowered earlier and reached peak flower earlier than group 2 plants. In group 1, the number of days to first flower (mean ± SE) of diploids and neopolyploids was 87.9 ± 3.0 and 90.9 ± 1.8, respectively, and the number of days to peak flower was 100.0 ± 2.4 and 100.35 ± 1.7, respectively. In group 2, the number of days to first flower of diploids and neopolyploids was 88.8 ± 3.6 and 100.5 ± 3.1, respectively, and the number of days to peak flower was 107.8 ± 2.6 and 109.7 ± 2.7, respectively. The total number of days flowering of neopolyploids completely overlapped with diploids (Fig. 5). The data used to generate Figure 5 comes from group 1 only, because group 2 flowers were harvested before the completion of their flowering cycle.

Self-fertilization

Self-fertilization was tested in 38 diploids and 54 neopolyploids. Both the hand and autonomous self-pollination treatments revealed a similar number of self-compatible individuals (hand-pollination = 6, autonomous self-pollination = 4), suggesting that pollen can reach the stigma of red clover in the absence of pollinators regardless of cytotype. Therefore, we pooled the results of the self-fertilization treatments. When we tested for differences in self-fertilization rates between diploids and neopolyploids, we found an approximately three-fold increase in individuals that were able to self-fertilize after genome duplication. For neopolyploids, 14.8% of individuals were able to set seed after self-fertilization, as opposed 5.2% of individuals for diploids. However, we did not find a significant difference between cytotypes in the proportion of individuals able to self-fertilize ($X^2 = 1.230$, $df = 1$, $p = 0.267$).

Flower visitors

We observed a total of 95 bee foraging behaviors over 18 observation periods lasting between one and four hours each, with the bees transitioning between 209 plants and 491 individual inflorescences. Overall, bees visited diploid and neopolyploid plants at similar frequencies; 54% of plants visited were diploids and 46% were neopolyploids. To test whether foraging behavior could lead to prezygotic isolation of neopolyploids, we looked for evidence of assortative mating of plants facilitated by bee behavior. Following Kennedy et al. (2006), we used a conservative measure of bee constancy (the tendency to preferentially visit either diploids or neopolyploids) to determine whether flower visitors could facilitate isolation. We used only the first transition between plants as our unit of measure to avoid complications of non-independence for the subsequent plant transitions in bee foraging bouts. We found that bee flights within (diploid to diploid, and neopolyploid to neopolyploid) and between (diploid to neopolyploid, and neopolyploid to diploid) cytotypes did not differ from flights that would be expected by random visitation ($X^2 = 6.767$, df = 3, p = 0.080). We also found that the bee communities visiting diploids and neopolyploids were very similar (Fig. 6). The most common bees in both diploid and neopolyploid communities were *Bombus* species. For diploids, the bee community consisted of 40.2% *Bombus* spp., 16.7% *Andrena* spp., 6.9% *Apis mellifera*, 5.6% *Colletes*, 4.2% *B. impatiens*, 1.4% *Megachile*, 25% unidentified, small bees (approximately 1cm or smaller). For neopolyploids, the bee community consisted of 49.1% *Bombus* spp., 13.5% *Andrena*, 6.8% *Colletes*, 5.1% *Apis mellifera*, 8.5% *B. impatiens*, 1.7% *Megachile*, and 15.3% unidentified, small bees (approximately 1cm or smaller). These bee groups did not visit one cytotype more frequently ($X^2 = 3.545$, df = 6, p = 0.738).

Discussion

Polyploidy is a common mode of speciation in plants, but despite its importance in plant evolution, surprisingly little is known about how neopolyploids become established. Theory predicts that neopolyploids will be unlikely to find a suitable mate and should quickly become extinct (Levin, 1975), yet polyploid species are extremely common (Barker et al., 2016). For neopolyploids to establish and persist in a predominantly diploid population, genome duplication must induce mechanisms that promote pre-zygotic reproductive barriers to facilitate assortative mating and avoid ineffective pollinations that would result in wasted gametes and scant offspring (Levin, 1975; Husband and Sabara, 2003; Husband et al., 2016). To best understand how neopolyploids become established, more studies examining the reproductive ecology in the generations immediately following speciation are needed. Here, we generated neopolyploid plants and compared them to diploids to determine if polyploidization directly altered aspects of the plant's reproductive biology that would lead to prezygotic isolation from diploids. We found that genome duplication did immediately impact floral morphology of our plants, but found no inherent changes associated with genome duplication that might facilitate prezygotic isolation.

In our study, we determined that flower size increased after whole genome duplication, in accord with the gigas effect observed in many other plant species, and there were also differences in flower shape (Chapter 1; Muntzing, 1936; Stebbins, 1971). These changes in floral morphology could cascade to a number of different effects important to plant reproductive ecology. For example, we know that tetraploid varietal lines of red clover can have larger flowers than diploids (Bender, 1999a, b; Vleugels et al., 2015) and that bee behavior can change depending on the cytotype (Bender, 1999a, b). Morphological changes associated with genome duplication could offer easier access to nectar or pollen rewards, and cause behavioral changes in

pollinators or attract different suites of pollinators altogether. However, in contrast to these expectations, our data suggest that despite the changes in flower morphology, bees were either unable to differentiate between neopolyploids and diploids, or the perceived differences were unimportant in flower selection. The results showed that there was no evidence of pollinator-mediated isolation due to flower visitor behavior or through changes in the composition of visiting bee communities. We are aware of only two other studies that have compared the community and behavior of pollinators of neopolyploids and diploids. Nghiem et al. (2011) observed that both diploids and neopolyploids were primarily visited by honeybees, and they showed that qualitatively, bees did not discriminate between diploid and neopolyploid plants. Another study conducted by Husband et al. (2016) also observed primarily honeybees (>90%) visiting both cytotypes, and found that pollinator behavior did not contribute to reproductive isolation of neopolyploids. Although most of the bees observed in our study were not honeybees, they were generalist species. If phenotypic changes do in fact play a role in prezygotic isolation of neopolyploids, perhaps that role is restricted to plant species with specialist pollinators.

Changes in flower size of neopolyploids also has the potential to impact phenological traits, if for instance, larger flowers require longer development times and results in later flowering (Cavalier-Smith, 1978; Ramsey and Ramsey, 2014). Indeed, we did find that neopolyploidy significantly delayed the time to first flower. This result is similar to some studies that have recorded flowering times of neopolyploids (Tulay and Unal, 2010; Oswald and Nuismer, 2011; Ramsey, 2011; Chae et al., 2013), but other studies have also found either that genome duplication does not alter flowering timing (Nghiem et al., 2011; Husband et al., 2016) or there are mixed results when neopolyploids are derived via hybridization (Hansen and Earle, 1994; Chen et al., 2002). Although our neopolyploids did take longer to begin flowering, this did

not translate into an overall shift in flowering phenology. Both diploids and neopolyploids reached peak flowering at the same time, and neopolyploid flowering did not extend past that of diploids (Fig. 4). This suggests that for red clover, neopolyploidy does cause changes in flowering initiation, but these changes would be unlikely to lead to reproductive isolation as the timing of neopolyploid flowering completely overlaps with diploids.

In addition to phenological and pollinator-based isolation, another potential isolating mechanism is self-fertilization. The propensity for genome duplication to break down self-incompatibility mechanisms is well documented, particularly for plants with gametophytic self-incompatibility systems; although the mechanisms behind the breakdown are poorly understood (Ramsey and Schemske, 1998; Mable, 2004; Barringer, 2007). The increased ability of neopolyploids to self-pollinate could be critical in preventing ineffectual pollinations with nearby diploids, and reducing the likelihood of succumbing to minority cytotype exclusion (Levin, 1975; Rodriguez, 1996; Baack, 2005; Rausch and Morgan, 2005; Fowler and Levin, 2016). In our experiment we found that red clover, which has a gametophytic self-incompatibility mechanism (Taylor and Smith, 1979), did experience a slight but non-significant increase (10%) in the proportion of self-compatible plants after genome duplication. However, our sample sizes may have been lower than necessary to detect differences at these frequencies.

We interpret our results with caution and would like to highlight two key limitations of this work. First, the results derived from studies using synthetic neopolyploids may not emulate the range of phenotypes observed in naturally derived neopolyploids. This is particularly true for neopolyploids generated by somatic doubling where genetic diversity is reduced compared to wild neopolyploids that would arise from sexual polyploidization. For example, it is possible that wild polyploids may only establish from unique genotypes, and so synthetically produced

neopolyploids may not recreate the genotypes and phenotypes that would facilitate establishment in nature (Ramsey, 2011; Ramsey and Ramsey, 2014). Despite these caveats, we argue that synthetic neopolyploids do provide us with the unique opportunity to observe phenotypes that stem directly from genome duplication, without the confounding effects of subsequent selection and drift associated with older, evolved polyploids (Ramsey and Ramsey, 2014). Second, we also used unrealistic proportions of neopolyploids and diploids in our flower visitor behavior arrays. In naturally derived neopolyploid populations, neopolyploids will be the minority cytotype rather than in equal proportions as used in our checkerboard array. Had we used more realistic proportions and randomized placement of cytotypes within the array, we would not be able to ensure that pollinator-mediated assortative mating was due to active pollinator preference alone and not influenced by spatial aggregation of cytotypes.

Together, the results of this study suggest that none of the prezygotic mechanisms that we tested are important in facilitating reproductive isolation of neopolyploid red clover. This is surprising given our original expectation that at least one of the mechanisms shown to be important in enacting reproductive isolation in established polyploids would be also be involved in reproductive isolation of neopolyploids. Although we observed shifts in floral morphology, these differences did not facilitate isolation of neopolyploids from diploids in self-pollination rates, flowering phenology, flower visitor behavior or flower visitor communities. These observations support the conclusions of Husband et al. (2016) that although neopolyploids often show immediate changes in floral phenotype, these changes on their own do not account for the reproductive barriers observed in natural, established populations.

One trait we did not examine that has been shown to strongly impact reproductive isolation in polyploids is geographic isolation. Studies that examine reproductive isolation of

established polyploids have found that geographic isolation is a primary contributor to isolation between cytotypes (Husband and Sabara, 2003; Pegoraro et al., 2016). In our study, we excluded geographic isolation as a potential prezygotic isolating mechanism because neopolyploids are expected to form within the distribution of their diploid progenitors. However, there is evidence that pollinators do facilitate assortative mating between cytotypes due to the spatial structure of cytotypes within populations (Husband and Schemske, 2000), and models suggest that limited seed and pollen dispersal can generate 'islands' within a larger, mixed cytotype populations where neopolyploids are not so greatly affected by minority exclusion (Baack, 2005). Therefore, small-scale spatial distribution of cytotypes could play an important role in pollinator-mediated isolation of neopolyploids, but was not considered in this study. Studies comparing relative success of neopolyploids in various spatial structures, and studies comparing the relative success of polyploids with differing dispersal mechanisms would broaden our understanding of the importance of geographic isolation as a factor contributing to neopolyploid establishment.

A major challenge is understanding the ubiquity of polyploids in nature and how they establish despite predictions that suggest they should be evolutionarily short-lived. Because polyploid establishment will occur in the generations immediately following formation, it is critical that we tackle this challenge using study systems that have not been altered through evolutionary processes such as selection and drift. Here, we show that three common modes of prezygotic isolation in established polyploids did not produce reproductive isolation of neopolyploids from diploids. More studies investigating multi-modal mechanisms of prezygotic isolation are needed to draw broad conclusions about the mechanisms that facilitate neopolyploid establishment.

Figures

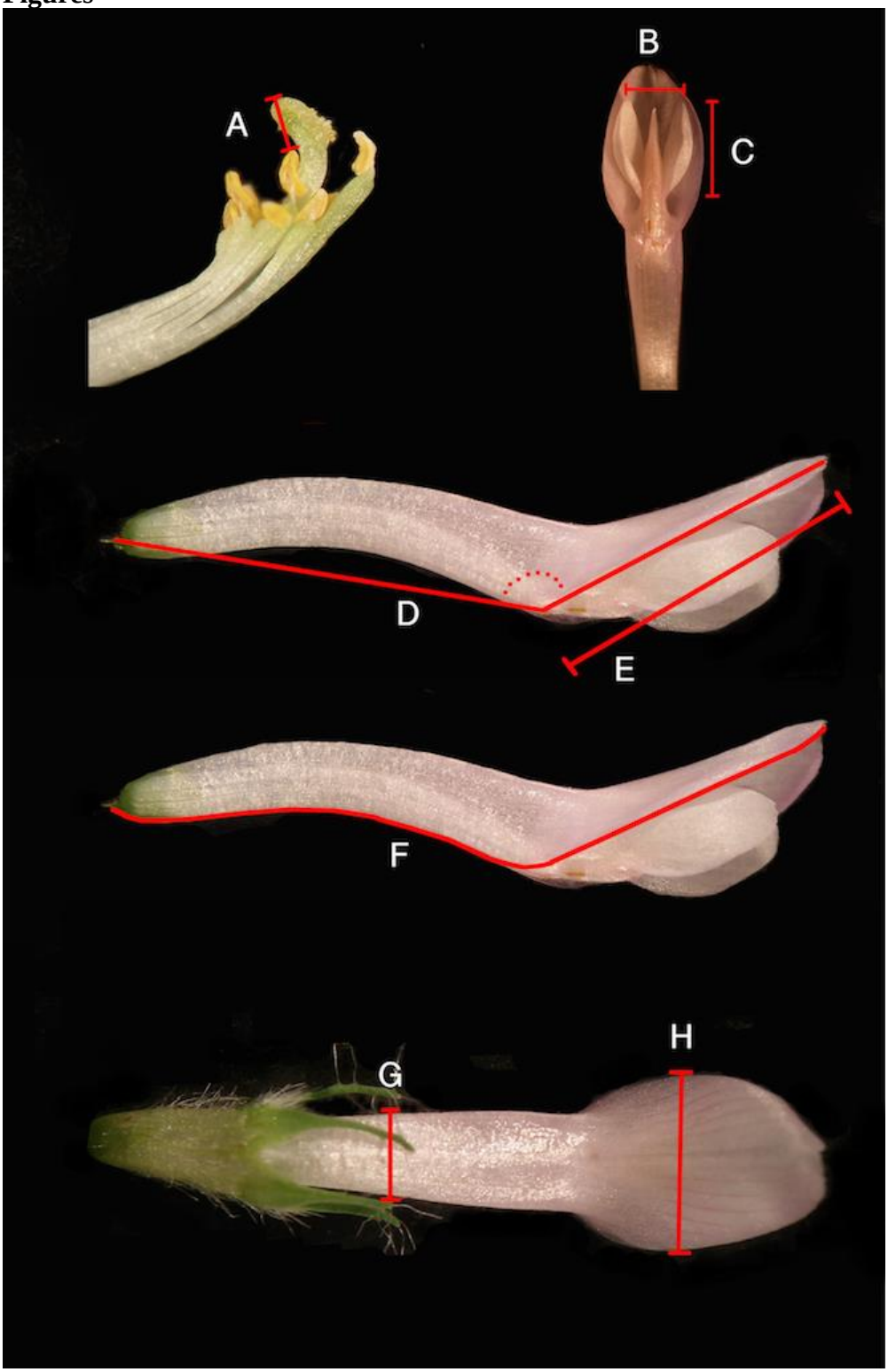

Figure 1. Flower measurements of *Trifolium pratense*. A, stigma-anther separation (SA); B, distance between wing petals (WD); C, wing petal length (WL); D, angle of the banner petal (AB); E, length of the banner petal (LB); F, total length of the flower (TL); G, width of the flower tube (WT); and H, width of the banner petal (BW).

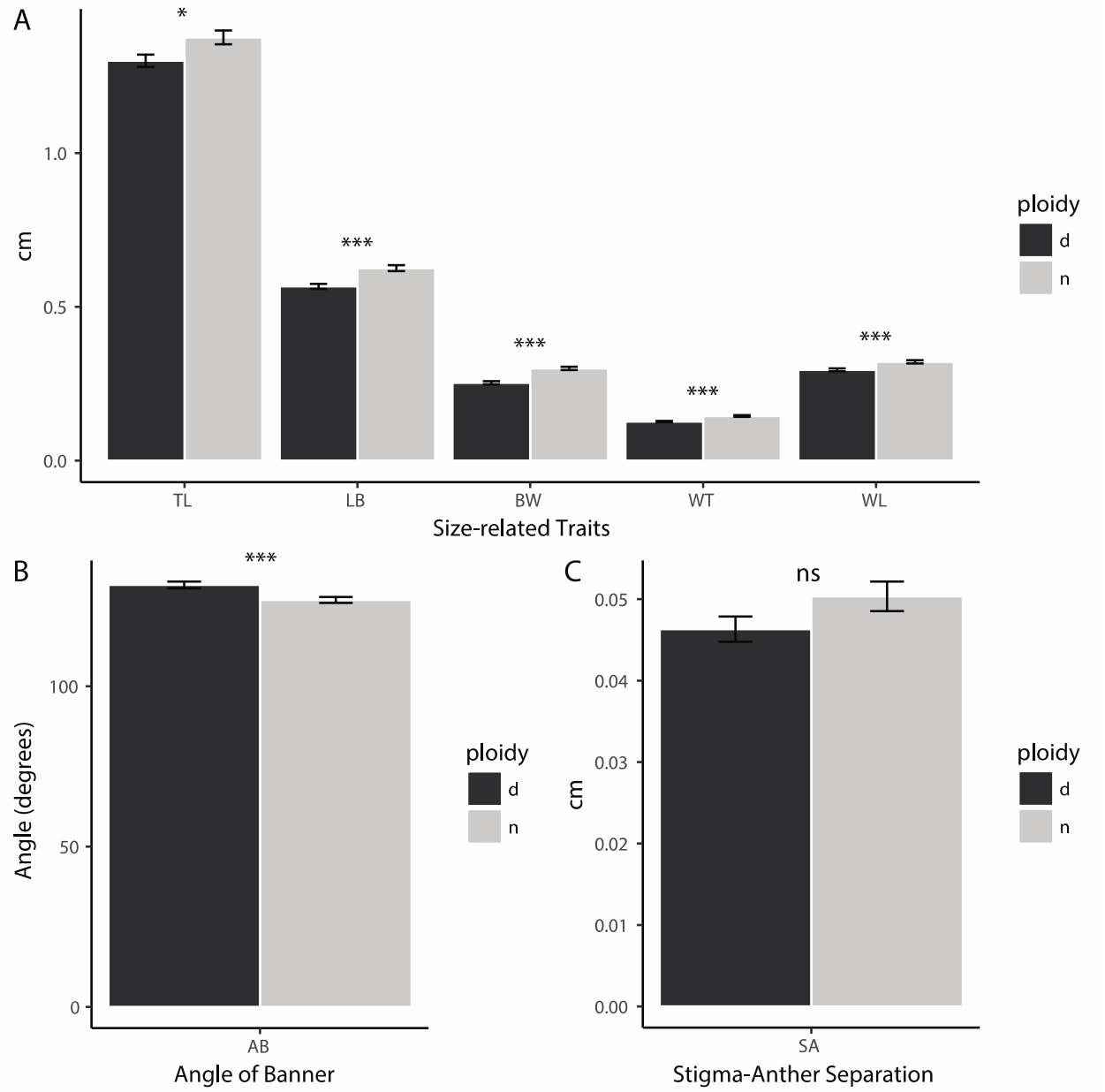

Figure 2. Comparisons of flower morphology between diploid (black) and neopolyploid (grey) *Trifolium pratense*. A) Size-related traits; TL (total length of flower), LB (length of the banner petal), BW (width of banner petal), WT (width of the flower tube), and WL (length of the wing petal). B) Angle of the banner petal relative to the flower tube. C) Distance between the tip of the stigma and nearest anther. Tukey's HSD post-hoc tests of pairwise significant differences between diploids and neopolyploids are indicated with asterisks. * $p < 0.05$, *** $p < 0.001$, ns not significant. Error bars are standard errors of the mean.

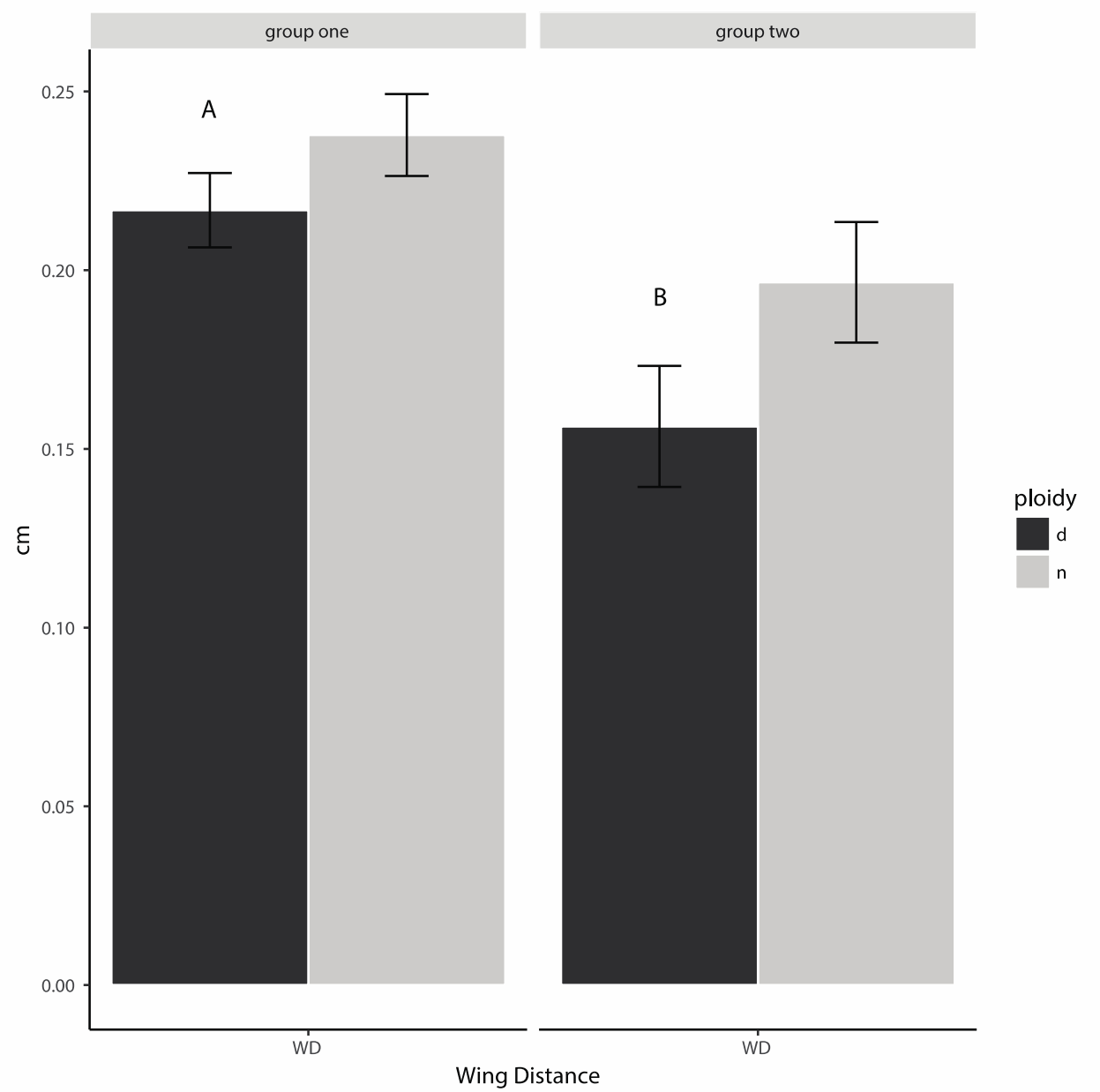

Figure 3. Comparison of the tip of wing petals of diploid (black) and neopolyploid (grey) *Trifolium pratense* of both groups one and two. Significant differences between diploids in group one and two using Tukey's HSD post-hoc tests are marked with different letters. Error bars are standard errors of the mean.

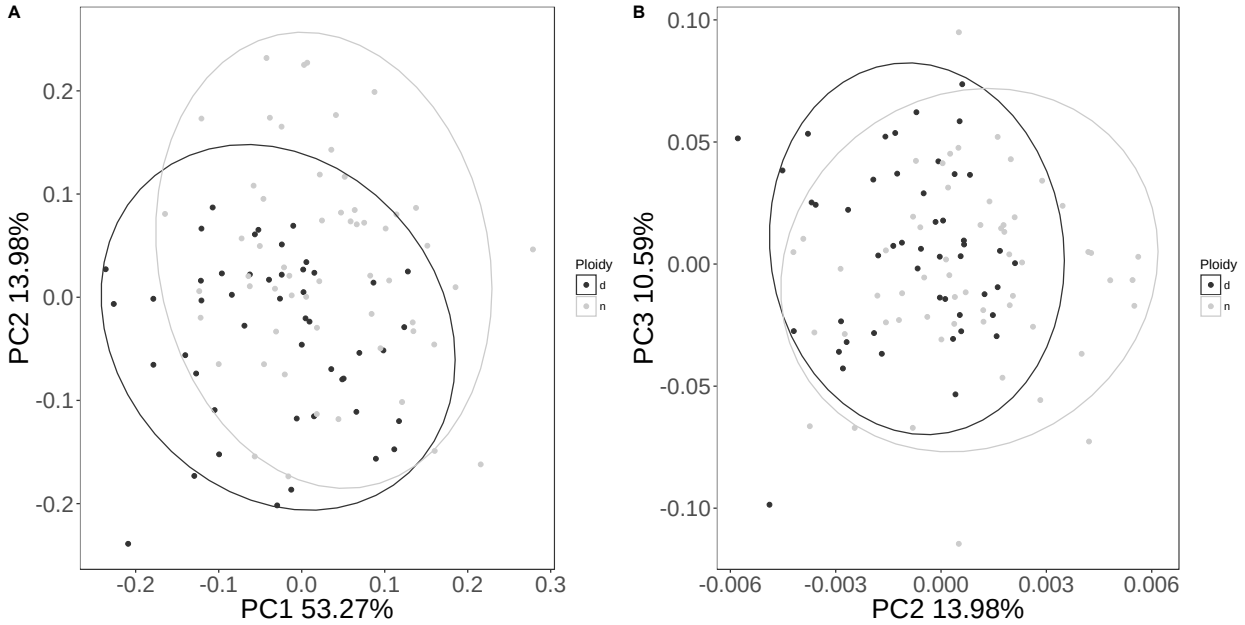

Figure 4. Principal components analysis of floral traits of diploid (dark grey) and neopolyploid (light grey) *Trifolium pratense*. A) PC1 and PC2. B) PC2 and PC3. Percentages of the total variance are indicated on the axes and circles represent 95% confidence estimates.

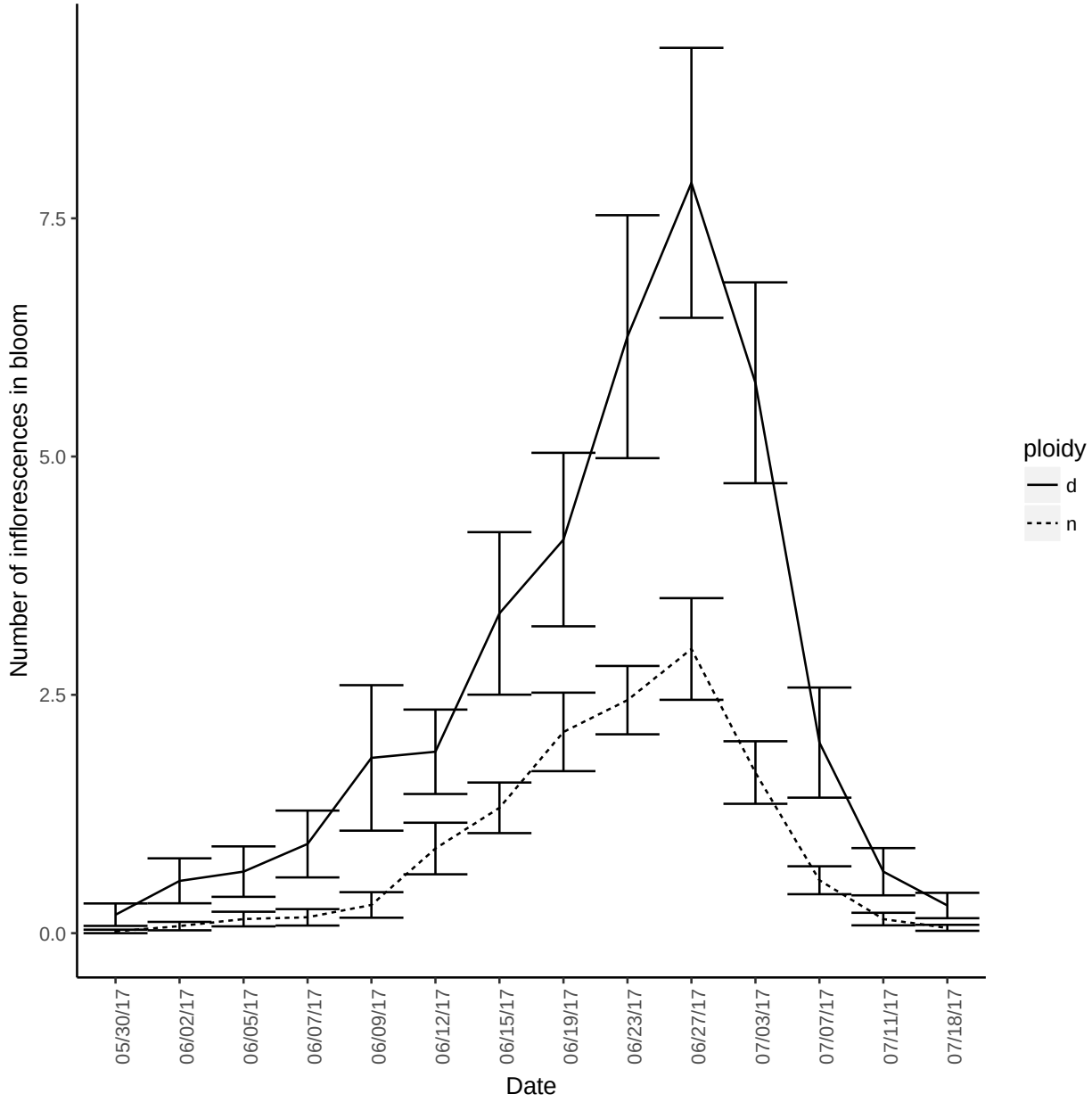

Figure 5. Floral phenology timeline of diploid and neopolyploid *Trifolium pratense*. Lines connect the mean and SE of the number of inflorescences in bloom of diploids (solid line) and neopolyploids (dotted line). Dates are MM/DD/YY.

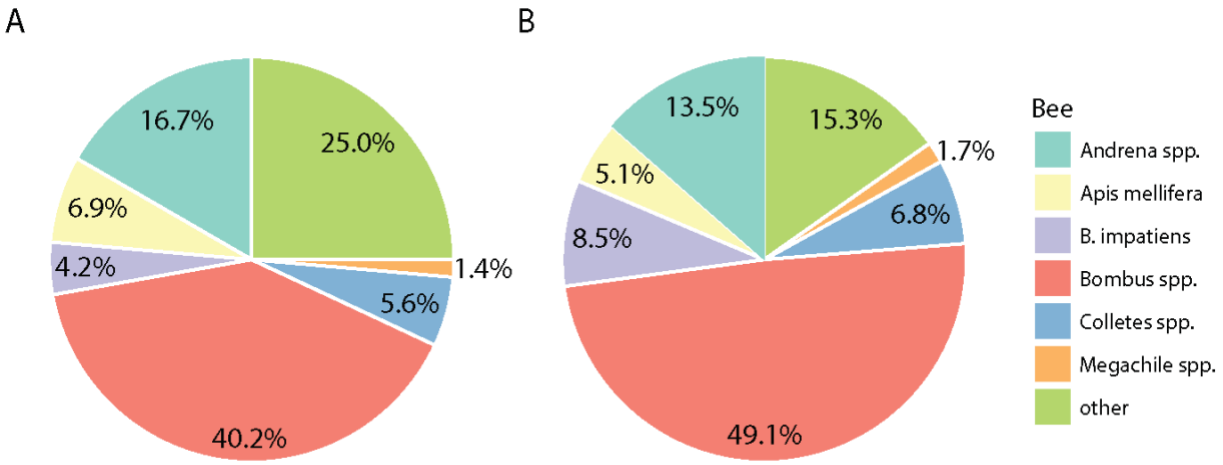

Figure 6. Visitor composition of diploid (A) and neopolyploid (B) *Trifolium pratense* bee communities. The total number of visitors to diploid plants was 72, and tetraploids 59.

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