



# DEPARTAMENTO DE CIÊNCIAS DA VIDA

FACULDADE DE CIÊNCIAS E TECNOLOGIA  
UNIVERSIDADE DE COIMBRA

Pollinator preference in a hybrid zone between  
two generalist plant species.

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José Cerca de Oliveira

2014



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Pollinator preference in a hybrid zone between two generalist plant species.

Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Ecologia, realizada sob a orientação científica do Professor Doutor João Loureiro (Universidade de Coimbra), da Doutora Sílvia Castro (Universidade de Coimbra) e do Doutor Rubén Torices (Universidade de Coimbra).

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Ao Nuno Lopes,

“De vez em quando a eternidade sai do teu interior e a contingência substitui-a com o seu pânico. São os amigos e conhecidos que vão desaparecendo e deixam um vazio irrespirável. Não é a sua ‘falta’ que falta, é o desmentido de que tu não morres.”

Virgílio Ferreira

*À minha família, namorada, amigos, orientadores e a todos os que directamente ou indirectamente  
contribuíram na minha formação ou na realização deste trabalho.*

O meu mais sincero e humilde obrigado.

*Choose a job you love, and you will never have to work a day in your life.*

Confucius, philosopher

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Abstract

Abstract

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The evolutionary mechanism behind flowers and its pollinators is generally understood to be a gradual co-adaptive process where the plant specializes to its most efficient pollinator, which exerts selective pressures on specific traits, driving floral evolution. Still, most flowering plants in nature are visited by a wide array of pollinator species, i.e. are generalist plants. However, the role of pollinators as significant drivers of floral evolution in generalist plants has been questioned due to the potential conflicting selection regimes exerted by different pollinators. Taking this into account, using a combination of observation and manipulative experiments, we assessed pollinator preference in a natural contact zone where the generalist rayed species *Anacyclus clavatus* and the rayless *A. valentinus* co-exist and hybridize, forming intermediate phenotypes. These contact areas show a remarkably high phenotypic variation, with the intermediate phenotype bridging both phenotypes and forming an exceptional micro-evolutive framework to explore how generalist pollinators could be driving the evolution of floral phenotypes. We found that the production of rays influenced the probability of being visited by specific insect groups, in particular by Dipteran groups; whereas bees showed no preference for rayed phenotypes and their visitation patterns were mainly driven by the number of capitula simultaneously blooming in the plant. In addition, we found support for the importance of the neighbours' phenotype when assessing pollinator preference on a focal individual. Rayed plants benefited from having other conspicuous neighbours, whereas rayless and intermediate phenotypes significantly competed for pollinators. In conclusion, all these differential behavioural patterns of floral visitors might affect gene flow within the hybrid zone between *A. clavatus* and *A. valentinus* influencing the degree of reproductive isolation and floral evolution between both species.

### Keywords

*Anacyclus*; Asteraceae; Discoid capitulum; Neighbourhood context; Pollinator behaviour;

Rayed capitulum;

Resumo

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Os mecanismos evolutivos que atuam nas flores e respetivos polinizadores são normalmente descritos como processos de co-adaptação gradual onde a planta se especializa no seu polinizador mais eficiente, que por sua vez, exerce pressões evolutivas em características específicas e dessa forma guia a evolução da flor. Ainda assim, a maior parte das plantas com flor são polinizadas por um leque diversificado de espécies de polinizadores, denominando-se assim plantas generalistas. No entanto, em plantas generalistas o papel dos polinizadores na evolução floral tem sido questionado devido a potenciais conflitos na selecção exercida pelas diferentes espécies de polinizadores que visitam a flor. Tendo isto em conta, abordagens observacionais e manipulativas foram utilizadas para avaliar as preferências dos polinizadores numa zona de contacto onde as espécies generalistas *Anacyclus clavatus* (com lígulas) e a espécie *A. valentinus* (sem lígulas) coexistem e hibridizam, formando fenótipos intermédios. Estas áreas possuem uma variação fenotípica notável, com o fenótipo intermédio a representar o cruzamento entre as duas espécies, garantindo um cenário microevolutivo excepcional para estudar de que forma os polinizadores conduzem a evolução fenotípica em espécies generalistas. Os resultados obtidos revelaram que a produção de lígulas influenciou a probabilidade das plantas serem visitadas por grupos específicos de insetos, em particular por dípteros; por sua vez, as abelhas não revelaram preferências por um fenótipo em particular, preferindo maioritariamente plantas com um elevado número de capítulos em flor. Além disto, os nossos resultados evidenciaram também que a composição fenotípica da vizinhança poderá desempenhar um papel importante na atração de uma planta focal específica; em particular, plantas liguladas beneficiaram em ter outros vizinhos com lígulas, enquanto que os fenótipos sem lígulas e intermédios competiram significativamente por polinizadores. Os diferentes padrões de comportamento diferentes por parte dos visitantes florais observados neste estudo podem afetar o fluxo genético na zona híbrida entre *A. clavatus* e *A. valentinus*, influenciando o grau de isolamento reprodutivo e evolução floral entre as duas espécies.

### Palavras-chave

Anacyclus; Asteraceae; Capítulo ligulado; Capítulo sem lígulas; Comportamento dos polinizadores;  
Contexto gerado pelos fenótipos vizinhos;

## Introduction

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Animal pollinated plants rely on pollinators for pollen removal and reception, depending strongly on the behavior and effectiveness of floral visitors for its reproductive success. Consequently, pollinators mediate floral evolution by exerting selective pressures on several floral traits (Wesselingh & Arnold 2000; Sánchez-Lafuente 2002; Campbell 2008; Gómez *et al.* 2008a; Parachnowitsch & Kessler 2010; Penet, Marion & Bonis 2012; van der Niet, Peakall & Johnson 2014). Most works focusing on flower evolution by pollinator pressures describe this evolutionary process as a gradual co-adaptive mechanism in which the plant evolves in response to its most efficient pollinator, assuming an unidirectional co-evolution that leads to the specialization for a specific pollinator or to a particular group of pollinators (Cope's rule of specialization; Cope 1896; Johnson & Steiner 2000; Aigner 2003; Gómez *et al.* 2007, 2014; Vereecken *et al.* 2012; Van der Niet *et al.* 2014). However, in generalist plants, some authors doubt that pollinators can act as significant drivers of floral evolution (Waser 2001; Kay & Sargent 2009), mostly because these plants can be visited by a numerous and diverse assemblage of pollinators (Herrera 2005), and because different types of floral visitors have shown distinct trait preferences and attributes as pollinators (Thompson 2001; Sánchez-Lafuente 2002; Castro *et al.* 2013), thus imposing conflicting selection regimes (Sahli & Conner 2011). Despite of that, recent studies on generalist plant species have found that inter-population variation on pollinator faunas can still exert selection patterns on corolla shape (Sánchez-Lafuente 2002; Gómez *et al.* 2008b, 2014), suggesting that floral traits of generalist plants may have been also shaped by the selection driven by pollinators. Nevertheless, with a few exceptions (e.g. see Sánchez-Lafuente 2002; Vereecken *et al.* 2012; Gómez *et al.* 2014), little is known about how and which pollinator groups select floral traits in generalist plant species.

The largest family of flowering plants, the sunflower family (Asteraceae), is a known example of a highly diverse lineage composed mainly by species with a broad assemblage of pollinators (Lane 1996). Plants of this family are easily recognised by its particular inflorescence, the capitulum (Funk *et al.* 2009), which functions as a single flower and serves as the basic unit of the plant's visual display to attract pollinators (Andersson 2001). Indeed, it is frequent that the main pollinators of a particular species are the most abundant floral visitors in that year or spatial area (Ollerton *et al.* 2007). However, some species with specialist pollination syndrome such as bird pollination are also known in this family (Lane 1996), including its oldest known fossil (Barreda *et al.* 2012).

Most of the research on pollinator interactions in Asteraceae has focused on understanding the ecological significance of the highly zygomorphic corolla produced by the flowers located on the outermost position of some capitula, i.e. ray florets in rayed capitula (Marshall & Abbott 1984; Stuessy *et al.* 1986; Celedón-Neghme, Gonzáles & Gianoli 2006; Nielsen, Siegismund & Hansen 2007; Andersson 2008). The presence of rays was shown to have significant consequences on pollination, primarily enhancing the attractiveness of capitula and consequently influencing the levels of outcrossing (Lack 1982; Marshall & Abbott 1984; Sun & Ganders 1990; Celedón-Neghme *et al.* 2006; Andersson 2008). Thus, petaloid rays seem to provide an advantage for attracting pollinators, although the pollination context, including pollinator's abundance and floral display, can reduce this effect (Andersson 1996; Nielsen, Philipp & Siegismund 2002).

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Despite the observed advantage of rayed capitula in attracting more insects, rayless species are frequent in Asteraceae. The several independent reversals towards rayless capitula in the evolution of this family, suggest that rayless capitula could also be adaptive (Bremer & Humphries 1993; Torices, Méndez & Gómez 2011). The production of rays might entail a cost by reducing available resources for fruit and seed production (Andersson 1999, 2001, 2008; Celedón-Neghme *et al.* 2006) and/or by attracting more seed predators (Fenner *et al.* 2002). Furthermore, as capitula are usually visited by a large number of pollinators, rayed capitula could be visited by a larger amount of less efficient pollinators than rayless capitula, reducing the amounts of pollen donation. Still, whether specific functional groups of pollinators show different preferences to rayed versus rayless phenotypes remains poorly explored (but see Stuessy *et al.* 1986).

Hybrid zones represent natural laboratories to understand ecological and evolutionary processes of reproductive isolation and selection on phenotypic traits (Barton & Hewitt 1985; Aldridge & Campbell 2006; Campbell & Waser 2007). These areas present a striking profusion of flower morphologies, allowing a better evaluation of pollinator preferences under natural conditions. Pollinator-mediated selection requires phenotypic variation, and studies in plant hybrid zones have already provided strong evidences on pollinated-mediated selection of floral traits (Hodges & Arnold 1994; Campbell, Waser & Melendez-Ackerman 1997; Campbell 2003, 2008). So far these studies were performed in plants with contrasting pollination syndromes such as bird versus insect plant pollinated species (Aldridge & Campbell 2006) in which pollen transference between related taxa was prevented by large differences in floral morphology, leading to a strong reproductive isolation due to pollinator behaviour (Schemske & Bradshaw 1999; Emms & Arnold 2000). However, little is known about hybrid zones involving generalist plant species, whose pollinator faunas highly overlap reducing the expected ethological isolation, as well as, about the role that generalist pollinators may have as selective agents in floral evolution and ethological isolation.

Within the Circum-Mediterranean genus *Anacyclus*, along the Western Mediterranean basin the rayed species *A. clavatus* co-exists with the rayless *A. valentinus* (Humphries 1979, 1981). In the sympatric areas, a large phenotypic diversity in the number and size of ray florets has been observed revealing the existence of a dynamic hybrid zone (Bello *et al.* 2013). In addition, a preliminary survey of floral visitors indicated that both plant species were visited by a large array of insects, most of them shared between both plant species (R. Torices, unpublished data). Hence, this hybrid zone provides an exceptional micro-evolutionary framework to explore whether different pollinator groups can preferentially select rayed versus rayless phenotypes and to explore whether hybrid zones between generalist plant species can be influenced by the behaviour of their floral visitors. Using a combination of observational and manipulative experiments we assessed the preferences of floral visitors in a contact zone between the two generalist plant species, *Anacyclus clavatus* (rayed phenotype) and *A. valentinus* (rayless phenotype). We performed phenotypic manipulations, simulating the rayed phenotype on an exclusive rayless site, and the rayless phenotype on an exclusive rayed site. In addition, to assess potential facilitation or competition effects between rayed and rayless phenotypes, we explored the effect

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of the intra-specific surrounding context by both characterizing quantitatively and manipulating the neighborhoods. Specifically, we aimed to determine: (i) how does the floral phenotype, floral display and surrounding context affect pollinator's attraction; (ii) how does capitulum size affect pollinator's attraction; and (iii) if there is an effect (facilitative or competitive) of any phenotype in the pollinator's visitation rates.



## Materials and Methods

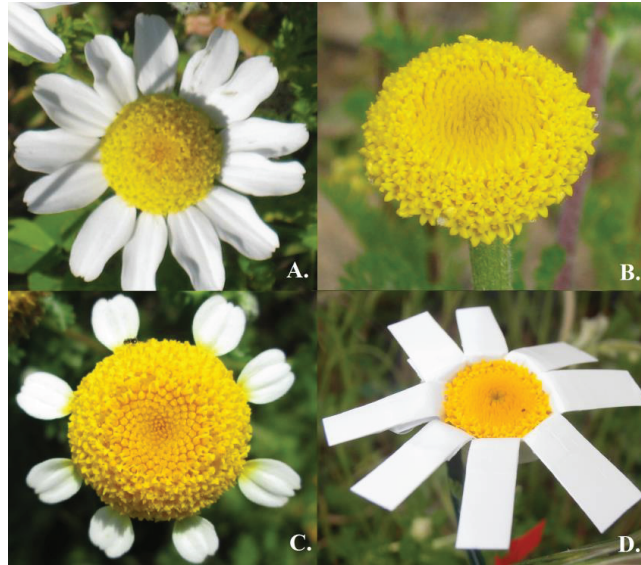
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### Study species

The genus *Anacyclus* L. (Anthemideae, Asteraceae) is composed of about 12 species of weedy annual herbs found in dry and disturbed habitats throughout the Mediterranean basin (Humphries 1981). This genus shows an extraordinary variation in reproductive traits and sexual expression within their capitula and among species, suggesting different evolutionary trends and hybridization events between some of its recent species (Figure 1; Humphries, 1981). One example is the species complex formed by *Anacyclus clavatus* (Desf.) Pers. and *A. valentinus* L. (Figure 1A and 1B). These two species present notable differences in floral morphology, however in areas where the two species coexist, morphological variation of the flowers is remarkably higher (e.g. in number and size of the rays; Bello *et al.* 2013), suggesting hybridization between both species.

*A. clavatus* is usually found in disturbed habitats, coastal beaches, fields and waste inland places, within the Circum-Mediterranean Basin (Humphries 1981). This plant has gynomonoecious capitula, with two types of flowers varying both in sex expression and morphology: rayed female florets with creamy-white ligules displayed in the outermost position of the capitulum and yellow bisexual disc-florets with a campanulate corolla and a narrow basal tube displayed in the central part of the capitulum (Figure 1A; Bello *et al.* 2013). *A. valentinus* is found in the Western part of the Mediterranean Basin (Morocco, Spain, Algeria and Tunisia), occurring in disturbed grounds, sandy and rocky places, lowlands, river banks, fields and roadsides (Humphries 1981). As *A. clavatus*, this species bears gynomonoecious capitula, however the female flowers displayed in the outermost positions usually lack rays and are fewer and inconspicuous, being the capitulum mostly represented by bisexual yellow disc-florets (Figure 1B; Humphries, 1979).

Both species are self-incompatible (I. Álvarez, personal communication) and commonly bloom from February to July. This long flowering season allows several generations of capitula to develop on the same individual. However, unsuitable conditions for flowering may often restrain the number of developing branches resulting in high variability in the number of capitula between individual plants (Humphries 1979).



**Figure 1.** The studied phenotypes.

A. *Anacyclus clavatus*, B. *Anacyclus valentinus*, C. Hybrid phenotype, D. Fake rays phenotype.

### Study sites

This study was conducted during the spring of 2013 in the contact zone between *A. clavatus* and *A. valentinus*, nearby Torre del Mar (Spain), at three different sites. The three selected populations included: 1) an open field, 1 m a.s.l., 210 m distance from the sea, where both species grow jointly and where an intermediate phenotype has been previously observed (hereafter *sympatric site*; +36° 43' 48.875" N, -4° 6' 8.154" W); 2) a road verge, 1 m a.s.l., 160 m away from the sea, being separated from the sympatric site by 100 m with buildings, only with *A. clavatus* (hereafter *rayed site*; +36° 45' 4.186" N, -4° 5' 58.289" W); 3) an open field area with planted palm trees, 16 m a.s.l., 1 km distance from the sea, only with *A. valentinus* (hereafter *rayless site*; +36° 43' 50.516" N, -4° 6' 4.697" W). The vegetation in the three sites was very similar, being characterized by ruderal herbaceous species such as *Leontodon longirostris* (Vill.) Mérat (Asteraceae), *Hirschfeldia incana* (L.) Lagr.-Foss. subsp. *incana* (Brassicaceae), *Chrysanthemum coronarium* L. (Asteraceae), and *Echium cretium* subsp. *granatense* (Coincy) Valdés (Boraginaceae). All populations had clear indications of the presence of livestock (J. Cerca de Oliveira, 2013, pers. obs.) and were chosen because they presented a high number of individuals of the desired study species growing in the same conditions.

### Experimental design

#### *Pollinator preferences in the sympatric site*

To assess floral visitor's preferences under natural conditions, we randomly selected and tagged 107 plants, including rayed, rayless and intermediate phenotypes (Figures 1A-C; Supplementary Table A). In order to maximize the efficiency of field observations, plants were monitored in groups of 2-7 individuals.

The selected plants were characterised phenotypically focusing on the individual plant characteristics (plant size and floral display), the capitulum traits (capitulum size, disk size, ray presence and ray number), and the intra-specific neighbourhood context. In particular, plant size was estimated as: (i) plant height, considering the distance from the ground to the tallest part of the plant, and as (ii) the plants' dimension, defined by a circular area, whose diameter was calculated by dividing the plants' largest diameter together with its perpendicular axis) by two. Floral display was defined as the total number of open capitula per individual at each observation day (quantified repeatedly through the field season). Capitula were characterized by: (i) the total diameter of the capitula (from the tip of a ray to the tip of the opposite ray), (ii) diameter of the disk and (iii) number of rays. Ray length was estimated by the following formula:  $(\text{diameter of the capitulum} - \text{diameter of the disk}) / 2$ . Finally, we measured intra-specific neighbourhood context using two proxies: (i) pollination context: the number of open capitula of *Anacyclus* within a 0.5 m radius, and (ii) neighbour density: the number of *Anacyclus* individuals within a 0.5 m radius. Neighborhood traits were surveyed at three different periods during the whole study. Floral visitors were monitored as described below in the *Floral visitor observations* section.

#### *Phenotypic manipulations at single-species sites*

To get further insights of the role of the rayed phenotype on the pollinator's preferences we performed two experiments of phenotype manipulation, one in the rayed site involving the removal of rays, and the other in the rayless site involving the addition of artificial rays to the capitula. Plants were characterized as described above. Floral visitors were monitored in all the experimental plots as described below in *Floral visitor observations* section.

## Materials and Methods

### *Rayed site: Ray removal experiment*

For this experiment we selected 30 pairs of nearby plants. The plants from each pair were carefully chosen to be similar in size, habit and number of capitula. One individual was set as the control and served as a rayed phenotype, while the other served as the rayless phenotype, with its rays being removed using tweezers. To maintain the paired individuals as similar as possible, we removed buds produced after the beginning of the experiment. Neighbourhood effects were studied using two approaches: First, pollination context and neighbourhood density were characterized in 20 pairs of plants to assess for its potential effects on pollinator's attraction using the variation in natural populations. For that, the number of surrounding *Anacyclus* plants and open capitula were counted in a radius of 0.5 m (see above). Second, in the remaining subset of 10 randomly selected pairs of plants we performed a manipulative experiment to assess the potential effects of the neighbourhood context on floral visitors' attraction, by manually removing all surrounding *Anacyclus* plants within a 1 m radius of the focal individuals.

As a procedural control for the ray addition experiment (see below), a third individual was selected near each pair and equipped with fake rays (Figure 1D). Visitation rate comparisons between the manipulated and naturally rayed phenotypes was performed by means of a Kruskal-Wallis test, and showed that fake rays marginally decreased the visitation rate to the capitula ( $\chi^2 = 3.06$ ,  $P = 0.08$ ) compared to naturally rayed phenotypes.

### *Rayless site: Ray addition experiment*

We carefully selected 30 pairs of individuals with similar characteristics, manipulating the individuals (adding fake rays and removing extra capitula buds) and the neighbourhood context (presence vs. absence of other *Anacyclus* plants) as described above. Within each pair, one individual served as the rayless individual (control) while the other was equipped with fake rays. Fake rays were made with synthetic paper and they were added to the capitula to mimic the rayed phenotype as realistic as possible (Figure 1 D; see statistical details in above sub-section), similarly to the approach by Nielsen and colleagues in the endemic *Scalesia* from the Galapagos islands (Nielsen *et al.* 2002). As in the rayed site, pollination context and neighbourhood density was characterized in 20 pairs of plants and in the remaining subset of 10 pairs of plants, all surrounding *Anacyclus* neighbours were removed within a 1 m radius.

### *Floral visitor observations*

A preliminary survey of pollinators was performed during the spring of 2012 in the contact zone of this study, to get insights about the pollinator fauna that was visiting *A. clavatus* and *A. valentinus*, and to collect insects for a reference collection of *Anacyclus* spp. floral visitors. In 2013, floral visitor observations were carried during the main flowering period of the study species, more specifically, during the central hours (from 10:30 to 18:00, GMT+1) of warm and sunny days from 30<sup>th</sup> of March to 26<sup>th</sup> of April. These observations were conducted similarly in the three studied sites. With the aid of small range-binoculars, plant groups were observed during intervals of five minutes, with the observer positioned at a considerable distance (1-2 m apart) from the plant group, to avoid disturbing the foraging activity of the insects, while recording all the insects that visited the tagged plants. A floral visit was only taken into account when there was a direct contact between the insect visitor and the sexual organs (anthers or stigmas) of the capitulum. Considering that these species are self-incompatible, the number of capitula visited per individual plant was not accounted. During observation intervals the overall weather conditions, the hour of the day and the surrounding insect activity were recorded for data quality assessment. A total of 1338 census were performed, corresponding to a total of 111.5 hours of net observation evenly divided by site. Insect identification was based on the reference collection gathered in 2012; still, whenever a new taxon was observed, it was collected with a capture net or a vacuum container for subsequent identification at the laboratory. Smaller insects were conserved in ethanol 70%, while bigger insects were air dried. All insects are being kept at the Centre for Functional Ecology (Faculty of Science and Technology, University of Coimbra). The pollinators were grouped into “functional groups” to facilitate the detection of general patterns. ‘Functional group’ was defined as a group of pollinators that tend to interact with flowers in a similar way. Following the methodology employed in Gomez *et al.* (2008b) we used criteria of similarity in size, proboscis length, foraging behaviour and feeding habits rather than taxonomic relationships. Also, given the low number of visits of each bee group, bees were grouped in the same group. In the end, the following 6 functional groups were established: ants, bees, beeflies, big flies, hoverflies and small flies.

### **Statistical analyses**

The effects of floral phenotypes, floral display and neighbourhood traits on pollinator attraction were assessed using general linear mixed models (GLMM). Overdispersion was calculated using Pearson residuals (Zuur *et al.* 2009), and is displayed for each model. All analyses were conducted using the lme4 package of the R 3.0.1 software. The statistical analyses were organized following our three main objectives:

### *1) How do the floral phenotype, floral display and surrounding context affect pollinator's attraction?*

We assessed the effect of floral phenotype (rayed, rayless and intermediate phenotypes), floral display and neighbourhood context on floral visitor rate fitting GLMMs for sympatric, rayed and rayless sites. We analysed the visits of all pollinators in one global model. Additional analyses were performed independently for each functional group. Each site had its own independent functional groups, established depending on the frequency and abundance of pollinator taxa.

Visitation rate was modelled with a Poisson distribution and a log link function. Floral phenotype, floral display and pollination context were included as explanatory variables, while plant identity was included as a random factor. Non-collinearity between explanatory variables was previously checked (Supplementary Table B). Differences between floral phenotypes were tested using least square means differences with the 'lsmeans' package. Models for rayed and rayless sites only considered the 20 selected pairs without manipulated neighbourhood conditions.

### *2) How does capitulum size affect pollinator's attraction?*

The previous section allowed to investigate the effect of rayed versus rayless phenotypes in the attraction of floral visitors. In this section we explored, within each phenotype from the sympatric population, which capitulum traits had an impact on floral visitors' attraction. First, capitulum size of rayed individuals including intermediate phenotypes was assessed in an exploratory analysis (Supplementary Table C). Afterwards, both capitulum components, disk size and ray length (this last one only for rayed and intermediate phenotypes), were analysed separately due to correlations between these variables (Supplementary Table B). In rayless individuals, only capitulum size, which is equivalent to disk size, was analysed. We fitted GLMMs for each phenotype using floral display, pollination context and capitulum traits as explanatory variables. Visits of all floral visitors were modelled with a Poisson distribution and analysed in a global model for all pollinator groups together. Additional models were fitted to the main pollinator groups of this site. Plant identity was set as a random factor.

## Materials and Methods

### 3) Is there a facilitation or a competing effect between rayed and rayless phenotypes?

After disentangling individual characteristics of phenotypes, we sought to understand how the ecological context of neighbours affected rayed and rayless phenotypes. For that, we followed two approaches: one based on the observational assessment in the sympatric site and the other based on the experimental manipulation of neighbourhoods on rayed and rayless sites. Firstly, on the sympatric site we assessed the effect of having rayed, intermediate or rayless neighbours on floral visitor rate on each phenotype separately, by means of GLMM models. Visitation rate was modelled with a Poisson distribution and a log link function. Floral display and the variable pollination context (either rayed, intermediate or rayless neighbours), were used as explanatory variables. In every model plant identity was set as a random factor. As for the previous analyses, visits of all pollinators were fitted in a global model and additional models were performed for main pollinator groups. Non-collinearity between explanatory variables has been previously assessed.

Secondly, in the rayed and rayless sites, we selected 20 and 18 pairs of plants (each pair including one rayed and one rayless plant), respectively. Half of these pairs had all *Anacyclus* neighbours removed, while the other half corresponded to the pairs with the highest pollination context (mean  $\pm$  SD (range): rayed site =  $47 \pm 17$  (25 - 102) neighbouring capitula; rayless site =  $89 \pm 58$  (19 - 201)). GLMM models included floral phenotype (rayed vs. rayless), pollination context (control versus neighbourhood removed) and its interaction as explanatory variables. Each pair of plants was included as a random factor. Visitation of floral visitors was modelled with a Poisson distribution and a log link function.



## Results

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### Floral visitors of *Anacyclus* species

We observed 128 different morphospecies, which accounted for a total of 640 interactions between *Anacyclus capitula* and its visitors within all three sites. There was a clear prevalence of Dipteran visitors, which accounted for almost two thirds of these visits (409 interactions; Table 1). The sympatric site was the site where most interactions were registered (408), with the rayed phenotype being the most visited out of the three phenotypes (Table 1). With respect to the rayless site, capitula with fake rays were visited 94 times while control plants were visited 81 times (Table 1). Hymenopteran and Dipteran visitors cover for around 92% of the interactions, with a high prevalence of ants, by far the most abundant Hymenopteran visiting *Anacyclus capitula* in the rayless site (52 interactions out of 82). This site was the only one where beeﬂies interacted with monitored plants, with 14 interactions in total. Finally, the rayed site had the lowest number of interactions (57 interactions), with Hymenoptera and Lepidoptera being responsible for 67% of the total number of visits (21 and 18, respectively), and only 10 visits were performed by Dipterans (Table 1).




### How do the floral phenotype, floral display and neighbourhood context affect pollinator attraction?

#### *Sympatric site*




Floral phenotype significantly affected the total number of visits when the overall assemblage of floral visitors was considered, whereas floral display and pollination context did not show significant effects (Table 2). Rayed phenotypes were visited at a significantly higher rate than rayless phenotype (Figure 2). The intermediate phenotype received less visits than rayed phenotypes but more than rayless one, not differing statistically from any of the phenotypes (Figure 2). The preference for rayed plants was also observed for each of the specific pollinator groups. Dipteran groups (hoverflies, small flies and big flies) were the main insects responsible for the differences in visitation rates according to the floral phenotype (Table 2). When analysed separately, in general, these Dipteran groups showed significant higher visitation rates to rayed phenotype in comparison with the rayless one (Table 2; Figure 2). Apart from the different preferences on floral phenotypes, when analysing pollinators groups separately, floral display and pollination context were relevant factors for specific pollinators groups. For example, floral display seemed to be an important factor driving the foraging behaviour of bees, which visited preferentially plants with larger floral displays (Table 2). On the other hand, hoverflies were impacted by the three variables, showing preference for rayed plants with a large floral display and with high number of *Anacyclus capitula* in the surrounding (Table 2; Figure 2)

**Table 1.** Absolute and relative frequencies of floral visitors on *Anacyclus* spp. capitula in each studied site. Sympatric site: rayed phenotype, intermediate phenotype and rayless phenotype, respectively. Rayless site: rayed phenotype (phenotypic manipulation) and the rayless phenotype (control), respectively. Rayed site, rayed phenotype (control) and the rayless phenotype (phenotypic manipulation), respectively.

(Page 1/4)

	Sympatric site				Rayless site				Rayed site											
	%		%	Total	%		%	Total	%		%	Total	%							
Coleoptera	10	4	2	3.1	2	2.2	14	3.4	6	6.4	1	1.2	7	4	2	7.1	0	0	2	3.5
Diptera	203	80.9	51	79.7	63	67.7	317	77.7	43	45.7	39	48.2	82	46.9	6	21.4	4	13.8	10	17.6
Hemiptera	1	0.4	0	0	1	1	2	0.5	0	0	1	1.2	1	0.6	2	7.1	4	13.8	6	10.5
Hymenoptera	36	14.3	11	17.2	22	23.7	69	16.9	42	44.7	37	45.7	79	45.1	11	39.4	10	34.4	21	36.8
Lepidoptera	1	0.4	0	0	5	5.4	6	1.5	3	3.2	3	3.7	6	3.4	7	25	11	38	18	31.6
Total	251	100	64	100	93	100	408	100	94	100	81	100	175	100	28	100	29	100	57	100
Coleoptera																				
Cetoniidae	0	0	0	0	0	0	0	0	1	14.3	0	0	1	14.3	0	0	0	0	0	0
<i>Oxythyrea funesta</i>																				
Cantharidae	0	0	0	0	0	0	0	0	2	28.6	0	0	2	28.6	0	0	0	0	0	0
<i>Rhagonicha fulva</i>																				
Malachiidae	1	7.1	0	0	1	7.1	2	14.2	0	0	1	14.3	1	14.3	0	0	0	0	0	0
<i>Clanoptilus abdominalis</i>																				
Oedemeridae	1	7.1	0	0	0	0	1	7.1	0	0	0	0	0	0	0	0	0	0	0	0
<i>Oedema simplex</i>																				
Dermestidae	0	0	1	7.1	0	0	1	7.1	0	0	0	0	0	0	0	0	0	0	0	0
<i>Attagenus</i> sp.																				
Others	8	57.2	1	7.1	1	7.1	10	71.6	3	42.8	0	0	3	42.8	2	100	0	0	2	100
non id.																				
Total	10	71.6	2	14.2	2	14.2	14	100	6	85.7	1	14.3	7	100	2	100	0	0	2	100




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	Sympatric site			Rayless site			Rayed site													
	%		Total %	%		Total %	%		Total %											
Diptera																				
Syrphidae																				
<i>Eristalis tenax</i>	23	7.5	4	1.3	8	2.6	35	11.4	3	3.7	0	0	3	3.7	2	20	2	20	4	40
<i>Eristalis arbustorum</i>	1	0.3	1	0.3	0	0	2	0.6	1	1.2	0	0	1	1.2	0	0	0	0	0	0
<i>Eupeodes</i> sp.	17	5.6	6	1.8	5	1.6	28	9	6	7.2	3	3.7	9	11	0	0	0	0	0	0
<i>Episirphus</i> sp.	0	0	0	0	5	1.6	5	1.6	1	1.2	1	1.2	2	2.5	0	0	0	0	0	0
<i>Sphaerophoria</i> sp.	51	16.3	15	4.8	19	6	85	27.1	6	7.2	7	8.6	13	15.9	1	10	0	0	1	10
<i>Syritta pipiens</i>	2	0.6	0	0	0	0	2	0.6	0	0	0	0	0	0	0	0	0	0	0	0
<i>Chrysotoxum</i> sp.	1	0.3	0	0	0	0	1	0.3	0	0	0	0	0	0	0	0	0	0	0	0
non id.	8	2.6	0	0	1	0.3	9	2.9	4	5	3	3.7	7	8.7	0	0	0	0	0	0
Bombyliidae																				
<i>Conophorus</i> sp.	0	0	0	0	0	0	0	0	6	7.2	3	3.7	9	11	0	0	0	0	0	0
non id.	0	0	0	0	0	0	0	0	4	4.9	1	1.2	5	6.1	0	0	0	0	0	0
Tachinidae																				
<i>Tachina fera</i>	0	0	1	0.3	1	0.3	2	0.6	0	0	0	0	0	0	0	0	0	0	0	0
Scathophagidae																				
<i>Scathophaga stercoraria</i>	1	0.3	0	0	0	0	1	0.3	2	2.4	1	1.2	3	3.6	0	0	0	0	0	0
non id. Miltogramminae	0	0	1	0.3	0	0	1	0.3	0	0	0	0	0	0	0	0	0	0	0	0
Calliphoridae																				
<i>Calliphora vomitoria</i>	3	1	2	0.6	0	0	5	1.6	1	1.2	2	2.4	3	3.6	0	0	0	0	0	0
<i>Lucilia caesar</i>	1	0.3	0	0	2	0.6	3	0.9	1	1.2	3	3.7	4	4.9	1	10	0	0	1	10
Anthomyzidae																				
non id.	12	3.9	2	0.6	3	1	17	5.5	0	0	1	1.2	1	1.2	0	0	0	0	0	0
Others																				
non id. Diptera	31	8.7	11	3.4	7	2.2	49	14.3	6	7.2	10	12.4	16	19.2	1	10	2	20	3	30
non id. Small Diptera	52	16.6	8	2.6	12	3.8	72	23	2	2.4	4	5	6	7.4	1	10	0	0	1	10
<b>Total</b>	<b>203</b>	<b>64</b>	<b>51</b>	<b>16</b>	<b>63</b>	<b>20</b>	<b>317</b>	<b>100</b>	<b>43</b>	<b>52.4</b>	<b>39</b>	<b>47.6</b>	<b>82</b>	<b>100</b>	<b>6</b>	<b>60</b>	<b>4</b>	<b>40</b>	<b>10</b>	<b>100</b>

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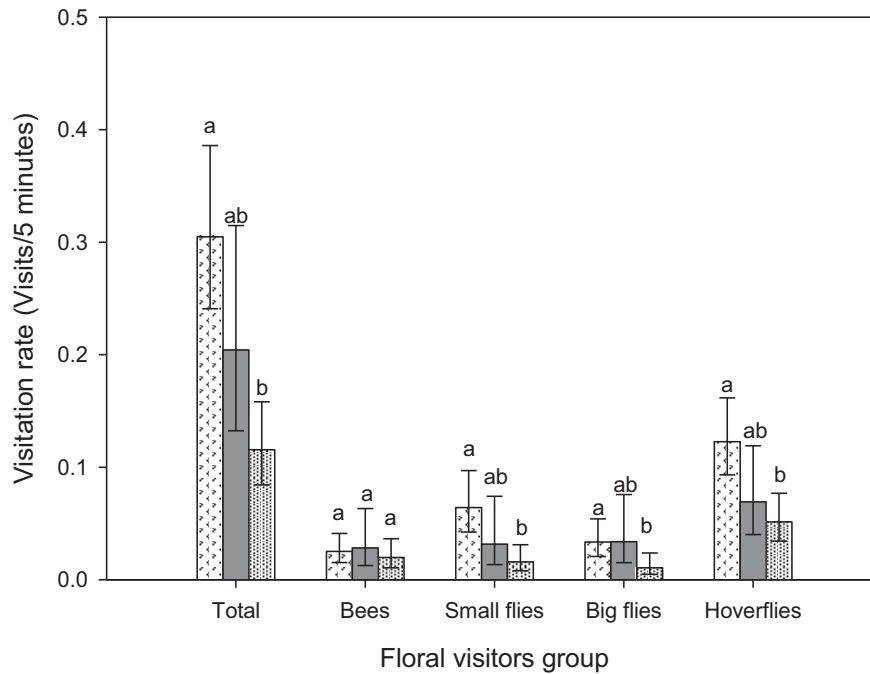
	Sympatric site				Rayless site				Rayed site											
	%	%	%	Total %	%	%	Total %	%	%	Total %	%	Total %								
<b>Hemiptera</b>																				
Others	1	50	0	0	1	50	2	100	0	0	1	100	2	33.3	4	66.7	6	100		
Total	1	50	0	0	1	50	2	100	0	0	1	100	2	33.3	4	66.7	6	100		
<b>Hymenoptera</b>																				
Formicidae	1	1.5	0	0	1	1.5	2	3	30	37.9	22	27.8	52	65.7	0	0	1	4.8	1	4.8
Apidae	12	17.4	4	5.8	11	15.8	27	39	1	1.3	5	6.3	6	7.6	6	28.6	7	33.2	13	61.8
<i>Apis mellifera</i>	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	9.5	0	0	2	9.5
<i>Anthophora</i> sp.	0	0	0	0	0	0	0	0	1	1.3	0	0	1	1.3	0	0	0	0	0	0
<i>Eucera longicornis</i>	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
<i>Armbobates</i> sp.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
non id.	0	0	0	0	2	3	2	3	0	0	0	0	0	0	0	0	0	0	0	0
Megachilidae	1	1.5	0	0	0	0	1	1.5	0	0	0	0	0	0	0	0	1	4.8	1	4.8
non id.	3	4.3	5	7.1	2	3	10	14.4	3	3.8	4	5.2	7	9	0	0	0	0	0	0
<i>Lasioglossum</i> sp.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	4.8	1	4.8
Sphecidae	19	27.5	2	3	6	8.6	27	39.1	7	8.9	6	7.5	13	16.4	3	14.3	0	0	3	14.3
non id.	36	52.2	11	15.9	22	31.9	69	100	42	53.2	37	46.8	79	100	11	52.4	10	47.6	21	100
Total	36	52.2	11	15.9	22	31.9	69	100	42	53.2	37	46.8	79	100	11	52.4	10	47.6	21	100

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	Sympatric site			Rayless site			Rayed site						
	%		%	Total	%		%	Total	%		%	Total	
Lepidoptera													
Pieridae													
<i>Colias croceus</i>	0	0	0	1	17	0	0	0	0	0	0	0	
Nymphalidae													
<i>Pararge aegeria</i>	1	17	0	0	0	0	0	0	0	0	0	0	
Others													
non id.	0	0	0	4	66	3	50	3	50	7	38.9	11	61.1
Total	1	17	0	5	83	3	50	3	50	7	38.9	11	61.1

**Table 2.** The effects of floral phenotype (rayed, intermediate and rayless phenotypes), floral display and pollination context on pollinator attraction for the entire pollinator assemblage (All groups) and for the different pollinator functional groups (Bees, Small flies, Big flies and Hoverflies) for the sympatric site. Plant identity was used as a random variable. Overdispersion index: a measure of overdispersion. Statistical significances ( $P < 0.05$ ) are shown in bold. Significantly positive effects are signed with (+).

Variables	Df	All groups			Bees			Small flies			Big flies			Hoverflies		
		$\chi^2$	P	SD	$\chi^2$	P	SD	$\chi^2$	P	SD	$\chi^2$	P	SD	$\chi^2$	P	SD
<i>Fixed</i>																
Floral phenotype	2	21.43	< <b>0.0001</b>		0.63	0.729		13.55	<b>0.001</b>		7.65	<b>0.022</b>		12.99	<b>0.002</b>	
Floral display	1	1.11	0.293		(+) 8.93	<b>0.003</b>		0.76	0.383		<0.01	0.986		(+) 4.69	<b>0.030</b>	
Pollination context	1	0.96	0.329		0.29	0.590		0.07	0.789		3.37	0.066		(+) 4.29	<b>0.038</b>	
<i>Random</i>																
Plant		0.40	0.63		0.39	0.62		0.78	0.88		0.83	0.96		0.36	0.60	
Overdispersion index			1.036			0.838		0.879			0.733			0.977		

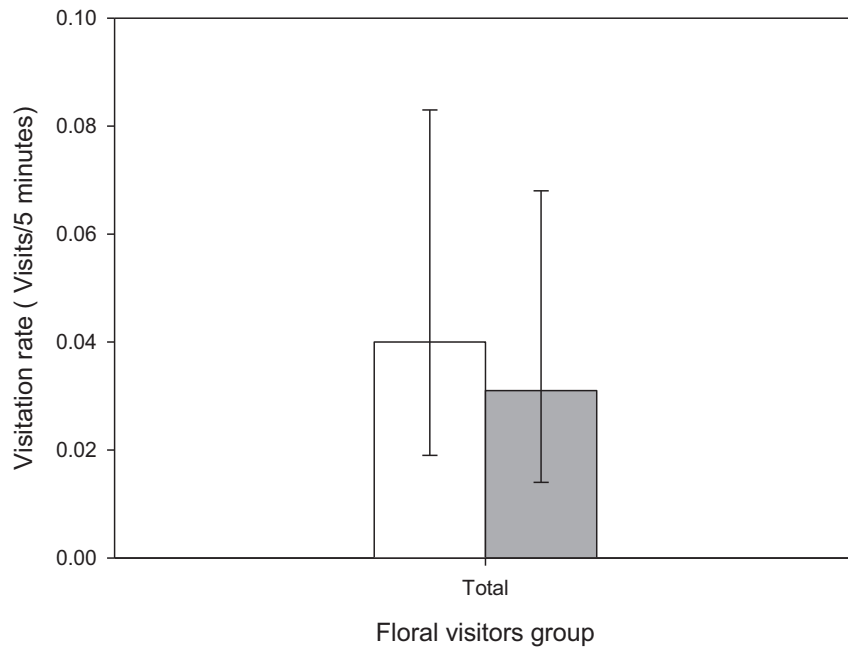


**Figure 2.** Least square means ( $\pm$  confidence intervals) of visitation rate (number of visits per 5 min intervals) in the sympatric site given for the entire pollinator assemblage (Total) and for each pollinator group observed (bees, small flies, big flies and hoverflies), according with the phenotypes present in the site: rayed (white bar), intermediate (grey bar) and rayless phenotypes (black bar). Means sharing the same letter were not significantly different at  $P < 0.05$ .

#### *Rayed site*

Due to the low number of interactions (Table 1), we could only fit a model with all groups of floral visitors together. Neither the ray removal factor, nor the pollination context had a significant effect on pollinator attraction in this site (floral phenotype:  $\chi^2 = 0.30$ ,  $P = 0.59$ ; pollination context:  $\chi^2 = 0.88$ ,  $P = 0.882$ ; Figure 3). Nevertheless, floral display showed a positive and significant effect on total visitation rates ( $\chi^2 = 22.88$ ,  $P < 0.0001$ ).

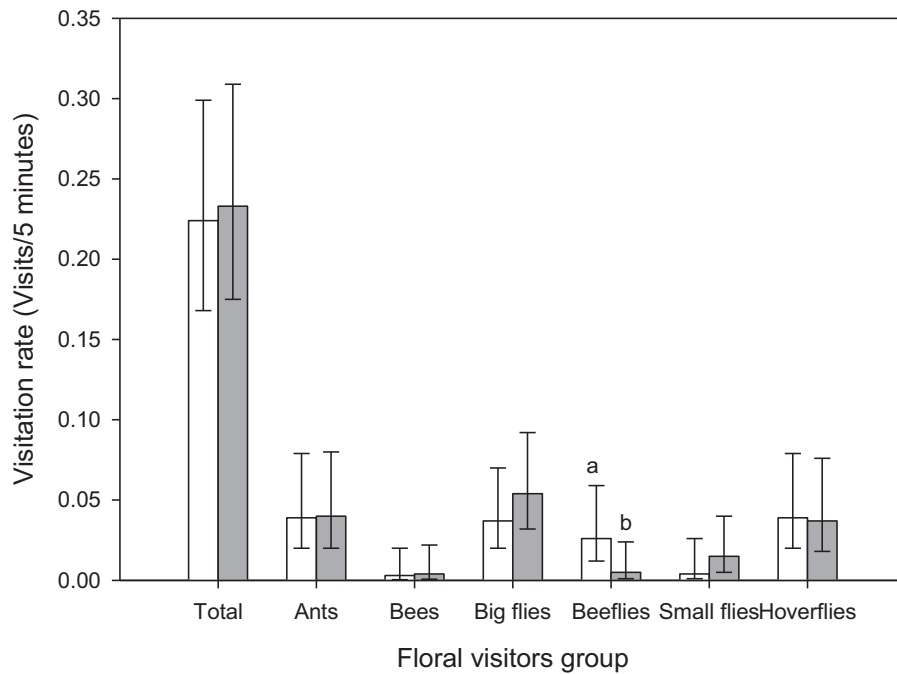




**Figure 3.** Least square means ( $\pm$  confidence intervals) of visitation rate (number of visits per 5 min intervals) in the rayed site given for the entire pollinator assemblage, according with the phenotypes present in this site: rayed (control; white bar) and rayless phenotypes (rays removed; grey bar). No statistically significant differences between phenotypes were found.

#### *Rayless site*

The rate of visits of the overall assemblage of pollinators was significantly affected by floral display, only, being unaffected by floral phenotype and pollination context (Table 3, Figure 4). The fake-rayed phenotype did not attract significantly more floral visitors than rayless ones in this population, affecting positively bees, only (Table 3, Figure 4). Besides this ray preference, bees and ants were significantly affected by floral display, preferring plants with a higher number of capitula (Table 3). Conversely, bees in this population showed a preference for plants with a more dense pollination context (Table 3).



**Figure 4.** Least square means ( $\pm$  confidence intervals) of the visitation rate (number of visits per 5 minutes intervals) in the rayless site given for the entire pollinator assemblage (Total) and for main pollinator groups (ants, bees, big flies, beeflies, small flies and hoverflies), according with the present phenotypes: rayless (control; white bar) and fake-rayed phenotype (grey bar). Significantly different LSmeans at  $P < 0.05$  are signalled with different letters.



### **How does capitulum size affect pollinator attraction?**

Larger capitulum sizes significantly increased visitation rates on rayed and intermediate individuals (Supplementary Table C), but not on rayless ones (Table 4). This increase in visitation rates was exclusively due to an increase in ray length (Table 5) and not due to disk size (Table 4). Longer rays significantly increased the visit of small flies and hoverflies, but not of bees and big flies (Table 5). Small flies were the functional group that showed a marginally negative significant relationship with larger disk sizes on rayed individuals (Table 4).

### **Is there a facilitation or a competing pattern between rayed and rayless phenotypes?**

#### *Natural variation in neighbourhood composition*

The impact of the pollination context varied according with the floral phenotypes. Two distinct patterns were observed in this study: a positive, facilitative pattern regarding the rayed phenotypes, and a negative, competitive pattern for the intermediate and rayless phenotypes. Rayed plants were significantly more visited by big flies and hoverflies when surrounded by rayed and intermediate neighbours (Table 6). By contrast, intermediate and rayless plants, showed no significant positive effects in visitation rates when grown surrounded by other plants (Table 6). Indeed, bees showed significantly lower visitation rates to intermediate plants in the presence of other intermediate phenotype plants, and to rayless plants when those were surrounded by neighbours of the same phenotype (Table 6).




**Table 4.** The effect of disk size on floral visitor attraction for the sympatric site. Disk size, floral display and pollination context were considered fixed explanatory variables of the rate of visits of the entire pollinator assemblage (All groups), and of different pollinator groups (Bees, Small flies, Big flies, Hoverflies). We analysed separately rayed individuals (including intermediate individuals) and rayless individuals. Plant identity was used as a random variable. Overdisp. index: a measure of overdispersion. Statistical significances (<0.05) are shown in bold. Positive significant effects are signed with (+).

Variables	All groups			Bees			Small flies			Big flies			Hoverflies		
	$\chi^2$	P	Df	$\chi^2$	P	Df	$\chi^2$	P	Df	$\chi^2$	P	Df	$\chi^2$	P	Df
<i>RAYED IND.</i>															
<i>Fixed</i>															
Disk size	0.42	0.516	1	0.70	0.403	1	(-) 2.87	0.090	1	0.35	0.556	1	0.16	0.685	1
Floral display	0.26	0.612	1	(+) 7.73	<b>&lt;0.01</b>	1	0.63	0.428	1	0.07	0.787	1	2.95	0.086	1
Pol. context	1.11	0.292	1	<0.01	0.986	1	0.24	0.621	1	(+) 4.32	<b>0.04</b>	1	3.26	0.071	1
<i>Random</i>															
Plant	0.52	0.72	1	0.68	0.82	1	1.14	1.07	1	0.99	0.99	1	0.49	0.70	1
Overdisp. index	1.093			0.864			0.951			0.739			0.999		
<i>RAYLESS IND.</i>															
<i>Fixed</i>															
Capitulum size	1.66	0.187	1	1.61	0.205	1	<0.01	0.945	1	0.16	0.694	1	0.51	0.477	1
Floral display	1.80	0.180	1	2.74	0.097	1	1.84	0.175	1	0.64	0.424	1	0.97	0.325	1
Pol. context	0.61	0.436	1	2.83	0.093	1	2.05	0.152	1	0.08	0.77	1	0.14	0.705	1
<i>Random</i>															
Plant	0.05	0.22	1	0	0	1	0	0	1	0	0	1	0	0	1
Overdisp. index	1.031			1.144			0.985			1.005			1.04		

**Table 5.** The effects of ray length, floral display and pollination context on pollinator attraction for the entire pollinator assemblage (All groups) and for different pollinator functional groups (Bees, Small flies, Big flies, Hoverflies) for the rayed individuals (including intermediate individuals) in the sympatric site. Plant identity was used as a random variable. Overdispersion index: a measure of overdispersion. Statistical significances (<0.05) are shown in bold. Positive significant effects are signed with (+).

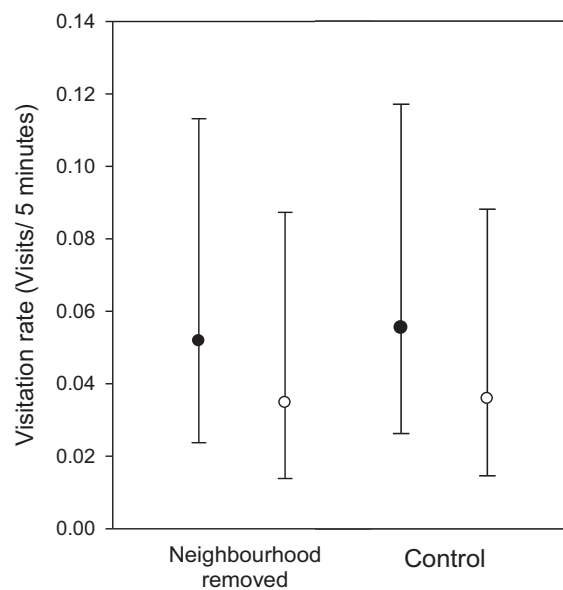
Variables	Df	All groups		Bees		Small flies		Big flies		Hoverflies	
		$\chi^2$	P	$\chi^2$	P	$\chi^2$	P	$\chi^2$	P	$\chi^2$	P
<i>Fixed</i>											
Ray length	1	(+) 6.64	<b>&lt;0.01</b>	0.26	0.614	(+) 9.91	<b>0.002</b>	1.72	0.189	(+) 4.91	<b>0.027</b>
Floral display	1	0.54	0.461	(+) 7.62	<b>&lt;0.010</b>	0.31	0.580	0.04	0.834	(+) 3.64	0.056
Pollination context	1	1.71	0.191	<0.01	0.950	0.28	0.600	(+) 4.71	<b>0.030</b>	(+) 3.74	0.053
<i>Random</i>											
Plant		0.43	0.65	0.70	0.84	0.91	0.95	0.89	0.95	0.39	0.63
Overdispersion index		1.107		0.849		0.967		0.755		1.021	

**Table 6.** The effect of particular neighbourhoods for different focal individuals (rayed, intermediate and rayless), for the entire pollinator assemblage (All groups), and for different pollinator guilds (Bees, Small flies, Big flies, Hoverflies). Statistical significances ( $<0.05$ ) are shown in bold. Ns:  $P > 0.10$ ;  $b$  = regression coefficient.

		All groups				Bees	Small flies	Big flies	Hoverflies
Focal rayed individuals 	Rayed	ns	ns	ns	ns	ns	$b = 0.05, P = \mathbf{0.045}$	$b = 0.02, P = \mathbf{0.023}$	
	Intermediate	ns	ns	ns	ns	ns	ns	$b = 0.12, P = \mathbf{0.011}$	
	Rayless	ns	ns	ns	ns	ns	$b = 0.06, P = 0.090$	ns	
Focal intermediate individuals 	Rayed	ns	ns	ns	ns	ns	ns	ns	
	Intermediate	ns	ns	$b = -0.65, P = \mathbf{0.023}$	ns	ns	ns	ns	
	Rayless	ns	ns	$b = -0.19, P = 0.055$	ns	ns	ns	ns	
Focal rayless individuals 	Rayed	ns	$b = -0.04, P = 0.071$	$b = -0.09, P = 0.109$	ns	ns	ns	ns	
	Intermediate	ns	ns	ns	ns	ns	ns	ns	
	Rayless	ns	$b = -0.51, P = \mathbf{0.022}$	ns	ns	ns	ns	ns	

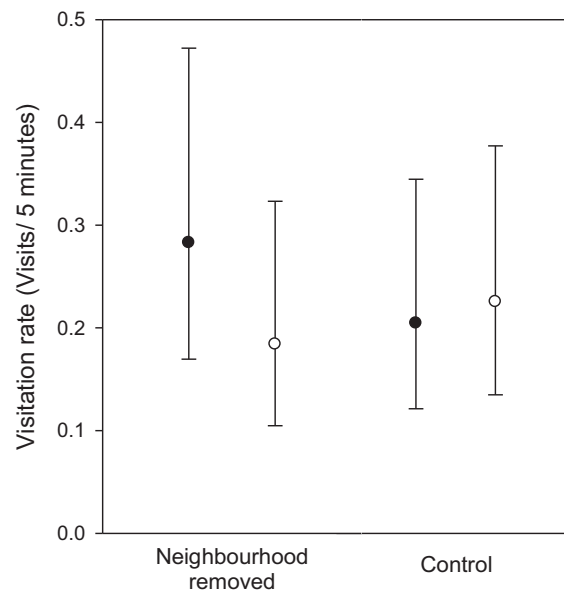
*Experimental manipulation of neighbour's density*

The removal of all *Anacyclus* neighbours did not significantly reduce the total visits of the floral visitors in both sites (Figure 5 and 6). However, in the rayless site, the removal significantly decreased the visitation rate of hoverflies to rayless plants (Figure 7). The other pollinator's functional groups were not affected by the experimental removal of the neighbourhood (results not shown).

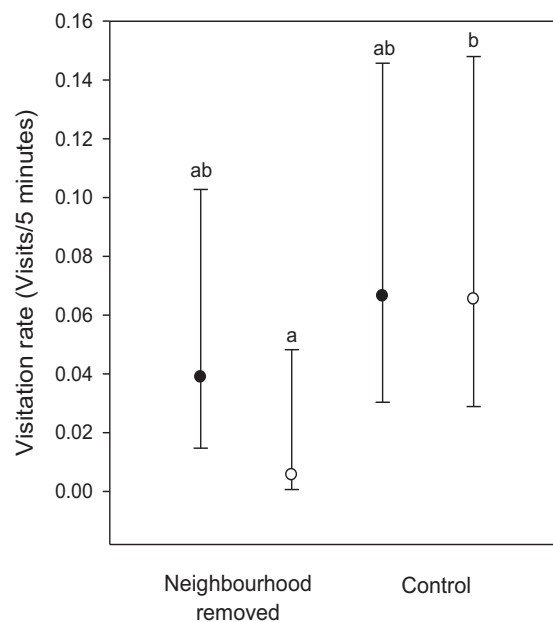


**Figure 5.** The effect of removing the neighbourhood on floral visitors within the rayed site. Least square means ( $\pm$  confidence intervals) of the visitation rate (number of visits per 5 minutes intervals) in the rayed site, given for the entire pollinator assemblage, with plants divided according with their surrounding context: neighbours removed and control. The black dots represent the rayed phenotype while the white dots represent the rayless phenotype. No statistically significant differences were found between groups.





**Figure 6.** The effect of removing the neighbourhood on floral visitors within the rayless site. Least square mean ( $\pm$  confidence intervals) of the visitation rate (number of visits per 5 minutes intervals) in the rayless site, given for the entire pollinator assemblage, with plants divided according with their surrounding context: neighbours removed and control. The black dots represent the rayed phenotype while the white dots represent the rayless phenotype. No statistically significant differences were found between groups.



**Figure 7.** The effect of removing the neighbourhood on hoverflies within the rayless site. Least square mean ( $\pm$  confidence intervals) of the visitation rate (number of visits per 5 minutes intervals) in the rayless site, given for the pollinator group hoverflies, with plants divided according with their surrounding context: neighbours removed and control. The black dots represent the rayed phenotype while the white dots represent the rayless phenotype. Different letters refer to statistically significant differences at  $P < 0.05$ .

## Discussion

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The two species of *Anacyclus* studied in this work are generalists that can be pollinated by a vast array of pollinators from different functional groups. Our observations have shown that *Anacyclus capitula* were visited by insects from at least 17 different families, from several orders (Table 1). However, three functional groups of diptera (hoverflies, big and small flies) and the bees (considering different functional groups together) were the most frequent floral visitors. Interestingly, diptera and bees showed different patterns of visitation and could be exerting contrasting patterns of selection, affecting the dynamics of the hybrid zone between these two species. Below, the implications of different preferences of the main floral visitors in the evolution and maintenance of floral rays and in the degree of reproductive isolation in the *A. clavatus* and *A. valentinus* contact zone are discussed.

Similarly to what was observed before in other studies, our results have shown that rays presented a positive effect in visitation rates. For example, diptera visited preferentially rayed individuals and, among them, individuals with larger rays, regardless of the size of the central yellow disk. This suggests that the production of conspicuous structures could be an adaptation to enhance pollinator attraction and guarantee high outcrossing rates between rayed phenotypes (Marshall & Abbott 1984; Stuessy *et al.* 1986; Sun & Ganders 1990; Nielsen *et al.* 2002; Celedón-Neghme *et al.* 2006; Penet *et al.* 2012). However, not all functional groups of pollinators showed a clear preference for rayed phenotypes. In the particular case of bees, this group of insects was indifferent to the presence of rays and visited preferentially larger plants that presented a higher number of capitula blooming simultaneously. In the absence of other forms of selection, visitation patterns of bees and dipteran visitors might cause a contrasting genetic flow in the hybrid zone, with insect abundance regulating this effect. Although the role of ethological isolation in speciation remains controversial (Aldridge & Campbell 2006), our results from the sympatric site suggest contrasting effects of pollinator's behaviour, with dipteran pollinators promoting assortative mating between rayed individuals, ultimately leading to an isolation pattern. By opposition, bees, by visiting plants independently of the phenotype, will mix pollen from both species, diminishing the pollinator-mediated effect of diptera (Schmid-Hempel & Speiser 1988; Thompson 2001). Since hybrids between the two plant species are able to produce viable seeds (I. Álvarez 2013, personal communication), this non-discriminated visitation pattern mediated by bees may ultimately cause introgression from one species to the other. Gómez and colleagues found a similar effect in *Erysimum mediohispanicum*, with some pollinator groups selecting for different corolla shapes, whereas some other groups visited flowers indiscriminately, leading to an attenuate effect and causing a contrasting selection (Gómez *et al.* 2008b). In this way, the levels of reproductive isolation and ultimately the dynamics of the hybrid zone will be mostly dependent on pollinator abundance (Emms & Arnold 2000; Thompson 2001). In order to better understand the potential selective role of these functional groups, we are developing studies focused in assessing the efficiency of these pollinators and their impact in plant fitness. Furthermore, future studies of fine-scale genetic patterns and parentage identification are needed to assess if differential behaviour between dipteran and bees are driving different mating patterns.

The different visitation patterns of floral visitors observed in the sympatric site were only partially supported by the phenotypic manipulations of rayed phenotypes. This phenotypic manipulations might

have failed maybe due to our simplistic ray models, which might not completely mimic the real shapes (i.e., manipulated rays had a straight cut shape, while real rays are ellipsoid; see Figure 1 A and D; Gómez *et al.* 2008b). Furthermore the manipulated rays showed some UV reflection, whereas natural ones do not reflect UVs (results not shown).

Regardless of being visited by one of the main pollinator groups, bees, rayless plants still received a lower number of visits than rayed ones. The lower visitation rate of rayless plants in the sympatric site suggests that rayed plants could be successfully competing with rayless plants for pollination services. Could we then expect that rayless phenotypes would slowly disappear in this contact zone? Considering the preliminary results of other studies that we are performing in the same contact zone, it seems that this is not probable, as both rayed and rayless plants were not pollen limited and both types of capitula showed similar levels of fruit set and absence of seed predation (R. Torices and J. Cerca de Oliveira, unpublished data). In addition, not every insect preferred rayed phenotypes. For example, bees in the sympatric site, and general pollinators in the remaining populations selected larger floral displays, independently of the floral phenotype. Floral visitors are opportunist insects with labile preferences that compete for floral rewards, thus they might bypass a crowded conspicuous flower for a less conspicuous one, if the flower has more resources (Wesselingh & Arnold 2000; Dilley, Wilson & Mesler 2000). Additionally, bigger plants guarantee a higher density of inflorescences in a small area, resulting in a bigger concentration of resources for pollinators, attracting pollinators regardless of their phenotype. Finally, beyond pollinator preferences, rayless plants might bear advantages in “stressful conditions”, as they do not have the cost to produce and subsequently maintain the extra structures (Chaplin & Walker 1982; Charlesworth & Charlesworth 1987; Galen, Sherry & Carroll 1999; Andersson 2001), reallocating resources for seed production (Andersson 1999).

Our observations also revealed that the phenotype of the neighbours plays an important role in driving pollinator’s behaviour. Pollinator attraction did not depend on the focal plant phenotype, only, with the phenotypes of the neighbouring plants affecting the visitation rate of the main floral visitors. When generalist co-flowering plant species occur in the same area they are often obliged to share pollinators, and inter-specific plant-plant interactions arise. These interactions can be neutral, competitive or facilitative (Landry 2013; Ye *et al.* 2013) and two opposing views emerged: 1) the struggle for existence should be greater between closely related species than between distantly related species, due to similarity in habits and constitution, causing a high overlapping and therefore a direct competition (Violle *et al.* 2011; Beltrán, Valiente-Banuet & Verdú 2012); 2) facilitation can happen between related species as a by-product of the similarities in the pollinator groups that they attract (Ghazoul 2006; Sargent & Ackerly 2008; Devaux & Lande 2009; Beltrán *et al.* 2012). Broadly, our results confirm the existence of the three types of interaction when analysing distinct functional pollinator groups. In the sympatric population, rayed phenotypes received more visits from big flies and hoverflies when in the presence of conspicuous rayed neighbours. By opposition, rayless and intermediate plants significantly competed for pollinators (bees). Using the Asteraceae species, *Lasthenia fremontii*, Sargent and colleagues (Sargent *et al.* 2011) found that this species would be less pollen limited when occurring in

## Discussion

communities composed of close relatives than when occurring in communities composed of more distant relatives. Therefore, disentangling the adaptive role of floral polymorphism should be assessed considering the potential interactions between the focal plant's phenotype and the phenotypes of their neighbours, which may affect pollinator behaviour and, most likely, plant fitness.

Conclusion

Conclusion

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## Conclusion

In conclusion, we found that the production of rays influenced the probability of being visited by insects in the studied sympatric site. However, not all groups of floral visitor showed a preference for the rayed phenotype. Also, contrasting selection was found between pollinator groups for the presence of rays, demonstrating that pollinators might be important agents of selection on floral traits for generalist plants. Finally, we found support for the importance of the neighbours' phenotype when assessing pollinator preference on a focal individual. Rayed plants benefited from having other conspicuous neighbours, whereas rayless and intermediate phenotypes significantly competed for pollinators. All these differential behavioural patterns of floral visitors might affect gene flow within the hybrid zone between *A. clavatus* and *A. valentinus* influencing its future dynamics.

## References

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## References

- Aigner, P.A. (2003) Optimality modeling and fitness trade offs: when should plants become pollinator specialists? *Oikos*, **95**, 177–184.
- Aldridge, G. & Campbell, D.R. (2006) Variation in pollinator preference between two *Ipomopsis* contact sites that differ in hybridization rate. *Evolution*, **61**, 99–110.
- Andersson, S. (1996) Floral display and pollination success in *Senecio jacobaea* (Asteraceae): Interactive effects of head and corymb size. *American Journal of Botany*, **83**, 71–75.
- Andersson, S. (1999) The cost of floral attractants in *Achillea ptarmica* (Asteraceae): evidence from a ray removal experiment. *Plant Biology*, **1**, 569–572.
- Andersson, S. (2001) Fitness consequences of floral variation in *Senecio jacobaea* (Asteraceae): evidence from a segregating hybrid population and a resource manipulation experiment. *Biological Journal of the Linnean Society*, **74**, 17–24.
- Andersson, S. (2008) Pollinator and nonpollinator selection on ray morphology in *Leucanthemum vulgare* (oxeye daisy, Asteraceae). *American Journal of Botany*, **95**, 1072–1078.
- Barreda, V.D., Palazzesi, L., Katinas, L., Crisci, J. V., Tellería, M.C., Bremer, K., Passalia, M.G., Passala, M.G., Bechis, F. & Corsolini, R. (2012) An extinct Eocene taxon of the daisy family (Asteraceae): evolutionary, ecological and biogeographical implications. *Annals of Botany*, **109**, 127–34.
- Barton, N.H. & Hewitt, G.M. (1985) Analysis of hybrid zones. *Annual Review of Ecology and Systematics*, **16**, 113–148.
- Bello, M.A., Álvarez, I., Torices, R. & Fuertes-Aguilar, J. (2013) Floral development and evolution of capitulum structure in *Anacyclus*. *Annals of Botany*, **112**, 1597–1612.
- Beltrán, E., Valiente-Banuet, A. & Verdú, M. (2012) Trait divergence and indirect interactions allow facilitation of congeneric species. *Annals of Botany*, **110**, 1369–76.
- Bremer, K. & Humphries, C.J. (1993) Generic monograph of the Asteraceae-Anthemideae. *Bulletin of the Natural History Museum (London)*, **23**, 73 – 179.
- Campbell, D.R. (2003) Natural selection in *Ipomopsis* hybrid zones: implications for ecological speciation. *New Phytologist*, **161**, 83–90.
- Campbell, D.R. (2008) Pollinator shifts and the origin and loss of plant species. *Annals of the Missouri Botanical Garden*, **95**, 264–274.
- Campbell, D.R. & Waser, N.M. (2007) Evolutionary dynamics of an *Ipomopsis* hybrid zone: confronting models with lifetime fitness data. *The American Naturalist*, **169**, 298–310.
- Campbell, D.R., Waser, N.M. & Melendez-Ackerman, E.J. (1997) Analyzing pollinator-mediated selection in a plant hybrid zone: hummingbird visitation patterns on three spatial scales. *The American Naturalist*, **149**, 295–315.
- Castro, S., Loureiro, J., Ferrero, V., Silveira, P. & Navarro, L. (2013) So many visitors and so few pollinators: variation in insect frequency and effectiveness governs the reproductive success of an endemic milkwort. *Plant Ecology*, **214**, 1233–1245.
- Celedón-Neghme, C., Gonzáles, W.L. & Gianoli, E. (2006) Cost and benefits of attractive floral traits in the annual species *Madia sativa* (Asteraceae). *Evolutionary Ecology*, **21**, 247–257.
- Chaplin, S.J. & Walker, J.L. (1982) Energetic constraints and adaptive significance of the floral display of a forest milkweed. *Ecology*, **63**, 1857–1870.
- Charlesworth, D. & Charlesworth, B. (1987) The effect of investment in attractive structures on allocation to male and female functions in plants. *Evolution*, **41**, 948–968.
- Cope, E.D. (1896) *The Primary Factors of Organic Evolution*. Open Court Publishing., Chicago, IL.

## References

- Devaux, C. & Lande, R. (2009) Displacement of flowering phenologies among plant species by competition for generalist pollinators. *Journal of Evolutionary Biology*, **22**, 1460–70.
- Dilley, J.D., Wilson, P. & Mesler, M.R. (2000) The radiation of Calochortus: generalist flowers moving through a mosaic of potential pollinators. *Oikos*, **89**, 209–222.
- Emms, S.K. & Arnold, M.L. (2000) Site-to-site differences in pollinator visitation patterns in a Louisiana iris hybrid zone. *Oikos*, **91**, 568–578.
- Fenner, M., Cresswell, J.E., Hurley, R.A. & Baldwin, T. (2002) Relationship between capitulum size and pre-dispersal seed predation by insect larvae in common Asteraceae. *Oecologia*, **130**, 72–77.
- Funk, V.A., Susanna, A., Stuessy, T.F. & Bayer, R.J. (2009) *Systematics, Evolution, and Biogeography of Compositae*. International Association for Plant Taxonomy, Vienna.
- Galen, C., Sherry, R.A. & Carroll, A.B. (1999) Are flowers physiological sinks or faucets? Costs and correlates of water use by flowers of *Polemonium viscosum*. *Oecologia*, **118**, 461–470.
- Ghazoul, J. (2006) Floral diversity and the facilitation of pollination. *Journal of Ecology*, **94**, 295–304.
- Gómez, J.M., Bosch, J., Perfectti, F., Fernández, J. & Abdelaziz, M. (2007) Pollinator diversity affects plant reproduction and recruitment: the tradeoffs of generalization. *Oecologia*, **153**, 597–605.
- Gómez, J.M., Bosch, J., Perfectti, F., Fernández, J.D., Abdelaziz, M. & Camacho, J.P.M. (2008a) Association between floral traits and rewards in *Erysimum mediohispanicum* (Brassicaceae). *Annals of botany*, **101**, 1413–20.
- Gómez, J.M., Bosch, J., Perfectti, F., Fernández, J.D., Abdelaziz, M. & Camacho, J.P.M. (2008b) Spatial variation in selection on corolla shape in a generalist plant is promoted by the preference patterns of its local pollinators. *Proceedings of the Royal Society B-Biological Sciences*, **275**, 2241–2249.
- Gómez, J.M., Muñoz-Pajares, A.J., Abdelaziz, M., Lorite, J. & Perfectti, F. (2014) Evolution of pollination niches and floral divergence in the generalist plant *Erysimum mediohispanicum*. *Annals of Botany*, **113**, 237–249.
- Herrera, C.M. (2005) Plant generalization on pollinators: species property or local phenomenon? *American Journal of Botany*, **92**, 13–20.
- Hodges, S.A. & Arnold, M.L. (1994) Floral and ecological isolation between *Aquilegia formosa* and *Aquilegia pubescens*. *Proceedings of the National Academy of Sciences of the United States of America*, **91**, 2493–6.
- Humphries, C.J. (1979) A revision of the genus *Anacyclus* L. (Compositae: Anthemideae). *Bulletin of the British Museum*, **7**, 83–142.
- Humphries, C.J. (1981) Cytogenetic and cladistic studies in *Anacyclus* (Compositae: Anthemideae). *Nordic Journal of Botany*, **1**, 83–96.
- Johnson, S.D. & Steiner, K. (2000) Generalization versus specialization in plant pollination systems. *Trends in Ecology & Evolution*, **15**, 140–143.
- Kay, K.M. & Sargent, R.D. (2009) The role of animal pollination in plant speciation: Integrating ecology, geography, and genetics. *Annual Review of Ecology, Evolution, and Systematics*, **40**, 637–656.
- Lack, A.J. (1982) Competition for pollinators in the ecology of *Centaurea scabiosa* L. and *Centaurea nigra* L. III. Insect visits and the number of successful pollinators. *New Phytologist*, **91**, 321–339.
- Landry, C.L. (2013) Pollinator-mediated competition between two co-flowering Neotropical mangrove species, *Avicennia germinans* (Avicenniaceae) and *Laguncularia racemosa* (Combretaceae). *Annals of Botany*, **111**, 207–214.
- Lane, M.A. (1996) Pollination biology of Compositae. *Compositae: Biology and Utilization, Proceedings of the International Compositae Conference* (eds P.D.S. Caligari & D.J.N. Hind), pp. 61–80. Royal Botanic Gardens.

## References

- Marshall, D.F. & Abbott, R.J. (1984) Polymorphism for outcrossing frequency at ray floret locus in *Senecio vulgaris* L., III. *Heredity*, **53**, 145–149.
- Nielsen, L.R., Philipp, M. & Siegismund, H.R. (2002) Selective advantage of ray florets in *Scalesia affinis* and *S. pedunculata* (Asteraceae), two endemic species from the Galápagos. *Evolutionary Ecology*, **16**, 139–153.
- Nielsen, L.R., Siegismund, H.R. & Hansen, T. (2007) Inbreeding depression in the partially self-incompatible endemic plant species *Scalesia affinis* (Asteraceae) from Galápagos islands. *Evolutionary Ecology*, **21**, 1–12.
- Van der Niet, T., Peakall, R. & Johnson, S.D. (2014) Pollinator-driven ecological speciation in plants: new evidence and future perspectives. *Annals of Botany*, **113**, 199–211.
- Ollerton, J., Killick, A., Lamborn, E., Watts, S. & Whiston, M. (2007) Multiple meanings and modes : on the many ways to be a generalist flower. *Taxon*, **56**, 717–728.
- Parachnowitsch, A.L. & Kessler, A. (2010) Pollinators exert natural selection on flower size and floral display in *Penstemon digitalis*. *New Phytologist*, **188**, 393–402.
- Penet, L., Marion, B. & Bonis, A. (2012) Impact of capitulum structure on reproductive success in the declining species *Centaurea cyanus* (Asteraceae): Small to self and big to flirt? *Journal of Pollination Ecology*, **8**, 52–58.
- Sahli, H.F. & Conner, J.K. (2011) Testing for conflicting and nonadditive selection: floral adaptation to multiple pollinators through male and female fitness. *Evolution*, **65**, 1457–1473.
- Sánchez-Lafuente, A.M. (2002) Floral variation in the generalist perennial herb *Paeonia broteroi* (Paeoniaceae): Differences between regions with different pollinators and herbivores. *American Journal of Botany*, **89**, 1260–1269.
- Sargent, R.D. & Ackerly, D.D. (2008) Plant-pollinator interactions and the assembly of plant communities. *Trends in Ecology & Evolution*, **23**, 123–130.
- Sargent, R.D., Kembel, S.W., Emery, N.C., Forrestel, E.J. & Ackerly, D.D. (2011) Effect of local community phylogenetic structure on pollen limitation in an obligately insect-pollinated plant. *American journal of botany*, **98**, 283–289.
- Schemske, D.W. & Bradshaw, H.D. (1999) Pollinator preference and the evolution of floral traits in monkeyflowers (*Mimulus*). *Proceedings of the National Academy of Sciences of the United States of America*, **96**, 11910–5.
- Schmid-Hempel, P. & Speiser, B. (1988) Effects of inflorescence size on pollination in *Epilobium angustifolium*. *Oikos*, **53**, 98–104.
- Stuessy, T.F., Spooner, D.M., Evans, K.A., Jan, N. & Stuessy, T.O.D.E. (1986) Adaptive Significance of Ray Corollas in *Helianthus grosseserratus* (Compositae). *American Midland Naturalist*, **115**, 191–197.
- Sun, M. & Ganders, F.R. (1990) Outcrossing rates and allozyme variation in rayed and rayless morphs of *Bidens pilosa*. *Heredity*, **64**, 139–143.
- Thompson, J.D. (2001) How do visitation patterns vary among pollinators in relation to floral display and floral design in a generalist pollination system? *Oecologia*, **126**, 386–394.
- Torices, R., Méndez, M. & Gómez, J.M. (2011) Where do monomorphic sexual systems fit in the evolution of dioecy? Insights from the largest family of angiosperms. *New Phytologist*, **190**, 234–248.
- Vereecken, N.J., Wilson, C. a, Höftling, S., Schulz, S., Banketov, S. a & Mardulyn, P. (2012) Pre-adaptations and the evolution of pollination by sexual deception: Cope's rule of specialization revisited. *Proceedings of the Royal Society B-Biological Sciences*, **279**, 4786–4794.
- Violle, C., Nemergut, D.R., Pu, Z. & Jiang, L. (2011) Phylogenetic limiting similarity and competitive exclusion. *Ecology Letters*, **14**, 782–787.

## References

- Waser, N.M. (2001) Pollinator behavior and plant speciation: looking beyond the ethological isolation paradigm. *Cognitive Ecology of Pollination: Animal Behavior and Floral Evolution* (eds L. Chittka & J.D. Thomson), pp. 318–336. Cambridge Univ. Press, Cambridge, NY.
- Wesselingh, R.A. & Arnold, M.L. (2000) Pollinator behaviour and the evolution of Louisiana iris hybrid zones. *Journal of Evolutionary Biology*, **13**, 171–180.
- Ye, Z.-M., Dai, W.-K., Jin, X.-F., Gituru, R.W., Wang, Q.-F. & Yang, C.-F. (2013) Competition and facilitation among plants for pollination: can pollinator abundance shift the plant–plant interactions? *Plant Ecology*, **215**, 3–13.
- Zuur, A.F., Ieno, E.N., Walker, N., Saveliev, A. & Smith, G.M. (2009) *Mixed Effects Models and Extensions in Ecology with R*. Springer, New York, NY.

Appendix

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**Supplementary Table A.** Plant, capitulum and surrounding traits of the *Anacyclus* plants in the sympatric site. Statistical significances (<0.05) obtained by a Kruskal-Wallis Test are shown in bold. n = 89 individuals.

Traits	All phenotypes			Rayed			Intermediate			Rayless			P	Df
	Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE			
<i>Plant</i>														
Floral display	5.99 ± 0.58	6.41 ± 0.89	7.91 ± 2.04	4.70 ± 0.62	0.39	2								
Height (mm)	220.53 ± 10.07	220.17 ± 15.01	223.57 ± 29.57	219.71 ± 15.38	0.10	2								
Plant dimension (cm <sup>2</sup> )	516.41 ± 79.02	568.96 ± 135.06	506.26 ± 144.96	457.23 ± 115.34	0.95	2								
<i>Capitulum</i>														
Number of rays	5.78 ± 0.52	9.40 ± 0.37	9.20 ± 0.63	0.00 ± 0.00	<0.0001	2								
Capitulum diameter (mm)	21.20 ± 0.87	28.89 ± 0.60	19.26 ± 1.27	12.72 ± 0.31	<0.0001	2								
Disk diameter (mm)	13.22 ± 0.54	13.24 ± 0.93	14.50 ± 2.03	12.68 ± 0.30	0.74	2								
Ray length (mm)	3.99 ± 0.51	7.83 ± 0.57	2.38 ± 1.33	0.00 ± 0.00	<0.0001	2								
<i>Neighbourhood</i>														
Pollination context	7.44 ± 1.19	6.25 ± 1.62	13.39 ± 4.07	6.44 ± 1.70	0.22	2								
Neighbour density	4.16 ± 0.64	4.24 ± 1.16	4.93 ± 1.42	3.74 ± 0.74	0.74	2								
n	89	41	14	34										

**Supplementary Table B:** Pearson correlation coefficients of plant, capitulum and surrounding traits for all plants in the sympatric site. Statistically significant Pearson correlation coefficients are highlighted in bold. All the *P* values were adjusted for multiple tests. Correlation coefficients for all plant traits and neighbourhood traits, additionally for capitulum and disk diameter were performed with data from all phenotypes (*n* = 89); while for correlation coefficients of the number of rays and ray length variables only intermediate and rayed phenotypes data were used (*n* = 55).

Traits	Plant			Capitulum			Neighbourhood
	Floral display	Height	Dimension	N <sup>o</sup> of rays	Capitulum diameter	Disk diameter	Pollination context
<i>Plant</i>							
Floral display							
Height	0.02						
Dimension	<b>0.79</b>	0.02					
<i>Capitulum</i>							
<i>Number of rays</i>	0.06	-0.04	0.09				
Capitulum diameter	0.14	0.08	0.10	0.15			
Disk diameter	-0.02	0.29	0.01	-0.31	0.01		
Ray length	0.00	-0.15	0.06	<b>0.31</b>	<b>0.74</b>	<b>-0.78</b>	
<i>Neighbourhood</i>							
Pollination context	-0.04	<b>0.64</b>	-0.05	0.06	0.02	0.13	-0.15
Neighbour density	-0.19	<b>0.42</b>	-0.20	0.04	0.12	0.10	<b>0.61</b>

**Supplementary Table C.** The effect of capitulum size, floral display and pollination context on pollinator attraction for the entire pollinator assemblage (Total), and for different pollinator guilds (Bees, Small flies, Big flies, Hoverflies) for the rayed individuals and intermediate individuals for the sympatric population. Plant identity was used as a random variable. A measure of overdispersion is also provided. Statistical significances (<0.05) are shown in bold. Positive and negative significances are signed with (+).

Variables	Df	All groups				Bees		Small flies		Big flies		Hoverflies	
		$\chi^2$	P	$\chi^2$	P	$\chi^2$	P	$\chi^2$	P	$\chi^2$	P	$\chi^2$	P
<i>Fixed</i>													
Capitulum size	1	(+) 11.04	<b>&lt;0.001</b>	0.03	0.865	(+) 7.69	<b>&lt;0.01</b>	1.98	0.160	(+) 9.23	<b>0.020</b>		
Floral display	1	0.83	0.362	(+) 7.62	<b>&lt;0.01</b>	0.29	0.588	0.03	0.871	(+) 4.24	<b>0.040</b>		
Pollination context	1	1.54	0.215	<0.01	0.936	0.18	0.673	(+) 4.44	<b>0.035</b>	(+) 3.11	0.078		
<i>Random</i>													
Plant		0.38	0.62	0.68	0.83	0.92	0.96	0.28	0.53	0.35	0.59		
Overdispersion index		1.120		0.859		0.982		0.760		1.014			