Pollination ecology of *Desmodium setigerum* (Fabaceae) in Uganda; do big bees do it better?

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Abstract—Explosive pollen release is documented in many plant families, including the Fabaceae. *Desmodium setigerum* E. Mey (Fabaeae) is a perennial herb with single trip explosive pollen release found in eastern Africa, and the unique ability to reverse floral colour change if insufficient pollination has occurred. However, little else is known about the pollination ecology of this species, what visitors can trigger explosive pollen release, and whether bee body size is related to pollination efficiency. We investigated: 1) the breeding system of *D. setigerum,* and whether it is pollen limited; 2) whether flowers are visited early in the day allowing sufficient time for a second opportunity for pollination; and 3) what insect species visit *D. setigerum* and the relative efficacy of different flower visitors in relation to visitor size and pollination success. We found that although self-compatible, *D. setigerum* requires insect visitation to set seed as explosive pollen release is needed even for selfing. Most flowers are initially visited before 1400h, and by 1800h nearly all flowers have been tripped. Flowers were not pollen limited in this study, and were visited primarily by bees. We observed 16 visiting species, and there was a wide variation (0-404 grains) in the amount of pollen deposited on stigmas. Although almost all bees deposited some pollen, the mean number of pollen grains deposited in a single visit per species was negatively related to body size. However, one particular megachilid species deposited significantly more pollen grains than any other visitor and so is likely an important pollinator of this species. This provides insights into the pollination biology of this unique plant species, and adds to increasing literature on the relationships between bee body size, explosive pollen release and pollination effectiveness.

Keywords: explosive release, Fabaceae, Leguminosae, pollen deposition, single visit, size matching

Introduction

Explosive pollen release, where pollen is rapidly expelled from a flower often following visitation, is presumed as an adaption to promote pollination by the "perfect" pollinator (Aluri & Reddi 1995). This trait has been recorded in over 17 plant families (Aluri & Reddi 1995), including the Fabaceae which contains 727 genera and 19,327 species (Lewis et al. 2005). Many Fabaceae display a tripping mechanism, where the 'mechanical' handling by the visitor is critical to release the pollen (Yeo 1993). When landing on a flower, visitors use the wing-keel arrangement as a landing platform, and probe for nectar using their proboscis beneath the base of the keel petals whilst forcing against the immoveable flag (Westerkamp 1997). The wing-keel complex is the only floral component that moves; it moves in a relative lowering movement away from the flag that is related to force applied as opposed to actual weight of the visitor, before returning to its original position (Westerkamp 1997). In some instances the mechanism is explosive with no return of the wing-keel complex to the original position – an 'all or nothing' response. Without this complicated manipulation of the flower resulting in the triggering mechanism, visitors cannot access flower reproductive parts. Although several studies have addressed the issue of how pollen release is triggered in papilionate flowers (e.g. Cordoba & Cocucci 2011; Stout 2000; Vivarelli et al. 2011), detailed information is still required on how different visitors and visitor size might affect various aspects of plant reproductive fitness in flowers of this type.

Desmodium setigerum E. Mey. is a common, scrambling perennial found in disturbed areas throughout eastern Africa (Fig. I). It has typical papilionate legume flowers that last a single day and 'trip' explosively when visited, with the keel petals remaining open and the filaments and gynoecium remaining uncovered. Unusually for species with flowers that only last a day (Van Doorn 1997), *D. setigerum* displays rapid floral colour change following visitation. The flowers are initially lilac but rapidly change colour becoming paler before turning white and eventually turquoise (Willmer et al. 2009). In addition to this, when tripped, flowers retain their tripped form, thus providing a morphological signal to signify prior visitation as well as the ensuing colour change. Perhaps the most striking feature of *D. setigerum* is that some flowers, if they have not been successfully pollinated, have the ability to regain some of their former colour to allow a second opportunity for pollination (Willmer et al. 2009). The ability to change colour and then reverse this change is so far unique to this species, and occurs over the short time frame of a single day. As a result we expect that *D. setigerum* has evolved this ability as it benefits from insect pollination, and that most flowers are visited early in the day so that there is ample time for a second chance at pollination following colour reversal; however, the reproductive ecology of this species has not been studied previously.

We investigate the pollination ecology of *D. setigerum* and use it as a model to determine what insect visitors elicit explosive pollen release, and whether there is any relationship between pollen deposition and visitor size. Although *D. setigerum* is plentiful and has been noted to receive visits by a wide range of different sized visitors (Willmer et al. 2009), the effectiveness of these visitors as pollinators has not been assessed. Specifically we asked the following questions:

I) What is the breeding system of *D. setigerum* and is it pollen limited? Are visits necessary for pollination success?

2) How does flower availability vary throughout the day?

3) Which visitors are most effective (i.e. can all visitors 'trip' flowers to cause explosive pollen release), and is their ability to deliver pollen to the floral stigma determined by pollen placement and/or body size?

MATERIALS AND METHODS

The study was conducted in Kibale Forest National Park, Uganda (0° 13' to 0° 41' N and 30° 19' to 30° 32' E) which lies north of the equator in the foothills of the Rwenzori Mountains. The area comprises 796 km2 of mid-altitude (1,590 m asl in the north to 1,110 m asl in the south) tropical moist forest. Rainfall pattern is bimodal with two rainy seasons, but with considerable variation between years (Struhsaker 1997). *D. setigerum* is plentiful in the northern part of the national park at forest edges and data were collected along trails, in forest gaps and in open areas in the vicinity of the Makerere University Biological Field Station (MUBFS) close to the village of Kanyawara (0° 35' N, 30° 20' E).

Breeding system and pollen limitation

To examine the breeding system of *D. setigerum,* and whether the species is pollen limited in our study sites, a manipulation experiment was conducted in August 2008. Nine sites were selected around MUBFS, and five pollination treatments were applied to six flowers in each site (N = 54 flowers per treatment), with each flower selected on a different spike. The treatments comprised: I) **self-pollinated**: manual tripping with addition of self-pollen; 2) **cross-pollinated**: manual tripping with addition of pollen from a neighbouring conspecific flower; 3) **natural pollination**: tripping and open pollination by insect visitors; 4) **artificial tripping with no pollen added**: pressure was applied to the base of the wing-keel complex with a dissecting needle to mimic explosive pollen release without visitation, and; 5) **control**: flowers were bagged (using fine netting) and remained untripped. The treated flowers were left for 5 days after which the developing seed pods were then collected and seeds counted in the field station laboratory. Where a flower had abscised without a seed pod being produced, seed set was recorded as zero.

Flower availability and tripping rates over time

To determine the rate at which flowers were visited, two I km transects were walked hourly through areas rich in *D. setigerum* multiple times throughout one day in August 2008. From 0800 h to 1800 h, untripped lilac flowers on the transect line were counted (transect walks lasted approximately 45 minutes each hour) to determine how many untripped flowers were available throughout the day.

Pollinator effectiveness

To examine which pollinators delivered the most pollen to the stigmas of *D. setigerum,* data were collected between the 13th and 18th July 2009. Our tripping rate observations and an earlier study (Willmer et al. 2009) had indicated that anthesis occurred between 0800 h and 0900 h with highest bee visitation from the latter time through to noon. Hence, sampling took place from 0830 h to 1100 h to capture peak pollinator activity. Individual bees were noted arriving on and foraging within randomly selected patches (ca. 5 × 5 m) of flowers (> 25 m apart) and followed during foraging bouts (up to 2 minutes) whilst tripping flowers. Preliminary observations indicated that not all bees were able to effect explosive pollen release; some small *Lasioglossum/Pseudapis* solitary bees (ca. 6-8 mm estimated body length from head to tip of abdomen) foraged for pollen at the tip of the keel petals rather than attempting to access nectar at the base of the flag (Fig.I; these bees were classed as "illegitimate visitors" and are not included in further analyses). To determine pollen deposition, visited and legitimately tripped flowers were immediately removed from the plants and fixed in 70% ethanol in small glass vials, ensuring that pollen deposition was the result of a single visit by a single bee, and the bee species recorded. We also collected stigmas from untripped flowers that had received visits from illegitimate

FIGURE 1.   A tripped and untripped flower of *D. setigerum* (above, photo DS), and a small solitary bee (*Lasioglossum*/*Pseudapis* sp.) foraging at tip of keel complex in untripped *D. setigerum*; an illegitimate visit that does not trigger explosive pollen release (below, photo CN).

visitors that foraged at the tip of the keel, attempting to prise apart the petals to access pollen. As we had found artificially tripped flowers did set some seed (and for comparison with pollen deposition by both legitimate and illegitimate visitors), we also artificially tripped (see treatment 4 above) 30 flowers and collected their stigmas to assess the background number of pollen grains deposited in this way. In the laboratory, we removed the stigma of each collected flower, made a squash preparation with glycerin jelly stained with fuchsin red (Brunel Micro Ltd. UK) and counted the number of pollen grains adhered to the stigma under a light microscope.

Single specimens of each visiting bee species were collected, and sent for identification (see acknowledgements). To quantify size of each species, morphometric measurements were made for any of the visitor species that were available in collections at the

Natural History Museum, London, UK (9 species out of 14 species, Tab. 1). Digital calipers (Vernier) were used to take measurements (mm) of mounted specimens ($N = 6 - 10$ individuals per species). Measurements made included head width (from a view of the lateral aspect of the head of the specimen from eye to eye at the widest point) and wing length (using the measurement from the base to the apex of the wing). We also measured dimensions of 50 *D. setigerum* flowers in the field.

Data analysis

To determine breeding system and pollen limitation in *D. setigerum*, we tested for differences in pollination treatments applied to flowers using generalized linear mixed effects models in the lme4 package (Bates et al. 2014) in R version 3.3.0 (R Core Team 2014). Pollination treatment was a fixed categorical effect with seed set as a response variable. Site was included as a random factor to control for spatial variance in the model, and a Poisson distribution specified as data were counts. Where statistically significant results were found between treatments, we used a Tukey HSD post-hoc test procedure to separate them by making pairwise comparisons between each treatment using the multcomp package (Hothorn et al. 2008).

We analysed pollinator effectiveness in two ways. Firstly, we used analysis of variance (ANOVA) to test for differences in the mean number of pollen grains deposited per species, and per pollen placement area. Secondly, we investigated linear relationships between morphometric measurements (for the 9 species where this was possible) and amount of pollen deposited. Head width and wing length were highly correlated (Pearson Product Moment Correlation; $t = 49.67$, df = 109, $P < 0.001$); therefore only head width was used in subsequent analyses as this variable resulted in models with a lower AIC value. As size and pollen deposition data were from different individuals, we calculated means of both measures per species. We then used a linear model to investigate the relationship between mean number of pollen grains deposited and mean head width per species. All models were validated by plotting standardized residuals versus fitted values, normal qq-plots, and histograms of residuals.

RESULTS

Breeding system and pollen limitation

There was a significant impact of treatment on the number of seeds produced (Generalized linear mixed effects model: $\chi^2 = 140.38$, df = 4, $P < 0.001$, Fig. 2). There was no difference in the number of seeds produced between naturally visited flowers and flowers manually tripped with cross pollen added, indicating that this species is currently not pollen limited in our study sites. The origin of pollen did not seem to be important; flowers set similar levels of seed when self-pollen was applied in comparison to cross pollen from another individual indicating that this species is self-compatible. However, no seed was set in flowers that remained untripped (apart from one isolated case), and very little in manually tripped flowers where no stigmatic contact occurred. This indicates that although *D. setigerum* is self-
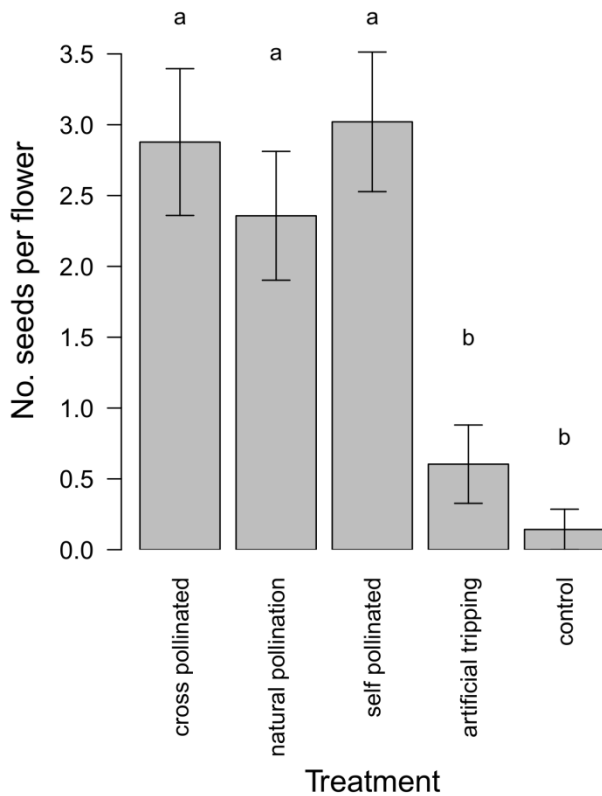
FIGURE 2. Mean number of seeds produced per flower of *D. setigerum* following pollination treatments. Error bars indicate standard error, and letters indicate significant differences ($P <$ 0.05). $N = 270$ flowers (54 flowers per treatment).

compatible it is visitor dependent, as without insect visitation to cause the tripping mechanism fertilization is unlikely to occur.

Flower availability and tripping rates over time

Flowers of *D. setigerum* were available throughout the day, with an increase in numbers as flowers emerged through the morning until 0900 h (transect 2) and 1100 h (transect 1). After this point, there was a rapid decline in the availability of untripped flowers (as tripping rates exceeded emergence rates). By 1400 h most flowers had been tripped, and by 1800 h almost all flowers were tripped (Fig. 3).

Pollinator effectiveness – visitor identity, size and behaviour

D. setigerum flowers were relatively small; the length of the keel was 9.45 mm ($\pm$ 0.50; range 8.40 - 10.30 mm; $N = 50$ flowers) from the base of the keel to the tip, and 6.67 mm ($\pm$ 0.45; range 5.50 - 7.75 mm; $N = 50$ flowers) from base to stigma tip. *D. setigerum* was only visited by bees, and 16 distinct bee species were recorded (although post-tripping visits by other taxa have been recorded at low frequency later in the day; Willmer et al. 2009). Of the 9 species that we could measure in the NHM collections, visitors ranged in body length (head to tip of abdomen) from 6.4 mm (*Anthidiellum* sp.) to 25.4 mm (*Xylocopa flavorufa*). In total, we counted 14,417 pollen grains attached to the stigmas of 184 flowers that were
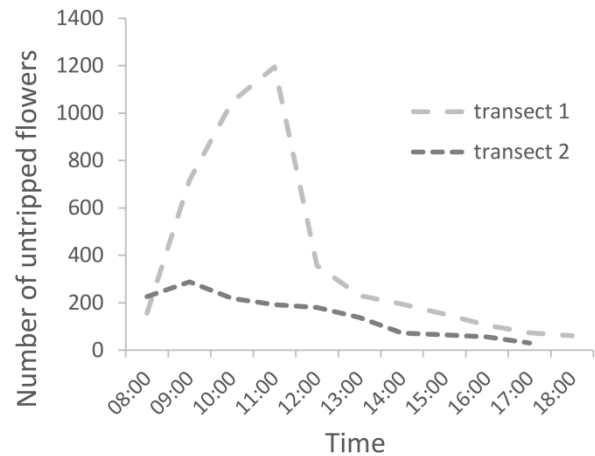

FIGURE 3. The number of untripped *D. setigerum* flowers available across the day in two transects. Numbers of flowers increase initially as flowers open in the morning, but steadily decrease during the day as nearly all flowers become tripped by 1800 h.

on stigmas from 30 further flowers that had been artificially tripped (Tab. 1). The number of pollen grains that adhered to stigmas ranged from 0 (hand-triggered; illegitimate visitors *Lasioglossum* sp. 2/*Pseudapis* sp.); to 404 (*Megachile* sp. 1), but pollen was deposited on at least some flowers by each visitor bee species represented in the analysis. Hand-triggered flowers had extremely low pollen deposition (6.8 $\pm$ 3.32 grains; $N = 30$ flowers), as did flowers receiving illegitimate visitation (37.4 $\pm$ 6.03; $N = 27$) (Tab. 1).

We found a highly significant effect of species on the number of pollen grains deposited (ANOVA: $F_{13,138} = 7.85$, $P < 0.001$, Fig. 4); "*Megachile* sp. 1" deposited a larger number of pollen grains than most other species. We also found an impact of pollen placement on the number of pollen grains deposited (ANOVA: $F_{4,147} = 7.31$, $P < 0.001$); bees that collected pollen on their abdomen deposited significantly more pollen grains than bees with pollen placed on other body parts (Fig. 5; although this was most likely driven by *Megachile* sp. 1). There was a significant negative relationship between head width and mean number of pollen grains deposited per species on floral stigmas (linear model: $F_{1,7} = 9.87$, $P = 0.02$, Fig. 6).

DISCUSSION

Our work has shed further light on the relationships between flower visitors, explosive pollen release, and single visit pollen deposition. We found that although *D. setigerum* is self-compatible and not pollen limited, insect pollination is required for substantial seed set. Although the species is visited and tripped by a diverse assemblage of bee species that all deposit pollen, bee size was negatively associated with pollen deposition and a particular megachilid bee species deposited more pollen than others.

That *D. setigerum* has evolved the trait of reversible floral colour change to allow a second opportunity for pollination (Willmer et al. 2009) suggests that insect pollination is of significant reproductive benefit to the

TABLE I. The 15 bee species collected visiting *D. setigerum*. Legend for Family: A = Apidae, H = Halictidae, M = Megachilidae, Legend for 'pollen placement: C = corbiculae; A = abdomen; HL = hind legs; T = thorax; S = scattered (i.e. noted on all four preceding parts). For analysis of pollen placement, the most important location was used for any species with more than one recorded, which is highlighted in bold. Size parameters (wing length and head with) are ranges recorded from approximately 10 specimens per species in the Natural History Museum, London.

Species	Family	Wing length range (mm)	Head width range (mm)	Pollen counts		Pollen placement
				Mean ± SE (no. of flowers)	Min-Max	
Anthidiellum sp.	M			56.3 ± 18.4 (4)	24-103	A
Illegitimate visitors*	H			37.4 ± 6 (27)	0-102	C
Megachile frontalis Smith	M	5.08 - 5.60	3.11-4.06	84.2 ± 17.6 (9)	30-170	A
Lasioglossum sp. I	H			39.9 ± 16.7 (10)	I-160	HL, **C**
Megachile semierma Vachal	M	7.60 - 10.07	3.79-4.52	106.3 ± 49.1 (3)	10-171	**A**, HL
Apis mellifera	A	7.43 - 9.17	3.64-4.22	84.1 ± 6.8 (31)	15-168	C
Amegilla fallax Smith	A	7.23 - 9.10	3.81-4.89	106.7 ± 39.3 (7)	29-316	S
Nomia sp. I	H			50.6 ± 11.5 (7)	20-112	A, **C**
Megachile sp. I	M			256.2 ± 38.4 (9)	95-404	A
Nomia sp. 2	H			69.1 ± 17.2 (11)	28-228	S
Xylocopa senior Vachal	A	14.05-15.39	5.74-7.34	46.3 ± 16.1 (7)	0-112	T
Xylocopa calens Lepeletier	A	15.04-17.71	6.37-7.73	78.4 ± 9.6 (38)	0-315	T
Megachile cincta Fabricius	M	14.12-15.94	5.35-6.16	53.3 ± 16.4 (10)	6-109	S
Xylocopa nigrita Fabricius	A	21.68-26.77	7.66-10.22	21.0 ± 4 (2)	17-25	HL
Xylocopa flavorufa DeGeer	A	24.08-28.37	7.66-9.02	62.1 ± 12.5 (9)	18-112	S
ARTIFICIAL 'Hand-triggered'				6.8 ± 3.3	0-75	-

Note: one additional bee species (*Pachyanthidium* sp.) was observed visiting *D. setigerum*, but no pollen deposition data was recorded, and two further bee species were recorded visiting and successfully tripping *D. setigerum* in 2009 that were not included in this study; *Megachile chrysopogon* Vachal (Megachilidae) and *Braunsapis* sp. (Apidae) (CN, unpublished data).
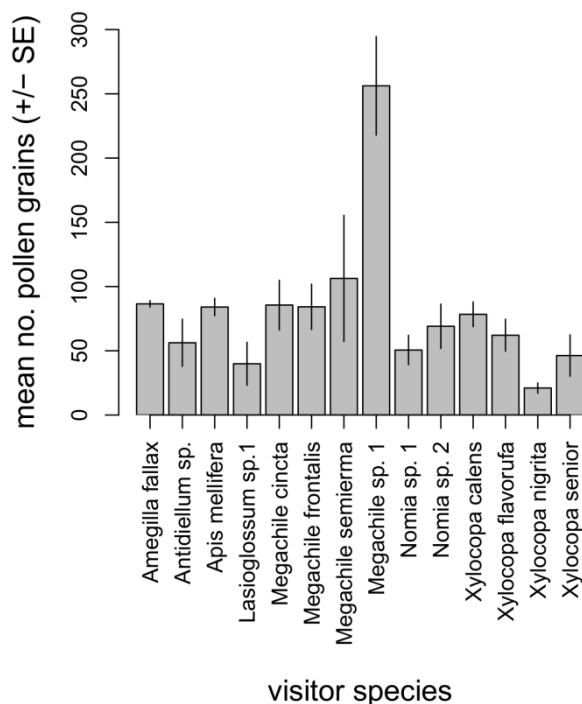*Lasioglossum* sp. 2; *Pseudapis* sp.

FIGURE 4. The mean (+/- standard error) number of pollen grains deposited on stigmas of *D. setigerum* by each visitor species. Significantly more pollen grains were deposited by *Megachile* sp. I than any of the other bee species. Error bars show standard error.

species, and that initial flower visitation happens early in the day to allow sufficient time for 'second round' pollination. Our findings show that *D. setigerum* is self-compatible; flowers artificially tripped and hand-pollinated with self-pollen set similar amounts of seed as those artificially tripped and hand-pollinated with cross-pollen. Self-compatibility has also been identified in other *Desmodium* species from South America (Alemán et al. 2014). In addition flowers that were artificially tripped but with no addition of pollen set some seed, suggesting that the act of tripping by hand leads to some deposition of self-pollen. However, although confirming self-compatibility, these circumstances are artificial and would not occur naturally. As flowers that were not visited by insects (and therefore not tripped) did not set any seed, and as those that were tripped and visited produced more seed than those tripped with no visits, this confirms that insect visitation is required for seed set. Notably, one outlier flower set seed without being tripped; as observations showed that small 'illegitimate' flower visitors can deposit pollen without tripping, this form of visitation may have occurred on this flower. Together, this shows that the process of insect visitation is fundamental to pollination and seed production. In addition, we found most flowers are initially visited prior to 1400 h. This gives substantial time before darkness (ca. 1800 h) and flower senescence for flowers that have not been successfully pollinated to have a second chance.
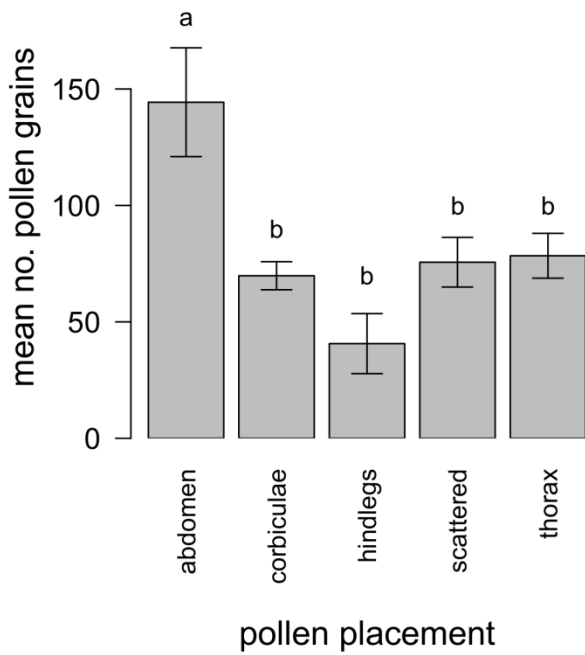
FIGURE 5. The mean (+/- standard error) number of pollen grains deposited by bees according to where pollen was predominantly placed on their bodies. $N = 152$ bees. Letters indicate significant differences ($P < 0.05$)
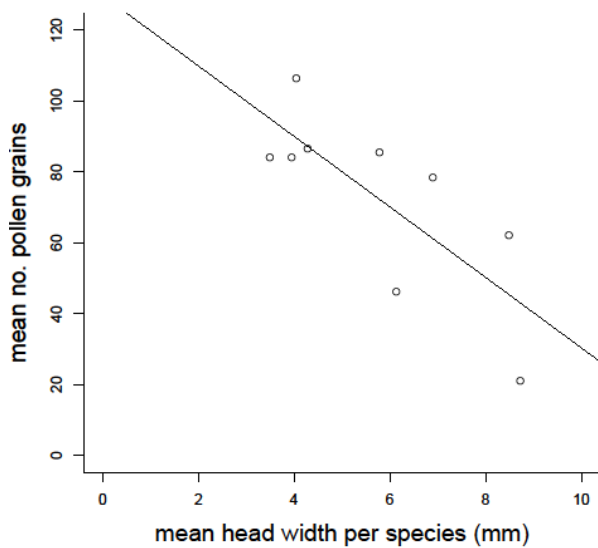

FIGURE 6. The relationship between the mean number of pollen grains deposited on stigmas and the head width (in mm) of the species visiting a flower. $N = 9$ bee species.

We found that all bees 'tripping' *D. setigerum* deposited some pollen, supporting the hypothesis that this explosive mechanism has evolved to 'filter' pollinators (Cordoba & Cocucci 2011). However, one species of megachilid (*Megachile* sp. 1, Tab. 1) deposited more pollen per single visit than most other visitors. This suggests that this plant species may be more specialized in terms of its flower visitors than usually assumed for papillionate flowers, and visits by other bees may result in insufficient pollen delivery requiring a second pollination event (see Willmer et al. 2009). There

are a number of reasons why this megachilid species may be more successful in pollen deposition than others; firstly, this family of bees has pollen-carrying hairs on the underside of the abdomen, potentially making visitors more likely to deposit pollen when positioning themselves on the keel of *D. setigerum*. Indeed, bees that had pollen placed under their abdomen (Tab. 1) deposited more pollen grains overall than bees with pollen in corbiculae, on the thorax or on the hind legs. Secondly, high deposition may also be due to morphological "fit" (or size matching) in that the size and shape of certain species is better for achieving contact with the reproductive parts of *D. setigerum*. Thirdly, it could be that this species behaves differently to others when handling flowers, thereby collecting and depositing more pollen.

We found a linear relationship between bee body size and pollen deposition, with smaller bees depositing more pollen than larger ones. Other work has also shown differently sized bees to be optimal in terms of pollen transfer/seed set in other Fabaceae species. Small bees (no measurement parameter documented) were found to have the lowest number of pollen grains on their bodies following non-tripping visits to *Pongamia pinnata*, which releases pollen explosively (Raju & Rao 2006). Using body length of bumblebees (*Bombus* spp.), Stout (2000) found that smaller bees were more successful in tripping flowers of the legume *Cytisus scoparius*; large queens were too big to physically effect tripping and this may have had impacts on plant reproduction. Vivarelli et al. (2011) reported lower seed set in *Ononis masquillierii* when visitation was mainly by smaller bees (halictids and megachilids) as measured by dry weight. *Ononis masquillierii* released pollen through a pump action as opposed to explosive release and a higher level of 'selfing' was apparent when small bees visited and pollen was released several times in a single visit (Vivarelli et al. 2011). Cordoba & Cocucci (2011) demonstrated that *Apis mellifera* was relatively weak compared to smaller (by weight) and stronger (measured force (mN)) megachilids which could trip flowers of high operative strength such as *Spartium junceum* that *A. mellifera* could not access. Investigating intra-specific variation in size in bumblebees (scored as large, medium or small) and pollination effectiveness in a non-leguminous plant, *Vinca minor* (Apocynaceae), Willmer & Finlayson (2014) found that pollen deposition to stigmas in single visits varied between individuals with larger bees depositing more grains.

In this work we only measured pollen deposition, a single component of pollinator "performance". Ne'eman et al (2010) reviewed the issue of pollinator performance, noting that plant reproductive success is not solely dependent on female fitness through pollen deposition on the stigma. They put forward a model that takes into account pollen deposition, visitation rates, and the necessity to continue any study to seed set to enable complete evaluation of reproductive success (Ne'eman et al 2010). Single visit deposition (SVD) of pollen has been put forward as the most practical measure of pollinator effectiveness (PE) in a range of tropical and temperate plant species (King et al. 2013). Pollinator effectiveness has been measured for relatively few individual plant species but is critical to the understanding of the relative importance of

flower visitors where many and varied visitors are involved. Only recently has the necessity to examine PE been highlighted in community-level studies of pollination; Ballantyne, Baldock & Willmer (2015) provided the first plant-pollinator network based on pollinator evidence rather than just visitation or pollen transport. Although we measured just a single component of the Ne'eman et al (2010) model, pollen deposition, *D. setigerum* is an ideal plant for further studies of this type. Pollen deposition by visitors can be assessed at species level through SVD (as visit frequency is not a factor here), and seed counts subsequently confirm overall individual pollinator performance. In addition, the structure of the bee assemblages and relative abundances of bees of different sizes could be taken into account in future studies.

D. setigerum has evolved a variety of cues to attract and direct pollinators. These include the papillionate legume tripping mechanism, but also floral colour change and ability to reverse this change. Our work shows that these mechanisms may have evolved as, although self-compatible, *D. setigerum* needs insect visitation to set seed. The large assemblage of bees that visit this bee-adapted flower are not equally important in one aspect of pollinator performance; one species appears to be significantly more successful in pollen deposition than others. As the majority of pollination studies focus on flower visitation and not single visit deposition (King et al. 2013), our work adds to the literature as it shows that not all pollinators are equal in terms of pollination efficiency, and that even individuals within the same genus (e.g. *Megachile*) can vary hugely in their pollen deposition. These findings give more insights into the pollination ecology of this papillionate legume, but also into explosive pollen release and the relationship between bee body size and pollination effectiveness.

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AUTHOR CONTRIBUTIONS

CN, DAS, ESB, KS, PW and TP designed and carried out the study; DAS and MO analysed the data; CN, DAS, KS and MO wrote the manuscript; all authors provided comments on subsequent manuscript versions.

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