Manuscript ID: JoVE62064

Title: Methods for collection and identification of pollen from honey bee colonies

We thank the Editor and the Reviewers for their valuable inputs to improve this manuscript. We addressed all comments with detailed responses in this document. Please note that all line numbers are referenced using the revised document in MS Word with track changes on (view as “all markup”).

Editorial comments

1. Reviewer comment: Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Please define all abbreviations at first use.

Author response: We thank the Editor for this suggestion. We proofread the entire manuscript for spelling and grammar errors.

2. Reviewer comment: Please revise the protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

Author response: We appreciate the Editor’s suggestion. We removed all personal pronouns in the protocol section.

3. Reviewer comment: Please highlight up to 3 pages of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

Author response: We thank the Editor for the suggestions and we reduced our highlighted portions of the protocol to fit the 3-page maximum limit required. However, we needed to remove color sorting (Step 2) and acetolysis (Step 3 and 4) of the protocol to satisfy this requirement. Therefore, our proposed video portion will only include our methodologies regarding pollen trapping (Step 1). The authors would appreciate if the Editor and the Editorial staff have any further suggestions for the video content.

4. Reviewer comment: Please include a scale bar for all images taken with a microscope to provide context to the magnification used. Define the scale in the appropriate Figure Legend.

Author response: We appreciate the helpful suggestions. We replaced the original Figure 7 with a new micrograph in the revised manuscript that includes a scale in the bottom righthand corner. The magnification is 40x. The figure legend has also been edited accordingly (line 491).

Reviewer #1

1. Reviewer comment: Further discuss the problems with pollen trapping in the introduction. What are the limitations and biases of the methods. What about small pollen loads? Nectar only forage sources? How do these affect the interpretation of the results (there was some of this in the discussion, but I would have liked to see an in-depth discussion in the introduction).

Author response: We thank the reviewer for the suggestions. We added a paragraph in the introduction discussing the limitations and biases of pollen traps as well as the other points raised by the reviewer (small pollen loads, bee body size, nectar only foragers, trap style, and hive equipment). Please see lines 91-101 in the revised manuscript.

2. Reviewer comment: Introduce the potential to harm colonies

Author response: We appreciate the reviewer's suggestions and have added additional content in the introduction to our paragraph on potential detrimental effects of pollen trapping. Please see lines 573-584 in the revised manuscript.

3. Reviewer comment: Can also be used to know what plants are blooming, or forage is available in the landscape (quantity, quality, and taxa).

Author response: We thank the reviewer for these suggestions. We added another sentence to our introductory paragraph stating further advantages of pollen trapping. Please see lines 80-82 in the revised manuscript.

4. Reviewer comment: please compare to molecular methods, visual id, pollen trapping, hand picking - a comparison table of time, $, accuracy, and ease would be a fantastic addition, and would help people decide on methods.

Author response: We appreciate the reviewer's suggestions. We created a new table (Table 1) to serve as our comparison chart for these different methods. Please see line 145 for the table placement and lines 83, 143, and 493-496 for reference to table in other sections of the revised manuscript.

5. Reviewer comment: Does line 90 contradict lines 82-83, or have I misunderstood?

Author response: We thank the reviewer for pointing this out. These two contradictory statements were an inadvertent error. We have rephrased lines 115-122 (formerly line 90) to dictate the complementary advantages morphological characterization methods have with color sorting methods and further explained our statement that multiple taxa are found in pollen pellets for clarification (lines 122-129) in the revised manuscript.

6. Reviewer comment: bungee cords can be useful for front mounting traps as well

Author response: We appreciate this fantastic suggestion from the reviewer. We added this information to step 1.3.1 of the protocol section in the revised manuscript. Please see line 188.

7. Reviewer comment: Please discuss how to avoid cross contaminating pollen samples for pesticide analysis. How to clean the traps between samples?

Author response: We appreciate the reviewer's suggestions and protocol steps 1.8.1. and 1.8.2. (lines 224-228) have been revised accordingly. The traps are cleaned by sterilizing in 5% bleach solution. Please see protocol step 1.10. of the revised manuscript for the details (lines 233-234).

8. Reviewer comment: consider adding a trouble shooting section. What if the bees pile up at the entrance? Wet samples? trouble with irrigation? How to store samples? cross contamination? no pollen? too much pollen?

Author response: We thank the reviewer for this suggestion. We added several paragraphs in the discussion section of the revised manuscript to discuss troubleshooting with pollen traps (lines 504-543). Specifically, please see lines 536-538 describing tips to avoid bees piling up at the entrance, lines 509-512, 512-515, and 520-522 for moisture contamination including field irrigation, steps 1.7 and 1.9 (lines 213-214 and 230-231) for proper storage of samples, lines 224-228, 233-234, and 547-549 for cross contamination issues, lines 546-558 for reducing risk of trapping no pollen, and lines 534-541 for reducing risk of trapping too much pollen.

9. Reviewer comment: how much variation in source or quantity is expected among colonies in an apiary? among apiaries?

Author response: We thank the reviewer for these suggestions and we added details discussing both of these statements in lines 85-89 and 562-564 of the revised manuscript.

10. Reviewer comment: If you limit your sample to 10g, please describe options / methods to ensure it is representative of the whole sample. How to homogenise and subsample larger samples?

Author response: We appreciate the reviewer's questions. We clarified step 2.2 and 2.4 of the protocol (lines 240-246) to add further details about removing a representative subsample from the whole. We also expanded our discussion of subsampling in lines 605-617 of the revised manuscript.

Reviewer #2

Reviewer #2 did not request any revisions.

Reviewer #3

1. Reviewer comment, line 89-92: Here it says that color sorting is not reliable because multiple plant taxa are typically found in a single pollen pellet. But the paragraph above discusses the flower constancy of honey bees and how a single pollen pellet therefore typically represents a single plant species. These are contradictory statements. I do agree with the statement that morphological traits of the pollen grain can provide further identification of the plant. The reason, though, is that several different plant species may have similarly colored pollen pellets; the number of distinct colors is limited relative to the huge number of plant species (even though in any one location and time period, the available number of plant species would be much more limited). Morphological characterization can help determine whether two similar shades of a color, for example, are of the same or different plants, to further then facilitate separation by color. And it is of course also needed in order to provide identification of the plant, and not just measuring number and diversity of plant species visited by the bees.

Author response: We thank the reviewer for pointing this out. The contradictions in these two statements were inadvertent. We also appreciate the reviewer's inputs on morphological characterization methods. We have rephrased lines 115-122 (formerly line 90) to dictate the complementary advantages morphological characterization methods have with color sorting

methods in the revised manuscript. We also further explained that multiple taxa are found in pollen pellets to clarify this statement (lines 122-129).

2. Reviewer comment, line 100-102: The same flower constancy issue arises here - do you mean estimating the proportions of different taxa within a single pollen pellet? If a pollen pellet is predominantly of one source, due to the high fidelity of honey bees, can these methods not determine which is the predominant taxon in a pollen pellet?

Author response: We appreciate the reviewer's concerns. Based on the reviewer's suggestions, we added further information explaining polyfloral pollen pellets and justifying the need for methods that estimate the proportions of different taxa within a pellet. This only applies to a few research goals/objectives. Please see lines 122-129 for added content in the revised manuscript.

3. Reviewer comment, protocol step 1.1. What do you mean by ideal conditions? Seems like you refer to ideal conditions for honey bees to be collecting pollen. This is different from the ideal climatic conditions for the researcher to be collecting pollen samples in a pollen trap. For example, high humidity or rainy weather may spoil pollen samples collected if the traps becomes wet and the pollen pellets become soaked and mushy. So on a winter day with several hours of nice sunny weather, followed by a short heavy rainstorm, conditions could be good for honey bees to be collecting lots of pollen, but not good for the condition of the pollen pellets sample in the trap.

Author response: We appreciate the reviewer's concerns. We added further details about selecting the ideal conditions for sample preservation in step 1.1 of the protocol (lines 163-164) and in the discussion section (lines 512-513) of the revised manuscript.

4. Reviewer comment, protocol step 1.2. Here it is also not clear what you mean by optimal colonies. If for example the researcher wants to assess the amount of pollen being collected by colonies in an apiary, they would want representative (average) colonies, not the strongest ones.
Same with 1.2.1.

Author response: We appreciate the reviewer's concerns. Even though we made no changes to our protocol steps (selecting large colonies to maximize the chance of trapping pollen from selected hives), we recognize that this may not be ideal for some research goals/objectives. Based on the reviewer's suggestions, we further discuss troubleshooting in the discussion section. Please see lines 534-536 and 541-543 in the revised manuscript.

5. Reviewer comment, protocol step 1.2.3. I don't understand why install traps on colonies facing same direction. [OK, I see an explanation later in line 440].

Author response: We thank the reviewer for this suggestion and we have now added more details in step 1.2.3. for reducing any potential reader confusion. Please see lines 175-177 in the revised manuscript.

6. Reviewer comment, protocol step 1.7.1 Important to state the duration of time between collection intervals. Colonies weaken if the traps are engaged for too long without recovery periods between engagement intervals.

Author response: We appreciate the reviewer's concerns. We found a published study (Hoover and Ovinge, 2018) that defines part-time pollen trapping as every other week. This recommendation supports a reduced risk of lower honey production when trapping. We added this information in lines 210-211 and 579-581 of the revised manuscript.

7. Reviewer comment, protocol step 1.7.2. Not clear why for diversity one collects for 72 h, but for pesticide residue up to 96h.

Author response: We thank the reviewer for pointing this out. In the original submission, Steps 1.7.1 and 1.7.2. were to serve as recommended collection intervals based on two different research objectives. In Step 1.7.1, we recommend 72 hours for landscape-level nutrition studies based on the findings of Dimou, 2006. Step 1.7.2. concerns pollen collection to analyze pesticide residues. It is standard for pollen to be collected with traps for a minimum of 24 hours if intended to analyze pesticide residues. We list the maximum collection interval as 96 hours to avoid pesticide degradation. We added a reference (Stoner and Eitzer, 2013) for the 96-hour maximum for step 1.7.2 (lines 219-220) to further clarify this in the revised manuscript.

8. Reviewer comment, protocol step 2.4 The instructions here implicitly focus on pollen weight, but pollen pellets number is also often of interest. Pellet number represents the number of bee visits to a particular plant. This would differ from weight, especially if there are systematic differences in weight of pollen pellets from different taxa (due to size, or to the amount of nectar bees added to each pellet during collection).

Author response: We appreciate the reviewer's suggestions, based on which we added an additional step in our protocol (step 2.8, lines 259-262) in the revised manuscript. The new step 2.8 addresses the act of counting the number of pellets in addition to weighing each color group and we further discuss details in lines 109-113. However, assessing pellet numbers to represent the number of bee visits to a particular plant would require recording forager visitation data. As foragers may visit flowers for resources other than pollen, exploring this research direction is beyond the scope of the present protocol.

9. Reviewer comment, protocol step 2.8. What is the "H"?

Author response: We thank the reviewer for pointing this out. The “H” was an inadvertent typographical error. We removed it from step 2.12 (formerly step 2.8) of the protocol section. Please see line 278 of the revised manuscript.

10. Reviewer comment, protocol step Line 431. Which type of pollen trap blocks the hive entrance, all of them?

Author response: We appreciate the reviewer’s concerns. All styles of pollen traps block the hive entrance. We clarified this statement in lines 545-546 in the revised manuscript.

11. Reviewer comment, protocol step Line 434. I agree that they should be given at least 24 h, but in the instructions above you wrote 12-24 h.

Author response: We thank the reviewer for this suggestion. We agree with the reviewer that this presented a continuity issue in the original submission. We changed step 1.6 (lines 205-206) to only list 24 h as listed in lines 549-550 in the revised manuscript.