bioRxiv preprint doi: https://doi.org/10.1101/2022.08.01.502348; this version posted August 3, 2022. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

Original Article

Self-interference and female advantage in chestnut

Clément Larue[a,b,*], Rémy J. Petit[a]

[a]Univ. Bordeaux, INRAE, BIOGECO, 33610 Cestas, France
[b]INVENIO, Maison Jeannette, 24140 Douville, France

***Corresponding author**
Clément Larue, INRAE, UMR Biodiversity Genes & Communities, 69 route d'Arcachon, 33610 Cestas, France.
Email: clement.larue@inrae.fr
Tel.: +33 535385314

Running title: Self-interference in chestnut

bioRxiv preprint doi: https://doi.org/10.1101/2022.08.01.502348; this version posted August 3, 2022. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

ABSTRACT

- *Background and Aim.* In gynodioecious plants, male-sterile individuals must have a female advantage to coexist with bisexual individuals. In European chestnuts, characterized by a form of late-acting self-incompatibility, male-fertile trees produce large amounts of pollen when female flowers are receptive, resulting in abundant self-pollination. Here we study whether male-sterile chestnut trees have an increased fruit set compared to male-fertile trees because they escape from the negative consequences of self-pollination on ovules, a case of self-interference.
- *Methods.* We measured fruit set of 242 male-fertile and male-sterile trees during three consecutive years. To test experimentally the hypothesis of negative interference of self-pollen on ovules as the origin of the female advantage of male-sterile trees, we emasculated male-fertile trees and compared their fruit set with that of control male-fertile trees. We performed the same experiment on male-sterile trees by removing the sterile but rewarding male catkins as a natural control to test for the effect of decreased insect attractiveness on fruit set.
- *Key Results.* The fruit set of male-sterile trees is higher than that of male-fertile trees by a factor of two in some years. Following emasculation, male-fertile trees have a higher fruit set than control male-fertile trees, pointing to a negative effect of the massive amount of self-pollen produced by fertile male catkins on fertilization probability of female flowers. In contrast, emasculated male-sterile trees had a lower fruit set than control male-sterile trees, pointing to a decrease in insect attractiveness after removing nectar-producing male catkins.
- *Conclusions.* Our emasculation experiment showed that sexual interference results in massive ovule discounting in male-fertile trees, sufficient for accounting for the persistence of female trees in natural populations of European chestnut trees.

Key-words: Fagaceae, *Castanea*, gynodioecy, late-acting self-incompatibility, sexual interference,

bioRxiv preprint doi: https://doi.org/10.1101/2022.08.01.502348; this version posted August 3, 2022. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

ovule discounting, emasculation experiment, self-pollen

INTRODUCTION

In angiosperms, most species have retained the ancestral floral organization, with male and female reproductive organs combined with the perianth (Yampolsky 1922). Starting with this already relatively complex arrangement, flowering plants have further evolved, resulting in an amazing variety of reproductive systems at the flower, inflorescence or whole plant level, constrained by the breeding system, pollination mode, and sexual interactions. For example, in gynodioeocious species, cosexual and unisexual (female) individuals coexist. The evolutionary mechanisms leading to the evolution of unisexuality in plants have represented one major research line since Darwin (1877). One enduring question is why cosexuality, in which transmission of genes of an individual can take place via both pollen grains and ovules, is sometimes replaced by unisexuality, in which transmission of genes can only be assured by one route, pollen grains or ovules (Barrett 2010; Dufay and Billard 2012).

A potential route to dioecy is the gynodioecy pathway (Barrett 2002; Dufay et al. 2014). For gynodioecious populations to be maintained in natural populations, female (i.e. male-sterile) individuals must have an advantage over bisexual (male-fertile) individuals. A first possible advantage is resources reallocation from the male to the female function, originally proposed by Darwin (1877). A second possible advantage is the avoidance of inbreeding due to obligate outcrossing in females, a view supported by the rarity of strictly allogamous gynodioecious species (Ross 1970; Arroyo and Raven 1975; Dufay and Billard 2012). However, even if they are a minority, fully allogamous gynodioecious species do exist (Kikuzawa 1989; Dufay and Billard 2012; Wang et al. 2021). In these species, outcrossing is enforced by self-incompatibility or complete dichogamy, ruling out inbreeding avoidance as the cause of female advantage. Another mechanism of female advantage that might apply to these outcrossing species but that is rarely

bioRxiv preprint doi: https://doi.org/10.1101/2022.08.01.502348; this version posted August 3, 2022. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

considered is the release from sexual interference. Sexual interference between maternal and paternal functions in cosexual plants was discovered relatively recently and remains understudied (Lloyd and Webb 1986; Barrett 2002). An example of sexual interference shown to promote the evolution of gynodioecy is sexual conflict over the length of male versus female maturity phases within hermaphroditic flowers (Tonnabel 2021; Wang et al. 2021). Sexual interference could also directly lead to gamete wastage and reduced fitness. For instance, self-pollination can decrease female fitness by diminishing seed set, even in self-incompatible species (de Jong et al. 1993). At least two mechanisms can be involved. First, self-pollen can clog stigmas and pre-empt space, thereby reducing the chances for compatible pollen grains to establish on stigmas and germinate. For example, Galen et al. (1989) showed, with the help of a pollen chase experiment, that pollen clogging reduces seed set of *Polemomium viscosum* by 40%. Second, pollen tubes from self-pollen might germinate, grow, penetrate the ovules and make them abort. This results in ovule discounting, i.e. the elimination of ovules rendered non-functional following interactions with self-pollen (Barrett et al. 1996). For example, Waser and Price (1991) applied a mix of self and outcross pollen on flowers of *Ipomopsis aggregata*. They showed that seed set decreased by 42% compared to the situation where they used only outcross pollen. These negative effects of self-pollen on seed set were reported in species with late-acting self-incompatibility systems (Seavey and Bawa 1986).

To test if sexual interference is taking place and results in seed discounting, emasculation experiments can be used. For instance, de Jong et al. (1992) showed that seed set of emasculated *Ipomopsis aggregata* individuals increased by 20% compare to the control. As the size of the plant increased, the negative effects of self-pollination increased (de Jong et al. 1993). However, only one study to date has shown that sexual interference through seed discounting following self-pollination can drive the evolution of gynodioecy. Kikuzawa (1989), working on the self-incompatible gynodioecious shrub *Daphne kamtchatica* in Japan, showed that emasculation strongly increased fruit set of hermaphrodite flowers. He proposed that clogging by pollen grains of its own flower (a

bioRxiv preprint doi: https://doi.org/10.1101/2022.08.01.502348; this version posted August 3, 2022. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

form of self-pollen interference) was a main factor driving the evolution towards gynodioecy in this species.

European chestnut (*Castanea sativa*) is an ecologically and economically important Fagaceae tree from the Mediterranean region characterized by massive and synchronous blooming with huge pollen production. This self-incompatible monoecious tree with non-rewarding female flowers is gynodioecious, as some natural populations include both male-fertile and male-sterile trees (Larue 2021 and unpublished observations). This suggests that male-sterile trees must have a female advantage to persist. One feature of special interest for the present study is that the male-sterile trees still harbour nectar-producing male catkins that attract pollinators (Larue et al. 2021a). Here, we first quantify female advantage in this species by comparing fruit set in male-fertile and male-sterile trees in a chestnut germplasm collection during three different years and using different sampling strategies. Second, we assess the importance of self-pollen interference in an experiment where we remove male catkins not only from male-fertile but also from male-sterile chestnut trees, a unique situation that should help disentangle the different functions of male organs on female fitness.

MATERIALS AND METHODS

Chestnut floral biology

The Fagaceae family includes wind-pollinated tree species such as oaks (*Quercus spp.*) and beeches (*Fagus spp.*) and insect-pollinated species such as chestnuts (*Castanea spp.*) (Larue et al. 2021a; Petit and Larue 2022). Chestnut trees are characterized by massive blooming, huge pollen production and the largest pollen:ovule ratio ever reported in plants (Larue et al. 2021a). They are characterized by multiple and complex mechanisms that limit self-pollination (Xiong et al. 2019;

bioRxiv preprint doi: https://doi.org/10.1101/2022.08.01.502348; this version posted August 3, 2022. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

Larue et al. 2022). At the flower level, chestnuts are monoecious: they have separate male and female flowers. At the inflorescence level, chestnuts are andromonoecious: they produce unisexual male catkins with only male flowers and bisexual catkins composed of one or two female inflorescences associated with a single male catkin (Larue et al. 2021a). They have a complex phenology called duodichogamy, characterized by two peaks of pollen emission (Stout 1928), which reduces the risk of self-pollination but does not eliminate it completely (Hasegawa et al. 2017). Unisexual male catkins are the first to bloom and release huge quantities of pollen, about 97% of the total amount (Larue et al. 2021a). Male flowers continue to emit pollen when non-rewarding female flowers of the same tree become receptive. In male-fertile trees, the great majority (>90%) of pollen deposited on stigmas of female flowers is self-pollen, even if the ultimate rate of self-fertilisation is very low (0.3%) (Hasegawa et al. 2009). Xiong et al. (2019) have demonstrated that self-incompatibility has two causes: first, a prezygotic late-acting self-incompatibility mechanism, with a lower percentage of ovule penetration and double fertilisation in selfed vs. crossed ovules; second, an early-acting inbreeding depression that results in the abortion of most of the self-fertilized ovules. Hence, the probability to observe ovule discounting following self-pollination in this self-incompatible mass-flowering tree species is very high.

In European chestnuts, some trees are completely or partly male-sterile (Solignat and Chapa 1975 ; Figure 1). This male sterility is likely of cytoplasmic origin (Sisco et al. 2014). It causes anomalies to male staminate flowers, ranging from male flowers with fully aborted stamens to flowers with short stamen filaments that produce scarce amounts of mostly non-functional pollen (Bounous et al. 1992). Interestingly, the corresponding male-sterile catkins continue to produce nectar and attract insects (Larue et al. 2021a).

Chestnut trees should be good models to study female advantage. First, some preliminary indications exist in the literature that male-sterile chestnut trees have higher fruit set than male-fertile trees (Pereira-Lorenzo and Ramos-Cabrer 2004). Second, fruit set is easy to estimate in

bioRxiv preprint doi: https://doi.org/10.1101/2022.08.01.502348; this version posted August 3, 2022. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

chestnuts. A female inflorescence is typically composed of three female flowers located side by side. The inflorescence develops into a burr; in each burr, each of the three flowers, if pollinated, gives a nut (a fruit with a single seed surrounded by a closed pericarp), and if not pollinated, an empty fruit (Figure 2). Multi-seeded chestnuts exist but are extremely rare (< 1%). Hence, the proportion of developed fruits per burr should provide an indication on pollination success. Third, because chestnut is monoecious and male and female inflorescences are physically separated (Figure 3), it is technically feasible to emasculate whole trees or branches to test the consequences of self-pollen on fruit production. There is no need to remove male flowers individually as they are packed together into inflorescences that can be removed as one unit, even in bisexual catkins, where all male flowers are located at the distal tip of the catkin.

Estimating female advantage

Study site

For this work, we used the INRAE chestnut germplasm collection located in Villenave d'Ornon (44.788319 N, −0.577062 E) (Larue et al. 2021b). There are 242 trees (ramets) corresponding to 92 different genotypes (clones) assigned to different chestnut species: the European chestnut *C. sativa*, the Japanese chestnut *C. crenata*, the Chinese chestnut *C. mollissima* and their interspecific hybrids. Among the clones, 62 are male-fertile and 30 are male-sterile, corresponding to 156 (64%) male-fertile and 86 (36%) male-sterile ramets.

Fruit set

To estimate fruit set, i.e. the proportion of female flowers giving a fruit, we sampled burrs from all trees from the INRAE chestnut germplasm collection in 2018 and 2019. In 2018, we aimed at assessing at least 20 burrs per tree; in 2019, we targeted at least 30 burrs to increase resolution. We

bioRxiv preprint doi: https://doi.org/10.1101/2022.08.01.502348; this version posted August 3, 2022. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

measured fruit set in all trees that had produced at least five burrs (i.e., ≥15 female flowers), corresponding to 196 trees in 2018 and 222 trees in 2019.

Burr set

In 2020, we measured fruit set differently on a subset of 18 trees, by tracking the fate of female inflorescences from spring to autumn. We estimated burr set by dividing the number of burrs counted in August by the number of female inflorescences counted during flowering in June. We then estimated the overall pollination success by multiplying burr set by fruit set. Among these 18 trees, there were nine male-fertile and nine male-sterile trees. For comparison, we also present fruit set estimates for these 18 trees in 2018 and in 2019.

Emasculation experiment

Study site

To check for self-interference, we carried out an emasculation experiment and compared fruit set of emasculated trees with that of control trees. This experiment was performed in 2019, in three orchards of the INVENIO experimental station in Douville (45.019723 N, 0.614637 W).

Plant material

We selected six hybrid varieties, three male-sterile ones (‘Bellefer’, ‘OG19’ and ‘Bouche de Bétizac’) and three male-fertile ones (‘Florifer’, ‘Maraval’ and ‘Marigoule’). They are all hybrids between *C. sativa* and *C. crenata* except ‘Bellefer’, which is a complex three-way hybrid. These trees are planted in three nearby orchards. The first orchard is composed of 8-year-old trees belonging to ‘Bellefer’, ‘OG19’, ‘Florifer’ and ‘Maraval’ varieties. The second orchard is composed of 20 m-high trees belonging to ‘Bouche de Bétizac’ and ‘Marigoule’ varieties. We replicated the experiment on ‘Marigoule’ variety in a third orchard, because ‘Marigoule’ is very

bioRxiv preprint doi: https://doi.org/10.1101/2022.08.01.502348; this version posted August 3, 2022. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

susceptible to cynips (*Dryocosmus kuriphilus*), which complicates fruit set measurements. Indeed, following cynips attacks, female flowers tend to proliferate, resulting in atypical inflorescences with more than three flowers that are difficult to use to estimate fruit set.

Modalities

To remove male flowers for the emasculation treatment, we cut all unisexual male catkins and the male part of bisexual catkins with a scissor (Figure 3). As these two types of flowers emerge at different times, we proceeded in three steps before trees started to flower. At the first pass, in the end of May, we removed all unisexual male catkins. At the second pass, we checked if no unisexual male catkins remained and we removed the male part of bisexual catkins. At the third and last pass, we removed the male flowers of bisexual catkins that remained.

We emasculated both male fertile and male sterile varieties. Male-sterile trees still have male catkins, which strongly resemble those of male-fertile trees except that they have aborted stamens. Even though they no longer produce pollen, these sterile catkins produce nectar and attract insects. We emasculated these male-sterile trees to evaluate the decrease in insect attractiveness or possible reallocation of resources in the female function following their removal.

For each modality, we selected five ‘OG19’ trees, six ‘Florifer’ trees, five ‘Bellefer’ trees and two ‘Maraval’ trees. Hence, there were 18 control and 18 emasculated chestnuts. For young trees, we applied this treatment to each individual tree. For older trees, we applied the treatment to single branches. In the first orchard, we selected five ‘Bouche de Bétizac’ and five ‘Marigoule’ trees. For each tree, we selected 20 branches and used ten for each modality. We replicated these two modalities on three more ‘Marigoule’ trees from another orchard.

bioRxiv preprint doi: https://doi.org/10.1101/2022.08.01.502348; this version posted August 3, 2022. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

Statistical analyses

Data analysis

We performed all analyses with R software (v3.6.6; R Core Team 2013) The corrected fruit set was calculated with basic functions implemented in R, while boxplots and histograms are computed with the ggplot2 (v3.6.3; Wickham 2016), ggthemes (v4.2.4; Arnold 2016) and cowplot (v1.1.1; Wilke 2020) packages.

Corrected fruit set

In the large-scale fruit set study of 2018 and 2019, we excluded rare atypical burrs containing fewer or more than three fruits. We also decided not to use burrs with zero fruits. These burrs with three empty fruits may abort during summer, so fruit set assessed in the fall may be biased. We therefore model fruit set using only burrs with at least one developed fruit. Pollination of each female flower is independent (Larue 2021). Thus, to obtain a fruit set estimate reflecting the probability for a flower to give a fruit, we used a zero-deflated binomial distribution to correct for the omission of empty burrs. For a given tree, typical burrs developed from 3-flowers inflorescences with one, two, or three developed fruits are noted x_1, x_2, and x_3 while the total number of harvested burrs is noted x_{tot}. Based on the definition of multinomial distribution, a maximum likelihood estimator reflecting pollination probability $\hat{p}$ is obtained (Annex 1):

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

All these estimates are automatically computed for all trees in R using the *apply()* function.

Estimation of female advantage

We represented the fruit set of male-fertile and male-sterile trees with two boxplots, one for each year, using *geom_boxplot()* function from ggplot2 package and ggthemes package to adjust graphic

bioRxiv preprint doi: https://doi.org/10.1101/2022.08.01.502348; this version posted August 3, 2022. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

colours. Female advantage is the difference in average fruit set between the two types of trees. We tested its significance with an Anova. On the subset of 18 trees on which we had counted flowers in 2020, we tested the difference in fruit set between male-sterile and male-fertile trees with a unilateral Student *t* test. We also calculated the female advantage as a ratio, by dividing the fruit set of male-sterile trees by the fruit set of male-fertile trees

Emasculation experiment

We drew histograms representing fruit set of control and emasculated trees with the *geom_bar()* function of ggplot2 package and adjust graphic colours with ggthemes package. We tested the significance of the difference between these two modalities with a Fisher exact test.

RESULTS

Female advantage

There were 176 trees in 2018 (110 male-fertile and 66 male-sterile) and 206 trees in 2019 (134 male-fertile and 72 male-sterile) that produced enough fruits to measure fruit set. We harvested 22 burrs (5-62) per tree in 2018 and 36 burrs (6-383) in 2019. In 2018, the average fruit set of male-fertile trees was 61% and that of male-sterile trees was 82% (figure 4, top), a highly significant difference (Anova, $p < 10^{-11}$). In 2019, the average fruit set of male-fertile trees was 55% and that of male-sterile trees was 83% (figure 4, bottom), again a very significant difference (Anova, $p < 10^{-16}$).

In the subsample of 18 trees, male-sterile trees also had a higher fruit set than that of male-fertile trees (Table 1, *t*-test, *p*-value < 0.008). For male-sterile trees, average fruit set was 0.83 in 2018, 0.77 in 2019 and 0.69 in 2020. For male-fertile trees, average fruit set was 0.64 in 2018, 0.49 in 2019 and 0.34 in 2020. Female advantage (male-sterile/male-fertile fruit set ratio) increased across the three years, from 1.30 in 2018, to 1.56 in 2019 and 2.01 in 2020. In 2020, we tracked the fate of

bioRxiv preprint doi: https://doi.org/10.1101/2022.08.01.502348; this version posted August 3, 2022. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

inflorescences to better estimate fruit set. Male-sterile trees had slightly more female inflorescences even though the difference was not significant (Table 1, *t*-test, $p > 0.3$). Similarly, the number of burrs was higher in male-sterile trees than in male-fertile ones but the difference was not significant (Table 1, *t*-test, $p > 0.19$). Hence, there was no evidence that burr set varies according to a tree's sexual type (Table 1, *t*-test, $p > 0.11$). To obtain the overall pollination success, we multiplied burr set by fruit set. We found that overall pollination success in 2020 is 0.65 for male-sterile trees and 0.28 for male-fertile trees, a female advantage of 2.3.

Emasculation experiment

The emasculation treatment resulted in a significant decrease in fruit set compared to controls for two of the three male-sterile varieties (figure 5, left). For 'Bellefer', fruit set in control trees is 91% whereas it is 81% for emasculated trees, a significant decrease (F test, $p < 10^{-5}$). Similarly, for 'Bouche de Bétizac', fruit set in control trees is 95% whereas it is 85% for emasculated trees, again a significant decrease (F test, $p < 10^{-7}$). Instead, for 'OG19', fruit set in control trees is 77% whereas it is 82% for emasculated trees, a non-significant increase (F test, $p > 0.82$). In contrast to what we found for male-sterile trees, the emasculation treatment resulted in a significant increase in fruit set compared to controls for two of the three male-fertile varieties (figure 5, right). For 'Marigoule', fruit set in control trees is 52% whereas it is 67% for emasculated trees, a significant increase (F test, $p < 10^{-5}$). Similarly, for 'Florifer', fruit set in control trees is 46% whereas it is 63% for emasculated trees, a significant increase (F test, $p < 10^{-10}$). For 'Maraval', fruit set of control trees is 73% compared to 78% for emasculated trees, a non-significant increase (F test, $p > 0.16$).

bioRxiv preprint doi: https://doi.org/10.1101/2022.08.01.502348; this version posted August 3, 2022. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

DISCUSSION

By comparing fruit set of a large array of male-sterile and male-fertile trees, we found that male-sterile trees have a strong female advantage over male-fertile ones: they produced 34% more fruits in 2018 and 55% more in 2019. In 2020, this female advantage was over 100%. When we consider burr set along with fruit set, the female advantage of male-sterile trees is even greater. This more than two-fold advantage is sufficient to explain the maintenance of male sterility in natural populations, regardless of the mode of inheritance of male sterility (Lewis 1941). Different chestnut species and their hybrids are included in our study but this should not affect the conclusions, as we could not find any evidence for differences in fruit set between the different species present in the orchard when controlling for sexual type (Larue 2021).

Results from the emasculation experiment point to a major role for self-pollen interference in the origin of this female advantage. When we removed male catkins from male-fertile trees, fruit set increased. In contrast, when we removed male flowers from male-sterile trees, fruit set decreased or remained unchanged. We argue that these opposite effects of emasculation on fruit set in these two types of trees are due to decreased self-pollination and decreased seed discounting in emasculated male-fertile trees, and reduced attractiveness to pollinators caused by the removal of nectar-producing male-sterile catkins in male-sterile trees. Together, the findings suggest that in male-fertile trees, the positive effect of reduced self-interference on fruit set generated by emasculation overcomes the negative effect of reduced insect attractiveness generated by emasculation, resulting overall in an increased fruit set. We also note that resource reallocation from the male to the female flowers is unlikely to cause increased fruit set. Indeed, following emasculation of male-sterile trees, we did not observed increased fruit set, as expected if reallocation of resources from male catkins to the female function was taking place. Instead, we found the opposite trend, suggesting that the removal of nectar-producing sterile male catkins does not benefit the female function. The lack of

bioRxiv preprint doi: https://doi.org/10.1101/2022.08.01.502348; this version posted August 3, 2022. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

transfer of resources from the male to the female function following emasculation makes sense as the catkins of male-sterile trees differ little from the catkins of male-fertile trees except for the abortion of the tiny stamens.

Overall, our results suggest that self-pollen interference has a strong negative impact on fruit set. In a related study, we used a process-based model to predict fruit set in the same site, combining fruit set measures with paternity analyses. The model was very accurate, but only when we accounted for self-pollen interference and associated ovule discounting (Larue et al. 2022). Following chemical emasculation of Chinese chestnut (*C. mollissima*), Zongyun and Kuiying (2009) have reported an increase fruit yield, by up to 39%, indicating that self-pollination has a strong negative effect on fruit production in this other chestnut species.

Interestingly, in our study, emasculation experiments of male-fertile trees involving either single branches or entire trees result in similar increases in fruit set. Insects such as beetles, which are particularly abundant in orchards under open conditions, can be seen walking back and forth on the crown, presumably bringing much self-pollen from nearby male catkins on female inflorescences. By emasculating entire branches, we remove sources of pollen near female inflorescences. Hence, we reduce self-pollination brought about by the behaviour of these insects, but not by that of insects flying among branches of the same tree. This suggests that insects that tend to walk on inflorescences rather than to fly from one place to another are major causes of self-pollination and eventually of self-interference and fruit set reduction.

We propose that this female advantage explains the persistence of male-sterile trees in wild population of European chestnut, making this tree one of the few gynodioecious tree species (Yampolsky 1922; Bawa and Opler 1975; Bawa 1980; Gibson and Diggle 1997; Dufay et al. 2014). Interestingly, this increased fruit set has been taken advantage of in chestnut cultivation in Europe,

bioRxiv preprint doi: https://doi.org/10.1101/2022.08.01.502348; this version posted August 3, 2022. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

as male-sterile varieties are overrepresented in chestnut orchards compared to male-fertile ones (Pereira-Lorenzo et al. 2006; Furones-Pérez and Fernández-López 2009; Martín et al. 2017).

Self-pollen interference resulting in ovule discounting has to date not been considered as a potential factor in the evolution of unisexuality, except in the pioneer study of Kikuzawa (1989) on *Daphne kamtchatica* in Japan. At this point, with just two studies in *Daphne* and in *Castanea* suggesting that self-interference could lead to the evolution of gynodioecy, it is difficult to appreciate the importance of self-interference in the evolution of gender polymorphism. To investigate the matter further, a review of the effect of emasculation experiments on seed set, especially in self-incompatible plants, would be useful. It could help evaluate the general importance of this under-studied mechanism.

FUNDING

This paper was part of the PhD and postdoc of CL. The Cifre thesis (Conventions Industrielles de Formation par la Recherche) was supported by the ANRT (Association Nationale de la Recherche de la Technologie, convention Cifre N° 2018/0179). It was funded by Invenio, the Région Nouvelle-Aquitaine (Regina project N° 22001415-00004759 and chestnut pollination project N° 2001216-00002632), by INRAE (Institut National de Recherche pour l'Agriculture et l'Environnement) and by ANR (project ANR-21-PRRD-0008-01).

ACKNOWLEDGEMENTS

We thank Teresa Barreneche (chestnut germplasm collection administrator) for her invaluable support. This study was possible thanks to the continuous support of INRAE experimental unit

bioRxiv preprint doi: https://doi.org/10.1101/2022.08.01.502348; this version posted August 3, 2022. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

Vigne Bordeaux (UEVB) in Villenave d'Ornon and of Xavier Capdevielle of UMR Biogeco who manage the experimental orchards used for this study. We thank the INVENIO chestnut team and the numerous seasonal workers who permitted to set up the emasculation experiment. Many thanks to Olivier Lepais and to Etienne Klein for developing a fruit set model. Mathilde Dufay comments greatly helped improve an earlier version of this manuscript.

CL, RJP, Yannick Mellerin, Céline Lalanne and Chantal Helou Bagate estimated fruit set in the INRAE chestnut germplasm collection. CL, Nathalie Lebarbier and William Genoud carried out the emasculation experiments in Invenio orchards. CL and RJP estimated fruit set in INVENIO orchards. CL, Etienne Klein and Olivier Lepais modelled fruit set. CL and RJP carried out statistical analyses. CL wrote the first draft of this article and RJP revised it.

LITERATURE CITED

Arnold JB (2016) ggthemes: extra themes, scales and geoms for “ggplot2”

Arroyo MTK, Raven PH (1975) The evolution of subdioecy in morphologically gynodioecious species of *Fuchsia* sect. *encliandra* (Onagraceae). Evolution 29:500–511. https://doi.org/10.2307/2407262

Barrett SCH (2010) Understanding plant reproductive diversity. Phil Trans R Soc B 365:99–109. https://doi.org/10.1098/rstb.2009.0199

Barrett SCH (2002) The evolution of plant sexual diversity. Nat Rev Genet 3:274–284. https://doi.org/10.1038/nrg776

Barrett SCH, Lloyd DG, Arroyo J (1996) Stylar polymorphisms and the evolution of heterostyly in *Narcissus* (Amaryllidaceae). In: Lloyd DG, Barrett SCH (eds) Floral Biology. Springer US, Boston, MA, pp 339–376

Bawa KS (1980) Mimicry of male by female flowers and intrasexual competition for pollinators in *Jacaratia dolichaula* (D. Smith) woodson (Caricaceae). Evolution 34:467–474. https://doi.org/10.2307/2408216

bioRxiv preprint doi: https://doi.org/10.1101/2022.08.01.502348; this version posted August 3, 2022. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

Bawa KS, Opler PA (1975) Dioecism in tropical forest trees. Evolution 29:167. https://doi.org/10.2307/2407150

Bounous G, Paglietta R, Peano C (1992) Methods for observing chestnut pollen viability, germinability and pollen tube growth. Proc Int Chestnut Conf, Morgantown, USA 76–78

Darwin C (1877) The Different Forms of Flowers on Plants of the Same Species. London UK

de Jong TJ, Waser NM, Klinkhamer PG (1993) Geitonogamy: The neglected side of selfing. Trends Ecol Evol 8:321–325. https://doi.org/10.1016/0169-5347(93)90239-L

de Jong TJ, Waser NM, Price MV, Ring RM (1992) Plant size, geitonogamy and seed set in *Ipomopsis aggregata*. Oecologia 89:310–315. https://doi.org/10.1007/BF00317407

Dufay M, Billard E (2012) How much better are females? The occurrence of female advantage, its proximal causes and its variation within and among gynodioecious species. Ann Bot 109:505–519. https://doi.org/10.1093/aob/mcr062

Dufay M, Champelovier P, Käfer J, et al (2014) An angiosperm-wide analysis of the gynodioecy–dioecy pathway. Ann Bot 114:539–548. https://doi.org/10.1093/aob/mcu134

Furones-Pérez P, Fernández-López J (2009) Morphological and phenological description of 38 sweet chestnut cultivars (*Castanea sativa* Miller) in a contemporary collection. Span J Agric Res 7:829–843. https://doi.org/10.5424/sjar/2009074-1097

Galen C, Gregory T, Galloway LF (1989) Costs of self-pollination in a self-incompatible plant, *Polemonium viscosum*. Am J Bot 76:1675–1680. https://doi.org/10.1002/j.1537-2197.1989.tb15152.x

Gibson JP, Diggle PK (1997) Structural analysis of female and hermaphroditic flowers of a gynodioecious tree, *Ocotea tenera* (Lauraceae). American Journal of Botany 84:298–307. https://doi.org/10.2307/2446003

Hasegawa Y, Suyama Y, Seiwa K (2017) Flowering phenology of a duodichogamous self-incompatible tree species, *Castanea crenata*. Japanese Journal of Ecology 67:31–39. https://doi.org/10.18960/seitai.67.1_31

Hasegawa Y, Suyama Y, Seiwa K (2009) Pollen donor composition during the early phases of reproduction revealed by DNA genotyping of pollen grains and seeds of *Castanea crenata*. New Phytol 182:994–1002. https://doi.org/10.1111/j.1469-8137.2009.02806.x

bioRxiv preprint doi: https://doi.org/10.1101/2022.08.01.502348; this version posted August 3, 2022. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

Kikuzawa K (1989) Floral biology and evolution of gynodioecism in *Daphne kamtchatica var. jezoensis*. Oikos 56:196–202. https://doi.org/10.2307/3565336

Larue C (2021) De la pollinisation à la formation des graines : le cas du châtaignier. PhD Thesis, Université de Bordeaux

Larue C, Austruy E, Basset G, Petit RJ (2021a) Revisiting pollination mode in chestnut (*Castanea* spp.): an integrated approach. Bot Lett 168:348–372. https://doi.org/10.1080/23818107.2021.1872041

Larue C, Barreneche T, Petit RJ (2021b) An intensive study plot to investigate chestnut tree reproduction. Ann For Sci 78:90. https://doi.org/10.1007/s13595-021-01104-w

Larue C, Klein E, Petit R (2022) Sexual interference revealed by joint study of male and female pollination success in chestnut. Preprints

Lewis D (1941) Male sterility in natural populations of hermaphrodite plants the equilibrium between females and hermaphrodites to be expected with different types of inheritance. New Phytol 40:56–63. https://doi.org/10.1111/j.1469-8137.1941.tb07028.x

Lloyd DG, Webb CJ (1986) The avoidance of interference between the presentation of pollen and stigmas in angiosperms I. Dichogamy. N Z J Bot 24:135–162. https://doi.org/10.1080/0028825X.1986.10409725

Martín MA, Monedero E, Martín LM (2017) Genetic monitoring of traditional chestnut orchards reveals a complex genetic structure. Annals of Forest Science 74:1–11. https://doi.org/10.1007/s13595-016-0610-1

Pereira-Lorenzo S, Ramos-Cabrer AM (2004) Chestnut, an ancient crop with future. In: Dris R, Jain SM (eds) Production Practices and Quality Assessment of Food Crops Volume 1: Preharvest Practice. Springer Netherlands, Dordrecht, pp 105–161

Pereira-Lorenzo S, Ramos-Cabrer AM, Díaz-Hernández MB, et al (2006) Chemical composition of chestnut cultivars from Spain. Sci Hortic 107:306–314. https://doi.org/10.1016/j.scienta.2005.08.008

Petit RJ, Larue C (2022) Confirmation that chestnuts are insect-pollinated. Botany Letters 0:1–5. https://doi.org/10.1080/23818107.2022.2088612

bioRxiv preprint doi: https://doi.org/10.1101/2022.08.01.502348; this version posted August 3, 2022. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

R Core Team (2013) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienne, Autriche. https://doi.org/URL https://www.R-project.org/

Ross MD (1970) Breeding systems in *Plantago*. Heredity 25:129–133. https://doi.org/10.1038/hdy.1970.13

Seavey SR, Bawa KS (1986) Late-acting self-incompatibility in angiosperms. Bot Rev 52:195–219. https://doi.org/10.1007/BF02861001

Sisco PH, Neel TC, Hebard FV, et al (2014) Cytoplasmic male sterility in interspecific hybrids between American and Asian Castanea species is correlated with the American D chloroplast haplotype. Acta Hortic 215–222. https://doi.org/10.17660/ActaHortic.2014.1019.32

Solignat G, Chapa J (1975) La Biologie Florale du Châtaignier. Institut National de Vulgarisation pour les Fruits Légumes et Champignons, Paris

Stout AB (1928) Dichogamy in flowering plants. Bulletin of the Torrey Botanical Club 55:141–153. https://doi.org/10.2307/2480605

Tonnabel J (2021) Digest: Sexual conflict as a novel hypothesis for the evolution of gynodioecy*. Evolution 75:557–558. https://doi.org/10.1111/evo.14161

Wang H, Barrett SCH, Li X, et al (2021) Sexual conflict in protandrous flowers and the evolution of gynodioecy*. Evolution 75:278–293. https://doi.org/10.1111/evo.14113

Waser NM, Price MV (1991) Reproductive costs of self-pollination in *Ipomopsis aggregata* (polemoniaceae): are ovules usurped? Am J Bot 78:1036–1043. https://doi.org/10.1002/j.1537-2197.1991.tb14511.x

Wickham H (2016) ggplot2: elegant graphics for data analysis. Springer-Verlag New York

Wilke CO (2020) cowplot: Streamlined Plot Theme and Plot Annotations for “ggplot2”

Xiong H, Zou F, Guo S, et al (2019) Self-sterility may be due to prezygotic late-acting self-incompatibility and early-acting inbreeding depression in Chinese chestnut. J Am Soc Hortic Sci 144:172–181. https://doi.org/10.21273/JASHS04634-18

Yampolsky C (1922) Distribution of Sex Forms in the Phanerogamic Flora, Gebrüder Borntraeger. Leipzig

Zongyun Z, Kuiying L (2009) Effect of chemical thinning catkins on Chinese chestnut yield and quality. Acta Hortic 457–460. https://doi.org/10.17660/ActaHortic.2009.844.64

bioRxiv preprint doi: https://doi.org/10.1101/2022.08.01.502348; this version posted August 3, 2022. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

bioRxiv preprint doi: https://doi.org/10.1101/2022.08.01.502348; this version posted August 3, 2022. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

Table 1: Fruit set measured during three consecutive year and detailed pollination success for 2020

			Fruits set			Details for 2020			
Clone[1]	Ramet[2]	♂-flowers[3]	2018[4]	2019	2020	#♀-inflo[5]	#Burrs[6]	Burr set[7]	*Ppol*[8]
Bétizac	A_58_O	S	0.97	0.92	0.89	16	17	1.06	0.95
Bétizac	E_80_E	S	NA	0.92	0.94	42	40	0.95	0.90
Dauphine	A_64_C	S	0.63	0.42	0.23	42	33	0.79	0.18
Dauphine	E_76_I	S	0.76	0.93	0.67	5	3	0.60	0.40
Marlhac	A_66_Q	S	0.80	0.75	0.62	254	230	0.91	0.56
Marlhac	E_73_D	S	0.77	0.75	0.72	96	88	0.92	0.66
Merle	A_45_E	S	0.85	0.50	0.26				
Précoce des Vans	E_40_R	S	0.96	0.92	0.95	57	46	0.81	0.77
Précoce des Vans	E_72_S	S	0.89	0.82	0.92	25	22	0.88	0.81
381	E_43_O	F	0.63	0.40	0.28	46	40	0.87	0.24
381	E_52_J	F	0.67	0.47	0.29	32	28	0.88	0.25
Despont n3	A_65_F	F	0.58	0.47	0.39	49	35	0.71	0.28
Despont n3	E_78_E	F	0.67	0.57	0.62	15	13	0.87	0.54
Maridonne	A_60_Q	F	0.64	0.51	0.28	128	63	0.49	0.14
Maridonne	E_50_P	F	0.73	0.75	0.62	63	47	0.75	0.46
Marigoule	A_42_N	F	0.34	0.29	0.17	44	42	0.95	0.16
Marigoule	A_44_Q	F	0.63	0.59	0.29	29	19	0.66	0.19
Vignols	A_55_S1	F	0.84	0.40	0.14				
		♂-**S**terile	0.83	0.77	0.69	67.1	59.9	0.86	0.65
		♂-**F**ertile	0.64	0.49	0.34	50.8	35.9	0.77	0.28
		Ratio S/F	**1.30**	**1.56**	**2.01**	**1.32**	**1.67**	**1.12**	**2.30**
		***t*-test**	***	***	***	**ns**	**ns**	**ns**	***

[1] Cultivar name of each studied tree
[2] Identification code of each studied tree (ramet)
[3] Sexual type: male-sterile (S) / male-fertile (F)
[4] Fruit sets for 2018, 2019 and 2020
[5] Number of female inflorescences counted during the spring
[6] Number of burrs counted in August
[7] Burr set calculated by dividing the number of burrs by the number of female inflorescences
[8] 2020 pollination success calculated by multiplying 2020 fruit set by the corresponding burr set

bioRxiv preprint doi: https://doi.org/10.1101/2022.08.01.502348; this version posted August 3, 2022. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

LIST OF FIGURES

Figure 1: Degrees of male-sterility observed. A) Fully male-sterile tree. Most stamens are aborted and do not produce pollen. B) Mostly male-sterile tree. Stamens do not protrude from glomerules. C) Partly male-sterile tree. Stamens slightly protrude from glomerules. D) Fully male-fertile tree. Stamen filaments are long and largely protrude from glomerules. These trees produce large amounts of pollen.

Figure 2: Each burr normally contains three fruits. Each pollinated female flower will give a developed fruit, otherwise the fruit will be empty. Empty burr (A) and burrs with one (B), two (C) or three developed fruits (D).

Figure 3: Chestnuts have two types of catkins: many unisexual male catkins that flower first (black) and few bisexual catkins (white) located at the tip of the branches that flower last.

Figure 4: Fruit set of male-fertile (left) and male-sterile trees (right) from INRAE chestnut collection calculated in 2018 (top) and 2019 (bottom).

Figure 5: Fruit set of control (red) and emasculated trees (blue). The three male-sterile varieties are on the left and the three male-fertile varieties on the right.

bioRxiv preprint doi: https://doi.org/10.1101/2022.08.01.502348; this version posted August 3, 2022. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

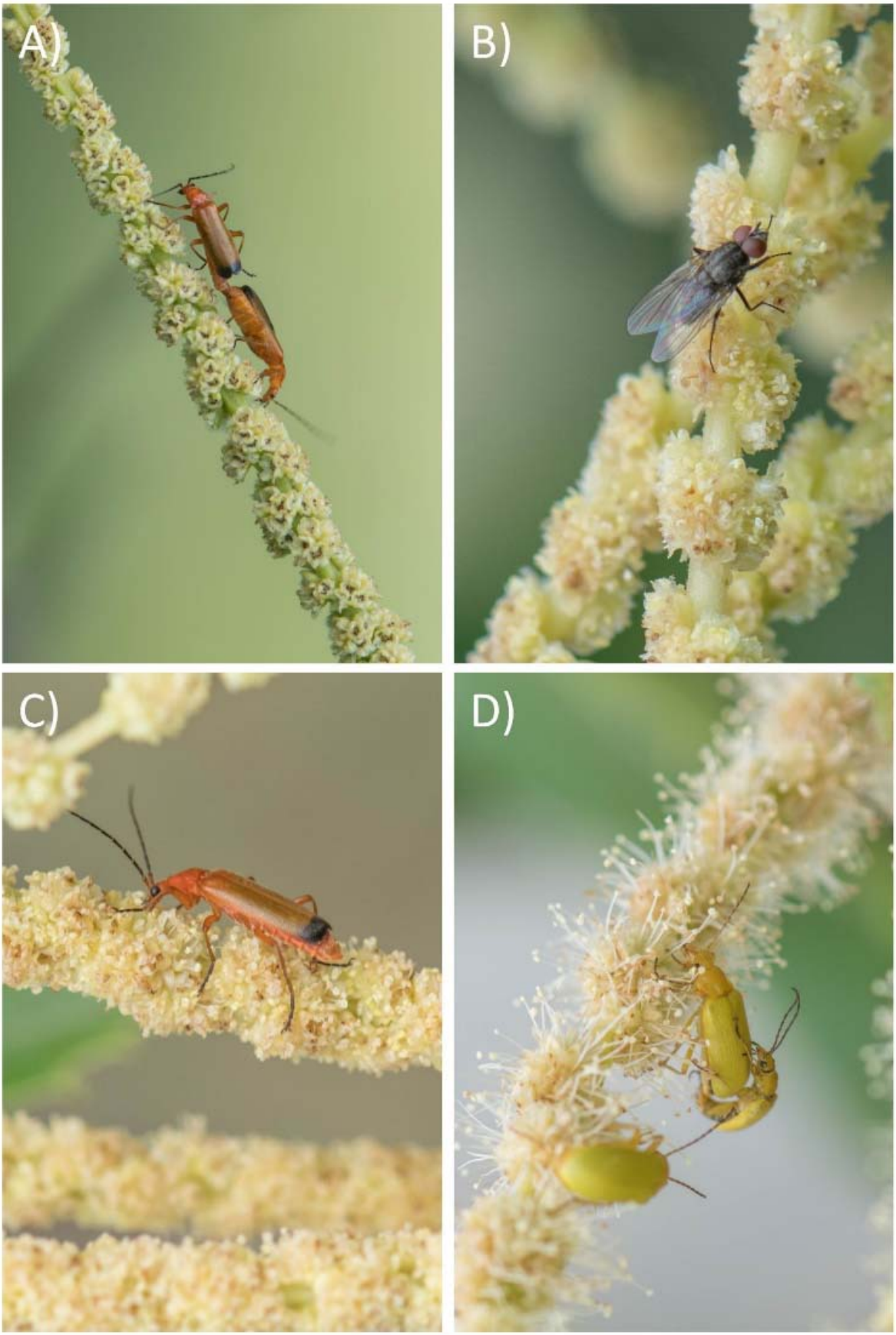


Figure 1: Degrees of male-sterility observed. A) Fully male-sterile tree. Most stamens are aborted and do not produce pollen. B) Mostly male-sterile tree. Stamens do not protrude from glomerules. C) Partly male-sterile tree. Stamens slightly protrude from glomerules. D) Fully male-fertile tree. Stamen filaments are long and largely protrude from glomerules. These trees produce large amounts of pollen.

bioRxiv preprint doi: https://doi.org/10.1101/2022.08.01.502348; this version posted August 3, 2022. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

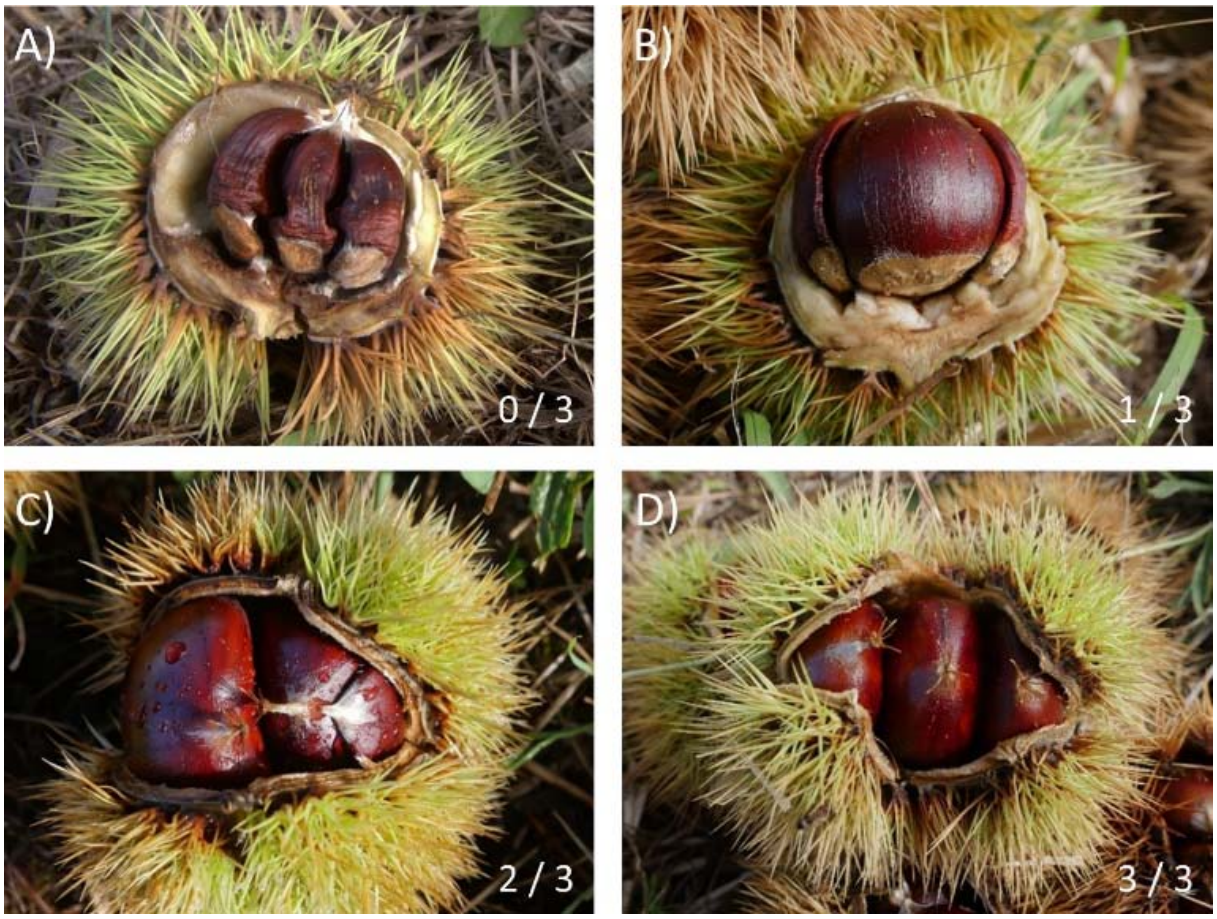


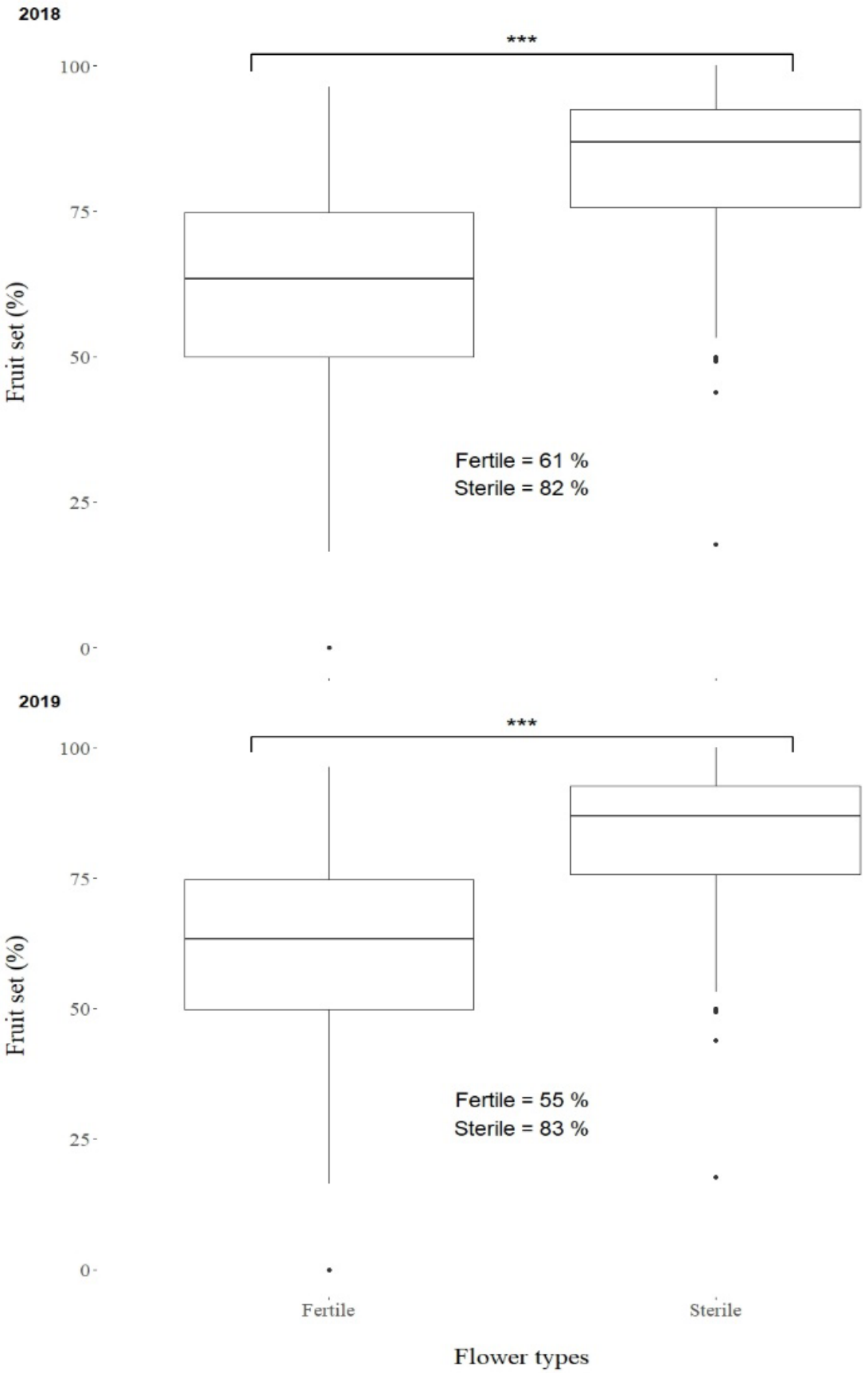
Figure 2: Each burr normally contains three fruits. Each pollinated female flower will give a developed fruit, otherwise the fruit will be empty. Empty burr (A) and burrs with one (B), two (C) or three developed fruits (D).

1

bioRxiv preprint doi: https://doi.org/10.1101/2022.08.01.502348; this version posted August 3, 2022. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

Figure 3: Chestnuts have two types of catkins: many unisexual male catkins that flower first (black) and few bisexual catkins (white) located at the tip of the branches that flower last.

bioRxiv preprint doi: https://doi.org/10.1101/2022.08.01.502348; this version posted August 3, 2022. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.



1 *Figure 4: Fruit set of male-fertile (left) and male-sterile trees (right) from INRAE chestnut collection calculated in 2018 (top) and 2019 (bottom).*

bioRxiv preprint doi: https://doi.org/10.1101/2022.08.01.502348; this version posted August 3, 2022. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

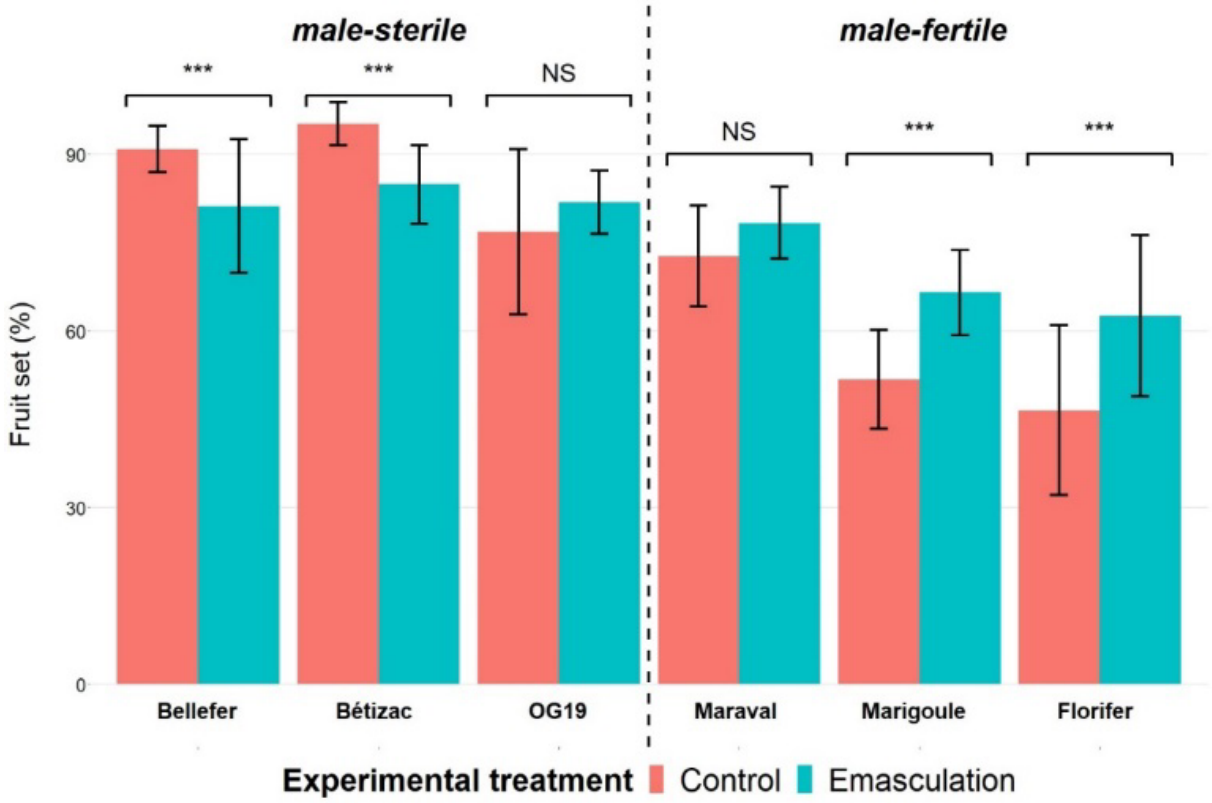


Figure 5: Fruit set of control (red) and emasculated trees (blue). The three male-sterile varieties are on the left and the three male-fertile varieties on the right.

1

bioRxiv preprint doi: https://doi.org/10.1101/2022.08.01.502348; this version posted August 3, 2022. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

ANNEX 1

How to estimate pollination success?

Proof.

This proof was written for this study by Etienne Klein, INRAE researcher at URFM Avignon.

The observations result from x_{tot} observed burrs among which (x_1, x_2, x_3) contain 1, 2 or 3 fruits. Since we did not collect burrs with 0 fruits, $x_1 + x_2 + x_3 = x_{tot}$.

Because we assume that each burr is independent of all the others, we can consider that (x_1, x_2, x_3) follows a multinomial distribution with parameters x_{tot} and (π_1, π_2, π_3), with

$$\pi_1 = \frac{3p(1-p)^2}{1-(1-p)^3}$$

$$\pi_2 = \frac{3p^2(1-p)}{1-(1-p)^3}$$

$$\pi_3 = \frac{p^3}{1-(1-p)^3}$$

where p is the probability for one seed to be formed, to be estimated. Following the definition of multinomial distributions, a maximum likelihood (ML) estimator of p is obtained by maximizing

$$logL(p) = log\left(\frac{x_{tot}!}{x_1!\,x_2!\,x_3!}\pi_1^{x_1}\pi_2^{x_2}\pi_3^{x_3}\right)$$

$$logL(p) = log\left(\frac{x_{tot}!}{x_1!\,x_2!\,x_3!}\right) + x_1 log(\pi_1) + x_2 log(\pi_2) + x_3 log(\pi_3)$$

$$logL(p) = log(K) + x_1 log\left(\frac{3p(1-p)^2}{1-(1-p)^3}\right) + x_2 log\left(\frac{3p^2(1-p)}{1-(1-p)^3}\right) + x_3 log\left(\frac{p^3}{1-(1-p)^3}\right)$$

$$logL(p) = log(K') + (x_1 + 2x_2 + 3x_3)log(p) + (2x_1 + x_2)log(1-p) - x_{tot}log(1-(1-p)^3)$$

The ML estimator can thus be found by solving

$$\frac{\partial logL(p)}{\partial p} = \frac{(x_1 + 2x_2 + 3x_3)}{p} - \frac{(2x_1 + x_2)}{1-p} - \frac{3x_{tot}(1-p)^2}{1-(1-p)^3} = 0$$

And with $q = (1-p)$,

$$(x_1 + 2x_2 + 3x_3)q(1-q^3) + (2x_1 + x_2)p(1-q^3) - 3x_{tot}pq^3 = 0$$

bioRxiv preprint doi: https://doi.org/10.1101/2022.08.01.502348; this version posted August 3, 2022. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

which becomes after several simplifications

$$(x_1 + 2x_2 + 3x_3)(1 + q + q^2) = 3x_{tot}$$

The only positive solution of this second order polynomial equation is

$$\hat{q} = \sqrt{3\frac{x_{tot}}{x_1 + 2x_2 + 3x_3} - \frac{3}{4}} - \frac{1}{2}$$

or

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