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ORIGINAL PAPER

Does hardness make flower love less promiscuous? Effect of biomechanical floral traits on visitation rates and pollination assemblages

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Abstract Visitation rates and assemblage composition of pollinators have often been related to environmental, ecological and phenotypic variables. However, the interaction between flowers and pollinators has not been evaluated in a biomechanical context. Floral rewards in keel flowers (Fabaceae, Faboideae) are concealed behind four joined petals, the keel-wing unit, and are accessible only if pollinators open this unit by exerting force on it. Force needed to open the flower is expected to affect the interaction with pollinators because pollinators must invest time and energy to open the keels. Consequently, plants with stiff flowers should be expected to experience diminished visitation frequency, particularly by weak visitors. To test this expectation of diminished visitation rates and of assemblage composition biased by pollinator strength, we measured the force needed to open the keel flowers of five co-occurring legume species and, using a canonical correspondence analysis (CCA), we tested their association with pollinator visitation rates and assemblage composition. We additionally included a size flag variable in CCA to test the effect of attractiveness on pollinator visits. There was no association between flower stiffness and visitation frequency. According to the CCA, pollinator assemblage compositions were associated with the force needed to open the keel and not flag size. As a general pattern, weak flowers are pollinated by an uneven assemblage of weak bees while the stiffest flowers are pollinated by an even assemblage of large and strong bees. These results supports the idea that force has an effect in controlling pollinator assemblage composition.

Introduction

Several angiosperms have a forcible flower mechanism that needs to be operated by pollinators to gain access to rewards. Consequently, pollination success in these flowers may be dependent on the ability of pollinators to overcome moveable obstacles. It is not as yet clear in these cases if flowers are imposing limits to the access to rewards as a means of controlling pollinator assemblage composition and visitation frequencies. However, for some Fabaceae, it is known that species of bees unable to exert enough force to open flowers cannot access rewards, acting as nectar robbers (Córdoba and Cocucci 2011).

The importance of functional biomechanics in pollen removal and its deposition on the pollinator body has been demonstrated for the staminal lever mechanism of *Salvia* (Claßen-Bockhoff et al. 2004a, b; Reith et al. 2007). In addition, the loss of functionality of the lever mechanism associated with pollinator shifts indicates the effect of pollinator-driven selection on biomechanical traits (Wester and Claßen-Bockhoff 2006).

A similar example is represented by the keel flowers (Fabaceae) where nectary and fertile organs are hidden behind petals that need to be forcibly turned aside by pollinators for the plant to be pollinated (Faegri and van der Pijl 1966). In these flowers, a moveable structural unit

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(which consists of four petals with hinge-like claws, the wings and the keel) protects nectar, stigma and stamens in such a way that pollinators must exert force on it to access the rewards (for more details see Córdoba et al. 2015). The mechanical functionality of keel flowers as well as the ability of pollinators to open flowers had been previously studied (Westerkamp and Weber 1999; Parker et al. 2002; Córdoba and Cocucci 2011; Aronne et al. 2012; Amaral-Neto et al. 2015). One particular trait, lever arm length, acts in combination with force in determining moment of force which measures the actual force exerted on the hinges when the keel-wing unit is rotated (Córdoba and Cocucci 2011).

From the foraging economics point of view, to minimize the energy and time invested to obtain rewards from keel flowers pollinators should be expected to selectively visit plant species with weak flowers. Additionally, selection on biomechanical properties such as hardness could indirectly impose selection on morphometric traits since the latter are dependent on the former; for example, the length of keel represents the lever arm of the forcible mechanism which could be an important aspect determining the floral functionality.

However, the fact that plants with a forcible mechanism have evolved several times among angiosperms casts doubts on an explanation purely based in pollination economics. For example, flower hardness could be a means of controlling theft or visitation by unsuitable pollinators (Córdoba and Cocucci 2011). Harder flowers could, thus, be pollinated by a more specialized and narrow assemblage of pollinators having the necessary strength to open them. In any case, it should not be ignored that pollinator visitation rates and assemblage composition could be affected by attractive features such as flag size in keel flowers, i.e. flowers with larger flags could receive more frequent visits and from a broader assemblage composition (Parker et al. 2002).

Here, we evaluated in five species of keel flowered legumes the association of two biomechanical traits, i.e. the force to open the keel-wing unit and lever arm length, and one attractiveness trait (flag size) with overall visitation rates and assemblage composition. If the foraging economics explanation were true, then the force needed to open the keel-wing unit is expected to have a negative association with visitation frequency. If the pollinator filtering explanation were true, then biomechanical features should be associated with pollinator assemblage diversity and composition. Flowers that are hard and that have long lever arms are then expected to be pollinated by a narrow assemblage of functionally specialized visitors. If the attractiveness explanation were true, then we expect to find a positive association between the assemblage composition and the flag size.

Materials and methods

Plant species and study site

We studied wild populations of the following five species of keel flowered legumes: *Collaea argentina, Desmodium cuneatum, Lathyrus pubescens, Rhynchosia edulis* and *Dalea elegans*. These occurred in two localities (Copina: 31°33′0″S, 64°42″W; Cuesta Blanca: 31°28′49″S, 64°34′26″W) within 8 km in the Sierras Grandes Mountain Range in Córdoba, Argentina. All these species present a typical papilionaceous architecture in which keel and wings protect stigma, stamens and rewards (Faegri and van der Pijl 1966). Consequently, pollinators must exert force on the keel-wing unit to move down petals and reveal the channel formed by the flag which functions as a tongue guide and facilitates access to nectar and pollen (Faegri and van der Pijl 1966; Westerkamp and Weber 1999).

Biomechanical and flag size variables

Two traits related with biomechanical floral aspects were estimated, the force needed to open the keel-wing unit and the lever arm length of floral mechanism represented by the total length of the keel. The force, expressed in mN, was measured in three virgin flowers per individual in a total of 30 individuals per plant species using the mean per individual in subsequent analysis. To estimate the force, a dynamometer with a range from 0.1 to 10 g (PESOLA, Baar, Switzerland, model 20010) was used, which was set on a vertical frame provided with a vertically sliding carriage and a metal tool holder. The flower whose force was to be measured was fixed on the holder to the dynamometer. In this setting, the metal tool simulates the flower visitor, while the carriage can be moved up or down by turning a threaded rod with a wheel to emulate the downward pressing movement of the pollinator (for more details see Córdoba and Cocucci 2011). A single measurement was obtained for each flower.

The total length of the keel was measured from the same flowers which were preserved in 70% ethanol immediately after force measurement. The preserved flowers were dissected, petals placed flat in a Petri dish, and photographed along with a reference scale using a single-lens reflex camera (Nikon D80, Tokyo, Japan) mounted and levered on a copy stand. Additionally, the length and width of the flag were obtained from the calibrated photographs. The product between flag length and width was used to estimate the flag size variable. All morphometric variables were obtained from the photographs using the ImageJ software (Rasband 2016).

Pollinator richness and visitation rates

Pollinator richness was characterized as the number of species of insects in each plant species through observations in situ. Visitation rates for each bee species estimated as the mean number of visits per flower per hour were calculated as: $V_f = V/F/T$, where V is the total number of visits to flowers, F the total number of flowers in the patch and T the observation time in minutes (Kearns and Inouye 1993). To this end, visits to individual flowers were recorded during 30-min observation periods from early morning to afternoon when the flowers closed. Visitations were recorded during the whole flowering season for 2 years in *R. edulis* and *D. uncinatum* and during the whole season of 1 year for the remaining species. No significant differences were detected when visitations were recorded for 2 years (results not shown). 15 to 41 observation patches and 400 to 1900 flowers were monitored for each plant species (see more details in Table 1). Visits were recorded only if the pollinators made contact with the fertile organs of the flowers.

To test differences in visitation rates among plants species, generalized linear models with a quasi-Poisson function were performed using the mean number of visits per flower per 100 h as response variable and pollinator species as predictor variable. To test the dominance of the pollinator assemblage in each plant species, Pielou's evenness index was estimated in R software Version 0.4–13 using the package *vegan* (Oksanen et al. 2008). This index ranges from zero to one to describe assemblages consisting of one dominant species to assemblages consisting of two or more equally dominant species, respectively.

Effect of biomechanical and flag size traits on pollinator assemblage and visitation rates

The relationship between biomechanical traits, flag size and visitation frequencies was analyzed with simple linear regressions across plant species, using total visitation rate as the response variable.

To identify the pattern of distribution of plant species using biomechanical traits and pollinator species abundances, a correspondence canonical analysis (CCA) was performed using force, lever arm length and flag size as predictor variables and visitation rates as measure of pollinator species abundance (Fig. 1). CCA is usually implemented in community ecology to describe the relationship between species abundance and environmental variables across communities (Ter Braak 1986). In this study, communities are represented by pollinator assemblages and points in the diagram represent pollinator species and plants species while vectors represent predictor variables. The CCA was performed in R software Version 0.4–13 using the package *vegan* (Oksanen et al. 2008). To test association between

pollinator assemblage groupings and flower force, a generalized procrustes analysis (Gower 1971) and permutation test were performed using distance matrices among plant species in pollinator assemblage composition and biomechanical force (PROTEST, Jackson 1995; Peres-Neto and Jackson 2001).

Results

Pollinator richness and visitation rates

Plant species included in this study are visited by a diverse group of bees (Hymenoptera, Apoidea, Table 1) including leaf-cutter bees (*Megachile* sp. and *Anthidium* sp.), carpenter bees (*Xylocopa* spp.), bumblebees (*Bombus* spp.), and honey bees (*Apis mellifera*); the pollinators carried the pollen on their ventral surfaces. All of them contacted the stamen and stigma when opening the keel flowers and, consequently, acted as pollinators. Though visits by *Xylocopa augusti* and *Centris tricolor* were occasionally observed on flowers of *C. argentina*, these never appeared in the observation patches and were not included in subsequent analyzes.

Except for *Apis mellifera*, the main pollinator of *D. elegans*, all visitors were native bees in the study area. Native pollinators showed a typical foraging behavior; that is, they landed on the keel-wing unit and pushed it down to access to rewards while pushing up the flag with the head contacting in all cases the stamens and stigma with their thorax and abdomen. Honeybees were observed as pollinators of *D. elegans* and also acted as nectar robbers of *C. argentina*. In *D. elegans*, petals are free from each other and do not build a solid keel-wing unit. Honeybees were able, thus, to access to rewards by rotating individual petals from one side of the flower.

Pollinator assemblage richness ranged between two and six bee species, with large flowered species (*C. argentina* y *L. pubescens*) having higher pollinator richnesses. On the other hand, *R. edulis* and *D. cuneatum* showed a similar pollinator assemblage though a not particularly diverse one.

Generalized linear models showed that plant species had one to three significantly more frequent pollinators: these were leaf-cutter bees for two plant species, *D. cuneatum* and *R. edulis*, the honeybee for *Dalea elegans*, the bumblebee for *L. pubescens* and carpenter bees plus leaf-cutter bees for *C. argentina* (Table 1). Pielou's evenness indexes ranged between 0.0926 and 0.7774. Plant species with a low Pielou's index presented a predominant pollinator, such as *D. elegans* which is pollinated mainly for *(A) mellifera, L. pubescens* with *(B) bellicosus* as the main pollinator and *D. cuneatum* predominantly pollinated by one *Megachile* species.

Table 1 Biomechanical variables, flag traits, pollinator composition and measures associated with pollination from five keel flower species

	Force (mN)	Lever arm length	Flag traits (mm)		Pollinators				Observation data	
			Length of flag lamina	Width of flag lamina	Species	Force (mN)	Visitation rates	Pielou's evenness index	Observation time (hour)	Observed flowers
L. pubescens	35.7±21.57	14.87±0.55	20.49±1.15	17.39±1.16	*B. bellicosus*	253.03±41.67	0.35[a]	0.3541	12	1745
					*Xylocopa sp.*1	–	0.03[b]			
					B. opifex	243.2±68.9	0.03[b]			
					Xylocopa sp. 2	–	0.03[b]			
					C. tricolor	–	0.002[b]			
					X. ordinaria	420.85±190.6	0.001[b]			
C. argentina	35.94±10.66	21.27±1.15	26.58±1.78	21.31±2.01	*X. ordinaria*	420.85±190.6	0.85[a]	0.7774	8	400
					Megachile sp. 1	62.01±13.8	0.51[a]			
					*Xylocopa sp.*1	–	0.4[ab]			
					B. opifex	243.2±68.9	0.07[b]			
					B. bellicosus	253.03±41.67	0.07[b]			
D. cuneatum	28.07±7.98	9.52±0.52	9.35±0.5	8.37±0.52	*Megachile sp.* 1	62.01±13.8	0.16[a]	0.6388	24	1960
					*Xylocopa sp.*1	–	0.05[b]			
					X. ordinaria	420.85±190.6	0.02[b]			
					*Anthidium sp.*1	66.08±14.11	0.01[b]			
R. edulis	6.59±1.47	9.86±0.31	11.13±0.51	7.75±0.55	*Anthidium sp.*1	66.08±14.11	0.14[a]	0.6882	20	950
					Megachile sp. 1	62.01±13.8	0.08[a]			
					*Xylocopa sp.*1	–	0.01[b]			
					X. ordinaria	420.85±190.6	0.01[b]			
D. elegans	1.08±0.56	10.02±0.7	8.36±0.61	5.09±0.44	*A. mellifera*	26.26±3.89	0.39[a]	0.0926	7	942
					B. opifex	243.2±68.9	0.005[b]			

Mean value and standard deviation is shown for force needed to open the keel-wing unit, lever arm, measurements of the flag (length and width) and maximum force exerted by pollinators. Visitation rate was estimated as the mean numbber of visits per flower per hour and Pielou's evenness index was calculated to test the dominance of the pollinator assemblage in each plant species. Different letters associated with visitation rates indicate significant differences obtained through generalized linear models with a quasi-Poisson function using visitation rate as the response variable and pollinator specie as the predictor variable
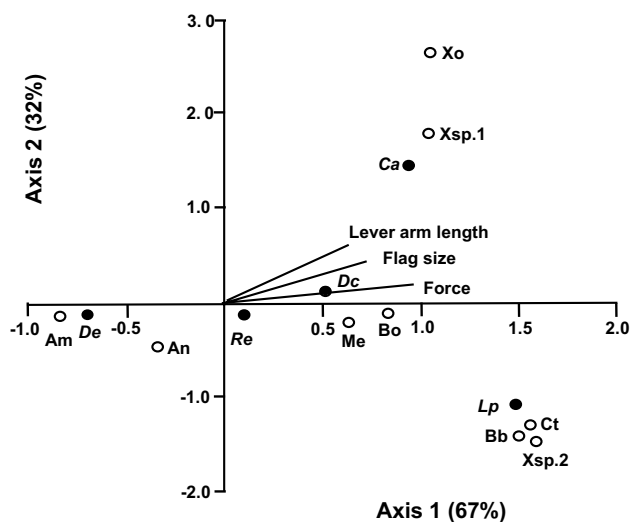
Fig. 1 Triplot graphic from canonical correspondence analysis. Vectors indicate predictor variables. *Filled circles* represent plant species while *open circles* indicate insect species. *Ct Centris tricolor; Bb Bombus bellicosus; Bo Bombus opifex; Am Apis mellifera; An Anthidium* sp1.; *Me Megachile* sp1; *Xsp1 Xylocopa* sp1; *Xo Xylocopa ordinaria; Xsp2 Xylocopa* sp2; *Lp Lathyrus pubescens; Re Rhynchosia edulis; Dc Desmodium cuneatum; Ca Collaea argentina; De Dalea elegans*

Effect of biomechanical and flag size traits on pollinator assemblage

No significant association between visitation rates and biomechanical variables was found ($R^2 = 0.3215$, $p = 0.3189$ to force; $R^2 = 0.01398$, $p = 0.8498$ to lever arm length). A similar result was obtained for the relationship between flag size and visitation frequency ($R^2 = 0.04859$, $p = 0.7216$).

On the other hand, the axis 1 from CCA was marginally significant ($p = 0.1$) and showed a high correlation with force ($r = 0.96$), explaining 67% of total variation while axis 2 and 3 were not significant ($p = 0.85$ and $p = 0.59$, respectively). Generalized procrustes analysis showed a strong ($r = 0.7$) and marginally significant association ($p = 0.08$) between force and the pollinator assemblage composition matrices.

An association pattern is evident between force needed to open the keel-wing unit and pollinator's assemblage composition. At one end of axis 1 is one species with soft flowers and low pollinator assemblage diversity and evenness (*D. elegans*) while at the opposite end is a plant species with stiff flowers and broader and less even pollinator assemblages (Fig. 1). Among the latter, are *C. argentina* with a pollinator assemblage consisting of two carpenter bee species and *L. pubescens* with a diverse pollinator assemblage (*B. bellicosus, C. tricolor* and *Xylocopa* sp. 2). Finally, at an intermediate position on this axis, are two species with intermediate to low flower stiffness (*D.*

cuneatum and *R. edulis*) which have *Megachile* sp.1 and *B. opifex* as pollinators.

Discussion

Diversity of pollinator assemblages for the studied species ranges from two to eight bee species. Each legume had at least one distinctly predominant pollinator. Except for *Apis mellifera*, the more frequent pollinator of *Dalea elegans*, the remaining pollinators were native solitary bees. *Xylocopa ordinaria* visited all studied legume species except for *Dalea elegans*. Flowers of *Dalea elegans* are comparatively small and delicate which would explain why these flowers do not support the relatively large bees such as large carpenter bees and *Bombus opifex* queens.

Two leaf-cutter bees, *Megachile* sp. and *Anthidium* sp., were more frequent pollinators of *D. cuneatum* and *R. edulis*, respectively. These two plant species flower one next to the other and exhibit staggered flowering times during the day, with *D. cuneatum* flowers opening early in the morning and *R. edulis* at mid-day.

Megachilid bees were recorded before as a pollinator of the exotic *Lathyrus odoratus* (Córdoba and Cocucci 2011). However, they were not seen pollinating the native species *L. pubescens* even when the force needed to open the keel was similar (35.7 mN for *L. pubescens* and 37.8 mN for *L. odoratus*). Presumably, preference of pollinators for these flowers could be associated with variables other than biomechanical ones, including floral display, phenology and reward content (Eckhart 1991; Grindeland et al. 2005; Karron et al. 2009; Dauber et al. 2010; Gibson et al. 2013 among other).

Apis mellifera only visited the relatively small-flowered *D. elegans* which also had the weakest flowers among the studied legumes. The behavior of the honey bee was not as expected for a keel flower, since it was able to open the flowers and access rewards laterally, not downward rotating the mechanism. This is possible because petals in this legume are not locked together in a keel-wing unit.

Even though *(A) mellifera* is a weak bee (Córdoba and Cocucci 2011), it is strong enough to open the mechanism of several legumes (Parker et al. 2002; Giovanetti and Aronne 2013), even plant species included in this study. These means that flowers potentially accessible were avoided by this bee. In addition, honey bees usually acted as nectar thieves of flowers they were not able to open, but could forage without making contact with the stigma and stamens (Aronne et al. 2012). Consequently, for this particular case, the foraging economics principle seems to apply since bees are avoiding flowers they could potentially open and only exploit them by circumventing the energy-demanding mechanism. We may speculate that, prior to

the introduction of the honey bee by European settlers, *Dalea elegans* must have been pollinated and must have evolved in association with native bees that are as weak as the honey bee, its current more frequent pollinator. One such bee, now largely displaced by the honey bee, could be *Bombus opifex*, the workers of which are only a little larger than the honey bee, and are expected for their small size to be also relatively weak (Córdoba and Cocucci 2011). It is known that the foraging behaviour of *(B) opifex* is affected by body size among this bee species (Stout 2000). Presumably, preference of *A. mellifera* for weak flowers could be related to the need of social bees to maintain colonies with rewards that are of low collecting cost (Westerkamp 1991).

Pollinator visitation frequencies were not associated with flower force, rejecting the notion that visitation is explained by bee foraging economics alone. Flower force and not flag size explained composition of pollinator assemblages in the CCA. On one hand, this indicates that it is not flower display size that explains which bees visit the flowers of a given species. On the other hand, this rather indicates that richness and relative visitation frequencies of bees in the pollinator assemblages were controlled by the difficulty flowers impose to gain access to rewards.

Previous study on the relationship between floral stiffness and pollination assemblages in the keel flowers of *Genista* and *Cytisus* shows that differences in flower mechanical force between these plants did not result in differences in pollinator assemblage (Parker et al. 2002). Contrary to the foraging economics principle, we found that the weakest flowers had narrower and more even pollinator assemblages while the stiffest flowers had relatively rich and uneven pollinator assemblages, apparently contradicting the initial expectation. However, pollinator assemblages of the stiffest flowers consisted of an even set of large and strong functionally equivalent bees (see Córdoba and Cocucci 2011). Consequently, the explanation of flower force as a filtering mechanism is still supported. But, it remains unexplained why flowers with low biomechanical force have narrow pollinator assemblages.

Main contributions

The biomechanics of floral functionality is an interesting aspect that could explain the interaction between plants and pollen vectors from an ecological and evolutionary perspective as an alternative to traditional floral variables to explain visitation rates and pollinator assemblages in a more integrated approach. Despite the study of floral biomechanics having an early beginning (Brantjes 1981), only recently has it been included in pollination biology studies (e.g. Claßen-Bockhoff et al. 2004a, b; Reith et al. 2006; Stöbbe et al. 2016). Additionally, in the keel flower family where flower mechanisms are prominent, flower

biomechanics has been almost completely neglected (see, however, Córdoba and Cocucci 2011; Córdoba et al. 2015). The present study contributes to the knowledge of keel flower functionality and opens a new perspective on their ecology and evolution.

In this study, we provide data suggesting that flower stiffness may work by filtering out weak bees and modulating the pollinator assemblage composition such that a guild of large and strong bees acts as the dominant pollinators of the stiffest flowers.

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