Brachiopod punctae: A complexity in shell biomineralisation

A. Pérez-Huerta [a,*], M. Cusack [a], S. McDonald [b,c], F. Marone [b], M. Stampanoni [b,d], S. MacKay [e]

[a] Dept. of Geographical & Earth Sciences, University of Glasgow, G12 8QQ Glasgow, UK
[b] Swiss Light Source, Paul Scherrer Institut, CH-5232 Villigen, Switzerland
[c] Dept. of Radiology, University of Laussane Medical School, CH-1005 Laussane, Switzerland
[d] Institute for Biomedical Engineering, University and ETH Zurich, CH-8092 Zurich, Switzerland
[e] Integrative and Systems Biology, Faculty of Biomedical and Life Sciences, University of Glasgow, G12 8QQ Glasgow, UK

ABSTRACT

Perforations ("punctae") are one of the most characteristic morphological shell features in calcite brachiopods. The significance of punctae is that they represent discontinuities in shell biomineralisation and thus add a level of complexity that must be accounted for in any model of brachiopod shell formation. A significant hindrance to understanding punctae growth and formation is the absence of sufficient information on volume, size and density. Here, we use synchrotron-radiation X-ray tomographic microscopy (SRXTM) to obtain three-dimensional information about punctae of five species of calcite brachiopods. X-ray tomography shows that punctae morphology is species-specific and reveals previously unknown levels of complexity for each species. This information is combined with previous data on morphology to discuss the function and growth of punctae. Overall the present study demonstrates the need to increase our understanding of discontinuities and the role of cell biology in the context of biomineralisation.

1. Introduction

Marine invertebrate organisms, such as corals and bivalve molluscs, have been a significant source of information about biomineralisation in calcium carbonate systems (Lowenstam and Weiner, 1989; Simkiss and Wilbur, 1989). Brachiopods, marine bivalved organisms comprising an independent phylum, are one of the least understood groups in terms of biomineralisation. Brachiopods can produce calcium phosphate (Subphylum Linguliformea) or calcium carbonate (Subphyla Craniiformea and Rhynchonelliformea) protective shells. Recent studies focused on calcite shells, the most common mineralised structures in brachiopods, have advanced our knowledge in terms of structural biology and calcification (e.g., Griesshaber et al., 2007; Pérez-Huerta and Cusack, 2008; Cusack et al., 2008). Despite this new information, little is known about the formation and growth of perforations ("punctae") in calcite shells of brachiopods, which are one of the most characteristic morphological shell features (Fig. 1). The significance of punctae is that they constitute a complexity in our understanding of shell biomineralisation. Punctae represent discontinuities in shell formation that must be accounted for in models that describe brachiopod biomineralisation.

A significant hindrance to understanding punctae growth and formation is the absence of sufficient information on their volume, size and density. Here, we use synchrotron-radiation X-ray tomographic microscopy (SRXTM) to obtain three-dimensional information about punctae of five species of calcite brachiopods. This information is combined with previous data on morphology to increase our knowledge of the formation of punctae in the context of shell mineralisation. Finally, the integration of all data prompts a discussion about the function and growth of punctae.

2. Material and methods

2.1. Material

Specimens of four recent species of terebratulide brachiopods, *Terebratalia transversa* (Puget Sound, USA), *Laqeus rubellus* (near Sagami Bay, Japan), *Calloria inconspicua* (Otago Shelf, New Zealand) and *Terebratulina retusa* (Firth of Lorn, Scotland), and a craniid brachiopod, *Novocrania anomala* (Firth of Lorn), similar to those previously studied and chemically characterised (Parkinson et al., 2005), were analysed. Dried and cleaned samples were sectioned longitudinally across the plane of symmetry, obtaining one half for imaging and the other half for X-ray tomographic microscopy. Thin slices of dorsal and ventral valves were cut longitudinally to obtain small shell fragments (~1 mm²) from anterior and posterior regions for synchrotron analyses.

* Corresponding author. Fax: +44 141 330 4894.
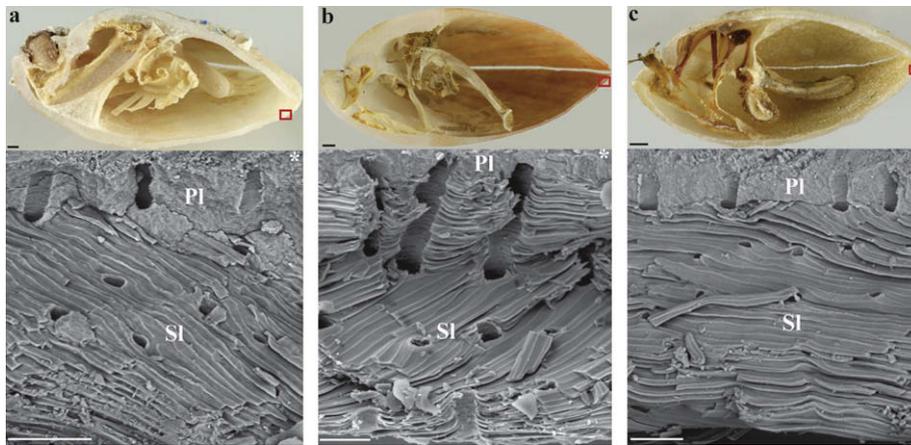E-mail address: Alberto.PerezHuerta@ges.gla.ac.uk (A. Pérez-Huerta).

Fig. 1. Photographs of longitudinal sections of brachiopod species ((a) *Terebratalia tranversa*; (b) *Laqeus rubellus*; (c) *Terebratulina retusa*; scale bars = 1 mm), showing the anterior region of ventral valves (red square) for corresponding SEM images of broken ventral valves showing punctae across the primary layer (Pl) and calcite fibres of the secondary layer (Sl) [scale bars = 50 μm; outer shell surface (white symbol)].

2.2. Scanning electron microscopy (SEM)

SEM images were obtained from gold coated samples of dorsal and ventral valves of brachiopod shells in an FEI Quanta 200F field-emission scanning electron microscope (SEM) using an accelerating voltage in high vacuum mode at 20 kV, aperture 4 and spot size 4. Fracture sections of *T. retusa*, *T. transversa* and *L. rubellus* were imaged to illustrate the distribution and morphology of punctae in 2D sections (Fig. 1). Polished surfaces of dorsal valves of *T. transversa* were etched with 10% HCl for 10 s and then gold coated for observation to show details of punctae morphology.

2.3. Synchrotron-radiation X-ray tomographic microscopy (SRXTM)

Shell fragments were imaged at the TOMCAT beamline of the Swiss Light Source (Paul Scherrer Institute) (Stampanoni et al., 2006). 1501 raw projections for each tomographic scan were acquired at an energy of 20 keV using a UPLAPO 20X objective [numerical aperture: 0.70; field of view: 0.75×0.75 mm^2; pixel size: 0.37×0.37 μm^2] and an exposure time of 600 ms. Projections were rearranged into corrected sinograms, subsequently reconstructed using an optimised algorithm. Reconstructed data were exported as TIFF images (8bit; 2×2 binned; isotropic voxel dimensions: 0.7 μm). Isosurface 3D renderings were performed using AMIRA 4.1 software.

3. Brachiopod punctae

3.1. Internal structure

All groups of living brachiopods have perforated shells with the exception of taxa belonging to the Order Rhynchonellida. Among calcitic brachiopods, there are two classical models that illustrate the internal structure of punctae in craniid and rhynchonelliform brachiopods (Fig. 2). Punctae in craniid brachiopods are complex structures (Williams and Wright, 1970), but two of the main differences with punctae in rhynchonelliform brachiopods are the presence of storage cells filling each puncta and the branching terminations towards the outer shell surface (Williams, 1997) (Fig. 2).

Brachiopod species of the Order Terebratulida have punctae (endopunctae *s.s.*) that contain papillose extensions of the mantle (caeca) that link the inner part of the outer epithelium and connective tissue with the outermost organic shell layer (periostracum)
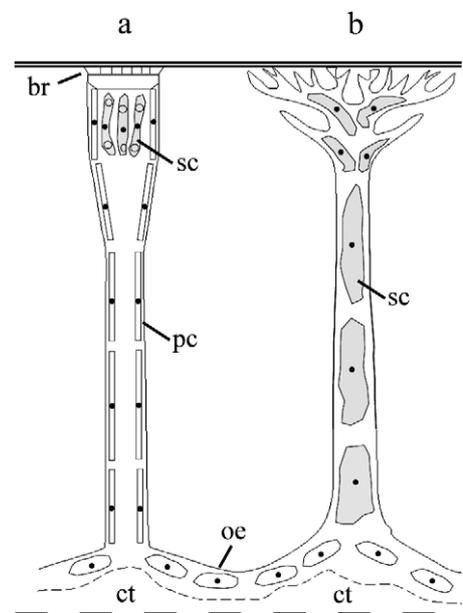

Fig. 2. Schematic and simplified representation of punctae in calcite brachiopods ((a) endopuncta in terebratulid brachiopod; (b) puncta in craniid brachiopod) [*Abbreviations:* br, brush; sc, storage cells; pc, peripheral cells; oe, outer epithelium; ct, connective tissue]. Figure modified and adapted from Williams (1997; p. 301).

(Williams et al., 1997) (Fig. 2). The connection between each puncta and the periostracum is through the brush that comprises fine tubular extensions of a proteinaceous membrane. In addition, each puncta contains peripheral cells linked to the outer epithelium, membrane secretions, lipids, proteins and polysaccharides (Williams et al., 1997; Pérez-Huerta et al., 2008).

3.2. External structure

Punctae are one of the most characteristic features of shell morphology in terebratulide brachiopods (Williams, 1997; Alvarez, 2005). In fracture and longitudinal sections of the shell (Figs. 1 and 3), punctae are evident as structures that perforate the calcitic primary and secondary layers (Fig. 1). A detailed study of acid-etched shell sections from the secondary layer shows that calcite fibres are clearly deflected by the punctae (Fig. 3) (see also Williams, 1968). Also, punctae are encased by biogenic calcite that is
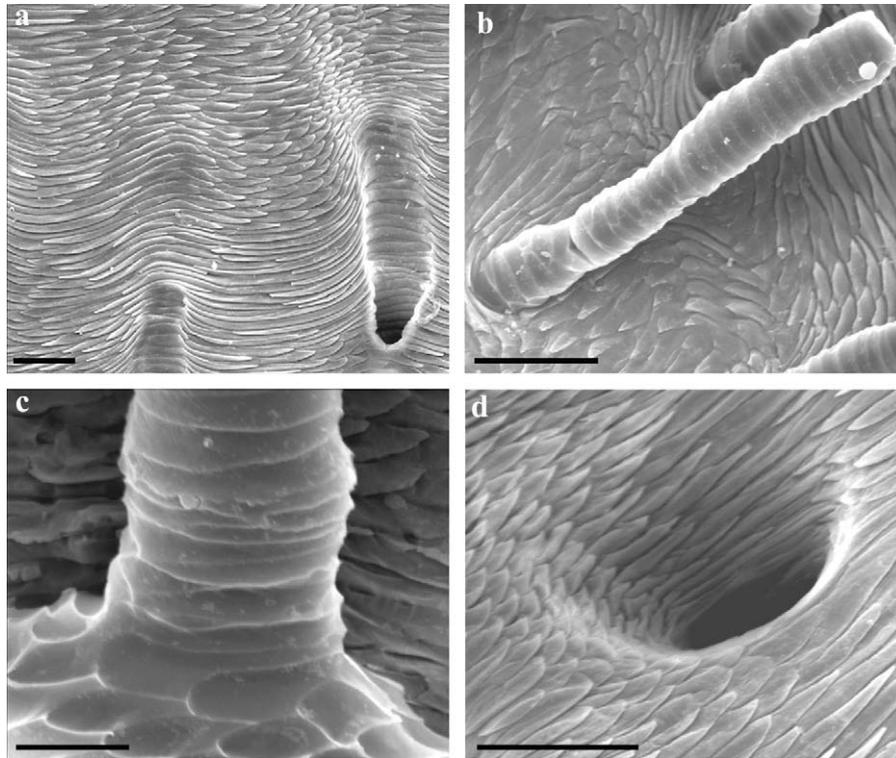
Fig. 3. SEM images of partially acid-etched sections of the dorsal valve of *Terebratalia transversa* showing details of endopunctae [(a) scale bar = 40 μm; (b) scale bars = 80 μm; (c) scale bar = 20 μm; (d) scale bars = 30 μm].

more resistant to acid etching than the calcite of the fibres (Fig. 3b). The calcitic sheath of punctae is divided into discrete ring-like structures, about 6–7 μm in width, that may reflect growth stages (Fig. 3b and c). These observations suggest that biomineralisation processes involved in the construction of punctae and fibres differ one from the other.

4. Results

4.1. 3D morphology of punctae

Conventional microscopic techniques (e.g., SEM) provide basic 2D images that characterise punctae of different terebratulide species as simple, cylindrical structures perforating the layers of calcite that comprise the shell (Fig. 1). Images of brachiopod punctae in three dimensions were obtained using the non-invasive technique of synchrotron-radiation X-ray tomographic microscopy (SRXTM), which allows data to be collected at submicron spatial resolution (Donoghue et al., 2006; Friis et al., 2008) (Figs. 4 and 5). With such resolution in 3D, SRXTM analyses reveal that each species of terebratulide has punctae of quite different morphology and complexity. This is in marked contrast to the images obtained by conventional 2D microscopy (Fig. 1).

Punctae in *Terebratalia tranversa* (Fig. 4a) are simple and uniformly thick cylindrical structures across the shell, while *L. rubellus* (Fig. 4b) shows a significant narrowing of punctae towards the inner shell surface. Specimens of *C. inconspicua* also display simple cylindrical structures but these are curved towards the posterior region of the shell (Fig. 4c). In contrast, *T. retusa* (Fig. 4d) displays a highly complex "tree-shaped" morphology with branching tubules coalescing into a single tubular structure at a precise level within the thickness of the shell. These results demonstrate that punctae morphologies are species-specific in all terebratulide brachiopods and of greater complexity that previously shown.

4.2. Comparison of punctae morphology in craniid and terebratulid brachiopods

Branching of punctae was thought to be only characteristic of craniid brachiopods (Williams and Wright, 1970; Alvarez, 2005), but tomographic data show that specimens of *T. retusa* also have a branching morphology (Fig. 4). The branching of punctae in craniid brachiopods is well understood and relates to the growth of the caeca (Williams and Wright, 1970). Several tubules, 75–100 nm in diameter and up to 2 μm long, are formed inside of the inner surface of the periostracum. Subsequently, tubules coalesce and form thicker branches concomitant with shell growth, since they are aligned with the radial axis of the shell, until they are incorporated in the primary layer (Williams and Wright, 1970). Following this developmental stage, punctae keep growing into the secondary layer (Fig. 4a).

The "tree-shaped" punctae of *T. retusa* have a different morphology (Fig. 4b). Usually four to five tubules, between 20 and 40 μm in diameter, grow from the shell exterior to interior and coalesce at a uniform point, about half way through the thickness, into a single tubule of similar diameter. The punctae morphology of *T. retusa* exemplifies the extent of biological control in punctae formation.

5. Discussion

5.1. Function of punctae

The function of the punctae has long been debated, and their role still remains unresolved, with suggested functions as sensory devices, storage compartments and in respiration. The idea of punctae serving as storage compartments is supported by the presence of storage cells as well as lipids and polysaccharides. Our results show that the morphology of punctae is species-specific in all
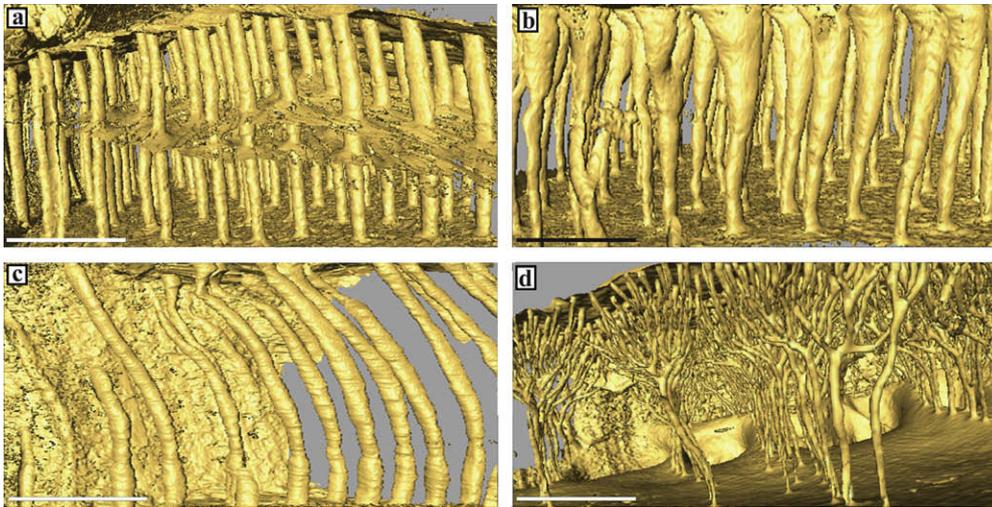
Fig. 4. Tomographic reconstruction of the 3D structure of brachiopod punctae of shell fragments from the anterior region of ventral valves of studied terebratulid brachiopod species [(a) *Terebratalia tranversa*; (b) *Laqeus rubellus*; (c) *Calloria inconspicua*; (d) *Terebratulina retusa*; scale bars = 160 μm; outer shell exterior at the top of each image].
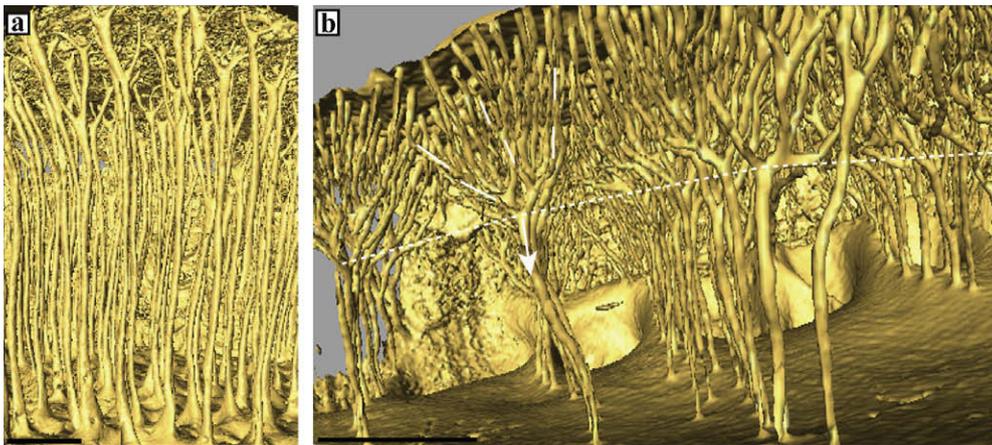

Fig. 5. Tomographic reconstruction of the 3D structure of brachiopod punctae of shell fragments from the anterior region of ventral valves of *Novocrania anomala* (a) and *Terebratulina retusa* (b) [scale bars = 160 μm; outer shell exterior at the top of each image and arrow indicating direction of growth in *Terebratulina retusa*].

terebratulide brachiopod studied requiring an additional explanation. Recent species of rhynchonelliform brachiopods have similar metabolic and physiological requirements (e.g., Peck, 2008), which would not support punctae having different storage capacity in terebratulide species. Thus, punctae are likely to have a different function from that of storage or at least a dual function.

A possibility is that punctae aid in respiration since their presence facilitates tolerance to low oxygen concentrations in terebratulide brachiopods (Thayer, 1986; Tunnicliffe and Wilson, 1988; Lee, 2008). Brachiopod species living in environments with dissimilar seawater oxygen availability would require a different surface area of punctae, explaining the observed differences in complexity of punctae morphology. Also, punctae functioning in oxygen exchange may also explain the wide distribution of terebratulides, present at all latitudes, and their high diversity and dominance, representing 76% of all rhynchonelliform genera (Lee, 2008). The hypothesis of brachiopod punctae acting as regulatory mechanisms for oxygen exchange has to be further tested, but it would draw interesting parallels with structures for ecological adaptation in taxa from evolutionary distant phyla. For example, calcite avian eggshells contain pores that control diffusivity of gases; a reduction of pore area occurs as an adaptation to altitude to prevent water loss (Rahn et al., 1977).

5.2. Growth of punctae

Knowledge of mechanisms involved in the growth of punctae is essential to understand the biomineralisation processes forming the brachiopod shell. Williams (1968) recognised this in his study of terebratulide brachiopods. It is difficult to reconcile the function and growth of punctae and the activity of the outer epithelium involved in shell secretion. This problem is increased with the observation of the three-dimensional morphology and complexity of punctae.

Previous studies show that the caecum originates at the mantle edge as an evagination of the outer epithelium and is then incorporated into the shell (Williams, 1968). Subsequently, peripheral cells, inside the punctae and connected to the outer epithelium, would contribute to the growth of punctae (Fig. 2). This interpretation requires that peripherial cells are only involved in the growth of caeca and, therefore of the formation of punctae, independently of the mineralisation of adjacent calcite structures (i.e., fibres). Alternatively, peripheral cells inside punctae could be involved in the secretion of calcite for the growth of fibres concomitant with the extension of punctae as the shell thickens. In both cases, these hypotheses would be against the idea that only calcifying cells are present at the generative zone at the outer ephithelium (Williams,
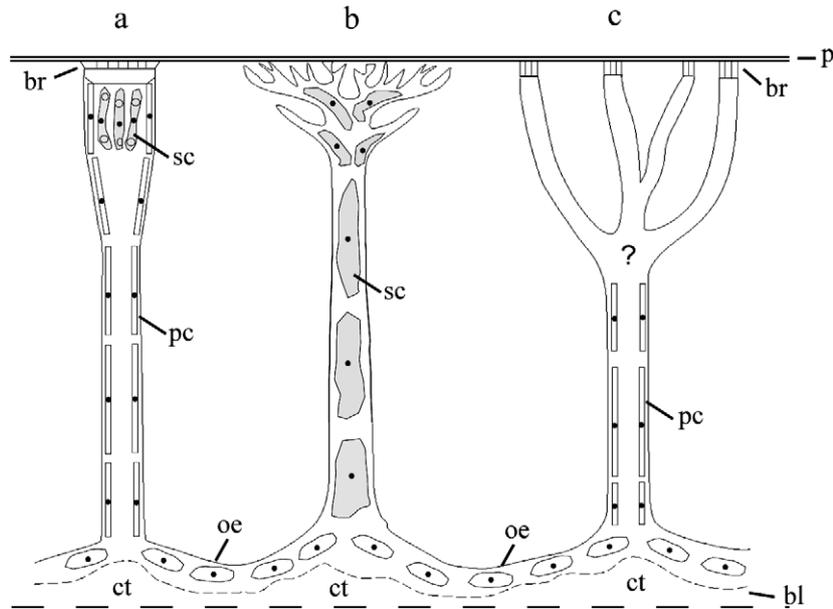
Fig. 6. Comparison of classical schematic representations of punctae in calcite brachiopods ((a) endopuncta in terebratulid brachiopod; (b) puncta in craniid brachiopod) with that of new observed morphology, after tomographic reconstruction, in *Terebratulina retusa* [*Abbreviations:* br, brush; sc, storage cells; pc, peripheral cells; oe, outer epithelium; ct, connective tissue; p, periostracum; bl, basal line].

1968) (Fig. 5). Also, these mechanisms cannot explain the complex 3D morphology of punctae in *T. retusa* (Figs. 5b and 6c). Tubules, growing from the shell exterior to interior, coalesce at a uniform point, which requires an interconnected system of cells involved in the formation of caeca. This growth of punctae cannot be explained in a similar manner to that of craniid brachiopods (Figs. 5a and 6b), where the "branching" is achieved by the formation of small tubules (<1 μm) inside the periostracum.

A plausible hypothesis for the formation and growth of punctae in terebratulid brachiopods, and other rhynchonelliform brachiopods, may be a mechanism similar to the development of Hertwig's epithelial root sheath (HERS) for teeth formation in vertebrates (see review in Luan et al., 2006). In the case of the HERS, a bilayered sheath of generative cells allows the formation of the root and contributes to the secretion of mineralised tissue. For brachiopods, a bilayered sheath of generative cells, resulting from an evagination of the outer ephithelium, would create the space for the caecum. The same cells would simultaneously contribute to the formation of caecum and the mineralisation of calcite in the primary layer and fibres of the secondary layer laterally, from the margins of the punctae. This mechanism would satisfy the observation of the caecum forming as an evagination of the outer ephithelium (Williams, 1968) and the location of peripherial cells inside the punctae (Figs. 5 and 6). These peripherial cells would be the residuals of the original sheath of generative cells. This hypothesis could explain the presence of "simple" punctae (Fig. 6), like those present in *T. transversa* (Fig. 4a), but it would be insufficient to explain the punctae morphology in *T. retusa*. Also, the presence of specialised cells that contribute to the formation of caeca and secretion of mineralised structures would determine that there are significant differences in shell formation mechanisms in terebratulids compared to rhynchonellids which lack punctae.

6. Concluding remarks

Using a combination of synchrotron-radiation X-ray tomographic microscopy (SRXTM) and previous histological and morphological data of punctae in brachiopod shells, this study demonstrates:

1. The necessity to obtain data about the formation and growth of punctae to understand the biomineralisation of brachiopods shells. Our data and methods used to obtain it can contribute to our understanding of discontinuities represented by perforations in other biomineralised carbonate structures (e.g., avian eggshells and freshwater bivalve shells).
2. The use of synchrotron-radiation X-ray tomographic microscopy (SRXTM), as well as microcomputer tomography (μCT) (e.g., Neues and Epple, 2008), can contribute significantly to a better understanding of biomineralisation, particularly when structures/components of different density are present.
3. A need to emphasise knowledge of cell biology in the context of biomineralisation (Weiner, 2008).

References

Alvarez, F., 2005. Lophophorata–Brachiopoda. Fauna Iberica 27, 57–207.
Cusack, M., Dauphin, Y., Chung, P., Pérez-Huerta, A., Cuif, J-P., 2008. Multiscale structure of calcite fibres of the shell of the brachiopod *Terebratulina retusa*. J. Struct. Biol. 164, 96–100.

Donoghue, P.C.J. et al., 2006. Synchrotron X-ray tomographic microscopy of fossil embryos. Nature 442, 680–683.

Friis, E.M. et al., 2008. Phase-contrast X-ray microtomography links Cretaceous seeds with Gnetales and Bennettitales. Nature 450, 549–553.

Griesshaber, E. et al., 2007. Crystallographic texture and microstructure of terebratulide shell calcite: an optimized materials design with hierarchical architecture. Am. Mineral. 92, 722–734.

Lee, D.E., 2008. The terebratulides: supreme brachiopod survivors. Fossils Strata 54, 241–249.

Lowenstam, H.A., Weiner, S., 1989. On Biomineralization. Oxford University Press, New York, 324 pp.

Luan, X., Ito, Y., Diekwisch, T.G.H., 2006. Evolution and development of Hertwig's epithelial root sheath. Dev. Dynam. 235, 1167–1180.

Neues, F., Epple, M., 2008. X-ray microcomputer tomography for the study of biomineralized endo- and exoskeletons of animals. Chem. Rev. 108, 4734–4741.

Parkinson, D., Curry, G.B., Cusack, M., Fallick, A.E., 2005. Shell structure, patterns and trends of oxygen and carbon stable isotopes in modern brachiopod shells. Chem. Geol. 219, 193–235.

Peck, L.C., 2008. Brachiopods and climate change. Earth Environ. Sci. Trans. R. Soc. Edinb. 98, 451–459.

Pérez-Huerta, A., Cusack, M., 2008. Common crystal nucleation mechanism in shell formation of two morphologically distinct calcite brachiopods. Zoology 111, 9–15.

Pérez-Huerta, A., Cusack, M., Ball, A., Williams, C.T., Mackay, S., 2008. Deciphering the distribution of organic components in brachiopod shells by confocal laser scanning microscopy. J. Microsc. 230, 94–99.

Stampanoni, M., et al., 2006. Trends in synchrotron-based tomographic imaging: the SLS experience. In: Bonse, B. (Ed.), Developments in X-ray Tomography V, Proc. of SPIE 6318, 63180M.

Simkiss, K., Wilbur, K.M., 1989. Biomineralization: Cell Biology and Mineral Deposition. Academic Press, London, 337 pp.

Thayer, C.W., 1986. Respiration and the function of brachiopod punctae. Lethaia 19, 23–31.

Tunnicliffe, V.J., Wilson, K., 1988. Brachiopod populations-distribution in Fjords of British Columbia and tolerance of low oxygen concentrations. Mar. Ecol. Prog. Ser. 47, 117–128.

Rahn, H., Carey, C., Balmas, K., Bathia, B., Paganelli, C., 1977. Reduction of pore area of avian eggshell as an adaptation to altitude. Proc. Natl. Acad. Sci. USA 74, 3095–3098.

Weiner, S., 2008. Biomineralization: a structural perspective. J. Struct. Biol. 163, 229–234.

Williams, A., 1968. Evolution of the shell structure of articulate brachiopods. Spec. Pap. Palaeontol. 2, 1–55.

Williams, A., 1997. Shell structure. In: Kaesler, R.L. (Ed.), Treatise on Invertebrate Paleontology Part H Brachiopoda [Revised 1: Introduction]. The Geological Society of America and The University of Kansas Press, Boulder (CO) and Lawrence (KS), pp. 267–320.

Williams, A., Wright, A.D., 1970. Shell structure of the Craniacea and other calcareous inarticulate brachiopoda. Spec. Pap. Palaeontol. 7, 1–51.

Williams, A., Brunton, C.H.C., MacKinnon, D.I., 1997. Morphology. In: Kaesler, R.L. (Ed.), Treatise on Invertebrate Paleontology Part H Brachiopoda [Revised 1: Introduction]. The Geological Society of America and The University of Kansas Press, Boulder (CO) and Lawrence (KS), pp. 321–422.