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SPECIAL ISSUE ARTICLE

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Does sorting by color using visible and high-energy violet light improve classification of taxa in honey bee pollen pellets?

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Abstract

Premise: Pollen collected by honey bees from different plant species often differs in color, and this has been used as a basis for plant identification. The objective of this study was to develop a new, low-cost protocol to sort pollen pellets by color using high-energy violet light and visible light to determine whether pollen pellet color is associated with variations in plant species identity.

Methods and Results: We identified 35 distinct colors and found that 52% of pollen subsamples ($n = 200$) were dominated by a single taxon. Among these near-pure pellets, only one color consistently represented a single pollen taxon (Asteraceae: Cichorioideae). Across the spectrum of colors spanning yellows, oranges, and browns, similarly colored pollen pellets contained pollen from multiple plant families ranging from two to 13 families per color.

Conclusions: Sorting pollen pellets illuminated under high-energy violet light lit from four directions within a custom-made light box aided in distinguishing pellet composition, especially in pellets within the same color.

KEYWORDS

acetolysis, *Apis mellifera*, high-energy violet light (HEVL), palynology, pollen

Most bees actively visit flowers to obtain nectar and pollen for energy and reproduction (Thorp, 2000). Pollen is a nutritionally diverse and highly valuable reward because it provides protein (Roulston et al., 2000) and other pollen nutrients, including lipids, fatty acids (Manning and Harvey, 2002), sterols (Vanderplanck et al., 2011), and micronutrients. Pollen is commonly collected from many different plant species and across many different land uses, including urban areas, agricultural areas, and natural areas (Requier et al., 2015). Upon collection, most bees transfer pollen to external structures specialized for pollen transport, including modified hairs forming corbiculae on legs (e.g., Apidae) or scopae on legs or other body parts (Thorp, 2000). The European honey bee (*Apis mellifera*) is a managed, generalist pollinator that visits a broad range of flower types, often readily collecting pollen from crop species (Kleijn et al., 2015). Consequently, *A. mellifera* is frequently deployed in hives in agricultural systems to meet the increasing demand for crop pollination (IPBES et al., 2016). However, even when flowers are highly abundant, such as in mass-flowering crops, honey bees often collect pollen preferentially from other plant species (Requier et al., 2015; Santos et al., 2022). Bees that forage for pollen from a diverse range of plant species benefit from obtaining a wider range of nutrients, including nutrients that occur in pollen in trace amounts (Donkersley et al., 2017).

As bee-collected corbicular pollen is often variable in color, this has been used as a basis to sort pellets based on pollen composition to determine plant species use (da Silveira, 1991; Conti et al., 2016; Salazar-González et al., 2018; Stoner et al., 2019; Topitzhofer et al., 2021; Hornby et al., 2022). Reliably categorizing pollen pellets by color requires standardization of color choice either by using an instrument to determine pellet color (Salazar-González

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et al., 2018; Hornby et al., 2022) or by comparing color to a standard set of color cards (Conti et al., 2016; Stoner et al., 2019; Topitzhofer et al., 2021). However, standardized and reproducible methods are lacking. For example, one frequently used method first takes a subsample of pellets, then sorts them by color (Barth et al., 2009; Conti et al., 2016; Topitzhofer et al., 2021). Subsampling pollen pellets prior to color sorting saves time but may exclude rare pellet colors, thereby underestimating the species richness of the pellet sample. It is also common in the literature to measure relative pollen proportions of a sample by counting the number of pollen grains in each pollen category using a microscope (pollen sorted to family, genus, etc.; Eckhardt et al., 2014; Requier et al., 2015; Salazar-González et al., 2018; Hornby et al., 2022; Santos et al., 2022). However, counting pollen grains and reporting relative proportions without accounting for pollen grain size may generate misleading results about pollen composition (Buchmann and O'Rourke, 1991; da Silveira, 1991; Conti et al., 2016). The categorization of pellet colors varies in the literature, with some authors reporting the red, green, and blue (RGB) values of reference colors (Conti et al., 2016) and others relying on photographs to convey color value to the reader (Newstrom-Lloyd et al., 2017); the former is an example of a digital, standardized color that any reader with access to a computer can re-generate and interpret, while the latter is an example of an unreproducible color categorization. Standardized methods are required to ensure representation of all plant species in samples, to determine the relative proportion of each pollen type, to validate color groups between observers or for a single observer, and to establish reproducible methods in future studies.

We collected honey bee pollen pellets from commercial blueberry orchards to evaluate how protective cover structures impact foraging behavior of *A. mellifera* (Kendall et al., 2021). As the aim of the study was to understand the richness of pollen collected by honey bees, we devised a new method to sort pollen by color to ensure the sampling method represents all colors as a potential proxy for palynological diversity. To determine pollen color accurately, we built a uniform lighting environment and used a set of color cards to reduce variation in color selection in this study. Some pollen pellets fluoresce when exposed to long-wavelength ultraviolet/high-energy violet light (~400 nm), thus ~400-nm wavelengths (here referred to as high-energy violet light [HEVL]) were included in the sorting criteria to help differentiate fluorescent pellets within colors. HEVL is emitted from many commercially available "blacklights," which tend to emit light in 385–415-nm wavelengths. To ensure that less-common colors were represented, we sorted all the pellets from each sample by color prior to acetolysis. Pollen count and volume were reported to estimate pellet composition and compensate for size differences among pollen types. To ensure repeatability, colors are provided as hexadecimal values as these are readily accessible in most computer graphics applications and are more succinct than reporting RGB values. We hypothesized that HEVL illumination would allow us to sort pellet samples into more groups and that the pellets in those groups would be more homogenous than if we had sorted under visible light alone.

METHODS AND RESULTS

The objective of the present study was to develop a new protocol for sorting *A. mellifera* pollen pellets by color that is both low-cost and easily standardized to facilitate repeatability. Our method uses HEVL as well as visible light to determine whether pollen pellet composition is associated with its color. To evaluate whether protective cover structures impacted honey bee foraging behavior (Kendall et al., 2021), we collected pollen from 16 *A. mellifera* hives at four different blueberry farms from 24 June to 20 August 2020 in the Coffs Harbour region of New South Wales, Australia (30°17′46.5936″S, 153°6′50.8896″E). Pollen pellets were collected in pollen tray collector traps designed to remove pollen from the corbiculae of forager bees as they entered the hive. Collected pollen pellets were dried at 40°C for 24 h in a food dehydrator (Food Dehydrator 10 Tray, model no. KA10DHDTIMA; Devanti, Hawthorn, Victoria, Australia), then weighed. To ensure representative sampling of all pollen pellet colors within each sample, whole pollen samples were first sorted by color to capture the presence and abundance of all colors, before being subsampled for acetolysis. To achieve even and uniform illumination of each sample, pollen pellets were transferred to plastic trays (31 cm × 22 cm × 5 cm; Quadrant, Melbourne, Victoria, Australia) lined with black matte vinyl and sorted by appearance inside a purpose-built 75 × 60 × 60 cm light box made with black corrugated plastic panels; black was selected as the background color to view HEVL fluorescence. HEVL light-emitting diode (LED) strip lights (395–400 nm; KXZM, Ziyang, China) and visible light LED strip lights (400–760 nm; Brilliant Lighting, Rowville, Victoria, Australia) were installed on the inside of the light box. To illuminate the sample close-up, a HEVL LED flashlight (390–410 nm, ≤500 lumen; unbranded) and a visible light LED lamp (500 lumen; Verve Design by Arlec, Scoresby, Victoria, Australia) were positioned 10–30 cm above the sample. Preliminary results indicated that pollen pellets reflected HEVL light to varying degrees, and pellets that glowed under HEVL illumination could be sorted into groups that were mainly unifloral in composition. For this reason, we sorted samples first under HEVL based on their relative strength and shade of fluorescence, then sorted them under visible light and assigned a color that matched one of 72 possible paint sample cards from four paint companies (Dulux, PPG, Taubmans, Wattyl). We observed pollen by switching back and forth between HEVL and visible (white) light until the sample was consistently sorted into homogenous color groupings.

Color groups are provided as hexadecimal colors to ensure repeatability of findings without the need to use the same set of paint cards (Appendix S1). Three pellets from each color group (mean mass ± standard error [SE], 0.018 ±

0.001 g) were withheld in individually labeled vials for acetolysis. Our methods for acetolysis follow those of Jones (2014) with some alterations (see Appendix 1). We disaggregated our samples in distilled water and dehydrated them in 3 mL of glacial acetic acid. Samples were then processed using acetolysis (3 mL of 9:1 acetic anhydride: sulfuric acid) for 5 min in a 90°C hot bath (BS-31 Shaking Water Bath; Wishmed, Blacktown, New South Wales, Australia). The acetolysis mixture was subsequently removed with a rinse of 3 mL of glacial acetic acid, then rinsed three times with 5 mL of water. Between each of these steps, we spun our samples in a swing-out centrifuge (ELMI CM-7S Plus Benchtop Centrifuge; POCD Scientific, North Rocks, New South Wales, Australia) at 1060 × *g* for 3 min. (See Appendix 1 for detailed protocol). Acetolysing multiple pellets from each color group allowed us to test the homogeneity of the predetermined color group. After acetolysis treatment was complete, the samples were resuspended in 3 mL of warm glycerin jelly (~80°C).

Twenty-five microliters of liquid sample jelly was transferred to slides and a cover slip applied. All slides were cured upside down on a slide warmer set to 80°C for 72 h, which allowed pollen to sink through the liquid medium to rest against the cover slip as it cured (Traverse, 2007). This method ensured pollen was in the same focal plane and greatly increased the quality of the photographs. We chose to suspend pollen in liquid glycerin jelly because its refractory index is similar to that of sporopollenin and therefore produces photographs with fewer artifacts than other mounting media (Traverse, 2007). Digital photos were taken of each sample using a Nikon Eclipse 90i compound microscope (Nikon, Tokyo, Japan). Pollen was counted and measured using the Count tool and Ruler tool, respectively, in Adobe Photoshop 23.4.2 (Adobe, San Jose, California, USA). We used relative volumes as a measure of pollen composition rather than relative pollen grain counts alone (Buchmann and O'Rourke, 1991; da Silveira, 1991; Conti et al., 2016) to account for the differences in pollen size (Appendix S2). We calculated the volume of pollen morphotypes by using formulas for the volume of spheres, ellipsoids, and various prisms. Representative photos of pollen morphotypes were identified by experts (S.H., K.C.B.S.S., and M.L.A.), and family-level identifications (or higher resolution, where possible) were assigned.

We identified 35 distinct color samples and found that 52% of pollen subsamples ($n = 200$) were dominated by a single pollen family (Appendix S3). Only one color consistently represented a single pollen taxon (Asteraceae: Cichorioideae; color 10, #FF9400). The use of an enclosed light box to provide illumination allowed us to sort pellets in a consistent lighting environment, regardless of the lighting conditions in the laboratory. Lighting pollen samples from four directions (from above, anteriorly, and laterally) gave us confidence that colors were assigned to pellets in a consistent manner, as evidenced by the composition of color 10 (orange, #FF9400). Similar to the findings of other authors, most of the pre-determined colors assigned under visible light were not strongly associated to plant taxa (Stoner et al., 2019). The use of HEVL illumination enabled greater differentiation of pellets within a color, which more consistently aligned with differences in pollen taxa. Hence, sorting pollen pellets under HEVL illumination significantly increased the number of pollen families found per color (mean = 5.229, standard deviation [SD] = 2.756) when compared to samples illuminated in visible light (mean = 4.343, SD = 2.496, $t(34) = -5.276$, $P < 0.001$; Figure 1). As it is known that HEVL can be harmful to the eyes, observers wore eye protection when viewing pollen pellets under HEVL illumination; however, it is unknown whether HEVL can be harmful to pollen pellets. Pollen pellets in our study were exposed to HEVL for the shortest duration possible, but more work is needed to test whether exposure to HEVL degrades DNA or other subcellular structures in pollen. By sorting pollen pellet samples by color first, we were able to measure the relative mass contribution of less-common pellet colors. Some pellet colors represented <1% of a sample's mass and would likely be excluded if a subsample of pellets were used for color sorting (Figure 2). Calculating pollen volume as well as count allowed large pollen grains to be contrasted more accurately with small pollen grains (Appendix S2).

To sort pollen pellets collected from *A. mellifera*, our method recommends standardization of illumination, sampling technique, calculation of pollen composition, and color reporting. Color sorting is an inherently subjective process; therefore, all means to standardize conditions should be taken for results to be relevant and reproducible. Studies that use ambient light, either by stating so (Stoner et al., 2019) or by inference (Conti et al., 2016; Topitzhofer et al., 2021), fail to account for the effect of shadows, daylight changes, and lighting source quality in their sorting and color assignments. By sorting and assigning colors inside a light box, we decreased variation in color perception and created a space where an observer could ostensibly re-sort a sample and achieve the same or similar result. Many researchers choose to sample prior to sorting (Conti et al., 2016; Stoner et al., 2019; Topitzhofer et al., 2021), but this technique risks omitting rare pollen colors. We advocate sorting into colors first before continuing to process pollen to ensure all color groups are accounted for in subsamples, and future studies could directly test this by comparing the conventional use of random subsamples to the sorting method we propose here to validate whether a greater number of rare colors are accounted for. Pollen pellet composition reported as the relative number of pollen grains can produce different results than when pollen volume is measured (Buchmann and O'Rourke, 1991; da Silveira, 1991; Conti et al., 2016; see also Appendix S2), and studies that use volume when measuring pollen collection by bees produce results that are more relevant to our understanding of how bees use pollen as a resource (Stoner et al., 2019). As mean pollen diameter ± SE in our study ranged from 16.24 ± 0.22 μm to

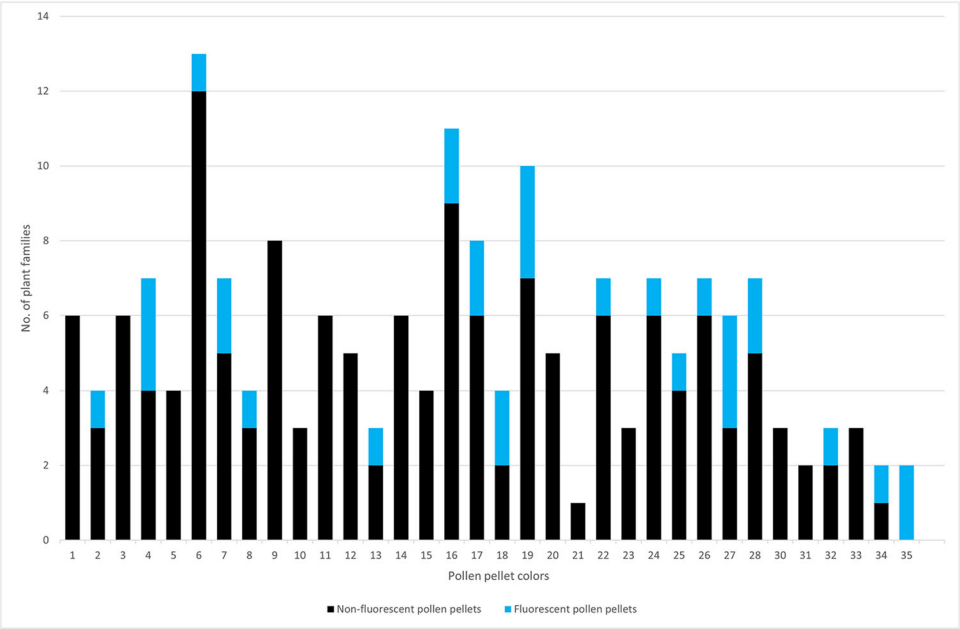

FIGURE 1 The number of additional pollen families revealed by HEVL fluorescence. Each bar in the graph represents the total number of distinct pollen families found in each pollen pellet color. The bright blue portion of the bar shows how many pollen families were found in HEVL fluorescent pollen pellets only. Sorting pollen pellets under HEVL illumination significantly increased the number of pollen families found (mean = 5.229, SD = 2.756) when compared to samples illuminated in visible light (mean = 4.343, SD = 2.496, $t(34) = -5.276$, $P < 0.001$).

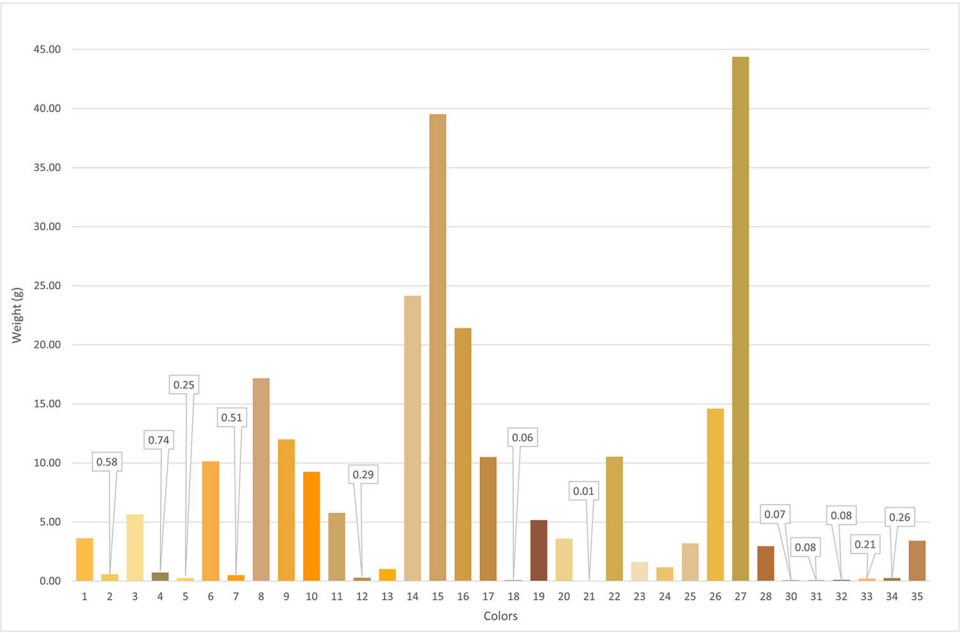

FIGURE 2 Relative proportion of pollen pellet colors collected. Each column in this graph represents the relative mass in grams of each pollen pellet color collected in this study. Colors with mass < 1.0 g have callout boxes showing the exact mass. Each bar is shaded with the hexadecimal color attributed to each pollen pellet color (Appendix S1).

107.29 ± 7.66 μm, the omission of pollen volume in pollen composition estimates would have greatly underestimated the contribution of large pollen grains. When studies fail to report the numerical value of colors, their methods are not easily reproducible (da Silveira, 1991; Newstrom-Lloyd et al., 2017; Topitzhofer et al., 2021). We thus report colors as hexadecimal numbers to describe color more accurately to other users (Conti et al., 2016; Hornby et al., 2022).

The above-mentioned method of sorting *A. mellifera* pollen pellets by color was variable in its capacity to predict

palynological diversity. In other highly visited crop plant species such as almond (*Prunus dulcis* (Mill.) D. A. Webb), pollen pellet color was highly correlated with pollen species (Santos et al., 2022). Reasons for the difference in predictive capacity among studies could include differences in the availability and identity of plant species available to worker bees in a given habitat. The blueberry crop sampled in this study (Ericaceae: *Vaccinium* L.) is rarely collected as a pollen source for honey bees (Javorek et al., 2002). Hence, although we did detect pollen in the Ericaceae, no colors were aligned with this plant family. Future studies using data from *A. mellifera* pollen pellets should thus be interpreted in the context of the habitat from which they were collected and extent of collection by bees. Pollen pellets collected by *A. mellifera* can darken in color over time due to oxidation (Barth et al., 2009). Because we dried pollen pellets prior to sorting them by color, it is possible that oxidation dulled distinct colors to similar shades, effectively masking some alignment between pellet color and palynological composition. Freezing pellets may alleviate this problem to preserve color, but more studies are needed to investigate how oxidation and dehydration affect pollen color when illuminated with HEVL and visible light. We analyzed the discarded supernatant after the first round of centrifugation during acetolysis in a number of samples and found that some contained floating pollen (Pinaceae) or pollen grains with an apparent low density. Samples from color number 5 (Appendix S1) sometimes contained pollen in the Pinaceae in the discarded supernatant, but limited time and resources prevented us from investigating this trend further. We believe that representation of Pinaceae pollen was greatly reduced in our results due to its loss during acetolysis.

CONCLUSIONS

The methods outlined in this study provide guidelines for the standardization of pollen pellet sorting and are inexpensive and reproducible. Consistent and uniform illumination must be used to sort pellets by color so that variations in pellet shades are perceptible. To measure chromatic and palynological diversity accurately, we advocate sample sorting by color first to capture rare pellet colors. The inclusion of pollen volume in calculations of pellet composition ensures representation of the contribution of large pollen grains. Lastly, colors should be reported in terms of their numerical composition (RGB or hexadecimal) to ensure reproducibility and to convey color values accurately to the reader.

AUTHOR CONTRIBUTIONS

C.P.B. and R.R. contributed to conception and design, as well as acquisition and analysis of data. C.A.S. and J.L.J. acquired physical data in the field. S.H., K.C.B.S.S., and M.L.A. identified pollen types to the highest taxonomic level. S.P. conducted preliminary studies on which this work was based. All authors approved the final version of the manuscript.

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OPEN PRACTICES BADGES

This article has earned an Open Materials badge for making publicly available the components of the research methodology needed to reproduce the reported procedure and analysis, and an Open Data badge for making publicly available the digitally shareable data necessary to reproduce the reported results. All materials are available within the article; the data are available at the Dryad Digital Repository (https://doi.org/10.5061/dryad.c866t1g9x).

DATA AVAILABILITY STATEMENT

The data from this study are publicly available at the Dryad Digital Repository at https://doi.org/10.5061/dryad.c866t1g9x (Bailey et al., 2023).

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

Appendix S1. Color chart showing colors assigned to each color group in the study.

Appendix S2. Pollen composition calculated by count and by volume, shown for subsample 006-4.

Appendix S3. Scatterplot of dominant pollen proportion.

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APPENDIX 1 Methods for sorting pollen pellets illuminated by HEVL and visible light.

Supply list

NOTE: Materials and equipment indicated here have been used in our laboratory. Substitutions with equivalent materials can be carried out as required.

1. Sorting container (shown in Figure A1; see instructions below)
   a. Plastic container (31 cm × 22 cm × 5 cm; Quadrant, Melbourne, Australia)
   b. Black matte adhesive vinyl
2. Light box (see instructions below)
   a. HEVL LED strip lights (395–400 nm; KXZM, Ziyang, China)

FIGURE A1 Tray for sorting pollen pellets. The top plastic container is lined with adhesive matte black vinyl. The holes in the top container allow pollen to be collected in the bottom container when gently pushed toward the holes with an artist's fan-shaped brush. The lid keeps the pellets secure inside during transport.

   b. Visible light LED strip lights (400–760 nm; Brilliant Lighting, Rowville, Victoria, Australia)
   c. HEVL LED flashlight (390–410 nm, ≤500 lumen) and visible light LED lamp (500 lumen; Verve Design by Arlec, Scoresby, Victoria, Australia)
3. Dehydrator or drying oven (Food Dehydrator 10 Tray, model no. KA10DHDTIMA; Devanti, Hawthorn, Victoria, Australia)
4. Paper bags
5. Resealable plastic bags
6. Colored silica gel beads
7. Petri dishes or weigh boats at least 100 mm in diameter
8. Paint cards in shades of yellow, brown, orange, green, black, etc.
9. Artist's fan-shaped brush (shown in Figure A2)
10. Featherweight forceps
11. Analytical balance
12. Envelopes or small cardboard jewelry-type boxes
13. Conical vials (5–10 mL)
14. Distilled water
15. Glacial acetic acid
16. Acetic anhydride
17. Sulfuric acid (>98%)
18. Vortex mixer
19. Swing-out centrifuge (ELMI CM-7S Plus Benchtop Centrifuge; POCD Scientific, North Rocks, New South Wales, Australia)
20. Heat block or hot water bath (BS-31 Shaking Water Bath; Wishmed, Blacktown, New South Wales, Australia)
21. Wooden skewers
22. Four to five glass beakers (500–1000 mL)
23. Volumetric pipette and bulb
24. Variable volume pipette (1–10 mL)
25. Two graduated cylinders
26. Glycerol
27. Gelatin powder
28. Glass microscope slides
29. Glass cover slips
30. Variable volume pipette (1–100 μL)
31. Filtered pipette tips
32. Slide warmer
33. Compound microscope equipped with camera (Nikon Eclipse 90i compound microscope; Nikon, Tokyo, Japan)

Instructions to build the sorting container and light box

To make the sorting container, use a box that is big enough to hold an entire sample from a pollen pellet trap and that is made from a material that will withstand repeated washing (e.g., plastic). Line the inside of the box with black matte material and cut 1–2-cm-diameter holes on the bottom of the container toward the edge (see Figure A1 for example). Make several of these sorting containers for increased efficiency.

FIGURE A2 The light box used to illuminate our samples. The size of the box accommodates the observer and provides enough space to sit upright comfortably and maneuver freely to sort the pellet samples (box measured 75 cm deep at the top [60 cm at the bottom] × 60 cm tall × 60 cm wide). In this photo, the HEVL LED light strips are illuminated, as is the HEVL flashlight held in the clamp of the retort stand. Visible on the right is the visible light lamp with adjustable flexible arm used to illuminate the sample in conjunction with the visible light LED strip lights. The low-speed fan pictured was used to cool the observer inside the box. The observer wore eye protection to shield their eyes from HEVL light.

We used a pair of 31 cm × 22 cm × 5 cm plastic containers and cut 1-cm-diameter holes in the bottom of one container. We lined the inside of the container with an adhesive matte black vinyl. The lined container with the holes fits on top of the second unaltered container so that pollen can be sorted in the top container and caught in the bottom container (Figure A1).

To build the light box, use materials that are lightweight but sturdy and construct the box to be large enough to work in comfortably. The box should have five sides (i.e., no back). For both the HEVL and visible light LEDs, affix LED strip lighting to the top, front, and both sides of the inside of the box. Place HEVL and visible light lamps inside the box so that they can illuminate the sorting container. Attach a piece of fabric to the open side of the box to act as the rear wall behind the observer (Figure A2).

> NOTE: We collected pollen from *Apis mellifera* hives at four different blueberry farms, from 24 June to 20 August 2020 in the Coffs Harbour region of New South Wales, Australia (30°17′ 46.5936″S, 153°6′50.8896″E). Pollen pellets were collected from 16 honey bee hives by installing generic plastic ventilated pollen tray collector traps. These traps removed pollen from the corbiculae of forager bees as they entered the hive.

> NOTE: It will take ~95 h to complete a single replicate of this method, assuming a sample with 12 colors (2–3 h for sorting, 3–4 h for acetolysis, 75–80 h for slide preparation, and ~6 h for photography). The slide preparation step includes a 72-h incubation period and can easily overlap with other steps in this protocol to increase efficiency.

Sorting pollen pellets

1. Transfer the pollen pellets from the pollen traps to paper bags and dry in a food dehydrator or drying oven at 40°C for 24 h, then store in paper bags placed inside resealable plastic bags containing colored silica gel beads that will absorb moisture from the air in the bag. Replace and recharge silica gel beads as necessary once they change color.
2. Rename all samples with randomly assigned numbers to blind the sample's provenance from the observer. Enter data into your datasheet using the new blinded sample numbers.
3. Weigh each whole sample using an analytical balance.
4. Transfer each sample to a clean sorting container, making sure not to damage the pellets. (Thoroughly wash and dry the sorting container between samples.)
5. Take sample (in sorting container) to light box.
6. Sort samples first under HEVL light based on the relative strength and shade of fluorescence (Figure A3).
7. Switch to visible light illumination, re-sort samples, if necessary, into homogenous color groups (Figure A3). Switch back and forth between HEVL and visible light illumination until all groups are homogenous in appearance. Assign a color that matches a paint sample card to each grouping.
8. Gently remove pellets from the tray by brushing them toward the holes on the right side of the tray with a nylon artist's fan-shaped brush, where they can be collected either into the bottom container or directly into a weighing vessel or Petri dish. Carefully transfer small groupings of pellets using featherweight forceps when necessary.
9. Weigh each color grouping as they are removed from the sorting tray.
10. Remove three pellets from each color grouping for acetolysis and weigh them also. Transfer acetolysis pellets to individually labeled 5–10-mL vials. Acetolysing multiple pellets from each color group tests the homogeneity of pollen pellets within a color group averaged across the trio of pellets (Figure A4).
11. Place the remaining pellets in each color grouping into a labeled envelope or small cardboard box and store in an airtight container with colored silica gel beads.

Acetolysis

Because the pollen samples were small, reagents were added to the sample in 3-mL volumes at each step, apart from 5 mL of water in the final three rinses.

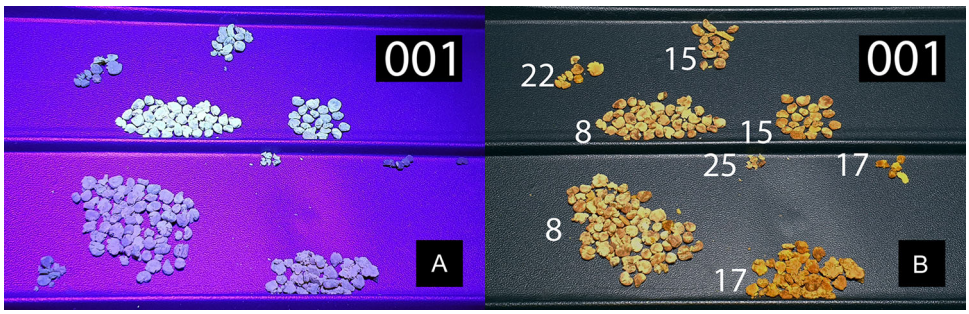

FIGURE A3 Photos of sample 001 under HEVL illumination (A) and visible light illumination (B). Numbers in B relate to the color associated with each grouping of pollen pellets (Appendix S1).

FIGURE A4 Color matching. The three pellets on the paint card were reserved for acetolysis, while the remainder of the pellets were stored in a labeled paper envelope.

1. Prepare the number of vials containing acetolysis pellets that your centrifuge can hold (typically 12–24). Refer also to Jones (2014) when following the acetolysis portion of this protocol.
2. Prepare aliquots of distilled water and glacial acetic acid in separate glass beakers, enough to complete acetolysis for the number of vials you have prepared.
3. To each vial, add 3 mL of distilled water and vortex for at least 30 s to break up the pollen pellets. If the pellets remain intact after vortexing, crush them with a wooden skewer and vortex again. Use a new clean skewer for each sample.
4. Centrifuge vials at 1060 × *g* for 3 min. Discard supernatant.
5. Add 3 mL of glacial acetic acid to each vial and vortex for 30 s.
6. Centrifuge vials at 1060 × *g* for 3 min. Discard supernatant.
7. With separate graduated cylinders or volumetric pipettes, measure out nine parts acetic anhydride and one part sulfuric acid. To a clean dry beaker, add acetic anhydride then slowly add sulfuric acid by tilting the beaker and pouring along the side of the beaker. This is the acetolysis mixture.
8. Add 3 mL of acetolysis mixture to each vial and vortex until the pellet is dissolved. Place vials into a heat block or hot water bath set to 90°C for 5 min.
9. Add 3 mL of glacial acetic acid to each vial and vortex for 30 s.
10. Centrifuge vials at 1060 × *g* for 3 min. Discard supernatant.
11. Add 5 mL of distilled water to each vial and centrifuge at 1060 × *g* for 5 min. Discard supernatant.
12. Repeat step 11 to rinse two more times.
13. Prepare glycerin jelly by dissolving 50 g of gelatin powder into 175 mL of warm water (40–80°C), stirring constantly. Once the gelatin is dissolved, add 150 mL of glycerol. Pour mixture into clean wide-mouth jars and use immediately, or refrigerate for up to 1 month.
14. To each vial containing acetolysis residue, add 3 mL of liquid glycerin jelly warmed to 80°C. Vortex until pellet is dissolved.

NOTE: We suspended the pollen in liquid glycerin jelly because its refractory index is similar to that of sporopollenin and therefore produces photographs with fewer artifacts than other mounting media (Traverse, 2007).

Slide preparation

1. If the samples have cooled since adding glycerin jelly, reheat them to 80°C and thoroughly mix using a vortex prior to mounting on glass slides. Bubbles will form in the glycerin jelly, but will quickly dissipate if kept at 70–80°C. (Omit this step if the samples will be mounted on slides directly after being resuspended in glycerin jelly.)
2. Using a variable volume pipette, pipette 25 µL of sample suspended in liquid glycerin jelly onto a glass microscope slide.

NOTE: We used filtered pipette tips to prevent the viscous liquid jelly from being aspirated into the pipette piston.

3. Cover with a glass cover slip (22 × 22 mm) and place slides upside down on a slide warmer set to 80°C for 72 h to cure. Hold each slide aloft with blank glass slides acting as spacers (spacer slides, Figure A5) under the ends to keep the sample slides from touching the surface of the warmer. While curing, pollen will sink through the liquid jelly to rest against the glass of the cover slip (Figure A5, inset B).
4. After the slides have cured, seal the cover slip edges with nail varnish.

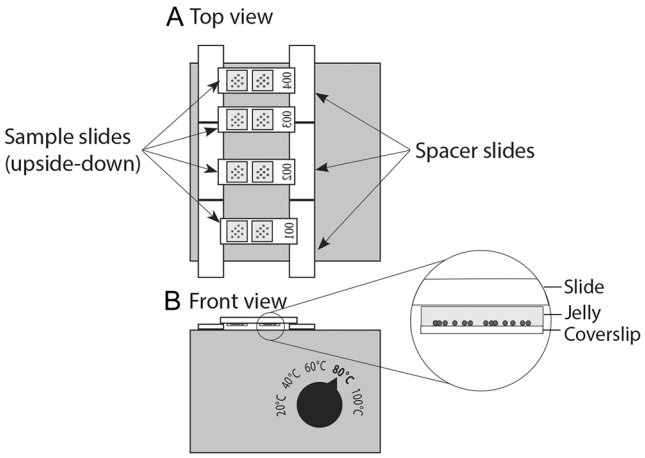

FIGURE A5 Curing slides on the slide warmer, viewed from above (A) and from the side (B). Slides were cured upside down so that pollen grains (small gray circles) sink in the liquid glycerin jelly to rest on the glass cover slip (close-up in B). Spacer slides were used to hold the edges of the slides so that the cover slip did not rest directly on the slide warmer (Traverse, 2007).

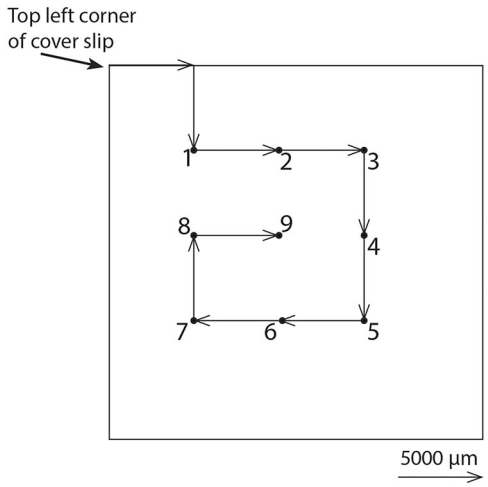

FIGURE A6 Photo sequence for all samples. Starting at the top left corner of the slide, we moved the stage to the right and up by 5000 µm, respectively. The first composite photo was taken at this location. In 5000-µm increments, the stage was moved to the remaining eight positions as outlined in the sequence illustrated here. Each arrow in this figure represents a stage movement of 5000 µm. Cover slip size is 22 × 22 mm.

Photography

1. Take photos of slides using a compound microscope outfitted with a camera. Photos should be taken with high enough magnification to see identifying features of the pollen such as exine morphological features and texture.
2. Make sure to take photos systematically to avoid observer bias. (See Figure A6 for an example.)
3. To allow slides to be revisited for pollen identification as needed, name the digital photo after its position on the slide. Each photo must include a scale bar.
4. Count and measure the photographed pollen using a computer application with count and measuring tools such as Adobe Photoshop or ImageJ (Schneider et al., 2012).

   NOTE: We used the Count tool in Adobe Photoshop 23.4.2 (Adobe, San Jose, California, USA) to tally pollen, and measured pollen using the same application with the Ruler tool to help distinguish between pollen morphotypes.

5. Consult with palynologists to identify pollen types to family level or higher.