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METHOD

Determination of squid age using upper beak rostrum sections: technique improvement and comparison with the statolith

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Abstract Analysis of growth increments in beak rostrum sagittal sections (RSSs) has been increasingly used for estimating octopus age. In this study, we develop an effective method to process and read the RSS of four oceanic ommastrephid squid species (*Dosidicus gigas*, *Ommastrephes bartramii*, *Illex argentinus* and *Sthenoteuthis oualaniensis*) and validate the daily deposition of the increments by comparing to corresponding statolith-determined ages. The proposed method of processing yielded readable rates ranging from 42.9 % in *I. argentinus* to 71.7 % in *D. gigas* for samples from *I. argentinus* to *D. gigas*. The high precision of the increment readings with low independent counting coefficient of variation indicates that the processing and

counting methods used are reliable. This study suggests that the RSS of the upper beak is an appropriate tool for estimating the age of *D. gigas*, *O. bartramii* and perhaps *S. oualaniensis*, although erosion of the rostral region may result in an underestimation of squid ages.

Introduction

Determination of age is extremely important for an understanding of cephalopod life history and stock assessment and management. Cephalopods, in particular oceanic ommastrephid squid, are not only considered pivotal components both as prey and predators in the marine ecosystems (Clarke 1996), but also support large cephalopod commercial fisheries (Csirke 2005). In general, oceanic ommastrephid squid are considered to be the only remaining abundant underexploited fishery resource in the world's oceans that can provide high-quality food protein (Jereb and Roper 2010). During the last two decades, global squid landings, including abundant oceanic species such as *Dosidicus gigas*, *Ommastrephes bartramii* and *Illex argentinus*, have varied from 1 to 2 million tonnes, accounting for about 50 % of the total world commercial cephalopod catch.

There are direct and indirect methods for estimating the age of cephalopods (Jackson 1994). The indirect method, using length frequency data analysis, has proved inappropriate for age determination of cephalopods because of their short life cycle, rapid growth rate and extended spawning season (Jackson 2004). Direct methods for measuring periodic growth increments have been adopted using hard structures such as statoliths (Lipinski 1978), beaks (Clarke 1993), gladius (Bizikov 1991) and crystalline lenses (Clarke 1993). These methods have proved to be

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useful for studying age and growth of cephalopods. Moreover, lipofuscin has been recently considered as an alternative material for estimating the age of cephalopods (Doubleday and Semmens 2011).

In the last three decades, the statolith has proved to be the most reliable material to define squid age (Arkhipkin and Shcherbich 2012), although frequent attempts have been made to use the gladius and beaks (Perez et al. 1996; Mereu et al. 2011). Growth increments within beaks were first observed in squid *Moroteuthis ingens* (Clarke 1965). The periodicity of increments was initially investigated by Perales-Raya and Hernández-González (1998), who suggested that regular increment deposition should be related to an individual's age. Subsequently, studies on the growth increments in the beak's rostrum sagittal sections (RSSs) and lateral wall surface (LWS) to determine octopod age have become increasingly popular (Hernández-López et al. 2001; Perales-Raya et al. 2010; Castanhari and Tomás 2012; Liu et al. 2014) because the statoliths of octopod are soft and chalk like leading to indiscernable growth increments. However, little research has been conducted on squid, apart from growth increments which were found and counted on the inner surfaces of the beak lateral wall for *Histioteuthis bonnellii* (Mereu et al. 2011).

An essential step in identifying the true age of a marine animal is to validate the periodicity of increments in that animal's hard structures. In recent years, laboratory culture and marking have been the most popular validation methods for examining the periodicity of increment formation in sepoid cuttlebone (Bettencourt and Guerra 2001; Chung and Wang 2013) and octopod stylet (Doubleday et al. 2006; Hermosilla et al. 2010), lenses (Rodríguez-Domínguez et al. 2013) and beaks (Oosthuizen 2003; Bárcenas et al. 2014). By comparing the increment number with the actual age and elapsed days after marking or between two marks, daily deposition of the growth increments in beaks was validated for two octopods, *Octopus vulgaris* (Oosthuizen 2003; Canali et al. 2011; Perales-Raya et al. 2014a, b) and *Octopus maya* (Rodríguez-Domínguez et al. 2013;

Bárcenas et al. 2014). Nevertheless, unlike sepoid and octopod, squid, especially adult individuals, are difficult to raise in the laboratory (Iglesias et al. 2014). Until now, the periodic deposition of growth increments in squid beaks was validated only in the paralarvae of some squid species such as *D. gigas*, *O. bartramii*, *Sthenoteuthis oualaniensis*, *I. argentinus* and *Todarodes pacificus* (Sakai et al. 2007).

Beaks are chitinous structures within the squid buccal mass (Clarke 1986). The greatest advantage of using beaks is that they are easy to extract and manipulate when compared with statoliths, although age information is recorded in both structures. In this study, we develop a complementary method to determine squid ages when other structures, especially the statolith, are not available. Thus, the objectives of this study are to develop a reproducible and effective methodology for observing growth increments in the RSS of the beak, to establish an appropriate protocol to read growth increments and to examine growth increment formation in the beaks of squid by comparing these estimations with the statolith-determined ages.

Materials and methods

Specimens

Four species of ommastrephid squid, *D. gigas*, *O. bartramii*, *I. argentinus* and *S. oualaniensis*, were obtained from different oceans during scientific fisheries survey by China (Table 1). Samples were frozen on boats and then defrosted in the laboratory, where beaks and statoliths were carefully removed, soaked in soapy water, washed and then separately stored in 75 % ethanol for further analyses. Meanwhile, mantle length, body weight, sex and maturity were also recorded.

Statolith processing and aging

The standard processing and aging methodology of statoliths was used in this study (Dawe and Natsukari 1991).

Table 1 Summary information for *D. gigas*, *O. bartramii*, *S. oualaniensis* and *I. argentinus* samples used in this study

Species	Catch area	Number of beaks		Readable rate (%)	Size range (mantle length, mm)
		Collected	Readable		
<i>D. gigas</i>	ECPO (Ecuador)	24	19	71.7	222–375
	SEPO (Chile)	29	19		386–465
<i>O. bartramii</i>	NWPO	30	21	70.0	230–450
<i>S. oualaniensis</i>	WCPO	20	12	60.0	115–204
<i>I. argentinus</i>	SWAO	28	12	42.9	186–240

ECPO east-central Pacific Ocean, SEPO southeastern Pacific Ocean, NWPO northwestern Pacific Ocean, WCPO west-central Pacific Ocean, SWAO southwestern Atlantic Ocean

An increment number was accepted when two independent counts differed by less than 10 % of the mean (Yatsu et al. 1997). The formation of growth increments in the statolith of these four species is assumed to be daily, and this has been accepted in previous studies (*D. gigas*, Chen

et al. 2011; *O. bartramii*, Yatsu et al. 1997; *I. argentinus*, Arkhipkin 1993; *S. oualaniensis*, Liu et al. 2009), taking into account that periodic deposition of increments was confirmed in several other Ommastrephidae squids (Hurley et al. 1985; Nakamura and Sakurai 1991; Uozumi and Ohara 1993).

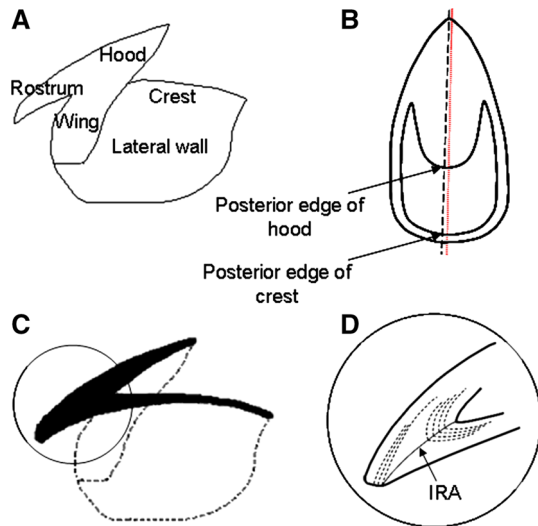
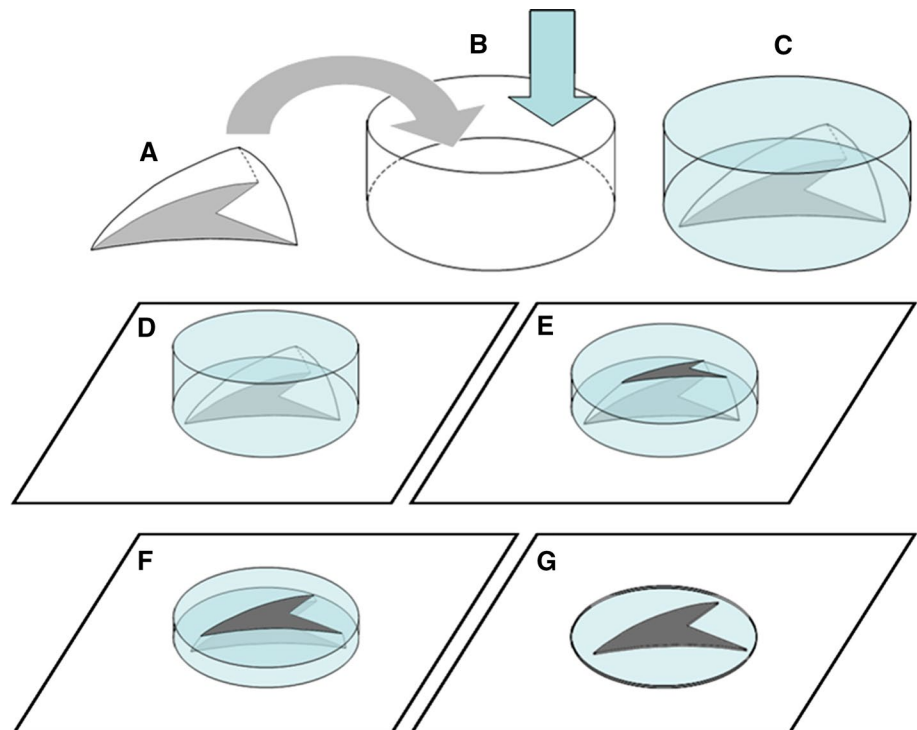


Fig. 1 Diagrams of upper beak of squid. **a** Lateral view of upper beak indicating the different parts; **b** dorsal view—the red line indicates the plane of section; **c** lateral view—medial surface (gray) after sagittal sectioning; **d** lateral view—beak rostrum sagittal sections showing growth increment in the dorsal (hood part) and ventral (crest part); IRA internal rostral axis

Fig. 2 Flowchart of upper beak rostrum sagittal section preparation. **a** Sectioned beak rostrum, **b** mold for embedding rostrum, **c** rostrum was embedded in the mold with epoxy mixed with hardener, **d–f** block was glued to microscope slide and then ground to the sagittal plane



Beak processing

Only those upper beaks with an intact rostrum (i.e., the rostrum tips were not obviously eroded or damaged) were selected for sectioning. The upper beak was first cut into two pieces along the posterior edge of the hood and crest to the rostral tip using a small cutting machine with a 0.3-mm blade (Fig. 1). Importantly, during this process, the cutting line was set slightly to one side (i.e., the cutting line was not in the middle) to prevent the sagittal plane (i.e., the central section plane which includes all of the growth increments) being cut through (Fig. 1). Then, the rostral area of the larger half of the upper beak was removed and embedded with the section plane face down in a small mold of epoxy mixed with a hardener (Fig. 2). The embedded mold was left for 24 h for hardening and then glued to a microscope slide after being cut into small blocks (~2–3 mm thickness). Each sample was ground gradually parallel to the cutting plane to approach the sagittal section surface with 240, 600 and 1200 grit waterproof sandpaper, and then, grinding was continued with

2000 grit waterproof sandpaper until the sagittal plane was exposed (Fig. 2). The block was then turned over, attached to the glass slide and ground again to the sagittal plane. During the grinding process, the blocks needed to be constantly checked with a microscope to avoid overgrinding into the sagittal plane. Finally, they were fine ground with 2500 grit waterproof sandpaper and polished with 0.05- μm aluminum oxide powder.

Counting experiment and periodicity verification

Increments within the RSS were observed at $\times 100$ and $\times 400$ magnifications using an Olympus light microscope, and digital images of the whole sections were captured with a HTC3.0 Camera (Shanghai Weituo Technology Development Co.) and then processed with PhotoShop7.0 software. The number of increments for each beak was counted three times independently by the same skilled person, one of the authors. The precision of the counted data was assessed with the coefficient of variation (CV), calculated as the ratio of the standard deviation over the mean (Luo and Wei 2011).

In order to find out the best direction for counting, a subsample of 10 well-processed RSS of *D. gigas* beaks was selected for counting the increments along 4 different directions: direction 1 being the shortest linear axis with the minimum increment width; direction 2 being the second shortest linear axis with a thin increment width; direction 3 being the second longest linear axis a little further away from the internal rostral axis (IRA) with a large increment width; and direction 4 being the longest axis with the maximum increment width as defined by Perales-Raya and Hernández-González (1998) (Fig. 3). The number of increments in the RSS was related to the statolith-determined age by a linear regression analysis to validate the hypothesis of “one day, one increment.”

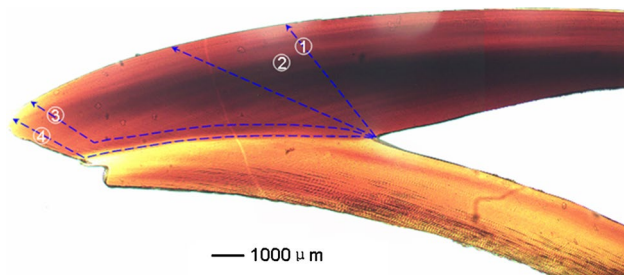


Fig. 3 Light micrograph of upper beak rostrum sagittal sections for one *O. bartramii*, showing four different directions to count increments

Results

Rostrum section microstructure

The RSS of the 4 species showed a distinct banding pattern from the rostrum tip to the joint of the dorsal (hood) and ventral part (crest), and the posterior portions of the banding were parallel to the rostrum edges. These bandings were composed of two units: a wider light increment and a thinner dark increment (Fig. 4). Compared to the crest, the growth increments in the hood were more discernable and clear where each increment near the IRA was the widest (Fig. 4a) and then the increments became gradually thinner further away from the axis (Fig. 4b). The increments in the tip region of the RSS were narrowest (Fig. 4c), and those in the medial part of the RSS were widest (Fig. 4d). The mean width of increments in the RSS was highest in *S. oualaniensis* (19.4 μm) compared to *D. gigas* (13.6–16.3 μm), *I. argentinus* (13.4 μm) and *O. bartramii* (12.4 μm) (Table 2). The mean width of increments in the RSS for *D. gigas* from the east-central Pacific Ocean (ECPO) was larger than that for *D. gigas* from the southeastern Pacific Ocean (SEPO) (Table 2).

Processing and counting

A total of 38 out of 53, 21 out of 30, 12 out of 20 and 12 out of 28 beaks were successfully processed and read for *D. gigas*, *O. bartramii*, *S. oualaniensis* and *I. argentinus*, respectively, which represented 71.7, 70.0, 60 and 42.9 % of the total samples (Table 1). Three independent counts for each species were thought to be precise with a CV lower than 5 % (Table 3). A relatively higher CV (4.95 %) in *I. argentinus* compared to the other three species indicated a lower precision (Table 3). The experiment for the evaluation of counting directions showed that the number of increments in direction 3 was the closest to the statolith-determined ages (Table 4).

Relationship between the number of increments of beak and statolith

Increments counted on the rostrum sections of *D. gigas* and *O. bartramii* were mostly similar to the statolith-determined ages, but those of *S. oualaniensis* and *I. argentinus* were less close to the statolith-determined ages, although counts for all four species were all slightly lower than the statolith-determined ages (Fig. 5). The number of growth increments in the upper beak RSS for the four species indicated significant linear relationships with the

Fig. 4 Increments near (a), far from (b) inner rostral axis for one *S. oualaniensis* beak, in the tip (c) and medial (d) part of rostrum section for one *O. bartramii* beak showing the difference in the width of increments

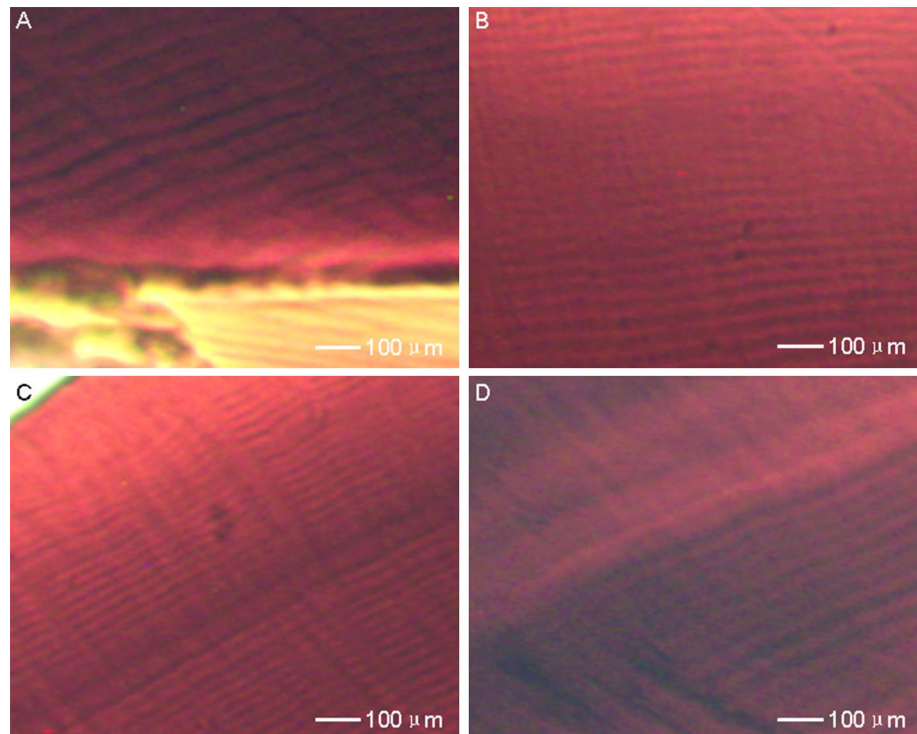


Table 2 Mean increment width in beaks and the relationship between statolith growth increments and beak growth increments for *D. gigas*, *O. bartramii*, *S. oualaniensis* and *I. argentinus*

Species	Catch area	Sample size	Regression slope		Width of increments (μm)	
			Mean	SD	Mean	SD
<i>D. gigas</i>	ECPO (Ecuador)	19	1.04	0.032	16.3	2.35
	SEPO (Chile)	19			13.6	1.46
<i>O. bartramii</i>	NWPO	21	1.02	0.039	12.4	1.47
<i>S. oualaniensis</i>	WCPO	12	1.10	0.086	19.4	2.56
<i>I. argentinus</i>	SWAO	12	1.18	0.112	13.4	2.28

ECPO east-central Pacific Ocean, SEPO southeastern Pacific Ocean, NWPO northwestern Pacific Ocean, WCPO west-central Pacific Ocean, SWAO southwestern Atlantic Ocean

Table 3 Precision of the three counts for upper beak rostrum sections in *D. gigas*, *O. bartramii*, *S. oualaniensis* and *I. argentinus*

Species	Sample size	Age range (days)	Mean CV	SD
<i>D. gigas</i>	38	127–357	2.86	0.96
<i>O. bartramii</i>	21	102–266	2.97	1.11
<i>S. oualaniensis</i>	12	51–120	3.42	1.25
<i>I. argentinus</i>	12	67–133	4.98	1.84

statolith-determined ages, with values of R^2 and slope close to 1, except for *I. argentinus* (Fig. 5). The regression equations were estimated as follows:

D. gigas: Increments = 1.0014 Age–9.1916 ($R^2 = 0.9943$, $n = 38$, $P < 0.001$)

O. bartramii: Increments = 1.0177 Age–6.6795 ($R^2 = 0.9693$, $n = 21$, $P < 0.001$)

S. oualaniensis: Increments = 1.0313 Age–10.239 ($R^2 = 0.9309$, $n = 12$, $P < 0.001$)

I. argentinus: Increments = 0.9245 Age–8.8241 ($R^2 = 0.8488$, $n = 12$, $P < 0.001$)

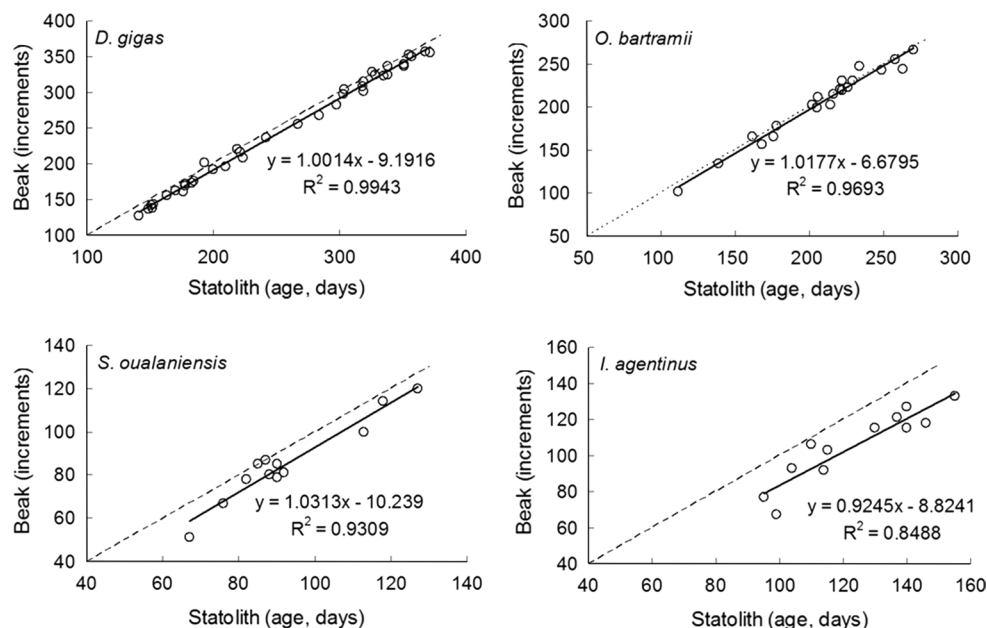
Discussion

In this study, we propose a effective technique to process RSS for estimating squid age. As is generally known, when

Table 4 The number of statolith growth increments corresponding to one beak growth increment in beak rostrum sagittal sections at different directions for 10 selected *D. gigas*

Specimen	Statolith-determined age	Number of growth increments counted in each direction			
		Direction 1	Direction 2	Direction 3	Direction 4
1	242	1.70	1.26	1.03	0.89
2	183	2.01	1.37	1.06	0.84
3	200	1.92	1.33	1.04	0.87
4	210	1.94	1.35	1.07	0.84
5	176	2.29	1.45	1.09	0.85
6	224	2.31	1.52	1.08	0.90
7	178	2.12	1.41	1.05	0.86
8	193	1.75	1.18	0.96	0.92
9	185	1.95	1.31	1.05	0.93
10	144	2.40	1.41	1.02	0.78
Average		2.04	1.36	1.05	0.87

Fig. 5 The relationship between number of increments counted in the direction three of beaks and statolith-determined age for *D. gigas*, *O. bartramii*, *S. oualaniensis* and *I. argentinus*



using hard tissue such as statolith to estimate squid ages, the selection of grinding plane is the most important factor affecting the section resolution. Perales-Raya and Hernández-González (1998) reported the best section plane for identifying growth increments. However, the final grinding plane achieved is never the optimum one because of bias generated in processing. For this reason, we developed a key procedure in the production of clear and accurate preparations which does not resemble any procedure reported in previous studies (Perales-Raya and Hernández-González 1998; Perales-Raya et al. 2010). We cut the beak into two pieces along the best section plane before further processing. This procedure makes it easy to keep the grinding plane always parallel to the section plane, after the embedded

block is attached to the slide with the section plane facing down. Significantly, for the fear of overcutting the increments in the central plane, the two beak pieces should not be cut symmetrically, but one should be a little larger than the other, and the larger one should be selected for further processing. In addition, we used a small, fine blade cutting machine to separate the beak instead of using scissors, to avoid damaging the section plane generated from the latter. Oosthuizen (2003) followed the method proposed by Perales-Raya and Hernández-González (1998), but the percentage of readable beaks obtained was much lower (18.8 %) than that achieved in this study (42.9–71.7 %).

Growth increments in the RSS of *D. gigas*, *O. bartramii*, *S. oualaniensis* and *I. argentinus* are always visible in

both hood and crest parts of the RSS as shown in octopods (Perales-Raya and Hernández-González 1998; Perales-Raya et al. 2010; Bárcenas et al. 2014; Perales-Raya et al. 2014a, b), but those in the hoods are more discernable and clearer than in the crests. However, the RSS microstructure of *D. gigas* and *O. bartramii* is clearer than in *S. oualaniensis* and *I. argentinus*, which results in a higher readable rate and lower independent counting CV. Similar to the statolith, the growth increments in the beak RSS of squid consist of light and dark lines. Here, we found that the mean width of increment in the squid RSS showed a significant interspecific and intraspecific variation (Table 2). *S. oualaniensis* collected from warm water have the widest increments compared to *I. argentinus* and *O. bartramii* that were obtained from cold waters and had thinner increments. In addition, *D. gigas* from warm waters (Ecuador) have wider increments than those from cold waters (Chile) (Table 2), as found in beaks of *O. vulgaris* LWS (Canali et al. 2011). Such a pattern is also congruent with the findings in statolith (Villanueva 2000) and in cuttlebone (Chung and Wang 2013).

Counting experiment data show that increment counts in the squid RSS for direction 3 were more accurate than for the other three directions, although previous studies have commonly counted along the IRA (direction 4) (Perales-Raya and Hernández-González 1998; Perales-Raya et al. 2010; Bárcenas et al. 2014). In this study, we found that the result was overestimated if counted along direction 4. One reason for this is that increments always branch into a couple of “false increments” in the region of the IRA (Fig. 6). Coincidentally, Oosthuizen (2003) counted the increments along the margin of the section (corresponding to our direction 3) rather than along the IRA.

Validation of the daily deposition of growth increments in beaks is widely studied in octopod *O. vulgaris* (Oosthuizen 2003; Canali et al. 2011; Perales-Raya et al. 2014a, b) and *O. maya* (Rodríguez-Domínguez et al. 2013; Bárcenas et al. 2014). Unfortunately, unlike octopods and sepioids, squid are difficult to rear in the laboratory (Iglesias et al. 2014). Thus, validating the periodicity of increment formation over their whole life by comparing to the statolith-determined ages might be an alternative method for a species whose statolith increment periodicity has been validated. This is the first attempt to validate the hypothesis of “one day, one increment” in the beak for the entire life of the squid, although it has been attempted in the paralarvae of several squid species (Sakai et al. 2007).

This study supports the hypothesis of daily deposition of growth increments at least in *D. gigas* and *O. bartramii* and even *S. oualaniensis* (Fig. 5), although the underestimation cannot be ignored (i.e., intercept less than zero). Feeding erosion of the rostrum and delay of hatching increment deposition might contribute to the undercounting of

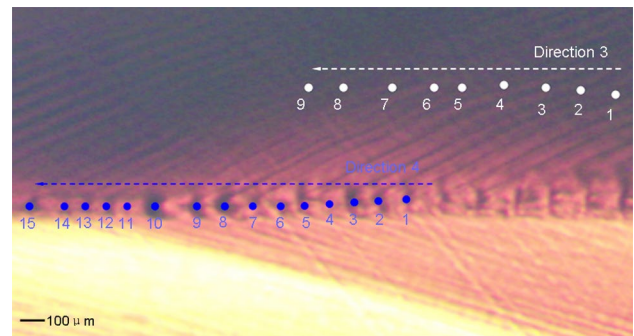


Fig. 6 Comparison of the number of increments at direction 3 and direction 4 in partial rostrum sagittal sections of one *D. gigas*

increments in the RSS (Perales-Raya et al. 2010; Bárcenas et al. 2014). The first increment in the rostrum for *D. gigas* and *O. bartramii* begins to form on the first day after hatching. Therefore, a major reason for this underestimation is the existence of some erosion in the rostral area used for biting and tearing prey (Hernández-López et al. 2001; Perales-Raya et al. 2010; Canali et al. 2011), although specimens analyzed in the current study were selected to minimize the effect of this erosion. However, such underestimation was not shown in the paralarvae of *O. vulgaris*, for which erosion is minimal (Hernández-López et al. 2001) and in laboratory raised *O. maya* that were fed on soft paste which reduced the beak’s erosion (Rodríguez-Domínguez et al. 2013; Bárcenas et al. 2014).

In summary, beaks have several advantages over statoliths because they are easy to extract, preserve and manipulate, and could provide additional or alternative data for determining squid ages, especially in cases where it is impossible to obtain statoliths (such as semi-digested prey or clean mandibles from stomachs of predators and plus from formalin-stored samples). Considering all the facts presented and discussed above, we suggest the upper beak RSS is an appropriate material for determining the age of *D. gigas*, *O. bartramii* and possible *S. oualaniensis*. However, possible erosion of the rostral region, which may result in an underestimation of squid ages, should be taken into account in determining ages. Further studies should be focused on growth increments in the LWS which has been demonstrated to be more reliable than the RSS for octopods (Perales-Raya et al. 2010).

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