Cleaning and preparing adult beetles (Coleoptera) for light and scanning electron microscopy

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The coleopterist Arnett (1947) concluded in a techniques paper that 'As with any attempt to outline technique, this is little more than a sketch of some of the points to be considered. Each technician must work out the details for himself.… Do not let the technique become the end, but rather carefully prepared material which will serve the best advantage of the worker in carrying out his research should be the end.' I concur but add that unless entomological techniques are documented, others have to reinvent the wheel, rather than refine it. Here I provide a summary of what is published and document the details that I have worked out for others to use as a starting point.

When one considers the advantages of, and the contribution that scanning electron microscopy (SEM) has made to beetle systematics (Beutel *et al.* 2009), it is surprising that literature lacks a concise techniques paper covering the cleaning, mounting, drying and sample preparation for beetles, or other hard-bodied arthropods for SEM. Those traced are included below: Nelson (1949) in his studies of Elmidae (Coleoptera) covered cleaning insects, where dry specimens are relaxed in Barber's relaxing fluid (Valentine 1934; May 1958) before being cleaned using a custom-made artist's fine paint brush and tri-sodium-phosphate (Na_3PO_4) as a route to removing the naturally occurring layer obscuring their microsculpture. Frank (1978) proposes the use of a 5 % solution of household liquid detergent and water to both kill and clean beetles prior to dry mounting or transferral into ethanol (C_2H_6O). He calls this 'auto-cleaning of captured beetles' and mentions the use of an ultrasonic cleaner, but without specifying how it should be used. Harris (1979) revisited entomological terminology and compiled 'a glossary of surface sculpturing', which remains a good source where invertebrate microsculpture is covered with text and clear SEM illustrations. His materials and methods included the use of soaking dirty specimens (of Hymenoptera) in ethanol or xylene (C_8H_{10}) prior to 10–20 seconds of sonication (note the very short sonication time for Hymenoptera in contrast to what more robust Coleoptera require). Corwin *et al.* (1979) working on ticks (Acari: Ixodida) used a common household glue, like a cosmetic face peal, to adhere to and then remove dirt in order to clean specimens prior to SEM, expanding on an aqueous cleaning technique proposed by Keirans *et al.* (1976). Speirs *et al.* (1986) used an ultrasonic cleaner with ethanol as the surfactant to clean beetle larvae for a SEM study. There are many other papers where cleaning is mentioned, but always so cursorily as to not provide any guidance to a novice trying to prepare dirty specimens. In a novel approach to removing small delicate arthropods collected on yellow sticky traps, Williams & O'Keeffe (1990) used an ultrasonic cleaner with xylene or ethyl acetate ($CH_3COOCH_2CH_3$) to dislodge and clean them. Álvarez-Padilla & Hormiga (2007/2008) provided a pancreatin protocol for digesting the soft internal tissue of spiders, and suggested the use of fine strands of Paraloid B-72 glue (http://en.wikipedia.org/wiki/Paraloid_B-72) in acetone ($(CH_3)_2CO$) as a mountant for small spider parts for SEM examination. More recently, Warner (2010a,b) described cleaning, relaxing and degreasing of beetle specimens.

Consequently, this protocol is written due to the absence of one covering the vitally important cleaning and preparation of especially geotaxic, fossorial beetles prior to light or SEM microscopy. However, much of it is equally applicable to other hard-bodied arthropods.

The following items are required (brand names of those used here are in brackets): specimens for preparation; heat- and vibration-resistant glass-

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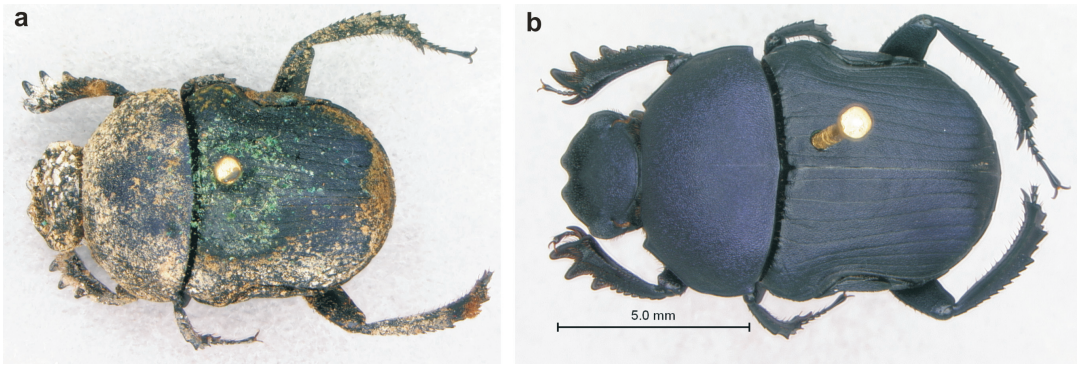

Fig. 1. Before and after photographs of a dung beetle. **a**, The 127-year-old syntype of *Gymnopleurus modestus* Péringuey, 1888, prior to cleaning. It was collected in 1885 by Rev. G.H.R. Fisk at Beaufort West (33°21′S 22°35′E) (Davis & Génier 2007). **b**, After cleaning and resetting (note new position of legs and tarsi) it has been restored to a state where all the external morphological characters are clearly visible, with even the rust on the brass insect pin being removed/retarded (insect pins rust from the specimens' body contents and residue from the hands which hold them). The fragile tarsi, which often break off if the limbs are outstretched, have been tucked closer to the specimen for protection during remounting.

ware (50–250 ml Pyrex® beakers); demineralized or distilled water; household kettle; kitchen fork; paper towel; permanent marker pens and high-quality paper; museum unit trays (50 × 100 mm); household liquid soap (Sunlight™ dishwashing liquid); an artist's fine paint brush; fine insect pins (sizes 0, 1); Herb Howard hackle pliers; surfactant (soapy water, Windolene® window cleaner, ethanol (C_2H_6O), acetone ($(CH_3)_2CO$) or hexane (C_6H_{14})); ultrasonic cleaner (Branson® B12); stereo microscope; fine-pointed and flexi tweezers; expanded polyethylene (EPX) foam mounting board; stainless steel insect pins (sizes 2, 3); plastic supporting cards (cut from plastic milk bottles); cardboard insect points; water-based glue; silica gel; sealable airtight boxes (Addisware™ Lockable Containers); and ethanol in concentrations of 25 %, 50 %, 75 % and absolute ethanol.

Specimen selection

View the specimen under a stereo microscope to assess its suitability for cleaning. Great care and a trial and error test phase is advised using replaceable specimens (of the same genus or family being studied) before using this technique with taxonomic type, unique or old specimens. This technique has been successfully used to clean beetle type specimens, as shown in Figs 1a and 1b, but only once it had been well-tested on non-type specimens. Use of the same type of ultrasonic cleaner is advisable, as the incorrect vibration, or vibration-setting (on adjustable models) can quickly and irreparably damage soft or fragile specimens. Additionally, it is advisable to clean only one individual of a type series leaving the others unmodified for future researchers. Specimens previously glued together, or where their connective tissue has been eaten by museum pests, *e.g.* Dermestidae, will undoubtedly disintegrate. Consequently, common sense and care needs to be applied during specimen selection.

Label data management

Remove paper labels from the specimen and pin these into a museum unit tray. Keep the same specimen, its labels and its working beaker together in the unit tray to prevent mislabelling. Reaffix the labels once the specimen preparation is complete. Refer to image file management (below) for recommendations on image names and folder archiving.

Rehydration

Whole, dry, previously pinned specimens are submerged in a suitably sized glass beaker of hot (90–95 °C) water (distilled or demineralized is preferred (Warner 2010a,b)) to rehydrate the muscle tissue. A kitchen fork is useful to hold and submerge the specimen, facilitating its rapid and uniform rehydration (a few drops of liquid detergent can be added with dirty specimens). Warner (2010a) includes some additional recommendations for dry, unpinned specimens. Rehydration makes the specimen soft enough for sonication and is the first step to softening dirt and allows for the specimens' limbs to be remounted facilitating

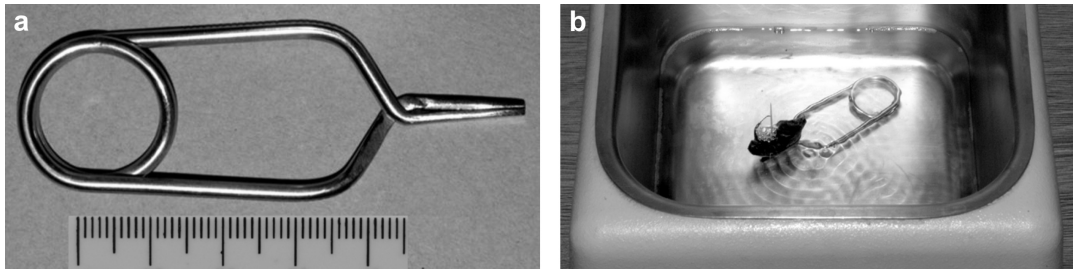

Fig. 2. **a**, Herb Howard hackle pliers (a sprung stainless steel fly-tying tool). **b**, The pliers holding the insect pin of a beetle during sonication. Hackle pliers are the ideal tool to hold and submerge a hard-bodied arthropod as they are flat, stable and light enough not to reduce the vibrating effect of the sonicator. Scale bar = 45 mm.

clear observation of these later on. Rehydration can take a few minutes for small specimens, or longer (10–30 min) for larger specimens. The specimens' appendages moving freely at their joints indicates sufficient rehydration.

Cleaning

The entire rehydrated specimen is soaked in liquid soap for 10–15 min in a suitably sized glass beaker. Considerable research goes into making domestic products highly effective surfactants, but their precise ingredients (and ratios) are retained as trade secrets (undiluted Sunlight® dishwashing liquid was used here). The specimen can be left in the soap for a few minutes, or sonicated immediately after submergence.

A novel and important step to prevent the specimen floating is to use Herb Howard hackle pliers (a fly-tying tool) to hold and submerse the specimen *via* its insect pin (Fig. 2a,b). This step is important as only the submerged part of the specimen is cleaned, and being held probably increases the cleaning effectiveness. Remove the specimen from the liquid soap, allowing the excess to drip-off. Clip on the hackle pliers and place the specimen upside down in a sonicator with an appropriate surfactant (Fig. 2b). Clear window cleaner (Windolene®) works well, but warm soapy water, ethanol, acetone or hexane can also be used. Ensure that the specimen remains submerged and sonicate for a few to 30 min depending on the state and size of the specimen.

A Branson® B12 ultrasonic cleaner (50/60 Hz, 80 W, 240 V) was used (Branson® Ultrasonics Corporation, Danbury, Connecticut, U.S.A.). The sonicator's wattage translates into vibration severity, so it is important to ensure that one is using an appropriately sized sonicator (industrial sized sonicators are probably too vigorous for arthropod use). Minimising the surfactant level maximizes the cleaning ability of the vibrating surfactant. The sonicator used by Williams & O'Keeffe (1990) on sticky trap specimens was operated at 117 V and 50/60 Hz.

Using tweezers remove the specimen and dab off on paper towel. Then view it under a stereomicroscope to see if all debris has been removed. A number '0' insect pin or fine paint brush can now be used to dislodge any stubborn particles. Repeat the cleaning steps above until the specimen is clean when viewed under a microscope. Once clean, rinse the specimen in 70 % ethanol, or distilled water (for slower setal drying) prior to mounting.

Long-lived beetles build up fat reserves which require a degreasing step. For greasy specimens the use of distilled or demineralized water prevents the formation of an insoluble white emulsification (Warner 2010a,b), which easily forms if mineralized water is used. Rehydrate greasy specimens (as indicated above) and then soak them overnight in acetone or hexane, prior to sonication in the same degreasing agent for 10 to 30 min. Acetone and hexane are highly volatile and this process should be done in a fume hood, or with the specimens inside a sealed chemically-resistant container, placed into a sonicator with a non-volatile surfactant (*e.g.* warm water). Thereafter they are rinsed in 70 % ethanol prior to window cleaner sonication. The labels on greasy specimens are often yellow and sticky prompting one to choose another specimen if a choice is available.

Dissection

Prior to remounting, drying and gold coating it is advisable to remove the genitalia, or any other parts required (*e.g.* mouthparts) for additional

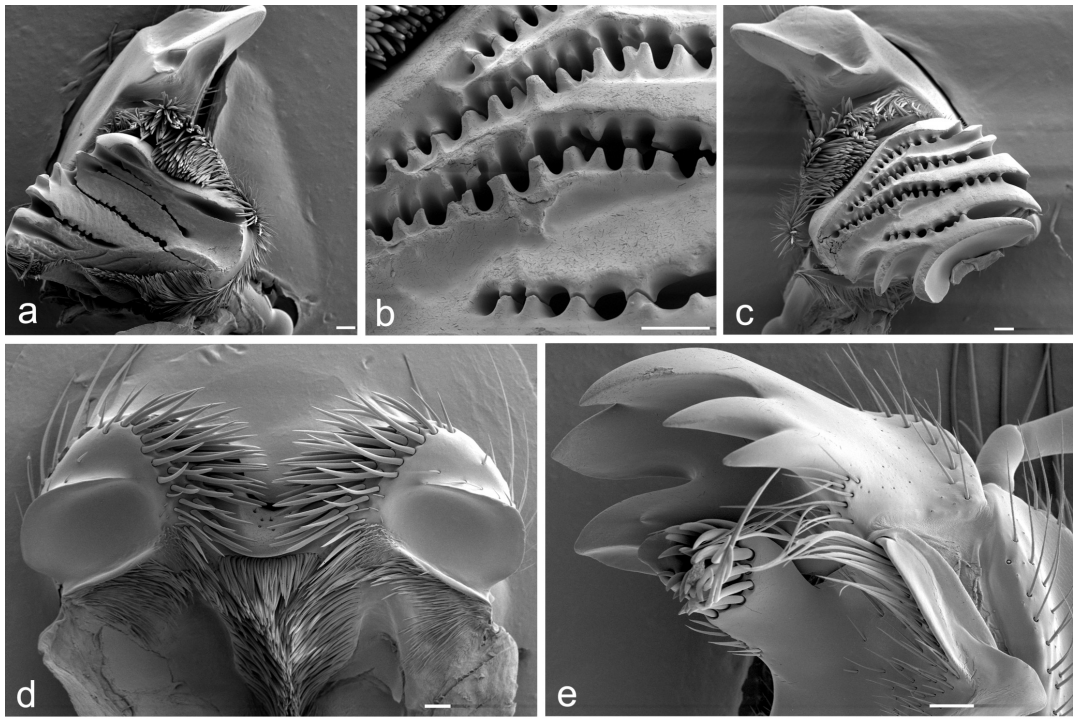

Fig. 3. Mouthparts of the leaf-feeding chafer beetle *Asthenopholis adspersa* (Boheman, 1857). **a**, Lateral view of left mandible; **b**, surface of right mandible's molar lobe; **c**, lateral view of right mandible; **d**, epipharynx in dorsal view; **e**, dorsal view of right maxillae. Note the absence of debris achieved with the technique presented here. Scale bars = 100 µm. (Previously published in Harrison 2009, *Zootaxa* 2225: plate 4; copyright Magnolia Press, reproduced with permission.)

morphological studies. This can be done using fine forceps, an insect pin and micro-scissors on the well-hydrated and cleaned specimens. Robust beetle genitalia and mouthparts (see Fig. 3a–e) can also be sonicated inside a vial of surfactant prior to rinsing in 70 % EtOH, dabbing dry on paper towel and mounting onto insect cards using a water-based glue, or placed in glycerol or 70 % ethanol in micro-vials. For detailed accounts on the preparation of beetle aedeagi and spermatheca refer to Arnett (1947), Barr (1961) and Smith (1979).

Remounting

If required remount the clean, hydrated specimen, manipulating its appendages for subsequent photo- or micrographing. Stainless steel insect pins and plastic supporting cards are used, allowing the specimen to be dehydrated in an ethanol series. The aim of remounting is to allow maximum visibility of the specimens' parts after drying, or gold coating, when they are no longer flexible.

Drying

Stainless steel pins, plastic supporting cards, and a small sheet of EPX foam are used so that the specimen can be dehydrated in an ascending series of ethanol in concentrations of 25 %, 50 %, 75 % and absolute ethanol inside an airtight container. These containers keep dust out and prevent the dehydrating ethanol from evaporating. After about a week the final absolute ethanol wash is drained and the specimen moved into a silica gel-filled airtight container for air-drying. Use of the same sized containers allows the EPX mounting board to easily be transferred from one container to another. Critical-point drying, or even wet viewing under SEM are other possibilities, but these techniques are not covered here. Bozzola & Russel (1999) provide coverage of these techniques and many other principles relating to the preparation of biological material for electron microscopy. However, in both of these alternative approaches one still requires clean specimens.

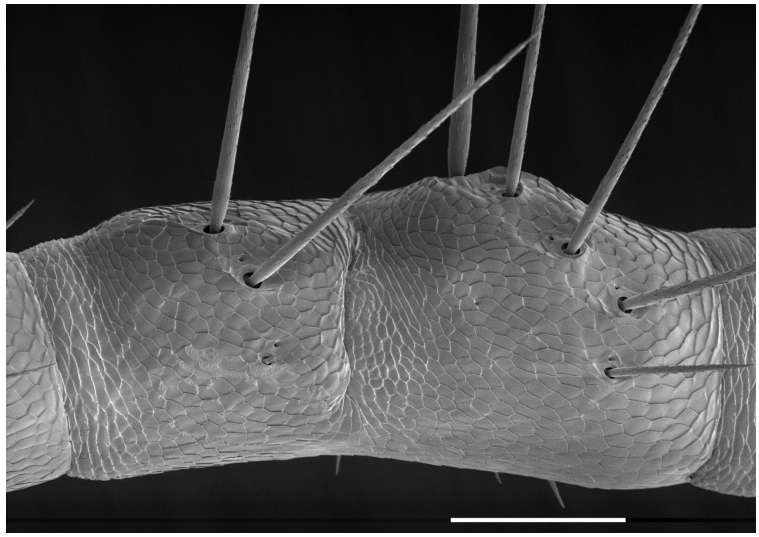

Fig. 4. Four antennal segments of the leaf chafer beetle *Pegylis pondoensis* Arrow, 1943 clearly showing the cuticular microsculpture, segment fusing and setae in their ball-and-socket joints at ×250 magnification. Note the absence of debris, and microsculpture clarity. Scale bar = 100 µm.

Sputter coating for SEM

Once dry, the specimen is removed and glued with aluminium tape, carbon dag or other preferred mounting medium onto aluminium stubs (25 × 40 mm) prior to gold coating in an E5200 sputter coater (Polaron Equipment Limited, Watford, England). Here specimens were gold coated five times for 10 s with intervals of 20 s between coats. Electron micrographs were taken using a JEOL JSM-840 (JEOL, Tokyo, Japan) scanning electron microscope, and images were captured with the aid of a frame-grabber and Orion version 6.60.4 software (Orion, Belgium). Álvarez-Padilla & Hormiga (2007/2008) provided a technique for digesting the internal soft tissue and subsequent mounting of spiders for SEM. They included a discussion of using Paraloid B-72 (http://en.wikipedia.org/wiki/Paraloid_B-72) solution in acetone as the glue to mount small spider parts onto stubs, *via* conductive glue strands.

Osmium tetroxide (OsO_4) vapour treatment (a few drops of aqueous OsO_4 placed in close proximity to the specimen in a sealed container for 1–2 h) prior to the gold sputter coating schedule used, is known to reduce any charging while being viewed in the SEM (J.F. Putterill, pers. comm.).

Specimen storage

When not in use all cleaned and prepared specimens for SEM are stored with silica gel in a Vaseline®-sealed glass desiccator, or an airtight lockable container. Those intended for light photography are stored in dust-free containers.

Image file management

Prior consideration needs to be given to a system of image file names and how they will be digitally achieved. I use taxon-specific folder names, and unambiguous, information-rich file names. For example, the folder and file name for the image used in Fig. 4 is 'Scarabaeidae/Melolonthinae/Pegylini/*Pegylis*/*pondoensis*' for folders and '*pondoensis* rhs antenna4.tif' as the file name. Additional information can be recorded in a notebook, text document or image database.

Capturing images (light photographs or SEM micrographs)

Knowing in advance exactly what images one requires and how one plans to use these, focuses ones research on important views and specific characteristics. Images for inter-/intraspecific comparisons are ideally taken at the same orientation, magnification, and using the same-sized scale bar as their scale reference. Harrison (2008a, 2009) provided examples of how these images translate into coherent comparative plates for a taxonomic paper. Habitus light photographs included in Fig. 1a–b were taken with a JVC Digital Camera (KY-F75U 3-CCD) mounted on a Leica Z16 APO

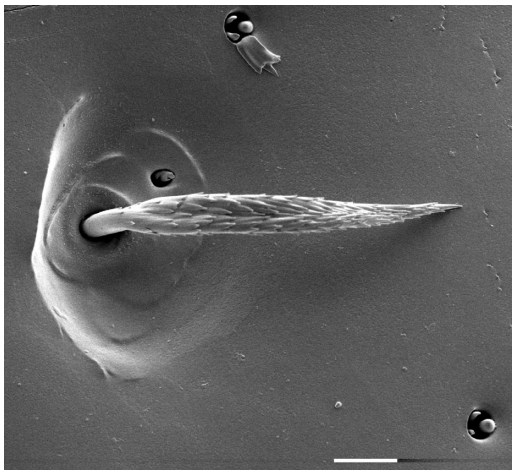

Fig. 5. The cuticle, a seta and two pores (the upper one with a glandular exudate) on the pronotum of the leaf chafer beetle *Pegylis sommeri* (Burmeister, 1855). Note almost no debris at ×1000 magnification. Scale bar = 10 µm.

via a computer directly into Cartograph Ver. 6.2.0, thereafter scale bars were added in Archimed Ver. 5.3.1. All figures presented here were processed using Adobe® Photoshop® 6.0.

Before (Fig. 1a) and after (Fig. 1b) light microscopy results using this technique are illustrated on a 127-year-old beetle specimen. Owing to the entire specimen being cleaned, both dorsal and ventral sides are equally clean. Scanning electron microscopy results from the technique are provided in Figs 3–5. These are ideal for interspecific taxonomic comparisons as done by Harrison (2009) in the revision of a morphologically ubiquitous chafer beetle genus.

Arthropod tarsi, claws and mouthparts are renowned for retaining debris during cleaning processes (J.F. Putterill, pers. comm.). Although it is difficult to have debris-free samples (even using this cleaning technique) it has consistently yielded almost debris-free specimens. However, it does allow the desired structures or microsculpture to be clearly seen. Example of this are indicated for the mouthparts of a leaf-feeding beetle in Fig. 3a–e, the clear view of fine antennal structure in Fig. 4 and cuticular surface with setae and glands in Fig. 5.

This technique was presented to the Technical Forum of the Microscopy Society of Southern Africa (Harrison 2008b) and results from it at an International Congress of Entomology (Harrison 2008c), and its taxonomic application has been published in Harrison (2008a, 2009). It is labour-intensive per individual sample, but allows one to maximize the outcome of the light photography and SEM study phase of the specimen/s and ensures high-quality images ready for final editing in programs like Adobe® Photoshop®, Illustrator® and Creative Suite®.

Clear images facilitate the dissemination of knowledge, and provide an observable point of reference for the text across many biological fields. The digital revolution has also resulted in poor quality images no longer being accepted by journal editors, resulting in rejection or reworking of papers until these are rectified. Ultimately a picture is worth more than 1000 words, especially when it is to be immortalized in print. Thus, the submission of high-quality figures or plates to journals can only expedite a paper's publication success, making appropriate sample preparation worth the effort.

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