OVERVIEW OF THE SHELL DEVELOPMENT OF THE COMMON CUTTLEFISH SEPIA OFFICINALIS DURING EARLY-LIFE STAGES

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ABSTRACT. – The cuttlefish’s inner shell, better known under the name “cuttlebone”, is a complex structure that has been studied by scientists since nearly two centuries. Indeed, this shell which is involved in tissue support and buoyancy regulation unites useful properties such as high mechanical resistance, porosity and permeability. Moreover, this ostensibly straight shell is unique in extant and known extinct cephalopods and thus of great interest to better understand the evolution of this mollusk group. However, despite various studies dedicated to the evolutionary history, the morphology, the microstructure, the composition and the functioning of this shell, a lot of open questions persist. Based on detailed pictures originating from adult and juvenile stages, this review aims to critically scrutinize data about the known structures of the Sepia officinalis shell and to describe its setting up during early-life stages in order to improve our understanding of the establishment of this elaborate structure.

INTRODUCTION

Among the extant cephalopod mollusks, chambered shells (also named phragmocone) are confined to the members of three families: the Nautilidae, which includes few species with an external coiled shell; the Spirulidae, which contains 1 species with an inner coiled shell; and the Sepiidae (i.e. the cuttlefish), which counts more than 100 species that form an inner straight shell (e.g. Denton, 1974). This latter shell (also called “cuttlebone”) has been thoroughly studied since nearly 200 years, highlighting a very intricate structure involved both in tissue support and buoyancy regulation (e.g. Voltz 1830, Appellöf 1893). Moreover, it possesses an exceptional combination of technologically desirable mechanical properties of high compressive strength (≥ 20 atm), high porosity (93 %) and high permeability sought by industry (Denton & Gilpin-Brown 1961a, 1973, Birchall & Thomas 1983, Ward & Boletzky 1984). However, despite numerous studies interested in the evolutionary history (Bandel & Boletzky 1979, Hewitt & Stait 1988, Lu 1998, Bonnaud et al. 2006), the morphology (Ward & Boletzky 1984, Ward 1991, Sherrard 2000), the microstructure (Bandel & Boletzky 1979, Dauphin 1981, Birchall & Thomas 1983, Checa et al. 2015), the composition (Okafor 1965, Dauphin & Marin 1995, Florek et al. 2009) and the functioning of this shell (Denton et al. 1961, Denton & Gilpin-Brown 1959, 1961a, b, c, 1964, Birchall & Thomas 1983), a lot of questions persist particularly considering the set up of this complex structure.

This review aims to homogenize the terminology related to cuttlefish shell with a particular emphasis on its establishment during early-life stages.

MORPHOLOGY AND STRUCTURE OF THE S. OFFICINALIS SHELL

Since the detailed description of the Sepia shell by Appellöf in 1893, various terms have been used to name the different parts of the cuttlefish shell. The terminology used in this article is largely adopted from the study of Bandel & Boletzky (1979).

The cuttlebone consists of two distinct regions: (i) the upper side called the dorsal shield (respectively named shield, horny layers and hypostracum by Dauphin (1981), Dauphin & Marin (1995) and Tiseau et al. (2005)), and (ii) the ventral chambered part (respectively called ventral process, lamellar matrix, ventral part and siphuncular zone by Dauphin (1981), Birchall & Thomas (1983), Dauphin & Marin (1995) and Tiseau et al. (2005), Fig. 1). The inorganic fraction of these two parts is made almost exclusively of calcium carbonate under its orthorhombic aragonite form (Bandel & Boletzky 1979, Birchall & Thomas 1983, Florek et al. 2009).

The dorsal shield is a dense and hard upper layer playing an important mechanical role, which consists of three
layers. The uppermost one covers only the intra-marginal (i.e. more central) part of the dorsal shield and is formed of calcareous tubercles. The central layer emerges at the rim of the dorsal shield and is characterized by organic and mineralized lamellae. The most ventral part of the dorsal shield, the inner layer, consists of two portions, an upper one with coarsely columnar prismatic crystals, and a lower one with spherulitic prismatic crystals. Finally, a poorly developed spine is situated on the mid-dorsal line on the convex dorsal shield, close to its posterior end (Fig. 1B, Appellöf 1893, Bandel & Boletzky 1979).

The chambered part is formed by the superposition of hollow chambers separated by septa, synthesized sequentially throughout the animal’s life from the posterior to the anterior part of the shell (Fig. 1C). The hitherto described chamber height varies between 100 and 800 µm, highlighting the wide plasticity of the shell structure even when studying animals from similar origin (i.e. Mediterranean Sea; Bandel & Boletzky 1979, Sherrard 2000, Florek et al. 2009). These chambers are filled with variable volumes of gas (mainly nitrogen) thus allowing the animal to adjust its buoyancy (Denton & Gilpin-Brown 1961b, 1964). Each chamber appears open posteriorly resulting in a striated area on the posterior ventral part of the shell, called siphuncular zone (named siphonal tube by Dauphin (1984) by homology with structure described in Spirulidae, Nautilidae and extinct shelled cephalopods).

The non-striated area corresponds to the ventral wall of the last formed chamber. The posterior portion of the chamber zone is embraced by a structure called the fork. The fork is broad posteriorly, narrows anteriorly on each side and ends near the apparent opening of the last formed chamber. It has been described as a complex of several separate layers made of finely laminated structure. In ventral view, it is possible to see that the dorsal shield forms a rim surrounding the fork and the chambered part, which is mainly calcified although associated with an uncalcified outer part (Fig. 1A, Bandel & Boletzky 1979).

Within the chambers, vertical pillars with round or straight sections form the supporting elements of the septa (Fig. 2A). These pillars are differently organized depending on the area observed in the chamber. They can be linked together forming a complex labyrinthine network as in the main part of the chamber (Fig. 3) or arranged in independent columns closely set in the posterior part of the chambers at the level of the siphuncular zone.
Each septum is made of a chamber roof and a chamber floor respectively on its lower and upper sides, which are microstructurally distinct (Fig. 2B). Indeed, whereas the chamber roof consists of a prismatic layer composed of rectangular, rod-like prisms, similar as in the pillar (and in the lower part of the dorsal shield’s inner layer) components, the chamber floor shows a lamellar structure (Bandel & Boletzky 1979). In addition, organic membranes are visible in the chamber as thin sheets coating the lamellar and pillar surfaces, as freely suspended sheets running parallel to the septa (and continuous into the pillars) and as vertical membranes joining the pillar network (Gutowska et al. 2010, Checa et al. 2015). Recently, it has been hypothesized that these membranes result from the merging of original nanomembranes involved in the setting up of the different chamber structures (i.e. pillar and septum; Checa et al. 2015), resulting in around 4-10 organic membranes parallel to the septa in each chamber (Bandel & Boletzky 1979, Checa et al. 2015). It is known that organic matter found in the mollusk shell is involved in the mineralization processes leading to the shell construction, as a template for the nucleation of the inorganic solid matter as well as to inhibit nucleation (Birchall & Thomas 1983). In the S. officinalis shell, the total amount of organic matter determined by thermogravimetric analyses is 9.8 % (Florek et al. 2009), which is relatively high compared to the amount found in the shells of other mollusks (less than 5 %; Marin et al. 2008). The major part of the organic matter is included in the dorsal shield whereas the chambered part contains between 3 and 4.5 % (Jeuniaux 1963, Birchall & Thomas 1983). The main components of S. officinalis shell organic matter are the polysaccharide β-chitin and proteins never described so far (Birchall & Thomas 1983, Dauphin & Marin 1995, Florek et al. 2009, Jia et al. 2009).

This complex inner structure is enclosed in a circular tissue called “shell sac”. This thin tissue sheet, consisting of one cell layer, fulfills the functions of mineralization and gas-liquid exchanges necessary to the buoyancy regulation (Denton & Gilpin-Brown 1961, Kawaguti & Oda...
formation and organization of this tissue is described in some detail below.

**THE SHELL SET UP AND DEVELOPMENT OF S. OFFICINALIS**

The staging system used in this review refers to the one proposed by Lemaire (1970).

The shell sac establishment

Although the future shell sac epithelium can be made out from stage 13, surrounded by an annular thickening that is the rudiment of the muscular mantle, the shell sac proper begins its formation at stage 18 as follows. First an annular ridge forms at the inner periphery of the mantle epithelium. This ridge then becomes a fold that grows centripetally over the mantle epithelium making a mantle invagination. The central opening of this mantle fold becomes slightly cornered, with one anterior (dorsal) and 2 lateral angles, before the shell sac is closed at stage 20-21 (Naef 1928; Lemaire 1970, Spiess 1972, Bandel & Boletzky 1979). The “scars” of the anterior and lateral angles will later differentiate into the organ of Hoyle, which acts as a hatching gland. Around stage 22, the posterior edge bends and grows outwards into a brim. This differentiation of the shell sac occurs synchronously with the ventral part of the mantle that is inserted on the lower face of this brim (Bandel & Boletzky 1979). During the whole closure process, the expansion of the invaginated epithelium remains low. Thereafter (i.e. from stage 22 to 24), the shell sac grows considerably and the primary epithelium secreting shell (i.e. ventral part of the shell sac) becomes thinner. If a large part of it has appeared 2- to 3-layered at stage 22, it has been clearly described as a monolayered epithelium by stage 24. It is conceivable that this rapid stretching is partly prepared by the formation of a groove that would represent a “surface reservoir”, and thus would rapidly enable the secretory activity set-up of this epithelium (Spiess 1972, Bandel & Boletzky 1979).

The different studies of the shell sac epithelium described up to 5 different cell-types, highlighting the important complexity of this tissue (Appellöf 1893, Denton & Gilpin-Brown 1961a, Spiess 1972, Bandel & Boletzky 1979). On the shell sac ventral part (i.e. the primary epithelium), 3 cell-types have been described: i) high, columnar cells (30 µm high, 2-3 µm wide) forming the calcareous material of the chamber zone (i.e. anterior ventral side), ornamented with a broad brush border and containing large vacuoles (Kawaguti & Oda 1963, Bandel & Boletzky 1979); ii) very low cells that secrete the mineralized (ventral) marginal zone of the dorsal shield (Bandel & Boletzky 1979); and iii) deeply folded siphuncular epithelium. This epithelium presents numerous ampullae on the side of the siphuncular zone, which are connected by very fine ducts to spaces in the basement connective tissue richly vascularized, allowing liquid exchanges (Denton & Gilpin-Brown 1961b, Bandel & Boletzky 1979). The secondary epithelium (i.e. dorsal part of the shell sac) that synthesizes the dorsal shield has been less thoroughly studied. The few descriptions published until now mention very flat cells becoming gradually thicker before they turn into the marginal part of the primary epithelium (Spiess 1972, Bandel & Boletzky 1979). The histological description of the shell brim cells, involved in the shell extension and synthesis of the organic brim, has never been thoroughly investigated.
The embryonic shell formation

The initial shell (or protoconch) synthesis is initiated as soon as the shell sac is closed (i.e. at stage 20-21). It is a simple organic membrane of about 0.9 mm long and 0.7 mm wide, without any growth lines and roughly spoon-shaped, with its deepest part near the embryo’s posterior end. During the following stages, the shell extension is ensured by the addition of organic material to the margins making its outline ovoid. Along with the deposition of more organic material, the posterior slope of the embryonic shell becomes steeper, until an angle of about 90° with the plane of the anterior dorsal shield is attained. In association with the shell sac’s fast growth, increments of organic material are rapidly deposited onto the protoconch rims (between 0.03 and 0.08 µm each) forming growth lines. These increments are broadest in the anterior part of the shell (Bandel & Boletzky 1979).

The calcification of the embryonic shell begins between stages 24 and 25, when the shell has a length of about 2-3 mm, afterwards one chamber by embryonic stage will be formed until stage 30 (Table I). The mineral composition of the cuttlefish shell formed during early-life stages seems similar to that of adult mainly compounds by aragonitic calcium carbonate (Bandel & Boletzky 1979, Florek et al. 2009, Gilles Luquet, pers com).

In its first phase, calcification is restricted to the ventral side of the future dorsal shield, where a continuous layer of minute aragonite crystallites that form needle lamellae is deposited. This first aragonitic layer covers entirely the inner ventral side of the shell except for a narrow marginal rim. This inorganic part is the basement for first pillars and subsequent first chamber, which is formed at stage 25 (Table I, Bandel & Boletzky 1979). Thus, pillar growth occurs dorso-ventrally (and not from the bottom to the top as proposed by Checa et al. 2015), by 15-30 µm layers delimited by the previously described organic horizontal nanomembranes (see part 2) in a liquid crystal matrix (Bandel & Boletzky 1979, Checa et al. 2015). Consistently, mineral apposition has been found only at the pillar uppermost portion, whereas further towards the base they do not grow in thickness (Bandel & Boletzky 1979), and pillar growth steps can be easily microscopically distinguished (Fig. 4, Birchall & Thomas 1983). These pillars are homogeneously distributed on the chamber ceiling, only the foremost ones forming walls that are radially arranged. The observation of pillar position in subsequent chambers highlighted their alignment (see Fig. 2, Bandel & Boletzky 1979, Checa et al. 2015). In the first formed chambers, pillars change in form during their growth. Indeed, towards their apices (in the direction of growth i.e. towards the ventral end), their initial columnar form tends to branch forming dotted or meandering lines on the chamber floor (Fig. 5). Recently, Checa et al. (2015) proposed an interesting hypothesis to explain such a phenomenon by viscous fingering.

As to the chamber floor, to the best of our knowledge its formation has never been precisely described.

The siphuncular zone of the first chamber is a small oval field (Fig. 5D) and those of every following chamber present a crescent-shaped band that shows the characteristic structure known of the cuttlefish shell. These areas differ from the anterior part of the chamber only by a denser distribution of pillars.

Table I. – Sepia officinalis shell development during early-life stages from first calcification (i.e. stage 24) to hatching. The numbers indicate the chamber building sequence. The arrowhead in the hatching shell picture indicates the nascent rostrum. Note the gradual loss of transparency of the first-formed chambers from stage 28 due to the dorsal shield upper part calcification. or = organic rim. Scale bar: 1 mm.

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<th>29</th>
<th>30</th>
<th>Hatchling</th>
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<td>5</td>
<td>6</td>
<td>8</td>
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<td>Shell dorsal view</td>
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The first calcification of the dorsal shield upper part appears around stage 28, i.e. after the formation of around 4 chambers (Table I, Bandel & Boletzky 1979). The crystallites of this initial dorsal cover do not show any particular orientation. They grow along with the formation of interspersed organic fibers. Except for the margin of the dorsal shield, the entire dorsal surface is rapidly covered by a continuous layer of aragonite crystals, and then organized in the known spherulitic arrangement of this dorsal layer (Bandel & Boletzky 1979).

In *S. officinalis*, the posterior region of the embryonic dorsal shield is first covered by the same initial layer as the anterior part. The earliest traces of a spine appear only towards the end of embryonic life (i.e. stage 30). The first crystallites that build the rudimentary spine are aragonite aggregations very similar to those forming the embryonic dorsal layer. The spine of *S. officinalis* shell emerges clearly few days after the hatching (Table I, Bandel & Boletzky 1979).

During this whole period, no fork synthesis has been described on *S. officinalis* shell and no function of this structure hypothesized.

At hatching time, the chamber number is around 8 highlighting a more important chamber synthesis during the last embryonic stage (i.e. stage 30, Table I, Choe 1963, Boletzky 1974, Bandel & Boletzky 1979).

**Later structure set up**

When the animals hatch the proportions of the shell differ markedly from those of adults. Its width to length ratio is about 1:2 at hatching, whereas it is 3:8 at the adult stage. Also, the relative length of the last septum, which is shorter than the siphuncular zone in young animals, will increase during the later development (Mangold 1966, Bandel & Boletzky 1979). It can be hypothesized that formation of the fork, which is synthesized during the first
month of post-hatching life, could be linked with this form change.

It has not been possible to link the chamber number of *S. officinalis* shell to the animal’s age because of the important interconnection of the temperature, the nutrition and the shell formation. Indeed, Richard (1969) highlighted the temperature influence in the rhythm of formation of the *S. officinalis* shell and the nutrition influence on the structure of newly formed chambers, both parameters being strongly linked. Moreover, Boletzky (1974) underlined a modification of the nutrition effect function of cuttlefish age. Consequently, this plasticity prevents the use of this steel as age-indicator in cuttlefish (Bettencourt & Guerra 2001, Choe 1963, Ré & Narciso 1994), yet some signs as zones of narrow winter chambers could be useful in order to make rough estimations of the age of the animal in geographical areas with contrasting seasons (e.g. *S. officinalis* from the English Channel, Hewitt & Stait 1988).

**CONCLUSION**

Despite the numerous studies focusing on cuttlefish shell, a number of unknown factors remain as for instance how the building of such complex architecture is regulated, how the chamber floor is set up, which are the protein compounds involved in its synthesis or whether the inner structures change during cuttlefish development. One of the major issues in our understanding of the cuttlefish shell set up is the lack of association with the shell sac, too often considered as being the mantle. Indeed, although since the nineteen eighties, several studies investigated the detailed mineral and organic composition of the cuttlefish shell (e.g. Dauphin & Marin 1995, Florek et al. 2009), its construction (Checa et al. 2015) and the effects of global warming on its synthesis (Gutowska & Melzner 2010, Gutowska et al. 2010, Dorey et al., 2013), the shell sac has been rarely included in the understanding of the shell construction processes. Yet, besides the unknown factors alluded to above, it would allow to better understand which repair mechanisms are set up to maintain the shell functions in case of fractures and would contribute to explain the great intraspecific plasticity of this mineralized structure (Boletzky & Overath 1991).

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