Experimental mineralization of invertebrate eggs and the preservation of Neoproterozoic embryos

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ABSTRACT

Understanding the processes that led to the mineralization of Precambrian metazoan eggs and embryos is essential to informed interpretations of such fossils and their significance in early metazoan evolution. Here we show that rapid mineralization of invertebrate eggs is possible under laboratory conditions. Under anaerobic conditions, eggs become coated in mainly calcium carbonate within three weeks. Preservation of the external morphology is comparable to that of fossil material, but no internal mineralization was observed in the laboratory. This is the first report of the laboratory mineralization of metazoan eggs in the absence of a decaying carcass, and demonstrates that eggs, and probably small embryos, can be preserved in the absence of larger organisms as a source of phosphorus or calcium. Thus, it is possible for organisms of this size to have been fossilized prior to the evolution of large metazoans.

Keywords: Neoproterozoic, eggs, Metazoa, fossilization, mineralization.

INTRODUCTION

The enigma of early metazoan evolution is the apparent discrepancy between the first appearance of abundant, unambiguous, fossilized metazoans in the Cambrian Period (543 Ma) and ''molecular clock'' estimates from ca. 670 Ma to 1500 Ma for the divergence of the Metazoa (Ayala et al., 1998; Wang et al., 1999; Hausdorf, 2000; Peterson et al., 2000). Possible reasons for the paucity of metazoans in the Precambrian fossil record include the lack of hard parts for fossilization and the possibility that Precambrian metazoans were small and thus easily degraded (Davidson et al., 1995; Fortey et al., 1996). Mineralization of soft tissue in phosphate preserves the finest detail and has been documented in the post-Cambrian fossil record (Martill, 1988, 1990; Wilby and Briggs, 1997), whereas fossils of putative metazoan eggs and embryos preserved in phosphate have been found in Cambrian deposits (Zhang and Pratt, 1994; Bengtson and Zhao, 1997). However, recent discoveries of three-dimensionally preserved metazoan eggs and embryos from Neoproterozoic deposits have begun to push back the metazoan fossil record into Precambrian time (Xiao et al., 1998; Xiao and Knoll, 1999). Fossilized embryological material, particularly at relatively advanced stages of development, will play an increasingly important role in unraveling the origins of the Metazoa and their embryology and developmental characteristics. Understanding their taphonomy is critical to (1) determining target lithologies for

collecting and processing, (2) resolving the degree to which the morphology has been altered in the mineralized fossil, and (3) establishing the mineralization potential of a range of taxa (Xiao and Knoll, 1999).

Mineralization must take place quickly to prevent collapse of soft tissues and to preserve them in three dimensions. Experiments provide unequivocal evidence of the conditions under which mineralization of soft tissues can take place; only inferences can be drawn from the fossils (Briggs, 1995). Rapid mineralization by calcium carbonate and apatite has been documented in the laboratory and is associated with enhanced bacterial activity and anaerobic decay. However, the extent of mineralization is variable, and phosphatization of labile tissues (e.g., muscle) has only been achieved experimentally in shrimps, usually adjacent to the exoskeleton (Briggs and Kear, 1993, 1994; Hof and Briggs, 1997; Sagemann et al., 1999).

Here we present experimental results that show that rapid mineralization of invertebrate eggs can be induced under laboratory conditions. This approach will enhance our understanding of the conditions under which embryos can become fossilized, which taxa are likely to be represented in this way in the fossil record, and the lithologies where Precambrian metazoan fossils are likely to be found.

METHODS

Fresh eggs (equivalent to 520 mg dry mass, ~350 eggs) from the European lobster (*Hom-arus gammarus* L.) were used in all experiments. The eggs were collected from the National Lobster Hatchery, Padstow, UK, and had been shed naturally by the female or accidentally rubbed off when moving gravid females during standard hatchery procedures. Any developing embryos contained within the eggs presumably died soon after being dislodged from the female because brooding was disrupted (Talbot, 1991). The eggs were stored at 2 ± 1 °C (to prevent excessive bacterial degradation) for as long as two weeks before use. The experiments were performed in crimp vials containing 30 mL of weakly buffered, oxic, artificial seawater (ASW) at pH 7.2, which replicated the salinity and buffer capacity of coastal and estuarine waters (Sagemann et al., 1999). The ASW was slightly modified by replacing $FeSO_4 \cdot 7H_2O$ with Na_2SO_4 to prevent excess precipitation of Fe-based minerals that could have interfered with morphological interpretation. Each vial was inoculated with 0.5 mL of mixed sediment obtained from the top 100 mm of sediment from the Tamar estuary, UK. The Tamar estuary is characterized by high levels of organic matter productivity (Upstill-Goddard et al., 1989). Vials were crimp sealed to prevent inward oxygen diffusion and incubated at 15 °C for 36 days. Two vials were used in each experimental run, and the experiment was repeated on two separate occasions.

The eggs were visually inspected every 5 days, and changes in color or surface texture were noted. After 36 days the pH in the me-

dium, at the bottom of the vial among the eggs, was measured with a pH microelectrode (Diamond General, Ann Arbor), and the eggs were prepared for scanning electron microscopy (SEM) by using the methods of Sagemann et al. (1999). Five eggs from each vial were sliced in half with a scalpel in order to determine the extent of any internal mineralization. The eggs were carbon coated, and SEM was performed on a Cambridge S250 Mk3 instrument with an acceleration voltage of 12–15 kV. An attached energy-dispersive X-ray (EDX) detector allowed qualitative determination of the elemental content of the minerals that formed.

RESULTS

In all of the vials, a white mineral precipitate was visible on most of the eggs after only 15 days decomposition. The mean pH after 36 days was 6.3; no pH gradient was observed in the vials, and no oxygen was present (preliminary investigations had shown that the vials become anaerobic within 1 day).

The outer envelope of a fresh lobster egg (Fig. 1A) is smooth, and remnants of the funiculus (attachment stalk) surround it. The mineralization preserved the shape of the egg, including the funiculus (Fig. 1B), and formed a coating to 50 μm thick that caused some loss in the fidelity of surface detail (cf. Figs. 1A and 1B). The mineralized coating is composed of a matrix of amorphous mineral and many small filaments and spheres, which indicate mineralized bacteria (Figs. 1C, 1D). The EDX analysis confirmed that bacteria were mineralized and revealed calcium peaks throughout the matrix. However, phosphorus peaks were absent, or very small, indicating that the majority of mineralization was most likely in calcium carbonate. The eggs used in this investigation were at an unknown stage of development, but the internal contents were presumably a mixture of yolk and possibly embryonic cells. Figure 1C shows that the inside of the egg is an amorphous solid mass, produced by fixation of the internal contents with glutaraldehyde during SEM preparation. No mineralization was evident encrusting or impregnating the inside of the egg, including the egg envelope, and this appearance was confirmed by EDX analysis.

DISCUSSION AND CONCLUSIONS

This experiment shows that lobster eggs can be mineralized under laboratory conditions in a manner that retains detail comparable to that preserved in fossil metazoan eggs (Bengtson and Zhao, 1997; Xiao and Knoll, 1999, 2000). Previously, mineralization of invertebrate soft tissue in experiments has been closely associated with the cuticle of the decaying organism (Briggs and Kear, 1994; Hof and Briggs, 1997). The cuticle may act as a source of calcium and phosphate in those species with a more heavily mineralized cuticle, e.g., *Neogonodactylus oerstedii* (Hof and Briggs, 1997). However, this association with the cuticle may be a function of location. Tissue adjacent to the cuticle is more likely to mineralize because it is the outermost soft tissue and will, therefore, be exposed to bacterial attack and degradation first. The cuticle may also act as a barrier to produce a microenvironment conducive to mineral precipitation (Briggs and Wilby, 1996). In this experiment, soft tissues were mineralized in the absence of any cuticle, biomineralized or not.

The only other report of laboratory mineralization of a metazoan egg is that of the shrimp *Palaemon* sp. (Briggs and Kear, 1993). In this case, decomposition was over a period of 20 weeks, and the entire animal carrying the egg mass was subjected to decay. The outer envelope of the egg decayed completely, and the egg was represented by granular apatite, which preserved no fine detail. The eggs were less completely mineralized than other soft tissues. In the present experiment, only the outer surface of the egg was preserved by a mineral coat. However, once the external morphology is stabilized, there is potential for mineral to infill the egg as well. Experiments performed over longer time periods and under various conditions are required to ascertain the subsequent morphological and diagenetic changes that occur, particularly possible increases in phosphorus and precipitation of apatite (Hof and Briggs, 1997).

Eggs from Neoproterozoic and Cambrian metazoans have been described from the Doushantuo Formation, China (Xiao et al., 1998; Xiao and Knoll, 1999), the basal Cambrian Pestrotsvet Formation, Russia (Bengtson and Zhao, 1997), the basal Cambrian Dengying Formation, China (Bengtson and Zhao, 1997), and the Middle Cambrian Gaotai Formation, China (Zhang and Pratt, 1994). Fossil metazoan egg envelopes are preserved either by impregnation with fine-grained apatite (<0.5 μm) (Fig. 1E; also see Fig. 6 in Xiao and Knoll, 1999) or by a coating of crystals on the inner (Fig. 1G) and/or outer surface (Fig. 1F), which indicates the original position of the envelope. The inner layer is thicker (to 20 μm) and is composed of an isopachous crust of crystalline apatite (Fig. 1F) or, in many cases, consists of spherules composed of fibrous crystals (Fig. 1G). The outer layer is thinner (to 5 μm) and is made up of an isopachous rim of crystals often growing perpendicular to the egg surface (Fig. 1F; also see Fig. 5C in Xiao and Knoll [1999] and Fig. 5.10 in Xiao and Knoll [2000]). Where both

an inner coating and outer coating are present in a fossil, the crystals growing from opposite directions, one must have formed directly on the egg envelope, and the other encrusted this initial coating after the organic envelope was destroyed by decay. The eggs in our experiments are encrusted on the outside of the egg envelope with a relatively thick layer of mostly amorphous calcium carbonate and mineralized bacteria (Figs. 1C, 1D). This result confirms the observation that bacteria may promote mineralization (Wilby and Briggs, 1997; Xiao and Knoll, 1999) and that this process would lead to encrustation of the egg tissue. However, fossil eggs show no signs of bacteria on the egg envelope (Figs. 1E, 1F), although the internal blastomeres are often coated with phosphatic spherules and filaments interpreted as mineralized bacteria (Xiao and Knoll, 1999) (Fig. 1H). Fossil material may also exhibit multiple generations of phosphatic coating, making putative bacterial filaments and egg envelopes appear larger than their original organic structure (e.g., see Figs. 5G and 5H in Xiao and Knoll, 1999). The coating on fossil blastomeres is comparable to the coating on the egg envelopes that have been mineralized in our experiments (cf. Figs. 1D and 1H), suggesting that the mechanics of mineral encrustation were broadly similar in both this investigation and the fossil material. However, the differences between laboratory-mineralized and fossil eggs and embryos still require further investigation to establish the cause of this variation. For example, the thickness and susceptibility to decay of the original envelope must play a role in the type of mineralization that forms, because the envelope is the surface that bacteria colonize first. In addition, the mechanism and the duration of mineral precipitation would influence both its thickness and final morphology.

No mineralization was evident inside the egg envelope; this is presumably due to the fact that the internal organic structure of the egg remained intact. The egg envelope of *H. gammarus* is thick ($\sim$40 μm), and it appears relatively intact even after 36 days of decomposition. The egg envelope may have acted as a barrier to bacterial degradation of the internal contents. However, it is interesting to note that a large proportion of the Doushantuo fossil eggs examined show no preservation of the internal bodies (S. Xiao, 2002, personal commun.); commonly only the envelope is preserved, as in the current experiments. Figure 1C shows ridges on the surface of the internal body of the egg. These resulted from shrinkage of the internal body, which is common in fossil material (Xiao and Knoll, 1999, 2000). However, we cannot exclude the possibility
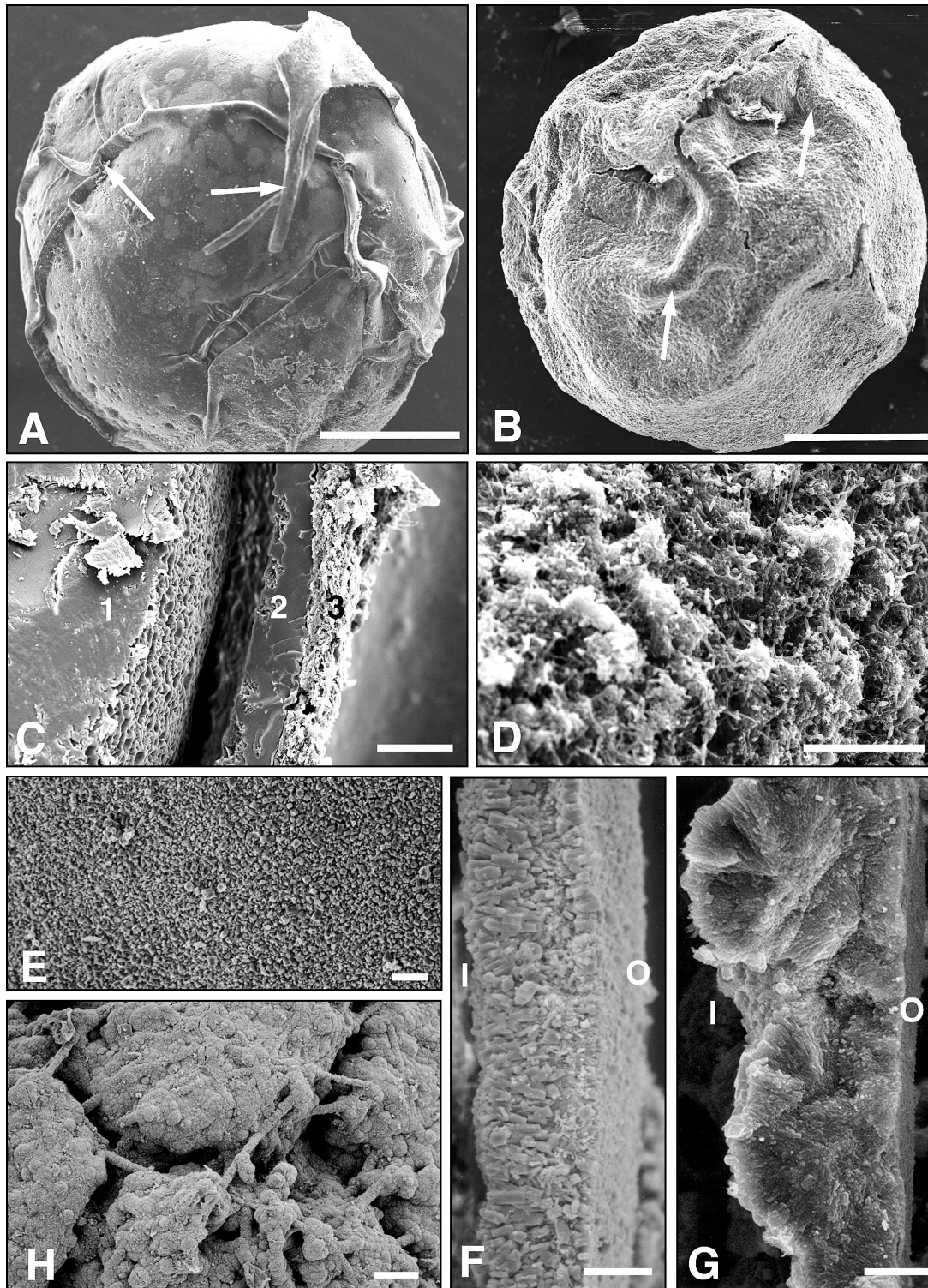
Figure 1. Scanning electron micrographs of lobster eggs (A–D) and Neoproterozoic (Doushantuo Formation, Weng'an) fossil eggs and embryos (E–H, reprinted from Xiao and Knoll [1999, Figs. 6F, 5E, 5F, and 7G, respectively]). A: Fresh egg showing remnants of funiculus (arrows) and relatively smooth outer egg envelope. B: Mineralized egg showing areas where morphology of funiculus has been preserved (arrows). C: Sliced egg exhibiting (1) internal contents, (2) egg envelope, and (3) mineralization on outer surface of envelope. D: Surface of egg (equivalent to layer 3 in C) displaying spherules and filaments of mineralized bacteria. E: Surface of fossil egg envelope (*Megasphaera* sp.) exhibiting preservation in fine-grained apatite. F: Cross section of fossilized egg envelope (*Parapandorina raphospissa*) showing coating growing both inward (I) and outward (O). G: Cross section of fossilized egg envelope (*Megasphaera* sp.) showing spherules composed of fibrous crystals on inner surface (I—inner surface, O—outer surface). H: Mineralized surface of blastomere (*P. raphospissa*) displaying spherules and filaments representing mineralized bacteria. Scale bar represents 500 μm in A and B; 50 μm in C; 20 μm in D and H; 5 μm in E, F, and G.

that the shrinkage was caused directly by SEM preparation, perhaps mediated by alteration to the internal contents during decomposition.

The specimens mineralized in our experiments are encrusted mainly with calcium carbonate, rather than the phosphate that is observed on the fossil specimens (Bengtson and Zhao, 1997; Xiao and Knoll, 1999, 2000). The final pH of 6.3 was similar to that observed in other experimental studies, where calcium phosphate precipitation was favored over calcium carbonate (Briggs and Kear, 1994; Sagemann et al., 1999). Lower pH alone (Briggs and Wilby, 1996), however, is insufficient to promote phosphatization if the concentration of phosphorous is too low. Briggs and Kear (1993) showed that the phosphate to carbonate ratio was lower in laboratory-mineralized eggs of *Palaemon* sp. than in mineralized body tissue, i.e., eggs were composed of a greater proportion of calcium carbonate than body tissue. Whether this phenomenon is unique to eggs or was a result of the position of the eggs external to the exoskeleton remains unclear. Clearly, calcium carbonate preserves the gross morphology of the eggs—a crucial stage in preservation. Longer decomposition times and diagenetic replacement with phosphorus may be required to obtain completely mineralized eggs similar to those in the Doushantuo material (e.g., Xiao and Knoll, 2000).

Our results demonstrate that (1) laboratory experiments can produce preservation in a style similar to that observed in fossils; (2) mineralization is produced under conditions of anoxia and lowered pH and is bacterially mediated, as illustrated by the texture in Figure 1D; (3) mineralization of eggs can be induced in the laboratory in the absence of a decaying carcass as a source of phosphorus or calcium, and consequently, it is possible for organisms of this size to have been fossilized prior to the evolution of large metazoans; and (4) this type of mineralization is very rapid, taking only 2–3 weeks to mineralize the eggs (in the Hof and Briggs [1997] experiment it took an order of magnitude longer).

This type of experimental approach provides a basis for more extensive investigations of the processes and conditions that lead to egg and embryo mineralization. Experiments such as these have considerable potential to aid the interpretation of fossilized eggs and embryos, notably those of Neoproterozoic and Cambrian age (e.g., Zhang and Pratt, 1994; Chen et al., 2000), and to assess their significance for analyzing the early stages in the radiation of metazoans.

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