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POLLEN ANALYSES FOR POLLINATION RESEARCH, UNACETOLYZED POLLEN

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Abstract—Pollinators feed on the pollen, nectar, and other plant exudates that are associated with flowers. As a result of this feeding activity, pollen becomes attached to them. Analysis of this pollen can reveal what they eat, their dispersal patterns in and around cropping systems, and their role in pollination. However, finding pollen on and or in a pollinator depends on the technique used to recover pollen. Two very easy techniques are described in detail that have been used to recover pollen from a variety of pollinators including beneficial and harmful insects, spiders, bats, and other pollinators. These techniques can be used to recover pollen from internal tissues (gut, alimentary canal, crop, etc.), external tissues (proboscis, legs, eyes, etc.), or both. By using the proper technique, better pollen recovery can be made and thus better data can be obtained about the pollinators, the foods they eat, the plants they pollinate, their migration routes and source zones.

Keywords: palynology, pollen recovery, pollinators, pollination biology

INTRODUCTION

For years, basic questions about flowers and their pollinators have been asked. Which species pollinate which plants? What can be done to increase the beneficial pollinators within a cropping system, habitat, region, etc.? How frequently do the different pollinators visit different flowers? What is the frequency and duration of visitation? Are single or multiple plant species visited? What times of the day are the different taxa visited? How do genetically modified plants (GMO) affect the pollinators, and their foraging habits? How far do the pollinators disperse GMO pollen? These types of questions can be answered from the identification of the pollen found on/in the pollinators that visit flowers.

Pollinators feed on the nectar, pollen and/or other plant exudates that are associated with flowers. As a result of this feeding activity, pollen becomes attached to them. Those that do not actively feed on nectar, pollen or plant exudates also can become "dusted" with pollen if they walk, crawl, and/or fly around or in the inflorescence stalks and/or flowers in search of food, mates, prey, or shelter. Thus, pollen becomes a natural marker on them indicating the plants on which they foraged and/or visited. The identification of this pollen can be used not only to determine the plant species that is being pollinated but also to determine a pollinator's migration routes, source zones, food sources, the habitats visited, the diversity of the habitat, and diversity of the food sources.

Furthermore, the data obtained from the identification of the pollen found on/in a pollinator are useful in a variety of areas. First, the yield of many crops (apples, almonds, peaches, melons, etc.) depends on and is improved by the pollinators that visit the crop's flowers. Knowing which species pollinate these crops is paramount for a successful harvest. Second, pollinators often feed on the pollen and nectar of flowers from non-crop plants. Having these plants surrounding the fields and orchards enables the pollinators to remain within the cropping system when the crops are not in flower. Third, some pollinators are pests of crops, garden plants, and orchards. Knowing what plants these pests go to besides the crops and if the pests "migrated' from somewhere else is pivotal for accurate management strategies. Finally, the habit, habitat, and life cycles of many pollinators are poorly known. Pollen found on/in them can indicate which plant species are visited, the dispersal distance potential of the pollinator, migration routes, and if the pollinators are visiting and dispersing genetically modified plants.

The literature is full of articles and books that state "methods" used to recover pollen from pollinators. However, the actual steps of these "methods" are usually short, incomplete, and omitted. Too often a technique is cited with no description of the technique at all or only a step or two briefly stated. The researcher is left to try and figure out each step, determine what steps were left out or implied, and the sequencing of the steps. This is frustrating, time consuming, and costly when a technique has to be tried and tried again to achieve the results needed.

The purpose of this manuscript and future manuscripts is to provide detailed, step-by-step techniques that recover pollen from any pollinator. These techniques have been used to recover pollen from a variety of insects (beetles, moths, butterflies, stink bugs, wasps, flies, stingless bees, honeybees, etc.), spiders, and bats. They can be used to recover pollen from the whole pollinator, the pollinator's internal (alimentary canal, midgut, crop, etc.), and/or the external tissues (the proboscis, head, feet, fur, etc.). These techniques

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were originally described by Jones (2012). However, they were written for palynologists who were familiar with the techniques. As such, they were written with some of the steps left out that the palynologists automatically understood. This and the future manuscripts fill in those gaps and provide step by step instructions for non-palynologists so that anyone can recover pollen from any pollinator. The manuscripts are not designed to teach pollen identification nor pollen data interpretation.

POLLEN CONTAMINATION

Pollen contamination is something that can be a problem with any sample, in any laboratory, and with anyone working with pollen. Pollen can enter a laboratory from open windows, ventilating systems, open doors, dirt, clothing, hands, etc. The pollinator can be accidentally contaminated with pollen when collected in traps, by sweep nets, aspirations, mist nets, or sitting in a trap waiting to be collected. Pollen contamination can occur due to unclean equipment such as dissecting pins, forceps, microscope stages, glassware, slides, etc.

It is important to be aware of any possible pollen contamination and to examine the laboratory thoroughly prior to any pollen research making sure that it is as free of pollen as possible. One way to determine if a laboratory has pollen contaminants is to place several glass slides on the counters, tables, under air conditioning vents, and near windows that have a drop or two of glycerin on them. Let the slides sit for a week. Add one drop of Safranin O stain (see staining pollen grains for light microscopy for making the stain) to the glycerin and mix well with an applicator stick. Cover the drop with a cover slip and seal (see slide preparation). Once the nail polish is dry examine the slide. If the slides are full of pollen, either wait for a couple of weeks and retest the laboratory or move to another laboratory that is pollen free. During the major pollen seasons (spring and fall), it is a good idea to double check the laboratory to ensure that there are no pollen contaminants coming into the laboratory. Other things that help prevent pollen contamination are to keep the laboratory as clean as possible, wear clean clothing, wash hands, keep all doors and windows shut especially while working with a sample. If necessary put cheesecloth or a filter over the air conditioning vents. The addition of an air purifier also helps as long as the filters are frequently changed. All collecting and dissecting equipment should be rinsed with ETOH prior to use, in between each use, and stored in pollen free areas or containers when not in use. Whenever pollinators are dissected, pollen contamination can easily occur if the insect is dissected in a liquid or on any substrate due to the tearing of the tissue. Pollen can contaminate not only the dissected pollinator but also all future pollinators unless the dissecting substrates are kept very clean.

LIGHT VERSUS SCANNING ELECTRON MICROSCOPY

Bryant et al. (1991) found that pollen was accurately separated from detritus 84.6% of the time when using a dissecting microscope but only correctly identified 15 % of the time. This indicates that a dissecting scope is neither adequate for determining the presence/absence of a particular type of pollen grain nor for the identification of pollen. Although a dissecting microscope can be used to remove pollen from a pollinator, it should never be used for any pollen identification.

There are two main types of microscopes that should be used for the pollen analyses of pollinators, a compound light microscope or a scanning electron microscope. Each type of microscope has its own pluses and minuses. Jones and Bryant (2007) detail many criteria for using either light (LM) or scanning electron microscopy (SEM). Light microscopy is more frequently used for pollen analyses regardless of the discipline. Because LM is the main microscope used for pollen analyses, there are many atlases, books, micrographs of pollen and pollen reference collections available on line and published that show pollen grains taken with LM (Roubik & Moreno P. 1991; Reille 1992; Herrera & Urrego 1996; Kapp et al. 2000; Beug 2004; to list only a few). The availability of the books, atlases, publications, and on line collections is a real plus when trying to identify unknown pollen grain or examining samples of a foreign origin. Another major advantage of using LM for pollen analyses is ease of access. Many laboratories contain a LM and it is available at any time day or night. If no one else uses the microscope, a glass slide can be left on the microscope's stage overnight or until the researcher returns to finish examining it.

Unfortunately, pollen identification can be more difficult with LM because of the lack of resolution compared to a SEM. Jones and Bryant (2007) found differences in the pollen diversity when the same sample was examined with LM and SEM. In their first count, they found 22 taxa with LM and 40 with SEM. This difference in the number of taxa was due in part to the increased resolution of SEM. When using SEM, they found multiple types of taxa that were not found when using LM. When the differences in the taxa were carefully examined, some of the differences could also be seen when they re-examined the samples with LM. However, some of the differences could not be seen with LM, and could only be seen with SEM.

A scanning electron microscope is better for seeing fine detail and differences among pollen grains because of the resolution of the image is increased (Jones & Bryant 2007). Regrettably, SEM can be expensive, may not be available to researchers, and may be time consuming if training is needed to operate the microscope. When using SEM, the pollen grains are "fixed" in one position and cannot be moved. Therefore, often the diagnostic characteristics of the pollen grains, such as the number of apertures (colpi or pores), and the aperture arrangement are not always visible. In addition debris can hide much of the pollen grain preventing possible identification.

When having to use the SEM repeatedly to count and identify the pollen grains of a single sample it can be impossible to replace the specimen in the exact location each time it is put into the SEM. When the sample is not in the exact same place each time, the same pollen grains can be counted multiple time and taxa missed. When this occurs, data becomes worthless. Jones and Bryant (2007) did figure

a way of marking the SEM stubs so that they could be replaced in the exact spot each time. Still, it takes additional time and additional SEM expertise to ensure that the specimen is in the same exact location each time.

Another drawback to using SEM for pollen identification and analyses is the lack of pollen micrographs photographed with SEM and the lack of SEM reference collections and material. Some modern palynological textbooks and atlases include SEM micrographs of pollen along with the LM micrographs (Ogden et al. 1974; Bassett et al. 1978; Nayar 1990; El-Ghazaly 1991; Moore et al. 1991; Moar 1993; Bingshan 2005). Only a few books are dedicated entirely to presenting SEM micrographs (Adams & Morton 1972, 1974, 1976, 1979; Bambara & Leidy 1991; Jones et al. 1995; Zhongxin 2003).

Historically, LM has been used to examine pollen from the internal tissues (gut, alimentary canal, crop, etc.) while SEM has been used to examine pollen from external tissues (legs, eyes, mouth parts, body, etc.). For example, most of the pollen analyses of boll weevils (*Anthonomus grandis* Boheman) examine the pollen found in the internal tissues (Benedict 1991; Jones et al. 1993; Hardee et al. 1999; Jones & Coppedge 1999; Greenberg et al. 2007, 2009). However, Jones & Coppedge (1998) used SEM to examine exoskeleton of boll weevils. They found a different pollen assemblage when examining the exoskeleton than what had been previously reported when the internal tissues were examined with LM.

Conversely, since moths and butterflies feed on the nectar, pollen adheres to their eyes, proboscis, and legs. Therefore, historically the external parts are examined with SEM. Jones & Lopez (2001) examined the crop of adult corn earworm moths (Lepidoptera), *Helicoverpa zea* (Boddie). They found that the crop contained different pollen grains from what had been found externally.

Unfortunately, except for some pollinators like honeybees and other Hymenoptera, there is usually not enough pollen recovered from a single pollinator to use both LM and SEM for the pollen analyses. If two or more samples are lumped together to make one "mega-sample", enough pollen can be recovered to use both LM and SEM. Lumping samples together increases the number of pollen grains recovered and the pollen diversity. The greater the number of samples examined, the greater the number of pollen grains recovered, and the greater the diversity of pollen taxa. However, combining the samples together prevents obtaining data per individual.

The decision whether to use LM, SEM, or both must be made prior to starting any pollen work. The techniques for LM are slightly different from those for SEM. When the samples are acetolyzed (chemically processed), some of the pollen residue can be placed onto SEM stubs at the end of the acetolyzation technique. However, if the decision is made to use SEM after the samples have been analysed, additional time and chemicals are needed to prepare the sample for SEM analyses. Any time a sample is re-prepared, there is a possibility of losing pollen. Similarly, if the samples are examined with SEM and at a later date it is decided to use LM, it is almost impossible to remove the coating needed for SEM from the pollen and then examine the those pollen grains with LM.

Regardless of the type of microscopy used, it is important to have a good quality microscope, a good photographic system, the knowledge to align the lenses, and the expertise to keep the lenses and other mechanical aspects of the microscope clean and free of debris and fingerprints. Debris, fingerprints, and other contaminants not only affect the quality of the subject in view but also the micrographs. What is seen as in focus while viewing a pollen grain may be actually out of focus when it is photographed due to the objectives being out of alignment or unclean, or contaminated with debris that interfere with mechanics of taking the micrograph.

For most part, when using LM, the objective lenses needed range between 10-100X. If the budget allows, an oil immersion lens or high-dri lens of 80-100X can be beneficial. For SEM, magnifications of 2000X are used for most pollen grains. To view and photographs larger pollen grains and pollen grains that are dispersed in clusters, such as *Acacia*, a magnification of 200X is sufficient. Smaller pollen grains and high magnification micrographs of the pollen's ornamentation need a resolution up to 7,500-10,000X.

INTERNAL VERSUS EXTERNAL POLLEN

Pollen can be recovered from the digestive tract (gut, alimentary canal, crop, etc.) (internal) or from legs, eyes, mouth parts, body, etc. (external). Determining whether the internal or external pollen or both will be investigated depends mainly on the questions being asked, the time available, availability of microscopes (LM or SEM), and the techniques used to recover the pollen.

Some pollen grains pass through the digestive tract whole (boll weevils, Cate and Skinner 1978) while others become cracked, folded, and collapsed (Roulston & Cane 2000). Cracked, folded, and collapsed pollen grains are more difficult to find and to identify. Pollen loss can be due to the destruction of the pollen grains from the actual mechanism of the digestive tract, mouth parts, or manner of feeding (Roulston & Cane 2000), digestive enzymes (Scott & Stojanovich 1963; Turner 1984; Rickson et al. 1990) or due to the pollen grains itself. Some pollen grains are thin walled or are more fragile than others. Cotton (*Gossypium hirsutum* C. Linnaeus) pollen for example is a very large pollen grain with long processes. However, cotton pollen easily breaks. Thus it is more often found in pieces rather than whole. In some cases, osmotic shock causes a pollen grain found internally to burst, reducing the possibility of finding and identifying the pollen grain (Kroon et al. 1974).

Todd & Vansell (1942) found that the number of pollen grains decreased the longer nectar remained in a bee's stomach (internal). Whole almond pollen grains were found in a boll weevil's gut for 96 h (Jones et al. 2007) and cotton pollen fragments as long as 120 h (Jones & Greenberg (2009). *Lycopodium* spores were found in the crop of corn earworm moths for three days (Westbrook et al. 1998).

Although Mikkola (1971) suggested that an examination of the Lepidoptera digestive system for pollen might be useful in the study of lepidopteran feeding. The idea that pollen can be drawn into the digestive system at the time of feeding is not frequently examined. Turnock et al. (1978) found pollen within the proboscis tube of Lepidoptera but did not examine the crop (insect organ that is the receptacle for food) or other parts of the digestive system for pollen. Jones & Lopez (2001) examined the crop of adult corn earworm moths and found a difference pollen assemblage between the pollen found internally and externally. This is not surprising and should be expected because of the nature of where the pollen is located.

External pollen is lost due to mechanical issues including flight, abrasion on flowers, flower parts, and plant parts, etc. *Colias eurytheme* (Boisduval), alfalfa caterpillar, lost 51% of *Phlox* spp. pollen grains after coiling and uncoiling its proboscis (Levin & Berube 1972). Asteraceae pollen was the most common taxa found on corn earworm adults (external) (Lingren et al. 1993; 1994). However pollen grains with processes (e.g. Asteraceae) were found less often on hawkmoths (Kislev et al. 1972).

Sonication is commonly used in palynology (Vaissiére 1991; Dafni 1992; Kannely 2003, 2005). Vaissiére (1991) and Dafni (1992) suggest soaking the pollen in acetone for 30 minutes prior to sonication. However, Kannely (2003, 2005) found that pollen would be released from lily anthers when the anthers were placed in distilled water and sonicated at 20 um amplitude for 12-15 s. How easily external pollen can be removed from a pollinator's exoskeleton or body parts when sonicated is the subject of future research.

SLIDES AND COVER SLIPS

The thickness, length, and width of slides and cover slips vary greatly. The stage of most LM microscopes easily handles a 15 X 75 mm glass slide or larger. The thickness of the slide and the cover slip make a big difference in the quality of the LM micrographs and the micrograph resolution. The thicker the slide and/or cover slip, the poorer the resolution because the more glass the transmitted light goes through, the more distorted the light becomes. Thicker slides and cover slips often prevent the use of oil immersion lenses because oil immersion lens are longer than 20 and 40X lenses and will hit the thicker slide or cover slip. For the best photographic results, slides should have a thickness of 0.93 - 1.05 mm. This thickness is thick enough to not easily break and yet thin enough for light to travel through it with a minimal amount of dispersion (Moore et al. 1991). An 18 mm square cover slip easily fits onto a 15 X 75 mm glass slide so that the slide can be labeled and the cover slip sealed to the slide. Cover slips should be a number 1 or 1.5 with a thickness of between 0.13 to 0.19 mm. Thinner cover slips are usually too fragile and brake easily, especially when tapping down on the cover slip to flatten any tissues below it. Thicker cover slips hold up better, but are not as good optically due to their thickness (Moore et al. 1991). Be sure to check the thickness of the slides and cover slips prior to purchase.

Slides and cover slips should be made of glass, not plastic. For the best identification and photography possible, the slide and cover slip must have a good refractive index (RI). Plastic slides and cover slips do not have as good of a refractive index as glass and scratch easily. The RI of air is 1.0, water is 1.34, glycerin is 1.41, Pyrex glass is 1.47, and most clear polycarbonates used for making plastic cover slips are 1.58-1.56. For optimal microphotography resolution, the indices of the slide, cover slip, and medium should match as closely to each other as possible. Large differences among the RI indices cause the loss of fine detail and resolution. When identifying pollen grains, resolution is of the upmost importance. Without good resolution, small details that separate one taxon from another cannot be seen.

Regardless of the manufacture claims, all slides and cover slips need to be cleaned just prior to use. Even "pre-cleaned" slides have a layer of oil on them that must be removed prior to use. If not cleaned, the slides often have debris on them that interfere with sealing the cover slip to the slide and any pollen identification and microphotography. The easiest way to clean slides and cover slips is to soak a clean lint-free towel or tech wipe with 95 -100 % ETOH and wipe the slide clean. For slides, it is easiest to hold one side of the slide and clean the other side. The specimen and cover slip are put on the cleaned half and the label on the other half. Place cleaned slides onto a clean cloth and cover with another clean cloth until use.

STAINING POLLEN GRAINS FOR LIGHT MICROSCOPY

The characteristics of pollen that differentiates one taxon from another are usually very subtle and often difficult to see through a compound light microscope (Fægri & Iversen, 1989). Fresh pollen is usually a light yellow color that is often difficult to separate from debris. Thus, pollen is usually stained to increase the contrast for microphotography and identification. The exine (outside layer) of the pollen grain absorbs certain stains. Pollen grains with a thick exine, such as cotton, absorb more stain than pollen grains with a thin exine, such as members of the Poaceae (grasses). When stained, the pollen grains are easier to see even at low magnification. Pollen grains that are fragmented or crumpled can be easily missed unless they are stained.

There are many stains available that increase the contrast of a pollen grain. These stains include but are not limited to methyl-green, fuchsin, nigrosin, and others (Wodehouse 1959; Beattie 1972; Moore et al. 1991; Kearns & Inouye 1993). However, Safranin O (Green 1991) is the "preferred" stain for palynological uses (Wood et al. 1996). Safranin O comes in a crystal form and easily dissolves in water and ETOH (Green 1991). It stains the pollen grains pink to red depending on the type of pollen and amount of stain. The pink/red color is pleasing to the eye, stands out, and has good contrast for microphotography (Fig. 1). In addition, most optical systems are corrected for green light and it is thought that the definition is best when the sample is a reddish-orange color (Traverse 1988).

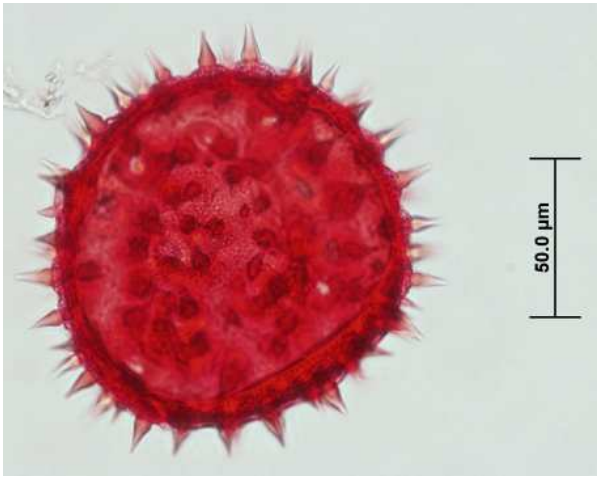

FIGURE 1. Cotton (*Gossypium hirsutum* C. Linnaeus) pollen taken with a compound light microscope. Bar = 50 micrometers. The ring of pores that occur near the polar end of the pollen grain can be seen in the micrograph.

Since Safranin O comes in crystal form, a stock solution should be made up prior to its need and kept ready. The amount of Safranin O crystals used to make the stock solution depends on how light or dark the stain is desired. Additional stain can always be added if the pollen grains are too light. To lighten pollen that is too darkly stained, bleach can but used but it will easily destroy the pollen. An easier was to lighten over stained pollen grains is to rinse them two to four or more times with 95% ETOH. To make up the stock solution, add 1 g of Safranin O crystals to 100 ml of 50% ETOH. To make a lighter stain, add 1 g of Safranin O crystals to 100 ml of 25% ETOH. Mix well and let it sit for 2-5 minutes. To make a 50% ETOH solution, add 50 mls of 100% ETOH to a graduated cylinder or beaker, then add 50 mls of distilled water and stir gently. To make a 25% ETOH solution, use 25 mls of 100% ETOH and add 75 mls of distilled water.

Normal tap water is not suitable to use for pollen analyses because tap water often has pollen in it. For all pollen work, use distilled or RO water. It would not hurt to double check the distilled water and RO water from time to time making sure that they are still pollen free.

GLYCERIN-STAIN STOCK SOLUTION

To make the glycerin-stain stock solution, fill a 2-dram vial with 10 ml of glycerin. Using a pipette, add 2-5 drops of Safranin O stock-stain to the 2-dram vial that has the glycerin in it. Mix well with a wooden, applicator stick, and then throw both the pipette and the applicator stick away. Screw the cap onto the 2-dram vial tightly and set aside. The more Safranin O stock-stain that is added, the darker the pollen grains will be stained. If the cap is tight, the glycerin-stain will last for years.

SEALING THE COVER SLIP TO THE SLIDE

Clean the slides just prior to use. Prepare the sample and place the sample onto one side of the slide. Putting the sample onto one side of the slide leaves enough room on the other side of the slide to add a label. There are a number of sealants that can be used. Two of the easiest sealants to use are nail polish and VALAP (McGee-Russel & Allen 1971). Both have their advantages and disadvantages. Nail polish is quick, inexpensive, and easy to find because it can be purchased anywhere at any time. VALAP is a wax-based sealant that dries very quickly and is easy to remove from the objective lenses of the microscope. Nail polish takes longer to dry and is very difficult to remove from the objective lenses. VALAP dries faster than it can be applied, takes longer to prepare, and must be kept in liquid form during the sealing stage. Over the years, many different types of sealants have been tried but fingernail polish remains the sealant of choice for pollen work.

When sealing the cover slip to the slide, first put a drop of the sealant on each of the four corners of the slide. Each drop should be on both the cover slip and the slide. This will prevent the cover slip from moving when the rest of the cover slip is sealed to the slide. Now, paint the peripheral edge of the cover slip with the nail polish/sealant allowing the nail polish/sealant to go onto the glass slide. If the drop of glycerin is too large, part of the glycerin with pollen in it will ooze out under the cover slip. This makes sealing the slide difficult because the nail polish will go on top of the glycerin and will not make contact with the surface of the slide. If the drop is too small, the nail polish will wick under the cover slip causing air to be trapped under the cover slip. Allow the nail polish/sealant to dry and then paint another coat over the first one.

Nail polish can be any color and any type. Some brands work better than others. Often pollen grains will spread under the sealant so using a clear top coat nail polish allows those pollen grains to be seen, identified and photographed. If using fingernail polish, make sure that it is dry before examining the slide with LM. More than one slide of the pollen residue will be needed to obtain a good representation of the pollen diversity for pollinators that do not collect large amounts of pollen.

If the cover slip is not sealed properly onto the slide, or if the material under the cover slip had extended beyond the cover slip prior to sealing, the slides will "weep". This means that some of the material underneath the slide will seep from the cover slip onto the slide or the upper edge of the cover slip. To fix this, take a dental wick and squirt 95% ETOH onto it and wipe the slide off where the liquid has seeped. Let dry and then reseal the cover slip to the slide.

FINDING AND DOCUMENTING POLLEN

How much pollen is recovered depends on the pollen retention in or on the pollinator, the habits of the pollinator, and the characteristics of the pollen grains. The potential for finding that pollen depends on the amount of the sample examined and the number of pollen grains actually counted (Traverse 1988). Pollen retention on/in a pollinator correlates with type of pollen grain, the amount of lipids on the pollen grain's surface, the ornamentation, size, and for ingested pollen, the strength of the pollen wall and the mechanisms of the pollinator's feeding and digestion.

Finding those pollen grains or fragments also depends on the type of microscope used, the person examining the sample, and the technique used to obtain the pollen (Bryant et al. 1991). Pollen grains that are not in "pristine" condition or that are folded, winkled, or broken add to the difficulty in finding and identifying them.

The following technique is for LM, is an easy technique to follow, and prevents pollen grains from being counted multiple times or being omitted. The addition of a didymium filter to a compound light microscope increases the color saturation intensity of brown, blue, and red-stained objects. If the pollen grains are stained with a red stain, the didymium filter makes them more easily seen.

First, clean the slide and the cover slip by wiping them with a dental wick that has been squirted with a small amount of 95% ethyl alcohol (ETOH) and allow them to dry. Next, put the slide onto the LM microscope stage and "clamp" into place. To scan for pollen use a 20X objective lens. If an objective lens is used of lesser power, pollen recognition is more difficult. If an objective lens of a higher power is used, it takes longer to scan the entire slide for pollen. However, when examining pollen from bees/bee pellets, or other pollinators that "collect" lots of pollen, use the 40X objective lens because of the high number of pollen grains.

A compound light microscope reverses the image so this technique is written as if one is looking through the microscope. While looking through the microscope, move the slide so that the upper left corner of the sealant or fingernail polish can be seen. This will be the starting point for scanning the entire slide for pollen. Before looking for any pollen, slowly move the slide to the right making sure that the sealant or fingernail polish is in the upper part of the field of view. Adjust the slide so that the sealant/fingernail polish is always at the top. Then move the slide back to left. Now start looking for pollen grains by slowly moving the slide to the right and focusing up and down through the slide (through focusing). Be sure to check the sealant/fingernail polish for pollen. Continue moving the slide to the right until the next corner comes into view or the sealant/fingernail polish is the only thing in the field of view. Look for and photograph any pollen grains along the way. Once the other corner or sealant/fingernail polish is in view, move the slide down one field of view by finding a small object at the very bottom of the field of view, then moving that object up to the very top of the field of view. Now, move the slide to the left until the sealant/fingernail polish on the other side comes into view. Move the slide down one field of view and go back to the right. Repeat this process until the entire slide has been examined. Remember to check the fingernail polish for pollen. This is especially important if clear nail polish is used. This technique is also used for finding pollen with SEM.

It is important to document each pollen type or what is thought of as a different pollen type by photographing it (Fig. 2). When a pollen grain is found, change the objective lens to a higher power lens if necessary and photograph the pollen grain. When using light microscopy, take four to six different views of the pollen grain. If the pollen is in a medium, like glycerin, the pollen grain can be rolled around so that the different diagnostic features can be seen and photographed. Light micrographs should include the entire grain in equatorial and polar view, the surface ornamentation on the top and the bottom of the pollen grain, the pollen grain in the "center" of the focus plane, any pores or colpi, and any unusual ornamentation such as the processes found on Malvaceae (cotton family) and Asteraceae (sunflower family) pollen.

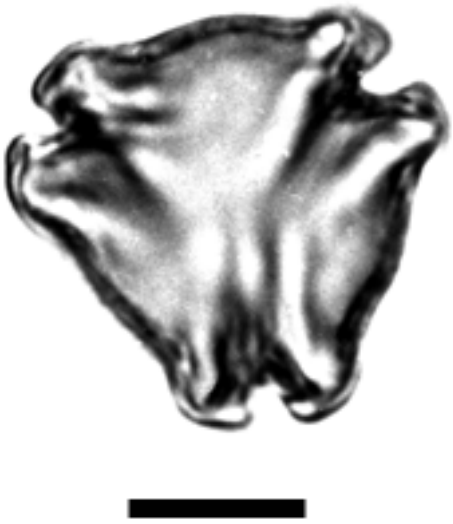

FIGURE 2. Flowering dogwood (*Cornus florida* C. Linnaeus) pollen taken with a compound light microscope. Bar - 20 micrometers.

For SEM, the pollen grains cannot be rolled around because they are fixed to the stub. Therefore, it is impossible to obtain the equatorial and polar view of the same pollen grain. When using SEM, take at least three micrographs; one of the entire pollen grain, regardless of its position, a close up (higher magnification) of the surface ornamentation, and any pores or colpi. Like microphotography with LM, include micrographs at a higher magnification of the processes or unusual ornamentation.

Each pollen type should be given a unique name or number so that it can be separated from other similar looking grains. This name/number, its characteristics (ornamentation, aperturation, size, shape, etc.), project number, slide/stub number, micrograph number(s), and microscope coordinates need to be recorded onto a data sheet. Once photographed, the pollen micrograph needs to be printed. Trying to identify pollen from LM or SEM micrographs while viewing them on a computer can be challenging because of trying to hold and look at books, reference material, other slides, other pollen grains in the project, etc. while sitting in front of a computer. Each printed micrograph should be labeled with its unique name or number, and the rest of the data recorded on the data sheet. Once labeled, the printed micrographs and data sheets can be placed into a notebook. This notebook becomes the photo representation of all the pollen types seen within the project. From the micrographs in this notebook, all of the pollen types can be compared with pollen grains within the

project, and compared with pollen grains found in atlases, on line, or in various pollen reference collections.

LIGHT MICROSCOPY TECHNIQUES

The following techniques, Light Microscopy Examination of Internal Tissues (LM1) and Light Microscopy of External Pollen (LM4) (Jones 2012) are easy to use, do not require a fume hood or specialized equipment (other than a compound light microscope), and do not use caustic chemicals. They can be conducted in any pollen free laboratory. Regrettably, these two techniques are the weakest for pollen recovery and pollen identification because the pollen grains are not acetolyzed and because any tissue put onto the slide can interfere with finding and identifying the pollen grains. Acetolyzation is a chemical process that removes the lipids from the pollen grains so that the surface ornamentation and fine detail of the pollen grain can be seen more easily. Acetolyzation techniques and SEM techniques will be covered in future articles. Since the slides made from these two techniques are in a liquid medium (glycerin), the slides need to be stored horizontally. If they are stored vertically, the glycerin will move down to the bottom of the slide.

LIGHT MICROSCOPY EXAMINATION OF INTERNAL TISSUES (LM1)

Not only can pollen from the internal tissues (crop, alimentary canal, mid-gut, etc.) of small pollinators such as insects, spiders, etc. be examined using this technique but also small amounts of excrement, honeybee pellets, and pieces of a hive or storage areas. If the presence or absence of pollen is the only thing that is needed, this technique is quick and easy (Jones 2012). Basically, the tissue or a small piece of the sample is put into a medium for microscopy, covered with a cover slip, sealed, and examined (Tab. 1). This technique has been used to recover pollen from insects such as the adults and larvae of the eucalypt nectar fly (Diptera) (Nicolson 1994), the spotted maize beetle (Melyridae) (Human & Nicolson 2003), ladybird beetles (Coleoptera: Coccinellidae), (Nalepa et al. 1992; Triltsch 1997, 1999), earwigs (Dermaptera), (Boukary et al. 1997), Systellognathan stoneflies (Plecoptera) (Tierno de Figueroa & Sánchez-Ortega 1999), hoverflies (Hickman et al. 1995; White et al. 1995; Wratten et al. 1995; Bowie et al. 1999; Irvin et al. 1999), syrphid flies (Ssymank & Gilbert 1993), and long-tongue flies (Diptera, Nemestrinidae) (Manning & Goldblatt 1996). The medium that is used varies from author to author. However, glycerin is easy to use, non-toxic, relatively inexpensive, and has good RI for light microscopy.

When looking at pollen pellets, excrement (Dettmann et al. 1995; East 1995; Quin et al. 1996; Van Tets 1997; Wester 2010), storage, or hive material, dry the sample prior to examination. Once dried, weigh the entire sample, and then weigh the piece that is going to be examined.

1. Clean slides and cover slips and set aside. Place a clean lint-free cloth over them if they are not going to be used immediately.
2. Using a clean glass slide, place one drop of the stock glycerin-stain onto the right side of it using either a pipette or an applicator stick. Throw the pipette or applicator stick away to prevent pollen contamination of the glycerin-stain.
3. Dissect the tissue from the pollinator, or remove a small amount of excrement, pollen pellet, or storage hive/comb from the original sample.
4. Place the tissue/excrement/bee pellet into the glycerin-stain drop on the glass slide and stir gently for a couple of seconds. For excrement, pellets, etc., stir for at least 5 s to break it up. Make sure that the drop is not enlarged so much that the cover slip will not cover the drop.
5. Place a cover slip over the drop. When covering the drop with a cover slip, place the cover slip at an angle to the drop and slowly lower the cover slip onto drop. The cover slip should touch the glycerin prior to being horizontal on the drop to remove any air bubbles that may have occurred.
6. Seal the cover slip following the instructions in "sealing the cover slip to the slide".
7. Once the fingernail polish has dried, paint another coat of fingernail polish over the first coat.
8. Store slides horizontally.
9. Prior to LM examination, take the eraser end of a pencil and gently push down and tap the cover slip over the tissue to flatten the tissue. Be careful not to push down too hard because the glass cover slip can break.

LIGHT MICROSCOPY EXAMINATION OF EXTERNAL POLLEN (LM4)

External pollen can be removed from specimen in a variety of ways. First, external pollen can be removed from specimen by combing or removing it manually with a probe, insect pins, forceps, or a camel hair brush. Insect pins are finer than a probe or an applicator stick and are easy to work with. Pollen was removed from the bodies of Lepidopteran insects by gently removing the grains with a dissecting needle, putting the pollen onto glass slides, and then mounting them in Calberla stain (Ogden et al. 1974; Goldblatt & Manning 2002). Pucci and Jones (2010) used this technique to examine the pollen from museum specimens of *Agathirsia* (Braconidae: Agathidinae) wasps. However, they acetolyzed the pollen after it was collected.

This is a good technique for mammals such as bats, birds, rodents, etc. It usually takes two people to comb a larger pollinator. One person is needed to hold the pollinator and the other to comb the pollinator or remove the pollen. A comb or a camel hair brush will not harm a live pollinator but a probe or insect pins creates a risk of injury to the pollinator. Flea combs work well for pollinators like bats that have fur, and camel hair brushes or forceps are the easiest to use for pollinators that have feathers. Prior to using a comb or camel hair brush, rinse it several time in 95% ETOH and then dry it with a clean lint free cloth, and re-

clean it in between samples. As one person holds the pollinator, the other person gently runs the comb or brush through the fir, feathers, or on the bill. Hold waxed paper under the area that is being "brushed" with one hand and brush the pollinator with the other. Have the pollen and debris fall onto the waxed paper. Small manila envelopes can also be used but they have a small opening into which the pollen and debris must fall, so sometimes allowing the pollen and debris to fall on the waxed paper is easier. Often pollen will stick to the brush or comb so it needs to be wiped off in the waxed paper and then cleaned in between samples. When the combing has been completed, fold the waxed paper up so that the pollen and debris are in the center and place the folded waxed paper into a labeled envelope for storage. Store the envelopes in a cool dry area. If there is no interest in pollen taxa or diversity collected at the locations of the pollinator, one sheet of waxed paper can be used for a single sample. However, if information is needed about where the most pollen was found, use different sheets of waxed paper or envelopes for the different parts of the pollinator. Often areas around the face, wings, "arm pits", stomach, and feet are the best places for finding pollen.

Second, pollen can be removed from a pollinator by dabbing the pollinator with glycerin jelly or agar or holding a live pollinator and wiping it with a filter paper. Beattie (1971) made glycerin jelly that was stained with basic fuchsin and dabbed it on the insects. The jelly was melted and the pollen examined. Similarly, Motten (1986) used 1 mm^3 agar cubes and dabbed them on the insects. The agar was dissolved and mixed in a drop of lacto-phenol with cotton blue stain. It is often best to squirt a little 95% ETOH on the filter paper prior to using it. If the pollinator is alive, make sure that the area on the pollinator dries before releasing it.

Third, various types of wipes or filters can be used to remove pollen from a pollinator. To examine the pollen from the tongue of live hawkmoths, Haber (1984) passed the tongue through a folded strip of filter paper. The pollen that was removed was transferred to glass slides that contained polyvinyl lactophenol with cotton blue stain. This technique can also be used for removing the pollen from the bill of hummingbirds and other birds. Adding ETOH to the filter paper just prior using it, will remove more pollen than if the filter paper was dry.

Baby wipes are frequently used to remove pollen from various substrates like furniture. If the wipes are not acetolyzed, they need to be rinsed several times in distilled water or 95% ETOH. However a centrifuge is needed to consolidate the pollen in the rinse. Unfortunately, baby wipes contain ETOH and other chemicals that may not be suitable for live insects and birds. The ETOH and/or other chemicals may clog the spiracles on an insect that allows air to enter the trachea depriving the insect of oxygen. If the wipe/filter is examined directly for pollen, it will need to be examined with a dissecting microscope. Most dissecting microscopes do not have high enough magnification to be used for pollen identification (Bryant et al. 1991).

Fourth, pollen can be removed from the pollinator's legs, proboscis, etc. by rolling the tissue (legs, proboscis, antennae, etc.) in the some type of liquid medium or by placing the appendages (legs, tongues, etc.) on a microscope slide. For live pollinators, it is better to either comb or brush the pollen off. Mikkola (1971) rolled the proboscis of noctuid moths (Lepidoptera) in Euparal. Likewise, the proboscis of *Colias eurytheme* Boisduval (Lepidoptera) was rolled in aniline-blue in lactophenol (Levin & Berube 1972). Pollen from the proboscis, palpi, parts of the head and thorax of the wood white butterfly (*Leptidea sinapsis* L.) were dipped in glycerin gelatin (Wiklund et al. 1979). Hickman et al. (1995) placed the head, thorax, and the dissected contents of hoverflies (*Melanostoma fasciatum*, Diptera: Syrphidae) onto a slide that contained two drops of aqueous Safranin, spread them over the slide, and examined them. Appendages of the cabbage butterfly (*Pieris rapae* L.) were placed onto a microscope slide, stained with methyl green, covered with glycerin jelly or Permount, covered with a cover slip and examined (Lazri & Barrows 1984).

Finally, pollen can be removed from the exoskeleton of an insect by washing the insect with water or ETOH then examining the rinse. Kendall and Solomon (1973) examined the pollen from a wide variety of insects visiting apple (*Malus*) flowers by washing the insects with 70% ETOH and then examined the rinse ETOH. Campbell et al. (1998) examined the pollen loads of hummingbirds by washing the pollen from the birds bill and face feathers. Again care needs to be taken when using this technique with live pollinators. The problem with leaving any pollen in ETOH is that the pollen becomes dehydrated and will crack, collapse, and break. This creates a problem with pollen identification and recognition. It is best to examine any pollen grains left in ETOH within 24 hours or pollen will be lost from the samples.

The following technique (LM4, Jones 2012) is a good technique to recover pollen from museum specimens (Pucci & Jones 2010; Jones & Pucci 2012), where the specimen cannot be destroyed for pollen analyses, or from larger pollinators like hawk moths, marsupials, bats, hummingbirds, etc. (Tab. 1). The technique is simple, however, manually removing the pollen is tedious and time consuming. Similar to LM1, this technique does not acetolyzed the pollen grains making the pollen grains more difficult to identify. If both internal and external pollen are needed, LM1 and LM4 can be used in conjunction with each other. It is best to remove the external pollen first and then do any dissection for internal pollen.

1. Clean slides and clover slips.
2. Prepare a glass slide by placing one drop of the glycerin-stain onto it (see Glycerin-stain Stock Solution)
3. For museum specimens, insects, and dead pollinators, comb or remove any pollen or what appears to be pollen from the insect with a clean pair of forceps, spatula, probe, or insect pin dropping the pollen into the glycerin-stain on the glass slide. When examining the proboscis, body, or appendages if possible, remove them, then roll them around in the glycerin. Make sure that the forceps, probe or pins are dry prior to the pollen removal each time they are used, and that all

instruments used to remove any pollen are cleaned between insects by rinsing them in 95% ETOH.

4. Repeat until "all" of the pollen has been removed.
5. For pollinators in which the pollen was collected and put into waxed paper or a manila envelope, "sprinkle" the drop with the pollen and stir with a wooden applicator stick to mix the pollen into the glycerin/stain drop.
6. Cover the glycerin-stain drop with a cover slip and seal the cover slip to the slide (see "sealing the cover slip to the slide").
7. Once the fingernail polish has dried, paint another coat of fingernail polish over the first coat.
8. Store slides horizontally. If stored vertically, the glycerin will move to the bottom of the slide.

Table 1. Generalized steps for the pollen recovery light microscopy examination technique of internal (LM1) and light microscopy examination technique of external pollen (LM4) for specimens that are dry, frozen, and fresh. An "X" in the box indicates a step that is needed for the pollen recovery of the pollinator.

	Dry	Frozen	Fresh
Thaw pollinator		X	
Clean slides and cover slips	X	X	X
Add 1 drop glycerin/stain to slide	X	X	X
Put sample into glycerin/stain drop	X	X	X
Cover drop with cover slip	X	X	X
Seal cover slip to slide	X	X	X
Let sealant dry	X	X	X
View with light microscopy	X	X	X
Identify and photograph all pollen grains	X	X	X
Set up photo documentation notebook	X	X	X

CONCLUSIONS

Joseph Kolreuter, during the 18th century, found that insects were necessary for the pollination of cucumbers, irises, and species of the Malvaceae (Proctor & Yeo 1972). Charles Darwin in 1858, found that seed set was poor, if insects were prevented from visiting pea flowers. In 1883, Bennett observed six species of butterflies, two species of hover-flies and three species of bees that visited flowers. He captured these insects and examined the contents of their abdomen. Some these insects were loaded with pollen while others were not. Some insects contained more than one pollen type while others carried only one.

Today, pollen found on/in an pollinator is used to determine the pollinator's food, its migratory activities, habitats, and source zones (i.e., Hagerup, 1950, 1951; Cate & Skinner 1978; Courtney et al. 1982; Hendrix et al. 1987; Benedict et al. 1991; Hendrix & Showers 1992; Gregg 1993; Lingren et al. 1993, 1994; Berkhousen & Shapiro 1994; Loublier et al. 1994; Boukary et al. 1997; Del Socorro & Gregg 2001). Pollen is used to determine the origin of boll weevil re-infestation of cotton in Texas (Kim et al. 2010). Pollen also has been found in fossilized Permian insects including Hypoperlidae (ancestral to bark-lice), Grylloblattida (distant relative of stoneflies), and Psocidiideae, the booklouse, *Parapsocidium uralicum* G. Zalessky (Krassilov & Rasnitsy 1997; Krassilov et al. 1999).

Finding pollen in or on a pollinator is essentially a "hit or miss" situation. Finding pollen depends on many factors including the availability and type of pollen, the amount of pollen produced by a flower, where the pollen is located on the pollinator, and mechanical loss due to flight (Jones & Greenberg 2009). Pollen retention also correlates with the type of the pollen grain, the amount of lipids on the pollen grain's surface, the ornamentation and size of the pollen grain, and for ingested pollen, the strength of the pollen wall. Furthermore, the feeding and digestion mechanisms of the pollinator can also affect the amount, condition, and type of pollen recovered. The potential for finding or missing a specific pollen type also depends on the number of samples examined and the number of pollen grains actually counted (Traverse 1988). Finally, finding pollen or pollen fragments on/in a pollinator depends on the type of microscope used, the person examining the sample, and the technique used to obtain the pollen (Bryant et al. 1991).

The techniques detailed in this manuscript have been successfully used on a variety of pollinator species to recover pollen from them. These techniques are the easiest of all pollen recovery techniques. They can be used in any laboratory, do not use specialized equipment or caustic chemicals, or need a fume hood. For the most part, they are quick and the pollen can be viewed as soon as the sealant has dried. These techniques work great when presence/absence questions need to be answered and when time is short. However, the identification of the pollen grains recovered from these techniques can be difficult because the pollen grains are unacetolyzed, usually contain lipids on them, and can be obscured by tissue or debris. When pollen identification is paramount for determining ecological zones, source zones, migration routes, honey typing, and feeding habitats, these pollen recovery techniques are inferior to those that acetolyzed the pollen grains.

The goal of this manuscript is to detail two easy to use techniques for recovering pollen from a variety of pollinators that anyone can use in any type of laboratory. Many of the questions asked by scientists about pollinators can be answered through pollen found on or in the pollinator. Unfortunately, without using the proper pollen recovery techniques and accurate pollen identification, the data are erroneous. Only by having and using the best techniques for recovering pollen can a full range of the pollen diversity on or in the pollinator be identified and be used to correctly answer the questions that are posed.

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