American Journal of
Botany

BOTANICAL SOCIETY OF AMERICA
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Fitness benefits and costs of floral advertising traits: insights from rayed and rayless phenotypes of *Anacyclus* (Asteraceae)

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PREMISE OF THE STUDY: Ray flowers commonly observed in daisies' flowering heads are a well-known example of advertising structures for enhancing pollinator attraction. Despite this, ray loss has occurred in multiple lineages, which still rely on pollinators, suggesting that rayless phenotypes could also be adaptive for animal-pollination. Here, we investigate the benefits and costs of these specialized floral advertising structures by comparing rayed and rayless phenotypes in two hybridizing closely related species.

METHODS: We assessed the advantages and costs of ray production in terms of floral visitor's attraction, pollen limitation, and female reproductive success using the broad natural variation on ray size and number at the contact zone of *A. clavatus* (rayed) and *A. valentinus* (rayless). In addition, we experimentally explored the effect of rays under controlled neighborhoods and the effect of ray removal on fruit production.

KEY RESULTS: In sympatry, rayed phenotypes attracted significantly more visitors than rayless plants, in which seed production was pollen limited. However, rayed phenotypes did not show higher fruit set or seed production than rayless phenotypes. Fruit set and seed production benefited from denser neighborhood displays and larger individual floral displays, respectively. The removal of ray florets did not appear to enable resource reallocation to fruit production.

CONCLUSIONS: Rayless heads compensated their lower visitation rate by means of a higher number of flowers per head achieving similar fecundity levels to rayed plants. The larger size of rayless heads might thus indicate an inflorescence-level trade-off between attraction and fertility.

KEY WORDS   Advertising-fertility trade-off; Asteraceae; Floral cost; Floral display; Inflorescence; Pollinator attraction; Neighborhood floral display; Ray florets; Rayless heads.

Animal-pollinated plants invest a considerable amount of resources to attract pollinators. These plants must have flowers that, in effect, advertise themselves to potential pollinators and that will compete with other surrounding floral displays. Under pollen limitation, when pollination services are below the full reproductive capacity of a plant, it is generally expected that plants with larger flowers are selected (Galen, 1989; Campbell, 1991; Herrera, 1993; Conner and Rush, 1996; Parachnowitsch and Kessler, 2010). However, the production of larger structures for attraction, such as petals, presents different types of costs. First, colorful petals represent an investment of biomass and costly molecules, such as pigments (Ashman and Schoen, 1997; Mendéz, 2001), potentially leading to costs and tradeoffs in terms of fruit set, seed quality, and seed germination (Andersson and Widén, 1993; Andersson, 1999, 2000, 2005, 2008;

Castro et al., 2008). Second, larger floral displays attract pollinators, but also herbivores (Knauer and Schiestl, 2017), nectar robbers (Rojas-Nossa et al., 2016), and/or inefficient pollinators (Lau and Galloway, 2004; Hargreaves et al., 2012; Koski et al., 2018). Finally, large floral displays frequently promote successive visits within the same plant, thus promoting geitonogamous pollination and ultimately reducing outcrossing, a phenomenon called "attractiveness dilemma" (Klinkhamer and de Jong, 1993; Karron et al., 2009).

Ray flowers of the daisy family (Asteraceae) are a well-known example of specialized structures that attract pollinators. This family is easily recognized by its inflorescence, the capitulum or head, which functions as a pollination unit (Burtt, 1977; Lane, 1996). In many species, heads are characterized by tubular

actinomorphic disc florets clustered together and surrounded by a ring of florets with zygomorphic corollas—the ray florets (Kim et al., 2008; Bello et al., 2013). The presence of rays has significant consequences on pollination success in a number of species, mainly enhancing the attractiveness of heads to pollinators (Lack, 1982; Celedón-Neghme et al., 2007; Andersson, 2008), and consequently influencing the pollination success and the levels of outcrossing (Marshall and Abbott, 1984; Sun and Ganders, 1990). Thus, the presence of rays seems to provide an advantage for attracting pollinators, although the pollination context, including pollinator abundance and individual floral display may reduce this effect (Andersson, 1996; Nielsen et al., 2002). Therefore, the efficiency of advertising structures, such as rays, may be context dependent.

Despite the observed advantages of rayed heads in attracting pollinators, rayless species are frequent in Asteraceae. Several independent reversals towards rayless heads have occurred in the evolution of this family, suggesting that rayless phenotypes could also be adaptive for animal pollination (Bremer and Humphries, 1993; Torices and Anderberg, 2009; Torices et al., 2011). Previous works have suggested that the production of rays might entail a reduction of the resources available for fruit and seed production (Andersson, 1999, 2008) and/or the attraction of more seed predators (Fenner et al., 2002). Furthermore, as heads are usually visited by a larger number of pollinators, rayed heads could attract a larger amount of less efficient pollinators than rayless heads, reducing pollen transfer efficiency. Still, whether specific groups of pollinators show a preference for different phenotypes (rayed vs. rayless) remains poorly explored (but see Stuessy et al., 1986). Considering the evolutionary lability of ray expression, ray polymorphisms represent a challenging model to explore the role of individual and contextual components on the costs and benefits of specialized attractive structures.

In this study, we provide an integrative approach combining observational and manipulative experiments to investigate simultaneously the benefits and costs of specialized floral advertising structures. Our approach centered on comparing rayed and rayless phenotypes in two closely related annual species of the genus *Anacyclus*: the rayed *A. clavatus*, and the rayless *A. valentinus*. These two species show a high degree of geographical overlap, and several hybrid areas had pronounced variation in ray size and number (Fig. 1; Humphries, 1979; Agudo, 2017). In sympatry, both rayed and rayless phenotypes showed mixed ancestry precluding the attribution of rayed and rayless phenotypes to its respective parental lineages (Agudo, 2017). The presence of substantial phenotypic variation, as commonly observed in hybrid zones, makes these areas ideal study cases to evaluate the effect of floral traits on plant fitness (Hodges and Arnold, 1994; Campbell et al., 1997; Campbell, 2003, 2008). Hence, using this broad natural variation in ray size and number, we assessed the advantages and costs of ray production in terms of pollinator attraction, pollen limitation, and female reproductive success, measured as average fruit set per head and the total number of seed produced per plant. Additionally, we experimentally explored the effects of rays on pollinator attraction and female reproductive success by performing manipulations on ray phenotype and on the floral display of neighboring congeners, i.e., the neighborhood floral display. Specifically, we asked four questions. (1) How do rays, individual floral display, and surrounding neighborhood floral display affect pollinator attraction? (2) Do rayed and rayless phenotypes differ in levels of pollen limitation?

(3) Does neighborhood floral display affect rayed and rayless phenotypes differently? (4) Do ray production and maintenance entail a cost in terms of fruit production? Manipulations were performed on the entire plant, and lifetime female fitness components were measured, providing estimates of the effect of this floral trait on plant fitness.

MATERIAL AND METHODS

Study species

Anacyclus L. (Anthemideae, Asteraceae) exhibits exceptional variation in floral morphology and sexual expression both within heads and among species, which arguably results from different evolutionary and hybridization events (Humphries, 1981). The species complex formed by *A. clavatus* (Desf.) Pers. and *A. valentinus* L. (Fig. 1A, B), shows notable differences in floral morphology and, in areas where both species coexist, a pronounced variation in the number and size of rays can be observed (e.g., Fig. 1G, H; Bello et al., 2013). *Anacyclus clavatus* is usually found in disturbed habitats, coastal beaches, open fields, and roadsides within the Circum-Mediterranean Basin (Humphries, 1979). This species has gynomonoecious heads (i.e., female and bisexual florets). Female florets are present at the outermost positions of the head, displaying white rays (Fig. 1A), whereas yellow, bisexual disc florets with a tubular-campanulate corolla are displayed in the central part of the head (Fig. 1A; Bello et al., 2013). *Anacyclus valentinus* is found in coastal areas in the Western Mediterranean, occurring in disturbed habitats, sandy areas, lowlands, river banks, open fields, and roadsides (Humphries, 1979). This species also bears gynomonoecious heads, having unisexual female flowers in the outermost positions of their heads, but these female flowers display inconspicuous or no rays (discoid-like or rayless head, Fig. 1B, Humphries, 1979; Bello et al., 2013). Both species are interfertile and self-incompatible (Humphries, 1981; Agudo, 2017), and bloom from February to July. After fertilization, flowering heads from both species produce two types of achenes, i.e., one-seeded dry fruits: flowers at the outermost positions, including both ray and disc outermost florets, produce winged and heavier achenes, whereas the innermost disc flowers produce lighter unwinged achenes (Torices et al., 2013; Afonso et al., 2014).

Study sites

This study was conducted during the spring of 2013 within the contact zone of both species, around Torre del Mar (Andalusia, Spain). Three sites with a high abundance of *Anacyclus* individuals were chosen, namely (1) sympatric site—an open field, where both species grew and where intermediate phenotypes had previously been observed (36°43′48.875″N, 4°6′8.154″W); (2) rayed site—an abandoned area on the edge of a road, containing only *A. clavatus* (36°45′4.186″N, 4°5′58.289″W); and (3) rayless site—an open field area next to planted palm trees, containing only *A. valentinus* (36°43′50.516″N, 4°6′4.697″W). Ecological conditions in these sites were similar. Vegetation was characterized by several ruderal herbaceous species such as *Leontodon longirostris* (Vill.) Mérat (Asteraceae), *Hirschfeldia incana* (L.) Lagr.-Foss. subsp. *incana* (Brassicaceae), *Chrysanthemum coronarium* L. (Asteraceae), and *Echium creticum* subsp. *granatense* (Coincy) Valdés (Boraginaceae).
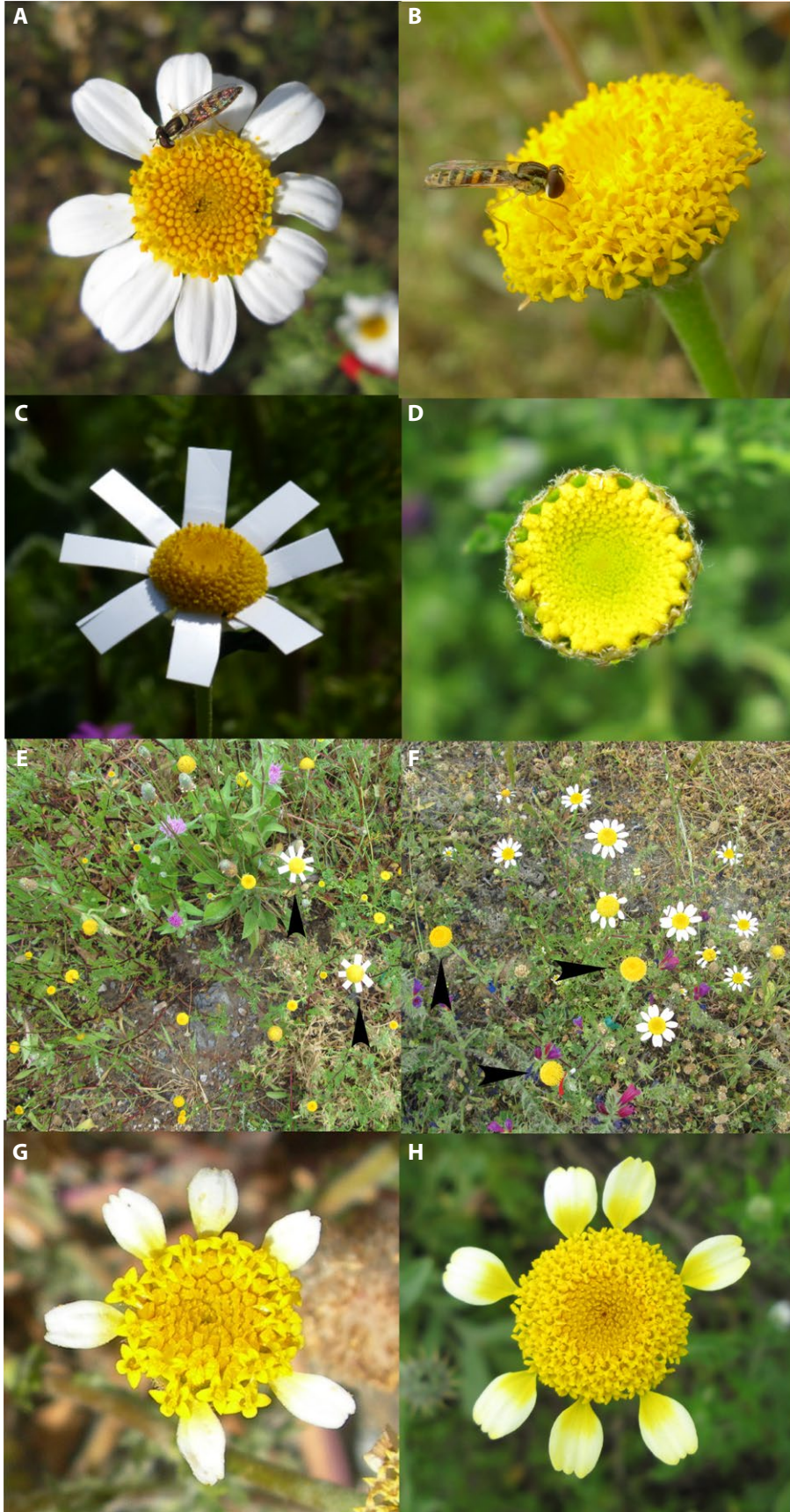
FIGURE 1. *Anacyclus* natural variation and manipulated set-up used in this study: (A) rayed head, *A. clavatus*; (B) rayless head, *A. valentinus*; (C) artificially rayed head; (D) artificially rayless head; (E) artificially rayed heads (highlighted with black arrows); (F) artificially rayless heads (highlighted with black arrows). (G–H) intermediate phenotypes observed in populations where *A. clavatus* and *A. valentinus* grow in sympatry.

Experimental design

We established two approaches: (1) a purely observational survey, and (2) an experimental manipulation of floral phenotypes. In both, we investigated the benefits and costs of rayed vs. rayless phenotypes. The observational survey was conducted in the sympatric site, where we observed plant–pollinator interactions with the minimum amount of disturbance, and characterized the naturally occurring neighborhood floral display, i.e., the floral display of neighboring congeners. In the rayed and rayless sites, we established the experimental manipulations of both floral phenotypes and neighboring plants to study both phenotypes (rayed vs. rayless) under two contrasting backgrounds. In addition, two complementary experiments were performed at all three sites to explore the extent of pollen limitation on fruit production and the potential costs of ray production in terms of fruit set and fruit size.

1. Observational survey at the sympatric site—The sympatric site was characterized by extraordinary phenotypic variability in ray traits including the presence of intermediate and both rayed and rayless phenotypes (Bello et al., 2013; Agudo, 2017). Previous surveys of the genetic structure in the contact zone between both species, including this site, showed that both rayed and rayless phenotypes frequently had mixed ancestries in sympatry (Agudo, 2017). Therefore, among hybrids in this site, ray number is highly variable and it is not indicative of the proportional genetic contribution from each parental lineage. Because floral phenotype is not useful to identify species in sympatric sites, we will always refer to rayed and rayless phenotypes instead of rayed and rayless species for this site. We established 27 patches including 2 to 7 plants, totaling to 103 selected plants. Selected plants were tagged and characterized based on plant and inflorescence traits and neighborhood floral display. Plant traits included plant height (the distance from the ground to the tallest part of the plant), plant volume (estimated as the plant's largest diameter, measured parallel to the ground, multiplied by its perpendicular axis, and by the plant's height) and individual floral display (the total number of blooming heads; quantified every 2–3 days throughout the field season). Inflorescence traits included head size (total diameter of the head, from the tip of a ray to the tip of the opposite ray), disc size (diameter of the yellow central disc), ray number, and ray length. In addition, we quantified neighborhood floral display by counting the number of open *Anacyclus* inflorescences surrounding each tagged plant within a 0.5 m radius (quantified also every 2–3 days throughout the field season).

2. Ray and neighborhood manipulations in rayed and rayless sites—In the single-phenotype sites, we experimentally manipulated the phenotypes and the neighborhood floral display. In the rayed site we removed rays to create rayless plants in a set of selected plants, while in the rayless site we added artificial rays to rayless plants (Fig. 1C–F).

At the rayed site, we selected 20 patches of three nearby plants, separated by ~1–2 m. Each triplet included one rayed phenotype (control phenotype), an individual that had its rays removed (experimental rayless phenotype; Fig. 1D, F) and one individual with

artificial rays (artificially rayed phenotype; Fig. 1C). In the experimental rayless phenotypes, ray florets were completely removed with tweezers when its elongation started and before its complete expansion and anthesis. Artificial rays were made with white synthetic paper (polyethylene; paper commonly used to print scientific posters) and were added to the heads to mimic the rayed phenotype (Fig. 1C) following a similar approach successfully employed by Nielsen et al., 2002. To minimize variation of plant traits and neighborhood structure, we selected plants with similar traits (height, dimension) and floral displays. In addition, we removed inflorescence buds produced after the beginning of the experiment to keep plants homogeneous during the experiment. The inclusion of an artificially rayed phenotype in the rayed site served as a procedural control to assess the effect of artificially rayed phenotypes at the rayless site.

At the rayless site, we selected 20 pairs of nearby plants, separated by ~1–2 m from each other. Within each pair, one individual remained as the rayless phenotype (control phenotype) while the other was equipped with artificial rays (artificially rayed phenotype; Fig. 1C, E). Similar plants were chosen and were kept homogeneous throughout the field season as described above for the rayed site.

The neighborhood floral display was studied in all the patches of plants selected above. Additionally, we removed neighboring *Anacyclus* plants in a 1 m radius in 10 additional plant pairs in both sites (the artificially rayed phenotype at the rayed site was not considered for this experiment) and compared solitary rayed and rayless phenotypes with plants with untouched neighboring conditions.

3. The effect of ray presence on pollen limitation—To determine whether pollen limitation differentially affects fruit production of rayed and rayless plants, we experimentally hand-supplemented flowering heads with pollen in the sympatric site (20 rayless and 20 rayed) and in the rayed and rayless sites (20 individuals in each). On each plant, we labeled two heads at the same phenological stage: one was hand pollinated with outcross pollen collected each day from at least five different plants across each site; the other was used as a control. The heads used in the experiment were selected from the central upper part of the plant to avoid other confounding effects. Additionally, control heads were further compared with heads from nearby unmanipulated plants and showed similar fruit set (results not shown), indicating that reallocation of resources for pollen-supplemented heads did not significantly decrease the number of fruits produced in control heads of manipulated plants (Wesselingh, 2007). Regrettably, most of the plants in the rayless site were eaten by cattle, and only four individuals ripened fruits, and therefore we did not get any estimates of pollen limitation for the rayless site. In the sympatric site, three plants (two rayless and one rayed) died before producing any fruit.

4. The effect of ray presence on fruit production—We assessed the cost associated to develop and maintain ray structures on fruit production by means of a ray removal experiment. For this, we selected 21 plants in the rayed site. For each plant, we selected three

heads in early developmental stages for the following treatments: (1) ray removal and hand-supplementation with outcross pollen, RR; (2) control and hand-supplementation with outcross pollen, PS; and (3) control with open pollination, C. Pollen supplementation assures that fruit production was not pollen limited, whereas the control left for open pollination was subjected to natural levels of pollination. At bud stage, ray florets were completely removed using tweezers, and thus, they were removed before disc florets developed completely. The number of fruits and fruit weight were quantified in the laboratory. Because *Anacyclus* species produce two types of fruits within one head (Torices et al., 2013), we tested the potential effect of ray removal on the size of both types of fruits separately.

Floral visitor censuses

A reference collection of *Anacyclus* floral visitors was gathered in a preliminary survey of pollinator assemblage within the contact zone in the spring of 2012. Floral visitor observations were performed during central hours (from 10:30–18:00, GMT+1) of warm and sunny days from 30 Mar to 26 April 2013 during the flowering period of both study species, throughout the three studied sites. Plant patches were observed during intervals of 5 min. Observers were positioned 1–2 m distance from the plant group and used small-range binoculars to avoid disturbing the foraging activity of floral visitors to tagged plants. A floral visit was only recorded when there was a direct contact between the forager and the sexual organs of the head (anthers and/or stigmas). Only approaches to each individual plant were considered, whereas consecutive visits within one individual plant were not considered. A total of 7885 min of observation time was performed in the sympatric site (75–95 min/plant; mean = 90 min). A total of 4265 min of observation time was performed in the rayed site (55–65 min/plant; mean = 60 min). A total of 3760 min of observation time was performed in the rayless site (50–80 min/plant; mean = 70 min). Insect identification was based on the reference collection. New pollinator taxa were collected with a capture net or a vacuum container for subsequent identification. Smaller insects were preserved in ethanol 70%, whereas larger ones were air-dried (Appendix S1).

Because of the high number and diversity of floral visitor species on *Anacyclus* plants (Appendix S1), we assessed preferences of particular pollinator groups for the studied plant traits. "Pollinator group" was defined as a group of pollinators that tended to interact with flowers in a similar way and was established following the methodology employed in Gomez et al. (2008). Using this, we obtained the following groups: ants, beeflies, beetles, bugs (Hemiptera), butterflies, hoverflies, large bees, large flies, small bees, small flies, and wasps. The relative abundance of some of these groups was very low (e.g., we observed only seven plant–Hemiptera interactions throughout the whole field season; Appendix S1), thus hindering the statistical analyses; therefore, we merged some groups and excluded others with very few interactions, ending up with four main represented groups: bees (including small and large bees), large flies, hoverflies, and small flies. Bees included individuals from approximately 2–12 mm (from head to abdomen), including members from the Apidae family such as *Apis mellifera*, *Anthophora* sp., and *Eucera longicornis*, but also *Lasioglossum* sp. (Halictidae) and some unidentified species from Megachilidae and Sphecidae. Hoverflies included individuals from approximately 9–15 mm

(from head to abdomen), from the Syrphidae. Specifically, this included *Eristalis tenax*, *Eristalis arbustorum*, *Eupeodes* sp., *Episirphus* sp., *Sphaerophoria* sp., *Syritta pipiens*, *Chrysotoxum* sp., and a few nonidentified hoverfly species. Large and small flies included members of the Calliphoridae, Anthomyzidae, Tachinidae, Scathophagidae, and few unidentified species. Large and small flies included individuals larger or smaller than 2 mm (from head to abdomen), respectively. Finally, in the rayed site there was very low visitation, and no pollinator groups were established because of statistical constraints. Detailed information about floral phenotypes and morphospecies interactions are displayed in Appendix S1.

Female reproductive success

We assessed two components of female reproductive success: the average fruit set per head of each individual, and the total number of seeds produced by the plant. Fruit set refers to the proportion of viable achenes considering the total number of flowers per head. After ripening, at least five flowering heads were sampled from each plant. The proportion of viable and nonviable achenes was investigated for all the sampled heads under a stereomicroscope. The number of achenes was quantified in one quarter of the head and extrapolated for the entire head. Preliminary exploration showed that estimation of seed production and fruit set in one quarter of the inflorescence was highly correlated with the total value obtained from the whole head (n = 10, r = 0.96, P ≤ 0.001). The total number of seeds in those plants with more than five heads was calculated by multiplying the average number of seeds per head from the five collected heads with the total number of heads produced by each individual plant. Relative fitness was calculated within site by dividing by the maximum seed number.

Statistical analyses

Broadly, data were analyzed using general linear mixed models (GLMM), using the 'lme4' package (Bates et al., 2014) in the R 3.0.1 software (R Core Team, 2013). Before fitting any model, we carefully analyzed and explored the data, searching for correlation and multicollinearity among variables (Appendices S2, S3). After fitting each model, we performed model validation routines plotting residuals against fitted values and against each explanatory variable in the model (Zuur et al., 2009), and estimating the overdispersion coefficient for Poisson models using parametric bootstrapping (Harrison, 2014). We included an observation-level random effect to deal with overdispersion when it was detected (Harrison, 2014). All analyses included 'plant', either alone or together with 'patch' as a random variable. Unless otherwise noted, deviance type-II tests of fixed factors were shown. In addition, differences between factor levels were assessed using least square means values of the models using pairwise comparisons with 'lsmeans' package (Lenth, 2013). Below, the statistical analyses followed in each experiment are presented in detail.

Observational survey at the sympatric site—First, we assessed the effect of floral phenotype (rayed vs. rayless) on floral visitors, fruit set, and total seed production. These three variables were fit as response variables in GLMMs wherein floral phenotype, and individual and neighborhood floral display were included as explanatory variables. Visitation rate was modeled with a Poisson

distribution and a log link function; fruit set was modeled with a binomial distribution and a logit link function; and total seed production using a Gaussian function with identity link. We also created additional models for each pollinator group: Bees, Large Flies, Hoverflies, and Small flies (Appendices S4, S5).

Second, we explored direct and indirect links between the three response variables by Structural Equation Modeling (SEM) using the 'lavaan' package (Rosseel, 2012). We fitted one model for each phenotype (rayed and rayless phenotype; Appendix S4). Direct effects were calculated through direct relationships between variables. Indirect effects were calculated through the multiplication of all the indirect effects between variables. In both models, floral visitation, fruit set and total seed production were considered as endogenous variables (i.e., response variables in these models). Ray size, disc size, and individual and neighborhood floral display were scaled and added as exogenous variables (i.e., independent variables in these models). Because disc size did not correlate with visitation rate, and neither significantly affected visitation rate (results not shown), we did not include the direct effect of disc size on visitation rate to avoid model saturation. In addition, ray number was not included in the model. Nevertheless, ray number was highly correlated with ray size (Appendix S3), and when both variables, number, and size were regressed against visitation rate, only ray size significantly affected floral visitors (results not shown). Model fit was analyzed through Root Mean Square Error of Approximation and its associated p-value (rayless model: $\chi^2 = 0.008$, df = 1, $p = 0.993$, RMSEA = 0.000; rayed model: $\chi^2 = 1.338$, df = 1, $p = 0.282$, RMSEA = 0.075).

Ray and neighborhood manipulations in rayed and rayless sites—We studied the effects of the established manipulations on floral visitors and fruit set. For that, all models included floral phenotype (rayed vs. rayless) and neighborhood floral display (control vs. neighborhood removed) as explanatory variables, and further
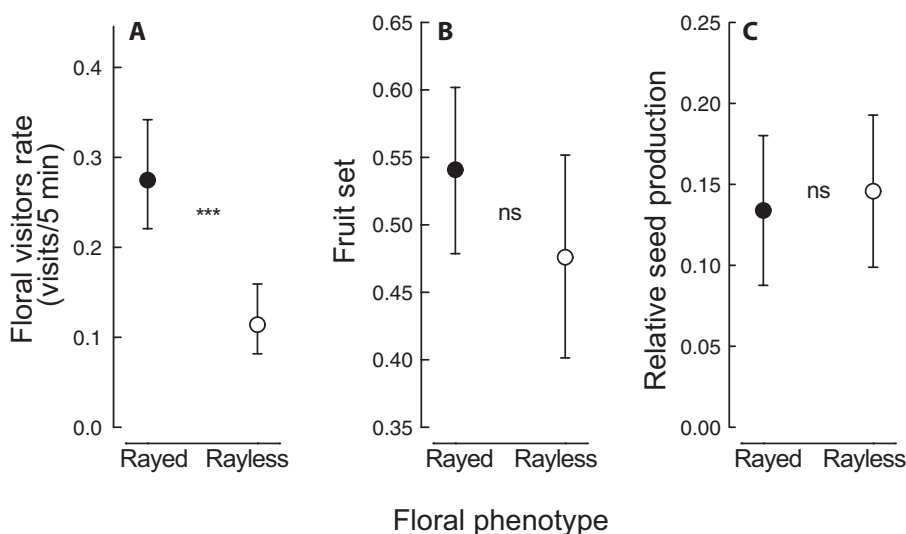
included the interaction between phenotype and neighborhood display. The interaction was assessed using type-III tests. As the interaction term was not statistically significant, main effects were assessed by type-II tests. In addition, we tested whether artificially rayed plants were efficiently simulating natural rays by assessing floral visitation rate and fruit set of both rayed phenotypes from the rayed site.

The effect of ray presence on pollen limitation—We fitted a GLMM where fruit set was modeled using a binomial distribution and a logit link function, wherein floral phenotype and pollen supplementation treatment were included as explanatory variables.

The cost of ray presence on fruit set and weight—We assessed the effect of ray removal on fertility and fruit weight in the rayed site. Fruit set was modeled with a binomial distribution, whereas fruit weight was modeled with a Gaussian distribution. The experimental treatment was included as the explanatory variable.

RESULTS

Observational survey at the sympatric site

Rayed vs. rayless phenotypes—Rayed phenotypes in the sympatric site produced a higher number of heads, and thus a higher individual floral display (Appendix S2). However, rayed heads had significantly smaller disc diameters and fewer disc flowers per head than rayless heads (Appendix S2). Consequently, the total number of disc flowers per plant did not differ between rayed and rayless plants (Appendix S2).

The presence of rays had a significant impact on floral visitor attraction at the sympatric site, so that rayed plants attracted significantly more floral visitors than rayless plants (Fig. 2A, Table 1). Dipteran groups (large flies, hoverflies, and small flies) drove this trend, because they visited rayed plants significantly more often compared with rayless ones (Appendices S5, S6). Bees visited plants with larger individual floral displays more frequently, irrespectively of its floral phenotype (Appendix S5).

Rayed phenotypes did not show a higher probability of setting fruits nor produced more seeds per plant than rayless phenotypes (Fig. 2B, C; Table 1). By contrast, as the number of neighboring heads increased—i.e., the neighborhood floral display, fruit set, and total seed production increased for both floral phenotypes (Table 1)—higher individual floral displays significantly increased total seed production in both phenotypes (Table 1).

Direct and indirect effects of floral traits, individual and neighborhood floral displays on floral visitors, and female reproductive success—For rayed phenotypes, ray length positively affected visitation rate of floral visitors, and had a direct effect on fruit set (Table 2; Appendix S4A).

FIGURE 2. The effect of floral phenotypes (rayed vs. rayless) on (A) floral visitation rate, (B) fruit set, and (C) total seed production in the sympatric site. Least square means (±95% confidence intervals) of visitation rate (floral visitors per 5 min), fruit set and total seed production. Total seed production was scaled by the highest value in the population. The details of the GLMM models are provided in Table 1 (ns: nonsignificant at $P > 0.05$, ***, $P < 0.001$).

TABLE 1. The effect of floral phenotypes (rayed and rayless) on floral visitors, fruit set and total seed production at the sympatric site based on generalized linear mixed model analyses. Floral phenotype (rayed vs. rayless), individual and neighborhood floral displays are included as explanatory variables. Plant identity was included as a random variable for floral visitation rate and fruit set, whereas patch was included for total seed production. An observation-level random effects (OLRE) was included to model floral visitation rate to cope with the overdispersion of this model. Degrees of freedom (*Df*), Chi-Square (χ^2), variance and standard deviation (SD) are provided. Statistically significant differences ($P < 0.05$) are shown in bold. A sign before the χ^2 value indicates the direction of the effects for significant individual and neighborhood floral displays.

Variables	*Df*	Floral visitation rate		Fruit set		Total seed production	
		χ^2	*P*	χ^2	*P*	χ^2	*P*
Fixed							
Floral phenotype	1	**21.57**	**<0.001**	1.64	0.201	1.21	0.272
Individual floral display	1	**(+) 4.88**	**0.027**	0.49	0.482	**(+) 194.0**	**<0.001**
Neighborhood floral display	1	1.33	0.249	**(+) 8.75**	**0.003**	**(+) 13.27**	**<0.001**
		Variance	SD	Variance	SD	Variance	SD
Random		0.23	0.48	0.94	0.97	0.01	0.96
OLRE		0.95	0.97	–	–	–	–

Nevertheless, the total effect of ray size on fruit set was not significant (Table 2; Appendix S4A). Individual floral display had a significant direct effect on floral visitors and total seed production (Table 2) and neighborhood floral display positively influenced floral visitors' attraction and fruit set, but not the total seed production (Table 2). The SEM for rayed phenotypes accounts for 39%, 22%, and 44% of variation of floral visitors, fruit set, and total seed production, respectively.

In rayless plants, individual floral display had a significantly positive effect on the visitation rate of floral visitors, fruit set and total seed production (Table 2; Appendix S4B). Disc size had a significant positive effect on fruit set and total seed production (Table 2). However, neighborhood floral display did not directly or indirectly affect either of the female reproductive success components (Table 2). The SEM for rayless phenotypes accounts for 24%, 26%, and 61% of variation of floral visitors, fruit set, and total seed production, respectively.

Ray and neighborhood manipulations in rayed and rayless sites

Experimental removal of rays in the rayed site had no significant effects on floral visitor attraction (Fig. 3A; Table 3), nonetheless ray removal significantly decreased fruit set (Fig. 3B; Table 3). In the rayless site, artificially rayed plants did not receive significantly more visits than control rayless individuals (Fig. 3C; Table 3); and, rayless plants had significantly higher fruit set than experimentally rayed ones (Fig. 3D; Table 3). Results concerning the artificially rayed phenotype should be considered cautiously because we observed that artificially rayed plants in the rayed site showed a lower (but only marginally significant) visitation rate than control naturally occurring rayed plants (Appendix S7A). They also had significantly lower fruit set (Appendix S7B) suggesting that the artificial ray addition might have some negative effect on both floral visitors and fruit set compared to naturally rayed plants.

Neighborhood removal affected visitation rate and fruit set in the rayless site (Table 3). In this rayless site, those plants in which their

TABLE 2. Total, direct, and indirect effects of neighborhood and individual floral display and floral traits on the attraction of floral visitors and plant reproductive success. Standardized direct (DE) and indirect (IE) effects and their sum (TE) were estimated by structural equation models separately for rayed and rayless plants in the sympatric site. Significant effects are shown in bold. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

| Variables	Floral visitation rate			Fruit set			Total seed production		
	DE	IE	TE	DE	IE	TE	DE	IE	TE
Rayed plants									
Individual floral display	**0.48***	-	**0.48***	–0.12	–0.08	–0.20	**0.66***	–0.07	**0.59***
Disc size	-	-	-	–0.09	-	–0.09	0.18	–0.02	0.16
Ray size	**0.28**	-	**0.28**	**0.24***	–0.05	0.19	0.16	0.03	0.19
Neighborhood floral display	**0.33**	-	**0.33**	**0.39**	–0.06	**0.33**	–0.12	0.06	–0.05
Floral visitation rate	-	-	-	–0.17	-	–0.17	–0.05	–0.04	–0.09
Fruit set	-	-	-	-	-	-	**0.24***	-	**0.24***
Rayless plants									
Individual floral display	**0.42**	-	**0.42**	**0.39**	–0.05	**0.34**	**0.42***	**0.23**	**0.65***
Disc size	-	-	-	**0.36**	-	**0.36**	**0.30**	**0.14***	**0.44***
Neighborhood floral display	–0.24	-	–0.24	**0.28***	0.03	**0.31***	0.04	0.06	0.1
Floral visitation rate	-	-	-	–0.12	-	–0.12	**0.23***	–0.05	0.18
Fruit set	-	-	-	-	-	-	**0.38**	-	**0.38**
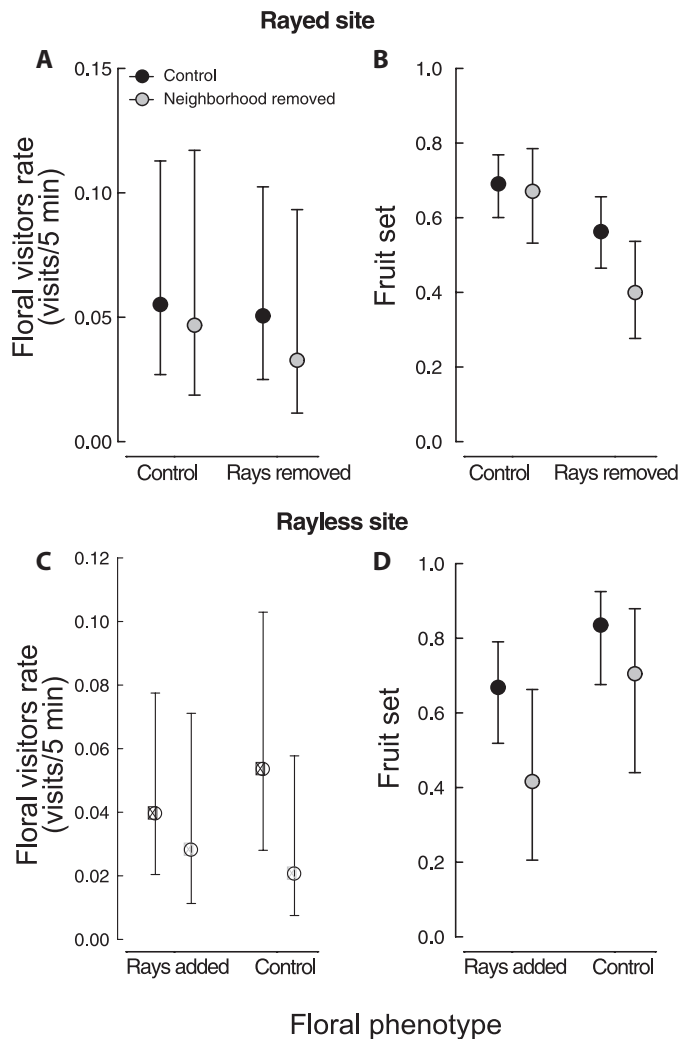
FIGURE 3. The effect of floral phenotype and neighborhood display manipulation on floral visitors and fruit set. Least square mean results (±95% confidence intervals) of visitation rate (A, C) and fruit set (B, D) for rayed and rayless phenotypes under control and removed neighborhoods in both: the rayed (A, B) and the rayless site (C, D). The statistical significance of each factor is indicated in Table 3.

neighboring flowering conspecifics were removed received marginally fewer floral visitors and produced significantly lower fruit sets than plants with control neighborhoods (Fig. 3C, D). Despite the lack of a significant interaction effect between ray manipulation and neighborhood removal (Table 3), solitary plants in which rays were either removed or added when they were growing in the other phenotype site showed the lowest mean fruit set compared to the rest of the experimental groups within each site (Fig. 3B, D).

The effect of ray presence on pollen limitation

Pollen supplementation significantly increased fruit set on rayless phenotypes of the sympatric site (n = 36, LRT = 9.06, P = 0.003), but not in the rayed phenotypes occurring at the same site (n = 38, LRT = 0.23, P = 0.634; Fig. 4). In the rayed site, pollen supplementation did not significantly increase fruit set compared to control

open-pollinated heads (n = 42, LRT = 1.14, P = 0.285), and we did not have sufficient data to determine the effect of pollen supplementation at the rayless site.

The effect of ray removal on fruit production

Ray removal increased neither fruit set nor fruit mass (Fig. 5, Appendix S8). However, outer-winged fruits in control open-pollinated heads were heavier than in heads that were pollen-supplemented, whether or not their rays were removed (Fig. 5B). This pattern was not observed for the inner-unwinged fruits, where no differences were observed between experimental groups (Fig. 5C).

DISCUSSION

This study was focused on understanding how the presence or absence of rays, in combination with the individual and the neighborhood floral displays, affect pollinator attraction and plant reproductive success. Additionally, we assessed whether rayed and rayless heads were pollen limited and explored potential costs of ray development in fruit production. We observed that when *Anacyclus clavatus* and *A. valentinus* naturally occur in sympatry, rayed phenotypes attracted significantly more floral visitors. Consequently, fruit production on rayed heads was not pollen-limited in contrast to rayless plants in which fruit set was limited by the available pollination resources. Despite this effect, larger individual floral displays, and denser local neighborhood floral displays, were more determinant factors driving plant reproductive success. Therefore, our analysis showed that the difference in attractiveness between rayed and rayless phenotypes in sympatry did not result in consequences for female fecundity at the plant level. Rayless plants were able to compensate the lack of rays by producing large heads with many florets. Thus, our results provide insights on the fitness consequences of advertising structures and on the potential inflorescence-level tradeoffs associated with the production of these structures.

Rays are effective advertising structures to attract different groups of insects. The presence of rays had a generally positive effect on pollinator attraction in several different study species (Lack, 1982; Marshall and Abbott, 1984; Sun and Ganders, 1990; Nielsen et al., 2002; Celedón-Neghme et al., 2007; Andersson, 2008). In agreement with these previous studies, we observed that in a natural hybrid site where ray size showed high variation, larger rays enhanced floral visitation rate and floral visitors preferred rayed phenotypes compared to rayless ones. However, our results also indicated that rays might not affect all pollinator groups in the same way. *Anacyclus* species were clearly generalist-pollinated, with flowers receiving visits from an extensive variety of taxonomic groups, including at least 17 different families from several insect orders (Appendix S1). Within this wide diversity, we found contrasting floral preferences in different pollinator groups. In the sympatric site, rays primarily attracted dipteran groups. Although it was suggested that hymenopterans are the main pollinators of Asteraceae (Lane, 1996), dipteran pollinators (mainly hoverflies) are also described as important pollinators of rayed species such as *Achillea ptarmica* (Andersson, 1991) and *Senecio vulgaris* (Abbott and Irwin, 1988). Moreover, a ray removal experiment in *Helianthus grosseserratus*—a rayed species pollinated mostly by dipteran and hymenopteran

TABLE 3. Experimental manipulation of floral phenotype and neighborhood display on floral visitors and fruit set at rayed and rayless sites. GLMMs were fitted independently for each response variable and for size to assess the effects of floral phenotype (rayed vs. rayless), neighborhood removal (neighborhood removed vs. control) and its interaction. The interaction was assessed using type-III tests. Because the interaction term was not statistically significant, main effects were assessed by type-II tests. Plant identity and patch were included as a random variable. Observation-level random effects (OLRE) were included to model floral visitation on the rayless site to cope with the overdispersion of this model. Degrees of freedom (*Df*), Chi-Square (χ^2), variance and standard deviation (SD) are provided. Statistically significant differences (*P* < 0.05) are shown in bold.

| | | Rayed site | | | | Rayless site | | | |
| | | Floral visitation rate | | Fruit set | | Floral visitation rate | | Fruit set | |
Variables	Df	χ^2	P	χ^2	P	χ^2	P	χ^2	P
Fixed									
Floral phenotype (F)	1	0.20	0.651	**9.86**	**0.002**	0.40	0.527	**5.14**	**0.023**
Neighborhood removal (R)	1	0.49	0.484	2.39	0.122	3.61	0.057	**3.90**	**0.048**
F x R	1	0.11	0.742	1.29	0.256	0.87	0.352	0.09	0.763
Random		**Variance**	**SD**	**Variance**	**SD**	**Variance**	**SD**	**Variance**	**SD**
Plant (Patch)		0.50	0.71	0.76	0.87	0.07	0.27	1.57	1.25
Patch		8.1×10^{-7}	8.9×10^{-4}	1.1×10^{-9}	3.3×10^{-5}	2.4×10^{-9}	4.9×10^{-5}	0	0
OLRE		–	–	–	–	2.84	1.69	–	–

species—resulted in a reduction in Diptera visitation rates, but not in Hymenoptera, revealing an important role of rays in the attraction of this particular group of insects (Stuessy et al., 1986). The differential effect of rays on the behavior of specific pollinator groups might thus result in different mating patterns not
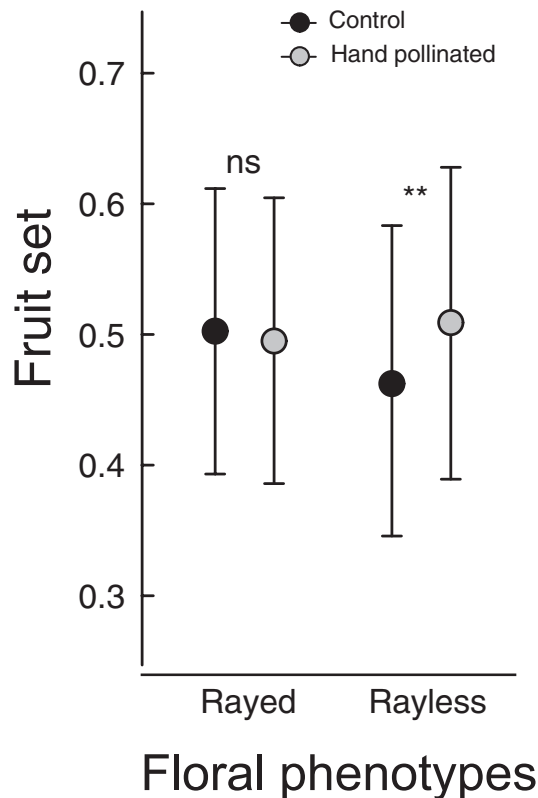

FIGURE 4. Hand supplementation with pollen to rayed and rayless plants in the sympatric site. Least-square means (±95% confidence intervals) of fruit set in control and hand-supplemented heads in both rayed (n = 38, LRT = 0.23, *P* = 0.634) and rayless phenotypes (n = 36, LRT = 9.06, *P* = 0.003). Statistical tests were performed independently for each phenotype: ns, nonsignificant at *P* > 0.05, **, *P* < 0.01.

only in single phenotype sites, but also between rayed and rayless phenotypes in sites of admixture.

The ray removal/addition experiment performed in this study focusing on two closely related species, one rayed and the other rayless, did not replicate the pattern observed in the sympatric site. This mismatch between the visitation rates observed in rayed and rayless phenotypes under natural and manipulated approaches might have resulted from unsuccessful manipulations, but also by a frequency-dependent effect of phenotype manipulations. Because manipulations were performed on sites where only one floral phenotype originally occurred, either rayed or rayless, pollinators might have been initially visiting these patches and choosing flowering heads driven by traits other than rays. In addition, although we followed the same approach of Nielsen et al. (2002) to create artificial rays, our ray addition manipulation produced unexpected negative effects in terms of both pollinator attraction and fruit set compared to naturally rayed plants (Appendix S7). This is most likely explained by the complexity of these inflorescences (Thomas et al., 2009), including complex ultraviolet (UV) reflection (Ron et al., 1977) and microcharacter variation (Lane, 1996), which can, independently or synergistically, influence pollinator attraction. That is, the microstructure of the chosen paper might not have effectively mimicked natural rays. Alternative solutions such as potted plants or artificial populations where the frequency of phenotypes also can be balanced should be used in future studies.

Despite the apparent disadvantage of rayless phenotypes compared to rayed ones in attracting pollinators in sympatric sites, rayless individuals showed the same female reproductive success levels as rayed plants. This outcome might result from compensation by other traits. In particular, rayless heads had significantly larger disc diameters and consequently more flowers per head than rayed heads (Appendix S2). This larger number of flowers of rayless heads might then offset its lower visitation rate resulting eventually in similar seed production when compared with the rayed phenotype. The higher numbers of flowers per head has two mating consequences: (1) an increase of available ovules to be fertilized per blooming unit, and (2) an increase of the temporal availability of these ovules to be fertilized. Heads are blooming units displaying several mature flowers at the same time and maturing sequentially (Harris, 1995; Bello
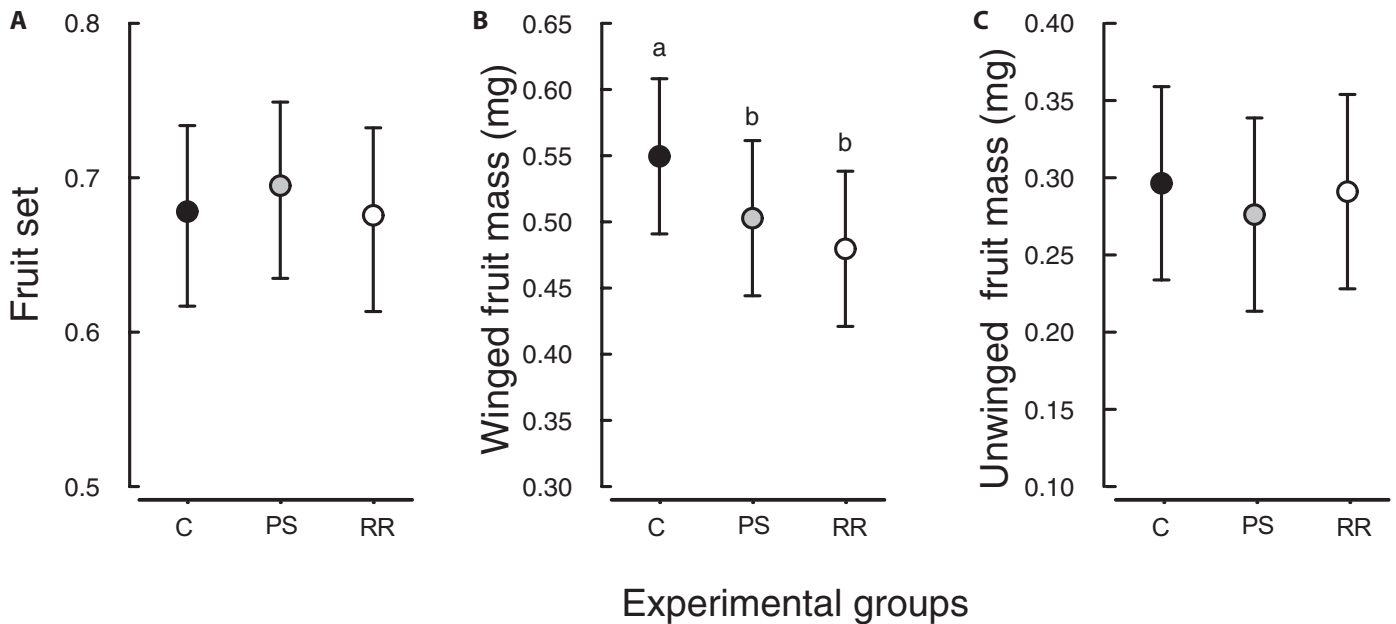
FIGURE 5. The effect of ray removal on fruit set (A) and fruit weight (B, C) in the rayed site. Least square means (±95% confidence intervals) of (A) fruit set and fruit weight of (B) winged and (C) unwinged fruits. C: control heads (closed dots); PS: pollen-supplemented inflorescences (gray dots); and, RR: pollen-supplemented inflorescence with rays removed (open dots). Means with different letters were significantly different at $P < 0.05$. No letter is displayed in those cases where no significant differences were found ($P > 0.05$). Statistical significance of each factor is indicated in Appendix S8.

et al., 2013). Single flowers are protandrous, releasing pollen grains during the first day, while the stigma is exposed only at the end of the first flowering day (Bello et al., 2013). Stigmas remain receptive during several days until they are fertilized with outcrossed pollen (Agudo, 2017). Within the same head, several rows of stigmas can thus be receptive, allowing a single pollinator visit to fertilize several flowers within a single head. Because rayless plants have more flowers per head, floral visitors to rayless heads might fertilize more ovules in a single visit than in rayed heads. Furthermore, a longer flowering duration of rayless heads might increase the total number of visits to each flower head, diminishing or cancelling the advantage conferred by higher attractiveness of the rayed phenotype. Consequently, plants receiving fewer visits per unit of time, such as rayless phenotypes, could still secure a sufficient amount of pollen, ultimately ensuring the fertilization of the same number or even more ovules per head.

The difference in number of flowers per head between rayed and rayless heads may also indicate a potential inflorescence-level tradeoff between advertising investment and head fertility. The pattern of rayless phenotypes having larger heads than rayed phenotypes was evident in the hybrid population at the sympatric site, but is also evident in the two pure species (Appendix S9). However, we did not observe a negative correlation between the ray length and the number of disc florets in the sympatric site as might be expected under this tradeoff. The study of tradeoffs using variation from natural populations can be misleading given the effect of variation on resource acquisition, which usually leads to positive correlations (Worley and Barrett, 2001). Thus, it is also expected that individuals growing in better microsites would have more resources, potentially resulting in larger heads with both longer rays and more flowers per head. Nonetheless, ray length

and the number of flowers per head or disc size were not significantly positively correlated either (Appendix S3). These genetic tradeoffs can be assessed by using controlled progenies grown under common garden conditions. In fact, the use of controlled progenies from experimental crossings between rayed and rayless plants of the daisy *Senecio jacobaea* has provided some evidence for this tradeoff between advertising investment and head fertility (Andersson, 2001). In the F2 and backcrossed populations of experimental hybrids between rayed and rayless plants, a principal component analysis showed that the ray traits (e.g., number, size, and width) and disc floret number loaded in opposite directions (Andersson, 2001). Additional support for this tradeoff between advertising investment and head fertility come from the analysis of allocation patterns in other currencies apart from biomass and interspecific comparisons of flower size and number per inflorescence. Phosphorous allocation to rays was negatively correlated with the allocation to the female sexual organs after controlling by inflorescence size (Torices and Méndez, 2014), and a negative correlation between flower size and the number of flowers per inflorescence has been detected across species (Sargent et al., 2007; Vasconcelos and Proença, 2015). Although theoretical models usually assume the existence of a tradeoff between advertising and primary sexual structures (Charlesworth and Charlesworth, 1987; Sakai, 2013), the evidence is still inconclusive.

The existence of this tradeoff was not supported by the ray removal experiment, and our study failed to find direct costs of ray production on female reproductive success (but see Andersson, 1999). Specifically, we did not find that removing rays increased the number of viable fruits. This result may suggest that *Anacyclus* plants might not be able to reallocate the resources from ray florets into the production of new fruits once

ray floret buds have been formed. Flower buds are developed sequentially, so the ray flower buds are formed before the disc flowers (Bello et al., 2013). After the determination of disc flower buds, the development of the ray starts, precluding any reallocation from rays to new flowers. Similar results were obtained in *Senecio jacobaea* (Andersson, 2001), where manipulative ray removal did not lead to an increase of head fertility. In *Achillea ptarmica*, ray removal had a stronger effect on pollinator attraction than in fruit set (Andersson, 1991). This might suggest also that pollen dispersal and siring success could be more affected than female fertility.

Plant reproductive success was strongly affected by the neighborhood floral display rather than by the focal-plant advertising phenotype. In both naturally rayed and rayless species, focal plants surrounded by a larger number of other conspecifics showed higher probabilities of setting fruits. However, the effects of the surrounding neighborhood floral display are very complex. Several studies have shown how neighborhood conditions affect a focal plant, concluding that factors such as spatial scale (Bartkowska and Johnston, 2014; Hegland, 2014), area and/or plant density combined with different population sizes (Williams, 2007; Dauber et al., 2010) and neighbor density (Makino et al., 2007; Hegland, 2014) affect pollinator visitation rates. Additionally, an increase in the allocation to pollinator attraction has been observed when plants are surrounded by kin rather than by unrelated individuals (Torices et al., 2018). The density of conspecifics may even interact with the individual floral display of focal plants. For example, in *Phlox hirsuta*, higher levels of conspecific density reversed the negative effects of larger individual floral displays at lower densities (Ruane et al., 2014). The neighborhood removal experiment presented here showed that the removal of *Anacyclus* neighborhood plants reduced plant fruit set, particularly in the less frequent floral phenotype. That is, rayless and rayed solitary plants in rayed and rayless sites, respectively, suffered a greater reduction in fruit number than the common floral phenotype in each site when conspecific neighbors were removed (Fig. 3B, D). Neighborhood floral display could have complex effects beyond mere density effects, altering pollinator behavior and individuals' floral display efficiency, and ultimately might also have consequences on mating patterns and the genetic structure of the populations.

CONCLUSIONS

Advertising traits, such as ray florets, are effective structures in enhancing pollinator attraction. However, plants can display different strategies to ensure ovule fertilization and maximize fecundity. Our study indicates that *Anacyclus* plants might have two distinct strategies to get their ovules fertilized: (1) increase pollinator attraction by means of larger rays, or (2) increase the number of reproductive units per blooming head. Despite the effect of the individual floral phenotype on pollinator attraction, the neighborhood floral display near each focal plant significantly determined female reproductive success. The complexity of the effect of the local context, accounting for many potential interactions between different phenotypes requires further studies. Experimental approaches aimed to manipulate and control these factors will be valuable steps towards understanding the functional consequences of intraspecific interactions on mating patterns and floral evolution.

AUTHOR CONTRIBUTIONS

R.T. and I.A. conceived the experiment and obtained funding; J.C., A.Ag., A.Af., S.C., and R.T. collected the data. J.C. and R.T. did the statistical analyses. J.C. and R.T. drafted the first version of the manuscript and the remaining authors contributed to its revision.

DATA ACCESSIBILITY

Data are archived in FigShare at https://doi.org/10.6084/m9.fig share.6839993.

SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

APPENDIX S1. Number of visits of floral visitors of *Anacyclus* spp. capitula in each studied site.

APPENDIX S2. Descriptive characterization of plant and inflorescence traits and pollination context of *Anacyclus* individuals in the sympatric site.

APPENDIX S3. Pearson correlation coefficient of plant and inflorescence traits and neighborhood structure for *Anacyclus* individuals in the sympatric site.

APPENDIX S4. Structural Equation Models for rayed plants (A) and rayless plants (B) at the sympatric site.

APPENDIX S5. The effect of floral phenotypes (rayed and rayless) on specific floral visitor groups at the sympatric site.

APPENDIX S6. Pollinator visits to rayed (closed dots) and rayless (open dots) phenotypes in the sympatric (A), rayless (B) and rayed site (C).

APPENDIX S7. Visitation rate and fruit set of artificial rayed heads compared to control rayed plants.

APPENDIX S8. The effect of ray removal on fruit production.

APPENDIX S9. Number of flowers and head diameter in allopatric populations of *Anacyclus clavatus* and *A. valentinus*.

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