







Ploidy effects on the relationship between floral phenotype, reproductive investment, and fitness in an autogamous species complex

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Abstract

Premise: The relationships between reproductive investment, phenotype, and fitness have been broadly studied in cross-pollinated plants in contrast to selfing species, which are considered less interesting in this area because they are supposed to be a dead end in any evolutionary pathway. Still, selfing plants are unique systems to study these questions since the position of reproductive structures and traits related to flower size play an important role in female and male pollination success.

Methods: *Erysimum incanum* s.l. is a selfing species complex that has three levels of ploidy (diploids, tetraploids, and hexaploids) and traits that are typically associated with the selfing syndrome. Here, we used 1609 plants belonging to these three ploidies to characterize the floral phenotype and spatial configuration of reproductive structures, reproductive investment (pollen and ovule production), and plant fitness. Then, we used structural equation modelling to analyze the relationship between all these variables across ploidy levels.

Results: An increase in ploidy level leads to bigger flowers with anthers exerted farther and more pollen and ovules. In addition, hexaploid plants had higher absolute values for herkogamy, which is positively correlated with fitness. Ovule production significantly mediated the natural selection acting on different phenotypic traits and pollen production, a pattern that is maintained across ploidies.

Conclusions: The changes in floral phenotypes, reproductive investment, and fitness with ploidy level suggest that genome duplication can be a driver for transitions in reproductive strategy by modifying the investment in pollen and ovules and linking them with plant phenotype and fitness.

KEYWORDS

anther exertion, *Erysimum*, floral phenotype, herkogamy, natural selection, pollen–ovule ratio, self-pollination

The importance of male and female reproductive investment (i.e., pollen and ovules, respectively) on plant reproduction have been broadly explored in flowering plants (Knight et al., 2005; Morales and Traveset, 2008; Breed et al., 2012; Wong and Frank, 2013). However, most studies have focused mainly on outcrossing plants whose

fertilization depends on different pollination mechanisms, leading to a broader understanding of biotic and abiotic interactions in these species. In contrast, selfing plant species have been overlooked in this area, despite the high frequency of self-pollinated hermaphroditic plants in the wild (Jarne and Charlesworth, 1993).

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Reproductive strategies based on self-pollination facilitate pollen grains reaching the stigma within the same flower, ensuring fertilization (Lloyd, 1992; Ashman et al., 2004; Eckert and Herlihy, 2004). Female reproductive investment is the limiting factor for reproductive success in selfing plants and reduces resource allocation to pollen compared to ovules (Lloyd, 1987; Michalski and Durka, 2009), resulting in a trade-off between pollen and ovules (Cruden, 1977). The pollen to ovule ratio (P:O ratio) was developed as an index to characterize different mating systems. High P:O ratios are expected in outcrossing plant species, for which male reproductive investment is a significant component of the plant's total fitness. Conversely, selfing plant species are expected to have a lower P:O ratio because pollen is only used to fertilize the ovules in their own flower. The P:O ratio has been broadly used to explore plant mating system evolution and is positively correlated with floral traits that drive the evolution of mating systems (e.g., corolla size) or with the relative spatial position of sexual organs (e.g., herkogamy) (Galloni et al., 2007).

Flower morphology and size are known to be subject to strong selection because they control the frequency and efficiency of plant reproduction in different taxa (Herrera, 1993; Caruso et al., 2003, 2019; Gómez et al., 2006). They can also have a significant effect on the mating system as a result of the relative spatial position of sexual organs (i.e., herkogamy; Lloyd, 1992; Eckert and Herlihy, 2004; Larrinaga et al., 2009). Positive herkogamy avoids self-pollination and exposes the stigma surface to pollinators. However, self-pollination is facilitated when herkogamy is negative (reverse herkogamy) or near zero (Larrinaga et al., 2009; Brys et al., 2011). Experimental studies have shown how selfing plants often have a lower degree of herkogamy with a low amount of pollen because there is a high possibility of their own pollen grains reaching the stigma (Johnston et al., 2009; Sicard and Lenhard, 2011). Apart from herkogamy, anther exertion can also affect the mating system because the exposed anthers enhance pollen export (Barrett, 2003; Medrano et al., 2005). Ultimately, the balance between pollen and ovule development per flower is the major characteristic describing the mating system (Cruden, 2000) and is closely related to plant fitness. Thus, male and female reproductive investments indirectly mediate selection on the other traits mentioned above, connecting them with fitness and constraining the possible selective scenarios.

Another level of complexity in plants is generated by whole-genome duplications. Approximately 35% of vascular plants have a polyploid origin, evidencing the importance of polyploidy in promoting speciation (Wood et al., 2009). Indeed, polyploidization events play an important role in the evolutionary history of angiosperms (Soltis and Soltis, 1999; Otto and Whitton, 2000; Soltis et al., 2004; Van der Meer and Scott., 2009; Leebens-Mack et al., 2019). Ploidy can also alter floral phenotypes in different dimensions (Jürgens et al., 2002) and influence flower size and position of sexual organs, which, in turn, might affect pollinator preferences (Te Beest et al., 2012;

Moghe and Shiu, 2014). Yet, studies often pay surprisingly little attention to the effect of ploidy variation on the reproductive output of selfing species (Jürgens et al., 2002).

Erysimum incanum is a self-compatible species complex of small-flowered, monocarpic, annual plants that differ in ploidy level (diploids, tetraploids and hexaploids; Favarger, 1979; Galland, 1988; Luque and Lifante, 1991) but have identical self-pollination mechanisms (Abdelaziz et al., 2019). Due to its self-reproductive strategy, *Erysimum incanum* is expected to increase the allocation of resources to female function compared to male function in its flowers (Charnov, 1987). Because of its short, monocarpic life history, the entire life production of pollen, ovules, and seed output can be measured to estimate fitness. We thus used this study system to (1) quantify the selective pressures shaping the adaptive trajectories in populations with different ploidy levels; (2) evaluate the effect of ploidy on the flower phenotype resulting from the selective scenarios; (3) explore the role of P:O ratio in constraining the total selection regimes resulting from the variations in the species complex; and (4) evaluate the effects of polyploidization on decoupling variations among traits in the *E. incanum* species complex.

MATERIALS AND METHODS

Study system

Species in the genus *Erysimum* L. are among the most diverse in the Brassicaceae family, and occur in Eurasia, North and Central America, and North Africa (Al-Shehbaz et al., 2006). Mechanisms promoting local adaptation and hybridization between lineages have been identified for different species in the genus (Gómez et al., 2009; Abdelaziz, 2013). *Erysimum incanum* can be considered a species complex; it includes annual and monocarpic species and subspecies inhabiting the Western Mediterranean basin (Nieto-Feliner et al., 1993; Abdelaziz et al., 2019). The complex harbors three ploidy levels: diploids ($2n = 2x = 16$ chromosomes), tetraploids ($2n = 4x = 32$; Nieto-Feliner et al., 1993) and hexaploids ($2n = 6x = 48$; M. Abdelaziz et al., unpublished manuscript). Diploids of *E. incanum* present a vicariant distribution in the Rif and the Pyrenees mountains, and tetraploids also have a vicariant distribution in the southwestern Iberian Peninsula and the Middle Atlas Mountains (Nieto-Feliner et al., 1993; Fennane et al., 1999). In contrast, hexaploid *E. incanum* plants were only found in the most southern ranges in Morocco (High Atlas and AntiAtlas; M. Abdelaziz et al., unpublished manuscript) (Figure 1A, B).

The predominant phenotype of the *E. incanum* species complex (but *E. wilczekianum*) is associated with a selfing syndrome: small, hermaphroditic, and self-compatible flowers (Feliner, 1991). An anther rubbing mechanism that we previously described promotes selfing in these plants (Abdelaziz et al., 2019).

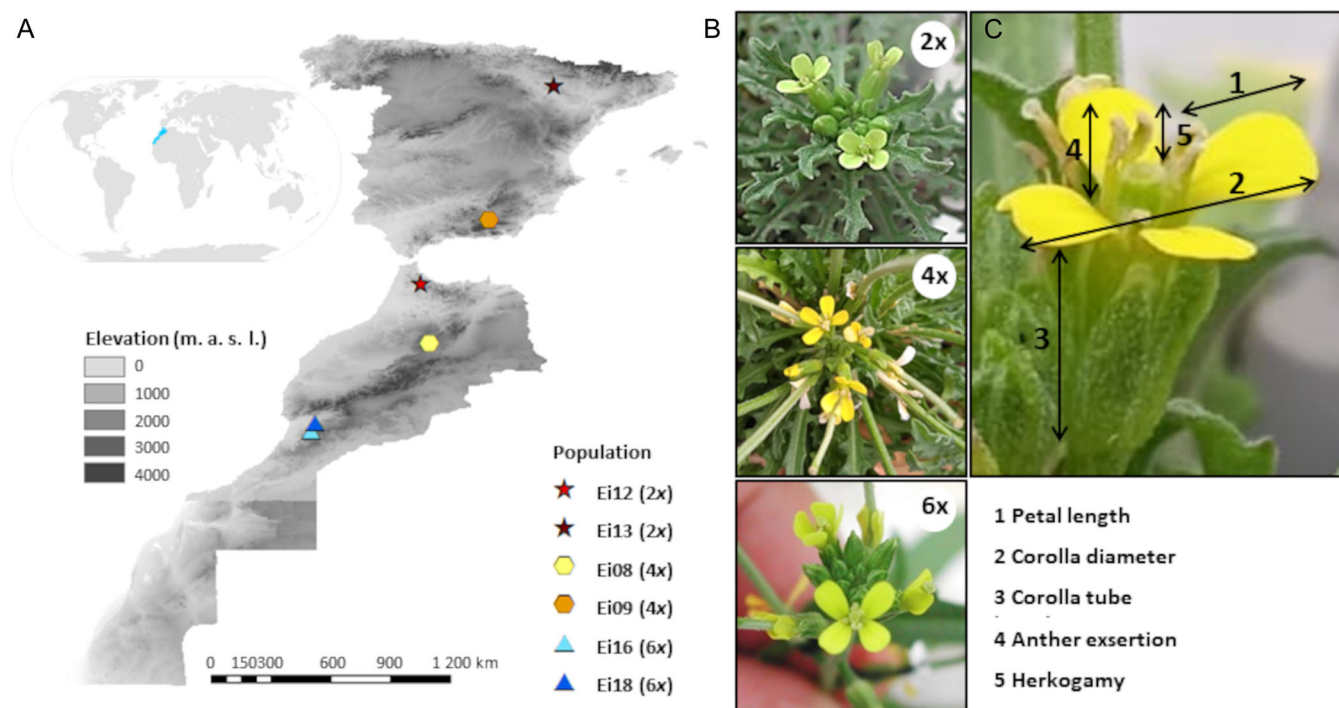


FIGURE 1 (A) Map of locations of the six studied populations in Morocco and Spain from *Erysimum incanum*. (B) Flowers from each ploidy level of *E. incanum* individuals. (C) Flower diagram showing the measured floral traits.

TABLE 1 Genome size estimations of each population. The number of plants phenotyped by population (Pop.) and ploidy, number of plant samples used for genome size analysis, genome size per population and ploidy, and geographic origin of each studied population are also given.

| Ploidy level | Pop. code | N: phenotyped | N: genome-sized | Mean \pm SE genome size (unit) | Origin |
|--------------|-----------|---------------|-----------------|----------------------------------|---------|
| 2x | Ei 12 | 289 | 21 | 0.35 \pm 0.01 | Morocco |
| | Ei 13 | 116 | 20 | 0.34 \pm 0.01 | Spain |
| 4x | Ei 08 | 513 | 25 | 0.78 \pm 0.10 | Morocco |
| | Ei 09 | 250 | 21 | 0.78 \pm 0.03 | Spain |
| 6x | Ei 16 | 230 | 21 | 1.10 \pm 0.03 | Morocco |
| | Ei 18 | 224 | 20 | 1.13 \pm 0.03 | Morocco |

Experimental design and phenotypic trait measurements

Seeds collected in two natural populations per ploidy (2x: Ei12 and Ei13; 4x: Ei08 and Ei09; 6x: Ei16 and Ei18; Figure 1A, Table 1) were sowed for three consecutive generations in a greenhouse at the University of Granada grow facilities to avoid any local effect. A total of 640 seeds per population were sowed in groups of four seeds in square plastic pots (11×11×11cm³) filled with Gramoflor Potting Soil (Gramoflor GmbH, Vechta, Germany).

During blooming of the plants, we measured a series of floral phenotypic traits on one flower on each 1622 plant using a digital caliper (Table 1, Figure 1C) Three of these traits were related to corolla size: petal length (distance

between the edge of the petal and the point where the petal starts to curve, shaping the corolla), corolla diameter (distance between the edge of the petal and the diametrically opposite one, in the flower) and corolla tube length (distance between the basis of the sepals and the corolla aperture point). We also measured the long stamen length (distance between the insertion point of the long filament and the anther) and the style height (distance between the style base and the stigma) to estimate the herkogamy value (difference between the style height and the long stamen length) and the anther exertion (difference between the long stamen length and the corolla tube length). Herkogamy indicates the relative ease for self-pollination, while anther exertion denotes the degree of anther exposure (and so the pollen grains) to floral visitors.

Genome size measurements

We estimated genome size using a young leaf and inferred the ploidy level using flow cytometry (FCM) as described by Muñoz-Pajares et al. (2018). At least 20 individuals were analyzed per population (Table 1). Because the ploidy level was homogeneous in each population studied, we assumed that each population mainly consisted of one cytotype. According to our previous knowledge of genome size variation in the *Erysimum* genus (Nieto-Feliner et al., 1993; Fennane et al., 1999) and knowing the three ploidy levels previously described for the complex, we could assign each individual and population to a specific ploidy level (Table 1) to be consistent with the main objectives of this study.

Reproductive investment estimates

We collected half of the stamens (i.e., two long stamens and one short stamen) from the same flower that we measured traits and preserved them in 70% v/v ethanol. To estimate male reproductive investment, we counted the pollen grains in each flower (“Pollen”) using the Multisizer Coulter Counter 3 particle counter (Beckman Coulter, Pasadena, CA, USA) provided by the morphometric lab of the Centro de Investigación, Tecnología e Innovación (CITIUS) at the University of Seville (Spain).

Once the plant was dried, we collected four mature fruits of each individual to count the number of viable and aborted seeds and the number of unfertilized ovules per fruit. These three numbers were added to equal the total number of ovules per flower (the female reproductive investment; “Ovules”). The P:O ratio was calculated as the number of pollen grains produced per flower divided by the number of ovules produced per plant.

Fitness estimation

To estimate fitness, we counted the number of fruits per plant and multiplied that total by the mean number of seeds estimated using four random fruits from the same plant. This way, we obtained the total seed production per individual plant used in the experiment. This value is an unbiased estimate of the individual fitness due to the monocarpic character of the plants, which complete their life cycle in only 10–12 weeks.

Overall, we considered eight variables grouped into four categories: floral size (petal length, corolla diameter, and corolla tube length), the spatial position of reproductive structures (anther exertion, herkogamy), reproductive investment (pollen, ovules), and fitness. Among the 1622 plants that completed their life cycle, we were able to measure floral size and spatial position traits for 1581 plants, male and female reproductive investment traits for 1380 and 1088, respectively, and seed production as a fitness component for 943 plants (Appendix S1).

Statistical analyses

Phenotypic traits

Structural equation modeling (SEM) is a powerful multivariate analysis tool, which has gained importance in ecological research in the last two decades (Fan et al., 2016). It also incorporates path analysis techniques that can be used to identify when a variable effect could be mediated by a different variable. Because we were interested in relationships among multiple variables, we used SEM to estimate the selection of traits related to floral size, the relative position of reproductive structures, and male and female reproductive investment. Pathway analysis allowed us to evaluate the direct and indirect effects of a series of traits that act simultaneously on fitness.

Based on a series of hypothesized mechanisms (Appendix S2, Table S2), we built an a priori model (Appendix S2, Figure S1). We tested several hypothesized models before we found a consensus model with a good fit regarding P and Akaike information criterion (AIC) values for all the populations (Appendix S3). In our model, fitness is directly connected to corolla traits, the relative position between sexual structures, and the male and female reproductive investment. Corolla size and position of sexual structures can promote or avoid self-pollination, which is why we connected them to fitness. These traits were linked to reproductive investment to test their influence on pollen and ovule production, which directly affect fitness. Moreover, we included a relevant covariance between pollen and ovule production, based on the trade-off between male and female sexual allocation (broadly described in flowering plants; e.g., Charlesworth and Charlesworth, 1981; Lloyd, 1984; Charnov, 1987), and on the P:O ratio hypothesis, which suggests that autogamous plant species reallocate resources to increase the production of ovules to the detriment of pollen grain production (Cruden, 1977). The rest of the covariances are detailed in Appendix S2.

Checking multicollinearity issues is important before statistical analyses based on regression models, specifically when variables are potentially correlated. We dealt with a strong correlation among variables by choosing measurements that were taken independently and not derived traits in our model. However, we inspected multicollinearity among all the variables by the variance influence factors (VIF) using the `vif` function from the R package `car` (Fox and Weisberg, 2019). The VIF value is an indicator of how much the variance of a coefficient in a regression model is inflated due to multicollinearity and is obtained by regressing one of our variables on all of the other variables (Miles, 2014). A VIF score lower than 5 means that variables are not strongly correlated among them (Joshi et al., 2012) (Appendix S4).

We evaluated the fit of the model for each population using Fisher's C and the P -value, which must be higher than 0.05 for accepting the hypothetical proposed model. Fisher's C is a fit index that is analogous to the χ^2 statistic and useful

for constructing AIC scores (Shipley, 2009, 2013; Lefcheck, 2016). To analyze the effect of ploidy variation on trait selection, we fitted the same model to each population and ploidy level separately and compared the resulting significant relationships between variables. Before these statistical analyses, we applied the scale function in R and performed log-transformed values for the pollen and fitness variables to fulfill normality assumptions.

Trait effects on fitness

Using the relationships of the traits with fitness in the SEM analysis, we calculated the direct, indirect, and total values of natural selection acting on each measured phenotypic and reproductive trait. Direct effects result from the unmediated relationship of the traits with fitness. Indirect effects were estimated as the product of different components of a path connecting any trait to fitness mediated by other traits. Finally, we added the indirect and direct effects to obtain the total value of natural selection.

Effect of P:O ratio on selection regimes

We evaluated the effect of the P:O ratio on the total selection on each of the phenotypic and reproductive traits, considering the total natural selection regimes experienced by the traits. For this, we performed a regression of the total fitness effect per population on the P:O ratio.

Effect of ploidy level on phenotypic and reproductive investment values

We performed linear models using the ploidy level as fixed factor to evaluate possible differences in phenotypic traits, reproductive investment, and P:O ratio between the three levels of ploidy of the *E. incanum* species complex. In addition, we performed linear models using the population as a nested factor within the ploidy level to assess the effect of ploidy level and population on the measured variables and did multiple comparisons using the R package lsmeans (Lenth and Lenth, 2018).

Phenotypic correlation among traits across ploidies

Phenotypic correlations between each pair of *E. incanum* phenotypic traits for each studied ploidies were analyzed using Pearson's correlation to estimate relationships independently between each pair of traits. In addition, we calculated the correlation for plants pooled by ploidy level. Since we carried out multiple comparisons, we applied the Bonferroni correction, with a new significance threshold of 0.0025 in the three ploidies. All the

statistical analyses described in this section were done using R version 4.0.3 (R Core Team, 2021), the R package piecewiseSEM version 1.2.0 (Lefcheck, 2016) for the structural equation modeling, and the R package stats for the other analyses.

RESULTS

Variation in phenotypic correlation among traits

The structural equation modeling that compared populations from each ploidy level showed heterogeneity in the relationships among traits. However, some patterns were maintained across populations (Figure 2). We found a significant negative relationship between corolla tube length and anther exertion in every population, probably because of anatomic coherence when anthers are hidden within a very long corolla tube, whereas anthers are longer than a short corolla tube and appear to extend from the flower. Similarly, we found a negative covariance between anther exertion and herkogamy across most populations from each ploidy. Since herkogamy had negative values (i.e., the difference between style height and long stamen length) in this species complex, we must keep in mind that a lower degree of herkogamy translates into greater separation of male and female structures and greater exertion of anthers above the corolla.

We also found that phenotypic traits related to flower size (petal length, corolla diameter and corolla tube length) were commonly negatively connected with herkogamy in all populations except for population Ei18, where the relationship is positive. In addition, corolla tube length was positively linked to female reproductive investment (ovules) across all populations. For the male reproductive investment (pollen), we only found a significant link with a floral trait for a diploid population (Ei12) and the tetraploid populations (Figure 2A, B).

Anther exertion was linked to male and female reproductive investment across all populations, so flowers with more exerted anthers might produce more pollen and ovules. On the other hand, herkogamy was only connected with female reproductive investment, meaning that flowers with less distance between male and female organs could produce more ovules (Figure 2).

In general, we found similar relationships patterns among traits across populations although the number of significant connections changes. For instance, the fewest relationships among traits were found for the diploid population Ei13, while the number of related traits increased with ploidy level, being maximum for populations Ei08 and Ei16, which are both polyploid populations. Population Ei18, which is hexaploid, had fewer links among variables, but we found several variables directly connected with fitness (Figure 2C).

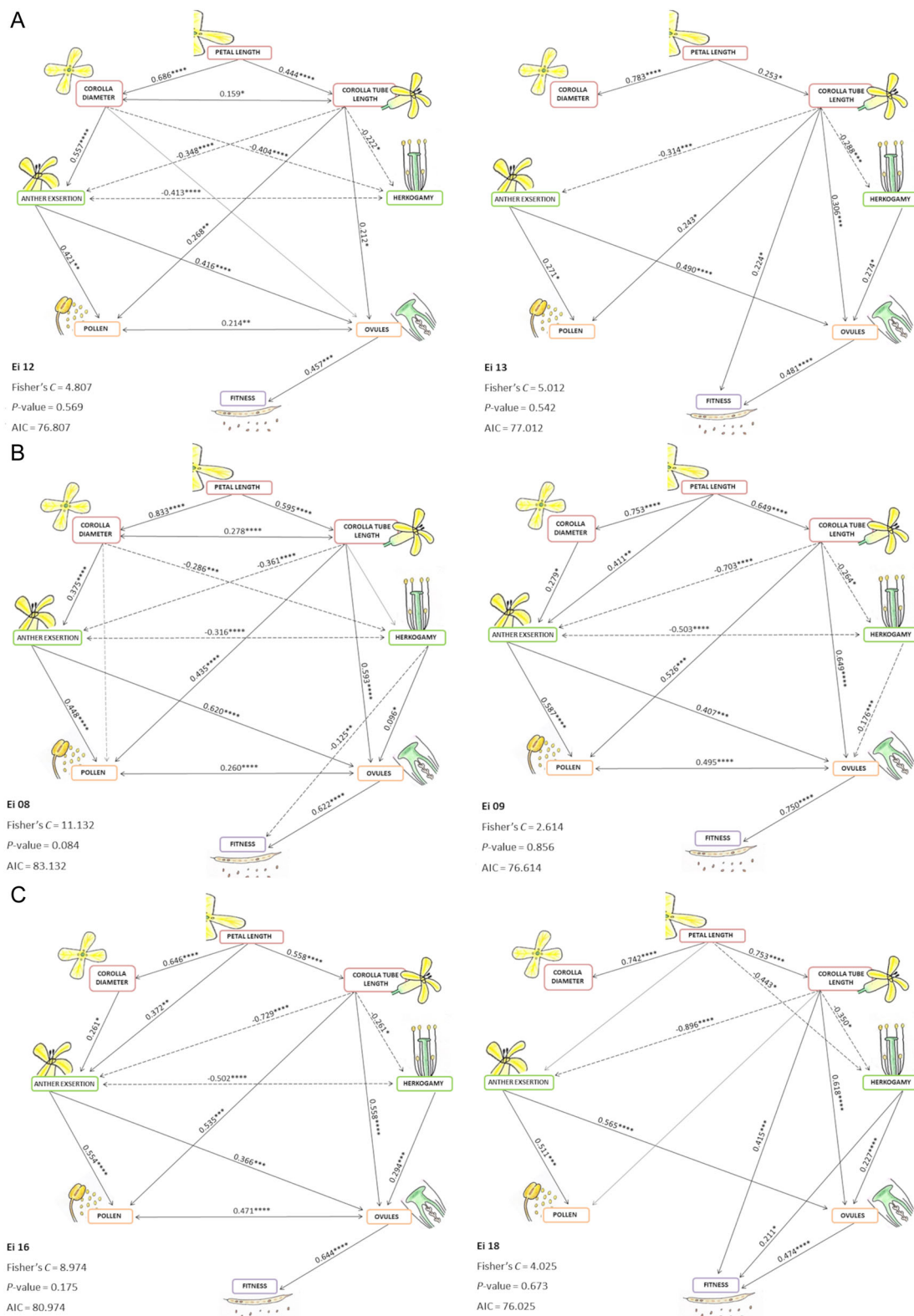


FIGURE 2 Path diagrams describing the effects of floral phenotypic traits (petal length, corolla diameter, corolla tube length), the spatial position of reproductive structures (anther exsertion and herkogamy) and reproductive investment (pollen and ovules) on fitness for (A) diploids, (B) tetraploids, and (C) hexaploids. Solid and dashed arrows indicate positive and negative relationships, respectively. For visual simplicity, only significant relationships are represented accompanied by the mean values of path coefficients and asterisks indicating the degree of significance. Grey lines indicate marginally significant relationships. (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$).

Variation in selection on phenotypic and reproductive traits

Patterns of trait selection across populations indicated that ovule amount was the only trait directly linked with fitness in all the studied populations (Figure 2). Due to this ovule–fitness relationship, traits linked with the female reproductive investment were indirectly selected. Male reproductive investment was directly (and negatively) selected only in hexaploid Ei18 (Figure 2C). Nevertheless, we found indirect selection acting on pollen mediated by its covariance with ovule amount.

Corolla tube length was under indirect selection in all populations because it is the trait with more established relationships with other traits directly linked with fitness (e.g., the ovules). The other flower size traits were indirectly selected for at least one population from each ploidy level (Figure 2). Herkogamy was directly selected, while anther exertion was selected indirectly through ovules and by its covariance with herkogamy (Figure 2). The estimated parameters and their standard errors resulting from the path analyses per population and ploidy are given in Appendix S5. The mean values of the direct, indirect and total effect of each studied attribute on fitness are shown in Appendix S6.

Effect of P:O ratio on selection regimes

We found a significant relationship between the P:O ratio and selective regimes on herkogamy across populations (Figure 3). The proportion of variance of selective regimes explained by the P:O ratio ($R^2 = 0.73$) was significant ($P = 0.03$) even when the number of populations included in the analysis was low. In populations where the P:O ratio was low, the approximation between anthers and stigma (this is, values of herkogamy closer to zero) was selected. While in populations where the P:O ratio was higher, the separation between anther and stigma (this is, more negative values of herkogamy) was positively selected (Figure 3, Table 2).

Effect of ploidy level on phenotypic and reproductive traits

Ploidy level and the interaction between ploidy and the population had a significant effect for all the measured variables but not for P:O ratio in the case of the ploidy–population interaction (Appendices S7, S8). More specifically, we found significant among-ploidy differences in all traits related to floral size (petal length, corolla

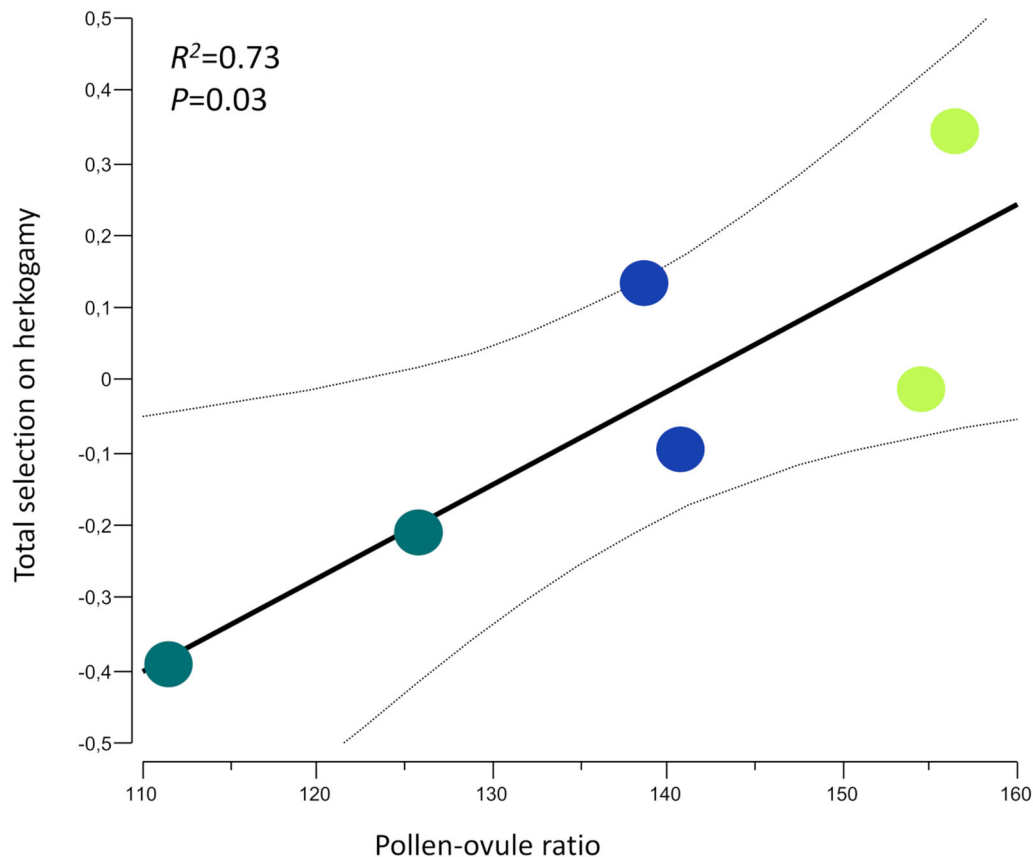


FIGURE 3 Effect of P:O ratio on selective strength. Response curves show the P:O ratio effect on the total selection on herkogamy. Each circle represents a single population: green circles refer to hexaploid populations, blue circles to diploid populations, and ocean circles to tetraploid populations. Grey lines represent 95% confidence intervals.

diameter, corolla tube length) separately (Figure 4). Plants with higher ploidy had bigger corollas, and this pattern is maintained for the corolla tube length.

Flowers of hexaploid plants have significantly more exerted anthers above the corolla compared with diploid and tetraploid plants. We found significant differences among diploid and polyploid plants. Diploid plants have reduced values of herkogamy compared to tetraploid and

hexaploid plants, which had more negative values of herkogamy (Figure 4).

Regarding reproductive investment, we found significant differences among ploidy levels but not the same pattern for pollen and ovules. Hexaploid plants produced a significantly more pollen, and diploids produced the least, with the tetraploids intermediate (Figure 4). However, hexaploids had significantly more ovules per flower than in the diploids and tetraploids, which did not differ significantly for this trait (Figure 4). When we evaluated the P:O ratio, hexaploids had the highest values. Still, diploids had intermediate values and tetraploids the lowest (Figure 4).

TABLE 2 Total selective effects on traits result from the sum of significant direct and indirect effects on fitness. The indirect effect results from the product of mediated on fitness according to SEM for each population.

| Traits | 2n | | 4n | | 6n | |
|---------------------|--------|-------|--------|--------|--------|-------|
| | Ei12 | Ei13 | Ei08 | Ei09 | Ei16 | Ei18 |
| Petal length | 0.175 | 0.147 | 0.449 | 0.815 | 0.310 | 0.130 |
| Corolla diameter | 0.171 | 0 | 0.313 | 0.212 | 0.081 | 0 |
| Corolla tube length | 0.173 | 0.259 | 0.316 | 0.302 | 0.247 | 0.357 |
| Anther exertion | 0.231 | 0.236 | 0.479 | 0.590 | 0.309 | 0.268 |
| Herkogamy | -0.095 | 0.132 | -0.210 | -0.295 | -0.013 | 0.342 |
| Pollen | 0.098 | 0 | 0.162 | 0.371 | 0.303 | 0 |
| Ovules | 0.349 | 0 | 0.622 | 0.586 | 0.644 | 0.474 |

Phenotypic correlation among traits across ploidies

We found significant differences between the phenotypic correlations among traits across ploidy levels. Diploids had significant correlations between every pair of measured traits. However, the number of significant correlations decreased in tetraploids, and hexaploids had even fewer (Table 3). There was a significant positive phenotypic correlation among all the attributes except for herkogamy, which negatively correlated with all the other traits. In tetraploids, most correlations were significant and positive; however, the correlation with

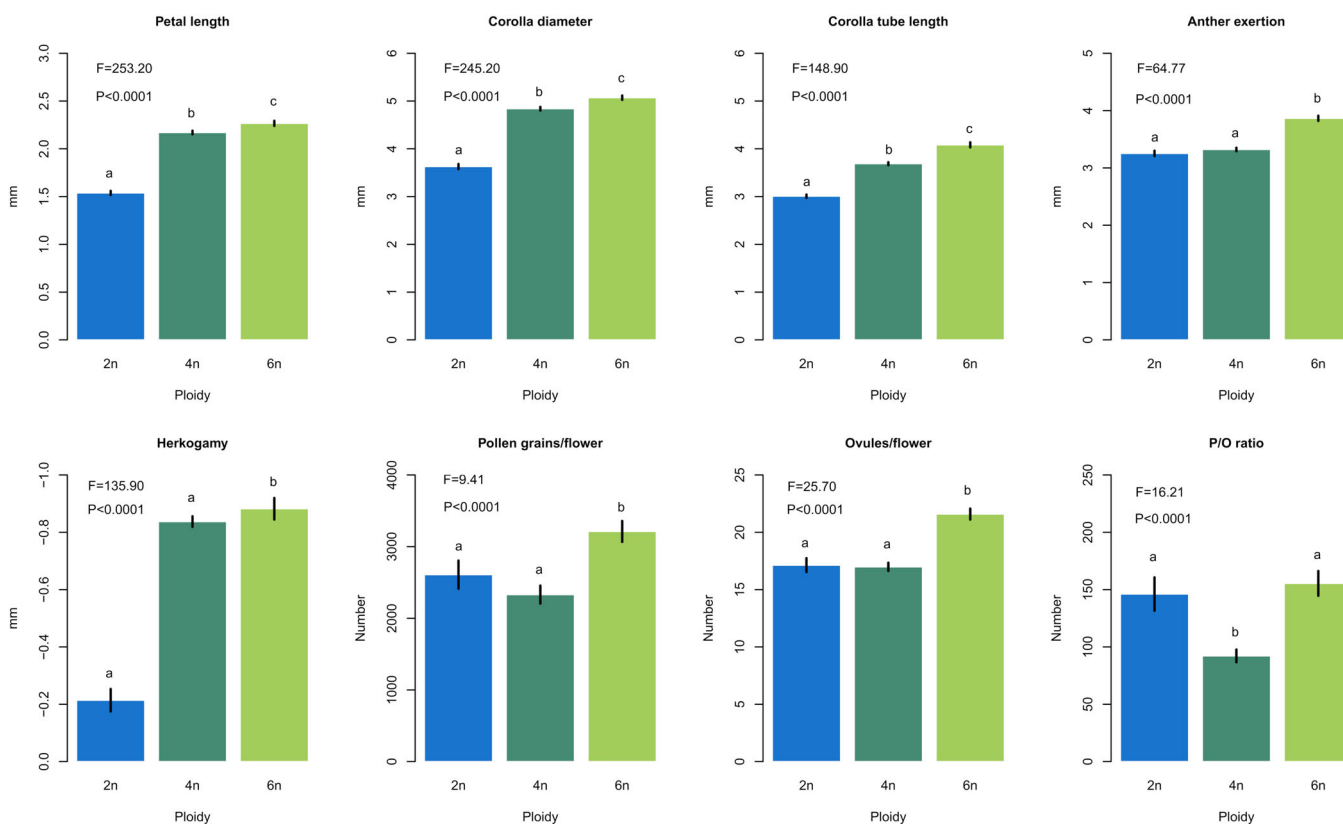


FIGURE 4 Mean and SE values for petal length, corolla diameter, corolla tube length, anther exertion, herkogamy, number of pollen and ovules per flower, and P:O ratio for each ploidy level based on linear models. Letters refer to these groups that are significantly different according to a pairwise test ($P < 0.05$).

herkogamy and the correlation between corolla tube length and exertion were negative. The correlations between corolla diameter–anther exertion and corolla tube length–herkogamy were not significant. Finally, we found significant positive correlations in hexaploids between petal length, corolla diameter, and corolla tube length. These floral attributes also had significant positive correlations with ovules. Corolla tube length and herkogamy, anther exertion and pollen grains, and pollen grains and ovules were also significant and positively correlated. There were significant negative correlations of anther exertion with petal length, corolla tube length and herkogamy, and between herkogamy and pollen production. The rest of the phenotypic correlations were not significant in hexaploid plants (Table 3).

DISCUSSION

The efficacy of natural selection acting on a selfing plant species has been explored at a molecular level (Arunkumar et al., 2015), mainly when the focus was natural selection

acting on phenotypic traits or reproductive investment, but has received less attention than it has in outcrossing species. By combining the study of relationships among traits, reproductive investment, and their direct and indirect effects on fitness across different ploidy levels, here we showed that changes in the amount of DNA significantly affected the configuration of the relationships established between the studied floral traits, modifying the phenotypic integration of these traits across ploidies. In this group of selfing species, the effect of flower traits on reproductive investment and self-pollination plays a vital role in plant fitness, shaping the floral patterns we found across ploidies. However, the strong selection of traits related to pollen export makes them key attributes driving changes in the evolution of reproductive strategies within a selfing multiploidy species complex.

Using path analyses, we were able to detect direct, indirect, and total effects of traits on fitness. These determinations were essential because indirect effects frequently pass undetected; traits interact with fitness and several selective agents simultaneously, which we cannot always control. Often, traits are mediated by natural

TABLE 3 Phenotypic correlations among *Erysimum incanum* phenotypic traits for each ploidy level. Positive correlations are highlighted in blue, while negative correlations are highlighted in red. The sample size is shown by ploidy. Significant values are shown in bold after Bonferroni corrections (P -value < 0.0025) while asterisks indicate significance levels without Bonferroni correction (** P < 0.001; *** P < 0.0001).

| | Corolla diameter | Corolla tube length | Anther exertion | Herkogamy | Pollen | Ovules |
|---------------------|------------------|---------------------|-------------------|-------------------|-------------------|-------------------|
| 2n (405) | | | | | | |
| Petal length | 0.7908*** | 0.6473*** | 0.3244*** | -0.3493*** | 0.2091*** | 0.4619*** |
| Corolla diameter | | 0.6140*** | 0.4343*** | -0.4103*** | 0.1770 | 0.5513*** |
| Corolla tube length | | | 0.1965*** | -0.3872*** | 0.1677 | 0.5558*** |
| Anther exertion | | | | -0.4908*** | 0.3563*** | 0.6124*** |
| Herkogamy | | | | | -0.2787*** | -0.4195*** |
| Pollen | | | | | | 0.3263*** |
| 4n (763) | | | | | | |
| Petal length | 0.6534*** | 0.5491*** | 0.1013 | -0.1011** | 0.2703*** | 0.3490*** |
| Corolla diameter | | 0.5109*** | 0.0874 | -0.1023** | 0.2420*** | 0.3122*** |
| Corolla tube length | | | -0.2622*** | -0.0214 | 0.1480*** | 0.4095*** |
| Anther exertion | | | | -0.3482*** | 0.4420*** | 0.3418*** |
| Herkogamy | | | | | -0.1906*** | -0.1887*** |
| Pollen | | | | | | 0.6691*** |
| 6n (454) | | | | | | |
| Petal length | 0.6956*** | 0.5492*** | -0.1456 | 0.0132 | 0.0544 | 0.2755*** |
| Corolla diameter | | 0.3668*** | -0.0109 | 0.0417 | -0.0614 | 0.2134 |
| Corolla tube length | | | -0.5936*** | 0.1248 | -0.0509 | 0.2855*** |
| Anther exertion | | | | -0.2226*** | 0.2622*** | 0.1174 |
| Herkogamy | | | | | -0.1408 | 0.0627 |
| Pollen | | | | | | 0.2861*** |

selection on other traits, modifying the selective pressure. For example, Gómez et al. (2009), applying SEM approximation to *Erysimum mediohispanicum* populations, described significant effects of plant phenotype by indirect selection on the total selection experienced by the plants. This work demonstrated that this indirect selection played a crucial role in the rise of the geographic mosaic of selection in *E. mediohispanicum* in the Sierra Nevada Mountains.

In our case, we found that traits related to corolla size are rarely under direct selection but more frequently under indirect selection. The cost of producing and maintaining the structure could explain the negative selection acting on some of these traits (Ashman and Schoen, 1994). However, bigger flowers enhance the exertion of the anthers, which has a positive effect on fitness (Gómez et al., 2009; Sahli and Conner, 2011). Evaluating the total effect on fitness, we found an existing counterbalance between different selective gradients. Previous studies have already shown that opposite selective pressures acting on the same trait are important to maintain phenotypic variation within a population (Siepielski and Benkman, 2010), which is essential for evolution. Additionally, differential responses to selection by the same trait could favor diverse evolutionary trends within a species (Zhou et al., 2020). Consequently, if the selected traits are related to the reproductive strategy, a transition in the mating system might be possible in the *E. incanum* complex.

Polyploidization has the potential to produce changes in flower morphology, size, and physiology (Müntzing, 1937; Stebbins, 1971; Levin, 2002; Clo and Kolář, 2021). Recent works comparing diploid and polyploid populations showed that plants with higher ploidy levels produce not only larger flowers but also larger pollen grains (Oliveira et al., 2022). In addition, studies in *Heuchera grossularifolia* (Segraves and Thompson, 1999) demonstrated that tetraploid populations had larger flowers that attracted a different pollinator assemblage than in the diploid populations. We also found a tendency for bigger flowers and more pollen grains as ploidy increased in *E. incanum*. Although we did not test for changes in pollinator visits, larger flowers associated with more pollen could increase floral advertisement and attraction, modifying pollinator interactions. Changes in metabolic routes could accompany these changes in ploidy level and flower size. An interesting example of such changes was described for the *Dianthus broteri* species complex; increasing floral scent variation was associated with polyploidization (Picazo-Aragón et al., 2020). In *E. incanum*, it would be interesting to evaluate changes in metabolites related to this increment in flower size and pollen grain production as a potential mechanism to attract floral visitors.

Stamen length plays an important role in the exposure (and subsequent pollen removal) of self-pollen and the reception of outcrossing pollen grains by attracting pollen vectors. In autogamous plants, Rosas-Guerrero et al. (2011) demonstrated a high degree of integration among floral traits that promote self-pollination, such as the length of the style and stamens. In addition, longer stamens and pistils

are associated with greater pollen exposure and stronger competition between self-pollen and the incoming outcross-pollen, as shown in classical studies on animal-pollinated plants (Campbell, 1989). Yet, stamen length is not necessarily associated with flower size, as observed here. According to our results, and contrasting with other autogamous mating systems, in *E. incanum*, high values for corolla traits, pollen amount, and anther exertion in polyploids might mean that outcrossing reproduction is more important as ploidy level increases. Despite the theoretical expectations that polyploids should self-fertilize more on average than their diploid progenitors to reduce frequency-dependent selection, autopolyploid species have been found on average to self-fertilize less than their diploid progenitors (Husband et al., 2008). Recent theoretical studies confirmed that avoiding selfing in neopolyploids promotes establishment, notably when polyploids have an initial decrease in fitness (Clo et al., 2022). In this sense, our results provide more evidence that the association between ploidy and selfing is probably less clear than previously thought.

Herkogamy is one of the primary mechanisms reducing selfing and driving mating system transitions in plants (Opedal et al., 2017). Plants of *Erysimum incanum* have reverse or negative herkogamy; i.e., the anthers are above the stigma surface to ensure self-pollen deposition in the stigma. However, reverse herkogamy is more accentuated when ploidy increases, with hexaploids having the greatest distances among sexual organs. Increasing values of herkogamy with greater anther exertion would promote pollen exportation more easily after pollinator visits, even with an anther-rubbing mechanism in *E. incanum* (Abdelaziz et al., 2019) that ensures self-pollination.

It is worth noting that reproductive investment indirectly mediates selection on most traits. Specifically, the most significant selection of traits was exclusively mediated via female function, i.e., ovule production. In addition, we found variability in pollen production associated with different phenotypes and significant differences in production between ploidy levels. So, to understand the full scope of these changes in sexual effort, we focused on the P:O ratio and its evolutionary consequences. On the basis of the sex allocation theory (Charnov, 1987; Brunet, 1992; Campbell, 2000), we expect a trade-off between male and female reproductive investment. Cruden (1977) described a trade-off mechanism between pollen and ovules production and proposed that low values in the P:O ratio would be associated with autogamous mating systems in plants, while high values would be characteristic of outcrossing species. This pattern has been described in *Silene* and *Dianthus* species, where lower levels of P:O ratio are associated with self-compatible species (Jürgens et al., 2002). However, the positive covariance between pollen and ovule production we found in our system means that plants that invest more in male reproduction also invest more in female reproduction. Our results demonstrate that a trade-off between male and female reproductive

investment does not exist in *Erysimum incanum*. This finding is unexpected for a selfing species.

We also detected a positive effect of the P:O ratio on the natural selection intensity on herkogamy across the analyzed populations. This result suggests that in populations with a lower P:O ratio, a reduction in herkogamy is favored. Nevertheless, in populations where the P:O ratio is higher, the separation between male and female reproductive organs is favored by selection. This outcome agrees with previous results in other species from the genus *Melochia* (Faife-Cabrera et al., 2018) and Fabaceae species (Galloni et al., 2007), where a positive correlation was described between P:O ratio and herkogamy. Our findings show that in populations where the proximity between male and female reproductive organs is selected, the P:O ratio decreases, probably because self-pollination is more efficient. Conversely, in a population where the separation between sexual organs is strongly selected, the P:O ratio increases, likely to increase the success of outcrossing. These findings support and provide a mechanistic explanation for Cruden's (1977) conclusion that the P:O ratio decreases with an efficient degree of self-fertilization.

Studying multiple traits that affect fitness provided a global view of the different selecting pressures acting on phenotypes. But it also allowed us to explore potential relationships between the measured traits and their integration into the phenotype. In this sense, the gradual reduction of significant correlations from the diploid to hexaploid plants suggests that increasing ploidy level significantly affects the covariation between plant traits (i.e., phenotypic integration). Phenotypic integration is thought to be adaptive when it results from convergent evolution in the values of functional traits to develop a common or independent function in the organism. However, we can also think of phenotypic integration as a constraint for future evolution of each trait's covariance (Pigliucci, 2003). For example, sets of vegetative or reproductive attributes sharing common functions would strongly covary and hence limit the independent variation of each other. Polyploidy was described as contributing to changes in covariation among traits in *Dianthus broteri* species complex (Balao et al., 2011) and *Brassica* allopolyploid species (Baker et al., 2017) and now in *E. incanum* complex (results herein). Reduced covariation values between traits may reduce and overcome the constraints imposed by phenotypic integration, allowing the new polyploid species to explore new trait spaces and potentially reach new or higher adaptive peaks unreached by their diploid counterparts (Hansen et al., 2019).

These findings in ecological variation between populations differing in ploidy level might shed light on the role of genetic mechanisms as drivers of the evolutionary trajectory followed even by a selfing species that generally has been considered to have a reduced ability to evolve (Igic and Busch, 2013). However, in recent years, new empirical and simulation studies have focused on discuss this traditional association between selfing species and a reduced adaptive

potential (Clo and Opedal, 2021; Opedal et al., 2023). Our study provides new results consistent with these last studies and highlights the importance of the genetics as a key factor enhancing the evolutionary dynamics within a selfing clade.

CONCLUSIONS

Changes in flower size, spatial position of sexual organs, and reproductive investment seem to be a direct consequence of genome duplications, with hexaploid plants having the highest values for every studied attribute. No trade-off was found between pollen and ovule production. Still, the number of ovules per flower was the trait under significant selective pressure in every population independently of the ploidy, evidence of the limiting role of ovules as a fitness component for these plants. In addition, ovules also significantly affected adaptive trajectories because of their relation with pollen production (P:O ratio), which constrains the selective pressures acting on important traits such as herkogamy. Finally, increasing ploidy values contributed significantly to decoupling variation among traits in *E. incanum* species complex, reducing phenotypic integration as ploidy increases. So, even working with a selfing species complex, we provide evidence that the variation promoted by polyploidization is a key factor in the reproductive strategy transitions by modifying the investment in pollen and ovules and linking them with plant fitness and phenotype evolution.

AUTHOR CONTRIBUTIONS

A.G.-M., C.F., A.J.M.-P., and M.A. conceived and designed the experiments. A.G.-M., C.F., and C.V.B. did the greenhouse experiments. J.L. and S.C. did the ploidy analyses. A.G.-M., A.J.M.-P., and M.A. did the statistical analyses and designed the tables and figures. C.V.B. constructed the figures. A.G.M. wrote the first draft of this manuscript; all other authors made significant contribution to the draft. A.J.M.-P., S.C., and M.A. obtained the funds to develop this study. A.J.M.-P. and M.A. supervised the study.

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DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included at the end of the article. Further inquiries can be directed to the corresponding author(s).

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

Appendix S1.

Table S1. Number of plant individuals per population for which we measured corolla traits (petal length, corolla diameter and corolla tube length), spatial position between reproductive structures (anther exertion and herkogamy), reproductive investment (pollen and ovules), and seed production or fitness.

Appendix S2.

Figure S1. A priori structural equation model to evaluate the effect of corolla traits, spatial position between reproductive structures, and male and female reproductive investment on plant fitness.

Table S2. Hypothesized mechanisms explaining the proposed links among measured variables in Figure S1.

Appendix S3.

Table S3. Comparison among proposed models based on P -value and AIC scores.

Appendix S4.

Table S4. Variance inflation factors (VIF) calculated from multiple linear regressions to test multicollinearity among variables.

Appendix S5.

Table S5. Estimated parameters and standard error of direct effects on reproductive structures position

(Anther exertion and Herkogamy) and male and female reproductive investment (Pollen and Ovules, respectively) based on structural equation modelling for each ploidy level.

Appendix S6.

Table S6. Values of each trait's direct and indirect and total effect on fitness for each population.

Appendix S7.

Table S7. Effect of ploidy and population on measured traits evaluated by linear models.

Appendix S8.

Table S8. Multiple comparisons of traits values between populations from the three ploidy levels based on linear models nesting population within ploidy.

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