PHOSPHATIC CARAPACE OF THE WAPTIID ARTHROPOD CHUANDIANELLA OVATA AND BIOMINERALIZATION OF ECYDYSOZOANS

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Abstract: Arthropods other than trilobites or bradoriids constitute a major component of many Cambrian Burgess-Shale-type Lagerstätten. Owing to their scarcity in other less well-preserved deposits, they are generally regarded as lightly sclerotized but non-mineralized. Here we show that Chuandianella ovata, one of the typical lightly sclerotized arthropods from the Chengjiang biota, reinforced its carapace with phosphatic mineralization. Multiple methods reveal a pitted microstructure and organophosphatic composition of the carapace. Nanograins of amorphous calcium phosphate (ACP) on the fossil carapace are comparable to those of recent crustacean microstructures, implying that arthropods may have a conserved mechanism of phosphatic mineralization since the Cambrian. The fossil record indicates that the mineralization of ecdysozoans, preceded by the mineralization of lophotrochozoans in the Terreneuvian (~541–521 Ma), appeared in Cambrian Stage 3 (~521–514 Ma) and surpassed the range of living counterparts in phylogenetic coverage. This phenomenon strengthens the view that biomineralization has evolved multiple times independently within the Metazoa. The sudden appearance of mineralized skeletons in the early history of metazoan evolution is frequently attributed to external environment drivers. However, this viewpoint cannot explain the minerology and appearance time of metazoan lineages with mineralized hard parts during the Cambrian explosion.

Key words: Chengjiang biota, Chuandianella ovata, biomineralization, phosphatic exoskeleton, Ecdysozoa, Cambrian explosion.

Arthropods are by far the most taxonomically diverse and ecologically successful group of living animals (Brusca et al. 2016, pp 733–784). Their success in diverse ecosystems is largely owed to the development of robust organic cuticle, which is an indispensable component of arthropod exoskeletons. Only some living crustaceans are known to have calcified cuticles, either carbonate or phosphate in composition (Bentov et al. 2016). In most cases, mineralized exoskeletons are composed of calcium carbonate minerals, although some elements are reinforced by incorporation of phosphatic minerals, such as in the claw of woodlice species such as Porcellio scaber (Vittori et al. 2016). In living arthropods, the barnacle Ibla cumingi has been known to construct a phosphatic carapace (Lowenstam & Weiner 1992).

Arthropod body fossils are known from Cambrian Stage 3 though their traces may extend back to the Fortunian (~541–521 Ma) (Mángano & Buatois 2014; Zhang et al. 2017). Arthropods dominate Cambrian soft-bodied biotas in both diversity and abundance as seen in Burgess Shale, Chengjiang and Qingjiang biotas (Briggs et al. 1994; Hou et al. 2017; Fu et al. 2019). Trilobites, phosphatocopids and bradoriids are well represented in the fossil record due to their mineralized exoskeletons (Tegler & Towe 1975; Müller 1979; Kouchinsky et al. 2012). However, in exceptionally preserved Burgess-Shale-type Lagerstätten (e.g. Chengjiang and Burgess Shale) and small carbonaceous deposits (Butterfield & Harvey 2012; Harvey et al. 2012) there are many arthropods with lightly sclerotized exoskeletons (e.g. most non-trilobitic taxa) which are unrepresented in normal fossil beds. Without fossil evidence, whether or not they had mineralized hard parts remains uncertain.

Here we analyse the composition and microstructures of the carapace of Chuandianella ovata (Lee 1975), a waptiid arthropod from the Chengjiang biota, Cambrian Stage 3. Unexpectedly, it has a mineralized carapace composed of calcium phosphate and preserves primary microstructures. This finding, combined with the fossil record of other Cambrian ecdysozoans, indicates that a number of ecdysozoan lineages had become biomineralized by Cambrian Stage 3.
MATERIAL AND METHOD

Material

A total of 1409 specimens from eight localities of the Chengjiang biota were analysed in this study (Fig. S1A). These have been deposited in the Shaanxi Key Laboratory of Early Life and Environments, Northwest University, Xi’an (LELE; although the specimen numbers retain the original prefix ELI). Specimens were collected from the Maotianshan Shale Member (Member 3) of the Yu’anshan Formation in Eastern Yunnan, South China (Zhang et al. 2007; Hou et al. 2017).

Imaging

All specimens were observed using a Nikon SMZ 100 stereomicroscope under various lighting conditions and photographed with a Canon EOS 5D Mark II digital camera under incandescent lamp. Morphological measurements were made on images with the software ImageJ v1.8.0. All images were processed in Adobe Photoshop CC 2019 and CorelDRAW X9.

Scanning electron microscopy

Selected specimens were coated with gold and examined with a FEI Quanta 450 FEG scanning electron microscope (SEM) in high vacuum and high accelerating voltage (20 kV) conditions and thermos Scientific Helios G4 UC focused ion beam (FIB) in high vacuum, 15 kV accelerating voltage and 0.4 nA electric current conditions for microstructures.

Element mapping

Uncoated selected specimens were analysed using an M4 Tornado micro x-ray fluorescence (XRF) spectrometer; gold-coated specimens were examined with an FEI Quanta 450 FEG energy-dispersive spectrometer (EDS) for element mapping.

Raman spectroscopy

Raman spectra were acquired on the LabRAM HR Evolution. Excitation was achieved with a green laser line (532 nm), a ×50 microscope objective, a 200 μm confocal aperture and a 600 mm−1 grating, with a resolution of 2–4 cm−1. The different spectra represent the Raman scattering from the outer mineralized surfaces of the C. ovata carapace and the molar teeth of the living crustacean Gonodactylus chiragra.

Geometric morphometric analysis

A total of 79 intact laterally compressed specimens without deformation were selected for geometric morphometric analyses. All selected specimens were imaged in lateral view with the anterior oriented to the right; those oriented to the left were mirrored (Fig. S2B). A total of 5 landmarks and 33 sliding semi-landmarks were digitized with the software tpsDig v2.16 (Rohlf 2010). Landmark 1 is at the posterodorsal corner of the valve and other four landmarks are established at the maximum bending points along the marginal rim of the valve, while 33 semi-landmarks were digitized along outline in equal intervals and in an anticlockwise direction (Fig. S2A, B). The software tpsUtil v1.44 was applied to transfer curves to landmarks and created the sliders file defining the semi-landmarks. A generalized Procrustes analysis (GPA) was performed to extract information regarding scale, position and orientation, and computed using tpsRelw v1.49. Relative warp analysis (RWA) was carried out to analysis of within-population morphometric variation based on landmark data (Rohlf 1993). The morphometric data was plotted by PAST v3.12 (Hammer et al. 2001).

RESULTS

Carapace microstructures

The carapace is folded about a median line, covering the anterior part of the body and offering lateral protection (Figure 1A, F). Two types of specimen were previously recognized based on presence and absence of ornament on the carapace, which turn out to be a preservational artefact (see Appendix S1). Ornament is more conspicuous on lightly altered or weathered specimens with a dark, rigid carapace, but is indistinct or absent on intensively pleated carapaces. Only a handful of wrinkled specimens are ornamented (Figure 1F, G). Ornament appears as evenly distributed pits with consistent dimensions (Figure 1B). Most specimens are preserved with moderate relief. Pitted ornaments are rounded on the central region (c. 30 μm in diameter) and elongate on the marginal region (Figure 1C). Equidistant holes, c. 5 μm in diameter are present on the surface (Figure 1D, E), possibly representing pore canals.

Chemical composition

Forty specimens were mapped using XRF. Our results show that Fe is enriched in all analysed specimens and concentrated on soft parts, such as appendages and abdomen, while unevenly distributed on the carapace, mostly in crevices (Figure 2D, H). This result is consistent with
those of previous studies, in which Fe concentration was thought to be late diagenetic in origin (Gabbott et al. 2004; Forchielli et al. 2013). Seven specimens show a significant enrichment of Ca and P on the carapace in a close spatial association but no enrichment on soft parts (Figure 2B, C; Fig. S4F, G). In specimens shown in Figure 2, Ca coupled with P occurs as patches on the carapace, which is unlikely to be phosphatized soft parts because the soft body is detached from the carapace. These phosphatic specimens range from moderate to the largest in size, are darker in colour, and have a rigid carapace with ornament (Figure 2A; Fig. S4E). There are seven specimens with an enrichment of P but an absence of Ca on the carapace (Fig. S4B, D), two of which are moderate in size and the other five large (Fig. S5). In contrast, Ca or P is hardly detected in the majority of specimens that have a thin, plastically deformed carapace, though P can be detected on the soft tissue areas (Fig. S4I–K).

Mineralogical composition

The specimens enriched with Ca and P were further analysed using multiple methods. SEM coupled with EDS analyses demonstrate that the carapace is enriched in Ca and P (Figure 3D, E), consistent with XRF results, and composed of densely colonized nanoscale granules (Figure 3F). In situ Raman spectroscopy reveals a band at ~ P\textsubscript{O\textsubscript{4}} 960 cm\textsuperscript{-1} attributed to phosphate and bands at ~1330 cm\textsuperscript{-1} (D-band) and ~1605 cm\textsuperscript{-1} (G-band) fit the characteristics of carbonaceous materials (Frezzotti et al. 2012). The results indicate that the carapace is composed of a composite material, containing both ACP minerals and organic remains (Figure 3B).

DISCUSSION

Phosphatic biomineralization

The enrichment of Ca in a close spatial association with P and the presence of ACP minerals indicate the carapace of *C. ovata* has a primary calcium phosphate composition based on the following arguments.

First, phosphatic lingulid shells from the Chengjiang biota were known to have survived diagenesis and weathering (Chen et al. 1996). Therefore, it is not surprising to find primary phosphatic minerals in Chengjiang fossils. Additionally, diagenetic phosphatization is not a principal
**FIG. 2.** Chemical composition of *Chuandianella ovata*. A–D, ELI SJZ-B25-924: A, optical image of a dark and rigid carapace with soft tissues rotated ventrally and protruded from the anterior part of carapace; B–D, XRF analyses of A, showing maps of Fe (D), Ca (B) and P (C). E–H, ELI SJZ-B08-825A: E, optical image, soft body rotated nearly 90° and protruded from the ventral of carapace: F–H, elemental maps of Fe (H), Ca (F) and P (G). Abbreviations: ap, appendages; c, carapace; t, trunk. Scale bars represent 4 mm (A, E).

**FIG. 3.** Mineralogy of the carapace; ELI SJZ-B25-924. A, optical image with arrow indicating the analysed area. B, Raman spectra, showing amorphous calcium phosphate (ACP) minerals and organic remains on the carapace. C, *in situ* SEM image. D–E, EDS Ca and P maps of C. F, enlargement of the square in C, showing c. 25 nm-sized granules. Scale bars represent: 10 μm (C); 1 μm (F).
preservation mechanism of either the Chengjiang biota or other Burgess Shale-type Lagerstätten (Gabbott et al. 2004; Gaines et al. 2008; Forchielli et al. 2013). It is known, however, to be responsible for the preservation of soft tissues, mostly digestive tracts, such as gut of *Mis- zhouia longicaudata* in the Chengjiang deposit (Vannier & Chen 2002). Yet, secondary phosphatization of cuticles or exoskeletons has not been found in any Burgess Shale-type deposit (Butterfield 2002; Gaines 2014). ACP granules (c. 25 nm in diameter) on the carapace of *C. ovata* are smaller than diagenetic phosphatic granules (c. 50 nm in width and 90 nm in length) of the Weng’an fossils (Xiao & Schiffbauer 2009). Hence, it is unlikely that the phosphatic carapace is diagenetic in origin. This is also supported by the fact that calcium phosphate is present only on the carapace but absent on adjacent body segments and matrix. In some cases, a slight enrichment of P associated with Fe but without Ca can be detected in abdomen regions (Fig. S4G–L), most probably representing organophosphorus released from the decay of organic matter. Gabbott et al. (2004) provided an explanation for the association of P and Fe, such that during oxidative weathering of the Chengjiang mudstones, sulphuric acid (H2SO4) from pyrite oxidization leached apatite and led to liberation of phosphate anions, forming a refractory chelate with iron hydroxide. Moreover, phosphatic carapaces are usually larger and harder specimens (Figs S4, S5), which is consistent with observations of living arthropods (Richards 1951) (see Appendix S1). This is convincingly interpreted as a primary phosphatic biomineralization of *C. ovata* cuticle, rather than a secondary phosphatization. Finally, nanoscale granules of ACP in our samples are analogous to those seen on the molar teeth in the mandible of the living crustacean *G. chiriagra*. The irregular accumulation of spherical structures is consistent with that of *C. ovata* in size, about 25 nm in diameter (compare Figure 3F with Fig. S6H), which hints at a similar mechanism of phosphatic mineralization in fossil and extant arthropods.

Phosphatic carapaces are rare, represented by 14 examples among 1409 specimens. This rarity may have resulted from decalcification and/or insufficient mineralized components in the cuticle (see Appendix S1) and might be a chief cause that the mineralization of *C. ovata* has long been overlooked. It is also a likely reason for the insufficient recognition of mineralization in Cambrian arthropods.

**Biomineralization of ecdysozoans**

A number of lines of evidence indicate that both microscopic and macroscopic soft-bodied ecdysozoans of different lineages were present in the Fortunian (~538.3–529 Ma). For instance, *Treptichnus pedum*, a sub-horizontal burrow that marks the base of the Cambrian, was thought to be produced by priapulids (Vannier et al. 2010). Additionally, millimeter-sized soft bodied fossils of scadilophorans, a group of ecdysozoans including priapulids, kinorhynchs and loriciferans, were recently recovered from the Fortunian Kuanhuaniu biota of South China (Liu et al. 2014, 2019; Zhang et al. 2015). Also, a number of studies demonstrated an early diversification of arthropods (Daley et al. 2018; Paterson et al. 2019).

However, ecdysozoans with mineralized hard parts are absent from the fossil record until the Cambrian Stage 3 (c. 521–514.5 Ma). Notably, trilobites are widely known to have low-magnesium calcite exoskeletons with phosphatic minerals incorporated only in rare cases (Teigler & Towe 1975). They appeared at the base of the Cambrian Stage 3 (Zhang et al. 2017). Other Cambrian arthropods that exploited calcium phosphate minerals to stiffen their exoskeletons include phosphatic carapaces of phosphatocopids (Müller 1979), some bradoriids (Kouchinsky et al. 2012) and *C. ovata* that first appeared in Cambrian Stage 3, while phosphatic *Phytophilaspiis* (Lin et al. 2010) is known from Cambrian Stage 4 (~514.5–509 Ma), and aglaspidids (Briggs & Fortey 1982) from the Furogian (~497–486.9 Ma) to the Ordovician. Apart from arthropods, palaeoscolecids, generally regarded as a stem group of the Priapulida, carry phosphatic sclerites, which have an extensive fossil record from the Cambrian Stage 3 to the Silurian (Kouchinsky et al. 2012). The lobopodians *Microdictyon* and *Onychodictyon* also bear paired, phosphatic net-like sclerites that are known from the Cambrian Stage 3 to Stage 4 (Hou et al. 2017; Kouchinsky et al. 2012). In contrast, mineralized skeletons of lophotrochozoans are well represented in the small shelly fossils (SSFs) of the Terreneuvian (~541–521 Ma), indicating an early biomineralization of lophotrochozoans (Kouchinsky et al. 2012).

In living phyla of the Ecdysozoa, only some crustacean arthropods have mineralized hard parts, and these prolifically use calcium carbonate to reinforce their skeletons (Bentov et al. 2016) (e.g. ostracods since the early Ordovician). Calcium phosphate is rarely used to build the carapace except in the barnacle *Ibla* (Lowenstam & Weiner 1992). However, it is frequently incorporated into specific structures to enhance physical properties, such as the hypermineralized hammer-like dactyl clubs of stomatopods (Currey et al. 1982) and the molar teeth in the mandible of crayfishes (Bentov et al. 2012).

It is evident that the distribution of Cambrian biomineralized ecdysozoans crosses a range of lineages (Figure 4). This pattern strengthens the assumption that biomineralization evolved independently in animals at the phylum level (Murdock & Donoghue 2011; Murdock 2020). Moreover, in term of phylogenetic coverage, Cambrian
FIG. 4. Distribution of biomineralization across ecdysozoan lineages with stratigraphical ranges.
ecdysozoans with mineralized components, including scalidophorans, lobopodians and euarthropods, were beyond the range of living groups among which only crustaceans are known to have mineralized body parts (Figure 4). In living ecdysozoans, a small number of crustaceans have calcitic exoskeletons. Phosphatic exoskeletons have been known in rare cases. In contrast, many Cambrian ecdysozoans carried calcitic or phosphatic hardparts (Figure 4), with trilobites carrying calcitic exoskeletons, while palaeostracoids, lobopodians and a number of arthropod groups adopted phosphatic hard parts (Figure 4). Therefore, mineralization originated multiple times during the early evolution of ecdysozoans in the Cambrian alone and phosphatic mineralization may have arisen multiple times in separate arthropod lineages. The diversification of ecdysozoans during the Cambrian explosion gave rise to a considerable number of forms of stem and crown groups. Some groups were armed with mineralized skeletons but did not survive to the present day. For example, calcitc trilobites went extinct at the end of the Palaeozoic and phosphatic aglas- parted phylum or activation of biomineralization toolkit genes in different lineages is a likely intrinsic explanation. For instance, silicification in a range of lineages was ascribed to incorporation of a foreign SIT gene (Marron et al. 2013). However, it is difficult to explain the synchronous appearance of mineralized ecdysozoans. Hence, the triggers of biomineralization remain open to discussion.

CONCLUSION

Pitted microstructures on the carapace of C. ovata are illustrated and described for the first time, and their absence in the minority of specimens attributed to a preservational artefact rather than inter or intraspecific variation. The nanostructure of ACP granules incorporated into the carapace of C. ovata is comparable to that seen in living arthropods and thus of primary origin. This finding broadens the taxonomic coverage of Cambrian mineralized arthropods and implies a conserved mechanism in constructing ACP hard parts. Also, phosphatic biomineralization may have had wider employment in the Cambrian because lightly sclerotized arthropods like C. ovata are abundant in Burgess-Shale-type Lagerstätten. The shelly fossil record indicates that biomineralization across ecdysozoan lineages initially took place during Cambrian Stage 3 and the phylogenetic coverage of mineralized ecdysozoans was broader in the Cambrian than in later geological times. The biomineralization among animal lineages during the Cambrian explosion is difficult to explain by external drivers alone.

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Author contributions. XLZ designed the research and CL performed analyses. XLZ and CL interpreted the data with input from DJF. CL prepared the first draft which was edited by XLZ.

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SUPPORTING INFORMATION

Additional Supporting Information can be found online in the online version of this article (https://doi.org/10.1111/pala.12570):

Appendix S1. Supplementary material including Figs S1–S6.
Appendix S2. Data for Fig. S2.
Appendix S3. Data for Fig. S3.

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