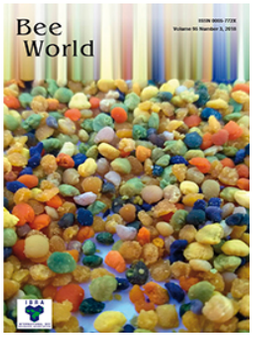

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What We Can (or Cannot) Learn from Multifloral Pollen Pellets

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Introduction

Pollen and its diversity, including the color of pollen pellets, have caught the attraction of bee keepers and researchers. Pollen is known to play an important role in the nutrition of honey bees as a source of proteins, amino acids, minerals, fats, starch, sterol, and vitamins (Brodschneider & Crailsheim, 2010; Roulston, Cane, & Buchmann, 2000; Stanley & Linskens, 1974). Since the chemical composition and nutritional value of pollen varies between botanical species, a diverse pollen diet is considered favorable for colonies (Conti et al., 2016; di Pasquale et al., 2013; Omar et al., 2017; Radev, 2018; Roulston et al., 2000). Knowing the phenology of the plants in the surrounding environment and the pollen pellet color could indicate the plant species upon which a bee forages (Kirk, 2006, 2018).

Few studies have revealed the existence of multifloral pollen pellets collected by corbiculate bees (Betts, 1920, 1935; Hodges, 1954; Sladen, 1913). Betts (1920) distinguished between two kinds of mixed loads of pollen – the segregated (S) mixture, on the one hand, and the mixed (M) one, on the other hand. An S-type mixture is defined as a pollen pellet with two or more distinct segments, a load with parti-colored bands results. This implies that the honey bee visited more than one plant species consecutively while foraging or that the pollen of the plant has two color forms. In comparison, an M-type mixture contains two or more kinds of pollen that are mingled. The origin of the pollen can be characterized either by making a chromatic assessment of the pollen pellets or a microscopic palynological analysis (Conti et al., 2016). In the present study, the latter method was used.

Although Betts (1920, 1935) and Hodges (1954) observed honey bees visiting two or more plant species during a single pollen-foraging trip, the behavior that had already been observed by Aristotle, that bees constantly visit one botanical species per flight is still considered to be the norm (Grant, 1950; Grüter, Moore, Firmin, Helanterä, & Ratnieks, 2011; Grüter & Ratnieks, 2011; Thorp, 1979). But, isn't there a saying that "exceptions make the rule?" We believe in this proverb and, for this reason, analyzed segregated (S-type) pollen pellets that were collected by *Apis mellifera* in two different sampling locations in Austria to learn more about their floral constancy.

Materials and methods

Segregated pollen pellets were found incidentally during a citizen science project on honey bee pollen collection (Van der Steen & Brodschneider, 2014). Pollen traps were mounted on honey bee colonies, and pollen was sorted by citizen scientists according to color. Segregated, multi-colored pollen pellets were separated upon discovery into individual laboratory tubes and then deep frozen until the palynological analysis was conducted. Altogether, 22 segregated pollen pellets were collected on three different sampling dates and from two different sampling locations. The results are aggregated to the five different sampling locations/dates. The sampling locations were in Altenmarkt (GPS ca. 47.365, 13.429, 936 meters above sea level) and Graz (47.077, 15.450, 377 m), Austria. Microscopic palynological analysis of pollen pellets was conducted following a method adapted from that of Barth et al. (2010), and pollen types were identified using the pollen database available via ponet.ages.at, which contains more than 6,000 reference specimens for comparison. 500 pollen grains were identified from each sample (Figure 1).

Results

In the segregated pollen pellets, we could identify between four (sample E) and ten (sample C) different pollen types, which are shown in Table 1. However, the percentage of the individual pollen types within a pellet was highly variable. These proportions vary between 0.2% (samples B, C, and D) and 83.0% (sample C). To exclude the possibility of contamination from "foreign" pollen grains and focus on major pollen compartments, a threshold value of 10.0% was set. We regarded pollen types with lower occurrences as minor contributors. The analysis of sample E revealed that it consisted mainly of "unknown" pollen grains (98.0%) and, furthermore, was treated as a single floral pellet.

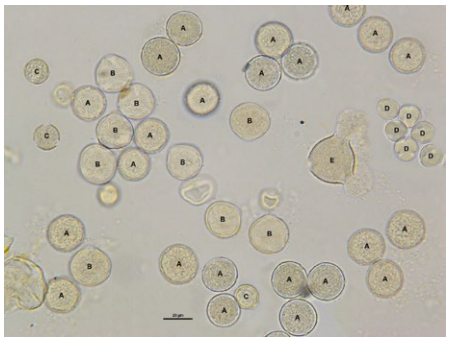

Figure 1. Image of pollen types from sample D. (A) Pollen from plantain (***Plantago*** spp.), (B) white clover (***Trifolium repens***), (C) meadowsweet (***Filipendula*** spp.), (D) Saint John's wort (***Hypericum*** spp.) and red clover (***Trifolium pratense***) are shown.

Not every pollen type could be assigned to species level due to the insufficiency of recognizable morphological characteristics as assessed using light microscopy. In this case, we allocated the pollen type to the respective genus or family level. Certain pollen could only be assigned to a group of species (e.g., *Malus* spp./*Pyrus* spp./*Crataegus* spp.). So far, not all Austrian plant species with their specific pollen characteristics have been included in the ponet-database and, therefore, some pollen types could not be allocated to a specific plant genus or species. Such pollen types were categorized as "unknown."

In sample A, three pollen types from three different plant families were found to be present in the pellet above the threshold value (mustard family: 40.2%, dandelion: 30.8% and mint family: 23.0%). In comparison, pollen types of samples B and C originated from only two main sources: mustard family and dandelion, respectively (Table 1). The highest pollen-type diversity was found

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Table 1. Sampled, mixed (S-type) pollen pellets and associated analyzed floral species.

Sample (location)	Sampling date	Identified pollen source (%)
A (Graz)	22 April 2016	***Brassicaceae*** (40.2); ***Taraxacum***-form (30.8); ***Lamiaceae***-form (23.0); ***Asteraceae*** (2.8); Unknown (1.0); ***Fagus sylvatica*** (0.8); ***Aesculus hippocastanum*** (0.6); ***Picea abies/Abies alba*** (0.4); ***Malus*** spp./***Pyrus*** spp./***Crataegus*** spp. (0.4)
B (Graz)	22 April 2016	***Brassicaceae*** (58.4); ***Taraxacum***-form (36.8); ***Asteraceae*** (2.2); ***A. hippocastanum*** (1.0); ***Hedera helix*** (0.6); ***Malus*** spp./***Pyrus*** spp./***Crataegus*** spp. (0.4); Unknown (0.2); ***Fagus sylvatica*** (0.2); ***Acer*** spp. (0.2)
C (Graz)	22 April 2016	***Brassicaceae*** (83.0); ***Taraxacum***-form (14.2); ***Anemone*** spp./***Clematis*** spp./***Pulsatilla*** spp./***Ranunculus*** spp. (1.2); ***Juglans*** spp. (0.4); Unknown (0.2); ***Quercus*** spp. (0.2); ***P. abies/Abies alba*** (0.2); ***F. sylvatica*** (0.2); ***Allium*** spp. (0.2); ***Acer*** spp. (0.2)
D (Altenmarkt)	28 April 2014	***Plantago*** spp. (53.4); ***Trifolium repens***-form (17.4); Unknown (13.8); ***Asteraceae*** (12.4); ***Filipendula*** spp. (12.4); ***T. pratense***-form (2.4); ***Hypericum*** spp. (0.4); ***Poaceae*** (0.2)
E (Altenmarkt)	08 June 2014	Unknown (98.6); ***Taraxacum***-form (0.6); ***T. pratense***-form (0.4); ***Acer*** spp. (0.4)

in sample D with five different pollen types above the threshold value: plantain, white clover, daisy family, meadowsweet, and an unknown pollen type.

Discussion

Our results clearly confirm and extend findings previously reported by other authors, namely, that segregated (S-type) pollen pellets can be composed of pollen from plants from different genera or families (Betts, 1920, 1935; Hodges, 1954). We identified the following matches in the analyzed pollen pellets: 1. mustard family (Brassicaceae) and dandelion (*Taraxacum* sp.) (samples B-C), 2. mustard family, mint family (Lamiaceae) and dandelion (sample A), 3. plantain (*Plantago* spp.) and white clover (*Trifolium repens*) (sample D). We classified the pollen load as multifloral if two or more floral pollen sources were present in amounts above the threshold value of 10.0%. The chosen threshold should help to distinguish between compartments of pollen that are deliberately collected by honey bees and pollen grains which are considered to be contamination from other sources. Sample E visually appeared to be a segregated pollen pellet, but the palynological analysis revealed that it almost completely consisted of one pollen type that could not be assigned to a taxon. It could have been made up from a single pollen source that provides pollen of different colors.

Parti-colored pollen pellets have been described before, but no clarity on the frequency of these is known, and it was also not the aim of this study to examine this phenomenon. Whereas Betts (1920) estimated the M-type mixtures to make up about 40 percent of pollen pellets collected by honey bees, truly segregated (S-type) pollen pellets with distinct color bands may occur much more rarely. We found minor contributors of pollen types to pollen pellets, which were comparable to those termed "doubtfuls" by Betts (1935). Most of these showed an occurrence of less than 1.0%, and often only one to three pollen grains were counted. We believe that these minor pollen types originate from other sources (i.e., not the active pollen collection) and pellet building in the pollen baskets (corbiculae). Betts (1920, 1935), among others, suggested that the contamination with such minor contributors in pollen pellets could be due to deposition at flowers by other insect pollinators visiting the same plant. However, we believe that there are probably two other means of contamination. First of all, pollen grains could remain on bees' hairs', picked up either during previous pollen or nectar foraging trips (Vaissière & Vinson, 1994), rubbing the body against parts of the hive, or as a result of bee-to-bee contact in the colony (e.g., social grooming) (Božič & Valentinčič, 1995). Secondly, single pollen grains could have become mixed into the samples through contamination in the pollen trap. Although we newly lined the drawers of the pollen traps during the sampling process, using a fresh sheet of paper towel for each sample, the analyzed mixed pollen pellets might have come into contact with other pellets collected by the same colony in the pollen trap drawer.

How can the genesis of multifloral pollen pellets be explained? Is it pure coincidence that bees randomly switch from one to another similar pollen source, or do they switch between plants for reasons of economy? The floral origins of the multifloral pollen pellets presented in this study are all new and hitherto unreported combinations. It is highly likely that researchers working in other areas will identify more and different combinations of pollen from plants that are not closely related in single mixed-pollen pellets of the S-type. Further published combinations are summarized in Table 2. It is striking that the conjointly collected pollens are frequently from distinctly different plant taxa. We, therefore, conclude that any hypothesis based on the coincidental collection of pollen from different plants due to high similarity of plants (e.g., form and color of flowers) should be rejected.

This leaves only explanations that address the flower constancy of at least a small number of pollen foragers. Betts (1935) hypothesized that it seems to be more usual for honey bees to forage on two or more assorted plant species during one trip, rather than constantly visiting one plant species and changing to another shortly afterwards. Bees obviously do sometimes switch between flowers on a single pollen foraging trip, and it is of interest to study whether these are bees that switch because pollen is no longer available from one flower source or there are other reasons. For example, inexperienced pollen-foraging bees may have problems with flower constancy, in contrast to experienced foragers. It could also be argued that the habitat richness of pollen sources could affect the frequency of mixed pollen loads, as more plant visits or farther flights between plants are needed to completely fill the corbiculae in environments that provide little pollen or habitats where high competition for pollen exists. However, bees living in a rich habitat may have a greater tendency toward floral constancy than those foraging in poor environments and may efficiently collect pollen

Table 2. Known botanical species that make up mixed pollen pellets.

Species	Bee species, country (reference)
Common Bird's-Foot Trefoil (***Lotus corniculatus***), White Clover (***T. repens***) and Common Cat's-Ear (***Hypochaeris radicata***)	***A. mellifera***, United Kingdom (Betts, 1935)
Heather (***Calluna vulgaris***) and Dwarf Gorse (***Ulex nanus***)	***A. mellifera***, United Kingdom (Betts, 1935)
Dandelion (***Taraxacum*** sp.) and Berberis (***Berberis*** spp.)	***A. mellifera***, United Kingdom (Hodges, 1952)
Indian warrior (***Pedicularis densiflora***) and Henderson's shooting stars (***Dodecatheon hendersonii***)	***Bombus*** spp., USA (Macior, 1986)
Dandelion (***Taraxacum*** sp.), Mint family (***Lamiaceae***) and Mustard Family (***Brassicaceae***)	***A. mellifera***, Austria (This study)
Dandelion (***Taraxacum*** sp.) and Mustard Family (***Brassicaceae***)	***A. mellifera***, Austria (This study)
Plantain (***Plantago*** spp.) and White Clover (***T. repens***)	***A. mellifera***, Austria (This study)

from the most abundant plant species. Regarding nectar foragers, such an effect of reward rates on flower constancy has been discussed (Grüter et al., 2011; Grüter & Ratnieks, 2011). The time for collecting and packing the pollen in pollen baskets, hence, should be minimized to achieve the optimum intake rates for the colony.

Mixed pollen loads have also been observed in other corbiculate bees (Macior, 1986; Sladen, 1913). It has been reported that bumble bees show less flower constancy than honey bees and, therefore, return to their nest with mixed pollen loads more frequently (Brian, 1952; Free, 1970; Sladen, 1913). According to Betts (1935), *Bombus terrestris* is known to collect 32% mixed pollen pellets, but she observed a frequency of only 3% in honey bees. One reason for this difference could be that bumble bees constantly visit a certain foraging site, but do not constantly visit certain floral species within that particular area, instead of collecting single floral pollen from several sites (Osborne et al., 1999).

Several lessons were learned during this study, but open questions about what we can (or cannot) learn from our findings and those already published still exist. First of all, a method that minimizes the contamination risk of pollen-pellet samples should be developed to increase the validity of results. This could be achieved by collecting pollen pellets directly from the bees' corbiculae or emptying pollen traps more frequently, which reduces the number of pollen pellets in the tray and, hence, the chance of cross contamination. We also want to point out that there is no information available on the general risk of contamination due to the bees' hair or pollen traps for single floral pollen pellets (Dag, Degani, & Gazit, 2001; Degrandi-Hoffman, Thorp, Loper, & Eisikowitch, 1992). One study investigated honey bee pollen pellets that were collected directly from bees foraging on gramineous weeds and found that they consisted of 100% pollen from the family Poaceae (Jones, 2014). We further suggest separating S-type pollen pellets with a scalpel and conducting palynological analysis of the separated subsamples to gain more information about the main contributors to the different colors of a pellet. It is possible that honey bee pollen foragers are not as constant to floral species as we have previously thought. Further investigations on the availability of pollen to honey bees in different habitats must be conducted to conclusively understand the behavior of multifloral pollen collection by honey bees and answer the question of whether exceptions really make the rule or vice versa.

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