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Thirty years' progress in age determination of squid using statoliths

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The discovery thirty years ago of daily growth increments in squid statoliths and the development of statolith ageing techniques gave new insight into squid age, growth and metabolism. The techniques have shown that the majority of recent coleoid cephalopods live in the 'fast lane', growing rapidly and completing their life cycles in a year or less. Surprisingly, these useful approaches to the study of age and growth in squid have not gained much momentum. Only approximately an eighth of more than 300 squid species have had their basic age assessed and described. Two dozen species are subject to continuing arguments about which increments to consider as daily growth increments. This paper outlines major problems encountered during age determination of squid and suggests ways to improve the techniques and make them applicable to a wider spectrum of species.

Keywords: squid, statoliths, growth increments, ageing techniques

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INTRODUCTION

Life cycle studies are incomplete without the knowledge of their duration (life span) and how fast the animals achieve maximum size (growth rates). In squid, these important biological parameters were first studied by the Petersen method, which involves the analysis of modal length progression over time (Verrill, 1881; Fredriksson, 1943). It was revealed that squid are rather short-living and fast-growing animals with life spans estimated to be of several years (Verrill, 1881; Raja, 1935; Fredriksson, 1943). But to confirm the results of these indirect studies, it was necessary to find a growth increment-bearing structure in squids' bodies that reliably recorded the animals' ages. In the mid-1960s, periodic growth increments were found in the statoliths, calcareous stones located in squid equilibrium organs called statocysts (Clarke, 1966). Growth increments have since been revealed in the statolith microstructure of all studied species of sepiids, sepiolids and squid (Jackson, 1994; Arkhipkin, 2005). The exception so far is octopod statoliths, which are made of the 'loosely bound, randomly arranged statoconia' without visible growth increments (Clarke, 1978). However, the periodicity of increment formation remained unknown. Moreover, some researchers questioned the presence of growth increments within the statolith microstructure (Dilly, 1976). After their discovery, twelve years passed before the first attempt was made to use statolith growth increments to estimate age and growth in squid (*Illex illecebrosus*, Lipinski, 1978 and *Loligo opalescens*, Spratt, 1978).

The first interpretations of growth increments in the statolith microstructure were uncertain. It was initially suggested that the first (inner) increments were laid around the structure's nucleus, on a daily basis, but those closer to the statolith periphery were laid on a monthly basis (Lipinski, 1978). The main reason for this was assumed to be a decrease in increment width; increments closer to the nucleus were wider and more discernible. These were called first-order increments. Each group of 14 to 28–30 first-order growth increments composed a second-order band. Close to the statolith periphery, the first-order increments were assumed to become so narrow that only the wide second-order (monthly?) bands could be observed (Spratt, 1978). Later studies comparing squid growth rates by the Petersen method with growth rates by statolith analysis also suggested the daily nature of growth increments in the statolith microstructure (Hurley *et al.*, 1979; Lipinski, 1980; Rosenberg *et al.*, 1980). The incorporation of a chemical mark (strontium or tetracycline) into statolith microstructure of captive squid confirmed the hypothesis of 'one growth increment–one day' in ommastrephid squid *Illex illecebrosus* (Dawe *et al.*, 1985; Hurley *et al.*, 1985) and *Todarodes pacificus* (Nakamura & Sakurai, 1991). The hypothesis was later confirmed further in more than a dozen other myopsid and oegopsid species (Jackson & O'Dor, 2001).

Examination of the statolith microstructure in hatchling squid has shown the starting point for growth increment counts: in oegopsid squid (*I. illecebrosus*) it is the nucleus periphery (Balch *et al.*, 1988); in myopsid squid (*Alloteuthis subulata*) it is the outer border of the post-nuclear zone (Morris, 1991). The total number of statolith growth increments from the starting point to the edge represents squid age with daily precision. Obviously, any extrapolation to other species of the validity of growth increment counting should be taken with caution, even within the same families (Lipinski & Durholtz, 1994).

Statoliths meet the essential criteria for a squid ageing tool because they contain interpretable growth increments that can be correlated with a regular determinate time scale; and they continue to grow at a measurable rate throughout the life of

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the squid (Beamish & McFarlane, 1983). Following these findings, a first workshop was held on the use of increment-bearing structures for cephalopod ageing at the Instituto di Tecnologia de Pesca e del Pescato (ITPP–CNR) in Mazara del Vallo, Italy, in 1989. The goal of the workshop was to create a platform for future studies of squid age and growth, and a variety of sampling and processing techniques was suggested to make the procedures less time and labour consuming (Dawe & Natsukari, 1991). Unfortunately, over the next 20 years the statolith ageing technique did not become a routine method to estimate age and growth in squid. Until now, many squid ageing studies are based only on a small sample size (less than one hundred squid; reviews: Jackson, 1994, 2004; Arkhipkin, 2004). Most studies have only analysed a limited part of the squid ontogenesis as, for example, the juvenile period in cranchiid squid (Arkhipkin, 1996) or sub-adult period in *I. illecebrosus* (Dawe & Beck, 1997).

The main aim of this paper is to reveal potential problems associated with squid statolith ageing techniques and present ways to resolve them based on our experience in processing and reading more than 15,000 statoliths from more than 50 different species. We also present some new hints that significantly improve the twenty-year-old techniques (Dawe & Natsukari, 1991) to make processing and reading easier and less dubious. Our goal is to develop statolith ageing as an effective and routine tool for the study of squid age and growth.

MATERIALS AND METHODS

Sampling

STATOLITH EXTRACTION

A transverse cut through the lower posterior part of the head cartilage exposes the statolith located on the anterior wall of the statocyst. The wing of the statolith is fragile, and we found that the easiest way to extract the statolith intact is to use a dental spoon with small-size head. Use of thin forceps may result in breaking fragile parts of the statolith such as the rostrum and wing.

Statoliths from larval and small juvenile squid are difficult to extract this way because of their small size (<0.5 mm). In these cases, the head of the squid should be placed on a microscope glass slide. The whole statocysts (which look like oval sacs through the transparent skull tissue) are dissected from the posterior part of the head and placed aside on the slide. The wall of the statocyst sac is broken using a preparation needle, and the statoliths are released from the cavity with the statocyst fluid. After cleaning away the debris of statocysts and skull, the two statoliths remain on the slide. The statoliths are then completely dried, embedded into Canada balsam, and covered with a cover glass for examination.

STORAGE

Statoliths are made of calcium carbonate, mainly in aragonite form (Radtke, 1983). Therefore, their crystalline microstructure is prone to damage in any acidic environment. When squid are left on deck for several hours before sampling, the surface of the statolith will disintegrate as the surrounding tissues start to rot. If statoliths cannot be extracted immediately, the squid should be frozen or iced for later extraction in the laboratory. After extraction, statoliths should be cleaned to remove any organic debris, then put either in separate vials (Eppendorf tubes) or water-proof paper envelopes. If they are stored dry, the humidity in the vials should be kept low to prevent microorganism growth on the statolith surfaces. The best storage is achieved by keeping the statoliths in 70–90% ethanol alcohol. The strength of the alcohol should be checked periodically for deterioration from moisture in the surrounding air.

If the statolith has been preserved correctly, it keeps its naturally translucent body and rostrum. If these parts instead are white (opaque), the statolith surface has been damaged and age readings near the statolith edge could be affected.

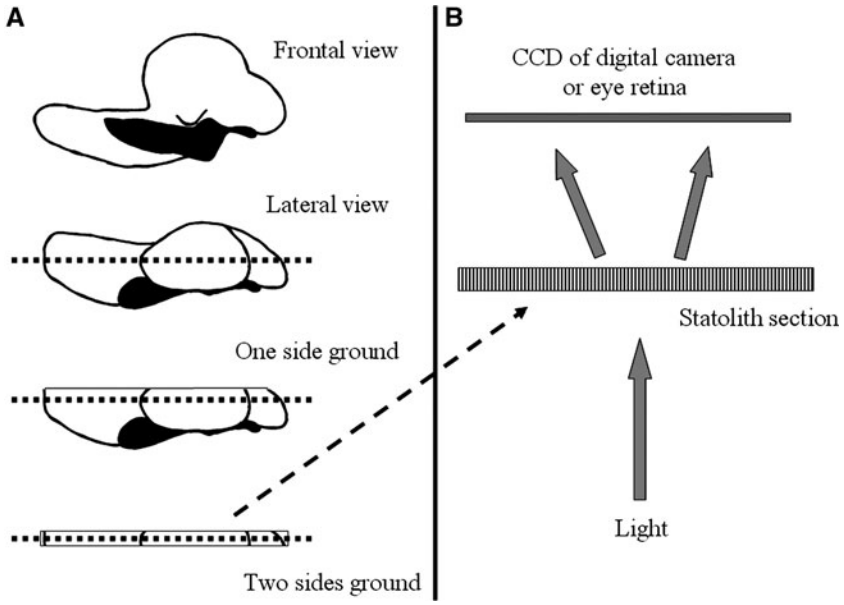

Fig. 1. General scheme of preparation of the statolith section of the squid (A) and its observation under transmitted light of a compound microscope (B).

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Preparation

CHOICE OF GRINDING PLANE

The shape of squid statoliths strongly varies among different families and genera (Clarke, 1978). It is important to choose a proper grinding plane to reveal (if possible) the whole sequence of growth increments from the statolith nucleus to the edge in one preparation. Statoliths grow three-dimensionally with uneven growth rates in different parts. In choosing the grinding plane, it is important to establish the direction in which growth increments are the widest, and therefore easiest to discern (Figure 1). In different squid, the widest growth increments are observed in the dorsal dome (Ommastrephidae, Onychoteuthidae, Dawe *et al.*, 1985; Arkhipkin, 2004), in the lateral dome (Loliginidae; Lipinski, 1986; Jackson, 1994; *Pterygioteuthis gemmata*, Arkhipkin, 1997a), or in the rostrum (Ancistrocheiridae, Arkhipkin, 1997b; *Berryteuthis magister*, Natsukari *et al.*, 1993).

Patterns of the microstructure may also vary in different statolith parts and should be taken into account when choosing the grinding plane. For example, in *Dosidicus gigas* (Ommastrephidae) the dorsal dome contains the widest increments, but its microstructure contains many internal cracks and additional centres of growth that obstruct the readability of growth increments. The statolith rostrum in *Dosidicus gigas* is shorter than the dorsal dome, but contains few internal cracks and therefore gives better readability than the dorsal dome.

The resolution of growth increments is the best when they are parallel to the direction of transmitted light from the microscope (Figure 2A–C). The resolution becomes worse as the angle between growth increments and transmitted light increases (Figure 2D–F). Because the direction of growth of various parts of the statolith changes during ontogenesis, it is almost impossible to choose a grinding plane in which all growth increments are parallel to the light, from the focus to the statolith edge. It is therefore preferable to use the least curved structure. In loliginid squids, for example, this is usually the rostrum; in most ommastrephid squids, it is the dorsal dome. In several species of loliginids such as chokka squid *Loligo vulgaris reynaudii* growth increments were best visible in the lateral dome (Lipinski & Durholtz, 1994). However, later in ontogenesis it becomes curved anteriorly preventing to observe all growth increments in focus if sectioned longitudinally. Despite being trickier to make compared with the longitudinal section, the transverse section of the lateral dome exposed the whole sequence of readable growth increments (Lipinski *et al.*, 1998).

In some squid the statoliths are so curved that it is practically impossible to make a section along one grinding plane. Statoliths of the giant squid *Architeuthis dux*, for example, have a depression in the middle of the dorsal dome (Jackson *et al.*, 1991; Lipinski, 1997). Therefore, the two statoliths of one individual should be prepared in a different grinding plane to read the whole sequence.

PREPARATION OF THE CROSS-SECTION

After choosing the best grinding plane, statoliths >0.5 mm total length should be ground and polished on both sides for growth increment reading. Smaller statoliths may be read whole if their surfaces are translucent. There are many ways to attach the statolith to the microscope slide for grinding, and even more mounting media. Some researchers prefer

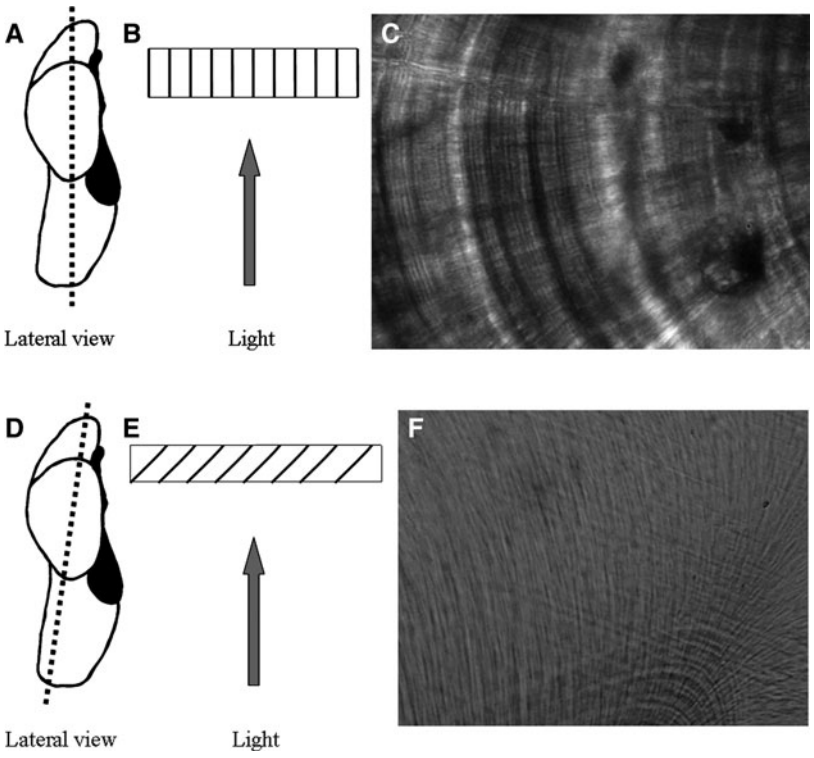

Fig. 2. Choice of grinding plane. Correct plane (A) with growth increments in the statolith section parallel to transmitted light (B), resulting in good readability of the growth increment sequence (C); incorrect plane (D) with growth increments in the statolith section at an angle to transmitted light (E), resulting in poor readability of the growth increment sequence (F).

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to mount the statoliths in adhesive blocks (similar to fish otoliths) (Natsukari *et al.*, 1993). However, with small statoliths it can be difficult to choose the right grinding plane. We have found that the best adhesive to mount and grind statoliths is the thermoplastic cement (Crystalbond 509 produced by Amber Aremco Products, Inc.). This cement has a high viscosity and relatively low melting temperature (121°C) that allows its usage with a hotplate. It is transparent in thin cross-sections, odourless, non-flammable and biodegradable.

A small amount of cement is put near the edge of the microscope slide and heated to melt. The statolith is then placed on the melted cement under a zoom microscope and left for a few seconds to harden. A big advantage of the thermoplastic is that an incorrectly mounted statolith can be re-positioned quickly by re-melting the cement. A statolith can also be easily flipped over to grind and polish one side, then the other, by re-melting the cement.

Grinding is done for each statolith individually. Unlike fish otoliths (Campana, 1999), it is almost impossible to make blocks containing several statoliths mounted in one grinding line. Statoliths are usually much smaller than otoliths, and the position of the nuclear area varies from individual to individual, making it extremely difficult to align multiple statoliths in the same block.

Grinding is usually done on waterproof sandpaper; holding the glass slide with attached statolith near its edge, and either grinding by hand with concentric movements or against a rotating disc. Water should be used for lubrication and to remove the calcium carbonate dust from the slide. Statoliths are ground first on coarse (600 grit) sandpaper for the larger part of the statolith layer, then finished on fine (800–1200 grit) sandpaper until the nucleus appears just under the surface of the cross-section. Some authors (Natsukari *et al.*, 1993; Jackson, 1994) use alumina powder to polish the sections, but we have found this unnecessary as small scratches are not visible if the section is embedded in mounting medium. The statolith section should then be flipped over and ground the same way from another side. It is important to periodically check the grinding depth under a zoom microscope in order not to over-grind the section. It is also important to remove the remnants of the statolith wing. The wing is composed mainly of vaterite calcium carbonate (Radtke, 1983), and its opaque microstructure could obscure the growth increments. This is especially important if the rostrum is chosen as the grinding section.

In most cases, the ground sections of statoliths are observed under transmitted light of a compound microscope. It is therefore important to choose the appropriate thickness of the section. As mentioned above, the statolith grows three-dimensionally, and new growth layers are laid successively over the nucleus as irregular spheres. If the statolith section is thick (>70 μm), parts of growth increments lying both above and below the focal plane of the microscope would create shadows (Figure 3A) that could result in doubling of the growth increment number. Ideally, the section should be the thickness (30–50 μm) that contains all growth increments parallel to the focal plane (Figure 3B). However, this never happens in reality as it is impossible to choose one grinding plane cutting perpendicular growth increments from the nucleus to the edge because the direction of growth of various statolith parts changes during ontogenesis. If the section is too thin (<30 μm), the resolution of growth increments deteriorates as there is not enough optical contrast between translucent and opaque growth rings (Figure 3C).

The prepared statolith sections should be left in thermoplastic and embedded in a mounting medium. In our experience, the most appropriate embedding medium is Canada balsam. It is translucent, preserves the statolith section from any contact with air, and also fills tiny scratches on the statolith surface, making them practically invisible. After embedding, the section is usually covered with thin cover glass for extra protection and a flat observation surface. To avoid trapping air bubbles under the cover glass, it should first be put in contact with one side of the mounting medium, and then gradually lowered to the other side until flat on the glass slide.

Canada balsam remains liquid at room temperature for a long time. It should be dried approximately 3–6 hours at 50–70°C depending on the thickness of the section under

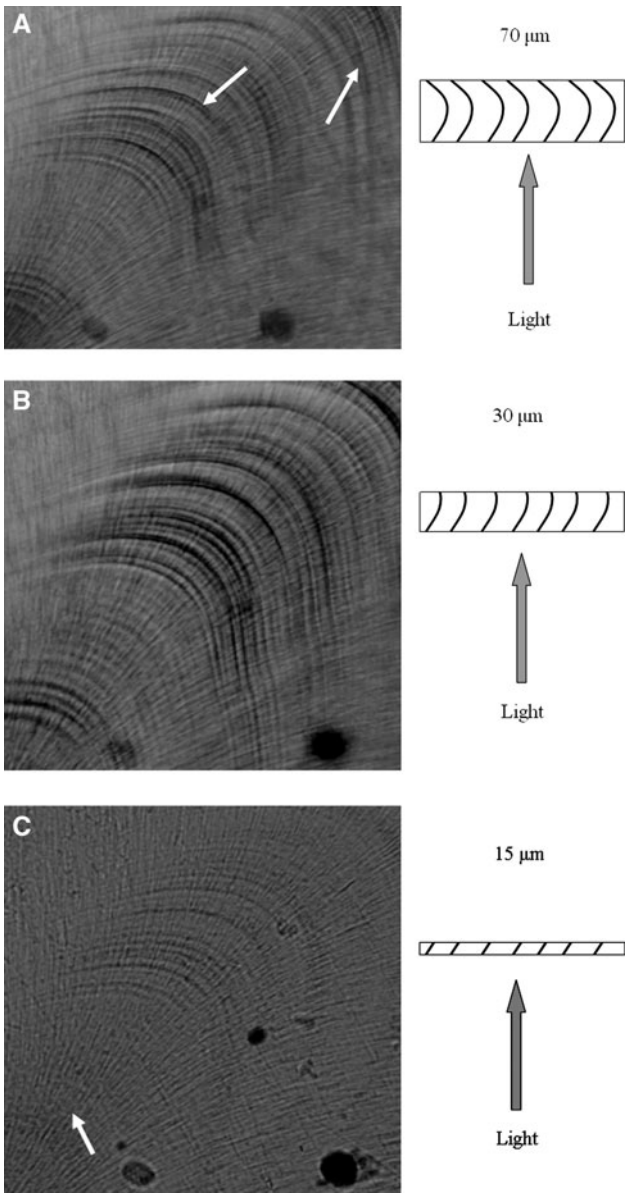

Fig. 3. Readability of growth increments at different section thicknesses of the same statolith. Thick section (>70 μm) with poor readability because of shadows from the deeper layers (A), medium section (~50 μm) with optimum readability (B), and thin section (<30 μm) with poor readability because of low contrast between opaque and translucent parts of growth increments (C).

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the cover glass. Higher temperature is not recommended as bubbles in the balsam may result.

Observation

OBSERVATION OF GROWTH INCREMENTS

Statoliths grow by laying down aragonite crystals of calcium carbonate within an organic matrix. Periods of intensive growth (at night: Bettencourt & Guerra, 2001) alternate with periods of decreased growth (during the day) and as a result, two rings of different optical quality are formed, one translucent (mainly calcium carbonate) and another opaque (mainly organic matrix). The translucent and opaque rings form one growth increment (Lipinski, 1993). Because of diurnal physiological periodicity of carbonate deposition in statoliths, it is assumed that one increment represents one day of the squid's life.

If the statolith section has been prepared properly, the periodic growth increments within its microstructure can be examined. Growth increments are narrow and are therefore best observed using a good quality compound microscope. Readability depends strongly on optimum lighting. For maximum light to go through the prepared section, the microscope field iris diaphragm ring should be adjusted to centre and inscribe the diaphragm image in the field view. To maximize the optical contrast between translucent and opaque rings, the microscope condenser should be equipped with an aperture iris diaphragm ring set to minimum opening (0.2). The phase-contrast Nomarski effect also improves resolution of the growth increments.

OPTICAL EFFECTS

Real growth increments (i.e. regions with different optical densities in the statolith microstructure) may be confused with optical effects that appear during observation under the compound microscope. This is mainly due to the width of statolith growth increments (2–5 μm) being close to the wave length of visible light, which ranges from 0.38 μm (violet) to 0.78 μm (red). As a result, the different optical densities of translucent and discontinuous growth rings cause diffraction and interference of the light transmitted through the statolith section. Light passing through a narrow slit makes a diffraction pattern of alternating light (maxima) and dark (minima) bands observed behind the slit. The many growth increments on a statolith section effectively act as a grid with multiple slits. The maxima of light that pass through all slits create a visible interference pattern. Depending on the width of the slits (i.e. the thickness of translucent bands), the light wavelength, and the number and distance between slits, the interference pattern may present varying numbers of secondary maxima between the primary maxima. When the statolith growth increments are wide (5–7 μm as in *Berryteuthis magister*, Gonatidae), it is often possible to see faint growth rings ('first-order' growth increments: Natsukari *et al.*, 1993) between well-resolved 'second order' bands (Figure 4). Interpretations of these growth increments have differed. Natsukari *et al.* (1993) assumed that the first order bands were daily and estimated the life span of *B. magister* to be 3–4 years. Arkhipkin *et al.* (1996) considered them to be sub-daily, or optical effects, and counted only 'second-order' increments, arriving at a 1-year life span for the same squid. Unfortunately, growth increments have not been validated yet for this species, but it would be of interest to study whether the 'first-order' growth rings are in fact interference patterns.

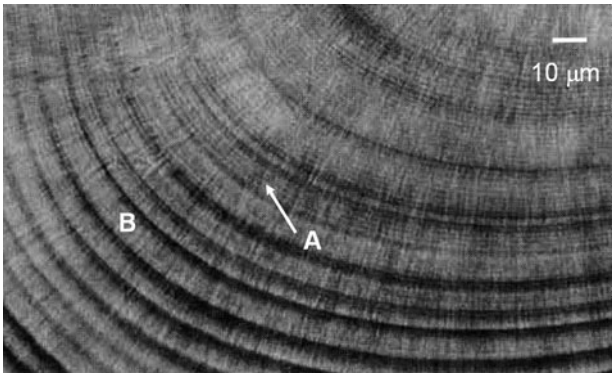

Fig. 4. First-order (A) and second-order (B) growth increments in the statolith of the squid *Berryteuthis magister*.

OPTICAL FEATURES OF THE MICROSCOPE

The resolution of growth increments can be enhanced by skilful use of the microscope optics. For example, using a larger numerical aperture (>0.3) of the microscope objective improves the resolution of the object examined, especially when its size is close to the wavelength of visible light. Microscope resolution also increases when using short wavelength monochromatic light (violet light) (http://microscopy.fsu.edu/primer/java/imageformation/airydiskbasics/index.html) (Fendt, 2003). Therefore, objectives with large numerical apertures and blue filters are recommended for studying statolith growth increments.

WRONG GRINDING PLANE

If a statolith is ground in the wrong plane, the growth increments in the thin section will be at an angle to the transmitted light, rather than parallel. The greater the angle, the poorer the resolution of growth increments as the section does not act as a 'grid' to the transmitted light. The best option is to change the grinding plane for the section being cut in a way that the majority of increments will be parallel to the transmitted light. However, growth increments cut in a wrong plane may still be readable under a different type of microscope, for example one using reflected light, or under a scanning electron microscope.

LIVE VERSUS FROZEN IMAGE

The development of digital cameras and imaging software for microscopes in recent years has enabled high quality images of the statolith microstructure to be transferred to the computer screen. It is visually easier to examine the microstructure patterns on-screen than via microscope oculars, but a certain caution should be taken into account when working with computer images. To judge which growth lines on the image represent real growth increments and which are optical diffractive lines or shadows, a researcher needs to 'play' with the focus adjustment knob of the microscope to ascend or descend the observation plane. Usually, real growth lines slightly change their position but stay constantly in the observation field, whereas optical effects and shadows tend to appear and disappear during the adjustment.

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Some studies have viewed, counted and measured statolith growth increments on a digital or video camera image (Jackson, 1994). As already mentioned, it is virtually impossible to get the whole sequence of growth increments in focus in one image, because statoliths grow three-dimensionally. In this case, it is difficult to tell from the captured 'frozen' image which increments are real and which are optical effects, as the focus cannot be adjusted (Figure 5). Various computer software packages (like TNPC Module in the Visilog environment, IFREMER, France) were developed to count and measure the growth increments by analysing black and white bands along the counting line on the image. Obviously, the software in this case counts all alternating black and white bands including optical effects, unless some special restrictions (necessarily subjective) are made about the range in growth increment width.

One way to avoid potential miscounting is to take pictures of sections of the statolith microstructure which are in focus, then stitch the images together using a software package available for counting the increments.

Validation

Before making definitive conclusions about a squid's age, the periodicity of the growth increment formation needs to be validated by one of the direct methods of chemical marking or known age comparison.

CHEMICAL MARKING

One method of growth increment validation is to add a chemical mark to the squid body either with food or water in the tank (strontium: Dawe *et al.*, 1985), or simply by injection (oxytetracycline solution: Nakamura & Sakurai, 1991). To serve as a mark, the chemical must quickly incorporate into the statolith microstructure, then quickly be destroyed or removed from the body to not confound the signal. Both strontium and oxytetracycline are suitable and have been used intensively for validation studies in captivity (Hurley *et al.*, 1985; Lipinski, 1986) and in the field (Lipinski *et al.*, 1998).

Oxytetracycline is the more popular of the two, and can easily be observed within the microstructure using UV-light. Usually, squid are injected or fed once or twice. The number of days elapsed between marking and re-capture (if once) or between first and second marking (if twice) is then compared to the number of growth increments between the chemical mark and the statolith edge, or between the two chemical marks. So far, growth increments that appeared within the statolith microstructure after hatching have been proven daily in all validation experiments (Jackson, 2004; Arkhipkin, 2005).

KNOWN AGE

Another method is to examine squid of known age (e.g. raised in aquaculture) and compare the number of growth increments in their statolith microstructure with the known age. By this method the daily periodicity of growth increments has been supported in Loliginidae species *Alloteuthis subulata* (Lipinski, 1986), *Sepioteuthis sepioidea* (Jackson *et al.*, 1993), *Lolliguncula brevis* (Jackson *et al.*, 1997), and others.

In some commercially exploited squid species, prominent modes in size distribution have been observed during the fisheries. By taking samples from the modal groups at different times, it was possible to compare the average differences in statolith microstructure formation with the number of days elapsed between samplings, and thereby confirm the daily periodicity of growth increments. This approach has been used for wild populations of *Illex illecebrosus* (Ommastrephidae: Uozumi & Shiba, 1993) and *Berryteuthis magister* (Gonatidae: Arkhipkin *et al.*, 1996). In studies like these, it is important to collect at least 25–30 animals from each modal group at every sampling period to account for variability in individual ages.

Capture is itself a stressful event for the squid, and often causes formation of so-called stress marks in the statolith microstructure. When squid could be kept alive for several days after capture, the periodicity of growth increments could be determined by comparing the number of growth increments between the stress mark and statolith edge with the number of days since capture (Arkhipkin & Bizikov, 1997).

Counting

UNDER THE MICROSCOPE

Once the growth increments in the statolith microstructure have been revealed and preferably validated, the next step is

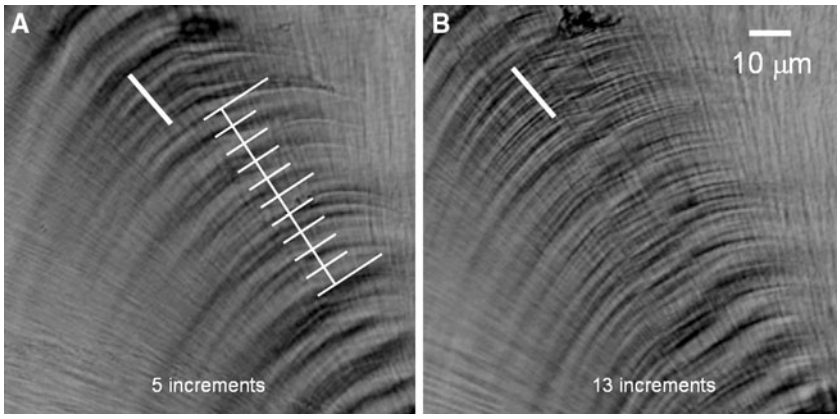

Fig. 5. Role of the focal adjustment of the compound microscope on resolution of growth increments. Correct focal plane revealing 5 daily growth increments (A), slightly off-focus (incorrect) plane with optical effects showing additional 'sub-daily' increments that obstruct reading of the real daily growth increments (B). Also shown in (A): position of ocular eyepiece to facilitate counting growth increments between the micrometer divisions.

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counting them to estimate the age of the animal. Until recently, the most common way to count (or 'read') increments has been direct observation of the statolith microstructure under the microscope. Continuous fine adjustment of focus helps to identify the real growth increments and to facilitate their counting. Due to their average width (2–5 μm in different species) the best magnification for reading should be ×400–600 using ×40 dry air objectives and either ×10 or ×15 oculars. The width of the growth increments in the observation field should be set to make them individually distinct by eye. Some scientists read whole sequences of increments without interruption. Others prefer to read growth increments between the checks (=stress marks, more prominent growth increments) to allow their eyes to rest and not lose the count, especially when moving the statolith section in the observation field.

Another way to count growth increments under the microscope is by using an ocular micrometer (eyepiece) (Arkhipkin, 2005). The ocular must be adjusted to see both the micrometer and the growth increment sequence in focus. Then, one end of the micrometer is placed at the initial count increment, and further counting is done along the micrometer (Figure 5). As the micrometer is marked into 100 divisions, it is easy to count how many growth increments lay in each of the 10 divisions. After counting growth increments along the whole micrometer, the observation field is relocated from the end to the start of the micrometer, and the next subset of growth increments is counted. The number of micrometer relocations will depend on magnification and the size of the statolith section. In this case, information is collected not only on the number, but also on the width of growth increments. It is relatively easy to change the direction of the counting line by, for example, moving the end of the micrometer along the check until it is aligned with a region of more clearly resolved growth increments. However, it is recommended to count growth increments in one statolith region along one counting line in all specimens from the sample (like in fish otoliths: Gauldie *et al.*, 1995), but trying to avoid possible inconsistencies in the microstructure such as fusion of several growth increments ('staggering growth') or cracks (Lipinski & Durholtz, 1994; Lipinski *et al.*, 1998).

A sequence of growth increments should be counted at least twice to minimize error. If the difference between the first and second count is <5%, the mean number of increments is accepted to represent the squid age measurement. If the difference is >5%, another count should be made. If the difference between the third and second counts is still >5%, the statolith should be rejected from further analysis as growth increments may not be sufficiently resolved to give a reliable age estimate.

ON THE COMPUTER SCREEN

The approaches to counting growth increments under the microscope are also applicable to counting from the image produced on a computer screen. But in this case it is important for the microscope digital camera to transmit a 'live' image on screen, with the option for focus adjustment.

The computer software (like TNPC Module mentioned above) is able to distinguish translucent and discontinuous growth bands on the screen, and thereby count peaks of various light intensity and measure the distance between them. Until recently, the main drawback of this software has been that it only worked with 'frozen' images, from which it was not possible to tell the difference between real growth increments and optical effects (Figure 5). Further progress in the development of image-processing software now allows growth increments to be flagged on a live image and then counted and measured digitally. Moreover, the counting line can be relocated from one sector of the microstructure to another to find the best resolution of growth increments. These advances in image analysis should enable a real breakthrough in the tedious, labour-consuming job of counting daily growth increments.

REGIONS WITH INVISIBLE INCREMENTS

Even with proper preparation in the right plane, some regions of a statolith can be poorly resolved or have invisible increments (due to either their narrow width or improper angle with the transmitted light). If these regions are small (not more than 5% of the counting line of the whole statolith) and growth increments are visible in statoliths from other individuals of the same species, it is possible to extrapolate the number of growth increments by measuring the region and dividing by the known mean width of each increment (Arkhipkin, 2005).

However, in some statoliths the increments are constantly invisible, especially close to the statolith edge. In every species, some special inference is required to reveal the seeming absence of growth increments. For example in the onychoteuthids *Onykia robusta* and *O. ingens*, it is possible to count growth increments in two structures: the statoliths and the gladius rostra (Bizikov & Arkhipkin, 1997). It was found that growth increment counts were the same in both structures in juvenile and immature adults. But when squid attain a certain size (>80–90 cm), statolith growth increments reduce their growth to less than 1 μm, and become impossible to discern under a light microscope. In contrast, the gladius rostrum continues growing regularly with the body of the adult animal. In this case, counts of gladius growth increments give more reliable age estimates of adult squid than any extrapolation of growth increments in the statolith (Bizikov & Arkhipkin, 1997).

DISCUSSION

Cephalopod statoliths are usually compared to fish otoliths, as both structures have similar positional sensory functions in the animals' equilibrium organs (statocysts in cephalopods, and inner ear in fish). Statoliths and otoliths have almost the same chemical composition of calcium carbonate crystals embedded in a protein matrix. Both structures are formed by periodic calcium carbonate deposition from surrounding fluid, resulting in translucent high $CaCO_3$ bands during rapid growth phases and discontinuous low $CaCO_3$ bands during slow growth phases.

Fish ageing with otoliths has become a routine technique worldwide over the past 20 years (Campana, 2005), using daily growth increments in larvae and juveniles, and annual growth bands in adults. However, squid ageing with statoliths is still in its infancy despite the fact that daily growth increments were revealed in both structures and first used for ageing at almost the same time (Panella, 1971 for fish; Spratt, 1978 and Lipinski, 1978 for squid). Why, despite all similarities, is the status of work with these two structures so different?

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Several problems pertain to statoliths. First, statoliths and otoliths are different in size. Common sizes of otoliths of adult fish vary from several mm to several cm, whereas statoliths rarely exceed 2 mm, being 1–1.5 mm even in the giant squid *Architeuthis dux* which can attain more than 100 kg body weight (Roeleveld & Lipinski, 1991). Fish otoliths are therefore easier to handle both during extraction and during processing. Big otolith sizes of adult fish enabled the development of processing routines that included embedding as many as several dozen otoliths in one resin block with their nuclei aligned along the cutting plane, then slicing, grinding, and polishing them simultaneously. If nuclei of some otoliths were displaced in one slice, it was usually possible to recover them on an adjacent slice that had been cut higher or lower. Unfortunately, a similar time-saving serial procedure has not been possible for statolith processing. Because of their small size and variable shape, multiple statoliths are extremely difficult to mount simultaneously in an optimal grinding plane. The position of each individual statolith must be monitored and adjusted to the grinding plane under the microscope, which would obviously not be possible with several statoliths embedded in one block.

Second, the shape of statoliths is much more complicated compared to fish otoliths of the same size. Fish otoliths $\leq$1 mm are usually either larval or juvenile, and at these ontogenetic stages they are round or oval shaped. Squid statoliths at this size are adult and are much more convoluted. Obviously, it is easier and quicker to cut round or oval otoliths than complex-shaped statoliths.

A third conspicuous difference between otoliths and statoliths is the width of the growth increments. Fish older than year-1 are aged by counting annual growth increments in their otolith microstructures. As the widths of annual growth increments vary in order from 100 to 1000 μm, optical effects do not interfere with identifying real annual growth increments like they do with daily growth increments of 1 to 5–7 μm in cephalopod statoliths, larval fish and year-0 fish. Numbers of growth increments also differ accordingly between fish otoliths and squid statoliths. It is much less time consuming to read tens of annual growth bands per otolith (rarely up to a hundred in long-living species such as orange roughy *Hoplostethus atlanticus*: Gauldie *et al.*, 1995), than hundreds of daily growth increments per statolith.

To conclude, in the 30-year period since statolith growth increments were first used for squid ageing, they have been found in the statolith microstructure of all studied species of squid, sepiolids and cuttlefish (Jackson, 2004; Arkhipkin, 2004). The daily periodicity of their deposition has been validated in approximately two dozen species, mostly loliginids (Jackson, 2004). Growth increments that have been validated in one or two species of a squid family can be assumed (albeit with caution) to have the same periodicity in other species of the same family, taking into account the co-familial similarities in growth increment morphology and physiological mechanisms of deposition.

Various techniques have been implemented to process statoliths, but they are generally time and labour consuming (Dawe & Natsukari, 1991). Innovations developed recently in the Fisheries Department of the Falkland Islands show promise for improving the procedures; for example the use of thermoplastic resin that enables statolith mounts to be adjusted to different planes during grinding, and the use of wet grinding to minimize scratching and reduce the need for polishing. Combined with advances in computer technology that allow increment counting from live images of statolith sections under microscope, a more streamlined process for squid ageing may be anticipated. The majority of squid have short life cycles (Jackson, 1994) and complicated population structures, requiring many specimens to be studied. Faster, better, and more accessible routines for age and growth analysis are strongly wanted in population dynamics and stock assessments of squid.

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