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EYE LENS STRUCTURE OF THE OCTOPUS *ENTEROCTOPUS MEGALOCYATHUS*: EVIDENCE OF GROWTH

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ABSTRACT The need for age determination for the proper management of octopus fisheries has directed research attention to the few hard structures in the octopod's soft body, beak, statoliths, and the vestigial shell. So far, none of these structures have proved reliable or practical for the purpose. Eye weight, pigment, and nitrogen content have been used successfully in age determination of vertebrates. Given the structural similarity of lenses between octopus and vertebrate, we explore the possibility of using octopus eye lens weight, diameter, and structural marks as age indicators. The lenses of 228 octopi were obtained from the commercial catch from Ancud and Queilén, in Chiloe, Chile. They were measured, weighed, and prepared for histological analysis of the concentric lines structure. The prehatching nucleus was identified, and the total number of lines counted. The lines start to form in the prehatching larvae. Lens diameter and weight showed a much smaller variation than body length or weight, with an apparent correspondence of size or weight classes of body measurements to a lens diameter or weight.

KEY WORDS: age, histology, octopod, eye, lens, octopus, *Enteroctopus*

INTRODUCTION

Age determination of soft-body organisms has always been a major problem for fishery biologists (Tesch 1971). Therefore, population size structure has been used as an alternative method (Gayalino et al. 1993), a technique that works for populations with a clearly defined recruitment and with a life span of several years (Allen 1966, Jones 1981). It has been used in cephalopods with reservation (Guerra 1979, Nepita & Defeo 2001), because they have short life spans of 1 or 2 y, and recruitment extends for several months, with a wide variation of sizes even from a single parent (Cortez et al. 1999, Boyle & Rodhouse 2005).

The lack of hard structures in the body of an octopus has prevented the determination of age. The beak, statoliths, and stylets have been proposed as structures where daily growth marks are registered (Raya et al. 1994, Raya & Hernández-García 1998, Perles-Raya et al. 2010, Doubleday et al. 2006). Although the technique for preparing and reading stylets has improved, its application to *Enteroctopus megalocyathus* has not given satisfactory age readings during our investigations, with age underestimated. The aim of this contribution is to present a technique to visualize the eye lens structure and to evaluate its application as a tool for age determination in *E. megalocyathus*. We present the correlation of *E. megalocyathus* eye lens weight and diameter to its mantle length, total length, and weight. We also present a technique to observe the structure of octopus lenses under light microscopy, discussing its potential for age determination.

METHODOLOGY

Two hundred twenty-eight individuals of *E. megalocyathus* were subsampled from a monthly sample of the commercial

catch of a fisheries population from the ports of Queilen and Ancud, Chiloe, Chile, carried out by the Instituto de Fomento Pesquero (IFOP). The organisms were measured and weighed fresh, and their eye lenses fixed. After fixation in neutral formalin (Luna 1969), the lenses were measured in diameter with vernier calipers and weighed with a digital plate scale with 0.01-g precision. For microscopic analysis, they were treated with decalcifying solution (Table 1) prior to dehydration and paraffin inclusion (Table 2). Sections were stained with Harry's hematoxylin-eosin stain (Luna 1969).

Plastic inclusion with polished thin sections was tried as an alternative to microtome sectioning. The lenses were dehydrated with the same technique recommended for paraffin inclusion, with five more baths to induce the penetration of the plastic solvent and the plastic resin (Table 3). Thin sections were polished manually with wet sandpaper nos. 100 and 400, and with metal polisher as a final step. Sections were mounted on microscope slides with microscope mounting media for final polishing and observation. An image of an embryo of unknown age was provided by IFOP in which the larvae eye lens is clearly visible.

Thin sections and microtome sections were observed in clear field and phase contrast microscopes. Digital images were taken with a Leica microscope (model DM 1000) with a camera (Leica Microsystems, Wetzlar, Germany). The program Leica Application Suit (v. 1.6.0) was used to capture and edit the images. ArcSoft Panorama Maker and PowerPoint were used to integrate the images. The concentric lines were counted manually. It is possible to count the number of concentric lines under 400× and 1,000×. In order not to duplicate the count of lines between consecutive images, it is important to identify marks that permit the continuity between images. Identifying marks are also necessary to stitch consecutive images into 1 single image (Fig. 1).

Lens diameter, and weight of left and right eyes were analyzed independently for their correlation to mantle and total length, and total fresh weight with Excel (Microsoft). Correlations

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TABLE 1.
Fixation and decalcification of *Enteroctopus megalocyathus* eye lenses for histological processing (Luna 1969).

Fixation	
Neutral formalin	
Formalin, 37–40%	100 mL
Distilled water	900 mL
Monobasic sodium phosphate	4.0 g
Anhydride dibasic sodium phosphate	6.5 g
Procedure	
<ul style="list-style-type: none"> ● Place the eye lenses in a minimum of 20–25 times their volume of fixative. Fixation is accomplished in 48–72 h. ● The eye can be fixed whole after making a few cuts with the scalpel, taking special care not to damage the eye lens. This allows easier cleaning after fixation. The eyes can be transferred to 70% methyl alcohol with glycerin at 1% for further conservation. ● Before proceeding with decalcification and dehydration, the eye lenses should be rinsed from the fixative or preservative solution with running tap water for 8–12 h. 	
Decalcification	
Solution A	
Sodium citrate	50 g
Distilled water	250 mL
Solution B	
Formic acid 90%	125 mL
Distilled water	125 mL
These two solutions are used in equal proportions.	
Procedure	
<ul style="list-style-type: none"> ● Decalcify with sodium citrate and formic acid for 48 h; change solution every 24 h. ● Wash with running tap water for 24 h. ● Impregnate in paraffin or your favorite inclusion media (Table 2). ● Place the eye lenses with the anteroposterior plane perpendicular to the cutting plane, including both anterior and posterior lenses. ● Expose the cutting area and humidify by immersion in distilled water. ● Keep both blade and block cold by rubbing with an ice cube. Cut with slow movements. ● Use 2 flotation baths: one with distilled water at ambient temperature, where the cut ribbons will be placed; and a second one approximately 10°C under normal flotation bath temperature. This second bath must be prepared with 3 teaspoons of 5% gelatin for every 1,000 mL distilled water. ● Note: The gelatin bath must be changed daily. The bath should be washed with disinfecting soap to prevent the formation of bacteria. ● Dry in the oven. 	

between eye lens body parameters were evaluated with Pearson's correlation index (Hampton & Havel 2006).

RESULTS

Lens Structure

Preliminary analysis of the octopus lenses showed concentric layers (Fig. 2), which are evident as dark, clear concentric bands.

TABLE 2.
Eye lens inclusion routine (Luna 1969).

	Automatic	Manual
Alcohol 95%	3 h	12 h (2 changes)
Alcohol 95%	2 Changes of 1 h each	12 h (2 changes)
Alcohol 100%	3 Changes of 1 h each	24 h (4 changes)
Chloroform	1 h	5 h (3 changes)
Chloroform	2 h	
Paraffin	2 Changes of 1 h each	5 h (3 changes)
Paraffin under vacuum	45 min	2 h (if you do not count with vacuum)

Under phase contrast microscopy, secondary lines are made evident (Fig. 2B). A nucleus is identified with a diameter of 130 µm (Fig. 3), which corresponds to the diameter of the eye lens in prehatching larvae (Fig. 4), but not to the diameter of the larval eye lens nucleus, which in Figure 3 was identified with a diameter of 50 µm.

TABLE 3.
Technique for the inclusion of octopus eye lens in synthetic resin, for the preparation of thin slides.

Regent	Time (h)
50% Chloroform, 50% *	4
Solvent	4
50% solvent, 50% resin	8
Resin without catalyst	8
Resin with catalyst	72, at ambient temperature (<20°C), or 8 h in the stove at 40–60°C

Note: Proceed with these baths after following the dehydration process described in Table 2 up to the second bath of chloroform.

* Solvent for synthetic resin.

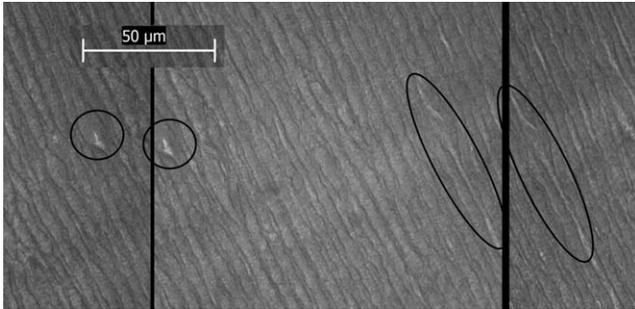


Figure 1. Identifying marks in individual images to integrate a mosaic and to count the concentric lines in the *Enteroctopus megalocyathus* crystalline structure (1,000 \times).

Preliminary analysis of line count of 3 organisms (Table 6) showed 67 lines for prehatching larvae of unknown age. So far, line count has varied between 542 and 571, which, if interpreted as day growth marks, would be equivalent to 13.9–14.6 mo (Fig. 5), and to 2.2 mo for the prehatching larvae (Fig. 4)

Biometry

Fresh weight of the organisms varied between 498 g and 5,750 g, with a total length between 46 cm and 127 cm, and a mantle length between 84 mm and 213 mm. The eye lens diameters varied between 2.32 mm and 9.82 mm, with a weight varying between 0.09 g and 0.56 g.

Variations between left and right lenses were observed during sample preparation (Table 4). These differences induced us to process each eye lens separately. These differences are not evident graphically (Fig. 6) or statistically (Table 4). However, an interesting association of variables shows up when the eye lens measurements are placed as the independent variable, and the organism lengths and weight are used as the dependent variables (Fig. 6). A range of mantle lengths or total lengths are scattered around every value of eye lens diameter or weight. The lens diameter–weight correlation was high ($R = 0.86$), with wider fluctuations for diameter than for weight (Table 4). A Pearson correlation analysis (Hampton & Havel 2006) showed higher correlation of female eye lens measurements to total length (Table 5).

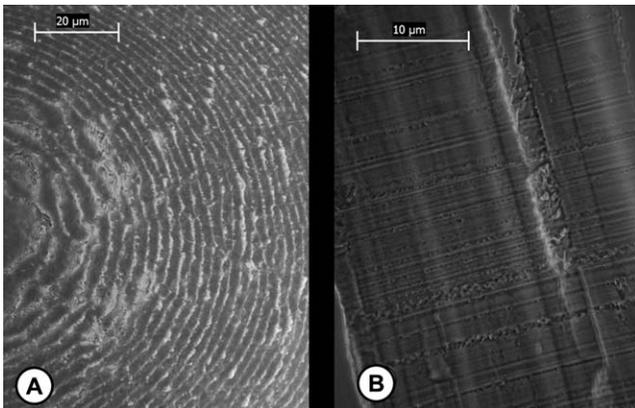


Figure 2. (A, B) Eye lenses of *Enteroctopus megalocyathus* concentric structure as presented in (A) polished surface of plastic resin embedding under phase contrast microscopy (400 \times ; A) and microtome section stained with hematoxylin–eosin under phase contrast microscopy (1,000 \times ; B).

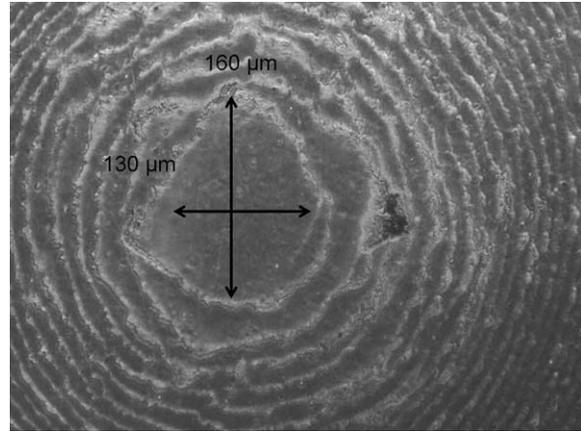


Figure 3. Eye lens nucleus of *Enteroctopus megalocyathus* from a polished surface of plastic resin embedding under phase contrast microscopy (400 \times).

DISCUSSION

Eye weight, pigments, and nitrogen contents have been used to determine age in rabbit (Wheeler & King 1980), kangaroo (Poole et al. 1982, Augusteyn et al. 2003, McLeod et al. 2006), cattle (Raines et al. 2008), and euphausiid crustaceans (Harvey et al. 2010). These are characteristics that keep constant, independent of diet and environmental variables. We have found a very low correlation of eye lens measurements with the organism weight and length. Even though eye measurements have a very low variability, the wide dispersion on body measurements could be the source of the lack of correlation, somehow observed graphically (Fig. 6), in which a range of weights or mantle length correspond to a value of eye measurement.

The numbers of lines counted on the preliminary samples of this study suggested a corresponding number of lines to days of age, and to eye weight and diameter, but this will have to be ascertained with a significant sample size. Wentworth and Muntz (1992) report that lens formation starts on embryonic

TABLE 4.

Measurements of sampled octopi and their eye lenses.

	Diameter (mm)	Weight (g)	Mantle (mm)	Length (cm)	Weight (g)
Right lens ($n = 228$)					
Maximum	9.82	0.51	213.00	127.00	5,750.00
Minimum	4.75	0.09	84.00	46.00	498.00
Mean	6.68	0.24	138.26	73.54	1,915.79
SD	1.01	0.09	25.33	15.96	896.62
Diameter	Cor. C.		0.55	0.59	0.57
Weight	Cor. C.		0.54	0.59	0.58
Left lens ($n = 205$)					
Maximum	9.57	0.56	213.00	127.00	5,750.00
Minimum	2.32	0.09	94.00	46.00	655.00
Mean	6.58	0.23	136.83	72.60	1,877.68
SD	0.97	0.09	23.95	15.04	849.48
Diameter	Cor. C.		0.49	0.51	0.50
Weight	Cor. C.		0.53	0.54	0.54

Cor. C., correlation coefficient for lenses vs body measurements.

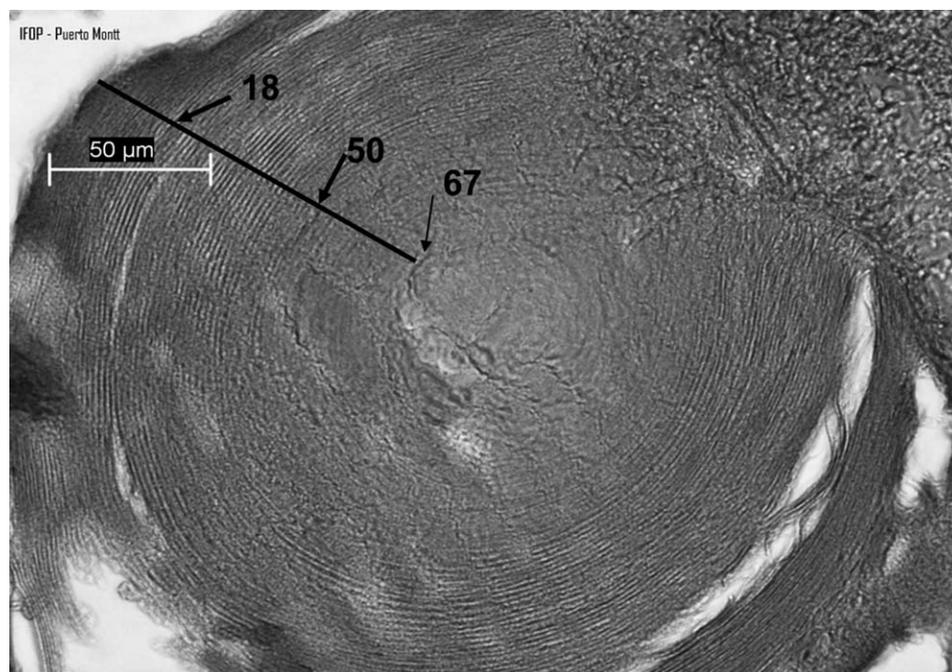


Figure 4. Prehatched larval eye of *Enteroctopus megalocyathus*. Lens with 65 growth lines and nucleus (400 \times).

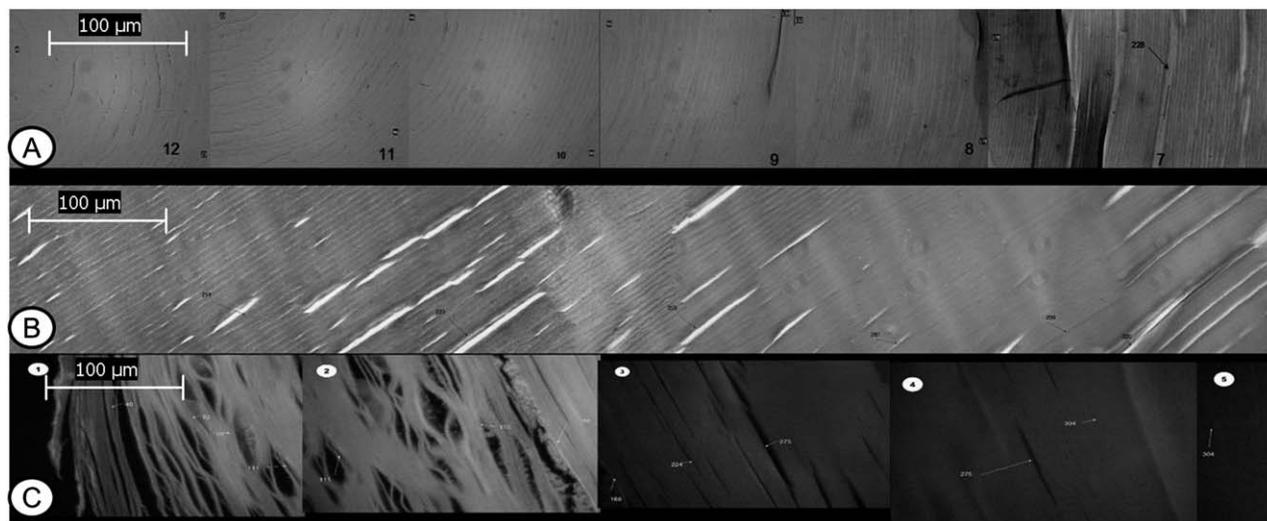


Figure 5. Integrated image of 3 lenses. (A) Sample Q1–6 mosaic of 16 sections, phase contrast without filter (400 \times). (B) Sample Q1–70 mosaic of 41 sections, phase contrast with green filter (1,000 \times). (C) Sample A2–34 mosaic of 13 sections, epifluorescence (400 \times).

stage IX, with the formation of a nucleus and overlying layers formed from processes of the lentigenic cells. The nucleus size observed in adult octopi does not correspond to the nucleus of prehatching larvae. Therefore, the nuclei so far observed may be an artifact of the level at which the slide was made. Figures 4 (for prehatching larvae), 5, and 6 for adult octopi show that the widths of the concentric lines are homogeneous, independent of the age of the organisms. Giuditta and Prozzo (1974) report an increase in nuclear number relative to increase in body weight, with nuclear density steadily increasing in *Octopus vulgaris* reared in tanks, but with differences between organisms reared

in dark and light. These authors proved that the rates of increment in number of nuclei and tissue weight became higher than normal in octopi reared in transparent boxes.

Given the small variability and ease of measurement of eye lens weight and diameter, we propose the use of this parameter as a tool to evaluate population structure through size frequency analysis, using a correlation factor with body size and weight estimated for every different population. These measurements could later be used for age estimation if the line number in the internal structure of the eye lenses is correlated to age, weight, and diameter.

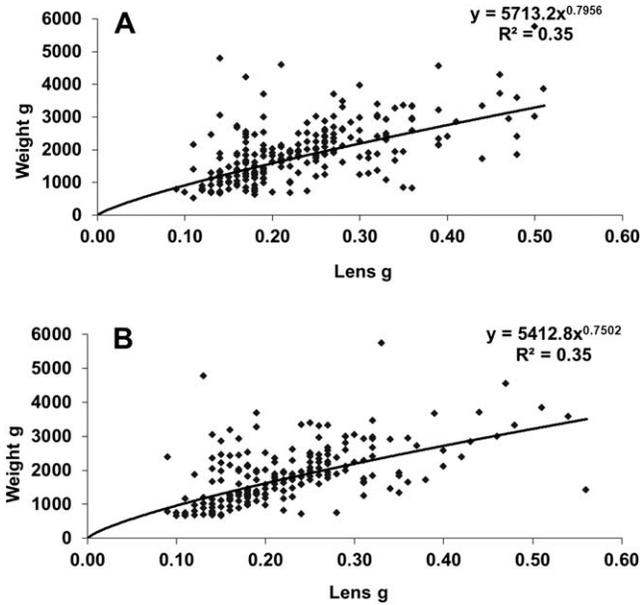


Figure 6. (A, B) *Enteroctopus megalocyathus* eye lenses weight regression to body weight: right eyes (A) and left eyes (B).

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TABLE 5.
Pearson correlation of eye lenses versus mantle, body length, and body weight $P < 0.05$.

	MI		TI		W	
	M	F	M	F	M	F
LI	0.54	0.53	0.56	0.59	0.58	0.36
Lw	0.54	0.54	0.56	0.61	0.59	0.54

MI, Mantle length; TI, total length; W, total fresh weight; LI, lenses diameter; Lw, lenses weight; M, males; F, females.

TABLE 6.
Weight and number of rings in crystalline structure of *Enteroctopus megalocyathus* from the south of Chile (corresponding to digital images in Fig. 5).

Identification	Weight (g)	Total Length (cm)	No. of Rings	Observations
A Q1-6	2,449	91	571	16 sections, phase contrast 40×
B A2-34	1,746	69	558	13 sections, epifluorescence 40×
C Q1-70	1,019	69	542	41 sections, phase contrast 100×

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