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Dioecy in a wind-pollinated herb explained by disruptive selection on sex allocation

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Summary

- The evolution of dioecy from hermaphroditism is widely thought to be a response to disruptive selection favouring males and females, driven by advantages of inbreeding avoidance, sexual specialisation, or both. However, it has hitherto been difficult to uncouple the importance of these two advantages of dioecy.
- Here, we estimate the selfing rate and fitness gain curves with and without inbreeding depression in wind-pollinated *Mercurialis annua*, based on variation in sex allocation among hermaphrodites that evolved from dioecy following the experimental removal of males. Because males had been removed, we could rule out any important contribution to disruptive selection due to advantages of sexual specialisation, allowing us to focus in inbreeding effects.
- Our results confirm a tradeoff between male and female allocation in *M. annua*, as assumed in sex-allocation theory. The individual selfing rates increased with male allocation, greatly altering the female and male fitness gain curves under strong inbreeding depression. There was strong disruptive selection on sex allocation due to its interaction with the mating system.
- Taken together, our study demonstrates that inbreeding avoidance on its own can lead to disruptive selection on sex allocation and thus the selection and maintenance of dioecy under wind pollination.

Keywords: anemophily; evolutionary branching; geitonogamy; mating system; monoecy; paternity; selection gradient; sexual conflict; sexual system

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Introduction

Why hermaphrodites should ever evolve towards dioecy has long intrigued evolutionary biologists (Darwin, 1877; Charnov *et al.*, 1976; Bawa, 1980; Thomson & Brunet, 1990; Renner & Ricklefs, 1995; Freeman *et al.*, 1997; Ashman, 2006; Käfer *et al.*, 2017). Two overarching explanations have been suggested. First, the 'inbreeding avoidance' hypothesis posits that unisexuality provides a fail-safe means of avoiding self-fertilization and the accompanying deleterious effects of inbreeding depression (Mather, 1940; Lewis, 1941; Charlesworth & Charlesworth, 1978). And second, the 'sexual specialisation' hypothesis recognises that the separation of sexes into different individuals allows males and females to express different trait values that optimize their respective fitness (Willson, 1979; Givnish, 1980), thus resolving sexual conflict and interference that may compromise fitness in simultaneous hermaphrodites (Abbott, 2011; Schärer *et al.*, 2015). These two explanations for dioecy are not mutually exclusive: populations may initially evolve dioecy in response to selection for inbreeding avoidance but then subsequently evolve sexually dimorphic traits that confer on each of the two sexes benefits of specialisation (Freeman *et al.*, 1997; Charlesworth, 2018); or dioecy may evolve gradually from hermaphroditism via divergence in sex allocation and sexual specialisation jointly (Lloyd, 1980a; Lesaffre *et al.*, 2024b). Accordingly, it has not been possible to isolate the role of one or other of the two leading explanations for the evolution and maintenance of dioecy in any single species to date.

A further, but related, reason for slow progress in understanding the evolution and maintenance of dioecy derives from the difficulty of comparing fitness among males, females and hermaphrodites with a range of different sex allocation strategies, because dioecious species typically comprise only males and females and the sex allocation in hermaphrodite populations is typically quite canalised. While the males and/or females of some dioecious show a degree of 'leaky' or inconstant sex expression (Delph & Wolf, 2005; Ehlers & Bataillon, 2007; Käfer *et al.*, 2022), the sex allocation of these leaky phenotypes is typically very close to that of the corresponding 'constant' (or pure) males or females (Cossard & Pannell, 2019). We thus lack the sort of natural variation within populations that is needed for comparisons of the fitness of phenotypes across a wide range of alternative sex-allocation strategies. More formally, we lack estimates of the shape of 'fitness gain curves' on which much of the theory for the evolution of dioecy versus hermaphroditism is based (Charnov *et al.*, 1976; Charlesworth & Charlesworth, 1981; Charnov, 1982; Campbell, 2000; Zhang, 2006;

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West, 2009; Fromhage & Kokko, 2010; Dorken & Van Drunen, 2018; Masaka & Takada, 2023; Lesaffre *et al.*, 2024a).

Fitness gain curves relate the fitness of an individual in a population through its male and female components of reproductive success to its sex allocation, i.e., to the fraction of reproductive resources allocated to its male versus female functions (Charnov, 1982). In the simplest terms, dioecy is predicted to be evolutionary stable when pure males and females have greater fitness than any intermediate (hermaphroditic) sex-allocation strategy, such that selection on sex allocation is disruptive and the curve relating the combined fitness through both male and female functions is u-shaped (Fig. **1**); such a scenario would be revealed, for example, by a positive second-order polynomial in a regression analysis (Lande & Arnold, 1983). In contrast, hermaphroditism should be stable to the invasion of sexual specialists (males or females, or individuals with male-biased or female-biased sex allocation) if selection is stabilizing and the fitness gain curve is n-shaped (revealed by a negative second-order polynomial, for instance; Fig. **1**; Lande & Arnold, 1983). These ideas were first applied to theory for the maintenance of dioecy versus hermaphroditism under the assumption of complete outcrossing of all phenotypes (e.g., hermaphrodites that are self-incompatible; Charnov et al. 1976; Lloyd, 1984a), but they were soon also applied explicitly to the case where hermaphrodites are partially self-fertilizing, with the fitness of selfed progeny potentially diminished by inbreeding depression (Charlesworth & Charlesworth, 1981). These models typically assumed a parametric fixed selfing rate independent of the distribution of sex allocation, yet the selfing rate might often depend on floral strategies, including relative allocation to male and female functions (Gregorius *et al.*, 1987; Holsinger, 1991; Damgaard & Abbott, 1995; Chen & Pannell, 2024b).

The selfing rate should be especially sensitive to sex allocation in wind-pollinated species, which are also enriched for dioecy (Weiblen *et al.*, 2000; Vamosi *et al.*, 2003; Friedman & Barrett, 2008; Renner, 2014); indeed, the association between dioecy and wind pollination is consistent with a likely link between the mode of pollination and selection on sex allocation (Bawa, 1980; Beach, 1981; Charlesworth, 1993). In wind-pollinated species, allocating more resources to male function likely results in a relatively linear increase in male fitness through outcrossing, with increases in pollen production leading to corresponding increases in siring success (Charnov, 1982; Campbell, 2000; Aljiboury & Friedman, 2022). However, in wind-pollinated hermaphrodites that are self-compatible, we should expect greater pollen

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production to increase the proportion of self-pollen in the pollen cloud around the plant's stigmas, causing greater self-fertilization through lottery competition or 'mass-action' mating (Gregorius *et al.*, 1987; Holsinger, 1991; Freeman *et al.*, 1997; Friedman & Barrett, 2009) and the potential reduction in the fitness of self-fertilized progeny by inbreeding depression (Charlesworth & Charlesworth, 1987a). While increased male allocation may therefore increase outcross siring success, it should also decrease female reproductive success as a result of ovule or seed discounting (Lloyd, 1992; de Jong *et al.*, 1999). Accordingly, when inbreeding depression is substantial, individuals allocating all their reproductive resources to either the male or female function should have higher fitness than those adopting a hermaphroditic strategy (Fig. **1**), leading to a u-shaped gain curve and the evolutionary stability of dioecy (de Jong *et al.*, 1999; de Jong & Geritz, 2001). This disruptive selection on sex allocation when selfing is increased by male allocation should be strongest for larger individuals in a population. This is because large individuals produce relatively more pollen, whereas small individuals might increase their male allocation with relative impunity, because their pollen contributes less to the pollen cloud around their stigmas than the pollen of large individuals. Small individuals are thus expected to experience less disruptive selection or even stabilizing selection than larger ones (de Jong *et al.*, 1999). While these ideas explicitly link sex-allocation theory with the inbreeding avoidance hypothesis for the evolution and maintenance of dioecy, they remain largely untested.

For empirical tests of theory for the stability of dioecy, we ultimately need to compare the fitness of individuals adopting a range of alternative sex-allocation strategies within a population, from fully male through hermaphroditism (ideally with a range of different sex allocations) to fully female (Fig. **1**). However, most plant species tend to be either dioecious or hermaphroditic, and species with intermediate sexual systems such as gynodioecy or androdioecy (where males or females, respectively, coexist with hermaphrodites) only allow comparisons between unisexuality and bisexuality for one of the sexual functions (Weller & Sakai, 2005; Spigler & Ashman, 2012; Varga, 2021; Laugier *et al.*, 2023). Although comparisons among related species with different sexual systems can provide valuable information (Steven & Waller, 2004; Sakai *et al.*, 2006; Soza *et al.*, 2012; Kwok & Dorken, 2022), they are often compromised by confounding trait variation and ecologies, and they do not replace comparisons among strategies within the same context. Comparisons among individuals expressing different sex-allocation strategies within the same population can be achieved by physical manipulation of the plants, e.g., through the removal of floral parts or

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flowers (Emms, 1993; Tomaszewski *et al.*, 2018; Aljiboury & Friedman, 2022; Larue & Petit, 2024; Chen & Pannell, 2024a), but such manipulations are often difficult to achieve realistically and may not generate phenotypes that would occur in nature. The dearth of standing variation in sex allocation for testing patterns of selection within a population has therefore been a barrier to studies of the evolution and stability of separate sexes.

Here, we test the inbreeding avoidance hypothesis for the evolution of dioecy by studying the fitness of individuals with highly varied sex allocation, across the full range from male to female, without confounding factors of sexual specialisation on ancillary traits. To do this, we estimate the fitness of individuals of a dioecious plant that have evolved intermediate sex-allocation strategies under experimental evolution, following the removal of males that differ in numerous vegetative traits from females (Cossard *et al.*, 2021; Gerchen *et al.*, 2024). Specifically, we used phenotypes expressed by females of the wind-pollinated dioecious annual plant *Mercurialis annua* that have evolved wide variation in the number of male flowers over the course of experimental evolution in the absence of males (Cossard *et al.*, 2021). Our experimental populations thus comprised naturally expressed phenotypes ranging in sex allocation from a male to a female extreme and covering the full sex-allocation spectrum in between (Fig. **2a**). Because males had been removed from these populations, the populations lacked the strong sexual dimorphism in vegetative traits that otherwise characterize natural populations of dioecious *M. annua*, allowing us to focus our attention specifically on the inbreeding avoidance hypothesis.

To advance our understanding of how selection for inbreeding avoidance affects the evolution and stability of separate sexes, we first confirmed the existence of a sex-allocation trade-off between male and female functions (Gerchen *et al.*, 2024), the fundamental assumption of sex-allocation theory on which its predictions are based (Charnov, 1982; West, 2009). We then asked (1) how the selfing rate depends on sex allocation and (2) how total fitness depends on the selfing rate of individuals with different sex allocation strategies under contrasting scenarios of inbreeding depression. For each individual and its sex-allocation phenotype, we estimated male reproductive success on the basis of paternity assignment for a large sample of progeny produced. We estimated female reproductive success in terms of a measure of seed production per individual and its corresponding phenotype, inferred as a function of inbreeding depression. With estimates of male, female, and total reproductive success, we finally inferred the shape of the respective fitness gain curves. Our study

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advances an understanding of the stability of dioecy to the invasion of alternative sex-allocation strategies on the basis of fitness comparisons over a range of physiologically realistic expressions of sex allocation that, to our knowledge, have hitherto not been realized in any study.

Materials and Methods

Plant materials and experimental populations

Mercurialis annua (Euphorbiaceae) is a wind-pollinated annual herb widely distributed around the Mediterranean Basin and throughout central and western Europe (Tutin *et al.*, 1976; Pannell *et al.*, 2004). Natural diploid populations are dioecious, with individuals producing either female or male unisexual flowers, though leaky sex expression in both sexes (where individuals produce a small number of unisexual flowers of the opposite sex) is not uncommon (Cossard & Pannell, 2019; Villamil *et al.*, 2022). Seeds of *M. annua* from a previous study of experimental evolution were sown in seedling trays in July 2022 and grown for five weeks in the greenhouse of the University of Lausanne, Switzerland (see Cossard et al. 2021). In mid-August, we set up three experimental populations as independent replicates in a five-layered hexagon comprising 61 individuals. The populations were set up at least 150 meters apart from each other on the University campus, minimizing gene flow among the populations. The seedlings were potted individually in 16-centimeter pots with soil (Ricoter substrate 140) and slow-release fertilizer (Hauert Tardit 6M pellets; 5 g fertilizer L–1 of soil) and were automatically watered throughout the period of the experiment of around 14 weeks. We harvested the aboveground parts of the plants and measured their sex allocation and other traits in mid-October when the night temperature dropped below 5 ºC and the plants ceased growing.

Phenotyping of sex allocation and biomass

To quantify the sex allocation of each individual, we counted the number of female (N_{F}) and male (N_{M}) flowers produced on the whole plant by detailed inspection throughout all the branches at the time of harvest. For each individual *i*, we further calculated its functional gender (*G*, hereafter gender) (*sensu* Lloyd, 1980) in terms of maleness as $G_i = N_{\mathrm{M},i} / \left(E \times N_{\mathrm{F},i} + N_{\mathrm{M},i}\right)$, where the equivalence factor, *E*, is the ratio of the number of total male flowers to total female flowers in the respective population. The inclusion of *E* in the formula guarantees that the mean gender of the population is 0.5, corresponding to the fact that half of

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all genes passed to progeny are via male and the other half via female function. After the phenotyping, all the harvested parts were kept in bread bags to dry at room temperature for three weeks, following which we measured their biomass and gathered and counted mature seeds that had been dispersed from capsules into the bags.

Paternity analysis and the estimate of the selfing rate

To estimate the selfing rate of each individual, we used variation at nine microsatellite markers to assign paternity to mature seeds (modified from Machado et al., 2017). Leaf samples of the parental individuals were collected upon harvest and dried in silica gel prior to DNA extraction. Five to ten mature seeds were arbitrarily selected for each individual that produced more than five seeds for DNA extraction; otherwise, all seeds were used. Total DNA was extracted from the leaves and seed samples using the BioSprint 96 DNA Plant Kit (Qiagen, Germany) according to the manufacturer's instructions and eluted in 100 µl of distilled water.

PCR amplification was carried out in a final volume of 10 µl, including 5 µl of 2× Multiplex PCR Master Mix (Qiagen, Germany), 0.2 µl of diluted DNA, 2.8 µl of distilled water, and 2.0 µl of multiplex containing variable primer concentrations. The nine microsatellite markers were grouped into two multiplexes modified from Machado et al. 2017. Thermal cycling was performed in a TProfessional Standard Thermocycler (Biometra GmbH, Göttingen, Germany) as follows: 95 °C for 15 min; 33 cycles at a temperature of 94 °C for 30 s, 90 °C for 90 s, and 72 °C for 90 s; and a final step at 72 °C for 10 min before cooling down to 4°C. PCR products were analyzed by capillary electrophoresis on an ABI3100 Genetic Analyzer (Applied Biosystems), with an internal size standard GeneScan-500 LIZ. Fragment length analyses and scoring were performed with GeneMapper v 6.0 (Applied Biosystems).

Paternity analyses were conducted for the three populations separately to assign the most likely father to each seed for which more than five loci were genotyped. Here, we used the software Cervus v 3.0.7, assuming a relaxed confidence level of 80% and an error rate of 0.01 (Kalinowski *et al.*, 2007). *M. annua* is self-compatible, and the selfing rate of each individual was estimated by the proportion of selfed seeds to the number of total seeds successfully genotyped for that individual.

Calculation of female, male, and total fitness

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The annual life cycle of *M. annua* allows us explicitly to proximate lifetime fitness by reproductive success estimated by seed production and the number of seeds sired as female and male fitness components, respectively. In this study, following the practice of most empirical studies (e.g., Karron & Mitchell, 2012; Briscoe Runquist *et al.*, 2017; Chen & Pannell, 2023; Hou *et al.*, 2024), we attributed the fitness gained via selfed progenies equally to female and male functions rather than attributing twice the fitness of selfed progeny to only the female function, as is typical in theoretical studies (e.g., Charlesworth & Charlesworth, 1987b; Lesaffre *et al.*, 2024a). However, not that inferences for total fitness based on the two contrasting approaches are entirely equivalent. Fitness components were estimated under two scenarios using selfing rates (S) estimated from the paternity analysis: one in which inbreeding depression (δ) was assumed to be zero; and one in which it was assumed to equal one. The actual level of inbreeding depression in wild populations of diploid *M. annua* remains unknown, with a previous study showing a negligible level of inbreeding depression estimated in hexaploid dioecious populations (Eppley & Pannell, 2009; Pujol *et al.*, 2009), and another unpublished study showing considerable inbreeding depression in diploid populations under experimental evolution (E. Le Faou and J.R. Pannell, unpublished data). The scenarios assumed in our analysis reflect the two extreme levels of inbreeding depression, allowing us to explicitly evaluate the effects of inbreeding depression on the patterns of selection on sex allocation.

We calculated female fitness of individual i as $W_{\mathrm{F},i} = N_{\mathrm{T},i} \times [S_i(1-\delta) + (1-S_i)]$. Under the scenario of $\delta = 0$, where selfing does not impose any cost in terms of fitness, female fitness equalled the number of mature seeds of the individual ($N_{\mathrm{T},i}$). When $\delta = 1$, selfed progenies do not contribute to the gene pool of the next generation at all.

We calculated male fitness of individual i using the paternity share of each dame derived from the paternity analysis as $W_{\mathrm{M},i} = \sum_{j \in F} \frac{n_{i,j}}{N_{\mathrm{G},j}} \times N_{\mathrm{T},j}$, where $n_{i,j}$ is the number of seeds sired by individual i on individual j, $N_{\mathrm{G},j}$ is the number of genotyped seeds of dame j, and $N_{\mathrm{T},j}$ is the total number of mature seeds of dame j. When $\delta = 1$, fitness gained via selfed progenies was zero (i.e., $j \in \mathrm{F}\ and\ j \neq i$). We calculated total fitness of individual i as $W_{\mathrm{T},i} = \mathrm{W}_{\mathrm{F},i} + W_{\mathrm{M},i}$ for each of the two scenarios of inbreeding depression.

Statistical analysis

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We conducted all the analyses within the *R* statistical framework v 4.0.3 (R Core Team, 2021). We checked the fit of the models with the package *DHARMa* (Hartig, 2019) and QQ plots. The detailed structures of each regression model can be found in the Supporting Information (Method S1). The general effects of the explanatory variables in each model were extracted using likelihood ratio tests using the *drop1* function.

To evaluate the trade-off between female and male flower numbers, we used a zero-inflated generalized linear mixed model (*glmmTMB* function in package g*lmmTMB*; Brooks et al., 2017), setting the number of female flowers as the response variable with a negative binomial distribution. Male flower number, plant size, and population were set as the explanatory variables with two-way and three-way interaction terms. For model convergence, the male flower number of each individual was standardized by the mean of each population and a standard deviation of one. Plant size was estimated by log-transforming the aboveground biomass (same as for the following analyses).

To investigate how the selfing rate depends on sex allocation (i.e., gender) and the size of focal plants in the three populations, we constructed a generalized linear mixed model (*glmer* function in package *lme4*, Bates et al. 2015) using the proportion of selfed seeds to genotyped seeds of each individual as a binomial response variable. Gender, plant size, and population were set as the explanatory variables with two-way and three-way interaction terms. We set individual identity as a random variable to take into account the non-independency of the seeds from the same individual. To assess how inbreeding depression affects the dependency of different components of fitness on gender and plant size under two inbreeding depression scenarios, we fitted relative female, male, and total fitness as the response variable in three separate linear mixed models (*lmer* function in package *lme4*, Bates et al. 2015).

The relative fitness of each individual was calculated by dividing the fitness by the mean of the focal population (Lande & Arnold, 1983). To detect the non-linear dependency of fitness on gender in each model, we set both linear and quadratic terms of gender as the explanatory variables accompanied by interactions with plant size, scenarios of inbreeding depression, and population. We weighted the variances by plant size to indicate that plants of different sizes have different variances (larger plants have larger variance in fitness; see Method S1). We set the identity of individuals as a random variable to take into account the fact that the fitness estimated under the two inbreeding depression scenarios was not independent (i.e., the same

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individual). We further used the *emtrends* function in the *emmeans* package to extract the linear and quadratic coefficients and their standard errors, with *P* values calculated from the fitted models (Lenth, 2020).

Results

Variation in sex allocation

Plants in the experimental population varied greatly in their size and their male and female reproductive allocations, ranging along a continuum from pure females to pure males (Fig. **2a**). Biomass and the male and female allocations showed no difference among populations (Table **S2**). Individuals produced an average of 238 ± 282 and 316 ± 449 female and male flowers, respectively (mean ± SD; $N = 180$; see Table **S2** for each population; Fig. **2a**). Larger plants produced more female and male flowers ($P < 0.001$). Our data also revealed a clear sex-allocation tradeoff, with a negative correlation between female and male flower numbers ($P < 0.001$), with the strength of the tradeoff being greater for larger plants (Fig. **2b** and Table **S3**). See Fig. **S1** for an evaluation of the non-linear sex-allocation tradeoff.

Dependence of self-fertilization on gender

In total, 948 seeds were successfully genotyped for at least five loci, for which paternity was assigned to a single father for 914 seeds (see also Table **S2** for details of each population). Overall, the average selfing rate was 29.6% (mean over $N = 172$ individuals; Table **S2**). The selfing rate was greater for individuals with greater male allocation (and greater male gender) ($P < 0.001$; Fig. **3**), with the dependence tending to be steeper for larger plants (Fig. **3**), although there was no significant interaction among gender, size, and population (Table **S4**). See also Fig. **S2** for the positive dependence of the selfing rate on absolute male allocation, i.e., male flower number.

Dependence of fitness on gender, plant size, and inbreeding depression

Patterns of mating and fitness tended to be similar across populations: because all higher-order interaction terms involving population were non-significant ($P > 0.05$; Table **S5**), we dropped interactions with population in presentation of the results (Table **1**; see also Fig. **S3** for a comparison among populations). The linear and quadratic coefficients of different components of fitness with gender under the two inbreeding depression scenarios for plants of different sizes can be found in Table **1**.

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The relationship between relative fitness and gender (maleness) depended strongly on plant size and scenarios of inbreeding depression, for both female function ($P < 0.001$ and < 0.01 for three-way interaction terms involving linear and quadratic terms of gender, respectively; Table **S5**; Fig. **4a**) and male function ($P > 0.05$ and < 0.01 for three-way interaction terms involving linear and quadratic terms of gender, respectively; Table **S5**; Fig. **4b**). For example, in small plants, relative male and female fitness components did not depend on gender for either scenarios of inbreeding depression ($P > 0.05$ for all coefficients; Table **1**; Fig. **4a** and **b**), whereas in large plants, relative female fitness negatively and convexly depended on gender for a scenario assuming $\delta = 1$ (for both sexual functions, $P < 0.001$ and < 0.05 for linear and quadratic terms respectively; Table **1**; Fig. **4b**), but it depended only linearly on gender for a scenario assuming $\delta = 0$ (for both sexual functions, $P < 0.01$ and > 0.05 for linear and quadratic terms respectively; Table **1**; Fig. **4b**).

When considering total fitness under different inbreeding depression scenarios, patterns of selection on gender were different for plants of different sizes (Table **S5**). Relative total fitness depended disruptively on gender in large plants under a scenario of $\delta = 1$ ($P < 0.001$ and < 0.001 for linear and quadratic terms respectively; Table **1**; Fig. **3c**), whereas the disruptive dependency was non-significant when assuming $\delta = 0$ ($P > 0.05$ for both terms; Table **1**; Fig. **3c**). In contrast, there was no detectable selection on gender in small plants regardless of the scenarios of inbreeding depression (Table **1**; Fig. **3c**)

Discussion

The phenotypic variation in sex allocation in our experimental populations provided an outstanding opportunity to assess the dependence of total plant fitness on sex allocation in a wind-pollinated plant. Three results stand out. First, we confirmed a clear evidence for a tradeoff between male and female allocation (Gerchen *et al.*, 2024), as typically assumed in theories of sex-allocation and life-history evolution (Charnov, 1982; West, 2009). Second, the selfing rate of individuals depended positively on male allocation and greatly altered the shape of female and male fitness gain curves under scenarios of strong inbreeding depression. And third, sexual interference caused by the interaction between sex allocation and the mating system led to consistent patterns of strong disruptive selection on sex allocation, a pattern predicted to favour the evolution and maintenance of dioecy (de Jong *et al.*, 1999; de Jong & Geritz, 2001).

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Non-linear trade-off in allocation between female and male function

We found a constant negative association between the number of male and the number of female flowers for each size class in the population. This result provides evidence for a sex-allocation trade-off for plants that likely had similar resource status, a fundamental assumption in sex-allocation theory (Charnov, 1982; West, 2009). In dioecious species, the production of sons versus daughters is usually a 'zero-sum game', such that sex-allocation tradeoffs are almost axiomatic. However, it has been difficult to demonstrate such tradeoffs in hermaphroditic species, likely because critical covariates of sex allocation such as resource status have been overlooked, or because male and female functions draw on different resources (de Jong, 1993; Campbell, 2000; Johnson & Nassrullah, 2024; summarized in Ashman, 2003; Mazer et al., 2007). A negative correlation between male and female functions has been found in some monoecious species that produce a mixture of unisexual flowers, e.g., *Astilbe biternata* (Olson & Antonovics, 2000), *Pinus silvestris* (Savolainen *et al.*, 1993), and *Zea mays* (Garnier *et al.*, 1993), but see *Begonia semiovata* (Agren & Schemske, 1995). Our confirmation of a tradeoff between the production of male and female flowers in *M. annua* (Gerchen *et al.*, 2024) adds substantially to this evidence.

Importantly, the sex-allocation tradeoff revealed for *M. annua* deviates strongly from the linear relation assumed in sex-allocation theory (e.g., Charlesworth and Charlesworth, 1981; Charnov, 1982), with its shape being significantly concave (see also Fig. **S1** for a supplementary analysis). This concavity would appear to reflect advantages of the 'economics of scale' for each sexual function and thus an advantage of specialisation (Reekie & Avila-Sakar, 2005; Saeki *et al.*, 2014), possibly linked to negative physiological interference between the sexual functions of individuals producing both male and female flowers, e.g., brought about by hormone regulation, gene expression, and nutrient acquisition (Diggle *et al.*, 2011; Golenberg & West, 2013; Sobral *et al.*, 2016; Jabbour *et al.*, 2022). Sex expression in *M. annua* is regulated by phytohormones at the early stage of floral development, with auxin and cytokinin inducing male and female floral buds, respectively (Louis *et al.*, 1990). It is thus plausible that the production of a mixture of male and female flowers by monoecious individuals may partially disrupt a finely tuned regulatory network required for the production of either male or female flowers (Durand & Durand, 1991; Golenberg & West, 2013). If so, such physiological interference would reduce the efficacy in flower production for individuals with intermediate allocation and should contribute to ecological advantages of sexual specialisation in allocation.

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Effect of selfing and inbreeding depression on female fitness

The selfing rate of individuals in our experimental populations increased with their relative and absolute allocation to male function. A positive dependence of selfing on male allocation has been demonstrated within a population for several insect-pollinated hermaphroditic species, both at the individual and flower levels (Damgaard & Abbott, 1995; Harder & Barrett, 1995; Karron *et al.*, 2004; Williams, 2007; Chen & Pannell, 2024b), but it has hitherto not been reported for any wind-pollinated angiosperm (though such a pattern has been reported for a gymnosperm; Denti & Schoen, 1988). Although the selfing rate should be particularly sensitive to male allocation in wind-pollinated plants, most wind-pollinated angiosperms are predominantly self-incompatible or dioecious (Friedman & Barrett, 2008), and indeed wild populations of *M. annua* are dioecious (Pannell *et al.*, 2004). Our study suggests, however, that the potentially monoecious or hermaphroditic precursors of currently dioecious wind-pollinated species may well have experienced the negative effects of self-fertilization, and that these might have contributed to selection for unisexuality.

The effect of sex allocation on the selfing rate also depended on the size of the plants considered. This finding corresponds to the predictions of the 'mass-action' model (Gregorius *et al.*, 1987; Holsinger, 1991), in which absolute allocation to male function increases the proportion of self-pollen in the local pollen cloud and thus increases the selfing rate of the seeds produced (see also Fig. **S2**). The positive dependence of selfing on sex allocation was steeper in larger plants, because larger plants produced more male flowers in absolute terms than smaller plants. In contrast, male flowers produced by small plants had a much milder effect on the selfing rate because of their smaller resource budget. As a result, most of the ovules produced by the small plants, regardless of their male allocation, were outcrossed by pollen produced by the larger neighbouring individuals.

The dependence of self-fertilization on male allocation could significantly alter the relationship between female fitness and sex allocation as a result of inbreeding depression, especially in mid- and large-sized plants. When inbreeding depression was assumed to be zero in our analyses, female fitness depended linearly and positively on the allocation to female function, implying a mostly linear female gain curve. Under high inbreeding depression, by contrast, an elevated level of ovule discounting in individuals with increased male allocation should impose substantial fitness costs on female function. As a result, females that avoid

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441 allocating to their male function should enjoy higher relative female fitness, leading to an
442 accelerating female gain curve (de Jong *et al.*, 1999). Our results thus provide some of the
443 first empirical evidence for the joint effect of the mating system and inbreeding depression on
444 the female fitness gain curves due to sexual interference, as modelled by Charlesworth &
445 Charlesworth (1981), de Jong *et al.* (1999), and Lesaffre *et al.* (2024a); see Chen & Pannell
446 (2024b) for another example in an insect-pollinated species.
447
448 *Implications for the evolution of sexual systems in wind-pollinated plants*
449 We found that male fitness did not level off with an increased allocation to male function,
450 indicating a linear (non-saturating) male fitness gain curve in wind-pollinated *M. annua*. Our
451 estimates of male fitness when inbreeding depression was assumed to be zero support the
452 general view that wind-pollinated species should have a linear male gain curve, because wind
453 is not easily saturated with pollen in the way an insect's body is (Charnov, 1982; Lloyd &
454 Bawa, 1984). The inferred linear male fitness gain curve for *M. annua* is thus similar to those
455 for the self-incompatible wind-pollinated *Ambrosia artemisiifolia* (Nakahara *et al.*, 2018;
456 Aljiboury & Friedman, 2022).
457
458 Our joint estimates of female and male fitness clearly indicate that dioecious sexual systems
459 should be favoured as an evolutionary response to promote male fitness and avoid self-
460 fertilization in self-compatible wind-pollinated species (de Jong *et al.*, 1999). Considering
461 total fitness gained via the two sex functions under strong inbreeding depression, we detected
462 strong disruptive selection on sex allocation that favours individuals of separated sexes in all
463 three of the experimental populations (Fig. **S3**). Although an accelerating female gain curve
464 due to ovule discounting may be common in both insect- and wind-pollinated species (Karron
465 *et al.*, 2004; Williams, 2007; Chen & Pannell, 2024b), the male gain curve is likely to be
466 saturating in insect-pollinated species (de Jong & Klinkhamer, 1994), potentially precluding
467 disruptive selection on sex allocation and rendering the evolutionary stable sexual system
468 hermaphroditic or gynodioecious (de Jong *et al.*, 1999; de Jong & Geritz, 2001). Our finding
469 of disruptive selection on sex allocation in *M. annua* may thus help to explain the common
470 association between dioecy and wind pollination in flowering plants (Renner & Ricklefs,
471 1995; Friedman & Barrett, 2009) and the frequent evolution of dioecy from hermaphroditism
472 following shifts to wind-pollination, e.g., in *Fraxinus* (Wallander, 2008), *Schiedea* (Weller *et*
473 *al.*, 1995), and *Thalictrum* (Soza *et al.*, 2012).
474

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We also found that disruptive selection on sex allocation was particularly strong for large plants and almost absent for small plants. This result would thus seem to imply that size-dependent sex allocation ought to be selected in *M. annua*, with the large individuals being strictly unisexual and the small ones expressing a wider range of sex allocation (de Jong *et al.*, 1999). The fact that wild individuals of *M. annua* are in fact predominantly unisexual, irrespective of their size, may indicate that dioecy has been stabilized largely by the selection on mid- and large-sized individuals, which produce almost all their parents' descendants. It is also possible that small individuals adopt a unisexual strategy due to the lack of any significant selection on sex allocation for small plants. Note that specialisation in sex allocation, as implied by the nonlinear resource trade-off, likely favours dioecy in *M. annua* irrespective of the effects of inbreeding, because selection on sex allocation is disruptive for large plants even in the absence of inbreeding depression.

Concluding remarks

Selection for inbreeding avoidance or selection for traits that promote advantages of sexual specialisation have long been identified as the two most likely mechanisms favouring the evolution of dioecy in plants (Bawa, 1980; Thomson & Brunet, 1990; Freeman *et al.*, 1997; Pannell & Jordan, 2022). Our results now indicate that disruptive selection on sex allocation, and thus the potential evolution of separate sexes, can arise in populations through the avoidance of the negative effects of selfing when the male fitness gain curve is not too saturating, even in the absence of advantages of sexual dimorphism. Thus, while sexual specialisation on ancillary traits may reinforce selection for separate sexes and the evolution of dioecy with sexual dimorphism (Lesaffre *et al.*, 2024b), our study demonstrates that inbreeding avoidance on its own can drive the evolution of dioecy (see Fig. **S4** for the effect size of ancillary traits on fitness from supplementary analyses), notably by distorting the shape of fitness gain curves such that selection on sex allocation becomes disruptive. Our study thus provides strong empirical evidence for fundamental elements of theory of sex allocation that have been difficult to test for lack of suitable phenotypic variation in populations.

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Competing interests

No competing interests were declared.

Author contributions

KHC and JRP designed the project, collected the data, and wrote the manuscript. KHC led the data analysis with input from JRP.

Data availability

The data that support the findings of this study is openly available in Zenodo (10.5281/zenodo.14542261).

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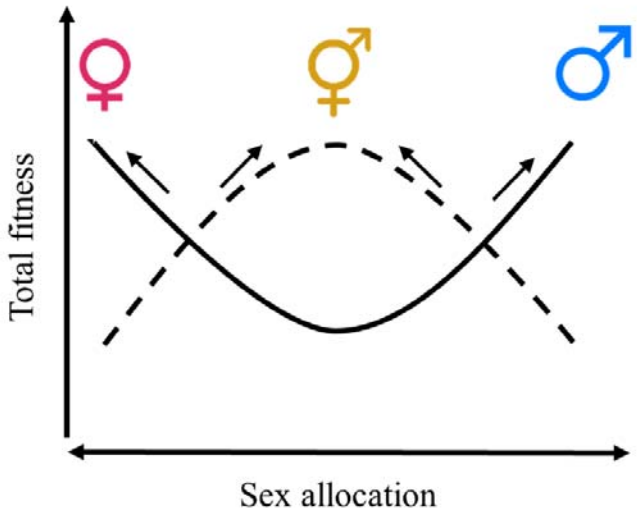
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762 **Table 1.** Comparisons of linear and quadratic coefficients of relative female (**a**), male (**b**), and
763 total (**c**) fitness on gender between the two scenarios of inbreeding depression (δ) in plants of
764 different sizes. Standard errors of the estimates of coefficients are indicated in the parenthesis.
765 A significant interaction indicates that the coefficients under the two scenarios are statistically
766 different. Significant coefficients and interactions were noted by asteroids.
767 Notes: $*$ $P < 0.05$, $**$ $P < 0.01$, $***$ $P < 0.001$

	$\delta = 0$	$\delta = 1$	Interaction
(a) Relative female fitness			
Small plants	Linear: 0.87 (1.86)	Linear: 0.33 (1.86)	
	Quadratic: -1.03 (1.91)	Quadratic: -0.54 (1.91)	
Medium plants	Linear: -2.01 (1.09)	Linear: -3.92 (1.09) ***	***
	Quadratic: 0.35 (1.1)	Quadratic: 1.97 (1.1)	***
Large plants	Linear: -4.92 (1.75) **	Linear: -8.21 (1.75) ***	***
	Quadratic: 1.74 (1.77)	Quadratic: 4.51 (1.77) *	***
(b) Relative male fitness			
Small plants	Linear: 1.58 (1.78)	Linear: 1.28 (1.78)	
	Quadratic: -1.54 (1.82)	Quadratic: -1.07 (1.82)	
Medium plants	Linear: 0.92 (1.05)	Linear: 0.08 (1.05)	*
	Quadratic: 0.43 (1.06)	Quadratic: 2.06 (1.06)	***
Large plants	Linear: 0.27 (1.68)	Linear: -1.11 (1.68)	
	Quadratic: 2.4 (1.71)	Quadratic: 5.19 (1.71) **	***
(c) Relative total fitness			
Small plants	Linear: 1.23 (1.32)	Linear: 0.81 (1.32)	
	Quadratic: -1.30 (1.35)	Quadratic: -0.83 (1.35)	
Medium plants	Linear: -0.55 (0.77)	Linear: -1.92 (0.77) *	***
	Quadratic: 0.38 (0.78)	Quadratic: 2 (0.78) *	***
Large plants	Linear: -2.35 (1.24)	Linear: -4.68 (1.24) ***	***
	Quadratic: 2.07 (1.26)	Quadratic: 4.86 (1.26) ***	***

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768

769 **Figure 1.** Conceptual diagram showing different selection schemes via total fitness on sex
770 allocation (gender) within a population. Unisexual female and male individuals are favoured
771 when the selection on sex allocation is disruptive (depicted by the solid line), rendering the
772 population dioecious. In contrast, when the selection on sex allocation is stabilizing (depicted
773 by the dashed line), hermaphroditism is favoured.

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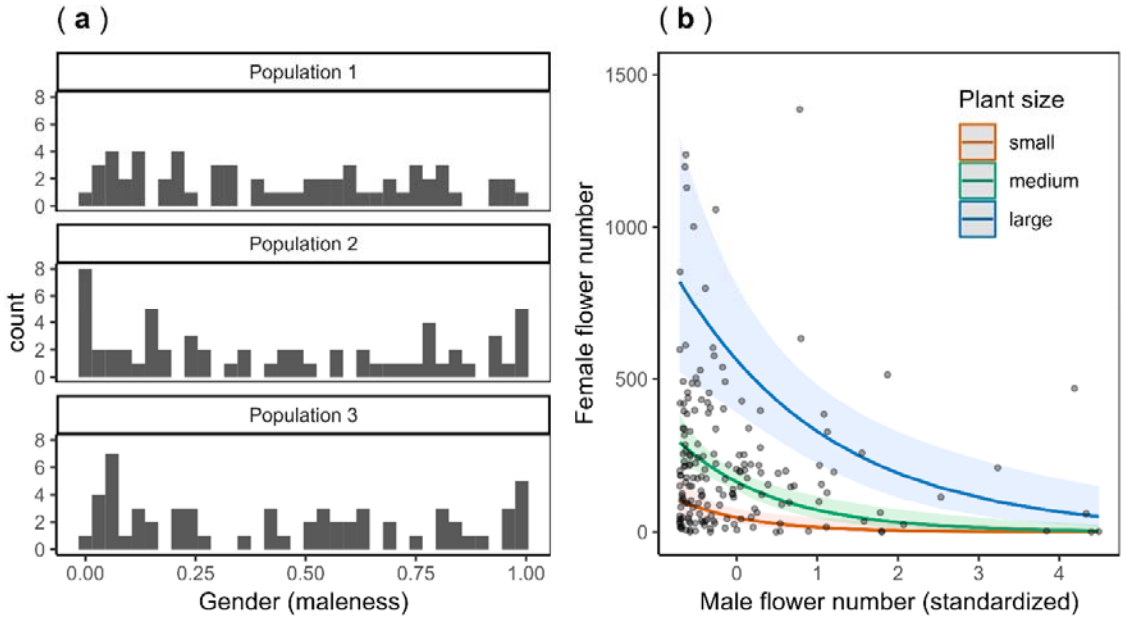


Figure 2. Plots showing the distribution of gender phenotypes in the studied populations (**a**) and the trade-off between female and male flower numbers of the individuals (**b**). In (**a**), a value of zero in gender indicates an individual with only female flowers, whereas a value of one indicates an individual with only male flowers. In (**b**), the trade-off lines for plants of different sizes were estimated by the generalized linear mixed model with the shaded ribbons indicating the 95% confidence interval of the corresponding regression lines. Hereafter to present the interactive effect with plant size, the regression lines for three levels of size (small, medium, and large) were shown, reflecting plants of mean size minus SD, mean size, and mean size plus SD, respectively. Note that one raw data point with an extreme number of female flowers (2108 female flowers) was not shown to avoid compression of the y-axis.

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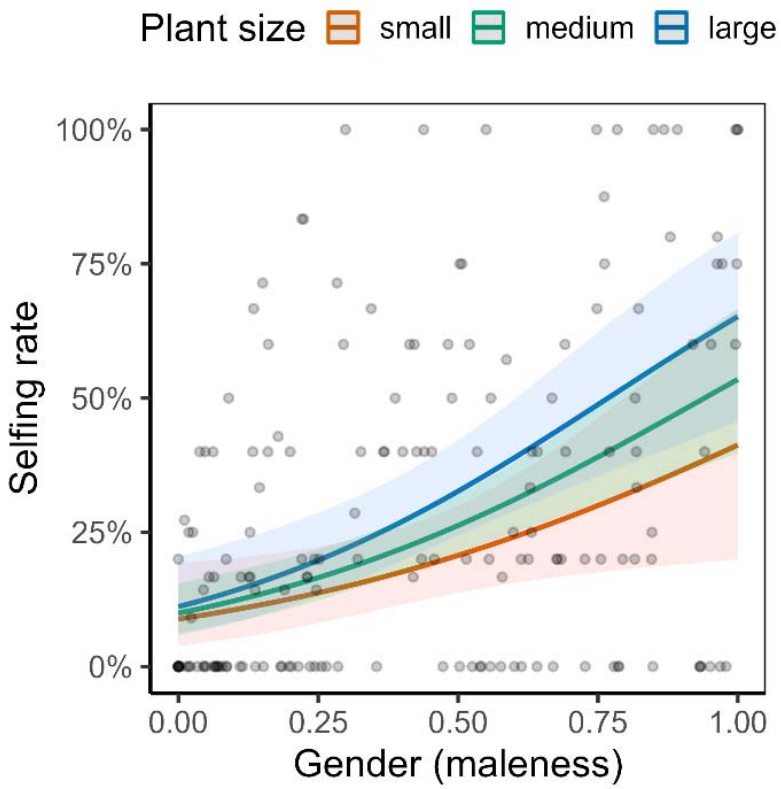


Figure 3. Plot showing the effect of gender (maleness) on the selfing rate of studied individuals (N = 172, individuals producing no mature seed were not included). Note that although the interaction with plant size was not significant, the slope trended to be steeper in larger plants. The shaded ribbon indicates the 95% confidence interval of the regression lines.

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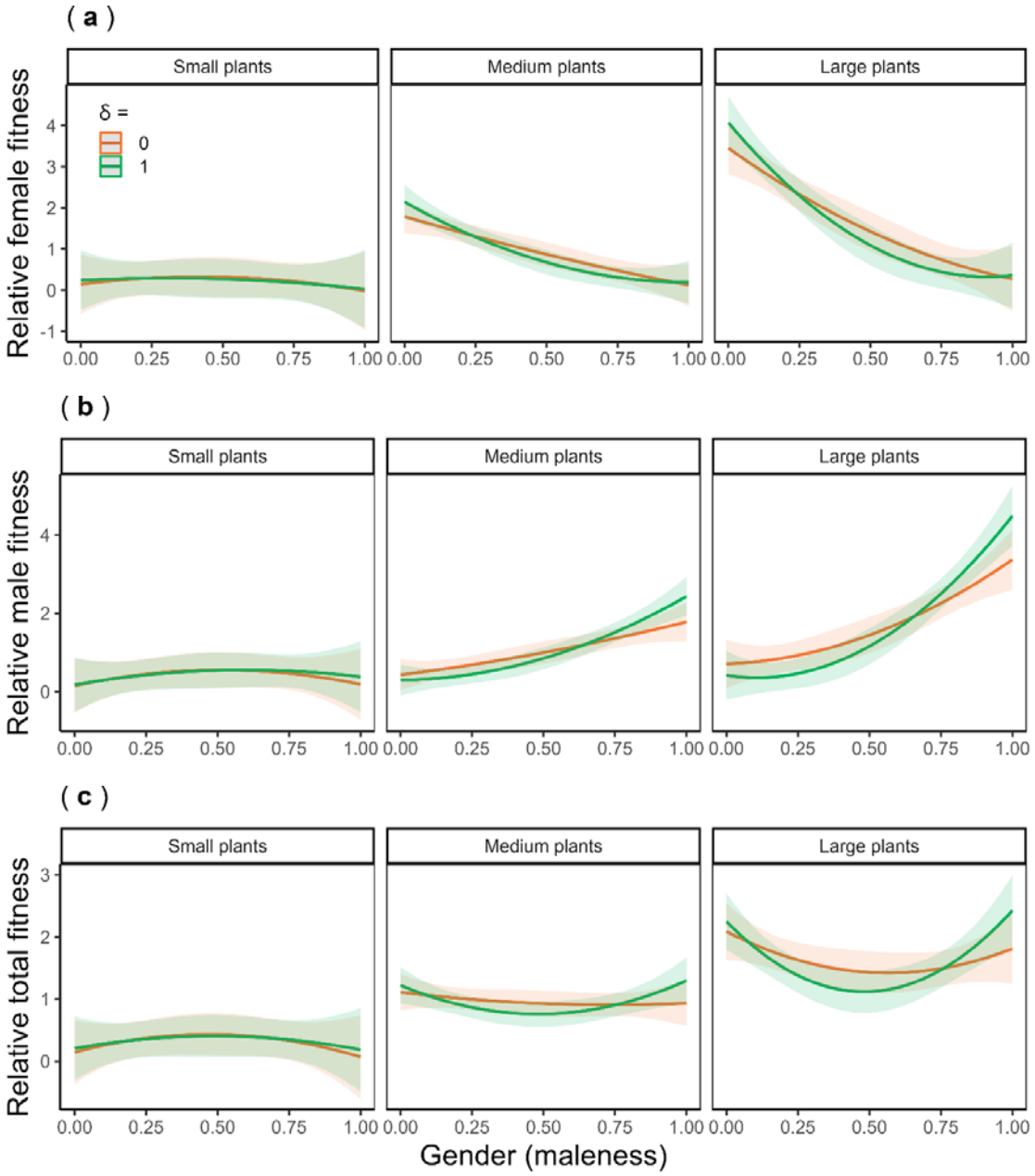


Figure 4. Plots showing the interactive effects of plant size, degree of inbreeding depression, and gender on relative female (**a**), male (**b**), and total (**c**) fitness of *Mercurialis annua*. Fitness was estimated under two scenarios of inbreeding depression (δ) of zero and one, depicted by orange and green lines, respectively. The shaded ribbons indicate the 95% confidence interval of the corresponding regression lines. The interactions with population were not significant thus the interactive effects with population were not shown (see Table **1** and Table **S4** for the *P* values).

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Supporting Information

Method S1. Detailed structure of each regression model used in this study.

Model aim	Response variable (model type, distribution)	Explanatory variables	Random variable	Sample size	Notes
1. Trade-off	Female flower number (*glmmTMB*, negative-binomial)	Male flower number x Size x Population	Identity (to take account for over-dispersion)	180 individuals	Zero-inflated model[1]
1. Selfing rate	Selfing rate (*glmer*, binomial)	Gender x Size x Population	Identity (to take into account for non-independency)	914 seeds from 173 individuals	
2. Fitness	Relative female fitness (*lmer*, Gaussian)	Gender x Size x Inbreeding depression x Population + Gender^2 x Size x Inbreeding depression x Population	Identity (to take into account for non-independency)	360 fitness estimates from 180 individuals	Variances weighted by plant size
	Relative male fitness (*lmer*, Gaussian)	Same as above	Same as above	Same as above	Same as above
	Relative total fitness (*lmer*, Gaussian)	Same as above	Same as above	Same as above	Same as above

Note: [1.] assuming that the probability of excess zeros in the response variable is the same across all levels of the predictor variables, regardless of their values (i.e., $zi \sim -1$).

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Table S1. Rotated factor matrix for PCA (principal component analysis) on eight ancillary traits and its correlation with gender (male sex allocation) in the individuals of *M. annua* used in this study. The eight ancillary traits were the number of later branches, total length of lateral branches, mean length of lateral branches, number of leaves, total leaf area, mean leaf area, leaf dry biomass, leaf specific area sampled from the top 15-centimeter part of each individual (N = 175; 5 individuals with incomplete measurements of traits were not included). PCA axes showed no or weak (i.e., PCA2) correlation with sex allocation, indicating no apparent sexual dimorphism.

	PCA1	PCA2	PCA3	PCA4	PCA5
Number of lateral branches	-0.10	-0.58	0.16	-0.64	0.05
Total length of lateral branches	-0.38	-0.40	-0.32	-0.11	0.33
Mean length of lateral branches	-0.36	-0.05	-0.52	0.40	0.33
Number of leaves	-0.38	-0.20	0.51	0.30	-0.01
Total leaf area	-0.50	0.21	0.20	-0.04	-0.17
Mean leaf area	-0.23	0.45	-0.39	-0.52	-0.20
Leaf dry biomass	-0.51	0.10	0.12	0.02	-0.37
Leaf specific area	-0.10	0.44	0.37	-0.22	0.76
Cumulative variance explained (%)	0.41	0.63	0.79	0.89	0.98
Correlation coefficients with phenotypic gender (p-value)	0.07 (n.s.)	-0.21(p < 0.001)	0.001 (n.s.)	0.02 (n.s.)	0.05 (n.s.)

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812 **Table S2.** Details of sex allocation, biomass, and paternity analyses of the three experimental
813 populations. Population showed no difference in all the variables tested ($P > 0.05$; see the
814 note below).

	Population1	Population 2	Population 3
Sample size	60 (one individual dead)	60 (biomass for one individual missing and thus excluded)	60 (one individual dead)
Mean female flower number (SD)[1]	239.7 (199.6)	247.3 (362.3)	227.1 (266.8)
Mean male flower number (SD)[1]	339.5 (443)	275.7 (416.7)	333.5 (488)
Mean above-ground biomass (SD) (g) [2]	5.08 (2.36)	6.36 (5.78)	5.37 (4.35)
Mean mature seed number (SD)[2]	98.3 (91.7)	108.4 (200.7)	111.3 (169.4)
Total successfully genotyped seed number	319	314	315
Paternity assignment rate	94%	98%	97%
Average geitonogamous selfing rate[3]	30.2% (N = 60)	25.5% (N = 58) [4]	33.4% (N = 54) [4]

815 Note: [1.] generalized model with negative binomial distribution; [2.] linear model with Gaussian
816 distribution; [3.] generalized mixed model with binomial distribution and plant identity as a
817 random factor; [4.] individuals producing no seed were excluded.

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818 **Table S3.** Summary table of the general effects of male flower number, size, and population
819 on female flower number estimated by a generalized linear mixed model. The *P* values were
820 extracted using likelihood ratio tests.
821 Notes: n.s. $P > 0.05$, $* P < 0.05$, $** P < 0.01$, $*** P < 0.001$

	AIC	LRT	*P* value
Male flower	2228.4	47.71	***
Size	2326.4	145.69	***
Population	2180.0	1.29	n.s.
Male flower x Size	2176.0	16.94	***
Male flower x Population	2165.5	8.45	*
Size x Population	2157.1	0.05	n.s.
Male flower x Size x Population	2161.1	1.73	n.s.

822

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Table S4. Summary table of the general effects of size, gender, and population on the selfing rate estimated by a generalized linear mixed model. The *P* values were extracted using likelihood ratio tests.

Notes: n.s. (non-significant) $P > 0.1$, m.n.s. (marginlly non-significant) $P < 0.1$, $*$ $P < 0.05$, $**$ $P < 0.01$, $***$ $P < 0.001$

	AIC	LRT	*P* value
Gender	565.8	30.5	***
Size	538.6	3.27	m.n.s. ($P = 0.07$)
Population	534.8	1.52	n.s.
Gender x Size	541.4	0.05	n.s.
Gender x Population	542.9	3.6	n.s.
Size x Population	539.7	0.37	n.s.
Gender x Size x Population	543.3	0.47	n.s.

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829 **Table S5.** Summary table of the general effects of size, gender (linear and quadratic terms),
830 scenarios of inbreeding depression (δ), and population on relative female, male, and total
831 fitness estimated by linear mixed models.
832 Notes: n.s. $P > 0.05$, $* P < 0.05$, $** P < 0.01$, $*** P < 0.001$

	Relative female fitness		Relative male fitness		Relative total fitness	
	Sum sq.	*P* value	Sum sq.	*P* value	Sum sq.	*P* value
Size x Gender x δ x Population	0.01	n.s.	0	n.s.	0	n.s.
Size x Gender^2 x δ x Population	0.01	n.s.	0.01	n.s.	0.01	n.s.
Size x Gender x δ	0.38	***	0.07	n.s.	0.19	**
Size x Gender x Population	0.06	n.s.	0.03	n.s.	0.06	n.s.
Size x δ x Population	0.01	n.s.	0.01	n.s.	0	n.s.
Gender x δ x Population	0.03	n.s.	0.01	n.s.	0.02	n.s.
Size x Gender^2 x δ	0.27	**	0.26	**	0.27	***
Size x Gender^2 x Population	0.04	n.s.	0.05	n.s.	0.06	n.s.
Gender^2 x δ x Population	0.04	n.s.	0.02	n.s.	0.03	n.s.
Size x Gender	0.09	n.s.	0	n.s.	0.05	n.s.
Size x δ	0	n.s.	0.15	*	0.03	n.s.
Gender x δ	0.43	***	0.06	n.s.	0.2	**
Size x Population	0.13	n.s.	0.04	n.s.	0.11	n.s.
Gender x Population	0	n.s.	0	n.s.	0	n.s.
δ x Population	0.01	n.s.	0.01	n.s.	0.01	n.s.
Size x Gender^2	0.01	n.s.	0.09	n.s.	0.05	n.s.
δ x Gender^2	0.3	***	0.32	**	0.31	***
Population x Gender^2	0	n.s.	0	n.s.	0	n.s.
Size	1.58	***	1.23	***	2.05	***

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Gender	0.24	**	0	n.s.	0.09	n.s.
δ	0	n.s.	0	n.s.	0	n.s.
Population	0.01	n.s.	0.01	n.s.	0.01	n.s.
Gender^2	0.04	n.s.	0.08	n.s.	0.08	n.s.

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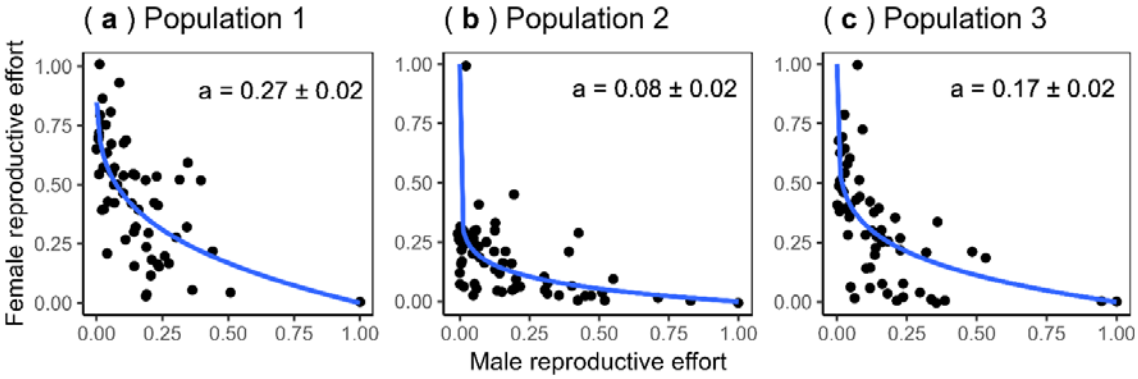


Figure S1. Plots showing the non-linear trade-off curves between female and male functions in studied populations. The non-linear curves were evaluated using non-linear least square regression (*nls* function in *nlme* package; Pinheiro et al., 2022) with the formula below. $RE_f = 1 - RE_m{}^a$, where RE_f and RE_m is the female and male reproductive efforts, respectively, defined as the number of flowers of that sex divided by the aboveground biomass of the plant and then relative to the plant with the highest effort in the population. The exponent *a* depicts the nonlinearity of the curves with $a < 1$, $= 1$, and > 1, indicating inward, linear, and outward trade-offs, respectively. The trade-offs in the three populations were all inward-shaped with the exponent *a* significantly smaller than one.

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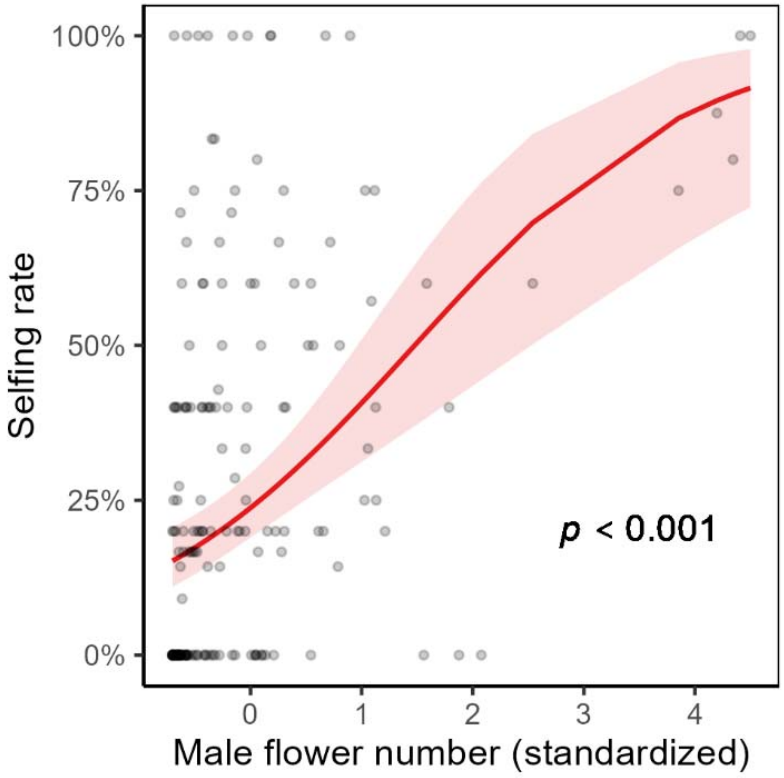


Figure S2. Plot showing the effect of the number of male flowers on the selfing rate in studied individuals (N = 172, individuals producing no mature seed were excluded). We used a generalized linear mixed model with a similar structure to the one presented in the main text (see Method S1) except that we replaced the explanatory variables of gender and size with male flower number here. The shaded ribbon indicates the 95% confidence interval of the regression lines.

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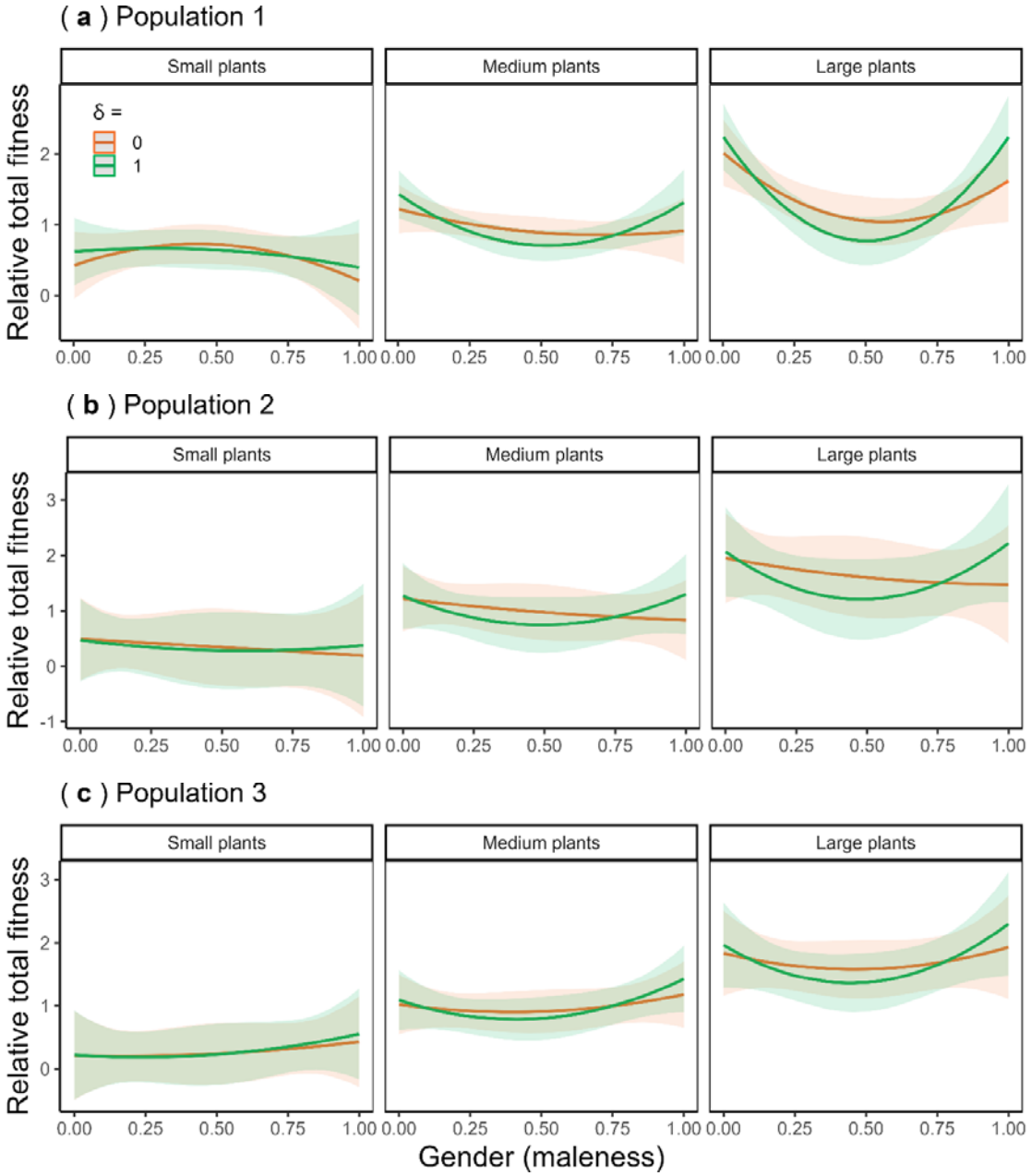


Figure S3. Plots showing the interactive effects of plant size, degree of inbreeding depression, and gender on relative total fitness of *Mercurialis annua* in three experimental populations. Fitness was estimated under two scenarios of inbreeding depression (δ) of zero and one, depicted by orange and green lines, respectively. The shaded ribbons indicate the 95% confidence interval of the corresponding regression lines.

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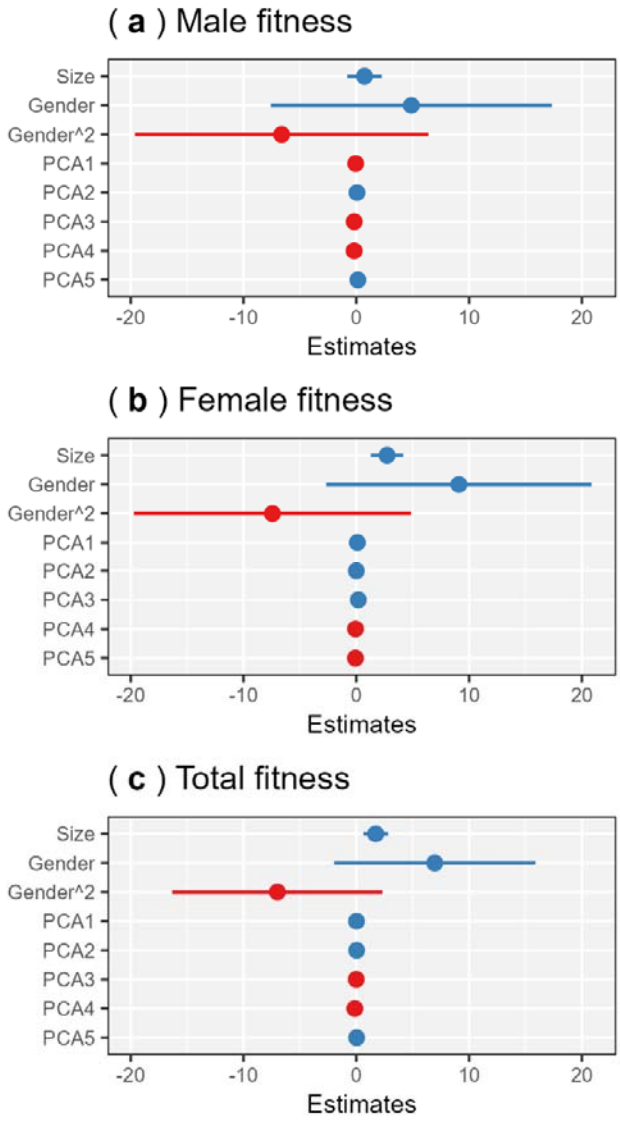


Figure S4. Plots showing the effect sizes of eight ancillary traits (compressed into 5 PCA axes; see Table **S1**), size, and gender (linear and quadratic terms) on relative male (**a**), female (**b**), and total (**c**) fitness estimated by linear mixed models (N = 175; 5 individuals with incomplete measurements of traits were not included). The PCA axes were added as single terms into the linear mixed models used for the analysis presented in the main text (see Method **S1** for their structures). For simplicity, only the effect sizes of a selected set of single terms were presented in the figures. The ancillary traits were thought to influence individual fitness via amelioration of pollen dispersal and/or pollen receipt. Nonetheless, according to the analyses, the ancillary traits likely played a minor role in determining individual fitness compared to sex allocation and size.