Pollen grain morphology is not exclusively responsible for pollen collectability in bumble bees

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Bee-pollinated plants face a dilemma in that bees both passively transport pollen grains among conspecific flowers and actively collect pollen to feed their larvae. Therefore, mechanisms that reduce pollen collection by bees have evolved in melittophilous plants. Malvaceae pollen is uncollectable for corbiculate bees which has previously been ascribed to pollen size, spines, and pollenkitt. We analysed the influence of pollen grain properties (diameter, spine length, spine density) on the collectability of echinate (spiny) pollen by bumble bees (Bombus terrestris). Workers individually foraging on one of eight plant species from six families performed significantly less pollen foraging on plants which have large, echinate pollen grains. Nevertheless, neither pollen grain size, spine length, nor spine density prove to be an absolute disqualifier for collectability. While pollen foragers did not shift to nectar collection but seized visiting flowers with uncollectable pollen, nectar foragers performed regular foraging bouts on these plants. Pollen that is uncollectable for corbiculate bees limits pollen depletion by generalist bumble bees and probably also honey bees while maintaining them as pollinators, which is an effective solution to the pollen dilemma. As previous assumptions about the impact of pollen morphology on its collectability are disproved, potentially determining factors are discussed.

Bees collect pollen as a protein source that is crucial for egg maturation1 and larval development2–4. Although many bees are efficient pollinators of a broad range of plant species, only a small fraction of the pollen adhering to a bee’s body contributes to pollination5–8. For instance, in Campanula rapunculus (Campanulaceae) only 3.7% of the pollen grains of one flower are transferred for pollination, although bees collect 95.5% of the pollen and leave 0.8% in the flower9. Despite legitimate pollinator activity, pollen is lost from pollination because of a variety of circumstances; for example, pollen foragers lose some pollen in the process of packing it and nectar foragers can actively remove accidentally acquired pollen grains by grooming. An arguably large proportion of pollen loss is caused by flower visitors frequenting different plant species during a foraging bout, thus leading to pollen being transferred to stigmas of flowers belonging to heterospecific plants or to non-fertile structures, and covering of the stigma with foreign pollen10–15. Pollen collected by corbiculate bees is agglutinated with regurgitated nectar, thus increasing adhesion between pollen grains16–19, but diminishing their viability and availability for stigmatic pollen deposition20.

The conflict of interest between a plant’s production of pollen for the purpose of reproduction and efficient pollen collection by bees to provide protein for their larvae, the “pollen dilemma”, underlies many plant-pollinator adaptations21,22. Several protective mechanisms that decrease pollen loss elicited by flower visitors and increase the chance of pollination have evolved. For instance, flowers of the family Fabaceae hide their pollen from sight with transformed petals, and flowers of the family Lamiaceae feature a staminal lever mechanism to transfer pollen to safe sites on a bee’s body that are difficult to groom or cannot be groomed at all23–25. Another protective measure is the echinate pollen grain structure of plants in various families26,27, which we examined in this study.

The generative and vegetative cells of pollen grains are coated with two layers: the inner intine, a thin layer composed of cellulose fibrils, and the outer exine, consisting largely of sporopollenin28–30. The sporopollenin forms the exterior structure of the pollen grain, producing, for example, an echinate (spiny), psilate (smooth), or reticulate (netlike) surface. The adhesion of pollen grains to flower visitors positively affects the transport distance and is ensured by both pollenkitt and echinate surface structures31,32, but also aided by non-morphological features such as electrical charge33.

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Previous laboratory tests demonstrated that pollen of *Alcea rosea* (Malvaceae) is mechanically protected from being compacted into the corbiculae of bumble bees by its long spines and the pollenkitt covering the grains. When spines were bent by vortexing or pollenkitt was removed by washing, workers of the buff-tailed bumble bee (*Bombus terrestris*, Apidae) could collect the pollen grains. This indicates that – independently of one another – pollenkitt and long spines on the pollen grain surface of *A. rosea* hinder pollen collection by bees. We sought to define pollen grain properties determining collectability for bumble bees by testing plants from different families under more natural conditions. We selected six plant species that differed in their pollen grain diameter, spine length, and density of spines – as these morphological characteristics are likely to physically impede pollen packing – to identify the cause of the bees' inability to pack echinate pollen into their corbiculae. We hypothesize that either one morphological trait or a combination of more than one trait (e.g. grain size and spine length) determines collectability of echinate pollen by bumble bees. To assess pollen collectability, we measured the number of visited flowers, the time that bumble bees spent collecting pollen, and the amount of pollen they gathered in their corbiculae in one foraging bout. We observed whether they cease to visit flowers with uncollectable pollen or if they shift to collecting nectar instead. Additionally, we compared the foraging behaviour of pollen- and nectar-collecting bumble bees on *Malva sylvestris* (Malvaceae).

**Materials and Methods**

**General conditions.** All tests were conducted in an outdoor flight cage, consisting of an aluminium frame covered with net fabric, in the Botanical Garden of the Heinrich Heine University Düsseldorf, Germany. A colony of *Bombus terrestris* (Biofa AG, Münsingen, Germany) was maintained in this flight cage and provided with diverse flowering plants from various families. Additionally, a feeder containing a 30% sugar solution provided a carbohydrate supply to the colony. To motivate bees to collect pollen from the plants in the flight cage, supplemental pollen was not provided. Most bumble bees collected both nectar and pollen, but during one foraging bout, they settled on one resource. During a given bout, foragers either visited flowers to collect nectar or they collected mainly pollen and also some nectar to pack the pollen grains in their corbiculae. According to their bout-based foraging task, we defined the bumble bees as nectar and pollen foragers for individual trials.

**Pollen characteristics.** To determine whether specific pollen grain characteristics of echinate pollen influence foraging behaviour of pollen-collecting bumble bees, we surveyed pollen collection from eight plant species. Six species in four families have echinate pollen grains: *Alcea ficifolia*, *Lavatera thuringiaca*, and *Malva sylvestris* (Malvaceae); *Knautia arvensis* (Dipsacaceae), *Cucurbita pepo* (Cucurbitaceae), and *Campanula alliariifolia* (Campanulaceae). Two species have non-echinate pollen: *Verbascum phlomoides* (Scrophulariaceae) and *Rosa arvensis* (Rosaceae). SEM micrographs of pollen samples from the six plant species with echinate pollen grains (Fig. 1) were obtained in the Centre for Advanced Imaging (CAi) of the Heinrich Heine University Düsseldorf. Images of the two non-echinate species were taken with a light microscope. All micrographs were analysed to quantify mean pollen grain diameter (n = 10) and mean spine length (n = 50) with the program ZEN 2012, blue edition (Carl Zeiss Microscopy GmbH, Jena, Germany). To extrapolate the total number of spines, we chose a circle with a diameter equal to the pollen grain radius, then counted the spines within the circle and multiplied that number by eight. Finally, spine density (n μm⁻²) was estimated by dividing spine number (n) by the pollen grain radius.
C four out of ten foragers collected pollen from fresh mass). These pollen grain properties (Table 1) were compared to their collectability by bumble bees.

### Pollen collectability experiment

Prior to testing, we covered plants with mosquito nets to ensure that the bumble bees were unexperienced with these flowers. For each test, one forager that had just left the nest was admitted in the net covering the plants of a selected species. The number of open flowers and amount of available resources enabled each worker to forage ad libitum.

The number of visited flowers and handling time during a foraging bout differed significantly between the tested plant species (Fig. 2a,b). Pollen-collecting bumble bees visited significantly more flowers of species with small pollen grains (23–34 μm) than flowers of plant species with large pollen grains (100–139 μm). The recorded handling time for all three Malvaceae species and *Rosa arvensis* was significantly shorter than for the other four plant species. Accordingly, the success of pollen collection – measured by the mean mass of collected pollen – also differed significantly between the tested plant species (Fig. 2c; Kruskal-Wallis test: \( \chi^2 = 51.897, df = 7, P < 0.0001 \)). Bees could not collect pollen from any Malvaceae species or *Rosa arvensis*, and only four out of ten foragers collected pollen from *C. pepo* flowers – with widely varying success (either <1 or >13 mg fresh mass).

### Effect of pollen grain characteristics

Comparing pollen grain characteristics (Table 1) and their collectability by bumble bees produces a mixed image (Fig. 3). A large pollen grain diameter is no exclusion criterion for collectability as shown by *C. pepo*, while spine length is not decisive either as evidenced by *K. arvensis*. Low spine density is found among species both with collectable and uncollectable pollen. Even when combining pollen grain characteristics, there is no common denominator that predicts pollen collectability: Pollen grains of the Malvaceae species and *C. pepo* share large grains with long spines and low spine density, but differ in collectability (with the limitation that only 40% of tested bumble bees collected *C. pepo* pollen).

### Statistical Methods

All data were analysed using R statistical software, version 3.5.1[^1]. After testing normality of the data with the Shapiro-Wilk test, Kruskal-Wallis test was used for analyses of multiple sets of non-parametric data with Mann-Whitney U test including fdr correction as post hoc analysis. Mann-Whitney U test (non-parametric data) or Student’s t-test (parametric data) were used for analyses between two sets of data.

### Results

#### Pollen collectability experiment

The number of visited flowers and handling time during a foraging bout differed significantly between the tested plant species (Fig. 2a,b; Kruskal-Wallis test: \( \chi^2 = 44.488, df = 7, P < 0.0001 \) and \( \chi^2 = 50.074, df = 7, P < 0.0001 \)).

#### Effect of pollen grain characteristics

| Plant species | Diameter [μm] | Spine length [μm] | Spine number [n] | Spine density [n μm⁻²] |
|---------------|--------------|------------------|------------------|------------------------|
| *Alcea ficifolia* | 134.10 ± 5.22 | 10.18 ± 1.39 | 210.67 ± 38.55 | 0.0024 ± 0.0001 |
| *Lavatera thuringiaca* | 111.03 ± 2.95 | 8.80 ± 1.12 | 126.67 ± 8.54 | 0.0033 ± 0.0004 |
| *Malva sylvestris* | 104.17 ± 6.94 | 6.77 ± 0.98 | 133.33 ± 5.96 | 0.0066 ± 0.0008 |
| *Knautia arvensis* | 99.77 ± 3.47 | 1.55 ± 0.35 | 130.67 ± 23.40 | 0.0041 ± 0.0007 |
| *Campanula alliariifolia* | 139.42 ± 15.41 | 5.98 ± 0.96 | 114.67 ± 25.16 | 0.0018 ± 0.0005 |
| *Cucumis sativus* | 34.01 ± 3.16 | 0.96 ± 0.12 | 184.00 ± 24.87 | 0.0517 ± 0.0109 |
| *Verbascum phlomoides* | 22.48 ± 1.02 | — | — | — |
| *Cucurbita pepo* | 159.28 ± 10.33 | 5.08 ± 0.85 | 130.67 ± 23.40 | 0.0041 ± 0.0007 |

Table 1. Pollen grain properties (mean ± SD) of eight tested plant species. Exine sculpturing is reticulate for *Verbascum phlomoides* and psilate for *Rosa arvensis*.

[^1]: R Core Team (2017). *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/
Pollen and nectar foragers. On average, nectar foragers visited significantly more flowers than pollen foragers on *M. sylvestris* (Fig. 4; Welch’s *t*-test: *t* = 3.948, df = 10.832, *P* = 0.0024) and spent longer handling flowers (on average 86 s; Mann-Whitney *U* test: *U* = 98.5, *P* = 0.0003). Pollen foragers attempted to pack pollen in their
corbiculae, but subsequently abandoned their effort and shed the pollen, whereas nectar foragers flew from flower to flower without making any effort to pack the pollen or to groom it off. When released from the net, unsuccessful pollen-collecting bees visited other plant species, whereas successful nectar foragers returned to the nest.

**Discussion**

One aim of this study was to link the inability of bumble bees to collect echinate pollen grains with specific pollen properties. Several somewhat divergent hypotheses can be found in the literature: Vansell deemed the viscid material agglutinating the grains responsible for the incapability of honey bees to gather pollen on cotton flowers (Gossypium sp., Malvaceae), Buchmann and Shipman considered pollen grain size to be the relevant factor. Vaissière and Vinson have found that honey bees cannot collect pollen of Abelmoschus esculentus (Malvaceae) and struggle to pack dried cotton pollen offered in a dish. They exclude pollen grain size and pollenkitt as crucial for packing efficiency and regard the length of the spines as the decisive factor. Lunau et al. have shown that bumble bees are not able to gather natural pollen of Alcea rosea from artificial flowers and ascribe this to the influence of pollenkitt and spines.

Testing the mechanical defence of echinate pollen grains against collection in a natural setting with unaltered pollen for the first time refutes the conclusions of some previous studies, e.g. concerning the importance of long spines. In our study, pollen-foraging bumble bees were incapable of collecting pollen from any of the three Malvaceae species or *Knautia arvensis* (Dipsacaceae). These species share the characteristic of relatively large pollen grains (100–134 µm in diameter) with spines whose length varies greatly. *Lavatera thuringiaca* possesses the longest spines (8.80 µm, 7.9% of the pollen grain diameter) and *K. arvensis* the second shortest (1.55 µm, 1.6% of the pollen grain diameter) among all species tested. In contrast, *Cucurbita pepo* pollen was successfully collected by some bumble bees, despite being the largest pollen grains tested with a diameter of 139 µm and spines measuring 5.98 µm (4.3% of the pollen grain diameter). It is known that Cucurbitaceae pollen is collected with varying success by different bee species. Thus, pollen size alone is not a disqualifier for pollen collectability, nor...
is the echinate exine structure (as shown by the collectability of spiny C. pepo and Campanula alliariifolia pollen). Echinate pollen can be found in several non-related orders of plants, and different exine structures even occur within some genera. Rowley et al.38 have found that anemophilous Asteraceae species possess pollen with tiny spines. Excluding all pollen properties tested in our experiment from the list of probable causes for uncollectable pollen stated by previous studies leaves only pollenkitt3,38. This viscous pollen coating enables adhesion of pollen grains to each other, to flower visitors, and to the stigma, with entomophilous species exhibiting both higher pollenkitt volumes and stronger adhesion than anemophilous species32. Consequently, the impact of pollenkitt on pollen packing in corbiculate bees remains to be tested.

Whereas polylectic bumble bees fail to collect echinate pollen of some plant species, oligolectic bee species are often specialized and morphologically adapted to collect such pollen grains. Ptilothrix plumata (Empirorrhini) preferentially forages on the large, spiny pollen grains of Pavonia sp. (Malvaceae), which are transported adhering to long hairs on the bees’ tibia38. Similar morphological adaptations, i.e. long and sparse hairs, can be found in several genera of bees. For example, the genus Peponapis (Eucerini) comprises specialist collectors of Cucurbitaceae pollen, whereas Andrena hattoriiana (Andreninae) is a specialist of a few species of Dipsacaceae39. Compared to honey bees and bumble bees that are used for pollination management40, native oligolectic bees are often more efficient pollinators of Solanaceae, forage legumes, and Cucurbitaceae37,41,42, owing to their visitation frequency (but see King et al.38; Ballantyne et al.43) – although these bees effectively deprive the plants of their pollen43,46.

In contrast to the scopae of these specialists, the bumble bees tested in our experiments compact pollen in the corbicula, a smooth depression on the tibia of the hind leg framed by rigid bristles. Although the collection of spiny pollen was not entirely inhibited, the eventual compaction of the pollen grains in the corbiculae was not successful when foraging on certain plant species. Owing in part to the size and spines of the pollen grains, which physically impede pollen packing, they presumably interact poorly with the regurgitated nectar used by bees to agglutinate their pollen load. The effort and amount of nectar required to completely envelop large pollen grains (especially with long spines) possibly exceeds the benefit of collecting the pollen, thus resulting in bees discarding the pollen. But as nectar is available in all tested plants with uncollectable pollen, nectar quantity is less likely to be a factor limiting pollen collection. The few individuals that were able to collect the large, echinate pollen of C. pepo might have been more experienced pollen foragers that had already learned to agglutinate pollen grains more efficiently. However, it is more likely that some other morphological or physiological trait, e.g. pollenkitt, has a species-dependent impact on pollen collectability27,35.

Although task specialization in Apis mellifera (Apidae) is well-studied (reviewed by Johnson45) and the propensity for nectar or pollen foraging has been shown to be connected to the maternal reproductive traits of workers46, little is known about the division of foraging tasks in bumble bees. Smith et al.38 have found that the sensitivity of sensory receptors, size of ovaries, and existing fat reserves do not correlate with foraging task specialization in workers of B. terrestris. Overall, bumble bee foragers are flexible in the collection of either floral resource45 and in adjusting to the colony’s needs regarding the supply of nectar and pollen51. We observed that workers focused on collecting either nectar or pollen during a foraging bout (description of the differentiation in methods section). However, pollen foragers also drank some nectar to compensate their energy demand and facilitate pollen collection by agglutinating the grains with regurgitated nectar.

Malva sylvestris offers abundant pollen, which covers all flower-visiting bumble bees independently of their foraging task. The echinate structure of Malvaceae pollen results in increased adhesion, thus causing it to strongly adhere to the hair of bees35,52,53. While pollen foragers soon ceased trying to collect pollen from M. sylvestris, they did not – during that foraging bout – shift to collecting nectar from these flowers instead, although they are a lucrative food source (indicated by the fact that nectar foragers readily forage on these flowers). The flowers are not able to train the bumble bees to change their foraging task during one bout, but constitute a rewarding food source for nectar foragers.

To acquire nectar at the base of flowers, foragers often positioned themselves on the column (the fused base of the stamens and pistil), presumably because they could not find a foothold on the smooth epidermal cells of the petal54,55. As the most convenient (and energy-efficient) way to gain access to the nectar is by scrambling down the column, the bumble bees regularly contacted the stigmas when approaching the flowers and became covered in pollen after sitting on the anthers. This behaviour enhances the chances of pollination considerably. Gorenflo et al.43; Ballantyne et al.43) – although these bees effectively deprive the plants of their pollen43,46.

Data Availability
All data generated and analysed during this study are provided as supplementary information (Supplementary Tables S1–4).
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**Author Contributions**

K. Lunau and S. Koethe designed the experiments, S. Koethe performed the experiments. S. Konzmann and S. Koethe analysed the data and wrote the manuscript. All authors reviewed the manuscript.

**Additional Information**

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