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Article in *Plant Ecology & Diversity* · April 2016

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SHORT COMMUNICATION

Heterospecific pollen transfer from an exotic plant to native plants: assessing reproductive consequences in an Andean grassland

Gastón O. Carvallo^{a*} and Rodrigo Medel^b

^aInstituto de Biología, Facultad de Ciencias, Pontificia Universidad Católica de Valparaíso, Valparaíso, Chile; ^bDepartamento de Ciencias Ecológicas, Facultad de Ciencias, Universidad de Chile, Santiago, Chile

(Received 8 June 2015; accepted 6 January 2016)

Background: The presence of exotic plants increases the heterospecific pollen (HP hereafter) received by native plants and reduces their reproductive output.

Aims: We assessed whether the exotic herb *Echium vulgare* (Boraginaceae) increased HP and reduced seed output of the native plants *Phacelia secunda* (Boraginaceae) and *Stachys albicaulis* (Lamiaceae) in an Andean locality, central Chile.

Methods: The presence of HP was studied in native plants growing with and without co-existence with the exotic *E. vulgare*. A complementary hand pollination experiment was carried out to assess whether *E. vulgare* pollen reduced the reproductive success of native plants.

Results: In the presence of *E. vulgare*, 17.3% and 3.7% of *P. secunda* and *S. albicaulis* individuals that coexisted with the exotic species received HP. For *P. secunda*, the number of conspecific pollen grains decreased in invaded patches compared with non-invaded patches; no differences were observed for *S. albicaulis*. The pollen of *E. vulgare* negatively affected the reproductive success of *S. albicaulis* but not that of *P. secunda*.

Conclusions: The presence of HP cannot be predicted from the presence of exotic plants alone, and other factors, such as flower morphology, could explain the greater HP transfer in *P. secunda* (actinomorphic flowers) than in *S. albicaulis* (zygomorphic flowers). A higher negative effect of *E. vulgare* pollen on *P. secunda* versus *S. albicaulis* could be related to the phylogenetic resemblance between the exotic donor and native recipient plant because pollen-stigma compatibility may be evolutionary conserved through common lineages.

Keywords: *Bombus*; stigma clogging; invasion; plant-pollinator; pollen tubes; pollination service

Introduction

Animal-mediated pollination is an essential ecological process because 87.5% of angiosperms benefit from animals for successful reproduction (Ollerton et al. 2011). Several factors are involved in the pollination process, among them, the identity and composition of the animal assemblages that visit the flowers (Arroyo et al. 1982, 1985), the number and frequency of visits (e.g., Faegri and van der Pijl 1979), the composition of pollen loads carried by the visitors (Gibson et al. 2006) and the pollen loads deposited onto the stigmas (Barrett and Glover 1985; Wang and Cruzan 1998). All of these factors impact the reproductive output of plants, affecting the selective regimes of the floral traits and possibly determining micro-evolutionary changes (Waser and Price 1981; Cresswell 1998; Jones and Reithel 2001; Alexandersson and Johnson 2002; Medel et al. 2003).

The introduction of an exotic plant may disrupt the pollination process of native plants (Traveset and Richardson 2006) because the presence of the exotic plants modifies pollinator preferences (Totland et al. 2006; Bjerknæs et al. 2007; Morales and Traveset 2009; Pyšek et al. 2011; King and Sargent 2012). As such, the number of visits to flowers can be reduced (pollen quantity; Campbell and Motten 1985; Brown and Mitchell

2001; Chittka and Schürkens 2001; Brown et al. 2002) or the proportion of conspecific pollen and heterospecific pollen (HP hereafter) received by the stigmas of native plants can be altered (pollen quality; e.g., Waser and Fugate 1986; Grabas and Lavery 1999; Brown and Mitchell 2001; Chittka and Schürkens 2001; Larson et al. 2006).

The deposition of HP has been estimated to be a common phenomenon in plants. For example, in a recent meta-analysis Ashman and Arceo-Gómez (2013) showed that 50% of the angiosperms studied ($N = 72$ species) received a deposit of HP > 10%. Heterospecific pollination can generate an allelopathic inhibition or physical inhibition to conspecific pollen (Waser 1978; Waser and Fugate 1986; Wilcock and Neiland 2002), which reduces female reproductive success (Ashman and Arceo-Gómez 2013) and acts as a selective force that alter the phenotypic selection of the floral traits of native species (McLernon et al. 1996; Bean and Roach 2015). Therefore, the transfer and deposition of HP could be considered a mechanism by which exotic plant species impact the reproductive performance of native plants.

Two non-mutual factors could limit HP transfer among plants: community composition and floral morphology. On the one hand, the spatial distribution (Yang

*Corresponding author. Email: gaston.carvallo@pucv.cl

et al. 2011), abundance (Lopezaraiza-Mikel et al. 2007) and diversity (Ghazoul 2006) of plants are important factors for determining the constancy of pollinators to flowers; these factors could define the amounts of HP received by a focal species. On the other hand, flower morphology facilitates pollinator recognition and favours pollinator constancy in flowers, which reduces HP transfer (Schaefer et al. 2004); additionally, flower morphology could act as a “barrier” to HP (Montgomery and Rathcke 2012; Ashman and Arceo-Gómez 2013). Specifically, HP deposit is less in bilateral flowers than in radial flowers (Montgomery and Rathcke 2012) because more open flowers are more susceptible to receiving pollen from different sources. Secondly, some characteristics of the pollen (e.g. size and aperture number) and pistils (e.g. stigma and style length) of donor plants can predict the impacts on the fitness on focal species (reviewed by Ashman and Arceo-Gómez 2013).

Although important progress has been made in understanding the effects of HP on plants (McLernon et al. 1996; Montgomery and Rathcke 2012; Ashman and Arceo-Gómez 2013), understanding the effects of HP in the context of exotic species is still an open question; these plants could act as pollen donors to co-flowering native plant species. In this study we assessed whether the presence of an invasive plant, *Echium vulgare* (Boraginaceae) increased the presence of HP in two native herbs in central Chile, *Phacelia secunda* (Boraginaceae) and *Stachys albicaulis* (Lamiaceae), and incorporated the floral traits of natives as a covariable to disentangle their role in pollen deposition. Specifically, our study answered the following questions: (i) is there any evidence of *E. vulgare* pollen is deposited on the stigmas of native plants, and (ii) does the pollen of *E. vulgare* reduce the reproductive success of native plants?

Materials and methods

Study area and species

The study was carried out in Termas de Chillán, central Chile (36° 54' 34" S, 71° 24' 46" W; 1834 m a.s.l.) during the austral summer in 2008. Our study focused on grasslands surrounded by deciduous forests of *Nothofagus pumilio* Krasser (Nothofagaceae) and *N. dombeyi* Oerst. The grasslands were dominated by the invasive plant *E. vulgare* (Carvallo et al. 2013), which was dispersed to the study site by hiker-related traffic (Valdebenito 2002). *Echium vulgare* L. (Boraginaceae) is a biannual herb that reaches 0.2–1.3 m in height with blue-violet flowers 10–19 mm in length (Matthei 1995); it is of European origin, was introduced in Chile in the late nineteenth century (Matthei 1995), and is widely distributed from 25° 17' S to 53° 01' S. Of the native plants, *Phacelia secunda* J.F. Gemel. (Boraginaceae: Hydrophylloideae) and *Stachys albicaulis* Lindl. (Lamiaceae) species with a high relative

abundance were studied. *Phacelia secunda* is a perennial herb with a basal vegetative rosette that reaches 0.4 m height; its flowers, 4–7 mm in length, are blue-violet, and arranged in a cyme. *Stachys albicaulis* is a perennial herb that reaches 0.4–0.8 m in height; reaching 10–12 mm in length, its flowers are pink, and arranged in whorls. A previous study conducted at the study site showed a facilitative effect of *E. vulgare* on the visitor richness for native species (Carvallo et al. 2013). The most common flower visitors at the site were the hymenopteran *Alloscirtetica gayi* (Apidae), the endemic bumblebee *Bombus dahlbomii* (Apidae) and the exotic bumblebee *Bombus terrestris* (Apidae) (Carvallo et al. 2013).

Pollen loads on stigmas

We studied the pollen loads on the stigmas of the native plants by comparing individuals inhabiting the non-invaded patches and the patches invaded by *E. vulgare*. Because natural variation exists among these types of patches (Carvallo et al. 2013), the numbers of non-invaded and invaded patches for each focal plant species were imbalanced (*P. secunda*: one non-invaded and two invaded patches; *S. albicaulis*: one non-invaded and three invaded patches). To address this imbalance in our analyses, all individuals pertaining to the invaded patches were pooled. Although this approximation could reduce the independence of data because spatial autocorrelation can exist among individuals within non-invaded patches versus in invaded patches, we discuss our results as a consequence of a natural experiment, which offers the opportunity to assess whether any HP transfer from exotic plant to focal natives exist.

We studied pollen deposition onto stigmas in the invaded and non-invaded patches. According to the number of individuals available in the patches, 90 and 150 individuals of *P. secunda* were tagged in the non-invaded and invaded patches, respectively, and 45 and 200 individuals of *S. albicaulis* were tagged in the non-invaded and invaded patches, respectively. For each individual, a floral bud was tagged. Four days after anthesis, the tagged flowers were removed from by cutting from the pedicel at the closest section to the branch. This time span allowed sufficient pollinator visits to the flowers because the anthesis of individual flower was in the range of 2 to 4 days (Carvallo 2011). Individual flower samples were deposited in vials with ethanol 70% v/v. For each collected flower, the style was dissected, and the number of pollen grains on the stigmas was quantified. To visualise the pollen grains, the styles were stained with aniline blue 0.1% dissolved in a sodium-phosphate buffer 0.07 M and pH = 9 (Kearns and Inouye 1993; Harder and Aizen 2004). Prior to staining, the styles were softened in a solution of 2 M NaOH for 24 h, and then the tissue was washed in distilled water and fixed with the stain for 48 h. After

the incubation time, the tips of the stigmas were cut, placed on a slide, squashed and observed with a fluorescence microscope Nikon Labophot 2 TM (Nikon Inc., Japan). Thus, the number of pollen grains on the stigmas was quantified, and the conspecific and HP sources were differentiated. The shape and size of the pollen grains allowed us to identify the pollen source plant because recognisable differences were exhibited among the species studied (size of pollen grains: mean \pm SE; *E. vulgare*: 27.7 $\mu\text{m} \pm 0.3$; *P. secunda*: 35.8 $\mu\text{m} \pm 0.2$; *S. albicaulis*: 49.6 $\mu\text{m} \pm 0.2$; in all $N = 9$ pollen grains, Carvallo 2011). For example, the pollen grains of *E. vulgare* are lengthened, tricolpates and without visible pores; *S. albicaulis* pollen is clearly distinguished by its large size and spherical shape; for *P. secunda*, the presence of ornamented pores and its spherical shape allow us to distinguish the pollen from *E. vulgare* pollen.

A Kruskal-Wallis test was used to assess whether the number of pollen grains on the stigmas varied between individuals from the invaded and non-invaded patches. To standardise the differences in the number of pollen grains deposited on the stigmas, each value was divided by the mean of the group. Because the number of pollen grains deposited on the stigmas can be influenced by floral morphology, some floral traits were included as covariables in our analyses (see below).

To determine the effects of flower morphology on HP transfer, we studied the corolla length, corolla width and anther-stigma distance in *P. secunda* (Appendix 1) and the corolla length, area of the lower petal, and anther-stigma distance in *S. albicaulis* (Appendix 1). These traits were chosen because they have been demonstrated to have a relationship with attracting pollinators (Barrett 2013; Schiestl and Johnson 2013) and reproductive assurance (Kalisz et al. 1999; Carvallo and Medel 2010). Floral traits were measured from digital photographs using Sigma Scan Pro 5.0 TM; photographs were taken by a Nikon TM D50 digital camera (Nikon Inc., Japan) using graph paper as the background. The relationship between each trait i to the number of pollen grains was tested using generalised linear models; all traits were z -standardised (z_i), and the number of pollen grains was standardised by dividing each individual value by the mean.

Heterospecific pollination effect on native plants

Individuals of *P. secunda* ($N = 40$) and *S. albicaulis* ($N = 60$) were tagged in non-invaded patches. On each individual, a stem that had flower buds (ranging between 1–5 flower buds in *P. secunda* and 1–11 in *S. albicaulis*) was covered with silk bags. As anthesis occurred, one of the following hand pollination treatments was applied to all flowers of the same individual: (i) conspecific pollination, (ii) heterospecific pollination, with pollen from *E. vulgare*, (iii) mixed pollination, using a mixture of focal species and *E. vulgare* pollen and (iv) the control (no-pollination). In all treatments, the flowers were emasculated the first day of anthesis. For each hand pollination

treatment, 15 and 10 individuals for *P. secunda* and *S. albicaulis* were used, respectively. Two anthers were used to generate a pollen bulk that was applied for pollination on five flowers. For heterospecific pollination an anther of *E. vulgare* and another of the focal native plant were used to generate the pollen bulk. As we did not count the number of pollen grains borne by each anther, we verified the maturity of the anthers (presence of pollen grains) by observing with a magnifying glass and transferred copious amounts of pollen to the focal flowers. To avoid geitonogamy, the anthers were not used to pollinate the same individual from which they were extracted. Hand pollination was carried out by using natural hair brushes n°1 (Artel TM, Santiago). After hand pollination, the flowers were recovered with silk bags to avoid further pollen deposition. Between 24 and 27 days after hand pollination, the fruits were collected in paper bags and carried to the laboratory, where the number of seeds per fruit was counted. Based on these data, the fruit set (the number of flowers that produced fruits with at least one seed) and the seed:ovule ratio (S:O ratio, hereafter) were estimated. The ovule number to *P. secunda* and *S. albicaulis* is a constant value ($N = 4$ ovules). To assess the changes in the fruit set and the S:O ratio, ANOVAs were carried out using a generalised linear model based on a quasi-binomial error term to address zero observations of our data (O'Hara and Kotze 2010).

Results

We detected the pollen of *E. vulgare* on the stigmas of 17.3% of the *P. secunda* and 3.7% of the *S. albicaulis* individuals in invaded patches. No individuals of the studied plants received HP in non-invaded patches. In invaded patches, 78.7% ($N = 70$) and 19.4% ($N = 37$) of *P. secunda* and *S. albicaulis* individuals received conspecific pollen, respectively. Regarding the number of conspecific pollen grains on stigmas, for *P. secunda* we detected 31% less in invaded than in non-invaded patches (Table 1); there were no significant differences for *S. albicaulis* (Table 1). We observed that the measured traits did not explain the number of pollen grains deposited onto the stigmas of the native plants (Appendix 2).

Regarding the hand-pollination experiment, the use of mixed hand pollination (conspecific and *E. vulgare* pollen) did not affect either the fruit set or the seed ovule ratio in *P. secunda* but reduced these reproductive estimators in *S. albicaulis* (Figure 1; Table 2). Hand pollination using HP reduced both the fruit set and the seed ovule ratio in *S. albicaulis* and *P. secunda* to levels equivalent to those of the control (no pollination).

Discussion

Our results suggest that there are limiting factors to pollen transfer from *E. vulgare* to native species in the invaded patches and that there is no probability of HP transfer from invaded to non-invaded patches. These results are

Table 1. Number of conspecific pollen grains (CP) and hetero-specific pollen (HP) deposited on the stigmas of the native plants *Phacelia secunda* (a) and *Stachys albicaulis* (b) inhabiting non-invaded and invaded patches by *Echium vulgare* (mean \pm SE), Termas de Chillán, central Chile. Descriptive statistics of the flower traits studied are included. Values in parentheses indicate the number of individuals analysed; X^2 is the result of a Kruskal-Wallis test that compared each variable between the non-invaded and invaded patches.

Traits	Non-invaded	Invaded	X^2
(a) <i>Phacelia secunda</i>			
CP	2.53 \pm 0.24 (89)	1.93 \pm 0.25 (133)	10.739***
HP	0 (89)	0.29 \pm 0.07 (133)	17.037***
Corolla length (mm)	7.21 \pm 0.08 (84)	7.16 \pm 0.07 (124)	0.408 ^{ns}
Corolla width (mm)	5.39 \pm 0.08 (84)	5.37 \pm 0.06 (112)	0.006 ^{ns}
Herkogamy (mm)	1.89 \pm 0.17 (61)	1.81 \pm 0.13 (84)	0.049 ^{ns}
(b) <i>Stachys albicaulis</i>			
CP	0.34 \pm 0.22 (29)	0.61 \pm 0.12 (191)	0.618 ^{ns}
HP	0 (29)	0.16 \pm 0.11 (191)	1.093 ^{ns}
Corolla length (mm)	16.47 \pm 0.33 (29)	16.11 \pm 0.15 (190)	0.079 ^{ns}
Lower petal area (mm ²)	6.31 \pm 0.26 (29)	6.62 \pm 0.10 (187)	1.182 ^{ns}
Herkogamy (mm)	2.48 \pm 0.35 (29)	2.61 \pm 0.13 (172)	0.443 ^{ns}

*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, not significantly different.

discussed from different pollination components. In invaded patches, there are several non-mutually exclusive factors that explain the low presence of *E. vulgare* pollen on the native plants. First, despite native plants and *E. vulgare* sharing the more abundant pollinators, which could contribute to HP transfer among individuals (Carvallo et al. 2013), these pollinators may act as ineffective pollen carriers. This could be the case for *Bombus terrestris* (exotic in Chile), which visits both native and exotic flowers in invaded patches (Carvallo et al. 2013) and has been determined to be an ineffective pollinator for other plant species in Mediterranean-type ecosystems of central Chile (Esterio et al. 2013) and several other geographic regions around world (Goulson 2003).

Another factor that may contribute obstructing the transfer of pollen from exotic to native plants is the pollen presentation, thus, the position and way that anthers release

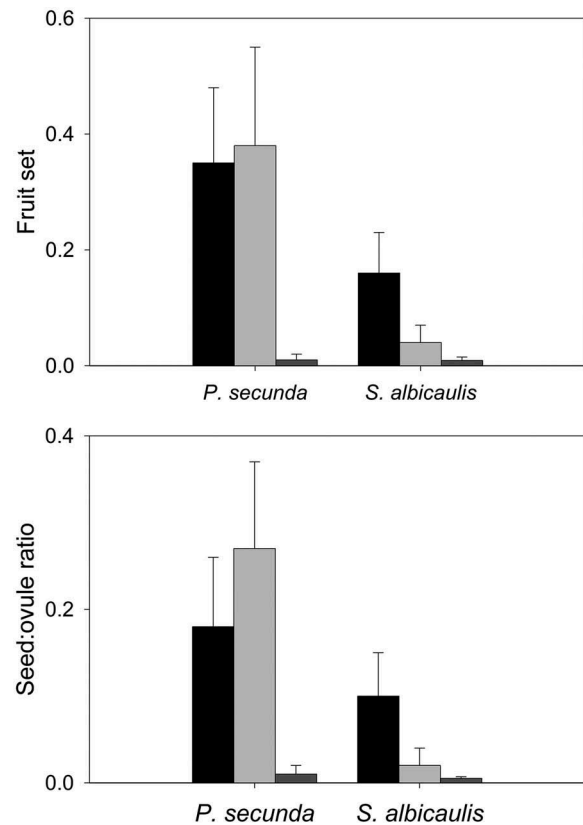


Figure 1. Reproductive output of native plant species after the hand-pollination experiment (mean \pm SE), Termas de Chillán, central Chile. The fruit set (a) and the seed:ovule ratio (b) of *Phacelia secunda* and *Stachys albicaulis* were estimated after applying the following treatments of pollination: conspecific pollination (black bars), mixed pollination (light grey bars) and heterospecific pollination (dark grey bars). The control treatments (absence of pollination) did not produce seeds (not shown). The generalised linear models that assessed differences among treatments are summarised in Table 2.

pollen (Castellanos et al. 2006) because it has been shown to prevent the adhesion of pollen to the body of pollinators (Castellanos et al. 2003). Flower traits contribute to preventing the entrance of HP; for example, stigma size in native plants is a key trait to hampering pollen deposit from exotic *Euphorbia esula* (Euphorbiaceae) (Montgomery and Rathcke 2012), or zygomorphic flowers receive more HP than actinomorphic flowers (McLernon et al. 1996). Traits did not explain the number of pollen grains deposited onto the stigmas of the native plants

Table 2. Results of the generalised linear model (quasi-binomial error term) that assessed the effects of hand pollination treatments on fruit set and the seed:ovule ratio of the native plants *Phacelia secunda* and *Stachys albicaulis*, Termas de Chillán, central Chile.

	Fruit set			Seed:ovule ratio		
	df	Residual deviance	F	df	Residual deviance	F
<i>P. secunda</i>	3	15.625	8.483***	3	7.543	9.598***
<i>S. albicaulis</i>	3	14.208	2.774	3	8.707	3.090*

*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

(Appendix 2). The zygomorphic morphology and narrow entrance to the flower tube in *S. albicaulis* is the most parsimonious explanation to understanding the low number of individuals and pollen amounts of both HP and conspecific sources. The narrow entrance to the flower tube has been shown to be a barrier to animals, and is related to specialised pollination systems (Hansen et al. 2012), an observation in line with the flower morphology of *S. albicaulis*, which has pollinators with long proboscises, such as acrocerid and nemestrinid flies that scarcely visit the exotic plant (Carvalho et al. 2013). On the other hand, the actinomorphic flowers of *P. secunda* could receive more HP than zygomorphic flowers; however, the low number of patch replicates used in our study limited our determination as to whether the absence of differences was a sample effect. Future studies are required to integrate a greater number of localities that permit a comparison between invaded and non-invaded patches and elucidate how the presence of exotic plants affects the phenotypic selection regimens in the flower traits of native plants.

Hand pollination using conspecific pollen demonstrated that the studied species showed some degree of a self-compatible reproductive system. The absence of pollen did not generate seeds in the studied species, which rules out any apomictic seed production mechanism. Our results suggest that the existence of pollen interference of *E. vulgare* on *S. albicaulis* but not on *P. secunda* (Figure 1). Several studies have shown a neutral effect of exotic HP deposition on the stigmas of native plant species (Moragues and Traveset 2005; Totland et al. 2006), suggesting that exotic plants are not often a threat to the reproduction of native plants. In this study, it is possible that the pollen grains of *E. vulgare* (small in comparison with those of the native species) could not block the stigma surface mechanically, allowing normal development of the pollen tube for the conspecific pollen of native species, at least for *P. secunda*. Additionally, *P. secunda* exhibits a higher degree of phylogenetic similarity to *E. vulgare* than *S. albicaulis* (both belong to the same order Boraginales; Nazaire and Hufford 2012), which could explain the absence of the negative effects of *E. vulgare* pollen on *P. secunda* pollination. The mechanisms of pollen-stigma compatibility have been recognised to possibly have some degree of phylogenetic conservatism (Morales and Traveset 2009; Ashman and Arceo-Gómez 2013) that would generate interference between less related plants (e.g. *E. vulgare* and *S. albicaulis*) through chemical inhibitions (Wilcock and Neiland 2002). Our study showed that the presence of HP cannot be predicted from the presence of exotic plants alone, as has been reported previously (Montgomery and Rathcke 2012) and this suggests that pollen transfer may depend on flower morphology, with zygomorphic flowers (e.g. *S. albicaulis*) receiving less pollen than actinomorphic flowers (e.g. *P. secunda*). Furthermore, we detected a weak negative effect of *E. vulgare* pollen on the reproduction of native plants, which may depend on the phylogenetic similarity between donor and recipient plants. Future integrative studies that elucidate the role of pollinators and the amounts of pollen

transferred from donor plants to animal bodies, and from animals to recipient plants will allow us to elucidate the invasion processes in angiosperms.

Acknowledgements

We thank to Alicia Arredondo and Alberto Veloso for help with pollen stain procedures; José M. Gómez and Luis Navarro gave invaluable inputs in early stages of this research, and two anonymous reviewers for their valuable comments.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This work was supported by the FONDECYT [grant number 3130399]. The authors declared that no competing interests exist.

Notes on contributors

Gastón O. Carvalho is an Assistant Professor. He is broadly interested in plant ecology and diversity from taxonomical, phylogenetic and functional approaches.

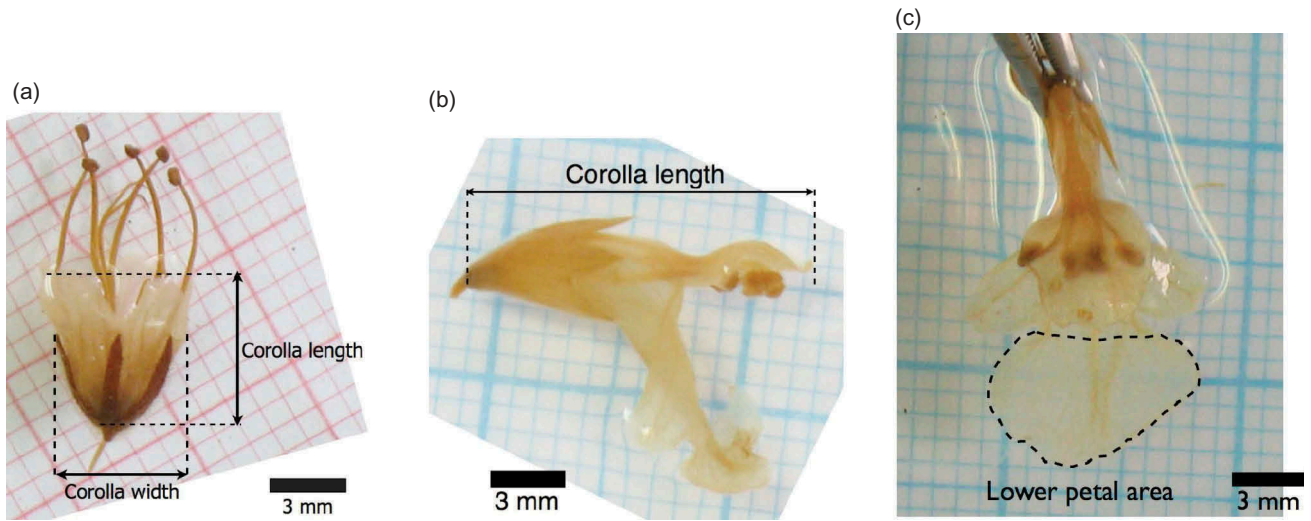
Rodrigo Medel is a Full Professor. His research consists on the evolutionary ecology of plant-animal relationships, with emphasis in antagonistic and mutualistic interactions.

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Appendix 1. Photograph that show floral traits measured for *P. secunda* (a) and *S. albicaulis* (b and c).



Appendix 2. Results of generalised linear models that assessed the effects of patch type (non-invaded or invaded by *E. vulgare*) and floral traits (as covariable) on the number of conspecific pollen grains of stigmas of *P. secunda* (a) and *S. albicaulis* (b). Estimator of each factor and standard error are reported.

Models (Covariable)	Patch type	Covariable	Interaction (Patch x Covariable)
(a) <i>P. secunda</i>			
Model 1	0.269 ± 0.091***		
Model 2 (Corolla length)	0.247 ± 0.096*	0.009 ± 0.066	-0.034 ± 0.096
Model 3 (Corolla width)	0.343 ± 0.101***	-0.107 ± 0.074	0.139 ± 0.102
Model 4 (Herkogamy)	0.386 ± 0.111***	0.186 ± 0.072**	-0.191 ± 0.105
(b) <i>S. albicaulis</i>			
Model 1	-0.566 ± 0.329		
Model 2 (Corolla length)	-0.785 ± 0.390*	0.013 ± 0.093	0.638 ± 0.328
Model 3 (Petal width)	-0.564 ± 0.334	0.211 ± 0.097*	-0.034 ± 0.342
Model 4 (Herkogamy)	-0.806 ± 0.390*	-0.159 ± 0.104	-0.503 ± 0.449

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.