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RESEARCH ARTICLE

Botany

Harmful self-pollination drives gynodioecy in European chestnut, a self-incompatible tree

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Abstract

Premise: Gynodioecy is a rare sexual system in which two genders (*sensu* Lloyd, 1980), cosexuals and females, coexist. To survive, female plants must compensate for their lack of siring capacity and male attractiveness. In European chestnut (*Castanea sativa*), an outcrossing tree, self-pollination reduces fruit set in cosexual individuals because of late-acting self-incompatibility and early inbreeding depression. Could this negative sexual interaction explain the presence of females in this species?

Methods: We studied gender variation in wild populations of European chestnut. In addition, we compared fruit set (the proportion of flowers giving fruits) and other key female fitness components as well as reproductive allocation between genders. We then performed emasculation experiments in cosexual trees, by removing nectar-producing fertile male inflorescences. We also removed sterile but nectar-producing male inflorescences from female trees, as a control.

Results: We found a highly variable proportion of male-sterile individuals in the wild in European chestnut. In the experimental plot, trees from each gender had similar size, flower density, and burr set, but different fruit set. Removing nectar-producing male inflorescences from branches or entire trees increased fruit set in cosexual but not in female trees.

Conclusions: These results show that self-pollination impairs fruit set in cosexual trees. Female trees avoid these problems as they do not produce pollen but continue to attract pollinators thanks to their rewarding male-sterile inflorescences, resulting in a much higher fruit set than in cosexuals. This demonstrates that even outcrossed plants can benefit from the cessation of self-pollination, to the point that unisexuality can evolve.

KEYWORDS

Castanea, emasculation experiment, Fagaceae, female advantage, late-acting self-incompatibility, ovule discounting, selfing avoidance, sexual interference

Most angiosperm species have retained the ancestral hermaphrodite floral organization (Yampolsky and Yampolsky, 1922). From this sexual system, well adapted for animal pollination (Sauquet et al., 2017), flowering plants have evolved in several directions, resulting in an amazing variety of reproductive systems at the flower, inflorescence, or whole plant level. For instance, gynodioecy is a rare but taxonomically widespread dimorphic sexual system featuring two "genders" (*sensu* Lloyd, 1980): cosexual and unisexual (female). The evolutionary mechanisms leading to such polymorphisms have fascinated researchers since Darwin (1877), not least because their study could help explain why the vast majority of flowering plants are in fact cosexual (Bawa, 1984). Studies on gynodioecious species provide unique opportunities to explore the consequences of the coexistence of both sexual functions within individuals. Indeed, comparing genders in gynodioecious plant species could reveal negative and positive interactions between sexual functions taking place in cosexual individuals (Webb, 1999). For instance,

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self-pollination can have either positive or negative consequences on plant fitness depending on the degree of self-compatibility (Wells, 1979). Similarly, either sexual conflicts or sexual facilitation can take place in hermaphrodite flowers or bisexual inflorescences (Tonnabel, 2021; Pauly et al., 2023). In particular, pollinator attraction is often attributable to male organs, resulting in intersexual mating facilitation (Darwin, 1877; Lloyd, 1975; Wise et al., 2011; van Etten and Chang, 2014; Pauly et al., 2023).

For females to evolve in these systems, they must have reproductive advantages over cosexuals that compensate for their own inability to sire offspring, as well as for any benefits conferred to the female function by the presence of male organs in cosexual plants. In species with nuclear gender inheritance, female persistence implies at least a twofold reproductive advantage over cosexuals (Lewis, 1941). Hence, for females to persist, the female advantage resulting from the release from negative sexual interactions taking place in cosexuals must be very large. This advantage is frequency dependent, decreasing when the proportion of females increases. It can take the form of increased lifetime seed production or superior offspring quality compared to cosexuals. For instance, females can produce more flowers, set more fruits, or produce more seeds that are larger and germinate better than those of cosexuals. Female advantage may be concentrated in a single female fitness component or it may be distributed over multiple fitness components across the plant's life cycle.

According to Givnish (1982), extending work from Charlesworth and Charlesworth (1978), mechanisms of female advantage can be divided in two categories, ecological and genetic. The most frequently cited ecological mechanism of female advantage is reallocation of resources to the female function from an abandoned male function (Darwin, 1877). Similarly, the most frequently cited genetic mechanism of female advantage is the outbreeding advantage due to the absence of selfing and associated inbreeding depression (i.e., selfing avoidance; Darwin, 1876; Mather, 1940; Baker, 1959; Lloyd, 1975). Outbreeding advantage has been given special attention because it is achieved immediately upon the emergence of a male sterility mutation, it is governed by straightforward principles, and it has high predictive power (Mather, 1940). In particular, it predicts that if cosexuals are partially self-fertilizing and produce lower-quality offspring because of inbreeding depression, females will be selected for in preference to males. This prediction is supported by the much greater prevalence of gynodioecy over androdioecy in flowering plants (Lloyd, 1975; Charlesworth and Charlesworth, 1978) and the relative scarcity of gynodioecious plants in which cosexuals are strictly outcrossed (Baker, 1959; Olson et al., 2016). However, a few predominantly outcrossed flowering plants are in fact gynodioecious (Dufaÿ and Billard, 2012), showing that enforced outcrossing is not the only source of female advantage.

Self-pollination can reduce female fitness in multiple ways, not merely by creating selfed offspring suffering from inbreeding depression (Table 1). In particular, in plant species with late-acting (ovarian) self-incompatibility (Seavey and Bawa, 1986), ovules are disabled by self-pollen tubes and thus excluded from cross-fertilization (Charlesworth, 1985; Sage et al., 1994; Gibbs, 2014; Johnson et al., 2019). This special case of male interference with the female function has been called ovule discounting or ovule usurpation (Barrett et al., 1996; Barrett, 2002; Duffy and Johnson, 2014). Self-pollen interference with ovules requires genetic mechanisms of self-recognition and ultimately limits inbreeding. If strong enough, it could drive the evolution of separate sexes.

A simple but powerful method to identify mechanisms providing instantaneous female advantage is emasculation of cosexual individuals (i.e., removing their anthers or stamens to simulate the emergence of a male-sterile mutant devoid of the pleiotropic effects of male-sterility genes and of the modifier genes selected after the establishment of sexual dimorphism; Sun and Ganders, 1986). However, a possible issue with emasculation experiments is that they also eliminate potential rewards (pollen or nectar) and visual cues for pollinators (stamens or anthers; Duffy and Johnson, 2011). Hence, special care is needed when choosing model species and controls (Charlesworth, 1993). Surprisingly, despite their great potential, few studies have used emasculation to investigate female fitness in gynodioecious plants (e.g., Kikuzawa, 1989; Pettersson, 1992; Alonso and Herrera, 2001; Wang et al., 2021), and none have attempted to control for the treatment's possible effects on pollinator attractiveness.

Trees are typically outcrossed (Petit and Hampe, 2006). Investigating female advantage in the few known gynodioecious trees (Gibson and Wheelwright, 1996; Dufaÿ and Billard, 2012; Caruso et al., 2016) could help explain how unisexuality evolves in obligatory outcrossing plants (Lloyd, 1975; Gibson and Wheelwright, 1996; Dufaÿ and Billard, 2012). For this study, we selected the European chestnut (Fagaceae: *Castanea sativa*) and its hybrids. These long-lived trees are monoecious and dichogamous, two sexual and flowering systems that limit self-pollination and promote outcrossing (Lloyd and Webb, 1986; Webb and Lloyd, 1986; Bertin and Newman, 1993; Routley et al., 2004; Koelling and Karoly, 2007). Moreover, chestnuts have a late-acting self-incompatibility system with major negative consequences on fruit set (Xiong et al., 2019; Larue et al., 2022). Studying female advantage in this outcrossed tree genus could help identify other sexual interactions taking place in cosexual individuals.

In this study, we first attempt to establish gynodioecy in the wild in the European chestnut. Spontaneous male-sterile variants have been reported in this species and its hybrids (Kaul, 1988; Soylu, 1992), but studies on gender variation in natural chestnut populations are lacking. Second, we compare several components or proxies of female fitness between genders across the trees' life cycle in an even-aged plantation and we study reproductive allocation in both genders. Third, to assess experimentally the importance of

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TABLE 1 Reproductive costs of self-pollination.

Stage	Mechanism	Details	Fitness consequence	Key reference	Evidence for female advantage	Reference
Self-pollination	Pollen clogging	Self-pollen restricts access of compatible pollen to stigma surface	Seed set	Barrett (2002)	Lacking	—
Self-pollen tubes	Pollen tube competition	Reduced efficacy of selection in the style	Offspring fitness[a]	Pélabon et al. (2016)	Weak	Shykoff (1992)
Fertilization	Late-acting self-incompatibility	Self-pollen tubes do not penetrate ovules or fail to achieve double-fertilization	Seed set	Gibbs (2014)	Lacking	–
Seed development	Early inbreeding depression	Embryo abortion	Seed set	Husband and schemske, (1996)	Strong	Collin et al. (2009)
Sapling	Inbreeding depression	Reduced germination, juvenile survival, and growth/reproduction	Offspring fitness[a]	Husband and schemske, (1996)	Strong	Dufaÿ and Billard (2012)

[a]Seed weight, germination rate, juvenile survival, growth/reproduction (flower production).

the reproductive cost of self-pollination, we remove male inflorescences from both cosexual and female chestnut trees. Both genders harbor rewarding, nectar-producing male inflorescences that attract pollinators; the only visible difference is that the stamens are aborted or greatly reduced in female trees (Larue et al., 2021a, 2022). This unique situation featuring female flowers associated with rewarding male inflorescences that are either fertile or sterile should help to disentangle the positive and negative effects of male function on female fitness. Finally, because in chestnuts male inflorescences are borne either on unisexual or on bisexual catkins, we perform partial emasculation experiments on cosexual trees, removing male inflorescences from unisexual catkins, from bisexual ones, or from both. Such studies should help evaluate whether self-pollen interference can drive the evolution of separate sexes, a largely neglected hypothesis.

MATERIALS AND METHODS

Chestnut reproductive biology

Chestnuts (*Castanea* spp.) are insect-pollinated trees characterized by massive blooming, huge pollen production, and the largest pollen:ovule ratio (~10–30 million) of any known plant (Larue et al., 2021a; Petit and Larue, 2022). Additionally, they have multiple mechanisms limiting self-pollination. First, they are monoecious, with separate male and female flowers distributed in two types of inflorescences: unisexual male catkins and bisexual catkins featuring one or two female inflorescences associated with a single nectar-producing male inflorescence (Figure 1; Larue et al., 2021a). Second, they have a late-acting self-incompatibility system (Xiong et al., 2019) but with rare occurrence of selfing (Larue et al., 2022), indicating leaky self-incompatibility. Third, they have a complex phenology known as duodichogamy, characterized by two successive pollen emission phases. Unisexual male catkins (Figure 1) bloom first, producing nectar and releasing huge amounts of pollen, ~97% of the total (Larue et al., 2021a). Non-rewarding female flowers then become receptive. Finally, male inflorescences from bisexual catkins start producing nectar and emitting pollen, generating a second, much smaller pollen emission phase involved in pollinator attraction and in pollen receipt on nearby female flowers (Pauly et al., 2023). This reduces but does not eliminate the risk of self-pollination (Hasegawa et al., 2017).

Indeed, cosexual chestnut trees experience high rates of self-pollination due to huge pollen production and frequent geitonogamy: 90% according to Hasegawa et al. (2009) and 74% according to Larue et al. (2022). Cytological studies and pollination experiments have shown that self-pollen tubes grow well in the styles, albeit less rapidly than cross-pollen tubes (Xiong et al., 2019). However, following self-pollination, fruit set (defined as the proportion of flowers from developed female inflorescences giving mature fruits) is very low for two

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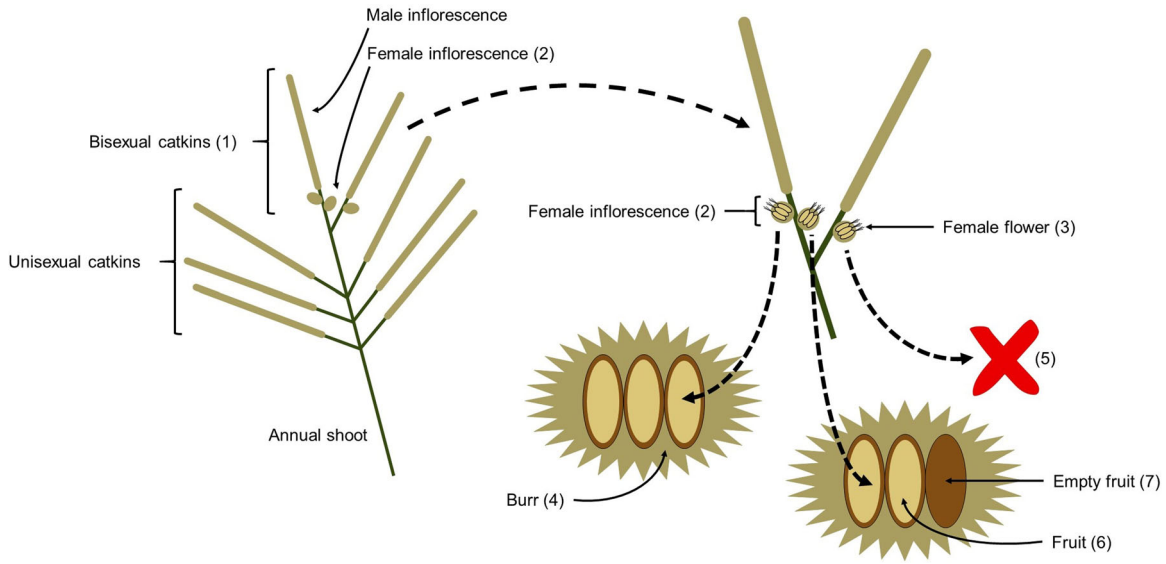

FIGURE 1 The fate of female flowers. (1) Each bisexual catkin can have one or two female inflorescences. (2) Each female inflorescence consists of three female flowers located side by side. (3) Each female flower becomes a nut (a dry fruit made typically of a single seed). (4) The female inflorescence becomes an infructescence: the floral bracts form the burr, which contains three fruits. (5) Some burrs may abort. (6) If a female flower is pollinated, the corresponding fruit contains at least one seed. (7) If the female flower is not pollinated, the fruit formed is empty. Burr set is defined as the proportion of female inflorescences that grow into mature burrs, and fruit set is defined as the proportion of flowers from mature inflorescences that have filled fruits.

main reasons. First, the rate of double fertilization after self-pollination is sevenfold lower than that following cross-pollination, resulting in massive ovule abortion (Xiong et al., 2019). Second, if self-fertilization nevertheless occurs, most of the resulting embryos abort at various stages before a mature seed is formed due to early inbreeding depression (Xiong et al., 2019). Therefore, self-fertilized seeds are very rare (<1% in Hasegawa et al., 2009; 4% in Larue et al., 2022; 5% in chestnut orchards in Larue and Petit, 2023). A process-based model (Larue et al., 2022) has confirmed that self-pollen is less competitive than cross-pollen in European chestnut and its hybrids. While the estimate for the average proportion of self-pollen reaching stigmas of male-fertile trees is 74%, a fivefold difference in competitive ability between self-pollen and cross-pollen results in a large decrease in the proportion of self-pollen reaching ovules, down to 48%. Most (95%) of these self-fertilized ovules abort before fruit formation, resulting in the loss of 46% of the fruit crop. These results suggest that the main cause of reduced reproductive potential in cosexual chestnut trees is sexual interference by self-pollen.

Two genders can be distinguished in European chestnuts and their hybrids: cosexuals, which are fully male-fertile trees, and at least partly male-sterile trees, henceforth called females (Larue et al., 2022; Figure 2). Only one gender (cosexual) is reported in Japanese and Chinese chestnuts planted in France. The female trees of European chestnuts or hybrids have dysfunctional staminate flowers with fully aborted stamens or with stamens borne on short filaments producing only small quantities of mostly nonfunctional pollen (Bounous et al., 1992). We have evaluated the male fertility of these different categories of trees using paternity



FIGURE 2 Chestnuts have two types of catkins: numerous unisexual male catkins that flower first and a few bisexual catkins located at the tip of the branches that flower later.

analyses, confirming the relevance of this classification (Larue et al., 2022).

All flowers on a tree and all grafted copies from a given clone have the same type of male flowers (Larue et al., 2021b, 2022). Male-sterile inflorescences continue to produce nectar and attract insects such as flies and beetles but not pollen-seeking insects such as hoverflies and bees (Larue et al., 2021a; Pauly et al., 2023). In crosses between European chestnut (*C. sativa*) and Japanese chestnut (*C. crenata*), only crosses with European chestnut as the mother generate female individuals (Bolvanský and Mendel, 1999; Sisco et al., 2014; Larue, 2021). However, segregation studies within European chestnut point to strict

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nuclear inheritance of male sterility involving a major recessive gene modified by one or more other genes (Soylu, 1992; Bolvanský and Mendel, 1999).

Each female inflorescence typically consists of three female flowers located side by side (Figure 1). It develops into a spiny infructescence called a burr (Figure 1). Each of the three female flowers forms a fruit. This fruit is either filled, if the female flower is pollinated, containing typically a single seed (multi-seeded fruits are very rare; Furones-Pérez and Fernández-López, 2009), or empty, with a pericarp but no seed inside (Figure 1). In rare cases, female inflorescences include more or fewer than three flowers (Breisch, 1995), resulting in a burr with more or fewer than three fruits.

Gender polymorphism in chestnut forests

We visited 14 naturally regenerated populations of European chestnut, one in Spain (described in Larue, 2021) and the rest in France. We selected stands that had the highest chances of being natural, avoiding ancient plantations as well as coppices, and focusing on regions identified as potential chestnut glacial refugia using paleoecological data (Krebs et al., 2019). In each stand, we estimated the proportion of three different types of trees: male-sterile, partly male-sterile, and male-fertile trees (Figure 3). In the subsequent analyses, the few partly male-sterile trees were pooled with male-sterile trees to form the category "female" used for gender comparison, as they tend to have low levels of male fertility (Larue et al., 2022).

Estimation of female advantage

Study site

We used the INRAE chestnut germplasm collection located in Villenave d'Ornon (44.788319N, −0.577062E) for these investigations (Larue et al., 2021b). The selected orchard is a 3.5 ha experimental plot planted in 1990 with 441 trees. In 2018, there were only 211 trees left, corresponding to 83 unique genotypes. We have previously assigned these genotypes to the following taxa: European chestnut (*C. sativa*; 50% of the trees), Japanese chestnut (*C. crenata*; 9%), Chinese chestnut (*C. mollissima*; 8%), and their interspecific hybrids, mostly Euro-Japanese hybrids (*C. sativa* × *C. crenata*; 27%) (Larue et al., 2021b). Most of the trees are grafted on one of three hybrid rootstocks: 'Marsol' (CA07; 56% of the trees), 'Maraval' (CA74; 27%), and 'Marlhac' (CA 118; 3%). The remaining trees (14%) grow on their own roots. Among the unique genotypes, 55 are male-fertile and 28 are partly or completely male-sterile, corresponding to 137 (65%) cosexual and 74 (35%) female trees distributed rather evenly throughout the plot (Appendix S1). This even-aged collection includes forest trees and clones recently selected for fruit production. Given the origin of this collection, the use of both female and cosexual clones in chestnut cultivation, and the recent introduction of selective breeding, systematic gender-biased effects of artificial selection on phenotypic traits were deemed unlikely. For our study, we used either the entire collection or a subset of 16–18 trees for the more labor-intensive measurements. The 16 trees correspond to eight clones, each with two clonal grafted copies: two cosexual and two female European chestnut clones and two cosexual and two female Euro-Japanese hybrid clones. For the study of three female fitness components—flower density, burr set (i.e., the proportion of female inflorescences that develop into mature burrs), and fruit set—we added two more trees, one from each gender (Table 2).

Investment in male function

The only apparent difference between male inflorescences of cosexual and female trees is the development of the stamens. To estimate the resources potentially saved by female trees lacking stamens or having underdeveloped stamens, we counted the numbers of male inflorescences on all 16 trees, measured their lengths, and weighed them. On each tree, we counted and measured the number and average length and weight of male inflorescences on five annual flowering shoots. In addition, to control for variation in shoot size, we measured the diameter of each corresponding annual shoot to calculate the average number of male inflorescences or the average weight of male inflorescences per square millimeter of branch cross section.

Female fitness components

We compared a total of eight female fitness components between genders, either across the entire chestnut collection or by focusing on a subset of trees.

We first studied, in 2019, three components of female fitness in all 211 existing trees: basal area, fruit set, and fruit weight. For basal area, we measured the diameter of the stem (or, for multi-stemmed individuals, of each stem) at breast height and derived the cross-sectional area. For fruit set, we measured the proportion of developed fruits per burr. For fruit weight, we collected 50 developed fruits per tree and weighed them.

We then studied, in 2020, four additional female fitness components on a subset of 16 trees. We first calculated burr set. On 10 marked branches per tree, we counted the number of female inflorescences during full bloom in June and the number of burrs formed at the end of the summer, in August. We estimated burr set by dividing the number of burrs by the number of female inflorescences. We then selected 10 additional branches on each of the same trees and counted the number of female inflorescences. We also measured the diameter of

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FIGURE 3 Degrees of male-sterility observed. Male-sterile tree: Most stamens are aborted and do not produce pollen (A). Mostly male-sterile tree: Stamens do not protrude from glomerules (B) or slightly protrude from glomerules (C). Fully male-fertile tree: Stamen filaments are long and produce large amounts of pollen (D).

each branch to calculate the cross-sectional area and standardize the number of female inflorescences per square millimeter of branch cross section, which we call "female inflorescences production." To study whether female trees invest more in fruit production than cosexual trees, we estimated fruit yield as follows:

$$\text{Fruit yield} = \text{female inflorescences production} \times \text{fruit set} \times 3 \times \text{mean fruit weight}$$

We estimated the number of female inflorescences, fruit set, and branch cross-sectional area on a subset of 18 trees and measured average fruit weight for each of the 211 trees.

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TABLE 2 Gender effect on female fitness components.

Fitness component	Number ♂[a]	Number ♀[a]	Mean ♂[b]	Mean ♀[b]	Test[c]
Basal area (cm^2)	137	74	308 (257)	290 (194)	ns (PERMANOVA)
Fruit set	115	61	0.57 (0.21)	0.84 (0.11)	$<10^{-15}$ (PERMANOVA)
Fruit weight (g)	115	61	10.5 (5.2)	7.7 (4.3)	0.0013 (PERMANOVA)
Burr set	9	9	0.77 (0.15)	0.87 (0.14)	ns (Student *t*-test)
Flower density	9	9	51 (34)	67 (80)	ns (Student *t*-test)
Female inflorescences production ($/mm^2$)	8	8	0.029 (0.010)	0.037 (0.023)	ns (PERMANOVA)
Fruit yield (g/mm^2)	8	8	0.40 (0.23)	0.76 (0.48)	0.02 (PERMANOVA)

[a]Number of cosexual (☿) and female (♀) trees.

[b]Mean value for cosexual and female trees (standard deviation).

[c]Significance of difference between cosexual and female means (test performed).

The female allocation variable obtained is in grams of fruit produced per square millimeter of branch.

To investigate interannual fluctuations in fruit set between genders, we measured fruit set in 2018, 2019, 2020, 2022, and 2023 for the 18 trees.

Mortality

The trees from the INRAE chestnut germplasm collection are no longer watered and there was a high rate of mortality in the orchard after 2018. We took advantage of this situation to investigate if female and cosexual trees differ in survival rates. We compared the survival of cosexual and female trees by counting the number of trees of each gender that died between 2018 and 2023. For these analyses, we focused on European chestnuts and on Euro-Japanese hybrids, the only taxa in which both genders are present. Rootstocks are known to control growth as well as resistance to pathogens and drought in chestnut (Solar et al., 2010; Camisón et al., 2021, 2023). To control for any possible rootstock effect, we considered it in the analyses.

Emasculation experiments

Emasculation creates trees or branches that do not produce pollen, thus helping explore the possible negative effect of self-pollen on fruit set.

Emasculation procedure

Male flowers of chestnuts are packed together into inflorescences that can easily be removed as one unit (Figure 1). In the emasculation treatments, we removed with scissors, together or separately, unisexual male catkins and the male inflorescence from each bisexual catkin (Figure 2). As these two types of male inflorescences flower at different times, we relied for total emasculation on a three-step procedure. In the first step, at the end of May, we removed all emerging unisexual male catkins. In the second step, we checked that no unisexual male catkin remained and removed the male inflorescences of bisexual catkins. Finally, in the third step, we removed the remaining male inflorescences of bisexual catkins. We also removed male-sterile inflorescences from female clones as a control, to evaluate potential reductions in insect attractiveness or reallocation of resources following the removal of these nectar-producing inflorescences.

In cosexual trees, removing male inflorescences implies removing the source of pollen present in the anthers, thus potentially eliminating self-pollen interference on ovules; however, it also implies removing the source of nectar, thus potentially reducing attractiveness to pollinating insects. The outcome is therefore hard to predict. In contrast, in male-sterile trees there is no release from self-pollination, only reduced insect attractiveness, so we predict that emasculation of such male-sterile trees should reduce fruit set.

First emasculation experiment

We performed a first set of emasculation experiments in 2019 in three orchards of the INVENIO experimental station in Douville (45.019723 N, 0.614637 W). When performed on plant parts rather than on whole plants, these experiments can be difficult to interpret due to the confounding effects of intra-individual resource reallocations (Knight et al., 2006; Runquist and Moeller, 2013). To avoid such biases, plants should be entirely emasculated. We therefore removed all male inflorescences from entire 8 yr-old trees (one emasculation treatment per tree). For larger trees, this was not possible, so we focused on branches within trees, using 10 branches for each emasculation treatment (emasculated or intact branches). For the emasculation experiments on small trees, we selected four hybrid clones ('Jeannette', 'Bellefer', 'Pollifer', and 'Maraval'). For each emasculation treatment (emasculated or

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intact trees), we used five trees for 'Jeannette', six for 'Bellefer', five for 'Pollifer', and two for 'Maraval'. For emasculation experiments on large trees, we selected two hybrid clones, a male-sterile one, 'Bouche de Bétizac' (five trees from a single orchard), and a male-fertile one, 'Marigoule' (eight trees distributed in two orchards).

Second emasculation experiment

We relied on partial emasculation treatments to disentangle the effects of the two types of male inflorescences on fruit set. We performed these experiments in 2023 on eight unrelated cosexual trees from the INRAE chestnut germplasm collection in Villenave d'Ornon—four European chestnut trees and four Euro-Japanese hybrids. We used four treatments: "Control" (two intact branches from each tree), "M1" (three branches in which we removed all unisexual catkins), "M2" (three branches in which we removed all male inflorescences from bisexual catkins), and "Total" (two branches in which we removed all male inflorescences).

Statistical analyses

Data analysis

We performed all analyses with R version 3.6.6 (R Core Team, 2013). We calculated the corrected fruit set using basic functions implemented in R. Violin plots were created using the R packages "ggplot2" version 3.6.3 (Wickham, 2016) and "ggthemes" version 4.2.4 (Arnold, 2019).

Estimation of female advantage

Investment in male function

We studied the investment in male function (the average weight of male inflorescences per square millimeter of branch section) and two of its components, the number of male inflorescences per square millimeter of branch section and the average length of male inflorescences. To compare these parameters between genders, we first checked for independence, normality, and homogeneity of residuals using the Durbin-Watson, Shapiro, and Bartlett tests. These conditions were not satisfied. Hence, we performed nonparametric permutational analysis of variance (PERMANOVA) while controlling for taxon identity using the *aovp()* function of the lmPerm package (Wheeler and Torchiano, 2016).

Female fitness components

We first compared female fitness components or proxies of female fitness components (i.e., basal area, fruit set, fruit weight, female inflorescence production, and female investment) between genders, while controlling for taxon and rootstock. Because the conditions for parametric tests were not satisfied, we used nonparametric permutational analysis of variance (PERMANOVA). We tested for differences between genders in female inflorescence density and in burr set using Student's *t*-test (unilateral for gender effect, and bilateral for taxon effect). We also checked for differences in fruit set between genders over 5 yr using the same approach.

Typical three-flower female inflorescences develop into burrs with zero, one, two, or three filled fruits. To model fruit set, we excluded atypical burrs (deriving from female inflorescences with fewer or more than three flowers) from the analyses, as they were quite rare. To avoid any bias resulting from burrs falling prematurely from trees (Figure 1), we used a zero-truncated binomial distribution to model fruit set (Larue et al., 2022). For a given tree, the numbers of burrs with one, two, and three developed fruits are denoted x_1, x_2, and x_3. Based on the definition of the multinomial distribution, a maximum likelihood estimator of a tree's pollination probability $\hat{p}$ that does not rely on information from empty burrs is (Annex 1):

$$\hat{p} = \frac{3}{2} - \sqrt{3\frac{x_1 + x_2 + x_3}{x_1 + 2x_2 + 3x_3} - \frac{3}{4}}$$

To estimate overall fruit set per tree, we thus aimed to monitor enough burrs with at least one developed fruit, ideally 30 or more. We computed pollination success for all trees in R using the *apply()* function.

Mortality

We modeled tree survival using a binomial distribution, counting the numbers of dead and living trees of each gender. To compare the survival of female and cosexual trees while controlling for taxon identity, we used a general linear model with binomial distribution and performed an analysis of variance.

Emasculation experiments

We modeled fruit set using a binomial distribution, counting the number of developed fruits and the total number of fruits contained in the burrs. We carried out two analyses: one for young trees that had been entirely emasculated and the other for adult trees on which 10 branches had been emasculated. For these two analyses, we used generalized linear mixed-effects models using the *glmer()* function of the package "lme4" (Bates et al., 2015). For young trees, we modeled fruit set as a function of emasculation treatment (intact or emasculated tree), gender (cosexual or female), and clone. We coded the first two as fixed variables and the last one as a random variable. For adult trees, we modeled fruit set as a function of emasculation treatment (intact or emasculated tree), clone (there are only two clones—'Marigoule', a cosexual clone, and 'Bouche de Bétizac', a female clone—so the clone effect is also a gender effect), and trees. We coded the first two as

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fixed variables and the last one as a random variable. We tested the significance of differences between groups using an analysis of variance. For partial emasculation experiments, we used generalized linear mixed-effects models using the *glmer()* function of "lme4" (Bates et al., 2015). We modeled fruit set as a function of emasculation treatment (four treatments: three types of emasculation plus intact branches as control) and individual tree (only cosexual trees were used). We coded emasculation treatment as a fixed variable and individual tree as a random variable. We then compared emasculation treatments using the *emmeans()* function in the "emmeans" package.

RESULTS

Gender polymorphism in chestnut forests

We monitored gender variation in 430 trees from 14 populations, sampling 26 to 42 trees per population (Appendix S2). There was no evidence of female sterility. On the other hand, 10 out of 14 populations included male-sterile (i.e., female) trees. Overall, 85% of the trees were cosexual (i.e., fully male-fertile) trees and the remaining 15% (up to 53% per population) were female, including 11% (up to 37%) fully male-sterile and 4% (up to 17%) partly male-sterile.

Estimation of female advantage in chestnut

Investment in male function

The average weight of male inflorescences per square millimeter of branch section differs significantly between genders. It is higher for cosexual than for female trees (Table 3; 0.54 g.mm^{-2} vs. 0.28 g.mm^{-2}, $p < 10^{-16}$). It is also higher in Euro-Japanese hybrids than in European chestnuts (Table 3; 0.56 g.mm^{-2} vs. 0.26 g.mm^{-2}, PERMANOVA, $p < 10^{-16}$). The number of male inflorescences per square millimeter of branch section does not differ significantly between genders. In contrast, Euro-Japanese hybrids have more male inflorescences per square millimeter of branch section than European chestnuts (0.72 g.mm^{-2} vs. 0.43 g.mm^{-2}, $p < 10^{-16}$). Male inflorescence length does not vary according to gender ($p > 0.06$). In contrast, it varies according to taxa: Euro-Japanese hybrids have longer inflorescences than European chestnuts (14.6 cm vs. 12.3 cm, $p < 0.005$). Thus, cosexual trees do not have denser or longer male inflorescences than female trees, but they have higher average weight of male inflorescences per square millimeter of branch.

Female fitness components

Comparison of cosexual and female trees allows us to identify the potential advantages of female trees.

TABLE 3 Effects of gender, species, and rootstock on each female fitness component (Permanova).

Fitness component	Source	df	R Sum Sq	R Mean Sq	Iterations	Pr(Prob)	
Basal area	Gender	1	8500	8500	51	1.0	
	Species	4	960,000	240,000	5000	$<10^{-15}$	***
	Rootstock	3	470,000	160,000	5000	0.006	**
	Residuals	202	8,600,000	43,000			
Fruit set	Gender	1	2.5	2.5	5000	$<10^{-15}$	***
	Species	4	0.05	0.013	160	1.0	
	Rootstock	3	0.13	0.04	319	0.3	
	Residuals	167	5.4	0.03			
Fruit weight	Gender	1	273	270	5000	$<10^{-15}$	***
	Species	4	141	35	717	0.3	
	Rootstock	3	83	28	1201	0.4	
	Residuals	167	4000	24			
Female inflorescences production	Gender	1	0.0003	0.0003	125	0.4	
	Species	1	0.001	0.001	2284	0.04	*
	Residuals	13	0.003	0.0002			
Fruit yield	Gender	1	0.5	0.06	5000	0.02	*
	Species	1	0.9	0.1	5000	0.003	**
	Residuals	13	0.1	0.08			

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Basal area, fruit set, and fruit weight

Basal area does not differ between genders; in contrast, it differs significantly among taxa and rootstocks (Tables 3 and 4; Appendix S3). Conversely, fruit set and fruit weight measured in 2019 differ between genders but not among taxa or rootstocks. Fruit set is much higher in females than in cosexual trees for both European chestnut and Euro-Japanese hybrids. In contrast, fruit weight is slightly lower in female than in cosexual trees for both taxa. Fruit set and fruit weight are slightly negatively correlated (Spearman rank-correlation coefficient: −0.15, $p = 0.04$).

Female inflorescence density and burr set

Female inflorescence density is highly variable among trees and there is no significant difference between genders (Tables 3 and 4; Appendix S4). Similarly, there is no difference in burr set between genders in our sample. Female trees therefore do not have more female inflorescences than cosexual trees and these female inflorescences are not more likely to form a burr. Hence, genders can differ in fruit production only if they differ in fruit set.

Female inflorescence production

We compared the number of female inflorescences per square millimeter of branch cross-section between genders. Female trees do not seem to invest more in the formation of female inflorescences than cosexual trees ($p > 0.44$). In contrast, female inflorescences production is twice as large in Euro-Japanese hybrids as in European chestnuts (0.04 vs. 0.02, PERMANOVA, $p < 0.05$; Table 2).

Fruit yield

Fruit yield (grams of fruit produced per square millimeter of branch) varies significantly according to gender: female trees have higher fruit yield than cosexual trees (0.76 vs. 0.40, $p < 0.02$). In addition, Euro-Japanese hybrids have higher fruit yield than European chestnuts (Table 2; PERMANOVA, 0.80 vs. 0.34, $p < 0.003$).

Fruit set across years

The average fruit set of female trees fluctuated over the study period, ranging from 0.69 in 2020 to 0.83 in 2018 (Figure 4; Appendix S4). In cosexual trees, it fluctuated more dramatically, ranging from 0.34 in 2022 to 0.64 in 2018. Fruit set was higher in females during all five fruiting episodes: the female:cosexual fruit set ratio was 1.3 in 2018, 1.6 in 2019, 2.0 in 2020, 2.5 in 2022, and 1.6 in 2023.

Survival

In 2018, there were 105 European chestnuts and 53 Euro-Japanese hybrids corresponding to 88 cosexual and 70 female trees. In 2023, there were only 66 European chestnuts and 32 Euro-Japanese hybrids left alive, corresponding to 51 cosexual and 47 female trees. There was no significant difference in mortality rates according to gender (Table 5; GLM, $p > 0.25$), taxon ($p > 0.76$), or rootstock ($p > 0.51$).

Emasculation experiments

First emasculation experiment

For young trees, the gender × treatment interaction is highly significant (Table 6; $p < 0.001$). Emasculation therefore has a different effect depending on the gender of the trees. Emasculation increased fruit set compared to controls in both cosexual clones. In contrast, emasculation reduced fruit set in one of the two studied female clones.

For adult trees, where emasculation involved branches rather than the whole tree, the gender × treatment interaction is significant (Table 7; GLMER, $p < 0.02$). Emasculation treatment therefore has a different effect according to gender. Emasculation increased fruit set in the cosexual clone and reduced fruit set in the female clone.

TABLE 4 Effects of gender, species and rootstock on investment in male function (Permanova).

Fitness component	Source	df	R Sum Sq	R Mean Sq	Iterations	Pr(Prob)	
Average weight of male inflorescences per mm² of branch section	Gender	1	1.3572	1.3572	5000	$<10^{-16}$	***
	Species	1	1.8727	1.8727	5000	$<10^{-16}$	***
	Residuals	77	5.1838	0.0673			
Number of male inflorescences per mm² of branch section	Gender	1	0.0396	0.0396	51	0.92	
	Species	1	1.5736	1.5736	5000	$<10^{-16}$	***
	Residuals	77	9.4320	0.1225			
Average length of male inflorescences	Gender	1	52.12	52.116	2711[a]	0.067[a]	
	Species	1	108.51	108.508	5000[a]	0.004[a]	**
	Residuals	77	972.67	12.632			

[a]Average value after performing 20 tests.

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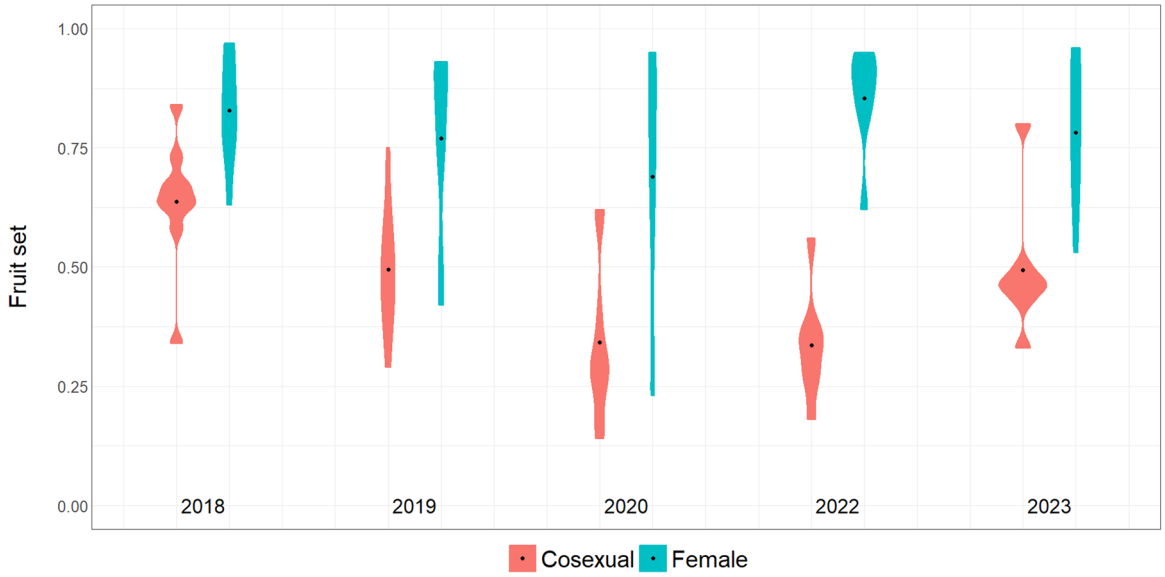

FIGURE 4 Interannual variation in fruit set in cosexual and female chestnut trees. Black dots indicate average fruit set.

TABLE 5 Effects of taxon, gender and rootstock on tree mortality (GLM).

Source	df	Deviance	Resid df	Resid Dev	Pr(>Chi)
Taxon	1	0.0917	9	9.8709	0.76
Gender	1	1.3231	8	8.5478	0.25
Rootstockt	2	1.3131	6	7.2348	0.52

TABLE 6 Effects of gender, treatment and their interaction on fruit set of young trees (GLMER).

Source	Chisq	df	Pr(Prob)	
Gender	6.9	1	0.009	**
Treatment	2.6	1	0.1	
Gender × treatment	11.6	1	0.0007	***

TABLE 7 Effects of gender, treatment, and their interaction on fruit set of adult trees (GLMER).

Source	Chisq	df	Pr(Prob)	
Gender	34.5	1	10^{-8}	***
Treatment	0.7	1	0.4	
Gender × treatment	6.3	1	0.01	*

Second emasculation experiment

We performed partial emasculation experiments on eight unrelated cosexual chestnut trees (Appendix S5). Fruit set varied significantly according to emasculation treatment (GLMER, $p < 10^{-4}$). It increased following emasculation, from 48% in controls to 58% following the emasculation of all male catkins. Fruit set of M1 (removal of unisexual catkins) was 63%, significantly higher than controls (Tukey, $p < 10^{-3}$), while fruit set of M2 (removal of male inflorescences from bisexual catkins) was 49%, not significantly different from that of controls (Tukey, $p > 0.99$).

DISCUSSION

We will argue here that the two mechanisms most frequently cited as drivers of female advantage in gynodioecious plants—namely, resource reallocation from the lost male function and outbreeding advantage—seem to play only a minor role in European chestnut, leaving "avoidance of self-pollen interference" as the single main driver of female advantage in this species. Indeed, the increased fruit set in the absence of pollen-producing stamens, observed both in females and in emasculated cosexual trees, suggests that a tree's own pollen negatively interferes with seed formation and causes selection for females.

Discovery of gynodioecy in chestnut

The discovery of gynodioecy in European chestnut was unexpected. In trees, while dioecy and monoecy are common, gynodioecy is rare (Olson et al., 2016; but see Steyn and Robbertse, 1990; Ellis and Sedgley, 1993; Gibson and Diggle, 1998; Penagos Zuluaga et al., 2020). European chestnut is an ecologically and economically important species widely cultivated for its nutritious nuts. The fact that gynodioecy in European chestnut had previously gone unnoticed might therefore seem surprising. However, reproductive traits of European chestnut have rarely been

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studied in the wild, possibly because the species' natural distribution is hard to assess with certainty due to a long history of cultivation and translocations. Moreover, if gender variation is studied in only a few populations, evidence for gynodioecy might be missed (Dufaÿ et al., 2014; Caruso et al., 2016). Our survey illustrates this point: we found a rather low (15%) but variable (0–53%) proportion of females, with half of the studied populations having <10% female trees. These figures are within the range of those found in other gynodioecious species (25% females, on average; Varga and Soulsbury, 2020). We also found some partly male-sterile trees, a frequent feature in gynodioecious species (Koelewijn and van Damme, 1996; Schultz, 2002). Further investigations of gender variation in other chestnut species and other entomophilous genera in Fagaceae may thus be rewarding.

Reallocation of resources

To study female advantage in chestnut, we measured several key female fitness components or proxies of fitness components over the species' complete life cycle (Philipp, 1980). Long-lived perennial plants must allocate more resources than short-lived plants to maintain vegetative structures (Obeso, 2002). Furthermore, the costs of female reproduction are higher than those of male reproduction in woody plants (Obeso, 2002; Thomas, 2011). Reallocation of resources from the male toward the female function should thus be limited in gynodioecious trees. Using a large grafted collection established 30 yr ago, we found no difference in basal area or survival rates between genders. In grafted trees, both rootstock and scion control growth (Camisón et al., 2021, 2023), so the lack of gender effect on scion growth is remarkable. We also found no difference in flower production between genders. These findings suggest that there is no significant reallocation of resources to the female function from the lost male function at these stages.

Female chestnut trees produce lighter male-sterile inflorescences, compared to cosexuals. Can they save the corresponding resources for fruit production? This does not seem likely, because female chestnut trees have fruits that are lighter, not heavier, than those of cosexual trees. We attribute this reduced fruit weight to higher competition for resources within female inflorescences caused by female trees having higher fruit sets (Xiong et al., 2019; Larue, 2021). However, these lighter fruits do not seem to have reduced fitness. In the only study we could find on the effect of fruit weight on offspring performance in European chestnut, Tumpa et al. (2021) reported no effect of fruit weight on germination performance. Despite their lighter fruits, female chestnut trees allocate more resources than cosexuals to overall fruit production, due to their higher fruit set. In principle, it is possible that reallocation of resources from the male to the female function plays some role in explaining female advantage, by keeping fruit weight above some threshold below which their fitness would be reduced. However, we have no indication that this is the case.

Outbreeding advantage

Chestnut trees are predominantly outcrossing. Based on paternity analysis, self-fertilization rates of cosexual and female trees were previously estimated to be 5% and 1%, respectively (Larue et al., 2022). Even assuming that all selfed seeds abort due to inbreeding depression, this difference in self-fertilization rates between the two genders is insufficient to drive the evolution of gynodioecy.

Avoidance of self-pollen interference

One clear advantage of females over cosexuals is their increased fruit set. This advantage is large and holds for both European chestnut and its hybrids. Japanese and Chinese chestnuts, in which evidence for gynodioecy is lacking, have fruit sets similar to those of cosexual European chestnut trees and hybrids. To our knowledge, gender effects on fruit set have not been investigated previously in chestnuts. However, there is some indication in the literature that gender effects exist, as female clones tend to have higher yields than cosexual clones and are overrepresented under cultivation (e.g., Pereira-Lorenzo and Ramos-Cabrer, 2004; Furones-Pérez and Fernández-López, 2009).

We observed higher and more stable fruit set in female than in cosexual trees across years. To persist in nature, females must have greater geometric lifetime fitness than cosexuals (Eckhart, 1992). Therefore, episodes of poor pollination during which cosexuals have particularly reduced fruit set compared to females (such as year 2022, marked by record heat and drought during pollination) will accentuate female advantage over cosexuals. The investigated orchard is composed of a relatively high proportion of females (35%), a value higher than that observed in most natural populations. The lower relative abundance of pollen donors should further reduce female advantage, so the conclusion for gender differences in fruit set measured in this orchard should be conservative. Overall, our data show that gender difference in fruit set is sufficient to explain female maintenance because it is close to the twofold threshold needed in the case of nuclear inheritance of male sterility (Lewis, 1941).

Emasculation experiments

Emasculation experiments support our hypothesis that increased fruit set is the main female advantage in chestnut and further suggest that reallocation of resources is not central in explaining female advantage.

When we remove male inflorescences (the sources of self-pollen) from cosexual trees, fruit set increases. Conversely, when we remove male-sterile inflorescences from female trees, fruit set decreases or remains unchanged. These contrasting effects suggest that the presence of pollen in anthers is the main factor of reduced fruit set of cosexual

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trees. An alternative explanation, reallocation of resources from male inflorescences toward fruits following removal of male inflorescences at an early stage, is implausible. Indeed, we removed male inflorescences from both cosexual and female trees, yet fruit set increased only in cosexual trees. We thus attribute the opposing effects of male inflorescence removal on fruit set in the two genders to two distinct processes: decreased self-pollination leading to increased cross-pollination success in cosexual trees, and reduced attractiveness to pollinators leading to reduced pollination success in female trees. These findings show that, in cosexual trees, the positive effect of emasculation caused by reduced self-pollination outweighs the negative effect of decreased pollinator attraction caused by eliminating rewarding male inflorescences. The partial emasculation experiment further suggests that it is the pollen produced by the numerous early-flowering male catkins that drive the decreased fruit set, whereas late-flowering nectar-producing male inflorescences from bisexual catkins play a key role in attracting insects close to female flowers (Pauly et al., 2023).

In European chestnut, females therefore have increased fruit set compared to cosexuals, an advantage that we could reproduce by emasculating cosexual trees. Interestingly, Zhao and Liu (2009) observed an increased fruit yield (of up to 39%) following the emasculation of Chinese chestnuts. Self-pollination thus reduces fruit production in this species not known to be gynodioecious. Similar reports of increased seed set following emasculation of self-incompatible cosexual plant species exist (e.g., Waser and Price, 1991; Vaughton and Ramsey, 2010; Duffy et al., 2013, 2021). In chestnut, an earlier study has shown that the main mechanism explaining the increased fruit set of females compared to cosexuals is an increased rate of double fertilization, due to a release from self-pollen interference. Early inbreeding depression plays only a minor role (Xiong et al., 2019). An obvious difference of self-pollen interference with ovules compared to inbreeding depression is that it prevents wasteful provisioning of low-quality progeny (Johnson et al., 2019; Larue et al., 2022). Other self-incompatibility mechanisms that preserve fruit set might seem at first sight more effective than late-acting self-incompatibility. However, late-acting self-incompatibility could have hidden benefits, such as improved mate choice or dilution of attacks by seed predators (Larue et al., 2022).

Emasculation increases fruit set in cosexual trees not only when performed on whole trees but also when performed on single branches. Beetles that walk back and forth within the crown are particularly abundant in sparse chestnut groves. As they walk, these insects presumably transport large quantities of self-pollen from nearby male inflorescences to female inflorescences (Larue et al., 2021a). By emasculating branches rather than entire trees, we reduce self-pollination mediated by such insects but not that mediated by insects such as flies that readily fly between branches of the same tree. This suggests that less mobile insects that tend to walk for extended periods on inflorescences are major causes of self-pollination and self-interference. Environmental conditions that increase the abundance of the least mobile pollinators involved in geitonogamous matings could thus facilitate the evolution of gynodioecy in chestnut by increasing self-pollination in cosexual trees and thus female relative advantage. It would be interesting to explore if the large variation observed in gender composition in extant natural European chestnut populations can be attributed to environmentally induced variation in pollinator assemblages.

Harmful consequences of self-pollination in the absence of selfing

In chestnut, previous studies have shown that self-pollination results in ovule usurpation, because of either reduced rate of double fertilization after self-pollination or, to a lesser extent, early inbreeding depression (Xiong et al., 2019; Larue et al., 2022). Together, these mechanisms usurp ~46% of the ovules (Larue et al., 2022), explaining the low rates of selfing despite very high rates of self-pollination. In this work, we have provided evidence showing that reduced fruit set caused by ovule usurpation can trigger gynodioecy in chestnut, an outcrossing species. Kikuzawa (1989) also concluded that the evolution of gynodioecy in a self-incompatible shrub in Japan resulted from the harmful consequences of self-pollination. Similarly, Ellis and Sedgley (1993) suggested that interference of self-pollen on stigmas could explain the evolution of gynodioecy in a eucalypt, an outcrossed species. The fact that self-pollination can be detrimental to plants even in the absence of selfing, to the point of selecting for unisexuality, was anticipated by Bawa and Opler (1975) and by Lloyd and Yates (1982), even if it has received little attention so far. Moreover, self-pollination can reduce seed set in outcrossed flowering plants (Burbidge and James, 1991; Broyles and Wyatt, 1993; Charlesworth, 1993). Furthermore, late-acting self-incompatibility, which has long been underestimated, is known to be common in angiosperms, especially in woody species (Seavey and Bawa, 1986; Sage et al., 1994; Gibbs, 2014; Johnson et al., 2019). We therefore argue that it is time to reconsider the evidence on the role of the effect of self-pollination in the evolution of gynodioecy.

Self-incompatibility, although probably the most effective outbreeding mechanism, is unique in that it does not reduce self-pollination. Interestingly, many self-incompatible plants are characterized by one or more other "outbreeding" mechanisms such as dichogamy, herkogamy, or monoecy (Lloyd and Webb, 1986). These mechanisms, initially seen as redundant anti-selfing mechanisms and later as mechanisms limiting pollen discounting (Barrett and Harder, 1996; Harder et al., 2007), might in fact be more accurately seen as anti-self-pollination mechanisms. The ultimate mechanism for avoiding self-pollination is unisexuality. Baker (1959), for whom the main cause of female advantage is outbreeding advantage, could see no reason why unisexuality can evolve in an already outcrossed species that avoids the deleterious

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effects of inbreeding depression. For him, "one might expect that in any hermaphrodite taxon where an incompatibility system is already established (or even a strongly effective outbreeding system of some other sort) there will be little likelihood of dioecism arising through direct natural selection." Here, contrary to Baker's prediction, we have shown that gynodioecy can evolve in already outcrossed plants because it helps avoid other deleterious consequences of self-pollination. We therefore suggest that we need a broader framework when studying the evolution of plant reproduction, by considering not just "selfing avoidance" but more broadly "self-pollination avoidance."

AUTHOR CONTRIBUTIONS

C.L.: conceptualization, investigation, data curation, formal analysis, visualization, writing. R.J.P.: conceptualization, investigation, formal analysis, writing, funding acquisition, supervision.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available at https://entrepot.recherche.data.gouv.fr/dataset.xhtml?persistentId=doi:10.57745/MOTPXM. They can be cited as Larue, Clément, 2022, "DATA: Harmful self-pollination drives gynodioecy in European chestnut, a self-incompatible tree," https://doi.org/10.57745/MOTPXM, Recherche Data Gouv, V1. The data on gender variation that support the findings of this study are openly available at https://entrepot.recherche.data.gouv.fr/dataset.xhtml?persistentId=doi:10.57745/LFZFT2. They can be cited as Bodénès, Catherine, 2023, "Conservation des ressources génétiques de châtaignier," Recherche Data Gouv, V1.

CONFLICT OF INTEREST STATEMENT

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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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. Map of the chestnut experimental plot 1.

Appendix S2. Estimation of gender polymorphism in chestnut forests.

Appendix S3. Gender effect on female fitness components by species.

Appendix S4. Fruit set measured over five consecutive years and details for 2020.

Appendix S5. Results of the second emasculation experiment. Fruit set following partial and complete emasculation treatments for the eight trees studied in 2023.

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