CHAPTER 38

Cephalopod Aging Using Hard Parts

1. Introduction

Cephalopods are exclusively marine mollusc (~800 species) characterised by a bilateral body, prominent head and set of arms. They play a key role in many marine ecosystems, both as predators and prey (Boyle & Rodhouse, 2005) and represent one of the most valuable commercial marine resources (Arkhipkin et al., 2015) contributing global catches of 3.6 million tonnes in 2018 (FAO, 2020). Cephalopods were fished from the Indian Seas as by-catch in shrimp trawls and currently contribute as one of the most important exploited marine fishery resources (CMFRI 2020) from India. During 1959, the annual catch of cephalopods that was 349 tonnes (Silas et al., 1982) increased drastically to 1.61 lakh tonnes in 2020. They are important resource in the Indian export trade, contributing to 15-20% annually.

Stock assessment challenges of cephalopods from Indian waters

The "live fast, die young" life history strategies of cephalopods present particular challenges for the stock assessment and management of squids. Most fishery models were developed for finfish that usually live much longer than cephalopods. They have a voracious appetite and grow fast, reaching commercial sizes in the first few months, which generally takes years in finfishes (Arkhipkin, 2020). Traditional modelling of stock assessment is generally unsuitable for cephalopods which are typically short-lived, with one or two generations present in the fishery at a given time. The poor stock-recruitment relationship strongly influenced by environmental factors (Arkhipkin, 2020), semelparity; continuous spawning contributing to microcohorts within (by hatching dates) each generation; the simultaneous presence of animals of different sizes and ages, having different growth trajectories; wide interannual fluctuations in abundance and mixed species nature of the tropical marine fisheries pose challenges in stock assessment. Meiyappan et al. (2000) pointed to several gaps that exist in the knowledge of cephalopods especially its life history and they argued for detailed studies from Indian waters.

The age composition and growth rate of fishery stocks are among the most important parameters for studying population biology, stock structure, life span and eventually for monitoring and managing the stocks appropriately. Reliable age and growth estimates are crucial parameters for better understanding of the population dynamics and for conducting a stock assessment, for which information on longevity, mortality rate, recruitment pattern, and age structure must be integrated (Andrade et al., 2019). The age and growth studies in squids were

first studied by the Petersen method (Verrill, 1881). This analysis required a substantial sample size over a short time. The length-frequency analysis gives a slow growth rate and high longevity (Jackson et al., 1997). However, recent studies based on culture and age estimation using hard parts demonstrated squids have a short lifespan and fast growth rate (Arkhipkin, 2004; Jackson, 2004). Moreover, many studies provide further evidence that length-frequency analysis is inappropriate for squids (Jackson et al., 1997).

Recent studies confirm length-frequency analysis over-estimate the lifespan and underestimate the growth rate of squids (Jackson et al., 1997). The evidence from statolith ageing (Jackson, 2004) and laboratory experiments (Forsythe et al., 2001) unequivocally supports short lifespans and non-asymptotic growth rather than long-lived asymptotic growth models.

2. Methods for age and growth studies

Age estimation gives details of the individual as well as the age structure of the entire population. Cephalopod growth is estimated by using indirect and direct methods

Different methods that are used for estimating the age of squid populations can be grouped into three categories.

2.1. Direct growth studies

The direct method for understanding cephalopod growth is by examining growth of known-age individuals or of laboratory-maintained field-caught individuals. Absence of a proper larval stage, the very rapid growth rates, the short lifespan and high nutritious value make cephalopods a highly promising species for aquaculture as food production (Nabhitabhata, 1995) and it also help us to understand age and growth rate of cephalopods. Shevtsova (1977) identified the cephalopods as a potential object for rearing under a controlled environment. The culture experiments of bigfin reef squid *Sepioteuthis lessoniana*, pharaoh cuttlefish *Sepia pharaonis* and *Sepiella inermis* has been conducted from the Indian waters (Sivalingam et al., 1993, 1999; Anil et al., 2005).

2.2. Tagging and recapture

To date, very little work has been reported for assessing squid growth using tagging and recapture. Direct methods of tag-recapture and laboratory are generally unrealistic because of low recapture rate and high mortality (Krstulovic-Sifner, 2008). The first tagging and marking experiments of cephalopods were conducted on pelagic species starting in 1927 with Soeda (1950), who studied the patterns for the establishment of migration models of *Todarodes pacificus*. Different kind of tags (Chemical, mechanical and electronic) were used for cephalopods. Despite extensive tagging efforts and intense commercial fisheries recapture rate of the squids have generally been lower.

The northern shortfin squid *Illex illecebrosus* tagged in offshore waters of Newfoundland did not yield any successful recapture. Many squid species such as Argentine shortfin squid *I. argentines*, European flying squid *Todarodes sagittatus*, neon flying squid *Ommastrephes bartramii*, Japanese flying squid *Todarodes pacificus* and jumbo squid *Dosidicus gigas* have been studied for age and growth by tagging and recapture method.

2.3. Indirect method for growth studies in squids

The length-frequency analysis method constructs a growth curve by connecting the modes or mean length values for successive time intervals. Verrill (1881) first demonstrated the growth of cephalopods by using this method over 130 years ago.

Analysis of length-frequency data has been the main method used to obtain estimates of the squid growth rate and longevities (Pauly,1985). The length-frequency analysis produces an asymptotic growth curve and a long lifespan (Mohamed, 1996). However, numerous studies have reported its errors and inadequacies (Alford & Jackson, 1993) since it underestimate growth in squids (Jackson et al., 2000).

2.4. Age and growth studies of squid by using hard structure

Almost all the hard parts such as statoliths, gladius, beaks and crystalline lens of squids have increments, except chitinous rings of arms and tentacles (Arkhipkin et al., 2018).

2.4.1. Gladius

The gladius is the internal shells of squid (suborders Oegopsida and Myopsida) and bobtail squid (order Sepiolida). Typically, it consists of inner, intermediate, and outer shell layers, but there are variations with respect to the number of layers in some families. These layers grow periodically and the increments or the striae are used in age estimation. Gladius processing for age estimation can be divided into four stages: extraction, preservation, sample preparation and reading. The intermediate layer is the most promising gladius layer for ageing studies.

2.4.2. Stylets

Statolith and shell analyses of octopus species are unsuitable for ageing. The increment analysis in the hard rod-like vestigial shells or the stylets are used for ageing octopus. However, stylet increment analysis is not suitable for all octopus species because of variation in stylet structure and increment readability

2.4.3. Beaks

The beaks are basically composed of a chitin–protein complex. Growth process takes place from the posterior border of the beak, where the most recent chitinized and hydrated material is deposited. Growth increments in cephalopod beaks were reported for the first time in the 1960s for the squid *Onykia ingens* using the inner surface of lateral walls. Beak increments have been used for age estimation in squid species in which daily deposition was confirmed by comparing with statolith-determined ages. Beak microstructure increment analysis is affected by processes such as feeding that wear down the beak, resulting in inaccurate estimates.

2.4.4. Sepion

Most attempts to age cuttlefish have concentrated on the cuttlebone. This structure functions as a dorsal backbone providing both support and buoyancy control. It consists of a thin, hard, calcified, dorsal shield and a ventral porous phragmocene comprised of numerous narrow chambers, delineated by chitinous septa. The cuttlefish controls its buoyancy by moving gas or liquid into or out of the chambers as required. As the cuttlefish grows, further septa are laid down at the anterior end. Early studies concluded that the periodicity of chamber formation was daily, however, recent studies found it was related to growth rate rather than chronological age. The growth rate of cephalopods is strongly influenced by temperature and food availability and thus subject to seasonal fluctuations. The width of individual chambers also varies with growth rate.

2.4.5. Crystalline lens

Few attempts have been made for tentative ageing of cephalopods with unreadable statoliths, like in octopus, from their crystalline eye lenses. They grow continuously throughout life by the addition of concentric layers of fiber cells to their outer surface.

The stained histological sections of lenses are observed for growth rings after decalcification and dehydration.

2.4.6. Statolith ageing

Statoliths are currently the most frequently used hard part for estimating the age and growth of squids (Jackson, 2004). They are paired calcified structures located inside the cephalopod's equilibrium organ called statocyst. When polished, their exposed microstructure reveals a series of concentric increments which have been frequently shown to be deposited at approximately a 24 h cycle (Jackson, 2004). During the last three decades, statoliths have been used for estimating age and growth of squids from all over the world (Arkhipkin, 2004; Sajikumar et al., 2020).

2.4.6.1. Statolith analysis

The sequences of statolith extraction and process for age estimation are shown in Fig.1.

2.4.6.2. Extraction of statolith

Statoliths are located just posterior and ventral to the eyes and were extracted by the following procedure: The squid is placed with the ventral side up for the removal of the funnel apparatus. In large squid, this is possible only after making the necessary incision on the mantle before removing the funnel. A transverse cut through the ventral portion of head cartilage is done by a surgical blade to exposes the statocyst. The statoliths are located at the anterior wall of statocyst. In squids the two statoliths are generally visible, appearing as white opaque objects lying side by side under a thin layer of transparent tissue and cartilage. The pair of visible statoliths were gently removed using a fine needle (Fig.2).

2.4.6.3. Statolith cleaning and storing

After extraction, statoliths were cleaned of organic debris using a fine brush and stored in vials (centrifuge tubes) with 70% alcohol.

2.4.6.4. Microscopic slide preparation

The coded clear glass ground edges slides (26×76 mm size) are used to fix the statoliths.

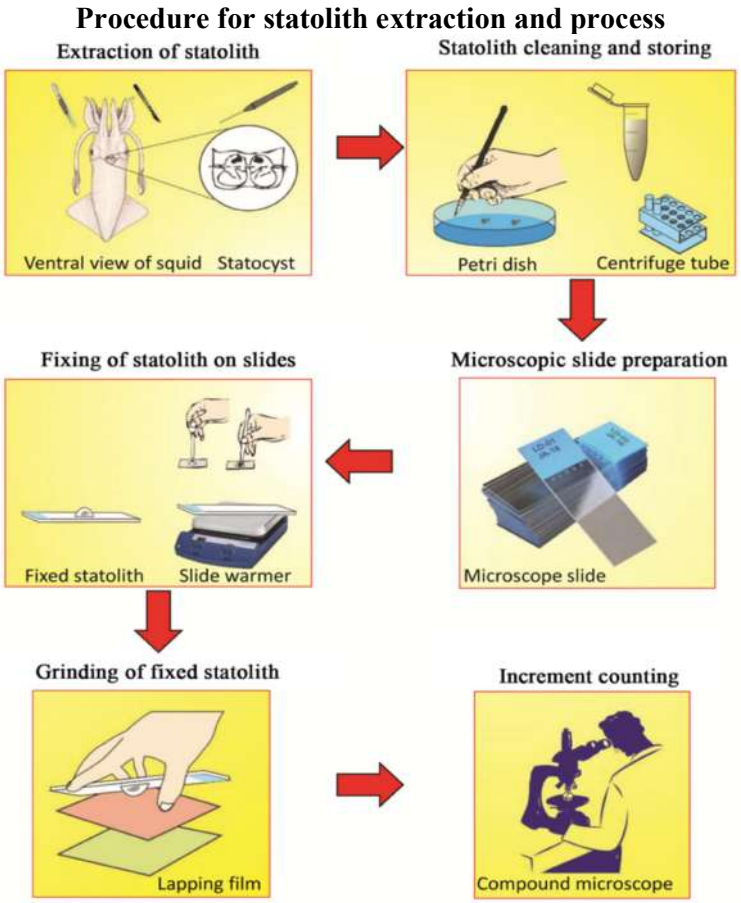

Fig. 1 Illustration of procedure for statolith extraction and process

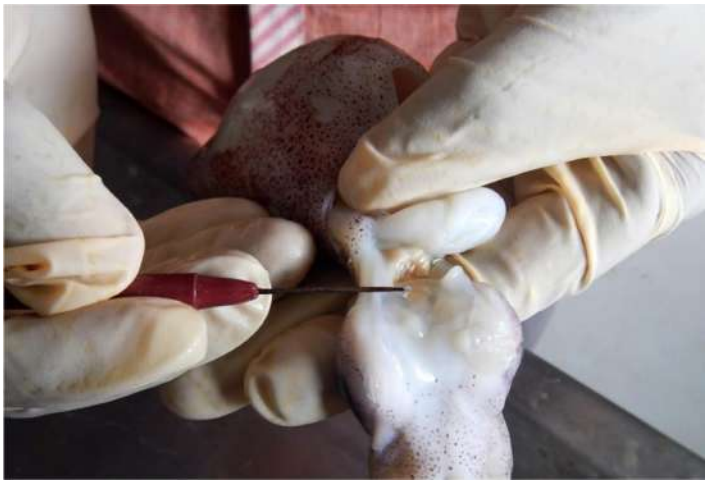

Fig. 2 Extraction of statolith from statocyst of squids

2.4.6.5. Statolith measurements and terminology

Statoliths are paired structures and are attached to the cartilage cavity called statocyst. The statolith size is usually less than 2 mm. The statolith consists of four parts, including dorsal dome, lateral dome, rostrum and wing. The first three parts are usually hard but the fourth part (wing) a fin-like extension is weak due to the presence of loosely packed crystals (Fig.3). The dorsal dome may be large or small, that clearly separated from the lateral dome (Fig.3). The surface of the dome is generally rough. The lateral dome is dorso-ventrally elongated. The rostrum is roughly cigar-shaped and the end may be pointed, rounded or broad (Fig.3). The attachment area or wing usually has a dorsal and ventral indentation separated by a spur.

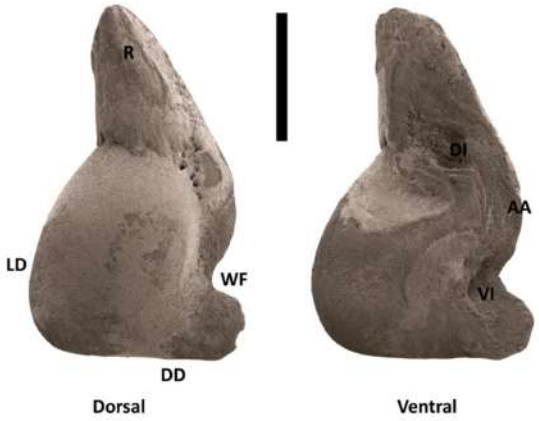

Fig. 3. Dorsal and ventral view of statolith of *Uroteuthis duvaucelii* (250 mm DML♂) DD= Dorsal dome, LD= Lateral dome, R=Rostrum, WF=Wing fissure, AA=Attachment area, DI=Dorsal indentation and VI= Ventral indentation (Scale bar=500µm).

The total statolith length (TSL) is measured from the edge of the dorsal dome to tip of the rostrum under the light microscope (Nikon Eclipse 85). The total statolith length (TSL) is measured to the nearest 0.01 mm.

2.4.6.6. Fixing of statolith on slides

The single statolith from one individual is generally enough for the estimation of age. Lipinski (1981) showed that both statoliths gave similar counts of increments. The dried statolith is mounted on a microscopic slide using thermoplastic cement (Crystalbond™). The thermoplastic cement Crystalbond™ is completely translucent, does not fluoresce under UV irradiation, and highly viscous. Statoliths can be easily turned-over and mounted using this cement as it melts at a low temperature (40 °C) and hardens relatively rapidly after removal from heat (Arkhipkin and Shcherbich, 2012.).

A small amount of thermoplastic cement is placed on the microscopic slides and warmed on a hotplate until it melts. After melting, the statoliths is placed over the cement. Both right and left statoliths can be placed on a single slide.

2.4.6.7. Grinding or polishing of fixed statoliths

Grinding is done for each statolith individually using waterproof sandpaper. Statoliths mounted on the slide are initially polished with coarse sandpaper (600 grit) followed by a fine paper (800-1200 grit) for 6-8 times.

2.4.6.8. Increment observation

Growth increments were examined under a compound microscope (Nikon, Eclipse-80i and Zeiss, Axiostar) under different magnification of 20×10, 40×10 and 60×10 X depending upon the size and visibility of statolith. When viewed under transmitted light, a growth increment is defined as the interface between an inner light and outer dark band (Fig.4). Each increments in statolith of squids comprised of two components, *i.e.,* one translucent layer and another opaque layer. The opaque layer is counted as a "ring" as described in Natsukari et al. (1993). Increments are counted from the first check (hatching ring) to the edge of the dorsal dome, where increments are generally most clearly visible (Villanueva, 1992; Dawe, 1985). However, it is sometimes necessary to extrapolate from adjacent areas to resolve increment counts in unclear areas. Growth increments are assumed to be daily, based on the validation studies in squids (Jackson, 2004; Arkhipkin, 2004).

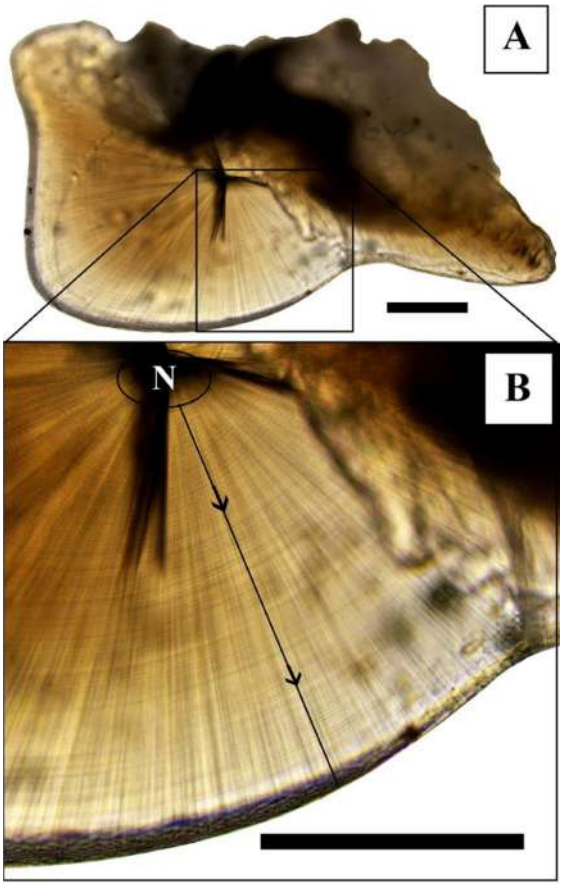

Fig.5. (A) Light micrograph of the ground statolith of *Uroteuthis duvaucelii* adult (male of 220 mm DML). (B) Magnified view of the area outlined by the rectangle showing growth increments. Scale bar= 200 um

A sequence of growth increments is counted more than once for minimizing the error. If the difference between first and second count is < 10%, the mean count is accepted. Counting is repeated when the difference is > 10%. However, if the final, difference is >10%, then the statolith is not used for increment analysis.

3. Summary

Determination of both age and growth are critical to understand the life history of harvested species and to model the dynamics of their populations, both of which are essential for assessment and management purposes. Successful age estimates have been achieved for many squid species by counting validated concentric daily increments found in statoliths. Recent years have seen the emergence of extensive studies of myopsid squid growth of the family Loliginidae. This has greatly advanced our understanding of their life histories. Growth data have accumulated from both statolith-based field studies and culture work. Validation studies on loliginids continue to support that statolith increments are laid down daily.

Ageing cuttlefish from statoliths has been less successful. In cuttlefish, the growth increments have proven difficult to distinguish due to the irregular and concentric deposition of the aragonite crystals, which result in a strong radial appearance, and the lower percentage of

organic matter, which results in weak dark rings. Statoliths of octopods contain randomly arranged statoconia, without any visible increments. This technique has failed to provide results for octopus due to the lack of growth rings and the morphology of octopus statoliths not possessing the same landmarks as those of squid and cuttlefish, which minimizes increment visualization. Stylets, however, do have concentric rings and have been validated for age estimation using *Octopus pallidus* of known age reared in captivity. At present there is no generally applicable method of age and growth determination for all cephalopods and several techniques are in their infancy necessitating continued research in finer refinements and validation.

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