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Baring it all – undressing Cambrian 'Orsten' phosphatocopine crustaceans using synchrotron X-ray tomographic microscopy

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Synchrotron X-ray tomographic microscopy (SRXTM) was used in order to virtually dissect and peel the shields off of the microscopic, bivalved phosphatocopine crustaceans in the Cambrian 'Orsten' type of preservation of Sweden. Doing so opened up for an array of concealed internal structures to be observed in a fully enclosed specimen of *Hesslandona ventrospinata* and a semi-enclosed specimen of *Hesslandona angustata*. For comparison also a head larva stage specimen of *H. angustata*, with shields in "butterfly position", was analysed. The X-ray tomographic data sets revealed excellently preserved structures, such as labrum, sternum, antennae, mandibular and postmandibular limbs with their minute setae, all of which were more or less disguised by the enclosing shields. This, moreover, allowed assignment to growth stages of the specimens, which is impossible based solely on external morphology and size. Microspherules observed inside the shields of the semi-enclosed *H. angustata* specimen may represent remains of food particles, and the feeding biology of phosphatocopines is discussed in detail. Our analyses suggest that phosphatocopines were particle feeders. The SRXTM technique offers the ability to three-dimensionally reconstruct the morphology in high resolution, construct virtual serial sections and study concealed structures. The resulting data allow for new structures to be revealed for previously known taxa and for new taxa to be identified, with the added benefit of not destroying the specimens in the process. Hence, we do not longer have to rely on serendipitous finds of broken and/or open phosphatocopine specimens in order to elucidate their diagnostic ventral morphology.

Keywords: Cambrian, 'Orsten', Phosphatocopina, ventral morphology, appendages, feeding biology, SRXTM

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Adding to the challenges posed by the incomplete fossil record, with its general lack of soft tissue structures, is the fact that reconstructing and understanding fossil organisms and ecosystems generally become increasingly difficult, yet no less intriguing, the deeper back in time we go. The Phanerozoic rock record, appropriately named in the 19th century for its abundance of "visible life", can be traced some 541 million years back in time. This coincides with the beginning of the Cambrian Period, a crucial time in the evolution of life on Earth with the advent of most modern phyla. Rapid and profound changes in ocean chemistry and substrate composition were coupled with the emergence of a wide array of new life forms and hard-shelled animals (e.g., Bengtson 1994; Marshall 2006; Smith & Harper 2013). Consequently, albeit incomplete, the Cambrian fossil record provides evidence for diverse marine ecosystems with well-developed and surprisingly complex trophic levels (Eriksson & Terfelt 2007; Vannier 2007, 2012; Vannier *et al.* 2007; Dunne *et al*. 2008).

This knowledge has primarily been acquired from Konservat Lagerstätten, of which there are unusually many in the Cambrian rock record (Zhu *et al*. 2006). One such example is the famous 'Orsten' Lagerstätte, or better 'Orsten' type of preservation, first discovered in southern Sweden (e.g., Müller 1979, 1982; Walossek & Müller 1991; Maas *et al*. 2006). It is characterized by remarkably well-preserved, minute fossils from the uppermost mid-Cambrian through Furongian (Series 3 and 4 of the Cambrian) bituminous limestones (colloquially known as 'Orsten'). Such 'Orsten' fossils have significantly improved our understanding of Cambrian marine ecosystems in general and arthropod evolution and phylogeny in particular. The fossils are represented by phosphatized material, most of which are arthropods, in the size range of 0.1–2 mm (Müller & Walossek 1991; Maas *et al*. 2006). Phosphatization is generally thought to have induced early diagenetic encrustation/impregnation of the external cuticular layer (non-chitinous epicuticle) of the animals, producing a pristine three-dimensional preservation, with the source of phosphorous possibly being coprolites (Maeda *et al*. 2011).

One of the most commonly represented and diverse faunal components in the 'Orsten' are the minute phosphatocopine crustaceans with bivalved head shields enclosing their body (Müller 1982; Maas *et al*. 2003, 2006). These Cambrian fossils had long been known before from their external shells only (Müller 1964). This has all changed, however, with the discovery of the Swedish 'Orsten' Lagerstätte, when Klaus Müller found beautifully preserved phosphatocopines, by that time assigned to ostracods, with ventral body parts including limbs and their setae still present (Müller 1979).

Because of the preservational bias among 'Orsten' fossils, the phosphatocopines range from the most common isolated shields through articulated specimens with completely enclosed shields, both categories with or without ventral body parts preserved. Although the species known from the 'Orsten' of Sweden are systematically diagnosed on their external shield characteristics (Maas *et al*. 2003) it is their ventral soft body or soft part morphology (i.e., according to Müller 1979, any details or fragile structures such as limbs, setae, eyes, the mouth and anus region etc.) – that lay the foundation for the phylogenetic and systematic position of the Phosphatocopina, representing an in-group of the Crustacea. Moreover, based on details of their soft body morphology, ontogenetic stages, development and mode of life can been deduced.

In this study we have used the cutting edge synchrotron radiation X-ray tomographic microscopy (SRXTM) technique to virtually dissect Cambrian 'Orsten' phosphatocopines in order to search for and investigate concealed, anatomical structures. We demonstrate how the resulting X-ray tomographic data sets can be used for high-resolution reconstructions of morphologies and discuss how such detailed visualization data can elucidate palaeobiological, palaeoecological and taxonomical queries. Based on the excellently preserved specimens at hand we also discuss the feeding biology of phosphatocopines.

Materials and methods

Geological setting and sampled localities

The samples collected for this study derive from classic 'Orsten'-yielding localities on Mount Kinnekulle, which is situated in the province of Västergötland, south-central Sweden. Mount Kinnekulle is an erosional outlier that comprises Cambrian to Silurian strata capped by dolerite intruded as sills during Permian times (Martinsson 1974; Andersson *et al*. 1985). Cambrian strata from

Series 3 to Furongian (= Series 4) of the Alum Shale Formation crop out in a few natural exposures and a number of abandoned alum shale quarries (e.g. Westergård 1922; Sundius *in* Johansson *et al*. 1943; Maas *et al*. 2003).

These strata typically consist of interfingering beds and layers of black alum shale and bituminous limestone. The latter are colloquially referred to as 'stinkstone' or 'Orsten' and represent the source rock of the exceptionally preserved microfossils. Polymerid trilobites, predominantly represented by olenids, and agnostoids (euarthropods with crustacean affinities; see Müller & Walossek 1987) occur abundantly in most parts of the succession and form the basis for the high-resolution biostratigraphic zonation (e.g., Westergård 1922, 1946; Henningsmoen 1957; Terfelt *et al*. 2008, 2011).

Biostratigraphically, the exposed Alum Shale Formation on Mount Kinnekulle spans the *Lejopyge laevigata* agnostoid Zone through the *Peltura costata* polymerid Zone (the *Acerocarina* Superzone of Weidner & Nielsen 2013) (Westergård 1922, 1947; Terfelt *et al*. 2011; Nielsen *et al*. 2014), although several stratigraphic gaps occur within the succession (Martinsson 1974). The bulk of the known Swedish Cambrian 'Orsten' material comes from the uppermost Guzhangian Stage (= Stage 7), or the *Agnostus pisiformis* Zone (which used to indicate the base of the traditional Upper Cambrian), today part of the *Paradoxides forchhammeri* Superzone *sensu* Nielsen *et al.* (2014). It represents the oldest 'Orsten' material from Sweden. Some 'Orsten' species, however, are only known from exceptionally preserved specimens from superjacent Furongian strata (Maas *et al*. 2003, their table 2, 2006; Eriksson & Terfelt 2012).

The material analysed herein was collected from the *A. pisiformis* Zone at Sandtorp (WGS 84: N 58° 32.723', E 13° 23.385'), southeastern Mount Kinnekulle, and the 'Transformatorstationen' locality (WGS 84: N 58º 32.558′; E 13º 19.910′) at Blomberg, south-western Mount Kinnekulle. For further information on these localities, see Maas *et al*. (2003) and Eriksson *et al*. (2012).

Sample processing and material

The 'Orsten' rock slabs were digested in pH-monitored buffered acetic acid, following the techniques described by Jeppsson *et al*. (1999). The pH was adjusted to >3.6 in order to avoid etching of phosphatic fossils. After digestion the resulting residue was rinsed through a 63 µm sieve and washed into a petri dish with deionized water. The residues were investigated for exceptionally preserved microfossils under a binocular light microscope. Specimens of interest were handpicked from the wet residues (c. 50 to 80 specimens per sample) using a fine brush and were stored submerged in water, to avoid damage prior to analyses.

Forty-five phosphatocopines (33 from Sandtorp and 13 from Transformatorstationen) were analysed using SRXTM in search of concealed ventral soft body parts. Although most of these proved to lack soft-part structures or only had a few and poorly preserved appendages within the shields, the specimens that are dealt with herein are nearly complete with abundant and excellently preserved soft tissue structures. The results and discussions are based on those specimens combined with published data.

All imaged 'Orsten' specimens are stored at the Department of Geology, Lund University, Lund, Sweden, with repository number LO (for Lund Original).

Synchrotron methods and settings

The fossil specimens were analysed by MEE, FT, FM, and AL using synchrotron radiation X-ray tomographic microscopy (SRXTM) at the TOMCAT beamline of the Swiss Light Source (SLS), Paul Scherrer Institute, Switzerland (e.g., Stampanoni *et al*. 2006). During our first analytical sessions at SLS, specimens were mounted on SEM stubs and/or stacked vertically in capillary glass tubes and separated by glass beads (see Eriksson & Terfelt 2012; Eriksson *et al*. 2012). Albeit functional, the latter method proved time-consuming during the data processing, since the fossil specimens had to be digitally removed from both the glass capillaries and glass beads. During consecutive analyses and refinements of sample handling, new methods for improvements in speed and efficiency have been developed, making optimal use of valuable beam time (Eriksson *et al*. 2013). Thus, the specimens are now attached onto a low light refractive 0.30 mm fishing line (Berkley; Trilene super strong; 100%

fluorocarbon) using a thin layer of water soluble spray-on glue. The fishing line with the specimens attached is then cut to appropriate length and mounted directly in a sample holder. This allows for sets of multiple specimens to be subsequently scanned without need to enter the experimental hutch and change the sample holder. Moreover, it significantly reduces time required for software data processing as the fishing line has such a low density that it can easily be excluded during rendering.

In order to optimise the contrast, the beam energy was set to 12 keV. The X-ray radiation transmitted by the sample was converted into visible light by a 20 µm thick Ce-doped LuAG scintillator screen (Crytur, Turnov, Czech Republic). Projection images were magnified by microscope optics and digitised by a high-resolution CCD camera with a 2048 × 2048 pixel chip and a pitch of 7.4 µm (PCO2000; PCO GmbH, Kelheim, Germany) for specimen LO 12165t, and a sCMOS camera called PCO.edge 5.5 with a 2560 × 2160 pixel chip with a 6.5 µm pitch and a 16 bit dynamic range, for the other specimens. The optical magnification was set to 20×, resulting in cubic voxels with a side of 0.37 µm in the reconstructed data sets. The tomographic reconstructions were performed on a 60-node Linux PC cluster using a highly optimised routine based on the Fourier transform method and a gridding procedure (Marone *et al*. 2010). The resulting tiff micro-tomograms, or slices, were imported and rendered into 3D-images, using the Voxler 2 and 3 software packages.

Scanning electron microscopy

For elemental mapping, the uncoated specimen LO 12163t was mounted on a sample stub and analysed in low vacuum in a Hitachi S-3400N scanning electron microscope (SEM) at the Department of Geology, Lund University, Sweden. This analysis was performed with an energy dispersive spectrometer (Inca X-sight, Oxford instruments) with a Si-detector; acceleration voltage 15 keV and beam current c. 2 mA. Subsequently this specimen and LO 12164t were gold-coated and photographed using a Hitachi SU3500 SEM at the Department of Biology, Lund University, Sweden.

Results

The Phosphatocopina

Phosphatocopines formed important faunal elements of the lower trophic levels in the Cambrian marine ecosystems (Maas *et al.* 2003). Their basal position in Cambrian food webs is suggested for example by their presence in coprolites (Eriksson & Terfelt 2007). They first appeared in Cambrian Epoch 2 and became diverse and abundant in the latest Guzhangian and Furongian and disappeared from the fossil record in provisional Age 10 of the Furongian Epoch (Williams *et al*. 2011). The taxon Phosphatocopina has been demonstrated to represent the sister-group of the Eucrustacea within the Labrophora *sensu* Siveter *et al*. (2003) based on a number of synapomorphic characters mainly concerning the food-intake and food-processing apparatus (see also Maas *et al*. 2003; Maas & Waloszek 2005).

Phosphatocopines are the most common and diversely represented ‘Orsten’ fossils. They are thought, like most or all of the Swedish ‘Orsten’ taxa, to have been part of the meiofauna (Maas *et al*. 2006) and appear to have been adapted to seawater hypoxia (Williams *et al*. 2011). In their monograph, Maas *et al*. (2003) (re-)described in detail 14 phosphatocopine species from the Cambrian Series 3 and Furongian Swedish ‘Orsten’, including the species analysed herein.

Similar to most other taxa known only from fossil material, phosphatocopine specimens are rarely preserved with soft parts; commonly only the isolated external shields are found. The ratio of specimens with soft parts (c. 2000) vs. those represented by isolated valves or shields (c. 50 000) in the material collected by Klaus Müller is approximately 1:25. Phosphatocopines most likely were able to enclose their body proper and all ventral soft body parts into the laterally strongly extended shield. In this respect they strikingly resemble ostracod crustaceans with their bivalved shields, which is why phosphatocopines were assigned to ostracods until the end of the last century (Maas *et al*. 2003). Specimens with ventral soft body parts preserved are predominantly known from the Cambrian Series 3 and Furongian Swedish ‘Orsten’ (Müller 1979, 1982; Maas *et al*. 2003, 2006; Eriksson *et al*. 2012). However, reports of such specimens are steadily increasing from other areas and discoveries have subsequently been made also from the “lower” through “upper” Cambrian of China (Dong *et al*.

2005a; Zhang *et al*. 2011, 2012; Zhang & Pratt 2012), Germany (Gründel & Buchholz 1981; Hinz-Schallreuter & Koppka 1996), Great Britain (Hinz 1987; Siveter *et al*. 2001, 2003, see also Zhang *et al*. 2012), Poland (Maas *et al*. 2006), Australia (Walossek *et al*. 1993), and Siberia (Müller *et al.* 1995). This wide geographical distribution, which is even greater including also the record of isolated shields, together with the short ranges of certain species, makes phosphatocopines potentially important biostratigraphic tools (Hinz-Schallreuter 2000; Maas & Waloszek 2005).

Some phosphatocopines could close their shields completely in life whereas others seem to have had permanently, more or less gaping valves, suggested also by the abundance of specimens preserved in "butterfly" condition (Fig. 1A; Müller 1982, 1985; Siveter *et al*. 2001; Maas *et al*. 2003; Williams *et al*. 2011). However, a muscle system that was able to open and close the valves – as is present in ostracod crustaceans – is lacking.

In well preserved specimens with their shields open, and in which exposed ventral body parts and limbs are common, it is a straight forward task to appreciate the complete morphology including soft part details. Data on the morphology of specimens with enclosed shields are restricted to those in which one of the shields is preservationally absent (Müller 1982; see also Maas et al. 2003, e.g., their pls. 3E, 7A, 10A). Morphological data of completely closed specimens could significantly add knowledge to the life style of phosphatocopines, since the exact orientation of the limbs and their setae inside the closed shields can be observed. For this study specimens belonging to two species, *Hesslandona angustata* Maas, Waloszek & Müller, 2003 and *H. ventrospinata* Gründel *in* Gründel & Buchholz, 1981, were analysed. For additional information on these species and full taxonomic descriptions, see also Maas *et al*. (2003) and Zhang *et al*. (2011, 2012).

Open phosphatocopine specimens

As an example of the general phosphatocopine morphology and for comparison with the enclosed varieties described below, a nicely preserved specimen (LO 12163t) with completely open shields, i.e., "butterfly position", is depicted in Fig. 1, illustrated using SEM (Fig. 1A, G), light microscopy (Fig. 1C) and SRXTM (Fig. 1B, D–F). The descriptive phosphatocopine terminology used follows that of Maas *et al*. (2003).

Specimen LO 12163t measures c. 150 µm along the hinge line (dorsal length) and has completely smooth external shields. The specimen has four pairs of appendages, i.e., the (broken-off) antennulae (or first antennae), the antennae (or second antennae), the mandibles and one postmandibular appendage (Fig. 1). Based on the small size and particularly the presence of appendages, the specimen at hand represents one of the earliest growth stages, maybe even the hatching stage, a so-called 'head-larva' (cf. Maas *et al*. 2003). The latter is characterized by its four pairs of appendages and, accordingly, four limb-bearing segments, which reflects the segmental composition of the head in the euarthropod ground pattern (see Walossek & Müller 1990).

It is difficult to assign very small phosphatocopine specimens, or the early ontogenetic growth stages, to specific species because the interspecific variability is considerably less expressed compared to that of larger specimens. However, based on the appearance of the limb stem of the mandible (Fig. 1), this specimen is assigned to *Hesslandona angustata*, for which Zhang *et al.* (2012) demonstrated a two-divided limb stem (coxa and basipod) for young larvae. By comparison, the morphologically closely similar and most well-known phosphatocopine species, *Hesslandona unisulcata*, have youngest growth stages possessing the adult condition of an undivided limb stem, i.e., coxa and basipod are fused to a degree that the basipod is only recognizable by its endite (Müller 1982; Maas *et al*. 2003). Moreover, the smooth shields of LO 12163t fit with those of *H. angustata*, whereas those of *H. unisulcata* have a prominent lobe in the anterior region (Maas *et al*. 2003). However, it should be noted that this condition could be attributed to the young growth stage.

Early phosphatocopine growth stages are overall rather poorly known and thus there are still some gaps in our knowledge of the detailed morphology of these stages, in particular with regards to the posterior part of the body (cf. Maas *et al*. 2003). Also for that reason specimen LO 12163t is interesting because it represents an early growth stage with well-preserved ventral soft body parts and a largely complete, albeit rather coarsely preserved, hind body (Fig. 1A, F). The hind body is triangular in shape, tapering posteriorly but no furca can be detected, which is present at least in more advanced stages of some species (Maas *et al.* 2003, their plates 8F, 12D, 36D).

The minute disc-shaped structures, c. 10 μm in diameter, present in the posteriormost region of the sternum (Fig. 1A) most probably comprise diagenetic artefacts that resulted in microgranular phosphate. Similar structures have been observed also in other 'Orsten' phosphatocopine specimens, see Maas *et al*. (2003, their plates 33A, B, 45A, B, fig. 62; see also below).

When observed under a light microscope (Fig. 1C) the appendages of LO 12163t are hyaline in appearance, as opposed to the phosphatized ventral body parts being brownish to black opaque as in specimens observed previously (personal observations by MEE, FT and AM). A semi-quantitative (EDX) elemental mapping proved them nonetheless, and as expected, to be composed of calcium phosphate (Fig. 1G) and the hyaline appearance may simply be a consequence of their small size. However, systematic analyses on additional specimens are needed in order to clarify if this feature could be attributed to different relations of calcium and phosphorous (and possibly also other elements).

Semi-enclosed phosphatocopine specimens

Several 'Orsten' phosphatocopines are found with their valves only slightly open. In many cases these specimens reveal the presence of preserved ventral soft body structures (= soft-cuticular structures). Herein, a nicely preserved semi-enclosed specimen (LO 12164t) of *Hesslandona angustata* was recovered from the same sample as LO 12163t. This specimen measures 285 μm (maximum dorsal length) along the hinge-line and possesses two smooth shields. A number of ventral soft tissue structures are preserved (Fig. 2A). These can be observed through the slightly gaping shields and, from anterior to posterior, part of an antennula, a nicely preserved labrum, and parts of the mandibles are protruding inwards and largely cover the sternum.

Using SRXTM to virtually open up the specimen, the full extent of the pristine preservation becomes clear and also allows closer inspection of the number of appendages and their morphology (Fig. 2E–G, H–L). The specimen has four to five pairs of appendages that are very well preserved. The limbs of the right side (Fig. 2A–C, E) seem to be flipped posteriorly, whereas those on the left side, at least antenna and mandible, are flipped anteriorly. The transition between the mandible and the sternum is uncertain. At least one additional pair of limbs is present, if not even two. Based on the presence of one or possibly two postmandibular limbs, combined with the size and general morphology, specimen LO 12164t belongs to possible growth stage II, if not stage III (see Maas *et al*. 2003).

Enclosed within the shields are three microspherules. The largest and most distinct one measures c. 30 μm and is situated between the antenna and the mandible close to the shield edge on the right side (Fig. 2E). The other two spherules are sub-spherical and approximately 17 μm in diameter. One is situated close to the anterior transition between hypostome and labrum and the other is sited posterior to the left antenna (Fig. 2F, G). The appearance of the spherules in the axial tomographic slices and semi-transparent SRXTM-data (Fig. 2E–G) suggests that they have the same density and thus a closely similar composition (calcium phosphate), as the phosphatocopine specimen itself (elemental mapping was not possible). Whereas the smaller spherules have a smooth surface, the largest one has a characteristic external pattern of minute disc-shaped structures or plates that seem to cover most of the surface of the spherule (Fig. 2O). The latter most likely represent preservational artefacts, as similar structures were observed both at the antero-dorsal edge of the labrum of this specimen and in the posterior part of the sternum of the open specimen LO 12163t, as mentioned above (see Fig. 1A).

Completely enclosed specimens

Phosphatocopines with their shields completely closed obviously reveal only the external shield morphology. In some specimens (such as LO 12165t), however, the shields are semi-hyaline and there is a possibility of observing at least the presence of ventral body parts and soft tissue structures already in a regular light microscope. However, their state of preservation and details of any sort are merely guesswork. In such cases SRXTM opens up new possibilities. It should be noted that most of the analysed, seemingly well-preserved and fully enclosed specimens with tightly fitting shields were completely barren of ventral soft tissue structures. This suggests that the chance of exceptional soft tissue preservation and phosphatisation increases when parts of the shields are ripped open (cf. Eriksson *et al*. 2012).

Specimen LO 12165t is well preserved although parts of the shields are slightly cracked and deformed (Fig. 3A–G). The specimen has smooth shields and measures 363 µm along the straight hinge line (maximum dorsal length). Based on the external shield morphology, the specimen is assigned to *Hesslandona ventrospinata*. Although the characteristic shield lobes of this species are lacking in specimen LO 12165t (most likely because of its young ontogenetic stage), the postero-ventral margin of both shields is slightly drawn out into short spines again being much less significant than in more advanced stages (Fig. 3; cf. Maas *et al.* 2003, their plates 19–21). The spines on the interdorsum in *H. ventrospinata* seem to appear in advanced stages and are less conspicuous or even lacking in early stages, as is the case in the specimen LO 12165t.

By virtually scraping off the shields a whole array of well-preserved, concealed soft tissue structures can be observed. Because the specimen seems to be filled also with some matrix, it is difficult to isolate all details, in spite of the fact that SRXTM is used. Viewed from the side and peeking through the shield, very nicely preserved limbs are exposed with their endites, curved exopods and complete setae (Figs. 3H, 4). The internal soft tissue structures seem to be hanging from the hinge line and has partially detached from the inner part of the shield (Fig. 3I). At least four but probably five postmandibular limbs can be observed in the specimen (Fig. 4). When virtually cutting down into the labrum a distinct pattern of string-like structures unfold (Fig. 4D, E). These probably represent paired labral muscles (cf. Eriksson *et al.* 2012; see also and below).

The ontogeny of the species is unknown, the previously smallest identified specimen is about 800 µm in length, but of which limb details are unknown (Maas *et al.* 2003, their plate 21D). Based on the presence of four or possibly five postmandibular limbs combined with the general morphology, specimen LO 12165t represents growth stage V or VI, despite its relatively small size.

Discussion

Microspherules and phosphatocopine feeding biology

The microspherules enveloped between the shields of specimen LO 12164t merit some comments as similar objects have not previously been recorded in phosphatocopines. The position and appearance of the microspherules, particularly the characteristic largest spherule 1 (Fig. 2O), could suggest that they represent eggs or embryos. This would make specimen LO 12164t a pregnant female. However, fossil arthropods with eggs and embryos *in situ* are exceptionally rare (Siveter *et al.* 2007, 2014; Duan *et al.* 2014). Moreover, the reproduction of phosphatocopines and at what ontogenetic stage they reached sexual maturity is not known, and there is no evidence for a clear distinction between sexes (cf. Maas *et al.* 2003). Given that specimen LO 12164t is an inferred larval stage – the adults may have reached sizes up to 3 mm – it is unlikely that the microspherules represent eggs or embryos. The distinctive external surface pattern of spherule 1 is reminiscent of the blastular cleavage pattern observed in some fossil and extant embryos (e.g., Dong *et al*. 2005b, fig. 1A, B; Raff *et al*. 2006, fig. 1). However, the detailed surface pattern configuration, irregularity and size, combined with a lack of internal structures in the SRXTM data (cf. Donoghue *et al*. 2006), rather suggests diagenetically formed microgranular phosphate.

Because the microspherules appear to have the same density and composition (Fig. 2E–G) as the phosphatocopine specimen they could simply represent diagenetic, authigenic phosphatic granules that formed within the shields. However, it is also possible that they represent phosphatised (green) algal remains that were trapped within the slightly gaping shields post mortem or undigested food particles. Extant crustacean individuals from marine or fresh-water environments are also frequently caught with food particles *in situ*. This brings us to the feeding biology of phosphatocopines.

The form of the mouth area with surrounding appendages allows a discussion of its function. A ventral view (Fig. 1A) of a young *H. angustata* exposes an enclosed space more or less rounded with a diameter of approximately 50 µm forming an oral cavity roofed by the paragnaths. The walls of this space are formed anteriorly by the labrum and followed posteriorly by the basal portions of the (second) antennae, mandibles, and the first postoral appendages. The basal portions of the appendages are wedge-shaped with the pointed side facing the cavity (Fig. 1A). Together the labrum and appendages seem to seal the space effectively (cf. Waloszek *et al.* 2007, e.g. fig 5). The coxa (only in

antenna and mandible) and basipods of the appendages are equipped with strong endites and spines (Figs. 1A, 2A; see also Maas *et al*. 2003, pl. 27) pointing towards the center of the enclosed space and spanning the area. A profuse setal armament of the exo- and endopods finishes the basket formed in front of the mouth. Later stages of this and other species retain this arrangement but the postoral feeding cavity is enlarged posteriorly by adding more appendages and extending the sternitic area. The result is a larger and more oval or elongated space (see Maas *et al*. 2003) still with the spines of the appendages meeting medially. The postmandibular limbs have anteromedially directed spines and setae which suggest that they could enable food to be passed orally and to prevent backflow.

Müller (1979) considered the phosphatocopines to be nektobenthic swimmers. The long exopods of the antennae and mandibles were considered to be used for locomotion and raking in plankton (sweep-net feeding). The rich supply of setae (bristles) on the appendages was supposed to monitor these two types of function.

Our interpretation of the feeding function is at variance with the original view of Müller in that the organization of the oral cavity and the morphology of the surrounding appendages indicate particle feeding. The support for this interpretation is the functional morphology of extant crustaceans being filter feeders and observations of feeding in small bivalved crustaceans.

Dahl (1956) showed that the topography of the head is correlated with the mode of feeding in extant Crustacea. Filter feeding is coupled with a large labrum directed posteriorly along the ventral surface. The labrum and body create a preoral cavity, which receives the filtered food driven anteriorly by the stream caused by the movements of the appendages. Gland secretion entangles the food which is then sucked into the foregut. The mechanism for this function is revealed in Cephalocarida (Elofsson *et al*. 1992) and Mystacocarida (Elofsson & Hessler 2010). A large labrum would hamper the access to larger particles and consequently in particle feeders the labrum is shorter and directed more ventrally which allows the food particles to be presented in front of the mouth.

A main feeding type among free-living extant crustaceans in the size range of the Phosphatocopina is filter feeding, as seen for example in some ostracods or cladoceran branchiopods which both have large bivalved shields. The mechanism for this function is discussed by Walossek (1993). Yet filter feeding demands the presence of a filter apparatus provided by, e.g., appendages with specific filter setae and sucking chambers. These are developed only in particular crustacean taxa, e.g., in branchiopods (e.g. Fryer 1987; Walossek 1993). The filter feeding apparatus of species of the cladoceran taxon *Daphnia,* which are about 2 mm in body size, is seemingly specialized on particles with an average size of 0.1–1.0 μm, with mean sizes of the filter meshes of about 0.4–0.7 μm provided by fine setulae on the main setae of the limbs (Gophen & Geller 1984).

The topography of the structures in the oral surroundings and the coarse setation and presence of strong spines on the antennal and mandibular endites in *H. angustata*, in contrast to the dense network of setae and setulae of *Daphnia* species, speaks in favor of particle feeding, rather than filter feeding, allowing for sizes below 50 μm to be handled. We presume that the animals used their strong endites and spines on the antennae, mandible (particularly those of the endopod portions) and postmandibular appendages for pushing food toward and stuffing it into the mouth opening. Large toothed median edges of the mandibular coxa of several phosphatocopine species (Maas *et al*. 2003, their pl. 25A), large paragnaths and fine bristles on the sternal surface speak in favor of particle feeding in phosphatocopines in general. The size of the appendages indicates strong muscles, a prerequisite for handling particle food. The inferred muscles detected in the labrum of *H. ventrospinata* (Fig. 4) and other *Hesslandona* species (Eriksson *et al*. 2012, fig. 4) are interpreted as being used for moving the labrum up and down and thus opening up the buccal cavity and facilitating ingestion. The microspherules found in the *H. angustata* specimen LO 12164t (Fig. 2), regardless whether they represent food particles or inorganic structures, fall within the size range (30 μm and less) for food manageable by the appendages with their coarse setation. The presumed nektobenthic habitat of phosphatocopines is also compatible with particle feeding. A possible dual function of the setae implicit in the suggestion by Müller (1979) is difficult to prove in the fossil record. The functions as rakes or paddles of extant crustaceans may, in some instances, differ with simple changes in ultrastructural design (see Cheer & Koehl 1987).

Cannon (1926) studied the feeding of the fresh water ostracod *Pinocypris vidua,* an animal in the size and shape of a phosphatocopine and occupying a habitat similar to that presumed for phosphatocopines. The ostracod stirs up most of the food to be eaten with the antennae. The particles

inside the shell are then transported towards the mouth by a current set up by vibratory plates on the mandibular palp and the exopodite maxillulae (the details of the process as well as auxiliary structures in the oral cavity are not elaborated on here). Depending on the number, size, morphology and setal armature of the limbs the path of food-loaded water into the feeding chamber and its further processing may differ between taxa (Walossek 1993, se his figure 49 for a comparison between a phyllocarid malacostracan and the 'Orsten' branchiopod *Rehbachidella kinnekullensis* Müller 1983). Gland secretion helps to keep particles together and it is highly probable that it was part of the feeding process also in phosphatocopines. The phosphatocopine labrum is equipped with pores (interpreted as glands) at the posterior side (Maas *et al*. 2003 their pl. 3G; Waloszek 2003a his fig 3; also Waloszek *et al*. 2007, their fig 5F) that may have been producing slime to entangle the food.

While the antennae and mandibles are inferably responsible for food capturing, the phopshatocopine antennula is not, in our opinion, involved in this process. Similarly, the antennulae of *P. vidua* are trailed over the back and comprise no swimming appendages, but are rather sensory (Cannon 1926, text-fig. 5). The observation by Cannon (1926) on the position and movements of the antennulae has implications on the antennulae of the phosphatocopines. Their basal portions do not take any part in the design of the oral basket and they are also directed more anteriorly and are less strong (e.g., Maas *et al.* 2003, pl. 9A) than the posterior appendages. A distinct separation has thus taken place in the function of the antennulae (first antennae) and antennae (second antennae). Whereas the antennae are involved in locomotion and food processing the antennulae are free for other tasks like sensory perception. This most likely happened in the ground pattern of Labrophora, when antenna and mandible became even more specialized for food gathering (see below).

The question now arises if the evaluation of their feeding procedure has bearing on phylogenetic discussions of the taxon Phosphatocopina. Fossil remains of fairly large early Cambrian arthropods from Mount Cap Formation, Northwest Territories in Canada, exhibits mouthparts allowing sophisticated handling of food particles (Harvey & Butterfield 2008). The degree of differentiation of the feeding appendages of the Mount Cap crustaceans is far more advanced than those seen in the phosphatocopines. This means that the particle feeding style advocated here for the phosphatocopines was not unique and had been successfully evolved already in the early Cambrian and is, most likely, already part of the ground pattern of Labrophora and, hence, plesiomorphic for Phosphatocopina and Eucrustacea.

According to Müller (1979) the antennula was engaged in the phosphatocopine food gathering. However, our re-evaluation of the phosphatocopine feeding mechanism suggests that this was not the case. In the ground pattern of Arthropoda s. str. the antennula was (still) the only appendage that was involved in food gathering (Waloszek *et al.* 2005, 2007), a task taken over into the crustacean ground pattern, but in which the succeeding two appendages autapomorphically added to this function (Haug *et al.* 2013). In the labrophoran ground pattern the cephalic feeding apparatus was further enhanced by the development of food-processing structures such as coxae on the antenna and mandible together with median prolongations and spines, enditic protrusions and a specific setation, the labrum and sternum with paragnaths (Maas *et al.* 2003; Siveter *et al.* 2003; Waloszek 2003b). The minute size of the antennula of the phosphatocopines with its few distal setae (Maas *et al.* 2003, their pl. 11A) deviates and their morphology and function has, for some reason, shifted. Maas *et al.* (2003) interpreted the antennular design as correlated with the presence of a bivalved shield, into which all appendages should fit when closed. Other factors may have been the presence of a huge median eye (Maas *et al.* 2003, their pl. 3B) and the necessity to guide water and food current behind the labrum. Later within eucrustacean evolution more appendages ("maxillula", "maxilla", "maxillipeds") became, partly independently, specialized and involved in food gathering and processing (cf. Haug *et al.* 2013), still being serial "post-mandibular" limbs in phophatocopines. Our suggestion of a shift in function of the antennulae to a predominantly sensory appendage, like in extant crustaceans, already in phosphatocopines merits consideration.

Synchrotron X-ray tomographic microscopy

Synchrotron X-ray tomographic microscopy (SRXTM) has been successfully applied to a number of different animal and plant fossils over the last few years, and has come to revolutionize the field of palaeontology and palaeobiology by making previously inaccessible views of internal structures possible (e.g., Donoghue *et al*. 2006; Tafforeau *et al.* 2006; Huldtgren *et al.* 2011; Eriksson & Terfelt 2012; Eriksson *et al*. 2012; Friis *et al*. 2014). The analyses and rendered X-ray tomographic datasets result in complete two and three-dimensional internal recordings at a sub-micrometre resolution. Thus, it has facilitated the study of concealed internal structures allowing increased understanding of not only functional morphology, animal anatomy, and digestive systems but also revealed structures crucial for our understanding of the phylogeny of long extinct organisms. Moreover, being a non-invasive technique, it allows for unique *Lagerstätte* fossils and/or type specimens to be studied without destroying them in the process.

The exceptionally preserved 'Orsten' fossils have a long and successful research history since Müller's discovery in 1979. Their potential also in answering questions concerning the evolution of arthropods has become evident shortly after the first works (Walossek & Müller 1990; see also review by Maas *et al*. 2006, and references therein). These fossils have traditionally been analysed using scanning electron microscopy (SEM) as the primary investigative tool. Over the years also other techniques have evolved, all with the aim of aiding in visualizing and understanding these extraordinary fossils, including the production of stereo SEM images and other 3D techniques using plasticine modeling (Müller & Walossek 1988), virtual modeling (Stein *et al*. 2008), light microscopy (Haug *et al*. 2009a), and autofluorescence microscopy (Haug *et al*. 2011).

SEM is still superior in producing high-resolution digital images of morphological details of the external surface but provides no information on internal structures, unless the specimens are fragmented or somehow can be sectioned. For those reasons SRXTM provides a powerful complementary tool with significant applications to material of the size, preservation and composition as that represented by the Cambrian 'Orsten' fossils. Thus, the method has great potential of improving our understanding of that biota (Eriksson & Terfelt 2012; Eriksson *et al*. 2012). For open phosphatocopine specimens SRXTM data allows not only reconstructions of external and inaccessible surfaces (as well as virtual serial sections; e.g., Zhang & Pratt, 2012) and concealed, internal soft tissue structures (Eriksson *et al*. 2012) but it also allows the specimen to be rotated, digitally manipulated and viewed from all angles. This is an advantage over SEM (the side, on which the specimen is glued to the stub cannot be investigated) and other conventional techniques as it does not require the fragile specimens to be moved on the sample stubs.

For phosphatocopines with closed or semi-closed shields the resulting tomographic data allows for new structures to be revealed in previously known taxa as well as for new taxa to be identified. Hence, we do not longer have to rely on, or await, serendipitous finds of specimens with partial shield preservation and/or open specimens of taxa that are known from closed shields only. This is of particular importance when we only have access to limited (type) material or have found one or very few unusual specimens. It also offers the possibility to isolate specific morphological structures and identify their ontogenetic growth stage, a difficult task based on external observations, particularly since size is not a good parameter for this (Maas *et al*. 2003).

Another significant advantage is that specimens require virtually no preparation for SRXTM and numerous specimens can be rapidly screened (in this study each specimen was screened in less than five minutes), whether searching for specific features and characters or studying aspects or quality of preservation (cf. Friis *et al*. 2014). On the downside, the post-screening data processing can be quite time-consuming and basically the effort of the investigator is thus shifted from preparation work in the laboratory to computer-based analysis and visualization. However, it offers the possibility of making attractive 3D images and animated movies that perfectly fit for both specialists and generalists and thus can be successfully used also for scientific outreach purposes, which are becoming increasingly important particularly with the growing demand of public engagement and knowledge utilization of funding bodies (Lautenschlager & Rücklin 2014).

Whereas SEM will probably continue to be the technique of choice for 'Orsten' fossils, due to its high resolution surface visualization capabilities allowing even for the detection of minute surface structures such as fine hairs, pores and sensilla (for phosphatocopines, see Maas *et al*. 2003; see also, e.g., Müller & Walossek 1987, 1988; Haug *et al.* 2009b, 2010), SRXTM is likely to prove particularly valuable for studying concealed structures. Moreover, because an important part of understanding of

the organization and structure of the 'Orsten' fossils is comparison with similar structures in putatively closely related extant taxa, SRXTM can be used to study preserved material of living microscopic arthropods to provide direct comparison with the fossil material. We predict that both techniques in combination open up new potentials and further consolidate the significance of 'Orsten' fossils in contributing data for phylogenetic and evolutionary considerations.

Conclusions

Synchrotron radiation X-ray tomographic microscopy (SRXTM) is a powerful micropalaeontological tool that can be successfully used to virtually dissect and non-destructively reveal inaccessible structures of 'Orsten' phosphatocopine crustaceans, and similar fossils, in high resolution. Combined with scanning electron microscopy (SEM), SRXTM allows new studies and increased understanding of the unique 'Orsten' fossils and their morphology and anatomy. The SRXTM method has the potential to not only unravel new information about these organisms per se; such data may serve also as templates for our understanding of the morphology of Cambrian organisms in general, and thereby also offer insights into the biology, functional morphology, and phylogeny of these half a billion year old organisms. In this study we also re-evaluated the feeding biology of phosphatocopines and suggest that they were particle feeders.

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FIGURES

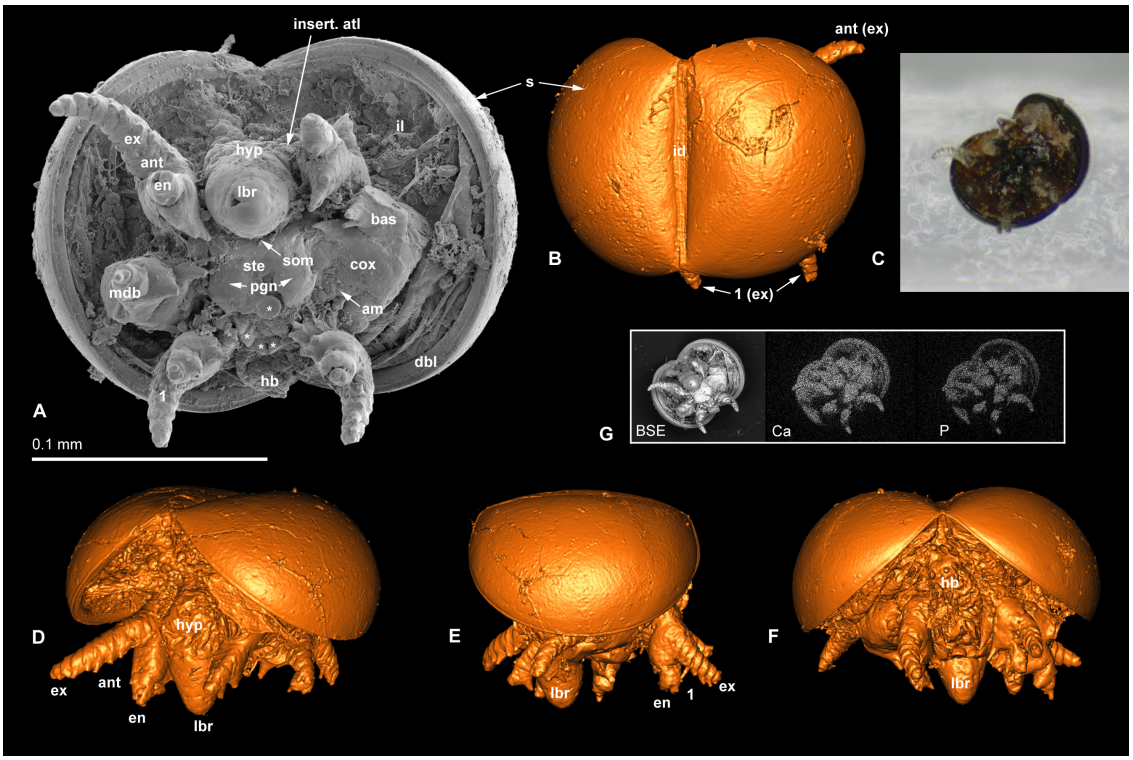

Fig. 1. A specimen of *Hesslandona angustata* (LO 12163t) with open shields ("butterfly position") from the uppermost Cambrian Series 3 (*Agnostus pisiformis* Zone) at Sandtorp, Mount Kinnekulle, Västergötland, Sweden. The well preserved specimen represents a very early ontogenetic stage, probably a head-larva stage. Antennula only present as insertion. A, SEM-micrograph in ventral view. Note that the exopod of the left antenna was accidentally broken when the specimen was removed from the fishing line and placed onto a SEM stub. B, D–F, 3D-rendering (isosurface) of a SRXTM dataset in different views; B, dorsal view. D, antero-lateral view. E, lateral view. F, posterior view. C, light microscopy photograph of specimen revealing the hyaline appearance of the appendages. G, elemental mapping including a backscatter SEM image (BSE) and the distribution of calcium (Ca) and phosphorous (P). The relative amount of an element is indicated by the brightness; the brighter the tone, the higher the level of a certain element. Abbreviated descriptive terms: am, arthrodial membrane; ant, antenna (or "second antenna"); bas, basipod; cox, coxa; dbl, doublure; en, endopod; ex, exopod; hb, hind body; hyp, hypostome; il, inner lamella; insert. atl, insertion of antennula (or "first antenna"); lbr, labrum; mdb, mandible; pgn, paragnaths; s, shield; som, site of mouth (located proximally on the posterior side of the labrum); ste, sternum; 1, first post mandibular limb. The asterisks (*) mark small diagenetic phosphatic microspherules.

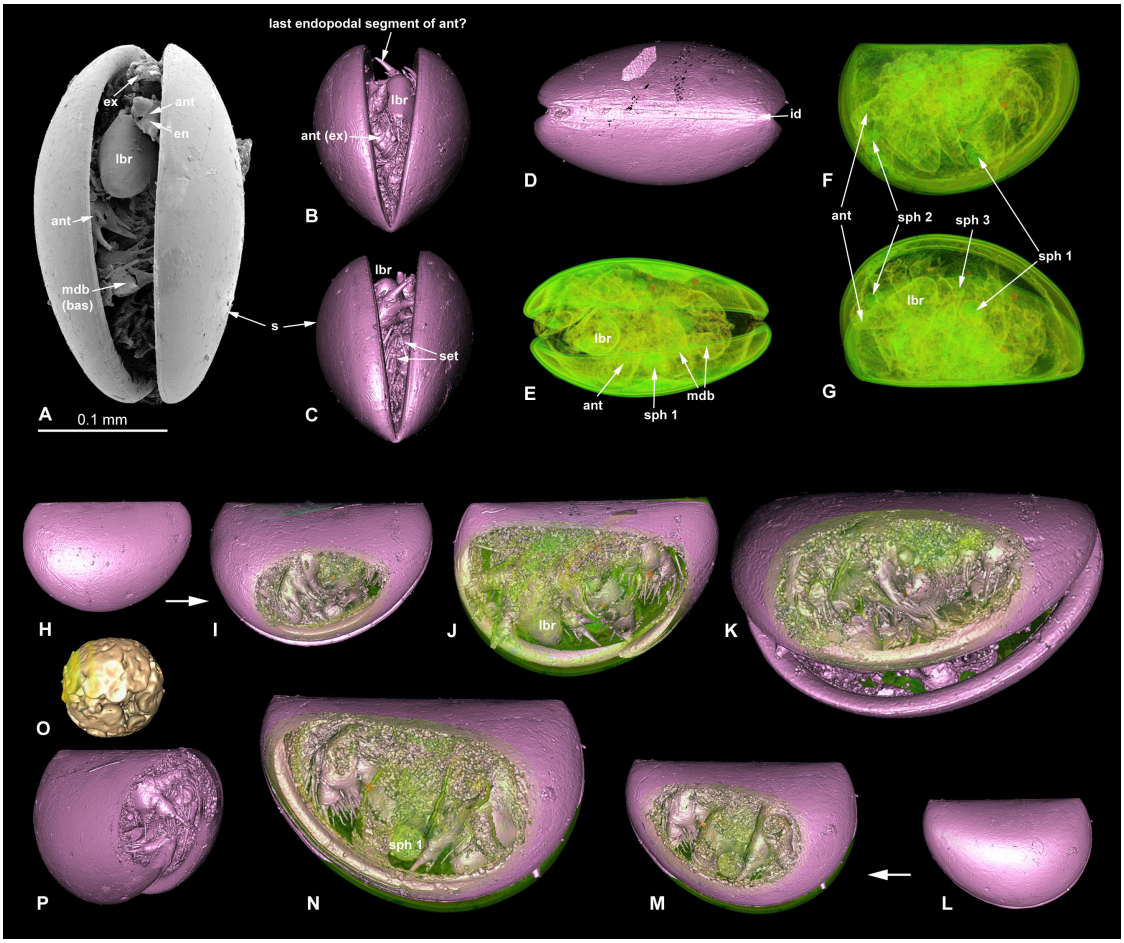

Fig. 2. A specimen of *Hesslandona angustata* (LO 12164t) with slightly open shields from the uppermost of Cambrian Series 3 (*Agnostus pisiformis* Zone) at Sandtorp, Mount Kinnekulle, Västergötland, Sweden. The specimen represents a possible growth stage II or III. A, SEM-micrograph of specimen in ventral view. B–L, 3D-rendering of a SRXTM dataset in different views; B, view from posterior. C, view from anterior. D, dorsal view. E–G, transparent images in ventral (E), lateral (F) and oblique lateral (G) views. The reddish dots represent heavier minerals, probably pyrite. H–N, a series of combined isosurface and transparent images with H–K, continuously deeper virtual cuts through lateral side with anterior portion facing left, and L–N, with anterior portion facing right. O, close-up of isolated spherule 1 (cf. Figs 2E–G, N). P, postero-lateral view displaying the hind body. Abbreviations as in Fig. 1, additionally: sph 1–3, spherule 1–3; set, setae. Scale bar refers to SEM-image (Fig. 2A).

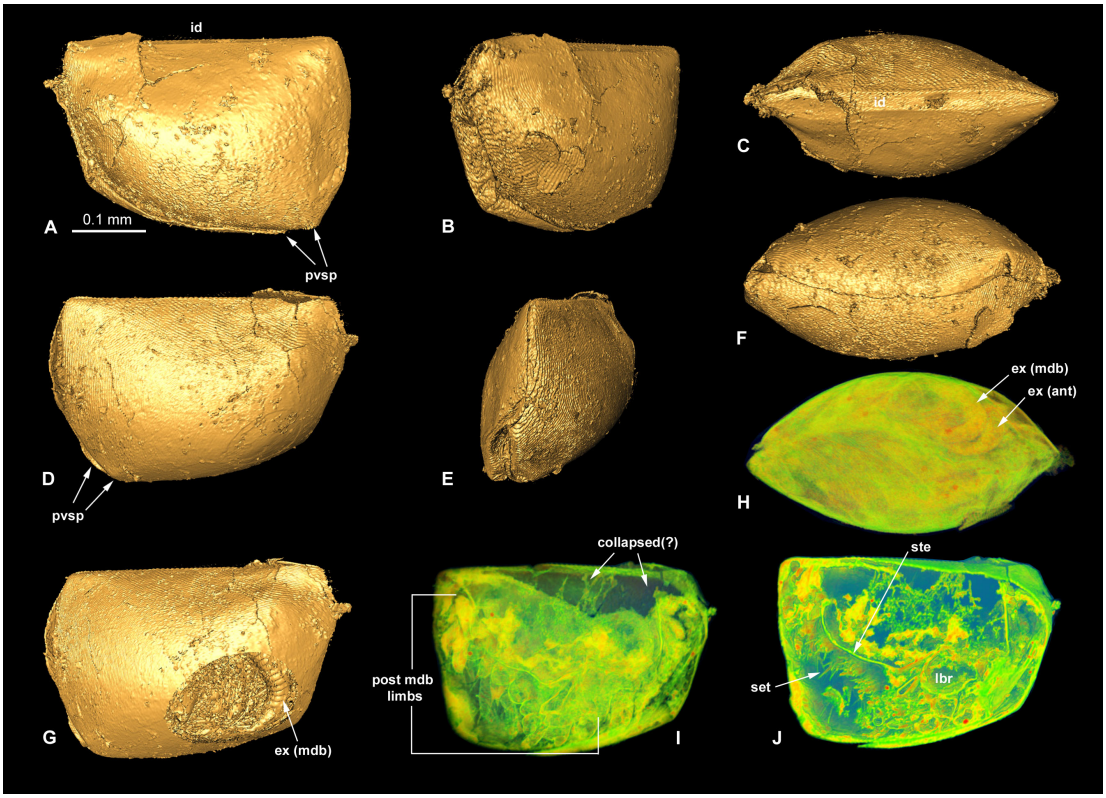

Fig. 3. A specimen of *Hesslandona ventrospinata* (LO 12165t) with completely enclosed shield from the uppermost of Cambrian Series 3 (*Agnostus pisiformis* Zone) at Transformatorstationen, Mount Kinnekulle, Västergötland, Sweden. A–G, 3D-rendering (isosurface) of a SRXTM dataset in different views; A, lateral view of left valve. B, antero-lateral view. C, dorsal view. D, lateral view of right valve. E, postero-lateral view. F, ventral view. G, lateral view with virtual cut through the shield and exhibiting parts of the mandibular exopod. H–J, 3D-rendering (semi-transparent images) of a SRXTM dataset in different views; H, ventral view. I, lateral view. J. lateral view, and specimen partly cut virtually. Abbreviations as in Figs. 1 and 2, additionally: pvsp, postero-ventral spine.

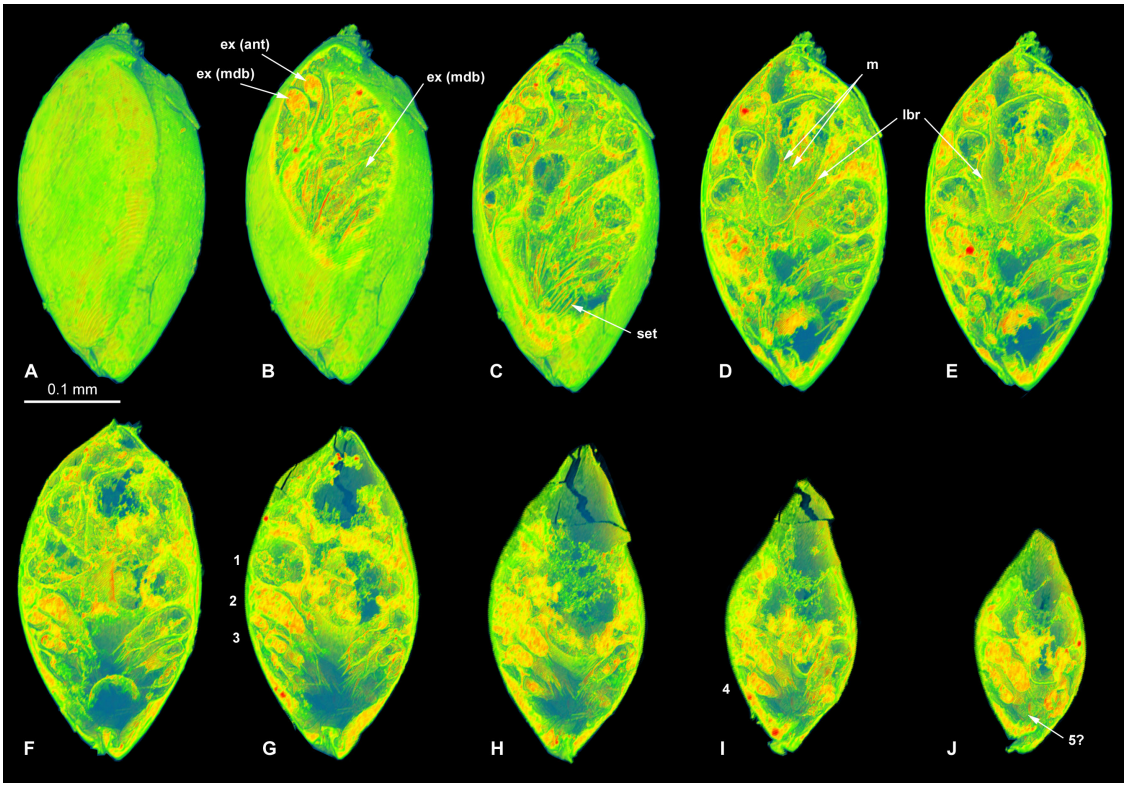

Fig. 4. 3D-rendering (semi-transparent images) of a SRXTM dataset of *Hesslandona ventrospinata* (LO 12165t); same specimen as in Fig. 3. A–J, a series of continuously deeper cuts from ventral toward dorsal presenting the internal soft tissue structures and appendages. Abbreviations as in Figs. 1 and 2, additionally: m, muscle; 1–5 refer to post-mandibular limbs.