Insect Preservation Methods Allow Quantification of Dispersing Microorganisms by Scanning Electron Microscopy

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Research Article

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Abstract

Insects may act as dispersal vectors for microbes where microbes can temporarily adhere to insect exoskeletons. Microbes carried by honey bees (*Apis mellifera*) may experience a range of dispersal outcomes, from successful colonisation of a new habitat to predation by grooming bees, partly depending on their location on the bee exoskeleton and the carried population size. I tested four methods for handling collected bees, and examined the bees in a scanning electron microscope, imaging and counting microbial cells attached to tarsal claws. Freeze-dried bees carried more microbial cells than bees that were pinned and air-dried, preserved in 96% ethanol then dried by CO₂ critical-point drying, or bees washed with potassium phosphate then preserved in 96% ethanol, but some microbes were found on bees from every treatment. The similarity in microbial passengers found between air-dried and ethanol-preserved bees suggests that examination by electron microscopy could be used to address questions regarding microbial dispersal by pollinators already present in collections associated with other pollination research.

Main Text

Diverse microbial communities have been found in flowers, short-lived habitats that include unique features such as nectar and pollen. Flowers are visited by insects and other animals, attracted by nectar, and serving to pollinate the plant, and providing a dispersal vector for microbes [1]. Pollinator-mediated dispersal has been studied in nectar yeasts and bacteria in a range of natural and agricultural environments, to address questions regarding infection dynamics e.g. [2], microbe-microbe interactions e.g. [3], and the role of dispersal in structuring patterns of biodiversity e.g. [4].

Studies of pollinator-carried microbes often remove living cells from animals such as birds and insects e.g. [5, 6] for later identification and characterisation. This washing removes information regarding the position and distribution of carried organisms on small animals. The position of a carried microbe on a pollinator's body affects the probability that the microbe will be deposited in a new habitat; microbes on mouthparts and legs are more likely to directly contact plant surfaces or be immersed in liquids such as nectar or water droplets compared to microbes on other body parts.

Insects including pollinators are frequently collected and stored during the course of a wide range of ecological and agricultural studies, and standardised collection and preservation methods are often employed. Insects and other small arthropods may be air-dried, typically on a pin and in a humidity-controlled environment [7] or may be collected directly into ethanol solutions that both quickly kill the animal and prevent decomposition [8]. These processes may dislodge externally-carried microbes or distort the shape of microbial cells beyond recognition. Freeze-drying can be used to dehydrate specimens without altering fine morphological features, and some microbes can survive the process on insect bodies [9], suggesting the possibility that many microbes on insect bodies may not be dislodged or distorted by freeze-drying.

The objective of this study was to test the feasibility of using insects collected and preserved by commonly-used techniques for locating and quantifying exoskeleton-attached microbes with scanning electron microscopy (SEM). SEM is a technique commonly employed by both entomologists and microbiologists and occasionally used to study microbes physically associated with insects e.g. [10].

Honey bees were obtained from a hive infected with American foulbrood; a disease caused by the Gram-positive bacterium *Paenibacillus larvae*. The whole hive was frozen, and adult worker bees were kept at -20 °C until treatment. Twenty bees were prepared for each treatment: pinned and allowed to *air-dry* in a vented cardboard box at room temperature (approximately 20 °C) for at least one week; *freeze-dried*; immersed in *96% ethanol*; or *washed* in 1 mL of 25 mM KH_2PO_4 solution and vortexed for 30 s, then briefly dried on a sterile paper wipe and immersed in 1 mL of 96% ethanol. Ethanol was removed by critical-point drying in CO_2 . All bees except air-dried were kept in sealed individual 1.5 mL microcentrifuge tubes at room temperature until dissection and mounting for SEM. The middle and rear legs from one side of each bee were removed with tools wiped with 96% ethanol and attached to an adhesive carbon disc on a SEM stub. Mounted bee legs were sputtered with gold to coat them. Stubs were arranged in the SEM (Jeol JSM 5800LV, Tokyo, Japan) and images of the tarsal claws were collected at 1000x magnification.

Four bees were removed from the analysis. One air-dried, one freeze-dried and one washed bee were damaged during dissection and poorly positioned on the SEM stub, leading to poor images that did not reach the measurable claw surface area minimum of $5 \times 10^6 \mu\text{m}^2$. One freeze-dried bee showed extensive fungal growth consistent with post-mortem proliferation.

Collected images were analysed using ImageJ [11] to quantify the 2D surface area of tarsal claw visible in each image, and manually counting microbial cells and other features. The total area examined on each bee was equal to the maximum area of an image, approximately $5 \times 10^6 \mu\text{m}^2$. Counts were square-root transformed to better fit the assumptions of a general linear model, conducted in R [12] and R-Studio version 1.4.1717 [13].

All treatments included bees with clearly visible microbial cells present on tarsal claws (Figure 1), as well as some bees without visible cells on examined tarsal claws. Bees that had been washed with KH_2PO_4 had significantly fewer cells than bees in other treatment groups ($p < 0.05$), but there were as many washed bees with cells as there were bees with cells from other treatments (χ^2 , $df = 3$, $p = 0.28$), and one freeze-dried bee had the highest cell count due to a large number of very small bacterial cells (Figure 3A). A possible *P. larvae* cell was found on the claw of an air-dried bee (Figure 3B).

Washing with KH_2PO_4 and similar solutions is used to remove microbial cells from surfaces such as plant leaves and insect bodies without lysing most cells [6]. This study has shown that such washing cannot be considered comprehensive or quantitative, as cells were found firmly attached to the smooth and exposed surfaces of bee tarsal claws, a surface that lacks the morphological complexity of many

other parts of the insect exoskeleton; many cells were observed on other parts of bee legs, often under a protective layer of bristles or within joints between leg segments.

The distribution of microbes between different habitats may be driven by their position on or in the body of the dispersal vector. Microbes attached to an insect's extremities, such as the tarsal claws, antennae, and mouthparts are more likely to dislodge onto surfaces contacted by the insect, such as flower petals, stigmas, anthers, and nectaries – all potential habitats for microbes. In contrast, microbes on other parts of the insect's exoskeleton may instead arrive at hives or nests and be removed by grooming or contact with other surfaces. This raises questions about the efficiency of biovectoring (using honey bees to distribute microbial biocontrols; [14]) and the potential adaptations of microbes that allow access to specialised habitats such as nectar or bee hive interiors [15].

Freeze-drying did not produce images with greater numbers of cells than either air-drying or ethanol preservation and is rarely used to preserve insects for scientific study. Thus, preparation of specimens that have been pinned or stored in ethanol for quantification of external microbes by SEM represents an opportunity to employ vast numbers of previously-collected pollinators to address questions of microbial ecology. The methods used here are well-placed to address such questions and the procedures are simple and inexpensive, using equipment commonly found at universities and other research institutions.

Declarations

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The author declares no conflict of interest. Ethics approval was not needed for this study of insects and microorganisms. No human subjects were involved and consent to participate was not needed.

The author consents for publication. **Availability of Data:** unedited photomicrographs created by the software associated with the scanning electron microscope have been submitted to the University of New England internal cloud server, along with count data. **Code availability:** No bespoke or custom software was developed or used in this study

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Figures

Figure 1

Example SEM images from treated bees, showing microbial cells on tarsal claws in each.

Figure 2

Cells of particular interest are circled. A. Abundant, very small bacterial cells on the claw of a freeze-dried bee. Each cell is approximately 1.6 µm long and 0.5 µm wide. B. A pair of possible vegetative cells of *P. larvae*, approximately 20 µm long and 2 µm wide.