Quantitative elemental imaging of octopus stylets using PIXE and the nuclear microprobe

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Abstract

By utilising targeted microprobe technology, the analysis of elements incorporated within the hard bio-mineralised structures of marine organisms has provided unique insights into the population biology of many species. As hard structures grow, elements from surrounding waters are incorporated effectively providing a natural 'tag' that is often unique to the animal’s particular location or habitat. The spatial distribution of elements within octopus stylets was investigated, using the nuclear microprobe, to assess their potential for determining dispersal and population structure in octopus populations. Proton Induced X-ray Emission (PIXE) was conducted using the Dynamic Analysis method and GeoPIXE software package, which produced high resolution, quantitative elemental maps of whole stylet cross-sections. Ten elements were detected within the stylets which were heterogeneously distributed throughout the microstructure. Although Ca decreased towards the section edge, this trend was consistent between individuals and remained homogeneous in the inner region of the stylet, and thus appears a suitable internal standard for future microprobe analyses. Additional analyses used to investigate the general composition of the stylet structure suggested that they are amorphous and largely organic, however, there was some evidence of phosphatic mineralisation. In conclusion, this study indicates that stylets are suitable for targeted elemental analysis, although this is currently limited to the inner hatch region of the microstructure.

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1. Introduction

The quantitative and spatial distribution of trace elements within the hard bio-mineralised structures of marine organisms provide a ‘story’ of an individual’s life history and movement patterns, as they commonly reflect changes in behaviour, physiology and the surrounding environment. However, such chronological information can only be accurately measured if the structure contains regular temporally- or age-related growth increments. The targeted microprobe-based analysis of trace elements incorporated within such hard structures, which has included fish otoliths [1], squid and cuttlefish statoliths [2,3], gastropod shells [4], and bivalve shells [5], has provided population-level information on many marine species. However, due to their crumbly conglomerate structure and lack of growth increments [6] octopus statoliths, unlike other cephalopod statoliths or teleost otoliths, are of little use for ageing and therefore time-specific trace element studies. On a global scale, there are many unresolved questions regarding the population biology and dispersal patterns of octopus, and this is largely due to the lack of available methods to study octopus populations. The development of such methods will be crucial for increasing our understanding of octopus populations and enable the sustainable management of increasingly exploited commercial species.
Stylets (also known as vestigial shells) are a little known structure unique to Octopoda and are thought to represent a remnant shell [7]. Stylets consist of a pair of fine, cartilage-like rods embedded within the dorsal mantle musculature. Their composition has been suggested to be based on a calcium phosphate compound, such as hydroxyapatite [8], or chitin [7]. The stylet microstructure of *Octopus pallidus* has been found to have distinct concentric regions, a visible pre-hatch nucleus, and age-related growth in the form of daily growth increments [9]. Due to this microstructure, like squid statoliths [2,3], are likely to incorporate elements from the environment on a chronological basis, and therefore, may be a useful tool to address ecological questions on the dispersal patterns of both juvenile and adult octopus.

Laser ablation inductively coupled plasma-mass spectrometry (LA ICP-MS) is one of the most widely employed microprobe techniques for examining bio-mineralised structures on a temporal level, as it requires relatively little sample preparation and is capable of measuring elements to the trace level [10]. However, LA ICP-MS requires compositional information and standard materials to accurately measure trace elemental concentrations. For example, internal standardisation, a calibration method used for the analysis of many biological materials, requires the presence of one element (usually Ca) of known concentration which is homogenously distributed throughout the microstructural region of interest [11].

The nuclear microprobe (NMP) is a powerful microanalytical tool for investigating the distribution of minor and trace elements in biological materials [12]. Proton induced X-ray emission (PIXE) is a reliable and effective NMP-based technique, which quantitatively maps the spatial distributions of elements within a structure [13]. PIXE analysis is also standardless, and therefore does not require compositional information or the use of a standard [14]. Although the use of the NMP for analysing the bio-mineralised structures of marine species is not common, studies have included the analysis of squid statoliths [15], fish otoliths [16], and octopus stylets [8].

This is the first time octopus stylets have been analysed with the CSIRO-GEMOC NMP (CSIRO Exploration and Mining) using PIXE and the Dynamic Analysis method of analysis, which allows for simultaneous multi-element analysis and quantitative imaging at a high spatial resolution (down to 1.8 μm) and sensitivity (detection limits have been recorded at 0.2 ppm) [14]. Standard comparisons, using accepted reference and secondary standards, have shown that the standardless PIXE method has an accuracy level of 5–10% for major and trace elements [17]. The Dynamic Analysis method produces ‘true’ quantitative images of the whole section which are resolved of elemental overlaps, background-subtracted, free of artefacts and generated in real-time [18]. This capability will enable the spatial distribution of elements of whole stylet sections to be quantitatively mapped. In comparison, the study by Napoli et al. [8] examined the elemental distributions by targeting the proton beam at a selected number of single 2–3 μm points across the section of the stylet. Using the Dynamic Analysis method this study assesses the potential of stylets as environmental time-recorders and the suitability of Ca as an internal standard for LA analysis. Furthermore, to broaden our understanding of the stylet structure and stoichiometry, general compositional analyses (including X-ray diffraction and infrared spectrometry) will also be conducted.

2. Materials and methods

All stylets were obtained from *O. pallidus*, a fully benthic shallow-water species found throughout south-east Australia. This species is the target of a small commercial fishery in northwest Tasmania, Australia. Stylets were removed from fresh mantle muscle and air-dried for 48 h.

2.1. General composition analyses

General composition analyses were performed on stylets collected from mature adults sourced from the commercial fishery in October 2005. Powder X-ray diffraction was conducted to determine mineral composition (Mineral Resources Tasmania, Australia). The dried stylect samples were ground to approximately 10–75 μm and pressed into a 25 mm sample holder. The samples were run on an automated Philips X-ray System and analysed with Diffraction Technology software. To determine total inorganic content ‘loss-on-ignition’ (combustion of organic material) was also conducted (Mineral Resources Tasmania, Australia). Secondly, infrared spectroscopic analysis was conducted on the stylet with most of the outer sheath removed (see [9] for more details on the microstructure). The sheath was removed to help identify, more clearly, the potential discriminating peaks characteristic of the calcified portion of the stylet. A crushed sample was mixed with anhydrous KBr and pressed into a 7 mm diameter pellet. The analysis was performed at 4 cm⁻¹ resolution using a Fourier Transform Bruker IFS66 infra-red spectrometer (Central Science Laboratory, University of Tasmania).

2.2. Nuclear microprobe analysis

Five stylets were sourced from adult octopus collected from the commercial fishery in October 2005. The mature adults were of similar size ranging from 550 to 760 g. Two juvenile stylets were also sourced from five-month-old aquaria-reared octopus in January 2006. These juveniles were reared in a natural seawater flow-through system with a simulated natural temperature regime at the Tasmanian Aquaculture and Fisheries Institute, Hobart, Australia. All stylets were removed from fresh mantle muscle and air-dried for 48 h. To remove excess tissue from the juvenile stylets they were soaked in a mixture of 30% H₂O₂ buffered with NaOH for 24 h, and then rinsed thoroughly in ultra-pure water (Milli-Q) prior to drying.
Transverse stylet sections, 3–4 mm in length, were cut from the post-rostral zone of the stylet, proximal to the bend, using Teflon-coated razor blades (see [9]). The stylet sections were mounted vertically in a 25 mm disc of epoxy resin, which was oven-dried for two hours at 60 °C. The epoxy disc was ground using 1500 grit carborundum paper lubricated with ultra-pure water until all sections were exposed and then polished using 0.3 μm alumina powder on a suede polishing disc. Once polishing was complete the mounts were ultra-sonicated for five minutes in ultra-pure water and then rinsed further to remove surface contaminants before being allowed to air dry. To prevent charge build-up each disc was carbon-coated using a standard sputter deposition technique.

Individual specimens to be scanned were viewed under a light microscope and then the respective x/y coordinates were digitised and subsequently translated to microprobe stage coordinates to permit the precise location of the beam for analysis. During analysis, the beam was rastered electrostatically in the y-direction while the stage was typically stepped in 1.6 μm increments in the x-direction (scan speed differed on some samples and was varied to optimise beam current integration). X-ray data was captured using a Canberra germanium detector.

The analyses were performed with 3 MeV protons using the CSIRO-GEMOC NMP and utilising PIXE. Beam spot size was nominally 1–2 μm using beam currents of 3.5 nA. A 47 μm Al filter was used to reduce the intensity of the Ca K X-ray lines while allowing for the simultaneous measurements of Ca and trace elements. Two sections were scanned using a 200 μm Al filter, this improved detection of heavier elements but suppressed Ca more than anticipated and were therefore not included in the final analysis. Scan size was determined according to the size of the individual specimens. The maximum scanned region was 1 × 1.5 mm (412 × 591 pixels). True quantitative elemental maps were developed with a spatial resolution of 2.5 μm using a rapid matrix transform method called Dynamic Analysis (incorporated within the GeoPIXE II software) [19], which directly relates X-ray yields obtained from the PIXE spectra to elemental concentrations [12]. A multi-element red–green–blue (RGB) image was also produced (in this study green was replaced with black for easier visualisation on a white background). Multi-elemental images, although quantitative, are primarily used to examine spatial distributions due to the difficulties associated with producing a concentration scale (additional colours are produced where elements overlap). Images were generated and analysed using the GeoPIXE II software package.

To determine whether Ca concentration varied significantly between individuals elemental values were taken every 5–7 μm along a transect line, which ran from edge to edge across the centre of each stylet cross-section, resulting in 40–90 data points (depending on cross-section width) per individual. A one-way analysis of variance (ANOVA) was then conducted on three individuals. One juvenile was not included in the analysis due to a detector artefact (variation in X-ray absorption caused by the stylet section being misaligned within the epoxy), resulting in unreliable values in a portion of the image. To further examine the relationship between Ca and Br distributions, elemental values obtained from the transect line of one adult was plotted on a scatter graph.

3. Results and discussion

No minerals were identified in the styles by X-ray diffraction, and as such the styles were therefore classified as amorphous. Loss-on-ignition showed a total inorganic content of 35%, although a portion of this may be due to the halite (salt) found in the structure from the X-ray diffraction. The infrared spectra showed amide I and II bands characteristic of α-chitin, which forms complexes with proteins in invertebrates [20,21] (Fig. 1). The spectra also indicated the presence of calcium phosphate. Napoleão et al. [8] found that although Ca and P both decreased from the core region to the edge of the styles of O. vulgaris, the P/Ca ratio along all sections remained consistent between individuals, suggesting that the major mineral constituent is a calcium phosphate compound, such as hydroxyapatite. Although our data suggests the presence of some calcium phosphates, it also indicates that styles are largely organic in nature and that any mineralised component consists of poorly crystallised or uncryrstallised material.

A suite of differentially incorporated elements were detected within the stylet microstructure (Table 1). Calcium, Sr, Zn, Fe, Br, Mn and Cu were detected in all sections and Co, Rb, As, Ni, and Ba were detected in much lower levels and in less than half of the sections. Some elemental concentrations varied between adult and pre-hatch regions of the microstructure (e.g. Ca, Sr, Mn and Ni), and others varied more significantly between individuals (e.g. Zn, Cu, Co, and Fe). Such heterogeneous distributions suggest, as stylet exhibit temporally-related concentric growth,
that element concentrations vary on a temporal scale in relation to changes in the surrounding environment and an individual’s physiology. Styles from the juvenile octopus had significantly more Zn than the adult styles (Fig. 2). This may be due to the different water chemistry to which the juveniles were exposed and/or different physiological requirements due to faster growth rates and greater metabolic need. In cuttlefish the assimilation efficiency of Zn (important for enzymatic function) was 22% greater in juveniles than adults [22]. Such trace metal concentrations in 

<table>
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<th>Adult 2</th>
<th>Juvenile 1</th>
<th>Juvenile 2</th>
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<td>Rb</td>
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(a) area selected within core ‘pre-hatch’ region, (b) area selected within outer ‘adult’ region.

Table 1

Average concentration (ppm), statistical error (based on total number of X-ray counts detected at each pixel) and detection limits (99% confidence; DL) of elements identified within a randomly selected 50 μm circle area of four individuals analysed. This highlights the importance of careful section preparation if outer regions are to be targeted for trace element analysis. There was a close relationship between Ca and Sr in all sections, with both elements decreasing from the pre-hatch region to the edge of the section (Fig. 2). A multi-element image revealed negligible Ca yield in the stylet’s gelatinous outer sheath, which also appears to be a region of relatively high Br concentration (Fig. 3). Although the Br is probably the result of saltwater evaporation, the lack of Ca suggests that this region is largely organic. Zinc showed an inverse relationship to Ca and Sr, showing a higher concentration in the outer regions of the section (Fig. 2). The trends observed for Ca, Sr and Zn were also found in the adult styles of 

O. vulgaris [8]. Such patterns may give insight into life cycle and diet changes of the individual, and movement between different environments, on both a long- and short-term scale. Such similar trends between species with differing life histories (O. vulgaris have planktonic young, whereas, O. pallidus have benthic young) suggest they are the result of physiological processes. However, diet, water chemistry, and the physical environment also significantly influence relative concentrations of elements in cephalopods. Although the number of studies are limited, diet and temperature has been found to significantly influence Ba concentrations in cuttlefish statoliths [24,25], and temperature has shown to negatively correlate with Sr concentration in squid statoliths [26]. Variation of such parameters on a geographic level, therefore, is likely to provide a useful indicator of a population’s movement and dispersal history.

Although styles are not composed of CaCO3, like the majority of bio-mineralised structures used for laser ablation studies, Ca still appears a suitable element for an internal standard. Table 1 indicates that Ca concentration within the core region of the microstructure only varied by 3%, regardless of stage of maturity and collection location. Although Ca decreased towards the edge of section and disappeared altogether in the sheath, this trend was relatively stable and consistent between individuals. The transect data showed that Ca was not significantly different between individuals (ANOVA: F2, 186 = 0.92, p = 0.40). In contrast to the 40% Ca standard used for CaCO3-based material, a 13% Ca standard for analysis of the inner region of the stylet should be adopted. Greater calcification and a potentially higher inorganic content in the inner region of the stylet, identified by the trends of Ca and P concentrations in 

O. vulgaris [8] and Ca in O. pallidus...
Fig. 2. Elemental images of two stylet cross-sections showing distributions of Ca, Sr, and Zn, (a) = stylet removed from adult, (b) = stylet removed from juvenile. Dashed circles indicate approximate location of the pre-hatch region. Scale bars represent concentration (ppm).

Fig. 3. (a) Multi-element image of a stylet cross-section (Ca = blue, Br = red, Fe = black). Dashed line indicates approximate position of the outer sheath. (b) Concentration profiles of Ca (●) and Br (○) along a transect region outlined in Fig. 3 (a). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
(Fig. 2), suggests that it is the most suitable area for microprobe analysis. Furthermore, as Ca is not homogeneously distributed through the microstructure, a different Ca concentration may be required if the mid-region of the stylet is to be targeted. The lack of calcification in the outer sheath indicates that edge analysis is currently not suitable for stylets.

These results support the potential of stylets as a useful tool for studying the dispersal and population structure of octopus populations through targeted trace element analysis, which has been applied so successfully to many fish species. Firstly, there was a suite of detectable elements incorporated heterogeneously within the microstructure (which are most likely linked to the individuals’ environmental and physiological history), and secondly, Ca appears to be a suitable internal standard for laser ablation analysis. The combined information from all analyses and from past studies [8] suggests that stylets are probably chitinious, with some inorganic component associated with phosphatic mineralisation. However, further analyses are required to define and quantify major organic and inorganic constituents more accurately. Additionally, this study further highlights the unique potential and efficacy of PIXE and Dynamic Analysis as a tool for examining the hard structures of marine species, providing insight into the physical and chemical structure, physiological processes involved in formation, and the biology and environmental history of the individuals analysed.

Acknowledgements

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