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Revisiting pollination mode in chestnut (*Castanea spp.*): an integrated approach

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ABSTRACT

Wind, insects, or both? The pollination mode of chestnuts, an important genus of nut-producing forest trees of the Fagaceae family, is still unclear. We revisit this old question using an integrated approach, focusing on cultivated *Castanea sativa* trees and hybrids in South-western France. We first conducted a large-scale insect isolation experiment. We then monitored 16 trees, focusing on flowering phenology, flower abundance and insect visits. Half of these trees are male-sterile, helping explore the role of pollen in insect attraction. Finally, we characterized the pollination syndrome of chestnuts and contrasted it with that of wind-pollinated oaks using original and published data. Chestnut female flowers have erect styles resembling stamens from male flowers, a probable case of intersexual mimicry. The tree's unusual phenology includes two peaks of pollen production. Pollinator exclusion experiments demonstrated a predominant role of insects in chestnut pollination. Flowering trees attract large numbers of beetles, bees and flies. In contrast, the few insects seen on female flowers (66 in 32 h of observation, <2% of the total) were mostly beetles. Compared to male-fertile trees, male-sterile trees attract fewer insects overall but their female flowers are more frequently visited and they have higher fruit set. All chestnut flower traits examined, such as the tiny pollen grains and the huge rate of pollen production, resulting in the highest pollen/ovule ratio ever reported in plants, are compatible with a beetle pollination syndrome. The high uncertainty of this pollination mode and its convergence with wind pollination explain the pervading confusion regarding chestnut pollination.

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Introduction

> The male flowers of the sweet chestnut were remarkably odoriferous. A fair sized bunch in a room would give fragrance to a whole house. Where would be the use of adding this powerful odour to flowers in mere arrangements for cross-fertilization by the aid of winds? (Meehan 1879).

To characterise accurately the pollination mode of a plant species, empirical observations and experimentations are essential. However, a focus on plants' "*pollination syndrome*" is also relevant, as illustrated by the above comment by Thomas Meehan. Pollination syndromes are recurring suites of floral traits, caused by convergent evolution, that are witnessing the functional pollinator group or abiotic agent servicing the plant (Dellinger 2020). They allow valuable predictive generalizations in pollination ecology (Faegri and Van Der Pijl 1979). The concept traces back to the Italian botanist Delpino (1868-1874), who recognized that unrelated plants that share common pollinators often exhibit similar suites of floral traits. The eco-evolutionary principle underlying pollination syndrome is the "*most effective pollinator principle*" (Stebbins 1970). It posits that, since natural selection is a quantitative process, those pollinators that visit a plant most frequently and effectively in a region should mould the characteristics of its flowers. This should favour correlations between traits, resulting in integrated syndromes. However, some traits can be difficult to interpret and mismatches between apparent pollination syndromes and current suite of pollinators exist, a decoupling that can help retrace recent pollinator shifts (Rosas-Guerrero et al. 2014). Pollination syndromes do not apply only to plants with specialized pollination systems (Stebbins 1970). Yet the question of the existence and prevalence of generalist or mixed pollination strategies (such as ambophily, i.e. adaptation to both wind and animal pollination) combining advantages of different strategies remains (Waser et al. 1996; Culley et al. 2002; Dellinger 2020; Timerman and Barrett 2020). When trade-offs exist between phenotypes that enhance the fitness contribution of one pollinator and phenotypes preferred by other pollination agents, optimization for generalized pollination is only possible when their joint contribution to global pollination fitness is greater than each individual contribution (Strelin et al. 2016). Hence, to interpret evolutionary changes in pollination-related traits, one should examine pollination syndromes with particular care, ideally by comparing related species differing in pollination mode.

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Discussions on pollination syndromes often neglect abiotic agents of pollination, yet wind-pollinated species represent a fair share of plant species on Earth. At least 16,700 angiosperm species are wind-pollinated, i.e. 5.5% to 6.4% of the estimated species of angiosperms (Renner 2014). Distinguishing animal- from wind-pollinated plants is generally considered straightforward. For example, Hall and Walter (2011) argue that "*the divide between wind and animal pollination seems relatively robust, given the specialist adaptations [...] required for wind pollination to be effective*". Similarly, Friedman and Barrett (2009) explain that "*the traits associated with wind pollination [...] are well established and tend to be less variable, and perhaps for this reason the notion of a 'wind pollination syndrome' has not attracted much scepticism*". One of the features most frequently associated with wind-pollinated species is that they invest heavily in pollen production, as wind pollination is an "*inherently wasteful process as the chances of pollination are meagre*" (Mangla and Gupta 2015).

Nevertheless, difficulties to distinguish wind from insect pollination exist. For instance, in the past, palms were considered to be mainly wind pollinated and to form the functional counterparts of dicotyledon catkin bearers (Delpino 1868-1874). Their inflorescence and the small size, plethoric production and particular morphology of their pollen grains supposedly fitted with a wind pollination syndrome. However, a revised assessment showed that palms are predominantly entomophilous and that two groups of pollinating beetles are closely associated with them (Henderson 1986). Similarly, field studies that have quantified airborne loads of *Cycas* pollen have all concluded that wind pollination could be effective in these plants. Yet Hall and Walter (2018) have shown that "*Cycas species are poorly adapted for wind pollination in ways that imply that insects have been the primary pollinators of these plants over a long evolutionary history*". These examples question the longstanding belief that wind- and animal-pollination syndromes are easy to tell apart. In fact, for Sargent and Otto (2004), "*forcing the data into the false dichotomy of biotic pollination [...] versus abiotic pollination may have obscured the true patterns. For example, selection by beetle pollinators may be as different from that of bee pollinators as they both are from abiotic pollination.*"

Like palms and cycads, chestnuts (genus *Castanea* Mill., Fagaceae) illustrate the difficulties to interpret plant traits in terms of evidence for one pollination vector over another. Both Sprengel (1811) and Delpino (1868-1874) suggested that chestnuts are wind-pollinated. Later, Groom (1909) argued that the showy, erect, scented male catkins and the numerous insect visitors point towards insect-pollination. Nevertheless, he considered that the inconspicuous and odourless female flowers, lacking reward for insect visitors, and bearing styles agreeing in size and position with the large stigmas of wind-pollinated flowers, suggest wind could also be involved. In one of the most complete investigation on chestnut pollination to date, Porsch (1950) summarized the attributes of this tree suggesting an entomophilous or anemophilous strategy. The former include the presence of nectar and nectaries, the stickiness of pollen, and the tiny size of the stigmas, as well as the frequent visits by beetles to flowering chestnuts. The latter include the massive production of male flowers, their somewhat reduced fragrance, the pollen that eventually becomes less sticky, as well as the lack of attractive power of female flowers. He concludes that chestnut exemplifies a case of ancient beetle-pollinated tree evolving towards wind-pollination, a stage already reached by some of its relatives, such as oaks and beeches. Subsequent investigations included various attempts to determine experimentally the pollination mode of chestnuts. In particular, in the USA, Clapper (1954) relied on emasculation and pollinator exclusion experiments and arrived at the strong but premature conclusion that chestnuts are largely wind pollinated. This work had much influence on subsequent studies. For instance, in Italy, Manino et al. (1991) observed many insects on chestnut flowers, more than half of which were honeybees (*Apis mellifera*), and performed experiments that showed a major role of insects in pollination. Nevertheless, they merely conclude that "*the action of insects may be considered useful above all in years when the climate does not favor an effective wind pollination*". Abrol (2015), summarizing the state of knowledge on chestnut pollination for fruit production purposes, insists, despite limited evidence, that most insects except honeybees do not occur in greater frequency during chestnut flowering than noted in other wind-pollinated plants. He recommends to establish honeybee-colonies in chestnut orchards and to include a sufficient proportion of well-distributed pollenizer trees for effective pollen dispersal by wind. Clearly, there is a need to re-evaluate chestnut pollination.

Chestnuts are particularly interesting models to investigate pollination. Chestnut species are monoecious, thereby facilitating the evaluation of pollinators' efficiency by determining those that visit not only male but also female flowers. Monoecy also allows exploring if rewardless female flowers have evolved to mimic rewarding male flowers to attract pollinators (Willson et al. 1989). Second, the presence in chestnuts of both male-sterile and male-fertile individuals (Pereira-Lorenzo and Ramos-Cabrer 2004) is ideal to test the role of pollen in insect attractiveness. Third, chestnuts have a complex flowering phenology, named duodichogamy, where each plant produces two batches of male flowers and a batch of female flowers that are temporally separated, an attribute shared by only a handful of species (Stout 1928; Renner 2014). This feature could help clarify pollination mechanism.

Finally, the existence of closely related and well-investigated wind-pollinated congeners, the oaks, is a nice opportunity to study the evolution of pollination modes.

Our objective in this paper is to re-examine the pollination mode of chestnut. This knowledge is key to better design and manage chestnut orchards, to evaluate ecological services provided by natural or semi-natural chestnut ecosystems, and to investigate the evolution of reproductive traits in Fagaceae. It should also help revisit pollination syndromes of species producing abundant wind-dispersed pollen. For this purpose, we use an integrated approach including an experimental set-up (classical pollinator exclusion experiments) and observations of insect visitors. We then focus on the role of pollen and of flowering phenology in insect attraction. Furthermore, we clarify if visits to female flowers are taking place and why. Finally, we make a new attempt to characterize the pollination syndrome of chestnuts by comparing it with that of wind-pollinated oaks.

Material and methods

Study species

Chestnuts belong to the Fagaceae family, which includes about 1000 species that dominate subtropical, Mediterranean and temperate forests of the Northern hemisphere. The Fagaceae family includes wind-pollinated tree species such as oaks *Quercus spp*. and beeches *Fagus spp*., and insect-pollinated ones, such as stone-oaks *Lithocarpus spp*., tanoaks *Notholithocarpus spp*. and Asian chinkapins *Castanopsis spp*. (Manos et al. 2001, 2008). With only seven species, the chestnut genus (*Castanea*) is among the smallest of the family. However, it encompasses economically and ecologically significant tree species, such as the American chestnut *C. dentata*, an iconic tree of the eastern part of North America that was devastated by the chestnut blight in the first half of the 20th century; the blight-resistant Chinese chestnut *C. mollissima*, an understory tree cultivated in East Asia for millennia for its nuts and currently the most widely cultivated chestnut worldwide; and the Japanese chestnut *C. crenata*, an important tree in Japan and elsewhere for its heavy production of sweet, edible nuts (Pereira-Lorenzo et al. 2012). All chestnut species have the same number of chromosomes, are self-incompatible and readily hybridize (Pereira-Lorenzo et al. 2019). According to Manos et al. (2001), the uniqueness of chestnuts lies in their female flowers, which always have six or more styles, compared to three in the other Fagaceae. The genus *Castanea* is closely related to *Castanopsis*. Both are sister to *Notholithocarpus* and to *Quercus*, the only purely wind-pollinated species in this clade (Oh and Manos 2008). A comparison of the pollination syndrome of chestnuts with that of the well-investigated oaks (genus *Quercus*) appears therefore particularly relevant.

The sweet chestnut (*Castanea sativa* Mill.) is the only native European chestnut species and an important multipurpose tree in the Mediterranean region. Used for its wood, fruit, honey, and tannin, it has played a major role in rural development. It still shapes the landscapes of several hilly Mediterranean regions in Italy, France, Portugal, Spain or Switzerland, covering an area of over 2.5 million hectares (Conedera et al. 2016). Named in France "arbre à pain" (Pitte 2014), it has been used for thousands of years for its nuts. Its domestication, based on grafting of trees selected in the field, is still ongoing (Pereira-Lorenzo et al. 2019). This has resulted in an intermingling of wild and domestic gene pools, to the point that it has become extremely difficult to trace its natural distribution (Conedera et al. 2004).

Chestnuts are mass-flowering trees (Figure 1) with small flowers grouped in inflorescences named catkins. At the flower level, chestnuts are monoecious. Instead, at the inflorescence level, chestnuts are andromonoecious. At the tree-level, the widespread occurrence of male-sterile and male-fertile trees (called respectively astaminate and longistaminate) along with trees with intermediate male fertility (Pereira-Lorenzo et al. 2016) suggest that *C. sativa* is gynodioecious (Figure 2). Crossing and chloroplast sequencing studies have demonstrated a cytoplasmic origin for this male sterility (Sisco et al. 2014). The male flowers of male-sterile trees have aborted anthers but still produce nectar, thereby remaining attractive to insects.

The female flowers of chestnuts are remarkable. They are grouped by three, each having 6–8 styles with tiny, crater-like wet stigmas at their tips. The number of styles matches with the number of locules in the ovary, each locule having its own conducting tissue and hosting two ovules (Feijó et al. 1999; Shi and Stösser 2005). Hence, there are about 12–16 ovules per ovary even if only one embryo is typically formed in a nut. Secretory cells layer the aperture of the stigma at full receptivity, which peaks several days after the onset of flowering (Nienstaedt 1956; Feijó et al. 1999). The receptivity period of each stigma is restricted to about two days, corresponding to the presence of a mucilaginous secretion. Only one style at a time becomes receptive, greatly increasing the overall receptivity period of the female flower (Feijó et al. 1999).

According to Cannon (2001), in the related stone oaks (*Lithocarpus*), the "*successful pollination of the female flowers is a mystery as they provide little attraction to pollinators. During many hours of observation, I recorded a single visit by a potential pollinator to a female spike, which appeared to be accidental*". The pollination of chestnut female flowers remains equally mysterious. For Johnson (1988), who studied the

Figure 1. Difference between male-fertile and male-sterile genotypes during flowering. This male-sterile tree received a graft from a male-fertile tree. The male-fertile genotype has long conspicuous catkins (right part of the canopy). The male-sterile genotype has less conspicuous green catkins (left part of the canopy).

pollination mode of *C. pumila* (L.) Mill. in the southeastern United States, "*diurnal and nocturnal observations failed to detect [insects] on pistillate flowers*". Giovanetti and Aronne (2011) tracked 23 honeybees visiting chestnut trees in an Italian orchard and recorded no visit to the female flowers. Zirkle (2017) surveyed both diurnal and nocturnal insects on *Castanea ozarkensis* Ashe and observed a single honeybee moving from a male catkin to a female flower of the same tree. However, Porsch (1950) argued that the tiny secretion produced by stigmas could represent a small reward for pollinating insects.

Insect exclusion experiment

Study site

We conducted this experiment in the late spring of 2019 in the chestnut orchard of the INVENIO experimental station in Douville (45.019723 N, 0.614637 W). This orchard, where several honeybee colonies are established, comprises 12 ha of chestnut plantations surrounded by deciduous broadleaved forests dominated by wild chestnuts and by pine plantations.

Plant material

We selected six *C. sativa* × *C. crenata* hybrid varieties: three male-fertile (staminate) varieties, Marigoule, Florifer and Maraval, and three male-sterile varieties, Bouche de Bétizac (hereinafter "Bétizac"), Bellefer and OG19. These trees grow in two orchards located side by side. The first is composed of ca. 20 m-high adult trees belonging to Marigoule and Bétizac varieties, while the second is composed of 8-year-old, ca. 6 m-high trees belonging to the four other varieties.

Experiment

To study the role of insects in chestnut pollination, we used two experimental groups. In the control group, open-pollinated flowers were accessible to both wind- and insect-transported pollen. In the treatment group, we enclosed all flowers in completely insect-proof polyester tulle nets with 400 µm × 700 µm openings (Diatex F550P; https://www.diatex.com/fr/diatex-produit/f510/). Hence, in the absence of apomixy (Shi and Stösser 2005), only airborne pollen can fertilize female flowers. The young trees received one modality each, with five trees per variety and per modality. Ten branches per tree were equipped with nets for the caged modality (Figure 3a). The Marigoule variety was represented by eight adult trees and Bétizac by five adult trees. Each of these trees received ten replicates of both treatments, allowing a more precise comparison between treatments than for the young trees (Figure 3b). Altogether, we used 65 trees. The insect-proof nets were set up in the spring before flowering. In the fall, we enclosed all burrs from both treatments in large nets to prevent fruit loss before evaluation of fruit set.

Fruit set and pollination success

In *C. sativa* and its hybrids, the female inflorescences are typically composed of three female flowers. If pollinated, each flower gives one nut typically including a single seed;

Figure 2. Difference between male-fertile and male-sterile trees (details). a) Bisexual catkin, with a female inflorescence (the future burr) at the basis and a fertile male inflorescence at the distal end of the catkin. b) Honeybee (*Apis mellifera*) collecting pollen on a male-fertile catkin, with tiny anthers borne on long stamens. c) Couple of red soldier beetles (*Rhagonycha fulva*) on a male-sterile catkin, with brown, aborted anthers borne on short stamens that do not protrude from the flowers.

otherwise, it gives an empty nut. Hence, to measure fruit set, we collected all the burrs in each treatment and counted the proportion of filled nuts. However, pollination success is the result of both fruit set (the average proportion of developed nuts per burr) and burr set (the proportion of inflorescences giving a burr). In our large-scale study, it was impractical to count the number of female inflorescences before setting up the nets. Instead, we estimated the reduction in pollination success by dividing the total number of developed fruits in the treatment by the total number of burrs produced in the control. Overall, to estimate fruit set and pollination success, we examined the content of over 1600 burrs.

Comparison with other insect exclusion experiments

We found mentions of five other pollinator exclusion experiments conducted on chestnuts, published in articles, conference reports or theses. In the USA, Clapper (1954) worked on Asian chestnuts species and hybrids, Johnson (1988) on *C. pumila* and Zirkle (2017) on *C. ozarkensis*, in Italy Manino et al. (1991) investigated *C. sativa, C. crenata* Siebold & Zucc. and *C. sativa* × *C. crenata* hybrids, and in Portugal de Oliveira et al. (2001) focused on *C. sativa* and *C. sativa* × *C. crenata* hybrids. All studies except that of Zirkle (2017) were performed in plantations. We discarded Johnson's (1988) study because no data was provided by the author

Figure 3. Insect-proof netting experiment. a) Nets on 8-years old trees. b) Nets on adult trees.

and Clapper's (1954) study because of the lack of repetitions and the questioning choice of control trees.

Flowering and insect monitoring

Study site and plant material

We carried out this part of the study in southwestern France (44.788319 N, −0.577062 E), in the INRAE chestnut genetic resources collection, which includes 237 trees belonging to *C. sativa, C. crenata, C. mollissima* Blume and their hybrids. These trees grow in two nearby orchards. The first was planted in 1970 and comprises 29 widely spaced trees on 2.3 ha. In 2019, six honeybee colonies were present in this orchard. The second orchard was planted in 1990. It includes 211 trees on 3.5 ha. We selected 16 trees in these orchards for flowering and insect monitoring, half of which are *C. sativa* and half *C. sativa* × *C. crenata* hybrids. In each taxa, there were four varieties, two male-fertile and two male-sterile ones, each represented by two ramets. The eight selected varieties were Marigoule (*C. sativa* × *C. crenata* hybrid, male-fertile), Maridonne (*C. sativa* × *C. crenata* hybrid, male-fertile), Bétizac (*C. sativa* × *C. crenata* hybrid, male-sterile), Marlhac (*C. sativa* × *C. crenata* hybrid, male-sterile), Despont n°3 (*C. sativa*, male-fertile), CA381 (*C. sativa*, male-fertile), Précoce des Vans (*C. sativa*, male-sterile), and Dauphine (*C. sativa*, male-sterile). The selected trees had easily accessible canopies to facilitate monitoring of visiting insects.

Phenology monitoring

To study the flowering phenology of the 16 trees, we used the approach outlined in Larue et al. (2021). We monitored each tree twice a week throughout the flowering period immediately before surveying arthropod visitors. We assigned three scores: one for male flowers of unisexual catkins, one for female flowers and one for male flowers of bisexual catkins (Figure 4). To identify flower-visiting arthropods (see below), we defined the flowering

Figure 4. Chestnut flowering shoot. There are two bisexual catkins at the tip, each with a single female inflorescence, and eight unisexual male catkins at the basis. We monitored the phenology of male unisexual catkins, of female inflorescences and of the male part of bisexual catkins. Note the large difference in the development of the two types of male catkins: whereas all but one unisexual catkins are in full bloom, the bisexual ones are still growing.

period as the period starting with the blooming of the first male flowers of unisexual catkins and finishing with the wilting of the male flowers of the bisexual catkins.

Flowering architecture of chestnut

To assess chestnut flowering architecture, we measured density and length of male catkins, density of female flowers, ratio of pollen-releasing and pollen-receptive reproductive surfaces, and relative importance of first and second peak of pollen emission. In spring 2020, we selected 10 branches per tree on each of the 16 trees monitored for insects in 2019. One of these trees did not produce any female flowers in 2020, so we replaced it with a clonal replicate from the same variety. On all these branches, we measured the branch section and counted the unisexual and bisexual catkins present on all annual shoot growth units. For bisexual catkins, we measured the length and diameter of the male catkin, the number and area (length × width) of female inflorescences (corresponding to three flowers each), and the distance between male and female inflorescence (herkogamy). We also cut five branches per tree. In the laboratory, we measured their section, the density of unisexual male catkins and their lengths. To estimate male flower density, we sampled four catkins per tree and for each of them counted all flowers on a 0.5 cm-long section under a binocular. For each parameter, we provide the mean, minimum and maximum values across all trees, unless otherwise specified.

The ratio of the number of pollen grains to the number of ovules in the flowers of a plant is an indicator of its breeding system as it reflects the likelihood of sufficient pollen grains reaching each stigma. The more efficient the transfer of pollen, the lower the pollen/ovule ratio (Cruden 2000). To estimate this ratio in chestnut, we assumed that each ovary harbours an average of 12 ovules (Feijó et al. 1999). We then relied on our estimates of the proportion of each type of flowers for the eight male-fertile trees, as described in the previous paragraph, along with published data on number of stamens per male flower and of number of pollen grains per anther (Mert and Soylu 2006). These authors studied four male-fertile chestnut cultivars and found a mean number of stamens per flower of 11.8 and an average of 4712 pollen grains per anther.

Insect observations

We monitored arthropod visits on the 16 trees before, during and after flowering in June and July 2019. We relied on a non-destructive approach using macrophotography. We visited all trees every 3 or 4 days during six weeks, inventorying all arthropods larger than 2 mm. The procedure was adapted from that used in the French citizen science program Spipoll (Deguines et al. 2012). One session on each tree lasted 20 min, during which we actively searched the accessible part of the canopy (< 2 m high) for insects and arachnids on flowers and leaves. During each session, we counted each arthropod making contact with the tree and photographed at least one individual of each taxon observed with an APS-C camera (Nikon D500, Nikon D7200 and Fujifilm X-T3) equipped with

a macro lens objective (AF-S VR Micro-Nikkor 105 mm f/2.8 G and Fujinon XF 80 mm f/2.8 R LM OIS W Macro) for subsequent taxonomic confirmation. We counted separately all insects making contacts with the female flowers. Each daily session lasted approximately from 10:00 to 15:00 hour, with two observers surveying eight trees each. The order of tree visits and their allocation to each observer differed each time. We then edited the photographs, annotated them (metadata included observer name, day and time of observation, identity of the tree surveyed and its phenological stage) and sorted them in a photo library for validation. For arthropod taxonomic identification, we relied on the Spipoll website (http://spipoll.snv.jussieu.fr/mkey/mkey-spipoll.html) and on "Le monde des Insectes" (https://www.insecte.org/).

Pollination syndrome

To establish the pollination syndrome of chestnut, we relied on previous descriptions of wind-, insect- and beetle-pollination syndromes (Gottsberger 1977; Faegri and Van Der Pijl 1979; Culley et al. 2002; Friedman and Barrett 2009; Mangla and Gupta 2015). We searched for relevant published data on chestnuts and oaks (genus *Quercus* L.) and completed this with our own observations. We distinguished directly observable and measurable plant traits typically used to establish pollination syndrome sensu stricto from other plant features (pollen dispersal ability, pollen nutritional value to insects, plant distribution, plant genetic structure, etc.) that indirectly help characterize the pollination mode.

Statistical analyses

We decided to use parametric or nonparametric statistical methods after applying a Shapiro-Wilk test to the data, which we visually inspected using four diagnostic plots: Residuals vs fitted values, Q-Q plot, Scale location plot and Cook's distance plot.

Insect exclusion experiments

To assess the role of tulle nets on fruit production in each experiment, we used a Fisher exact test on a two-way table containing the number of developed and empty fruits for the control and the treatment. To compare fruit set between varieties, we performed a bidirectional Wilcoxon rank-sum test to compare fruit set across ramets of each variety.

Insect monitoring

To test if flowering trees attract arthropods, we compared the average number of arthropods per collection seen on each tree during and outside the flowering period. We then performed a unidirectional paired-Wilcoxon rank-sum test across all 16 trees to check if arthropods were indeed more abundant during flowering. To test if some insect species were relatively more frequent on female flowers than on whole trees, we applied a Fisher's exact test on a two-way table with the abundance of each insect versus all other insects on trees versus on female flowers. To check whether some insect species were more attracted by male-fertile than by male-sterile trees, we applied a bidirectional unpaired Wilcoxon rank-sum test to compare insect mean abundance per collection across the two groups of trees. We then used a Fisher's exact test to check if insect preferences for male-fertile versus male-sterile trees varied according to the scale of observation: whole tree or female flowers. Finally, we investigated when insects visit female flowers. For each tree, we subdivided all collections made during the flowering period into two groups. The first group included all collections where we saw at least one insect on female flowers. The second group included all collections where we saw no insect on female flowers. We then compared the number of days elapsed since the onset of flowering between the two sets of collections using a bidirectional Wilcoxon rank-sum test. All statistical analyses were performed in R (R Foundation for Statistical Computing, Vienna, Austria), and all graphics were constructed with the package ggplot2, ggthemes and cowplot.

Results

Insect exclusion experiment

Fruit set of the six studied varieties

Average fruit set was 69% in the control (open pollination) and only 22% for the netted branches (Figure 5, Appendix 1). Fruit set thus dropped by 70% when preventing insects to contact female flowers (Fisher's exact test, $p < 10^{-15}$). In open-pollinated branches of male-fertile trees, average fruit set was 48% compared to 89% in male-sterile ones. In netted branches compared to open-pollinated ones, fruit set decreased by 57% in male-fertile trees and by 75% in male-sterile ones.

Comparison with other studies

Previous insect exclusion studies also reported a marked decrease in fruit set in chestnuts (Figure 6). In Manino et al. (1991) experiment, overall fruit set was quite low: 18% for the controls and 5% for netted branches, representing a 74% drop in fruit set (Fisher's exact test, $p < 10^{-8}$). In the de Oliveira et al. (2001) experiment, the control fruit set was 57% and the netted fruit set 12%, a drop of 79%. In the study of Zirkle (2017), the control fruit set was 95% while the netted fruit set was 52%, a drop of 54% (Fisher's exact test, $p < 10^{-5}$). de Oliveira et al. (2001) used two types of nets differing in mesh size. The average fruit set of

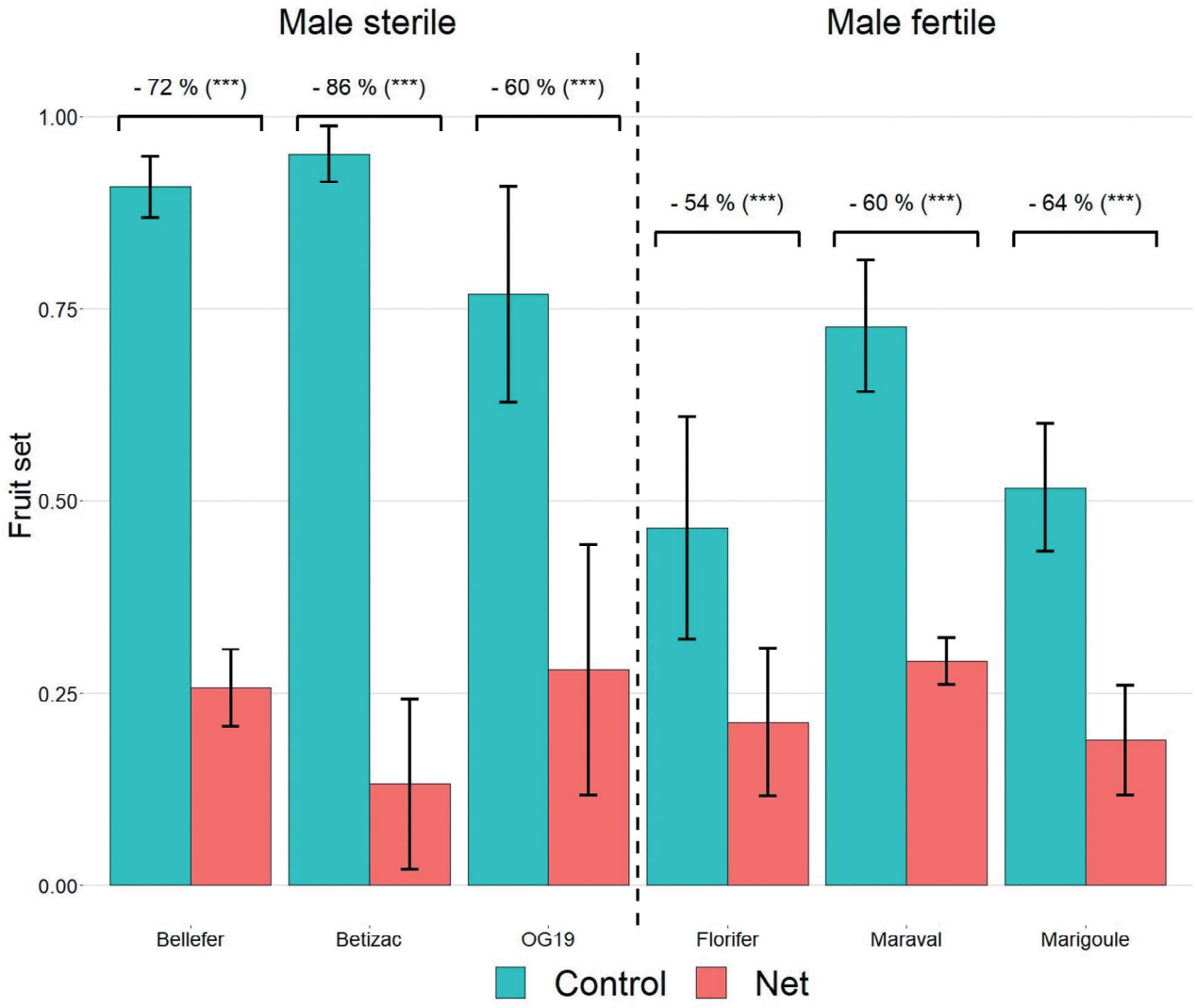

Figure 5. Insect exclusion experiment: comparison between male-sterile trees (on the left) and male-fertile trees (on the right). Average fruit set and standard deviation are measured on control and netted branches of six chestnut varieties including three male-sterile varieties and three male-fertile ones.

the fine net treatment was 7% compared to 12% for the net with larger mesh size, a significant difference in three of the eight studied varieties (results not shown). Zirkle's (2017) study also included another treatment (pollination bags). The fruit set in the bags (40%) did not differ significantly from that found in the netting treatment (52%).

Pollination success

For Marigoule and Bétizac cultivars, we could correct for differences in burr set, providing a better estimate of overall pollination success (see Material and Methods). Pollination success for the treatment was larger for Marigoule (19%) than for Bétizac (9%). However, there were large differences in pollination success across individual trees (i.e. ramets) of each variety, so the difference was not significant (Wilcoxon test, $p = 0.14$). The overall reduction in pollination success was 70% for Marigoule and 91% for Bétizac (Figure 7). These values are comparable to those of de Oliveira et al. (2001) and Zirkle (2017), who assessed both burr set and fruit set per burr. In the de Oliveira's experiment, pollination success was 41% for the control, 5% for the large net treatment and 3% for the fine net treatment. The average reduction in pollination success between the control and large net treatments reached 88%. In Zirkle's experiments, the pollination success was 72% for the control, 18% for the netting treatment and 12% for the bagging treatment. The average loss in pollination success between control and netting treatment reached 75%.

Monitoring flowering phenology and pollination

Flowering phenology

We monitored phenology from early June (day 152, shortly after the earliest trees had started to bloom), to mid-July (day 190) (Figure 8). The latest trees were in full bloom 10 days after the earliest. Male unisexual catkins bloomed first (peak 1). About 15 days later,

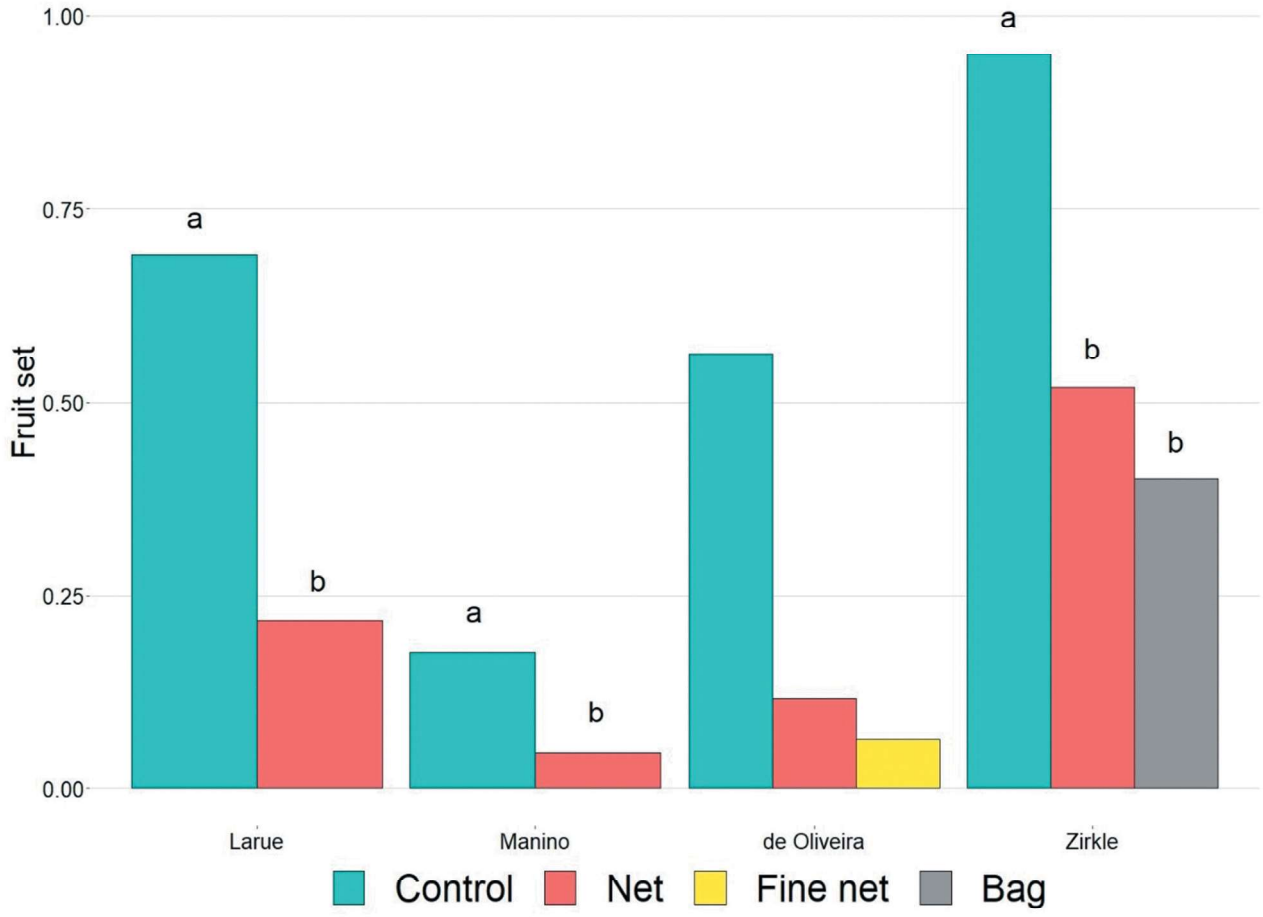

Figure 6. Comparison of fruit set in four different insect exclusion experiments (this study; Manino et al. 1991; de Oliveira et al. 2001; Zirkle 2017). The x-axis gives the name of the first author of the study. Different letters indicate significant differences within an experiment.

male flowers of bisexual catkins started to bloom (peak 2). The female flowers were receptive during two to three weeks, resulting in a long overlap between the male and female flowering periods. There was no clear difference in flowering phenology between male-fertile and male-sterile trees.

Flowering characteristics of chestnut trees

We sampled an average of 47 (7–157) bisexual catkins and 978 (274–1779) unisexual male catkins per tree. Mean length of the male part of bisexual catkins was 9.5 cm (6.6–13.0) and mean length of unisexual male catkins was 13.1 cm (6.7–17.7). Both types of male catkins harboured an average of 40 (31–48) male flowers per centimetre of catkin. The mean circumference of these male catkins in male-fertile plants was 1.3 cm (1.1–1.5). There were generally one and up to three female inflorescences (on average 1.3) per bisexual catkin, comprising typically three female flowers each. The upper receptive surface of a female inflorescence (about 20 styles, 5–8 per flower) was 68 mm2 (44–89). On a bisexual catkin, the average distance separating the female flowers from the male flowers was 1.6 cm (1.1–2.1). In terms of sex ratio, there were 29 (11–78) unisexual male catkins for every bisexual catkin. Hence, for each female flower, there were on average 4000 male flowers (1300–6200). In terms of area that would be visible to insects (surface of the cylindrical male catkin versus surface of the female inflorescences) in male-fertile trees, the ratio was 2300 (1100–3800).

The ratio of male flowers (and hence of pollen and/or nectar) produced in peak 1 versus peak 2 was 41 (13–109), that is, most male flowering (>97%) took place during peak 1. The pollen/ovule ratio, estimated in eight male-fertile chestnut trees, ranged from 10 million to 29 million, with an average of 21 million.

The female flowers are strongly reminiscent of male flowers: their numerous white erect styles are similar in appearance and colour to the stamens of male flowers (Figure 9c). The clustering of female flowers in small inflorescences reinforces the impression that we are dealing with a portion of a male catkin. We found some rare catkins with only female flowers. When comparing such female catkins with normal bisexual ones, the similarity between male and female flowers was particularly striking (Figure 9(a,b)).

Arthropod visitors

Overview. We observed and counted arthropods on the 16 trees during 162 sessions of 20 minutes,

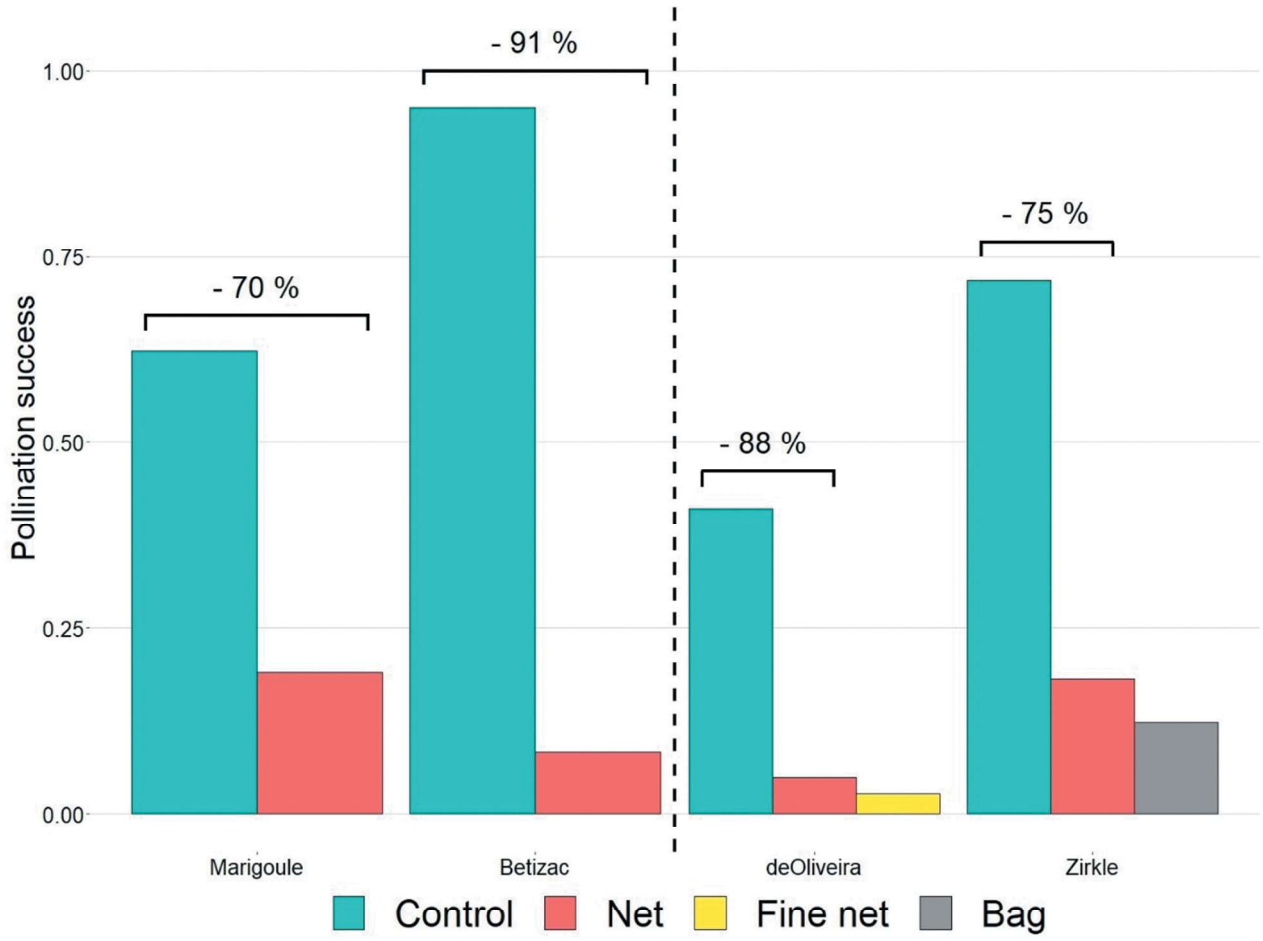

Figure 7. Comparison of pollination success (combining the effects of burr set and of fruit set/burr) in different insect exclusion experiments. On the left, results from this study for two varieties, a male-fertile one (Marigoule) and a male-sterile one (Bétizac). On the right, experiments by de Oliveira et al. (2001) and by Zirkle (2017).

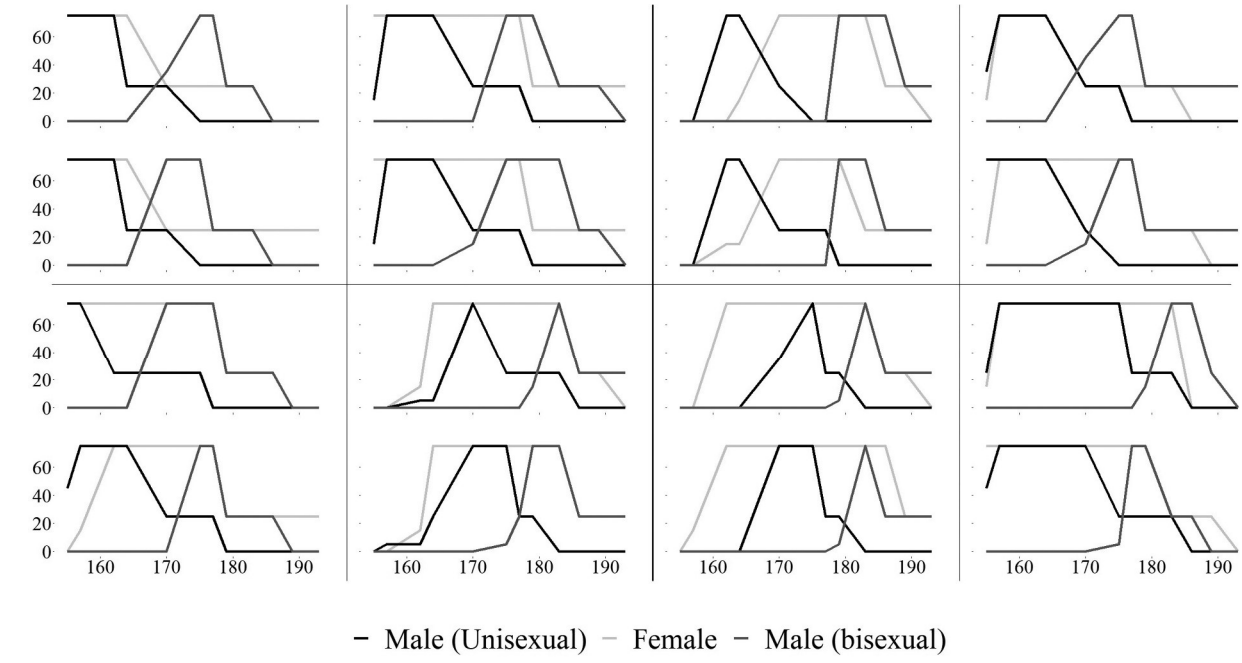

Figure 8. Phenology of the 16 monitored chestnut trees. Left, male-fertile trees. Right, male-sterile trees. The two clonal replicates from each of the eight varieties are represented one above the other. For each diagram, x-axis is expressed in Julian days and y-axis in percentage of open flowers.

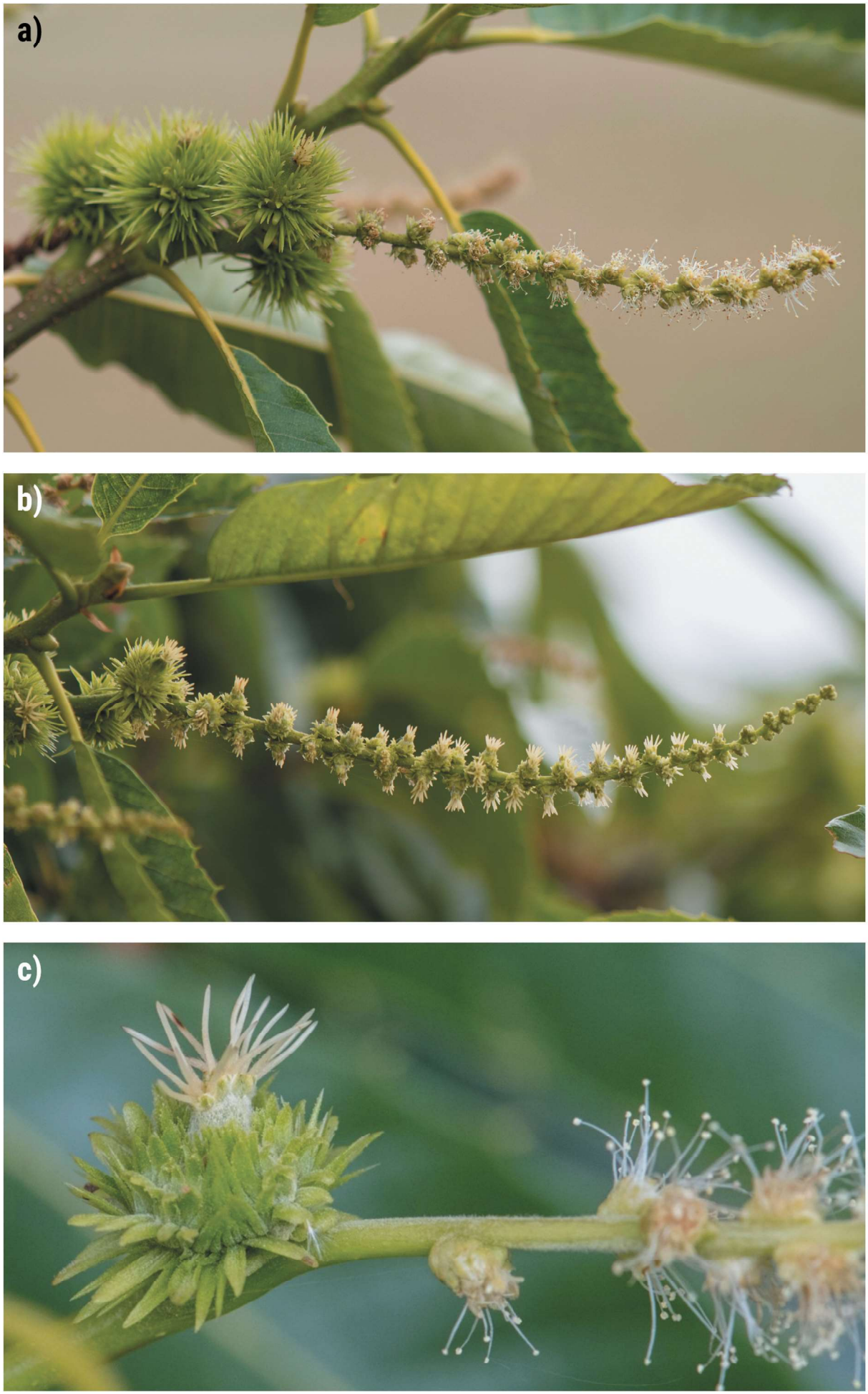

Figure 9. The case for automimicry. a) Normal bisexual catkin, with male flowers in bloom. b) Abnormal catkin, where male flowers are replaced by female flowers. Note the overall similarity with the normal bisexual catkin above. c) Close-up of the basis of a bisexual catkin. Note the similar aspect of the styles and stamens (length, colour, and grouping).

Table 1. List of the main arthropod taxa observed on chestnut trees and on female flowers, and comparison of their abundance on flowering and non-flowering trees and on male-fertile and male-sterile trees.

Taxon[1]	Order	Family	#coll	N	N/col	x flower	flower	test	w pol	w/o pol	test	♀ fl
Synema globosum	Arachnida	Thomisidae	32	37	0.23	0.11	0.28	*	0.29	0.26	ns	0
Arachnida	**Arachnida**		**63**	**99**	**0.61**	**0.54**	**0.64**	**ns**	**0.66**	**0.61**	**ns**	**0**
Coccinella septempunctata	Coleoptera	Coccinellidae	27	69	0.43	0.02	0.59	**	0.58	0.60	ns	2
ladybird	Coleoptera	Coccinellidae	13	16	0.10	0.02	0.13	*	0.10	0.16	ns	1
ladybird larva	Coleoptera	Coccinellidae	47	96	0.59	0.74	0.53	ns	0.59	0.47	ns	1
Paracorymbia fulva	Coleoptera	Cerambycidae	16	34	0.21	0.00	0.29	**	0.54	0.04	ns	1
Oedemerid beetle	Coleoptera	Oedemeridae	13	17	0.10	0.02	0.14	*	0.20	0.07	ns	0
Cteniopus sulphureus	Coleoptera	Tenebrionidae	28	203	1.25	0.00	1.75	**	3.10	0.35	ns	2
Rhagonycha fulva	Coleoptera	Cantharidae	110	1500	9.3	1.5	12.3	***	11.4	13.3	ns	42
Small beetles	**Coleoptera**		**46**	**56**	**0.35**	**0.07**	**0.46**	***	**0.66**	**0.25**	**ns**	**0**
Larger beetles	**Coleoptera**		**74**	**275**	**1.70**	**0.07**	**2.34**	**	**4.14**	**0.49**	*	**3**
Coccinellidae	**Coleoptera**		**97**	**229**	**1.4**	**1.0**	**1.6**	**	**1.6**	**1.5**	**ns**	**6**
Coleoptera	**Coleoptera**		**140**	**2062**	**12.7**	**2.7**	**16.7**	***	**15.6**	**17.8**	**ns**	**51**
Sphaerophoria scripta	Diptera	Syrphidae	28	45	0.28	0.09	0.35	*	0.25	0.46	ns	1
Syrphid flies	Diptera	Syrphidae	12	19	0.12	0.07	0.14	ns	0.25	0.02	*	0
Tachinid fly #1	Diptera	Tachinidae	85	218	1.35	1.13	1.43	*	1.25	1.61	ns	2
Minettia	Diptera	Lauxaniidae	35	46	0.28	0.15	0.34	*	0.37	0.30	ns	1
Helina reversio	Diptera	Muscidae	35	50	0.31	0.39	0.28	ns	0.37	0.18	ns	2
Sarcophaga	Diptera	Sarcophagidae	14	21	0.13	0.11	0.14	ns	0.19	0.09	ns	1
Pachygaster atra	Diptera	Stratiomyidae	28	48	0.30	0.39	0.26	ns	0.12	0.40	ns	0
Fly	Diptera		82	156	0.96	0.87	1.00	ns	0.80	1.21	ns	3
small black fly	Diptera		27	36	0.22	0.15	0.25	ns	0.29	0.21	ns	0
Diptera	**Diptera**		**153**	**752**	**4.6**	**4.0**	**4.9**	**ns**	**5.2**	**4.7**	**ns**	**11**
Apis mellifera	Hymenoptera	Apidae	47	330	2.0	0.0	2.8	***	4.8	0.8	**	1
wild bees	Hymenoptera	Apidae	22	26	0.16	0.09	0.19	**	0.22	0.16	ns	0
Halictid bees	Hymenoptera	Halictidae	36	109	0.67	0.04	0.92	***	1.7	0.1	**	0
Bombus terrestris	Hymenoptera	Apidae	28	60	0.37	0.04	0.50	*	0.97	0.02	***	0
Anthophila	**Hymenoptera**		**73**	**533**	**3.3**	**0.22**	**4.51**	***	**7.8**	**1.1**	***	**2**
Ichneumonidae	Hymenoptera	Ichneumonidae	24	24	0.15	0.20	0.13	ns	0.08	0.18	ns	0
Microhymenoptera	Hymenoptera		18	60	0.37	0.89	0.16	ns	0.05	0.28	ns	0
Tenthredinoidea	Hymenoptera		19	20	0.12	0.07	0.15	*	0.12	0.18	ns	0
Other Hymenoptera	**Hymenoptera**		**61**	**131**	**0.81**	**1.43**	**0.56**	**ns**	**0.37**	**0.75**	**ns**	**0**
Formica	Hymenoptera	Formicidae	24	60	0.37	0.20	0.44	ns	0.31	0.58	ns	0
Formica sanguinea	Hymenoptera	Formicidae	11	35	0.22	0.04	0.28	*	0.19	0.39	ns	0
Formicinae	Hymenoptera	Formicidae	44	208	1.28	1.67	1.13	ns	0.80	1.47	ns	1
Myrmicinae	Hymenoptera	Formicidae	17	105	0.65	0.63	0.66	ns	0.61	0.70	ns	1
Formicidae	**Hymenoptera**	**Formicidae**	**90**	**422**	**2.6**	**2.7**	**2.6**	**ns**	**2.0**	**3.2**	**ns**	**2**
Hymenoptera	**Hymenoptera**		**135**	**1086**	**6.7**	**4.3**	**7.6**	*	**10.2**	**5.0**	*	**4**
Cicadellidae	Hemiptera	Cicadellidae	36	46	0.28	0.41	0.23	ns	0.32	0.14	*	0
Other insects			**72**	**111**	**0.69**	**0.76**	**0.66**	**ns**	**0.83**	**0.47**	**ns**	**0**
pupa of ladybird	Coleoptera	Coccinellidae	18	27	0.17	0.09	0.20	*	0.29	0.11	ns	0
Lymantria dispar caterpil.	Lepidoptera	Erebidae	30	49	0.30	0.41	0.26	ns	0.25	0.26	ns	0
Larva			**53**	**93**	**0.57**	**0.57**	**0.58**	**ns**	**0.76**	**0.39**	**ns**	**0**
Total			**162**	**4203**	**25.94**	**12.8**	**31.1**	***	**34.9**	**27.3**	**ns**	**66**
Total w/o *Raghonycha*			**161**	**2703**	**16.69**	**11.3**	**18.8**	**	**23.5**	**13.9**	*	**24**
Richness			**162**	**1371**	**8.46**	**6.7**	**9.2**	**	**10.3**	**8.0**	*	

[1]Headings: Taxon studied; Order; Family; #coll: number of collections where the taxon was detected; N: total number of individuals observed; N/col: mean number of individuals per collection; x flower: mean number of individuals per collection for non-flowering trees; flower: idem for flowering trees; Test: Wilcoxon paired test of greater abundance of the taxon on flowering than on non-flowering trees; w pol: mean number of individuals per collection for male-fertile trees; w/o pol: idem for male-sterile trees; Test: Wilcoxon test of the difference in mean abundance between male-fertile and male-sterile trees; ♀ fl.: number of insects seen on female flowers. In each comparison, significantly higher values are underlined.

corresponding to a total of 32 hours and 20 minutes. We surveyed each tree about 10 times, starting on June 4. At that time, only three trees had started to flower. We stopped the survey when all trees had finished flowering, on July 12. Altogether, we made 15 collections on trees that had not yet flowered, 54 during the first peak of male flowering, 18 between peak 1 and peak 2, 44 during peak 2 and 31 afterwards. That is, 28% of the collections were outside the flowering period of the trees, enabling to test if flowering trees attract arthropods (Table 1, Appendix 2).

We inventoried 4203 arthropods grouped in 129 taxa corresponding to at least 101 species. The most abundant orders were Coleoptera (2062 individuals and 21 taxa) followed by Hymenoptera (1086, 36 taxa) and Diptera (752, 36 taxa). The most abundant insect species by far was the common red soldier beetle (*Rhagonycha fulva*, 1500 individuals), followed by the honeybee (330 individuals), a Tachinid fly (218 individuals), and the sulphur beetle (*Cteniopus sulphureus*, 203 individuals).

There were nearly three times as many arthropods found on flowering trees than on non-flowering trees (31.1 compared to 12.8, $p < 0.001$). Among all arthropod taxa with more than 15 individuals, 25 out of 34 were more abundant on flowering trees and 18 significantly so. The flower-visiting species included a crab spider (*Synema globosum*), seven Coleoptera, including several ladybirds as well as the red soldier beetle and the sulphur beetle, three bees (the honeybee, a bumblebee (*Bombus terrestris*), and halictid bees), a syrphid fly (*Sphaerophoria scripta*) and a Tachinid fly. The mean arthropod richness measured on flowering trees was also significantly higher

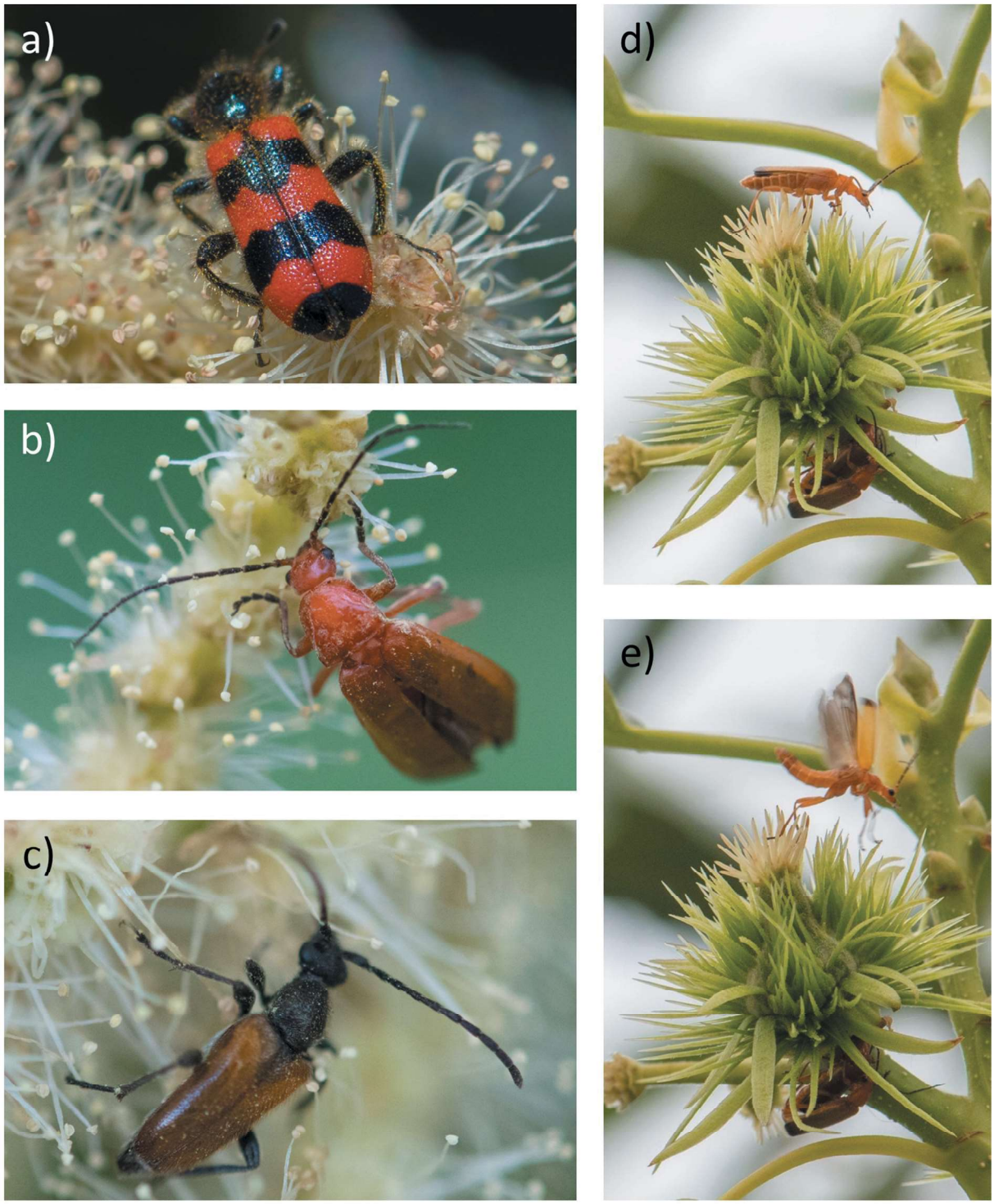

Figure 10. Beetles on chestnut flowers. Beetles foraging in male catkins and abundantly covered with sticky chestnut pollen: (a) bee beetle (*Trichodes alvearius*), (b) soldier red beetle (*Rhagonycha fulva*) and (c) tawny longhorn beetle (*Paracorymbia fulva*). Soldier red beetle standing on (d) and taking off from (e) a female inflorescence. Notice also the couple underneath the female inflorescence: the abundance of mating insects seen on chestnut trees at the end of the flowering season strongly suggests that these insects use trees as mating rendezvous.

than on non-flowering trees (9.2 versus 6.7, $p< 0.001$). On flowering trees, insects visiting male catkins were readily dusted with chestnut pollen (Figure 10(a,b,c)).

In total, we observed 66 insects on female flowers (Figures 10 and 11). This represents a small fraction (1.8%) of the insects observed on flowering trees. They were typically observed landing on or taking-off from the styles (Figure 10(d,e)), walking on the tip of the styles (Figure 11(a,d,e)), or apparently licking the tip of the styles (Figure 11(b,c)). In other cases, the contact with the female flower seemed purely accidental (Figure 11f). We found 51 beetles on female flowers (42 red soldier beetles, 6 ladybirds, 2 sulphur beetles,

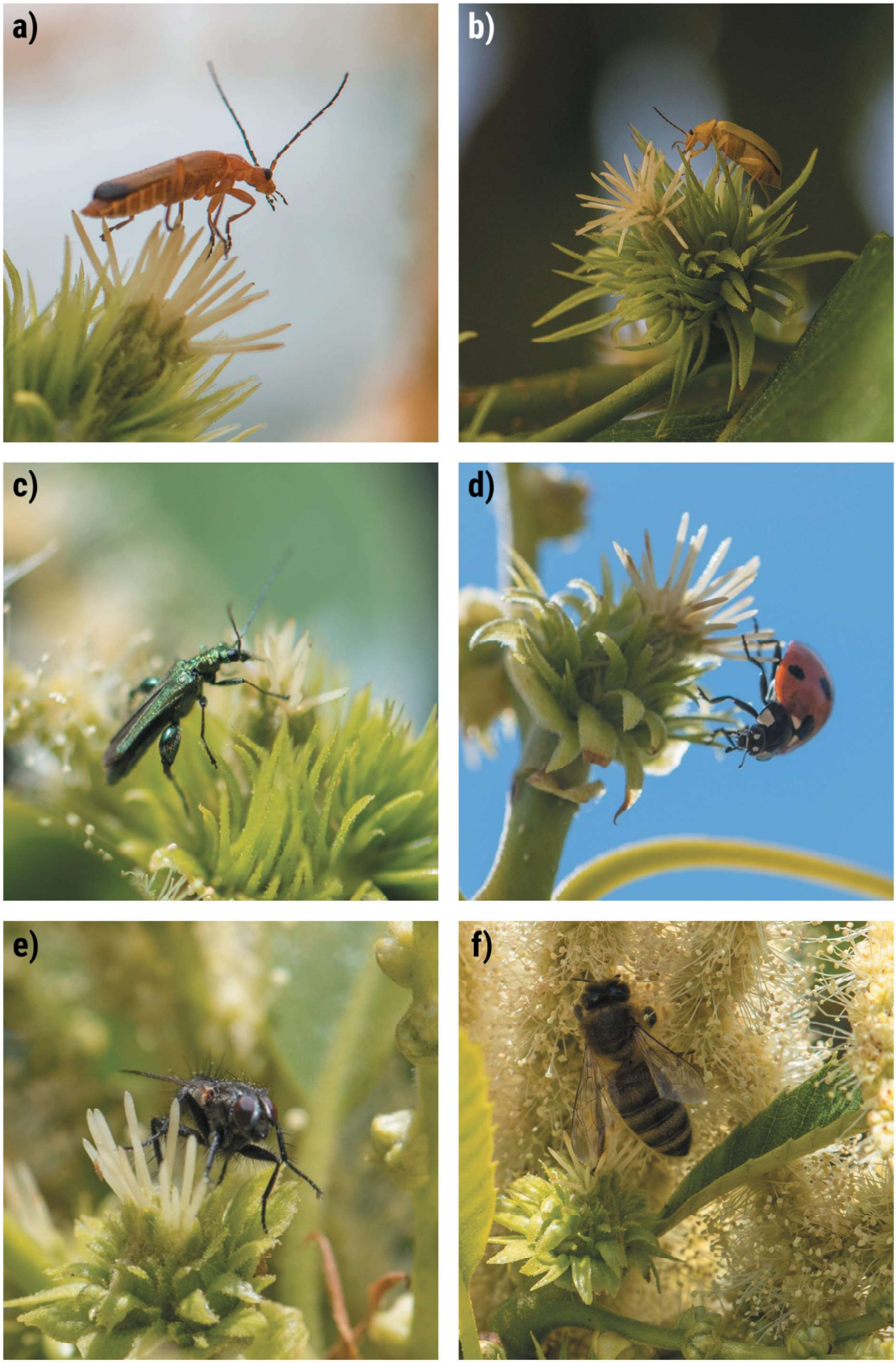

Figure 11. Insects visiting female flowers. a) Soldier red beetle (*Rhagonycha fulva*) standing on the extremities of the styles of a female inflorescence. b) Sulphur beetle (*Cteniopus sulphureus*) licking the stigmatic portion of a style. c) Idem for a swollen-thighed beetle *(Oedomera sp.*). d) Adult ladybird walking on a female flower. e) Fly perched on a female flower. f) Pollen-collecting honeybee (*Apis mellifera*) accidentally touching a female flower.

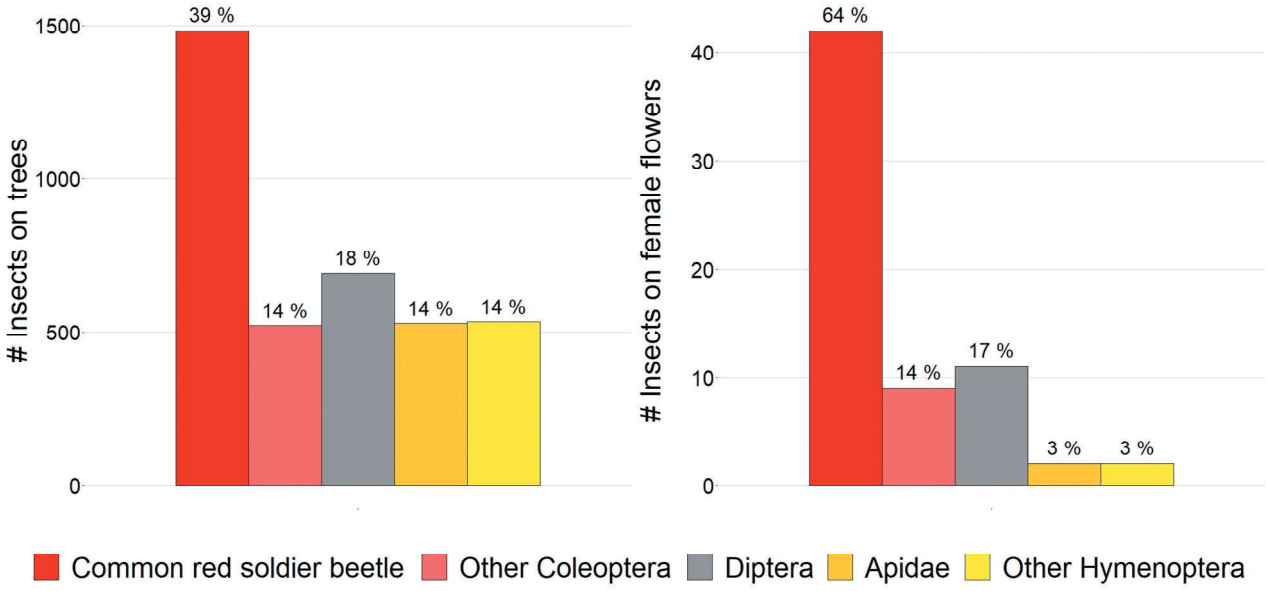

Figure 12. Abundance of insects on chestnut trees versus on female flowers.

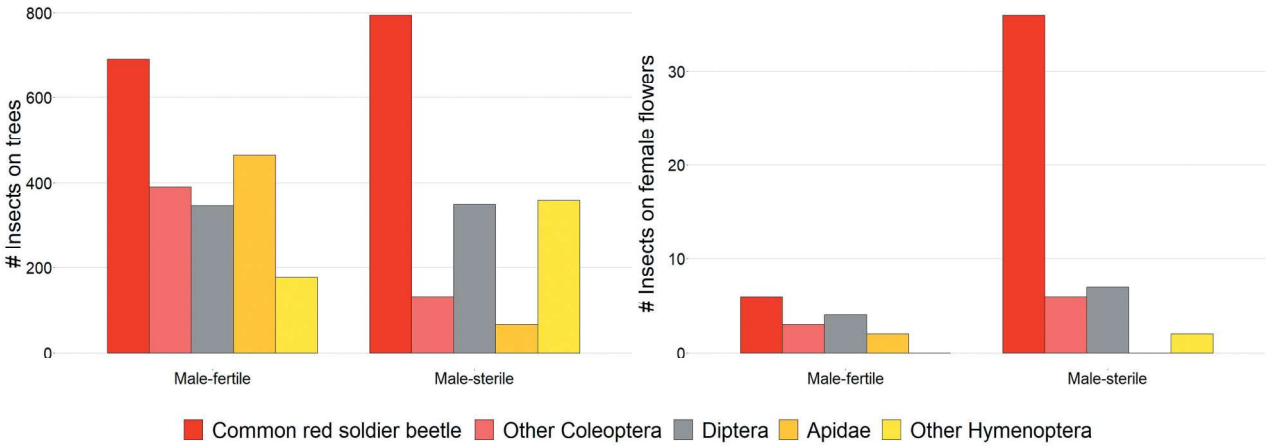

Figure 13. Abundance of insects on chestnut trees versus on female flowers, as a function of tree gender.

C. sulphureus, and 1 tawny longhorn beetle, *Paracorymbia fulva*), representing 77% of the total, a higher proportion than on trees (52%, see Figure 12). We also found 11 flies and 4 Hymenoptera on female flowers, including 2 ants and 2 bees, one of which was a honeybee.

We then compared the proportion of Coleoptera, Diptera and Hymenoptera on trees versus on female flowers. In Coleoptera, we distinguished the common red soldier beetle from other species. In Hymenoptera, we contrasted bees (*Anthophila*) with other species. We found that red soldier beetles were over-represented on female flowers (Fisher's exact test, $p = 10^{-4}$) and Hymenoptera under-represented on female flowers (Fisher's exact tests, $p = 0.006$ for bees and $p = 0.006$ for other Hymenoptera). Other beetles as well as Diptera were neither overrepresented nor underrepresented on female flowers (Fisher's exact tests, $p > 0.05$) (Figure 12).

Male-fertile versus male-sterile trees. We compared insects present on flowering male-fertile and male-sterile trees, predicting that if a visiting insect searched for pollen, it should be more abundant on male-fertile trees. Overall, we found no significant difference (Figure 13, Table 1 and Appendix 2), but the difference became significant after excluding red soldier beetles (23.5 insects on male-fertile trees and 13.9 on male-sterile ones, $p< 0.05$). Insects showing a preference for male-fertile trees included larger beetles (all beetles larger than 0.7 mm except red soldier beetles), syrphid flies, and three bees: the honeybee, halictid bees and bumblebees. Overall, arthropod richness was higher on male-fertile trees than on male-sterile ones (10.3 versus 8.0, $p< 0.05$).

Contrasting the relative abundance of insects on trees versus on female flowers, we found that red soldier beetles as well as the other beetles were over-represented on female flowers from male-sterile trees

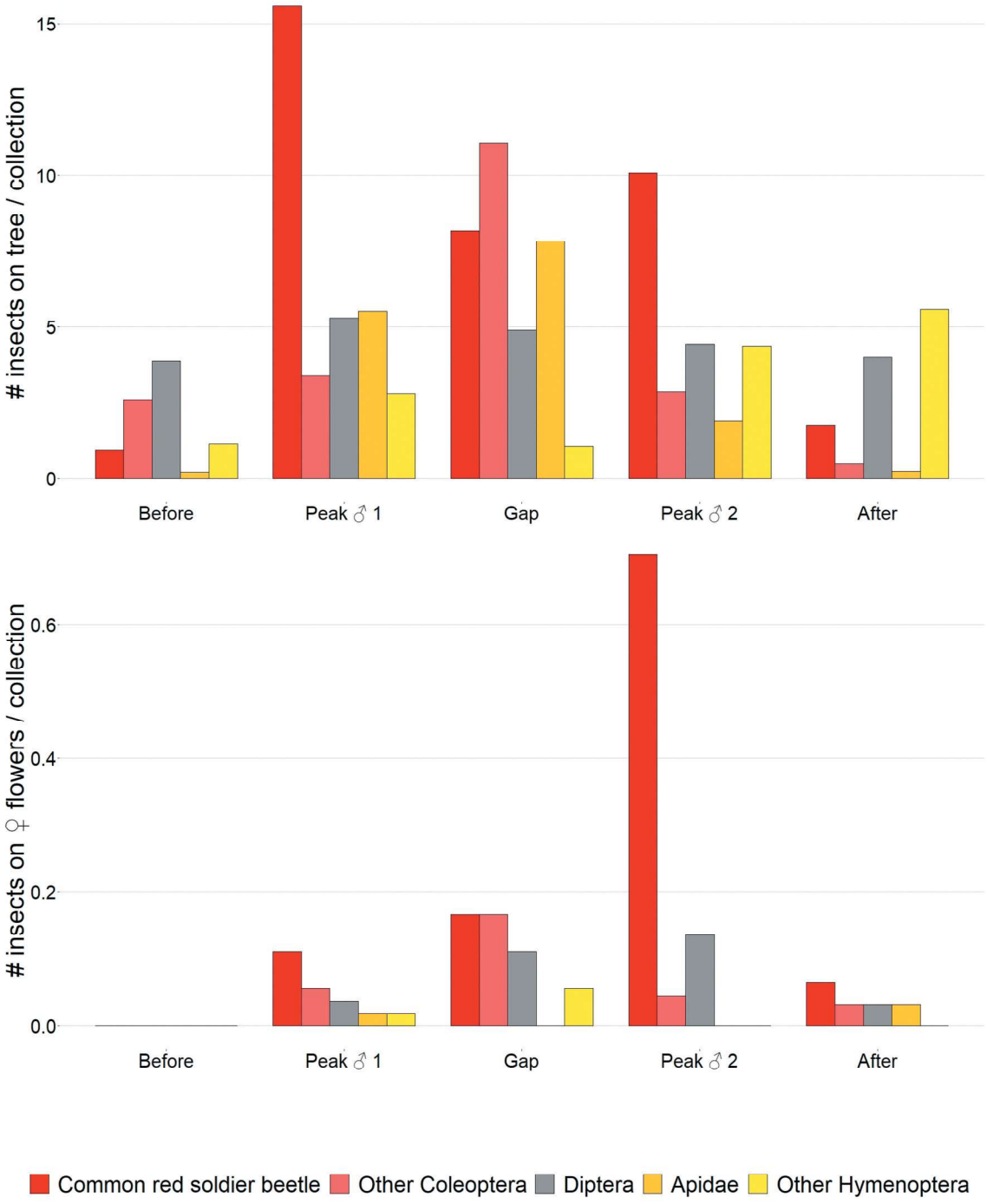

Figure 14. Abundance of insects on chestnut trees and on female flowers, as a function of the flowering stage of the trees.

(Fisher's exact tests, $p = 2.10^{-5}$ for soldier beetles, 0.01 for the other beetles). This is particularly remarkable for the other beetles because they were more numerous on male-fertile trees. The relative abundance of other insects (Diptera and Hymenoptera) did not differ significantly between trees and female flowers (Fisher's exact tests, $p > 0.05$).

Insect visits as a function of the flowering stage of trees. Trees undergo important changes during the flowering period. The most massive flowering display takes place during the first flowering peak. At that time, about 97% of the male flowers were in bloom, resulting in the production of large amounts of reward in the form of nectar and (in male-fertile trees) of pollen. Yet insect visits to trees were maximal during peak 1 only for red soldier beetles and to a smaller extent for Diptera (Figure 14). For the other beetles and for bees, insects were most abundant during the flowering gap between peak 1 and 2, when male flowers from unisexual catkins had started to fade and those from bisexual catkins had not yet started to bloom. During peak 2, corresponding to the flowering

Table 2. Chestnut pollination syndrome in comparison with oak.

Chestnut	Oak	Syndrome	Comments	References
Plant traits				
Massive showy white inflorescence	Yellow inflorescence	insect		Groom 1909; Porsch 1950
Strong aminoid semen-like fragrance	Odorless	beetle, fly		Porsch 1950; Zhang et al. 2019
Late flowering after leaves unfold	Early spring flowering before leaves unfold	insect	Late flowering coincides with maximum insect activity and loss of airborne pollen due to foliage interception	Groom 1909; Kaul 1986; Millerón et al. 2012; Bastl et al. 2020
Open flat blossoms, reduced perianth	Reduced perianth	beetle, wind		Faegri and Van Der Pijl 1979
Nectar produced by male catkins (~14 kg/ha)	Nectarless	insect		Baude et al. 2016
Hexose rich or hexose dominant floral nectar	/	Short-	tongued pollinators	Long-tongued pollinators prefer sucrose rich nectar, short-tongued pollinators prefer hexose rich nectar
Baker and Baker 1983; Kim et al. 2020				
Long, conspicuous, erect stamens, brush type	Shorter pendulous flexible stamens	beetle	Erect stamens less appropriate for pollen take off by wind	Groom 1909; Faegri and Van Der Pijl 1979
12 stamens per flower	6 stamens per flower	beetle	Polyandry is often considered a shared trait of beetle-pollinated systems	Gottsberger 1977
Tiny butterfly-shaped anthers (<0.01 mm^3)	100 x larger anthers (>1 mm^3)	beetle	Diffuse packaging of pollen to help cope with pollen-consuming beetles	Molina et al. 1996; Manos et al. 2001; Zhong et al. 2020
Huge pollen production: 2.0×10^{12} pollen grains/ha/yr	Slightly lower pollen production: 1.2×10^{12} pollen grains/ha/yr	beetle, wind	Beetles are rather clumsy pollinators that "waste" and consume pollen	Kiyonaga 1991, Kiyonaga 1995; Faegri and Van Der Pijl 1979
Huge pollen/ovule ratio: 21.10^6, all the more remarkable since ovary is pluri-ovulate	Unknown but presumably lower pollen/ovule ratio	beetle, wind	Highest pollen/ovule value reported to date. Some beetle-pollinated trees also have high ratios	This study; Cruden 2000; Erbar and Langlotz 2005
Tiny, smooth prolate pollen grains (15x11µm)	Pollen grains 10 x larger (31x26 µm), subprolate	beetle, wind	Pollen too small for effective capture on stigmas	Whitehead 1983; Diethart and Bouchal 2018; Halbritter et al. 2016
Abundant pollenkitt, pollen grains sticky clumping together in masses	Less pollenkitt, pollen dry and powdery	insect	Sticky pollen adapted to insect-pollination	Groom 1909; McKay 1972; Hesse 1978
No visible release of pollen when inflorescences are touched	Pollen readily released when inflorescences are touched	insect		Groom 1909; Porsch 1950
Pollen grains eventually dry: pollen export by insects precedes pollen peak in the air	Pollen dry and powdery	mixed	Is the change in stickiness truly a wind adaptation?	Groom 1909; Porsch 1950; Clapper 1954; Sabugosa-Madeira et al. 2008
Bisexual protogynous catkins	Absence of bisexual catkins	insect	Cosexuality favors insect pollination when female flowers have no reward	McKay 1972; Kaul 1986; Zirkle 2017
Ovaries and ovules protected by hardened spiny bracts and by the close massing together of the flowers	Ovaries more accessible to herbivorous insects	beetle	Adaptations against damage to the ovules expected in flowers of beetle-pollinated plants	Grant 1950; Proctor et al. 1996
5–8 milky-white needle-shaped styles per flower; brush-like aspect reminiscent of stamen brush	3 slightly-recurved styles	insect	"Mistake" pollination by insects of auto-mimetic female flowers, female flowers used as take-off/landing platform	This study; Nakamura 1992; Fan et al. 2015
Tiny hollow stigmas (~0,005 mm2)	10x larger capitate stigmas (~0,05 mm2)	insect		Nakamura 1992; Boavida et al. 1999
Stigma's aperture layered with secretory cells	Much larger dry stigma	insect	Stigmatic secretions used as reward for insects?	Porsch 1950; Feijó et al. 1999; this study
12–16+ ovules/ovary	6 ovules/ovary	insect	Few large clustered flowers with many ovules not optimal to maximize airborne pollen capture	Porsch 1950; Nakamura 1992; Friedman and Barrett 2011
Other features				
Occurrence in comparatively species-rich, warm and humid conditions	Can occur further north, in colder or drier conditions	insect	Reduced chance for wind-transported pollen reaching conspecific flowers in species-rich communities; less constraints on nectar production in warm and wet habitats; less effective wind dispersal in humid areas	Rech et al. 2016; Thurm et al. 2018
Excellent nutritive value of pollen for bees, with positive effects on insects health	Good nutritive value for bees, but reduced digestibility	insect		Tasei and Aupinel 2008; Di Pasquale et al. 2013; Ghosh and Jung 2017
Stokes's terminal velocity of pollen ~1 cm/sec	terminal velocity 3–4 cm/sec	wind		Stanley and Linskens 1974; Tampieri et al. 1977
Long-distance pollen transport by wind	Idem	wind		Frei 1997; Peeters and Zoller 1988
Sharp decline of pollen concentration in the air away from trees; chestnut pollen often under-represented away from source	Oak pollen over-represented in lake samples	insect	Pollen decline away from trees caused by foliage interception or pollen clumping	Paillet et al. 1991; Conedera et al. 2006; López-Sáez et al. 2017; Fang et al. 2019; Jiang et al. 2020
Regular seed mast (no "masting")	Masting driven by pollen dynamics linked with spring weather conditions	insect	In wind-pollinated plants, seed production dynamics is fluctuating across years and synchronised among individuals	Zirkle 2017; Schermer et al. 2019
Relatively high genetic structure in *C. sativa*: F_{ST} = 0.15 (Spain), 0.17 (Europe), 0.18 (Turkey)	Typically lower genetic structure: F_{ST} = 0.02 (*Q. robur*), 0.03 (*Q. petraea*), 0.10 (*Q. ilex*), 0.07 (*Q. suber*)	small insects	Pollination by small insects leads to greater differentiation compared to pollination by large insects, vertebrates or wind	Michaud et al. 1995; Toumi and Lumaret 1998; Villani et al. 1999; Mattioni et al. 2008; Petit et al. 2003; Martín et al. 2012; Gamba and Muchhala 2020

peak of male flowers from bisexual catkins, beetles were nearly as abundant as during peak 1 but the abundance of bees strongly declined.

We observed few insects on female flowers during peak 1 (Figure 14). Insects, mostly beetles and flies, were most frequent on female flowers during the flowering gap and during peak 2, with a marked increase of red soldier beetles during peak 2. On average, we found insects on female flowers 16.3 days after the onset of flowering. This is significantly later than expected by chance (12.1 days; Wilcoxon test, $p = .14$), and nearly so if we exclude those collections where female flowers were not yet fully receptive (Wilcoxon test, $p = 0.07$).

Chestnut pollination syndrome

We found data for 21 plant traits useful to infer pollination syndromes in both chestnuts and oaks (Table 2). Among them, 16 point exclusively towards a biotic pollination syndrome, including at least five associated with cantharophily (beetle-pollination). Four others are equivocal, representing possible adaptation to either wind pollination or to beetle-pollination (small flowers with reduced perianth, huge pollen production, tiny pollen grains and high pollen/ovule ratio). Only one is suggestive of ambophily: change from initially sticky to eventually dry pollen, allowing wind-dispersal of pollen grains not collected by insects. No trait unequivocally points exclusively towards wind pollination. Regarding the seven other attributes of chestnuts, five are suggestive of insect pollination and two of wind pollination.

Discussion

Insect exclusion experiments, performed on different continents, on different chestnut tree species and in different contexts, have all shown that fruit set is dramatically reduced (by about 80%) when insects cannot reach female flowers. This clearly points to a minor role for airborne pollen in chestnut pollination. The results also show that the role of airborne pollen can be easily overestimated if burr set is not taken into account. In fact, netting resulted in a particularly reduced burr set in the case of *C. ozarkensis* (Zirkle 2017). In this species, each burr is made of a single flower, not three as in other species of chestnuts, so pollination success depends to a larger extent on burr set than in other chestnut species.

Where does the pollen that formed the few fruits found in the nets come from? Airborne pollen that fertilizes seeds inside the nets can be brought by wind but also by insects that lose pollen during their flights. Pierre et al. (2010) demonstrated experimentally the effectiveness of insect-assisted wind pollination in rapeseed, showing that at close range, honeybees participate in pollination without touching the female flowers by releasing pollen from their bodies. This hypothesis would be worth testing in chestnuts, as Hasegawa et al. (2015) have shown that bees carry high loads of outcrossed chestnut pollen. Selfing could also explain the origin of some of the nuts found inside the nets, as bagging had nearly the same effect than netting in Zirkle's (2017) experiment. Self-fertilization is typically a rare event in chestnut (Stout 1926; Hasegawa et al. 2009; Xiong et al. 2019), but selfing rate could increase in the absence of outcross pollen. Paternity analyses could help clarify this issue (Wright and Dodd 2013).

In principle, insect exclusion experiments could underestimate pollination by wind, as fine nets can reduce to some extent incoming wind flow (e.g. Ramsay et al. 2003; Bartomeus et al. 2014). Alternatively, exclusion experiments performed in orchards could overestimate pollination by wind, for several reasons. First, pollination is a frequency- and density-dependent process (Klein et al. 2017). Because airborne pollen has to compete with pollen brought by insects in the control but not in the treatment, a simple additive model might be misleading. Second, in chestnut orchards, tree density is typically much higher than under natural conditions, favoring wind pollination (Zirkle 2017). Third, in large orchards, the massive and abrupt flowering might overwhelm insect pollination capacities (Brittain et al. 2013). Hence, the figures obtained in orchards probably overestimate the importance of wind pollination compared to natural conditions. Overall, the results therefore point to a major role of insects in chestnut pollination, raising the question of the mechanisms favoring insect pollination.

In this study, we decided to monitor arthropod visits before, during and after blooming, to establish which species flowering trees attract. The overall abundance of arthropods greatly increased during flowering. In particular, all adult beetles and all bees increased in abundance, but only a few of the flies and a single arachnid did so. We saw 66 insects on female flowers, hence quantitatively documenting for the first time insect pollination in chestnuts. Beetles, which represented 52% of all insects seen on flowering chestnut trees, increased their share to 77% on female flowers, suggesting that they represent the main pollinators of chestnut, as already proposed by Porsch (1950). Many of these flower-visiting beetles are hairy and covered with chestnut pollen. To confirm that these insects can indeed pollinate chestnuts, experimental approaches could be used in future investigations (e.g. Chifflet et al. 2011). In our study site, the red soldier beetle was particularly abundant on flowering chestnuts. However, we surveyed pollination in a single season, in 2019, and at a single site. The results may thus reflect seasonal and spatial stochasticity in pollinator assemblages. In fact, we found that another beetle, the sulphur beetle, was the most

abundant insect on flowering chestnuts in another locality located just 20 km away. In Austria, Porsch (1950) also noted that the most abundant beetle species on flowering chestnuts differed across localities.

Interestingly, in chestnut, female flowers look like male flowers of male-fertile trees. The clustered styles evoke a piece of male catkin with erect stamens, suggesting automimicry, i.e. imitation of male flowers by female flowers to attract pollinating insects searching for rewarding male flowers (Willson et al. 1989). The case for intersexual mimicry is stronger when the structures of one sex are modified to resemble non-homologous structures of the other sex (Bawa 1980; Dukas 1987; Willson et al. 1989). This is clearly the case in chestnut. A prerequisite of mimicry systems, the rarity of the mimic in comparison to the model, also applies: the male-to-female ratio is very large, over 2000 when expressed in terms of surface accessible to insects. Automimicry in plants was discovered relatively recently (Gilbert 1975; Baker 1976; Bawa 1977), so early students of chestnut pollination had no reference to look for it. Automimicry is in fact quite common in plants, especially in diclinous species. For instance, Lunau et al. (2017) identified 124 cases of stamen-like pistils in the Alpine flora (10% of the species). Wind-pollinated species, in which male and female flowers have no selective pressure to share signals, tend to exhibit greater sexual dimorphism (Johnson and Schiestl 2016). Since the many styles of the female flowers of chestnuts represent a unique and probably derived feature (Manos et al. 2001), it would be interesting to investigate the evolution of female flowers across both insect-pollinated and wind-pollinated Fagaceae.

In contrast to beetles, bees avoid chestnut female flowers, as already noted by Giovanetti and Aronne (2011). In particular, the honeybee, the second most abundant insect visiting chestnut flowers in our study site, was observed only once on a female flower and this was clearly accidental. Honeybees discriminate better than other insects against non-rewarding female flowers (e.g. Dukas 1987). During the flowering period, a subset of insect species, including bees and syrphid flies, neglected male-sterile chestnut trees. This suggests that pollen represents a major reward for these insect visitors. Given that male-sterile trees are at least as well if not better pollinated than male-fertile trees, these insects are likely not the main chestnut pollinators. Instead, red soldier beetles congregated in similarly great numbers on male-fertile and male-sterile trees. In the future, it would be interesting to check if this species, which disperses to relatively large distances (Rodwell et al. 2018), uses chestnuts trees as rendezvous sites, as noted in other beetle-pollinated plants (Faegri and Van Der Pijl 1979).

Remarkably, we observed more insects on female flowers of male-sterile than of male-fertile trees even though the latter received more visits from insects. In male-sterile trees, the only flowers that look like staminate flowers are the female flowers. If insect visits to female flowers are inversely related to the local abundance of staminate male flowers, this would explain this trend. Such process could contribute to the greater fruit set of male-sterile trees (Pereira-Lorenzo and Ramos-Cabrer 2004) and hence to female maintenance in gynodioecious chestnuts, a topic worthy of further investigations.

There was nearly no overlap between the two peaks of pollen production in chestnut. In contrast, both overlapped with the production of female flowers, supporting Hasegawa et al. (2017) view that duodichogamy may "*promote outcrossing [...] rather than prevent self-pollination*". The extreme imbalance between the two peaks of flowering in terms of male flowers displayed and reward produced does not translate into an equally dramatic imbalance in number of insect visitors. In fact, honeybees were only twice more numerous at peak 1 than at peak 2, red soldier beetles only 50% more so and the other insects were roughly equally abundant during both peaks of pollen production. For bees and for the other beetles, abundances peaked between the two peaks. It is unclear why insects would not take more advantage of the full male bloom of chestnut trees.

Visits to female flowers were most numerous after the first pollen peak, on average about 16 days after the onset of flowering. This coincides rather well with the time of maximum stigmatic receptivity reported for chestnut (between 9 and 17 days after flowering had started, Nienstaedt 1956). According to McKay (1972), one of the strongest arguments in favour of insect-pollination of chestnut is the existence of bisexual catkins. For him, they seem to be "*a device to attract insects from other trees to the mixed catkins of a given tree at the time the female flower is receptive*". Insect visits to female flowers increase when the rewarding male catkins become relatively less abundant, suggesting that female flowers compete with male flowers for insect visits. This would explain the greater number of visits to female flowers once the first massive pollen peak is over. Interestingly, in one of the first published reports of automimicry in plants, Bawa (1977) described a self-incompatible duodichogamous tropical tree species from the Sapindaceae family in which rewardless female flowers mimic male flowers. He argued that the low frequency of the female flowers and their appearance for only a short duration of time are critical for the mimicry system to operate, otherwise insects would readily learn to discriminate between male and female flowers.

Insect interactions with female flowers are comparatively rare in chestnut. In diclinous plant species, this is not surprising, as a single insect visit to a female flower is often sufficient to fertilize almost all available

ovules (Barrett and Hough 2013). Nevertheless, we were able to document a sizable number of visits to female flowers, providing some clues on how pollination is achieved. Compared to bees, beetles tend to stay longer on the plant, wandering on catkins, leaves and branches, thereby increasing the chances that they touch female flowers. Moreover, the stiff, upright borne styles located close to rewarding male flowers present "*an ideal take-off and landing platform for flying insects*" (Zirkle 2017), especially for some beetles and flies. Such imprecise pollination mode has been termed the "mess and soil" principle of pollination to emphasize its lack of precision compared to cases where the insect deposits the pollen in small amounts on a specific receptive area of the plant (Faegri and Van Der Pijl 1979). However, we also saw beetles licking the tip of the styles, giving some credence to the proposal that the stigmatic secretion could represent a small reward for some insects (Porsch 1950).

In view of the rarity of insect visits to chestnut female flowers, an extended period of receptivity of female flowers could help secure pollination (Schoen and Ashman 1995). In line with this prediction, female chestnut flowers possess 6–8 styles that are successively receptive for about two days each, resulting in a long overall receptivity period (Feijó et al. 1999). This system, combined with a delayed fertilization of about six weeks due to the retarded development of ovules, provides an opportunity for all pollen tubes to fertilize the ovules regardless of their times of arrival on the stigmas (Fan et al. 2015). This should maximize the chances that outcross pollen fertilizes at least one ovule from each female flower. For this to happen, insects must carry enough compatible pollen. Hasegawa et al. (2015) have investigated pollen diversity on the bodies of insects visiting *C. crenata*, using single pollen genotyping. They found that all insects carry loads of pollen, with outcross-pollen rate being highest in bumblebees (66%), followed by small bees (35%), flies (31%), and small beetles (18%). However, our own results indicate that some of these insects, including bumblebees and honeybees, seldom visit female flowers, so their pollen load is irrelevant for pollination.

No reproductive trait of chestnut unambiguously and exclusively points towards wind pollination. In particular, chestnut pollen grains, which are much smaller than those of related wind-pollinated Fagaceae and of most other wind-pollinated plants, are probably too small for effective capture in the air by stigmatic surfaces (Whitehead 1983). In contrast, their small size increases the odds that the legs and mouthparts of insects likely to encounter the tiny stigmas will bear at least some pollen grains. Hence, we should not consider massive pollen production and apparent wastage as the exclusive prerogative of wind-pollinated plants. Convergence caused by high uncertainty of pollen delivery could explain the superficial similarity between wind- and beetle-pollination syndromes. Under this logic, the low settling velocity and long-distance dispersal potential of the chestnut pollen grains in the air should not be interpreted as direct adaptation to wind pollination, just as nutritive pollen actively collected by insects is not proof for insect pollination in oaks (Manos et al. 2001; Oh and Manos 2008; Saunders and Packer 2018).

A growing number of cases of ambophily have been reported in recent years (reviewed in Culley et al. 2002; Friedman and Barrett 2009). This is surprising, as Stebbins (1970) most effective pollinator principle states that plants should evolve to increase the efficiency of their main pollinating agent. In studying potentially ambophilous species, one should go beyond the mere realization that both animals and air bring pollen to the stigma. Instead, one should try to establish if the plant is indeed adapted to both pollination agents. To date, it is still unclear whether ambophily can be an evolutionarily stable strategy (Friedman and Barrett 2009). In the case of chestnuts, all well-performed insect-exclusion experiments have shown that insects play a major role for pollination, even in conditions favouring wind pollination. This leaves little room for the evolution of wind-pollinated traits. To support the hypothesis that chestnuts are ambophilous rather than entomophilous, one would need to identify traits favouring wind pollination that have evolved across chestnut species or populations in response to environmental gradients favouring this mode of pollen dispersal. Until then, we argue that the intuition of Thomas Meehan was correct (Meehan 1879) and that chestnuts ought to be considered as one of the most important insect-pollinated forest tree species in the northern hemisphere.

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CL performed all insect isolation experiments with Invenio staff in 2019. GB, RJP and CL surveyed insect visitors in 2019, GB established the photo library and performed all insect identifications from the photographs, EA and RJP carried out the measures of flower abundance with the help of two students from Bordeaux University, Julien Bonnier and Tanguy Menthonnex. RJP compiled information on chestnut's pollination syndrome. CL and EA performed the statistical analyses, CL designed the figures in R. RJP and CL wrote the paper, with inputs from the other authors. Photographs of Figure 3 are by CL and all others are by RJP.

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