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A method for preparing lightly sclerotized mites for examination by transmission electron microscopy

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Abstract

Modifications to established protocols for preparing Acari for transmission electron microscopy are described. These modifications were found to improve the results obtained in a study of the ultrastructure of lightly sclerotized eupodoid mites.

Key words: methodology, transmission electron microscopy, ultrastructure, mites, Eupodoidea.

Introduction

There have been numerous studies of mite ultrastructure using transmission electron microscopy (TEM) (Alberti & Coons 1999), but the majority of taxa have received little or no attention to date. This lack of anatomical information is a hindrance to determining the function of structures and also to systematic analyses that require comparative data.

Current ultrastructural information about members of the Eupodoidea (Acari: Prostigmata) is insufficient for inter- or intra-taxon comparison (Alberti 1984; Alberti & Storch 1977; Di Palma 1995; Ehrnsberger 1977, 1979, 1984). As part of revisionary work on the superfamily, the present authors examined several characters by TEM in order to assess the systematic value of obtaining ultrastructural data to complement external details seen by scanning electron and light microscopy (Baker 1990, 1995). Established TEM preparation protocols were largely followed (Crooker 1985, Albert & Nuzzaci 1996), but we found that the incorporation of a number of modifications and additions better met the particular challenges posed by preparing such fragile and rapidly-moving mites, and produced good results routinely (examples given in Figs. 1–4). This methodology was developed in the light of research by the senior author into techniques for preparing dinoflagellate resting cysts for TEM (Kennaway & Lewis, in press), where the same problem of dehydration through a thick cell wall was presented. The main advantages of the methodology we used for eupodoids are that it is relatively quick, reliable and damage to tissue is avoided. Also, it could be used to prepare other small, lightly sclerotized and active arthropods for ultrastructural studies, especially where refractory material is known to cause problems.

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Material and methods

SPECIMEN COLLECTION

Live mites were extracted from moss or leaf litter samples through modified Berlese funnels. The samples were dried slowly at room temperature (RT) and specimens collected onto moist paper towelling. Adults of one species of the genera *Eupodes* (family Eupodidae), *Penthaleus* (Penthaleidae) and *Rhagidia* (Rhagidiidae) were obtained, plus two immature stages (larva and nymph) of the *Penthaleus* species. Based on trial and error, the optimum number of specimens to prepare for TEM examination is five, the minimum safe number three. This is because there are so many steps during TEM preparation where one small error or omission renders the specimen useless (e.g., during dissection at the dehydration stage or misalignment during sectioning).

SPECIMEN HANDLING

The mites were fragile and, in the case of the *Eupodes* and *Rhagidia* species, exceedingly fast-moving, and so needed to be collected singly. The specimens were picked up on the tip of a paintbrush moistened with saliva or glycerine, which restrained them sufficiently, and without causing damage, to be immersed in the primary fixative. However, the hydrophobic qualities of their exoskeleton and their high activity increased the difficulty of keeping specimens submerged but intact. To overcome this, live specimens were first encapsulated in molten low temperature gelling agarose Type 1X (www.Sigma-Aldrich.com). Once on the brush tip, the specimen was submerged in a small drop of aqueous 2% agarose and kept under the surface of the gel so the specimen was thoroughly enrobed. A warm plastic pipette should be used to dispense molten agarose and specimens must be encapsulated singly. The coated specimens were put in the refrigerator or cold room (below 15°C) for the agarose to gel. The solidified agarose was cut into a block with the specimen at the centre. Blocks must be kept small to ensure penetration of the reagents. With larger, globular specimens that needed to be bisected at the dehydration stage (see below), the blocks were kept relatively large for easier handling. Once in their individual blocks, the specimens were fixed and dehydrated in individual glass vials and embedded in individual blocks. Species and their life stages were kept separate.

TEM PREPARATION PROTOCOL

A trial run followed a standard protocol (Alberti & Nuzzaci 1996) to prepare whole mites for TEM. Using this method, we found that fixation of all specimens was good but that the dehydration results were variable. The evidence for incomplete dehydration is holes in ultrathin sections of the specimen caused by the hydrophobic nature of some resins. In the small *Eupodes* (ca. 250µm long), dehydration was complete (Fig.1), but, in the larger *Rhagidia* (ca. 820µm long), a few small holes indicated incomplete dehydration, and, in *Penthaleus* (600–710µm long), sections were unusable due to poor dehydration. Extending dehydration times was sufficient to complete the process in *Rhagidia* (Figs. 3, 4), but not in *Penthaleus*. Although the specimens of *Penthaleus* were shorter than those of *Rhagidia*, their maximum width and depth were respectively two-thirds and just over one half of the idiosomal length, as opposed to approximately one third in *Rhagidia*. Consequently, to overcome the problem of the lower surface area to volume ratio in the large, globular *Penthaleus*, complete dehydration was achieved by bisecting the specimen before beginning this step (see below) (Fig. 2). An important advantage of cutting the exoskeleton after fixation is that the tissue is firm and material is not lost or displaced.

There are three major steps in the routine preparation of biological material for TEM: 1) fixation; 2) dehydration, and 3) infiltration and embedding. Each step will be presented with the modifications we found to be successful and general comments as necessary.

1. Fixation

General comments. Fixatives containing both paraformaldehyde and gluteraldehyde give better results over a wide range of tissue than either aldehyde alone. Formaldehyde penetrates the tissue faster than gluteraldehyde, temporarily stabilising structures which are subsequently cross-linked by gluteraldehyde (Karnovsky 1965). Fixation times were increased to ensure penetration of the reagents through the sclerotized exoskeleton and agarose block.

Primary fixation. The primary fixative contained 2% paraformaldehyde and 2.5% gluteraldehyde (Glauert 1987) in 0.1M PIPES buffer (www.Sigma-Aldrich.com). Fixation time was extended to 15 hours (hr) at 4–5°C. Samples were rinsed in 0.1M buffer (3 X 15 minutes) on a rotary mixer.

Secondary fixation. A small volume (ca. 2–3ml) of aqueous 2% OsO_4 was added to each vial and rotated slowly for 5–6 hr at RT. The samples were rinsed in 0.1M buffer (2 X 15 minutes) with a final rinse in distilled water. Samples may be left in the osmium fixative overnight at 5°C. If left at RT overnight, the agarose and specimen blacken and are indistinguishable, making alignment impossible.

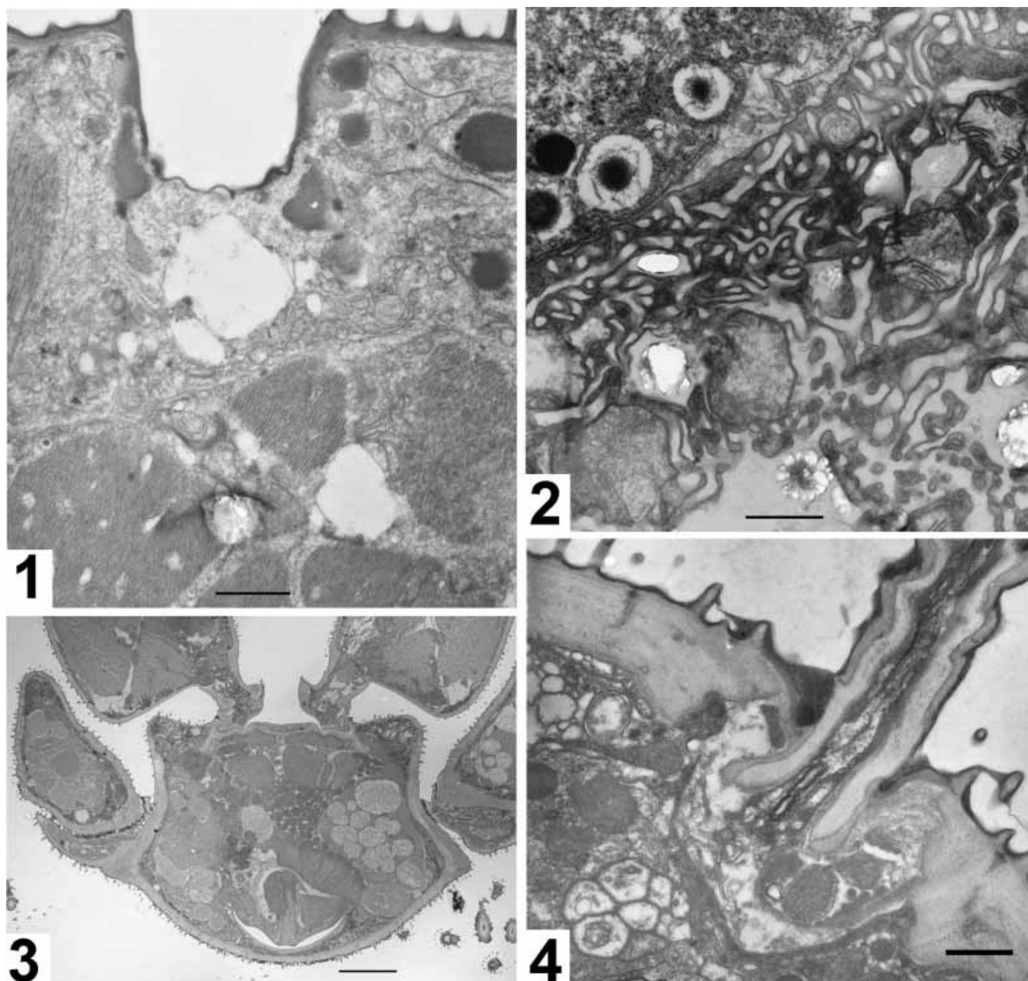

FIGURE 1–4. 1, *Eupodes* sp. (adult), vertical section through bothridium of prodorsal seta sc_1 (scale bar: 1 micrometre); 2, *Penthaleus* sp. (adult), vertical section through gut in region below dorsal anus (scale bar: 1 micrometre); 3, *Rhagidia* sp. (adult), longitudinal section through anterolateral ventral seta of tarsus I (scale bar: 833 nanometres); 4, *Rhagidia* sp (adult), vertical section through gnathosoma (scale bar: 6.7 micrometres).

2. Dehydration

General comments. Modifications of the standard dehydration schedule were fundamental to achieving good results. These were 1) the use of ice-cold acetone, 2) extended dehydration times and 3) bisecting the body of larger organisms after fixation. Cold acetone was used in preference to ethanol as lipid extraction is reportedly less (Ashworth *et al.* 1966) and with increased dehydration times this was important. In addition, acetone and Spurr resin are miscible, so the propylene oxide step, which potentially extracts lipids, can be omitted. Dehydration causes shrinkage of the specimen (ca. 30%), which must be taken into account when locating the area of interest to be sectioned.

Preparation of *Penthaleus* specimens. Each agar block was laid on a flat smooth surface so that the mite was ventral side uppermost. Specimen and block were then bisected at a level just posterior to legs IV by a single cut made with a razor blade at right angles to the block. This step required practice as the sample tended to slide about and the single cut must be clean. Routinely, the posterior of the specimen fell out of the agarose, but, because the sample was fixed, robust and large enough to see with the naked eye, it was processed as it was, but could be re-encapsulated at this stage. Anterior and posterior parts of each specimen were placed in different, clearly marked vials and processed separately.

Dehydration protocol. A concentration series of aqueous acetone (30%, 50% 70%, 80%, 90%, 95%, 100%, 100% over a molecular sieve) was used. All dehydration steps were carried out in the refrigerator (ca. 5°C) and solutions exchanged over ice or quickly in the refrigerator. Samples were gently rotated for 30 minutes in each solution. Solutions were exchanged quickly to prevent the sample drying. The volume of the solution must be roughly ten times that of the specimen. If the samples cannot be processed straight through to the resin stage, they must be left in 70% acetone in the cold (ca. 5°C) overnight. When the specimens were in the 95% solution, the samples were brought to RT. The final 100% acetone must be carefully dispensed as particles from the molecular sieve could get into the resin and damage the diamond knife.

3. Infiltration and embedding

Infiltration

General comments. The epoxy resin Spurr was used in this study because of its low viscosity (Spurr 1969, Glauert & Lewis 1998a,b). This resin is known to be harmful so must only be used after familiarisation with health and safety regulations for its use.

Protocol. A hard Spurr resin mix was used (www.taab.co.uk) and infiltrated into the specimens slowly and incrementally at RT on a rotary mixer (Table 1).

TABLE 1. Resin infiltration schedule.

Resin:acetone ratio	Time (hours)	Comment
1:2	5.0 – overnight	
1:1	7.0 – overnight	
2:1	7.0	
100% resin	overnight	
change 100% resin	7.0	
change 100% resin	overnight	
make fresh 100% resin	embed for 2.0 at RT before polymerising	polymerise at 70°C for 24hr

Embedding and polymerisation

General comments

Specimens to be sectioned in a particular orientation are most conveniently embedded in a small rectangular block. Silicon rubber moulds can be obtained commercially (www.taab.co.uk), but there are several other methods (Bozzola & Russell 1999a).

ULTRAMICROTOMY

This subject is covered comprehensively by Bozzolla and Russell (1999b), which should be read before embarking on this step for the first time.

Each block was examined under a light microscope for the position of the specimen in relation to the cutting face. In cases where complete reorientation was required, the new orientation was marked with lines using a fine permanent pen on the face of the block (specimen at the centre). The new lines were scored into the resin with a diamond saw (or fine hacksaw blade) and the area containing the specimen then carefully cut off the main block. The chunk of resin containing the specimen should be kept relatively large at this stage because it can easily be lost. The orientation must be rechecked as the excised area is glued into place on a spare resin block, using 2-part, slow-cure Araldite adhesive (www.adhesives.vantico.com). Rapid drying Araldite is unsuitable for this purpose as it is not stable under the electron beam. If the orientation needs further adjustment, place the resin fragment on some strongly adhesive tape to prevent it flying off, and re-trim with a new razor blade. Allow a twelve-hour curing period at RT before shaping the block and preparing the mesa. Small adjustments of the specimen arm on the microtome may be needed to perfect the alignment of the specimen with the knife. If the correct orientation is difficult to achieve, it is possible, though not entirely advisable, to re-trim and re-glue more than once.

Semithin sections

It is important to remember at this stage that the specimen will have shrunk during dehydration, maybe by as much as 30%, so care must be taken in estimating distances on the specimen. A standard ultramicrotomy 4mm diamond knife (Diatome) was used to cut the semi- and ultrathin sections.

Sections were cut on a Reichert Ultracut S microtome. Semithin sections were used to check orientation of the specimen and proximity of the target area to the cutting face. This is particularly important when very small features are to be studied. Several semithins (ca. 700nm each) were cut, picked up with a loop and placed on a drop of water on a clean microscope slide. The position of the sections was ringed with a permanent pen and the slide laid on cocktail sticks (or similar) over a hot plate (64°C). When the water had evaporated, a mixture of azure II and methylene blue (Richardson *et al.* 1960) was spread over the sections and the preparation left on the heat until a metallic ring formed round the edge of the dye (ca. 40 seconds). The slide was rinsed of all traces of dye under slow running tap water, dried and examined under a light microscope so that the orientation of the specimen and proximity of the target area could be established. Permanent mounts were made for storage by covering the sections with DPX and sealing with a coverslip.

Preparation of coated grids

Although grids with a large mesh and narrow grid bars may be used, the risk of the target structure being obscured by a grid bar remains high. In this study, because some of the features of interest were less than 1.0µm in diameter and serial sections were required to build up a three-dimensional picture of the morphology, the risks were considered too high. Consequently, slot grids with a support film were used. This method is not without its own problems (e.g., wrinkled sections obscuring the target area, the film splitting under the electron beam), but the risks were considered to be lower. A quick and reliable method for coating slot grids with Formvar was developed.

Copper grids with a 1mm X 0.5mm slot were chosen (www.taab.co.uk). To ensure a stable support film, 'sticky grids' 'were prepared by dipping each grid in 2% Formvar (in chloroform), drying them dull side up on filter paper and storing in a covered glass petri dish. Another glass petri dish was put in a deep freeze to get very cold. New clean glass microscope slides were polished with velin (lint-free) tissue and checked for a dust free surface. They were immersed singly in 2% Formvar, withdrawn and dried upright on filter paper for approximately 45 seconds. A margin was cut round the coated slide (ca. 5mm at the bottom, 2–3mm on each side and 5mm below the Formvar level at the top of the slide) to facilitate loosening of the plastic film. The trimmed slide was then placed in the cold petri dish in the freezer. After 5 minutes, the slide was taken out and held at RT for approximately 30 seconds before floating the film off in a cylindrical trough of water (ca. 700ml at RT) (Bozzola & Russell 1999b). The slide must be introduced at an angle of 30–35° to the water surface and pushed in smoothly until the film floats free. If the film was slow to release, the slide/ water interface was gently breathed on, but, as a general rule, if the film did not then separate easily, that attempt was discarded. The water in the trough was changed for each film. Forceps were used to place 'sticky grids' dull side down on the Formvar film, each grid surrounded by a 2–3 mm gap. It is important to use the dull side of grids as they have a marginally rougher surface than the polished side. The film was then lifted from the water surface on a rectangular piece of card. The card was placed on one end of the film and pushed under the surface at an angle of 45° for the length of the film. As the card was submerged, the film rested on top with the grids touching the card. Excess water was removed and the card placed in a glass petri dish in an oven to dry (ca. 30°C). A carbon evaporative coater (Edwards Auto306) was used to deposit a thin layer of carbon over the film on the dull side of the grids. A baffle was used to protect the plastic film during the process. The layer of carbon prevents the film from charging under the electron beam. Carbon coated grids were covered and stored in an oven (ca. 30°C). Individual film-coated grids were carefully separated with a needle or forcep tips and kept dull side uppermost. It is important to remember that coated grids are fragile and collect dust so should be stored under glass (e.g., in a covered petri dish) in a warm dry oven (ca. 30°C). Plastic should not be used because the static attracts dust particles. As this method of producing coated grids is quick and reliable, it is unnecessary to produce more grids than can be used over several days because the quality of the film can deteriorate during storage.

Ultrathin sections

Ultrathin (80nm) serial sections were cut because of the small size of the target areas and to build up a detailed picture of the structures. In order to keep the sections consecutive, a maximum of six sections were cut at a time. Although time-consuming, it did ensure that changes in the morphology of the smallest structures were recorded. Sections were stretched with chloroform, lined up and the dull side of the grid gently lowered onto them. Sections may be picked up in a variety of ways but the minimum number of wrinkles per section is the objective. Grids were stored on numbered silica rubber mats (www.taab.co.uk). After counter-staining with 2% uranyl acetate and lead citrate (Reynolds 1963), grids were examined on a Hitachi H7100 TEM operating at 100 kV.

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