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Validation of growth increments in stylets, beaks and lenses as ageing tools in *Octopus maya*

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ABSTRACT

Octopus hard structures have recently been used as ageing tools. Thirty-two *Octopus maya* were raised in captivity in four age groups, ranging from 124 to 233 days old. Their stylets, beaks and eye lenses were analyzed in order to validate the periodicity of growth increments during all the octopus life. Transverse sections of stylets were mounted in glycerin jelly, beaks were sagitally cut to analyze their lateral walls and eye lenses were processed by histological techniques and mounted in resin. Growth increments in each structure were observed and counted under a microscope. Stylet increments were successfully validated as their counts were closely related to age in days. Beak increments from the two younger age groups showed a close relationship with age, suggesting a daily deposition. However, persistence of lower increment counts strongly suggests that not all growth increments could be counted, probably due to erosion during feeding. Eye lens increment counts did not show a relation with age, although periodicity, if any, might be subdaily. The use of stylets is recommended for *O. maya* growth and ageing studies as they also showed a high precision in increment counts between readers.

1. Introduction

Age and growth estimations are important tools for the development of fisheries management. Knowing the age and growth of commercially exploited species is essential for evaluating parameters as age structure, growth rate, longevity, hatching date, recruitment, and survival rate of different age classes and hence contribute to the rational exploitation of populations (Campana, 2001, Doubleday et al., 2006).

Octopus maya is an endemic species of the Yucatan Peninsula, Mexico. It is the third marine fishery resource by value and the seventh by volume in Mexico (CONAPESCA, 2011), and is the largest octopus fishery in the Americas (FAO, 2013). Age and growth of wild populations have been estimated through indirect methods such as mantle length (ML) modal analysis (Arreguín-Sánchez, 1992; Nepita-Villanueva and Defeo, 2001). They suggest larger longevities (1–2.5 years) than those observed under culture (1 year, Van Heukelem, 1983). High individual variability in octopus growth produces a lack of relationship between their size and age (Leporati et al., 2008; Semmens et al., 2004; Van Heukelem, 1983), stressing the need of direct ageing tools.

Direct methods for age estimation have been recently implemented in octopus through the analysis of hard structures. Stylets represent the vestigial shell in octopods (Bizikov, 2004) and their growth increments have been analyzed in several studies (Doubleday et al., 2006; Leporati et al., 2008; Sousa Reis and Fernandes, 2002). Daily deposition of these increments have been validated in known-age *Octopus pallidus* (Doubleday et al., 2006) and using tetracycline stains in *Octopus vulgaris* (Hermosilla et al., 2010). Growth increments in octopus upper beaks have been studied mainly in *O. vulgaris* (Canali et al., 2011; Castanhari and Tómas, 2012; Hernández-López et al., 2001; Perales-Raya et al., 2010; Raya and Hernández-González, 1998). Daily deposition has been confirmed in their paralarvae (Hernández-López et al., 2001), and adults (Canali et al., 2011). A recent study validated the increments in young (≤122 days old) *O. maya* (Villegas-Bárcenas et al., in press). Clarke (1993) and Baqueiro-Cárdenas et al. (2011) described and counted concentric lines in the eye lenses of *Sepia officinalis* and *Enteroctopus megalocyathus*, respectively, suggesting its possible use for age estimation.

A validated direct method to determine *O. maya* age would improve population evaluations, from its biology and ecology to fisheries management (Campana, 2001; Doubleday et al., 2006; Hermosilla et al., 2010). In this sense the aim of this study is to validate the periodicity of increment deposition in the stylets, beaks and eye lenses of *O. maya* for age estimations.

2. Materials and methods

2.1. Specimen collection

O. maya produces large, benthic hatchlings, a fact that has encouraged its use for cultivation studies (Domingues et al., 2012; Rosas et al.,

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2012). Thirty-two *O. maya* individuals were raised in captivity from four females at different periods, conforming four age groups: Group 1, n = 5, 124 days; Group 2, n = 6, 162 days; Group 3, n = 14, 208 days; and Group 4, n = 7, 233 days. Each age group was kept in a circular tank (5 m in diameter) with an open sea water flow and a recirculation system (each age group was kept in a different tank) at ambient water temperature (26–27 °C) and natural photoperiod. Three PVC tubes per animal were placed in each tank as a refugee. Groups 1 and 2 were fed twice a day with a soft paste made of shrimp and squid (Rosas et al., 2012), while group 3 and 4 were initially fed with soft paste and then diet was switched to frozen blue crabs (*Callinectes* sp.). Octopuses of each group were sacrificed simultaneously using 5% ethanol in seawater as terminal anesthesia (Moltschaniwskyj et al., 2007) and their body weight (BW) was measured to the nearest g.

2.2. Structure preparation

Stylets were extracted using watchmaker forceps and surgical scissors. They were placed in labeled histocassettes and preserved in 4% formalin. To avoid dehydration, stylets were washed with fresh water prior to cutting. They were weighed and their anterior, posterior and total lengths and central width were measured (Doubleday et al., 2006). Stylets were prepared following Bizikov's (1991) technique, placing them between two polystyrene blocks (1.0 cm × 1.5 cm × 5.0 cm) corresponding to the size of the stylet. They were cut by hand with a razor blade in the bending region or near it (Doubleday et al., 2006). Several cuts were made in each stylet and they were mounted in glycerol jelly on a microscope slide. A small amount of jelly was placed in a slide and heated until it melted (avoiding boiling). Stylet sections were embedded in the jelly and a coverslide was placed on (Bizikov, 1991; Doubleday et al., 2006). Samples were observed at 400× magnification with a compound microscope. Increments were counted from the first visible increment surrounding the nucleus (considered the first post-hatch increment, Doubleday et al., 2006) to the stylet periphery with a hand counter.

Beaks were removed and placed in labeled histocassettes and kept in water at 4 °C in a refrigerator. Upper beaks were weighed and the following measures were taken: hood length, rostral length, crest length and height (Perales-Raya et al., 2010; Raya and Hernández-González, 1998). The outer section (crest and hood) of the upper beaks was sagitally cut with scissors; each section was clean by hand with water to remove the mucus inside the beak (Hernández-López et al., 2001). Increments were counted under a stereoscopic microscope at 50× magnification and a hand counter from the rostral tip to the opposite end of the lateral wall (Hernández-López et al., 2001). Beak was left to dry prior counting to allow the visibility of increments.

Eye lenses were extracted and processed following Luna's (1968) technique with some modifications, significantly shortening the preparation time developed by Baqueiro-Cárdenas et al. (2011) (Table 1). Fixation and decalcification solutions were prepared as in Luna (1968). Eye lenses embedded in paraffin cubes were placed in distilled water from one to two days for softening. Sections of 7 μm were cut using a LEICA rotary microtome. Sections were placed in a water bath at 40–45 °C for stretching and then mounted on a slide. Samples were stained with Harry's hematoxilyn eosin protocol (modified from Luna, 1968 and López-Ripoll, 2010) (Table 2), mounted with J.T. Baker polymer resin and covered with a coverslide. Samples were left to dry for one day. Increment counting was not possible to be done under microscope as contiguous increments were hard to appreciate. Furthermore, in several samples increments could not be visualized until photos were taken and edited. Digital photos were taken using a Nikon Eclipse E600 compound microscope with a DS-5M-L1 camera at 400× magnification. To improve the visibility of the increments, photos were edited using Picasa 3 program. Increment counting was performed manually on the screen.

Increment counting in the three structures was performed by two independent readers. Each reader counted the increments at least two times, with the exception of eye lenses, which were read only once. Growth increment widths in some samples of all structures were measured in characteristic regions: anterior or inner, mid-region and posterior or outer.

2.3. Data analysis

Counts were compared to determine the reproducibility of the procedure (Campana, 2001). Coefficient of variation (CV) (Chang, 1982) was calculated as a measure of precision and reproducibility of any pair of readings. Count pairs with CV < 10% were taken as valid and their averages were considered as the number of increments for a given octopus sample. CVs for each age group were averaged (Campana et al., 1995) to detect any bias by age.

Increment counts in the three structures were related to octopus age in days through a linear regression. Regression slopes were tested if they were not significantly different from one (an increment per day hypothesis) with a Student's two tailed *t*-test following Zar (1984). The one sample Kolmogorov–Smirnov test was used to assess normal distribution and Levene's test for homogeneity of variances. When normality and/or homogeneity of variances were not satisfied a Spearman's correlation test was performed.

Table 1
Eye lens preparation for paraffin inclusion histological technique (modified from Luna, 1968).

Process	Step	Reagent	Time
Fixation	1	Neutral formalin	72 h
	Optional	Ethanol, 70% for conservation	
	2	Tap water	72 h
Decalcification	3	Decalcifying solution (sodium citrate solution and formic acid solution)	24 h
	4	Decalcifying solution	24 h
	5	Tap water	24 h
Dehydration	6	Alcohol, 95%	1.5 h
	7	Alcohol, 95%	30 min
	8	Alcohol, 95%	30 min
	9	Alcohol, 100%	30 min
	10	Alcohol, 100%	30 min
	11	Alcohol, 100%	30 min
Clearing	12	Ultraclear	30 min
	13	Ultraclear	1 h
Impregnating	14	Paraffin	1 h
	15	Paraffin	1 h
	16	Paraffin	2 h