

Morphological and chemical description of the stylets of the red octopus, *Enteroctopus megalocyathus* (Mollusca: Cephalopoda)

FEDERICO MÁRQUEZ^{1,2,3} & MARÍA EDITH RÉ¹

¹*Biología y Manejo de Recursos Acuáticos, Centro Nacional Patagónico, Consejo Nacional de Investigaciones Científicas y Técnicas, Boulevard Brown 2915, 9120 Puerto Madryn, Argentina. Email: fede@cenpat.edu.ar*

²*Universidad Nacional de Córdoba.*

³*Universidad Nacional de la Patagonia San Juan Bosco.*

Abstract

This paper provides the first morphological and chemical description of the stylets (vestigial shell) of *Enteroctopus megalocyathus*, a large, commercially and ecologically important benthic species that is fished along the southern coasts of Argentina and Chile. The stylets are thin, semi-transparent curved structures embedded in the muscular tissue of the dorso-lateral regions of the mantle, and placed inside an epithelial sac. Rostral and post-rostral parts and a bend could be distinguished. Differences in the curvature of the post-rostral parts allow the discrimination of left and right stylets. In recent years these structures have received attention because of their potential use as a tool for age determination, based on interior growth rings. The elemental composition inside stylets can also assist in reconstructing environmental histories, and in determining dispersal and population structure. The length of the different parts and the maximum diameter at the bend were measured and compared between sexes and between left and right stylets. Transverse cuts were used to describe histological structures, the nucleus and the white core that surrounds it, and growth increments. Constituent chemical elements were analysed with a scanning electron microscope equipped with an X-ray energy-scattering microanalyzer. No significant differences between sexes were detected in the relative length of the post-rostral and rostral parts of the stylets. These relative lengths could, however, be of taxonomic value at the generic level. Two types of concentric increments were observed; the embryonic nucleus of the stylets was amorphous and did not present growth increments. The distribution of chemical elements in the stylets is also described.

Key words: Vestigial shell, growth increments; chemical elements

Introduction

The calcareous phragmacone of ancient coleoid cephalopods is retained in some orders but lost in others, the latter being the case for the Teuthida and Octopoda. In the Teuthida it is represented by a dorsal internal structure, the gladius, while in the Octopoda the vestigial shell is reduced to a pair of stylets or is absent (Donovan and Toll 1988).

The vestigial shell has been described for few species of the suborder Incirrata (Isgrove 1909; Wells 1978; Sousa-Reis and Fernandes 2002; Bizikov 2004). The form and structure of the stylets vary among species (Bizikov 2004). In *Eledone cirrhosa* (Lamarck, 1798) they have been described as a pair of chitinous oval rods, colorless and semi-transparent, which curve and taper at both ends. They are enclosed in and secreted by the walls of an epithelial sac (Isgrove 1909). The stylets of *Octopus vulgaris* Cuvier, 1797 have been characterized as a vestigial shell reduced to two slender and long structures embedded in the musculature of the mantle, near the insertion of the gills (Wells 1978; Sousa-Reis and Fernandes 2002). Those of *Enteroctopus dofleini* (Wülker, 1910) are thin, translucent rods with pointed ends lying on the dorsal-lateral sides of the mantle, sharply bent, formed by a cartilage-like structure of chitinous texture that is laid down in concentric layers (Bizikov 2004). The stylets of *Octopus pallidus* Hoyle, 1885 are similar to the latter (Doubleday *et al.* 2006). For other species, including *Benthoctopus johnsoniana* Allcock, Strugnell, Ruggiero & Collins, 2006, *Benthoctopus normani* (Massy, 1907), *Microeledone mangoldi* Norman, Hochberg & Boucher-Rodoni, 2004, *Thaumeledone gunteri* (Robson, 1930),

Thaumeledone peninsulae Allcock, Collins, Piatkowski & Vecchione, 2004, *Thaumeledone rotunda* (Hoyle, 1885) and *Cistopus sp.*, drawings or brief descriptions of the stylets were included as part of taxonomic descriptions (Norman *et al.* 2004; Allcock *et al.* 2004; Allcock *et al.* 2006; Liao 2003).

Appellöf (1898) proposed that the epithelial sacs are formed by the shell gland in *Octopus* Lamarck, 1798. This gland, after closing and sinking below the external surface of the mantle, divides into two halves; each of them takes up a lateral position and secretes a stylet. These structures consist of a calcified organic matrix, as is the case for other mollusc shells (Sousa-Reis and Fernandes 2002), its function being to act as a support for muscles and internal organs (Bizikov 2004).

A few studies have suggested the potential use of stylets for age determination based on the observation of concentric increments in cross-sections (Moriyasu and Benhalima 1993; Sousa-Reis and Fernandes 2002; Doubleday *et al.* 2006). The periodicity of the increments was recently validated using laboratory-raised *O. pallidus* of known-age (Doubleday *et al.* 2006). Research by Napoleão *et al.* (2005) and Doubleday *et al.* (2008) demonstrated that this structure incorporated elements from the environment on a chronological basis, and therefore used the elements inside the stylets as a tool to provide information about population structure and dispersal.

The Patagonian red octopus, *Enteroctopus megalocyathus* (Gould, 1852), is distributed in the southwestern Atlantic Ocean from the San Matías Gulf to the Beagle Channel, the Falkland (Malvinas) Islands and

Burdwood Bank (Ré, 1998ab), and northwards to Central Chile in the southeastern Pacific. A relatively large species, it is caught by fishermen along the coasts of Argentine Patagonia, mainly between March and November. In southern Chile it supports a small scale octopus fishery ~300 tons per year (Chong *et al.* 2001; Rocha and Vega 2003; Olguín and Jerez 2003; Hermosilla 2004; SERNAPESCA 2004). There are no estimations of the size of the Patagonian red octopus stocks from the Argentine shelf. However, Koen *et al.* (1993) estimated that the annual consumption of *E. megalocyathus* by sea lions [*Otaria flavescens* (Shaw, 1800)] between 41° and 47° S, could reach 34,000 tons, suggesting that the population is large.

The aim of this paper is to describe for the first time the morphological and chemical characteristics of the stylets of *E. megalocyathus*.

Materials and Methods

A survey of the *Enteroctopus megalocyathus* population from Nuevo Gulf (42°47'S 65°03'W), Argentina, was conducted from August 2003 to October 2005. A total of 305 individuals was collected by scuba divers, including 154 females (total weight 115 to 3150 g) and 151 males (total weight 68 to 3550 g). Sex was determined by examination of the gonads. Total body weight was recorded to the nearest 0.1 g. Stylets were removed: the white cores were observed for their presence or absence, and the relative lengths were recorded. They were then preserved in 5% seawater formaldehyde solution, keeping the right and left separate. Broken stylets were discarded from the analyses. A total of 276 pairs of stylets were analysed.

For the biometric study of the vestigial shell the following parts were defined (Fig. 1): the rostral (RP), between the center of the point of inflection ('bend') and the tip of the shortest branch, and post-rostral (PRP), between the center of the bend and the tip of the longest branch. Rostral length (RL), post-rostral length (PRL), maximum diameter (MD) and total length of the stylet (TLS = RL + PRL) were measured on each stylet to the nearest 0.01 mm with Vernier calipers, or using a stereoscopic microscope. While specimens were being dissected, the position of the stylets in the mantle, and of the organs and muscle associated with them were recorded. To describe the morphology of the stylets, comparisons were made of TLS, PRL, RL, MD, and the PRL/RL ratio between right and left stylets by sex. A parametric t-test or a nonparametric Willcoxon paired-sample test was used depending on the nature of the data (Zar 1996). Furthermore to compare the left and right stylets for each sex a nonparametric Kolmogorov-Smirnov test or a t-test for non-paired samples was used.

The conventional haematoxylin-eosine staining was used in microscopic observations on cross-sections made at the point of maximum diameter of the stylet. In order to determine the presence and type of growth increments, cross-sections of the stylets were made using razor blades under a stereoscopic microscope (16x magnification). The diameter

of the nucleus and of the white core that surrounds it (when present) was measured on each section with a graduated ocular (400x magnification).

The proportion of inorganic matter and water content of the stylets was estimated from two samples (3.4 g and 2.15 g) taken randomly, dried at 105 °C, and calcinated in a muffle at 550°C; ashes were subsequently weighed.

Four transverse sections of the stylets were made on the PRP, near the bend; two were chosen with the white core and the other two without it, to identify the elemental chemistry and distribution patterns qualitatively. All the sections were observed with scanning electron microscopy (Jeol JSM 6460 LV equipped with EDAX PW 7757/78 X-ray energy-scattering microanalyzer). The location of the most abundant chemical elements was determined by scanning the transverse cross-section surface of the stylets.

Results

The stylets of *Enteroctopus megalocyathus* are located on the dorsal-lateral sides of the mantle, one at each side of the axial plane, placed inside the epithelial sac with their concave surface in contact with the dorsal part of the mantle. Relative to the RP, the PRP is more superficially embedded in the mantle. Both the PRP and RP are projected towards the central axis of the mantle. The bend is the thickest part of the stylet; the variable obtuse angle is oriented dorsally. The retractor muscle of the funnel, which gives support to the gills, is inserted on the ventral part of the bend (Fig. 1); the area of attachment is clearly identifiable. The RP is short and nearly straight, while the PRP is longer and curved. There are differences in the curvature of the PRP between the left and right stylets of the same specimen. When placing both stylets with the ventral surface of the bend up, it was observed that the PRP of the right stylet is curved upwards, while the left is curved downwards. This was more evident in the largest specimens.

The stylets are semi-transparent fine structures, often with a central and opaque rod ('white core') longer than 50% of TLS. The rod was present in 61.5% of the right ($n = 291$) and 57.6% of the left stylets ($n = 288$). Of the 276 pairs of stylets observed, 46 (16.7%) did not have a white core, 32 (11.6%) presented it in at least one of the stylets, and 198 (71.74%) presented it in both stylets. The tip of the PRP generally showed a zigzag pattern on its external part and in the central white core. We did not find significant differences ($p > 0.05$) between the RL, PRL, MD and TLS of the right and left stylets of males. However, we found significant differences ($p < 0.05$) between the TLS, PRL and MD of the left and right stylets of females; no significant difference was found in RL. Comparisons between sexes showed a significant difference only in the right RL. The PRL/RL ratio of females and males (both stylets) was, respectively, 2.03 (SD = 0.25) and 2.08 (SD = 0.27). No significant difference was detected between the left and right stylets in the PRL/RL ratio, either in males or females.

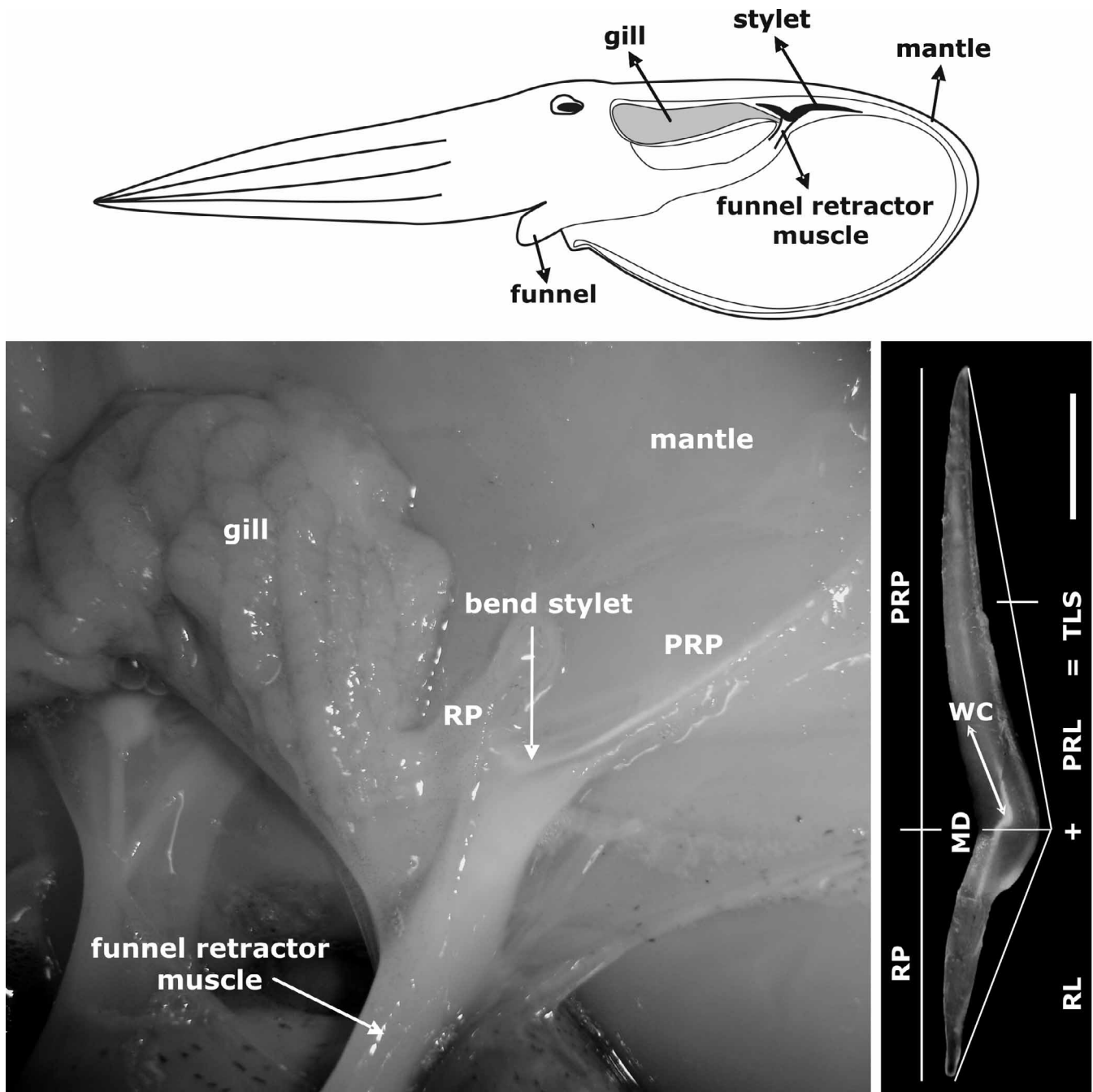


FIGURE 1. Internal morphology of *Enteroctopus megalocyathus* showing the location of the stylet (upper and left) and general view of the stylet (right) with the recorded measurements. Abbreviations: MD, maximum diameter at the bend level; PRP, post rostral part; PRL, post-rostral length; RP, rostral part; RL, rostral length; TLS, total length stylet (RL + PRL); WC, white core. Scale bar 1 cm.

Histologically, the stylet sac has a simple epithelium of narrow cells (depending on the state of secretion). The latter has basophilic cytoplasm, a rounded nucleus located in the basal third, and many nucleoli. Below this epithelium there is a dense conjunctive tissue layer irrigated by blood vessels, surrounded by longitudinal and circular smooth muscle fibers. Some deeply stained nuclei were observed randomly distributed between the concentric increments.

Two types of growth increments were observed in stylet cross-sections: wide rings with diffuse boundaries ('second-order increments') and thin rings constituted by a dark and a

light band ('first-order increments'). The latter were packed inside the second-order increments (Fig. 2). The increments located in the area of insertion of the retractor muscle of the funnel presented a zigzag (meander) pattern. No significant difference ($p > 0.05$) was found between males and females in the diameter of the nucleus of the stylet (males: mean = 0.028 mm, SD = 0.007, $n = 39$; females: mean = 0.038 mm, SD = 0.016, $n = 33$) or the diameter of the white core that surrounds the nucleus (males: mean = 0.174 mm, SD = 0.119, $n = 26$; females: mean = 0.167 mm, SD = 0.063, $n = 26$) (Fig. 2).

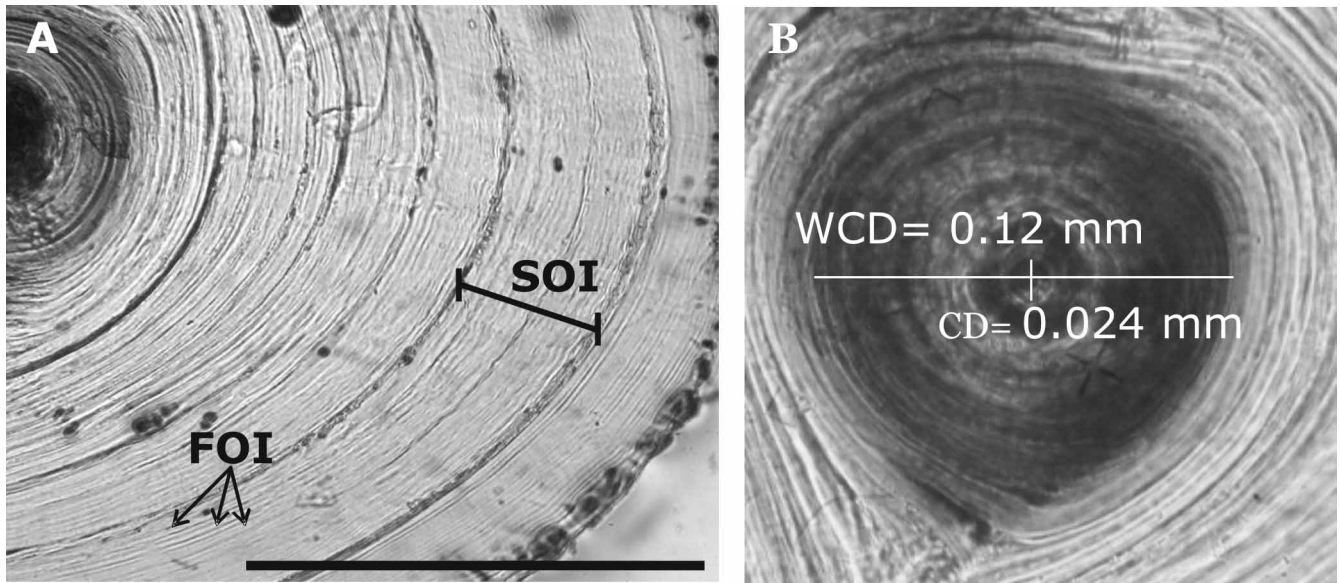


FIGURE 2. Cross-sections of the stylet of *Enteroctopus megalocyathus*. **A.** Cross-sections through the bend near the post-rostral part of the stylet. **B.** Detail of the stylet center. Abbreviations: FOI, first-order increments; SOI, second-order increment; CD, nucleus diameter; WCD, white core diameter. Scale bar: A = 0.5 mm.

The stylet has a high water and organic content (82.3 to 86%) and scarce inorganic material (ashes: 4 to 9.8%). There were different chemical elements in the cross-sections of the stylets analyzed with and without the white core in place. The scanning of the cut surface (white core included) revealed that carbon is located mainly in the periphery, while most of the phosphorus and calcium is concentrated in the center, their presence decreases closer to the cross-section edge. Oxygen showed the most homogeneous distribution. Sections without a white core showed that carbon, oxygen, phosphorus and calcium are uniformly distributed, although a remarkable decrease of the latter two elements was observed in comparison to the cuts with the white core included (Fig. 3). In all the samples of stylets analysed there were also smaller absorption peaks of magnesium, sodium and chlorine.

Discussion

The location of the stylets of *Enteroctopus megalocyathus* and the insertion of the funnel retractor muscle on the ventral part of the bend were consistent with those reported for *Enteroctopus dofleini* and *Eledone cirrhosa* (Isgrove 1909; Moriyasu and Benhalima 1993; Bizikov 2004). The position of the stylets in the mantle, with the PRP directed posteriorly, the bend ventrally enlarged, and the RP anteriorly, suggests the function of the stylets in the Octopodidae Orbigny, 1845: indirect support for the visceral mass and a point of attachment for the funnel retractor muscle. This last function would be very important because during the contraction of the muscle the stylet bends to take up the load from the contraction forces (Bizikov 2004). The left and right stylets of *E. megalocyathus* differed in the angle of the bend of the PRP. This finding is relevant, as it may have implications for morphometric studies.

The variability of the presence or absence of the white core in at least one of the stylets of an octopod is specified here for the first time. Bizikov (2004) found in *E. dofleini* that one or several layers (diverging from the bend along the axes of both shoulders) become whitish and opaque in some stylets, apparently because of past injuries. However, the author did not give the percentage of the total sample of stylets that presented this white core. In this study, 83.34% of the stylets of *E. megalocyathus* had a white core in at least one of the stylets. Since it is unlikely that more than 80% of the individuals of a population were 'injured', it is suggested that the presence of the white core is not a product of lesions. Moreover, it is suggested that the core adds strength to the stylet, necessary for its support of the visceral mass and the insertion of the funnel retractor muscle. The right and left stylets of males did not show significant morphometric differences; the differences were significant for the TLS, PRL and MD in the case of females. These findings suggest that either left or right stylets of males can be used in future studies of *E. megalocyathus* but in females left and right stylets must be studied separately. These results differ from those found in *E. dofleini* and *E. cirrhosa* (Bizikov 2004; Moriyasu and Benhalima 1993). Although these authors indicated ontogenetic individual variability for RL, PRL, TLS and MD, the differences observed were not statistically significant. Inter-sexual differences were not tested for these species. In *E. megalocyathus*, as well as in *E. dofleini*, PRL is twice RL. Bizikov (2004) pointed out that the ratio between PRL and RL does not change with age in *E. dofleini*, as was also the case in *E. megalocyathus*. In *E. cirrhosa* RL is approximately one third PRL (Moriyasu and Benhalima 1993). Therefore, differences in the ratio between these measurements could be of significance in taxonomic studies of other octopodid genera.

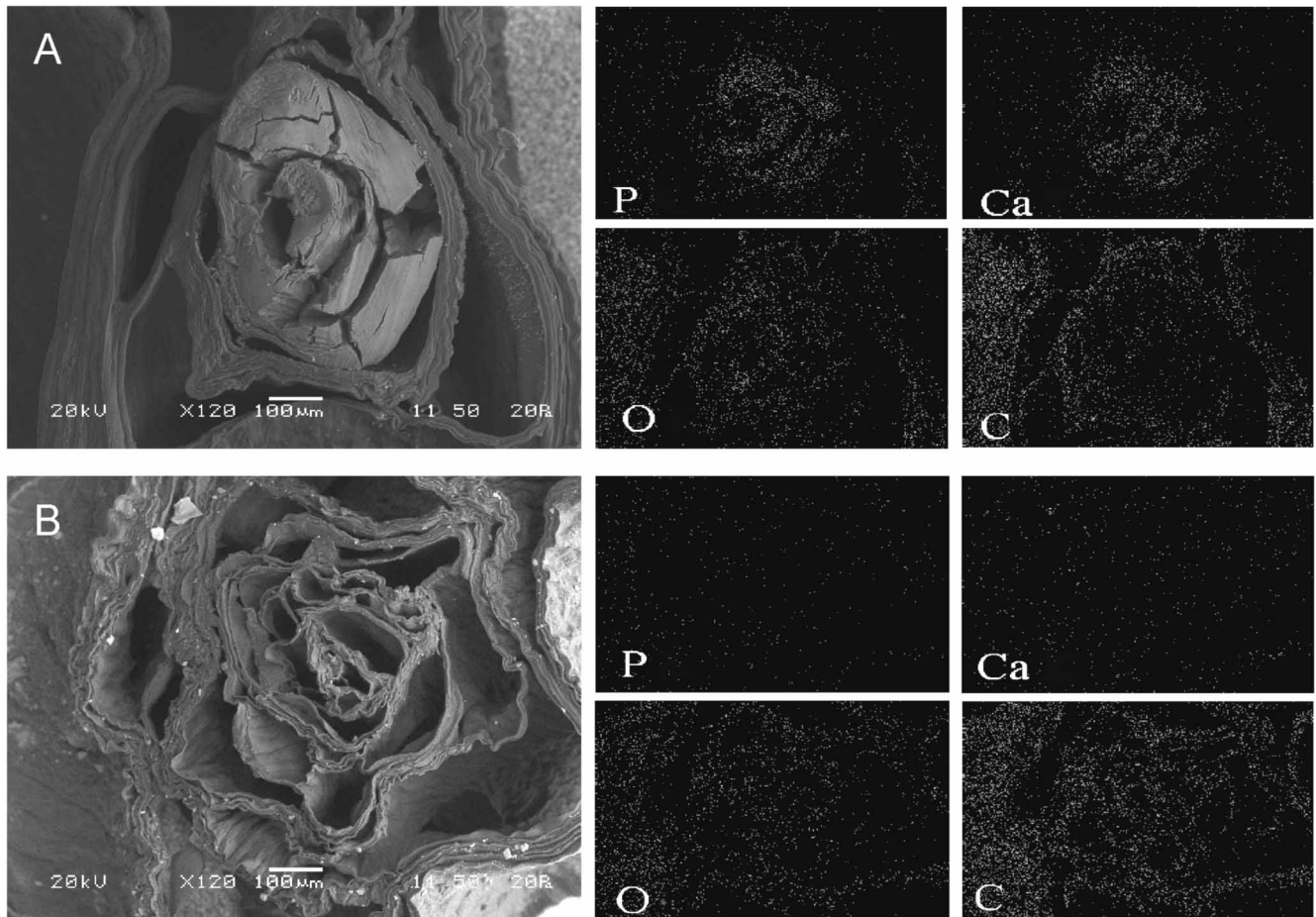


FIGURE 3. Transversal cross-section surface of the stylets observed under a scanning electron microscope showing the constituent chemical elements and their locations in a transverse cut. **A.** With white core. **B.** Without white core. Abbreviations: C, carbon; Ca, calcium; O, oxygen; P, phosphorus. White point, presence of the chemical elements. Scale bars: A and B = 100 µm.

Enteroctopus megalocyathus is similar to *Eledone* Leach, 1817 in the disposition of the histological tissues described for the stylet sac by Isgrove (1909). In both cases the stylets are enclosed in, and secreted by, the walls of an epithelial sac. The few rounded bodies found in cross-sections of *Eledone* stylets were distributed randomly between the concentric layers of chitinous material (rings) secreted by the epithelial sac. It has been postulated that they correspond to nuclei of deteriorated cells of the epithelial sac (Isgrove 1909). Basophilic structures with these characteristics were also observed in *E. megalocyathus*. The disposition of the secreting epithelial tissue corroborates the direction of deposition of growth increments, with the oldest, deposited during early ontogeny, located at the center of the stylet.

There were two types of concentric increments seen in cross-sections of *E. megalocyathus* stylets: wide rings that do not exhibit a regular pattern ('second-order increments') and thin rings ('first-order increments') with a more regular pattern, that are packed within the former. These rings were also observed in stylets of *E. dofleini*, *Octopus vulgaris* and *O. pallidus* (Sousa-Reis and Fernandes 2002; Bizikov 2004; Doubleday *et al.* 2006). The origin of the second-order increments is unknown; further study is needed as they could provide valuable information on growth. The deposition of

growth increments observed in the cross-sections of the stylets of *E. megalocyathus* showed, as in *E. dofleini*, a zigzag pattern in the proximity of the most external ventral layers of the bend, where the funnel retractor muscle is inserted. Bizikov (2004) pointed out that this zigzag pattern was due to the growth of the stylet on the 'early minute knobs' (Bizikov's term) that are located on the ventral (inner) side of the bend, where the funnel retractor muscles insert. These protuberances modified the circular pattern of deposition of increments on the ventral area. *E. megalocyathus* also presents minute knobs in this area, as well as having the zigzag pattern in the increments. As this area of the stylet supports the strongest traction during the contraction of the funnel retractor muscles the zigzag pattern could add flexibility to the bend.

Bizikov (2004) described the middle region of the cross-sections of *E. dofleini* stylets as the embryonic nucleus in and its center of growth. It is amorphous, opaque and about 0.1 mm in diameter. In the stylet of *E. megalocyathus* the embryonic nucleus has a diameter of 0.028 mm in males and 0.038 mm in females, is also amorphous, and does not present growth increments. Contrary to *E. dofleini*, this nucleus is translucent when the white core that surrounds it is absent. When it is present, it has an oval outline, is opaque, approximately 0.17 mm in diameter in both sexes, and has

growth increments near the periphery. These increments in the nucleus were not mentioned for *E. dofleini*, and its opaque condition suggests that the white core and the embryonic nucleus have been described as a single structure by Bizikov (2004).

Bizikov (2004) described the stylets of *E. dofleini* as a cartilage-like chitinous substance that is laid down in concentric layers. Wells (1978) hypothesized that the stylet of *O. vulgaris* is formed by an organic matrix impregnated with calcium carbonate, as in other mollusc shells and Napoleão *et al.* (2005) indicated that the chemical composition of the stylets of this species could be a calcium phosphate compound, such as Hydroxiapatite. This last hypothesis is supported by our results and the presence of calcium phosphate has been documented in other Octopoda Leach, 1818 such as *O. pallidus* by Doubleday *et al.* (2008). In *E. megalocyathus* and *O. vulgaris* (Napoleão *et al.* 2005), the phosphorus and calcium decreased from the center to the edge. However, when the stylet does not have a white core, the phosphorus and calcium were uniformly distributed in the section, and are less abundant compared with samples with a white core.

This is the first time a description of the presence and absence variability of the white core in at least one of the stylets of the same individual has been recorded. As well this study provides the first detailed information on the stylets of *E. megalocyathus*, including the distribution and description of the chemical elements, and thus provides baseline data for the use of stylets as a tool in future fishery research on this species.

Acknowledgments

We thank 'Lobo' Orenzens, Augusto Crespi, Nicolás Ortiz, Silvina Van der Molen and Flavio Quintana for comments on the manuscript, and German Pérez for help with fieldwork. We are very grateful to Aluminio Argentino S.A.I.C and the staff of the electron microscope unit, especially to Jaime Grosier. Institutional support was given by Centro Nacional Patagónico (CONICET). This study was supported by Agencia Nacional de Promoción Científica y Tecnológica, PICT 2002 No. 12737. The manuscript was greatly improved by the comments and constructive criticisms of two anonymous reviewers.

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