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Problems associated with ageing squid from their statoliths: towards a more structured approach

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Abstract: It appears that squid statoliths cannot yet be regarded as accurate an ageing tool as fish otoliths. Statoliths from the same pair, prepared differently for viewing and counting increments, were compared. Increment counts do not imply age in days, because this was not validated. One statolith from each pair was examined by light microscopy (LM) after preparation following a new method. The other was viewed by Scanning Electron Microscopy (SEM) with a modified etching solution. Shape of each statolith was similar when compared by multiple regression analysis (11 variables, $n = 53$). There was a weak but significant difference between sexes (statoliths of females were slightly larger). All other differences were insignificant. Microscopic observation and increment counts of increments were successfully carried out for 37 pairs of statoliths. Significant differences between two independent counts were found for the LM method, but no significant differences were found between two independent SEM counts. Counts were significantly different when interpreted by both LM and SEM, probably because of poor resolution in the LM readings and over-resolution (growth layers prominent and numerous) in those read by SEM. Recommendations are made on how ageing studies, based on statoliths, should be structured and the results evaluated.

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Introduction

After a slow start (Young 1960, Clarke 1966, Spratt 1978, Lipinski 1978), squid statolith research has gained momentum, mainly in respect of analysis of age and growth (Bigelow 1992, Villanueva 1992, Arkhipkin & Mikheev 1992, Jackson & Choat 1992, Natsukari & Komine 1992, Arkhipkin 1993). Despite growing enthusiasm, however, many problems remain unresolved. Nothing has yet been published on systematic improvement of increment resolution for light microscopy (LM) or on criteria for differentiation between increments and other rings such as growth layers, seen by scanning electron microscopy (SEM) (Lipinski 1993). The problem of verification, as outlined by Wilson *et al.* (1983), has not been sufficiently researched for squid. Similar problems have been tackled successfully in fish (Campana 1992).

Validation experiments to date have dealt with <20 squid reared in aquaria. Data from just two squid (*Loligo chinensis*) have been presented as validation (Jackson 1990), making statistical analysis impossible (see Rice 1987). The most complete analyses to date (Nakamura & Sakurai 1991, Jackson *et al.* 1993) involved 17 and some 20 individuals respectively. In the latter analysis, 38 squid were reared in aquaria, but fewer pairs of statoliths were prepared successfully (the exact number was not given and the performance of the method used was not assessed clearly). Based on this rather limited evidence, many authors have assumed that statolith increments (see Clarke 1978, Lipinski *et al.* 1991, Lipinski 1993 for terminology) are deposited daily, even for species for which no direct validation has been carried out (Natsukari *et al.* 1988, Natsukari & Komine 1992, Arkhipkin & Mikheev 1992, Villanueva 1992, Arkhipkin 1993). Criticism of this practice (Natsukari *et al.* 1991) has usually been disregarded.

As a result, grossly incompatible increment counts for the same species from similar areas have been reported: *Berryteuthis magister* (Berry 1913) from the North Pacific (Arkhipkin as reported by Nesis 1993 vs. Natsukari *et al.* 1993) and *Todarodes angolensis* Adam, 1962 from the South Atlantic (Villanueva 1992 vs. Lipinski *et al.* 1993). This state of affairs emphasizes the need for rigorous statolith analysis, including the clearest possible definition of increments, an unambiguous interpretation of their arrangement, i.e., verification (see Geffen 1992 p. 101, for more elaborate definition and explanations) and statistical analysis of growth.

In this paper, an attempt is made at highlighting some of the problems associated with the first two elements of statolith analysis, investigating the possible cause of large differences between various increment counts. The last two elements (validation and growth modelling) will be adressed subsequently.

Material and methods

Chokka squid (*Loligo vulgaris reynaudii,* d'Orbigny, 1845) were caught either by jigging on their spawning grounds in November 1991 or by trawling in a much larger area between Cape Agulhas and East London (Fig. 1) in September 1992. In all, 32 males, 14 females and 8 unsexed juveniles were examined. Dorsal mantle length (ML) was measured to 1 mm and most of the sexed individuals weighed to the nearest gramme. Maturity

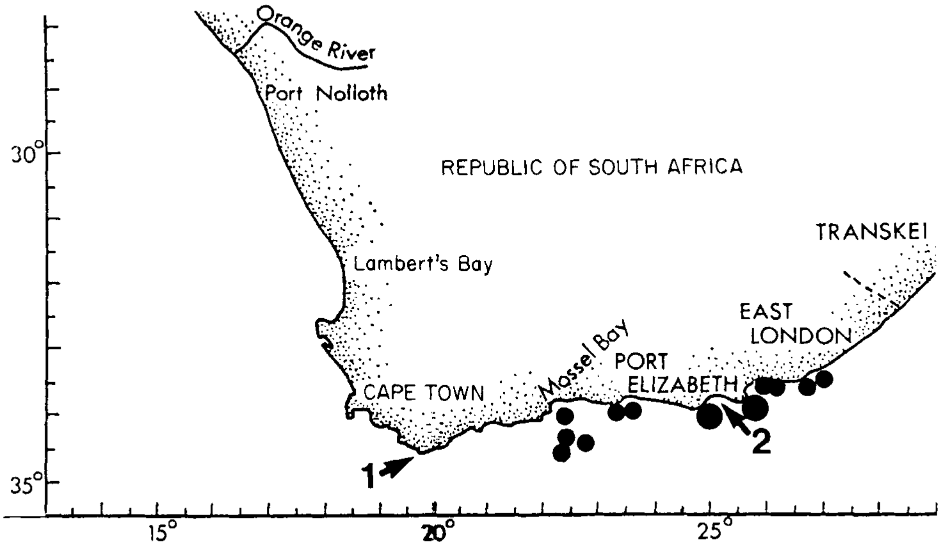

Fig. 1. Sampling stations. Large circles: spawning grounds, November 1991; small circles: trawling stations, September 1992. Arrow 1 - Cape Agulhas; arrow 2- St. Francis Bay.

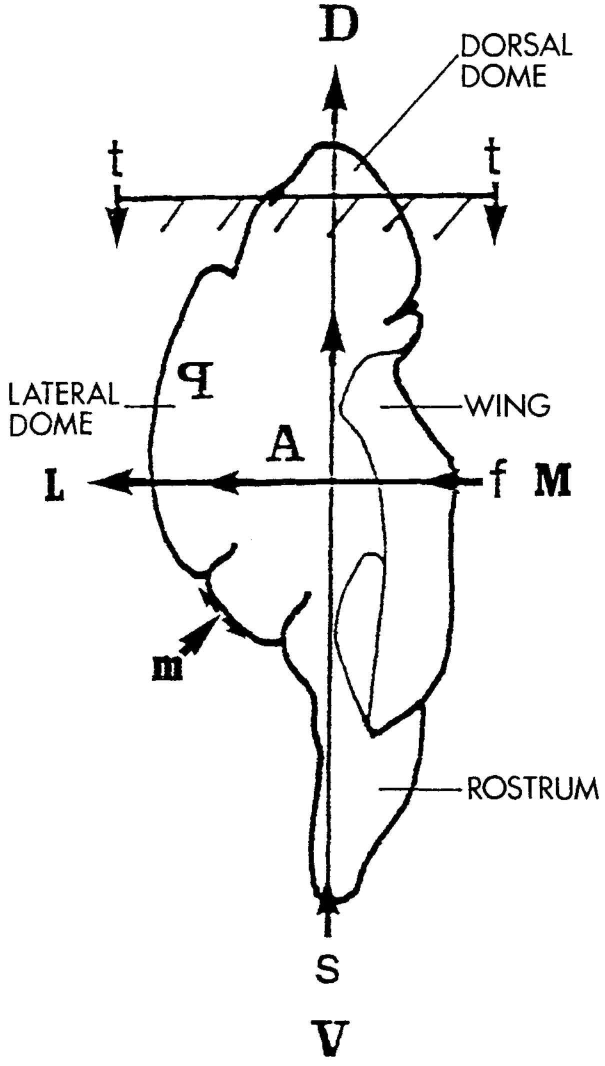

Fig. 2. Statolith terminology and orientation: A, anterior; D, dorsal; L, lateral; M, medial; P (mirror image), posterior; V, ventral; f, frontal; m, marginal; s, sagittal; t, transverse (Lipinski, 1993).

was determined according to the scale published by Lipinski (1979).

Statoliths were dissected from fresh animals (Lipinski 1981) and stored in 70% ethanol. Both left and right statoliths were later dried for 24 h at 40°C, weighed to the nearest μg and measured independently (by different readers) using variables described by Lipinski *et al.* (1993), but with slight modifications. The statolith orientation is given on Fig. 2 and the measurement definitions in Fig. 3a. Multiple regression analysis (Genstat5 Committee 1987) was applied to all individuals with 11 response variates (variables) and the following fitted terms: male-female; left-right statolith; area+year1-area+year2 (Fig. 1); person1-person2; mantle length.

One statolith from each pair was then prepared for light microscopy using a modification of the method of Natsukari (Dawe & Natsukari 1991, pp. 90–92). The modifications are summarized in Fig. 3b. Gelatine capsules of 0 size (not cut), supported in Cooke microtitre trays, were used as moulds when embedding statoliths in resin. Heat-curing orthodontic acrylic resin was used (Japanese Bioresin). All statoliths were sectioned in the oblique frontal plane (Fig. 2, 3c) and ground on both sides. The plane of the section was controlled precisely under the stereomicroscope so as to pass through the focus and the most lateral point of the lateral dome. The dorsal part of the statolith was ground first. Use of the oblique frontal plane of grinding facilitated comparison with statoliths prepared for SEM, which were broken in a similar plane. An additional advantage was the relative ease with which sections incorporating both the focus and the margin in the same plane could be obtained, a difficulty often encountered with the more commonly used transverse and sagittal sections (Fig. 2). The other statolith from each pair was prepared for SEM (Lipinski 1991, Lipinski *et al.* 1993) using an etching solution of 9 ml of 1% HCl with 1 ml of absolute ethanol and 100 mg of $SrCl_2$ x $6H_2O$.

Prepared statoliths were photographed and the increments counted from the photographs (each increment taken to consist

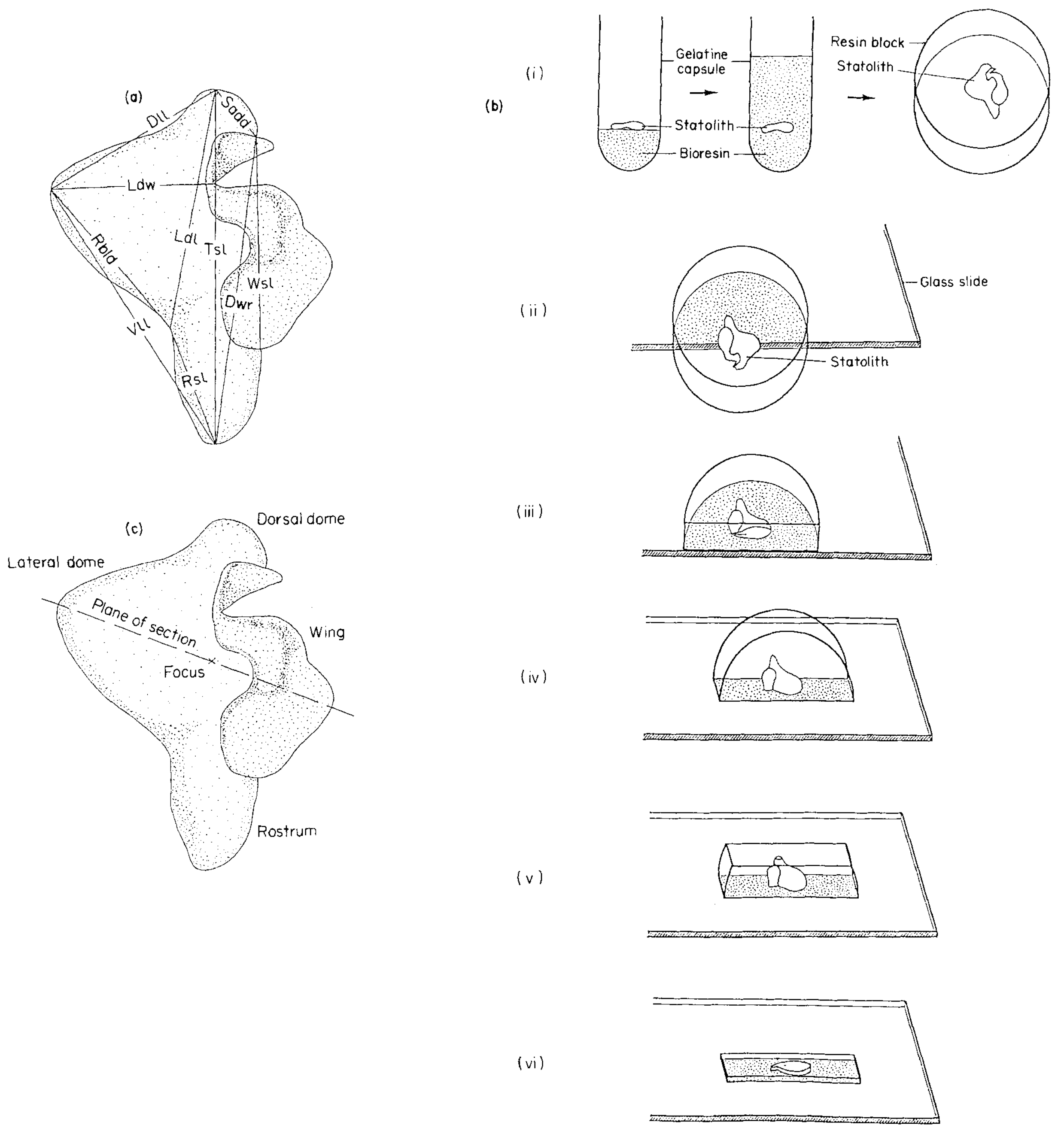

Fig. 3. a. Measurements recorded for each statolith: TSL, total statolith length; LDL, lateral plus dorsal dome length; DWR, dorsal wing to tip of rostrum; WSL, wing length; DLL, dorso-lateral length; RSL, rostral length; LDW, lateral dome width; RBLD, rostral base to lateral tip of lateral dome; VLL, ventro-lateral length; SADD, dorsal tip to dorsal wing. **b.** Statolith preparation for LM: (i) each statolith is embedded horizontally in resin, using a gelatine capsule as a mould. Once cured, the resin block is trimmed and polished; (ii) the block is attached to a glass slide (such that the anterior surface of the statolith faces up) by means of a nail varnish, and orientated so that the edge of the slide governs the desired plane of sectioning; (iii) the resin block is ground until the edge of the slide has been reached. The ground surface is then polished; (iv) the block is removed and remounted on a clean glass slide (ground surface down); (v) the block is ground in a plane parallel to that of the surface of the slide; (vi) when the specimen has reached the desired thickness, the surface is polished and the specimen is ready for viewing. **c.** Plane of sectioning used for light microscopy (LM).

Table I. Multiple regression analysis results for *Loligo vulgaris reynaudii* (see Fig. 3a for definitions).

Correlation matrix

	Mass	TSL	LDL	DWR	WEL	DLL	SADD	RSL	LDW	RBLD	VLL	Mantle
Mass	1.000											
TSL	0.959	1.000										
LDL	0.947	0.980	1.000									
DWR	0.956	0.991	0.975	1.000								
WSL	0.928	0.967	0.952	0.967	1.000							
DLL	0.925	0.925	0.906	0.924	0.888	1.000						
SADD	0.818	0.843	0.824	0.815	0.780	0.794	1.000					
RSL	0.879	0.915	0.843	0.908	0.890	0.854	0.753	1.000				
LDW	0.872	0.860	0.860	0.852	0.824	0.823	0.773	0.759	1.000			
RBLD	0.765	0.812	0.845	0.808	0.797	0.693	0.672	0.622	0.719	1.000		
VLL	0.922	0.968	0.941	0.963	0.947	0.864	0.809	0.913	0.828	0.866	1.000	
Mantle	0.930	0.908	0.879	0.900	0.861	0.905	0.749	0.853	0.814	0.653	0.854	1.000

Response variate: TSL

Degrees of freedom: regression 4, residual 101

	estimate	s.e.	*t*
Constant	1.2502	0.0300	41.63
sex 2	0.1099	0.0248	4.43
ltrt 2	-0.0092	0.0212	-0.43
md 2	0.0117	0.0212	0.55
mantle	0.002629	0.000109	24.19

Response variate: TSL (males only)

Degrees of freedom: regression 4, residual 57

	estimate	s.e.	*t*
Constant	1.4352	0.0393	36.51
ltrt 2	-0.0033	0.0240	-0.14
md 2	0.0174	0.0240	0.73
mantle	0.001848	0.000141	13.15
month	0.0411	0.0253	1.62

Sex 2: males-females
ltrt 2: left-right statolith
md 2: two different persons
mantle: ML
month: area+year1-area+year2 (see Fig. 1)

of one strong dark and one strong light ring; see Lipinski *et al.* 1991, Lipinski 1993). Two readers made one count each for every statolith. Counts within and between each method were compared by means of paired *t*-tests and the relationships between ML and number of increments was investigated by regression analyses. Slopes and elevations of regression lines were compared following the method of Zar (1984), using Student's *t* statistics.

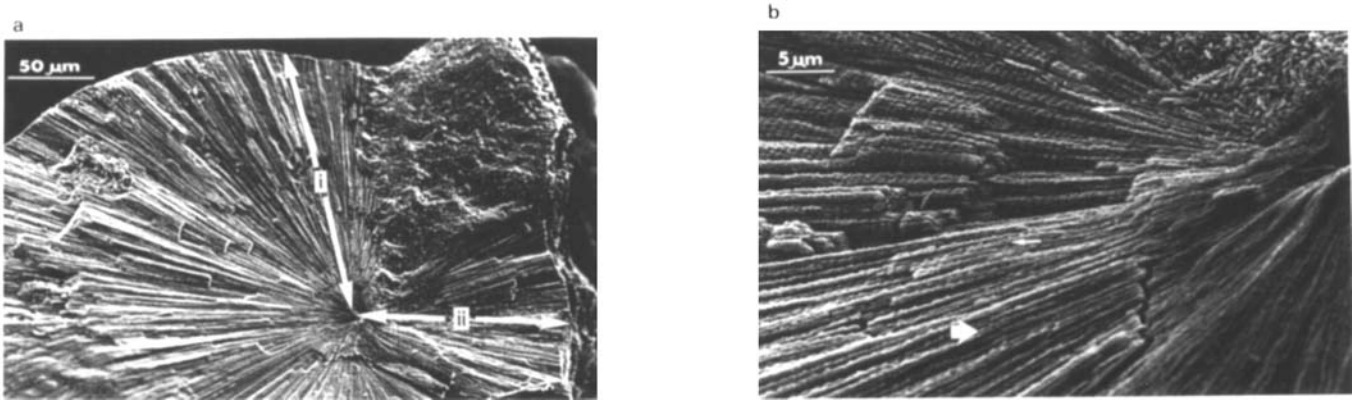

Fig. 4. a. Frontal break through the lateral dome, viewed under SEM (the same individual as in Fig.5); i - longer axis of increment counting (close to wing); ii - shorter axis (on the other side of the wing). **b.** Changing widths of increments (SEM). Thick arrow indicate a wide section of an increment, while small arrows indicate the narrow part of the same increment. ML = 82 mm.

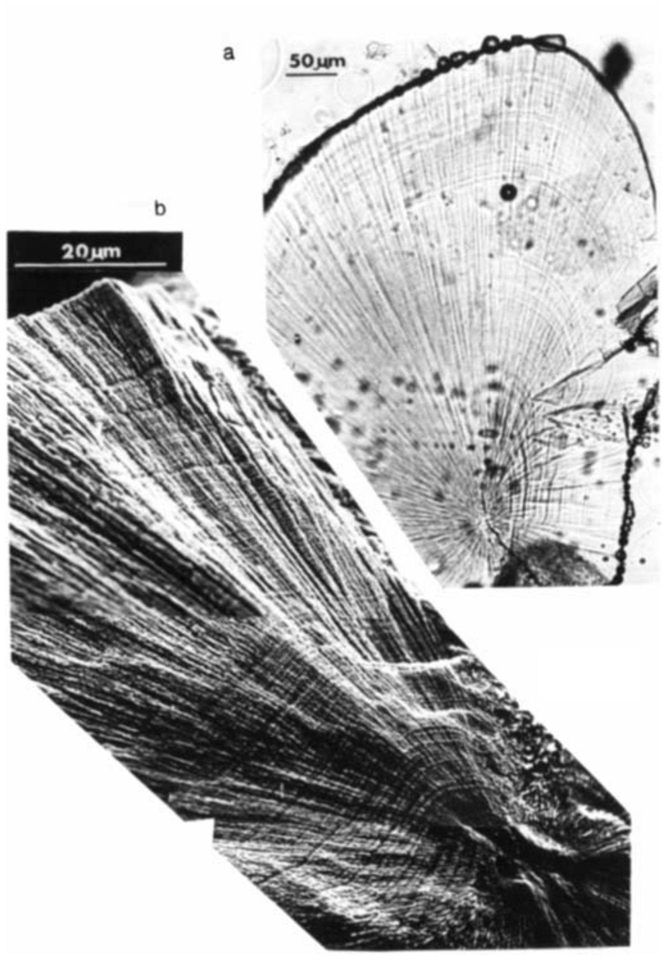

Fig. 5. a. A statolith viewed under LM (right statolith of a pair). **b.** A statolith viewed under SEM (left statolith of a pair). ML = 60 mm, TW = 11 g, juvenile male.

Results and discussion

Comparisons of natural or experimentally induced microstructures between members of a pair of statoliths or otoliths should first consider whether their shape and microstructure are similar.

Results of multiple regression analyses (Table I) revealed strong correlation between all variables investigated. Total statolith length (TSL) is given (Table I) as an example of the relationship between variables and fitted terms. Differences between statoliths of male and female squid, although small, were significant, the female statoliths being slightly larger. Differences in ML were highly significant, as would be expected (Lipinski *et al.* 1993), but all other multiple regression differences were insignificant. Of particular relevance is the absence of any significant difference between right and left statoliths for all variables, supporting the view that length (Lipinski 1981) and structures (Natsukari *et al.* 1988) of statoliths of the same pair are similar. Published observations of statolith microstructures are scant, however, and may be insufficient to draw such a conclusion.

In particular, patterns of increment width, as well as "zones" (Morris & Aldrich 1985, Arkhipkin 1988, Arkhipkin & Mikheev 1992, Arkhipkin 1993) change according to the axis of viewing (Fig. 4). For example, the number of increments along axis "a" of Fig. 4a should be the same as along axis "b", but the width and the distribution pattern of the increments will obviously differ between the two axes. Furthermore, in the same plane, increment width may change. In Fig. 4b, a wide increment (thick arrow) narrows gradually (thin arrows). This is further complicated by staggered growth phenomena (a number of rings joining together and some of them disappearing; Lipinski 1993), which may potentially invalidate the use of measurements and the technique of switching direction of counting employed (Morris & Aldrich

Table II. The stratified success level for the statolith analysis.
n = 54

Number	Success level	LM	SEM
1	Statoliths destroyed/lost	5	4
2	Statoliths not readable (rejected)	6	7
3	Statoliths require considerable extrapolation during counting	15	22
4	Statoliths need little extrapolation	5	11
5	Statoliths readable from nucleus/protostatolith to the margin	23 (42.6%)	10 (18.5%)
6	Significant agreement between readers (paired *t*-test)	$t = 5.934$, d.f. = 36. Counts significantly different	$t = 1.233$, d.f. = 39. Counts significantly similar
7	Significant agreement between different methods (paired *t*-test)	$t = 2.931$, d.f. = 36 $p < 0.01$. Significant difference between methods (based on a first count only)	
8	Provisional validation (no statistical verification possible; see Rice 1987)	–	–
9	Full validation (both aquarium and field material; statistical verification completed	–	–

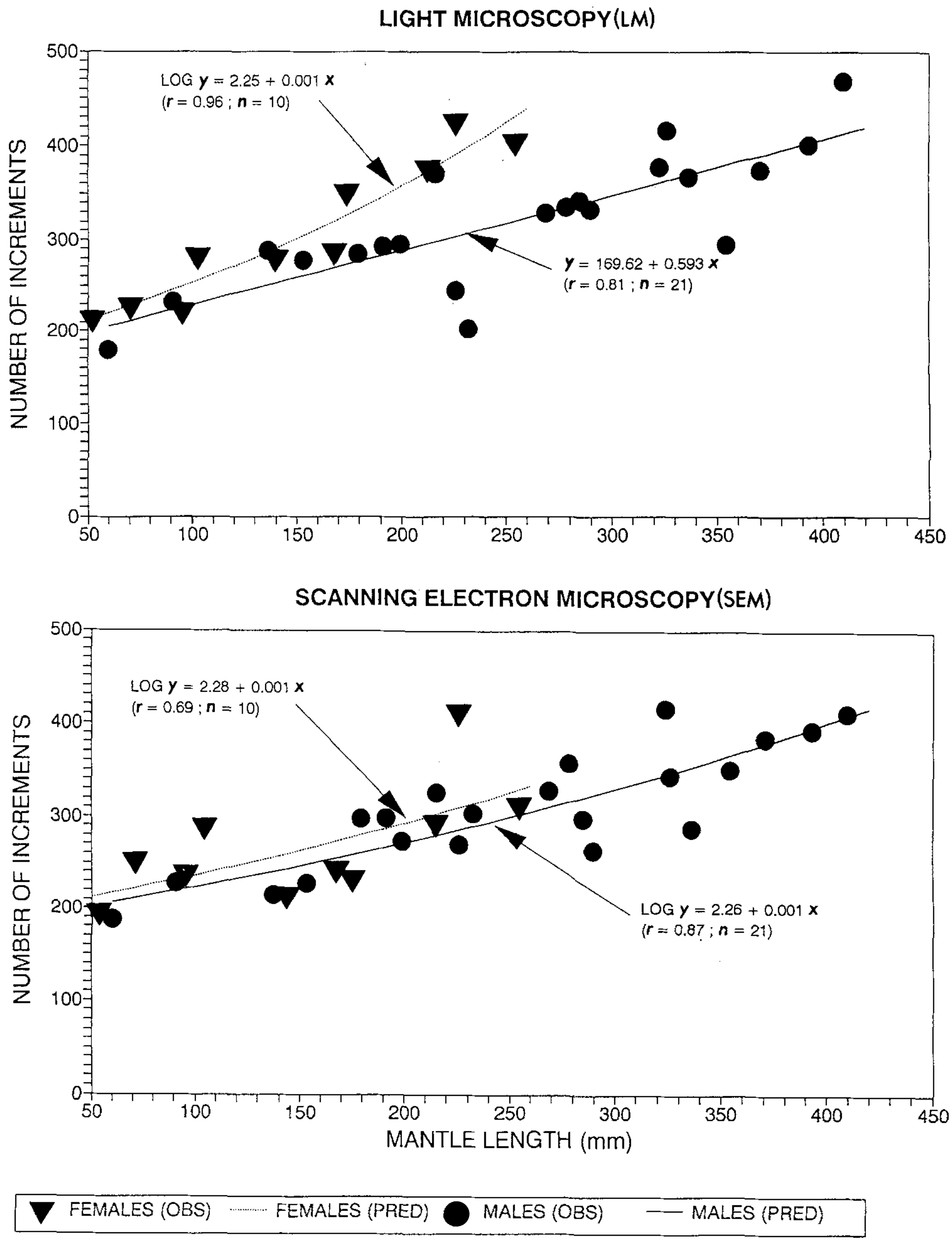

Fig. 6. Comparison of increment counts against mantle lengths between methods and sexes.

1985, Fig. 7) and advocated by Ralston (1985). The squid statolith may show patterns of zonation but this may be more complicated than previously thought.

Are these patterns identical in both statoliths from the pair? This question needs to be investigated. Nevertheless, on the basis of results of multiple regression analyses, it can be assumed that increment counts between right and left statoliths can be compared meaningfully. Results of these comparisons are presented in Figs 5, 6 and Table II. LM occasionally reveals substantially fewer increments than SEM (Fig. 5). Together with some interpolations and extrapolations (especially at the margin) this results in significantly different counts between different methods (Table II). Nevertheless, the two methods do provide similar approximations of statolith growth (expressed as the number of increments counted) in terms of increase in mantle length (Fig. 6).

Slopes and the elevations of regressions for males were not significantly different between methods ($p > 0.50$), after a semi-log equation for male statoliths examined under LM has been calculated ($\log y = 2.28 + 0.001x$; $r = 0.79$; $n = 21$). Slopes ($p > 0.10$) and the elevations for females were also not statistically different ($p > 0.50$). Differences between sexes were, however, significant for LM (slopes, $p < 0.05$; elevations, $p < 0.005$) though not for SEM (slopes, $p > 0.50$; elevations, $p > 0.20$).

It is believed that ML will be the most frequently used independent variable (x); properly validated numbers of increments (= days) will be rare. Because of this, ML and the number of increments are set differently on Fig. 6 than in most of the published work (see, for example, Villanueva 1992). To facilitate comparison with other papers, however, "reversed" equations are given below:

$$\text{LM males: } y = -96.53 + 1.09x \ (r{=}0.81, n{=}21)$$
$$\text{or } y = -607.42 + 746.19 \log x \ (r{=}0.79);$$
$$\text{LM females: } y = -1371.57 + 614.12 \log x \ (r{=}0.96, n{=}10);$$
$$\text{SEM males: } y = -1959.02 + 892.55 \log x \ (r{=}0.87, n{=}21);$$
$$\text{SEM females: } y = -1094.96 + 514.40 \log x \ (r{=}0.69, n{=}10).$$

Bello (1991) strongly advocated use of total weight (TW) instead of ML for assessing growth, particularly for females. To check the merit of his recommendation in the light of growth of squid statoliths, relationships between ML and TW of squid against SW (statolith weight) were calculated for both sexes by linear regression ($n = 10$).

The formulae obtained were as follows:

$$\text{Females: } \mathrm{SW} = 0.08587 + 0.00606\mathrm{ML},\ r = 0.99;$$
$$\mathrm{SW} = 0.55118 + 0.00348\mathrm{TW},\ r = 0.95.$$
$$\text{Males: } \mathrm{SW} = 0.29853 + 0.00390\mathrm{ML},\ r = 0.97;$$
$$\mathrm{SW} = 0.71645 + 0.00158\mathrm{TW},\ r = 0.94.$$

It appears that both ML and TW reflect statolith growth very well, although ML is marginally better.

The problems known from otolith studies (poor resolution in LM, over-resolution in SEM; Campana, 1992) are also evident when interpreting statoliths, but they are currently much less investigated and understood. The preparation success of statoliths may be reasonably high (Table II, LM, numbers 1–5), but their verification confidence is poor (Table II, LM, numbers 6–7) and *vice versa* (Table II, SEM, numbers 1–7). Therefore, no recommendation can be made at present as to which method (LM or SEM) is better. Both should be used and improved. In Table II a summary is given as to how ageing studies based on statoliths should be structured and the results evaluated. A full study should deal with all points listed under numbers 1–9. The results of such a study have not yet been published.

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