BIOMINERALIZATION IN LOLIGINID SQUID STATOCYSTS AND THE USE OF STATOLITHS TO ESTIMATE AGE

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DECLARATION

In this thesis, I report the results of original research carried out by myself between 1993 and 1998. The data collected during the experiments are my own. The ideas and conclusions presented are my own, but developed under the supervision and criticism of Dr. Marek Lipinski (Sea Fisheries Research Institute), Prof. John Field (University of Cape Town) and Prof. Peter Cook (University of Cape Town).

Dr. Marek Lipinski prepared all of the specimens which were analyzed by scanning electron microscopy, as well as viewing and photographing the specimens using the SEM at the University of Cape Town Electron Microscope Unit. Dr. Lipinski also prepared the specimens for the nuclear microprobe investigation. The analyses of this material using the microprobe were conducted jointly by myself, Dr. Lipinski and Dr. Przybylowicz of the National Accelerator Center. All other specimens were prepared and analyzed by myself.

The tagging and marking of squid during the field validation study (Chapter 3) was conducted by Dr. Lipinski and members of the demersal section of the Sea Fisheries Research Institute. Marked squid were recovered from commercial catches by Dr. W.H.H. Sauer. Marking, tagging and subsequent maintenance of squid during the laboratory study was conducted jointly by myself and Dr. Lipinski.

Dr. Nancy Alcock (University of Texas Medical Branch, Galveston, USA) conducted the atomic absorption spectrometry analyses on samples I collected during the experiments described in Chapter 6. Dr. M. Kinter (University of Virginia, Charlottesville, USA) conducted the liquid chromatography and mass spectrometry analyses described in Chapter 7.

Dr. L.G. Underhill ran the generalized linear models for the error modelling excersise described in Chapter 3. All other statistical analyses were conducted by myself, using Statistica, a commercially available statistical software package.

Publications which have been generated by this work are listed in the Acknowledgements section.

This work has not been submitted for a degree at any other university, and any assistance I received during the course of its completion is fully acknowledged.

D.

Marius Deon Durholtz

DEDICATION

To my parents, who have made all things possible.

ABSTRACT

The use of statoliths to estimate the age of loliginid squid is critically assessed. Alternating "light" and "dark" layers visible in statoliths viewed with a light microscope are proposed to reflect a daily cycle in statolith mineralization. A "light" and adjacent "dark" layer, known as an increment, are hypothesized to be deposited over a 24 hour period, and hence can be used to estimate the age of squid. Research was directed to the methodological aspects of the application of the statolith age estimation technique, as well as the physiological processes of statolith mineralization.

A method of preparing statoliths of chokka squid *Loligo vulgaris reynaudii* for increment counting using light microscopy (LM) is described. The performance of the method is assessed by comparing the results with those obtained using scanning electron microscopy (SEM). The LM method is superior to the SEM method in that fewer specimens which require interpolation or extrapolation of increment numbers is obtained. For any given reader, the null hypothesis that increment counts obtained using LM were similar to those obtained using SEM could not be rejected. Either method can be used to count increments in chokka statoliths with similar effectiveness. Between-reader differences in increment interpretation and counting were apparent in the LM results, but not in SEM. The problem of interpretation of statolith microstructure may introduce a significant source of error when using LM rather than SEM.

The hypothesis that increments in squid statoliths are deposited with a daily frequency was tested for the first time in wild populations, using statolith marking experiments. The results are consistent with daily increment production in adult males of *Loligo vulgaris reynaudii*. The same result was obtained for the single adult female included in the study. Analysis of the errors involved in increment counting highlighted the importance of proper statolith preparation. The error in counting increments in a poorly prepared statolith may be three times higher than that in a well-prepared statolith. The results of statolith marking experiments conducted on *Loligo vulgaris reynaudii* in the laboratory were consistent with daily increment production in males, but not in females. Increment deposition rates in males were similar in both field and laboratory conditions, indicating that the results of laboratory experiments of this nature can be justifiably extrapolated to wild individuals.

The hypothesis that temperature influences statolith growth and resultant microstructure was tested in a laboratory study of the loliginid squid *Lolliguncula brevis*. Groups of squid were subjected to various temperature regimes for extended periods, and the regions of the statolith deposited during the experiments were subsequently examined using laser scanning microscopy. Checks deposited in response to stressful events such as capture and handling are shown to consist of a series of prominent increments, rather than reflecting a period of interrupted statolith growth. Constant temperature conditions appeared to reduce increment contrast and clarity, presumably due to a dampening of the metabolic cycles associated with increment formation. Average statolith growth rates observed over the course of the experiment showed a strong positive relationship to ambient temperature. A significant sex effect was also apparent, with statoliths of female squid generally growing faster than those of males. Statolith growth rates at low temperatures (15 °C) indicated that the widths of daily increments produced under these conditions approach the resolution limits of a light microscope (less than 1 μm). The implications for studies using increment numbers to estimate age are discussed.

Statolith mineralization in *Loligo vulgaris reynaudii* was investigated using a nuclear microprobe. Quantitative maps of calcium (Ca) and strontium (Sr) distribution in the frontal plane were obtained, and the measurements were complemented by LM and SEM observations. Ca and Sr were uniformly distributed in the statoliths of paralarval squid. In contrast, Sr was concentrated in the wing and adjacent areas of juvenile and adult statoliths, whereas Ca concentration was highest on the edge of the lateral dome. Although temperature, sex, stage of maturity and the administration of oxytetracycline may influence the quantitative distribution of Ca and Sr in the statoliths of adult squid, they do not appear to affect this general pattern. Increments showed the best definition in the areas anterior and adjacent to the wing, corresponding to areas of high Sr content. This finding supports the hypothesis linking strontium with statolith deposition and the formation of clearly defined increments.

It is proposed that increment formation in squid statoliths is a function of, or reflected by, fluctuations in the chemistry of the surrounding statocyst endolymph. The hypothesis that increments are deposited with a daily frequency was tested at the physiological level by monitoring the levels of endolymph calcium and magnesium ion content in *Lolliguncula brevis* over periods ranging from 24 to 48 hours. Attempts to measure strontium ion concentration and pH were unsuccessful. The data were analysed using a non-linear regression model. Endolymph calcium and magnesium ion concentrations displayed periodic fluctuations, but evidence suggesting that these fluctuations occurred with a daily frequency was weak, particularly in the case of calcium. In general, endolymph magnesium ion concentrations peaked at night, while calcium ion concentration peaked during the day. The implications of the results in terms of increment formation in statoliths are discussed, and recommendations for future studies made.

A preliminary study aimed at purifying and characterizing the organic matrix proteins from statoliths of the loliginid squid *Lolliguncula brevis* is described. Proteins extracted from the statoliths by a simple decalcification technique were separated into two fractions, insoluble and soluble in aqueous solutions, and purified using gel electrophoresis. Glycoprotein enzyme immunoassays indicated that the major proteins of both fractions are glycosylated. Molecular weight and partial sequence data of peptides from five of the insoluble and one of the soluble proteins were obtained using liquid chromatography - electrospray - tandem mass spectroscopy. These data were used in a database search for possible homologs. No matches were found, suggesting that these proteins may belong to a class of hitherto undescribed organic matrix compounds. It is proposed that the differential secretion of the insoluble fraction proteins (or their sub-components) into the statocyst endolymph is responsible for the bipartite nature of statolith increments.

It is concluded that statoliths remain a viable tool for estimating the age of loliginid squid, providing the level of error inherent in the age estimates are quantified for the species and life stages being assessed.

CHAPTER 1 - GENERAL INTRODUCTION

The research described in this thesis investigated aspects of biomineralization in loliginid squid statocysts, and specifically the formation of statoliths, in an attempt to assess the use of these structures to obtain age and growth information from individual squid.

1.1. WHY AGE SQUID?

Knowledge of age and growth of individuals in exploited populations is fundamental to fisheries science. Information concerning age structure is required to clarify growth, survival, recruitment and the effects of fishing pressure. Classical stock assessment theory in fisheries is largely based on age-structured models using an annual time scale (Caddy, 1991). A number of techniques are used to obtain this information, the most common being various size-frequency methods. For example, length-frequency distributions obtained from sequential samples are separated into age groups or cohorts (identified as peaks in the size-frequency distribution). The changes in size and relative proportions of each cohort over time can then be used to estimate annual growth and mortality rates. The development of methods to estimate the age of individuals directly from time-related marks in various hard structures has greatly facilitated this process, particularly in cases where cohorts are not readily identifiable.

In the context of exploited squid populations, these techniques are usually not suitable for stock assessment purposes. The development of effective management strategies for exploited squid stocks has consequently been hampered in the past by the lack of reliable age structure information. Most commercially exploited squid species appear to have short life spans, rarely exceeding two years (see Jackson, 1994a). Any form of annual marks are therefore of limited use. Further, the conventional size-based analyses applied to fin fish populations have been found to be inappropriate for squid stock assessment procedures. Several properties of squid populations combine to complicate such analyses of population structure (Caddy, 1991; Hatfield and Rodhouse, 1994; Brodziak and Macy, 1996). These include:

(i) Rapid, highly variable growth rates. Squid are very susceptible to environmental change, and growth rates reflect this, being very plastic in response to local environmental conditions.

(ii) Short life spans.

(iii) Migrations can introduce errors if several migrating groups are mixed in the population, but not identified as such (i.e. the assumption was made that the groups were successive modes from the same stock). Hatfield and Rodhouse (1994) demonstrated this effect in a *Loligo gahi* population in the Falkland Islands. Dawe and Beck (1997) noted that migrations in *Illex illecebrosus* would result in misleading results if attempts to estimate growth rates were made by modelling size data alone.

(iv) Extended spawning seasons, with several peaks in spawning activity which lead to the presence of several microcohorts, each with characteristic growth and survival rates.

Modal analysis of size-frequency distributions involves considerable bias if modal groups or cohorts are not identified (Pierce and Guerra, 1994). These problems and biases are insurmountable without supplementary age data to clarify the age structure of the population (Caddy, 1991). In an evaluation of mathematical stock assessment models applied to the South African chokka squid (*Loligo vulgaris reynaudii*) fishery, Augustyn *et al.* (1993) recommended that cohorts entering the fishery be treated as independent stock units, each of which is assessed and subsequently monitored. This approach requires sophisticated cohort-separation procedures which can only be based on reliable age information obtained from the stock. Ageing techniques represent the only valid means of testing population models and elucidating details of squid life history (O'Dor and Coelho, 1993).

The discovery of daily growth rings in the otoliths of teleost fish (Panella, 1971) initiated a new era in studies of age and growth. Analysis of these features has subsequently become a widely accepted method for estimating the age of individuals on a daily time scale. Various structures in squid have been identified for this purpose, including the beak (Clarke, 1965), gladius (LaRoe, 1971; Bizikov, 1991), crystalline lens of the eye (Willekens *et al.*, 1984) and the statoliths (Lipinski, 1978; Spratt, 1978; Hurley *et al.*, 1979). The latter structures appear to be the most promising, and a great deal of attention has been directed at the statolith ageing technique and its application to squid stock assessments.

1.2. STATOLITHS, STATOCYSTS AND THE DAILY INCREMENT AGEING TECHNIQUE

The statoliths of squid are small calcified structures, a single statolith occurring in each of the paired statocysts (see Dilly, 1976 and Clarke, 1978 for descriptions of statolith morphology). The statocysts are fluid-filled cavities located ventro-posteriorly in the cephalic cartilage (see Stephens and Young, 1982 for a description of the morphology and structure of the statocysts of loliginid squids). The statocysts are sensory organs responsible for the detection of both angular and linear acceleration of the animal, and can thus be considered as the functional analogue of the vestibular system of vertebrates. Statocyst structure and function in various cephalopod taxa are described by Budelmann (1975, 1977, 1980, 1994), and is comprehensively reviewed in the cephalopods by Young (1989). The statoliths are situated in the anterior region of the statocyst, and together with two statoconial layers (layers of numerous fine crystals), provide the animal with information regarding its attitude in space, essentially functioning as a gravity receptor. Each statolith is positioned vertically in the transverse plane, and is loosely attached to a region of sensory epithelium containing numerous hair cells known as the macula statica princeps. Attachment is by means of a mass of fine fibrils attached to the surfaces of the hair cells of the macula, with large numbers of minute elongated crystals embedded among the fibrils (Stephens and Young, 1982). The two statoconial layers cover two other maculae known as the macula neglecta superior and the macula neglecta inferior. All three maculae are arranged at roughly right angles to one another. The statoliths are compact structures, approximately 1mm long in adult *Loligo vulgaris reynaudii*.

Statolith morphology is highly complex, and is species specific (Clarke and Maddock, 1988a, 1988b). Statoliths are composed of four main parts, referred to as the dorsal and lateral domes, the rostrum and the wing (Fig. 1.1).

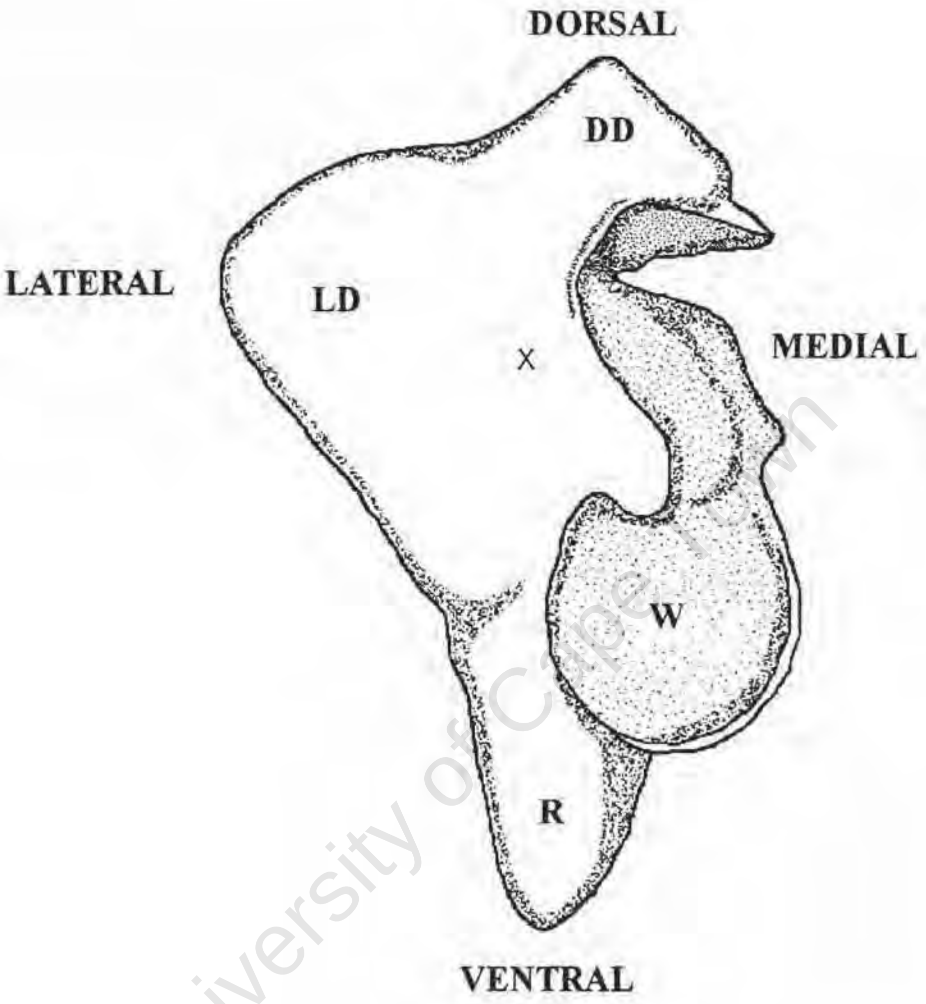


Figure 1.1: Diagram showing an anterior view of the right statolith of a loliginid squid. DD - dorsal dome; LD - lateral dome; R - rostrum; W - wing; X - position of focus; D - dorsal; V - ventral; L - lateral; M - medial.

The wing marks the area of attachment of the statolith to the macula, and its shape corresponds closely to that of the macula (the shape of a "3"). The wing shows a different microstructure to the rest of the statolith, in that it is composed of loosely packed irregular crystals, as opposed to the well-ordered crystal arrangement in the rest of the statolith. Apart from the wing area, the remainder of the statolith is freely suspended in the endolymphatic fluid of the statocyst. Squid statoliths are composed of crystals of aragonitic calcium carbonate deposited on a proteinaceous organic matrix. It has been suggested that the organic matrix of biologically calcified tissues may function as a template for crystal deposition (Lowenstam, 1981). Statolith formation is an unusual example of biomineralization. In most biological systems, the formation of calcareous structures is associated with some form of secretory surface which is in direct contact with the forming mineral (Wilbur, 1980). In contrast, the squid statolith appears to represent a case where ions are secreted into solution (the statocyst endolymph), and then deposited on the mineral at some point removed from the secretion site (Morris, 1988, 1991a). Examination of the internal microstructure of squid statoliths under transmitted light reveals a series of alternating light and dark rings, extending concentrically from a central focus (Figure 1.2).

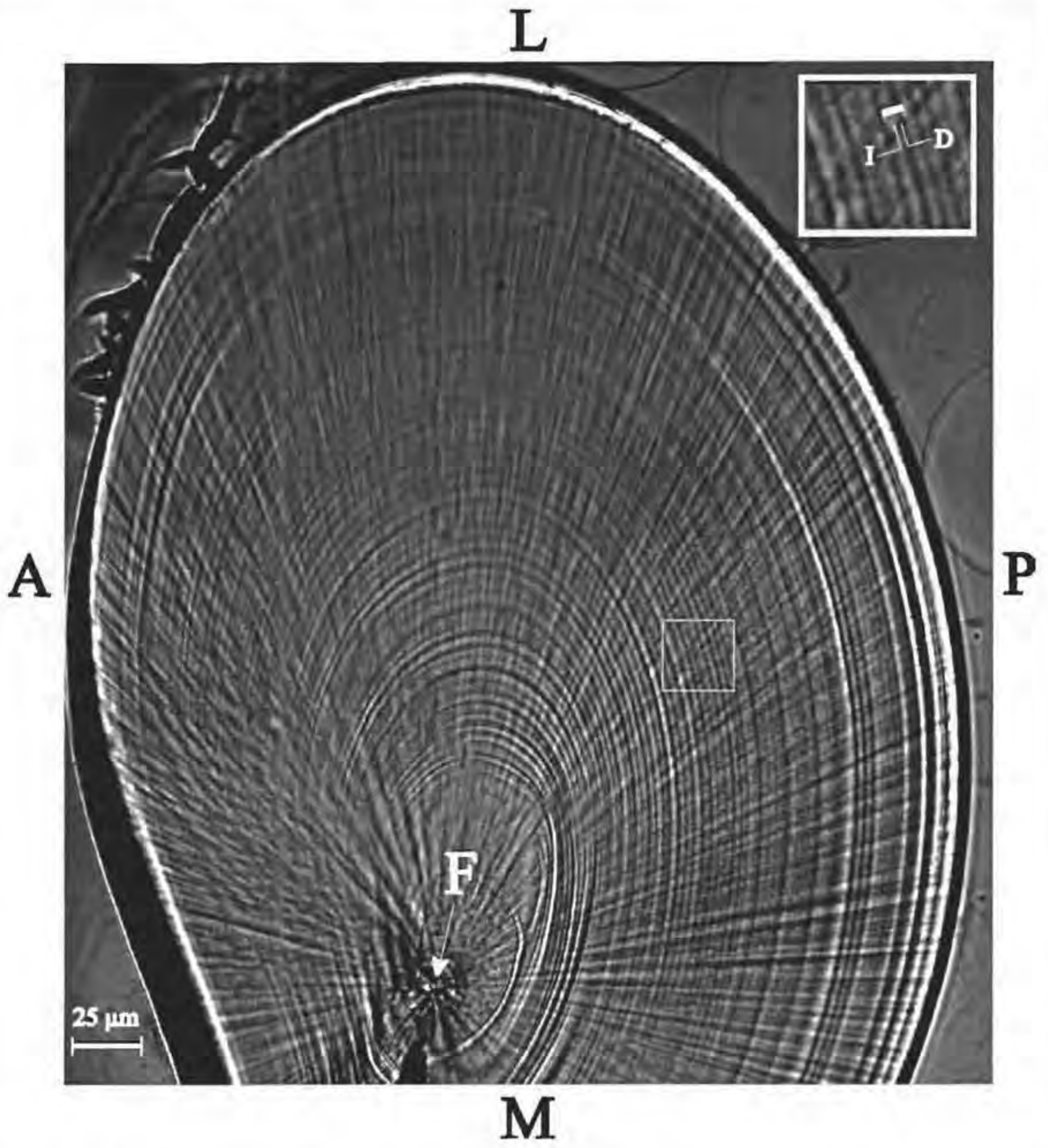


Figure 1.2: Light micrograph showing the dorsal view of the left statolith from an adult female *Lolliguncula brevis* (dorsal mantle length = 51 mm), illustrating the internal microstructure. The statolith has been ground and polished in an oblique frontal section. Increments are visible as alternating light and dark layers. F - focus; L - lateral; M - medial; A - anterior; P - posterior. Inset: one increment is bracketed, I - incremental zone; D - discontinuous zone.

These features have been described in a number of taxa as "rings", "growth rings", "lamellae", "microincrements" and "increments". Current convention defines a "light" and adjacent "dark" ring as an "increment" (Lipinski *et al*., 1991), and this terminology will be adopted throughout this thesis. As in fish otoliths, the light and dark components of each increment are referred to as incremental and discontinuous zones respectively (Mugiya *et al*., 1981). In fish otoliths, the incremental zones have been shown to contain relatively more mineral and less protein than the discontinuous zones (Degens *et al*., 1969; Dunkelberger *et al*., 1980), and reflect periods of rapid otolith growth, whereas the discontinuous zones contain relatively more protein and reflect periods of slow otolith growth (Mugiya and Satoh, 1995). Increments in cephalopod statoliths were first observed in *Octopus vulgaris* and *Alloteuthis subulata* by Young (1960), who suggested that these structures might be of use in studies of age and growth. Clarke (1966) reported similar findings for ommastrephid squids, but was unable to relate the "growth laminations" to any time-scale.

The observation of approximately 360 increments within each annulus of temperate fish otoliths led Panella (1971) to propose the daily increment hypothesis. This hypothesis suggests that increments reflect the presence of a daily cycle in otolith growth, manifest as the deposition of a light and dark layer every 24 hours. The total number of increments present in the otolith of an individual is therefore presumed to be equivalent to the age of the individual in days. The implications of this hypothesis for fisheries scientists are profound, permitting biologists to estimate the age of individuals with a resolution previously impossible. Subsequent research has also demonstrated that daily increments can be used to infer the timing of life history events and elucidate historical growth patterns (e.g. Campana and Jones, 1992; Jones, 1992), although shortcomings inherent in the latter limit its application (e.g. Morales-Nin, 1988).

Following Panella's discovery, it has been suggested that increments in squid statoliths also represent a daily cycle in statolith growth, and hence can be used to estimate the age of individual squid (Lipinski, 1978; Spratt, 1978; Hurley *et al*., 1979). The "1 increment = 1 day" hypothesis is a landmark in squid population studies, presenting an opportunity to avoid the problems and biases implicit in the conventional stock assessment procedures discussed above. Ensuing research has been aimed at evaluating the validity of the daily increment hypothesis, and at applying it to studies of squid population dynamics.

At this point, it is important to make the distinction between the daily increment hypothesis itself, and its application as an age estimation technique. The hypothesis pertains only to the physiological cycle that regulates increment formation, whereas an ageing technique encompasses the methodological aspects of age estimation. This distinction may perhaps be considered pedantic, but it is fundamental to the philosophy underlying tests of the validity of the hypothesis and its application. The physiological and methodological aspects have to be considered separately in any critical assessment of the use of putative daily increments to estimate age.

1.2.1. Physiological aspects:

The daily increment hypothesis rests on two premises:

Premise 1: There is a daily cycle in statolith formation that persists throughout the life cycle *of the animal.*

The initial component of this premise is of obvious relevance, since, if a daily cycle does not exist, statolith microstructure will have no chronometric significance. The latter component is of critical importance for age estimation purposes. If the daily cycle does not persist for the entire life span, interpretation of the microstructural end products of the cycle will have no chronological meaning, unless the observer has some *a priori* indication of when the alteration in cycle frequency occurred, and what the new frequency is.

This premise implies the presence of a circadian rhythm, and this has indeed been invoked by several authors to explain the daily cycle in both statolith (Lipinski, 1993) and otolith (Campana and Neilson, 1985) growth, and the formation of daily increments. The term circadian rhythm is used in the strictest sense, in that it defines an endogenous, genetically encoded, temperature-compensated rhythm which is entrained by a single environmental variable known as a "zeitgeber" (Bunning, 1967), and which persists (free-runs) with a period of approximately twenty four hours in the absence of any environmental periodicities. In other words, circadian rhythms can be considered to have all the characteristics of an internal biological clock (Saunders, 1977).

Whether circadian rhythms are in fact endogenously controlled, or purely a response to some exogenous environmental variable remains controversial, although current opinion favours the former (Hastings *et al.*, 1991). It is important that the daily increment hypothesis rests on a circadian rhythm, rather than an exogenously controlled cycle. The rhythm, and by inference, the production of putative daily increments, has to be independent of any exogenous (and potentially non-daily) periodicities other than the entraining zeitgeber, which by definition has to cycle with a daily periodicity. It is crucial for age estimation purposes that the rhythm persists in spite of non-daily cycles in the environment.

Premise 2: The daily cycle in statolith growth results in the formation of one increment every 24 hours.

Although this premise may be considered trivial, the assumption of the daily nature of increments does not necessarily follow from the first premise. It is possible that while a daily cycle may exist in statolith mineralization, increment formation does not reflect this cycle (i.e. increments are deposited in response to some other aspect of the physiology of the organism).

In view of these two premises, it is clear that confirmation of the daily increment hypothesis rests firstly on knowledge of the biomineralization processes responsible for the formation of increments, and secondly on proof that these processes cycle with a daily frequency.

Understanding of the physiological and molecular processes of statolith mineralization and increment formation is currently very limited. Considerable research has been directed to biological calcification, but most of this work has investigated shell formation in various molluscs, particularly bivalves. The commonality of the underlying organization of fish otoliths and many mollusc shells prompted Gauldie and Nelson (1990b) to suggest that there is some property common to all biologically mineralized aragonite tissues. Certainly, there are many structural and functional parallels between squid statoliths and teleost otoliths which justify considering these systems collectively. However, the mollusc shell mineralization system differs from those of both statoliths and otoliths. As was mentioned above, statoliths are deposited from solution (the endolymph), rather than in direct contact with a secretory surface (Morris, 1988, 1991a). Secondly, statoliths are composed entirely of aragonite (Lipinski, 1986, 1993), with no evidence of the calcite phases that occur in many mollusc shells. In view of these differences, whether the processes that have been elucidated in mollusc shell formation apply to the formation of squid statoliths, remains to be established.

The emerging picture from studies of mollusc shells appears to be one of "matrix-mediated mineralization" (Lowenstam, 1981). Most biologically calcified tissues, including statoliths, contain an organic matrix composed primarily of proteins and glycoproteins, that is closely associated with the mineral phase. The matrix appears to be capable of actively regulating crystal growth (Weiner *et al.*, 1983; Wheeler and Sikes, 1984; Addadi and Weiner, 1985; Albeck *et al.*, 1996) by control of crystal morph (Falini *et al.*, 1996), crystal nucleation, orientation and morphology (see Simkiss and Wilbur, 1989).

Increment formation in bivalve mollusc shells is postulated to be a function of respiratory stress (Lutz and Rhoads, 1977, 1980). During periods of shell closure, respiration of the animal shifts to anaerobiosis with the associated accumultion of acidic end products. $CaCO_3$ from the shell is dissolved to buffer the acids. The higher proportion of organic material in the discontinuous zones therefore reflects periods of shell dissolution corresponding to periods to anaerobic respiration.

In contrast, the hypothesis of increment formation in squid statoliths proposed by Morris (1988, 1991a) suggests that the bipartite nature of increments reflects periods of reduced calcification, rather than mineral dissolution. There is an organic component of the statocyst endolymph that reversibly polymerizes with increasing pH, inhibiting calcification. Morris suggests that activity-induced pH fluctuations resulting from the accumulation of metabolic end products either promote or inhibit calcification via this organic component, explaining the bipartite nature of increments. Lipinski (1993), however, argues that pH fluctuations of the magnitude suggested by the hypothesis of Morris are unikely in the generally well-buffered endolymph, and presents an alternative hypothesis that implicates strontium as the key factor regulating increment formation, possibly linked with the organic component.

Strontium appears to play a central role in squid statolith mineralization. Larvae of cephalopod species reared in artificial sea water lacking strontium exhibit aberrant swimming behaviour resulting from the either the abnormal development, or complete lack of statoliths in these individuals (Hanlon *et al.*, 1989). Similar observations have been reported for the opisthobranch mollusc *Aplysia californica* (Bidwell *et al.*, 1990). Further, the discontinuous zones of increments in teleost otoliths show higher strontium:calcium ratios than do the incremental zones (Mugiya and Satoh, 1995), suggesting that strontium is involved in increment formation. Further research investigating this element is needed.

Information concerning otolith formation in teleost fish has been obtained primarily from results of morphological and structural studies. The aragonite crystals of teleost otoliths are twinned (Gauldie and Nelson, 1988). By regulating the preferred direction of crystal growth, twinning controls crystal morphology and orientation, whereas neuroproteins secreted at the macula control the rate of crystal growth, and possibly crystal size (Gauldie and Nelson, 1988). A later study suggested that pH also plays a role (Gauldie and Nelson, 1990a). Fluctuations in pH which result from CO_2 driven reactions catalyzed by the enzyme carbonic anhydrase, cause both mobilization of calcium and its subsequent precipitation as $CaCO_3$.

From the preceding discussion, it is clear that while some information concerning statolith mineralization is available, understanding of the mechanisms and their role in increment formation is still limited. This also applies to evidence concerning daily cycles in biomineralization processes. Several studies have demonstrated circadian rhythms in the behaviour of both fish (Gibson, 1973; Eriksson and van Veen, 1980) and cephalopods (Cobb *et al.*, 1995), and the presence of a circadian pacemaker has been demonstrated in the mollusc *Aplysia* (Jacklet, 1991). However, the presence of a circadian rhythm in the biomineralization processes leading to increment formation in both fish and squid has yet to be conclusively established, and the zeitgeber remains unknown. Photoperiod has been suggested as the most likely candidate for a zeitgeber (Campana and Neilson, 1985), but this hypothesis has yet to be confirmed.

Some evidence for daily cycles in teleost otolith deposition and the formation of increments has been accumulated, although it should be stressed that none of these studies can be considered as a rigorous test of the presence of a circadian rhythm. The different mineral to protein ratios in the incremental and discontinuous zones of increments (Degens *et al.*, 1969; Dunkelberger *et al.*, 1980) suggest that the bipartite nature of daily increment structure reflects daily cycles in either calcium carbonate accretion or organic matrix formation, or both (Mugiya *et al.*, 1981; Watabe *et al.*, 1982; Campana and Neilson, 1985; Mugiya, 1987).

The most convincing evidence for a daily cycle in $CaCO_3$ accretion is the diurnal rhythm in the rate of ^{45}Ca uptake by the otoliths of juvenile goldfish *Carassius auratus* (Mugiya *et al.*, 1981), postulated to be the result of reduced calcium secretion from cells of the macula. Morphological observations that showed that the discontinuous zones of increments were formed during periods of reduced calcification (Mugiya *et al.*, 1981) link the cycle in otolith growth with increment formation. The same diurnal rhythm in calcium uptake by otoliths was observed in an *in vitro* study using isolated preparations of otolith-containing sacculi of rainbow trout *Salmo gairdneri* (Mugiya, 1984). In both studies, the cycle in the rate of calcium uptake parallelled a diurnal cycle in levels of plasma calcium, which, it is suggested, is a secondary effect of a diurnal rhythm in branchial calcium uptake. Considering the well known role of calcium ions to trigger the activity of secretory cells, the decreasing phase of plasma calcium may represent a decrease in trigger calcium, with consequent reduced calcium secretion by the macula and hence reduced otolith growth. Several studies cited by Romanek and Gauldie (1996), however, have indicated that endolymph calcium concentrations are independent of plasma calcium, casting some doubt on this argument.

Mugiya (1987) established that the deposition of calcium and organic matrix on the otoliths of rainbow trout varied diurnally in antiphase, and concluded that the incremental zones are formed primarily by calcium deposition, while the discontinuous zones result from reduced calcium and substantially increased matrix deposition on the otoliths. Further support for this argument is provided by Gauldie and Nelson (1988), who found that neurosecretory activity in the cells of the macula showed a daily cycle corresponding to the appearance of daily increment bands. They suggested that the secretion of the neuroprotein is continuously modulated so that a narrow, mineral-deficient band and a wider mineral-dense band constitute each daily increment.

The hypothesis of teleost otolith increment formation proposed by Campana and Neilson (1985) rests on the basic premise that daily increment production is linked to an endocrine-driven, endogenous circadian rhythm that is entrained at an early age by photoperiod. Endocrine secretion controls many physiological processes, including skeletal deposition (Simpson, 1978), and displays a circadian periodicity in many animals (Simpson, 1978; Jacklet, 1981). Plasma calcium levels are under endocrine control (Oguro and Pang, 1982, cited by Mugiya, 1987). A circadian rhythm in the endocrine system could result in daily cycles in otolith deposition, acting through the link between plasma calcium and otolith $CaCO_3$ accretion discussed above. Recent research has contributed to this concept. Surgical removal of the pituitary gland causes a reduction in otolith growth (Mugiya, 1990) that can be restored by injection of pituitary extract (Simmons, 1971, cited by Wright *et al*., 1992).

The majority of the studies investigating daily increment formation have employed validation experiments. These experiments compare counts of increments deposited over a known time period with the elapsed number of days. The daily frequency of increment production is considered to be confirmed when the increment counts closely approximate the elapsed period. The results of validation studies conducted on both squid and fish are reviewed by Jackson (1994) and Geffen (1992) respectively. In almost all cases, the conclusion has been drawn that increments are deposited with a daily frequency. However, there are limitations inherent in this approach and its underlying philosophy. These are explored in more detail in Chapter 3 of this thesis.

The results of validation experiments conducted under controlled conditions are used to infer the circadian nature of daily increment formation. The rationale is that one can infer that a circadian rhythm is free-running when daily increment production persists in spite of either non-daily environmental cycles or constant conditions. Daily increments were deposited in spite of non-daily cycles in photoperiod and temperature that were imposed on juvenile starry flounders (*Platichthys stellatus*) that had been preconditioned to a natural environmental regime (Campana and Neilson, 1982). Other experimental studies have reported that daily increment formation persisted in constant light (Campana and Neilson, 1982; Geffen, 1982; Neilson and Geen, 1982; Wright *et al*., 1991; Jackson *et al*., 1993), darkness (Neilson and Geen, 1982) and temperature (Taubert and Coble, 1977; Campana and Neilson, 1982; Neilson and Geen, 1982; Radtke and Dean, 1982; Campana, 1984b), suggesting a free-running endogenous rhythm in increment production. The same conclusion can be drawn from the presence of daily increments in fishes from the polar regions (Townsend and Shaw, 1982; Radtke and Hourigan, 1990) where diel light and temperature cues are often absent. However, the possibility that some uncontrolled cyclical variable is entraining increment production cannot be excluded, and any conclusions drawn from such results can only be tentative, unless all other possible variables have been excluded by the experimental design. As

Campana and Neilson (1985) stated, "proof of the existence of an endogenous rhythm will probably never be obtained, due to the possible existence of uncontrolled variables in any experimental design".

A final factor that links the physiological and methodological aspects of the daily increment hypothesis and its application concerns the influence of environmental and biotic factors on the biomineralization processes and resultant microstructure. Few workers have considered environmental modification of microstructural features (Campana and Neilson, 1985) that may influence the manner in which these features are interpreted.

1.2.2. Methodological aspects:

The application of the daily increment hypothesis to age estimation procedures suffers from a number of methodological shortcomings. These shortcomings can be broadly separated into theoretical and purely technical problems.

Theoretical:

The interpretation of statolith microstructure remains an unresolved problem. Increments are not always clear, unambiguous structures. Various environmental and biological factors may influence statolith microstructure to the extent that the interpretation of daily increments is compromised. Because the history of the individual being analyzed is usually unknown, a lack of understanding of the effects of such factors on statolith microstructure will limit that ability of the investigator to recognise and interpret the results of these effects, introducing an unknown level of error into the age estimates.

Campana and Neilson (1985) refer to such factors as "masking agents". Factors other than the entraining zeitgeber can "mask" the rhythm, obscuring the underlying daily features by inducing a non-daily cycle with a frequency equivalent to that of the masking agent. Although the masking agent does not phase-shift or alter the period of the endogenous daily rhythm, which still persists, the microstructural results of the masking cycle overlay and obscure those of the innate daily rhythm.

A major problem facing the daily increment ageing technique are sub-daily increments in many species of fish (Campana and Neilson, 1985; Zhang and Runham, 1992a) and squid (Natsukari *et al.*, 1993b; Jackson *et al.*, 1993; Jackson, 1994; Arkhipkin *et al.*, 1996). These features may be interpreted as daily increments, resulting in over-estimates of age and under-estimates of somatic growth rate (Neilson, 1992). Identification of these features are a particular cause for concern (Jones, 1992; Campana, 1992; Neilson, 1992). At present, subjective criteria are used to identify these features, resulting in substantial disagreements between age estimates obtained by different researchers (Campana, 1992; Jackson, 1994). Objective criteria that characterize daily increments and distinguish them from other non-daily features appears to be the only manner in which this problem can be solved. An integral part of such an increment recognition concept is identification of the biological and environmental factors that may influence statolith microstructure, and hence the identification and interpretation of daily versus non-daily features. Very little research exploring potential modifiers of squid statolith microstructure has been conducted. Most of the available information has been obtained from studies of teleost otoliths.

The environmental and physiological factors most commonly viewed as potential modifiers of otolith microstructure are photoperiod, temperature, feeding and growth (Campana and Neilson, 1985; Jones, 1986). Reversal of the light dark cycle reverses the order of formation of discontinuous and incremental zones in goldfish otoliths (Tanaka *et al.*, 1981). An abnormally short photoperiod of 6L:6D induces the formation of two increments per day (Wright *et al.*, 1991). Several studies have shown that daily increments are not produced in the absence of a 24 hour light-dark cycle (Taubert and Coble, 1977; Radtke and Dean, 1982). These results, however, conflict with the studies mentioned earlier which indicated that daily increment production persisted in spite of constant light or dark (Campana and Neilson, 1982; Neilson and Geen, 1982; Wright *et al.*, 1991; Jackson *et al.*, 1993).

Short-term temperature fluctuations are capable of inducing the formation of non-daily increments (Brothers, 1981; Neilson and Geen, 1985), but constant temperature does not appear to inhibit daily increment production, although it may reduce the clarity or "sharpness" of the increments (Campana, 1984b; Neilson and Geen, 1985; Ré *et al.*, 1986). Increments formed under constant temperature conditions (such as those usually imposed in the laboratory) may consequently be more difficult to detect.

The influence of feeding is more difficult to assess. While young chinook salmon fed more than once a day produced significantly more increments than those fed only once daily (Neilson and Geen, 1982, 1985), other studies have indicated that multiple daily feedings did not affect daily increment production (Taubert and Coble, 1977; Campana, 1984b; Tzeng and Yu, 1992). Campana and Neilson (1985) attributed such discrepancies to the interpretation problem. The studies of Geen and Neilson (1982; 1985) made no attempt to distinguish between daily and sub-daily increments, all increments were enumerated. The opposing studies differentiated between daily and sub-daily increments, only enumerating those that were considered to be daily. Campana (1983a) noted that the incidence of sub-daily increments appeared to increase with feeding frequency, which suggests that these features were responsible for the elevated increment counts reported by Neilson and Geen (1982, 1985).

Campana and Neilson (1985) have discussed the issue of sub-daily increment recognition, arguing that the regularity of the growth structures produced in response to the daily rhythm (in terms of width and contrast) , as opposed to the irregular structures produced in response to the masking agent (the degree of calcium uptake, and hence the appearance of the growth structure will be a function of the strength of the masking agent) provides a potential means of recognising sub-daily from daily increments. It is precisely this aspect of increment appearance that is often used as a subjective criterion characterizing daily versus sub-daily increments (e.g. Taubert and Coble, 1977; Campana and Neilson, 1982; Marshall and Parker, 1982; Campana, 1992).

Although quantification of these criteria has been attempted (Campana, 1984b), such classifications can be difficult and imprecise (Campana and Neilson, 1985). Campana (1984b) offered the suggestion that by using the relationship between otolith size and known-age data, one can forecast what the width of increments are likely to be in areas where there are problems with making the distinction between daily and sub-daily increments. This approach was used with some success in common sole larvae by Lagardère and Troadec (1997). The suggestion that daily increments in larval fish otoliths can be readily distinguished from subdaily increments by altering the focus of the microscope (Narimatsu and Munehara, 1997) does not extend to older individuals. Imprecise age estimates obtained from older fish during the study of Narimatsu and Munehara (1997) were attributed to difficulties in recognizing sub-daily increments.

An additional problem often encountered in analyses of daily increments are regions where increments are either poorly defined, or apparently absent. These regions could reflect periods of constant environmental conditions, which have been shown to reduce increment clarity (Campana, 1984b; Neilson and Geen, 1984; Ré *et al.*, 1986). Alternatively, these regions may reflect periods of deposition of very narrow increments (perhaps in response to conditions such as low temperature or poor food availability), the widths of which are below the resolution limits of the microscope, or periods where no daily increments are deposited at all. Poorly defined or narrow increments are more difficult to detect and count, introducing error. Further, narrow increments may be interpreted as subdaily rather than daily, again introducing error. In many cases, interpolation from adjacent areas of clear increments is used to estimate increment width and numbers (Ralston, 1985). Morales-Nin (1988) has criticized this practice, demonstrating how bias will be introduced into subsequent growth parameter estimates.

Pauly (in press), referring to the theory of increment formation proposed by Lutz and Rhoads (1977; 1980), suggested that periods of respiratory stress prevent the formation of daily increments, leading to "blurred zones" in otoliths, and presumably statoliths. He further suggested that slower-growing individuals will be more prone to respiratory stress of this magnitude than will faster-growing individuals, and will therefore contain a higher proportion of these blurred zones. Otoliths where a large proportion of blurred zones occur along the couting axis are often discarded during the analyses, biasing growth parameter estimates towards faster growing individuals. This argument must be investigated further if increment counts are to be utilized with any degree of confidence.

Technical:

Technical limitations are a major obstacle currently impeding the wide-spread application of the daily increment ageing technique. A representative evaluation of the age structure of a population relies on rapid collection of accurate increment counts from as large a sample of statoliths as possible. The time factor is of great importance in analyses of exploited squid stocks, where short term fluctuations in abundance are common.

Microscopic examination using either a light microscope (LM) or a scanning electron microscope (SEM) is essential to visualize daily increments. Increments are not always clearly visible in intact statoliths in their natural state (particularly in adults). Various procedures are required to prepare statoliths for microscopic examination. Specific techniques will vary depending on the method of observation and the properties of the statoliths of the species being analysed. A number of workers have described methods of statolith and otolith preparation for LM, including Dawe and Natsukari (1991) for squid statoliths and Secor *et al.* (1992) for teleost otoliths. Lipinski (1991) describes a procedure of squid statolith preparation for examination by SEM. These procedures, and the subsequent counting of increments, are time consuming, labour intensive, and often expensive. Preparation success rate is often very low with many specimens being destroyed or damaged. These constraints limit the number of specimens that can be analyzed, and sampling protocols have to take this into account. Improvement of the methods of statolith preparation (both in terms of time and success rate, as well as the cost factor) is essential to maximize the potential of the daily increment technique.

In summary, neither of the premises implicit in the daily increment hypothesis have been subjected to sufficiently stringent testing, particularly in the case of exploited squid species. Although much of the evidence reviewed above provides considerable support for the premises, most of the results can only be considered as circumstantial. The possibility still remains that increments are merely structural features that are coincidentally deposited at a rate of approximately one per day, and are not in any way related to any daily physiological cycle (Rodhouse, 1991). Considering the importance of the daily increment ageing technique and its already widespread application based on the presumed validity of the daily increment hypothesis, it is surprising that the hypothesis has not been subjected to more rigorous examination. This is perhaps understandable in view of the urgent requirement for age information for populations that are under severe fishing pressure, but is not acceptable if the results are to be used in the formulation of management strategies which could have far-reaching consequences.

Further research needs to be aimed at elucidating the processes of statolith growth and increment formation, as well as environmental influences on these processes (Rodhouse and Hatfield, 1990). Carefully designed experiments testing the daily cycle in increment formation need to be conducted at the physiological level. Only through such stringent tests can the daily increment hypothesis be unequivocally established as valid, justifying the analysis of microstructure as a robust age estimation tool. Our ability to identify daily increments clearly needs drastic improvement. The occurrence of sub-daily increments, and the factors leading to their formation, needs to be assessed, and their structural characteristics clearly defined. Solving this (and other) interpretation problems clearly rests on a detailed understanding of the physiological processes of statolith growth and, particularly, those processes that result in the formation of the various microstructural features that may complicate the interpretation process. Improvements in the methodological aspects are crucial. The time and labour constraints associated with the statolith ageing technique are probably the major factors limiting its widespread application and its potential.

1.3. OBJECTIVES

The research described in this thesis was aimed at providing information towards resolving several of the shortcomings discussed above, particularly with respect to the use of statoliths to estimate the age of the South African chokka squid, *Loligo vulgaris reynaudii*. This species is a relatively large loliginid squid (males can reach mantle lengths of up to 440 mm) which occurs in coastal waters and along the continental shelf around Southern Africa. Adults spawn in shallow coastal waters (in depths of less than 50 meters) along the south and east coasts of South Africa, where they form large spawning aggregations that are the target of an intensive hand-jigging fishery (Sauer, 1993). This species, however, is very difficult to maintain in captivity for extended periods. The thumbstall squid, *Lolliguncula brevis*, was consequently used as an experimental subject in several of the studies described in this thesis. This latter species is a smaller animal that occurs in shallow water in the western Atlantic Ocean, and the Gulf of Mexico. It is readily obtainable in Galveston Bay (USA) through most of the year, and with the facilities and expertise developed by the National Resource Centre for Cephalopods (a division of the Marine Biomedical Institute of the University of Texas Medical Branch at Galveston), can be maintained in the laboratory for extended periods.

The basic objectives of the research were two-fold. Firstly, to improve the technical and methodological aspects of the statolith ageing technique. Secondly, to address the physiological processes of statolith mineralization, with the intention of testing the daily increment hypothesis at a physiological level, as well as to provide information contributing towards resolving the interpretation problems discussed above.

The technical aspect of the ageing technique, and specifically the preparation of statoliths for examination by light microscopy is addressed in Chapter 2. An attempt is made to develop a preparation technique that improves the success rate of the procedure, generating sections in which increments are clearly visible from the nucleus to the margin. The performance of the technique is assessed relative to a method of statolith analysis using scanning electron microscopy. The hypothesis that the two methods generate similar estimates of total increment numbers is tested.

The underlying assumption inherent in the statolith ageing technique, namely the hypothesis that increments are deposited with a daily frequency is tested in Chapter 3 by a series of validation experiments employing a statolith marking technique, using the South African chokka squid *Loligo vulgaris reynaudii*. The experiments were conducted both in the laboratory and in the field. The latter represent the first attempt to conduct field experiments of this nature in any cephalopod species. The results are assessed in terms of a test of the daily increment hypothesis, as well as the extrapolation of laboratory results to natural conditions. Multiple replicate estimates of the number of increments deposited over a known period obtained by three different researchers were used to quantify the sources of error in increment counting, as well as an assessment of differences in the interpretative abilities of the three researchers.

The influence of ambient temperature on statolith growth and microstructure is examined in Chapter 4 in an attempt to identify how this environmental factor may influence the interpretation of statolith microstructure. The hypothesis that temperature directly influences statolith growth is tested in a laboratory study of the loliginid squid *Lolliguncula brevis*.

Chapter 5 investigates the mineralization of statoliths of chokka squid *Loligo vulgaris reynaudii* by using a nuclear microprobe. The distribution and concentration of a number of elements in sectioned statoliths were mapped and analyzed. The influence of temperature, sex, stage of maturity and the use of oxytetracycline to mark statoliths during validation experiments, on the elemental composition of statoliths is examined.

An attempt to test the daily increment hypothesis at the physiological level is described in Chapter 6. Aspects of the chemistry of the statocyst endolymph of the loliginid squid *Lolliguncula brevis* were monitored over a daily period to test the hypothesis that these variables fluctuate with a daily periodicity. The approach rests on the assumption that increment formation is a result of fluctuations in the chemistry of the endolymph at the statolith surface. Calcium and magnesium ion concentrations were measured, and an attempt to measure pH and strontium ion concentration is described.

A preliminary study investigating the role of the organic matrix in statolith mineralization is presented in Chapter 7. The results of biochemical studies aimed at characterizing the protein component of squid statoliths are discussed in relation to current knowledge regarding the organic matrices of calcified structures in other taxa. A synthesis of the work is presented in Chapter 8.

CHAPTER 2 - STATOLITH PREPARATION FOR INCREMENT ENUMERATION USING LIGHT MICROSCOPY, AND AN EVALUATION OF THE RESULTS

ABSTRACT

A method of preparing statoliths of chokka squid *Loligo vulgaris reynaudii* for increment counting using light microscopy (LM) is described. Statoliths embedded in orthodontic resin are sectioned in the oblique frontal plane by grinding from both the dorsal and ventral surfaces. Use of this plane allows sections incorporating clear increments extending from the nucleus to the margin of the statolith to be obtained with relative ease. The performance of the method is assessed by comparison of the results with those obtained using scanning electron microscopy (SEM) techniques. The method is superior to the SEM technique, in that fewer specimens requiring interpolation or extrapolation of increment numbers in obscured regions are obtained. Morphometric analyses of chokka statoliths indicated no differences between statoliths from the same squid in terms of size or shape. It was assumed that increment counts could therefore be justifiably compared between statoliths from the same pair. Increment counts obtained using LM showed no significant differences from those obtained by the same reader using SEM, suggesting that either method of examination can be used with similar effectiveness. Between-reader differences in increment interpretation and counting were apparent in the LM results, but not in SEM. The problem of interpreting statolith microstructure could therefore introduce a significant source of error when using LM examination.

2.1. INTRODUCTION

The application of the daily increment hypothesis as an age estimation technique is based on the assumption that the total number of putative daily increments in a statolith is equivalent to the age of the animal in days. As was pointed out in Chapter 1, several methodological shortcomings limit the widespread application of the technique. The study described in this chapter was aimed at addressing two of these shortcomings.

Firstly, the technical aspects of statolith analysis need to be improved. All increments between the nucleus and the margin of the statolith should be clearly visible. Microscopic examination is essential for this purpose, because increments in squid statoliths typically show widths of the order of 1 µm. Statoliths can be examined by either light microscopy (LM) or scanning electron microscopy (SEM). Both techniques require a degree of sample preparation prior to microscopic examination of the internal microstructure.

For examination by SEM, statoliths have to be broken or sectioned, and then treated with a weak acid or calcium-chelating agent such as EDTA, to differentially etch the incremental and discontinuous zones of increments. This process generates a contoured surface which can be examined topographically (Campana and Neilson, 1985). That the features apparent in this contoured surface are homologous to the structures visible using LM is clearly illustrated in Figure 4 (pg. 1025) of Campana and Neilson (1985). Lipinski (1991) describes the application of this technique to squid statoliths. Although SEM has the advantage of higher resolution than LM, several disadvantages limit its use. The preparation and viewing of specimens is time consuming and costly. Etching has to be conducted with great care to prevent over-etching and loss of detail. Viewing is relatively inflexible, being confined to the surface of the specimen. Considering that the objective of statolith ageing studies is to generate data for stock assessment purposes, many specimens would have to be analysed. The cost and time constraints are major obstacles to the use of SEM techniques for this purpose.

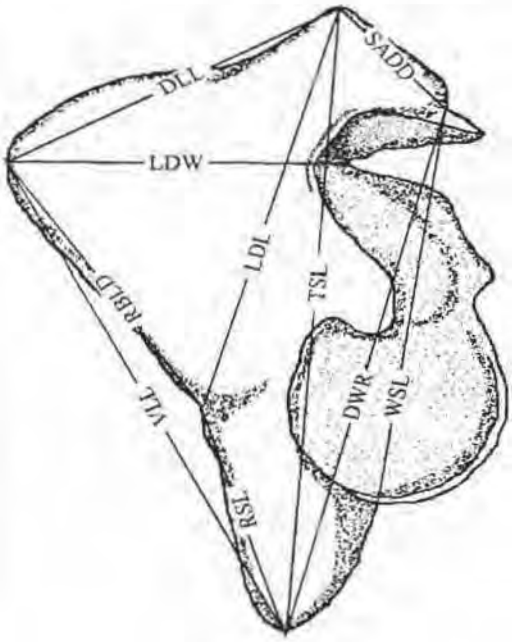
Because statoliths are generally optically opaque in their natural state, LM examination also requires that specimens be sectioned in order to visualize internal microstructure. This is usually achieved by grinding and polishing. Although also time consuming and labour intensive, statolith preparation and observation by LM is usually more rapid and flexible than by SEM. Specimens can be readily observed during the sectioning process to monitor progress. Viewing is rapid and not as costly as SEM. The focal plane can be adjusted to visualize features below the sectioned surface. There are, however, several limitations to LM examination. The resolution limits of normal transmitted light preclude detection of fine features. Optical artefacts that obscure detail can be a problem, particularly in the marginal areas of the section. In spite of these disadvantages, LM techniques are felt to be more suitable for the large-scale sample processing inherent in a stock assessment.

The second methodological shortcoming explored in this study arises from incompatible increment counts obtained for the same species from similar areas that have been reported in the literature (e.g. Villanueva, 1992 vs. Lipinski *et al.*, 1993 for *Todarodes angolensis*; Bizikov, 1990 cited by Jackson, 1994 vs. Natsukari *et al.*, 1993a for *Berryteuthis magister*). The problem here is one of reproducibility, (or verification, see Geffen, 1992 p. 101 for a more detailed definition), arising from inconsistencies in reader interpretation of daily increments.

This chapter describes a technique developed for the preparation of statoliths from the South African chokka squid *Loligo vulgaris reynaudii* (d'Orbigny, 1845) for increment counting using light microscopy. The performance of the technique is evaluated by comparing increment counts obtained using the technique with those obtained using scanning electron microscopy. The issue of reproducibility is investigated by comparing increment counts obtained by different researchers from the same material.

2.2. METHODS

Statoliths from 32 males, 14 females and 8 unsexed juveniles were used. The squid were obtained either by jigging on their spawning grounds near Port Elizabeth, or from trawls in the area between Cape Agulhas and East London. The dorsal mantle length (DML, mm) of all the individuals was measured, and the sex and stage of maturity assessed (Lipinski, 1979). Most of the sexed individuals were weighed (TW, g). The statoliths were dissected from fresh animals (Lipinski, 1981) and stored in 70 % ethanol. Both statoliths of each pair were later dried for 24 hours at 40 °C and weighed (SW, µg). A series of morphological measurements modified from Lipinski *et al.* (1993) (see Fig. 2.1) were then recorded for each statolith, using an eyepiece micrometer mounted in a stereo microscope.



TSL - total statolith length
LDL - lateral dome length
DWR - dorsal point of wing to rostral tip
WSL - wing length
DLL - dorso - lateral length
SADD - tip of dorsal dome to dorsal point of wing
RSL - rostrum length
LDW - lateral dome width
RBLD - base of rostrum to lateral edge of lateral dome
VLL - ventro - lateral length

Figure 2.1: Diagram showing the anterior view of the right statolith from an adult *Loligo vulgaris reynaudii* illustrating the morphometric measurements recorded on each statolith.

Measurements from the left and right statoliths of each pair were recorded by two different researchers independently. The data were analysed by multiple regression analyses (Genstat5 Committee 1987), and measurements compared between left and right statoliths using paired *t* - tests. One statolith of each pair was then prepared for examination by SEM (Lipinski, 1991; Lipinski *et al.*, 1993). The statoliths were broken in the frontal plane, and the broken surface then etched using a solution comprising 9 ml of 1 % HCl, 1 ml absolute ethanol and 100 mg of $SrCl_2 \times 6H_2O$. The other statolith of each pair was prepared for examination by light microscopy using a modification of the method of Natsukari (Dawe and Natsukari, 1991, p. 90 - 92).

Preliminary trials indicated that clear, unambiguous increment sequences extending from the nucleus to the margin of the statolith depend to a large extent on the plane of observation. Several different planes of sectioning were assessed during these trials, and the oblique frontal plane (see Fig. 2.2) was selected as the most suitable. Use of this plane has an advantage over the more commonly employed transverse and sagittal planes in that sections along an axis extending from the nucleus to the margin of the lateral dome can be obtained with relative ease. This axis shows no evidence of the staggered growth phenomena observed in several studies of statolith microstructure (e.g. Lipinski, 1993). Such features complicate interpretation and accurate counting of increment sequences. Use of this plane facilitated comparisons with statoliths that were prepared for SEM using a similar plane.

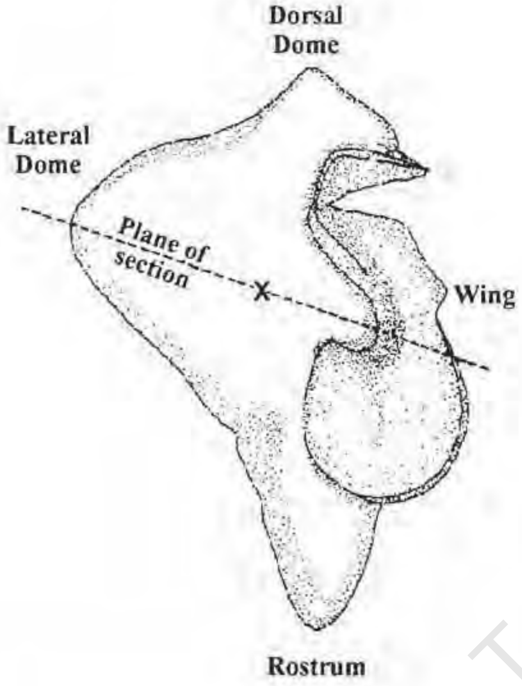


Figure 2.2: Diagram of a squid statolith illustrating the oblique frontal plane of sectioning used in this study. X indicates the position of the focus.

The statoliths were sectioned by grinding and polishing following the method illustrated in Fig. 2.3. Statoliths were embedded in clear heat-curing orthodontic acrylic resin (Japanese Bioresin), using gelatine capsules (0 size) supported in Cooke microtitre trays as moulds (Fig. 2.3 A - C). Embedding statoliths in a resin block facilitates manipulation of the specimens during the procedure, as well as "clearing" the external surface, permitting a certain degree of examination of the internal microstructure of the intact statolith.

Once the resin had cured, the resin block containing the statolith was trimmed and polished. The block was then attached to a glass microscope slide (with the anterior surface of the statolith facing up) using clear nail varnish. The block was orientated (while being viewed under a stereomicroscope) such that the edge of the slide governed the plane of the section, with the statolith being ground from the dorsal surface first (Fig. 2.3 D). The varnish was allowed to cure overnight.

Grinding of the resin block was carried out using 1200 grit sandpaper. The specimen was periodically checked under a stereomicroscope to ensure the plane was correct. When the desired plane was reached (Fig. 2.3 E), the specimen was polished using aluminium powder on a wet felt polishing cloth and removed from the slide. Gentle heating of the slide enabled the specimen to be easily detached. The block was then mounted ground surface down on a fresh glass slide using superglue (Fig. 2.3 F), and left overnight for the glue to cure.

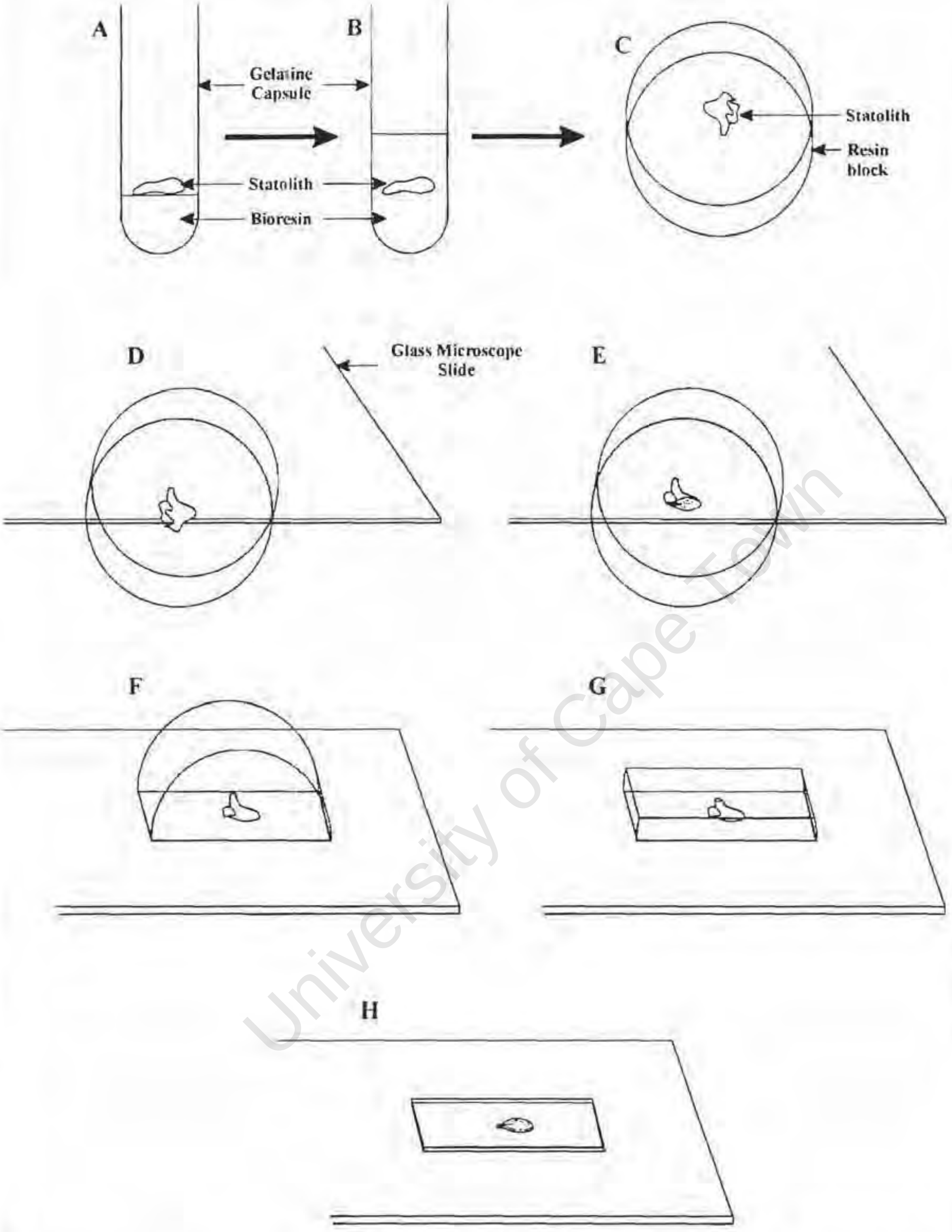


Figure 2.3: Diagrams illustrating the preparation of statoliths for examination by light microscopy. A - B: each statolith is embedded horizontally in resin using a gelatine capsule as a mould. C: the resin block is trimmed and polished. D: the block is attached to a glass microscope slide with nail varnish, such that the statolith is positioned anterior side up, and the side of the slide governs the plane of the section. E: the block is ground and polished until the edge of the slide is reached. F: the block is detached and remounted, ground surface down, on a fresh glass slide using superglue. G: the block is then ground further in a plane parallel to the surface of the slide. H: when the specimen has reached the desired thickness, the surface is polished, and the section is ready for viewing.

Grinding continued in a plane parallel to the surface of the slide (Fig. 2.3 G), such that the statolith was now being ground from the ventral surface. During this part of the procedure, the specimen was frequently polished and viewed under a compound microscope to ensure that no over-grinding occurred. Care was taken that the specimen was not ground too thin, since this increases the risk of damage to the specimen. Thin sections often become detached from the slide during grinding, and are destroyed. Further, increment clarity is reduced in very fine sections. Once the specimen had reached a satisfactory thickness, the surface was polished, and the section was ready for viewing. Use of short wavelength illumination (e.g. green light) was found to improve the contrast of increments.

Statolith sections (both SEM and LM preparations) were photographed and the total number of increments counted from the micrographs. In regions of the sections where no increments were visible, the number of increments were estimated by interpolation (or extrapolation in the marginal areas) using the widths of increments in areas immediately adjacent to the obscured regions. A single count of the total number of increments in each specimen (both SEM and LM preparations) was recorded by each of two readers.

The influence of animal size (dorsal mantle length, DML) and sex, the method of examination, and the readers of the statoliths on the increment counts was examined using a multiple regression analysis. Before being included in the analysis, each explanatory variable was regressed against the dependent variable, and the standardized residuals examined for departures from normality and for homoscedasticity. It was found that logarithmic transformation of the dependent variable (increment count) was required. Comparisons of the animal size versus increment count relationship between sex, method of examination and reader were made using simple linear regression analyses. The relationships were generally found to be exponential, and the dependent variable was consequently logarithmically transformed before being included in the analysis. Note that in this analysis, the dependent variable was the increment count recorded from each specimen. In most studies, increment counts are considered to reflect age (i.e. a measure of time), and are consequently analyzed as the explanatory variable (e.g. Campana and Jones, 1992, Villanueva, 1992). At the time of this study, daily increments in statoliths of *Loligo vulgaris reynaudii* had not been validated (see Chapter 3), and the increment counts could not be considered to reflect the age of the squid. Further, an assumption of Model I regression analysis is that the explanatory variable is measured without error (Sokal and Rohlf, 1981). This was true for the measurements of dorsal mantle length, but not for the increment counts. The former variable, rather than the increment counts, was therefore set as the explanatory variable. Slopes and elevations of regression lines were compared following the method of Zar (1984).

2.3 RESULTS

The technique of statolith preparation for examination by LM developed during this study, while labour intensive and time consuming, generated specimens that were generally of superior quality to those prepared for SEM. Examples of preparations of statoliths from the same squid are illustrated in Fig. 2.4.

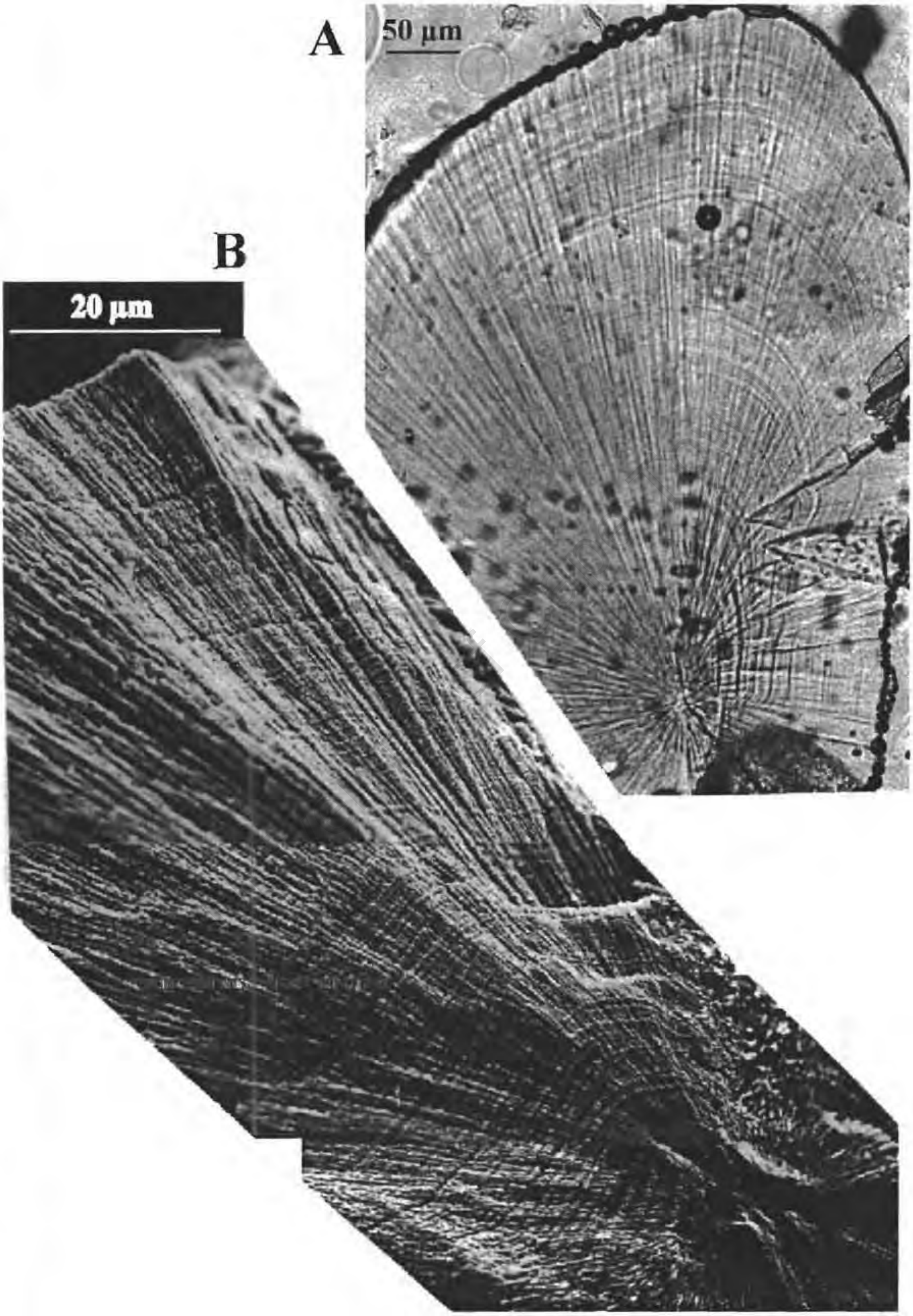


Figure 2.4: Micrographs of sections of statoliths from the same squid. A - viewed under LM (right statolith). B - viewed under SEM (left statolith). Juvenile male, ML = 60 mm, TW = 11 g.

The techniques showed similar success levels in terms of the number of statoliths that were destroyed or lost during preparation, or rejected as unreadable (Table 2.1). However, the technique developed for LM compared favourably with SEM examination in terms of increment clarity. Fewer of the sections obtained using the LM technique required extrapolation or interpolation during counting, and a larger proportion of specimens displaying clear increments from the nucleus to the margin were generated by the technique. The morphometric data collected from the statoliths used in this study were used to test whether statoliths from the same pair are similar in size and shape. All of the morphometric variables are strongly correlated with each other and with animal size (DML) (Table 2.2).

Table 2.1: The performance of the LM preparation technique compared to that of SEM examination. The number of statoliths in each success category is indicated for LM and SEM techniques.

Success Level	LM	SEM
Statoliths destroyed or lost during preparation	5	4
Statoliths not readable (rejected)	6	7
Statoliths requiring considerable extrapolation or interpolation during counting	15	22
Statoliths requiring little extrapolation or interpolation	5	11
Statoliths readable from the nucleus to the margin	23	10
Total	54	54

Table 2.2: Correlation matrix comparing morphometric measurements recorded on statoliths of *Loligo vulgaris reynaudii* during this study (see Fig. 2.1 for definitions). SW = statolith weight, DML = dorsal mantle length. All correlation coefficients are significant at $p < 0.05$. n = 105.

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

Total statolith length (TSL) was used as a measure of statolith size in a multiple regression analysis investigating influences on this variable. The results (Table 2.3) revealed that while both animal size and sex significantly influence statolith size, no significant differences are apparent between the measurements recorded by different readers, or between the left and right statoliths of each pair.

Table 2.3: Coefficients and associated statistics of the multiple regression model fitted to the TSL measurements recorded on statoliths from *Loligo vulgaris reynaudii* (n = 106, $r^2 = 0.8480$, $F_{4,101} = 146.98$, $p = 0.0000$)

Variable	Coefficient	S.E.	t_{101}	p
Constant	1.2502	0.0300	41.63	0.0000
Sex	0.1099	0.0248	4.43	0.0000
Left / Right	- 0.0092	0.0212	- 0.43	0.6681
Reader	0.0117	0.0212	0.55	0.5835
DML	0.0026	0.0001	24.19	0.0000

The latter conclusion was confirmed by comparisons of all the morphometric variables between left and right statoliths of each pair (paired t - tests for dependent samples). With the exception of DWR, no significant differences between left and right statoliths can be detected for any of the morphometric variables, including statolith weight (SW):

SW: $t_{50} = 0.4037, p = 0.6882$ TSL: $t_{52} = -1.7526, p = 0.0856$
LDL: $t_{52} = -0.6738, p = 0.5034$ DWR: $t_{52} = -3.9462, p = 0.0002$
WSL: $t_{52} = 1.9220, p = 0.0601$ DLL: $t_{52} = 0.0168, p = 0.9867$
SADD: $t_{52} = 0.7030, p = 0.4852$ RSL: $t_{52} = 0.7476, p = 0.4581$
LDW: $t_{52} = -1.4054, p = 0.1658$ RBLD: $t_{52} = -1.5908, p = 0.1177$
VLL: $t_{52} = -1.8475, p = 0.0704$

In view of these ultrastructural similarities, it appears that statoliths from the same squid show similar growth patterns. It was consequently assumed that increment counts can be meaningfully compared between statoliths of the same pair. There is a significant sex effect, however (statoliths of female squid were slightly larger than those of male squid of the same size, Table 2.3), and microstructural comparisons should take this into account. The logarithmically transformed increment counts (see section 2.2) obtained by the two readers using the two methods of examination were subjected to a multiple regression analysis (Table 2.4). The model is highly significant, explaining about 52 % of the variation in increment counts. As expected, increment counts are strongly related to animal size (expressed as DML), the positive coefficient indicating that statoliths of larger squid contain more increments.

Table 2.4: Results of the multiple regression analysis conducted on the logarithmically transformed increment counts. n = 137, $r^2 = 0.5221$, $F_{4,132} = 36.0560$, $p = 0.0000$.

Variable	Coefficient	S.E.	t_{132}	p
Constant	- 7.8366	4.6275	- 1.6935	0.0927
DML	0.0019	0.0002	11.4309	0.0000
Male - Female	0.1050	0.0356	2.9490	0.0038
LM - SEM	0.0267	0.0291	0.9168	0.3609
Reader 1 - Reader 2	- 0.1013	0.0291	- 3.4824	0.0007

Sex also has a significant effect, the positive coefficient indicating that statoliths of female squid have more increments than do those of males. An unexpected result is that the method of examination does not appear to influence increment counting. Although SEM counts tended to be slightly higher than LM counts, this difference is not significant. Increment counts were strongly related to the reader doing the counting. Reader 1 tended to count more increments in a given statolith than did reader 2. This discrepancy arises from differences in the interpretation and counting of increments between the two readers.

Further examination of the data using linear regression analyses suggests that these differences in interpretation may be related to the method of examination. Regressions of log-transformed increment counts on dorsal mantle length (Table 2.5, Fig. 2.5) revealed that SEM counts tended to be more consistent between the readers than did the LM counts. The regression models fitted to the LM data displayed no significant differences in slopes between readers for either males ($t_{44} = 0.3974, p = 0.6930$) or females ($t_{14} = 1.1955, p = 0.2518$), but displayed differences in elevation between the readers that were significant for males ($t_{45} = 4.5163, p < 0.0001$), and almost significant for females ($t_{15} = 1.7743, p = 0.0963$). In contrast, the models fitted to the SEM data showed no differences in the slopes or elevations between the readers for either females (slopes: $t_{18} = -0.9279, p = 0.3657$; elevations: $t_{19} = 0.3247, p = 0.7490$) or males (slopes: $t_{45} = -1.6438, p = 0.1072$; elevations: $t_{46} = 1.2832, p = 0.2058$). The readers consequently interpreted increments in SEM preparations in a similar fashion, generating similar results. In contrast, the readers differed in their interpretation of increments in LM preparations, resulting in the discrepancies discussed above.

Table 2.5: Results of the regression analyses conducted on the logarithmically transformed increment counts recorded by readers 1 and 2. Results are shown for regressions fitted to female (F) and male (M) data obtained from the light microscopy (LM) and scanning electron microscopy (SEM) preparations. The models are plotted in Fig. 2.5.

Data	Sex	Reader	n	r^2	Intercept			Slope		
					a (± 1 SE)	t	p	b (± 1 SE)	t	p
LM	F	1	11	0.9243	5.1947 (± 0.0552)	94.0966	0.0000	0.0034 (± 0.0003)	10.4811	0.0000
		2	7	0.1778	5.3846 (± 0.3593)	14.9875	0.0000	0.0018 (± 0.0017)	1.0397	0.3461
	M	1	25	0.5987	5.2721 (± 0.0857)	61.4989	0.0000	0.0018 (± 0.0003)	5.8583	0.0000
		2	23	0.5300	5.1268 (± 0.0946)	54.1910	0.0000	0.0016 (± 0.0003)	4.8667	0.0001
SEM	F	1	11	0.1851	5.3755 (± 0.1267)	42.4219	0.0000	0.0011 (± 0.0008)	1.4300	0.1865
		2	11	0.3831	5.1787 (± 0.1554)	33.3272	0.0000	0.0022 (± 0.0009)	2.3643	0.0423
	M	1	25	0.3990	5.3614 (± 0.1069)	50.1686	0.0000	0.0016 (± 0.0004)	3.9073	0.0007
		2	24	0.7785	5.1204 (± 0.0723)	70.8642	0.0000	0.0023 (± 0.0003)	8.7932	0.0000

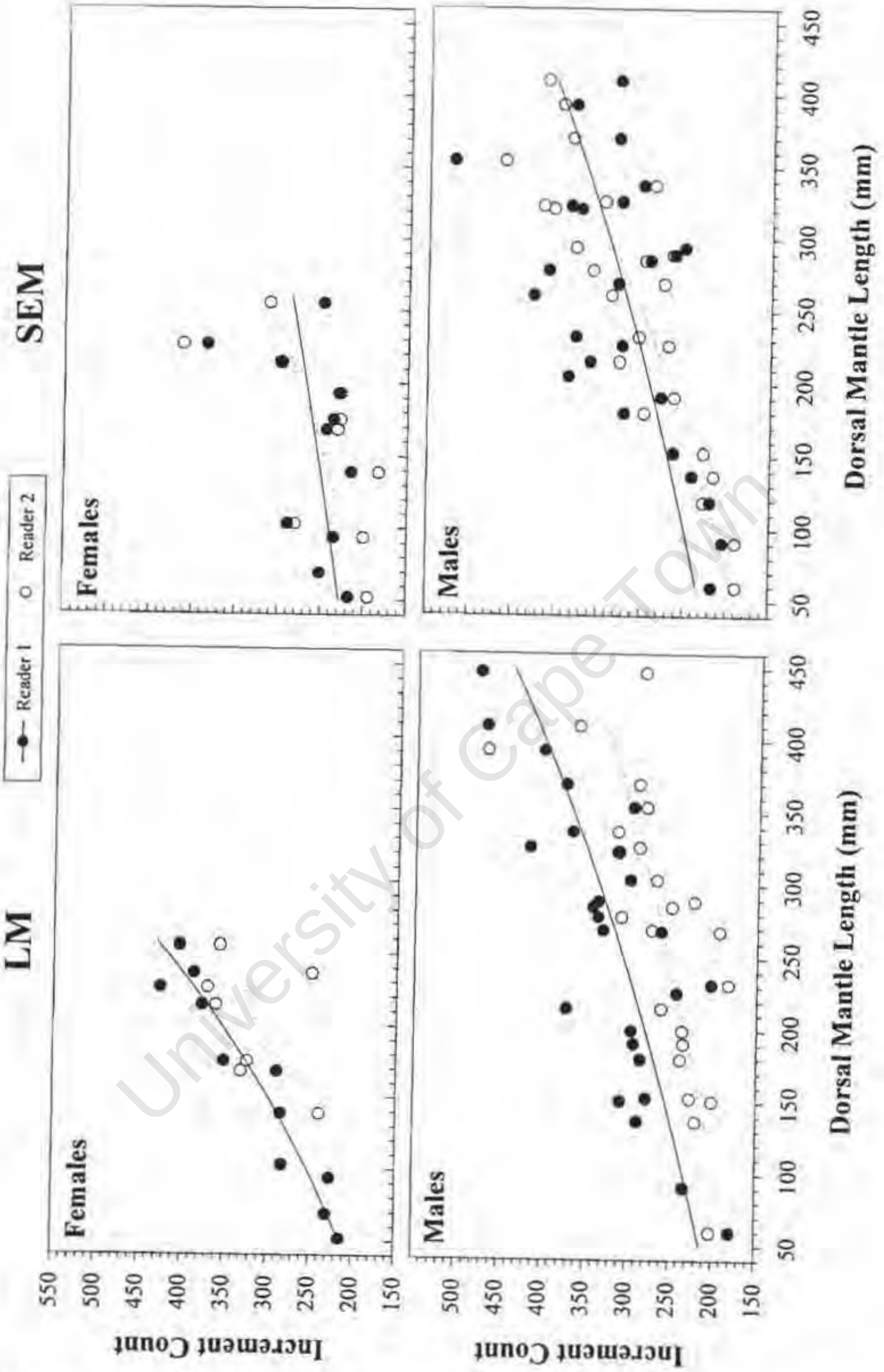


Figure 2.5: Comparisons of increment counts recorded from statoliths of *Loligo vulgaris reynaudii* between readers and examination methods. Individual counts of the total number of increments recorded by readers 1 (dots) and 2 (circles) using LM and SEM are plotted against dorsal mantle length for male and female specimens separately. The regression models fitted to the data are illustrated with solid and dotted lines for readers 1 and 2 respectively. Note that although the regressions were conducted on logarithmically transformed data, the models are plotted on an arithmetic scale. The regression statistics are given in Table 2.5.

2.4 DISCUSSION

The technique of statolith preparation for increment counting by LM developed in this study, while generating satisfactory results, clearly requires improvement. Although the performance of the technique is superior to that of preparation for SEM examination (generating twice as many specimens that were readable along the entire counting axis from nucleus to margin), the time and labour constraints are still a limitation. Of more concern is the overall success rate of the technique. If the criterion for a successful preparation is that all increments are visible along the entire counting axis, the technique resulted in a success rate of only about 42 %. In other words, for any given sample of statoliths, less than half of the specimens will be successfully prepared using this technique. This has serious implications for effective stock assessments in terms of the number of individuals that would have to be sampled, given the expected success rate indicated by these results. Further attention should be directed to improving and refining the technique, to increase the success rate and decrease the time and labour constraints.

The approach used in this study involved comparisons of increment counts obtained by the two examination methods (LM and SEM) between statoliths from the same squid. Comparisons of natural or experimentally induced microstructural features between statoliths of the same pair should first establish whether the statoliths are similar in terms of their microstructure. No information concerning microstructural comparisons between statoliths of the same pair appears to have been published. In the absence of such data, attention was directed to statolith size and shape. The morphometric data collected from the statoliths indicated that the left and right statoliths from the same pair showed no differences in terms of their size and shape. Similar conclusions have been reported by Lipinski (1981) and Natsukari *et al.* (1988). These results suggest that growth patterns of paired statoliths are the same. Based on this conclusion, it was assumed that microstructural features, and specifically increment counts, can be meaningfully compared between the left and right statoliths from the same squid. The finding that statoliths of female squid tend to be slightly larger than those of male squid of similar size suggests that such comparisons should treat males and females independently.

The results of these comparisons generated two major conclusions. Firstly, the method of examination (LM or SEM) did not significantly influence increment counting. Although SEM tended to yield slightly higher counts than LM, the differences are negligible. Consequently, either method can be used to count increments in chokka squid. However, the method of examination does impact on the second major finding, namely the issue of microstructural interpretation and hence reproducibility. The method of examination influences the manner in which increments are interpreted and counted, resulting in often substantial differences between the results obtained by different readers. In this regard, SEM examination was superior, in that the readers obtained similar results from the SEM preparations. In contrast, the LM preparations yielded different results for the two readers, with reader 1 tending to count more increments in the statoliths of any given squid. Considering that squid ageing studies using statolith microstructural analyses generally employ light microscopy, this finding is cause for some concern. Results obtained by different researchers may not be comparable, contributing to the discrepancies in age estimates that were discussed in the opening section of this chapter. It is crucial that further research be directed to the issue of microstructural interpretation in order to resolve this problem.

No inferences regarding the age of the squid are drawn from the increment counts recorded in this study. Because tests of the possible daily frequency of increment production in statoliths of *Loligo vulgaris reynaudii* have not been performed at the time of the study reported in this chapter, deductions concerning the time period represented by counts of total increment numbers cannot justifiably be made. The next chapter (Chapter 3) addresses this lack, and describes a series of validation experiments employing statolith marking techniques, aimed at investigating the putative daily nature of increments in statoliths of chokka squid.

CHAPTER 3 - FIELD AND LABORATORY VALIDATION OF DAILY INCREMENTS IN THE STATOLITHS OF THE SOUTH AFRICAN CHOKKA SQUID *Loligo vulgaris reynaudii*

ABSTRACT

Field and laboratory validation experiments aimed at testing the daily increment hypothesis in statoliths of the chokka squid (*Loligo vulgaris reynaudii*) are described. The field experiments are the first successful studies of this nature to be conducted on any cephalopod. Seven hundred and forty-two squid were captured on the spawning grounds, tagged, injected with oxytetracycline to mark the statoliths, and released back into the sea. Sixty-three individuals were recovered from commercial catches after periods ranging from 4 to 20 days. Statoliths from recaptured animals were prepared for examination by light and UV fluorescence microscopy. Forty-seven statoliths, of which only one was from a female squid, were successfully prepared for microscopic examination. Three different readers conducted three replicate counts of the number of post-marking increments in each statolith. The results support the daily frequency of increment production in chokka males in the size range 290 - 370 mm dorsal mantle length (DML), and the same was true for a 173 mm DML female. Estimates of the statistical power of this conclusion were very low, primarily as a result of small sample sizes. Obervations of the quality of each statolith preparation recorded by each of the readers were used in an analysis of the errors involved in increment counting. Proper statolith preparation, increment identification and subsequent counting are crucial for obtaining accurate age estimates. The error in counting a poorly prepared statolith may be three times higher than that for a well prepared statolith. One hundred and two squid were obtained during the laboratory study. The statoliths of 75 squid, comprising 36 males and 39 females ranging from 1 to 29 days post-marking, were prepared successfully. The results supported the daily increment hypothesis in male squid, but not in females. Increment deposition rates in males were similar under both field and laboratory conditions, indicating that the results of laboratory experiments can be extended to squid in the wild. The assumptions underlying the use of validation experiments to test the daily increment hypothesis are discussed.

3.1. INTRODUCTION

The importance of critically evaluating the validity of an ageing method for any given species has been emphasized by Beamish and McFarlane (1983). They further stressed the requirement that all ages must be validated for the technique in question, criticizing the practice of extrapolating beyond the maximum validated age for any given species or population. These warnings are, however, frequently ignored. For example Narimatsu and Munehara (1997), having validated daily increment formation in larvae of *Hypoptychus dybowskii*, assumed increments form daily throughout the life cycle. Several studies of squid population dynamics have used increments in statoliths for age estimation purposes without validating the estimates (e.g. Natsukari *et al.*, 1988; Natsukari and Komine, 1992; Arkhipkin and

Mikheev, 1992; Villaneuva, 1992; Arkhipkin, 1993), usually justifying this approach with results of validation experiments conducted on other species. Again, criticism of this practice (Natsukari *et al.*, 1991) has usually been disregarded. If age estimates are to be used in stock assessment procedures which will form the basis for management decisions which are likely to have profound implications for the status of the exploited species, it is critical that the estimates be validated.

What precisely is meant by the term "validation" as regards the daily increment ageing technique? There appear to be several terminological and philosophical inconsistencies in its use in the literature. Wilson *et al.* (1983) defined validation to be the confirmation of the temporal meaning of an increment. In this sense, validation is a test of the daily increment hypothesis. In contrast, Beamish and McFarlane (1983) are of the opinion that validation means proving a technique is accurate. Francis (1995) considers this broader definition a more useful concept, but qualifies it by saying that the validation of an ageing technique should estimate the accuracy of the procedure, rather than whether it is accurate. An explicit, quantitative measure of accuracy is required. As Francis (1995) states, the acceptable level of accuracy of any given ageing procedure should be evaluated within the framework of how the age estimates will be used. It is the opinion of Francis (1995) that the term "validation" should therefore encompass the process of estimating the accuracy of an age determination method, the first stage in this process being the confirmation of the temporal meaning of the ring.

A further inconsistency in terminology is introduced with the term "verification", which is frequently confused with "validation" (Wilson *et al.*, 1983). "Verification" has been used to describe the process of confirming the daily periodicity of increment formation (e.g. Narimatsu and Munehara, 1997). Wilson *et al.* (1983), however, define "verification" as the repeatability of a numerical interpretation that may be independent of age (i.e. precision). It is important to stress here that precision has no bearing on the level of accuracy, but relates to the level of reproducibility of several replicate age estimates, rather than on how close the age estimates are to the true age of the individual. A high level of precision does not imply that the estimates are accurate. Similarly, the mean of a number of replicate age estimates may be very accurate (i.e. very close to the true age of the individual), but have a very low precision (i.e. a high degree of variability).

Whatever the terminology being used, there are two aspects or phases to any attempt to assess the validity of any given age determination method, and these should be clearly stated and distinguished between. The first aspect is that of testing the chronometric significance of the microstructural features that are being used to estimate age, namely the putative daily increments. The second aspect concerns the applicability of these features in terms of the accuracy (or conversely, the degree of error) of the age estimates that are obtained from them. This must include the development of an objective increment recognition concept (which can only be based on an understanding of the underlying physiology of increment formation), as well as quantification of the errors (or the level of accuracy) inherent in the subsequent increment enumeration.

Almost all so-called validation studies described in the literature have attemped to confirm the daily frequency of increment production, very few attempting to assess the level of error associated with the ageing technique. The approach used in these studies has been to compare the number of increments deposited over some known time period with the elapsed number of days, employing either direct or indirect techniques. Indirect techniques involve comparison of the increase in increment numbers in the modal groups of sequential samples with the time period between samples. Such inferences are, however, subject to the biases discussed in Chapter 1. Another indirect method is the marginal increment technique. The state of completion of the outermost increment in samples taken at intervals over a 24 hour period may indicate whether an increment is deposited with a daily frequency (e.g Ré, 1984; Ré *et al.*, 1985). This technique is usually not suitable for squid statoliths, since the outermost growth increment is often very difficult to detect or measure.

Direct validation methods involve either increment enumeration in animals of known age (usually individuals reared in a laboratory), or statolith (or otolith) marking techniques (Geffen, 1992). Statolith marking techniques rely on the introduction of a temporal reference mark into the statolith at a known date. The number of increments bracketed by this mark and the edge of the statolith are then compared with the period elapsed between marking and the demise of the animal. The reference mark can be introduced by administering a chemical that is incorporated into the statolith structure and which can then be detected at a later date. Compounds that have been used for this purpose include oxytetracycline (e.g. Dawe *et al.*, 1985; Lipinski, 1986), alizarin complexone (Narimatsu and Munehara, 1997) and strontium chloride (Dawe *et al.*, 1985). Marks can also be introduced in the form of checks (prominent increments), which are produced in response to stress-related events that are imposed on the animal (Geffen, 1992 has reviewed a number of validation studies employing this approach). This technique should be applied with caution, however, since uncontrolled check formation in response to other events may complicate the identification of the dated check associated with the marking procedure.

Whatever the method used, data obtained are increment counts that are then compared to the elapsed number of days by means of various statistical tests, usually either t - tests or simple linear regression. Results where no significant differences are found between the number of increments deposited and the elapsed number of days are then considered to confirm the daily rate of increment production in the species concerned. The majority of such studies, particularly in the case of studies conducted on various squid species, have concluded that increments are deposited with a daily frequency (see Jackson, 1994 for a review of validation studies on squid). In spite of results such as these, validation studies are subject to a number of shortcomings. Different ageing techniques that may yield up to a four times difference in maximum ages can still legitimately claim to have been 'validated' (Gauldie, 1994). Jackson (1994) has summarized conflicting age results that have arisen from statolith ageing studies for several squid species. In several cases, individuals of similar dorsal mantle lengths were assigned ages differing by more than a year. Gauldie (1994, p. 2341) attributes these difficulties "to a fundamental problem in the general dogma of age estimation: that of 'validation' ". In Gauldie's opinion, the widely held belief that "validation", "accuracy" and "precision" can be brought together as a scientifically acceptable way to test an age estimation hypothesis is incorrect. These reservations have to be considered seriously, because they address the philosophy underlying the experimental approach of the majority of the studies exploring the use of daily increments for age estimation.

To date, no attempts to measure age determination accuracy or precision have been documented for squid, and very few for fish. The majority of direct validation studies on squid have been conducted in the laboratory, with limited success (e.g. Lipinski, 1986) until recently (e.g. Nakamura and Sakurai, 1991; Jackson *et al.*, 1993). Laboratory studies, while cost-effective and easily conducted, have been criticized on the basis of the artificial conditions imposed during the experiments, and the applicability of the results of such experiments to wild populations has been questioned (Campana and Neilson, 1982; Jones, 1986; Campana and Moskness, 1991). The question of whether the conditions in the laboratory, which can be either constant or cyclical (often with a daily frequency) could influence the rate of increment production has yet to be resolved. Further, laboratory conditions are known to influence the appearance of increments (e.g. Campana and Neilson, 1985; Jackson *et al.*, 1993), which differ markedly from natural conditions. The interpretation of increments in laboratory reared individuals may therefore differ from those of wild individuals (Campana and Moskness, 1991). The only studies investigating increment deposition in squid under natural conditions are those of Morris (1988, 1991b, 1993). The results of experiments where squid embryos were incubated in the field showed no relationship between increment counts and the elapsed time period, suggesting that the criticisms mentioned above have some basis.

The study described in this chapter was undertaken in an attempt to address these issues, and critically examine the proposed daily periodicity in increment deposition in the statoliths of the chokka squid *Loligo vulgaris reynaudii*. The nature of the squid fishery on the south east coast of southern Africa is suitable for field validation studies. Fishing (hand jigging) is conducted on spawning concentrations of squid in shallow (< 50m) inshore waters in spring and summer (Sauer, 1993). Squid remain in the area for some time during the spawning season (Sauer, 1993), increasing the chances of recapture of marked individuals. The analysis of statoliths of such individuals could provide information concerning the viability of the statolith ageing technique. A laboratory validation study was also conducted to establish whether the results of such experiments are applicable to wild populations.

3.2. METHODS

The validation approach used in this study was that of marking squid statoliths with oxytetracycline (OTC) to provide a reference mark in the progress of statolith growth. OTC binds to the $CaCO_3$ crystals and to the protein matrix of squid statoliths during the deposition process, resulting in a fluorescent band when viewed under ultraviolet light (Lipinski, 1986). This technique has been used frequently for both fish (e.g. Campana and Neilson, 1982; Tzeng and Yu, 1992) and squid (e.g. Lipinski, 1986; Jackson, 1989, 1990a, 1990b). Live squid were injected in the ventral mantle musculature with up to 0.1 ml of an aqueous solution of OTC (modified from Lipinski, 1986). To establish the post-injection period required for OTC to be incorporated into the statoliths, injected squid were sacrificed at one-hour intervals after injection, and the statoliths observed under UV light. OTC fluorescence was evident in the statoliths after one hour, and a well developed OTC band was visible after 2 to 3 hours.

3.2.1. Field study

This study was conducted in the Cape St. Francis area on the south-east coast of southern Africa in November 1993. Squid were captured by hand jigging, injected with OTC, tagged with Japanese filament tags for identification, and released. Simultaneous underwater observations made during the procedure showed that the marked squid immediately re-engaged in normal spawning activity after release (Sauer, pers.comm.), indicating that capture and marking did not disrupt normal behaviour. Marked squid were subsequently recovered from commercial catches and frozen for later processing and analysis. The squid were subsequently thawed, the dorsal mantle length, wet weight, sex and maturity stage (assessed according to the scale of Lipinski and Underhill, 1995) recorded, and the statoliths extracted and stored in liquid paraffin for spectroscopy (Merck #7161) until processing. This storage medium does not damage the surface layers of the statoliths, a feature which is of crucial importance, because it is the outermost layers of the statoliths that are of interest. Damage to these layers would generate erroneous increment counts. All statoliths were stored in darkness to minimize the fading of the OTC fluorescence (Geffen, 1992).

3.2.2. Laboratory study

Squid were captured by hand jigging in False Bay, and transported to the laboratory in large bins filled with continuously aerated sea water. Each squid was placed in a plastic bag suspended in the water to minimize movement and damage to the animals during transport. The plastic bags were perforated with small holes to prevent suffocation of the animals. Decreasing the water temperature in the bins by about 8 to 10 °C using blocks of frozen sea water substantially reduced mortalities during transport, presumably by decreasing the metabolic rate and hence the stress response of the animals to capture. Once in the laboratory, the squid were injected with OTC, and released into 2 000 liter tanks supplied with a continuous flow of fresh sea water. Squid were fed *ad libitum* with live mullet (*Liza richardsoni*) at least twice a day, and the tanks were cleaned daily. The tanks were exposed to an ambient light cycle. Dead squid were removed from the tank, the biological information recorded, and the statoliths extracted and stored. During the course of the laboratory experiments, two squid survived for a period exceeding 25 days. These two animals were labelled for a second time with OTC, providing a double-check on the frequency of increment formation during the course of the experiment.

3.2.3. Statolith preparation and analysis

One statolith of each pair was randomly selected, washed in warm water and a mild detergent, air dried and prepared for light microscopy following the procedures described in chapter 2, but using a slightly modified plane of sectioning. The statoliths were ground from in the oblique frontal plane (Fig. 3.1 A), targeting the area superficial and anterior to the medial inclusion of the wing (Fig. 3.1 B). This was the only region of statoliths of *Loligo vulgaris reynaudii* where increments are consistently visible to the margin (Fig. 3.1 C and D).

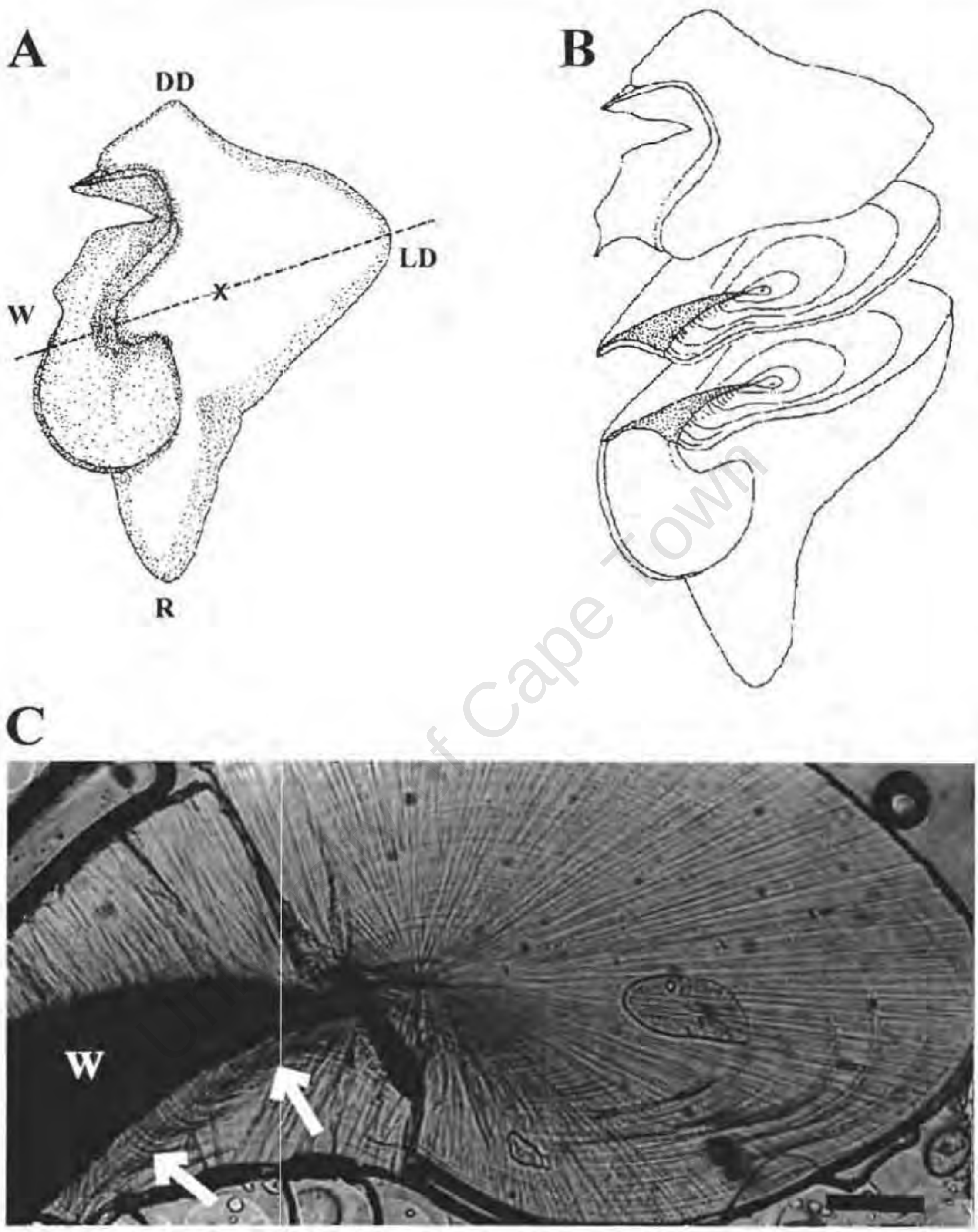


Figure 3.1: Diagrams illustrating the sectioning of statoliths of Loligo vulgaris reynaudii. A - the oblique frontal plane of the sections, indicated with a dashed line (X = position of the focus, DD = dorsal dome, LD = lateral dome, R = rostrum, W = wing). B - position of the section. C - light micrograph showing the results of the sectioning procedure. The axis of clear increments is indicated with arrows. Scale bar = 70 µm. (Continued overleaf).

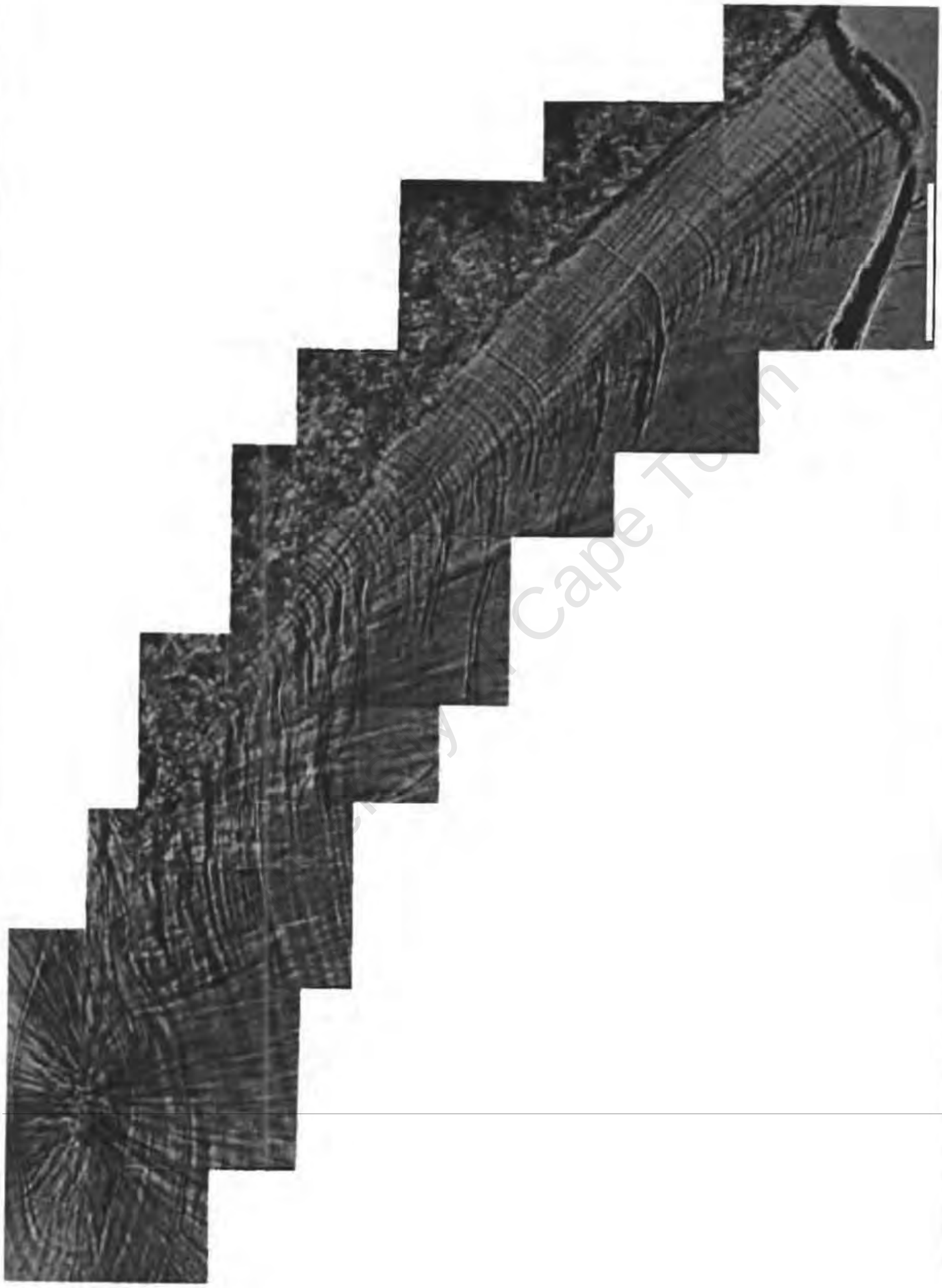


Figure 3.1 (continued): D - light micrograph showing the axis of clear increments extending along the wing inclusion

Completed sections were viewed under a Zeiss epifluorescent microscope equipped with a Zeiss 48-77-05 filter combination, which incorporated a BP 450-490 exciter filter, a FT 510 chromatic beam splitter and an LP 520 barrier filter. Each section was first viewed under normal transmitted light to identify regions of clearest increments extending to the margin (Fig. 3.2 A). The section was then viewed under UV light to identify the location of the OTC mark (Fig. 3.2 B). OTC is clearly metabolized over an extended period, since statoliths extracted from squid that had been marked several weeks previously exhibited the OTC mark as a broad band extending over 10 to 20 increments (see Fig. 3.4).

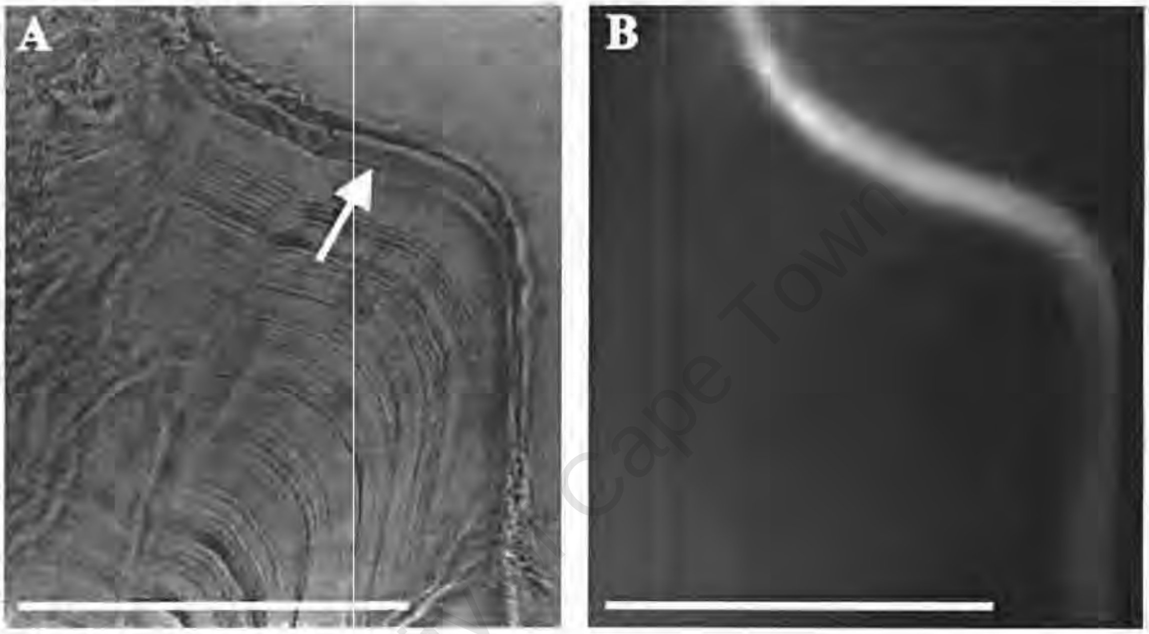


Figure 3.2: Photomicrographs of a ground and polished section of an OTC-marked statolith. A - Viewed under normal transmitted light. The prominent check associated with the OTC mark is indicated with an arrow. B - Viewed under ultraviolet (UV) light, showing the fluorescent OTC band. Statolith from a male squid (DML = 303 mm, ♂V) recaptured 5 days after marking. Scale bars = 50 µm.

The innermost edge of the OTC band (indicating the time of marking) was usually clearly defined, and could be located within one to two increments. The band gradually became more diffuse towards the margin, eventually disappearing in statoliths marked more than 20 days previously. In many cases, the innermost edge of the OTC band was defined by a clear check (i.e. a prominent discontinuity in the increment progression, Fig. 3.2), indicating that the marking procedure involved a certain degree of physiological stress to the animal. Similar observations have been reported previously (e.g. Jackson, 1989). Once the OTC mark had been located, the number of increments between the mark and the margin of the statolith were counted.

Three replicate counts were conducted on each successfully prepared statolith. In the case of the field validation material, the author and two additional readers (readers 2 and 3) each recorded three replicate counts on each statolith. Reader 2 was experienced in counting increments in otoliths from larvae of various pelagic fish species, while reader 3 was experienced in increment enumeration in squid statoliths, primarily by means of SEM techniques. In the case of the laboratory material, statoliths were only analysed by the author (reader 1). It should be emphasized that all statoliths from both the field and laboratory studies were prepared and sectioned by the author. To avoid bias, all counts were conducted without prior knowledge of the post-marking interval of the statoliths, and without reference to previous counts. Preliminary observations indicated that the quality of the preparations varied considerably, either as a function of the preparation process (over- or under-grinding, damage to the specimen), or of the specimen itself. Consequently, the level of confidence in the accuracy of each count varied between statoliths.

Three properties of the statolith preparation were identified to account for the quality of each specimen.

(i) The clarity and definition of the increments between the OTC mark and the margin. Increments were often faint or invisible along portions of the counting axis, requiring either interpolation or extrapolation during counting, with the associated uncertainty in the increment count.

(ii) The definition of the inner edge of the OTC mark. A "smearing" effect was often apparent, possibly resulting from the sectioning procedure (during grinding, loose crystals containing OTC may re-adhere to the ground surface at points removed from the fluorescent band itself, resulting in a "smear" of spread-out fluorescence (Fig. 3.3).

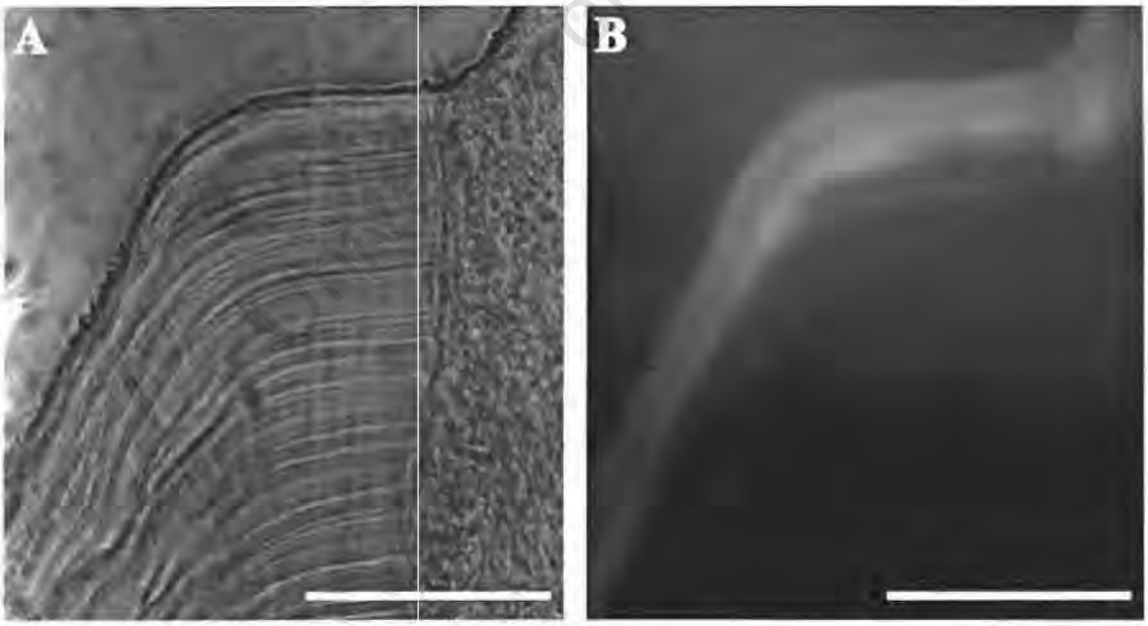


Figure 3.3: Photomicrographs of a ground and polished OTC-marked statolith illustrating a poorly defined OTC mark. A - Normal, transmitted light. B - UV light. Statolith from a squid (DML = 316 mm, ♂V) recaptured 5 days after marking. Scale bars = 50 µm.

Statoliths that had not been ground sufficiently thin during preparation also showed a layering effect in the OTC fluorescence due to the curvature of the marginal surface, resulting in very diffuse fluorescence in the innermost regions of the OTC band. This diffuse glow prevented clear identification of the beginning of the OTC mark, and hence location of the exact point where post-mark increments were deposited.

(iii) The clarity and definition of the margin. In many cases, the margin of the statolith was either damaged or obscured by debris resulting from grinding (Fig. 3.4). Edge effects resulting from light refraction by the curved marginal surface (Campana, 1992) and accumulated debris further complicated increment identification in the marginal regions. These effects closely resembled increments, but could usually be distinguished from increments by altering the focal plane. However, these features usually obscured the outermost regions of the counting axis, again requiring extrapolation of increment numbers.

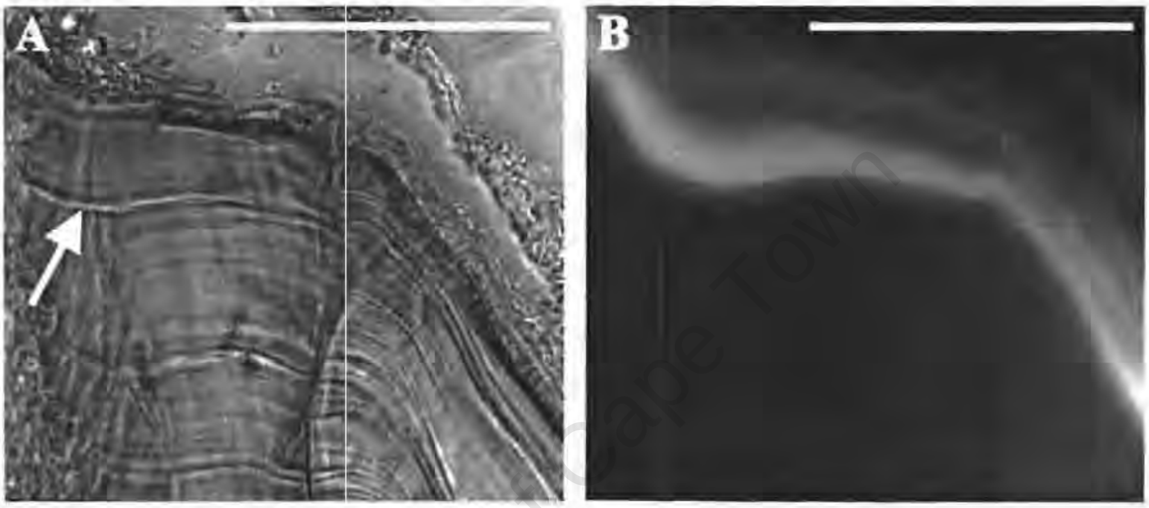


Figure 3.4: Photomicrographs of a ground and polished statolith where the margin has been damaged during the preparation procedure. A - Normal, transmitted light. The check induced by marking is clearly visible (arrow). B - UV light, showing the clear band of OTC fluorescence. Statolith from a squid (DML = 276 mm, ♂V) recaptured 18 days after marking. Scale bars = 50 µm.

These three quality factors, namely increment clarity (I), OTC band definition (O) and margin clarity (M) were used by each reader to quantify the quality of each specimen in each replication. Each specimen was assigned a rank for each factor according to the scale: 1 (poor; increments faint or not visible in parts; OTC band badly smudged and could not identify inner edge; margin chipped or obscured), 2 (average: counting possible, but interpretation of some increments unclear; inner edge of OTC band diffused over several increments; margin irregular in places) or 3 (good: all three structures clear and unambiguous).

3.2.4. Data analyses

To test the daily increment hypothesis, the relationship between elapsed time and increment counts was quantified by fitting simple linear regression model to the data:

$$Y = a + bX$$

where:

Y = number of increments distal to the OTC mark

X = number of days after OTC marking
and a and b are the intercept and slope of the fitted relationship respectively.

Several studies have constrained the regression model to pass through the origin, on the assumption that time zero corresponds to zero increments (e.g. Wilson and Larkin, 1980). This approach is flawed because the model will not account for any potential delay in increment deposition that may occur after marking, yielding an inaccurate estimate of the rate of increment deposition. Separate analyses were conducted on the data collected by each reader. The average of the three replicate counts obtained by each reader for each statolith yielded a single estimate of the number of post-mark increments (the dependent variable). An assumption implicit in regression analysis is that the values of the dependent variable are independently distributed for any given value of the independent (explanatory) variable (Sokal and Rohlf, 1981). Treating each replicate count on each statolith as an independent datum would violate this assumption. This would also artificially increase the number of data points included in the analysis, reducing the standard error associated with each parameter estimate, and artificially inflate the level of statistical significance. For the two individuals that were labelled twice with OTC during the laboratory study, the two estimates of increment deposition frequency obtained from each statolith were treated independently.

To account for the level of confidence each reader had in the accuracy of their estimate of the number of post-mark increments, linear models were fitted to data that were filtered using the three quality factors. Statoliths that were rated as "average or better" (i.e. $I \geq 2$, $O \geq 2$ and $M \geq 2$) for all three replicate counts were analysed separately. The daily increment hypothesis is usually then tested as the null hypothesis:

H_o: b (slope) = 1, against the alternative hypothesis:
H_A: $b \neq 1$

If the null hypothesis cannot be rejected at a set level of statistical significance, it is usually concluded that increments are deposited with a daily frequency (e.g. Moksness and Wespestad, 1989; Bigelow, 1992). This approach has been criticized by Rice (1987) on the basis that the "acceptance" of the null hypothesis at a set level of Type I error (α) greatly increases the risk of Type II error (β), i.e. failing to reject a false null hypothesis. Statistical power, equivalent to $1 - \beta$, measures the probability of avoiding Type II error. By calculating the power of the test of the null hypothesis, one can determine the likelihood that a significant difference in slope would be detected if one really existed (Rice, 1987). In this study, the power of the test to detect a deviation of 0.1 in either direction from unity (a two-tailed test) was estimated, so as to be comparable with other studies (e.g. Ahrenholz, 1994; Legardère and Troadec, 1997). Power was estimated for those tests where the null hypothesis could not be rejected at $\alpha = 0.05$, using the equations given by Rice (1987) for two-tailed tests:

$$\text{Power} = P\,(t > t_{\mu} - \theta) + P\,(t < -t_{\mu} - \theta)$$

where

$$\theta = \frac{\textit{deviation of regression slope from } 1.0}{s_b}$$

(Note: s_b = standard error of the slope estimate)

and $t_u = t_{\alpha(2)(n-2)}$

3.2.5. Error modelling

By assessing the quality of the statolith preparations during each reading, the factors that may have contributed to errors in the increment counting process could be examined. The results of the field validation study were used for this purpose. The "error" in the increment counts was calculated for each replicate by each reader as the absolute value of the difference between the number of increments counted and the actual number of days since marking. These errors were modelled as functions of a number of explanatory variables using a generalized linear model (McCullagh and Nelder, 1989). Because the errors are counts, the Poisson distribution, with the associated logarithmic link function provided an appropriate model.

A number of explanatory variables were considered for the generalized linear modelling of the errors. Firstly, the quality factors increment visibility (I), OTC mark definition and clarity (O) and margin quality (M). The nine values for each of these factors (three readers, three replicates) were averaged to obtain a single value of each factor for each statolith. The interactions between the quality factors (represented as the products I*O, I*M, O*M and I*O*M) were also included because the various activities related to the preparation of statoliths might not be independent. For example, grinding and polishing, which determined the definition of increments (I), was also responsible for the quality of the margin (M) and a possible smudging effect on the OTC band (O). Finally, the number of days between marking and capture was considered as an explanatory variable because errors in counts are likely to be larger for longer intervals. Using the Poisson distribution for the generalized linear modelling accommodates the tendency for larger counts to have larger variances. Because different variables may be involved in under- and over-estimation of increment counts, separate generalized linear models were considered for those statoliths where the counts under-estimated the actual number of days (errors ≤ 0), and those where the counts over-estimated the number of days (errors ≥ 0).

3.3. RESULTS

3.3.1. Field study

Sixty three OTC labelled squid were recovered from commercial catches, 62 from the November 1993 survey, and one from a preliminary study conducted in November 1992. All statoliths showed a flourescent band around the perimeter, indicating that OTC had been incorporated into the statoliths. Of the 63 statoliths processed, 16 were destroyed or severely damaged during the preparation procedure, the remaining 47 (75%) being considered satisfactory for enumeration purposes. Two of these specimens were excluded from further analysis on the assumption that the recapture dates were incorrectly reported. These specimens were the only two recovered from a boat operating in the Port Alfred area, and both displayed post-marking intervals substantially narrower than what was typical for the reported 15 and 20 day post-marking periods.

The biological information, increment counts and associated quality factors recorded by the three readers on the remaining 45 statoliths obtained from the field study are provided in Appendix I. This material included only one female squid, the remaining specimens all being mature males. Most of the specimens (62%) fall into the 5 days post-marking interval (see Appendix I), the remaining specimens being relatively evenly distributed over 6 to 18 days post-marking periods. This bias results from the majority of the squid being marked five days before the end of the November 1993 closed season. Intensive fishing for squid on the spawning grounds resumed immediately after the closed season, and the majority of the recaptures were collected on the first day that fishing resumed.

Reader 1

The relationship between the mean of the post-mark increment counts recorded by reader 1 from each statolith, and the number of days after marking, is illustrated in Fig. 3.5 (the regression statistics are provided in Table 3.1).

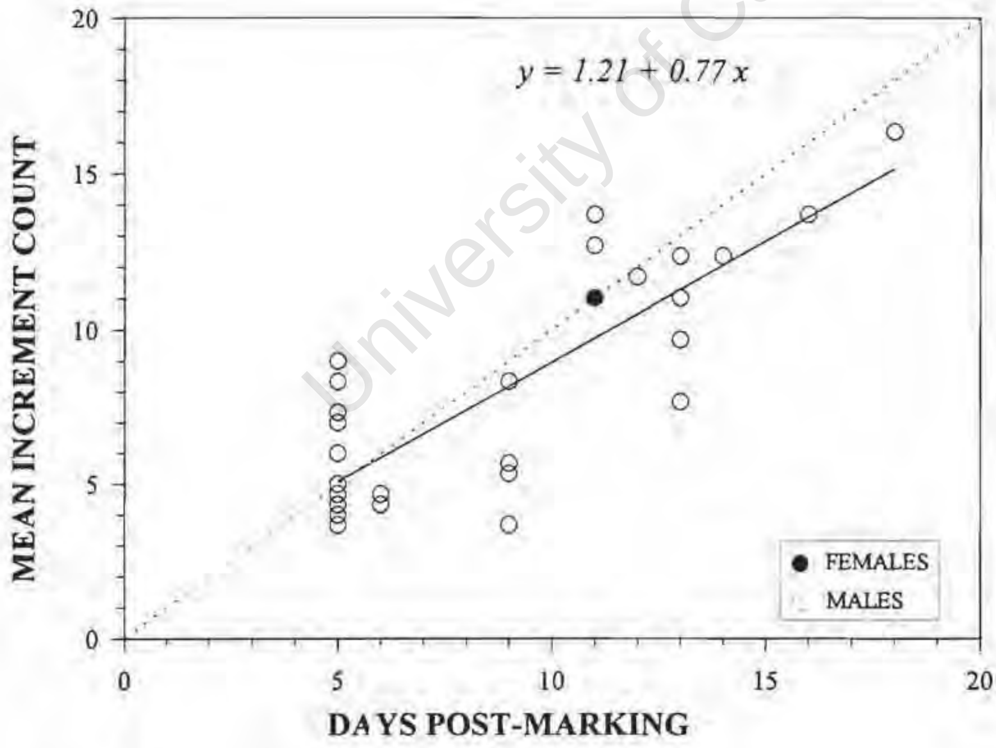


Figure 3.5: Relationship between the increment counts recorded by reader 1 and days post-marking for the field validation study. Data are the means of three replicate counts of post-mark increments recorded for each statolith. The solid line illustrates the regression model fitted to the data (the equation describing the regression line is given in italics), while the dotted line shows the 1:1 relationship (the daily increment hypothesis). The regression statistics are provided in Table 3.1.

Table 3.1: Results of the regression analyses conducted on the field and laboratory validation data. Statistics are shown for analyses conducted on both the original (All) and the filtered data sets collected by the three readers for each statolith.

Reader	Data	Intercept		Slope		n	r^2	Ho: $b = 1$		
		$a \pm$ SE	p	$b \pm$ SE	p			t	p	Power
Field Study										
1	All	1.2109 ± 0.6111	0.0539	0.7733 ± 0.0742	0.0000	45	0.7166	3.0568	0.0038	-
	Filtered	0.7274 ± 1.3829	0.6267	0.9190 ± 0.1347	0.0024	6	0.9208	0.6011	0.5802	0.0681
2	All	5.6913 ± 0.9056	0.0000	0.2950 ± 0.1099	0.0000	45	0.1435	6.4147	0.0000	-
	Filtered	-0.9887 ± 2.8295	0.7370	1.1831 ± 0.3670	0.0146	9	0.5975	0.4988	0.6332	0.0542
3	All	6.2837 ± 0.8658	0.0000	0.2890 ± 0.1051	0.0087	45	0.1496	6.7662	0.0000	-
	Filtered	6.4692 ± 1.2754	0.0001	0.2001 ± 0.1716	0.2573	22	0.0637	4.6630	0.0002	-
Laboratory Study										
1	All	0.3200± 0.3718	0.3922	0.9263 ± 0.0329	0.0000	77	0.9136	2.2396	0.0281	-
	Filtered	0.0092 ± 0.5085	0.9856	0.9476 ± 0.0394	0.0000	50	0.9233	1.3303	0.1897	0.6994

Mean increment counts tended to over-estimate the number of days in the lower post-marking intervals (particularly in the 5 days post-marking interval), whereas the reverse is true at higher post-marking intervals. The regression model fitted to these data does not support the daily production of increments (Table 3.1). The slope of the regression line ($b = 0.7733$) is significantly different from unity ($p = 0.0038$), indicating that the data do not conform to the daily increment hypothesis.

The regression analysis conducted on the data from statoliths that fulfilled the quality criteria (see sections 3.2.3 and 3.2.4) led to the opposite conclusion. Only six statoliths (10% of the total), one of which was from the only female squid, were considered to be of a quality sufficient for accurate increment counts. The means of the increment counts obtained from these statoliths by reader 1 are plotted against the post-mark period in Fig. 3.6.

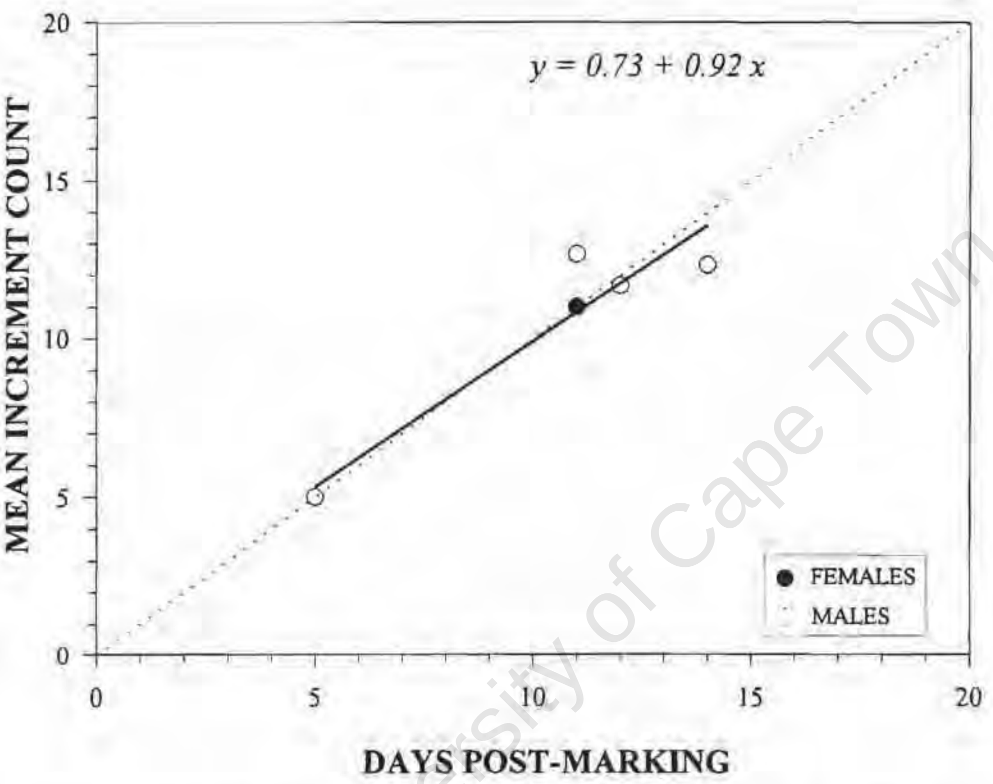


Figure 3.6: Relationship between the mean increment counts recorded by reader 1 from the field validation statoliths that fulfilled the filtering criteria, and days post-marking. The regression fitted to the data is illustrated with a solid line (expression in italics, see Table 3.1 for the associated statistics). The dotted line illustrates a 1:1 relationship between the variables. Note that two data points are superimposed at 5 days post-marking.

The regression model fitted to these data has a slope ($b = 0.9190$) that is not significantly different from unity ($p = 0.5802$, Table 3.1), supporting the daily deposition of increments. The reason for this is apparent from a comparison of Figures 3.5 and 3.6. The filtering procedure excluded all of the scatter in the unfiltered data at the 5 and 9 day post-marking intervals and most of the scatter at the higher intervals. This implies that statolith preparations that were considered to be satisfactory for accurate increment counting generated estimates of the number of post-mark increments that closely approximated the elapsed time period. In contrast, poor preparations did not. The power of the test to detect a relatively large deviation of 0.1 from a slope of 1.0 is less than 7%, indicating that the probability of accepting a false null hypothesis is unacceptably high. The conclusion that the data support the daily increment hypothesis is therefore not statistically robust. This is a direct function of the stringency of the filtering criteria, which excluded 87% of the original data set. This reduction in sample size profoundly influences the estimation of power, primarily through its influence on the standard error of the slope estimate.

Other readers

Mean increment counts calculated from the data of readers 2 and 3 are plotted in Fig. 3.7.

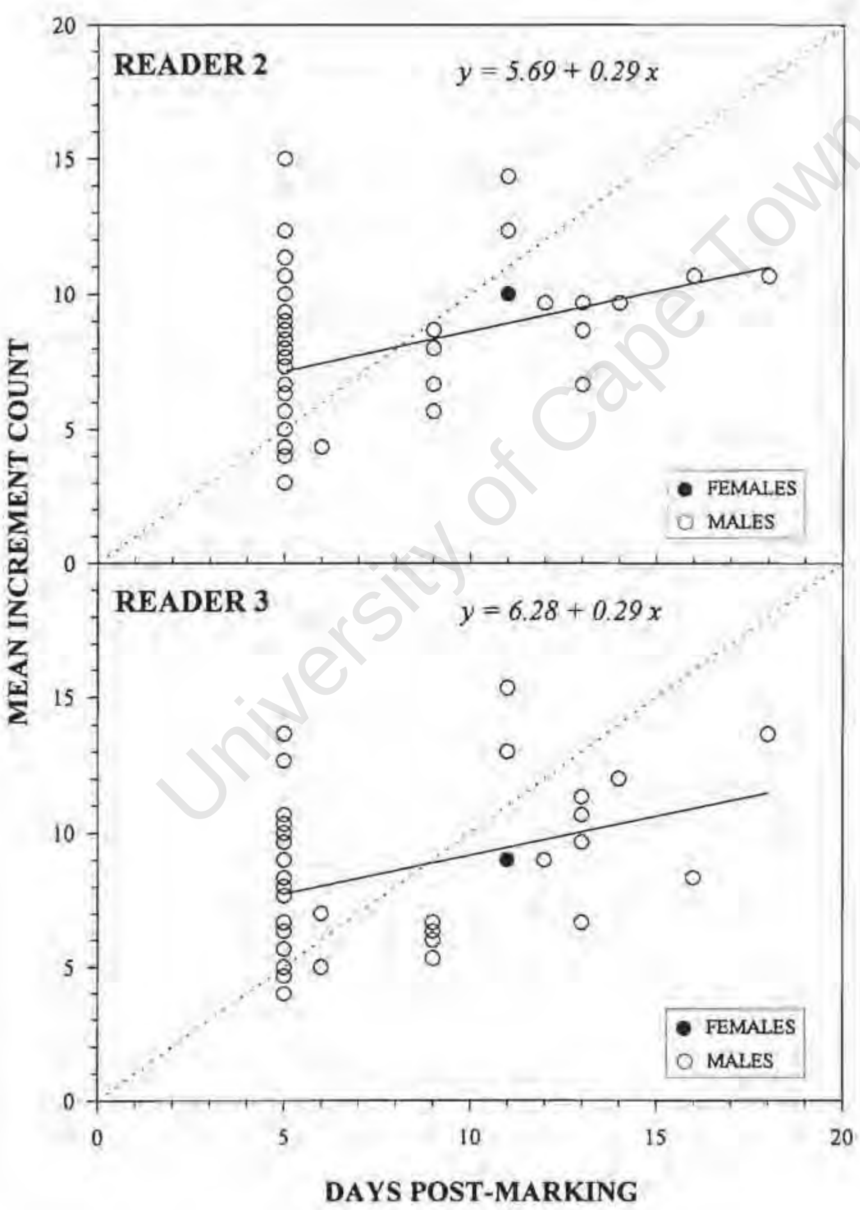


Figure 3.7: Relationships between the mean increment counts and days post-marking obtained from the field validation statoliths by readers 2 (upper plot) and 3 (lower plot). The regressions fitted to the data are illustrated with solid lines (the relevant expressions are shown in italics, see Table 3.1 for the associated statistics). The daily increment hypothesis is illustrated with a dotted line in each plot.

The data are clearly more variable than those illustrated in Fig. 3.5 for reader 1. Further, the tendency to for increment counts to over-estimate the number of days at low post-marking intervals, and under-estimate at the higher post-marking intervals, is considerably more marked for these two readers than it was for reader 1. The regression models fitted to the data consequently show elevated intercepts and reduced slopes. The regression analyses conducted on the data recorded by both readers 2 and 3 provided no support for the daily increment hypothesis. The slopes of the regression models fitted to the data for readers 2 and 3 ($b = 0.2950$ and $b = 0.2890$ respectively) were both significantly different from one (Table 3.1).

Excluding the data from those statoliths that readers 2 and 3 did not consider to fulfill the quality criteria yielded conflicting results. In the case of reader 2, the filtering procedure excluded 36 of the 45 statoliths that were successfully prepared (a success rate of 15%, similar to that of reader 1). A plot of the filtered data (Fig. 3.8) showed that, although more variable than the data of reader 1, the mean increment counts approximated the daily increment hypothesis reasonably well, with most of the "over-estimates" at low post-marking intervals, and "under-estimates" at the higher intervals having been removed.

In spite of the variability in the data, the regression analysis supported the daily increment hypothesis (Table 3.1). The slope of the fitted regression ($b = 1.1831$) was not significantly different from unity ($p = 0.6332$). The power of the test to detect a slope deviation of 0.1 from one, about 5%, was even lower than that for reader 1, primarily due to the higher variability inherent in the data combined with the small sample size. The conclusion that the increments were deposited on a daily basis has to be rejected in view of the high probability of committing a type II error.

In the opinion of reader 3, 23 of the statoliths did not fulfill the quality criteria, implying a preparation success rate of about 36%. The regression model fitted to the filtered data describes a weak relationship between the mean increment count and the elapsed number of days (Fig. 3.8, Table 3.1). The slope of the model ($b = 0.2001$) was significantly different from one ($p = 0.0002$), indicating that increments were not formed with a daily frequency. Examination of a plot of the data (Fig. 3.8) indicates that this conclusion is a direct result of what reader 3 considered to be a satisfactory statolith preparation. Unlike readers 1 and 2, the filtering of the increment counts recorded by reader 3 retained most of the over-estimates in the lower post-marking categories, and many of the under-estimates in the higher post-marking categories, retaining much of the variability that was evident in the original data set.

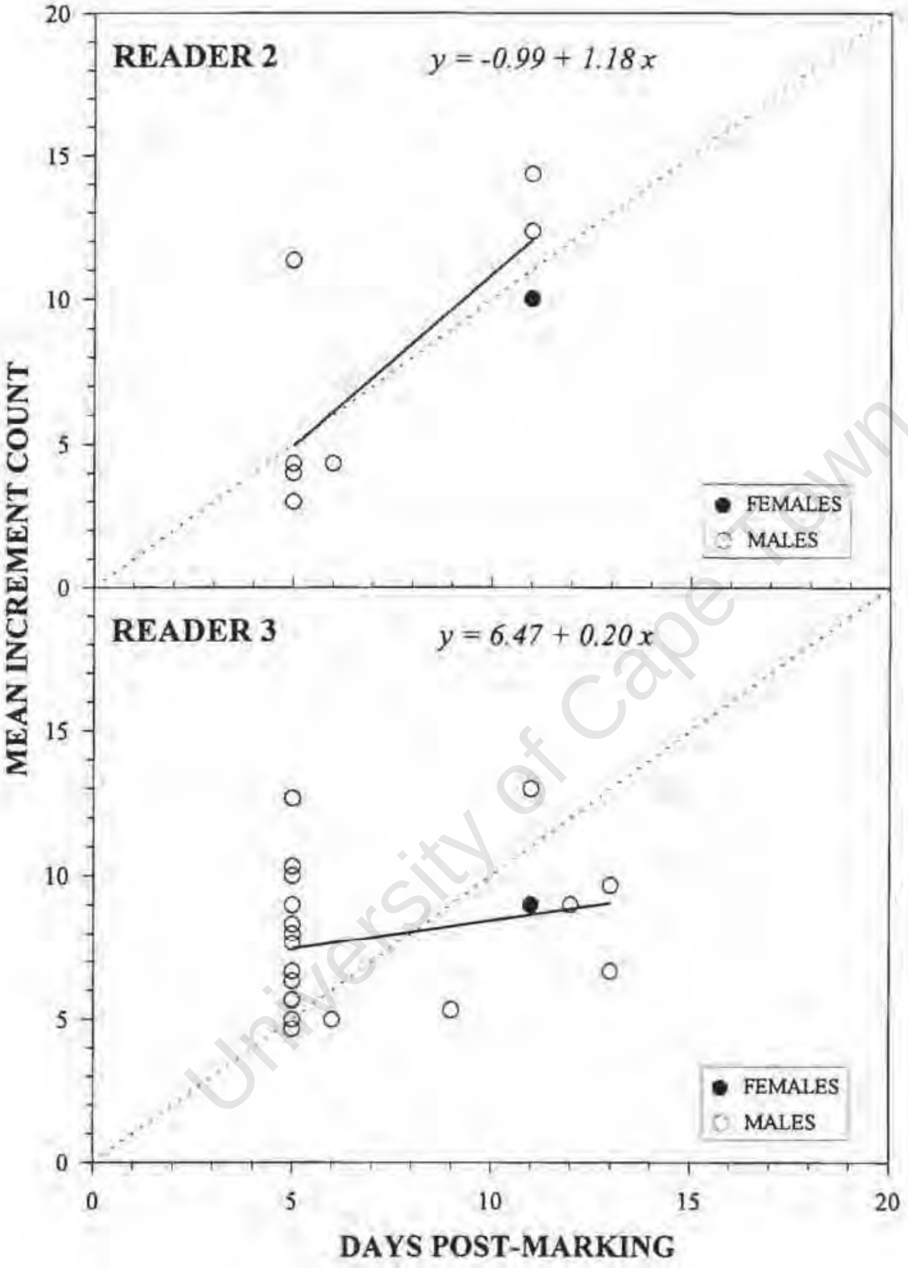


Figure 3.8: Relationships between mean increment counts and days post-marking obtained from the field validation statoliths that fulfilled the quality criteria (i.e. filtered data) by readers 2 (upper plot) and 3 (lower plot). The regressions fitted to the data are illustrated with solid lines (equations in italics, see Table 3.1 for the associated statistics). The daily increment hypothesis is illustrated with a dotted line in each plot.

3.3.2. Laboratory study

OTC-marked statoliths from 102 squid were obtained from this study. 75 of the statoliths (including the two double-labelled specimens) were prepared successfully successfully (73.5%), the remainder were damaged during the preparation procedure. The material comprised statoliths from 39 females and 36 males, with post-marking periods ranging from 1 to 21 days and 1 to 29 days respectively. The biological data, increment counts and quality factors recorded by reader 1 are given in Appendix II. Mean increment counts closely approximate the post-marking periods (Fig. 3.9).

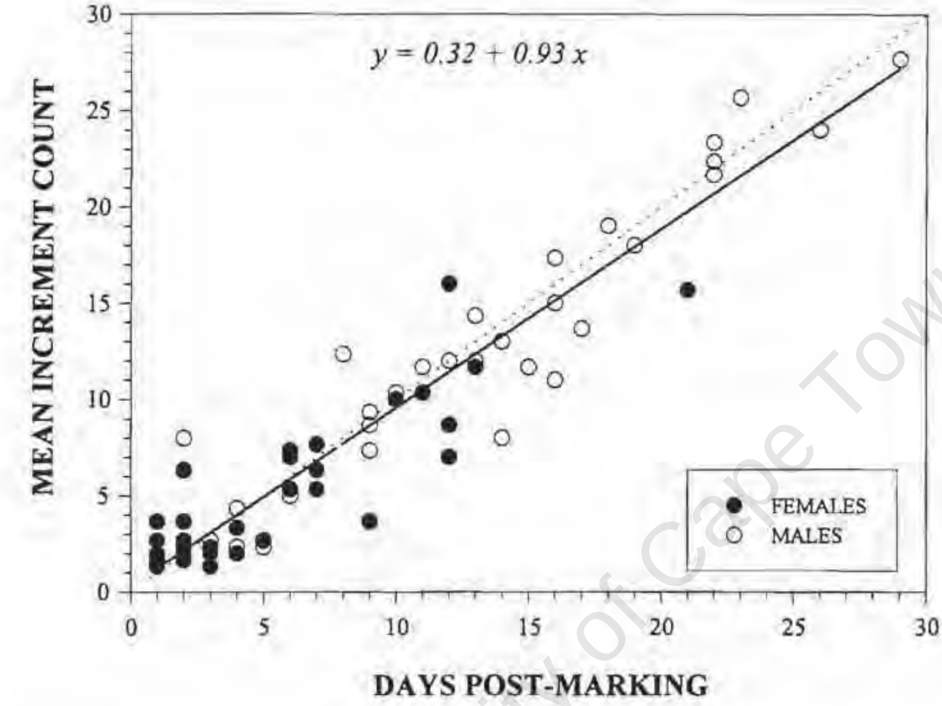


Figure 3.9: Relationship between mean counts of the number of post-mark increments and days post-marking, obtained by reader 1 from the laboratory validation study. The regression model fitted to the data is illustrated with a solid line (expression in italics, associated statistics given in Table 3.1). The 1:1 relationship between the variables (i.e. the daily increment hypothesis) is shown with a dotted line.

The regression model fitted to the data, however, does not support daily increment formation. The slope ($b = 0.9263$) is significantly different from one ($p = 0.0281$,Table 3.1). The tendency for over-estimation at low post-marking intervals and under-estimation at higher post-marking intervals that was observed in the field data was not apparent in the laboratory data set.

Of the 75 successfully prepared statoliths, 49 fulfilled the quality criteria. This reflects a success rate of 48%, which is considerably higher than the 10% success rate in the preparation of the field validation statoliths. Although a plot of the filtered data (Fig. 3.10) does not differ markedly from that of the unfiltered data shown in Fig. 3.9, these data, in contrast, do support daily increment formation. The regression model fitted to the filtered data has a slope of 0.9476, which is not significantly different from 1.0 ($p = 0.1897$, Table 3.1). The estimated power of the test to detect a deviation in slope of 0.1 from 1.0 (70%) was considerably higher than that estimated for the field validation data, primarily due to the larger sample size combined with lower variability in the data. The estimate of power indicates a 30%

probability of incurring Type II error. This value is arguably too high for any level of confidence to be expressed in the conclusion that the data support daily increment production.

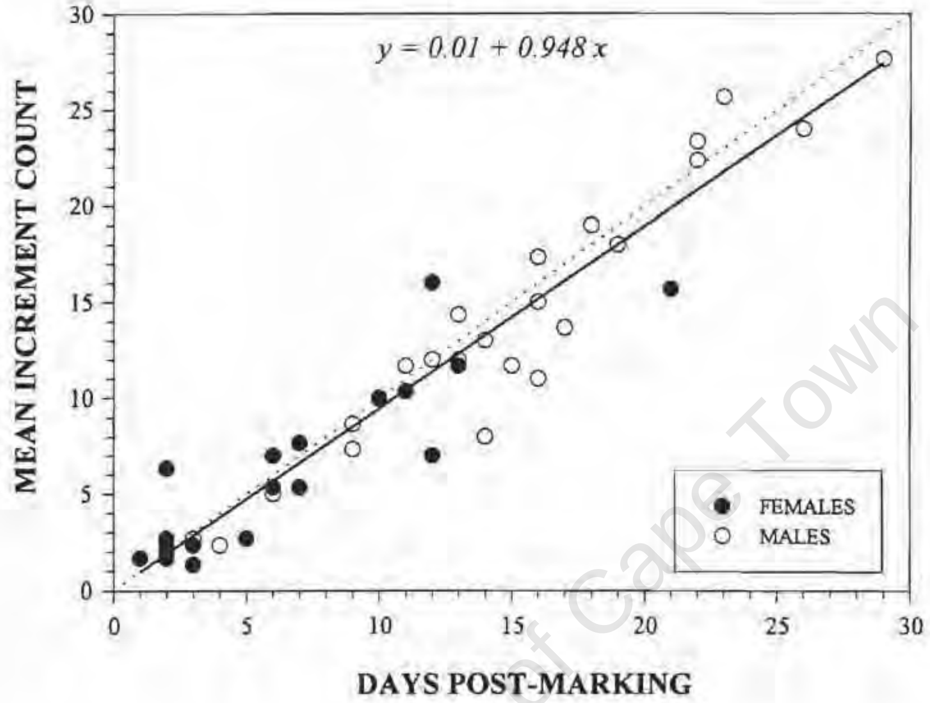


Figure 3.10: Relationship between mean post-mark increment counts and the number of days post-marking obtained by reader 1 from the laboratory validation statoliths that fulfilled the quality criteria (i.e. filtered data). The regression model fitted to the data is illustrated with a solid line (expression in italics, associated statistics in Table 3.1), whereas the daily increment hypothesis is shown with a dotted line.

When the filtered data were examined for each sex independently, the model fitted to the female data has a slope significantly different from unity ($p = 0.0249$, Table 3.2), suggesting that increment formation in female squid does not occur with a daily frequency. In contrast, the model fitted to the male squid does support daily increment formation ($p = 0.9086$), but the power associated with this test is relatively low (40%). The slope of the model fitted to the male data was compared to that fitted to the filtered data collected from male squid by reader 1 during the field study, using the procedure described by Zar (1984, pg. 292). The slopes were not significantly different ($t = 0.4248$, $p = 0.6741$), suggesting that male squid deposit increments with a daily frequency under both laboratory and field conditions. This conclusion cannot be applied to female squid because the slope of the model fitted to the female data obtained during the laboratory study was significantly different from unity (see above), and also significantly different from the slope of the model fitted to the male data ($t = -2.0933$, $p = 0.0419$).

Table 3.2: Results of regression analyses conducted on the data from female and male squid statoliths obtained from the laboratory study that filfilled the quality criteria (i.e. filtered data).

Data	Intercept		Slope		n	r^2	Ho: $b = 1$		
	a (± SE)	p	b (± SE)	p			t	p	Power
Females	0.8871 (± 0.6605)	0.1943	0.7956 (± 0.0839)	0.0000	22	0.8182	-2.4255	0.0249	-
Males	-0.7935 (± 0.8699)	0.3700	1.0064 (± 0.0552)	0.0000	28	0.9275	0.1159	0.9086	0.4049

3.3.4. Error Modelling

The 45 statoliths obtained from the field study that were used in this analysis generated 405 replications from the three readers. In 66 of the replicates, the differences between the number of increments counted and the observed number of days (i.e. the "error") was zero (Fig. 3.11).

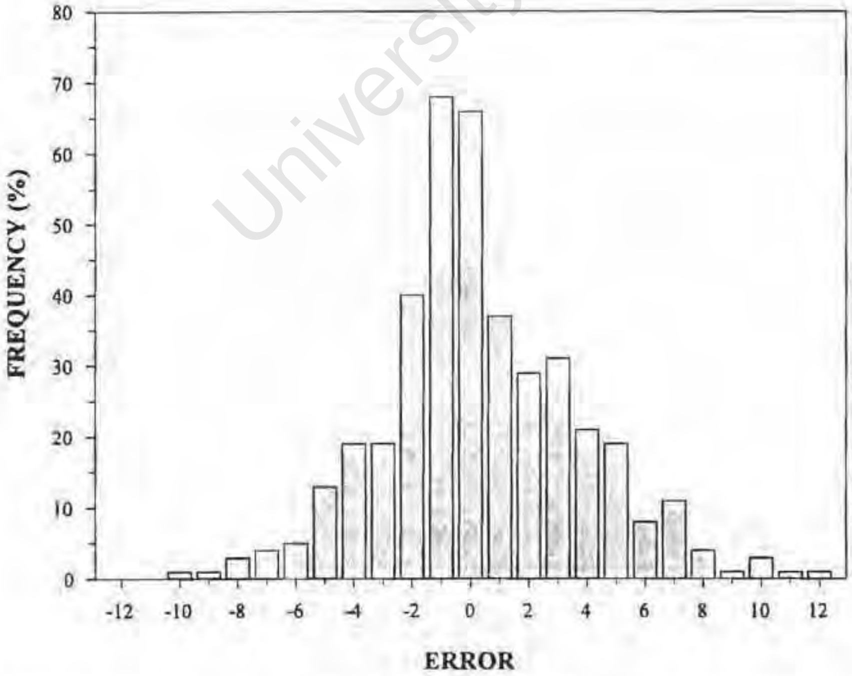


Figure 3.11: Histogram showing the distribution of errors (the difference between the number of counted increments and actual number of elapsed days) for all replications.

In 166 of the replications, the observed counts were larger than the number of days, and in the remaining 173 replications the observed counts were less than the true number of days. The mean of the differences was -0.3, very close to zero. The approximate symmetry of Fig. 3.11 justifies the decision to model the absolute values of the differences as a Poisson random variable. This symmetry was tested using the t-test; differences were not significant ($t_{404} = -1.14, p = 0.2550$).

There is, however, a complication in the data. Most of the counts obtained by readers 2 and 3 for squid recaptured five days after marking are over-estimates, whereas counts obtained from squid with longer post-marking intervals are mostly under-estimates (see Fig. 3.7). Consequently, Figure 3.11 is composed of two halves of possibly heterogeneous material, with explanatory variables for accounted error possibly differing from the analysis of all material, and from each other. These possibilities were taken into consideration during the modelling process, and the results are presented below.

In modelling the absolute values of the differences using the proposed suite of explanatory variables, the most biologically meaningful generalized linear model (GLM) included increment visibility (I) and margin quality (M), as well as indicator variables to show differences between the three observers, and the number of days after marking (Table 3.3).

Table 3.3: Coefficients of the generalized linear model (GLM) relating absolute errors between the counted number of increments and elapsed days. The GLM has a Poisson distribution; the logarithmic link function was fitted using Genstat 5.3 (Genstat 5 Committee, 1995). $F_{(5, 399)} = 17.62, p < 0.005$.

Explanatory variable	**Regression coefficient**	**Standard error**	*t*	*p*
Constant	1.281	0.237	5.4	0.000
I	-0.166	0.078	-2.1	0.036
M	-0.421	0.087	-4.9	0.000
Days	0.036	0.008	4.4	0.000
Reader 2	0.664	0.085	7.8	0.000
Reader 3	0.693	0.085	8.2	0.000

The GLM, through the logarithmic link function, predicted that the absolute error for given values of the explanatory variables for a given statolith and reader 1 is a Poisson variable with mean λ given by the expression

$$\log \lambda = 1.281 - 0.166\,(I) - 0.421\,(M) + 0.036\,(\text{Days})$$

or:

$$\lambda = e^{[1.281 - 0.166\,(I) - 0.421\,(M) + 0.036\,(\text{Days})]}$$

$$\lambda = (\,3.60\,)\,(\,0.089^{I}\,)\,(\,0.656^{M}\,)\,(\,1.037^{\text{Days}}\,)$$

In comparison with reader 1, readers 2 and 3 made, on average, errors that were about twice as large:

Reader 2: $e^{0.664} = 1.94$

Reader 3: $e^{0.693} = 2.00$

The positive regression coefficient for the actual number of days showed that the magnitude of the errors increased as the size of the potential error increased. In other words, the longer the post-mark duration, the greater the degree of error. The values for the explanatory variables "increment visibility" (I) and "margin quality" (M) lay in the range 1 (poor) to 3 (good). The negative signs for the regression coefficients of these two variables showed that as the quality of the margin and the increment visibility improved, the absolute differences between the observed count and the actual number of days decreased. Expressed quantitatively, the expected improvement between a poorly prepared statolith with I = 1 and M = 1, and a well prepared statolith with I = 3 and M = 3 can be calculated from the regression coefficients in Table 3.3 as the ratio

$$e^{[(-0.166)(1)-(0.421)(1)]} \div e^{[(-0.166)(3)-(0.421)(3)]} = 3.23$$

In other words, the model estimates that the absolute error in counting a poor statolith is more than three times the absolute error for a good statolith.

When the product I*M was included in the model, no improvement in the quality of the fit was achieved. This was also true when the third quality factor O (definition and clarity of the OTC mark) was included. This was probably due to the correlation between the variables I and O ($r = 0.45$; $p < 0.0001$). If I was replaced in the model of Table 3.3 by O, the coefficient for O (-0.06) had a negative sign, as expected, but the fit of the model was slightly poorer. The variable "margin quality" (M) seems to be the most significant explanatory variable for overall errors in counting increments (Table 3.3).

Results of the separate GLM's for under-estimated values and over-estimated values are presented in Tables 3.4 and 3.5 respectively.

Table 3.4: Coefficients of the generalized linear model (GLM) relating absolute errors between the counted number of increments and elapsed days, for which the counted number of increments was under-estimated. $F_{(4,234)} = 50.32$, $p < 0.005$.

Explanatory variable	Regression coefficient	Standard error	t	p
Constant	0.193	0.332	0.58	0.563
M	-0.580	0.142	-4.10	0.000
Days	0.126	0.012	10.50	0.000
Reader 2	0.400	0.116	3.50	0.001
Reader 3	0.271	0.122	2.20	0.029

The post-marking duration was the most significant variable explaining under-estimates, followed by margin quality (Table 3.4), whereas the interaction between margin quality and OTC mark clarity (i.e. O*M) seems to be the most significant explanatory variable for over-estimates (Table 3.5).

Table 3.5: Coefficients of the generalized linear model (GLM) relating absolute errors between the counted number of increments and elapsed days, for which the counted number of increments was over-estimated. $F_{(5, 219)} = 8.38, p < 0.005$.

Explanatory variable	Regression coefficient	Standard error	t	p
Constant	-2.890	1.140	-2.53	0.012
O	1.802	0.583	3.09	0.002
M	1.580	0.538	2.94	0.004
O*M	-0.933	0.274	-3.40	0.001
Reader 2	0.890	0.137	6.51	0.000
Reader 3	0.925	0.134	6.90	0.000

Good margin preparation is therefore one of the most important factors contributing to the "accuracy" of the increment counts. That the length of the counting axis (i.e. the post-marking period) has a significant influence on under-estimates is an important finding that has implications for increment counting in general. The older the squid, the more likely it is that total increment numbers will be under-estimated. Reasons for over-estimates are possibly more complex than those for under-estimates, and it is likely that they were not fully evaluated in this study.

3.4. DISCUSSION

The research described in this chapter represents the first successful attempt to conduct statolith marking experiments in field conditions, and compares the results of these experiments with those obtained from a laboratory experiment conducted on the same species. Experiments of this nature are usually conducted with the intention of testing the daily frequency of increment production. The results presented in this chapter provide limited support for this concept. Data obtained from statoliths that were felt to be of a satisfactory quality for increment counting conformed to the daily increment hypothesis in some cases (field data recorded by readers 1 and 2, laboratory data recorded by reader 1), but not in others (field data obtained by reader 3). However, in those cases where estimates of increment deposition rate were not significantly different from daily, the null hypothesis had to be rejected on the basis of unacceptably high probabilities of incurring Type II error. This result is a function of the variability inherent in the data, combined with the generally small sample sizes.

The variability in the data has a number of possible sources. Firstly, the assumption that the post-marking durations are true is not neccessarily met in the case of the field data. Although the date of marking of each animal was known, the squid were recaptured by commercial fishermen during periods of intense fishing, and recovered during later processing on shore. The possibility that recapture dates were incorrectly reported cannot therefore be discounted. The case of the two individuals recaptured near Port Alfred are an example of this source of error.

A major factor contributing to the variability in the increment counts is the quality of the statolith preparations. The practice of assessing specimen quality in terms of the criteria of increment visibility, OTC mark definition, and the state of the margin of the specimens, generated interesting insights into potential errors in the increment enumeration process. The GLM fitted to the "errors" in increment counts indicated that the error associated with a poorly prepared statolith could be as much as three times higher than that associated with a well-prepared specimen. The GLM modelling the "over-estimates" (which dominated in squid recaptured five days after marking) suggested that difficulties in increment recognition are linked with the quality of the margin of the specimens, as well as the definition of the OTC mark. This is most likely a combination of edge effects (Campana, 1992) and smearing of the OTC bands. The "under-estimates", which dominated in squid recaptured after longer periods, were also partially explained by margin quality, but primarily by the length of the counting axis (reflected in the variable "days"). This latter finding, namely that the longer the axis of enumeration, the higher the error, has serious implications for obtaining age estimates from total increment counts for stock assessment purposes. The older the animal, the more the increment count will under-estimate true age, introducing an age-related bias into the data set.

An additional factor that may have contributed to the variability in increment enumeration, but which was not included in the analysis, is the magnification at which increments are counted. A subset of nine statoliths (ranging from 5 to 20 days post-marking) were re-analysed by reader 1 using firstly a 40x objective and subsequently a 100x oil-immersion objective. The mean of the counts recorded using the 100x objective was significantly higher than that obtained using the 40x objective (*t*-test, $p = 0.0191$). In terms of accuracy, use of the 40x objective resulted in average under-estimate of about 1.5 days, in contrast to a mean under-estimate of only 0.3 days obtained using the 100x objective. Unfortunately, the magnification at which increments were counted was not recorded by the three readers during the field study, preventing the inclusion of this potential explanatory variable into the modelling of the counting errors. Attention should be directed to the magnitude of this potential source of bias in age estimation procedures, and a record maintained of the magnification being used during increment counting.

An additional factor contributing to the variability in the data is that of reader subjectivity. The results of the validation excersise depend on how statolith microstructure is interpreted. Campana (1992, pg. 65) observed that in any otolith preparation, only a fraction of the structures that are evident are daily growth increments, and further stated that "even practised workers can differ (sometimes substantially) in their interpretation of a given increment sequence. This element of subjectivity is one of the most significant sources of error in otolith microstructure examination...". Comparison of the results recorded by the three different readers from the field validation material demonstrates that this problem is not trivial. Estimates of increment deposition showed substantial differences, ranging from 0.20 d^{-1} (reader 3) to 1.18 d^{-1} (reader 2). These differences indicate that the three readers differed markedly in what they considered to be a daily increment, introducing a high degree of subjectivity into the increment enumeration process. In view of this subjectivity, it is not suprising that the tests of the daily increment hypothesis yielded inconsistent results, with the data of readers 1 and 2 supporting daily increment production, while those of reader 3 did not. Clearly, verification as outlined by Wilson *et al.* (1983) has not been achieved in the field study. The three readers often do not agree with each other as regards the number of post-mark increments in any given statolith, and occasionally do not agree with themselves. Further research directed at developing an objective increment recognition concept is required.

The element of subjectivity is also clearly apparent in the assessments of the quality of the statoliths obtained from the field study. The three readers agreed on only three out of the 45 statoliths as fulfilling the quality criteria. Readers 1 and 2 were more discriminatory in their interpretation of what constituted a "satisfactory" preparation, since the filtering of these data excluded more than 80% of the statolith preparations from further analysis, in contrast to the 50% in the case of reader 3.

These observations could be used to argue that increments were in fact deposited with a daily frequency during the experiments, and that the high probability of Type II error that was obtained from the analyses is a function of the small sample sizes. Good statolith preparations would generate accurate estimates of the numbers of post-mark increments. If increments were being deposited daily, accurate estimates of the number of post-mark increments obtained from good statolith preparations would approximate the number of days post-marking more closely than estimates obtained from poor statolith preparations. One could argue that reader 3, being less critical of statolith quality, included a higher proportion of erroneous counts in the filtered data set, hence the negative result (i.e. rejection of the daily increment hypothesis) obtained from these data. This argument could be taken further, in that the more stringent the filtering criteria, the more accurate the increment counts (i.e. the closer the counts approximate the true number of increments), and the closer these increment counts conform to the elapsed number of days. A point in support of this argument is that all three readers agreed on only three of the 45 statoliths (one of which was from the only female) as fulfilling the quality criteria in all cases. The counts recorded from these statoliths never deviated by more than two from the elapsed number of days in any of the nine replicates recorded by the three readers.

The success rates of the statolith preparations that were obtained during this study are major cause for concern. The approach adopted in the analyses described above was that only those statoliths that fulfilled a given set of criteria were considered to be successfully prepared, and hence would generate and accurate estimate of the number of increments. The number of successfully prepared statoliths was very low, particularly in the field material. This has serious implications for the application of the ageing technique to stock assessments. Considering the best case scenario of the laboratory material, less than half of the statoliths sampled will be prepared satisfactorily. In other words, the number of samples required by any given sampling protocol would have to be doubled to obtain the required number of age estimates with any level of confidence, as would the time and labour involved in preparing the samples for increment enumeration. Since an effective stock assessment procedure would more than likely involve several hundred samples, the low preparation success indicated by these results is highly unsatisfactory.

Further effort should be directed to improving statolith preparation techniques, both in terms of time and success rate.

The majority of validation experiments testing the daily increment ageing technique by means of direct methods are conducted in the laboratory, and the applicability of information collected from such experiments to wild populations has been questioned (Morris, 1988). If the reservations concerning laboratory studies are valid, the value of such research is considerably diminished. A comparison of the estimates of increment deposition rate obtained by reader 1 between the field and laboratory material suggested that these reservations are not valid. No significant differences could be detected between the slopes of the regression models fitted to the data from male squid, suggesting that male squid deposit increments at the same rate under both field and laboratory conditions. This conclusion does not apply to female squid. Data from only one female was obtained from the field study, precluding such a comparison. Further, the estimate of increment deposition rate obtained for female squid from the laboratory study was significantly different from daily (see Table 3.2), and also significantly different from that obtained for males. Further research should be aimed at testing whether this difference between the sexes is real or an artefact. It is possible that this difference is a function of interpretation. Female chokka squid tend to be smaller than males, and considering that statolith size scales with body size, the statoliths of a female squid will be smaller than those from a male of the same age. The implication of this argument is that the widths of increments in will differ between the sexes, perhaps explaining why increment counts recorded from female squid statoliths tended to under-estimate the post-marking periods.

A point that should be mentioned arises from the observation mentioned in section 3.1, namely that laboratory conditions have been known to influence the appearance of increments, and hence their interpretation during microstructural analysis. If one considers that the assessment of statolith "quality" would reflect any such potential differences in increment appearance, there is perhaps some basis for this argument in the results of this study. The higher success rate of the laboratory statolith preparations (48% of the total number of statoliths prepared were considered to be satisfactory, in comparison to the 10% of the field validation statoliths) could conceivably arise from such an occurrence. It should be emphasized, however, that the field validation statoliths were prepared before those obtained from the laboratory study, and the preparation technique was still being developed and perfected during this period. The higher preparation success rate of the laboratory material could arguably reflect improved techniques and experience, rather than a physiological effect (although the latter cannot be discounted).

Reference was made in the opening section of this chapter to the reservations of Gauldie (1994) concerning the use of validation to test age estimation hypotheses. Gauldie bases his arguments on the anti-verificationist philosophy advocated by Karl Popper (1983), which rejects the verificationist approach of looking for instances or observations that confirm a theory or hypothesis. These reservations arise from the philosophical inconsistencies in the use of the term "validation" that were discussed in the introduction of this chapter. The distinction between validation as a test of the daily increment hypothesis,as opposed to validation as a test of the accuracy and precision of an age estimation technique is usually disregarded. This distinction is crucial. If one adopts the suggestion of Francis (1995), validation is a two-stage process. The first stage is confirmation of the temporal significance of the features being used to estimate age, in other words, a rigorous test of the daily increment hypothesis. This can only be achieved at the physiological/molecular level, as was discussed in Chapter 1. The physiological processes responsible for the formation of increments have to be elucidated, followed by clear evidence (always subject to refutation) that these processes cycle with a daily frequency throughout the life-span of the animal. Once the periodicity and structural characteristics of daily increments have been demonstrated at the physiological level, the second stage of the validation process can then test the application of the hypothesis in terms of the accuracy and precision of the age estimates that are obtained (Gauldie, 1994).

Most validation studies such as those described in this chapter, are conducted with the intention of testing the daily increment hypothesis. In other words, they are aimed at the first stage of the validation process outlined above. The application of such studies to acheive this aim is, however, limited. Experiments of this nature cannot be considered to be a rigorous test of the daily increment hypothesis. Even if the problems associated with the approach (e.g. reader subjectivity, inaccurate estimates of total increment numbers) are resolved, any daily relationship between counts of increments and elapsed time that is detected can only be considered circumstantial. No conclusions concerning cause and effect can be drawn, and the possibility that the increments were coincidentally deposited at a rate of approximately one per day during the experiment cannot be discounted.

Studies such as those decribed here, should rather be aimed at the second stage of the validation process, namely the testing of the accuracy and precision of the age estimation technique, assuming the increments are being deposited daily. Several factors have to be taken into account when interpreting the results of such studies. Firstly, the results only apply to that part of the life span (or counting axis) covered by the experiment. Increment enumeration for age determination purposes has to cover the entire statolith axis from nucleus to margin. The level of accuracy that is realised by any given ageing technique therefore has to be calculated over the entire axis. It is unlikely that the error associated with the entire axis will be the same as that associated with only the outermost portion, and extrapolation of the degree of accuracy associated with increment counting in this part of the statolith to the rest of the statolith cannot be justified. This argument is strengthened by the results of the error modelling discussed above, where the number of days that had elapsed after marking showed a positive relationship with understimates (i.e. the longer the post-marking period, and hence the counting axis, the larger the degree of underestimation). Valid estimates of the levels of accuracy and precision associated with a given ageing technique can only be obtained from individuals where the true age is known, such as animals reared from hatching. Further, the estimates should cover the entire age range that is likely to be encountered during the stock assessment procedure.

Statolith marking experiments generate valuable material for training purposes. The study of statoliths where the post-mark duration is known could assist researchers in identifying daily increments, and lead to the development of an objective increment recognition concept. As Campana (1992, pg. 65) states, validation "is more important as a check on the interpretive skills of the worker, than as check on the true frequency of increment formation".

In conclusion, I feel that statoliths are a valuable tool for applied squid fisheries research. Increments appear to be deposited with a daily frequency, although further rigorously controlled experiments and conservative statistical analyses are required to conclusively test this hypothesis for all life stages of both male and female squid.

CHAPTER 4 - THE INFLUENCE OF TEMPERATURE ON THE MICROSTRUCTURE OF STATOLITHS OF *Lolliguncula brevis*

ABSTRACT

A study investigating the influence of temperature on the microstructure of statoliths of *Lolliguncula brevis* is described. Squid captured in Galveston Bay were transferred to the laboratory and subjected to various temperature regimes for extended periods. Two groups of squid were maintained at constant temperatures of 15 °C and 20 °C respectively for the duration of the experiment. Two other groups were initially maintained at these temperatures, and then switched between the two regimes midway through the experiment. Statoliths extracted from the squid were examined using a confocal microscope in laser scanning mode. The parts of the statoliths deposited during the course of the experiments were identified using either putative daily increment counts, or from the patterns of clear checks that were formed in reponse to capture and handling. Checks deposited in response to stressful events such as capture and handling are shown to consist of a series of prominent increments, rather than an interrupted period of statolith growth. Increments generally displayed reduced contrast and clarity during the experiment, presumably in response to the constant conditions imposed in the laboratory. Average statolith growth rates observed over the course of the experiment were strongly related to ambient temperature, increasing with increasing temperature. A significant sex effect was apparent, with statoliths of female squid generally growing faster than those of males at either temperature. Statolith growth rates at 15 °C were generally below 1 $\mu m.d^{-1}$, implying that the widths of daily increments produced under these conditions approach the resolution limits of a light microscope. The implications for studies using increment numbers to estimate age are discussed.

4.1. INTRODUCTION

A researcher, when confronted with the plethora of microstructural features apparent in squid statoliths and fish otoliths, must identify and interpret the various features in order to derive any biological meaning from the observed microstructure (Lagardère and Troadec, 1997). A substantial element of subjectivity is apparent in the interpretation process (Campana, 1992), leading to considerable variation in the results obtained by different researchers. The study of Campana and Moksness (1991) illustrates this point as regards fish otolith studies, while the results presented in Chapter 3 of this thesis demonstrate that this problem also applies to analyses of squid statoliths. Considering that the increment counts recorded during the study described in Chapter 3 only covered a fraction of the entire growth axis of the statolith, the potential for error in counts of the total number of increments within any given statolith is cause for concern. If age estimates derived from total increment counts are to be obtained with a satisfactory level of accuracy and precision, this problem must be resolved.

Three main factors concerning the interpretation of growth increments need to be addressed.

Firstly, the distinction between daily and sub-daily increments. Considerable attention has been directed towards this problem in fish otoliths (see Campana, 1992), but remains largely unexplored in squid statoliths. Secondly, the occurrence of prominent checks and discontinuities in statoliths needs to be investigated. The possibility that checks may represent an unknown period of interrupted increment formation complicates the accurate interpretation of a growth sequence (Campana and Neilson, 1985). Very little is known about the physiological processes of check formation. Knowledge of the factors that result in the formation of these features, and whether they do in fact represent periods of interrupted increment formation would contribute considerably to enhancing the value of statolith microstructural analysis. Finally, information concerning environmental influences on statolith microstructure, and increment formation in particular, is vital. How various environmental factors (both abiotic and biotic) impact on increment formation to the extent that their interpretation is compromised, needs to be elucidated.

Towards this latter objective, the study described in this chapter was aimed at investigating the influence of temperature on statolith growth and resultant microstructure. While factors such as feeding (e.g. Maillet and Checkley, 1989; Lagardère and Troadec, 1997) and activity-induced respiratory stress (Pauly, in press) have been implicated in modifying increment structure and appearance, temperature has been identified as probably the most significant environmental factor influencing otolith growth in fish (Gauldie and Nelson, 1990b). Considering that squid growth is highly temperature dependent (O'Dor and Wells, 1987; Forsythe and Hanlon, 1989; Forsythe, 1993), it is very likely that statolith growth will also be profoundly influenced by ambient temperature. This argument is strongly supported by seasonal differences in the statolith:body size relationship reported by Jackson (1995).

Although temperature does not appear to influence the periodicity with which increments are deposited, it has been described as a strong masking agent (Campana and Neilson, 1985) capable of affecting increment structure, probably acting at the level of metabolic rate. Further, temperature fluctuations have the greatest potential for the introduction of sub-daily increments into the growth sequence (Campana and Neilson, 1985).

The approach used during the study was to subject adult thumbstall squid (*Lolliguncula brevis*) to various temperature regimes for extended periods, and then to examine the microstructure of the regions of the statolith that had been deposited during the experimental period. The data obtained from the analysis were then used to test the hypothesis that temperature has a direct influence on statolith growth rate, and hence increment width and appearance. A crucial requirement of this approach is that squid be maintained in the laboratory for as long as possible. *Lolliguncula brevis* was used because this species is readily obtainable in Galveston Bay (Texas, USA), and can be maintained in relatively large numbers in the laboratory for extended time periods. The experiments were conducted in the laboratories of the National Resource Center for Cephalopods (NRCC), a division of the Marine Biomedical Institute of the University of Texas Medical Branch in Galveston, USA.

4.2. METHODS

Live squid were collected in Galveston Bay by brief bottom trawls. Animals that were in a satisfactory condition were transferred to a holding tank on board the vessel, which was supplied with a continuous flow of air and ambient sea water. On returning to the laboratory, the entire holding tank was transferred from the vessel to the laboratory, and supplied with a continuous flow of air and recirculated filtered sea water. The squid were maintained in the holding tank for a minimum of 24 hours, after which all dead and moribund animals were removed. In some instances, squid were maintained in the holding tanks for periods of up to 12 days, to ensure that sufficient numbers of animals were available for the experiment. Water temperature in the holding tank was not monitored during this period. Surviving squid that were in a good condition were transferred to the experimental tanks.

In order to ensure minimal disruption to statolith deposition during the experiment, the statoliths were not marked with oxytetracycline (OTC) at the beginning of the experiment. The results of the study described in Chapter 3 indicated that administering OTC does influence statolith microstructure to some extent. Further, this practice may contribute to increased mortality rates. It was felt that increment counts, combined with stress-induced checks resulting from capture and transfer between tanks would generate adequate reference marks bracketing the section of the statoliths deposited during the experiment. The use of stress-induced checks as benchmarks during otolith growth has been discussed by Campana and Neilson (1985). Capture and handling of squid involve high levels of physiological stress, often resulting in mortalities. Stress at this level has a profound influence on the metabolism of the animal, and is very likely to be reflected in statolith microstructure as a prominent check, which can then be used as a reference point in the progression of statolith growth. Several of the specimens obtained during the validation studies described in Chapter 3 displayed prominent checks corresponding to the time of marking of the animal (the clear flourescent band produced by incorporation of OTC into the statolith confirmed this observation), supporting the use of this approach.

During the course of the experiment, squid were exposed to a natural ambient photoperiod, and were fed *ad libitum* with live penaeid prawns and fish. The tanks were cleaned once a day, and any dead squid removed.

The experimental system consisted of four 400 litre experimental tanks supplied with a continuous flow of air and recirculated, filtered sea water. The tanks were divided into two separate systems, each system including a separate conditioning tank in which the water temperature was controlled by thermostatically regulated heating and cooling elements (Fig. 4.1). Two of the tanks (experimental tanks A and B) were supplied with water maintained at 15 °C, while experimental tanks C and D were supplied with water from a second conditioning tank, which was maintained at 20 °C. About halfway through the experiment, the squid in tanks B (15 °C) and D (20 °C) were exchanged. The experiment consequently involved four groups of squid. Groups 1 and 3 were respectively maintained at 15 °C (tank A) and 20 °C (tank C) for the entire experiment. Group 2 was maintained at 15 °C (tank B), and then transferred to

20 °C (tank D) after a period of 43 days. Group 4 was maintained at 20 °C (tank D) and then transferred to 15 °C (tank B) after 38 days. Squid in tanks B and D that died before the transfer dates were assigned to groups 1 and 2 respectively.

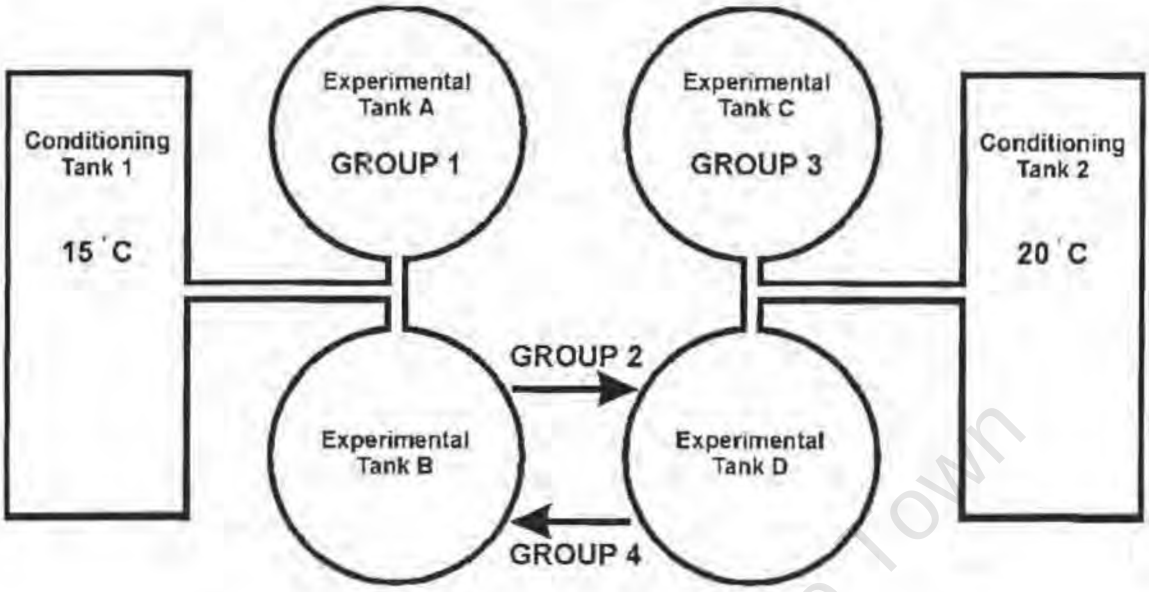


Figure 4.1: Diagram illustrating the experimental system (see text).

The temperatures that were imposed during the experiment were selected on the basis of the temperature tolerance of *Lolliguncula brevis*, that ranges between about 15 °C and 27 °C. The lower temperature treatment therefore corresponds to the lowest temperatures that the species is likely to encounter in the wild. Temperatures approaching the upper limit of the tolerance range are likely to result in elevated mortality rates due to physiological stress. Because it was important that the squid survive for as long as possible, the mid-point of the tempertature tolerance range was selected as the higher temperature treatment in this study.

Squid that died during the experiment, or were sampled at the end of the experiment, were weighed (body wet weight), measured (dorsal mantle length), the sex and stage of maturity assessed according to the scale of Lipinski and Underhill (1995), and the statoliths extracted. Statoliths were washed in fresh distilled water, air dried and stored frozen.

Before being prepared for microscopic examination, the total statolith length (TSL) and the lateral dome width (FLD) were measured for each statolith (see Fig. 4.2), using an eye-piece micrometer mounted in a stereo microscope.

After measurement, the statoliths were embedded in clear orthodontic resin, and sectioned in the oblique frontal plane using the procedure described in Chapter 2. Completed sections were viewed using a Zeiss LSM 410 confocal microscope, operated in laser scanning mode rather than in confocal mode. The microscope incorporated a high resolution video camera interfaced with a computer. Images in the field of view could be manipulated with image analysis software to enhance features of interest, and then saved to disk in Tagged Image File Format (TIFF) for later analysis.

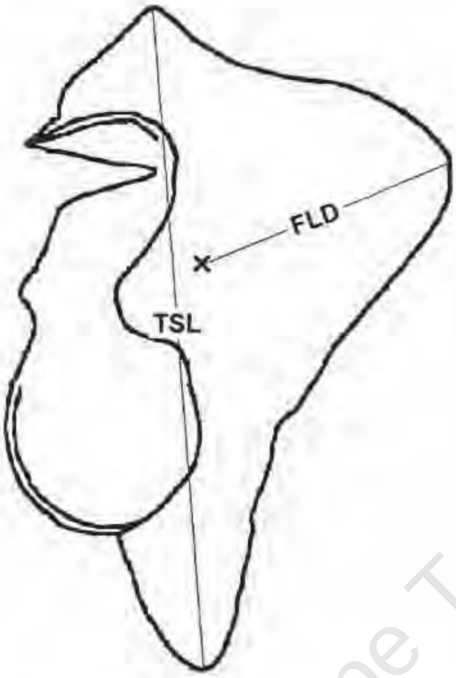


Figure 4.2: Diagram illustrating the morphological measurements recorded on the intact statoliths obtained from the controlled temperature experiment. X - position of the focus

Statolith growth over the experimental period was inferred from either daily increment counts (which have been validated for this species by Jackson *et al.*, 1997), or other microstructural features, specifically checks. Statolith growth that occurred during the experimental treatments was measured along the longest axis extending from the focus to the lateral tip of the lateral dome in each section. The distance between the point at which the treatment began and the margin was divided by the duration of the experimental treatment to generate an average daily statolith growth rate integrated over the duration of the treatment. Because statolith growth varies in different regions of the statolith, and the sections differed between specimens in terms of their plane through the lateral dome, these measurements are not directly comparable between specimens. In order to allow such comparisons, the measurements of statolith growth recorded from each specimen were standardized relative to the width of the lateral dome (FLD in Fig. 4.2) recorded in the intact statolith, assuming proportionality.

Estimates of statolith growth corresponding to the various treatments were compared using a simple one-way ANOVA. Because temperature may not be the only variable influencing statolith growth, the influence of a number of other potential modifiers of statolith growth rate was assessed using a multiple regression analysis. Before being included in the analysis, each explanatory variable was regressed against the dependent variable, and the standardized residuals examined for departures from normality and homoscedasticity. Where appropriate, the variables were logarithmically transformed. The multiple regression analysis was conducted following the "step-down" approach described by Zar (1984, p. 342).

4.3 RESULTS

Statoliths from 54 squid were obtained from the experiment. To ensure that the results were representative of the imposed temperature regime, only statoliths from individuals that survived for periods exceeding 30 days were analyzed. The information associated with the specimens that were successfully prepared for microscopic examination is summarised in Table 4.1.

Table 4.1: Table summarising the material obtained from the controlled temperature experiments. The dorsal mantle length (DML), body wet weight (Wt) and sex of each squid are given, as are the dates of capture (C), introduction (I), transfer (T) and sampling (S). The periods for which each squid was maintained at the given temperatures (T °C) are indicated in the treatment columns.

No	DML (mm)	Wt (g)	Sex	Date C	Date I	Date T	Date S	Treatment 1 Days	Treatment 1 T °C	Treatment 2 Days	Treatment 2 T °C
Group 1											
33	56	12.69	F	18/10/95	30/10/95	-	14/12/95	45	15	-	-
50	69	23.13	F	18/10/95	30/10/95	-	6/01/96	68	15	-	-
51	75	30.81	F	18/10/95	30/10/95	-	7/01/96	69	15	-	-
53	58	14.76	F	18/10/95	30/10/95	-	9/01/96	71	15	-	-
55	42	6.09	M	18/10/95	30/10/95	-	9/01/96	71	15	-	-
56	48	8.10	F	18/10/95	30/10/95	-	9/01/96	71	15	-	-
Group 2											
41	72	28.82	F	18/10/95	30/10/95	12/12/95	20/12/95	43	15	8	20
45	79	27.70	F	18/10/95	30/10/95	12/12/95	26/12/95	43	15	14	20
46	71	24.70	F	18/10/95	30/10/95	12/12/95	28/12/95	43	15	16	20
48	67	20.11	F	18/10/95	30/10/95	12/12/95	01/01/96	43	15	20	20
57	65	19.41	M	18/10/95	30/10/95	12/12/95	09/01/96	43	15	28	20
Group 3											
26	59	12.14	F	08/11/95	09/11/95	-	09/12/95	30	20	-	-
31	52	8.74	F	08/11/95	09/11/95	-	11/12/95	32	20	-	-
32	69	22.50	M	01/11/95	04/11/95	-	13/12/95	39	20	-	-
34	65	24.43	F	01/11/95	04/11/95	-	15/12/95	41	20	-	-
38	71	25.78	F	01/11/95	04/11/95	-	18/12/95	44	20	-	-
43	77	33.15	F	01/11/95	04/11/95	-	22/12/95	48	20	-	-
Group 4											
36	70	29.19	F	1/11/95	4/11/95	12/12/95	16/12/95	38	20	4	15
39	78	33.77	F	1/11/95	4/11/95	12/12/95	19/12/95	38	20	7	15
52	66	20.64	M	1/11/95	4/11/95	12/12/95	09/01/96	38	20	28	15

Statoliths of *Lolliguncula brevis* do not display the sequence of clear increments extending along the anterior edge of the medial inclusion of wing crystals that are apparent in statoliths of *Loligo vulgaris reynaudii* (see Chapter 3). Examination was consequently directed to the lateral dome. With the exception of three specimens, marginal increments were very faint, or not visible. In these specimens, the region of the statolith corresponding to the experimental treatments could not be identified from putative daily increment counts. As an alternative, examination was directed to the checks that were apparent in all the specimens (see Fig. 4.3).

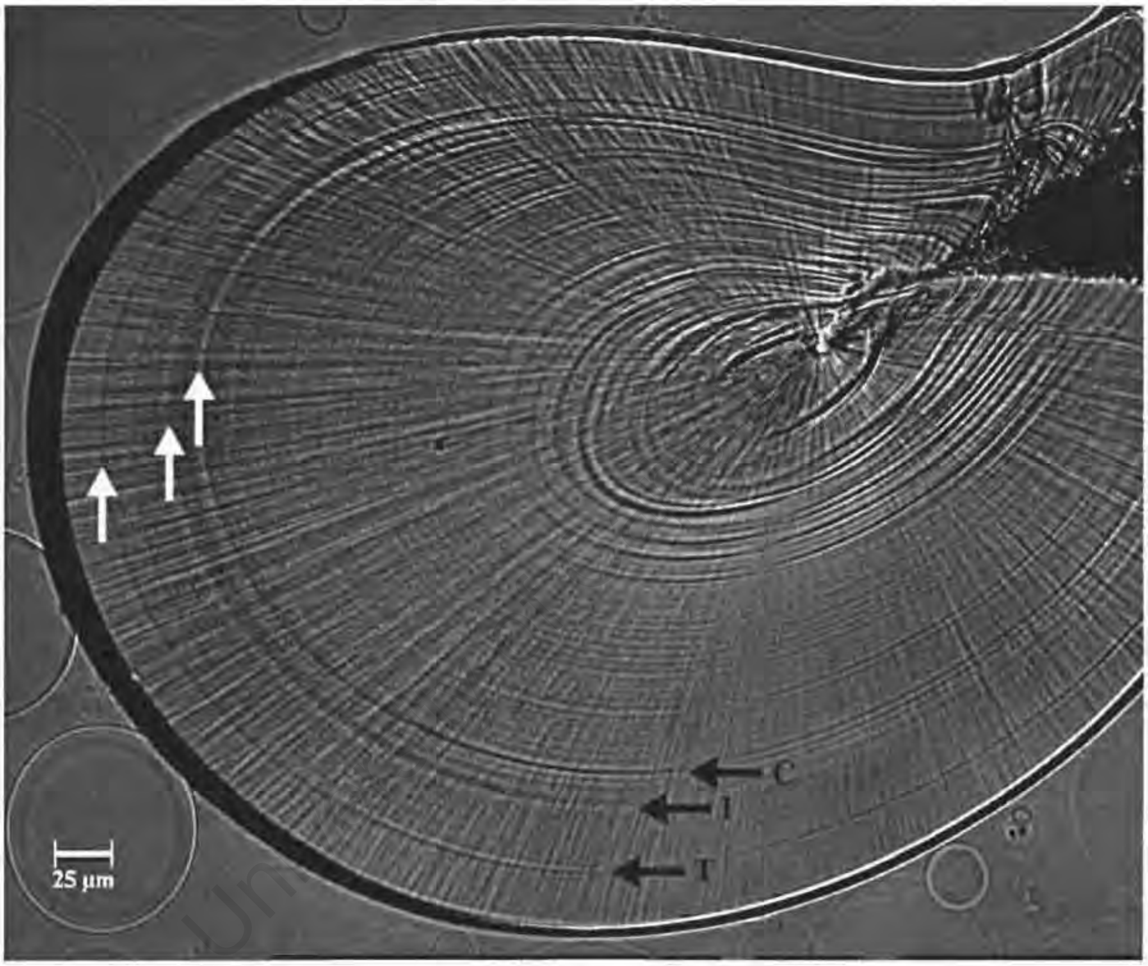


Figure 4.3: Digitized light micrograph of a frontal section of a statolith from Lolliguncula brevis (specimen no. 57 in Table 4.1) illustrating the series of checks considered to correspond to capture (C), introduction (I) and transfer (T). All checks are very clear in the posterior regions of the lateral dome (black arrows), but the introduction and transfer checks gradually fade in the lateral regions of the lateral dome, while the capture check retains its prominence (white arrows).

Considering the high levels of stress associated with capture, and, to a lesser extent, introduction to and transfer between experimental tanks, it was considered very likely that the checks closest to the margin would correspond to these events. Although the level of stress experienced was not quantified, one would expect that capture would involve the highest levels of stress, followed by introduction to and transfer between holding tanks. If this argument is valid, checks associated with capture should be more prominent than those associated with introduction and transfer. In many of the specimens, the checks closest to the margin appeared to support this argument. Fig. 4.3 illustrates a specimen introduced into a 15 °C experimental tank 12 days after capture, then transferred to a 20 °C tank 43 days later, where it survived for a further 28 days before sampling (specimen no. 57 in Table 4.1). This sequence of events is reflected in the series of checks evident in the statolith, particularly in the posterior regions of the lateral dome (see Chapter 2 for a description of statolith orientation).

In most of the specimens, the posterior regions of the lateral dome were found to be most suitable for the interpretation of check series. The microstructural features in the lateral regions were often diffuse and faint, while the anterior regions, due to the short axis of growth, always displayed a "merging" effect, which complicated the interpretation of microstructural features (in fish otoliths, this phenomenon has been referred to as "staggered" growth). The check interpreted to correspond to capture is a very prominent feature, which maintains its structural clarity throughout the section (Fig. 4.3). The checks presumably associated with introduction and transfer, although of similar prominence in the posterior regions, display a gradual decrease in definition and clarity towards the lateral regions of the lateral dome.

In many of the specimens, microstructural changes distal to the checks that were considered to reflect capture, introduction and transfer events, appeared to support these interpretations. The regions distal to the proposed introduction check generally displayed a more homogeneous appearance in comparison to the regions between the focus and the introduction check. Increments were very faint, and the complex series of "light" and "dark" zones, checks and increment patterns evident in the "wild" regions of the statolith were either absent, or dampened. The transition between these scenarios was very abrupt, and demarcated by the proposed capture and/or introduction checks. This was particularly evident in the statoliths exposed to the 15 °C treatments (examples are illustrated in Fig. 4.4). These observations were interpreted as manifesting the transition from natural, and probably highly variable environmental conditions, to the constant temperature conditions imposed in the laboratory. Constant temperature conditions in the laboratory have been shown to reduce the contrast between the incremental and discontinuous zones of increments in fish otoliths (Brothers, 1981; Campana, 1984b; Neilson and Geen, 1985).

In the three specimens where marginal increment counts could be obtained, the numbers of increments counted between the checks closely matched the number of days between events. Fig. 4.5 illustrates a specimen from a squid (no. 39 in Table 4.1) that was introduced into a 20 °C tank three days after capture, and transferred to a 15 °C tank after 38 days, where it survived for a further 7 days. The capture and introduction checks are clearly apparent in the micrograph of the whole section (Fig. 4.5 A), while the transfer check is very faint and poorly-defined. Closer examination of the anterior and lateral regions of the lateral dome (Fig. 4.5 B) reveals a series of relatively clear increments between the checks. The very prominent capture and introduction checks are separated by two increments (corresponding to the period between capture and introduction). 35 increments were counted between the introduction and transfer checks (an underestimate of 3 days), and a further 7 increments are apparent between the transfer check and the margin, matching the period between transfer to the 15 °C tank and the death of the squid. In the other two specimens where marginal increments were clearly visible, increment counts never deviated from the number of days between events by more than one.

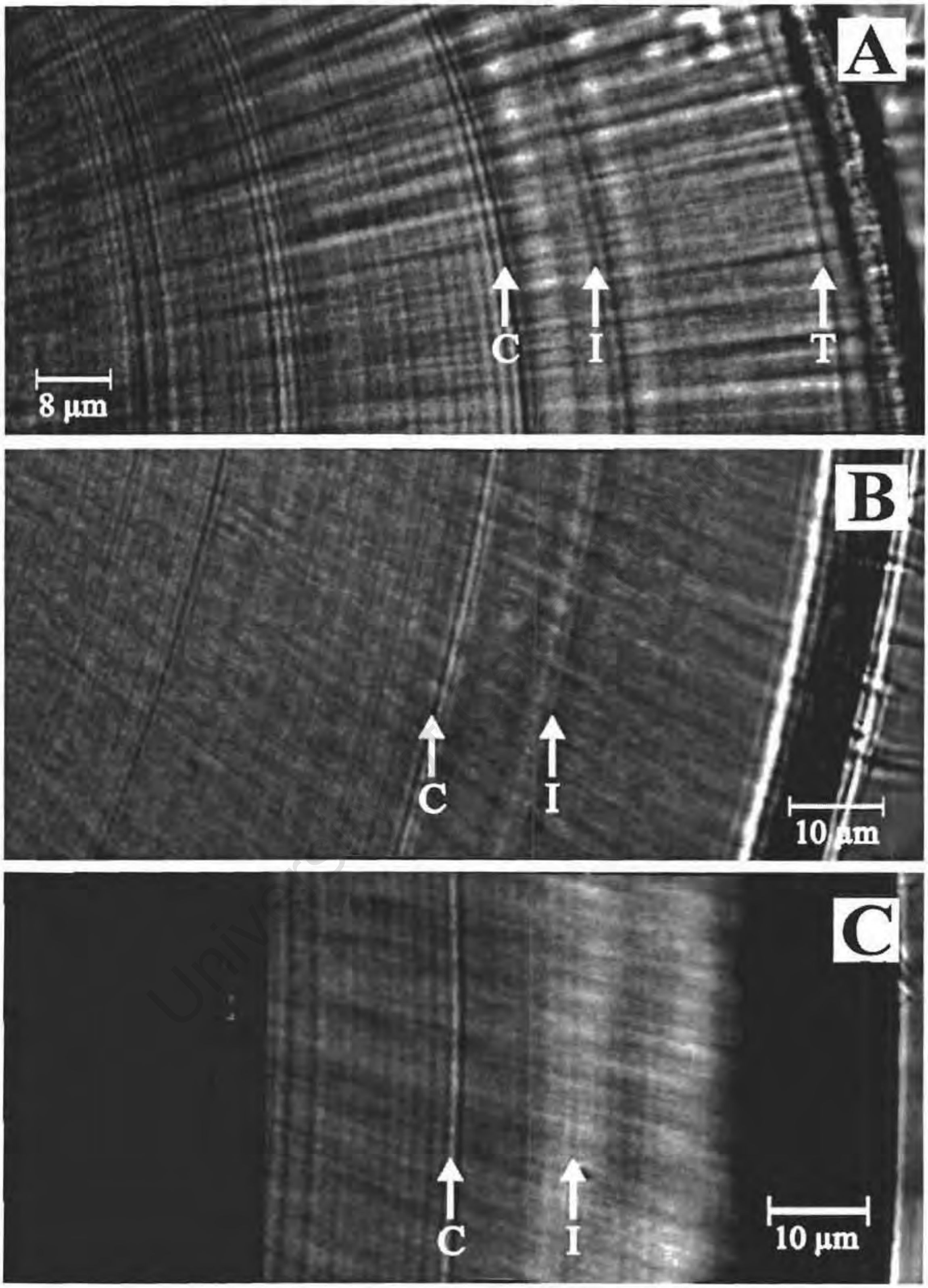


Figure 4.4: Light micrographs illustrating "fading" increments in the regions of the statolith corresponding to the experimental treatments. A - specimen no. 41. B - specimen no. 50. C - specimen no. 51 (see Table 4.1). Checks corresponding to capture (C), introduction (I) and transfer (T) are indicated with arrows.

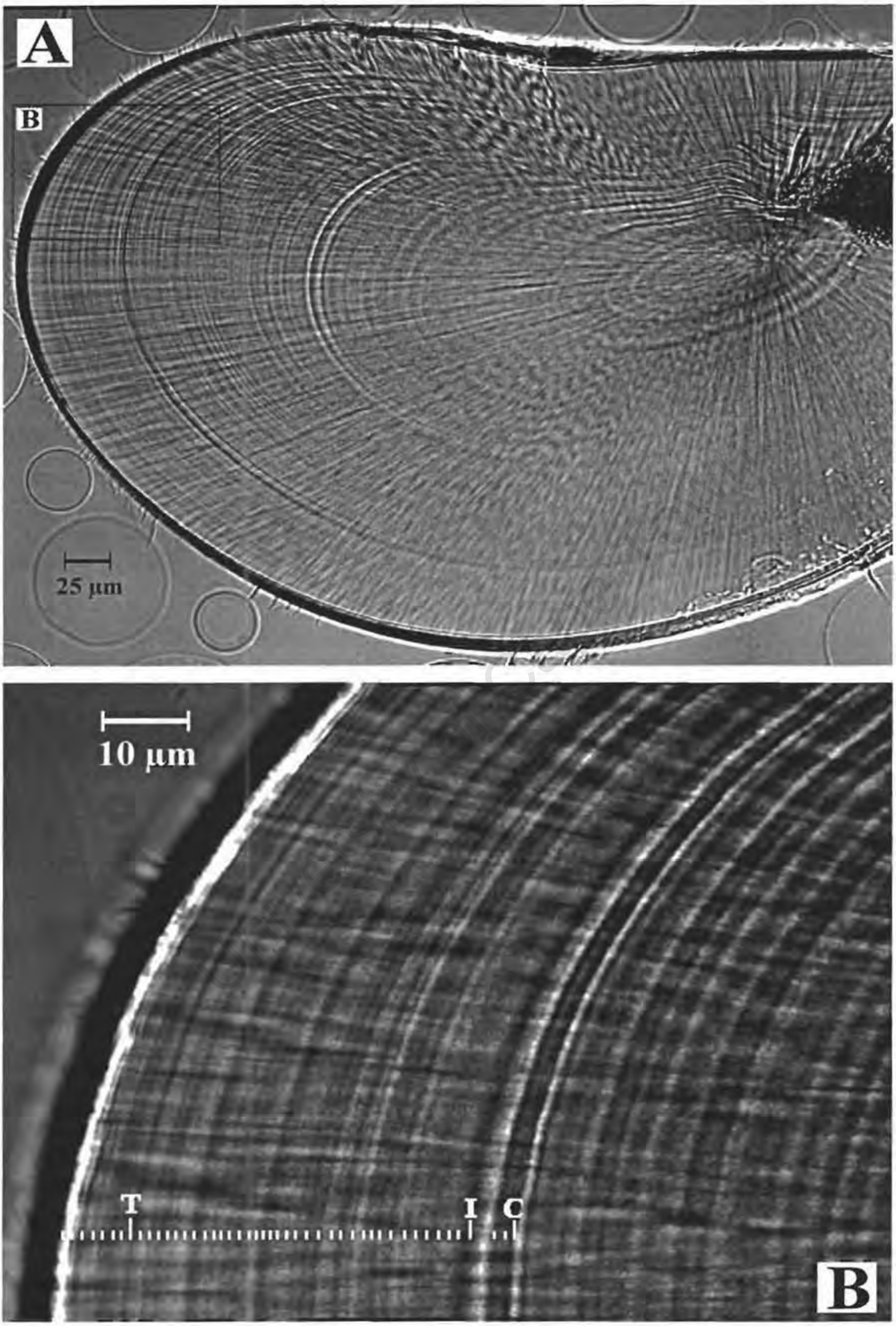


Figure 4.5: Light micrographs illustrating a statolith (specimen no. 39 in Table 4.1) where increments are visible in the regions corresponding to the experimental treatments. A - whole specimen. B - magnified view of inset in A. Checks corresponding to capture (C), introduction (I) and transfer (T) are indicated. Increments are indicated with tick marks.

In view of these results, it was felt that use of the checks described above as reference points corresponding to capture, introduction and transfer was justified. These features were consequently used to estimate statolith growth rates over the duration of the various experimental treatments. Average statolith growth rates computed from the specimens exposed to the constant temperature treatments are plotted in Fig. 4.6 (the values are given in Table 4.2).

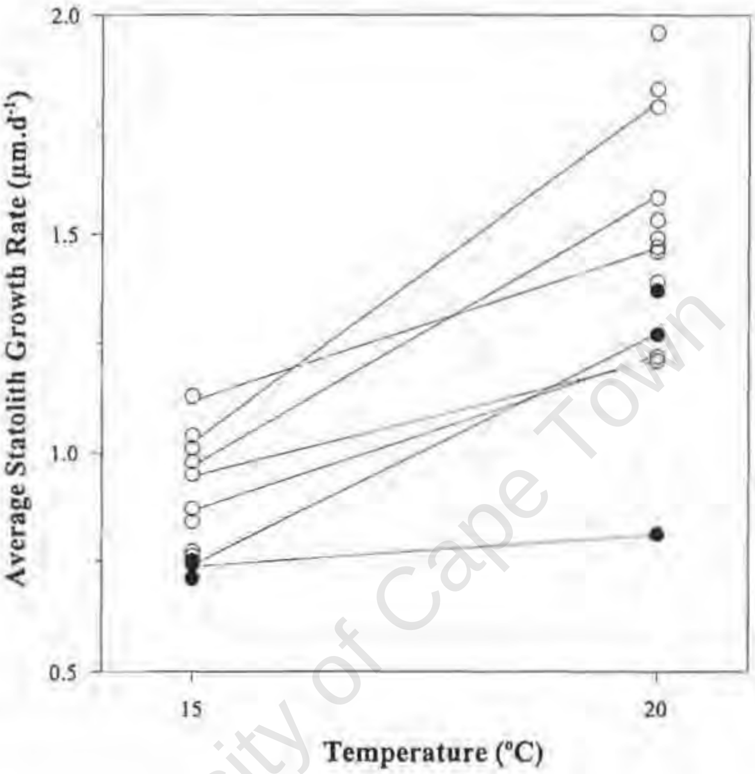


Figure 4.6: Average statolith growth rates estimated from statoliths of squid subjected to 15 °C and 20 °C treatments. Circles = females; dots = males. The solid lines join estimates that were obtained from the same statolith (i.e. those individuals that were subjected to both treatments consecutively).

Note in Table 4.2. that specimen no. 36 does not have an estimate of statolith growth corresponding to the 15 °C treatment, and that the growth estimate corresponding to the 20 °C treatment is integrated over a 36 day period, rather than the full 38 days of the treatment. Marginal artefacts obscured the outermost region of the axis, including the entire 15 °C treatment area. Statolith growth was consequently integrated over the 36 increments that were countable after the introduction check (assuming the increments were daily). Also note that estimates of statolith growth rates were obtained from both statoliths of individuals 43 and 57. The estimates were very similar between the statoliths from each individual, differing by a maximum of 0.17 $\mu m.d^{-1}$. Because the estimates came from the same individuals, and could therefore not be considered independent, only the values obtained from the left statoliths of these individuals were used in subsequent analyses.

Table 4.2: Table showing estimates of statolith growth obtained from squid subjected to the various temperature treatments. Observed (Obs., μm.d^{-1}) and standardized (Stan., μm.d^{-1}) statolith growth estimates associated with each treatment are provided for the relevant statolith (L = left, R = right) from each squid.

No.	ML	Wt	Sex	Stat.	Treatment 1				Treatment 2			
					Days	T°C	Obs.	Stan.	Days	T°C	Obs.	Stan.
Group 1												
33	56	12.69	F	R	45	15	0.61	0.75	-	-	-	-
50	69	23.13	F	R	68	15	0.61	0.84	-	-	-	-
51	75	30.81	F	R	69	15	0.64	0.76	-	-	-	-
53	58	14.76	F	R	71	15	0.60	0.77	-	-	-	-
55	42	6.09	M	R	71	15	0.61	0.71	-	-	-	-
56	48	8.10	F	R	71	15	0.87	1.01	-	-	-	-
Group 2												
41	72	28.82	F	R	43	15	0.90	1.04	8	20	1.54	1.79
45	79	27.70	F	R	43	15	0.88	1.13	14	20	1.14	1.47
46	71	24.70	F	L	43	15	0.84	0.87	16	20	1.18	1.22
48	67	20.11	F	L	43	15	0.81	0.98	20	20	1.31	1.58
57	65	19.41	M	R	43	15	0.66	0.77	28	20	0.94	1.10
57	65	19.41	M	L	43	15	0.65	0.75	28	20	1.09	1.27
Group 3												
26	59	12.14	F	R	30	20	1.37	1.49	-	-	-	-
31	52	8.74	F	R	32	20	1.35	1.53	-	-	-	-
32	69	22.50	M	L	39	20	1.10	1.37	-	-	-	-
34	65	24.43	F	L	41	20	1.56	1.96	-	-	-	-
38	71	25.78	F	R	44	20	1.19	1.39	-	-	-	-
43	77	33.15	F	R	48	20	1.49	1.74	-	-	-	-
43	77	33.15	F	L	48	20	1.49	1.83	-	-	-	-
Group 4												
36	70	29.19	F	R	36	20	1.30	1.46	4	15	-	-
39	78	33.77	F	L	38	20	1.06	1.21	7	15	0.83	0.95
52	66	20.64	M	L	38	20	0.75	0.81	28	15	0.68	0.74

A clear difference in average statolith growth rates is apparent between the temperature treatments, with statoliths growing considerably faster at 20°C than at 15°C. A sex effect is also apparent: statoliths of female squid generally grew faster than those of males within each temperature treatment. A 2-way ANOVA indicated that both sex and temperature effects on average statolith growth rates were significant, whereas their interaction was not:

Sex:	$F_{1,23} = 9.4589$	$p = 0.0053$
Temperature:	$F_{1,23} = 32.3179$	$p = 0.0000$
Sex and temperature:	$F_{1,23} = 1.3335$	$p = 0.2600$

Although the influence of temperature on average statolith growth rates is highly significant, the possibility that the results are a function of the size / age of the animal was also investigated. The relationship between statolith size and squid body size is generally best described by a power curve (Fig. 4.7), implying that as the animal gets larger and older, statolith growth rate decreases.

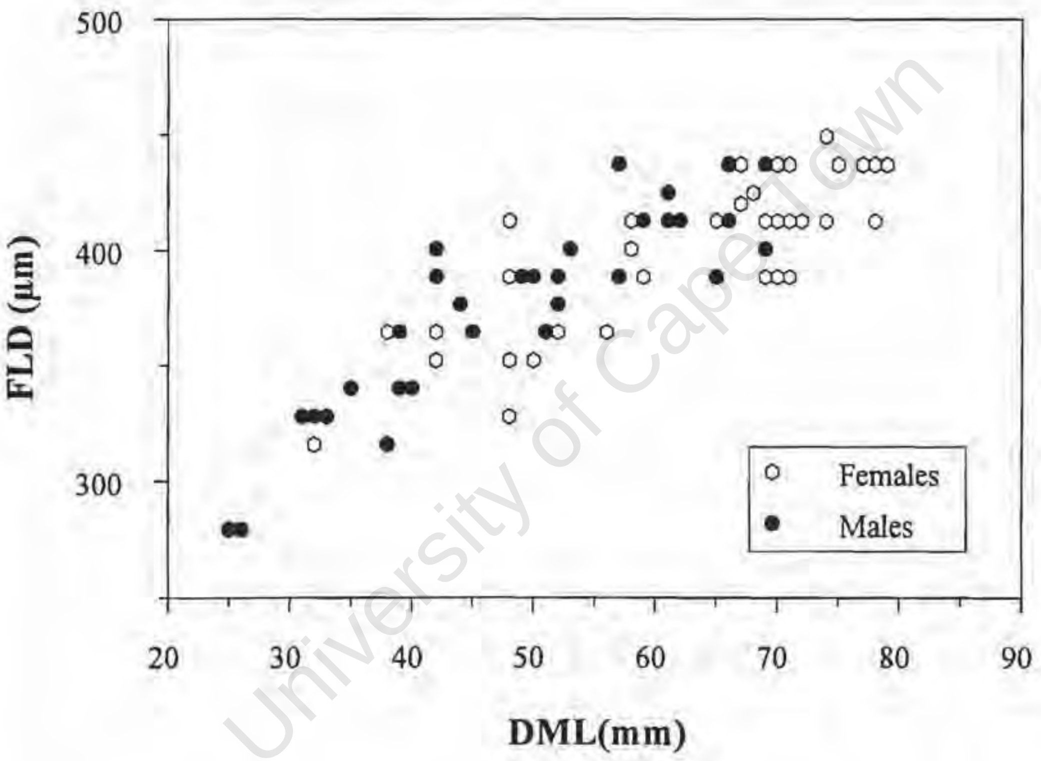


Figure 4.7: The relationship between squid dorsal mantle length (DML) and the width of the statolith lateral dome (FLD in Fig. 4.2).

A multiple regression analysis was used to assess this possibility. Because no age estimates were obtained for the squid, and no measurements of body size were recorded at the beginning of each treatment, an indirect index of the size / age factor was required. The point in statolith growth at which each treatment began was selected as the most viable option. The distance along the longest axis extending from the focus to the margin of the lateral dome at which the introduction and transfer checks were situated was measured. These values were standardized relative to the lateral dome width of the intact statolith (FLD in Fig. 4.2), assuming proportionality. The other explanatory variables included in the analysis were the sex of the individual, and the temperature of the treatment corresponding to the estimate of statolith growth. The dependent variable (average statolith growth rate) was logarithmically transformed to fulfill the requirements of normality and homoscedasticity. The results of the analysis are given in Table 4.3.

Table 4.3: Results of the multiple regression analysis conducted on data obtained from the controlled temperature experiments. Note that the dependent variable included in the analysis was the natural logarithm of the average statolith growth rate estimated in each case. n = 27, $r^2 = 0.7718$, $F_{2,24} = 40.5910$, $p = 0.0000$.

Explanatory Variable	Regression Coefficient	Standard Error	t_{24}	p
Intercept	24.0870	7.3539	3.2754	0.0032
Sex	- 0.2569	0.0733	- 3.5040	0.0018
Temperature	0.1004	0.0122	8.2298	0.0000

The results of the analysis support the conclusion drawn above, namely that temperature, followed by sex, is the most significant variable influencing average statolith growth rates. The positive coefficient for temperature indicates that statolith growth rate increases as temperature increases. The distance along the axis of statolith growth at which the treatments were conducted had no significant influence on the statolith growth rate estimates. This latter result suggests that the influence of temperature over-rides any age or size dependence of statolith growth.

4.4. DISCUSSION

The results of this study demonstrate that temperature has a profound impact on statolith growth and microstructure, and consequently on the interpretation of putative daily increments. The effect of temperature is most likely at the level of metabolic rate, given the strong temperature dependence of poikilothermic organisms such as squid and fish. Two aspects of ambient temperature are of relevance in terms of resultant statolith microstructure.

Firstly, whether the ambient temperature is constant or fluctuating. Metabolic rate controls the differential deposition rates of calcium and protein, resulting in the bipartite nature of a daily increment (Campana and Neilson, 1985). Constant temperatures, with a concomitant dampening effect on metabolic rate, would therefore be expected to dampen the cycles of calcium and protein deposition, reducing the contrast between adjacent incremental and discontinuous zones of increments, and hence their visual clarity. This is precisely what was observed in the specimens in this study. The parts of the statolith that were deposited during the constant temperature treatments showed a substantial reduction in microstructural detail when compared to the regions corresponding to wild conditions, and displayed a blurred appearance, with no clear microstructural features. In most of the specimens, increments in these regions were either faint, or not visible at all. Similar observations have been reported studies of both fish otoliths (Brothers, 1981; Campana, 1984b; Neilson and Geen, 1985; Alhossaini and Pitcher, 1988) and squid statoliths (Jackson *et al.*, 1993). This effect of constant temperature conditions complicates the interpretation of a growth sequence, and could introduce a significant level of error into increment counts. A point worth emphasising is that, in spite of the constant temperature conditions imposed during the experiments, increments were still being produced with a daily frequency. Three of the specimens analysed displayed clear increments corresponding to the experimental period, and the increment counts obtained from these statoliths closely corresponded to the elapsed number of days.

The second effect of temperature concerns its influence on statolith growth rate. Integrated statolith growth rates estimated in this study were strongly dependent on the ambient temperature, confirming the suggestion of Jackson (1995). Statolith growth rates estimated from squid maintained at 20 °C ranged between 0.75 and 1.56 $\mu m.d^{-1}$, with only two of the estimates (both from males) falling below 1 $\mu m.d^{-1}$. In contrast, animals maintained at 15 °C displayed average statolith growth rates ranging between 0.60 and 0.90 $\mu m.d^{-1}$. These values imply that daily increment widths in the latter specimens would fall to below 1 μm. The resolving power of a light microscope, assuming perfect optics, high numerical aperture of the objective lens, ideal sample preparation and short-wavelength illumination (e.g. green light), is theoretically in the region of 0.2 μm (Campana and Neilson, 1985). In practice, this value is probably closer to 1 μm. Consequently, if a squid is exposed to low temperatures, the increments that are deposited during this period may well fall below the resolving power of a light microscope, and will consequently not be detected during the interpretation of the growth increment sequence, introducing errors into the increment count. If the animal is exposed to such low temperatures for an appreciable length of time, a blurred zone that appears to have no increments will be deposited in the statolith.

Pauly (in press) suggests that such "blurred" zones may reflect periods of respiratory stress experienced by the squid. Anaerobic metabolism during such periods, with the associated accmulation of acidic by-products, dampens the pH cycles that have been proposed to be responsible for increment formation (Morris, 1988, 1991a; Lipinski, 1993). Whatever their origins, such blurred zones present a serious problem to the researcher attempting to interpret the increment sequence. An option frequently employed by researchers when confronted by such features, is to interpolate the number of increments in the blurred zone, using the widths of adjacent increments. If the blurring is a result of constant temperature conditions, or respiratory stress as suggested by Pauly (in press), interpolation is a viable option (provided that the deposition rate does not change over the blurred zone). If the blurring is due to a series of narrow increments falling below the resolving power of the microscope, the interpolation will underestimate the number of increments in the zone, introducing error into the age estimate derived from the specimen. This has further implications regarding the use of the age estimates in subsequent growth modelling of the population (Morales-Nin, 1988 discusses this aspect in more detail). Where the occurrence of such zones is a common feature in a sample of animals collected for stock assessment purposes, further examination of the specimens using scanning electron microscopy to establish whether the zones are a function of narrow increments, rather than poorly-defined increments, may be appropriate.

The stress experienced by the squid during capture and subsequent handling resulted in the production of clear checks or prominent increments. The stress associated with capture had the most profound influence on check formation. Checks of this nature have been considered to perhaps reflect periods of interrupted increment deposition. The results of this study indicate that this is not the case. These checks in fact reflect a series of prominent increments. Figure 4.8 illustrates an example of this observation.

In the anterior region of the lateral dome, the check associated with capture appears as a prominent disruption, with what appears to be abnormally wide "light" and "dark" zones (white arrow labelled C in Fig. 4.8). Examination of the lateral region of the lateral dome demonstrates that this check is in fact composed of a series of three prominent increments (see inset in Fig. 4.8). In the anterior region of the lateral dome, the increments become compressed into the shorter growth axis of the statolith in this region, producing the wide light and dark zones effect. Little is known of the physiological processes of check formation. The enhanced visibility of checks prompted Campana and Neilson (1985) to suggest that some aspect of otolith deposition has been disrupted, and checks may therefore reflect anomalous incorporation of either calcium, or protein, or both. Check formation has been associated with reduced calcium deposition (Campana, 1983b), but no monitoring of protein deposition has been attempted. The visual clarity of an increment is a function of the difference in the calcium:protein ratios between its component incremental and discontinuous zones. Enhancement of these differences (perhaps by reduced calcium deposition during periods of stress) would enhance the visual clarity of the increment (Campana and Neilson, 1985).

Whatever the causes of prominent increments, the effects of stressful events (such as capture in the case of the specimen illustrated in Fig. 4.8) clearly last for several days, generating a series of prominent increments. When observed in a region of the statolith where the growth axis is short (such as the anterior and posterior regions of the lateral dome), these increments are compressed, resulting in a marked discontinuity in statolith growth. When such features are encountered, they should not be interpreted as a period of interrupted increment formation. Examination should always be directed to the longest growth axis of the specimen, because it is here where the component increments of the check are most likely to be separated.

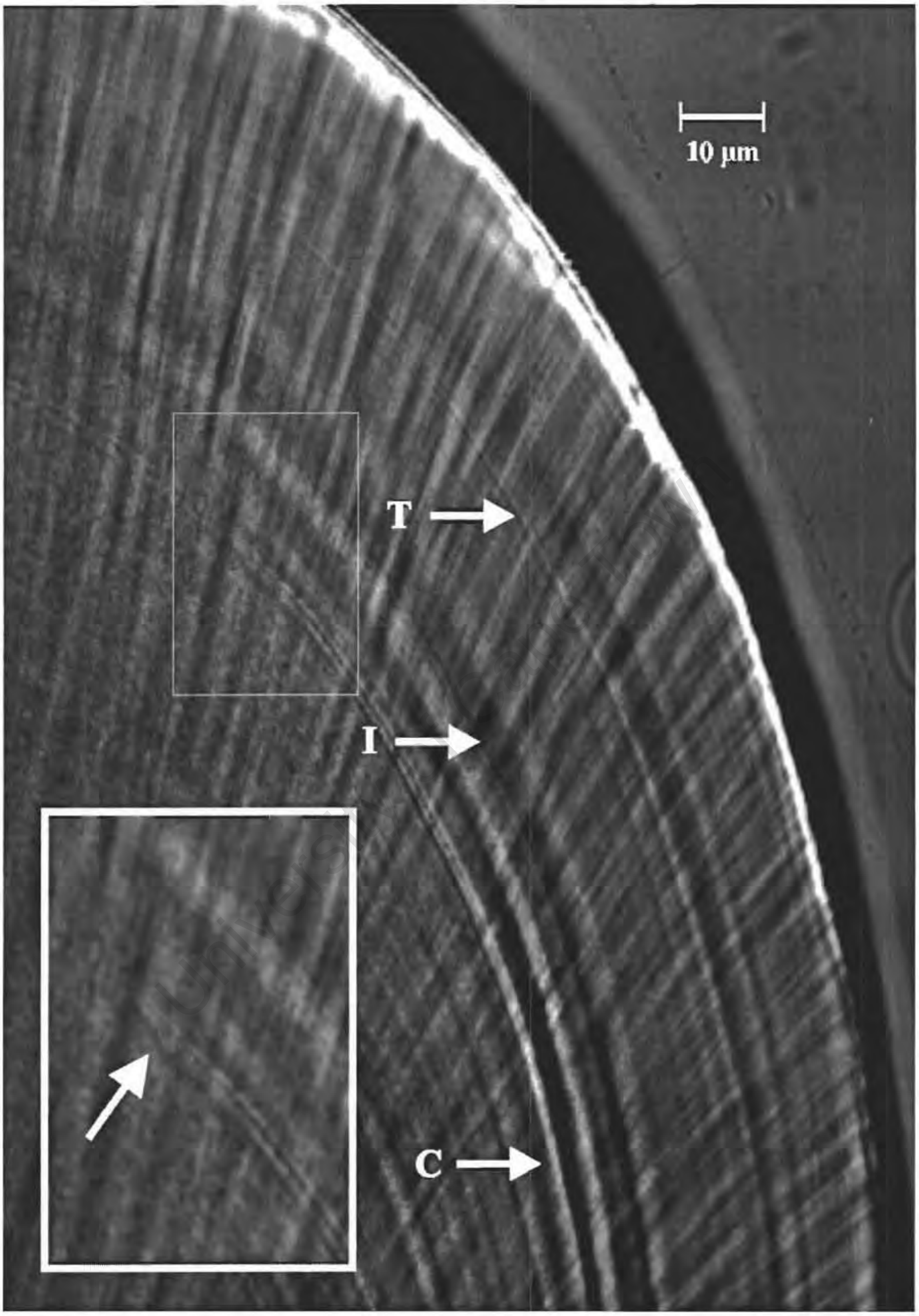


Figure 4.8: Light micrograph of the anterior and lateral regions of the frontal section of a statolith from specimen no. 57 (see Table 4.1), illustrating a prominent check associated with capture (C), which separates into a series of three prominent increments in the lateral region of the lateral dome. Introduction (I) and transfer (T) checks are indicated. Inset: high magnification image of rectangular area in main image. Arrow indicates the series of prominent increments comprising the capture check indicated in the main image.

CHAPTER 5 - NUCLEAR MICROPROBE MAPPING OF STATOLITHS OF CHOKKA SQUID *Loligo vulgaris reynaudii*

ABSTRACT

Loliginid squid statoliths were broken in half and their elemental composition investigated using a nuclear microprobe. Proton induced X-ray emission and proton backscattering were used simultaneously. True, quantitative maps of calcium and strontium distribution in the frontal plane were obtained using a rapid-matrix-transform method called Dynamic Analysis. These measurements were complemented by observations using scanning electron microscopy and light microscopy. In juvenile and adult statoliths, strontium (Sr) was concentrated in the wing and adjacent areas, whereas the calcium (Ca) concentration was highest on the edge of the lateral dome. In contrast, Sr and Ca were uniformly distributed in the statoliths of paralarval squid. Increments showed the best definition in the areas anterior and adjacent to the wing, corresponding to areas of high Sr content. Although temperature, sex, stage of maturity and the administration of oxytetracycline may influence the quantitative distribution of Ca and Sr in the statoliths of adult squid, they do not appear to affect the general pattern described above. The finding that Sr is concentrated in regions adjacent to the macula where the clearest increments are found in loliginid statoliths supports the hypothesis linking strontium with the regulation of statolith deposition and the definition of daily increments.

5.1. INTRODUCTION

The problems associated with the presence of non-daily microstructural features in squid statoliths have been highlighted in previous chapters of this thesis. In other areas of biology, many difficulties in the interpretation of changes in the frequency of events have been overcome by obtaining an understanding the physiology of their underlying causal mechanisms (Gauldie *et al.*, 1995). In their paper on modelling of otolith growth, Romanek and Gauldie (1996) stated: "Theoretical models of otolith growth would greatly assist in establishing criteria for the validation of age marks, but they are surprisingly uncommon given the extent of disagreement between age estimation techniques". Research towards this goal is relatively well advanced in terms of fish otoliths (Gauldie and Nelson, 1990a; Gauldie *et al.*, 1995; Romanek and Gauldie, 1996), but very little attention has been directed to the physiological mechanisms of squid statolith formation, apart from the studies of Morris (1988, 1991a) and Lipinski (1986, 1993).

One area of concern is the insufficiency of basic data concerning the elemental composition and dynamics of various components of the vestibular systems of fish and squid (e.g. otoliths/statoliths, endolymph fluid, maculae etc.) to improve the existing models. Particularly important are data concerning the statolith deposition process, as emphasised by Romanek and Gauldie (1996). Such an understanding could provide valuable insights into the nature and operation of the presumed circadian rhythm implicit in the deposition of daily increments, as well as clarifying their structural and functional characteristics.

This latter aspect is felt to be of crucial importance in the application of the statolith ageing technique. A great deal of information contributing to this understanding with respect to fish otoliths has been obtained from investigations of the elemental composition of otoliths. For example, Gauldie *et al.* (1992, 1995) used a proton microprobe to evaluate otolith age estimates of two fish species by comparing patterns of checks, cycles in microincrement width, and cycles in strontium and calcium composition. Mugiya and Satoh (1995) were able to analyse calcium and strontium levels within microincrements using X-ray microanalysis. Unfortunately, the goal of the latter study cannot be realized in loliginid squid because the increment widths in the statoliths are typically of the order of 1 μm or less, below the resolution limits of current microanalytical techniques. However, as has been demonstrated by Gauldie *et al.* (1992, 1995), microanalysis of elemental composition on a larger scale than that of microincrements can generate valuable information contributing to our understanding of the deposition of otoliths and statoliths, potentially clarifying questions regarding the formation of microincrements.

The objectives of the work reported in this chapter were firstly to detect any qualitative associations between statolith microstructure (specifically increment appearance) and the distribution of calcium and strontium within squid statoliths, and secondly to assess whether the patterns discovered (if any) were consistent between sexes, stages of development and ambient temperature. Ratios of strontium to calcium content in biogenic calcium carbonates are proposed to be related to ambient temperature at the time of crystal formation offering the potential for reconstructing the thermal history of individuals (e.g. Radtke, 1989; Radtke and Morales-Nin, 1989; Townsend *et al.*, 1989). This possibility was investigated in the present study using statoliths from individuals collected in warm and cold waters around the coast of Southern Africa. Further, the influence of oxytetracycline (OTC) marking on elemental composition of statoliths was also considered. Results of validation experiments employing this substance as a chemical marker indicated that its incorporation into the statoliths may have been associated with some physiological stress, and hence may have had some influence on the crystals deposited while the OTC was being metabolised (see Chapter 3).

Strontium concentrations in biogenic calcium carbonates are often a limiting factor in studies of this nature. The proton microprobe system selected for this investigation was able to measure elements at levels approaching a few ppm, with the further advantage of a data acquisition system that permitted quantitative mapping with full spectrum processing at every pixel in the image.

5.2. METHODS

5.2.1. Statolith collection and preparation

Statoliths of chokka squid (*Loligo vulgaris reynaudii*) were collected during several summer cruises of RS "Africana", RS "Algoa" and various commercial vessels in the years 1992-1994. The area of collection (Fig. 5.1) extended from St. Helena Bay on the west coast of South Africa to Port Alfred on the south-east coast. The material used in this study is listed in Table 5.1.

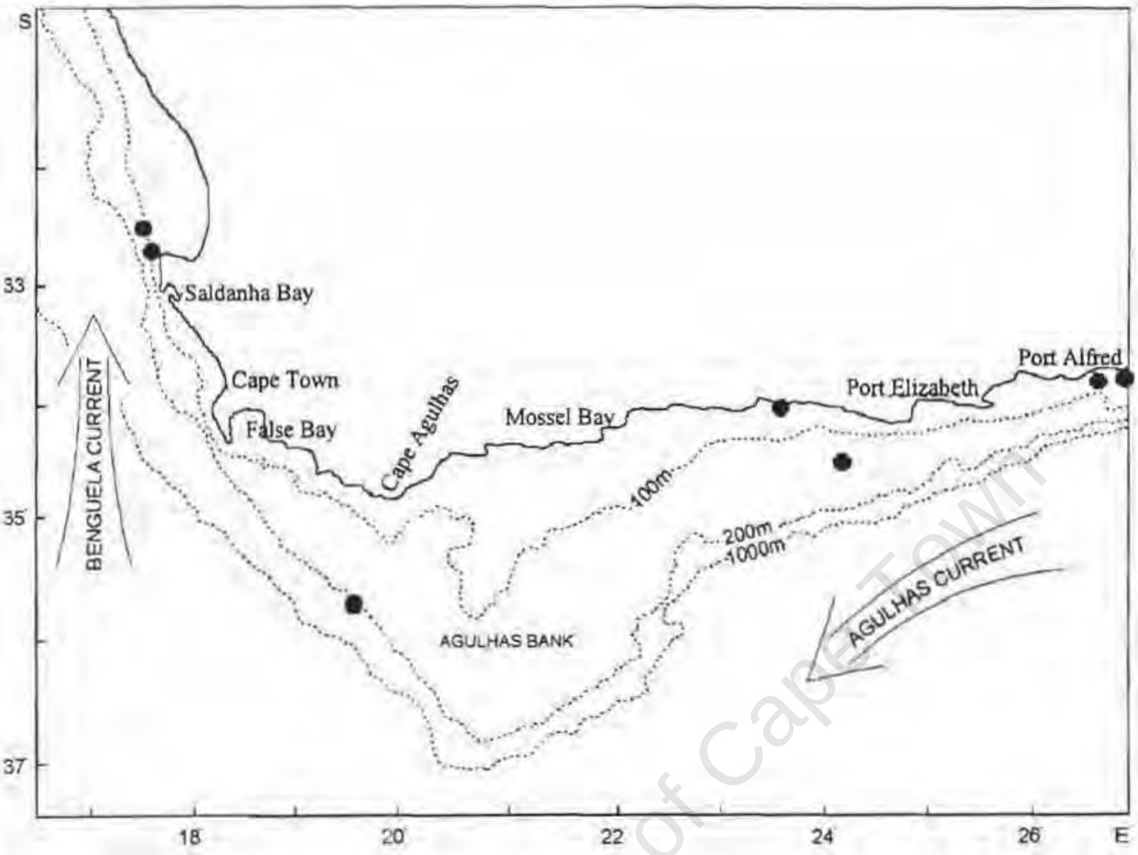


Figure 5.1: Distribution of squid sampling sites.

The three squid from warm waters were collected at 30-meter depths at sites near Port Alfred, influenced by the warm Agulhas Current. The bottom temperature at these sites was 17.6 °C at the time of collection. In contrast, the three squid from colder waters were collected in cold upwelled water near St. Helena Bay at depths of 100 m, the bottom temperature at these sites being 9.1 °C at the time of collection.

The OTC marked statoliths were obtained from laboratory validation studies (see Chapter 3), where squid were injected with up to 0.1 ml of an OTC solution (Lipinski, 1986) into the ventral mantle musculature, so as to mark the statoliths with a fluorescent label. After marking, the squid were maintained for periods ranging from 2 to 30 days in aquaria supplied with a continuous flow of fresh sea water, and exposed to the ambient light regime. The squid were fed a diet of live fish *ad libitum* for the duration of the experiments.

Statoliths were dissected according to the method of Lipinski (1981) and stored dry in small plastic tubes. They were fractured in the frontal plane using a clean and sterilized scalpel blade. The line of the break was determined by the approximate position of the nucleus and varied relatively little (Fig. 5.2). Ten statoliths however, were broken below the nucleus where the rostrum forms an angle with the lateral dome (plane 3 in Fig. 5.2), and ten statoliths were broken above the nucleus (plane 1 in Fig. 5.2). The surface of the break was viewed under a stereomicroscope and only statoliths with relatively clean and even surfaces were accepted for further preparation.

Table 5.1: Description of the material used in the PIXE analysis.

Type of material	n	ML (mm)	Date of capture	Location	Sex and Maturity
paralarvae	4	<2	10/10/91	Kromme Bay	
juveniles	2	65	15/09/92	Plettenberg Bay	M I
		78	15/09/92	Plettenberg Bay	M I
adults	6	154	15/09/92	Plettenberg Bay	M III
(mixed material)		175	11/11/93	Kromme Bay	F III
		198	16/09/92	S. of TCNP	M III
		250	05/01/94	Agulhas Bank	M II
		263	12/11/93	Kromme Bay	M V
		350	11/11/93	Kromme Bay	M V
adults	5	171	01/03/93	aquarium*	F III
(OTC marked)		182	14/11/93	aquarium*	M IV
		200	01/03/93	aquarium*	M III
		240	22/12/93	aquarium*	M V
		324	15/11/93	Kromme Bay	M V
adults	3	182	20/09/92	Port Alfred	F V
(warm water)		214	20/09/92	Port Alfred	M III
		268	20/09/92	Port Alfred	M V
adults	3	179	13/01/94	St. Helena Bay	F II
(cold water)		202	13/01/94	St. Helena Bay	M II
		225	13/01/94	St. Helena Bay	M III

(* aquarium maintenance material was all captured in False Bay, see Fig. 5.1)

The statolith halves were then mounted with Araldite Rapid glue on a formvar membrane spread on a thin aluminium frame. A second frame with formvar was glued on top of the statolith's frontal surface and coated with a thin layer of carbon. Once the nuclear microprobe observations had been completed, the samples were re-mounted on aluminium stubs, re-coated with carbon and viewed under a Stereoscan 200 scanning electron microscope. To locate areas of most prominent increments, statoliths were prepared following the procedure described in Chapter 2, viewed and photographed under a Zeiss light microscope. Terminology is that of Clarke (1978) and Lipinski *et al.* (1991).

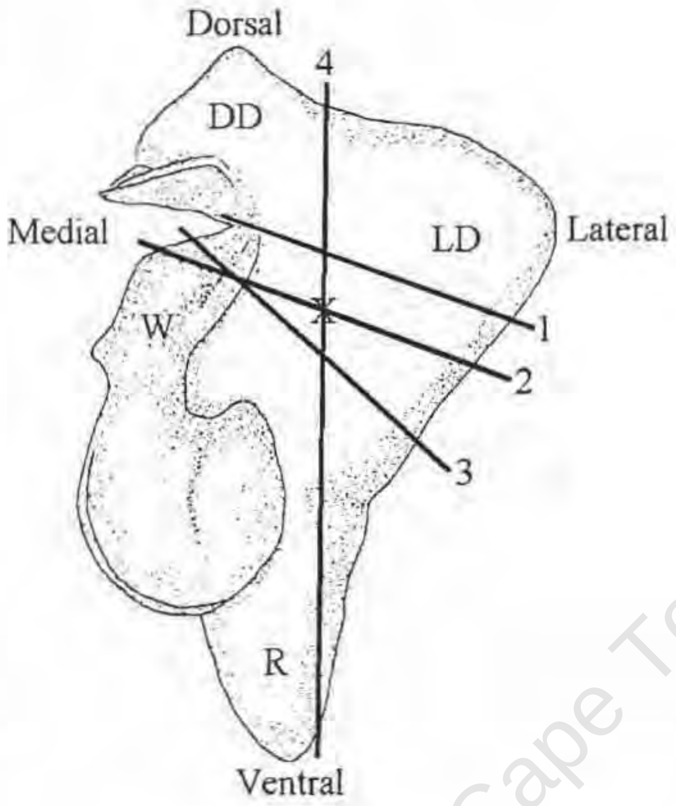


Figure 5.2: Diagram illustrating an anterior view of the left statolith of a chokka squid showing the orientation and parts of the statolith, as well as the break and section planes used in this study. DD - dorsal dome; LD - lateral dome; R - rostrum; W - wing; X - position of the nucleus. 1 - frontal break above the nucleus; 2 - frontal break through the nucleus; 3 - oblique frontal break below the nucleus; 4 - sagittal section through the nucleus.

5.2.2. Microprobe measurements

The selection of the microanalytical technique for this study was based on two criteria, namely lateral resolution and sensitivity. Ideally, a technique with a lateral resolution of about 0.5 µm or less (the spatial scale of increments in squid statoliths), with a sensitivity permitting quantitative mapping of the elements of interest is required. Since strontium concentration was of particular interest in this study, the analytical technique had to be capable of measuring this element at levels approaching a few parts per million. Although the proton microprobe is capable of this level of sensitivity, it cannot fulfill the requirement for lateral resolution. Techniques that provide such high levels of resolution (such as electron microprobes in energy dispersed spectrometry mode, or electron energy loss spectroscopy) require very thin samples. These are almost impossible to obtain from squid statoliths, which tend to disintegrate when very fine sectioning is attempted. Furthermore, it is doubtful whether such techniques are suitably sensitive for strontium analysis. No technique fulfilling both the sensitivity and resolution criteria appears to exist at present. The use of the proton microprobe represents a compromise between these two criteria.

The analyses were performed with 3.0 MeV protons, using the nuclear microprobe of the National Accelerator Centre (NAC). The details of the experimental setup have been reported previously (Tapper *et al.*, 1993, Prozesky *et al.*, 1995). Two complementary techniques, Proton Induced X-ray Emission (PIXE) and proton BackScattering (BS) were used simultaneously. The beam spot size was ca. 3 μm x 3 μm for proton currents of 200 - 400 pA. The proton currents were maintained within this range because higher currents resulted in destruction of the formvar layer mounted on top of the statolith's frontal surface, affecting the charge collection. PIXE spectra were registered using a Si(Li) X-ray detector situated about 37 mm away from the target at 135° to the incoming beam direction and separated from the evacuated specimen chamber by means of an intervening 25 μm Mylar window. An additional 40 μm Al absorber was used to reduce the intensity of Ca K X-ray lines and allowed the simultaneous measurements of calcium as well as minor and trace elements. The setup was calibrated using thick pure elements and glass standards as well as thin standards from Micromatter(TM) (Prozesky *et al.*, 1995; van Achtebergh, 1995). PIXE spectra were analysed using the GeoPIXE suite of programs (Ryan *et al.*, 1990; Ryan and Jamieson, 1993). This package allows the analysis of X-ray spectra with complete corrections for beam stopping, X-ray attenuation, and secondary fluorescence of thick targets.

Backscattered protons were registered with an annular silicon surface barrier detector, situated at 176° to the incoming beam direction. The simulations of proton backscattered spectra were performed with a modified version of RUMP (Doolittle 1986) using experimental cross-sections for isotopically natural carbon and oxygen at a laboratory angle of 170° (Amirikas *et al.*, 1993). No correction was made for this slight difference in angle between the experimental and simulated geometry. Elemental maps were made using a recently developed rapid matrix transform method called Dynamic Analysis (DA) (Ryan and Jamieson, 1993; Ryan *et al.*, 1995). This is a part of the GeoPIXE package and allows for the production of true elemental images. The images are inherently overlap-resolved and background-subtracted, and the maps are generated on-line. Final maps give quantitative elemental images and the intensity is given in ppm.

Mapping was performed using a systematic approach. First one or more point analyses were made in selected regions of the statolith in order to find a representative PIXE spectrum with all present elements and to evaluate the specimen thickness for proper matrix corrections. It was found that the specimens were infinitely thick for microanalytical purposes; i.e. their thickness exceeded the depth of penetration of the proton beam, and approximation of the sample composition as $CaCO_3$ was sufficient for matrix-correction purposes. The influence of the formvar layer on top of specimens (ca. 0.5 um thick) was found to be negligible and the correction for this layer was not included. Scan size was selected according to the size of individual specimens. Scanned regions were divided into 64 x 64 pixels. Data presentation was made by the Interactive Data Language (IDL) package (Anonymous 1993) and the maps are contours linking pixels with similar values.

5.2.3. Statistical analysis of the influence of temperature

A preliminary investigation of the possible influence of temperature on calcium and strontium levels in squid statoliths was conducted using the data from three individuals of similar size and maturity from each of the two temperature regimes. Raw data files obtained from the microprobe analyses provided measurements of calcium and strontium concentration within each pixel of the maps. To ensure that the data were representative of the temperature regime at which the squid were captured, only points around the periphery of the lateral dome (i.e. the most recently deposited mineral) were used. Concentrations of both calcium and strontium were obtained for each pixel around the periphery, Sr : Ca ratios were calculated for each pixel, and the data subjected to an analysis of variance (ANOVA) to test for differences between the statoliths and between the temperature regimes. All statistical procedures were carried out using "STATISTICA", a commercially available statistical package.

5.3. RESULTS

5.3.1. Morphology of the surface of the statolith frontal break

Scanning electron microscope (SEM) observations of the surfaces of the frontal breaks illustrated in Fig. 5.2, show aragonite crystals radiating outwards from a central point (Fig. 5.3). In the breaks through the nucleus (plane 2, Fig. 5.2), this central point is clearly the focus. However, in breaks above and below the nucleus (planes 1 and 3, Fig. 5.2), the central point is not the focus, suggesting the presence of a previously undescribed axis of crystallization extending dorso-ventrally from the focus. Light microscopy (LM) observations of ground and polished sagittal sections (plane 4 in Fig. 5.2) through the focus clearly show the axis extending ventrally from the focus beyond the nucleus and possibly the protostatolith, into the rostrum (Fig. 5.4). The dorsal component of the axis above the focus is not clearly apparent, and probably does not extend very far, if at all, beyond the nucleus.

The breaks differed, however, with respect to the medial inclusion of the irregularly orientated wing crystals into the lateral dome. In the planes above and below the focus, the inclusion of wing crystals does not reach the axis of crystallization (Fig. 5.3A and 5.3C). In contrast, when a statolith is broken through the focus, it is clear that the triangular wing inclusion originates at the focus.

LM observations of the frontal break in the same plane as the nucleus showed that the clearest increments were located in the regions adjacent and anterior to the triangular inclusion of wing crystals (Fig. 5.3D).

5.3.2. Maps of calcium and strontium distribution in loliginid statoliths: ontogenetic changes

In chokka hatchlings, calcium (Ca) and strontium (Sr) seem to concentrate in the middle of the statolith, but this may be an artefact related to the thickness of the sample (see Methods). It is more likely that these elements are distributed uniformly through the break. Sr levels were considerably higher than those measured in the lateral domes of older squid, but either comparable to levels in the wings of both juvenile and adult squid, or intermediate (Table 5.2, Figs. 5.5 and 5.6).

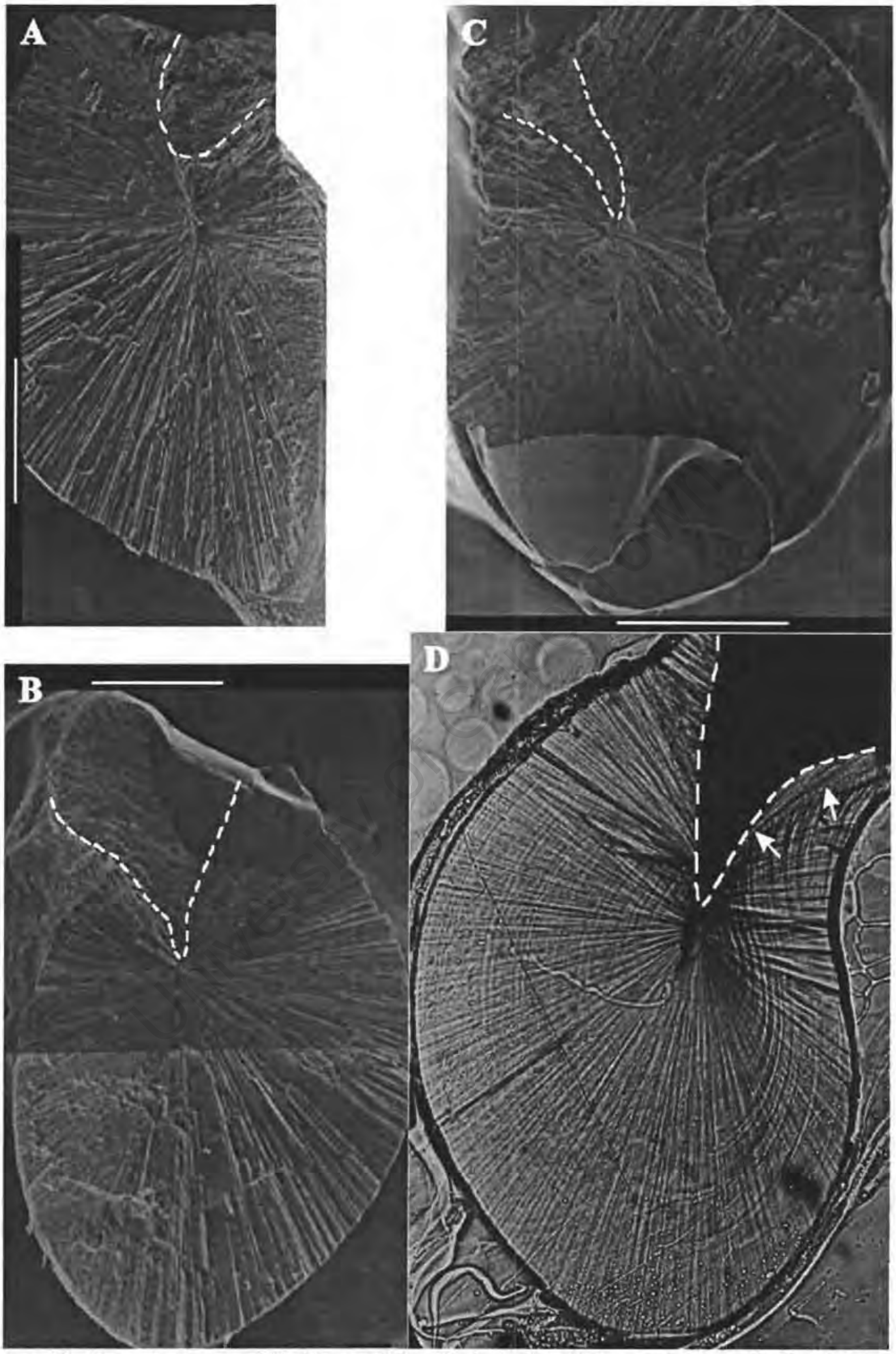


Figure 5.3: Internal structure of chokka statoliths. The inclusion of wing crystals is outlined with a dotted line. (A) Scanning electron micrograph (SEM) of statolith (ML = 175 mm, ♀V) broken below the nucleus (plane 3 in Fig. 5.2); (B) SEM of statolith (ML = 268 mm, ♂V) broken at the level of the nucleus (plane 2 in Fig. 5.2); (C) SEM of statolith (ML = 179 mm, ♀II) broken above the nucleus (plane 1 in Fig. 5.2); (D) Light micrograph of a ground and polished frontal section (ML = 294 mm, ♂V) showing the wing and position of clearest increments (arrows).

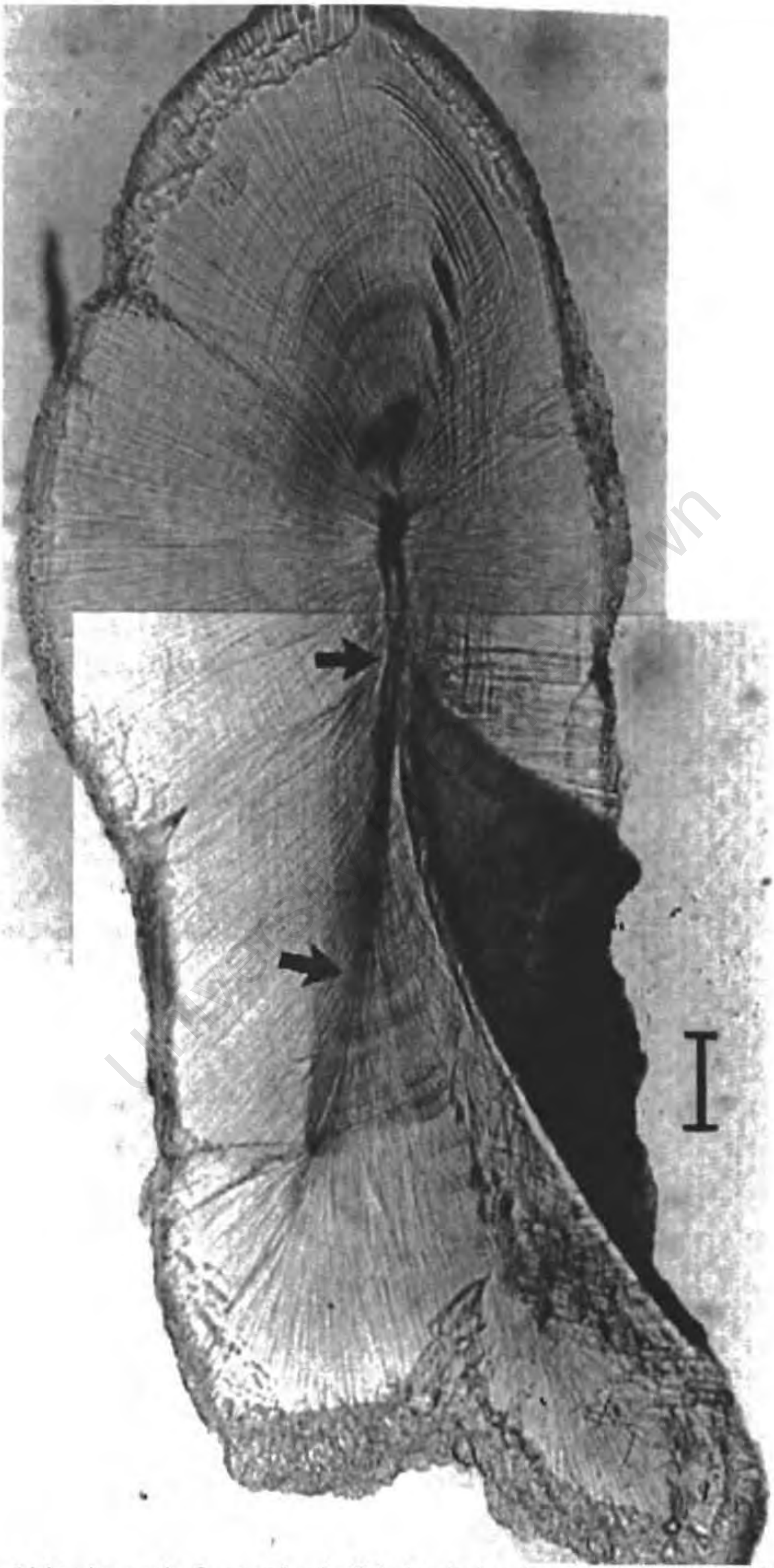


Figure 5.4: Light micrograph of a ground and polished sagittal section of a statolith (ML = 320 mm, ♂V) showing the transverse axis of crystallization (arrows). The scale bar is 100 µm.

Table 5.2: Calcium (Ca) and strontium (Sr) composition of squid statoliths.

Statolith	Element	Concentration (ppm)		Sr : Ca (%)
		Mean	S.D.	
paralarvae	Ca	3.763×10^5	5.304×10^4	2.25
	Sr	8.451×10^3	157	
	Ca	3.374×10^5	4.779×10^4	2.34
	Sr	7.892×10^3	164	
	Ca	3.266×10^5	4.550×10^4	3.03
	Sr	9.906×10^3	231	
	Ca	3.468×10^5	4.894×10^4	2.20
	Sr	7.614×10^3	143	
ML 324mm (OTC*) (lateral dome)	Ca	4.415×10^5	6.230×10^4	1.88
	Sr	8.321×10^3	149	
ML 324mm (OTC*) (wing)	Ca	3.461×10^5	4.789×10^4	3.39
	Sr	1.174×10^4	206	

* Statoliths that were labelled with oxytetracycline

In juveniles and adults of mantle lengths 65 mm and larger, patterns of Ca and Sr are generally very similar between individuals. The distribution of Sr showed the most striking and consistent pattern. In all planes investigated, Sr exhibited uniformly high concentrations in the wing crystals and adjacent areas, with patches of high concentration extending along a curved axis into the anterior regions of the lateral dome (Figs. 5.5, 5.6 and 5.8). Ca distribution patterns were less clearly defined, showing great variability between individuals. In general, however, Ca concentrations were higher in the lateral domes (particularly in the peripheral regions) and lower in the wing areas (Figs. 5.5, 5.6 and 5.7).

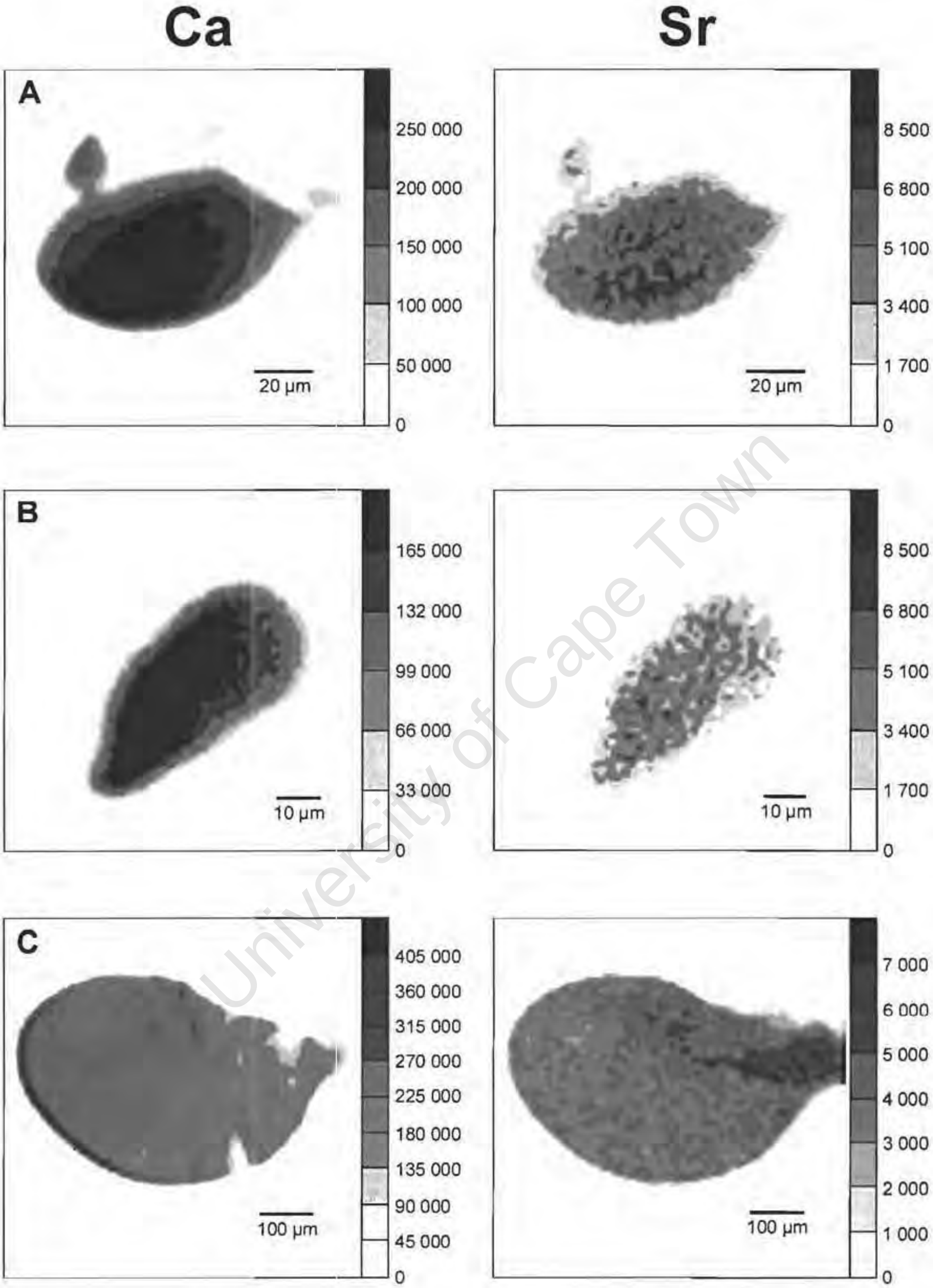


Figure 5.5: Ontogenetic changes in calcium and strontium concentrations in squid statoliths (values in ppm). (A) paralarva; (B) paralarva; (C) DML = 65 mm, ♂I (continued overleaf)

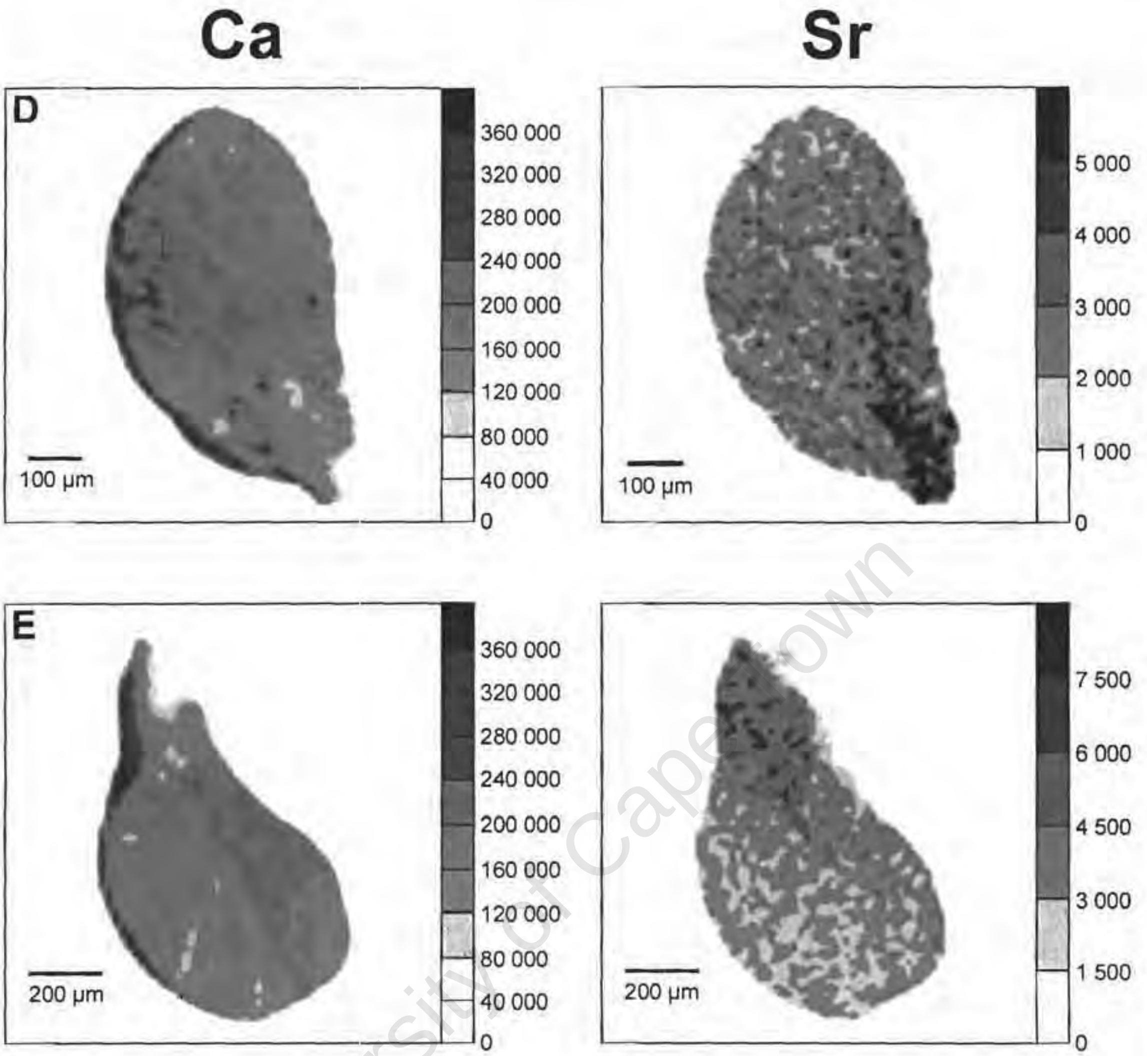


Figure 5.5 (continued): Ontogenetic changes in calcium and strontium concentrations in squid statoliths (values in ppm). (D) DML = 78 mm, ♂I; (E) DML = 214 mm, ♂III.

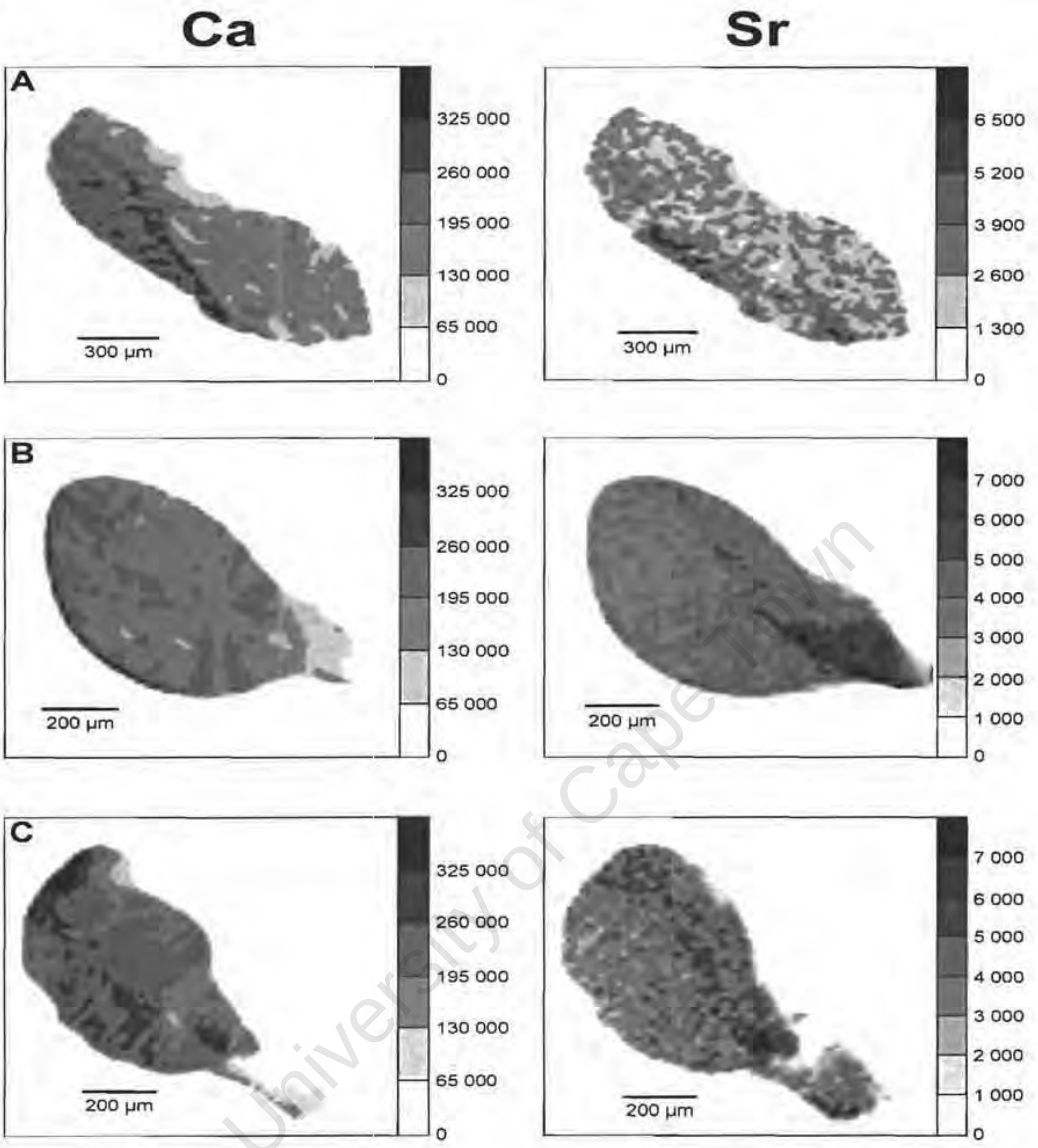


Figure 5.6: Calcium and strontium concentrations (values in ppm) in transverse sections of squid statoliths at levels (A) below the nucleus (ML = 175 mm, ♀V), (B) at the nucleus (ML = 182 mm, ♂IV, OTC marked squid, 19 days post-marking) and (C) above the nucleus (ML = 179 mm, ♀II).

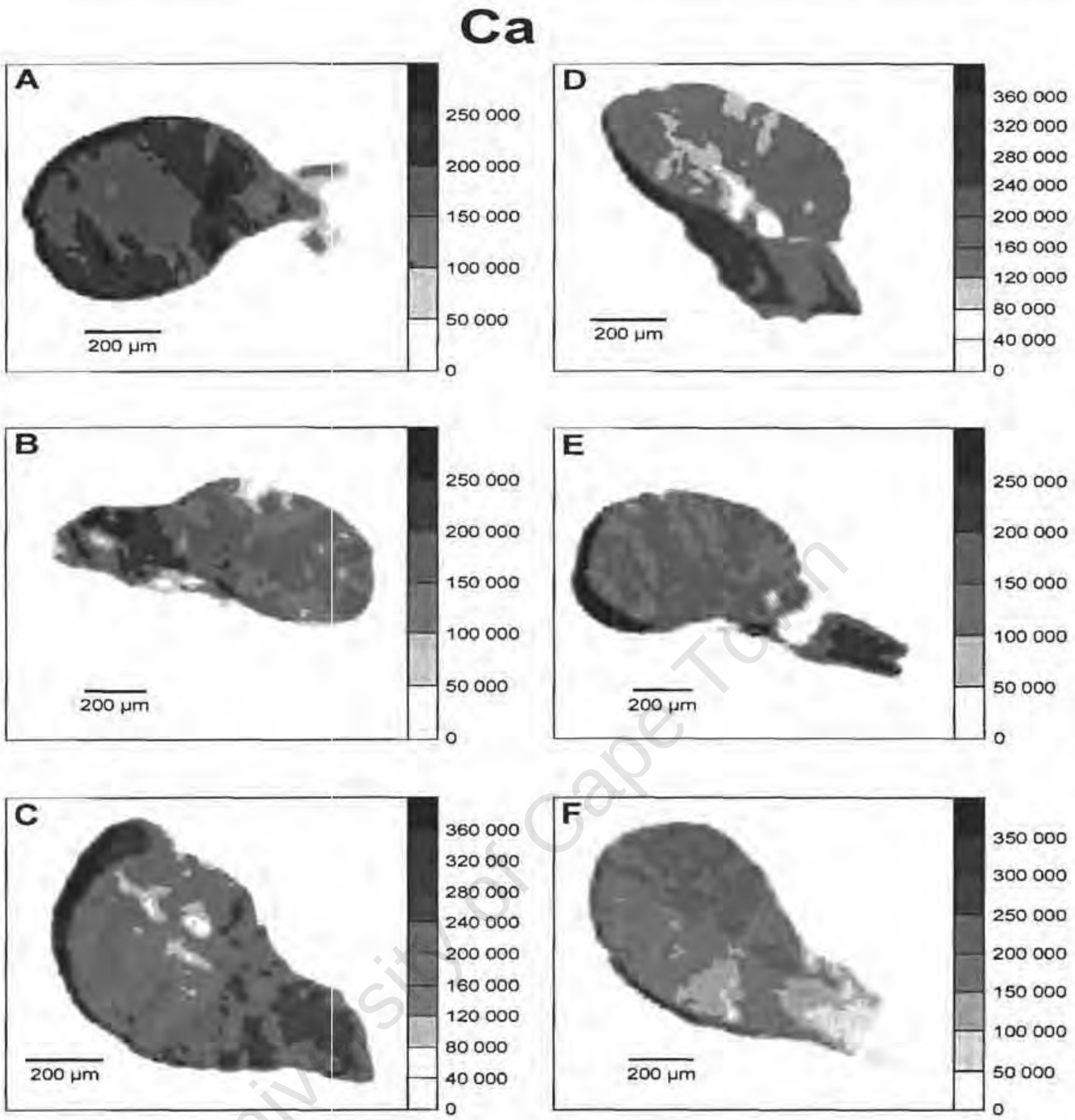


Figure 5.7: Calcium concentrations (values in ppm) in statoliths from cold waters (A - C) and warm waters (D - F). (A) ML = 179 mm, ♀II; (B) ML = 202 mm, ♂II; (C) ML = 225 mm, ♂III; (D) ML = 182 mm, ♀V; (E) ML = 214 mm, ♂III; (F) ML = 268 mm, ♂V.

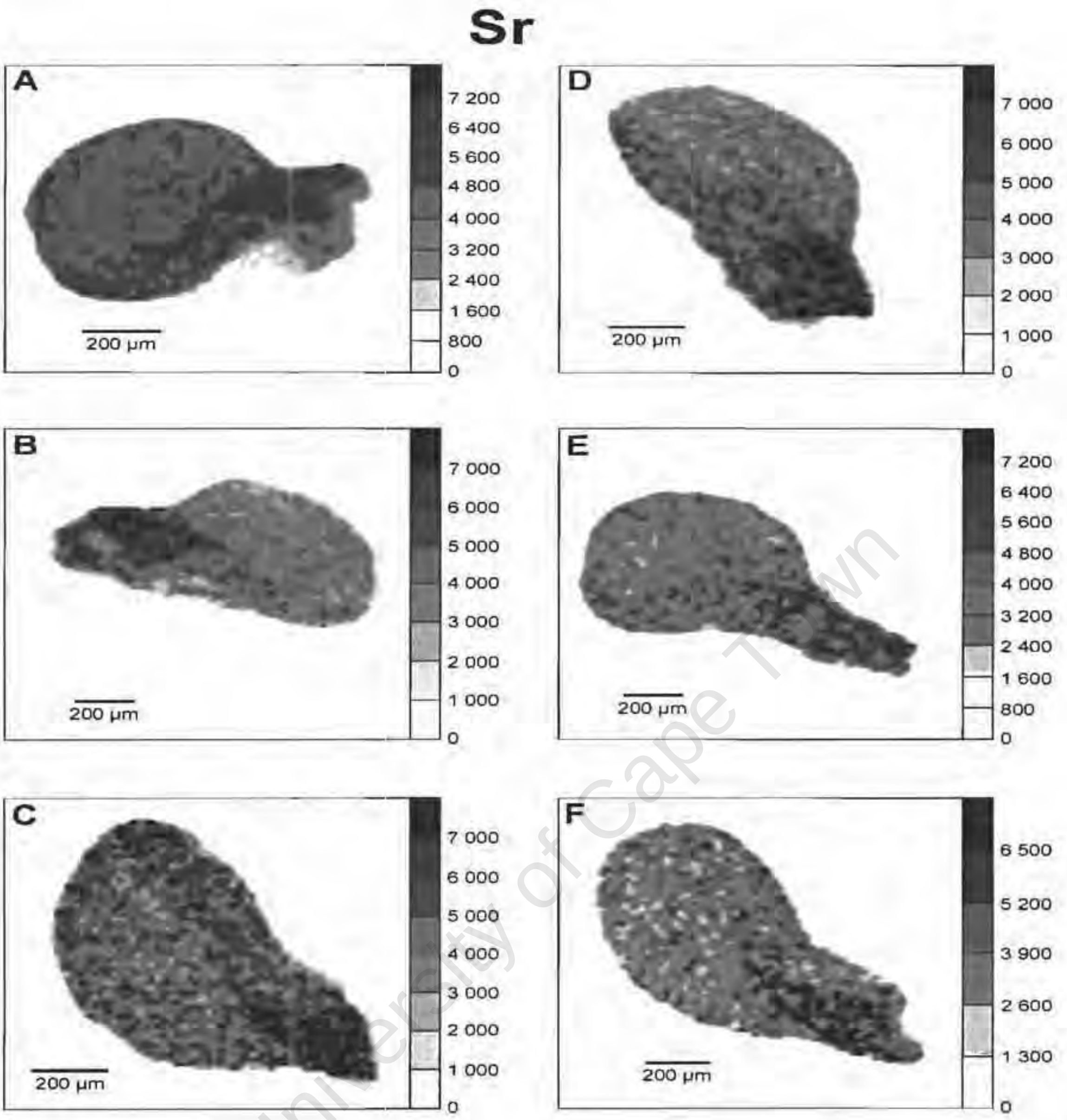


Figure 5.8: Strontium concentrations (values in ppm) in statoliths from cold waters (A - C) and warm waters (D - F). Same individuals as in Figure 5.7.

5.3.3. Temperature, sex and OTC injection effects

The maps of statoliths collected in both warm and cold water showed considerable variability in Ca and Sr content, but the general structural patterns described above did not appear to differ between the two temperature regimes (Fig. 5.7 and 5.8). The peripheral regions of statoliths from cold waters contained significantly more Ca and slightly more Sr than those of statoliths from warm waters (Table 5.3). Corresponding Sr:Ca ratios in statoliths from warmer waters were significantly higher than those in statoliths from cold waters. Although these results suggest a significant temperature effect, particularly in the case of statolith Ca levels, the statoliths within each temperature group also differed significantly from each other in terms of both Ca and Sr levels, as well as the corresponding Sr:Ca ratios (Table 5.4).

The results of *a posteriori* Tukey multiple comparison tests showed no clear pattern in the differences in Ca, Sr and Sr:Ca levels between the various statoliths that could possibly be attributed to a temperature effect (Table 5.5). No consistent sex- and maturity-related differences were detected (Fig. 5.7 and 5.8). OTC incorporation into the statolith had no apparent effect on the structural patterns observed (Fig 5.7B vs. Figs. 5.8 and 5.9).

Table 5.3: Comparison of mean calcium concentrations (ppm), mean strontium concentrations (ppm) and Sr:Ca ratios between warm- and cold-water statoliths. The standard deviation about each mean is italicised in parentheses below each value.

	Calcium		Strontium		Sr : Ca	
	Mean	ANOVA	Mean	ANOVA	Mean	ANOVA
WARM (n = 419)	247 378 *(52 606)*	$F = 76.497$ $p < 0.001$	5 210 *(1 212)*	$F = 3.779$ $p = 0.052$	0.0221 *(0.0075)*	$F = 14.114$ $p < 0.001$
COLD (n = 394)	281 161 *(57 518)*		5 370 *(1 131)*		0.0201 *(0.0074)*	

Table 5.4: Comparison of mean calcium and strontium concentrations (ppm), and Sr / Ca ratios within warm- and cold-water groups of statoliths. The dorsal mantle length (mm) of each specimen is provided in parentheses. The standard deviation about each mean is italicised in parentheses below each value.

	n	Calcium		Strontium		Sr : Ca	
		Mean	ANOVA	Mean	ANOVA	Mean	ANOVA
Warm							
W 1 (268 mm)	123	267 247 *(52 294)*		5 207 *(1 337)*		0.0200 *(0.0059)*	
W 2 (182 mm)	138	229 668 *(63 690)*	$F = 17.916$ $p < 0.001$	5 430 *(1 227)*	$F = 4.286$ $p = 0.014$	0.0256 *(0.0098)*	$F = 25.817$ $p < 0.001$
W 3 (214 mm)	158	247 361 *(33 452)*		5 020 *(1 011)*		0.0206 *(0.0047)*	
Cold							
C 1 (202 mm)	128	282 303 *(31 393)*		5 023 *(858)*		0.0179 *(0.0032)*	
C 2 (179 mm)	139	292 511 *(46 495)*	$F = 6.441$ $p < 0.001$	5 396 *(957)*	$F = 11.854$ $p < 0.001$	0.0189 *(0.0044)*	$F = 25.381$ $p < 0.001$
C3 (225 mm)	127	267 588 *(81 416)*		5 693 *(1 420)*		0.0237 *(0.0109)*	

Table 5.5: Results of the Tukey multiple comparison tests comparing calcium and strontium data from warm (W1, W2 and W3) and cold water (C1, C2 and C3) statoliths (the same specimens as in Table 5.4). Probabilities significant at the 5% level are indicated in bold type.

	Calcium				
	C 2	**C 3**	**W 1**	**W 2**	**W 3**
C 1	0.650	0.244	0.237	**0.000**	**0.000**
C 2		**0.003**	**0.003**	**0.000**	**0.000**
C 3			1.000	**0.000**	**0.032**
W 1				**0.000**	**0.043**
W 2					0.068

	Strontium				
	C 2	**C 3**	**W 1**	**W 2**	**W 3**
C 1	0.102	**0.000**	0.810	0.054	1.000
C 2		0.313	0.798	0.999	0.073
C 3			**0.013**	0.458	**0.000**
W 1				0.656	0.799
W 2					**0.037**

	Sr : Ca				
	C 2	**C 3**	**W 1**	**W 2**	**W 3**
C 1	0.893	**0.000**	0.180	**0.000**	**0.028**
C 2		**0.000**	0.793	**0.000**	0.309
C 3			**0.001**	0.253	**0.006**
W 1				**0.000**	0.987
W 2					**0.000**

5.3.4. Other elements present:

In addition to calcium and strontium, the elements detected by PIXE were Cr, Fe, Zn, Cu and Br. Cr and Fe were confined to the extreme borders of the specimens, with very low concentrations within the body of the statolith. It was therefore felt that these elements were in fact experimental artefacts introduced during the preparation process, rather than genuine components of the statoliths. Here mapping shows a distinct advantage over point analyses, because this conclusion could be drawn from the distribution patterns of these elements, compared to the shape of the statolith contour. The remaining elements (Zn, Cu and Br) were present in only some of the statoliths (Figs. 5.9 and 5.10), but when present, their distribution suggested that they were not impurities.

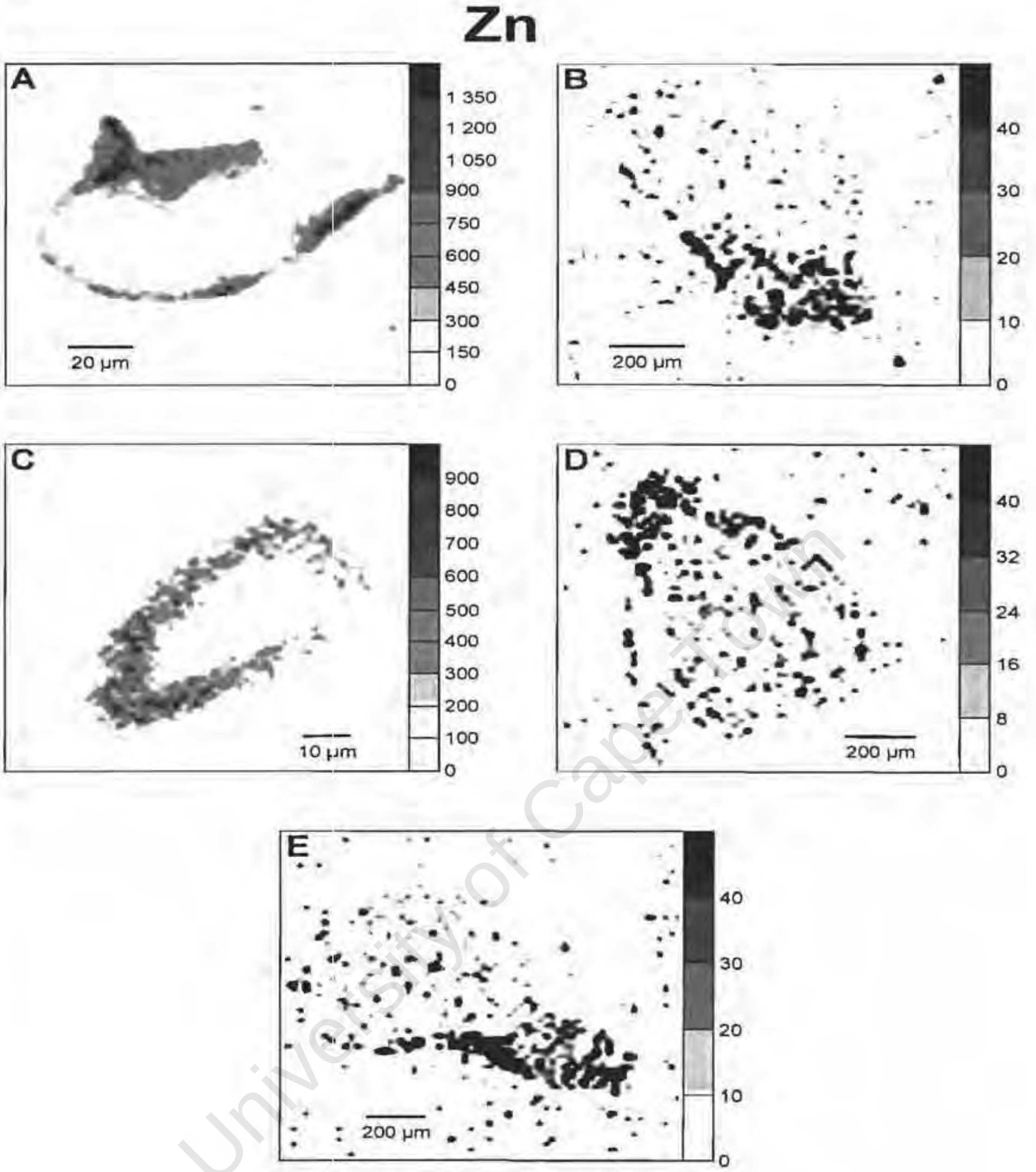


Figure 5.9: Zinc concentrations (values in ppm) in selected squid statoliths. (A) paralarva; (B) ML = 182 mm, ♀V; (C) paralarva; (D) ML = 214 mm, ♂III; (E) ML = 214 mm, ♂II.

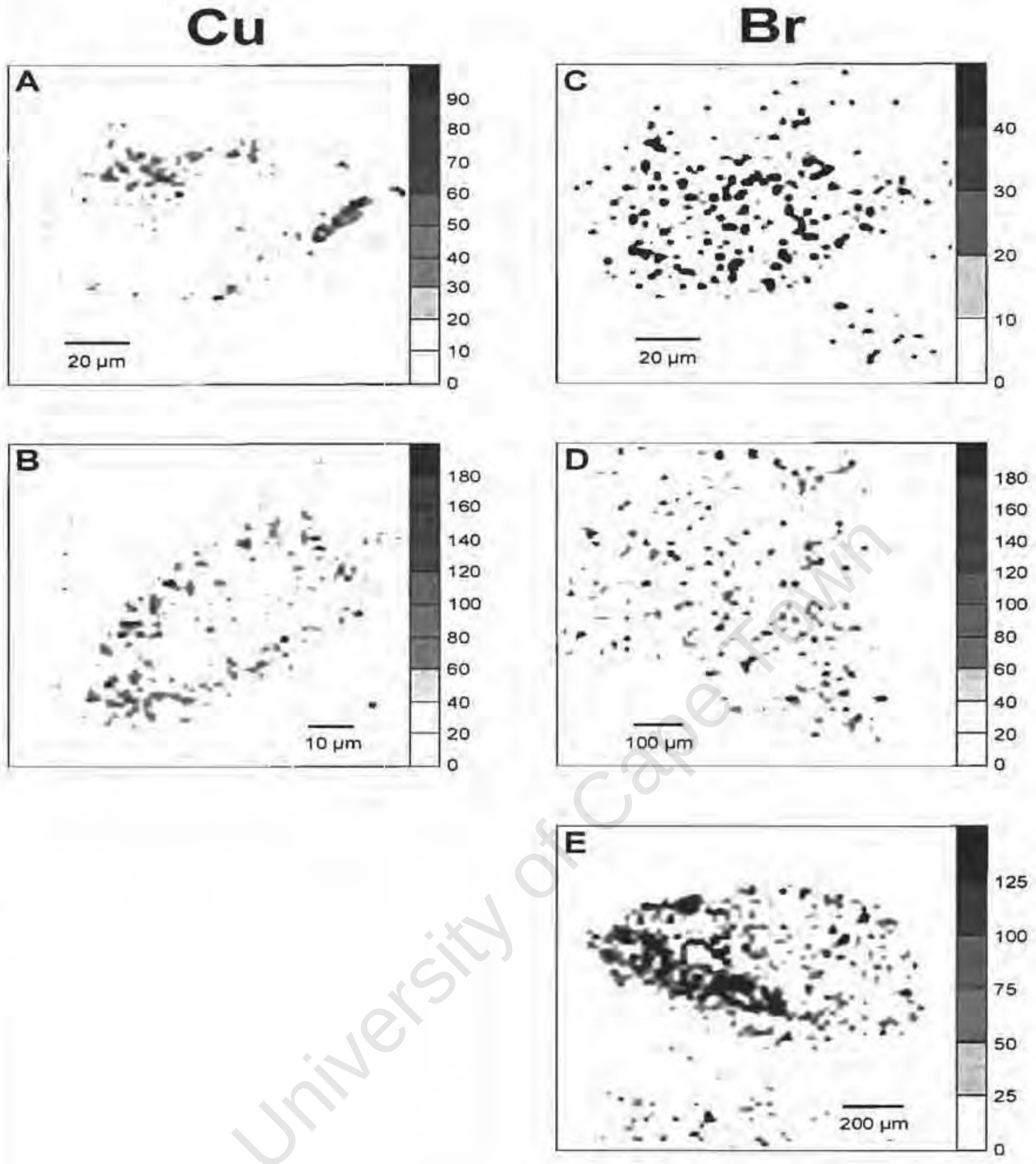


Figure 5.10: Copper and bromium concentrations (values in ppm) in selected squid statoliths. (A - C) paralarvae; (D) ML = 78 mm, ♂I; (E) ML = 202 mm, ♂II.

5.4. DISCUSSION

5.4.1. Morphology

Interpretation of previously reported data of statolith structure (Lipinski *et al.*, 1991; Lipinski, 1991, 1993) suggests that there is a single nucleation site, with calcification radiating from that site. The present results, however, indicate an alternative, namely that although there is a single nucleation site, further calcification proceeds along an axis extending dorso-ventrally from the focus. This type of calcification applies to loliginids only; calcification in ommastrephids appears to proceed according to the concentric mode (Balch *et al.*, 1988). Further research should be directed at this axis, specifically at the potential complications in interpretation of increment patterns and increment counts.

5.4.2. Maps: ontogenetic changes of calcium and strontium content

The ontogenetic changes in calcium and strontium distribution patterns in *Loligo vulgaris reynaudii* statoliths that have been described in this study provide valuable information concerning the deposition of squid statoliths. Paralarval statoliths showed uniformly high concentrations of Ca and Sr. With continuing growth of the animal, and deposition of statolith layers, there is a shift to increasing Ca concentration in peripheral areas of the statolith (especially posterior), distant from the wing area. Sr, on the other hand, shows consistently high concentrations in the wing crystals themselves, in areas adjacent and anterior to the wing, and in the anterior regions of the lateral dome.

These observations are consistent with the hypothesis of biomineralization in the squid statocyst proposed by Lipinski (1993), namely that Sr is secreted into the endolymph from the cells of the macula, whereas Ca is supplied from sites distant to the macula (Morris, 1988). With the onset of deposition in squid embryos, Ca and Sr precipitation occurs at the macula (reactions according to Lipinski, 1993), accounting for the high concentrations in paralarval statoliths. As statolith deposition progresses and the statolith increases in size, deposition sites on the posterior and lateral areas of the statolith become increasingly distant from the Sr supply in the macula, hence low Sr concentrations in these areas. Since Ca supply sites are distant from the macula, Ca availability does not become limited as the statolith increases in size, hence the higher Ca levels in the peripheral area of the lateral dome.

The relevance of these deductions to incremental growth of squid statoliths rests in the importance of strontium. Lipinski (1993, p. 259) suggested that good increment visibility is associated with high Sr concentration in squid statoliths. The Sr distribution patterns described above support this hypothesis. The preparation procedures developed for the purpose of increment enumeration (see Chapter 2) target the area adjacent and anterior to the cone shaped intrusion of wing crystals, since increments are consistently visible from nucleus to margin in this area (Fig. 5.3D). This region is the area of the adult statolith that is closest to the cells of the macula and hence the Sr supply, and shows high Sr contents in all microprobe maps of adult statoliths. Clear increments also occur along a curved axis extending from the nucleus into the anterior regions of the lateral dome, again corresponding to areas of high Sr concentration observed in this study. In contrast, marginal areas of the lateral dome of adult statoliths generally show no increments, corresponding to areas of low Sr concentration.

The relationship between Sr distribution patterns and increment visibility can be explained by the results of Mugiya and Satoh (1995), who documented relatively higher Sr concentrations in the discontinuous zones of increments in fish otoliths. Each increment comprises a "light" incremental zone and a "dark" discontinuous zone. Clear increments are a product of clearly defined discontinuous zones, and hence layers of high Sr content. As the statolith grows, sites removed from the macula will experience a reduced supply of Sr, and hence less clear increment definition in marginal areas. The physiological processes linking Sr content to statolith growth can only be speculated on, but that Sr availability is critical to normal statolith growth has been clearly demonstrated by Hanlon *et al.* (1989). A lack of Sr in ambient sea water resulted in either a complete absence of statoliths, or the formation of grossly deformed statoliths in a number of cephalopod species. It is very likely that the organic (protein) component of squid statoliths plays a key role in the deposition process, and it may be that Sr is intimately linked to this component.

A view commonly expressed in the literature attributes the appearance of the discontinuous zones of increments to a relatively higher protein content of the aragonite crystals in comparison to the incremental zones, suggesting a link between protein and Sr in increment formation. Mugiya and Tanaka (1995) indicated that in fish otoliths, Sr may successfully compete with Ca in binding to some kinds of protein. One could argue that a Sr-protein complex stabilises the aragonite wing crystals in the reverse reaction of deposition and resorption and that these crystals then serve as a template for the subsequent deposition of other parts of the statolith. It would therefore appear that statolith formation is a process that is triggered, and possibly controlled by Sr levels in the statocyst linked with the protein component. Details of this process, as well as the extent to which it is coupled or modified by other factors (such as pH), needs to be investigated.

5.4.3. Temperature, sex and OTC injection effects

Statoliths from individuals collected more than 600 km apart in cold (9°C) and warm (18°C) waters showed no major differences in Ca and Sr distribution patterns, indicating that temperature does not influence the basic biomineralization processes occurring in squid statocysts. Ca concentrations in the peripheral regions of the lateral domes of statoliths from the cold water sites were significantly higher than those from warm waters, resulting in significantly higher Sr:Ca ratios in the latter, a result that contradicts the negative relationship between Sr:Ca ratios and temperature often reported in the literature (Radtke, 1989; Radtke and Morales-Nin, 1989; Townsend *et al.*, 1989; Radtke *et al.*, 1990). It should be emphasised, however, that the nature and validity of the Sr:Ca - temperature relationship remains controversial (Kalish, 1989). Although the results of some studies on fish otoliths have shown no relationship between Sr:Ca ratios and ambient temperature (e.g. Gallahar and Kingsford, 1996; Kawakami *et al.*, 1998; Otake and Uchida, 1998), Townsend *et al.* (1995) have reported that the concentration ratios of Sr to Ca in otoliths of larval cod (*Gadus morhua*) are related to water temperature at the time of otolith precipitation. The situation in squid statoliths remains inconclusive (e.g. Rodhouse *et al.*, 1994).

Although the results presented here would appear to indicate that temperature does influence the levels of Ca and Sr in squid statoliths, variation in elemental composition between statoliths from squid collected at the same temperature was such that this conclusion cannot be drawn from the limited data set used in the analysis.

There was no apparent difference in general patterns nor in details of Sr and Ca distributions between males and females. The variability of the detected contents was considerable however, and the size of the sample may have been insufficient to detect any fine patterns. Although the relationship between maturity and Sr and Ca concentrations were not investigated in detail, the material used did contain a number of immature (stage I-II) and fully mature (stage V) males and females (maturity scale according to Lipinski and Underhill, 1995), and these showed no difference in the basic pattern of Sr and Ca distribution between statoliths.

The data obtained from the five OTC-marked statoliths indicated that the incorporation of this compound did not have a detectable influence on Ca and Sr levels in the statolith. If any effects did occur, they were on a scale below the resolution limits of the microprobe.

5.4.4. Other elements present

Many different elements have been investigated for various reasons in otoliths, mollusc shells, ostracods and brachiopods by means of a proton microprobe (Gauldie *et al.*, 1992, 1995; Sie and Thresher, 1992; Coote and Trompetter, 1995; Dai *et al.*, 1995; Nystrom *et al.*, 1995; Bruckschen *et al.*, 1995). The present study concentrated on Ca and Sr, but other elements such as Zn, Cu and Br were also detected and were found to be genuine parts of the statolith composition. Their concentration was highly variable (e.g. Zn) and they were detected only in certain statoliths. Explanation and description of the presence-absence patterns concerning these elements must await further investigation. It must also be pointed out that some elements of possible significance (e.g. F; see Coote and Trompetter, 1995) were not investigated at all due to the constraints of the method used.

CHAPTER 6 - FLUCTUATIONS IN THE CHEMISTRY OF THE STATOCYST ENDOLYMPH OF *Lolliguncula brevis*: DAILY RHYTHMS?

ABSTRACT

This chapter describes a study investigating elements of the chemistry of the statocyst endolymph in *Lolliguncula brevis*. Endolymph calcium and magnesium ion concentrations were monitored at intervals over periods ranging from 24 to 48 hours, in an attempt to establish whether these parameters fluctuated with a daily frequency. Attempts to measure strontium ion concentration and pH were unsuccessful. Multiple regression analyses conducted separately on results from male and female squid were used to account for variability in the data attributable to temperature, size and stage of maturity. Predicted endolymph calcium and magnesium ion concentrations were computed using the models, and these data analysed using a non-linear regression model. Endolymph calcium and magnesium ion concentrations displayed periodic fluctuations, but evidence suggesting that these fluctuations occurred with a daily frequency was weak, particularly in the case of calcium. In general, endolymph magnesium ion concentrations peaked at night, while calcium ion concentration peaked during the day. The implications of the results in terms of increment formation in statoliths are discussed, and recommendations for future studies made.

6.1. INTRODUCTION

Chapter 3 of this thesis demonstrated that the validation experiments generally employed to test the daily frequency of increment production suffer from short-comings that leave the conclusions drawn from such experiments open to question. It was concluded that the only reliable test of the daily increment hypothesis is to establish whether or not a daily cycle is apparent in the physiological and molecular processes of statolith calcification, and if so, whether this cycle is manifested as the differential deposition of the incremental and discontinuous zones that constitute each increment. Information of this nature is very limited for teleost fish, and does not exist for squid. The only studies investigating the deposition process in squid statoliths are those of Morris (1988, 1991a) and Lipinski (1986, 1993). No studies have rigorously investigated the proposed daily cycle in the deposition process. Morris (1991a) did compare ionic concentrations and pH in the statocyst endolymph between day and night samples, but found no significant differences. He attributed these findings to the high degree of physiological stress experienced by the experimental animals as a result of captivity.

The experiments described in this chapter were aimed at establishing whether daily cycles occur in various parameters of squid statocyst endolymph chemistry. This approach rests on the assumption that the processes of statolith mineralization, and particularly increment deposition, are occurring in the endolymphatic fluid surrounding the statolith. The rationale is based on the unusual nature of biomineralization in the coleoid cephalopod statocyst (Morris, 1988, 1991a). Most of the statolith is freely suspended in the statocyst cavity, surrounded by the endolymph. Only the wing area is in contact with any epithelial tissue, namely the cells of the macula (Stephens and Young, 1982). Since statolith growth occurs in all directions from the central focus, mineral must be deposited on the statolith surface from the endolymph. The components of the statolith must be secreted into the endolymph from the walls of the statocyst, and then be deposited at points distant from the secretory site, as opposed to the more common scenario where mineralization occurs in contact with some secretory surface. While this latter scenario clearly applies to statolith growth at the wing/macula interface, it appears that the mineralization processes that occur in this region differ from those in the rest of the statolith. The $CaCO_3$ crystals in the wing show a very disordered structure with no evidence of the microstructural features (particularly increments) that are apparent in the rest of the statolith (Lipinski, 1986). In other words, the areas of the statolith deposited from the endolymph show increments and other microstructural features, whereas the area deposited in contact with the macula does not (Lipinski, 1993).

The processes that result in increment production must therefore be occurring in the endolymph adjacent to the statolith surface. It could consequently be argued that the differential deposition of the incremental and discontinuous zones of increments is a reflection of changes in the chemistry of the surrounding endolymph. Several studies have demonstrated intimate links between the chemistry of the endolymph and the growth of teleost otoliths. A strong linear relationship between calcium:strontium ratios in the endolymph and the outermost layers of the otoliths of twelve species of fish led to the suggestion that solution chemistry is a major factor in determing these ratios in fish otoliths (Kalish, 1989). Romanek and Gauldie (1996) proposed that endolymph chemistry can be used to predict otolith growth. Observed increment widths in several species of fish fell within the boundaries of increment widths that were predicted using a model based purely on the inorganic ion content of the endolymph and aragonite precipitation kinetics. Considering the many structural and functional similarities between fish otoliths and squid statoliths, it is likely that similar relationships apply in squid statoliths. Analyses of the endolymph could consequently provide information concerning the deposition of increments, and particularly whether or not a daily cycle in these processes is occurring. As Morris (1991a) pointed out, the statocyst endolymph presents a relatively large volume of fluid available for investigation into the biomineralization processes. Samples of this fluid can be easily obtained with the minimum of dissection and analyzed.

The research described in this chapter adopted this approach, and attempted to quantify temporal fluctuations in various components of the statocyst that were considered to be involved in statolith deposition. Four aspects were identified as most likely being of central importance, namely calcium, magnesium, strontium and pH. A fifth aspect, the organic component, is examined in Chapter 7. Calcium is the fundamental building block of aragonitic crystals, and as such should be included in any exploratory investigations of this nature. Magnesium has been implicated in biological calcification, but its action and relevance have yet to be elucidated. An inhibitory effect on calcification by magnesium was indicated by the results of Morris (1991a). $CaCO_3$ precipitation rate decreased when magnesium concentration was increased. The results of several studies described previously (e.g. Lipinski, 1986 and

1993), including the study described in Chapter 5, suggest that strontium is intimately involved in the deposition process, although the mechanism is unknown. Further evidence implicating strontium in statolith mineralization was documented by Hanlon *et al.* (1989). Larval cephalopods reared in the absence of strontium possessed either statoliths displaying severe structural abnormalities, or no statoliths at all. Similar findings were reported for the opisthobranch mollusc *Aplysia californica* (Bidwell *et al.*, 1990).

The role of pH is also unknown, but this parameter was central to the hypothesis of statolith mineralization put forward by Morris (1988, 1991a), and also forms an integral component of the models of statolith and otolith mineralization put forward by Gauldie and Nelson (1990b) and Lipinski (1993). Morris (1988, 1991a) detected an organic component of the endolymph of the squid *Alloteuthis subulata* that is sensitive to pH, and proposed that this organic component reversibly polymerizes with increasing pH, inhibiting calcification. Morris further proposes that alterations in activity levels linked with protein metabolism are responsible for the pH fluctuations, and hence periods of reduced or enhanced statolith growth. Lipinski (1993) rejects this hypothesis, arguing that activity-induced alterations of pH of the magnitude suggested by Morris (1988, 1991a) are unlikely. Lipinski proposed that pH changes are rather a result of alterations in the balance of inorganic ions in the endolymph (particularly strontium), and these alterations drive the mechanism of increment formation. Whatever the mechanism, pH is clearly an important factor in statolith biomineralization, either in a passive role as part of the environment that will either favour or inhibit calcification, or through a more active role via its influence on the organic component.

The approach employed in this study was to sample individual squid at various intervals over time, and obtain measurements of pH and ionic concentrations of calcium, magnesium and strontium in the statocyst endolymph. The data were then tested for any evidence of a daily periodicity in the fluctuations (if any) of these variables.

6.2. METHODS

Due to the location and function of the squid statocyst, individuals have to be sacrificed to obtain endolymph samples, precluding the monitoring of the statocyst endolymph in a single individual over a period of time. Attempts to remove endolymph samples from live animals were unsuccessful, resulting in the death of the squid shortly after the samples were removed. Further, considering that the process is extremely stressful to the animal, it is unlikely that such samples would reflect the true status of the endolymph at the time of sampling. Consequently, the experimental approach employed was to maintain large numbers of squid in several tanks in the laboratory, with several individuals being sampled at periodic intervals. Each individual was sacrificed, and the endolymph sample extracted. This approach rests on the assumption that if some cycle is present in endolymph chemistry, the periodicity will be the same in all individuals in the population, and all individuals will be in phase with each other.

The loliginid squid *Lolliguncula brevis* was used as study animal, since it is relatively simple to obtain and maintain large numbers of animals in the laboratory. The experiments were conducted in the laboratories of the National Resource Center for Cephalopods, a division of the Marine Biomedical Institute at the University of Texas Medical Branch in Galveston, U.S.A.

6.2.1. Squid collection and maintenance

Live squid were collected in Galveston Bay by brief bottom trawls. Animals that were in a satisfactory condition were transferred immediately to a holding tank on board the vessel that was supplied with a continuous flow of air and ambient sea water. On returning to the laboratory, the entire holding tank was moved into the laboratory. The squid were maintained for a minimum of twenty-four hours in the holding tank, after which all dead and moribund individuals were removed. Squid that were in a good condition were then transferred to the experimental tanks, which comprised five 400 liter tanks supplied with a continuous air supply, and a continuous flow of recirculated, filtered sea water. Salinity and temperature in the tanks were adjusted to reflect ambient conditions at the time of capture. The squid were fed *ad libitum* with live penaeid prawns and fish, and were allowed a minimum of one week to acclimate to the conditions in the laboratory before any experiments were initiated. The squid were exposed to a natural ambient photoperiod during the acclimation and experimental periods.

6.2.2. Experimental procedure

Squid were sampled at periodic intervals over periods ranging from 24 to 48 hours. Water temperature was measured at each interval, and water samples were taken for the analysis of ambient calcium, magnesium and strontium ion concentration. The animals were removed from each tank using a net. Each animal was immediately submerged in liquid nitrogen, the entire procedure from capture to immersion never lasting more than twenty seconds. This precaution was adopted in an attempt to minimize changes in the chemical status of the endolymph resulting from passive diffusion of ions from the surrounding tissues. Studies investigating the chemistry of the endolymph in both squid (Morris, 1991a) and fish (see Romanek and Gauldie, 1996) have observed large differences in the ionic composition of the blood plasma and endolymph. These gradients are maintained by active transport. The ATP pump responsible for maintaining the integrity of the various fluid compartments degrades rapidly after the death of the animal, placing a time constraint on the period in which representative samples of the endolymph can be obtained (Romanek and Gauldie, 1996). Further, the stress associated with capture and subsequent sampling of the endolymph from a live animal can also be expected to influence the endolymph chemistry. The procedure employed in this study immediately arrests all metabolic activity, and prevents passive diffusion of ions across the tissue barrier surrounding the statocyst. After a few minutes, the frozen squid was removed from the liquid nitrogen, and transferred to a -74 °C freezer until further processing.

Each squid was individually thawed, and the tissue covering the ventral surface of the statocyst dissected away. The exposed cartilage was washed with distilled water and gently dried with tissue paper. The statocyst was then punctured with a pH microelectrode (NMPH - 1 "Beetrode" pH microelectrode, WPI electronics) and pH measured in each statocyst. An AgCl reference electrode manufactured in the laboratory was applied to the outer surface of the statocyst. The pH microelectrode was re-calibrated before each measurement in a pH 7 sea water solution buffered with 20 mM Tris. After the pH measurement had been obtained, an aliquot of known volume of the statocyst endolymph was withdrawn using a fine glass capillary tube attached to a Hamilton syringe of 5 μl capacity, and transferred to a Falcon tube for later analysis of Ca, Mg and Sr content.

Since sample volumes were typically of the order of 1 to 2 μl, each sample was transferred into a known volume of deionized water to ensure that the entire sample was transferred. The syringe and capillary tube were washed several times between each sample to ensure that the samples were not contaminated with the previous sample. During the sampling procedure, care was taken that no loose statoconia were included with the sample. Statoconia are fine $CaCO_3$ crystals covering two of the maculae in the statocyst (see Chapter 1), and readily become detached from the maculae after freezing. Considering that the samples were transferred into DI water, any statoconia contaminating the sample would release an unknown quantity of the ions into solution. Blanks of the deionized (DI) water were analyzed to control for any ions that may have been present in the DI water, and to correct the final measurements of ionic concentrations. The samples were stored at -74 °C until analysis of ionic content using an atomic absorption spectrometer. After the sample had been obtained, the dorsal mantle length (DML), sex and stage of maturity (assessed according to the scale of Lipinski and Underhill, 1995) were recorded for each individual. Four separate experiments were conducted. The duration, conditions and sampling intervals of each experiment are given in Table 6.1.

A minimum of 4 individuals were sampled at each sampling interval. In several cases, however, the statocysts were found to be damaged after the specimens were thawed. This was presumably a result of the rapid freezing of the animals in the liquid nitrogen. It is possible that expansion of the fluid in the statocyst during the freezing process cracked the cartilagenous walls of the statocysts. This represents a drawback of the technique, because damage of this nature would result in the contamination of the endolymph by surrounding body fluids. Each specimen was carefully examined under a stereo microscope prior to sampling the endolymph to ensure that the statocyst was intact and uncontaminated. Damage to the statocyst was readily apparent. The endolymph generally displayed a cloudy appearance when contaminated by surrounding fluids, and/or contained bubbles. No samples for ionic concentration measurements were taken from these specimens, because the measurements would not reflect the true chemical status of the endolymph at the time of sampling.

Table 6.1: Details of the endolymph chemistry experiments. The duration of each experiment is indicated in parentheses below the experiment number.

Experiment	Acclimation period	Temp. (°C)	Salinity $(^{\circ}/_{oo})$	Photoperiod	Sampling
1 (48h)	14 days	14	25	Sunrise - 07:30 Sunset - 19:15	6h intervals, starting at 13:00
2 (48h)	14 days	16	30	Sunrise - 07:00 Sunset - 19:46	Midnight, sunrise, noon and sunset
3 (24h)	9 days	19	29	Sunrise - 06:30 Sunset - 20:07	Noon, sunset, midnight, sunrise
4 (36h)	7 days	19	25	Sunrise - 06:25 Sunset - 20:13	Sunrise, 3h after sunrise, noon, 3h before sunset, sunset, midnight, 3h before sunrise

6.2.3. Data analyses

Measurements of total calcium and magnesium ion contents of each endolymph sample were converted to units of concentration (mM), and corrected for the ionic content of the DI water into which the samples were originally decanted. All statistical analyses were conducted using "Statistica", a commercially available statistical software package. Differences in mean calcium, magnesium and pH levels between experiments, salinity treatments, sex, maturity and photoperiod (day - night samples) were examined using simple one-way ANOVAs. Where necessary, *post hoc* Tukey HSD tests were performed to identify where the differences occurred. Relationships between the three dependent variables and a suite of explanatory variables were investigated using multiple regression analyses. All of the analyses listed above were conducted for all data pooled over all experiments, as well as for each experiment individually, and for each sex independently.

The objective of the experiments conducted during this study was to establish whether or not a daily cycle could be detected in the investigated parameters of endolymph chemistry. The null hypothesis was proposed that the period of the cyclical fluctuations (if any) was 24 hours. This was tested by fitting a non-linear regression model to the data. The model incorporated a linear component to account for any overall increasing or decreasing trends, as well as a cyclical component in the form of a sine curve to account for a daily cycle in the dependent variable. The model

$$Y = a + bX + c \sin [d (X + e)]$$

where

Y = either pH or calcium or magnesium concentration

X = time (expressed in radians)

was fitted to the data using the non-linear estimation facility of "Statistica". The parameters of the model were estimated using a quasi - Newton iterative procedure with a least-squares approach. The parameters of the model determine:

a - the elevation
b - the slope of the overall trend
c - the amplitude of the cycle
d - the period of the cycle
e - the phase of the cycle

Since the model used a sine curve to describe the possible cycle, a daily cycle (i.e. period = 24 hours) would be described if the period parameter (d) was equivalent to unity. The null hypothesis (H_o: $d = 1$) was tested using a t - test, in a manner similar to that described in Chapter 3. This approach suffers from the same problems regarding type II error as those experienced during the validation studies described in chapter 3. Consequently, power estimates for each test of H_o: $d = 1$ were computed using the methods described in section 3.2.4. The power of each regression to detect a deviation in period of ± 3 hours (an arbitrarily selected value for the purposes of comparison) from a daily period was calculated for each model, and the probability of incurring type II error assessed in each instance.

In most cases, the raw data sets showed a high degree of variability in the dependent variables, which considerably decreased the "goodness of fit" of the models. Because an unknown portion of this variability was due to factors other than time, the multiple regression results were used to account for variability in the data resulting from temperature, animal size (expressed as the dorsal mantle length, DML) and stage of maturity. Before being included in the multiple regression analysis, each explanatory variable was regressed against the dependent variables, and the standardized residuals examined for departures from normality (using the normal plot facility of Statistica) and for homoscedasticity. In all cases, linear models were found to be satisfactory, and no data transformations were applied. The multiple regression models were used to compute predicted values of the dependent variables corresponding to the relevant measurements of the three explanatory variables. These computations were performed for each experiment separately, and independently for males and females. The non-linear regression analyses were then conducted on both the original observations, as well as on the second data set comprising the predicted values. The non-linear model in which the highest proportion of the variance in the dependent variable was explained by time was then employed to test the daily cycle hypothesis as described above. In all but two cases, the models fitted to the predicted values explained a greater proportion of the variance than did those fitted to the original data set. In the cases of the two exceptions, the models fitted to the original data sets were used to test the daily cycle hypothesis.

6.3. RESULTS

Measurements of calcium and magnesium ion concentration were obtained from all four experiments. The raw data are presented in Appendix III. Measurement of strontium concentration proved problematic, and no strontium data were obtained from any of the experiments. Because strontium ions in the endolymph are present in trace quantities (Kalish, 1991 documents levels below $10\ \mu M.l^{-1}$ in bearded rock cod endolymph), and the sample volumes in this study were typically of the order of $1\ \mu l$, the technique employed to measure strontium concentration was incapable of detecting the low quantities of the ion.

Measurements of endolymph pH were only obtained from experiment 2, damage to the microelectrode precluded measurement of pH during the other three experiments. Measurements of endolymph pH averaged 6.82 ± 0.20 (ranging from 6.39 to 7.24). This value is substantially lower than the average of 8.08 ± 0.19 (ranging between 7.65 and 8.43) that was reported for *Alloteuthis subulata* endolymph by Morris (1991a). By normal biological standards, the pH levels measured during this study are very low, some values falling within the range 6.0 to 6.5 that Romanek and Gauldie (1996) consider would result in the dissolution of aragonite rather than its precipitation, and which further would result in tissue degradation if maintained for extended periods. The low pH measurements recorded during this study may have been a result of post-mortality CO_2 diffusion between the endolymph and surrounding tissues while the animals were thawing, with a concomitant effect on pH in the endolymph. An alternative explanation is that the process of sampling the squid induced significant levels of stress-induced anaerobiosis, with associated production of acidic waste products. Whatever the reason for the low pH observations, they are undoubtably not representative of the endolymph pH at the time of sampling. It was consequently concluded that the pH data incorporated a substantial degree of error, and that further analyses of the data could not be justified. No further discussion of the pH results will therefore be included in this chapter.

6.3.1. Calcium

Calcium ion concentrations measured in the statocyst endolymph of *Lolliguncula brevis* show considerable variation, fluctuating between 2.11 mM and 37.49 mM. Three of the measurements are clearly outliers, and were attributed to contamination of the samples, either by statoconia, or by surrounding body fluids. All data from these individuals were consequently excluded from further analysis. The remaining calcium measurements ranged between 2.11 mM and 16.83 mM. When the data were examined for all experiments collectively, mean calcium levels showed significant differences between experiments (ANOVA, $F_{3,130} = 13.2551$, $p = 0.0000$), between salinities (ANOVA, $F_{2,131} = 12.8864$, $p = 0.0000$), and between sexes (ANOVA, $F_{1,132} = 6.8022$, $p = 0.0102$). *Post hoc* Tukey tests indicated that differences in overall mean calcium levels cannot be attributed to either salinity or temperature, since no patterns in the differences were detected. The difference between sexes is primarily a function of the first experiment, which included only two males (see section 6.3.1.1). The other three experiments, examined independently, showed no differences in mean endolymph calcium levels between male and female squid. Photoperiod did not appear to influence mean calcium levels when data from all experiments were analyzed collectively. No differences were evident between mean calcium levels recorded during the day and night (ANOVA, $F_{1,132} = 0.2482$, $p = 0.6192$), even when males and females were examined separately. It is possible that variability in the data resulting from differences in conditions between experiments could obscure any underlying temporal patterns in the data, leading to the negative results regarding day - night differences discussed above. Further analyses were therefore conducted on the data from each experiment independently in an attempt to minimize between - experiment variability.

6.3.1.1. Experiment 1

Measurements of endolymph calcium concentration were obtained from 31 squid during this experiment (Fig. 6.1). Note that the data set contains measurements from only two male squid (52 mm and 56 mm DML respectively), the remaining 29 individuals that were successfully sampled were female, ranging in size from 48 mm to 75 mm DML. Temperature showed an increase from 13 °C to 14.8 °C during the experiment (Fig. 6.1 B). This was a result of a poor temperature control system. The experimental tanks were maintained in a laboratory where ambient temperature was not controlled. The system that was used to control water temperature could not counteract the effects of a dramatic increase in ambient temperature that occurred during the experiment. Calcium levels, with the exception of one measurement from the second 19h00 sampling interval, ranged from 2.11 mM to 8.91 mM, the majority of the measurements falling within a relatively narrow range of 2 mM to 5 mM (Fig. 6.1 A). The outlier in the second 19h00 sampling interval (18.33 mM) was attributed to contamination of the sample, and all data from this individual were excluded from further analyses.

Mean calcium levels show significant differences between males and females (ANOVA, $F_{1,28} = 6.5459$, $p = 0.0162$), but this is most likely due to the fact that only two males were sampled during this experiment. No differences in mean calcium levels are apparent between females at different stages of maturity (ANOVA, $F_{3,24} = 2.0548$, $p = 0.1330$). Since only two males were sampled, the influence of maturity could not be assessed. Examination of the data in Fig. 6.1 suggests the presence of a daily cycle in endolymph calcium levels. Measurements obtained during the day are slightly higher and more variable than those obtained during the night. However, these differences are not significant (ANOVA, $F_{1,28} = 3.0411$, $p = 0.092$). Further, no differences in mean calcium concentrations occur between the individual sampling intervals (ANOVA, $F_{8,21} = 1.2538$, $p = 0.319$). The same result is obtained when males and females are assessed separately. This is most likely due to the fact that, with the exception of the very first sampling interval, elevated mean calcium concentrations (and variability) in the midday samples are the result of a single elevated datum in each case. In an effort to remove variability in the data associated with temperature, size, sex and stage of maturity, the female data were subjected to a multiple regression analysis. No such analysis could be conducted for the male data because only two males were sampled. Neither the regression nor the coefficients are statistically significant (Table 6.2).

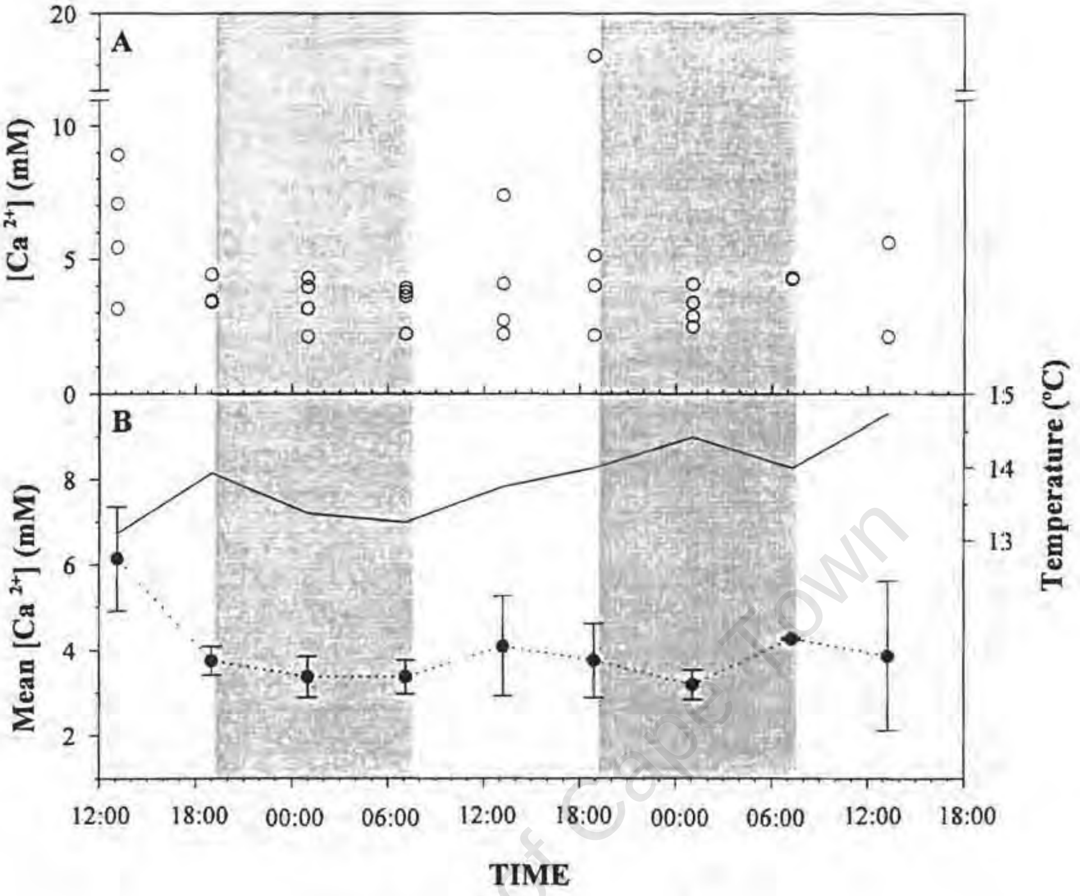


Figure 6.1: Calcium concentrations recorded during experiment 1. (A) Individual measurements; (B) mean calcium concentrations (± 1 standard error) at each sampling interval (note that the outlier in the upper plot has been excluded). Temperature is indicated with a solid line in B. Shaded areas represent periods of darkness.

Table 6.2: Coefficients and associated statistics of the multiple regression model fitted to the calcium data obtained from female squid during experiment 1 (n = 28; $r^2 = 0.1063$, $F_{3,24} = 0.9519$, $p = 0.4313$).

Coefficient	Estimate	Standard Error	t_{24}	p
Intercept	6.414	7.2637	0.8830	0.3860
Temperature	- 0.090	0.5379	- 0.1671	0.8687
DML	- 0.052	0.0827	- 0.6293	0.5351
Maturity	0.538	0.4890	1.1735	0.2521

In spite of this, the coefficients of the model were used to calculate predicted calcium concentrations for each female. The original data for the two males was included in this data set. The non-linear model described in section 6.2.4 was fitted to both the original and the predicted data sets.

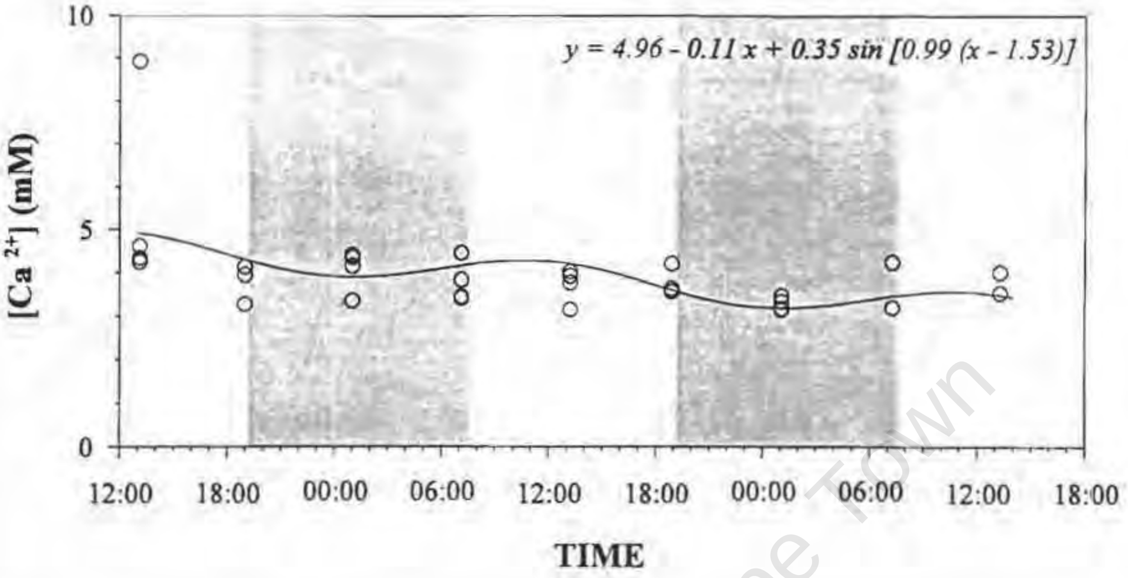


Figure 6.2: Graph illustrating the non-linear model fitted to the calcium data obtained in experiment 1. The expression describing the model is given in the graph, and the associated statistics are given in Table 6.3. Note that the data points are predicted calcium concentrations computed from the multiple regression results shown in Table 6.2 (see text).

Table 6.3: Parameter estimates and associated statistics of the non-linear regression model fitted to the calcium data obtained during experiment 1 (n = 30; $r^2 = 0.2563$, $F_{1,28} = 9.6516$, $p = 0.0043$). Note that with the exception of the two male squid, the data used in the analysis were predicted values of calcium concentration computed from the multiple regression analysis.

Parameter	Estimate	Standard Error	t_{25}	p
a	4.961	0.4688	10.5831	0.0000
b	- 0.111	0.0473	- 2.3368	0.0278
c	0.351	0.2343	1.4970	0.1469
d	0.999	0.1321	7.5701	0.0000
e	- 1.5343	1.1400	- 1.3459	0.1904

Both models were significant, but the model generated by the predicted data set (Fig. 6.2, Table 6.3) shows a slightly better fit than that derived from the original data ($r^2_{\text{pred}} = 0.2563$ vs. $r^2_{\text{obs}} = 0.2071$), and was consequently used to test the daily cycle hypothesis. The model describes a daily cycle in calcium concentrations peaking during the day and decreasing to a minimum at night, superimposed over a gradually decreasing trend. The period of the cycle (d = 0.999, equivalent to 24.02 hours) is not significantly different from one ($t_{25} = 0.0017$, $p = 0.9987$), and it is concluded that the fluctuations in endolymph calcium levels are consistent with a daily cycle. The estimated power of the test (14.27%), however, indicates a very high probability of incurring type II error. The conclusion should consequently be viewed with some scepticism.

6.3.1.2. Experiment 2:

Measurements of calcium concentration (Fig. 6.3) were obtained from 18 female (40 mm to 80 mm DML) and 12 male squid (41 mm to 57 mm DML).

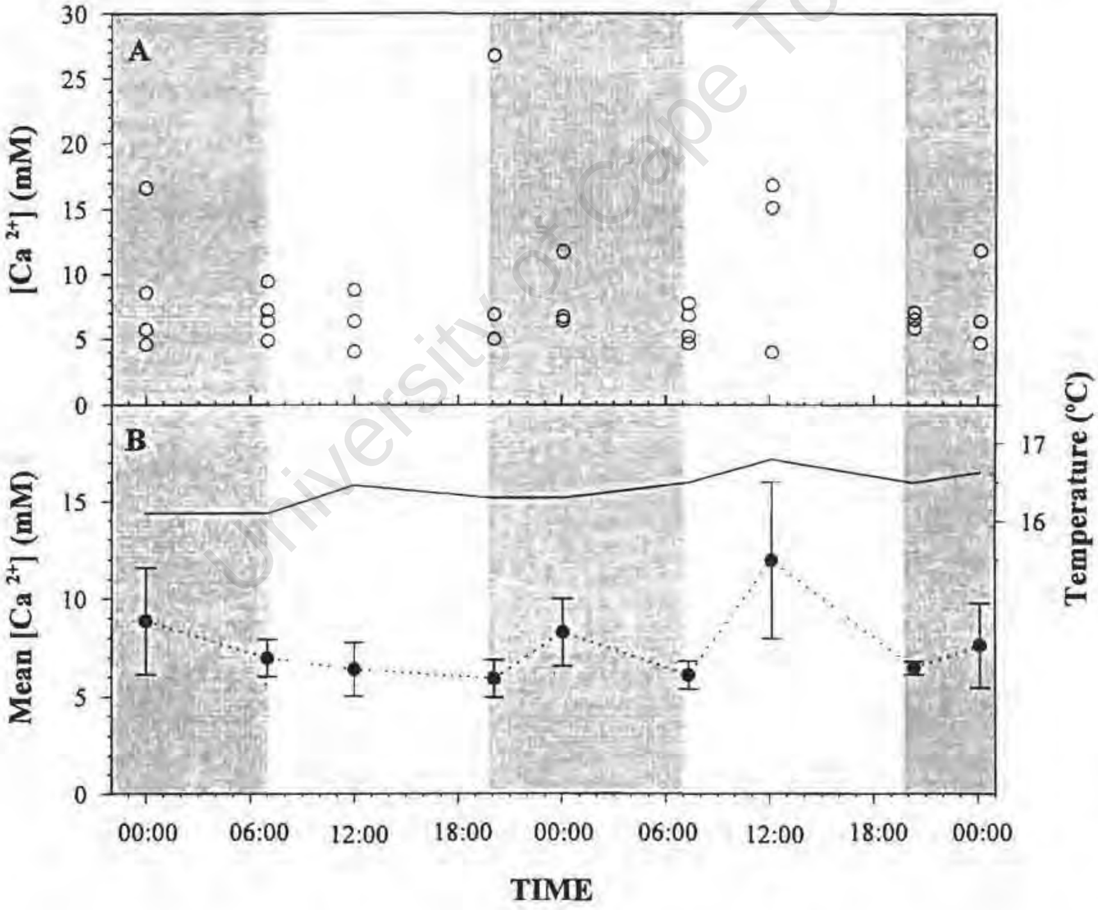


Figure 6.3: Calcium ion concentrations recorded during experiment 2. (A) Individual measurements; (B) mean concentrations (± 1 SE) for each interval (note that the outlier in A has been excluded). Temperature is indicated with a solid line in B. Shaded areas represent periods of darkness.

Temperature increased from 16.1 °C to 16.8 °C during the experiment, with a daily fluctuation peaking at noon (Fig. 6.3 B). Modification of the temperature control system subsequent to experiment 1 improved temperature control, but was still not satisfactory. The data contained a single outlier in which the concentration (26.73 mM) was considerably higher than the other measurements. This individual was excluded on the basis of sample contamination. The remaining data ranged between 3.98 mM and 16.83 mM, the majority of the measurements falling between 4 mM and 10 mM (Fig. 6.3). No differences in mean calcium levels were evident between sexes (ANOVA, $F_{1,27} = 0.7942$, $p = 0.3807$) or stages of maturity (ANOVA. Females: $F_{2,14} = 0.0476$, $p = 0.6308$. Males: $F_{1,10} = 0.2024$, $p = 0.6624$).

The pattern in calcium fluctuations that was observed in experiment 1 is not apparent in the results of this experiment. Peaks occur in all midnight intervals, in contrast to the noon peaks evident in the first experiment. No differences in mean calcium concentrations are apparent between day and night samples (ANOVA, $F_{1,27} = 0.0009$, $p = 0.9765$) or between the sampling intervals (ANOVA, $F_{8,20} = 0.8541$, $p = 0.5687$). The multiple regression analyses conducted separately on the female and male data generated the models shown in Tables 6.4 and 6.5 respectively.

Table 6.4: Coefficients and associated statistics of the multiple regression model fitted to the calcium data obtained from female squid during experiment 2 (n = 16; $r^2 = 0.1684$, $F_{3,12} = 0.8105$, $p = 0.5122$).

Coefficient	**Estimate**	**Standard Error**	t_{12}	p
Intercept	- 26.082	75.3853	- 0.3460	0.7353
Temperature	2.246	4.5450	0.4942	0.6301
DML	- 0.182	0.2076	- 0.8768	0.3978
Maturity	2.026	1.5770	1.2848	0.2231

Table 6.5: Coefficients and associated statistics of the multiple regression model fitted to the calcium data obtained from male squid during experiment 2 (n = 12; $r^2 = 0.3529$, $F_{3,8} = 1.4545$, $p = 0.2980$).

Coefficient	**Estimate**	**Standard Error**	t_8	p
Intercept	- 40.4506	64.8724	- 0.6235	0.5503
Temperature	3.136	3.8035	0.8246	0.4335
DML	- 0.353	0.1818	- 1.9395	0.0884
Maturity	2.989	2.5105	1.1905	0.2680

Neither of the models are significant, and none of the coefficients indicate a significant influence on endolymph calcium levels. In spite of this, predicted endolymph calcium concentrations were computed for each squid using the coefficients. The non-linear regressions were conducted on these values, as well as on the original data set. The non-linear model fitted to the predicted values (Fig. 6.4,

Table 6.6) shows a slightly better fit than that fitted to the original data ($r^2_{pred} = 0.1693$ vs. $r^2_{obs} = 0.1188$).

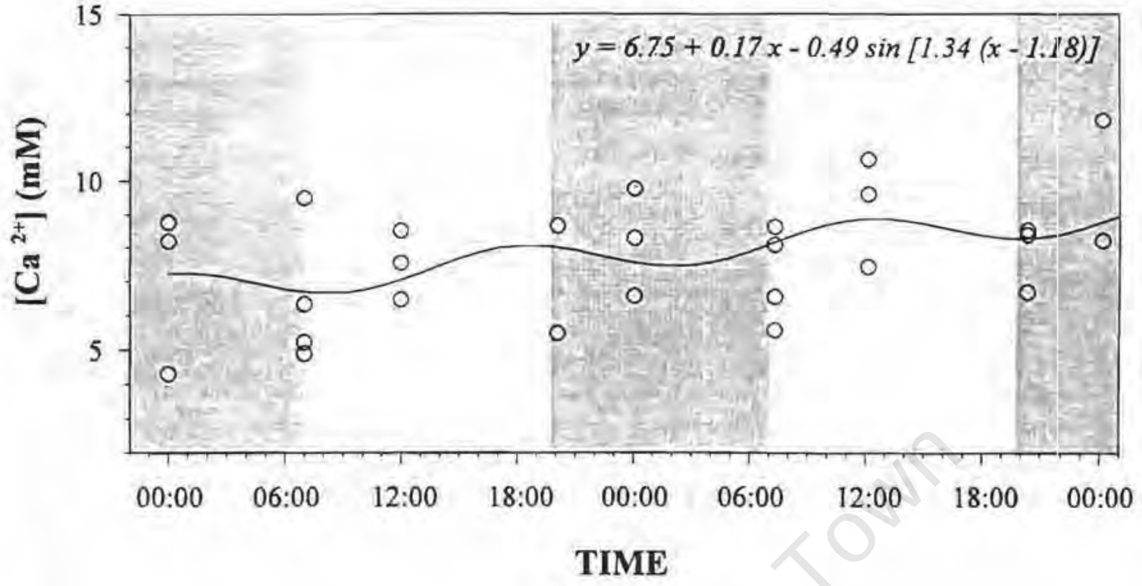


Figure 6.4: Graph illustrating the non-linear model fitted to the calcium data obtained during experiment 2. The expression describing the model is shown in the plot, and the relevant statistics are provided in Table 6.6. Note that the data points in the plot are predicted endolymph calcium concentrations computed from the multiple regression results provided in Tables 6.4 and 6.5.

Table 6.6: Parameter estimates and associated statistics of the non-linear regression model fitted to the calcium data obtained during experiment 2 (n = 28; $r^2 = 0.1693$, $F_{1,26} = 5.2989$, $p = 0.0296$). Note that the calcium data used in the non-linear modelling were predicted values of calcium concentration computed from the multiple regression analyses.

Parameter	Estimate	Standard Error	t_{23}	p
a	6.753	0.3127	11.0232	0.0000
b	0.172	0.0975	1.7636	0.0911
c	- 0.487	0.5877	- 0.8282	0.4161
d	1.344	0.3035	4.4291	0.0002
e	- 1.1817	1.4722	- 0.8027	0.4304

Further, the former model is statistically significant ($F_{1,26} = 5.2989$, $p = 0.0296$), whereas the model fitted to the original data is not ($F_{1,27} = 3.6397$, $p = 0.0671$). The model fitted to the predicted values was consequently used to test the daily cycle hypothesis. The period parameter (d = 1.344, equivalent to a cycle period of 17.9 hours) indicates that calcium concentrations fluctuate over a period substantially shorter than one day. Although the estimated period is not significantly different from unity ($t_{23} = 1.1336$, $p = 0.2686$), this result was primarily due to the high standard error associated with the estimate, and this is reflected in the low power of the test (6.64%). The conclusion that the calcium data support the daily cycle hypothesis is therefore questionable. Owing to the short period, the pattern described by the model differs from that observed in the previous experiment. The first peak in endolymph calcium levels occurred near midnight, followed by a peak late the next afternoon, with the last peak evident at noon on the last day of the experiment. This pattern conflicts with the regular noon peaks evident during experiment 1. Further, an increasing linear trend underlying the calcium fluctuations is apparent in the data from experiment 2, as opposed to the gradually decreasing trend evident during the first experiment. These inconsistencies between the experiments cast further doubts on the reliability of the results, particularly with respect to the conclusion that the data are consistent with a daily cycle in endolymph calcium concentration.

6.3.1.3. Experiment 3:

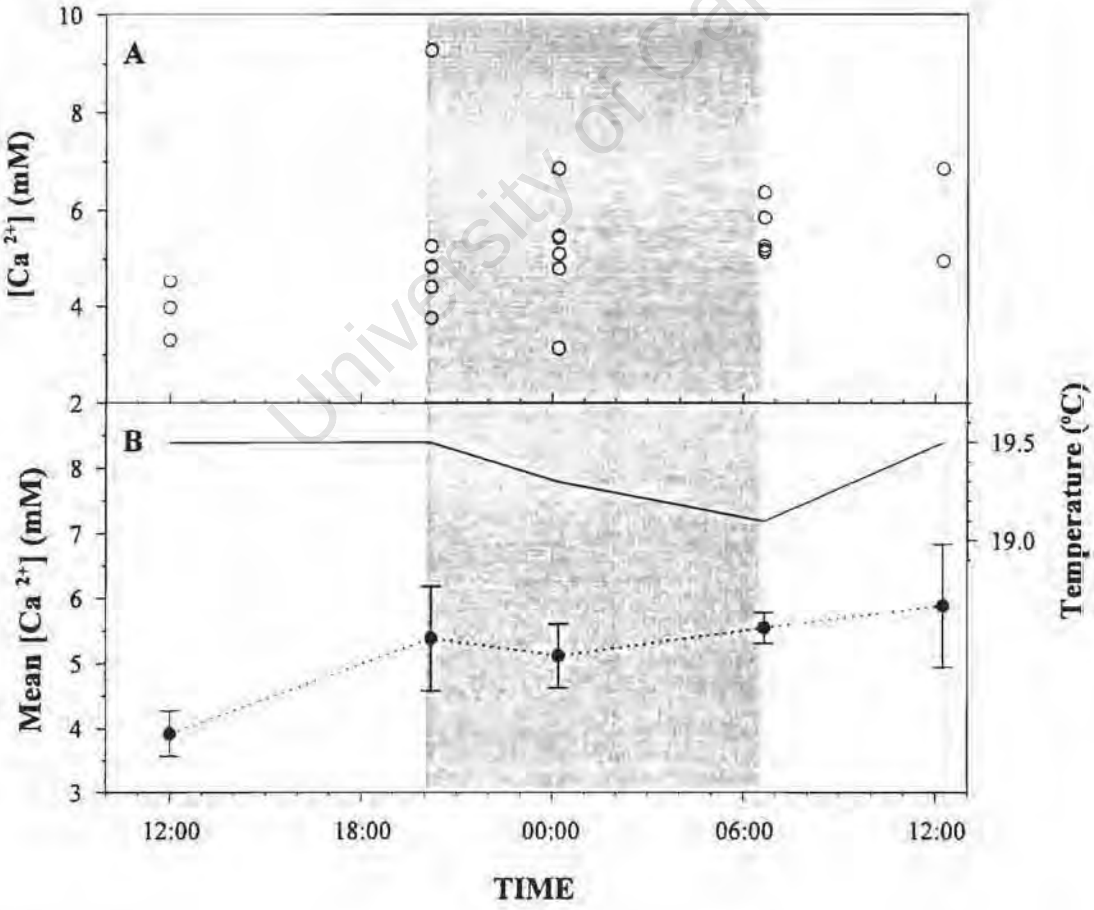


Figure 6.5: Calcium concentrations recorded during experiment 3. (A) Individual measurements; (B) mean concentrations (± 1 SE) for each interval. Temperature is illustrated with a solid line in B. Shaded areas represent periods of darkness.

Measurements of calcium concentration were obtained from eleven female (41 mm to 81 mm DML) and eleven male squid (44 mm to 67 mm DML) sampled over a 24 hour period (Fig. 6.5). Ambient temperature fluctuated between 19.1 °C and 19.5 °C and displayed a daily cycle, with temperatures decreasing at night, and increasing to a maximum during the day (Fig. 6.5 B). Calcium concentrations fluctuated between 3.11 mM and 9.26 mM (Fig. 6.5 A), the majority of the data falling in the range 3 mM to 7 mM. No differences in mean calcium levels were apparent between sexes (ANOVA, $F_{1,20} = 0.0045$, $p = 0.9470$) or stages of maturity (ANOVA. Females: $F_{2,8} = 3.1247$, $p = 0.0994$. Males: $F_{1,9} = 0.0030$, $p = 0.9574$). The data show no evidence for a daily cycle in endolymph calcium levels. Rather, an almost linear increasing trend is apparent (Fig. 6.5 B). Mean calcium levels show no significant differences between day and night samples (ANOVA, $F_{1,20} = 0.0426$, $p = 0.8386$) or between individual intervals (ANOVA, $F_{4,17} = 0.9388$, $p = 0.4653$). The results of the multiple regression analyses conducted separately on the data from female and male squid are shown in Tables 6.7 and 6.8 respectively.

Table 6.7: Coefficients and associated statistics of the multiple regression model fitted to the calcium measurements recorded in female squid during experiment 3 (n = 11; $r^2 = 0.7526$, $F_{3,7} = 7.0963$, $p = 0.0158$).

Coefficient	Estimate	Standard Error	t_7	p
Intercept	- 32.926	23.3649	- 1.4092	0.2016
Temperature	2.210	1.2158	1.8175	0.1120
DML	- 0.066	0.0291	- 2.2775	0.0568
Maturity	- 0.078	0.2589	- 0.3026	0.7710

Table 6.8: Coefficients and associated statistics of the multiple regression model fitted to the calcium measurements recorded in male squid during experiment 3 (n = 11; $r^2 = 0.1773$, $F_{3,7} = 0.5027$, $p = 0.6924$).

Coefficient	Estimate	Standard Error	t_7	p
Intercept	44.584	68.4880	0.6510	0.5358
Temperature	- 2.368	3.5162	- 0.6734	0.5223
DML	0.086	0.0901	0.9499	0.3738
Maturity	0.324	0.954	0.3395	0.7442

The analyses generated a significant model for the female data, but not for males. None of the explanatory variables exert a significant influence on calcium levels, although an almost significant negative size effect is apparent in females. The coefficients from the models were used to predict endolymph calcium levels for each squid, and these values, as well as the original data set were analysed by non-linear regression. No meaningful non-linear model could be fitted to the original data set. A simple linear model fitted to these data explained 12% of the variance. The non-linear model fitted to the predicted values (Fig. 6.6), however, describes a significant relationship between time and endolymph calcium levels (Table 6.9).

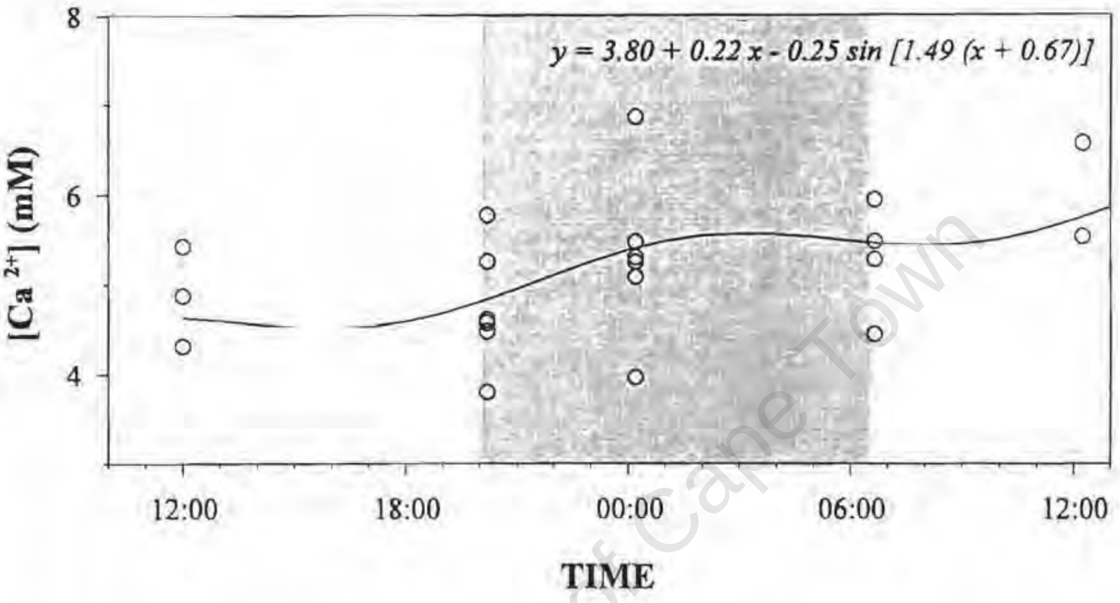


Figure 6.6: Graph illustrating the non-linear model fitted to the calcium data obtained during experiment 3. The expression describing the model is shown, and the associated statistics are given in Table 6.9. Note that data points are predicted concentrations computed from the multiple regression results in Tables 6.7 and 6.8.

Table 6.9: Parameter estimates and associated statistics of the non-linear regression model fitted to the calcium data obtained during experiment 3 ($n = 22$; $r^2 = 0.2293$, $F_{1,20} = 5.9501$, $p = 0.0242$). Note that the calcium data were predicted values of calcium concentration computed from the multiple regression analyses.

Parameter	Estimate	Standard Error	t_{17}	p
a	3.797	0.7353	5.1647	0.0001
b	0.220	0.1123	1.9605	0.0665
c	- 0.251	0.3439	- 0.7310	0.4747
d	1.4910	0.7104	2.0988	0.0511
e	0.671	3.2020	0.2095	0.8365

The period of the model (d = 1.491, equivalent to 16.1 hours) is considerably less than 24 hours, but owing to the high standard error associated with the estimate, no significant difference from a daily period was detected ($t_{17} = 0.6911, p = 0.4988$). This is reflected in the low power of the test (about 5%). The conclusion that the model supports a daily cycle has to be viewed with considerable scepticism. The model predicts that calcium concentrations peak during the night, which does not correspond to the day maxima observed in the previous two experiments. Further, the increasing linear trend underlying the cycle, while consistent with that observed in the previous experiment, conflicts with the decreasing trend observed in experiment 1.

6.3.1.4. Experiment 4:

Measurements of calcium ion concentrations were obtained from thirty-eight female (61 mm to 90 mm DML) and 16 male squid (54 mm to 74 mm DML) sampled over a 36 hour period (Figure 6.7).

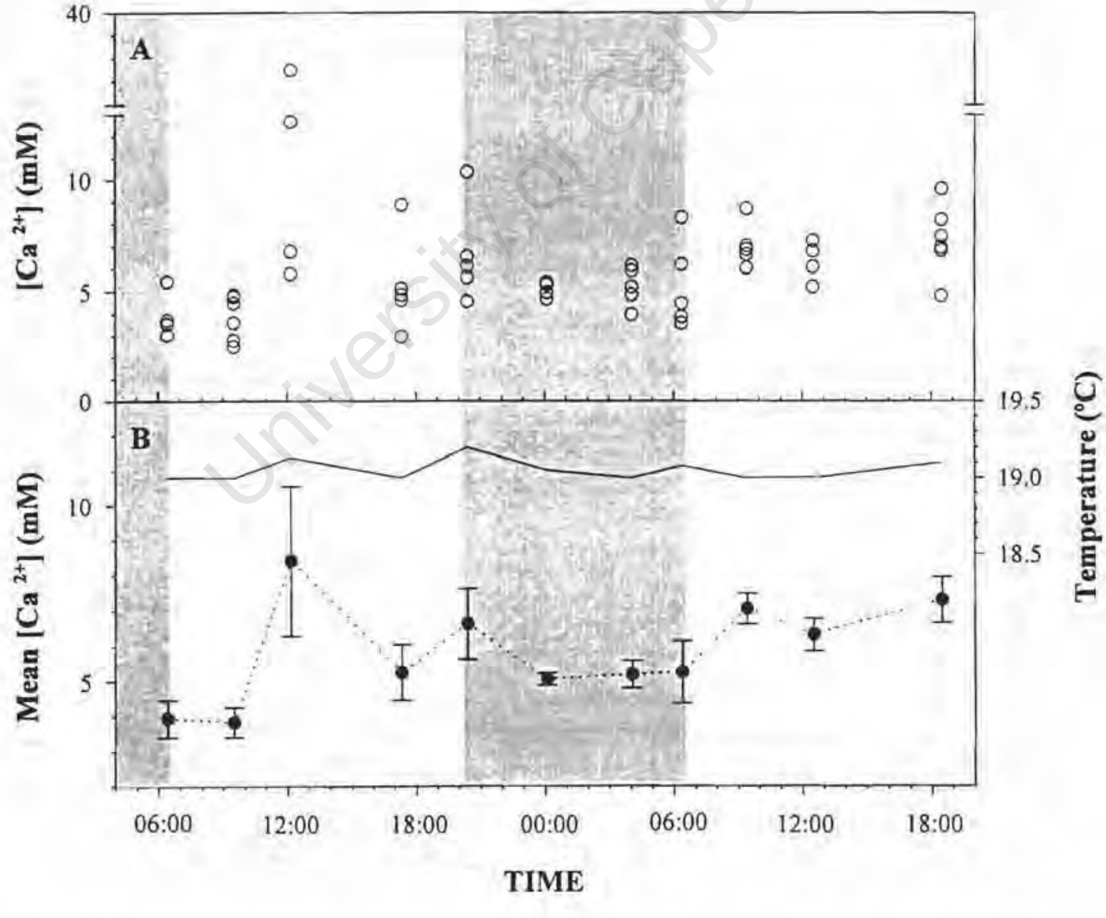


Figure 6.7: Calcium concentrations recorded during experiment 4. (A) Individual measurements; (B) mean concentrations (± 1 SE) for each interval (note that the outlier in A has been excluded). Temperature is indicated with a solid line in B. Shaded areas represent periods of darkness.

Temperature fluctuated by only 0.2 °C over the course of the experiment (Fig. 6.7 B). No daily cycle in the temperature fluctuations was apparent. Calcium concentrations measured during this experiment display considerable variation, particularly during the first 24 hours. An outlier was obtained in the first noon sample (37.49 mM, Fig. 6.7 A). All data from this individual were excluded from further analyses on the basis of probable sample contamination. The remaining measurements fluctuated between 2.51 mM and 12.66 mM, with the majority falling in the range 2.5 mM to 8.0 mM.

No differences in mean calcium concentrations were detected between sexes (ANOVA, $F_{1,51}$ = 0.6319, p = 0.4304), maturity stages (ANOVA. Females: $F_{4,32}$ = 1.9326, p = 0.1290. Males: $F_{1,14}$ = 3.3919, p = 0.0868) or between day and night samples (ANOVA, $F_{1,51}$ = 0.0407, p = 0.8409). There are significant differences between intervals (ANOVA, $F_{10,42}$ = 3.3689, p = 0.0027), but a *post hoc* Tukey test indicated that this was due to the mean for the second interval (09:30) differing from those of the third (12:00) and the last (18:30) intervals. There are no temporal patterns indicating day - night differences. The multiple regression analyses conducted separately on the female and male data generated the results given in Tables 6.10 and 6.11 respectively. Neither model is significant, and the only significant effect is a positive relationship between temperature and female endolymph calcium levels (Table 6.10).

Table 6.10: Coefficients and associated statistics of the multiple regression model fitted to the calcium measurements recorded in female squid during experiment 4 (n = 35; r^2 = 0.1446, $F_{3,31}$ = 1.7473, p = 0.1771).

Coefficient	Estimate	Standard Error	t_{31}	p
Intercept	- 194.645	89.7987	- 2.1676	0.0380
Temperature	10.363	4.6861	2.2115	0.0345
DML	0.049	0.0507	0.9603	0.3444
Maturity	- 0.159	0.3891	- 0.4075	0.6864

Table 6.11: Coefficients and associated statistics of the multiple regression model fitted to the calcium measurements recorded in male squid during experiment 4 (n = 16; r^2 = 0.2736, $F_{3,12}$ = 1.5068, p = 0.2629).

Coefficient	Estimate	Standard Error	t_7	p
Intercept	- 94.078	102.3300	- 0.9194	0.3760
Temperature	6.077	5.4288	1.1194	0.2849
DML	- 0.008	0.0762	- 0.1042	0.9187
Maturity	- 3.067	1.6830	- 1.8223	0.0934

Predicted calcium contents were calculated for each squid using the coefficients. These values, together with the original data set, were subjected to the non-linear regression analyses. Both non-linear models were highly significant, but, in contrast to the previous three experiments, the original data set yielded a "better" model than did the predicted values ($r^2_{obs} = 0.2285$ vs $r^2_{pred} = 0.1346$). The former model (Fig. 6.8, Table 6.12) was consequently used to test the daily cycle hypothesis.

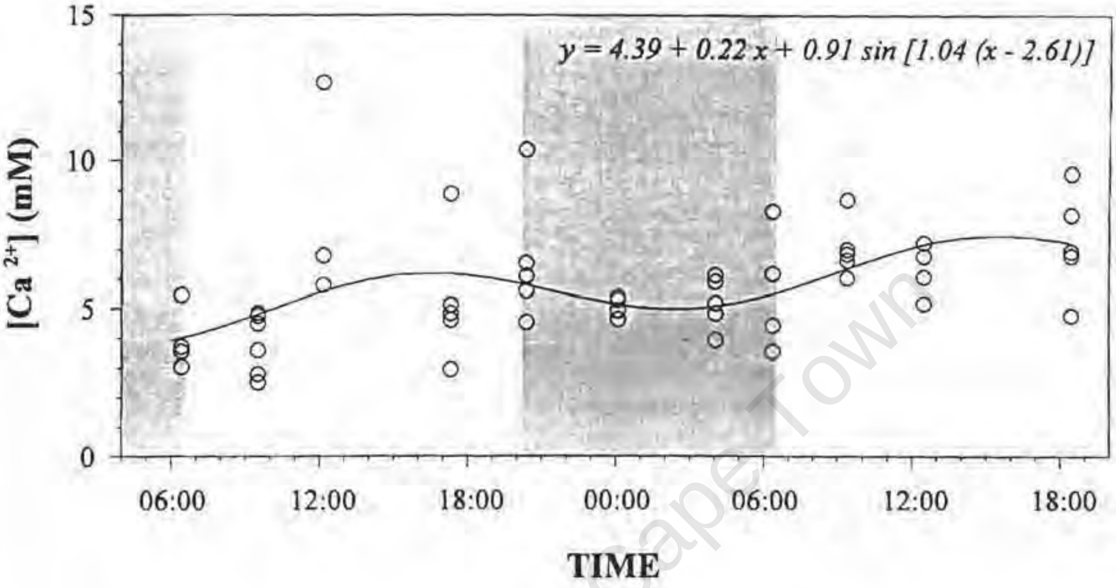


Figure 6.8: Graph illustrating the non-linear model fitted to the calcium data from experiment 4. The expression describing the model is shown in the plot, and the relevant statistics are provided in Table 6.12. Note that the data points in the plot are the original measurements of calcium concentration.

Table 6.12: Parameter estimates and associated statistics of the non-linear regression model fitted to the calcium data obtained during experiment 4 (n =53; $r^2 = 0.2285$, $F_{1,51} = 15.1016$, $p = 0.0003$). Note that the calcium data used in the non-linear modelling were the original measurements of calcium concentration recorded during the experiment.

Parameter	Estimate	Standard Error	t_{48}	p
a	4.393	0.6606	6.6500	0.0000
b	0.215	0.0894	2.4023	0.0202
c	0.909	0.5257	1.7285	0.0903
d	1.044	0.1626	6.4196	0.0000
e	- 2.6113	0.8459	- 3.0871	0.0034

The period parameter of the model (d = 1.044) describes a 23 hour cycle. This value shows no significant difference from a daily cycle ($t_{48} = 0.2742, p = 0.7851$). The data are therefore concluded to be consistent with daily cycle in endolymph calcium content. In spite of the low deviation of the cycle from a daily period, the estimated power of the test is low (about 11 %), indicating a high probability that the null hypothesis is in fact false. The pattern described by the model is similar to those in experiments 1 and 2, with peaks in endolymph calcium concentration occurring during the day, followed by decreased levels at night. The increasing linear trend underlying the cycle corresponds to those in experiments 2 and 3, but not to the decreasing trend apparent in experiment 1.

6.3.2. Magnesium:

Magnesium ion concentrations in the statocyst endolymph were generally considerably higher than calcium, fluctuating between 7.50 mM and about 30 mM. Mean levels are significantly different between experiments (ANOVA, $F_{3,130} = 16.2637, p = 0.0000$), but not between salinities (ANOVA, $F_{2,131} = 2.1678$, $p = 0.1185$). No differences in overall magnesium levels are evident between the sexes (ANOVA, $F_{1,132} = 0.6308, p = 0.4285$). Photoperiod does not appear to influence mean magnesium levels pooled over all experiments, since samples obtained at night showed no differences from those obtained during the day (ANOVA, $F_{1,132} = 0.4760$, $p = 0.4914$), even when females and males are examined independently. Further analyses were conducted for each experiment independently in an attempt to minimize between-experiment variability.

6.3.2.1. Experiment 1:

Magnesium concentrations fluctuated between 10.74 mM and 20.74 mM, with most of the data falling in the 10 mM to 17 mM range (Fig. 6.9).

Mean magnesium levels calculated for each sampling interval (Fig. 6.9 B) suggest a clear daily cycle in this variable. Highest concentrations occurred near midnight, dropping to a minimum during the day (the reverse pattern to calcium fluctuations). Only the last sampling interval deviates from this pattern, possibly due to the sudden increase in temperature that occurred prior to this interval. These temporal differences are not significant, either in terms of day-night comparisons (ANOVA. $F_{1,28} = 3.9711, p = 0.056$) or between intervals (ANOVA. $F_{8,21} = 1.9537, p = 0.105$). The multiple regression analysis fitted to the female data generated a model (Table 6.13) that was not significant.

None of the explanatory variables had a significant effect on female endolymph magnesium levels. In spite of this result, the model was used to compute predicted magnesium levels for each female squid (the original data from the two males were included in this data set). These values, as well as the original data set were analyzed using the non-linear regression procedure.

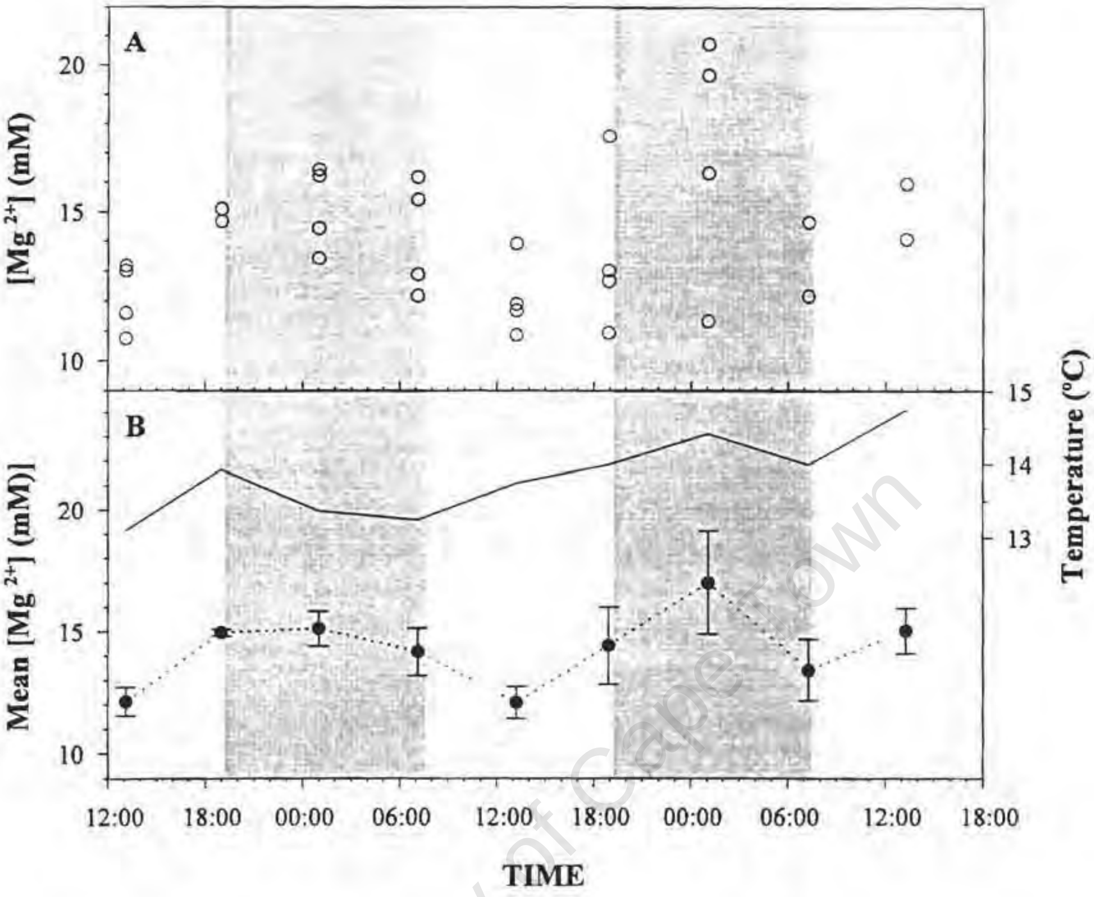


Figure 6.9: Magnesium concentrations recorded during experiment 1. (A) Individual measurements; (B) mean concentrations (± 1 SE) for each interval. Temperature at each interval is shown with a solid line. The shaded areas represent periods of darkness.

Table 6.13: Coefficients and associated statistics of the multiple regression model fitted to the magnesium data obtained from female squid during experiment 1 (n = 28; $r^2 = 0.1567$, $F_{3,24} = 1.4866$, $p = 0.2434$).

Coefficient	Estimate	Standard Error	t_{24}	p
Intercept	- 6.531	12.5933	- 0.5186	0.6088
Temperature	0.982	0.9326	1.0528	0.3029
DML	0.171	0.1434	1.1933	0.2444
Maturity	- 0.958	0.7958	- 1.2044	0.2402

The model fitted to the predicted values (Fig. 6.10, Table 6.14) shows a better fit than does the model fitted to the original data set ($r^2_{pred} = 0.5057$ vs. $r^2_{obs} = 0.3469$).

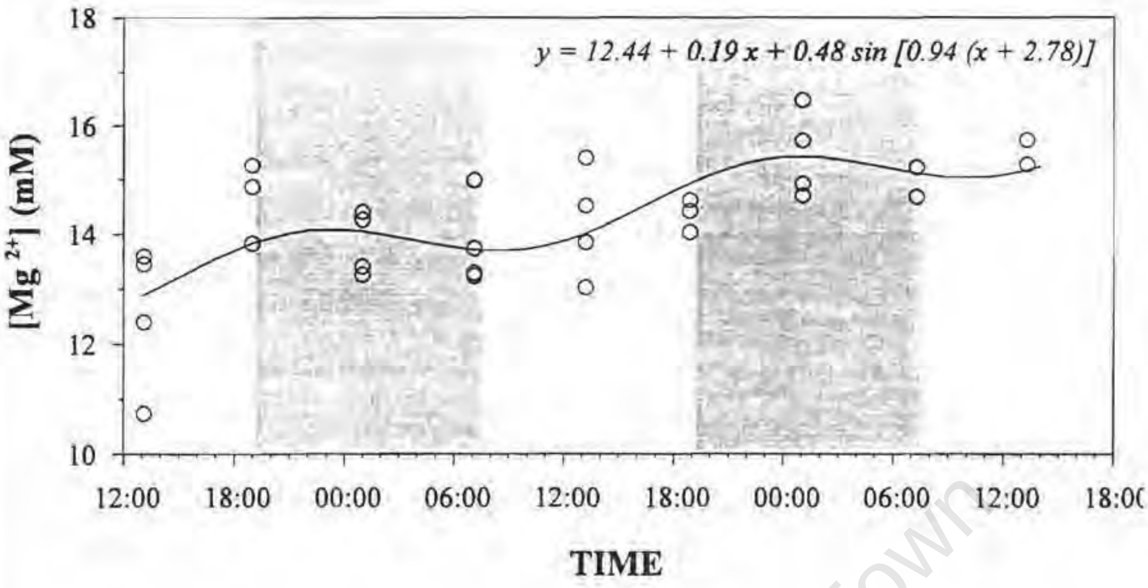


Figure 6.10: Graph illustrating the non-linear model fitted to the magnesium data obtained during experiment 1. The expression describing the model is given in the graph, and the associated statistics are shown in Table 6.14. Note that the data points in the plot are predicted magnesium concentrations calculated from the multiple regression results given in Table 6.13

Table 6.14: Parameter estimates and associated statistics of the non-linear regression model fitted to the magnesium data obtained during experiment 1 (n = 30; $r^2 = 0.5057$, $F_{1,28} = 28.6446$, $p = 0.000$). Note that the magnesium data used in the analysis were predicted magnesium concentrations computed from the results of the multiple regression analysis (see text).

Parameter	Estimate	Standard Error	t_{25}	p
a	12.436	0.4948	25.1348	0.0000
b	0.199	0.0491	4.0503	0.0004
c	0.484	0.2453	1.9721	0.0598
d	0.936	0.1671	5.6030	0.0000
e	2.775	2.0265	1.3693	0.1831

The period of the cycle (d = 0.936, equivalent to 25.6 hours) is not significantly different from unity ($t_{25} = 0.3813$, $p = 0.7062$), supporting a daily cycle in endolymph magnesium concentration. However, the power estimate associated with this test (10.67%) is even lower than that computed for the calcium non-linear regression, casting some doubt on the conclusion. According to the model, magnesium levels in the endolymph peak during the night, dropping to a minimum during the day. This is the opposite trend to that observed in the calcium data, suggesting a negative relationship between calcium and magnesium levels. However, no significant correlation was detected between calcium and magnesium concentrations ($r = -0.2227, p = 0.2370$). The model describes an increasing linear trend underlying the daily cycle, in contrast to the decreasing trend evident in the calcium data. Although these trends suggest a relationship between these two variables and temperature (which increased steadily over the course of the experiment), no significant correlations between temperature and either calcium or magnesium were detected (Calcium: $r = -0.2328, p = 0.2160$; Magnesium: $r = 0.3573, p = 0.0530$), a result also evident in the multiple regression models presented above.

6.3.2.2. Experiment 2:

Magnesium concentrations ranged between 7.50 mM and 20.00 mM during this experiment (Fig. 6.11 A).

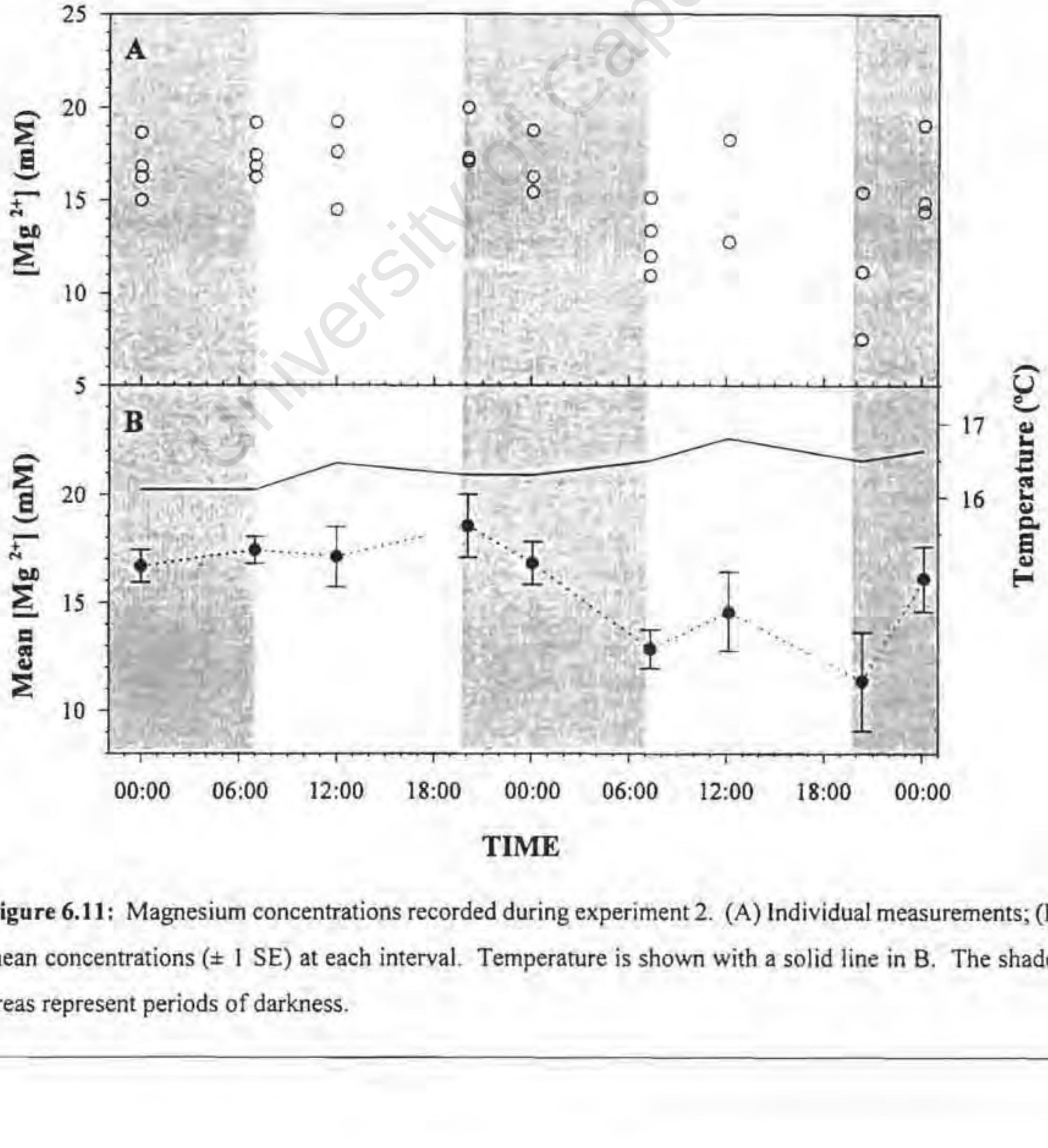


Figure 6.11: Magnesium concentrations recorded during experiment 2. (A) Individual measurements; (B) mean concentrations (± 1 SE) at each interval. Temperature is shown with a solid line in B. The shaded areas represent periods of darkness.

The majority of the measurements fluctuated over a range of 15 mM and 20 mM during the first 24 hours of the experiment, and over a much broader range of between 7 mM and 19 mM during the second 24 hours. No differences in mean magnesium concentrations were detected between sexes (ANOVA, $F_{1,27} = 0.4297$, $p = 0.5177$) or stages of maturity (ANOVA. Females: $F_{2,14} = 1.1490$, $p = 0.3451$. Males: $F_{1,10} = 1.2230$, $p = 0.2947$). Although a significant difference was detected between the individual sampling intervals (ANOVA, $F_{8,20} = 3.1043$, $p = 0.0190$), a *post hoc* Tukey test indicated that this result was primarily due to the eighth interval (i.e. second sunset sample), and not a result of any pattern of day - night differences. Mean magnesium concentrations show no significant differences between day and night samples (ANOVA, $F_{1,27} = 0.0913$, $p = 0.7648$), even when females and males are analyzed separately. The multiple regression analyses generated the models shown in Tables 6.15 and 6.16. Neither model is significant, and the only significant effect is a strong negative relationship between temperature and female magnesium levels (Table 6.15).

Table 6.15: Coefficients and associated statistics of the multiple regression model fitted to the magnesium data obtained from female squid during experiment 2 (n = 16; $r^2 = 0.3241$, $F_{3,12} = 1.9182$, $p = 0.1805$).

Coefficient	Estimate	Standard Error	t_{12}	p
Intercept	159.076	60.5511	2.6271	0.0221
Temperature	- 8.725	3.6506	- 2.3899	0.0341
DML	- 0.023	0.1667	- 0.1359	0.8942
Maturity	0.103	1.2667	0.0814	0.9364

Table 6.16: Coefficients and associated statistics of the multiple regression model fitted to the magnesium data obtained from male squid during experiment 2 (n = 12; $r^2 = 0.1608$, $F_{3,8} = 0.5109$, $p = 0.6859$).

Coefficient	Estimate	Standard Error	t_8	p
Intercept	35.222	54.3717	0.6478	0.5353
Temperature	- 1.599	3.1879	- 0.5017	0.6294
DML	- 0.065	0.1524	- 0.4286	0.6795
Maturity	2.120	2.1042	1.0075	0.3432

Predicted endolymph magnesium levels were calculated for each squid, and the non-linear model then fitted to both the original and the predicted data sets. The models fitted to both data sets were highly significant, but the model fitted to the predicted values (Fig. 6.12, Table 6.17) explained considerably more of the variance than did that fitted to the original data, and was consequently used to test the daily cycle hypothesis.

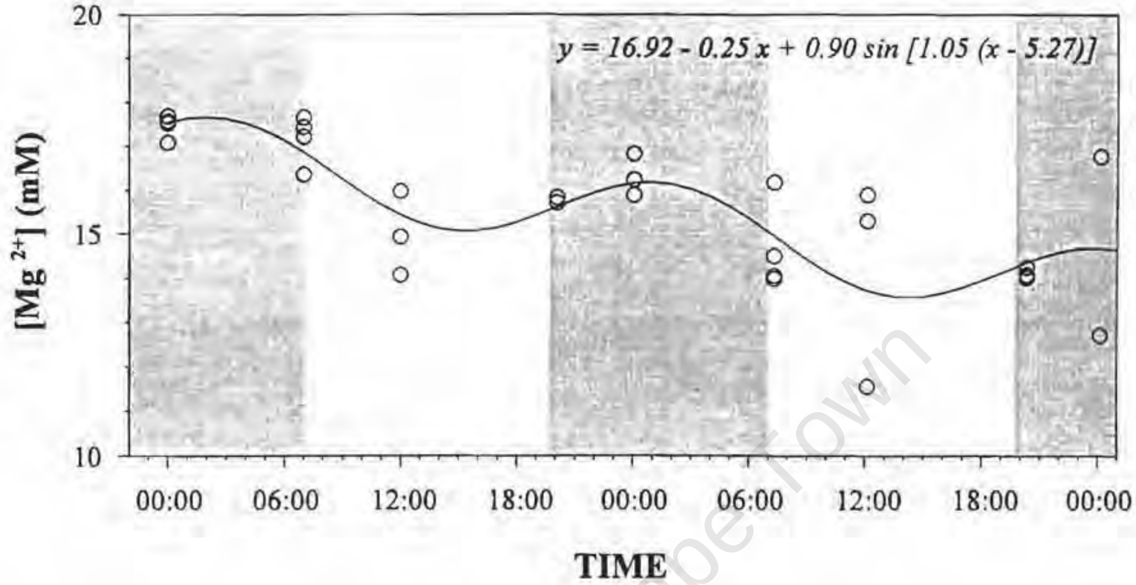


Figure 6.12: Graph illustrating the non-linear model fitted to the magnesium data obtained during experiment 2. The expression describing the model is shown in the plot, and the associated statistics are given in Table 6.17. Note that the data points are predicted magnesium concentrations computed from the multiple regression results given in Tables 6.15 and 6.16.

Table 6.17: Parameter estimates and associated statistics of the non-linear regression model fitted to the magnesium data obtained during experiment 2 (n = 29; $r^2 = 0.5913$, $F_{1,26} = 37.6179$, $p = 0.0000$). Note that the magnesium data employed in the non-linear modelling were predicted values of magnesium concentration computed from the multiple regression analyses (see text).

Parameter	Estimate	Standard Error	t_{23}	p
a	16.919	0.4049	41.7879	0.0000
b	- 0.247	0.0554	- 4.4563	0.0002
c	0.899	0.3654	2.4606	0.0218
d	1.048	0.0947	11.0691	0.0000
e	- 5.271	0.3105	- 16.9777	0.0000

The model describes a cycle in magnesium concentration with a period close to daily (d = 1.048, equivalent to 22.9 hours). The period is not significantly different from 24 hours ($t_{23} = 0.5099$, $p = 0.6150$), supporting the daily cycle hypothesis. The power of the test was estimated to be 23.55%, and although substantially higher than that associated with the calcium regression, indicates an unacceptably high probability of incurring type II error. The model is consistent with that fitted to the magnesium data obtained during experiment 1, in that the cycle peaks at night. The underlying decreasing trend does not, however, correspond to the increasing trend evident in the results of the first experiment.

6.3.2.3. Experiment 3:

Magnesium concentrations fluctuated between 10.53 mM and 21.29 mM, with most of the data falling in the range 15 mM to 21 mM (Fig. 6.13).

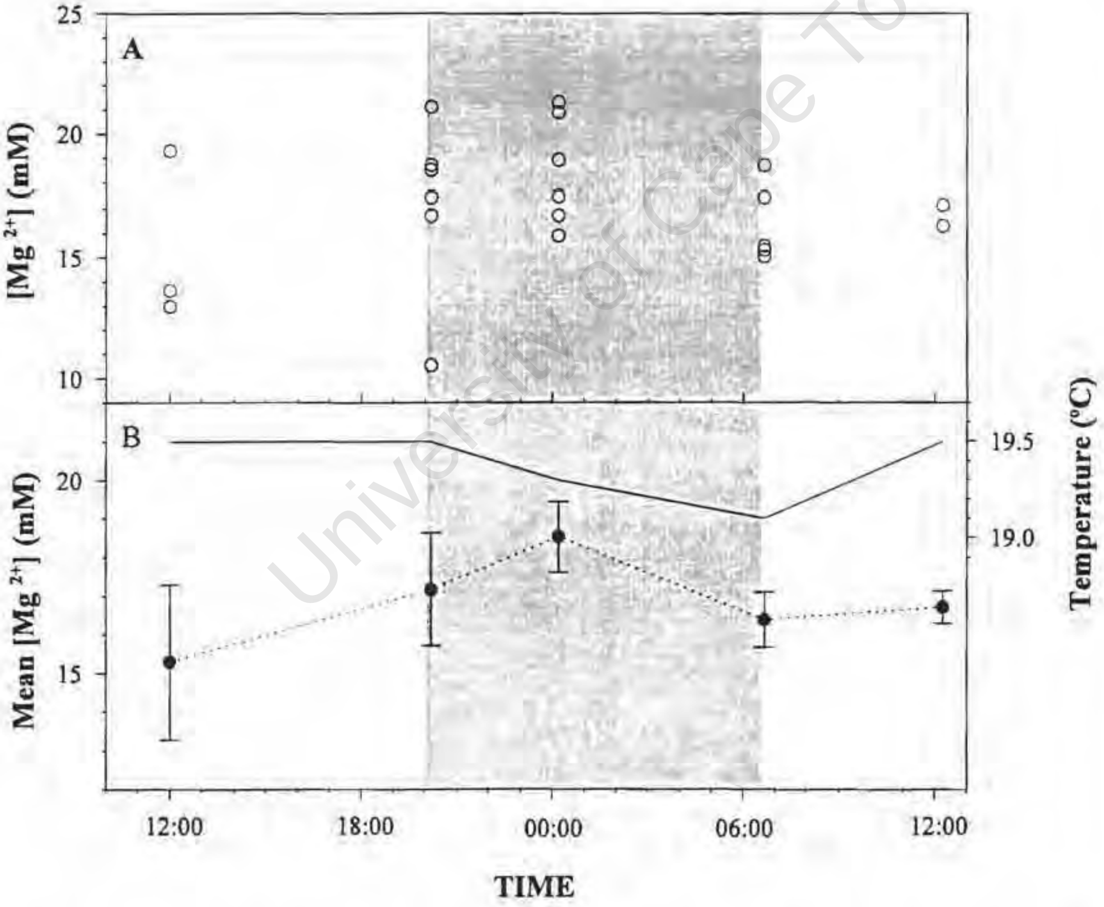


Figure 6.13: Magnesium concentrations recorded during experiment 3. (A) Individual measurements; (B) mean concentrations (± 1 SE) at each interval. Temperature is shown with a solid line in B. Shaded areas represent periods of darkness.

The data show a very similar pattern to that observed in experiment 1, with mean magnesium concentrations peaking during the night and decreasing during the day (Fig. 6.13 B). However, these patterns are not statistically significant. Mean magnesium levels in the endolymph show no differences between day and night periods (ANOVA, $F_{1,20} = 2.4589$, $p = 0.1325$), or between the sampling intervals (ANOVA, $F_{4,17} = 0.8562$, $p = 0.5096$). No differences in mean magnesium levels are apparent between sexes (ANOVA, $F_{1,20} = 0.2730$, $p = 0.6071$), or between maturity stages (ANOVA. Females: $F_{2,8} = 1.4655$, $p = 0.2869$. Males: $F_{1,9} = 0.5057$, $p = 0.4950$).

The multiple regression model generated by the female data (Table 6.18) is not significant, and explains only about 5% of the variance in the data. All of the coefficients show very high probability levels, indicating a large degree of redundancy. The male data, however, resulted in a significant model that explains about 66% of the variance, primarily due to a highly significant size effect (Table 6.19). Predicted endolymph magnesium levels were computed for each squid using the coefficients of the models, and these values, as well as the original data set, were subjected to the non-linear regression analysis.

Table 6.18: Coefficients and associated statistics of the multiple regression model fitted to the magnesium measurements recorded in female squid during experiment 3 (n = 11; $r^2 = 0.0485$, $F_{3,7} = 0.1190$, $p = 0.9461$).

Coefficient	Estimate	Standard Error	t_7	p
Intercept	3.698	101.1766	0.0365	0.9719
Temperature	0.620	5.2645	0.1178	0.9095
DML	0.064	0.1259	0.5093	0.6262
Maturity	- 0.668	1.1213	- 0.5955	0.5703

Table 6.19: Coefficients and associated statistics of the multiple regression model fitted to the magnesium measurements recorded in male squid during experiment 3 (n = 11; $r^2 = 0.6601$, $F_{3,7} = 4.5318$, $p = 0.0457$).

Coefficient	Estimate	Standard Error	t_7	P
Intercept	- 30.504	83.3522	- 0.3660	0.7252
Temperature	1.401	4.2794	0.3273	0.7530
DML	0.389	0.1097	3.5353	0.0095
Maturity	- 0.424	1.1616	- 0.3647	0.7261

The results of the non-linear regression differ from those described previously, in that the original data set generated a model (Fig. 6.14, Table 6.20) that explained a higher proportion of the variance in the data than did that derived from the predicted values ($r^2_{obs} = 0.1677$ vs. $r^2_{pred} = 0.0160$). The model fitted to the original data indicates that endolymph magnesium concentrations cycle over an 18.8 hour period (d = 1.278).

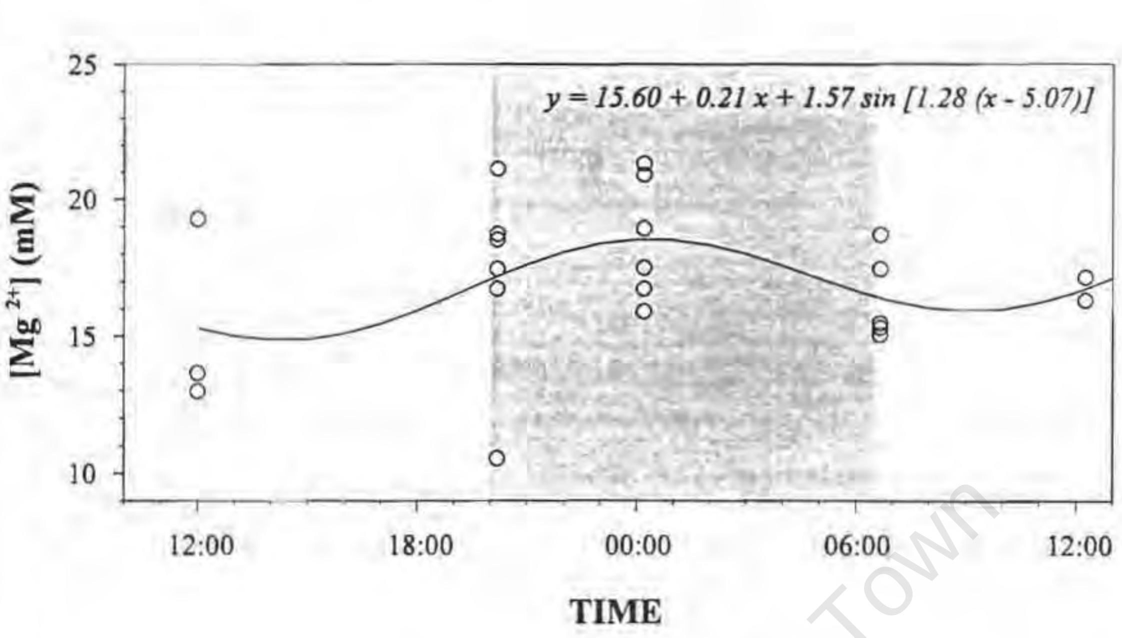


Figure 6.14: Graph illustrating the non-linear model fitted to the magnesium data obtained during experiment 3. The expression describing the model is given in the graph, the associated statistics are given in Table 6.20. Note that the data points are the original measurements of magnesium concentration .

Table 6.20: Parameter estimates and associated statistics of the non-linear regression model fitted to the magnesium data obtained during experiment 3 (n = 22; $r^2 = 0.1677$, $F_{1,20} = 4.0289$, $p = 0.0584$). The magnesium data used in the modelling procedure were the original measurements recorded from the experiment (see text).

Parameter	Estimate	Standard Error	t_{17}	p
a	15.599	2.1470	7.2657	0.0000
b	0.214	0.3267	0.6569	0.5201
c	1.568	0.8709	1.8004	0.0896
d	1.278	0.2352	5.4355	0.0000
e	- 5.069	0.3731	- 13.5859	0.0000

Although the daily cycle hypothesis could not be rejected ($t_{17} = 1.1832$, $p \doteq 0.2530$), a high probability of type II error is indicated by the low level of power (7%) associated with the test. The model describes the same pattern found in the previous two experiments (higher levels at night, lower levels during the day). A slight linear increase underlying the cycle is evident, although this is not significant (Table 6.20).

6.3.2.4. Experiment 4:

Magnesium levels fluctuated between 9.67 mM and 29.67 mM, the majority of the measurements falling between 13 mM and 25 mM (Fig. 6.15).

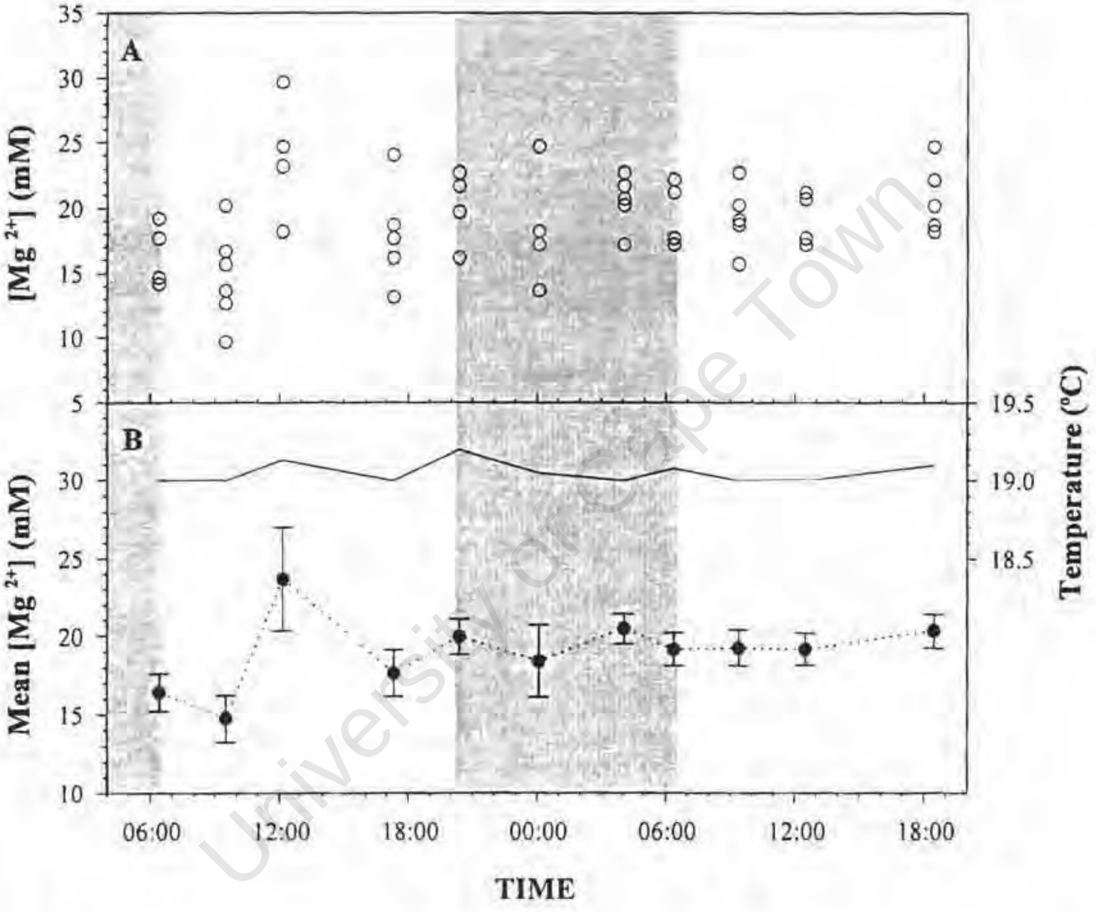


Figure 6.15: Magnesium concentrations recorded during experiment 4. (A) Individual measurements; (B) mean concentrations (± 1 SE) at each interval. Temperature is shown with a solid line in B. Shaded areas represent periods of darkness.

No differences in mean magnesium concentrations are apparent between sexes (ANOVA, $F_{1,51} = 0.0055$, $p = 0.9413$), maturity stages (ANOVA. Females: $F_{4,32} = 1.1248$, $p = 0.3622$. Males: $F_{1,14} = 0.0032$, $p = 0.9559$), or between day and night samples (ANOVA. $F_{1,51} = 1.1783$, $p = 0.2828$). Although differences are apparent between sampling intervals (ANOVA. $F_{10,42} = 2.4072$, $p = 0.0230$), this is primarily due to the difference between the means of the second (09:30) and third (12:00) intervals, and not a function of any day - night differences. Neither of the multiple regression models fitted to the data are significant (Tables 6.21 and 6.22), and the probability levels of the estimated coefficients indicate that none of the explanatory variables included in the analyses have any significant effect on magnesium concentrations in either females or males.

Table 6.21: Coefficients and associated statistics of the multiple regression model fitted to the magnesium measurements recorded in female squid during experiment 4 (n = 35; $r^2 = 0.0946$, $F_{3,31} = 1.0793$, $p = 0.3723$).

Coefficient	Estimate	Standard Error	t_{31}	p
Intercept	- 126.586	168.1832	- 0.7527	0.4573
Temperature	7.102	8.7765	0.8092	0.4246
DML	0.116	0.0950	1.2220	0.2309
Maturity	0.345	0.7287	0.4729	0.6396

Table 6.22: Coefficients and associated statistics of the multiple regression model fitted to the magnesium measurements recorded in male squid during experiment 4 (n = 16; $r^2 = 0.2967$, $F_{3,12} = 1.6876$, $p = 0.2224$).

Coefficient	Estimate	Standard Error	t_{12}	p
Intercept	- 248.046	187.3509	- 1.3240	0.2102
Temperature	14.566	9.9394	1.4655	0.1685
DML	- 0.217	0.1395	- 1.5576	0.1453
Maturity	0.574	3.0814	0.1862	0.8554

Both the original data set and the predicted values computed using the multiple regression models generated significant non-linear models, but the latter (Fig. 6.16, Table 6.23) accounts for more of the variance in the data than does the model derived from the original data ($r^2_{\text{pred}} = 0.1900$ vs. $r^2_{\text{obs}} = 0.1379$).

The model fitted to the predicted values describes a cycle with a period of 29 hours (d = 0.829), that is not significantly different from a daily cycle ($t_{46} = 0.8719$, $p = 0.3878$). The power of the regression was very low, however (9.36%), casting some doubt on the validity of the conclusion. The pattern described by the model over the experimental period indicates that magnesium concentrations peak during the night, the same pattern as was detected in the previous three experiments. The slight increasing trend underlying the model corresponds to the increasing trends evident in the magnesium data from experiments 1 and 3, but not to the decreasing trend displayed by the data from experiment 2.

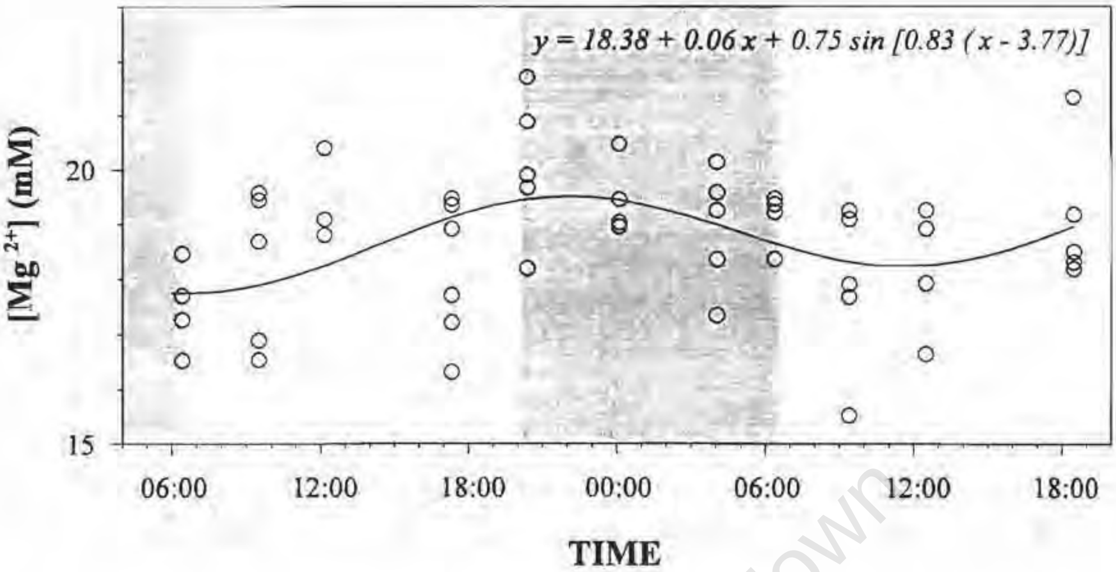


Figure 6.16: Graph illustrating the non-linear model fitted to the magnesium data from experiment 4. The expression describing the model is given in the plot, and the associated statistics are provided in Table 6.23. Note that the data points in the plot are predicted magnesium concentrations calculated from the multiple regression results shown in Tables 6.21 and 6.22.

Table 6.23: Parameter estimates and associated statistics of the non-linear regression model fitted to the magnesium data obtained during experiment 4 (n =53; $r^2 = 0.1900$, $F_{1,49} = 11.4968$, $p = 0.0014$). Note that the magnesium data used in the non-linear modelling were predicted values of magnesium concentration computed from the results of the multiple regression analyses.

Parameter	Estimate	Standard Error	t_{48}	p
a	18.382	0.6253	29.3970	0.0000
b	0.062	0.0898	0.6936	0.4914
c	0.752	0.2483	3.0308	0.0040
d	0.829	0.1964	4.2188	0.0001
e	- 3.769	1.0650	- 3.5389	0.0009

6.4. DISCUSSION

The experiments described in this chapter were aimed at monitoring various components of the chemistry of the statocyst endolymph of *Lolliguncula brevis* in an attempt to establish whether or not these components displayed periodic fluctuation, and if so, whether the fluctuations occurred with a daily frequency. The lack of success in obtaining pH and strontium data is a shortcoming of the study. Both of these components have been implicated in statolith mineralization (see Chapter 1 and section 6.1), and further attention should be directed to elucidating their roles.

All four of the experiments conducted in this study yielded measurements of endolymph calcium and magnesium ion concentration. The levels of these ions fluctuated to some extent, but the evidence suggesting that these fluctuations occurred with a daily frequency was weak. The possibility that the differential deposition of the incremental and discontinuous zones of increments is not a reflection of fluctuations in the levels of calcium and magnesium ions (or *vice versa*) cannot be discounted.

A further possibility is that the assumptions implicit in the experimental approach are not valid. Two assumptions warrant discussion.

(i) If some cycle in endolymph chemistry is in fact operative, this cycle will be of the same frequency and phase in all individuals in the population. If this is not the case, the approach of sampling several individuals at each interval will yield estimates of ionic content that are not applicable to the population. Considering that the squid used in each experiment had all been exposed to similar conditions in the wild, and were further acclimated to identical conditions in the laboratory for a minimum of seven days, it is felt that this assumption is valid.

(ii) The endolymph samples extracted from the statocysts are representative of the endolymph at the statolith surface. In the opening discussion of this chapter, it was argued that the processes of increment formation are occurring in the fluid immediately adjacent to the statolith surface. Since mixing of the samples undoubtably occurred during the sampling procedure, the measurements of ion content are an average of the entire endolymph. The approach consequently assumes that the endolymph has a homogeneous chemistry. It is possible that micro-gradients do occur in the vicinity of the statolith, particularly if elements are secreted in close proximity to the statolith (e.g. the cells of the macula) and are almost immediately deposited on to the growing surface. However, considering the size of the statolith, and that $CaCO_3$ deposition is also occurring at points distant from the macula (such as at the edge of the lateral and dorsal domes, and the tip of the rostrum), and bearing in mind that the function of the statocyst relies on fluid movement in the cavity, it was felt that the overall chemistry of the endolymph is representative of the conditions over most of the statolith surface, and particularly the increment-bearing regions of the statolith.

If these assumptions are valid, the most likely alternative explanation for the absence of clear cycles in the raw data is that variability inherent in the data is obscuring any temporal patterns that may be present. This variability has two sources; experimental error, and natural variability attributable to diverse environmental and biological parameters. Experimental error would contribute to the variability in the data if the measurements of ionic concentration were not representative of the true concentration in the endolymph at the time of sampling.

One source of experimental error that has been discussed previously is contamination of the samples with either statoconia, or body fluids surrounding the statocyst. The outliers observed in the data (see section 6.3.1) were excluded on this basis. If the samples were contaminated by the surrounding body fluids, one would expect that both calcium and magnesium levels would be elevated, considering the large concentration gradients that exist between the endolymph and the plasma (Morris, 1991a, Romanek and Gauldie, 1996). Examination of the data presented in Appendix III shows that this is not the case; elevated calcium levels apparent in the outliers did not correspond to elevated magnesium ion concentration. This suggests that the outlier samples were contaminated by statoconia. Since very low amounts of magnesium are incorporated into precipitating calcium carbonate, statoconia represent a significant source of calcium, but not magnesium. If some statoconia were included in the endolymph sample, and subsequently dissolved in the DI water, substantially more calcium than magnesium would be released into solution, explaining the elevated calcium but similar magnesium levels in the outliers compared to the other individuals. Where statoconial contamination did occur, this was immediately apparent purely by comparison with the other data. Considering the magnitude of the differences between the outliers and the remaining data, the possibility that the outliers fall within natural variability is unlikely.

Another potential source of experimental error is apparent when the results of this study are compared with those reported in the literature. Only one study has examined calcium and magnesium concentrations in squid statocyst endolymph. Mean endolymph ionic concentrations in *Alloteuthis subulata* were 8.17 ± 0.48 mM (range 1.0 - 15.5 mM) for calcium and 0.45 ± 0.22 mM (range 0.05 - 1.0 mM) for magnesium (Morris, 1988, 1991a). Measurements of calcium concentration in the endolymph of *Lolliguncula brevis* obtained in this study (5.68 ± 0.22 mM; range 2.11 - 16.83 mM) were comparable to those obtained by Morris (1991a), but magnesium concentrations (16.81 ± 0.31 mM; range 7.50 - 29.67 mM) were considerably higher. Ambient sea water concentrations measured in this study ranged between 9.01 mM and 9.07 mM calcium, and 42.33 mM to 46.25 mM magnesium. These values are comparable to the ambient levels levels measured by Morris (1991a) (16.1 mM for calcium and 48.7 mM for magnesium). The difference in magnesium content between the two studies cannot be attributed to differences in ambient levels.

A possible explanation for the differences is post-mortality ion diffusion that may have occurred while the specimens were being thawed. Because the ATP pump responsible for maintaining the ionic integrity of the endolymph compartment was inactive, passive diffusion of magnesium ions down the concentration gradient from the plasma into the endolymph could conceivably have elevated the magnesium content of the endolymph samples. However, calcium concentration gradients between the plasma and endolymph, while not of the same magnitude as magnesium gradients, also exist (Morris, 1991a). If passive ion diffusion did occur to any appreciable extent, endolymph calcium content would also be elevated. This is clearly not the case; calcium contents measured in this study are on average lower than those reported by Morris (1991a). Species differences may be an explanation for the discrepancy.

The major source of variability inherent in the data is most likely natural variability attributable to various biological and environmental factors. Several physical and biological parameters interact to influence the chemistry of the endolymph (Kalish, 1989, 1991). Considering that the squid sampled in this study encompassed a broad size range of both sexes at various stages of maturity, it is not suprising that the raw data contained a great deal of intrisic variability. The multiple regression analyses were aimed at accounting for variability attributable to temperature, size, sex and stage of maturity. In most cases, the resulting models did not explain an appreciable amount of the variance in the data. Further, the explanatory variables generally did not have a significant influence on the ion content of the endolymph. Where significant relationships were detected, there were inconsistencies in their nature and magnitude. As a result of this, considerable variance was still present in the data after the multiple regression results were applied.

Factors that were not controlled for in the experimental design probably influenced statocyst endolymph chemistry to varying degrees, contributing to the variance in the data sets. It is crucial that such factors be taken into account when conducting further experiments of this nature. The success of attempts to isolate temporal fluctuations in whatever parameters are being investigated would be greatly enhanced by controlling for as many potentially confounding factors as possible. In view of the low coefficients of determination (r^2) associated with most of the multiple regression models, the experiments conducted during this study clearly did not achieve this goal to any great extent. In spite of these results, application of the models accounted for some of the variance in the data, and in most cases revealed patterns that were not immediately apparent in the raw data sets.

The non-linear regression analyses modelling these patterns provided some support for the daily cycle hypothesis, but the evidence is weak. All of the non-linear models described periodic fluctuations in both calcium and magnesium concentrations, but the periods of the cycles deviated from a daily period by as much as 8 hours in the case of calcium, and 5 hours in the case of magnesium. Further, the calcium models displayed phasic inconsistencies. Three of the models indicated that endolymph calcium levels peaked during the day, while the remaining model, owing to the short period parameter, indicated that peaks occurred at night. In the case of magnesium, all of the models consistently peaked at night.

Daily cycles were demonstrated in the fluctuations of both calcium and magnesium concentrations in all four experiments. These results are encouraging, but can only be considered as tentative. Although the period of several of the models fitted to the data deviated substantially from a daily period, the tests of the null hypothesis indicated no difference between the estimated period of the model and a daily period. These results were primarily due to the high standard errors of the period parameters, which were a function of the variability remaining in the data. Note that the conclusion that the data supported the daily cycle hypothesis was based on non-rejection of the null hypothesis. This approach raises the issue of type II error, specifically the probability of accepting a false null hypothesis (see Chapter 3). This aspect was assessed by estimating the level of statistical power associated with each test. The parameters and statistics pertaining to the tests of the daily cycle hypothesis are summarised in Table 6.24.

Although all of the non-linear models were highly significant, they generally explained low proportions of the variance in the data, particularly in the case of the calcium models. The estimates of statistical power that were computed for each test of the daily cycle hypothesis (Table 6.24) reflected this. The power of the various models to detect a difference in period of ± 3 hours from daily were extremely low, ranging from about 5% to a maximum of 25%. These values indicate an unacceptably high probability of type II error, suggesting that although the null hypothesis was accepted in all cases, there was considerable risk of accepting a false null hypothesis.

Table 6.24: Table summarising the results and relevant statistics of the tests of the daily cycle hypothesis for each experiment. The period (d) parameter estimate from each model, and the deviation from a period of 24 hours are given, as are the standard error of the period parameter estimate (SE_d), the r^2 values corresponding to each model, and the power of each model to detect a deviation of 3 hours from a daily period.

Variable	Experiment	Period (hours)	Deviation (hours)	SE_d	r^2	Power
Ca^{2+}	1	24.02	+ 0.02	0.1321	0.2563	0.1427
	2	17.9	- 6.1	0.3035	0.1693	0.0664
	3	16.1	- 7.9	0.7104	0.2293	0.0528
	4	23.0	- 1.0	0.1626	0.2285	0.1143
Mg^{2+}	1	25.6	+ 1.6	0.1671	0.5057	0.1067
	2	22.9	- 1.1	0.0947	0.5913	0.2355
	3	18.8	- 5.2	0.2352	0.1677	0.0761
	4	29.0	+ 5.0	0.1964	0.1900	0.0936

A means of increasing power is to increase the sample size (Rice, 1987). In those experiments where attempts were made to increase the sample numbers taken at each interval (namely experiments 3 and 4), the power estimates were not appreciably improved. It is worthwhile noting that in those instances where the models explained relatively higher proportions of the variance in the data, the period parameter estimates tended to show less of a deviation from a daily cycle, and also corresponded to higher power estimates (due to the lower standard errors of the period parameter estimates). Note that the calculation of power is not influenced by the magnitude of the parameter estimate, but rather by its standard error. The key factor is clearly the variability attributable to factors other than time that was not accounted for in the regression analyses. Because of this variability, the conclusion that calcium and magnesium levels in the statocyst endolymph fluctuate with a daily frequency can only be considered as tentative. Further research testing the daily cycle hypothesis must control for as much of this variability as possible before robust conclusions can be drawn from the results.

The non-linear modelling approach employed in this has several limitations. Firstly, the use of a sine wave to model the data assumes a regular cycle. A scenario where the molecular / physiological processes associated with statolith growth show a spike (i.e. a short-term, rapid fluctuation, followed by relatively constant conditions over extended periods) would not be very well approximated by a model of this nature. However, the sampling intervals employed (which were a direct function of the number of animals that could be maintained in the laboratory for extended periods) precluded identification of such an occurrence. That cycles in the parameters were detected, even using a relatively temporally low resolution sampling protocol, suggests that such rapid short-term fluctuations are not occurring. Secondly, a model of this nature can be fitted to almost any data set with a high level of "goodness of fit", purely by manipulating the period / frequency and amplitude parameters of the model. For an extreme example, by forcing the model to cycle with a frequency of a few minutes, the data obtained in this study could have been explained by time alone, with a very high level of statistical significance. The parameter estimates resulting from the modelling procedure rely to a large extent on the initial parameter estimates set by the researcher. Initial parameter estimates were set at levels consistent with what was observed by examination of the raw data, thereby preventing unrealistic parameter estimates, which, although perhaps of not much biological meaning, would have generated very high r^2 values.

All of the models described linear trends underlying the cycles. With only two exceptions (the models fitted to the calcium data from experiment 1 and the magnesium data from experiment 2), the trend was an increasing one. Although this suggests some correspondence with temperature, increasing trends were apparent in the two experiments where temperature did not increase (experiments 3 and 4). Further, no correlations were detected between the dependent variables and temperature, and the multiple regression results generally further supported this. An alternative explanation concerns the level of stress to which the animals were exposed. Squid are very sensitive to the conditions imposed in the laboratory. During the experiments, the squid were frequently subjected to periodic disturbances associated with the removal of animals at each sampling interval. It is very likely that these disturbances added to the stress being experienced by the squid, and hence a stress-related increase in metabolic rate. Elevated metabolic rates may have influenced the ionic content of the endolymphatic fluid, generating the increasing trends that were observed.

The inverse relationship detected between calcium and magnesium cycles (i.e. magnesium concentrations peaking at night, while calcium peaks during the day) provides a basis for some speculation concerning the role of magnesium in statolith mineralization. Magnesium has an inhibitory affect on calcification (Morris, 1988, 1991). It could therefore be argued that daily episodes of elevated magnesium levels represent a regulatory mechanism to prevent runaway deposition of aragonite crystals. Fish otoliths are composed of twinned aragonite crystals (Gauldie and Nelson, 1988). Twinning represents an efficient mechanism for rapid crystal growth. If such crystallization is permitted to continue unimpeded, the function of the statolith / otolith would become compromised through the accumulation of excess mass. Periods of elevated magnesium levels (leading to reduced calcification, perhaps corresponding to the formation of the discontinuous zones of increments) could conceivably represent a regulatory mechanism to periodically limit statolith / otolith growth. This argument suggests that the discontinuous zones are formed at night (when peaks in magnesium content of the statocyst endolymph occurred), while the incremental zones (which are presumed to represent periods of increased $CaCO_3$ deposition) form during the day, when relatively higher levels of free Ca^{2+} ions are available for crystallization.

This argument does not account for the roles of either the protein component, or strontium. The proposed roles of the organic component (see Chapter 7), imply a strong regulatory influence on crystallization, while the importance of strontium is emphasized by the results of Hanlon *et al.* (1989) and Bidwell *et al.* (1990) which have been discussed previously. There also appears to be an intimate link between strontium and the protein matrix. The discontinuous zones of otolith increments contain relatively higher strontium contents than do the incremental zones (Mugiya and Satoh, 1995), corresponding to the higher organic content of the discontinuous zones relative to the incremental zones.

Based on available information, no clear conclusions concerning cause and effect in increment production can be drawn as yet. The information reviewed above could reflect two scenarios. Firstly, the formation and deposition of the protein matrix is constant, but due to periodic elevations in magnesium content (and perhaps decreases in pH), aragonite deposition fluctuates as a function of the conditions in the endolymph. The relatively higher protein and strontium contents of the discontinuous zones of increments are then merely an incidental by-product of periods of reduced mineral deposition. Alternatively, the capacity of the endolymph to precipitate aragonite crystal is constant, but fluctuations in the formation of protein matrix (described by Gauldie and Nelson, 1988), perhaps linked with strontium, regulate the rate of crystallization. Fluctations in the inorganic components may well be merely a reflection of periods of reduced or enhanced mineral deposition. If the hypothesis of Morris (1988, 1991a) is valid, increased protein formation/deposition represents an inhibitory phase, where the protein actively inhibits crystallization, resulting in the relatively higher protein to mineral ratios observed in the discontinuous zones.

Clearly, the physiological and chemical process associated with increment formation are not understood, and further research is required to elucidate these interactions. This information is crucial to not only conclusively establish the daily nature of increment formation at a physiological level, but also to gain an understanding of the processes of non-daily increment formation, hopefully generating objective criteria that can be used to identify and distinguish these confounding features from true daily increments.

In conclusion, the results of this study suggest that daily cycles in the levels of endolymph calcium and magnesium ions may be occurring. For the first time, this finding provides support, albeit weak, for the concept of a daily cycle in squid statolith growth at a physiological / molecular level. Both elements have been implicated in statolith mineralization (see section 6.1 and Chapter 1). In view of the intimate link between endolymph chemistry and statolith / otolith composition (Kalish, 1989; Romanek and Gauldie, 1996), daily cycles in the levels of calcium and magnesium ions suggest that there may be daily cycle in statolith growth. It should be stressed, however, that the presence of a daily cycle in the chemistry of the endolymph does not imply that the formation of increments corresponds to this cycle. No conclusions concerning the link between alterations in the chemical status of the endolymph, and the differential formation of the incremental and discontinuous zones of increments can be drawn from these results. Further carefully controlled experiments exploring this potential link have to be conducted before the daily formation of increments can be established at a physiological / molecular level.

CHAPTER 7 - PRELIMINARY CHARACTERIZATION OF THE ORGANIC MATRIX PROTEINS FROM STATOLITHS OF THE LOLIGINID SQUID *Lolliguncula brevis*

ABSTRACT

A preliminary study aimed at purifying and characterizing the organic matrix proteins from statoliths of the loliginid squid *Lolliguncula brevis* is described. Proteins extracted from the statoliths by a simple decalcification technique were separated into two fractions, insoluble and soluble in aqueous solutions. Gel electrophoresis indicated that the insoluble fraction is composed of at least eight major proteins, ranging in size from 25 000 to more than 200 000 Da. A glycoprotein enzyme immunoassay indicated that the five largest insoluble proteins were glycosylated. In contrast, the soluble fraction was composed of a single dominant protein of approximately 100 000 Da, and two other major proteins of higher molecular weight. All three soluble proteins were glycosylated. Molecular weight and partial sequence data of peptides from five of the insoluble and one of the soluble proteins were obtained using liquid chromatography - electrospray - tandem mass spectroscopy. These data were used in a database search for possible homologs. No matches were found, suggesting that these proteins may belong to a class of hitherto undescribed organic matrix compounds.

7.1. INTRODUCTION

Statoliths, as do most biologically calcified structures, contain an organic matrix closely associated with the mineral phase. The importance of the organic matrix in biological calcification has been highlighted in a number of publications (e.g. Lowenstam, 1981; Weiner *et al.*, 1983; Wheeler and Sikes, 1984; Addadi and Weiner, 1985; Sikes and Wheeler, 1986; Simkiss and Wilbur, 1989; Albeck *et al.*, 1996), including those investigating statolith mineralization (Morris, 1988, 1991a; Lipinski 1986, 1993). Lowenstam (1981) first put forward the concept of "matrix mediated mineralization", suggesting that crystal growth within a preformed organic matrix acting as a structural framework is the basic mode of skeletal formation adopted by many organisms. Subsequent studies have indicated that the organic matrix plays more than a merely structural role, and is in fact capable of actively regulating crystal growth (Weiner *et al.*, 1983; Wheeler and Sikes, 1984; Addadi and Weiner, 1985; Simkiss and Wilbur, 1989; Albeck *et al.*, 1996). Although understanding of the precise mechanisms of the regulatory function of the organic matrix is still limited, a number of functional roles have been proposed, including control of crystal morph (Falini *et al.*, 1996), nucleation, orientation and morphology (see Simkiss and Wilbur, 1989).

The organic matrix appears to play an important role in the formation of increments in both squid statoliths and teleost otoliths. Increments are clearly visible in the organic matrix after the mineral phase had been removed by means of treatment with a weak acid or calcium chelating agent (Kristensen, 1980;

Dean *et al.*, 1983; Lipinski, 1986). In contrast, no increments are apparent in the mineral when the organic matrix is removed by treatment with NaOH (Kristensen, 1980). These observations suggest that it is the organic matrix that is responsible for regulating the architecture of statolith microstructure. The mechanisms of this regulation are not known. Morris (1988, 1991a) proposed that an organic component of the statocyst endolymph of the squid *Alloteuthis subulata* reversibly polymerises with increasing pH, inhibiting $CaCO_3$ precipitation. No further research testing this hypothesis has been reported. Many studies have examined the role of the organic matrix in biocalcification, but most of the studies have been conducted on the shells of bivalve molluscs.

Mineralization of the cephalopod statolith differs from that of the molluscan shell in two respects. Firstly, statoliths are composed entirely of aragonite (Lipinski, 1986, 1993), with no evidence of the calcite phases that are apparent in many mollusc shells. Secondly, as argued by Morris (1988, 1991a) and discussed previously in this thesis, most of the coleoid cephalopod statolith, and particularly the increment-bearing regions, is not in contact with any form of secretory surface. The processes responsible for crystallization and increment formation must be occurring in the endolymph adjacent to the statolith surface. Recent results reported by Takagi and Takahashi (*in prep.*) support this argument. Components of the organic matrix of teleost otoliths are produced in cells lining the sacculus, and secreted into solution in the endolymphatic fluid, from where they are subsequently deposited onto the otolith. In view of these differences, information from investigations of mollusc shells may not be applicable to squid statolith mineralization.

An understanding of the role of the organic matrix in statolith growth could contribute to resolving the problems experienced with the interpretation of statolith microstructure that have been discussed previously. Before the regulatory role of the matrix can be elucidated, a detailed knowledge of the nature of the component macromolecules has to be obtained. Available data are restricted to amino acid composition analyses (Radtke, 1983; Lipinski, 1986; Morris, 1988). No primary structure or sequence information for squid statolith matrix proteins exists. In fact, very little primary structure data appear to exist for any vestibular matrix compounds. Complete sequence data are available only for the major protein from aragonitic frog otoconia ("otoconin - 22", Pote *et al.*, 1996), and partial sequence data for various mollusc shell matrix proteins have been reported (Donachy *et al.*, 1992; Keith *et al.*, 1993; Halloran and Donachy, 1995). This chapter describes preliminary work aimed at purifying and characterizing the organic matrix proteins from statoliths of the loliginid squid *Lolliguncula brevis*, with the objective of gaining an understanding of the nature and function of these proteins in statolith mineralization, and particularly increment formation.

7.2. METHODS

Squid were captured in Galveston Bay by brief bottom trawls. Immediately after capture, the animals were decapitated, and the heads frozen and stored at -20°C. After thawing, the statoliths were extracted from the statocysts and subjected to a stringent cleaning procedure using a 5% sodium dodecyl sulphate (SDS) / 0.5% dithiothreitol (DTT) solution. The statoliths were then washed several times in fresh double-distilled water and stored frozen at -10°C.

Statolith organic matrix proteins were extracted by a simple decalcification procedure and separated into two fractions, soluble and insoluble in aqueous solutions. Previous studies have suggested that the two fractions are biochemically and functionally distinct, and fulfill different roles in the mineralization process (Weiner *et al.*, 1983; Wheeler *et al.*, 1988; Simkiss and Wilbur, 1989). Clean, intact statoliths were transferred to a silinated glass microcentrifuge tube and immersed in a 1.0 M EGTA solution (pH 8.0) at 4°C. When the entire mineral phase was in solution (assessed by microscopic observation under polarised light), the tube was centrifuged for 5 minutes at 10,000 g. The supernatant (containing the soluble fraction) was removed and dialysed for 24 hours against double-distilled water using dialysis tubing with a 3.5 kDa cut-off, to remove the EGTA and calcium from the solution. The dialysate was then lyophilized, resuspended in 20 µl double-distilled water and stored at -10°C. The insoluble fraction remaining in the centrifuge tube after decalcification and centrifugation was washed several times in fresh distilled water to remove remaining EGTA and calcium, and stored at -10°C.

A number of analyses were performed on the organic matrix (OM) extracted from the statoliths:

1. Proteins in both the insoluble (IOM) and soluble (SOM) fractions were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE).
2. Proteins separated by SDS-PAGE were tested for glycoprotein content.
3. Molecular weights and partial sequence information of proteins in the dominant bands observed in the SDS-PAGE gels were obtained using liquid chromatography - electrospray - tandem mass spectrometry (LC - MS). These data were then used in a database search for possible homologs.

7.2.1. SDS-PAGE

The SDS-PAGE was run on a mini-gel system at 4°C. A 15% polyacrylamide gel mix (1:174 N,N'-methylenebisacrylamide: acrylamide) was used under highly reducing conditions (1% DTT), because this was found to provide satisfactory protein separation over a range of 18 000 to 200 000 Da. Preliminary electrophoresis runs indicated that a negligible amount of material fell outside of this range. The protein from thirty statoliths was extracted as described above. The solution containing the SOM fraction was combined with 20 µl 2x SDS sample buffer, heated at 80°C for 5 minutes and loaded on to the gel. The IOM fraction was dissolved in 20 µl of a 10% SDS / 1% DTT solution for 20 minutes at 100°C. The solution was then combined with 20 µl of the 2x SDS sample buffer, heated for a further 5 minutes at 80°C, centrifuged and loaded on to the gel. Samples were run against a series of markers covering a range of 18 kDa to 200 kDa. After each run, the gels were removed from the apparatus and stained with Coommassie Blue.

7.2.2. Glycoprotein analysis

The SOM and IOM fractions from 30 statoliths were extracted and loaded onto SDS gels as described above. Solutions of fetuin and lysozyme were loaded as positive and negative controls respectively. After electrophoresis, each gel was removed from the apparatus, soaked in transfer buffer and western blotted onto a nitrocellulose membrane. The gel was stained using a conventional $AgNO_3$ staining procedure to identify all bands on the gel. The proteins that had been immobilized on the nitrocellulose were tested for glycosylation using a commercially available DIG glycan detection kit (Boehringer-Mannheim Cat.No.1142 372). Positive results were indicated by the relevant band being stained a dark grey colour. Comparison of the silver stained gel with the treated nitrocellulose indicated which of the proteins were glycosylated.

7.2.3. Liquid chromatography - electrospray - tandem mass spectroscopy

IOM and SOM fractions from 30 statoliths were separated by SDS-PAGE and stained with Coommassie Blue. Bands of interest were cut out of the gels, minimizing the amount of extra polyacrylamide associated with each band. Each band was cut into smaller pieces. The gel pieces were washed and destained in 500 µl 50% methanol overnight. They were then dehydrated in acetonitrile, rehydrated in 50 µl of 10 mM DTT in 0.1 M ammonium bicarbonate and reduced at 55°C for 1 hour. The DTT solution was removed and the sample alkylated in 50 µl 50 mM iodoacetamide / 0.1 M ammonium bicarbonate at room temperature for 1 hour in the dark. The reagent was removed and the gel pieces washed with 100 µl 0.1 M ammonium bicarbonate and dehydrated in 100 µl acetonitrile for 5 minutes. The acetonitrile was removed and the gel pieces rehydrated in in 100 µl 0.1 M ammonium bicarbonate, then dehydrated in 100 µl acetonitrile, the acetonitrile removed and the pieces completely dried by vacuum centrifugation. The gel pieces were then rehydrated in 12.5 ng/µl trypsin in 50 mM ammonium bicarbonate and incubated on ice for 45 minutes. Any excess trypsin solution was removed and 20 µl 50 mM ammonium bicarbonate added. The sample was digested overnight at 37°C. Peptides were extracted from the polyacrylamide in two 200 µl aliquots of 50% acetonitrile / 5% formic acid. These extracts were combined and evaporated to less than 20 µl for liquid chromatography - mass spectrometry (LC-MS) analysis.

The LC-MS system consists of a Finnigan-MAT TSQ7000 system with an electrospray ion source interfaced to a 10 cm x 75 µm id POROS 10 RC reversed phase capillary column. One µl volumes of the extracts are injected and the peptides eluted from the column by an acetonitrile / 0.1 M acetic acid gradient at a flow rate of 0.6 $\mu l.min^{-1}$. The electrospray ion source is operated at 4.5 kV with a 1.2 $\mu l.min^{-1}$ coaxial sheath liquid flow of 70% methanol / 30% water / 0.125% acetic acid and a coaxial nitrogen flow adjusted as needed for optimum sensitivity. The digest was analysed by capillary LC-electrospray mass spectrometry to measure the molecular weight of the peptides present in the digest. Peptide sequences were determined by collisionally activated dissociation (CAD) using LC-electrospray-tandem mass spectrometry with argon as the collision gas. Database searches were conducted using peptide molecular weight information (mass mapping by MSFit) and partial peptide sequences (BLAST, Altschul *et al.*, 1997) in an attempt to identify any protein that could produce the peptides.

7.3. RESULTS

7.3.1. SDS-PAGE

SDS-PAGE runs of the insoluble fraction (IOM) show eight resolved bands (IF1 to IF8) that stained with Coomassie Blue (Fig. 7.1A).

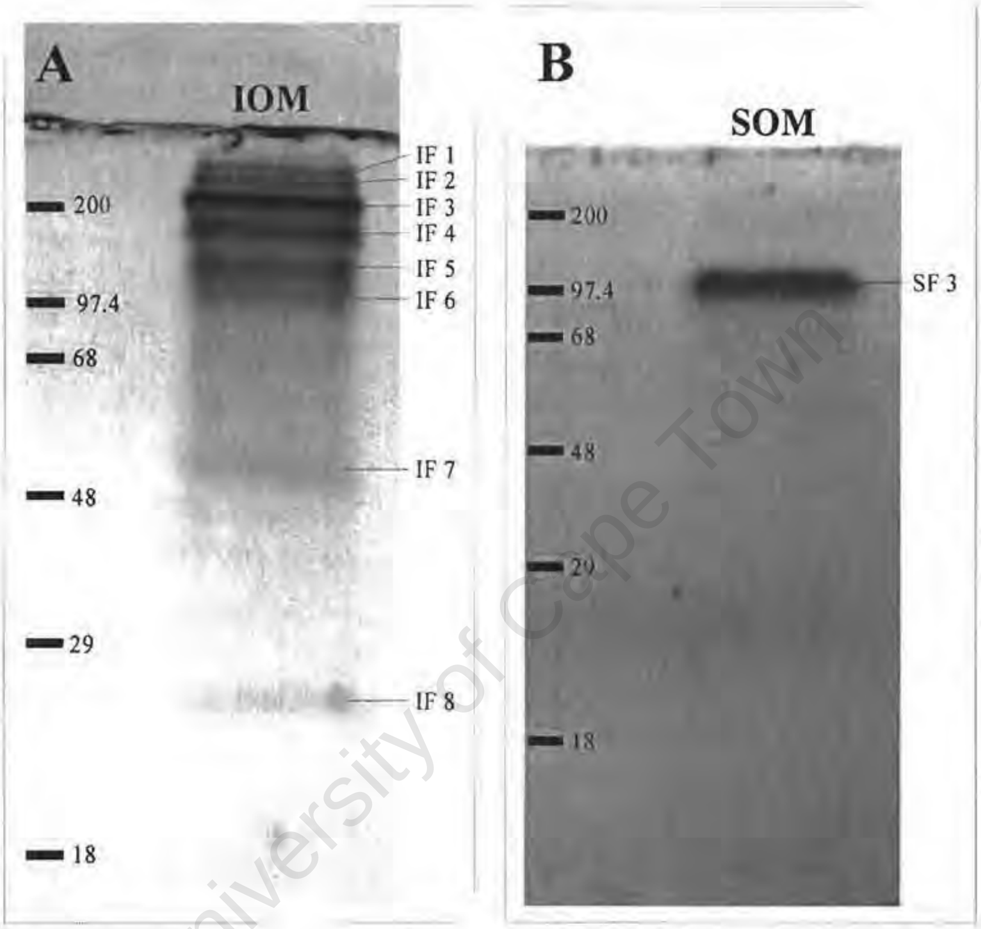


Figure 7.1: Results of the SDS-PAGE showing the composition of the organic matrix ectracted from *Lolliguncula brevis* statoliths. A - Insoluble fraction (IOM), showing the eight resolved bands labelled sequentially IF1 to IF8. B - Soluble fraction (SOM) showing the dominant band (SF3). Bands SF1 and SF2 stain weakly with Coommassie Blue, and are not visible in this gel. Markers are shown in kilodaltons.

The bands ranged in molecular weight from over 200 000 Da (IF1) to about 25 000 Da (IF8). In contrast, SDS-PAGE of the soluble fraction (SOM) showed a simpler composition (Fig. 7.1B), with a single dominant band (SF3) at about 100 000 Da. Bands SF1 and SF2, both over 100 000 Da, stain very weakly with Coomassie Blue, but are clearly visible in the glycoprotein assay (see below).

7.3.2. Glycoprotein content

The high molecular weight SDS-PAGE bands of the insoluble OM fraction (IF1 - IF5) all stain positive with the glycoprotein enzyme immunoassay (Fig. 7 2A). In contrast, the three lower molecular weight bands (IF6 to IF8) do not. The dominant band of the soluble fraction (SF3) stains positive for glycoprotein (Fig. 7.2B), as do bands SF1 and SF2, although staining very weakly with Coomassie Blue.

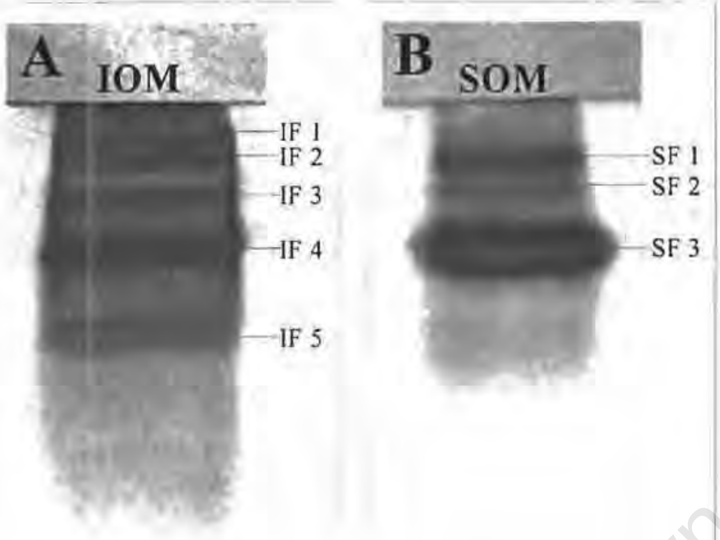


Figure 7.2: Results of the glycoprotein detection assays. Glycoprotein content is indicated with a dark stain. A - Insoluble fraction (IOM). B - Soluble fraction (SOM). Bands SF1 and SF2, which were not visible in the SDS-PAGE results are clearly visible here.

7.3.3. LC - mass spectrometry analysis

Bands IF1 to IF5 from the IOM, and SF3 from the SOM were analyzed by LC-MS. IF5 appears to be a doublet, but was treated as a single band. The remaining bands were excluded because there was insufficient protein present. Trypsin digestion of the proteins in the bands listed above yielded 31, 11, 20, 28, 13 and 8 peptides respectively. The molecular weights and sequences determined from the CAD spectra of each peptide are given in Table 7.1. Some overlap between the IOM proteins is evident. Six of the peptides in IF1 were also present in IF2, specifically those of molecular weight 925, 1022, 1189, 1433, 1608 and 1832 Da. Similarly, the peptides of molecular weight 860, 988 and 1229 Da from IF3 were also present in IF4. Only four of the peptides in band SF3 generated CAD spectra that could be interpreted with any level of confidence. The database searches for proteins containing peptide sequences homologous to those determined for peptides from both the IOM and SOM generated no matches.

Table 7.1: Amino acid sequences and molecular weights (in parentheses) of the peptides obtained from trypsin digests of five of the insoluble fraction (IF1 to IF5) and one of the soluble fraction (SF3) proteins extracted from statoliths of *Lolliguncula brevis*. *(Continued overleaf).*

#	IF1	IF2	IF3	IF4	IF5	SF3
1	VFXGVR (691)	FXAXGXYK (925)	uninterpretable (633)	AFXXK (592)	--ddser (872)	SXFFR (668.8)
2	MAXAQAK (733)	=F=PXK (963)	--p=K (745)	VFX--R (691)	uninterpretable (953)	AAFNXVXEK
3	VTXNNEK (818)	dvXYQVASK (1023)	XTFYSTK (860)	NVXSFK (708)	--HX=R (1083)	--FNXV--K (1023.0)
4	XWXEASR (875)	--NN=K (1163)	YTF= xR (988)	XTFYSTK (860)	--EW= (1335)	--EHEXNQR
5	XPX--VNK (884)	--SFTYPMVR (1189)	--XQ=xyK (1001)	VNXgvmXK (874)	=R (1370)	uninterpretable
6	XTGV---K (915)	--XEXN=QK (1345)	uninterpretable (1007)	XWXEasR (875)	--ENX =DK (1417)	=yqnxer (2265.0)
7	FXAXGXYK (925)	--VVSG=K (1432)	--XXMQNVVK (1132)	xhGEN-- K (912)	WGAGXHFNSK	uninterpretable
8	--VA=R (926)	uninterpretable (1535)	YXQVYFGDK (1133)	WXN-- PK (915)	uninterpretable	uninterpretable
9	DVXYQVASK (1023)	XXAXDGFGE=ER	YAVG =VASXK (1168)	--FXP-- K (949)	uninterpretable	
10	NPDW=dK (1052)	=NF=XS=K (1609)	VDXSP=K (1229)	YXVYFPR (958)	uninterpretable	
11	VAEYMFYAK (1122)	--NSFEYATVSXWVR	WYADVVP =R (1285)	--XNVSXVR (987)	--NDEGTXGhn=	
12	---X=R (1131)		SFVGPYVnnGDK	YTF=R (989)	-- ydnfy= (2758)	
13	--XSXXhdfK (1162)		YXXNMVQPXTNK	EXXP--XAK	--sqx (3136)	
14	--DX=K (1164)		yeNEVFEp--R (1444)	XVXYaasasK		
15	--SFTYPMVR (1189)		--VSAGD=psR (1518)	HXX= (1039)		

Table 7.1: (continued)

#	IF1	IF2	IF3	IF4	IF5	SF3
16	--TSghx=K (1255)		WEVTXGXYS =R	uninterpretable		
17	---XSX=K (1290)		rxDXGVVG-TGVTYR	XFGXYADXR		
18	uninterpretable (1335)		uninterpretable (1694)	--TEV=K (1132)		
19	--XEXN= K (1364)		uninterpretable (1941)	--NX=K (1160)		
20	uninterpretable (1397)		uninterpretable (1947)	NGSAdnxFEAK		
21	uninterpretable (1399)			uninterpretable		
22	--VVS=K (1433)			VDXSP=K (1229)		
23	uninterpretable (1482)			uninterpretable		
24	NXX=R (1485)			uninterpretable		
25	--TH=K (1505)			VTEDXS=DK		
26	uninterpretable (1564)			uninterpretable		
27	---VVSE=R (1583)			uninterpretable		
28	--DFG=K (1607)			uninterpretable		
29	XXAXDGFG=EER					
30	--XSFEYA=R (1833)					
31	--EVXT-- S= (2286)					

Note: X designates either Leu or Ile, which cannot be distinguished by low energy CAD. Lower case letters indicate tentative assignments. - designates a single unknown amino acid. = designates an unknown number of unknown amino acids.

7.4. DISCUSSION

The cephalopod statocyst represents a very attractive model for biomineralization studies in that the processes of statolith mineralization occur primarily in the fluid of the statocyst endolymph. A relatively large volume of fluid separating the secretory surface from the deposition site is consequently available for study (Morris, 1991a). The objective of the research described in this chapter was to provide information towards elucidating the role of the organic component in the statocyst in statolith mineralization, and increment formation in particular. Considering that the mineralization processes are occurring in the endolymph, attention was initially directed to the organic component in this fluid. Preliminary gel electrophoresis of endolymph samples demonstrated a highly complex and heterogeneous ensemble of polypeptides, a similar result being obtained by Takagi and Takahashi (in prep.) in the endolymph of trout. It is unlikely that all of these compounds are involved in statolith mineralization. Identification of those proteins that are involved must therefore begin with characterization of the proteins present in the statolith itself, i.e. the organic matrix. Once this information is available, attention can be directed to the biochemical processes occurring in the endolymph.

The organic matrix of most calcified tissues contains two sub-components, the insoluble and soluble fractions. Because different functional roles have been ascribed to these fractions, they were investigated separately in this study. Very little attention has been directed to insoluble matrix proteins from vestibular structures, and no primary structure data appears to exist for these compounds. The insoluble OM fraction from statoliths of *Lolliguncula brevis* separated in this study contained at least eight major components, ranging from about 25 kDa to in excess of 200 kDa. These results show a more complex composition than the three major IOM components detected in calcitic shell layers of oysters by gel electrophoresis (Kawaguchi and Watabe, 1993). These differences could be attributed to differences in the treatments used to solubilize the proteins, combined with taxonomic and mineralogical differences. It appears that the number and sizes of matrix proteins differ between biogenic calcite and aragonite (Belcher *et al.*, 1996).

The sequence data obtained for five of the insoluble fraction proteins, although incomplete, represents the first attempt to elucidate the primary structure of insoluble matrix proteins from molluscan mineralized tissues. Based on X-ray diffraction patterns, the insoluble matrix in molluscan shells has been described as a silk fibroin - like protein (Weiner and Traub, 1980). That no homologs were found in the database searches suggests that the IOM proteins investigated in this study may very well belong to a previously undescribed class of macromolecules.

In comparison to the IOM, gel electrophoresis of the SOM showed a much simpler composition, with only one dominant protein, and two minor proteins. Considerably more data exist for SOM proteins, drawn primarily from studies of mollusc shells. The numbers and molecular weights of SOM proteins reported in the literature vary substantially, ranging from six major (5 to 95 kDa) and five minor (19 to 200 kDa) proteins in mussel shell SOM (Keith *et al.*, 1993), to two major (67 and 62 kDa) and two to four minor (ranging up to 140 kDa) proteins in oyster shell layers (Kawaguchi and Watabe, 1993). Again, this could be a function of different experimental treatments, combined with taxonomic differences, although mineralogical differences are probably the major contributing factor. The three major proteins extracted from the aragonitic statoliths of *Lolliguncula brevis* in this study match the three dominant proteins isolated from abalone shell composites by Belcher *et al.* (1996). Only one dominant protein was detected by SDS-PAGE in aragonitic teleost otolith matrix (Takagi and Takahashi, *in prep.*). In view of the poor staining of the higher molecular weight SOM proteins that was observed in this study, the possibility that other proteins were not detected by Takagi and Takahashi cannot be discounted.

Trypsin digestion of the dominant SOM band generated eight peptides. Partial sequencing of these peptides showed no evidence of the dominant $(D - X)_n$ sequence (where X is glycine or serine) suggested for molluscan shell soluble matrix (Halloran and Donachy, 1995), or any correspondence to the sequences determined for the most acidic protein component in scallop shell soluble fraction (Halloran and Donachy, 1995). Further, the derived sequences contained no aspartic acid and very few glutamic acid residues, both of which are presumed to dominate SOM proteins (e.g. Weiner and Traub, 1984). This result, combined with the lack of success in identifying any homologs for any of the SOM or IOM peptides in the database searches, suggests that squid statolith OM proteins belong to a class of previously undescribed macromolecules. Different proteins may therefore be involved in biomineralization processes in different organisms.

The majority of the proteins in both the insoluble and soluble matrix fractions contained glycoprotein. This result is consistent with those obtained from many investigations of vestibular organic matrices (e.g. Wright, 1991; Pote *et al.*, 1993; Albeck *et al.*, 1996). Glycoproteins are considered to be fundamental to the regulatory nature of the OM proteins (Albeck *et al.*, 1996; Falini *et al.*, 1996). It is worthwhile noting that the molecular weight estimates of these proteins obtained by comparison with the markers may not be a true reflection of the mass of the polypeptide chain, since glycosylation of proteins is known to influence the rate of their migration through polyacrylamide gels (Sambrook, Fritsch and Maniatis, 1989).

The preliminary results presented here, when combined with previously reported observations, permit some speculation concerning the role of the OM in increment formation in squid statoliths. SEM observations of statoliths and otoliths that have been etched with weak acids or calcium chelating agents reveal increments as alternating deeply etched areas between prominent lightly etched areas. The former correspond to the discontinuous zones of increments, while the latter correspond to the incremental zones (terminology of Mugiya *et al.*, 1981). These observations have led to the inference that the incremental zones represent regions of relatively higher mineral to protein ratios, while the reverse is true for the discontinuous zones. An alternative explanation could be that the incremental zones contain a higher proportion of insoluble matrix proteins relative to the discontinuous zones.

In the opening section of this chapter, mention was made that when the mineral phase is removed from a statolith, the remaining organic matrix (visible as a transluscent gelatinous "ghost" - Lipinski, 1986) retains the overall morphology of the statolith, including the incremental and other microstructural features that are apparent in the intact statolith. It should be noted that the treatments that are used to decalcify statoliths also remove the soluble organic matrix fraction. In other words, the residual matrix "ghost" is composed entirely of the insoluble fraction. This would suggest that it is the insoluble fraction that regulates the overall architecture and microstructure of the statolith, including the increments.

Aragonite crystals in teleost otoliths are twinned (Gauldie and Nelson, 1988). Twinning often occurs in the presence of foreign ions, and represents an efficient mechanism for rapid crystal growth. Some mechanism must be in place to prevent runaway crystallization. The observation of a daily rhythm in the secretory activity of the cells of the macula led to the suggestion that neuroproteins secreted at the macula control the rate of crystal growth and possibly crystal size (Gauldie and Nelson, 1988). If these proteins comprise the structural framework of the mineral (i.e. the insoluble matrix), reduced secretion of the protein would periodically limit crystal growth, resulting in a check on runaway calcification. This check would be manifested as the deposition of the discontinuous zone of each increment. This zone is considered to reflect periods of slow growth of teleost otoliths (Mugiya *et al.*, 1981). The implication of this argument is that it is the insoluble matrix proteins that regulate statolith formation on an incremental scale. While the soluble matrix proteins presumably regulate crystal growth at a molecular level, it may be the differential secretion of the insoluble matrix proteins over time that regulate increment periodicity and width (i.e. appearance).

Further research directed at possible daily cycles in the production, secretion and/or deposition of the insoluble matrix proteins is required to test this hypothesis. If confirmed, further detailed characterization of these proteins may provide a link between the production of these compounds and the circadian rhythm that is presumably regulating their secretion into the statocyst lumen. It is hoped that the preliminary results presented here will contribute to further developments in this field.

CHAPTER 8 - CONCLUSION

Many studies employing the statolith ageing technique have been conducted over the past decade, highlighting the value of the information that can be derived from statolith microstructure for elucidating squid population dynamics. For example, life spans have been suggested to be considerably shorter than previously assumed (e.g. Jackson, 1994; Macey, 1995; Brodziak and Macey, 1996), and initial estimates of squid growth rates have had to be revised. Squid growth appears much more rapid than initially thought, and the evidence strongly suggests that the form of growth is exponential (e.g. Jackson and Choat, 1992), rather than asymptotic, as is assumed in many of the growth models conventionally used in stock assessments. The implications of such revisions in terms of management strategies and decision-making are profound, and need no further discussion.

However, criticisms of the daily increment ageing technique in terms of the validity of the results and subsequent conclusions have been voiced in the literature. For example, Jarre *et al.* (1991) argue that growth rates derived from statolith ageing studies considerably over-estimate true squid growth. Dawe and Beck (1997) have assessed this argument, however, and concluded that several biases implicit in the study of Jarre *et al.* (1991) seriously weaken their argument. Other criticisms present a more serious obstacle. Morris (1988) challenges the assumption of the daily periodicity of increment formation in squid statoliths. This issue had yet to be resolved, as will be discussed below. Pauly (in press) and Balgos and Pauly (in press) question the validity of squid growth estimates obtained from statolith analyses. Their argument is based on the currently unresolved issue of statolith microstructure interpretation, and specifically the biases inherent in inaccurate increment recognition and enumeration. Such biases could lead to inaccurate estimation of population growth parameters (Morales-Nin, 1988).

These criticisms emphasise the need for further research investigating the application of the daily increment ageing technique to squid population studies. The studies described in this thesis were aimed at critically assessing the use of statoliths to estimate age, and attempted to provide information that could contribute to resolving the issues discussed in the opening chapter.

The first aspect that was addressed was the technical limitations of the statolith ageing technique, and specifically the preparation and subsequent analysis of statoliths using light microscopy (LM). The method of preparing loliginid squid statoliths for increment counting that was described in Chapter 2 and refined in Chapter 3, generates specimens in which increments can be visualized from the nucleus to the margin of the statolith. The overall performance of the method in terms of success rate appears to be superior to that used for scanning electron microscopy (SEM). An unexpected finding is that increment counts obtained using the LM method are not significantly different from those obtained using SEM, suggesting that either method could be used to estimate increment numbers in *Loligo vulgaris reynaudii* with equal effectiveness. Considering the greater resolving power of SEM, one would expect that substantially larger increment counts would be obtained using SEM. This was not the case. A possible explanation for this finding is that increments in the statoliths of the squid used in the study did not fall below the resolving power of the light microscope. Larger numbers of squid sampled from all areas of their habitat are required to rigorously test this hypothesis.

Two limitations of the LM technique require discussion. Firstly, increments were not interpreted as consistently by different readers as they were using the SEM technique. This problem could introduce considerable bias into age estimates if several different readers are involved in statolith analysis during a stock assessment. The results obtained by the three different readers during the field validation study described in Chapter 3 clearly illustrate this shortcoming. This is a crucial issue, and material of the nature of that collected during the validation experiments is vital to resolving this problem. Well-prepared statoliths from squid of known age (or marked statoliths of known post-marking duration), encompassing as broad a size and age range of both sexes as is possible, are required as training material to familiarize readers with increment structure and appearance. Material of this nature is also invaluable for assessing the level of error associated with the age estimates obtained by different readers.

The second limitation of the technique concerns the time and labour constraints imposed by the successful preparation and subsequent analysis of the specimens. The time constraint is of critical importance in assessments of commercially exploited squid stocks. If the rapid growth rates and short life-spans currently felt to apply to such squid species are valid, management decisions have to be made in the short term, requiring that age information be made available as quickly as possible. Further, an effective stock assessment relies on age estimates representative of the entire population. This is particularly important in assessments of squid stocks, considering the spatial and temporal heterogeneity of squid populations (O'Dor, 1998; Lipinski *et al.*, in press). The sampling strategy would therefore have to cover as broad a range of individuals from as wide an area as possible, and would consequently involve many samples. The time needed to successfully prepare the required number of samples has to be minimized. The low success rates of the preparation technique indicated by the results of Chapters 2 and 3 exacerbate the problem further, since more samples will have to be collected and prepared than may be specified by the stock assessment protocol.

Although less time-consuming and labour-intensive than the SEM preparations, these constraints on the LM preparation technique are still considerable. Experience with the technique will improve efficiency and success rates, but further research should explore other possibilities. Advances in image analysis technology hold some promise for facilitating the second stage of the process, namely the acquisition of data from the specimens. Images of specimens can be obtained by high-resolution video cameras mounted in the microscope, and subesquently downloaded onto a personal computer. Techniques to enhance these images, as well as automate increment counting from the images, have been developed (Campana, 1987; Troadec, 1991; Macy, 1995; Lagardère and Troadec, 1997; Arkhipkin *et al.*, in press). Images can be readily downloaded and archived on disk, eliminating the requirement for photography (which is comparatively inflexible, and often results in a loss of image quality).

From the above discussion, it is clear that an assessment of the success rate of any given ageing technique is crucial to developing a representative sampling protocol (and specifically, estimating the numbers of samples that will be required) for a stock assessment. For this purpose, it is useful to quantify success in terms of the quality of the statolith preparations that are generated. In Chapter 2, this was loosely assessed by the degree of interpolation or extrapolation required to obtain an estimate of total increment numbers from a given statolith. In Chapter 3, a more rigorous (albeit subjective) quantification of preparation quality was based on various criteria influencing the confidence with which a reader counted increments deposited after oxytetracycline marking of the statoliths. Although useful for the purposes of the validation study, this latter approach has no relevance to estimates of total increment numbers. In ageing studies, it would perhaps be more appropriate to adopt the approach used in Chapter 2, but assign each specimen a value expressing the level of confidence associated with the estimate of increment numbers. The proportion of the counting axis that required extrapolation/interpolation could be expressed as a percentage, for this purpose. The error modelling exercise described in Chapter 3 illustrates how such information can then be used to quantify the relationship between specimen quality and the level of error. The number of specimens that will provide estimates of increment numbers with a specified level of accuracy can then be estimated for the preparation method.

The second major issue addressed in this thesis is that of the fundamental hypothesis underlying the application of the daily increment ageing technique, namely that increments in squid statoliths reflect a daily cycle in statolith growth. This hypothesis has to be confirmed if the technique is to have any relevance to ageing studies. In comparison to studies of teleost otoliths, relatively little research has been directed to this question in squid statoliths. Usually, tests of the daily increment hypothesis in cephalopod statoliths have employed statolith marking experiments (e.g. Hurley *et al.*, 1985; Dawe *et al.*, 1985; Lipinski, 1986; Nakamura and Sakurai, 1990, 1991; Jackson, 1990a, 1990b). Without exception, these studies have concluded that increments are deposited with a daily frequency. However, all of these studies have been conducted in the laboratory. The applicability of the results of laboratory studies to wild populations has been questioned (Campana and Neilson, 1982; Jones, 1986; Campana and Moskness, 1991). The validation studies described in this thesis (Chapter 3) addressed this issue, and are the first statolith marking experiments to be conducted under field conditions, and compare the results with those of laboratory experiments conducted on the same species.

The results of the experiments were, to a certain extent, consistent with daily increment production under both field and laboratory conditions. Counts of the number of post-mark increments recorded by two out of three readers from field specimens that fulfilled stringent quality criteria, closely approximated the elapsed number of days. This was not the case for the third reader. However, it was observed that this reader was considerably less rigorous in the identification of a well-prepared statolith. It is argued that the negative result obtained from this reader is a function of the poorly-prepared statoliths that were considered by the reader to be suitable for accurate increment counting, but which in fact were not. In other words, the result was due to observer error in ranking the specimens according to their quality. Where the readers were more critical in their assessment of specimen quality, the results were consistent with daily increment production.

This conclusion was drawn in spite of the high estimates of the level of type II error inherent in the tests of the hypothesis. The approach used in validation studies of this nature yields a positive conclusion (i.e. in support of daily increment production) by non-rejection of the null hypothesis. This approach raises the issue of statistical power, and the probability of accepting a false null hypothesis. The low estimates of power corresponding to the tests of the daily increment hypothesis in this study indicated an unacceptably high risk of incurring this source of error. It is argued that this result is a direct function of the small sample sizes involved, particularly in the case of the field study. In support of this argument, the laboratory study (which generated a larger number of successful statolith preparations) resulted in a substantial increase in the level of power associated with the hypothesis test. It is felt that with increased experience in the preparation technique, and the acquisition of a larger number of satisfactorily prepared specimens, tests of the hypothesis can be conducted with a greater level of statistical power, increasing the level of confidence in the conclusions.

Comparison of data between field and laboratory conditions indicated that the results of laboratory studies can be justifiably extended to wild populations of South African chokka squid. This conclusion, however, only applies to adult males. Data from only one female was available in the field study, and the differences between male and female results in the laboratory study indicated that the same conclusion cannot be made for female squid. That the results from the female squid in the laboratory study are not consistent with daily increment production is a cause for some concern. Research should be directed to establishing whether this is a function of physiological differences between the sexes, or merely an experimental artefact. A further limitation of the validation studies is that the conclusions only apply to mature adults. No inferences concerning the frequency of increment deposition in paralarvae and juveniles can be drawn from the results. Since an assumption inherent in the application of the daily increment technique is that daily increment production persists throughout the lifespan of the squid, attention should be directed to testing this hypothesis in the younger life stages.

The preceding discussion has highlighted the inaccuracies in increment interpretation and counting that result from poor preparation procedures. Similar inaccuracies can also result from physiological effects on statolith deposition. Various environmental factors can influence the deposition of increments to the extent that their interpretation and subsequent enumeration is compromised. The factors of relevence in this regard are those influencing increment appearance in terms of width and clarity. Chapter 4 explores the effects of probably the most significant of these factors, namely temperature. This environmental variable has a direct, positive effect on statolith growth rate in the thumbstall squid (*Lolliguncula brevis*), and hence on putative daily increment width. Low temperatures (15 °C) were demonstrated to result in statolith growth rates of below 1 μm.d^{-1}. Increment widths of this magnitude approach the resolution limits of a light microscope. It is very likely that increments deposited under such conditions would not be detected using a light microscope, or alternatively, would be interpreted as sub-daily in nature, either of which would introduce errors into the age estimates obtained, leading to under-estimates of age and over-estimates of somatic growth.

If such low-temperature conditions are maintained for extended periods, the region of the statolith deposited over this period would display a "blurred" appearance, with no clear incremental structures. Researchers confronted with such zones in a statolith generally resort to one of two options. The number of increments in these zones can be interpolated or extrapolated using the widths of adjacent increments.

Alternatively, and particularly in cases where such zones occur over a significant proportion of the statolith growth axis, the statoliths are discarded as unreadable. If such zones are a result of narrow increments, interpolation or extrapolation would generate an inaccurate estimate of increment numbers, with a concomitant effect on growth parameter estimation (Morales-Nin, 1988). If such zones are a common occurrence in a sample of statoliths, it is strongly recommended that a sub-sample be examined using SEM to establish whether the zones comprise a series of narrow increments as opposed to a series of low-contrast increments resulting from either poor statolith preparation, or constant environmental conditions (see below).

The constant temperature conditions imposed during the experiments resulted in a general reduction in increment contrast and clarity. This effect is presumably due to a dampening of the metabolic cycles associated with increment production, resulting in a decrease in the contrast between adjacent incremental and discontinuous zones of increments. As was evident in Chapter 4, areas of poorly-defined increments complicate increment interpretation and counting, increasing the level of error associated with estimates of total increment numbers. Whether such constant conditions can occur for any appreciable length of time in wild populations remains to be explored.

In the closing section of Chapter 3, the philosophy underlying the use of validation experiments such as those described in this study to rigorously test the daily increment hypothesis was questioned. The argument was put forward that the results of such experiments, while perhaps demonstrating a correspondence between increment counts and elapsed time that may be considered to support the hypothesis, cannot discount the possibility that increments are not a reflection of a circadian rhythm in statolith growth, but were coincidentally deposited at a rate of approximately one per day in response to some other factor(s) over the course of the experiment. In other words, no conclusions concerning cause and effect can be inferred from the results of such studies. Such conclusions, it is suggested, can only be derived from studies investigating the physiological processes of statolith deposition, with clear proof that these processes cycle with a circadian rhythmicity. Information of this nature is very limited with regard to squid statolith deposition, and this deficiency has to be addressed if the daily increment ageing technique is to be applied with any level of confidence.

Towards this objective, the studies described in Chapters 5, 6 and 7 investigated various aspects that were felt to be of relevance in statolith growth. The results of the nuclear microprobe study (Chapter 5) investigating the distribution of elements in statoliths of the South African squid *Loligo vulgaris reynaudii* were consistent with the hypothesis of biomineralization of squid statoliths proposed by Lipinski (1993), namely that strontium is secreted into the endolymph from the cells of the macula, whereas calcium is supplied from sites distant to the macula. The correspondance between regions of high strontium content and clear increments provide support for the hypothesis that this element is intimately involved in statolith deposition, and specifically the formation of clear, well-defined increments (Lipinski, 1993). The nature of this involvement with regard to whether strontium actively regulates increment deposition rather than fulfilling a passive role in the differential deposition of increments, remains speculative. The observations of Hanlon *et al.* (1989) and Bidwell *et al.* (1990)

suggest that strontium plays an active role.

An attempt to elucidate the role of this element in statolith deposition, as well as that of calcium, magnesium and pH, is described in Chapter 6. The experiments conducted during this study are the first to explore the possibility of daily cycles in the chemistry of the statocyst endolymph of loliginid squid. The approach of the study rests on the assumption that the processes of statolith growth, manifest as the differential formation of increments, is a function of fluctuations in the chemistry of the statocyst endolymph. If such fluctuations occur with a daily frequency, some physiological basis for the daily increment hypothesis can be inferred. Clearly, the link between such fluctuations and increment formation would have to be elucidated, and clear proof that these fluctuations occur with a circadian rhythmicity would have to be obtained before conclusive deductions concerning the daily frequency of increment formation can be drawn.

Such objectives were beyond the scope of the study, which can only be considered preliminary. The lack of success in obtaining data for strontium and pH is a serious shortcoming. Further attention should be directed to these parameters. The data that were obtained for calcium and magnesium levels, however, represent the first indication that the daily increment hypothesis has some physiological basis in squid. The daily cycles that were evident in these two parameters may reflect the differential deposition of increments in squid statoliths. The evidence is weak, however, primarily due to the considerable variability apparent in the data. Attempts to account for this variability met with limited success. It is recommended that further experiments of this nature be very precisely controlled, with as many potentially confounding variables as is possible being accounted for.

The anti-phasic nature of the calcium and magnesium fluctuations provide some basis for speculation concerning the roles of these elements, and magnesium in particular, in increment formation. Magnesium has been shown to have an inhibitory effect on calcification (Morris, 1988, 1991). The elevations of endolymph magnesium content at night may reflect a regulatory mechanism to periodically limit runaway calcification. This argument does not account for the roles of strontium and the organic component, both of which have been implicated in regulating biological calcification. Further research is required to explore the proposed regulatory roles of these components.

Before the role of the organic component can be elucidated, the macromolecules comprising the organic matrix have to be characterized. No such information exists for squid statolith organic matrices, apart from the amino acid composition studies of Radtke (1983), Lipinski (1986) and Morris (1988). This deficiency was addressed in the study described in Chapter 7. The primary structure information obtained during the study represents the first attempt to characterize the organic matrix proteins of squid statoliths, and the first primary structure data for the insoluble fraction from any molluscan mineralized tissues. The majority of the proteins (both soluble and insoluble) were glycosylated, a feature common to most proteins from biologically calcified structures. However, the primary structure data indicated that these proteins are unique, and belong to a class of previously undescribed organic macromolecules. This finding suggests that different proteins may be involved in biomineralization in different organisms. In terms of the role of the organic matrix proteins in increment formation, it is proposed that while the soluble proteins may regulate crystallization at a molecular level, it is the insoluble proteins that regulate statolith growth at the incremental level. I suggest that differential secretion of these insoluble proteins (or their sub-components) over time governs the differential deposition of the incremental and discontinuous zones of increments. If this hypothesis can be confirmed, the cephalopod statocyst presents an attractive model for testing the proposed daily cycle in insoluble protein secretion, and linking this cycle to the circadian rhythm that is hypothesized to underly daily increment formation in squid statoliths. I consequently recommend that further research investigating the physiology of statolith formation be aimed at the insoluble matrix component, and specifically at potential daily cycles in the production and/or secretion of these proteins.

In summary, the work described in this thesis has attempted to contribute to resolving several of the issues currently limiting the application of the daily increment ageing technique to squid stock assessments. A method of preparing statoliths from chokka squid has been developed, but requires further refinement. Recommendations are made to facilitate the collection and analysis of data from statolith examination. Support for the daily frequency of increment formation in chokka squid has been obtained, but further tests of the hypothesis in other life stages are required. Laboratory experiments appear to be capable of generating meaningful results applicable to wild populations, but this has to be researched further, both for adult females and for the younger life stages. The influence of temperature on statolith microstructure is assessed, and is concluded to be capable of significantly influencing the interpretation of increments via its effects on increment width and contrast. A nuclear microprobe study mapping the elemental composition of statoliths indicates that strontium is closely linked to increment formation. It is recommended that further attention be directed to this element. The results of preliminary studies investigating the physiological processes of statolith mineralization yielded the tentative conclusion that these processes may display daily cycles, but no deductions concerning cause and effect can be expressed. Many questions concerning these processes remain unanswered, and it is recommended that carefully designed and controlled experiments address the physiological processes and cycles of increment production.

It is concluded that statoliths remain a valuable, and at present, the only tool for estimating the age of squid. Considering that squid populations generally display substantial short-term fluctuations in abundance, coupled with complicated migration patterns, the spatial and temporal heterogeneity of squid stocks dictate that effective stock assessments incorporate extensive age structure information that is representative of the entire stock. Statolith analysis is currently the only tool that can provide this information. Although the statolith ageing technique still has several shortcomings, these can be overcome by rigorous sampling, data collection and analysis protocols. Carefully controlled and properly conducted validation experiments are crucial to quantify the level of error (or conversely, the level of accuracy) inherent in the age estimates obtained from the species and life stages in question. Recommendations to management can then be made with a specified level of confidence in the results of the stock assessment.

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APPENDIX I: Results of the field validation study. Counts of the number of post-mark increments and the values assigned to each quality factor (I, O and M) are shown for each replicate (1, 2 and 3) conducted by readers 1, 2 and 3 on each statolith. ML = dorsal mantle length (mm); WT = body wet weight (g); SEX = sex and maturity (assessed according to the scale of Lipinski and Underhill, 1995); D = days post-marking. Data fulfilling the filtering criteria (see text) are shaded. (Continued overleaf)

BIOLOGICAL				READER 1												READER 2												READER 3											
				COUNTS			I			O			M			COUNTS			I			O			M			COUNTS			I			O			M		
ML	WT	SEX	D	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
306	532	♂ 5	5	5	5	5	3	3	3	3	3	3	2	3	3	4	5	4	2	2	2	3	2	3	2	2	2	7	5	5	3	3	2	3	3	3	3	3	1
303	427	♂ 5	5	5	5	5	3	3	3	3	3	3	2	3	3	4	4	4	3	3	2	3	3	3	3	3	2	5	5	5	3	3	3	3	3	3	3	3	3
290	406	♂ 5	5	4	4	3	3	3	3	1	1	2	2	2	2	3	3	3	3	3	3	3	3	3	3	2	2	5	5	4	3	3	3	3	2	2	2	2	2
260	270	♂ 5	5	4	5	4	2	2	1	1	1	1	2	1	1	5	5	5	2	1	1	2	2	2	2	2	2	6	6	5	2	2	3	3	2	2	2	2	3
295	446	♂ 5	5	5	5	4	2	1	2	3	3	2	3	2	2	7	6	6	2	1	1	3	2	3	1	2	2	5	6	8	3	2	2	3	3	3	3	2	2
173	134	♀ 5	11	11	10	12	2	2	2	3	3	3	3	3	3	10	10	10	2	2	2	3	3	3	2	2	2	9	9	9	3	3	2	3	3	3	2	2	2
293	413	♂ 5	5	6	4	5	2	1	1	1	1	1	3	3	2	7	4	4	3	1	1	3	2	2	2	2	2	5	6	6	2	2	2	1	3	1	3	3	3
318	416	♂ 5	5	4	4	5	2	1	2	2	2	2	1	1	1	5	6	6	2	2	2	2	2	2	1	1	2	7	6	10	3	3	3	3	3	3	2	3	3
316	433	♂ 5	5	6	5	7	3	3	3	1	2	1	2	2	3	11	12	11	3	3	3	2	2	2	2	3	2	8	10	9	3	3	3	2	3	2	3	3	3
390	657	♂ 6	9	4	4	3	2	1	2	2	3	2	2	2	1	7	10	7	2	2	3	3	2	3	1	2	2	5	6	5	3	3	3	3	3	3	2	3	2
280	334	♂ 5	11	15	14	12	2	1	1	1	1	2	2	2	2	14	15	14	2	2	2	2	2	2	2	2	2	16	18	12	3	2	2	1	2	1	2	2	3
288	330	♂ 5	14	13	12	12	2	2	2	3	3	3	3	2	2	10	9	10	1	2	2	3	3	3	1	2	2	13	11	12	3	2	2	3	3	3	1	2	2
282	333	♂ 5	9	8	8	9	2	3	2	1	1	1	1	1	1	7	6	7	2	2	2	2	2	1	1	1	1	6	7	6	3	2	2	3	2	2	2	1	2
321	450	♂ 6	9	5	6	5	2	1	1	1	1	1	2	3	2	9	10	7	2	1	1	1	2	2	3	2	1	7	6	7	2	2	2	1	3	2	2	2	2
290	298	♂ 5	11	13	11	14	2	2	2	3	3	3	3	2	2	11	14	12	2	2	2	3	3	3	2	2	2	14	13	12	2	2	3	3	3	3	3	3	3
276	300	♂ 5	18	15	16	18	3	2	2	3	3	3	2	1	1	8	10	14	2	2	2	2	3	3	2	1	1	17	15	9	3	3	2	3	3	3	2	1	1
291	265	♂ 5	9	6	6	5	3	2	2	3	3	3	1	1	2	6	5	6	3	2	2	3	2	3	1	1	1	6	5	7	3	2	2	3	3	2	1	2	1
245*	202	♂ 5	13	10	11	12	2	2	2	3	3	3	2	2	1	9	9	8	1	1	2	2	3	2	1	2	2	8	11	10	3	2	2	3	3	3	3	3	2
291	300	♂ 5	13	8	7	8	1	2	2	3	3	3	1	1	1	6	7	7	2	2	2	2	3	2	1	1	2	6	6	8	3	2	3	2	3	3	2	2	2
325	431	♂ 5	13	11	13	13	1	2	2	2	2	1	2	2	1	8	11	10	2	3	3	1	1	1	2	1	2	12	11	9	3	3	2	2	1	1	3	1	2
352	504	♂ 5	6	5	4	4	2	2	2	2	2	2	1	1	2	4	4	5	3	2	2	3	3	2	2	2	2	5	5	5	3	3	3	3	3	2	2	3	2
350	424	♂ 5	6	5	4	5	2	1	2	1	1	2	2	2	2	4	5	4	2	2	2	2	2	2	2	2	2	6	6	9	3	2	3	1	1	1	2	2	2

APPENDIX I: (continued)

BIOLOGICAL				READER 1												READER 2												READER 3											
				COUNTS			I			O			M			COUNTS			I			O			M			COUNTS			I			O			M		
ML	WT	SEX	D	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
326	353	♂5	13	9	11	9	1	1	1	1	1	1	1	1	1	8	9	9	1	2	2	1	1	1	2	2	1	10	13	11	3	3	1	2	1	1	3	2	1
302	370	♂5	12	11	13	11	2	2	2	3	3	3	3	2	2	7	11	11	1	2	2	3	3	2	2	2	2	8	9	10	3	3	3	3	3	3	2	2	2
291	360	♂5	5	4	4	4	1	1	2	2	2	2	3	2	2	4	5	3	1	1	1	2	2	2	2	1	2	5	5	7	2	2	2	1	3	2	1	2	1
340	563	♂5	5	8	7	6	3	2	2	2	2	1	3	2	2	7	8	10	3	2	2	3	2	2	1	1	1	8	7	9	3	3	3	3	2	3	2	2	3
332	486	♂5	5	5	4	4	2	2	2	2	1	1	2	2	2	9	8	9	2	3	2	3	2	1	2	1	2	9	9	12	3	3	3	3	2	2	2	3	3
381	627	♂5	5	5	5	5	1	2	2	1	1	2	2	1	1	6	6	3	2	1	2	2	2	2	1	2	2	7	6	7	2	2	2	2	2	2	2	2	2
353	544	♂5	5	10	6	6	1	2	2	2	2	1	2	2	2	7	8	7	2	1	1	2	2	1	1	2	2	10	9	8	3	3	1	2	3	2	1	2	2
321	473	♂5	5	6	4	5	1	1	2	1	1	1	2	1	2	12	16	17	2	1	1	2	2	2	1	2	2	15	15	11	2	2	2	3	3	2	2	2	1
392	673	♂5	5	6	5	4	1	1	1	1	1	1	2	2	2	12	6	8	2	2	1	2	2	1	2	2	2	12	8	12	2	3	2	1	2	2	3	3	3
337	416	♂5	5	7	9	6	2	2	3	3	2	2	2	1	1	14	12	11	2	1	2	2	3	2	1	1	1	8	11	12	3	2	2	3	3	3	3	3	3
349	407	♂5	5	7	5	6	2	1	1	1	2	1	2	1	1	8	10	6	1	1	1	2	2	1	2	2	2	8	7	10	2	2	2	3	3	3	2	2	3
316	342	♂5	5	5	4	4	1	2	1	1	1	1	2	2	2	5	5	5	2	2	2	3	1	1	2	2	1	5	5	7	2	3	2	2	3	2	2	3	2
330	482	♂5	5	4	5	6	2	2	3	2	1	1	1	2	1	6	9	5	2	2	2	1	1	1	3	2	2	9	9	12	3	2	2	1	1	1	3	2	2
370	587	♂5	5	7	7	7	2	2	2	1	2	1	1	2	2	9	13	10	2	2	2	2	2	2	2	2	1	10	15	13	2	3	2	3	3	3	3	3	3
378	602	♂5	5	5	5	4	2	2	2	2	1	1	2	1	1	8	10	5	2	2	1	2	2	2	1	1	1	10	9	10	2	2	2	2	3	2	1	1	1
335	461	♂5	5	9	8	8	2	2	3	2	2	1	3	1	2	8	8	11	2	2	1	1	2	2	2	1	2	7	8	8	3	3	2	3	2	2	2	1	1
270	259	♂5	5	13	7	7	1	1	1	3	2	2	2	2	2	10	9	9	1	1	1	2	2	2	1	2	2	10	7	10	2	3	2	3	3	3	3	3	3
351	500	♂5	5	4	4	3	1	1	1	1	1	1	1	2	2	8	9	9	1	1	1	1	1	2	2	2	2	13	10	8	1	1	1	1	1	2	2	3	1
412	762	♂5	5	5	4	4	1	2	2	1	1	1	1	1	1	12	8	10	1	2	1	2	1	2	1	2	2	10	7	8	3	2	2	1	1	2	1	2	1
321	433	♂5	5	4	4	4	2	1	1	1	1	1	1	2	2	4	4	4	2	2	1	3	1	2	1	2	2	4	4	4	1	2	1	1	1	1	1	2	2
359	601	♂5	5	4	5	4	2	2	2	2	2	1	3	2	3	4	6	5	3	2	2	3	2	2	3	1	2	6	6	5	3	2	3	2	2	3	3	3	3
336	456	♂5	5	5	5	3	1	2	1	1	1	1	3	3	3	11	5	7	1	1	1	1	1	1	2	1	2	7	11	9	1	1	1	1	1	1	3	2	2
308	359	♂5	16	12	15	14	2	2	1	1	1	1	1	2	1	11	10	11	2	2	2	2	1	1	2	2	2	8	9	8	2	2	2	1	2	1	1	1	2

APPENDIX II

Results of the laboratory validation study. Counts of the number of post-mark increments and the values assigned to each quality factor (I, O and M) are shown for each replicate (1, 2 and 3) conducted on each statolith. ML = dorsal mantle length (mm); WT = body wet weight (g); SEX = sex and stage of maturity (assessed according to the scale of Lipinski and Underhill, 1995); D = days post-marking. Data fulfilling the filtering criteria (see text) are indicated in shaded blocks. The two individuals labelled twice with OTC (see text) are indicated with * and # respectively. (Continued overleaf).

BIOLOGICAL				COUNTS			I			O			M		
ML	WT	SEX	D	1	2	3	1	2	3	1	2	3	1	2	3
181	132	♀3	2	6	7	6	3	3	3	2	2	2	3	3	3
200	192	♂3	4	4	4	5	2	2	3	2	2	2	1	2	2
171	131	♀3	4	3	3	4	2	2	2	3	3	3	1	2	3
180		♀4	7	7	8	8	3	2	2	3	3	3	2	2	2
203		♀5	7	5	6	8	1	2	2	2	2	2	1	2	2
335	613	♂5	14	12	13	14	3	2	3	3	3	3	2	2	2
222		♀5	6	8	6	7	3	3	3	2	3	2	2	3	2
185	148	♀5	2	2	2	2	2	2	2	2	3	3	2	2	2
237	236	♂5	3	2	3	3	3	2	2	3	3	3	2	2	2
200	146	♂5	2	10	7	7	2	3	3	1	3	2	1	2	2
310	389	♂5	8	12	13	12	1	2	1	3	3	3	1	2	2
164	115	♀5	2	2	2	2	3	3	3	3	3	3	2	2	2
143	83	♂2	2	1	2	2	1	1	1	1	1	2	1	1	2
253	212	♂5	4	2	3	2	3	3	2	3	2	3	2	2	2
188	167	♀5	6	8	5	9	2	3	3	2	2	3	1	2	2
199	162	♀5	3	2	2	2	2	3	3	2	2	3	2	1	2
180	126	♀5	3	1	1	2	3	2	2	2	2	3	2	2	2
363	516	♂5	5	3	2	2	2	3	2	1	2	2	2	2	2
177	145	♀5	7	5	5	6	3	3	3	2	3	2	2	3	3
164	118	♀5	9	3	3	5	3	3	2	1	1	1	2	2	2
183	123	♂3	9	10	10	8	1	2	3	2	2	2	2	2	2
269	286	♂5	9	8	6	8	3	3	3	2	3	2	2	3	2
208	154	♂5	10	11	10	10	1	2	3	2	2	3	2	2	2
338	419	♂5	17	16	13	12	2	2	3	3	2	3	2	2	2
194	168	♀5	2	3	3	2	2	3	2	3	3	3	2	2	2
198	176	♀5	2	2	2	7	2	3	3	1	1	1	2	3	2
188	131	♀4	2	1	2	2	2	1	2	2	1	2	2	2	2
174	125	♀4	2	2	2	3	2	3	2	3	3	2	2	2	2
178	152	♀4	3	3	2	2	2	1	2	2	3	2	2	2	2
185	153	♀5	4	2	2	2	1	1	2	2	3	2	1	2	2
189	139	♂2	9	10	7	9	2	2	2	2	2	2	2	2	2
243	230	♂5	11	12	12	11	2	3	2	2	2	2	3	2	2
273	400	♂5	12	11	13	12	2	2	2	3	3	3	2	2	2

APPENDIX II (continued)

BIOLOGICAL				COUNTS			I			O			M		
DML	WT	SEX	DAYS	1	2	3	1	2	3	1	2	3	1	2	3
249	256	♂ 5	13	11	11	13	2	2	2	3	3	3	2	2	2
303	430	♂ 5	13	14	14	15	2	2	3	3	3	3	2	3	2
194	165	♂ 3	13	11	12	13	3	3	3	2	2	2	2	3	2
265	304	♂ 5	16	15	15	15	3	3	3	3	3	3	2	3	3
291	335	♂ 5	16	17	18	17	2	3	2	2	2	2	2	2	2
219	250	♀ 5	1	3	3	5	1	3	3	2	2	3	1	3	2
291	371	♂ 4	1	2	1	2	3	3	3	3	3	3	3	3	3
207	195	♀ 5	1	1	1	2	1	1	2	2	2	2	2	1	1
183	141	♀ 3	1	2	2	2	1	1	2	1	1	2	1	1	1
227	293	♀ 5	1	2	2	1	2	3	3	2	2	2	2	3	2
195	155	♀ 3	1	3	2	3	2	3	3	1	2	2	2	2	2
204	210	♀ 5	1	1	2	2	1	3	2	2	2	2	1	2	2
170	115	♀ 2	1	1	1	2	2	3	3	1	2	2	2	2	2
218	245	♀ 5	2	2	2	2	2	3	3	3	2	2	2	2	2
192	142	♂ 5	2	2	2	2	3	3	3	2	2	3	3	2	3
202	154	♀ 5	2	1	2	2	3	3	2	2	2	3	2	2	3
226	243	♀ 5	2	2	2	3	3	3	3	2	2	2	2	2	2
192	181	♀ 4	2	2	2	2	3	3	3	1	2	2	2	3	3
203	233	♀ 5	6	6	5	5	2	3	3	2	2	2	2	2	2
150	89	♂ 3	19	18	18	18	2	2	2	2	2	2	2	2	2
194	163	♂ 5	22	25	20	20	1	2	2	3	3	3	1	2	2
183	124	♂ 4	23	25	27	25	3	3	3	3	2	3	2	2	3
147	81	♀ 5	10	8	12	10	3	2	3	2	3	3	2	2	2
159	110	♀ 5	11	9	12	10	2	2	2	2	2	2	2	2	2
172	143	♀ 5	12	7	8	6	3	3	3	3	2	2	3	3	3
159	118	♀ 5	12	15	18	15	2	3	3	2	2	2	2	2	2
167	128	♀ 5	13	8	15	12	2	2	3	2	2	3	2	2	3
276	332	♂ 5	14	8	8	8	3	3	3	2	2	2	2	3	2
174	121	♂ 4	16	10	11	12	2	2	3	3	2	2	2	2	2
194	177	♂ 3	16	17	14	21	2	2	2	3	3	3	2	2	3
182	145	♂ 4	19	17	17	20	2	2	3	3	3	2	1	2	3
180	172	♀ 5	21	13	16	18	3	3	3	3	3	3	2	2	2
190	166	♀ 5	3	2	3	2	2	3	2	3	2	3	2	2	2
177	136	♀ 4	5	2	2	4	2	2	2	2	2	2	2	2	2
184	144	♀ 5	12	7	10	9	1	2	2	2	2	2	2	2	2
214	183	♂ 5	15	12	13	10	3	2	3	3	3	3	2	3	3
271	325	♂ 5	18	18	21	18	2	2	3	3	2	3	2	2	3
240	256	♂ 5	18	18	19	20	2	2	2	3	2	3	2	3	2
240	288	♂ 5	22	23	27	20	3	3	3	3	3	3	2	2	2
297	433	♂ 5	22	23	22	22	3	3	3	3	3	3	2	2	3
225^{*}	216	♂ 5	3	2	3	3	1	2	3	3	3	3	2	3	2
225^{*}	216	♂ 5	26	24	26	22	3	2	3	3	3	3	2	3	2
$182^{\#}$	142	♂ 3	6	5	5	5	2	2	3	2	2	3	2	2	3
$182^{\#}$	142	♂ 3	29	28	27	28	2	2	3	3	2	2	2	2	3

APPENDIX III

Results of the endolymph chemistry experiments. Data in bold type indicate samples excluded from the analysis on the basis of sample contamination (see section 5.2.2.)

Time	Temp. (°C)	DML (mm)	Sex and Maturity	[Ca $^{2+}$] (mM)	[Mg $^{2+}$] (mM)
Experiment I					
13:00	13.0	71	F 5	5.43	11.59
13:05	13.0	64	F 5	7.07	13.00
13:10	13.2	56	M 4	8.91	10.74
13:15	13.2	69	F 5	3.15	13.17
18:55	14.0	75	F 5	3.45	15.08
19:03	14.0	61	F 4	4.42	15.11
19:09	13.8	57	F 2	3.39	14.67
00:50	13.5	72	F 5	2.11	14.44
00:55	13.5	67	F 5	3.15	16.42
01:03	13.0	69	F 5	4.28	16.21
01:10	13.5	56	F 2	3.95	13.42
07:00	13.5	65	F 3	3.90	16.17
07:05	13.0	55	F 2	2.20	15.42
07:10	13.50	66	F 5	3.76	12.87
07:16	13.0	58	F 3	3.60	12.17
13:05	13.5	64	F 4	7.38	10.84
13:10	14.0	65	F 4	4.07	11.67
13:17	13.5	48	F 2	2.70	13.92
13:22	14.0	59	F 2	2.20	11.89
18:45	14.0	60	F 3	4.00	13.00
18:50	**13.5**	**53**	**F 2**	**18.33**	**10.92**
18:57	14.0	51	F 2	5.12	17.58
19:03	14.0	70	F 5	2.15	12.67
00:57	14.5	68	F 3	4.06	20.74
01:00	14.2	55	F 2	2.87	16.33
01:08	14.5	52	F 2	2.47	11.33
01:12	14.5	58	F 2	3.37	19.67
07:15	14.0	58	F 2	4.30	12.17
07:20	14.0	52	M 5	4.25	14.67
13:14	14.5	61	F 3	2.12	14.09
13:18	15.0	72	F 5	5.62	15.98

Appendix III (continued).

Time	Temp. (°C)	DML (mm)	Sex and Maturity	[Ca^{2+}] (mM)	[Mg^{2+}] (mM)
Experiment 2					
23:52	16.1	54	F 2	4.58	16.81
00:00	16.1	63	F 5	5.70	15.00
00:00	16.1	46	M 5	8.56	16.25
00:06	16.1	66	F 5	16.63	18.65
06:57	16.1	49	F 2	7.22	19.17
06:57	16.1	54	F 3	6.36	16.83
07:02	16.1	44	M 5	9.43	16.25
07:02	16.1	57	M 5	4.87	17.42
11:57	16.5	53	M 5	6.36	17.60
11:57	16.5	47	F 2	8.78	14.46
12:05	16.4	68	F 5	4.03	19.25
20:00	**16.3**	**50**	**F 3**	**26.73**	**17.25**
20:00	16.3	50	F 2	4.97	17.08
20:11	16.3	66	F 5	6.88	20.00
00:04	16.3	68	F 5	6.41	16.25
00:04	16.3	45	M 5	6.72	15.42
00:04	16.3	54	M 5	11.73	18.75
07:14	16.5	80	F 5	5.20	10.94
07:14	16.5	43	M 4	7.72	13.33
07:20	16.5	52	F 2	4.63	15.11
07:23	16.5	50	M 5	6.84	11.99
12:06	16.8	67	F 5	16.83	12.75
12:10	16.8	47	M 5	15.13	18.25
12:10	16.8	56	M 5	3.98	12.75
20:14	16.5	47	F 3	6.51	7.50
20:22	16.5	47	M 4	7.05	15.42
20:26	16.5	70	F 5	5.85	11.15
00:03	16.5	41	M 5	11.82	19.06
00:07	16.7	40	F 2	6.36	14.86
00:14	16.7		F 5	4.68	14.38

Appendix III (continued).

Time	Temp (°C)	DML (mm)	Sex and Maturity	$[Ca^{2+}]$ (mM)	$[Mg^{2+}]$ (mM)
Experiment 3					
11:58	19.5	74	F 5	4.51	13.63
12:01	19.5	50	M 5	3.28	12.96
12:01	19.5	63	M 5	3.96	19.28
20:08	19.5	67	M 5	4.38	18.51
20:08	19.5	80	F 5	5.25	21.10
20:13	19.5	61	M 5	9.26	18.72
20:13	19.5	44	M 5	3.73	10.53
20:17	19.5	78	F 5	4.84	17.44
20:17	19.5	53	M 5	4.81	16.71
00:06	19.3	41	F 2	6.83	17.48
00:10	19.3	56	M 5	5.41	16.71
00:10	19.3	58	M 5	4.78	21.29
00:16	19.3	81	F 5	3.11	15.88
00:19	19.3	64	F 3	5.45	20.88
00:19	19.3	61	M 3	5.08	18.91
06:38	19.1	58	M 5	5.83	15.29
06:38	19.1	58	M 5	6.35	15.46
06:38	19.1	67	F 5	5.17	17.44
06:43	19.1	58	F 2	5.12	15.04
06:43	19.1	55	F 2	5.25	18.69
12:15	19.5	64	F 5	4.94	16.29
12:15	19.5	52	F 2	6.83	17.13
Experiment 4					
06:24	19.0	64	M 5	5.46	19.17
06:24	19.0	75	F 5	3.54	14.67
06:29	19.0	67	F 2	3.04	17.67
06:29	19.0	66	M 5	3.71	14.17
09:30	19.0	77	F 5	4.49	20.17
09:30	19.0	64	F 3	2.51	9.67
09:32	19.0	56	M 5	4.76	15.67
09:32	19.0	64	F 3	2.79	12.67
09:32	19.0	61	F 5	4.86	16.67
09:32	19.0	85	F 5	3.59	13.67

Appendix III (continued)

Time	Temp. (°C)	DML (mm)	Sex and Maturity	$[Ca^{2+}]$ (mM)	$[Mg^{2+}]$ (mM)
Experiment 4 (continued)					
12:07	**19.2**	**79**	**F 6**	**37.49**	**24.67**
12:07	19.2	74	F 3	12.66	18.17
12:14	19.0	78	F 5	6.79	29.67
12:14	19.2	65	M 5	5.79	23.17
17:18	19.0	86	F 4	2.96	13.17
17:18	19.0	64	M 5	5.14	16.17
17:21	19.0	62	F 3	4.84	16.17
17:21	19.0	79	F 5	5.11	18.67
17:21	19.0	84	F 5	4.61	17.67
17:21	19.0	70	F 3	8.87	24.05
20:19	19.2	66	F 3	5.61	16.17
20:19	19.2	73	F 5	4.54	19.67
20:19	19.2	84	F 5	6.54	22.67
20:27	19.2	72	F 6	10.36	21.67
20:27	19.2	59	M 5	6.11	19.67
00:05	19.1	58	M 5	4.94	17.17
00:05	19.1	65	M 5	4.64	18.17
00:07	19.0	56	M 5	5.39	24.67
00:07	19.0	80	F 5	5.29	13.67
04:00	19.0	82	F 5	5.91	20.67
04:02	19.0	85	F 5	6.14	21.67
04:02	19.0	90	F 5	5.19	22.67
04:07	19.0	65	F 5	3.94	17.17
04:07	19.0	74	F 5	4.84	20.17
06:20	19.2	69	F 5	3.56	17.67
06:20	19.2		F 3	3.86	17.17
06:21	19.0	83	F 5	4.44	17.67
06:27	19.0	84	F 5	8.29	22.17
06:27	19.0	61	M 5	6.19	21.17
09:21	19.0	55	M 4	8.69	19.05
09:21	19.0	82	F 5	6.64	20.17
09:21	19.0	70	F 5	7.01	22.67
09:21	19.0	74	M 5	6.04	15.67
09:27	19.0	68	F 5	6.86	18.67

Appendix III (continued)

Time	Temp. (°C)	DML (mm)	Sex and Maturity	[Ca $^{2+}$] (mM)	[Mg $^{2+}$] (mM)
Experiment 4 (continued)					
12:29	19.0	82	F 5	5.19	17.67
12:31	19.0	63	M 5	6.09	17.17
12:31	19.0	79	F 5	6.79	21.17
12:31	19.0	68	F 2	7.24	20.67
18:17	19.1	54	M 5	8.19	22.17
18:21	19.1	66	F 5	4.79	18.17
18:24	19.1	75	F 3	6.96	24.67
18:24	19.1		F 5	7.44	18.17
18:35	19.1	68	M 5	6.81	18.67
18:40	19.1	64	M 5	9.59	20.17