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Research article

Polyploidy-induced floral changes lead to unexpected pollinator behavior in *Arabidopsis arenosa*

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The paradox between the ubiquity of polyploid lineages in plants and the early obstacles to the establishment of polyploids is a long-studied yet unresolved question in evolutionary biology. It is assumed that to successfully persist after emergence, newly formed polyploids need to display certain fitness advantages and show a certain extent of reproductive isolation with their diploid progenitors. In this study, we tested whether immediate floral changes following polyploidization can improve pollinator visitation and enable pollinator preference leading to assortative mating, i.e. build a premating reproductive barrier between diploids and polyploids. For this purpose, we generated synthetic tetraploids of *Arabidopsis arenosa* and measured insect visitor behavior on diploids and synthetic tetraploids. We found that the increased floral size that accompanied polyploidization did not lead to a measurable increase in visitor preference, with insects visiting diploid and tetraploid plants at equal frequency. Despite this observation, tetraploids set more fruits than diploids, suggesting a positive impact of polyploidization on pollen transfer via other means. In addition, polyploidization did not lead to assortative mating but instead promoted interploidy pollen exchange, since visitors preferentially switched between cytotypes rather than preferring one. Consistent with this switching behavior, most of the progeny from tetraploid plants were triploid. Our data suggest that polyploidization has an immediate impact on mating in plants, but in a more complex way than has been assumed previously.

Keywords: *Arabidopsis arenosa*, floral display, interploidy barrier, pollinator behavior, polyploid establishment, reproductive success

Introduction

Polyploidy is widespread across angiosperms, with a large number of successfully established polyploid lineages (Wood et al. 2009, Barker et al. 2016). This remains

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a paradox, as upon emergence, polyploids face numerous obstacles to their establishment, such as reduced mating opportunities, also known as minority cytotype exclusion (Levin 1975). Polyploids may overcome their starting disadvantage by displaying novel traits increasing their fitness. One of these traits is the so-called gigas effect, where polyploids often have larger cells, leading in particular to an increase in the size of their floral organs (Müntzing 1936, Levin 1983). Flower size is known to influence flower visitation by pollinators, either increasing the visitation rate (Klinkhamer et al. 1989, Andersson 1991, Conner and Rush 1996), decreasing it (Andersson 1988, Klinkhamer and de Jong 1990) or having no obvious impact on it (Vaughton and Ramsey 1998, Ohashi and Yahara 2002). Thus, larger flowers due to the gigas effect may influence pollinator visitation of newly formed polyploids (neopolyploids), potentially compensating for the rarity of mating opportunities of neopolyploids. Experimental work has shown different floral morphology and pollinator behaviors between natural polyploids (i.e. polyploids established in populations in the wild) and their presumed diploid progenitors, but not necessarily increased visitation of polyploids by pollinators (Segraves and Thompson 1999, Palmqvist et al. 2021). However, such polyploids have undergone natural selection, and the direct effect of whole genome duplication cannot be derived from such experiments. Whether the increase in flower size by the gigas effect is enough to affect the visitation of newly formed polyploids by pollinators remains to be tested.

The floral changes brought on by whole genome duplication may impact the foraging preference of pollinators and potentially lead to assortative mating, enabling a reproductive barrier between diploids and newly formed polyploids. Reproductive isolation is an important factor contributing to the establishment of a polyploid lineage from diploids. Triploid block, or the inviability of hybrid seeds between diploids and polyploids, is often evoked as an instant reproductive barrier between cytotypes (Köhler et al. 2010). However, triploid block is often 'leaky' with interploidy seeds surviving to differing extents (Burton and Husband 2000, Meeus et al. 2020, Morgan et al. 2021). Thus, other mechanisms are likely to contribute to reproductive isolation between diploids and newly formed polyploids in early stages, such as assortative mating driven by pollinator shift. Differences in floral morphology and pollinator preference between diploids and established polyploids have been shown, leading to assortative mating (Segraves and Thompson 1999, Palmqvist et al. 2021). Again, these may be explained by late-acting selective processes rather than polyploidy itself. A few studies used synthetic polyploids to address whether assortative mating mediated by pollinator preference for a specific cytotype could arise immediately after polyploidization, in *Acacia mangium*, *Chamerion angustifolium* and *Trifolium pratense* (Nghiem et al. 2011, Husband et al. 2016, Porturas and Segraves 2020). All three studies did not find assortative mating following polyploidization, questioning whether assortative mating can be established directly after the formation of new polyploids, and the low number of available studies on this topic calls for additional work.

From all possible systems to study these questions, *Arabidopsis arenosa* (Brassicaceae) appears as a relevant one. *Arabidopsis arenosa* is self-incompatible (Säll et al. 2004) and, as the rest of the Brassicaceae family, is likely to be a generalist when it comes to pollinator attraction (Gómez et al. 2016). It is a species complex including diploids and established autotetraploids. Their distribution ranges from southeastern Europe to Scandinavia, with a primary contact zone between the cytotypes in central Europe (Carpathian Mountains) and a few secondary contact zones in southeastern Europe (Monnahan et al. 2019, Morgan et al. 2020). The origin of tetraploids is estimated to have occurred in the Carpathian Mountains 11 000–30 000 generations ago (Arnold et al. 2015) or, alternatively, 226 000 generations ago (Marburger et al. 2019), making tetraploid *A. arenosa* a relatively young lineage. This may explain why no significant niche shift has been observed in the contact zones between cytotypes (Morgan et al. 2020). Nevertheless, triploid block is strong in this species, at least in the direction of diploid mother × tetraploid father (Morgan et al. 2021). Also, common garden open pollination experiments have shown that, despite being in physical proximity, plants of the two cytotypes barely produced any triploid seeds (Morgan et al. 2021), suggesting that prezygotic barriers arose during establishment of the tetraploid lineage. This raises the question whether such barriers arose as a direct byproduct of polyploidization, or whether these barriers arose later on during the tetraploid lineage evolution.

In this study, we tested whether newly formed polyploids show an advantage in insect visitor attraction due to the changes mediated by whole genome duplication in *A. arenosa*. A higher reproductive output may offset the deleterious impacts of minority cytotype exclusion by increasing the size of the polyploid's brood, even if the progeny are triploid due to the mating with surrounding diploids (triploid bridge; Ramsey and Schemske 1998). We also tested whether polyploidy-induced floral changes induce assortative mating between individuals of the same ploidy.

Material and methods

Plant material and polyploid induction

In this study, we focused on a diploid population of *A. arenosa* from the western Carpathian lineage (Kolář et al. 2016b), which was shown to be the ancestor of the natural autotetraploids (Arnold et al. 2015). Seeds of the diploid population were collected in the Veľká Fatra Mountains, Slovakia (48°50'4.128''N, 19°0'48.528''E) in August 2018 (Supporting information). Autotetraploid individuals of *A. arenosa* were derived from the diploid population treated with colchicine by following the method modified

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from a previous study (Lafon-Placette et al. 2017). First, seeds collected from the field were surface-sterilized (with 5% NaClO, 0.01% (v/v) Triton X-100 and sterile water) and sown on an agar plate (containing: 1× MS-Salts, MES hydrate and 0.8% (w/v) plant agar; pH 5.8). Then, plates were transferred and kept under controlled conditions (12 h at 23°C/day and 12 h at 13°C/night) for two weeks to initiate germination. After two weeks, germinated seeds were transferred to small pots filled with damp soil and were grown for two weeks under the same conditions to provide time for the seedlings to be large enough for the colchicine treatment. Following that, the shoot apical meristems of diploid seedlings were treated with 0.025% colchicine solution by dropping 10 µl on to them with a pipette. Colchicine-treated seedlings were then transferred to the vernalization chamber and were grown for six weeks in short-day conditions (8 h of light per day) at 4°C to induce flowering. Following vernalization, colchicine-treated seedlings were transferred to long-day conditions (16 h of light per day) at 21°C day/15°C night. Ploidy levels of colchicine-treated individuals were estimated using flow cytometry. We found a rare occurrence of tetraploids among all the treated plants, in addition to chimeras (different stems with different ploidies) and other ploidy levels (Supporting information). Successfully induced autotetraploid individuals were selected to produce F1 seeds, to reduce the toxic effect of colchicine on the phenotype. For that, manual pollinations were performed within the populations for both diploids and induced autotetraploids. To avoid genetic differences between the diploids and induced autotetraploids, the same maternal and paternal donors were used to perform manual pollination for both diploids and induced autotetraploids (Supporting information). This was possible since these plants were chimeras, producing both diploid and tetraploid stems. For all the crosses, designated female partners were emasculated before anthesis, and the pistils were hand-pollinated two days after emasculation. Even though *A. arenosa* is self-incompatible (Säll et al. 2004), emasculation was performed to avoid any potential mentor effect (forced self-pollination made possible with the deposition of foreign pollen). For both crosses, 20 manual pollinations were performed. Five weeks after pollination, fully maturated siliques from the first generation of diploids and induced autotetraploids were harvested and were stored at 4°C under dry and dark conditions for six weeks to break dormancy. These seeds were used to grow the plants for the experiment. Seeds were germinated and the plants were vernalized applying the same conditions as explained above. For acclimatization, seedlings were then transferred to big pots filled with damp soil and exposed to long-day conditions (16 h of light per day) at 21°C day/15°C night for two weeks. During this period, the ploidy level (relative DNA content) of all induced autotetraploid plants was estimated using DAPI flow cytometry (Doležel et al. 2007), and unambiguously tetraploid plants (Supporting information) were kept for the experiment. Finally, all of the plants were transferred to the field for the visitor experiment.

Ploidy level detection

We measured the ploidy level of induced plants and their progeny using DAPI flow cytometry (Doležel et al. 2007). For the induced polyploid plants, we collected petals from different stems of each plant, to account for potential chimeras. For their progeny, we collected rosette leaves. All the samples were measured individually, using *Carex acutiformis* (Veselý et al. 2012) as an internal standard. Samples were prepared using Otto I and Otto II buffers containing the fluorescent DAPI dye according to the protocol of Doležel et al. (2007). The samples were analysed on a CyFlow ML instrument (excitation source: UV LED chip 365 nm). The fluorescence of at least 3000 nuclei (for ploidy level detection) per sample was recorded, and we used a maximum coefficient of variation (CV) of 5% as the threshold for valid measurements. All data were evaluated using the Partec FloMax 2.4d program (Partec GmbH, Münster, Germany). A comparison with DAPI flow cytometric data of diploid and tetraploid *A. arenosa* from Kolář et al. (2016a) allowed for unambiguous ploidy determination.

Flower visitors experiment

Experimental design

A common garden for the purpose of the visitor experiment was established in Průhonice, Czech Republic (49°59'41.042''N, 14°33'55.27''E). This common garden has a similar climate to the original location of the *A. arenosa* populations (central Europe), and tetraploid *A. arenosa* grows naturally within a few kilometers of this common garden (Padilla-García et al. 2023). Therefore, we did not expect major changes in the insect visitor community between the common garden and the original locations of the *A. arenosa* populations, even though we were unable to exclude it. Two experimental plots for the purpose of floral visitation observation were established; these two plots were 70 m apart from each other. Each plot consisted of 16 diploid and 16 synthetic tetraploid plants of *A. arenosa* arranged in a regular chessboard pattern, with four blank spaces left in the center of the square design (Supporting information). Blank spaces were included due to the unavailability of enough healthy representatives of both cytotypes to fill the full square design in both plots. This design of the plot ensured that the representatives of both cytotypes were equally accessible to floral visitors. The distance between a pair of two laterally adjacent plants within a plot was set to 50 cm. The surface of the plots was formed by open soil, as we removed all vegetation prior to the experiment to avoid interaction of the experimental plants or floral visitors with other plant species within the plot.

Plants were moved to the experimental plots from the cultivation chamber prior to anthesis, typically once they had formed a well-developed basal rosette. The plants were buried in their original pots in the ground according to the scheme in the Supporting information; we did so as planting them in the common garden substrate may have damaged their root system and impacted their subsequent performance.

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All experimental plants within both plots were treated with identical watering management. From the time of establishing the plots until the time of flowering, the plants showed individual differences in growth vigor, and we classified them as small, medium or large; we labelled as 'small' and 'large' any plants that were obvious and extreme outliers in their growth (reduced and increased, respectively) by simple observation, and labelled as medium the other plants. We did not move extreme plants to different positions in the plots.

Data collection

To assess visitor foraging, the visit duration (i.e. time spent per plant) and visitation sequence (i.e. the identity of plants visited during a visitation bout) for each individual visitor were recorded. We used a digital stopwatch to estimate visit duration, measured as the time a floral visitor spent on a plant before moving to another plant in the plot, and we recorded the visitation sequence until a floral visitor left the experimental plot. These measurements were done 'blindly' without knowledge of the ploidy of the plants to avoid any bias related to collector expectations. For each plot, three observation periods with at least 30 visitations each were set across a time range of 2.5 weeks (17 June–5 July 2022) between 11:00 and 16:00. The date and hour of the visitor observations, as well as weather conditions, were recorded.

Analysis of insect visitation

To determine whether floral visitors preferentially spend more time visiting plants of a given ploidy level, we used a linear mixed effects model with visit duration (natural log transformed) as the response variable, ploidy, plant size (small, medium or large) and plot as fixed effects, and bout ID and plant ID as random effects. The model was fit using the package 'lme4' (Bates et al. 2015), and the statistical significance of the fixed effects was calculated with 'lmerTest' (Kuznetsova et al. 2017) in R ver. 4.2.2 (www.r-project.org).

To test whether floral visitors sequentially visited plants independently of ploidy level, we calculated a 2 × 2 transition matrix for all visitor movements between two plants within a single pollination bout. This transition matrix was calculated for each of the two plots separately. We used a X^2 test to evaluate the hypotheses that transitions between ploidy levels are 1) dependent only on the frequency of plants of a given ploidy in the plot, and 2) a function of the frequency of plants of a given ploidy considering only the nearest neighbours (plants adjacent to a focal plant). Due to the experimental design, each plant was surrounded by a plant from the opposite ploidy in the vertical and horizontal axes, and by a plant of the same ploidy along the diagonals (Supporting information). Thus, we used a correction factor when calculating the expected transition matrix to account for the fact that plants along the diagonal (same ploidy) are further away than those along the vertical and horizontal axes (different ploidy). From the Pythagoras theorem, the distance along the axes is equal to h = square root (2) × c, where c is the horizontal and vertical distance among plants, and h is the diagonal distance. Finally, we also calculated Gegear and Thomson (2004) constancy index CI, where values range from −1 (complete inconstancy) to 0 (random foraging) to +1 (complete constancy).

Plant traits measurements

All the plants used for the phenotypic analyses (floral traits, fruit set and progeny characterization) were also included in the pollinator experiment.

Floral traits

Ten randomly selected plants of both ploidy levels were used for morphometric measurements of floral traits. We collected three well-developed flowers per individual plant (total 60 flowers). Immediately after collecting, individual flower parts, i.e. petals, sepals, stamens and pistil, were separated and attached onto a paper sheet of contrasting color with transparent adhesive tape. Sheets with flower parts were scanned with an attached scale bar. From the scanned material, and two weeks after collection, seven floral traits comprising all floral organs were measured on each analyzed flower using ImageJ 1.53e (Schneider et al. 2012): petal length, petal width, sepal length, sepal width, pistil length, filament length and anther length. For measurements of each morphological trait, we chose the longest flower part within a particular flower (i.e. the longest petal, sepal, filament and anther). As flowers are not biological replicates, we averaged the values of three flowers to obtain a single value per plant, and this was used for statistical analyses.

A Shapiro–Wilk test and Mardia's skewness and kurtosis test were used to determine multivariate normality of the dataset comprising the seven morphological traits. A MANOVA was used to test the significance of morphological difference between plants of the two ploidy levels, followed by the post hoc univariate ANOVA tests to determine the differentiation among each trait individually.

Statistics were performed in R ver. 4.2.2 (www.r-project.org) utilizing the packages 'mvnormtest' (Jarek 2012) and 'mvnormalTest' (Zhou and Shao 2014), while the package 'ggplot2' (Wickham 2016) was used for creation of box plots.

Fruit set

At the end of the flowering period, we measured the total number of peduncles and the number of developed siliques (longer than 1 cm) on 20 randomly selected plants per ploidy out of all the open-pollinated plants of the experimental plot. The measurements were done in a double-blind manner to avoid any bias. The relative fruit set was calculated by dividing the total number of developed siliques by the total number of peduncles for each plant.

A Wilcoxon rank sum test was used to compare the relative fruit set between diploids and tetraploids using the 'R Stats' package.

Seed progeny germination rates

Open pollinated, mature siliques were collected from five mother plants in the experimental plot per cytotype and

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kept in a paper bag. Seeds collected from these siliques were arranged on white paper and were photographed under a stereo microscope (Leica M205 C with Fusion Optics). Normal-looking seeds were identified as brownish-yellow and plump, with variable sizes. Collapsed seeds were dark brown/black, shriveled and irregularly shaped. Counting seeds for each category was done using ImageJ (Schneider et al. 2012). The rate of normal-looking seeds was obtained by dividing the number of normal-looking seeds by the total number of seeds.

After phenotype assessment, seeds were surface sterilized (with 0.5% NaClO and 0.01% (v/v) of Triton X-100) and plated on MS medium (containing: 1 × MS-Salts and 0.8% (w/v) plant agar, at pH 5.8). Then, plates were transferred and kept for two weeks in a growth chamber (12 h at 23°C/day and 12 h at 13°C/night). Germination rates were assessed by counting seeds with a ruptured seed coat and protruding radicles.

A Wilcoxon rank sum test was used as non-parametric test to compare the rate of normal-looking seeds/germination between diploids and tetraploids using the 'R Stats' package.

Seedling progeny ploidy measurements

Following seed germination, we investigated the ploidy level of germinated progenies (three-week-old seedlings). To do this, we collected rosette leaves of 10–15 seedlings per mother plant (five mother plants) for each cytotype and measured the ploidy level following the same procedure as explained in the 'Ploidy level detection' section.

Results

Larger floral organs in synthetic polyploids

The Shapiro–Wilk and Mardia's skewness and kurtosis test confirmed multivariate normality of the floral dataset. A MANOVA revealed significant morphological differentiation between plants of the two ploidy levels ($F(7,12) = 18.85$, $p < 0.0005$; $n = 10$ plants per cytotype), and the inter-cytotype difference in individual traits was consistently confirmed also in post hoc tests, with p-values below 0.005 in all seven variables (Fig. 1). On average, flower parts of tetraploids were 38% longer or wider than diploids. The most significant difference was observed in petal width (on average, tetraploids had 1.8 times wider petals compared to diploids), while the smallest relative difference was observed in filament length, with tetraploids having on average 1.2 times longer filaments than diploids. The number of flowers opened at the same time could influence overall floral display and attractiveness to visitors but did not significantly differ between diploids and tetraploids (average of 10.9 flowers for diploids and 13.4 for tetraploids).

Higher mating success in synthetic polyploids

The number of fruits produced per plant was significantly higher in induced autotetraploids than in diploids (Wilcoxon rank sum test [W] = 97, p-value = 0.009338, n = 19–20 per ploidy). A similar trend was observed for the flower number between cytotypes, but the differences were not significant (W = 123.5, p-value = 0.06365, n = 19–20 per ploidy).

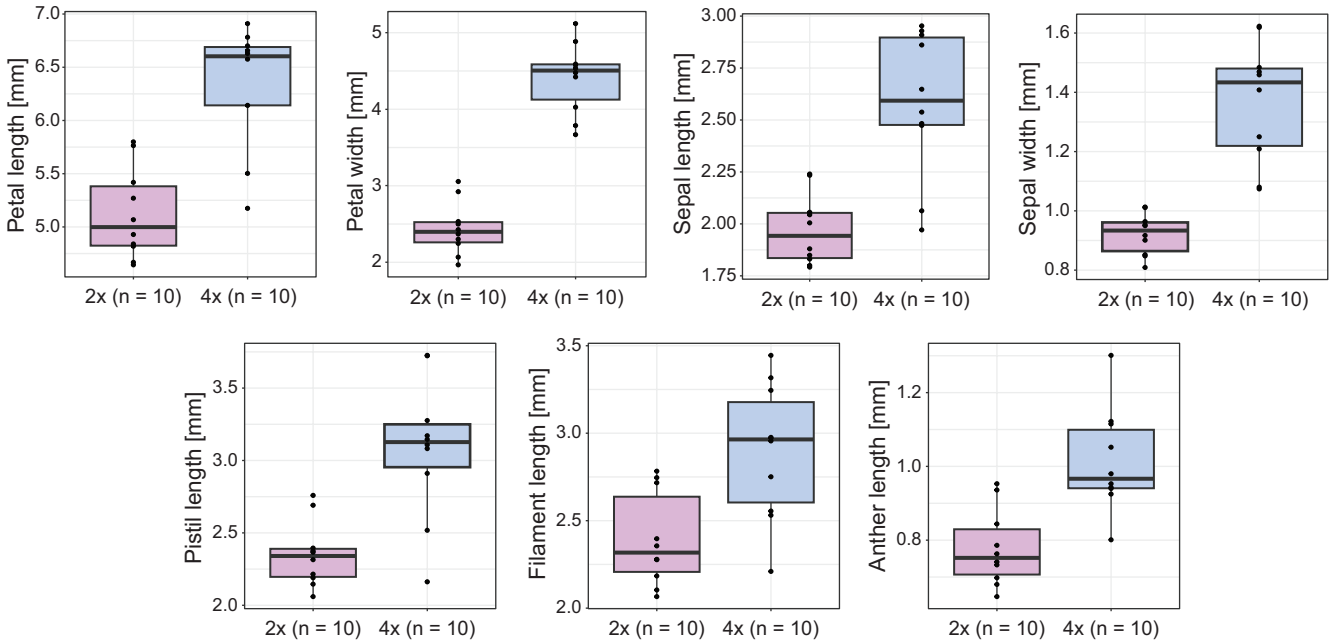


Figure 1. Box plots displaying the variability of the morphological traits in diploid and induced autotetraploid *Arabidopsis arenosa*. The number of individuals (n) is shown for each cytotype.

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This resulted in induced autotetraploids showing a significantly higher relative fruit set (64.2%) compared to diploids (50.34%; W = 76.5, p-value = 0.001482, n = 19–20 per ploidy; Fig. 2).

Floral visitation to diploids and tetraploids

We found a diversity of insects visiting the plants, mostly species of bees and flies (Supporting information). We found no statistically significant difference in the amount of time that floral visitors spent on plants of different ploidy levels (n = 162–169 visitors per ploidy; Table 1, Fig. 3). On average, floral visitors spent 36.494 ± 46.437 s (mean ± SD) and 30.048 ± 33.613 s in diploid versus tetraploid plants, respectively. In contrast, plant size significantly influenced the time floral visitors spent on a plant, with larger plants receiving overall longer visits (Table 1).

Movement of floral visitors among plants was highly inconstant (CI = −0.637 and −0.710 for plots A and B, respectively), suggesting that visitors moved mostly between, rather than within, ploidy levels (Supporting information). Both χ^2 tests using either the frequency of ploidies in each plot ($\chi^2 = 107.04$ and 150.41 for plots A and B, respectively; df = 1, $p < 0.001$), or the corrected expectation based on ploidy-specific distances to the nearest neighbor ($\chi^2 = 60.287$ and 93.568 for plots A and B, respectively; df = 3, $p < 0.001$) support visit sequences that are not independent of ploidy level.

Progeny of open-pollinated plants

Mature seeds from open-pollinated diploid and tetraploid plants were collected. The rate of normal-looking seeds, potentially indicating the presence of triploid seeds, was of 47% in average for diploid plants and 13% for tetraploid plants (n = 5 maternal plants, total 431 seeds for diploid plants and 409 seeds for tetraploids; Fig. 4a, Supporting information). This rate was significantly different between cytotypes (W = 25, p-value = 0.01219). The germination rate, however, was not significantly different between cytotypes, with a mean of 33% for diploid plants and 36% for tetraploid plants (W = 12, p-value = 0.1, n = 5 maternal plants, 315 seeds for diploids and 291 seeds for tetraploids; Fig. 4a, Supporting information). Strikingly, while only diploid seedlings were obtained from germinated seeds of diploid maternal plants, germinated seeds from tetraploid maternal plants

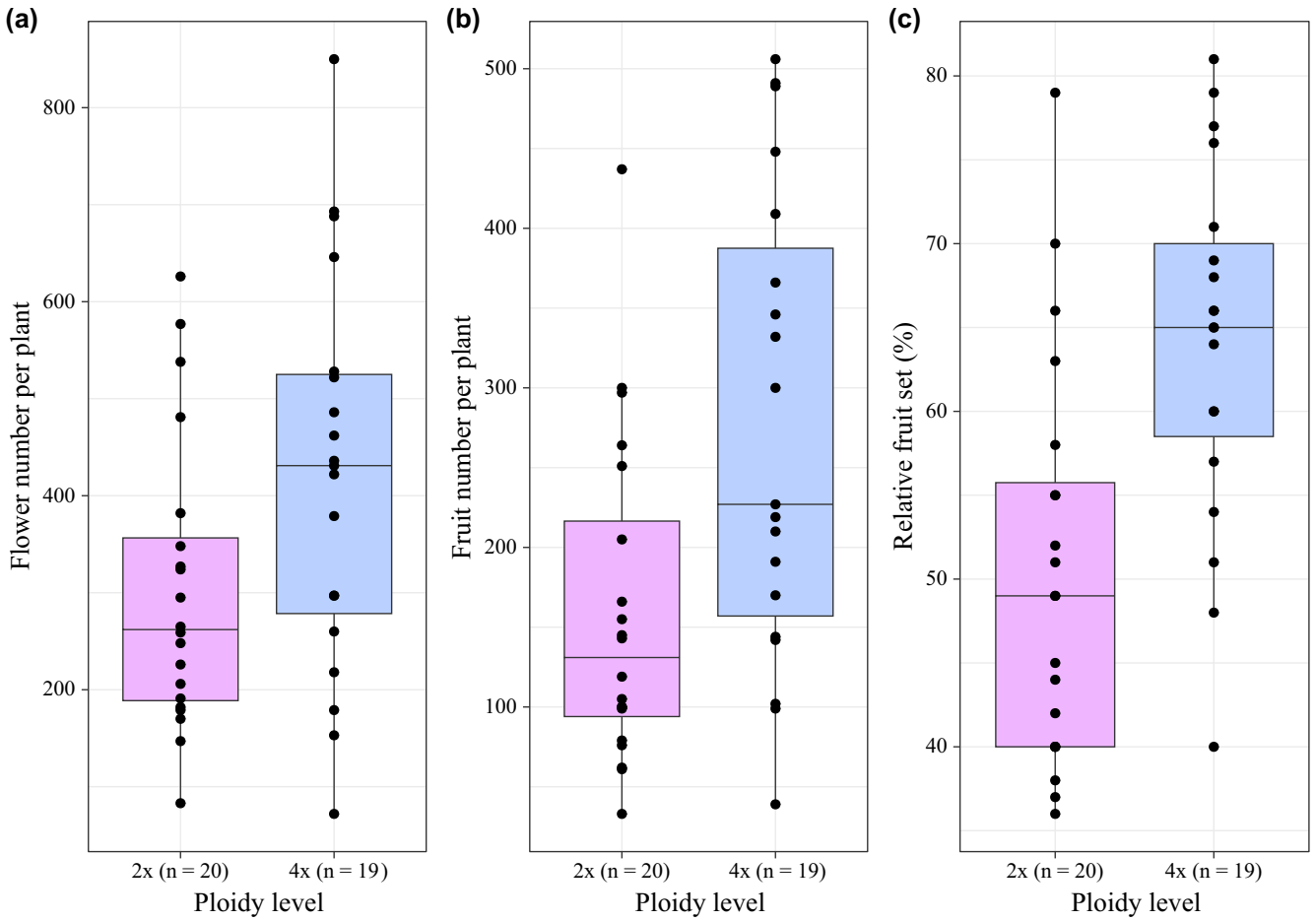


Figure 2. Reproductive success of diploid and induced autotetraploid *Arabidopsis arenosa*. The total number of flowers (a), the number of fruits per plant (b), and the relative fruit set per plant (c) is indicated for each cytotype, together with the number of individuals (n).

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Table 1. Coefficient estimates, standard errors and statistical significance of main effects for pollinator visit durations.

	Estimate	SE	p
Intercept	3.113	0.093	
Ploidy			0.236
4x	−0.112	0.094	
Size			< 0.001
Large	0.319	0.160	
Medium	−0.314	0.104	
Small	−0.686	0.157	
Plot			0.940
B	0.007	0.097	

gave rise to a vast majority of triploid seedlings (55 out of 56 seedlings, n = 5 maternal plants; Fig. 4b).

Discussion

The early acting factors contributing to the establishment of new polyploids remain largely unknown. These factors may either influence the fitness of newly formed polyploid individuals and/or build reproductive isolation between cytotypes, leading to the emergence of genetically independent polyploid lineages. In this study, using synthetic *A. arenosa* tetraploids, we tested whether instant floral changes due to polyploidization could improve reproductive success and could lead to reproductive isolation between diploids and tetraploids, in the form of assortative mating mediated by changes in insect visitation.

Polyploidy-induced floral changes are not more attractive to visitors, but do improve mating success

As often reported in the literature, we found that synthetic polyploids had larger organs, including petals (Abdoli et al. 2013, Zahumenická et al. 2018, Meeus et al. 2020), an important trait for pollinator attraction (Moyroud and Glover 2017). However, this did not translate into preferential visitation of tetraploids by insects in our study. This finding is consistent with the few available studies on this topic, yet in different plant families, in the species *A. mangium*, *C. angustifolium* and *T. pratense* (Nghiem et al. 2011, Husband et al. 2016, Porturas and Segraves 2020), which also did not find any impact of induced polyploidy on insect visitation. However, these studies did not test the quantitative preference of visitors (visit duration) between cytotypes but instead tested if assortative mating occurred, acting as an interploidy reproductive barrier. Here, we show that the floral changes induced by polyploidy in *A. arenosa* do not lead to differences in visit duration between diploids and tetraploids. These results, accompanied by previous results of the studies mentioned above, have not provided evidence to support the hypothesis that instant floral changes provoked by polyploidization contribute to reproductive isolation of new polyploids from their diploid progenitors. With this in mind, we must consider that synthetic polyploids may not perfectly emulate the formation of neopolyploids in nature. In particular, the formation of unreduced gametes in nature and subsequent outcrossing may capture more allelic diversity than with a somatic doubling induced by chemicals (De Storme and Geelen 2013). This may have consequences for the emergence of novel floral phenotypes in natural neopolyploids that may have stronger impact on visitor behavior which we cannot recapitulate in our study. For future research, working with species that are prone to unreduced gametes formation, such as *Cardamine* species (Kreiner et al. 2017), to produce neopolyploids may be a way to mimic better what happens in nature.

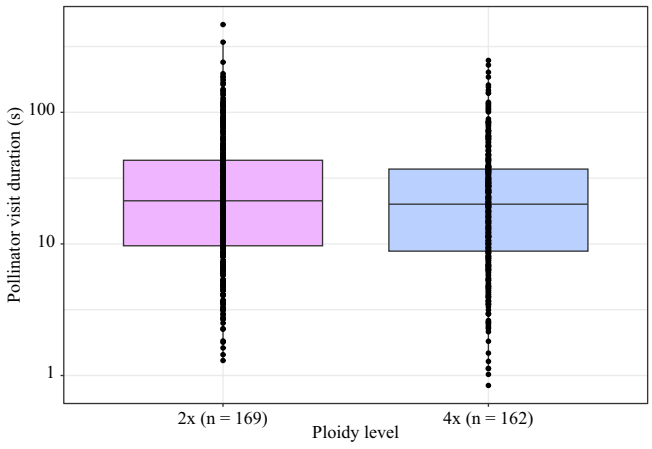


Figure 3. Box plots of visit duration of diploid and induced autotetraploid *Arabidopsis arenosa*. Duration values in seconds (s) are $\log_{10}$-transformed for readability purposes. The total number of pollinator individuals (n) is indicated for each plant cytotype.

Despite no clear visitor preference, we found that the tetraploids set more fruits (both total number and relative fruit set) than the diploids, suggesting higher pollination rates. A higher pollination rate might still be explained by higher visitation rates, as visitations also happened outside our observation period. We performed the visitor observations only for 2.5 weeks, while the flowering period of *A. arenosa* is approximately two months. Therefore, the insect visitor community could have changed during the flowering period, and the tetraploids might have attracted specific visitors outside the time windows of the measurements. Also, we did not test for different foraging behavior between visitors as we did not identify pollinator species in detail, and it could be that specific visitor species were more attracted to tetraploids than others. Alternatively, the size of floral organs like the style has been shown to influence the efficiency of pollen transfer from the pollinator body to the stigma (Mazer et al. 2019), and it could be that the larger floral organs (including the pistil) of synthetic polyploids improved the pollen transfer and, consequently, increased fruit set. More generally, we studied visitors and not actual pollinators, and differences in fruit set between cytotypes could be due to cytotype-specific foraging behaviors/pollen transfer efficiency of certain insect visitors. Polyploidization could lead to the breakdown of self-incompatibility (SI; Ramsey and Schemske 1998, Mable 2004), and thus higher fruit set could be explained

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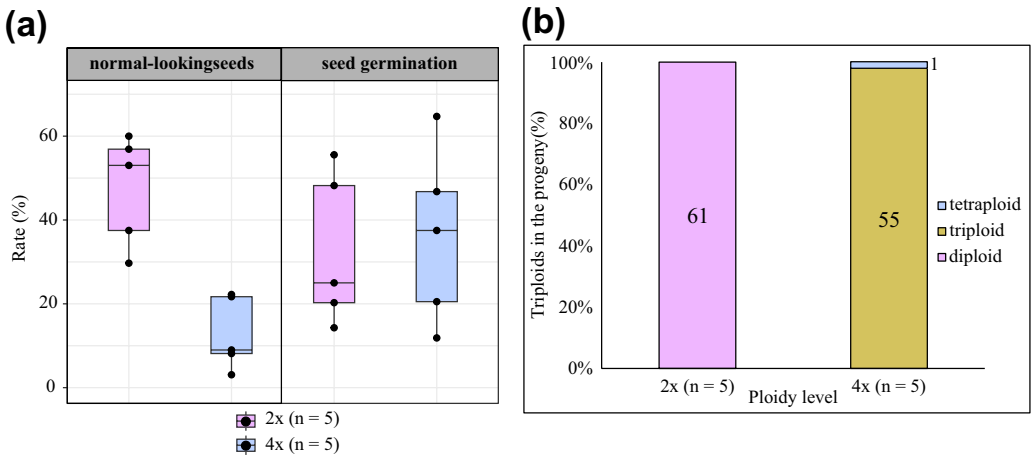


Figure 4. Progeny assessment of diploid and induced autotetraploid *Arabidopsis arenosa* mother plants. (a) Number of normal-looking seeds obtained from diploid (n = 431 seeds) and induced autotetraploid (n = 409 seeds) *A. arenosa* mother plants; seed germination rate among the diploid (n = 315 seeds) and induced autotetraploid (n = 291 seeds). Dots represent the individual mother plants (n = 5 mother plants per cytotype). (b) Proportion of triploid seedlings in the progeny of diploid and tetraploid mother plants. The number of mother plants (n) is shown for each cytotype. The numbers inside the bars indicate the number of seedlings in the progeny matching a given ploidy level (diploid: pink; triploid: brown; tetraploid: blue).

by selfing. However, SI breakdown by polyploidy occurs in the gametophytic SI system and not for the sporophytic one (Mable 2004). Combined with the fact that *Arabidopsis* species have a sporophytic SI system (Kusaba et al. 2001), and that natural *A. arenosa* tetraploids are self-incompatible (Kiefer et al. 2023), selfing is unlikely to explain our results. In addition, the vast majority of the progeny in tetraploid plants were triploid, which is inconsistent with a selfing scenario. Independently of the mechanisms that led to higher fruit set in tetraploids as compared to diploids, the fitness of tetraploids may not increase as a result, as triploids may have reduced fertility as often reported (Farco and Dematteis 2014, Morgan et al. 2021). Further studies should be performed to understand the causes of higher fruit set in synthetic *A. arenosa* tetraploids.

Polyploidy-induced floral changes may promote, rather than prevent, interploidy gene flow

We also tested whether floral changes provoked by polyploidization could lead to assortative mating via pollinators preferentially visiting plant individuals of the same cytotype rather than between cytotypes. We did not find evidence for assortative mating, consistent with previous studies on synthetic polyploids and their diploid progenitors (Nghiem et al. 2011, Husband et al. 2016, Porturas and Segraves 2020). Several other studies, however, focusing on natural polyploids, found partially different pollinator preferences in diploids versus polyploids (Segraves and Thompson 1999, Husband and Schemske 2000a, Thompson and Merg 2008), suggesting that pollinator shift is not acting as an early reproductive barrier causing the genetic divergence between diploid and polyploid lineages, but rather coming into play at a later stage. Importantly, the chessboard design we used, while helpful to normalize the probability of visitor visitation between cytotypes, is not representative of natural conditions. Tetraploids are likely to be grouped spatially in their early emergence and later, and the patchy distribution of cytotypes has been shown to influence between-cytotype pollinator visitation (Husband and Schemske 2000b). In general, the size of floral patches has been shown to influence the amount of visitation as well as the pollinator movement between patches (Santos et al. 2021). It would thus be interesting to carry out an experiment closer to natural conditions of the emergence of neopolyploids, with small patches of polyploids interspersed with larger patches of diploids, and to observe the impact of this design on pollinator behavior.

Although polyploidy-induced floral changes might eventually lead to a reproductive barrier between ploidies, our results suggest that they may alternatively promote interploidy gene flow. Indeed, we observed that visitors preferentially moved between cytotypes rather than within cytotypes. While this could be attributed to the chessboard design, which places a heteroploid plant as the closest one, we still observed a preferential movement between cytotypes even after statistically correcting for this spatial bias. Although puzzling, this type of disassortative mating in mixed ploidy experimental plots with similar experimental design as ours has been observed before. A study in *C. angustifolium* using a different statistical approach to measure pollinator movements also found that pollinators were more likely to move between plants of different cytotypes rather than within the same cytotype (Husband et al. 2016). The biological explanation for this behavior remains obscure. However, taken at face value, our results and those of Husband et al. (2016) suggest that this kind of disassortative mating could, on one hand, hinder the establishment of neopolyploids through exacerbating the effects of minority cytotype exclusion, which disfavors the initially rare polyploid cytotype. On the other hand, if pollinators preferentially move between diploids and

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neopolyploids, this would promote interploidy gene flow and the formation of a triploid bridge (Harlan and deWet 1975, Husband 2004), potentially helping neopolyploids to establish.

The scenario of a triploid bridge is somewhat supported by the vast majority of the germinated seeds from tetraploid plants being triploid, likely a consequence of the visitors preferentially switching between cytotypes. A very similar design to the present study was performed in the same location, growing diploid and natural tetraploid *A. arenosa* in a common garden in a chessboard manner (Morgan et al. 2021). The results were opposite to ours: less than 10% of seeds from natural tetraploid plants were triploids, the rest were tetraploid. This suggests that since the origin of tetraploids 11 000–30 000 or 226 000 generations ago (Arnold et al. 2015, Marburger et al. 2019) until now, additional reproductive barriers arose, preventing the formation of triploid seeds even when diploid and tetraploid plants are in direct contact. These barriers could be pollinator preferences and/or postmating prezygotic barriers such as pollen rejection. On the other hand, while we did not find triploids in the germinated progeny of diploid plants in the current study, it does not necessarily mean that no triploid seeds were formed: crosses performed between diploid maternal plants and tetraploid pollen donors in *A. arenosa* produced triploid seeds, but > 90% of these seeds were inviable (triploid seeds are mostly viable in the other cross direction, tetraploid mother × diploid father; Morgan et al. 2021). In our study, seeds from diploid plants germinated to a rate of 33%. It is likely that triploid seeds largely contributed to the remaining 67% that did not germinate.

In conclusion, our study suggests that floral changes upon polyploidization impact pollinator behavior and mating success in ways that are more complex than a mere increase in insect visitation or the rise of an interploidy reproductive barrier. Ploidy-induced floral changes increased visitation between cytotypes, suggesting the opposite of a reproductive barrier, and higher reproductive success of the induced tetraploids was found, implying a pervasive fitness effect of whole genome duplication. Future works may disentangle the mechanisms underlying reproductive success of newly formed polyploids.

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Data availability statement

Data are available from the Figshare Digital Repository: https://doi.org/10.6084/m9.figshare.23302055.v1 (Schmickl et al. 2024).

Supporting information

The Supporting information associated with this article is available with the online version.

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