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Procedures for preparation and conservation of whole insect permanent microscope slide mounts within the Department of Entomology, The Natural History Museum, London

- Paul A Brown & Emma De Boise

The Natural Sciences Collections Association (NatSCA) held a workshop entitled 'Insect Collections for Natural History Curators and Conservators' at The Natural History Museum, London on 19th January 2004. This paper reflects information presented at this meeting and is published here to increase the circulation of our slide mounting preparation and conservation techniques beyond the usual expert-entomologist readership, to the wider natural history conservator and curator community.

Many slide-making methods have been published in limited-circulation taxonomic papers and in curatorial manuals (such as Wagstaffe and Fidler, 1970) but other, more general works (such as Carter and Walker, 1999), do not cover slide-making techniques at all. Anyone wishing to study a group of organisms, should refer to the relevant literature and experts working on the group concerned, to learn the latest and best preparation and dissection techniques. Such experts may or may not know about the long-term performance of their slide preparations, the chemistry involved or the effects on the specimens.

Preparation for microscopical examination for Homoptera bug whole mounts.

Many small insect specimens can only be identified accurately by studying the exo-sceletal features with transmitted light. Thus their body contents must be removed before they are mounted on microscope slides. Specimens preserved in formalin cannot have their body contents removed and so are almost useless for taxonomic study. Standard 75 x 25 mm slides, preferably of 0.8-1.2 mm thickness, are normally used. Cover-slips should be the thinnest possible, no.0 grade. Many workers have their own preferences for cover-slip dimensions. We use circular cover-slips of 13 mm, 16mm, 19mm and 22mm diameter, depending on the size of the specimens.

For rapid identification of a specimen, almost any mounting medium with a contrasting refractive index can be used. Using transmitted light, bright field microscopy, the refractive index of the mountant should contrast with insect cuticle so that the specimen can be seen clearly. The refractive index of Canada balsam contrasts with Insect cuticle when still wet at 1.48 but changes to 1.52 after the xylene has evaporated to become similar to insect cuticle at 1.52 and the specimen becomes difficult to see. Phase contrast microscopy alleviates this problem.

For permanent museum preparations, it is important that an archival quality mountant is used. There are many slide mounting media available, as listed in Brown (1997). After much experience of mountant deterioration (as mentioned below) we suggest that the best choices for permanent preparations are Canada balsam and Euparal, because of their proven long-term performance. Canada balsam is used for Hemiptera, Thysanoptera (Mound & Pitkin, 1972), Phthiraptera (Palma, 1978), Psocoptera, Trichoptera, (Mosely, 1943), and small Hymenoptera whole mounts (Noyes, 1982) and is manufactured from the resin of the conifer *Abies balsamea,* which is usually thinned with xylene. Canada balsam is known to be stable for over 150 years. Euparal, as devised by G. Gilson of Louvain, Belgium, is manufactured by ASCO Laboratories, Manchester and is used for Diptera whole mounts and Lepidoptera genitalia mounts, none of which have exhibited deterioration in over 50 years. Euparal is reportedly a recipe of Eucalyptus oil, methyl salicylate, camsal, sandarac and paraldehyde but the recipe is a trade secret. Euparal has a contrasting refractive index of 1.48 when dry and so is more suitable for bright field microscopy. Methods for Lepidoptera genitalia preparation using Euparal are described by Robinson (1976) and Pitkin (1986).

The technique used at the NHM for whole mounting small Hemipteran bugs (aphids, whiteflies and coccids) is documented below and closely follows the method published by Martin (1999). For many small insects, all stages of this method can be carried out in a solid, square-based watch glass or similar small receptacle with a wide top and a lid. Decanting fluids between stages of the procedure needs to be done whilst observing the pipette tip through the dissecting microscope (to avoid the loss of specimens); the use of test tubes is not recommended. The United Kingdom COSHH regulations for some of the reagents and chemicals used in this technique, demand the use of suitably ventilated working areas, preferably with fume ducting or fume hoods (Fig.1).

PREPARATION METHODOLOGY

1. The removal or maceration of body contents is carried out by warming to around 80°C in a 10 % potassium hydroxide solution (an alkali) for 5-10 minutes, or longer, until the insect cuticle is clearly visible. A small puncture may be made in the ventral surface of each specimen in order to speed up this and subsequent processes, and to help prevent osmotic collapse.
2. Decant excess potassium hydroxide macerant.
3. If the insect is naturally waxy, de-waxing of cuticle is carried out by gently warming specimens in a medium such as carbol-xylol (xylene with 10% dissolved phenol), carbol/Histo-Clear (Histo-Clear with dissolved phenol) or chloral-phenol (equal weights of phenol and chloral hydrate warmed to liquefy and remaining liquid when cooled).
4. Decant de-waxing fluid, making sure that as much chloral phenol, if used, is removed as possible as this might cause blackening problems in the future!
5. a) for specimens with opaque black cuticle, rinse in strong alcohol and then partially bleach cuticle by immersing in a freshly prepared mixture of cold 25% ammonia solution and 30-volume hydrogen peroxide solution. Bleaching should be monitored as it can be very rapid, and may be stopped quickly by adding a few drops of a water-soluble acid. Domestic bleach is unsatisfactory for controlled cuticular bleaching.

   OR

   b) for very pale un-sclerotised specimens, staining may be carried out by adding an excess of glacial acetic acid* or acid alcohol and a few drops of acid fuchsin stain solution. Staining is carried out cold and usually only takes a few minutes. Failure of staining may result if de-waxing has been inadequate (see stage 3).
6. Decant bleach or stain and twice rinse specimens in glacial acetic acid* or 95% Ethanol.
7. Final dehydration of specimens may be carried out by soaking in glacial acetic acid* or absolute ethanol for a few minutes. Decant dehydration fluid.
8. Add a few drops of clove oil for Canada balsam or Euparal Essence for Euparal.
9. Place specimen(s) on a pre-cleaned and polished slide, in a drop of Canada balsam or Euparal and arrange specimens as required. It is a good idea to place some specimens dorsum-upwards and some venter-upwards as this aids, particularly, in the resolution of ventral characters of species with very ornate dorsa. When mountant has partially dried, gently a lower cover-slip with a small amount of fresh mountant: the drier mountant will hold the specimens in place, while the fresh mountant will spread to cover the entire lower surface of the cover-slip. A little practice will be needed to perfect the amount of mountant needed to provide a preparation which does not cause distortion through over-flattening, but is also not so thick that its optical quality is impaired.
10. Slides must be adequately dried, especially if vertical storage is to be employed. With Canada balsam, slides may require up to two months at 35-45°C prior to permanent vertical storage. If slides are provided with a pair of thick card labels, they can then be stacked immediately, and the cover-slip will also be protected throughout the life of the slide: for this reason, paper labels are not recommended for permanent collections.

* The choice between using glacial acetic acid, or a series of alcohols for dehydration, is influenced by economic and safety factors. Glacial acetic acid has the advantage of being a cheap means of neutralising alkalis and vigorously dehydrating material, and provides the acid medium necessary for staining; it has the disadvantages of its unpleasant, breath-catching smell and ability to cause skin burns. Whilst 95% industrial ethanol (IMS) is cheap when it can be readily obtained, absolute (100%) ethanol is extremely expensive, and both are more pleasant to work with than acetic acid. Ethanol is extremely hygroscopic, however, and many workers prefer 100% iso-propanol, which is not. Iso-propyl alcohol is therefore, a better choice for dehydration in humid environments, as well being cheaper than absolute ethanol.

Labels, glues and inks should all be selected with archival quality in mind. Suitable materials and methods are suggested by Carter & Walker (1999) for slide-mounted material.

Conservation of Deteriorating microscope slide preparations

Upton (1993) and Brown (1997) have drawn attention to the problems of gum chloral and plastic mounted slides deteriorating over time. We here discuss the procedure we have developed to rescue such material from the slide collections held in the Department of Entomology of The Natural History Museum, London.

Remounting of slide material should only be done by a well-trained slide-preparator or conservator. When in doubt please employ an expert to assess the problem first and to train staff if necessary or subcontract the work to a conservator! Carter and Walker (1999) briefly mention restoration of insect specimens on slides but the procedure mentions "easing the cover-slip off the slide" which we would not recommend as damage to the specimen(s) may occur.

There are many Berlese recipe gum chloral mounts within the NHM Aphidoidea collection which are actively deteriorating. Due to lack of time and man-power, only deteriorating 'type' slides and slides of species not well represented in the collection (i.e. if there are less than 50 slides of a given species) are chosen for remounting. Slides selected for remounting are those gum chloral mounts showing signs of phenol blackening with pink, bluish or black areas emanating from the specimens (Fig. 2). Other gum chloral mounts showing signs of crystallisation are also selected where chloral hydrate crystallises from the edge of the cover-slip as water evaporates from the Berlese due to a failed sealant ring. Sometimes crystallising slides can be reversed by removing the sealant ring and placing in a warm and damp environment, so that the mountant re-hydrolyses and dissolves the chloral hydrate crystals. Such slides can then be re-ringed.

Blackening in the Diptera collection slides has been blamed by some as a reaction of the ringing medium Euparal with gum chloral. In the aphid slides, blackening and bleaching might be caused by insufficient washing of the clearing mixture of chloral phenol from the specimens before placing in the Berlese mountant, as the blackening emanates from the specimens and not from the edge of the cover-slip. Phenol is used in photography as a blackening agent! Both aphids and Diptera were mounted in the same Berlese recipe that as quoted in Eastop & Van Emden (1972):-

gum arabic	48g.
chloral hydrate	80g.
50% w/w glucose syrup	20ml.
glacial acetic acid	20 ml.
distilled water	120 ml.

Berlese and other new mounting media were chosen because of the contrast in refractive index with insect cuticle, Berlese having a Refractive Index of 1.48.

Phenol balsam slides in the Diptera collection are satisfactory at the moment but a small number of aphid slides in this medium have turned black with cuticular degradation and have been rescued by soaking out in xylene. This may indicate a future problem for this mountant. After dehydration, the specimens are soaked in a phenol/100% ethanol mix before placing in Canada balsam, which is dissolved in phenol/ethanol instead of xylene.

Plastic (polyvinyl lactophenol) and gelatine mounts, showing signs of shrinking where air is entering under the cover-slips in long fingers, are also rescued. Canada balsam mounts are also occasionally rescued when the slide is broken or when the body contents need to be cleared for further taxonomic study using warm 10% potassium hydroxide. The Canada balsam slides in the Entomology Department are mostly not showing signs of deterioration apart from yellowing and many are of a great age.

CONSERVATION METHODOLOGY

1. Forty slides make up one batch and these are prepared by scraping off the ringing medium (Euparal and Murrayite) with a sharp scalpel, being careful not to damage the cover-slip in the process (Fig.2).
2. These slides are then marked into three using a diamond stylus and the thirds are carefully snapped along the score lines, which usually do not splinter.
3. The central third with the specimens under the cover-slip are placed into a watch glass with 30% ethyl alcohol which discourages fungal growth during the soak.
4. The two ends are put into water in a tub on top of the watch glass so as to keep the labels associated with the specimens (Fig 3).
5. The labels float off the glass and are affixed to a new slide, which is again placed with the associated specimens and watch-glass. If the labels are paper, these are glued to an already carded slide using neutral pH Lineco PVA adhesive. If the labels are card, these are fixed with the same glue to an un-carded slide and if the card de-laminates in the water, the top label surface can be glued to a new card square. Future removal of such labels is done by carefully removing a layer of the card below the label, which strengthens it for further affixing. Occasionally non-permanent ink runs in this treatment so the soaking is watched carefully so those labels deteriorating can be removed quickly and then removed with careful use of a sharp scalpel. The newly labelled but as yet blank slides are then left to dry on top of the watch glass with the associated specimens. Especially when dealing with 40 slides, one must be careful not to disassociate the specimens from the labels! A dedicated slide mounting-conservation area is prerequisite to avoid other people disturbing this system.
6. The specimens are left for a few days to soak in watch glasses with in 30% ethyl alcohol (the alcohol stopping fungal growth). These are placed in trays with suitable warning signs to avoid disturbance and covered to reduce evaporation and exposure to light. Some water-soluble mounts soak out quickly and the slide-square and cover-slip can easily be removed and disposed of in the sharps bin.
7. Other mounts often need a further soak in cold 10% potassium hydroxide that digests the remaining mountant without damaging the specimens. This soak can be for 5 to 30 minutes. If the mountant is still intransigent, a further soak in warm 10% potassium hydroxide, or warm acetone will usually work. Acetone has a low boiling point so care must be taken to avoid over-evaporation or fire. The much thicker Lewis Diptera slides mounts in the NHM have successfully been soaked out of the possibly, slightly different gum chloral mountant by using warm acetone. The insect cuticle in this mountant has not deteriorated or bleached to the same extant as in the aphid 'Berlese' mountant.
8. Occasionally damaged Canada balsam slides can be soaked in Histo-Clear 'orange' oil † and or in xylene but the latter should be done in a fume hood. Cover-slips and specimens should not be helped out of the mountant as this often can damage the specimen with appendages breaking off.
9. Often old Canada balsam slides have un-cleared specimens with body contents still opaquely present. For taxonomic study, features of the cuticle need to be viewed so the body contents should be cleared. A careful heating in 10% potassium hydroxide can clear these specimens before they are washed and dehydrated. We re-iterate that specimens that have previously been stored in formalin before mounting will not clear as the body contents are preserved!
10. The freed specimens can then be soaked in 30% alcohol in the watch glasses for a further period to wash away any remaining potassium hydroxide.
11. The 30% alcohol is then decanted and glacial acetic acid added for a short period of 2-5 minutes or changes of 50%, 80%, 95% and 100% alcohol, to dehydrate the specimens.
12. If the cuticle of the specimens has been badly bleached by the deterioration process, acid fuchsin can be added in the glacial acetic stage to stain them.
13. The acid or alcohol is then decanted off and drops of clove oil are added to the specimens in the watch glass. From the clove oil the specimens can be removed to a drop of clove oil on the new slide and the appendages arranged suitably if the specimen is not already too rigid to allow this. Do not force the specimen if rigid as damage may occur. Different needle forceps, flattened pins and small spatulas can be used to remove the specimens from the watch glass to the slide, which is the most delicate part of the operation. Any appendages that become detached, should be placed on the slide and close to the specimen from which they came.
14. The clove oil is then carefully soaked from the arranged specimens using the rolled corner of a tissue, taking care not to remove the specimens or their appendages in the process. Sometimes speci-

mens will disintegrate through no fault of the conservator. If this occurs, place the fragments on the slide as even these can still be of taxonomic use.

15. Add a drop of Canada balsam to the specimens and ensure that they are still arranged correctly and then place a cover-slip over the balsam and specimens ensuring that there are no or few air bubbles. Small air bubbles will often vanish when the slide is placed in the oven. Attempting to remove bubbles by pressing on the cover-slip may cause damage to the specimens. This process is routinely carried out in a fume hood, especially if glacial acetic acid or xylene is used (Fig.1).
16. Place the slides in an oven at 30°C for three-four weeks to harden. If the balsam slides are not incubated then the balsam may well never harden sufficiently so that, if the slides are to be stored vertically, the mountant will run to the bottom of the slide under the influence of gravity. Very large numbers of 'thin-mount' slides are stored vertically in the NHM Entomology collections and no properly hardened slides have slumped. Thick mounts should always be stored horizontally as the centre of a mount rarely hardens sufficiently even after baking.

† It has been reported (Laurence Mound pers. comm.) that Histo-Clear mixed with Canada balsam instead of xylene has prevented total hardening of the balsam even after oven baking. If Histo-Clear is used to liquefy Canada balsam, ensure all remnants of Histo-Clear is wicked off the specimen before re-mounting via clove oil to new balsam. A mount that never hardens will always be prone to slippage or accidental moving of the cover-slip and distortion or destruction of the specimens.

Liquid mount conservation is not discussed here, as we do not have such mounts in our direct care. Moore (1979) discusses the conservation of liquid slide mounts in his paper reporting on a project undertaken to save drying slides held within the Royal College of Surgeons in London.

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Fig.1 Hazardous chemicals such as glacial acetic acid, phenol and xylene require the use of a portable fume hood.

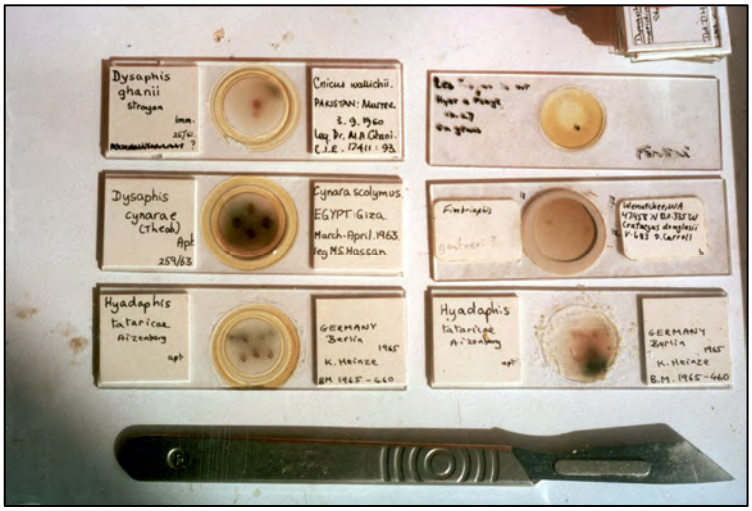

Fig. 2 Deteriorating aphid slides showing blackening and crystallisation of Berlese gum chloral mountant and the removal of the sealant ring with scalpel.

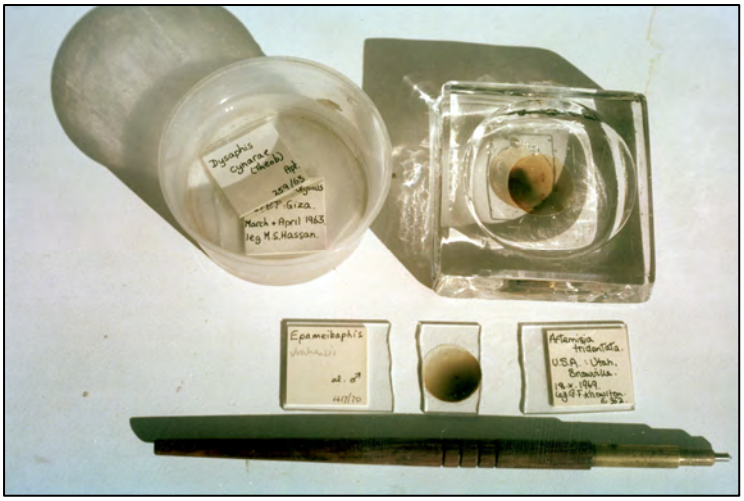

Fig.3 Rescue of a microscope slide mount. Cutting of slide with diamond stylus and soaking off of labels and specimens from mounting medium (beware water-soluble inks).