Growth increments and biomineralization process in cephalopod statoliths

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Abstract

A study on morphological, structural and biochemical composition of Sepia officinalis and Loligo vulgaris statoliths and statocyst endolymph was undertaken with the aim of determining the major factors affecting the deposition process of statolith formation and to clarify the cause for the poor definition of the growth increments in S. officinalis statoliths. It is suggested that the different biochemical composition of the statocyst endolymph found in the two species accounts for distinct statolith crystallisation processes, which results in a different microstructure. This explains the better definition of growth increments in L. vulgaris statoliths comparing with those of S. officinalis. The protein content as well as Ca$^{2+}$ and Mg$^{2+}$ concentrations in the endolymph are more implicated in growth increments formation than Sr$^{2+}$ ion concentration. Moreover, the daily variations of the three factors mentioned, allowed us to formulate a working hypothesis to explain the daily deposition of growth increments: a dark ring (rich in organic matter) is deposited during daylight whereas a light ring (rich in CaCO$_3$) during darkness. These results are discussed in the light of alternative hypotheses explaining the deposition mechanisms in statoliths. © 2000 Elsevier Science B.V. All rights reserved.

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1. Introduction

Cephalopod statolith formation results from the deposition of a mineral structure in a living creature. This mineralization process in a biological environment is called biomineralization. Gautron (1994) defined biomineralization as a sequence of events,
which the minerals secreted by the cells crystallised and formed a structure ensemble following a defined plan of construction. The resulting structures are composed of a mineral matrix and an organic matrix, generally made up of a mixture of proteins, phosphoproteins, glycoproteins, proteoglucids and polysaccharides.

The presence of several substances in a mineral structure inhibits or favours the primary or the secondary nucleation and controls the crystalline structure formation. These regulation factors combined with the limits fixed by the space result in the elaboration of a methodical mineral structure of a determined configuration.

Although several studies have been carried out on age and growth of cephalopods using growth increments in the statoliths (Morris and Aldrich, 1985; Natsukari et al., 1988; Arkhipkin, 1993; Bettencourt et al., 1996; Jackson, 1998; Lipinski et al., 1998, among others) little is known about the processes involved in increment deposition in the statoliths. However, the clarification of this point is essential, since it could provide answers to several questions about the probable daily origin of the increments, allowing us to use the statoliths as an extremely useful tool for bioecological studies in the majority of cephalopod species.

Cephalopod statoliths are paired calcareous concretions essentially composed of calcium carbonate crystallised as aragonite with only a small percentage of organic material that has been ascertained to be protein (Radtke, 1983).

Presently, two alternative hypotheses to explain the deposition mechanisms in statoliths have been developed. Morris (1988, 1991a,b) hypothesised that the low concentration of Mg ions in the statocyst fluid allows the CaCO₃ precipitation in the form of aragonite, the process being controlled by the pH of the statocyst endolymph and the organic matrix. Lipinski (1993) proposed that strontium is directly or indirectly responsible for the definitions of growth layers and increments in the statoliths.

Considering both hypotheses, a study on morphological, structural and biochemical composition of the statoliths and statocyst endolymph of cuttlefish *Sepia officinalis* and the long-finned squid *Loligo vulgaris* was undertaken. Our goals in this paper are to determine major factors affecting the deposition process in the statolith and to determine the causes for the poor definition of the growth increments in *S. officinalis* statoliths compared to those of *L. vulgaris*.

2. Material and methods

2.1. Specimen collection and terminology used

*Sepia officinalis* (Linnaeus, 1758) and *Loligo vulgaris* (Lamarck, 1797) specimens were collected from commercial fisheries in the Galician Rias (NW Iberian Peninsula) in summer of 1996.

The terminology used in the present study is as follows: the four mains parts of the statolith, dorsal dome, lateral dome, rostrum and wing as defined by Clarke (1978). ‘Focus’ is defined as the starting point of crystallisation (Kristensen, 1980; Lipinski et al., 1991). ‘Nucleus’ is an area inside the first layer of proteinaceous template (Dawe et al., 1985; Lipinski et al., 1991). ‘Ring’ or ‘line’ is a general term which applies to all
linear structures visible inside the statolith (Lipinski, 1993), the ‘light rings’ are essentially composed of CaCO$_3$ and the ‘dark rings’ are composed of organic matter. Finally, an ‘increment’ or a ‘growth increment’ is an area of the statolith composed of a light ring and a dark ring (Dawe et al., 1985, Rodhouse et al., 1994).

2.2. Scanning electronic microscopy (SEM)

Ten statoliths of Sepia officinalis and ten of Loligo vulgaris were prepared for SEM (Philips SC-30). The statoliths were extracted from the cephalic cartilage (Lipinski, 1980) and stored in 96% ethanol. After removal, statoliths were cleaned in distilled water and then broken along the frontal plane (terminology after Clarke, 1978) using a scalpel. The statoliths were then etched in HCl (0.27 M) for 2–3 min and again cleaned in distilled water. Time differences in etching were random. The statoliths were then mounted on an aluminium stub and vacuum-coated with gold before viewing in the SEM. Magnifications did not usually exceed 1000×.

2.3. Energy dispersive X-ray analyses

Twenty statoliths of S. officinalis and ten statoliths of L. vulgaris covering the widest length range possible were used. Statoliths were prepared as for SEM. Energy dispersive X-ray analyses were performed on an EDAX analyser attached to the SEM. The analyses were taken in duplicate from two distinct points of each statolith. One point was located in the lateral dome at 50 μm from the exterior margin and the other one chosen as a central point in the concave side of the wing (Fig. 1). A Student’s $t$-test was used to compare data between wing and lateral dome of both species.

2.4. Atomic absorption spectrophotometry

Calcium, magnesium and strontium concentrations in the endolymph of both species were determined using an atomic absorption spectrophotometer (Varian 250 Plus). Six samples of each species were taken. Three samples of 40 individuals each were collected at 07:00–10:00 h (morning specimens) on distinct days, and another three samples of 40 individuals each were taken at 19:00–21:00 h (evening specimens). After capture, individuals were immediately processed and 10–20 μl of endolymph were extracted from the statocyst with the help of a microsyringe. The statoliths were removed and then frozen at $-20\,^{\circ}\mathrm{C}$ until required for protein analysis.

Organic matter was removed from the endolymph with nitric acid. Lanthanum chloride was then added to avoid ionisation interference. The solutions thus obtained were taken to the spectrophotometer to determined calcium and magnesium concentrations. Strontium was measured in a nitric/acetylene oxide flame under atomic emission spectrophotometry. ANOVAs were used to check differences between species and for each species between morning and evening specimens.
2.5. Organic matter determination

Statoliths were ashed in order to determine the percentage of organic matter present. Water cleaned statoliths were dried at 60°C for 24 h and then weighed individually. Dried statoliths were placed in a muffle furnace at a temperature of 500°C for 3 h and again weighed. The organic content was expressed as the percentage of weight lost due to ashing compared to the original dry weight. The percentage of organic matter and size of the individuals relationship was determined and the differences between species were tested using an ANCOVA.

2.6. Protein content

Protein content analyses were carried out on the endolymph and on the statoliths of both species. The total protein content in the endolymph was determined by the Bradford and Smith method (Smith et al., 1985). The protein content in the statolith was
determined together, in the morning and in the evening specimens, since the protein in the statolith accumulated throughout growth. The total protein content was determined by quantification of the total nitrogen using the Havilah et al. (1977) method. The soluble protein content was determined by the Bradford and Smith (Smith et al., 1985) method. However, first it was necessary to solublise and purify the sample, etching the statoliths in 1 M EGTA for 32 h. The solution was dialysed (MWCO: 3,500d) against ultra-pure water for 24 h and finally lyophilised (Christ Beta 2-16) at −80°C for 18 h. ANOVAs test were used to check differences between species and between morning and evening specimens it was used an ANOVAs

2.7. Biomineralization in vitro

A comparative study of the crystallisation rate of dissolved calcium in the endolymph was carried out in both species. The crystallisation rate was determined by measuring pH (Sentron 1001pH) variation in time (Wheeler et al. (1981) in Wright, 1991; Garcia-Ruiz, pers. commun.). The in vitro crystallisation method consists of diffusing ammonia, coming from a deposit-solution of ammonium bicarbonate, through a solution of calcium chloride and endolymph, in a closed system. The gradual dissolution of NH₃ raised the pH of CaCl₂ solution until it reached basic values, allowing the conversion of CO₂ to carbonate. All the assays were completed in triplicate.

3. Results

3.1. Scanning electronic microscopy (SEM)

Specific morphological differences between the statoliths of the two species were observed. Thus, *S. officinalis* statoliths have the four main parts: lateral dome, dorsal dome, rostrum and wing, very well defined and separated by pronounced angles. However, these parts are not so well defined in *L. vulgaris* statoliths and they are not separated by pronounced angles, but by smooth outlines (Fig. 1). The crystalline microstructure of the external surfaces of *S. officinalis* statoliths is very heterogeneous with crystals showing prominent angles and being deposited irregularly. In contrast, the crystalline microstructure of the external surfaces of *L. vulgaris* statoliths is quite homogeneous with crystals regularly deposited and forming slight curvatures (Fig. 2). These crystal curvatures provide a relatively broad surface, continual and uniform, that allows organic matter deposition, developing a more conspicuous and homogeneous dark ring than in crystal growth of *S. officinalis* statoliths where their pronounced angles prevent the formation of any broad and uniform surface. This seems to be the explanation for the better visualisation of increments in *L. vulgaris* statoliths than in those of *S. officinalis* (Fig. 3). The focus is clearly visible in the statoliths of *S. officinalis*. Nevertheless, growth increments are very difficult to visualise, being obvious only in the lateral dome (Fig. 4a). In this species, crystals are deposited concentrically with their large axes oriented parallel to the statolith growth direction (Fig. 4b). The crystals in the rostrum of *S. officinalis* are jumbled; therefore a standard defined
deposition does not exist (Fig. 4c). Consequently, it is practically impossible to visualise growth increments in this part of the statolith.

3.2. Energy dispersive X-ray analyses

Fig. 5 shows the relationship between Sr/Ca ratios in the wing and in the lateral dome of *S. officinalis* and *L. vulgaris*. There was significantly more strontium in the wing than in lateral dome of statoliths of both species (*t* = 3.55, *P* < 0.05 in *S. officinalis* and *t* = 2.64, *P* < 0.05 in *L. vulgaris*). Although, there was more calcium in the lateral dome than in the wing of both species, these differences were non-significant (*P* > 0.05). There were no significant differences in absolute concentration of Sr$^{2+}$ and Mg$^{2+}$ ion between the two species, but the Sr/Ca ratio was significantly higher in the wing than in the lateral dome of both species (*t* = 3.66, *P* < 0.05 in *S. officinalis* and *t* = 2.71, *P* < 0.05 in *L. vulgaris*). The Sr/Ca ratio was also higher in the wing (*t* = −2.09, *P* < 0.05) and in the lateral dome (*t* = −1.81, *P* < 0.05) of *L. vulgaris* than in *S. officinalis*. This ratio did not depend on the length of the individuals, in either species.

3.3. Atomic absorption spectrophotometry

Calcium, magnesium and strontium concentrations in the endolymph of *S. officinalis* and *L. vulgaris* are shown in Table 1. There were significantly higher values of calcium in the morning specimens than in the evening ones in both species, and the opposite trend was seen for magnesium ions. Comparing these ion concentrations between species and considering specimens captured at the same period of the day it can be seen that both, calcium and magnesium showed significantly higher concentrations in *S. officinalis* than in *L. vulgaris*. Furthermore, strontium was more concentrated in the morning specimens of *S. officinalis*, but more concentrated in the evening individuals of *L. vulgaris*.
Fig. 3. SEM picture showing the growth increments in the rostrum of a *Loligo vulgaris* statolith.
Fig. 4. SEM pictures of *Sepia officinalis* statoliths: (a) lateral dome where the focus and some increments can be observed, (b) lateral dome showing very sharp aragonite crystals concentrically deposited, and (c) rostrum where the irregular pattern of CaCO₃ deposition can be seen.
3.4. Organic matter determination

The percentage of organic matter in the statoliths of both species decreased as a power of the size of the individual (Fig. 6). However, the percentage of organic matter was significantly higher in the statoliths of *L. vulgaris* than in *S. officinalis* ones ($F = 224.7$, $P < 0.0001$).

Table 1

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Sampling period (h)</th>
<th>Sepia officinalis</th>
<th>Loligo vulgaris</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>07:00–10:00</td>
<td>19:00–21:00</td>
<td>07:00–10:00</td>
</tr>
<tr>
<td>Endolymph (µg ml⁻¹)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>502.6±36.1*</td>
<td>835.8±24.7*</td>
<td>372.6±9.9*</td>
</tr>
<tr>
<td>Mg²⁺</td>
<td>89.0±2.6*</td>
<td>71.9±2.4*</td>
<td>42.8±3.1*</td>
</tr>
<tr>
<td>Sr²⁺</td>
<td>4.3±0.64*</td>
<td>1.5±0.14*</td>
<td>1.6±0.19*</td>
</tr>
<tr>
<td>Protein</td>
<td>1820.0±468.8*</td>
<td>661.7±204.6*</td>
<td>5566.0±552.2*</td>
</tr>
<tr>
<td>Statolith (µg mg⁻¹)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total protein</td>
<td>43.7±14.5⁺⁺</td>
<td></td>
<td>53.3±10.4⁺⁺</td>
</tr>
<tr>
<td>Protein soluble</td>
<td>15.2±0.3*</td>
<td></td>
<td>18.6±0.5*</td>
</tr>
</tbody>
</table>

* Significantly different at $P < 0.0001$; n.s., not significant.

Fig. 5. Relationship between Sr/Ca ratio, in the wing and lateral dome of *Sepia officinalis* and *Loligo vulgaris* statoliths and the size ML (mm) of the individuals.
3.5. Protein content

The total protein content in *L. vulgaris* endolymph was significantly higher than in *S. officinalis*, independent of the hour of the day (Table 1). Protein content was significantly higher in the morning than in the evening, in both species. Although the total protein content was not significantly higher in the statoliths of *L. vulgaris* than in those of *S. officinalis*, the soluble protein in *L. vulgaris* statoliths was significantly higher than that of *S. officinalis*.

3.6. Biomineralization in vitro

The rate of crystallisation was measured by pH variation in time (Fig. 7). The increase of pH was very fast in the blank assay, reaching a maximum of 9.48 after 10 min. Subsequently, pH decreased slow. This revealed a very short induction time for nucleation, followed by a rapid crystallisation rate. The pH variation followed a different pattern in the *S. officinalis* endolymph assay. First, the pH decreased a little and then started to increase gradually, reaching maximum of 7.89 after 120 min. The induction time was longer and the crystallisation rate slower than in blank assay. The overall pH variation in *L. vulgaris* endolymph assay was similar to *S. officinalis*. However, the increase of pH was slower and not so constant. Induction of nucleation required 150 min and after that, the crystallisation process occurred very slowly as indicated by the very low rate of reduction of pH.

4. Discussion

The crystalline structure of *S. officinalis* statoliths has a very irregular form. This irregular form coupled with the lower percentage of organic matter in *S. officinalis* statoliths compared with *L. vulgaris* could explain the poor visualisation of growth increments in *S. officinalis* statoliths. Furthermore, the statoliths of both species become
Fig. 7. Rate of crystallisation (mean and standard deviation) measured by the pH variation in time in a closed system. (a) Blank assay, (b) Sepia officinalis endolymph assay, and (c) Loligo vulgaris endolymph assay. The decrease in pH trend was significant ($P < 0.0001$) in all assays.

more calcified as individuals grow since the proportion of organic matter declines with age. Consequently, ageing results in a lost of increments definition.

The results of this study showed a higher level of strontium in the wing than in the lateral dome of the statoliths of both species, but increments did not have better definition in the anterior or in the adjacent area of the wing than in the lateral dome. This is contrary to the results of Durholtz et al. (1997), who found better visualisation of
increments in areas of the wing corresponding with zones of high strontium concentration, supporting Lipinski’s hypothesis. On the other hand, results of the present study also showed that growth increments are more visible in the lateral dome of S. officinalis statoliths and in the rostrum of L. vulgaris statoliths, than in the adjacent areas of the wing. These findings contradict Lipinski’s hypothesis. This hypothesis alone would predict that the better defined growth increments in L. vulgaris statoliths should have more strontium than the S. officinalis statoliths. However, strontium differences are non-significant either in the wing or in the lateral dome.

The identification of the origin of strontium in the statoliths of cephalopods is controversial. As in fish otoliths, strontium in statoliths could be the result of a combination of exogenous factors (temperature, salinity, strontium dissolved in seawater and feeding) and of endogenous factors, such as physiological responses associated with growth, stress and reproductive cycles (Gallahar and Kingsford, 1996). Since all the individuals used in our analysis were collected at the same time and place, it seems less likely that environmental factors could be the potential source of variation of strontium. Our results showed that the Sr/Ca ratio is not related to growth in either species. Therefore the hypothesis that strontium is responsible for growth increments definition is, by it self, inadequate to explain the differences.

Most of the authors that have studied the biomineralization process (Crenshaw, 1982; Weiner and Traub, 1984; Williams, 1984 and Simkiss and Wilbur, 1989; among others) indicated that the organic matrix could form the substrate to, firstly, promote and secondly, regulate the direction of growth of the crystals and also could act as inhibitor of crystallisation. It has been demonstrated (Addadi and Weiner, 1985) that inhibition of crystal growth occurs when proteins interact with some axes of the crystal, converting its form. Moreover, Wright (1991) showed the existence of a glycoprotein in the soluble matrix of the otoliths that could inhibit the crystal nucleation, even after crystallisation has started. Our findings are in agreement with these results. Thus, addition of endolymph to the crystallisation system increased the required time to begin the crystallisation process and decreased the precipitation rate. The delay in the increase of pH and subsequently slow decrease in the assay for L. vulgaris endolymph as compared with the assay using S. officinalis endolymph suggested a longer induction time and a subsequent reduction in the crystallisation rate. This delay could be explained because the total protein content is higher in L. vulgaris endolymph than in S. officinalis, either in the morning or in the evening specimens. The fact that the L. vulgaris curve is not unimodal could suggest the presence of multiple proteins or activities sites. Furthermore, growth increments could be more defined because the aragonite crystal edges are smoothened in presence of more proteins, allowing for a uniform and continuous deposition of organic matter.

The relationship between calcium and protein concentrations in the endolymph inverted between the morning and the evening individuals of both species. The evening specimens showed more calcium and less protein than the morning ones. This seems to support the existence of a rapid precipitation of the aragonite crystals during the night, which agrees with the increase of feeding activities of S. officinalis species during the darkness in Ria de Vigo (Castro and Guerra, 1989). Sauer et al. (1997) also showed that L. vulgaris reynaudii off South Africa leave mating and spawning areas at night,
presumably to feed. Calcium concentration in the endolymph decreased towards dawn while protein contents increased. These proteins probably inhibit the precipitation of CaCO₃ crystals and modify the form of the crystals allowing a uniform deposition of the organic matter during daylight. This modification accounted for the formation of growth increments constituted by alternate light rings (rich in CaCO₃) and dark rings (rich in organic matter). These results agree with the findings of Mugiya et al. (1981) who indicated that the concentration of total calcium in the endolymph of gold fish, Carassius auratus, showed a clear daily variation and calcium precipitation decreased or stopped at sunrise and resumed in 3 h.

The role of Mg²⁺ and Sr²⁺ ions in the deposition process of the statolith is also controversial. Magnesium is considered by Morris (1988, 1991a) to be the key factor in calcification, whereas statolith biomineralization is controlled by strontium concentration in the endolymph, according to Lipinski (1993). With the results of the present study we can not reject any of the hypotheses, since the concentrations of both ions in the endolymph were within the range of values that affect calcification in a crystallisation system. However, Sr²⁺ showed a different pattern of concentration in the endolymph of the two species. Although Sr²⁺ ions are vital for the development of cephalopods’ statoliths (Hanlon et al., 1989), Mg²⁺ ions seem to be more closely related with growth increment definition, since their concentration changes inversely in relation to calcium concentration, in the endolymph of both species. In this way, the Mg²⁺ ions were more concentrated, and associated with low Ca²⁺ concentration during daylight allowing organic matter deposition while delaying calcification. The inverse process would occur at sunset when Ca²⁺ ion concentration was high, protein content was low and Mg²⁺ ion concentration was low. Conjunction of these three factors gives the possibility of a rapid CaCO₃ precipitation in aragonite form.

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