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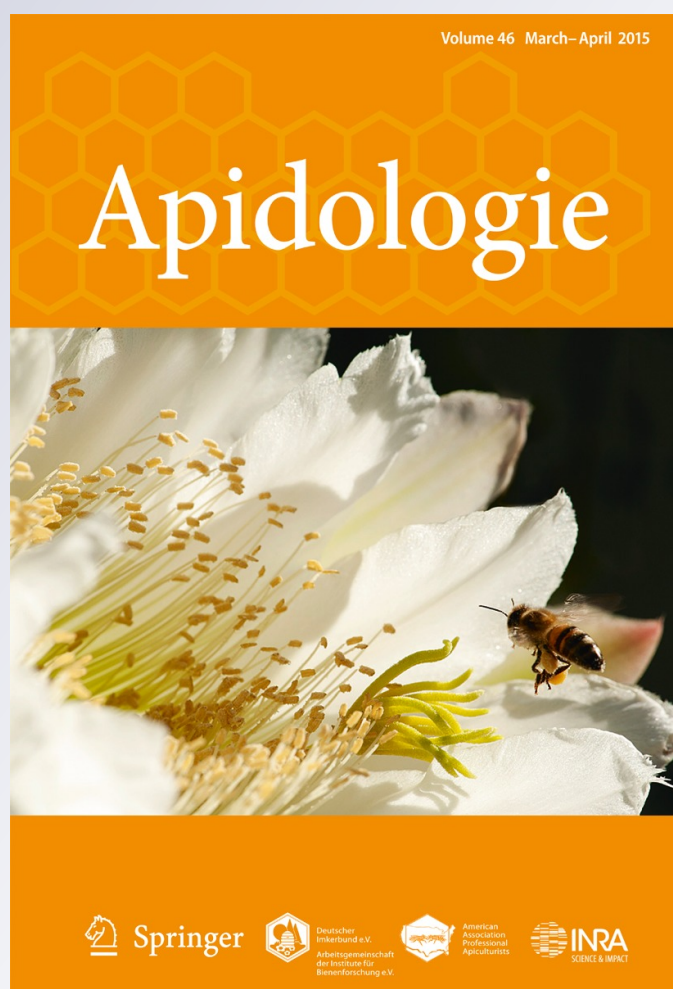
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Just spines—mechanical defense of malvaceous pollen against collection by corbiculate bees

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Abstract – Bee-pollinated plants face the dilemma that bees do not only transport pollen grains between flowers as pollen vectors but also collect large amounts of pollen that is withdrawn from pollination. Here, we show that pollen of the common hollyhock, *Alcea rosea*, is mechanically protected against collection by corbiculate bees. In a laboratory setup, bumblebees did not collect the large, sticky, and spinose pollen grains of *A. rosea* from artificial flowers unless following manipulation of the spines or the pollenkitt. Following removal of the sticky pollenkitt or bending the spines by vortexing the bumblebees readily collected the pollen. Our results show that the pollen of *A. rosea* is not bitter-tasting or toxic, but mechanically protected by the spines against being collected by corbiculate bees. Light microscopic (LM)-micrographs indicate that the long spines of malvaceous pollen grains are not covered by pollenkitt suggesting a new mechanism of mechanical defense against collection: pollenkitt-free spines and lipophilic pollenkitt prevent compacting pollen grains into pollen storage organs of corbiculate bees. The experimental data fit to observations at flowers of *A. rosea* and other malvaceous plants that honeybees and bumblebees are densely dusted with pollen but discard the pollen while packing it into their corbiculae.

pollen / pollenkitt / mechanical defense / corbiculate bees / Malvaceae

1. INTRODUCTION

The pollen dilemma of bee-pollinated plants results from the twofold function of pollen grains as a transport unit in sexual reproduction and as a reward for flower visitors (Thorp 1979; Harder and Thomson 1989; Westerkamp 1996; Lunau et al. 2011). Bees, known as effective pollen vectors, collect large amounts of pollen to provision their offspring. The pollen that has been collected by bees and stored in the scopa, the pollen-carrying

apparatus, is no longer available for pollination (Thorp 2000). Bee-pollinated plants might thus benefit from defending their pollen against collection (Westerkamp 1996; Hargreaves et al. 2009). Known pollen-saving strategies of flowers include orchid pollinaria bearing coherent masses of pollen grains that bees are unable to eat or collect (Johnson and Edwards 2000), pollen placement on safe sites that bees are unable to groom effectively (Westerkamp and Claßen-Bockhoff 2007), concealment of pollen in the corolla tube (Lunau 2000), heteranthy, within-flower stamen polymorphism, resulting in feeding and pollination stamens (Luo et al. 2008), and toxic pollen less suited to serve as larval food for bees (Detzel and Wink 1993; Roulston and Cane 2000; Praz et al. 2008).

In advance of this study, we observed honeybees and bumblebees visiting the flowers of the common hollyhock (*Alcea rosea*, Malvaceae) that

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were densely dusted with pollen, but discarded the pollen while trying to pack it into the corbiculae of the hind legs. Pollen discarding behavior of bees has also been observed at other malvaceous flowers (Azo'o et al. 2011). We hypothesize that a property of the pollen grains associated with either chemical or mechanical defense might have impeded the storing of the pollen into the corbiculae. The toxic-pollen hypothesis assumes that bees reject *A. rosea* pollen, because they sense bitter or toxic substances while collecting the pollen. The mechanical-defense hypothesis assumes that bees fail packaging and storing the pollen of *A. rosea* in their corbiculae, because biometrical parameters of pollen grains prevent a dense arrangement. The mechanism of this mechanical defense, however, is not known. In a comparative study, Vaissière and Vinson (1994) suggested that the physical interference of the spines in the pollen aggregation process might be responsible for the fact that bees fail to package their pollen pellets which fits to long known uncommonness of honeybees to collect cotton pollen (Eisikowitch and Loper 1984). The structure of the pollen grains of *A. rosea* and other malvaceous plants is outstanding because of the large size and the long spines (Christensen 1986; Shaheen et al. 2010).

In this study, we manipulated *A. rosea* pollen in order to test whether the pollenkitt contains bitter-tasting or toxic substances preventing collection and, alternatively, whether the spines mechanically defend the pollen against collection by bumblebees. We developed an experimental setting in which bumblebees of a laboratory colony collect pollen through buzzing from pipette tips attached to emasculated flowers. For testing the mechanical-defense hypothesis, the spines of fresh pollen were bent by persistent vortexing. For testing the toxic-pollen hypothesis, the pollenkitt was removed by washing with a solvent. The collection of pollenkitt-free pollen grains, pollen grains with bent spines, and natural pollen by bumblebees was quantitatively compared. In addition, the collection of natural *Pinus* pollen with an admixed bitter-tasting substance was monitored in order to test the bumblebees' response to bitter-tasting pollen. Because also the failure of an essential chemical cue might cause the refusal to collect pollen, the collection of a

chemically inert pollen surrogate, glass powder, by bumblebees was tested.

2. MATERIAL AND METHODS

The behavioral tests were done with individually marked workers of a laboratory-based bumblebee colony (*Bombus terrestris*) (see Papiorek et al. 2013). The *A. rosea* pollen collection experiment was conducted in a flight cage (0.6×0.6×0.8 m) illuminated by four fluorescent tubes. The flight cage offered four emasculated flowers of *Oenothera biennis* or *Jasminum nudiflorum* that carried a dispenser made of a 10- μ m pipette tip completely filled with pollen. The dispensers were presented in a manner that the bumblebees were able to buzz the dispenser when clinging to the flower. Three treatments were offered subsequently: natural: pollen of *A. rosea* was hand-collected, frozen, and unfrozen before experimental testing. Pollenkitt-free: Natural pollen of *A. rosea* was washed three times with *n*-hexane, washed three times with water, dried at 30 °C, and powdered before experimental testing. Pollen with bent spines: Natural pollen of *A. rosea* was vortexed for 30 min together with one pellet of silica gel before experimental testing. Conventional scanning electron microscopy of dried and gold-sputtered pollen probes was used to check how the treatments changed the appearance of the pollen grains. Up to four experienced bumblebees were released in the flight cage in which four emasculated flowers offering the same treatment were presented and monitored for 10 min. The rationale of testing a small group of bumblebees was their increased activity and their ability to stimulate each other for foraging (Leadbeater and Chittka 2007). Each of 10 individual bumblebee workers was tested only once.

To test whether buzzing bumblebees are able to detect bitter-tasting substances while collecting pollen, an experiment was set up with three treatments, which were natural, hand-collected *Pinus mugo* pollen, a 100:1, and a 1:1 mixture of *P. mugo* pollen/quinine powder. A total of 10 bumblebee workers was tested.

To test whether bumblebees readily collect a chemically inert substance, glass powder (Worf Glaskugeln GmbH) was used as a pollen surrogate. Four treatments were offered to the bumblebees which were artificial flower (made of blue foam rubber) with glass pellets (average diameter 55 μ m); artificial flower with pollen (*P. mugo*); natural, emasculated flower (*O. biennis*) with glass pellets; and natural, emasculated flower with pollen

(*P. mugo*). The testing procedure was similar to that in the previous experiment. Each individual bumblebee was tested 10 min for all treatments, but the sequence was varied. A total of 28 bumblebee workers was tested.

Conventional light microscopy was combined with batch image processing in order to analyze the coverage of pollen grains by pollenkitt of *Hibiscus rosa-sinensis* pollen, because this species has yellow pollenkitt well contrasting against the whitish spines. The number of spines per pollen grain was determined using light microscopic (LM)-photographs of single pollen grains from which the number of spines in a circular area of the half diameter of the pollen grain (representing one eighth of the surface area of the pollen grain) was counted and multiplied by 8. Conventional SEM microscopy (LEO 1430 VP, LEO Elektronenmikroskopie GmbH, Oberkochen) of pollen probes sputtered with gold was used to verify the manipulations of the pollen grains.

3. RESULTS

In the experiment with *A. rosea* pollen, all bumblebees tested visited and buzzed the flowers independent of the treatment. All bumblebees failed packing the natural pollen into the corbiculae (Figure 1a), but collected without problems washed pollen (Figure 1a) as well as vortexed pollen (Figure 1a). Ninety percent of the bumblebees tested collected the pure *P. mugo* pollen (Figure 1b), but only 40 % collected *Pinus* pollen embittered with 1 % quinine and only 20 % collected *Pinus* pollen embittered with 50 % quinine (Figure 1b). Three bumblebees that had collected embittered *Pinus* pollen discarded the collected pollen before the end of the test (Figure 1b). Out of 28 bumblebees tested, 15 buzzed at least one of the four types of flowers. Only one bumblebee buzzed an artificial flower, whereas 10 and 11 bumblebees buzzed natural flowers offering *P. mugo* pollen and glass powder, respectively; among those four bumblebees, each buzzed only natural flowers with glass powder and natural flowers with *Pinus* pollen (Figure 1c).

The pollen grains of *A. rosea* ($n=11$) are $123.47 \pm 4.32 \mu\text{m}$ in diameter and possess 153 ± 18 spines with a mean length of $11.81 \pm 0.58 \mu\text{m}$ as revealed by LM-micrographs. SEM-micrographs show that pollenkitt threads do not end at the tip of a spine, that in washed pollen the pollenkitt has been completely

removed but spines are unaffected, and that in vortexed pollen the pollen grains possess bent spines but still have its pollenkitt coating (Figure 1a).

4. DISCUSSION

The results of the experiments are not consistent with the hypothesis that the pollen of *A. rosea* is toxic or contains bitter-tasting substances, because the bumblebees readily collected vortexed *A. rosea* pollen still covered with pollenkitt, the solvent of hypothetical allelochemicals. Moreover, the bumblebees stopped collecting *Pinus* pollen embittered with quinine indicating that buzzing bumblebees sense bitter-tasting substances of the collected pollen. The fact that not all bumblebees immediately stopped collecting embittered pollen suggests that buzzing bumblebees from time to time probe the pollen rather than sense bitter-tasting substances when regurgitating nectar onto the pollen or when dusted with pollen. The fact that the bumblebees readily collected glass powder, which is visually inconspicuous and scentless, as often as *Pinus* pollen, indicates that no chemical stimulus is needed to induce pollen collection.

The results of the experiments support the hypothesis that the pollen of *A. rosea* is mechanically defended against collection by corbiculate bees and that both, spines and pollenkitt, are involved in the mechanical defense mechanism. The prediction of the toxic-pollen hypothesis that bumblebees collect pollenkitt-free pollen, provided that the toxic substances are in the pollenkitt, was not verified by the results. The predictions of the mechanical-defense hypothesis that bumblebees do not collect neither pollen with bent spines nor pollenkitt-free pollen were supported by the experimental data. Furthermore, the inability of the bumblebees to collect pollen with bent spines or pollenkitt-free pollen suggests that the interaction between spines and pollenkitt is involved in this mechanical defense mechanism.

Interestingly, the ability to carry single pollen grains at the bristles of the bees' body does not interfere with the inability to store the pollen in the corbiculae (Figure 2a). Furthermore, oligolectic bees that are specialized on pollen of Malvaceae

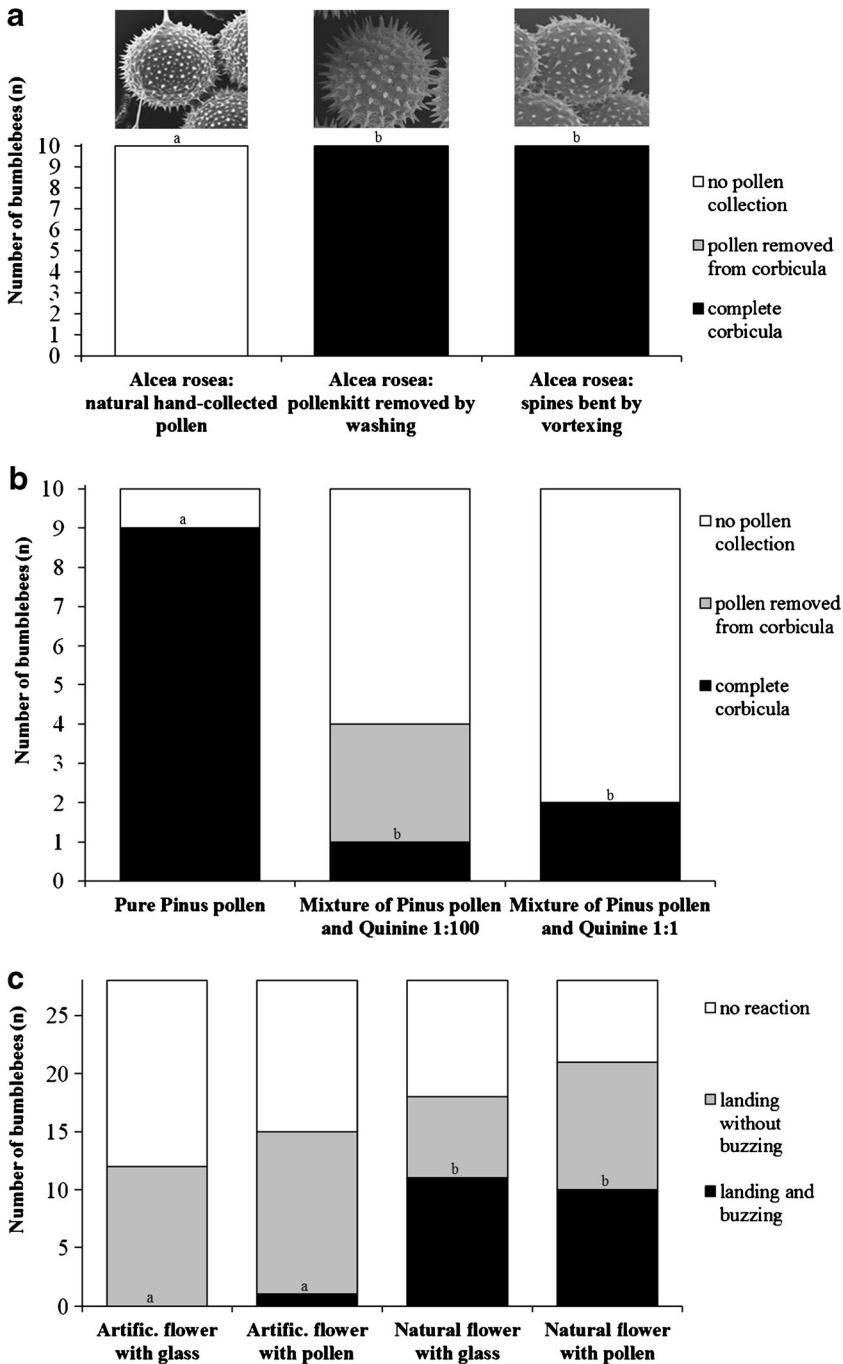


Figure 1. Collection of natural and manipulated pollen by bumblebees. *Different letters refer to significant differences ($p < 0.01$, two-sided Fisher's exact test).* **a** Collection of natural and manipulated pollen of *Alcea rosea* from artificial feeders on emasculated flowers. *Insets:* SEM-micrographs of natural and manipulated *Alcea rosea* pollen grains. **b** Collection of natural and embittered pollen of *Pinus mugo*. **c** Collection of natural pollen of *Pinus mugo* and glass powder.

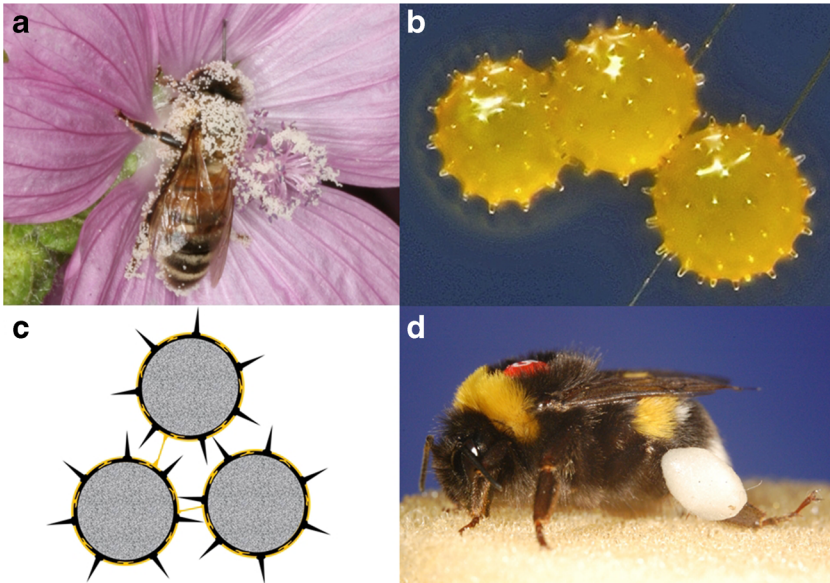


Figure 2. **a** Honeybee dusted with pollen of *Lavatera* spec. **b** Batch image processed LM-micrograph of pollen grains of *Hibiscus rosa-sinensis*. **c** Sketch of hypothetical interactions between malvaceous pollen gains (in cross section) indicating interactions between pollen wall (black) and pollenkitt (yellow). **d** Bumblebee with pure glass powder collected in the corbiculae.

seem to transport pollen not as a compacted mass rather than as a multitude of loose pollen grains adhering to the sparse bristles (Gaglianone 2000; Schlindwein et al. 2009).

Among the many functions of pollen wall and pollenkitt (Pacini 2000; Pacini and Hesse 2005), defense against collection has not yet been described. The focus-stacked LM-micrograph of pollen grains of *H. rosa-sinensis* shows that the pollenkitt covers the entire pollen wall, but not the spines. This is clearly seen at those spines viewed from the side, in which the yellow pollenkitt covers the basis but not the clear tip of the spines (Figure 2b). The SEM-micrograph of *A. rosea* pollen grains shows that the pollenkitt produces only few threads of pollenkitt that connect the pollen grains (Figure 1). Pollenkitt-free spines have been described for *Dahlia* (Knoll 1930) and *Lavatera* (Nepi and Franchi 2000). Modeling the interaction between spines of one pollen grain and pollenkitt of a butting pollen grain indeed shows that the poor bond between pollen grains due to the interaction between pollenkitt-free spines and pollenkitt-covered exine may impede the compacting within

pollen storage organs (Figure 2c). The spines obviously reduce the contact zone between butting pollen grains, because only one or few spines interact with the pollen wall of a butting pollen grain. The large size of the pollen grains may contribute to the effect of a reduced contact zone (Figure 2c). Further studies will provide more details of the role of regurgitated nectar, which is not able to glue natural malvaceous spiny pollen grains together but inert pellets of glass powder (Figure 2d)

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Justement des épines – Défense mécanique du pollen de malvacées contre la récolte par des abeilles corbiculées

Mécanisme de défense / récolte du pollen / *Alcea rosea* / pollenkitt / corbeille à pollen / abeille / *Bombus terrestris*

Ausgerechnet Stacheln – Mechanischer Schutz von Malvaceae-Pollen vor dem Sammeln durch corbiculate Bienen

Pollen / Pollenkitt / mechanischer Schutz / corbiculate Bienen / *Alcea rosea*

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