Northwest Atlantic Fisheries Organization

Serial No. N907

NAFO SCR Doc. 84/IX/110

SCIENTIFIC COUNCIL MEETING - SEPTEMBER 1984

Development of an Ageing Technique for the Short-finned Squid (*Illex illecebrosus*)

by

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SPECIAL SESSION ON SQUIDS

Abstract

A technique has been developed which improves the accuracy and efficiency of ageing squid over previously described methods. The spatial pattern of growth increments is studied using light and scanning electron microscopy techniques.

Daily growth increments in statoliths are validated by employing chemical 'time' markers e.g. strontium and tetracycline, and known-age laboratory-reared animals. Increment formation is suggested to be intrinsically controlled. Increments begin to form immediately after hatching.

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* Work performed by Hurley Fisheries Consulting under contract to Fisheries and Oceans and Supply and Services Canada.

Introduction

To date, attempts at validating the age of the short-finned squid (Illex illecebrosus) have been made indirectly by comparing the difference in statolith increment counts and the number of days elapsed between samples of squid thought to belong to the same cohort (Hurley and Beck, 1979; Lipinski, 1981; Morris, 1983; and Radtke, 1983). Interpretation of the results of these analyses is complicated by variation in the technical procedures used to prepare and analyze statoliths for study, and the possible irregularities in increment formation caused by physiological stress or the presence of mixed age groups within a single year-class (Dawe, 1981).

A more direct approach paralleling the methods normally used in fish age validation studies, i.e. the use of known age material or chemical labelling of otoliths (Brothers et al., 1976; Campana and Neilson, 1982 and others) has been recommended by Hurley and Beck (1979) and Dawe (1981) to validate daily growth increments in squid statoliths. They stressed the importance of identifying the factors e.g. photoperiod and feeding rate which may be involved in the formation of increments.

The objectives of this study were:

1) to test the accuracy and improve the efficiency of preparing squid statoliths for ageing.

2) to employ strontium (Hurley et al., in press) and tetracycline as temporal markers to validate daily growth increments under different laboratory feeding and photoperiod conditions.

3) to determine the age when increment formation began by examining the statoliths of laboratory-reared known-age larvae.

4) to directly evaluate the importance of feeding regime on the rate of increment formation by comparing increment counts between samples of inshore and offshore squid. The former are thought to feed opportunisticly (Ennis and Collins, 1979) and the latter on a 24-hour cycle (Amarantunga et al., 1979). By selecting squid having a mantle length which corresponds to the precise mode (within one cm) of the sample length frequency distribution (compared to random sampling or

modal approximation in earlier studies), a single cohort of squid could be more effectively studied as they progressed through the season.

The description and discussion of statolith preparation techniques is included in the 'Materials and Methods' section. The 'Results and Discussion' section deals with biological aspects of the study.

Materials and Methods

Marking experiments

Squid for the marking experiments were obtained from a trap net located in St. Margaret's Bay, near Halifax, Nova Scotia during October and November of 1982 and 1983. They were transported to Dalhousie University in Halifax and were placed in the pool tank at the Aquatron Laboratory, a description of which is given by O'Dor et al., (1977). They were maintained in the tank at a constant increased photoperiod of 16 hours light:8 hours dark during 1982 and under natural photoperiod in 1983. The water temperature was kept between 12 and 15°C. Salinity varied from 30.8 to 32.1% during the experiments.

Squid to be marked with strontium, were fed one or two whole, cooked shrimp which had been soaked for 24 hours in a solution of 1.2 grams of strontium chloride per ml of distilled water. In 1982 squid to be marked with tetracycline were fed shrimp stuffed with 75 mg of oxytetracycline while in 1983, they were force fed a solution of 0.5 ml of oxytetracycline in 1.5 ml of seawater. Individual identification was made possible by recognition of specific skin abrasion patterns peculiar to each animal. During the 1982 marking experiments, normal feeding after marking was ad libitum with untreated shrimp. In 1983, squid were not fed after marking in order to determine if growth increments were laid down during a period of starvation. In both years, the length of time that individual marked squid were maintained from the time of marking until they died ranged from 3-24 days (Table 1). They were then sexed, routine body morphometrics were recorded according to Amaratunga et al., (1978) and statoliths were extracted and prepared as described below.

Known-age specimens

Two larval squid developed from an egg mass which was spawned on November 7, 1983. The eggs were maintained at 25°C and both hatched six days later. One squid died on November 15, 1983 which was 2 days after hatching while the other died on November 16, 1983 or 3 days after hatching probably due to lack of food (O'Dor, 1983).

Each squid was preserved immediately after death in 75% ethanol. They were processed for histological examination by immersion in a succession of ethanol rinses of increasing concentrations from 80 to 100%, cleared in xylene, and embedded in paraffin wax. Microtome sections of 6 m were prepared and stained using Mallory's Triple Stain (Pantin, 1960).

Inshore and offshore samples

Four samples of squid from the inshore Newfoundland jigger fishery were collected at Holyrood, Conception Bay from July 23 to August 31, 1982. Samples of squid caught by Canadian research and foreign commercial otter trawlers in Nova Scotia offshore waters were obtained in 1982 - 2 samples on June 1 and August 18 and in 1983 - 5 samples between June 14 and November 1.

Where possible, statoliths from 10 animals of each sex belonging to the modal length groups (in cm) and 2 animals from each of the remaining cm - length groupings were extracted (Figure 1). Only the left statolith from each squid was analyzed since increment counts did not differ significantly between left and right statoliths (this study; Lipinski, 1981. P 0.05).

Statolith preparation

Extraction of the statoliths was performed by making a single transverse cut through the statocyst exposing the paired statoliths in their maculae.

After removal, the statolith was immersed in distilled water for approximately 24 hours before mounting and viewing under the light microscope. Other premounting treatments (i.e. trypsin/papain, 15 min - 24 hours at 35°C; absolute ethanol; glycerin) were equally effective

in preventing the thin outer membrane from air drying which rendered it opaque to transmitted light. Prolonged exposure to sodium hypochorite, which has been used successfully to 'chemically' extract statoliths from the skull (Hurley and Beck, 1979) had a bleaching effect on the statoliths making them unreadable. Several mounting media namely Permount, Protexx, Cover Bond, EPON, Flotexx and Canada Balsam were found to be suitable for viewing growth increments. However, Protexx was used throughout this study for several reasons: it resisted cracking and chipping during grinding (unlike Permount and Cover Bond); it hardened relatively quickly--within 24 hours (unlike Canada Balsam); it did not require formula preparation or thermal setting (unlike EPON); it was locally available (unlike Eukitt).

In experimental trials, the total number of growth increments could be counted in about about 5% of statoliths without grinding by viewing mounted specimens with the concave, anterior side of the statolith facing upwards. In most of these whole mounts, the lack of complete readability was due to the presence of occulting crystals and fragments of the outer membrane obscuring the increments (Figure 2a). The occulting crystals were located on the anterior surface of the statolith about 40 to 70 increments from the nucleus. They interferred with the determination of total increment counts in all but statoliths from very small juvenile squid (Figure 2b). To investigate the microstructure of the occulting crystals and statolith membrane, the scanning electron microscope (SEM) was employed. A statolith was first fixed immediately after extraction in glutaraldehyde buffered with seawater, followed by dehydration in a series of increasing concentrations (50 - 100%) of ethanol, and critical-point drying. The statolith was glued to an aluminum SEM stub using 5-minute Epoxy and then coated with gold. It was viewed in a Bausch and Lomb Nanolab 2000 SEM. The membrane of the statolith was shown to peel off the main portion of the statolith in patches (Figure 3a) exposing the tips of the underlying calcium carbonate crystals which met the surface at right angles (Figure 3b). The occulting crystals appeared as prominent clumps of crystals which were partially covered by the irregularly arranged crystals of the wing (Figures 3c). The latter made the wing appear dark in light micrographs (Figure 2a) since they did not transmit light.

Morris (1983) pointed out that when the concave side of the statolith faced upwards, grinding through the occulting crystals often obliterated the increments below. A new technique is proposed here which facilitates the viewing of all increments including those in the region of the occulting crystals. The statolith was glued to the glass slide so that the posterior, convex side of the statolith faced upwards. Grinding was necessary in order to derive a total increment count since only a few of the outer growth increments in the whole mount could be seen. Grinding proceeded by using a succession of increasingly finer grit carborundum papers down to 3/0 polishing paper until the increments close to the nucleus (Figure 4a) and those lying above the occulting crystals (Figure 4b) were clearly visible by focusing down through the statolith. The process was speeded up considerably by grinding several statoliths which had been mounted on a single slide, at the same time. Up to six statoliths were prepared successfully for ageing in this way. A thin film of water over the ground surface helped to improve the resolution of the increments by filling in small cracks and acting as a 'liquid' covership. For the marked statoliths, grinding continued until the increments distal to the marker were exposed along the maximum radius.

The growth increments were best viewed with the microscope properly adjusted to ensure Kohler illumination, and a sub-stage aperture which was almost completely closed. A single polarizing filter rotated for maximum clarity in the direction of interest and a green filter also helped.

Optical sectioning (i.e. focusing to the plane of maximum clarity) (Figures 2b, 4b), indicated that all increments were not visible on the same plane. As further proof of this, the statolith was ground down to the nucleus and the increments were exposed under SEM by acid etching. Acid etching was carried out by first polishing the ground surface of the statolith with 1 μm diamond paste, immersing the statolith in 1% HCl for 90 - 200 seconds ringing and coating it with gold. Increments were not visible in several areas particularly along the maximum radius (Figure 5a). The plane on which the nucleus itself (Figure 5b) was

exposed using this technique was difficult to locate suggesting that the nucleus was relatively thin. By viewing a specimen which had been fractured along its postero-dorsal axis, i.e. perpendicular to the normal grinding plane (Figure 6), the calcium carbonate crystals are shown to radiate out in different directions from a small core area (the nucleus) to meet the surface of the statolith at right angles. Presumably only increments which were oriented in the same direction as the ground surface were successfully etched in Figures 5a and b.

Counting procedure

For each statolith, a map of the increments along the maximum radius was prepared using a Zeiss photomicroscope and a drawing arm set at 680X magnification. This method allowed for fine focusing during counting and provided a permanent record of the result similar to that produced by Morris (1983) using a microprojector. An Apple IIE microcomputer was programmed to count and measure the individual distances between increments with the aid of a graphics tablet. Data was held temporarily on diskette storage before being loaded on to the Cyber facility at Dalhousie University for statistical analysis using MINITAB.

The method of detection (i.e. using a line profile superimposed on a back-scattered electron image) and counting procedure for the statoliths marked with strontium have been outlined by Hurley et al. (in press). Tetracycline fluorescence was detected using a No. 2 exciter filter (350 nm) and a No. 50 barrier filter (500 nm). For illustrative purposes paired UV and bright light micrographs were prepared (Figure 7) but age validation counts were made by projecting the UV image on its respective drawing arm map and counting the number of increments from the proximal edge of the fluorescent band to the outer edge of the statolith. An example of a statolith which had been marked successively with both strontium and tetracycline is given in Figures 8a-c.

Results and Discussion

Known-age squid

A section through the statolith of a larval squid (Figure 9a) which hatched two days previously shows an inner dark band and a faint

outer band. The two measured about 11 and 16 µm respectively from the centre of the statolith. Their dimensions
roughly correspond with the outer limit of the nucleus and the first growth increment measured on acid etched statoliths of adult specimens (Figure 5b and Hurley and Beck, 1979). In a whole mount view of the statolith attached to the statocyst wall, the nucleus can be seen as a dark core within the statolith (Figure 9b). Lim (1973) suggested that the nucleus was made up of mucopolysaccharide and mucoprotein which probably accounts for its optical dissimilarity with the rest of the statolith. These observations support the conclusion of Radtke (1983) that increment deposition begins at the time of hatching but were inconsistent with the hypothesis of Morris (1983) that the first 38 increments are laid down prior to hatching.

Marked squid

There were a total of 8 squid which had statoliths successfully labelled with chemical markers (Table 1). Overall the agreement between the number of growth increments and the time elapsed in days was excellent. Where deviations occurred, they were underestimates of the time elapsed to a maximum of 3 days. This may have been due to not seeing very faint increments or errors in locating the precise counting start and end points on the surface of the statolith. In this regard, Hurley et al (in press) discussed the relative merits of strontium compared to tetracycline. Daily growth increments were shown to be laid down regardless of feeding regime. Since water temperature in the pool tank did not fluctuate regularly on a daily basis and the squid could not make vertical diurnal migrations, the deposition of increments must be controlled either by photoperiod or intrinsically. Campana and Neilson (1982) and Campana (1983) reported an intrinsic controlling mechanism for flounder and trout species. This augurs well for a direct relation between increment counts and age.

Comparison of increment counts between inshore and offshore samples

Modal length analyses comparing growth increments and time elapsed between samples taken from inshore Newfoundland in 1982 and offshore Nova Scotia in 1982 and 1983 are presented in Figure 10. The box and whisker plots are to be interpreted as follows:

- upper limit of counts from all animals
- upper limit of counts from modal-length animals
- mean of counts from modal-length animals
- lower limit of counts from modal-length animals
- lower limit of counts from all animals

Assuming that squid sampled at a particular location at successive times throughout the fishing season were taken from the same cohort (born on the same day), then the modal range of increment counts and at the very least the total range should intersect the line representing the expected number of increments if the 'one increment per day' hypothesis can be considered valid.

Based on an examination of the modal ranges, there was a general trend in all cases for the increment counts to fit this relationship reasonably well early in the fishing season but to increasingly underestimate the expected number of increments later in the year. An explanation for this may be the presence of mixed age groups or migration off the fishing grounds late in the year.

The results, which agree substantially with those previously reported by Hurley and Beck (1979) and Morris (1983) for inshore Newfoundland in 1978 and 1981 respectively, and Lipinski (1981) for offshore Nova Scotia in 1977, is not entirely surprising as these researchers employed light microscopy methods for counting. The method of selecting a sample of statoliths i.e. random sampling (Lipinski (1981); Morris (1983)); from a range of modal length animals (Hurley and Beck (1979); from animals having the precise modal length (this study, did not appear to be important.

The pattern of narrow (2-3 μm), closely spaced increments near the nucleus, followed by a zone of wide (3-5 μm), evenly spaced increments out to about 100 increments from the nucleus, and increments of irregular width (1-5 μm) toward the periphery of the statolith (Figure 12) correspond closely with the R_1 and R_2 zones respectively of Morris (1983) and the pattern of acid etched increments shown in SEM micrographs (Figure 5a). Statoliths from squid captured late in the fishing season (Oct./Nov.) have a third zone of increments (corresponding to the R_3 zone of Morris (1983)) which are faint, narrow and evenly spaced (Figure 8a).

Radtke (1983) validated daily growth increments in random samples of Newfoundland squid over the entire season--June to September--by making counts of acid etched increments using scanning electron micrographs. His increment counts for smaller size squid underestimated considerably those given in other studies (Figure 11). A partial explanation for the difference may be found in SEM micrographs of etched statoliths (Figure 5a) which reveal large areas where increments do not appear.
Estimating increment numbers across these areas is uncertain at best and this technique should be considered inappropriate for ageing purposes.

Acknowledgements

Special thanks to J. Dawson who aided in the statolith preparation and the light microsope counts. The authors wish to acknowledge E. Dyer and C. Collings of the Atlantic Research Laboratory and C. Nielsen of the JEOL application laboratory in Peabody, Massachusetts for their assistance in the strontium labelling and C. Mason, M. Willison, and S. Fry, Dalhousie University for scanning electron and fluorescent microscopy consultation. N. Balch, manager of the Aquatron, provided salinity and temperature records for the pool tank and assisted in the rearing of larval squid. T. Chandler designed the computer program for the graphics tablet and H. Tucker assisted with the computer tabulation and statistical analysis of the data.

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Table 1

Specimen code	Sex	Mantle length (cm)	Date marked	Method*	Date died	Time between marking and death (days)	No. of increments	Average increment width (μm)
SM-29/11-1-1	M	22.0	Nov 13/82	str	Nov 27/82	14	11	3.2
			Nov 19/82	tetra		8	8	3.1
SM-06/12-1-2	F	24.0	Nov 13/82	str	Dec 4/82	21	20	1.1
			Nov 15/82	str		19	18	1,1
			Nov 19/82	tetra		15	13	1.4
SM-23/11-1-3	M	21.5	Nov 19/82	tetra	Nov 23/82	4	4	2.5
SM-13/12-1-5	F	24.8	Nov 19/82	tetra	Dec 13/82	24	24	2.1
			Dec 1/82	str		13	13	2.0
SM-06/12/-2-7	F	26.5	Nov 17/82	str	Dec 4/82	17	17	2.2
SM-29/11-1-9	F	24.0	Nov 19/82	tetra	Dec 8/82	19	16	2.6
SM-20/11-83-2	F	26.0	Nov 17/83	tetra	Nov 20/83	3	3	3.6
SM-27/11/83-1	F	28.0	Nov 21/83	tetra	Nov 27/83	6	6	1.8

*all specimens marked in 1982 were held under constant, increased photoperiod with feeding ad libitum. In 1983 marked squid were held under natural photoperiod with no feeding.

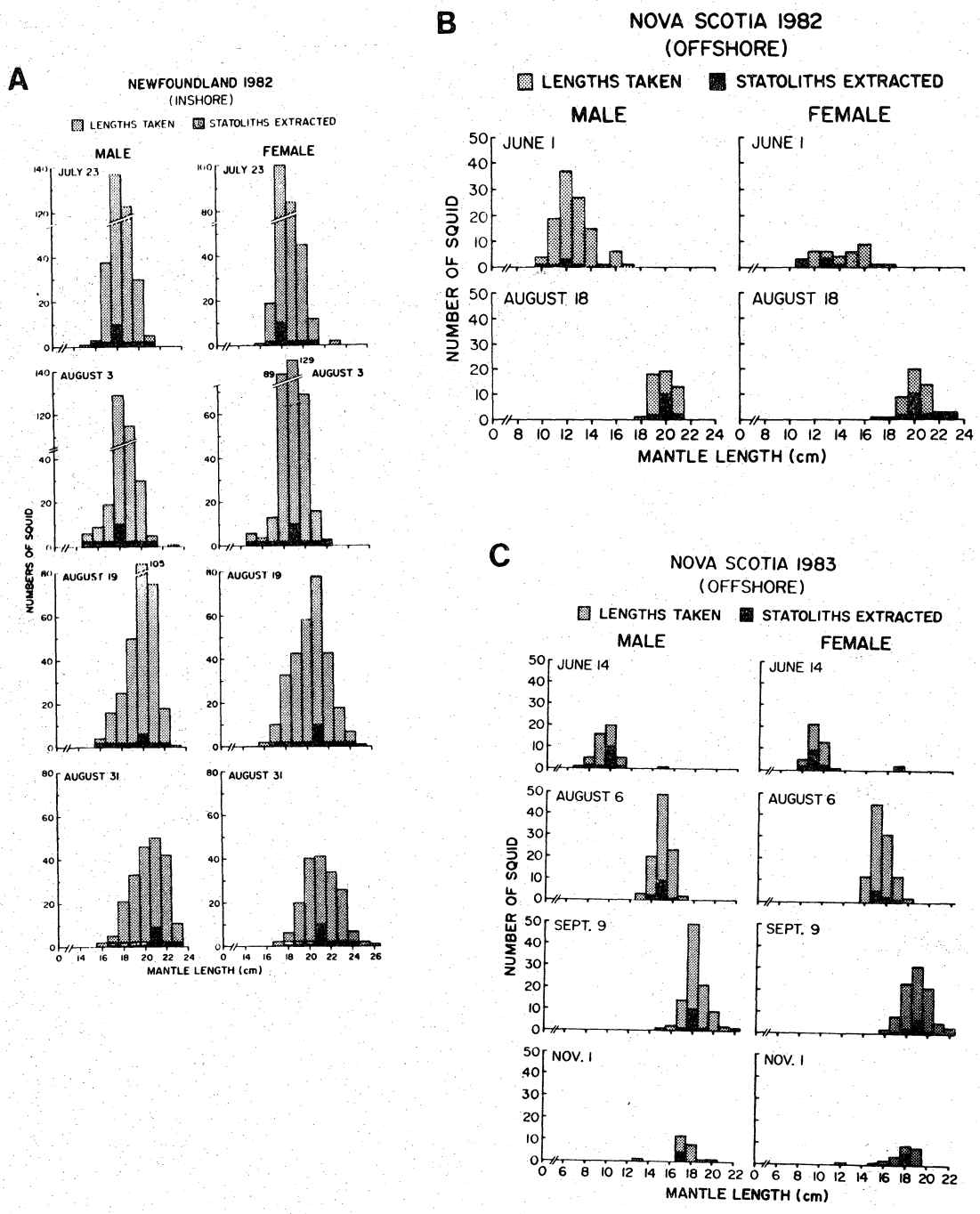

Figure 1 The number of mantle lengths measured and statoliths extracted for male and female squid sampled at inshore and offshore locations.

a) Newfoundland 1982 (inshore)

b) Nova Scotia 1982 (offshore)

c) Nova Scotia 1983 (offshore)

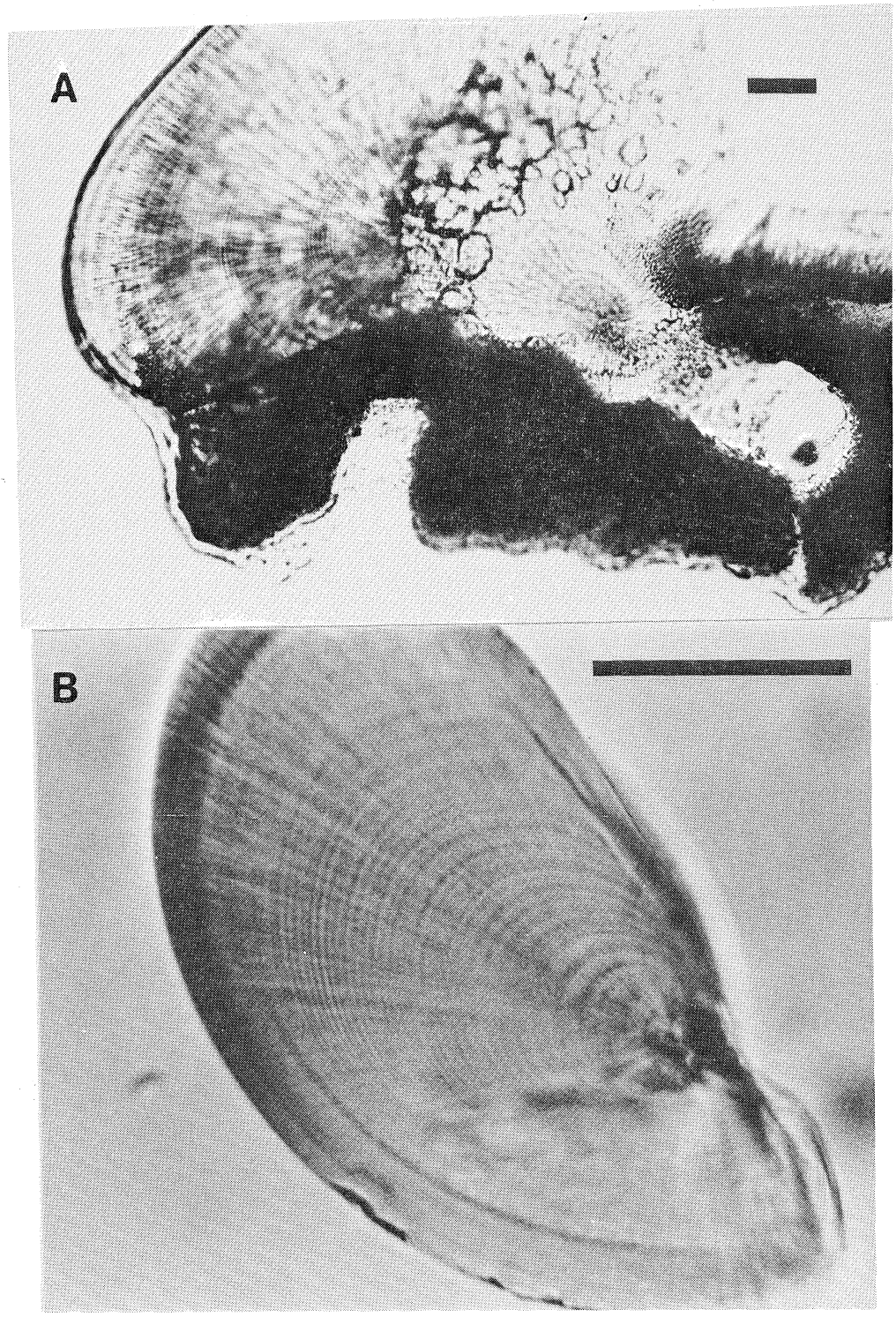

Fig. 2. a) Light micrographs showing a whole mount statolith, from a squid of 12 cm mantle length. View from the concave, anterior aspect. The presence of occulting crystals and fragments of the statolith membrane obscure some increments. Bar = 62.9 μm.

b) Light micrograph showing a whole mount statolith, from a squid 1.1 cm mantle length. View from the concave, anteior aspect. The occulting crystals have not yet formed on the surface of the statolith. Bar = 100 μm.

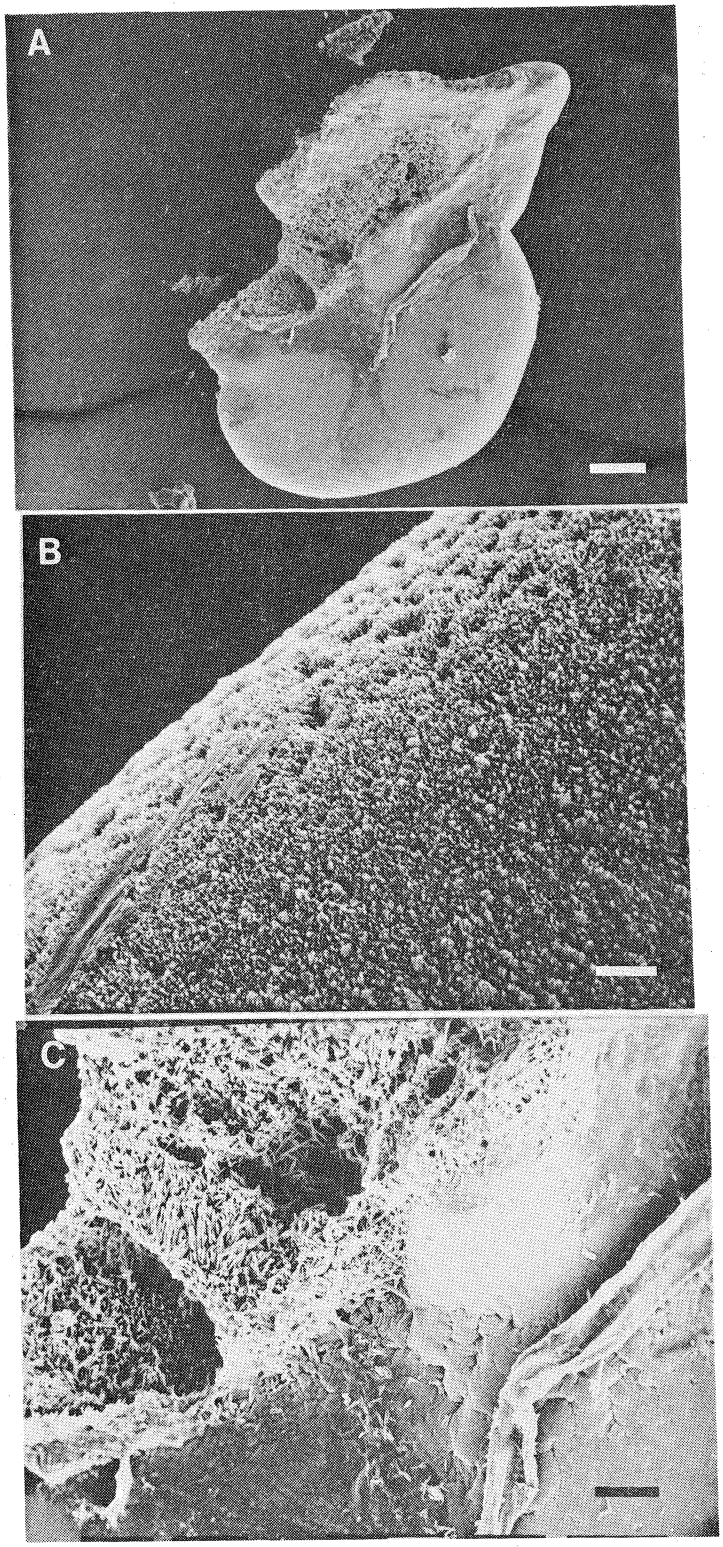

Fig. 3. a) SEM micrograph of the anterior right side of a statolith from a squid of 22 cm mantle length. Patches of the outer membrane are shown to have peeled off the main body of the statolith. Bar = 152 µm.

b) SEM micrograph of the same statolith showing a residual portion of the statolith membrane and the tips of projecting calcium carbonate crystals. Bar = 7.51 µm.

c) SEM micrograph of the same statolith showing occulting crystals and random arrangements of crystals in the wing. Bar = 32 µm.

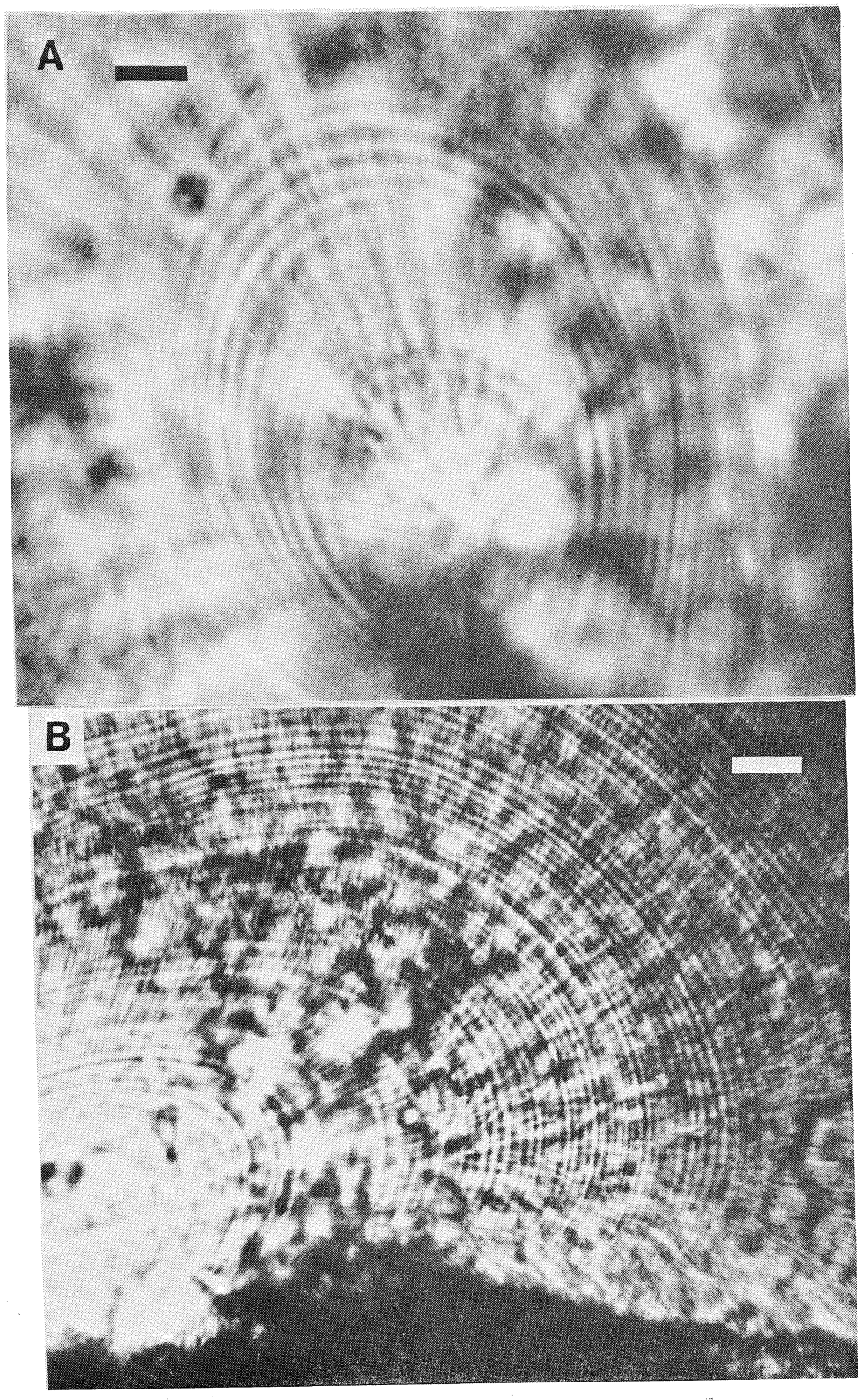

Fig. 4. a) Light micrograph of increments in the immediate vicinity of the nucleus. Notice there are no increments in the nucleus itself. Bar = 9.2 µm.

b) Light micrograph focused on increments lying above the occulting crystals. Statolith was ground on the convex, posterior side.

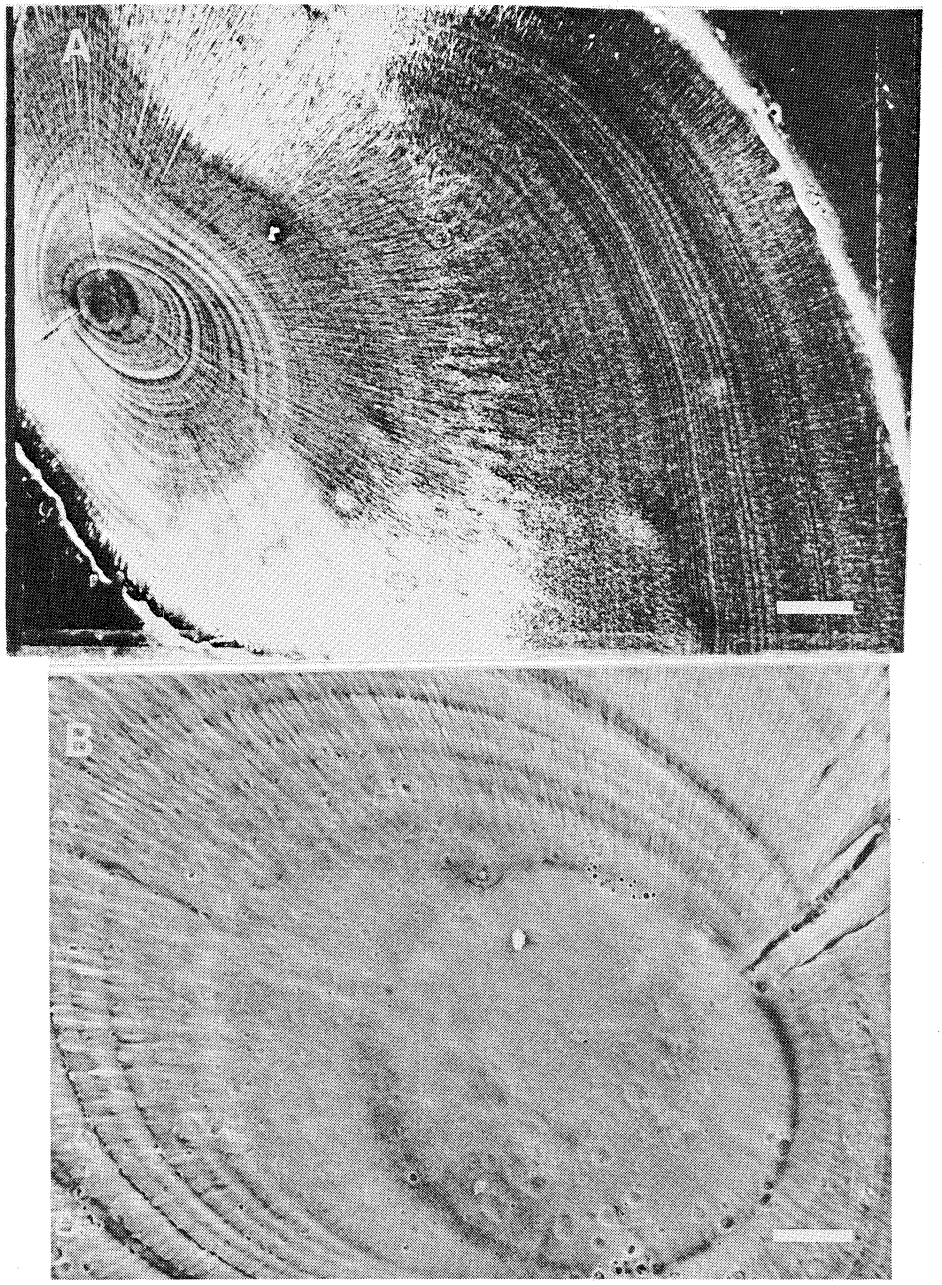

Fig. 5. a) SEM micrograph of acid etched increments on the ground surface of the statolith from a squid of mantle length = 19 cm on a plane through the nucleus. Bar = 34.4 µm.

b) SEM micrograph of acid etched increments in the immediate vicinity of nucleus from the same specimen as above. Bar = 12.2 µm.

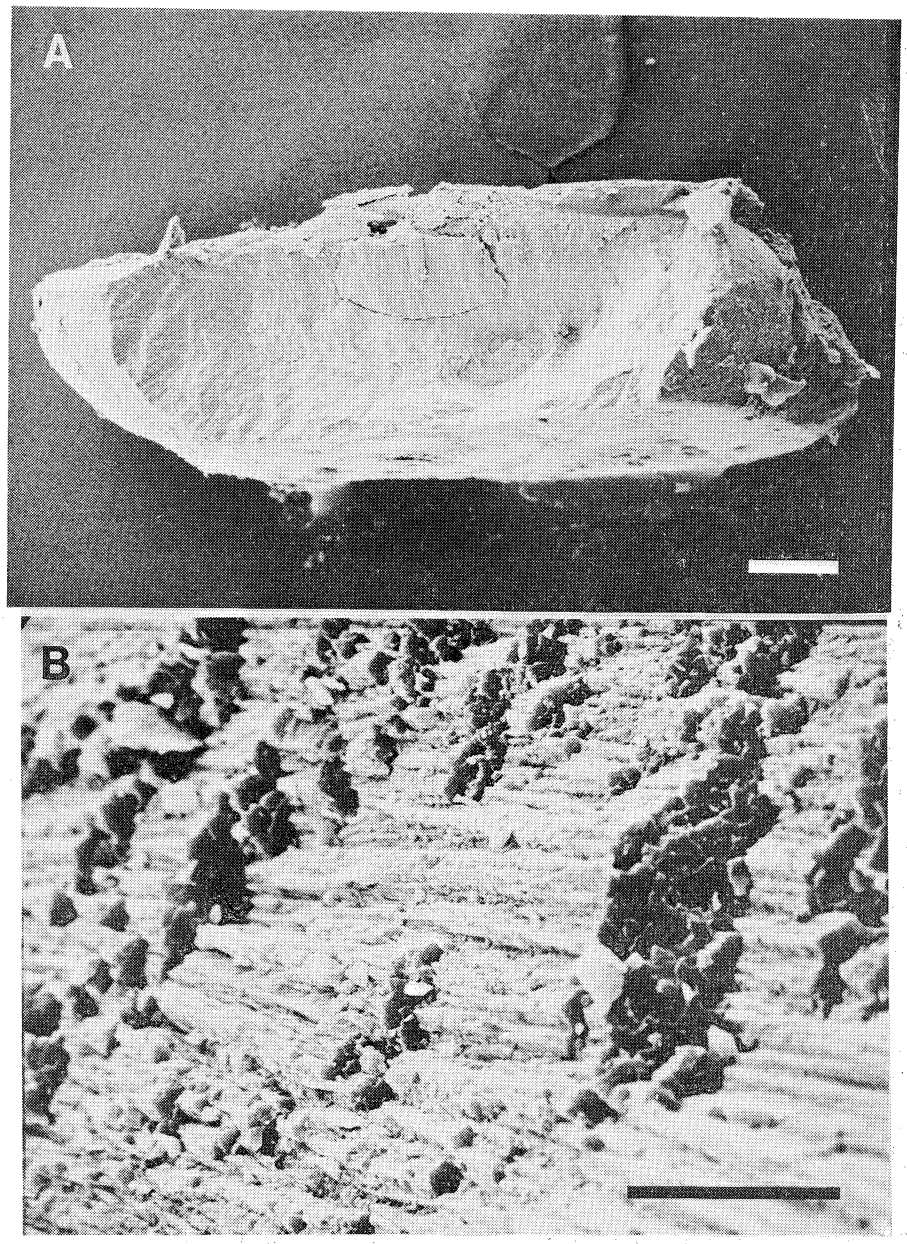

Fig. 6. a) SEM micrograph of a statolith fractured along its postero-dorsal axis. Calcium carbonate crystals are shown to radiate out from a central core (nucleus). Only a narrow band of these crystals on the same plane as the nucleus are approximately parallel to the ground surface. Bar = 100 μm.

b) SEM micrograph showing detail of crystals in 5(a).

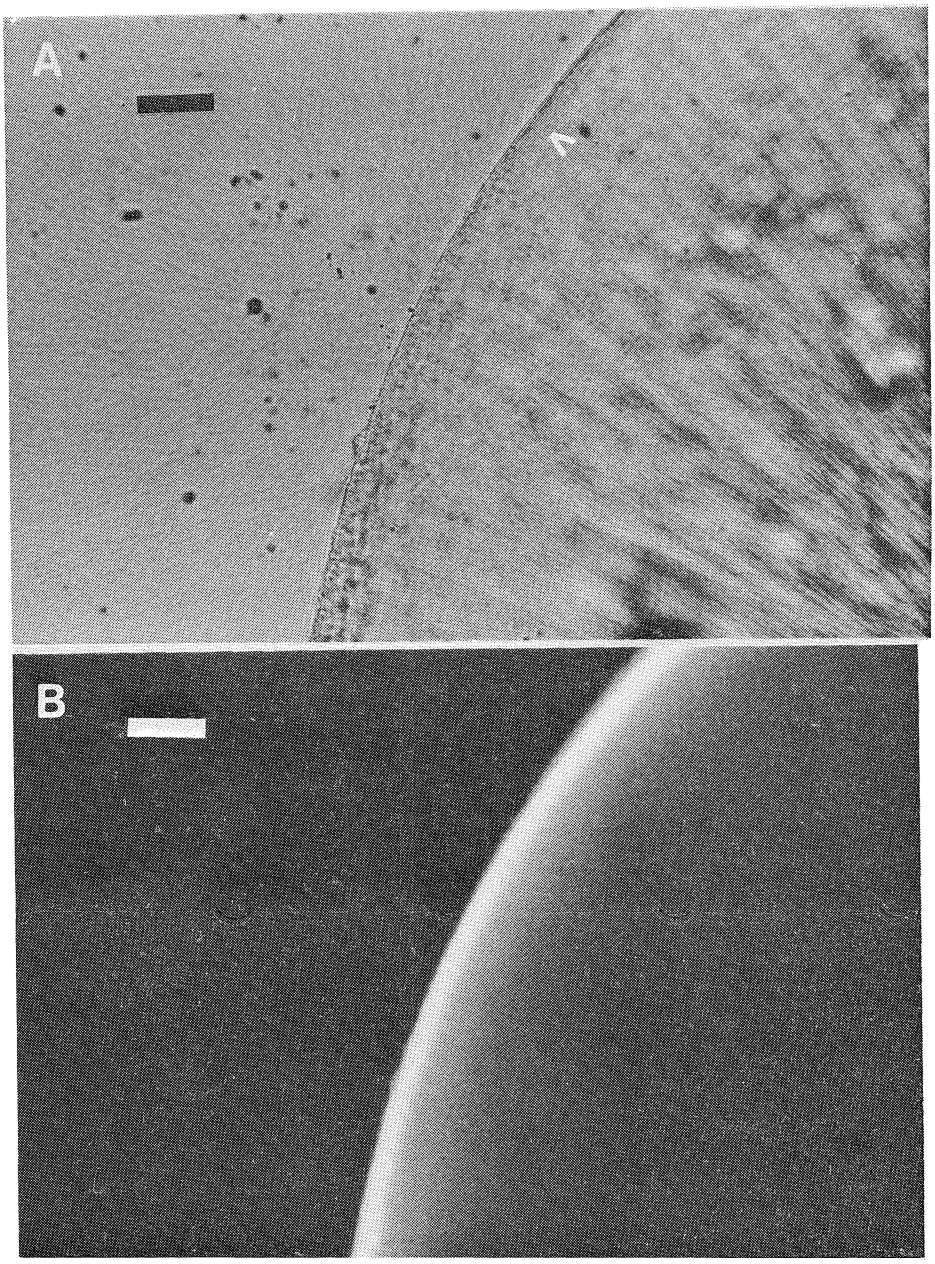

Fig. 7. a) Peripheral view of a ground statolith under bright field illumination showing six daily growth increments laid down after injection with tetracycline (marked with an arrow) under conditions of starvation and natural photoperiod. Bar = 27.7 µm.

b) Same as above under ultraviolet light. Proximal side of fluorescent band marks the site of first incorporation of tetracycline. Bar = 27.7 µm.

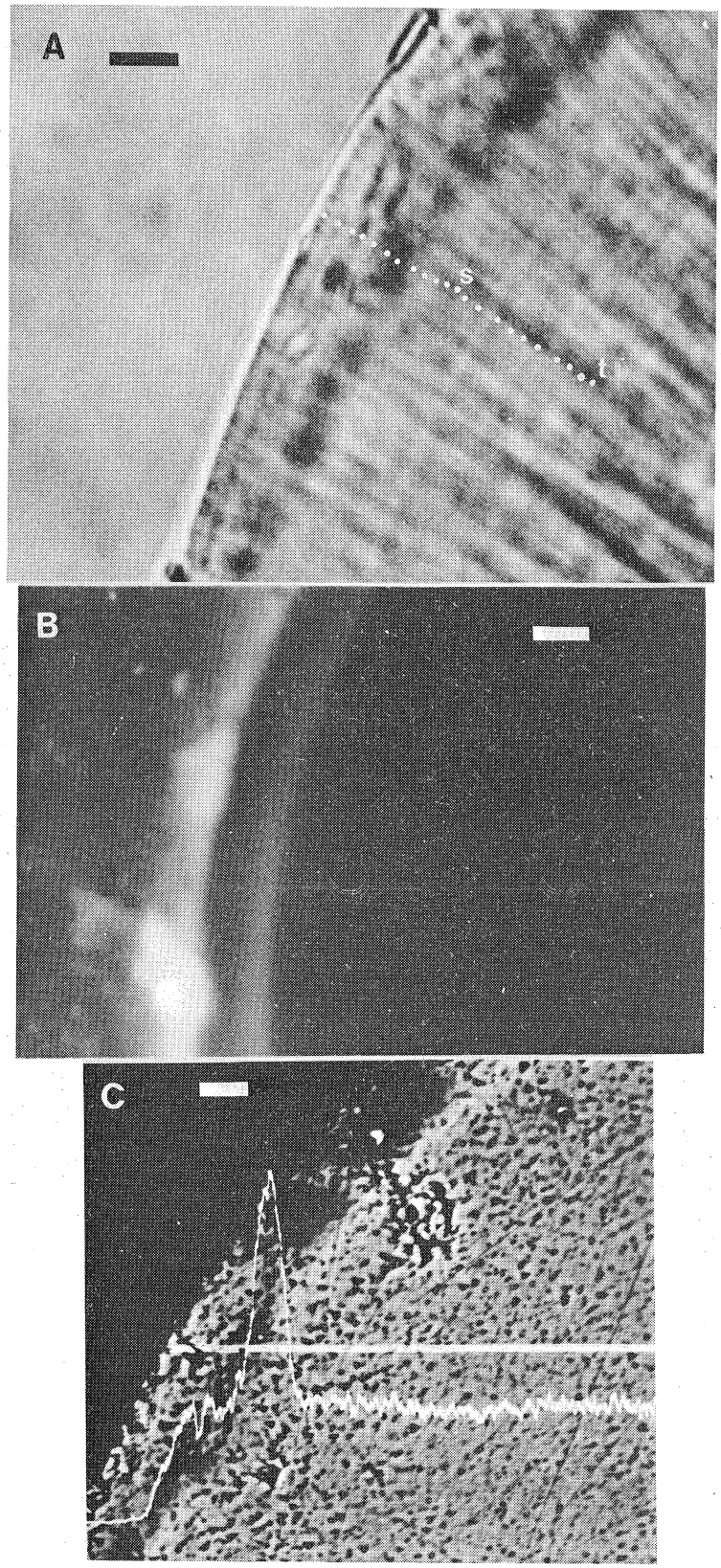

Fig. 8. a) Light micrograph of a marked specimen code number SM-13/12-1-5 (Table 1) under bright field illumination showing daily increments. Symbols s and t represent locations of the strontium and tetracycline labels respectively. Bar = 10 µm.

b) Light micrograph of the same specimen under ultraviolet light. Tetracycline fluorescence shows as a bright band. Bar = 27.7 µm.

c) SEM micrograph of the same specimen showing a line profile/back scattered electron image of the strontium band. Bar = 10 µm.

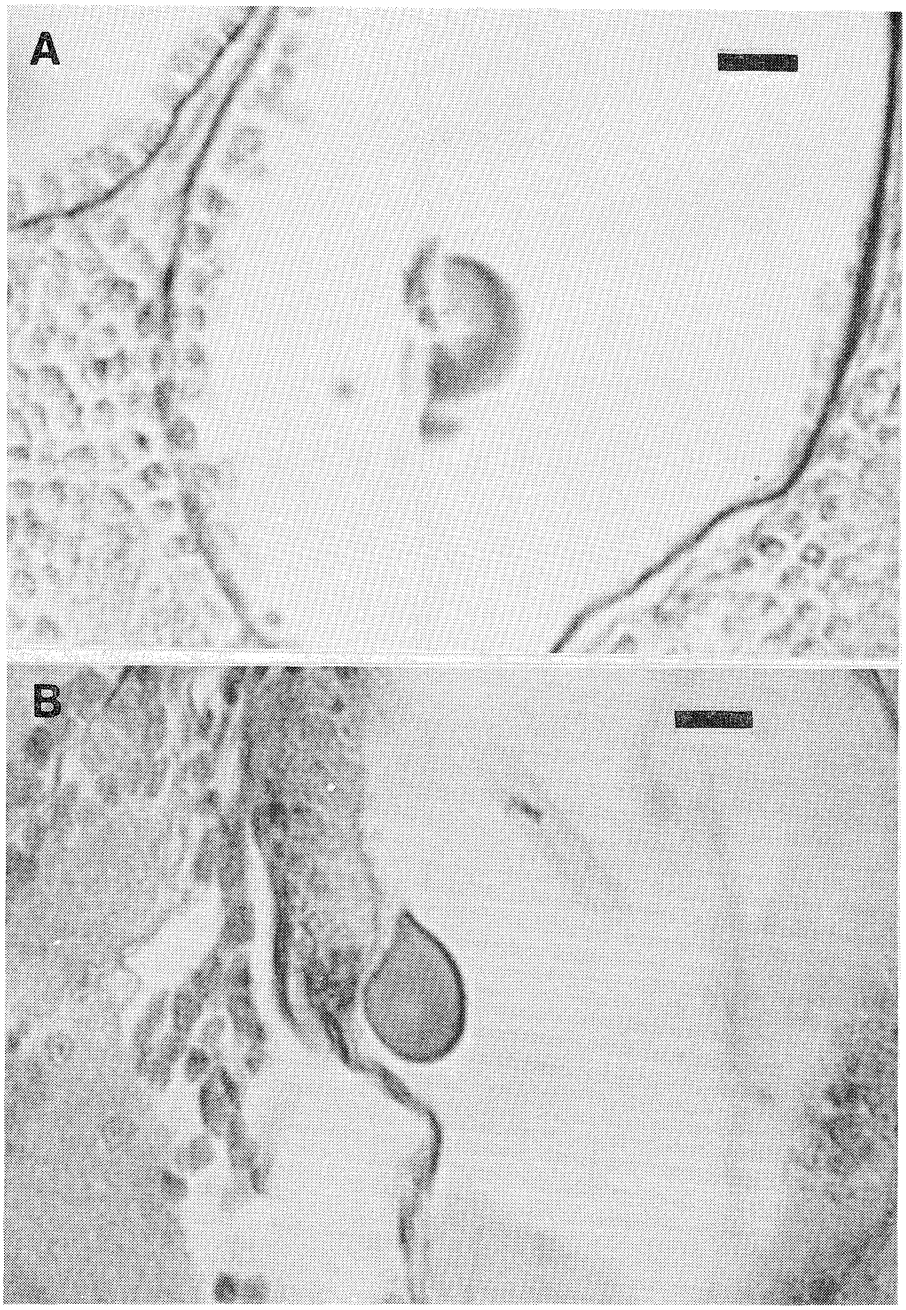

Fig. 9. a) Light micrograph of a 6 µm histological section showing a sectioned statolith from a 8 day old laboratory-reared larval squid which died two days after hatching. A dark band corresponding to the border of the nucleus and a fainter outer band are visible. Bar = 13.7 µm.

b) Light micrograph of a 6 µm histological section of a statolith from a 9 day old laboratory-reared larval squid. Although the increments are not visible, the nucleus can be seen as a dark, central core. Bar = 13.7 µm.

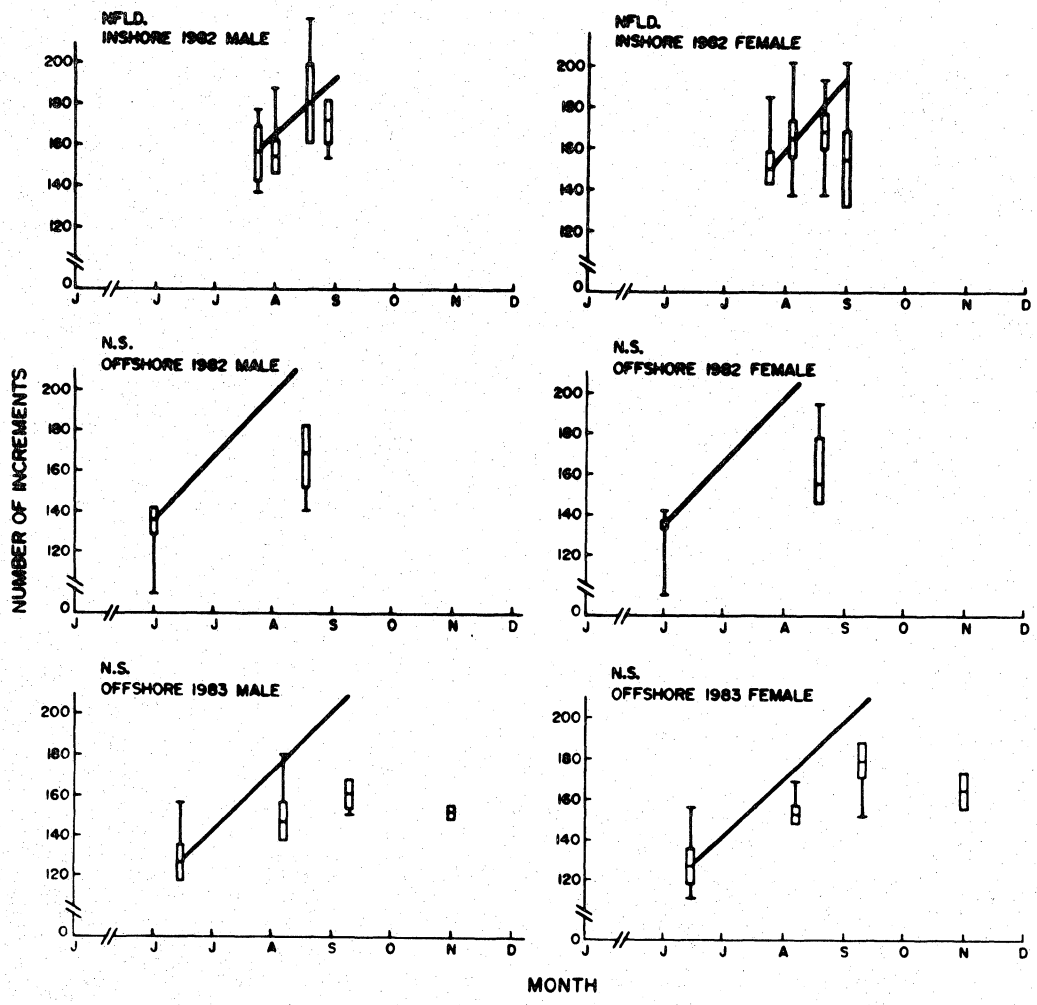

Figure 10 Mean and range of increment counts from inshore and offshore sample locations. Oblique solid line represents the expected number of daily increments. For explanation of the box and whisker plots see text.

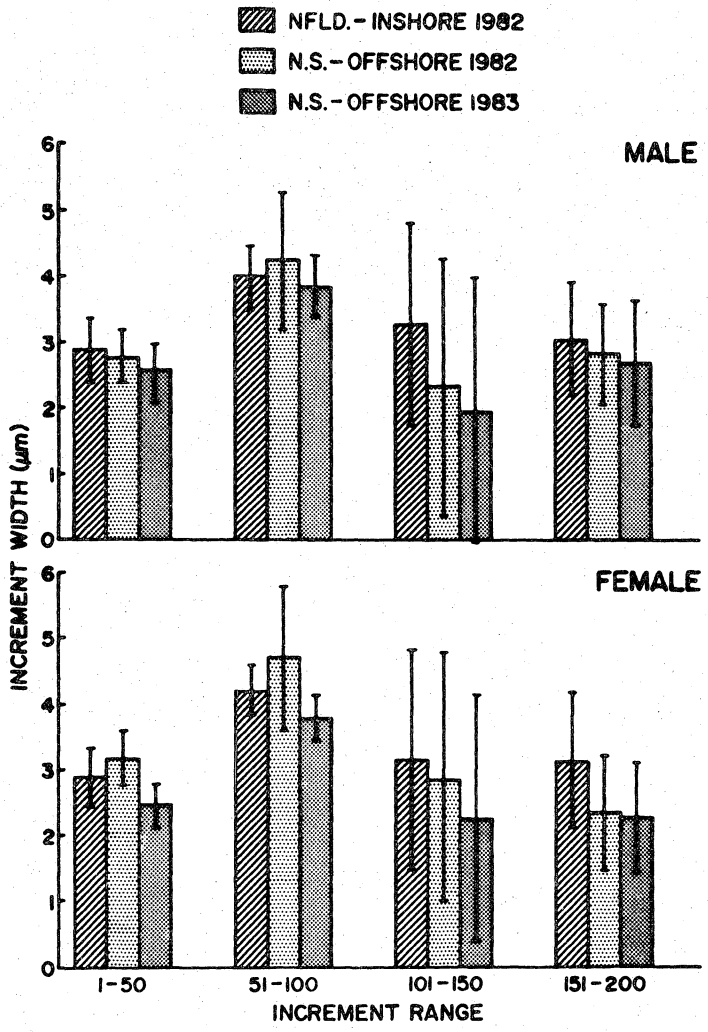

Figure 11 Summary of mean increment widths of all statoliths analyzed in Figure 10. Range = one standard deviation of the mean.

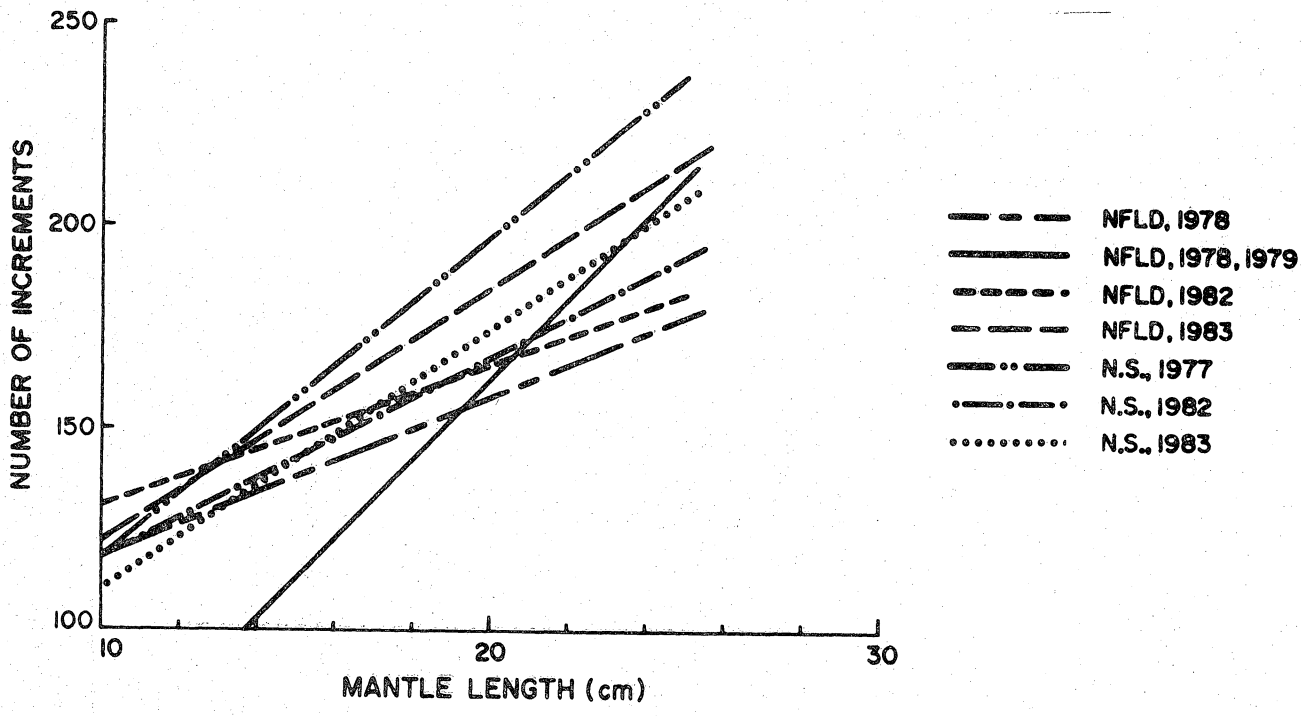

Figure 12 Relationship of dorsal mantle length and increment count from various studies .

Nfld 1978, 79 - Radtke (1983) - derived from his equation $y = 3.87 + 0.10x$, y = length (cm) and x = number of increments; r = 0.77

Nfld 1978 - Hurley and Beck (1979) - derived from theiq equation $y = 3.07 \times 10^{-4} x^{2.19}$, y = length (cm) and x = number of increments; r = 0.94.

N.S. 1977 - from Lipinski (1980) - $y = 2.9 \times {}^{0.8}$, r = (not given).

Nfld 1981 - from Morris (1983) - $y = 0.625x + 60.1$; r = 0.98.

Nfld 1982 - this study - $y = 4.47x + 77.2$; r = 0.64

N.S. 1982 - this study - $y = 4.77x + 71.8$; r = 0.87

N.S. 1983 - this study - $y = 4.09x + 92.1$; r = 0.80