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STUDIES OF BIVALVE LARVAE USING THE SCANNING ELECTRON MICROSCOPE AND CRITICAL POINT DRYING

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INTRODUCTION

The study of minute organisms using the Scanning Electron Microscope (SEM) is now routine but the quality of the results is directly proportional to the care with which specimens are selected and prepared. Consequently the techniques used and the care taken in cleaning, drying and mounting specimens are most important for successful SEM work.

The purpose of this paper is to point out some of the problems that may be encountered working with bivalve larvae and to present techniques which we have found to be successful. Our research involves problems which we have found can best be accomplished and illustrated using the SEM: 1) Studies on the variation in the sculpture and hinge structure of the valves of pediveliger larvae and their importance in systematic work both on an inter and intra specific level 2) Studies of larval transport by currents based on the ability to identify larvae to species 3) The morphology of the foot, velum, apical "flagellum", cilia and microvilli of pediveliger larvae to aid in an understanding of their function and their possible use in systematic work.

PREPARATION OF SHELLS

Bivalve larvae form a major component of the plankton (Thorson 1946; Thiede 1974) and though the percentage varies greatly with season and locality, large numbers are routinely taken in plankton tows. Most of these larvae, unfortunately, remain unidentified or are determined only to family or possible genus. McBride (1914) believed that the veliger larvae of bivalves were so similar that they could not be distinguished. Since that time many papers on bivalve larvae have been published, the more notable recent ones being those of Jorgensen (1946), Rees (1950) and Loosanoff, and Davis (1963), Loosanoff, Davis and Chanley (1966), Chanley and Andres (1971). All of these authors, however, depended largely on shell shape and size to distinguish species, the hinge structure and sculpture generally being beyond the ability of the instruments then available to examine and photograph them. The shells of bivalve larvae seldom reach 400 microns in length, most are strongly convex and many are nearly spherical, characters which make them nearly impossible to photograph through the light microscope.

Turner and Johnson (1969) and Scheltema (1971) using the SEM illustrated two species of teredinids which showed marked differences in sculpture and hinge structure. We have now examined 6 additional species of teredinids and 3 pholads using the SEM and on the basis of these micrographs it is apparent that the sculpture and hinge structure can be effectively illustrated and that larvae of species within the same genus can be readily distinguished. All of the larvae which we have used in our SEM studies are reared in the laboratory from known parents so that identification to species is positive.

Cleaning the tiny larval shells is one of the major problems in SEM work. The soft parts can be removed from preserved or relaxed living specimens by teasing with fine needles, soaking in a weak base or digestive enzyme, or by sonicating. These methods are not always successful, particularly if the valves are tightly closed, and many of the specimens may be lost in the process. If living larvae are available feeding them to small anemones is an effective way of cleaning them. The anemones digest the soft parts and egest empty, gaping valves. The sea anemones, all less than 1 cm in diameter, are kept in a series of petri dishes and the larvae are pipetted directly onto the tentacles. To insure complete removal of all soft tissue the anemones should not be over fed, so no more than five larvae should be given at one time. The gaping valves should be picked up immediately on release from the anemones before they become covered with bacteria and debris. (Culliney, Boyle and Turner, 1975).

To further clean the valves they should be washed 5 or 6 times by gently shaking them for 3-4 minutes in a small vial of distilled water to which a minute drop of Teel or other detergent can be added. After each wash the water should be decanted or pipetted off the valves immediately after they settled to the bottom. The process should then be repeated an

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equal number of times using 75 to 80% alcohol. The specimens can then be stored in 95% alcohol as recommended by Thiriot-Quiévreux (1972).

If the shells, on inspection with a compound microscope, look completely clean they can be mounted on metal stubs using double coated tape or other adhesives, coated with gold-palladium and examined under the SEM.

This process, however, may be unsatisfactory for three reasons: 1) The valves are seldom clean enough for the high magnifications possible with the SEM 2) Mounting individual valves on the stub is extremely time consuming and specimens may be damaged or pick up dirt in the process 3) Specimens mounted on double coated tape or other media available at the present time are, in a matter of a few months, enveloped by the medium and so lost. Consequently, rare specimens or those needed for a permanent museum record should not be examined with the SEM.

The procedure used in our studies began with the cleaning of the shells as outlined above and storing them in 95% ethyl alcohol. Final preparation for SEM examination consisted of rinsing the specimens in distilled water, placing them in an ultra filtration cell (Amicon Model 12 without the stirrer) using a PM 30 Diaglo ultra filter. The filter was numbered for identification purposes with a fine Esterbrook Perm-color pen, and placed in the bottom of the ultrafiltration cell. The cell was filled 1/2 to 2/3 with distilled water, the specimens pipetted in and the top clamped into place. The cell was connected to a nitrogen tank and then submerged in a water bath in a 3M sonicator (Plate 1, fig. 5). After sonicating for about 1 minute, the nitrogen was turned on gradually and the water was slowly replaced by nitrogen. As soon as the nitrogen began to bubble out of the vent on the bottom of the cell it was removed from the bath and the nitrogen allowed to continue to pass through the filter until it appeared dry. This deposited the specimens on the surface of the plastic filter and they adhered firmly to it. The filter was removed, after drying the outside of the cell, and examined under a dissecting microscope. If it was still damp some rearranging of the specimens was possible using a fine needle. When the filter was dry (this is very important for successful coating) it was attached to the stub with conductive paint and placed in the sputter coater, the pressure raised to 200 psi and the specimens coated with gold-palladium for about 1-1/2 minutes. The specimens were then ready for examination with the SEM. After examination and photographing, the filter was removed from the stub and stored in a small plastic box as a permanent part of the collection (Plate 1, figs. 1-2).

The advantages of this system are: 1) It is relatively easy to examine populations of larvae, 2) The plastic filters do not "consume" the specimens, so the picture is not the only permanent record of the material examined thus permitting the examination of rare specimens 3) It is possible to re-examine the specimens years later.

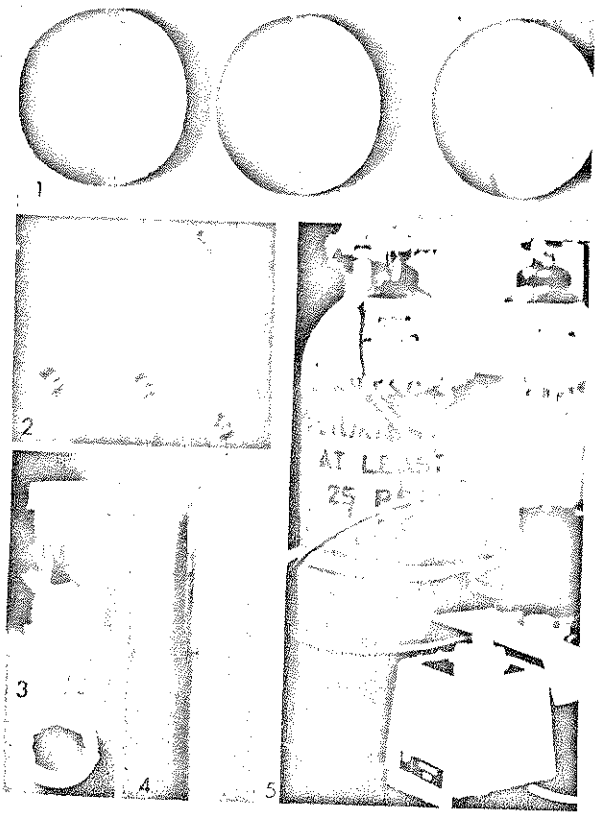

Plate I

Fig. 1. Filters with specimens attached after examination with SEM and ready for storage in the collection.

Fig. 2. Close up of filter showing coated larvae.

Fig. 3. Capsule made from pyrex tubing, plastic caps and nytex for use in the critical point dryer.

Fig. 4. Pick-up tool made from applicator stick and double coated tape.

Fig. 5. Ultrafiltration cell in sonicator and attached to nitrogen tank.

PREPARATION OF SOFT TISSUE

The examination of soft tissue with the SEM is relatively new and to date has been confined mainly to bacteria, protozoa, phytoplankton, small invertebrates and isolated tissue (Anderson, 1951, 1956; Hollenberg and Erickson 1973 – review and bibliography; Paerl and Shimp 1973; Por and Bromley 1974). An effective and commonly used procedure for preparing soft tissue for examination with the SEM is by critical point drying, the procedure used in this study. To our knowledge this is the first time relaxed bivalve larvae have been examined in this

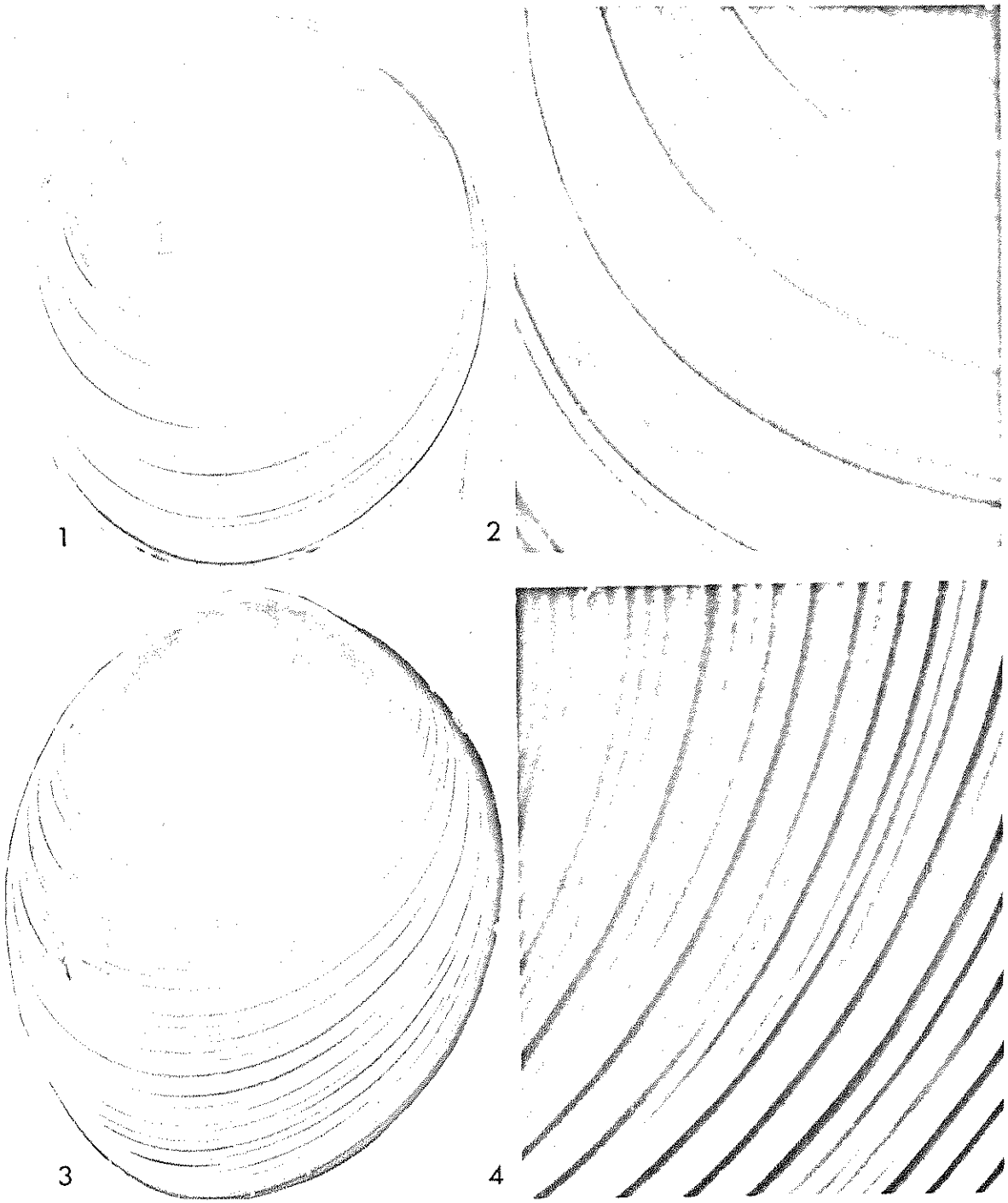

Plate II

Fig. 1. *Teredo navalis* Linnaeus, valve of pediveliger (360 X).

Fig. 2. Sculpture of the same (1000 X).

Fig. 3. *Lyrodus pedicellatus* (Quatrefages), valve of pediveliger (260 X).

Fig. 4. Sculpture of the same (1000 X).

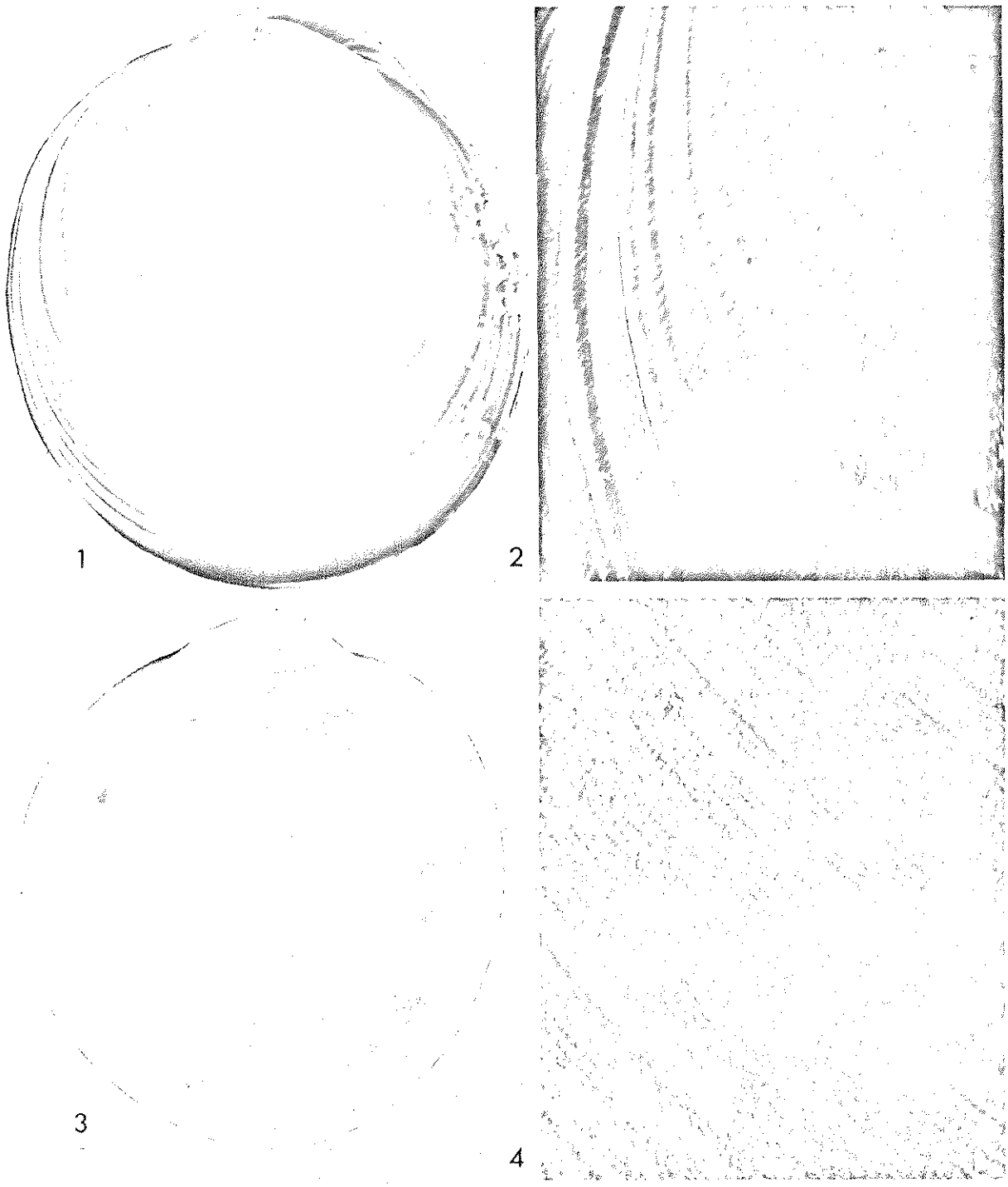

Plate III

Fig. 1. *Nototeredo knoxi* Bartsch, valve of pediveliger (600 X).
Fig. 2. Sculpture of same (2000 X).
Fig. 3. *Teredo furcifera* von Martens, valve of pediveliger (300 X).
Fig. 4. Sculpture of same (3000 X).

way. The following procedures were used in preparing of the specimens illustrated on Plate 4.

Relaxation of specimens. To obtain fully relaxed specimens 100-200 active larvae were placed in a petri dish with 50 ml sea water and 8 ml of 7.5% $MgCl_2$ made with distilled water. They were left undisturbed for 1 hour, or until most larvae were swimming, then another 8 ml of the $MgCl_2$ solution was added and within an hour the specimens were fully relaxed. Experience, however, has shown that the reaction of larvae differ. *Lyrodus pedicellatus* relaxed readily with $MgCl_2$ but not with propylene phenoxetol while *Martesia striata* relaxed in both solutions. Experimentation is needed to find the best relaxant for a given species. When the larvae were fully relaxed the solution was pipetted off leaving only enough to cover the specimens. Further immobilization was accomplished by placing the dish in the refrigerator for 15 minutes and then in the freezing compartment until the first ice crystals began to form at the edge of the dish. This must be watched carefully because the specimens must not be allowed to freeze.

Fixation. The specimens were fixed by flooding them with chilled 2% glutaraldehyde buffered with sodium cacodylate (0.025 M in seawater). We dissolve 1.07g sodium cacodylate in 200 ml of 1μ filtered sea water; add 8.0 ml of 25% glutaraldehyde to 92 ml of the buffered sea water. This solution was changed 5 times at 5 minute intervals. At this stage the specimens can be stored for a few weeks *unfrozen* in the refrigerator.

Dehydration. Dehydration of the specimens is accomplished by running them up through a series of miscible liquids to liquid CO_2 (such that at no time is there a liquid-liquid phase boundary which would distort the specimens). This is accomplished in 3 steps. 1) The alcohol series. The water is gradually replaced by alcohol to 100%. Concentrations, time intervals and the number of changes per concentration are shown in Table 1. The alcohol solutions from 7.5% to 50% were made with 3.5% sodium chloride solution adjusted to pH 8 which is isotonic with sea water to prevent osmic shock to the fixed tissue. Alcohols from 75% to 95% were made with distilled water to prevent the precipitation of salts on the surface. If necessary, specimens can be stored for a short time in 95% alcohol. 2) The amyl acetate series. The alcohol was gradually replaced with amyl acetate, (which is miscible with both alcohol and liquid carbon dioxide) according to Table II. After the last change in 100% amyl acetate the specimens were transferred to a container made to fit the "bomb" chamber of the critical point dryer. This container was made from pyrex tubing fitted at each end with a plastic cap from which the center had been removed. A disc of nylon screen (nytex) of a mesh size small enough to contain the specimens was placed over the ends of the tube and the plastic cap pushed firmly into place. The tube containing the specimens to be dried was submerged in a bottle of 100% amyl acetate until it was placed in the critical point dryer.

Replacement of amyl acetate by liquid CO_2. After checking the critical point dryer to be sure that everything was functioning properly the container of specimens was removed from the jar of 100% amyl acetate, drained briefly on a piece of paper towel to remove excess liquid and sealed quickly into the bomb which must be at a temperature of 25°C or below. For a discussion of the principle behind the functioning of a critical point dryer, the critical temperature and pressure, see Anderson (1951, 1956). There are now several models of CPD's on the market, each with its own manual of operation and this should be studied carefully before the specimens are placed in the bomb chamber.

After removal of the specimens from the dryer they should be mounted immediately in as dry and clean an atmosphere as possible so that the specimens do not pick up moisture or dirt. If they cannot be mounted immediately they should be left in the container and placed in a dessicator.

Attachment of specimens to the stub. Two dissecting microscopes were used for this process: under

Table I — Time Table — The Alcohol Series

% ETOH	Time (min.)	# of Changes	Notes
7.5	1	1	Made with 3.5% NaCl solution adjusted to pH 8
7.5	20	1	" "
15	1	1	" "
15	20	1	" "
30	1	1	" "
30	20	1	" "
50	1	1	" "
50	20	1	" "
75	5	6	Made with distilled water unbuffered
85	5	8	" "
95	5	10	" "
			Can store for short period
100	6	3	

Table II — Time Table — Amyl acetate Series

% Amyl acetate	Time	No. of Changes
20	1 min.	1
20	6	1
40	6	1
60	6	1
80	6	1
100	6	3

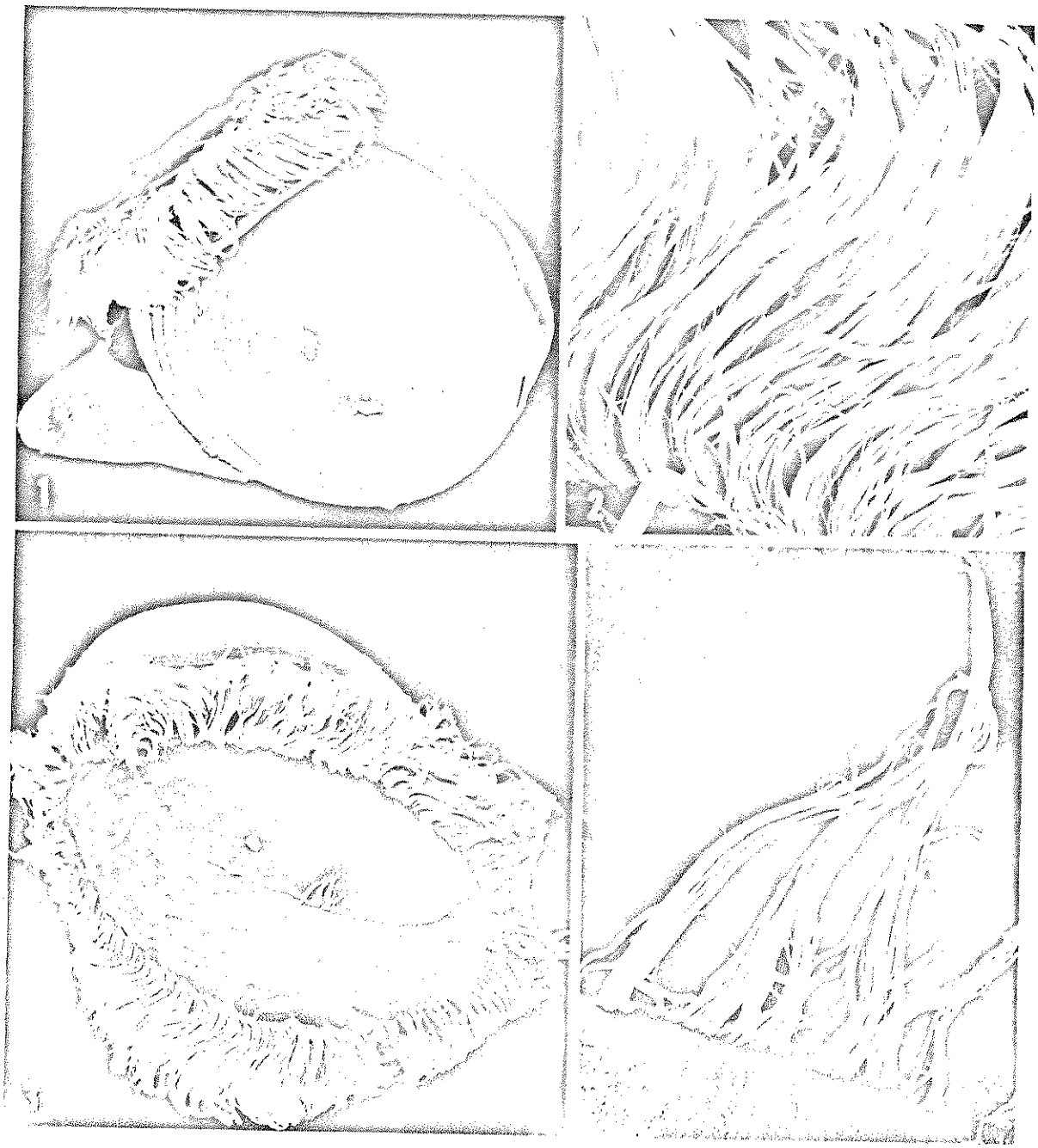

Plate IV – Critical point dried *Lyrodus pedicellatus* (Quatrefages).
Fig. 1. Side view of entire larvae with foot and velum extended, note the cilia covering the foot (180 X).
Fig. 2. Enlargement of cilia on the velum (1000 X).
Fig. 3. Anterior view of vellum showing the 'apical flagellum' (200 X).
Fig. 4. Enlargement of the 'apical flagellum' (2000 X).

one we had the loose dried specimens and under the other the stub with double coated tape on which they were to be mounted. The specimens were transferred from the tube to a slide a few at a time by gently tapping the side of the tube. They were picked up one at a time using a piece of double coated tape which had been cut to a fine point, wrapped around the end of a small applicator stick and then trimmed to the finest possible point. By gently touching the point of the double stick tape to the dried specimen it was easily picked up, transferred to the stub and placed in the desired position without damage to the specimen. The specimens should be distributed on the stub so that they do not interfere with each other during coating or when viewing with the SEM. The best medium to date for the mounting of critical point dried specimens is double coated tape because it allows time for the careful transfer and placement of specimens not possible with the quick drying liquids. The disadvantage is that it will eventually "consume" the specimens. However, if, after examination, the stubs are kept in a dessicator in a cool place they should last for a considerable time. When all specimens were mounted on the stub the tape was attached to the stub with conductive paint to reduce charging. It was then placed in a dessicator and kept in a cool, dry place until ready for coating.

Coating and examination. The specimens were coated using a mini sputter coater and viewed with a JEOL Inc. Scanning Electron Microscope model JSM 35.

CONCLUSIONS

Variation in sculpture of four species of Teredinidae is shown on Plates 2 and 3. The high magnification possible with the SEM clearly shows the finest growth lines on the valves. Since we know the exact age of these laboratory reared larvae, careful counting of these growth lines should give us some indication of their periodicity.

The most exciting result from the examination of the critical point dried larvae was the demonstration that the apical flagellum is not a single element but a bundle of large cilia (Plate 4).

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