

ONTOGENY AND TAPHONOMY: AN EXPERIMENTAL TAPHONOMY STUDY OF THE DEVELOPMENT OF THE BRINE SHRIMP *ARTEMIA SALINA*

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Abstract: Although the relationship between ontogeny and phylogeny has been of long-standing interest to palaeontologists, the fossil record has provided little insight into the development of long extinct organisms. This has changed with the discovery of numerous assemblages of fossilized invertebrate embryos and larvae, but realising their evolutionary significance is hampered by a paucity of data on the relationship between ontogeny and taphonomy. We describe the results of an experimental taphonomy study of the development of the anostracan brine shrimp *Artemia salina*, which show that in conditions of aqueous aerobic and anaerobic autolysis and microbial decay, the developmental stages exhibit differential preservation potential. The most decay resistant developmental stage is the diapause cyst, encapsulating the gastrula, in which the gross morphology of the embryo can be maintained for 18 months or more in simple anaerobic conditions. Otherwise, the embryo shrinks within the cyst and cellular and tissue detail of breaks down as lipid droplets coalesce. Postembryonic excysted larvae decay more rapidly. The rate of decay is similar among all larval stages

with the exception of the L₄ larva, which resists cuticle failure for longer than later developmental stages. The larvae decay leading to liquefaction of the muscles and viscera, leaving an intact but empty and progressively shrunken and distorted cuticle that eventually loses structural integrity and collapses. Our experimental results provide an explanatory model for the phenomenal abundance of putative diapause stage embryos, in the absence of postembryonic stages, as seen in the Ediacaran Doushantuo Formation of South China and the incompleteness of fossilized developmental sequences of embryos and larvae more generally. It also cautions against the association of developmental stages in fossil deposits without additional evidence. Finally, the pattern of decay seen in larvae provides an explanation for the preservation style of Orsten-type Lagerstätten where preservation of cuticular detail can be astonishingly fine, but extends internally to muscles and viscera only rarely.

Keywords: experimental taphonomy, preservation, ontogeny, development, embryos, Crustacea, Doushantuo.

THE relationship between ontogeny and phylogeny has been of enduring interest to palaeontologists. This interest has increased in recent years as developmental genetics has facilitated a molecular approach to determine why different organisms follow different embryological and ontogenetic trajectories. Palaeontological evidence has been used to constrain models of developmental evolution, revealing the pattern of character evolution intermediate of the otherwise disparate bodyplans of living relatives, such as between agnathans and gnathostomes (Donoghue and Purnell 2005), fishes and tetrapods (Coates *et al.* 2002), crocodiles and birds (Padian and Chiappe 1998). However, the fossil record has rarely provided direct insight into the process of development.

This is not to say that ontogenetic information cannot be obtained from the fossil record. In fact, it abounds, in

the sclerochronological record of accretionary skeletons, in the larval shells of molluscs and brachiopods, the instars of arthropods, the ossification sequences in juvenile amphibians and reptiles and the boney embryos of fishes and archosaurs (Donoghue and Dong 2005). However, such data are generally limited to bones and shells and they are invariably restricted to features of late ontogeny.

The dearth of embryological data in the fossil record has limited the scope of palaeontology to identifying the evolutionary patterns (e.g. heterochronies), rather than the mechanisms. Indeed, the limitations of palaeontology have even been exploited in the development of hypotheses that are effectively untestable. For instance, De Beer (1958) argued that gaps in the fossil record inferred from the absence of 'transitional forms' between major grades of organization were more apparent than real. Instead, he

argued, they represented episodes of 'clandestine evolution' in which large-scale change was facilitated through ontogenetic repatterning in embryonic stages that are effectively invisible to the fossil record.

However, embryology is not invisible to the fossil record. Fossilized embryos, representing even the earliest and seemingly more fragile stages of development, are a rare phenomenon, but where they are found, they can be abundant (Donoghue *et al.* 2006a) and exquisitely preserved (Donoghue *et al.* 2006b). Fossil embryos are a recent discovery, but ontogenies for Cambrian microcrustaceans have been known for decades (Müller 1979) from animals fossilized in three dimensions with all their appendages to the resolution of the smallest seta.

The interpretation of such data appears simple because the fossils resemble living analogues. However, interpretation is not straightforward because the record is biased, both in terms of what anatomical features of the embryos and larvae are preserved and in terms of what embryological and larval stages are not. All assemblages of fossil embryos show evidence of bias. The Ediacaran Doushantuo Formation is dominated by what is interpreted as single cell diapause cysts (Yin *et al.* 2007); later cleavage stages are increasingly rare (Dornbos *et al.* 2005a) and only a few probable pre-hatchling forms have been identified (Xiao *et al.* 2007b). The Lower Cambrian Kuanchuanpu assemblage is more balanced, preserving a range of developmental stages for a variety of organisms (Bengtson and Yue 1997; Steiner *et al.* 2004). Embryos from more recent strata are dominated by the scalidophoran *Markuelia* (Donoghue *et al.* 2006a), for which only a few cleavage embryos are known among the thousands of pre-hatchling larvae (Dong *et al.* 2005a; Dong *et al.* 2004) and hatched larvae are yet to be reported. The spectacular Orsten fauna of microcrustaceans is also not without bias, in that their embryonic and adult stages are absent although complete sequences of instars are known for the larvae (Maas *et al.* 2006; Walossek 1995).

Understanding the bases of these biases through experimental taphonomic analyses is integral to interpreting this fossil record of development. Vast atlases, devoted to the study of decaying organisms, serve as the guides to interpreting fossils and the fossil record (Dix and Graham 1999; Schäfer 1962). However, none addresses the most pervasive of all biases in the fossil record, the bias against embryonic and early larval stages. Preliminary attempts have been made to uncover the biases affecting the decay and preservation of the earliest stages of embryonic development (Gostling *et al.* 2008; Raff *et al.* 2006, 2008), but we lack a coherent understanding of the relative preservation potential of different stages of embryology and ontogeny. We sought to obtain such an understanding through an experimental taphonomic study of the anostracan brine shrimp *Artemia salina*. Our aim was limited to

identifying bias in the rate of decay and, thus, how preservational potential varies between different embryonic and larval developmental stages. These results were compared with patterns revealed by fossil assemblages of embryonic and larval animals. However, in contrast to the majority of contemporary experimental taphonomy studies (Briggs 1996), our aim was not to simulate the process of fossilization.

MATERIALS AND METHODS

Artemia salina is an ideal model for interpreting embryos from the Ediacaran Doushantuo Formation. The Doushantuo fossils have recently been interpreted as diapause cysts containing eggs and embryos (Yin *et al.* 2007). Indeed, there is already evidence that subcellular features of the diapause cysts of *Artemia* can be preserved over a timescale of tens of thousands of years (Clegg and Jackson 1997).

Furthermore, as an anostracan crustacean, it represents a good analogue (with respect to body plan and size) for the stem- and crown-crustaceans from the Orsten fauna.

Experimental conditions

Encysted embryos and hatched larvae were euthanized via exposure to deoxygenated artificial sea water (ASW; 35 ppt). Forty-eight hours exposure was sufficient to euthanize the larvae and adults, but cysts were still viable. Clegg (2007) reported that cysts can remain viable for as much as 4 years in anoxic water. Experimentation identified a period of 2 weeks as sufficient to euthanize the cysts provided additional organic matter was available to promote reducing conditions. Commensal bacteria, living in the carapaces of adult shrimp produce H₂S when exposed to anoxic conditions. This is toxic to cysts, larvae and adults alike. Death was established by exposing a sub-sample to oxygenated ASW and observing that no cysts hatched. Approximately 100 mg of euthanized cysts, larvae and adults was then placed into Wheaton crimp vials with 30 mL of either oxic or anoxic ASW. These two environmental conditions were chosen to constrain the effects of aerobic vs. anaerobic microbial decay. The two systems were maintained at a constant temperature of 15°C in a refrigerated incubator throughout the experiment. The oxic system was maintained by allowing open diffusion between the atmosphere inside and outside the vial. The anoxic system was maintained by sealing the vial with a rubber septum and aluminium crimp to prevent oxygen diffusion. Neither system was sterile, as a small amount of the ASW in which the shrimps were growing was carried across into the experimental systems and, indeed, the embryos, larvae and adults would have carried endo-

genous microbes. Thus, the results reflect both autolytic and microbial decay processes in combination.

Experiments were replicated (three vials per week for each environment) to allow the degree of postmortem decay to be recorded at weekly intervals for the first 6 weeks and, subsequently, at 6, 10 and 18 months. Each experiment was terminated by fixing the contents of the vial with either 4% paraformaldehyde before thin sectioning or 2.5% Gluteraldehyde before critical point drying.

Critical point drying

Material was fixed in 2.5% (w/v) Gluteraldehyde, 0.1 M Cacodylate buffer, 10 mM CaCl_2 (pH 7.4) for 2 h at room temperature, or overnight at 4°C. The sample was then washed in 0.1 M Cacodylate buffer (pH 7.4) and dehydrated using a graded series of ethanol washes of 10%, 20%, 30%, 40%, 50%, 70%, 90%, 100%, followed by two washes in absolute ethanol to exchange the sample's water content for ethanol. The samples were then transferred to the critical point drying chamber with approximately 5 mL of absolute ethanol. Once sealed in the chamber, ethanol was flushed from both the chamber and sample using pressurized carbon dioxide. This process was repeated until no further ethanol was released from the specimens into the chamber. The sample was then fixed at 37°C for 90 min in the critical point dryer. This dried the sample thoroughly preventing further decay and degradation.

Scanning electron and optical microscopy

Specimens were analysed using a Hitachi S-3500N Scanning Electron Microscope, at an accelerating voltage of 15 kV, or thin sectioned and examined using a Zeiss Photomicroscope III fitted with Nomarski Differential Interference Contrast optics and a Q-Imaging 5.0 Megapixel Micropublisher firewire camera.

Thin sectioning

Samples were first washed three times, each for 5 min, in phosphate buffered saline (PBS) on a shaker. A postfixation step of 2 h, in 2% Osmium Tetroxide, followed this. Samples were then washed (three times, each for 5 min) in PBS. The samples were dehydrated for 2 h in 70% ethanol, followed by 2 h in 96% ethanol and a final 1 h wash in 100% ethanol, at room temperature on a shaker. The samples were placed in a 1:1 solution of 100% ethanol and Technovit solution A and left for 8 h. This was followed by a 30 h infiltration step in Technovit

solution A. The samples were embedded in Technovit 7100 Solution B and polymerization took place over the following 2 h. The polymerized samples were bonded to blocks for sectioning using Technovit 3040. The material was thin sectioned, producing 50 μm sections and mounted using DPX Microscopy Mounting Medium, onto glass slides under a cover slip.

NORMAL DEVELOPMENT IN ARTEMIA

Artemia salina has a life cycle of approximately 1 year and grows to a maximum size of approximately 10 mm. As the name suggests, *A. salina* lives in saline conditions. Under normal conditions, females are ovoviviparous, releasing nauplii larvae. However, in adverse conditions, such as dessication, females are oviparous, producing encysted embryos (Criel and Macrae 2002). The development of the embryo pauses at the stage of gastrulation (Spooner *et al.* 1994) and remains viable for months or even years within this diapause state (Clegg 2007), dessicated within a protective cyst. When conditions are once again favourable, the cyst quickly rehydrates and development continues, the cyst ruptures and a nauplius larva emerges. This is achieved within 24 h.

The seventeen developmental stages after hatching begin with the Nauplius I/Larva 1 ($=L_1$), a 400 μm long teardrop-shaped structure that hangs below the cyst for several hours within the hatching membrane (Cohen *et al.* 1998). Eventually the larva becomes detached, free of the membrane and thus is capable of free swimming. Nauplii possess only head appendages. This first larval stage has two pairs of appendages (L_1 antenna and L_1 antennule) that elongate as the larva develops. At stage L_2 , the trunk elongates, the first outgrowths of trunk appendages become visible and the labrum elongates considerably. By L_4 (Metanauplius II), the antennae and antennulae are noticeably longer relative to the size of the head and the antenna is highly branched with many distal outgrowths. The outgrowths, from distal to proximal, are the setae bearing an exo- and endopodite, curved spine, protopodite and the proximal enditic process. From L_1 to L_4 these appendages begin to ramify and the antennae are used for propulsion. By L_6 (Postmetanauplius I) the larva has well defined limb buds on a lengthening trunk.

The major changes in the succeeding 11 developmental stages involve the loss of setae from the exopodites and endopodites of the antennae, with the role of propulsion being taken over by the thoracopods and the replacement by two large lateral compound eyes of the single simple median larval eye (that started to develop at stage L_5). The genitalia of both sexes become more apparent with the male genitalia developing spines, while the ovaries of the female show oocyte development. The body axis

becomes elongated to accommodate the thoracopods producing adults in the order of 7–10 mm.

For the purposes of our study, we examined the pattern of decay within five broad developmental stages: (i) prehatching cysts and cyst cases; (ii) L₁ Nauplius I; (iii) L₂ Nauplius II; (iv) L₄, Metanauplius II; (v) L₆ Postmetanauplius I/adults (Text-fig. 1). While we aimed to achieve quantitative results for developmental stages i–iv, the small numbers of adults (two to three) incorporated into the experiments facilitated only qualitative consideration of the pattern of decay exhibited by this developmental stage.

RESULTS: MORPHOLOGICAL STAGES OF DECAY

The pattern of decay was similar in the different populations of brine shrimp under both oxic and anoxic conditions. The most significant difference observed was rate, with material in oxic conditions degraded to a shrivelled mass of amorphous organic material within 6 weeks. Even after 18 months decay in the anoxic system, the gross morphology of the embryo remained intact within the cyst, although cellular and tissue detail had been lost and the fabric of the embryo was dominated by aggregated

and coalesced lipid droplets. We provide below a description of the pattern and stages of decay exhibited, organized into their developmental stages for both oxic and anoxic conditions. These descriptions are supported by illustrations (Text-figs 2–7) and the results are summarized graphically in Text-fig. 8.

Cysts

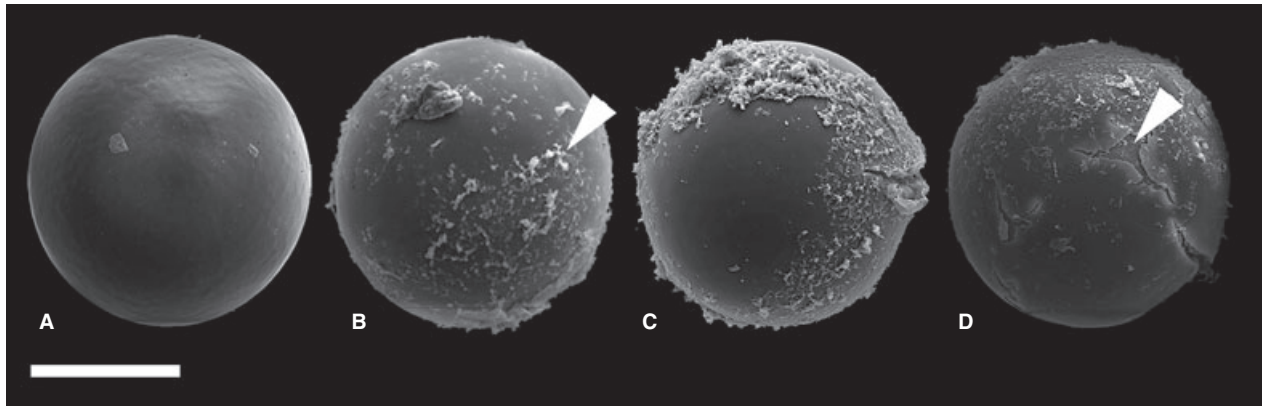
Stage 1 – freshly euthanized cysts. The rehydrated, freshly euthanized cysts appear healthy (Text-fig. 2A). They are golden brown with brighter iridescent flecks. The cyst is filled with the developing embryo (Text-fig. 3A), itself surrounded by a membrane, and the cyst wall comprises several distinct layers.

Stage 2 – initial degradation of the embryo. The embryo starts to shrink away from the cyst wall (only observable in section; Text-fig. 3B–I). The layers of the cyst wall also show signs of degradation and become less distinct from each other. A white coating is observed on many cysts (determined to be composed of carbon, oxygen and hydrogen by Energy Dispersive X-ray (EDAX) analysis, data not presented) that is likely to be a carbohydrate/mucilage decay product (Text-fig. 2B).

Stage 3 – further breakdown of embryonic tissues. Gaps appear in the tissues of the embryo (Text-fig. 3F), accompanied by the



TEXT-FIG. 1. The principal developmental stages of *Artemia salina* considered in the study. A, Diapause cyst, B, Nauplius I/L₁, C, Nauplius II/L₂, D, Metanauplius II/L₄, E, Postmetanauplius/L₆ – adults. Scale bar: A, 178 μ m; B, 177 μ m; C, 150 μ m; D, 250 μ m; E, 3.5 mm.



TEXT-FIG. 2. Progressive decay of brine shrimp cysts. A, Freshly euthanized cyst, B, as decay progresses the first signs are seen as a build up of a white granular substance, probably a carbohydrate generated by decay (arrow). This build up is seen most around cracks, or mechanical damage to cysts, C, D, eventually the cyst wall begins to delaminate (D, arrow) in areas close to cracks that appear to be the result of embryo eruption. Unhatched cysts show little or no signs of decay with SEM after 18 months. Scale bar: 100 μ m.

appearance of lipid droplets. Embryos shrink to approximately half their volume.

Stage 4 – collapse of the cyst wall. The cyst wall is distorted and has lost its spherical shape (Text-fig. 3H). Many of the cysts become translucent, allowing the embryo within to be observed (Text-fig. 3J). Cracked or damaged cysts show extensive build up of white coating (Text-fig. 2C).

Stage 5 – breakdown of the embryo. The embryo collapses leaving a shrunken mass that is not readily recognizable as an embryo either in whole mount, or thin section (Text-fig. 3L). Asymmetric shrinkage of the embryo means that it is often absent from sections, depending on the plane of section. Cysts show extensive delamination of the wall layers (Text-fig. 2D).

Stage 6 – presence of amorphous organic matter. The embryos break down until all that remains is amorphous organic matter (AOM) (Text-fig. 8).

Pattern of decay. Under oxic conditions, decay progressed rapidly (Text-fig. 8). Cysts were initially filled entirely by the embryo, but after 2 weeks, a majority exhibited extensive shrinkage of the embryo. The embryos broke down rapidly thereafter. The final product was unrecognizable as an embryo (stage 5). After 6 weeks decay, approximately one tenth of the cysts observed contained any identifiable embryonic material. The cysts also became coated in a white substance. After 2 weeks, about one quarter of the cysts in any given population had a white deposit on the surface. After 6 weeks, only about a quarter were free of such a deposit. A full observation of the pattern of decay (at 15°C) was possible only under anoxic conditions, as decay proceeded too rapidly under oxic conditions.

Under anoxic conditions, the layers of the cyst wall became less distinct and there was some loss of sphericity in many specimens (Text-fig. 2B). Subsequently, the embryonic tissue itself began to break down with rents appearing in the cyst wall

(Text-fig. 2C). This progressed through weeks 4 and 5 postmortem (Text-fig. 3D, E) until the cracks became obvious gaps at 6 weeks (Text-fig. 3F). The most significant change over the ensuing 4 months was the coalescence of lipid droplets in the embryonic tissues (Text-fig. 3G). There was no obvious change in the embryonic membranes and even after 18 months (Text-fig. 3I) many embryos still exhibited an intact cyst wall, embryonic membrane and the gross morphology of the embryo itself. At any given time, various decay stages co-existed (Text-fig. 8). At 18 months, a roughly equal number of cysts appeared pristine (Text-fig. 3J, yellow arrow; Text-fig. 3L) and altered (Text-fig. 3J, white arrow; Text-fig. 3L) becoming translucent revealing the embryonic material within.

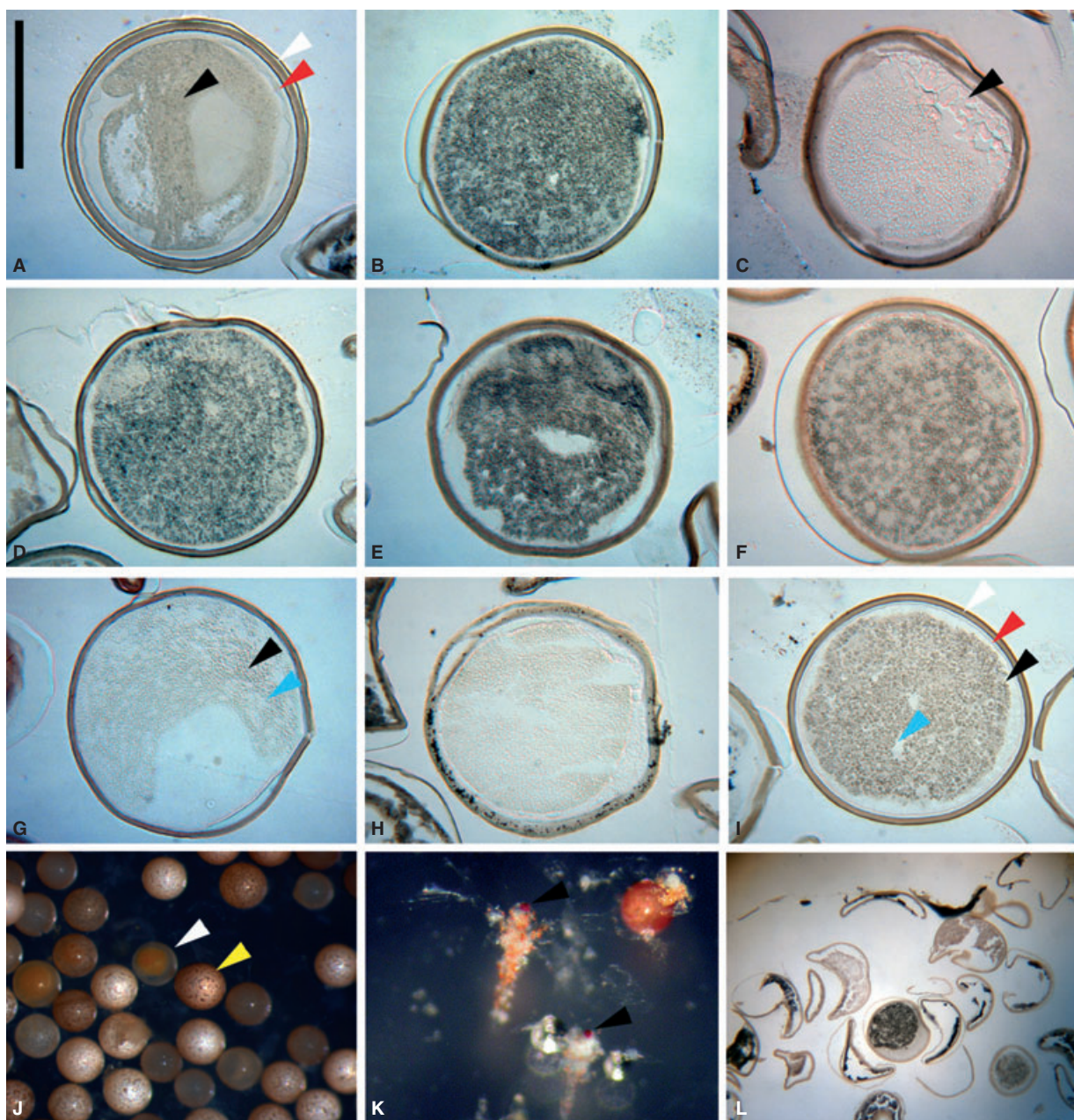
L₁ larva (nauplius I)

Stage 1 – freshly euthanized L₁ larva. The L₁ Larva hangs below its cyst, all membranes are intact and the appendages are clearly visible through the hatching membrane.

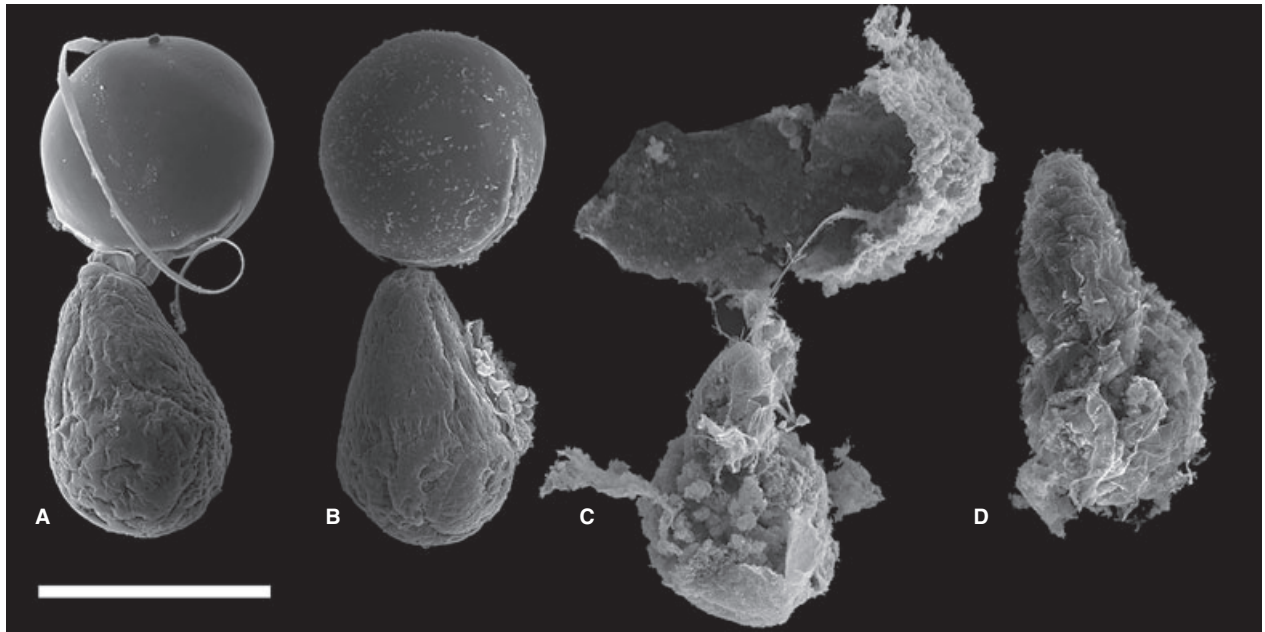
Stage 2 – deterioration of the hatching membrane and embryo. Holes appear in the hatching membrane revealing the embryo which begins to shrink.

Stage 3 – continued shrinkage, and initial disarticulation of the larva. Holes in the hatching membrane are extensive, with up to 50% loss, the now extremely shrunken embryo also shows signs of disarticulation. There is a loss of cuticle and epidermis in the embryos even though the hatching membrane remains intact.

Stage 4 – detachment of larva from the cyst. Embryos, still within fragmentary membranes, detach from their cysts. Limbs are missing. The cysts show extensive build up of a white granular material over the external surface and around the edges of cracks.



TEXT-FIG. 3. A–J, L, Thin sections of 1–6 week, and 6, 10, and 18 month postmortem brine shrimp cysts. J–K, 18 month postmortem cysts and larvae. A, After 1 week postmortem the cyst wall (white arrow head) has distinct layers. The gastrula-stage embryo (black arrow head) is inside a membrane (red arrow head). B, After 2 weeks the cyst wall has less obvious layers and there is a loss of sphericity in some specimens. C, After 3 weeks under anoxic conditions the embryo within the cyst started to look a little less intact, with spaces appearing with the embryonic tissue (blue arrow head). D, E, This progressed through weeks 4 and 5. F, Distinct spaces were visible in the embryonic tissue at 6 weeks postmortem. Very little changed beyond this over the following months and year. G, After 6 months droplets of lipid were clearly visible. H, There was little further change after 10 months. I, Even after 18 months many embryos displayed a layered cyst wall, embryonic membranes and embryos within. J, Externally, many cysts appeared unscathed after 18 months postmortem (yellow arrow); however, many showed changes in the cyst wall, becoming translucent, revealing the shrunken embryo within (white arrow). K, L_4 larvae also persisted for over 18 months after death; the single red eyespot was most clearly visible (black arrows) along with some internal structures, seen through the translucent cuticle. Decaying remains became extremely fragile and the slightest movement resulted in their disintegration. L, Although some extremely well preserved specimens were seen after 18 months postmortem, a mosaic of preservational qualities was observed, with collapsed specimens, those with shrunken embryos, and distorted morphologies were observed. Scale bar: A–I, 100 μm ; J, 600 μm ; K, 600 μm ; L, 80 μm .



TEXT-FIG. 4. A, In the hatching larvae the fresh hatchling is a tightly bound embryo within a membrane. Limbs are clearly observable through the membrane. B, As decay progresses the embryo becomes less well defined through the membrane, which becomes prone to damage. C, D, Subsequently, the embryo may become detached from the cyst (C), and shows extensive damage (D). Scale bar: 200 μ m.

Stage 5 – L₁ larva free of the membranous sack. The membranous sack has disintegrated. L₁ larvae show a loss of appendages and disintegrate easily, when disturbed.

Stage 6 – isolated larvae unidentifiable as more than amorphous organic matter. The larvae that are free of their membranes have undergone such extensive decay that they are only recognizable by their gross morphology (size and shape).

Pattern of decay. Fresh L₁ larvae (Text-fig. 4A) decay quickly, notably even under anoxic conditions. The embryos began to shrink and holes formed in the membrane that wrapped the embryo (Stage 2) after 2 weeks under anoxic conditions. Under oxic conditions, this was evident in approximately one third of the specimens after only 1 week (Text-fig. 8).

Under anoxic conditions, nauplii became extremely shrivelled (Stage 3) and the membrane around the hatchling also showed signs of shrinkage. At 4 weeks, the membrane around the L₁ larva showed signs of degradation with tears and holes, exposing the shrivelled embryo within (Text-fig. 4B). At 5 weeks, the L₁ larva within was exposed, with about half of the sack lost in a majority of specimens (Text-fig. 4C). By 6 weeks, the decay had reached an extent such that the ectoderm/cuticle of the fully exposed larva showed significant signs of decomposition, beyond shrinkage of the whole organism (Text-fig. 4D). The next observed stage of decay was disassociation of the L₁ larva (still within its membrane) from its cyst (stage 3), followed by disintegration of the membrane (stage 4). L₁ larvae were still present in the anoxic system after 12 months of decay, although the cuticle was extremely fragile (stage 5).

L₁ larvae that had not become free of their sack before death, were also still present, although in significantly lower numbers. They were seen still attached to their original cyst, some with an intact membranous sack, but they were very shrunken inside this membrane, so as to be unrecognizable in isolation (stage 6). No L₁ larvae were recovered at 18 months from the anoxic system (Text-fig. 8); despite their presence in the vials, they were too fragile to be recovered from the surrounding media.

L₂ larva (= nauplius II); L₄ larva (metanauplius II); L₆ larva (postmetanauplius)–adults

Stage 1. The loss/matting/shrinkage of setae on the exopodite and endopodites of the antenna and appendages.

Stage 2. Contortion of the body.

Stage 3. Loss of segments along the length of the appendage, loss of cuticle.

Stage 4. Collapse of internal organs, leaving only cuticle and pigmented structures obvious, as the cuticle becomes transparent.

Stage 5. Disintegration of the body into cuticular fragments.

Stage 6. All that remains of the embryos is amorphous organic matter.



TEXT-FIG. 5. A, The freshly euthanized larva I (L_1) have three pairs of appendages, each branching distally. B, The first stages of decay involve the matting of these exopodites, and in some cases loss of them. C, As decay progresses losing limbs/limb segments is common. D, Initially, the limbs lose distal segments, but eventually entire limbs detach from individuals. E, This is often coupled to extensive deterioration of the cuticle. F, As the cuticle is damaged, internal surfaces of the animal are exposed. Scale bar: 150 μm .

Pattern of decay – L_2 larva. L_2 Larvae (Text-fig. 5A) showed signs of rapid degradation (stage 1), the antenna degraded after only 1 week of the experiment, under both oxic and anoxic conditions. The setae of the exopodite and endopodites (at the terminus of the antenna) appeared to be matted, showed evidence of shrinkage and also the loss of the terminal segments of appendages (Text-fig. 5B).

The whole larva went through a stage of shrinkage or deformation (twisting and contortions of normal morphology (stage 2). The next stage of decay involved the loss of entire appendages (Text-fig. 5C). During early decay stages, the appendages disarticulated along their joints, principally in a distal to proximal sequence. However, during later stages of decay, the appendages detached entirely or at any point along their length (Text-fig. 5D). In approximately a third of the specimens observed, the epidermis and cuticle were entirely absent from large areas of the organism (stage 3), exposing the internal anatomy (Text-fig. 5E, F). At 6 weeks the experiment had progressed so that only fragments of L_2 larvae remained in the oxic system. Recognizable L_2 larvae were present in the anoxic system after 12 months of decay, but they were too fragile to be recovered

from the media. Thereafter, the cuticle was transparent and contained shrivelled internal organs, but the large red eyespot was still apparent (stage 4). However, these specimens were very fragile; disturbance of the surrounding medium caused their immediate disaggregation/disintegration into a mass of cuticular fragments (stage 5). Few L_2 were successfully recovered after 18 months, although the morphology was poor, such that it was difficult to identify individuals as larvae (Text-fig. 8). Decay stage 1 was the longest in L_2 larvae with some specimens failing to progress to later decay stages within the first 6 weeks of the experiment, an interval of time in which specimens representative of all other developmental stages had achieved later decay stages.

L_4 larvae (metanauplius II) – L_4 type specimens. The shrimp (Text-fig. 6A) first showed evidence of degradation in the distal elements of their appendages (stage 1; Text-fig. 6B). Only about a quarter of the specimens showed any sign of contortion of their bodies by 2 weeks (stage 2; Text-fig. 6C). After 3 weeks of oxic decay, more than half of the specimens had lost approximately half of the cuticle (stage 3; Text-fig. 6D, E). This was fol-



TEXT-FIG. 6. A, Larva II (L_2) shows a decay profile similar to that of L_1 . B, Initially the filamentous exopodites become matted. C, This is followed by a collapse of the body. D, E, There is continued damage to the appendages, again coupled with a loss of cuticle. F, Finally all that remains is a collection of disarticulated limbs. Scale bar: 300 μm .

lowed by collapse and degradation of the whole organism by 5 weeks (stage 5). After 6 weeks of oxic decay, the larvae were highly contorted and lacked the exopodite extensions of the antenna (Text-fig. 6F). However, specimens representative of stage 3 were still present in the anoxic system after 12 months. After 18 months, few L_4 larvae were recovered, from the anoxic system, but among those remaining, some were relatively intact. (Text-figs 3K, 8). The progression through stage 1 was rapid, compared to subsequent decay stages.

L₆ larva (postmetanauplius) and adults. The distal ends of the thoracopods were initially the first part of the L_6 /adult (Text-fig. 7A) to degrade. As decay progressed, the cuticle shrank and further disrupted normal morphology (Text-fig. 7B–ventral and Text-fig. 7C–dorsal). After 10 days of oxic decay, complete cuticle failure had occurred and no adults could be recovered from the medium for examination. Analysis *in situ* showed that the shrimp continued to decay and the anterior and posterior ends curled exposing the ventral side (Text-fig. 7D). After 6 weeks, no whole individual was present. Under anoxic conditions adults decayed more slowly, but even after the first week, in some cases, the morphology was already showing signs of abnormality

from the *in vivo* morphology. No complete adult was recovered from the anoxic system at 12 months; however, the media was seen to contain very large amounts of cuticular debris from all stages of brine shrimp development (Text-fig. 8).

Comparative taphonomy of development

The results of our experiments are summarized graphically in Text-fig. 8. There is an obvious and fundamental bias in preservation potential of different developmental stages of *Artemia*. The higher preservation potential of the diapause cysts must reflect the factors that make *Artemia* among the most environmentally tolerant of all living animals. Indeed, there is a research industry developed around exploring the limits of tolerance of the encysted embryo, including space flight, dessication, thermal extremes, exposure to anoxia on a timescale of years, high dosage UV and ionizing radiation (Clegg 2005). This high degree of tolerance occurs because of a number of factors. These include the impermeability of



TEXT-FIG. 7. Adults are subject to decay profiles similar to that of larvae. A, Freshly euthanized adult. B, After death the cuticle shrinks. C, Subsequently, limb segments become matted and lost. D, The adult then becomes shrivelled and difficult to discern. In most cases, the adults fall to pieces and all that is observed are the disjointed fragments of bodies. Relative scale bar: A, 2 mm; B–D, 1 mm.

the cyst wall to microbes and nonvolatile solutes. A high proportion of the volume of the embryo is accounted for by a chaparone protein, which maintains the conformation of other proteins and by glycerol, which stabilises DNA (Clegg 2005; Clegg and Jackson 1997). Among these factors, we suggest that the impermeability of the cyst wall to exogenous microbes and the availability of glycerol must be integral to maintaining the structural integrity of the embryos postmortem on our experimental timescales.

The timescale over which the structural integrity of the excysted larvae and adults is maintained is approximately comparable across postembryonic development. All stages exhibit a similar pattern of decay in which the finer setae become tangled, the internal organs and tissues decay and shrivel; later the epidermis and cuticle begins to show evidence of disintegration, with appendages disarticulating at joints and fragments of cuticle falling away to reveal and essentially empty interior. Eventually, the carcass disintegrates into minute fragments if the fluid medium is disturbed.

The slight exception to this common pattern of decay occurs in the L_4 larvae, specimens of which maintained structural integrity longer than for any of the other postembryonic developmental stages.

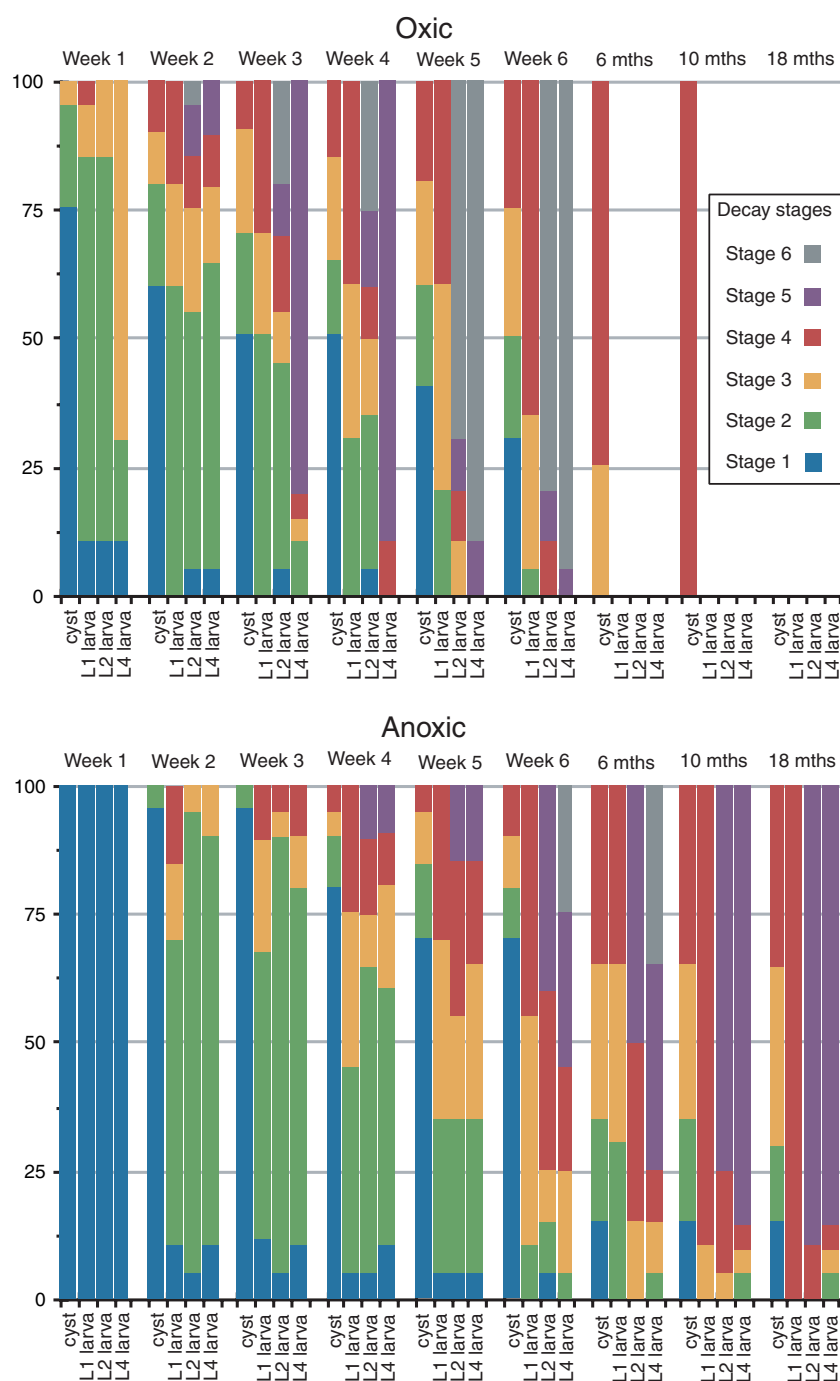
DISCUSSION

Comparative experimental taphonomy of eggs and embryos

Martin *et al.* (2003, 2004, 2005) have demonstrated that it is possible to maintain the structural integrity of *Homarus*, *Limulus*, and *Nephrops* egg cases for at least a year, inducing fossilization via a suite of mineral systems, but with no evidence of replication of the embryo.

Raff *et al.* (2006) and Gostling *et al.* (2008) undertook experimental studies of the pattern and processes of decay in the embryos of sea urchins, a tunicate, an annelid, a gastropod and a bivalve mollusc. Embryos decayed rapidly under both oxic and anoxic environmental conditions and only AOM remained after 6 weeks. Their

TEXT-FIG. 8. The progression of decay, with time, is shown under different decay conditions. A, Oxidic conditions. B, Anoxic conditions. The bars of the histograms present stacked percentages of the decay stages represented per developmental stage, per sampling stage. In all instances, the progression of decay is more rapid under oxidic conditions. A mosaic of decay states is seen in all developmental stages at all time points. The graph betrays complexity in the decay process that is not apparent from qualitative observational consideration of the results of the experiments. L_4 larvae are the slowest to decay of all the larval stages, however, L_4 larvae reach decay stage six earliest within the first 6 months of the experiment (both environmental conditions).



fossilization potential was minimal; conditions at actual time of death would have had to favour replication in calcium phosphate extremely rapidly (Martill 1989; Martill and Harper 1990). Under strongly reducing conditions, however, the structural integrity of the embryos maintained longer; i.e. there is a longer time interval during which replication could occur. Its duration is compatible with conditions conducive to authigenic mineralization via calcium phosphate being initiated

during, and as a result of, the initial stages of decay (Briggs and Kear 1993), or via a change in the chemistry of the external environment. Although strongly reducing conditions are precisely those under which authigenically mediated calcium phosphate mineralization occurs (Briggs *et al.* 1993; Briggs and Wilby 1996) and fossilization of these embryos is, thus, feasible, none of the fossil embryos discovered to date can be reliably attributed to any group on which the experiments were based (Gostling, *et al.* 2008).

The results of our experiments are broadly consistent with those of previous studies (Gostling, *et al.* 2008; Raff, *et al.* 2006), particularly with regard to the pattern of decay and the extrinsic variable that impacts upon timing. The volume of the embryo within the cyst decreases, separating it from the cyst wall, lipid droplets coalesce within the embryo and the cells/tissues break down and the embryo shrinks further until it is an amorphous mass that remains within the cyst, which has itself become distorted. As in previous experiments, the rate at which an embryo passes through these stages of decay varies considerably between oxic and anoxic environmental conditions. However, in *Artemia*, the difference in rate is much more extensively skewed. Indeed, under anoxic conditions, *Artemia* showed less evidence of decay after 18 months than the majority of specimens in previous studies that were decayed under strongly reducing conditions after only 6 weeks. This is not a factor unique to *Artemia*, but to its encysted diapause stage, as evidenced by the fact that excysted larval and adult stages underwent much more rapid decay under anoxic conditions. This indicates that the factors that promote survival of the diapause stage under adverse conditions also promote maintenance of the structural integrity of the embryo postmortem.

Experimental results inform the interpretation of putative embryos from the Doushantuo formation

Our experimental results may prove informative in interpreting the fossil record of embryonic development, not the least because it indicates that diapause cysts may facilitate maintenance of the structural integrity of embryos on a timescale of years, which far outstrips the timescale necessary for the initiation of conditions conducive to authigenically-mediated calcium phosphate mineral replacement of biological structure. This potentially explains the phenomenal accumulation of embryos in the Ediacaran Doushantuo Formation, where they are not just abundant, but literally rock forming and are mined commercially so that the calcium phosphate that has replaced biological structure to sub-cellular fidelity (Hagadorn *et al.* 2006) may be used as an agricultural fertilizer.

Interpretation of the Doushantuo fossils as embryos is controversial. They were initially interpreted as algae (Xue *et al.* 1995) and subsequently as animal embryos (Xiao *et al.* 1998), mainly on the basis that the proposed blastomeres show evidence of flexible membranes, rather than rigid cell walls (Xiao and Knoll 1999b). The pattern of cleavage has subsequently been elucidated (Hagadorn *et al.* 2006), their complex egg case interpreted as a cyst and, thus, the embryos represent a diapause stage (Xiao *et al.* 2007a; Yin *et al.* 2007) of an organism for which

possible hatchlings have recently been identified (Xiao *et al.* 2007b). Alternatively, (Bailey *et al.* 2007a, b) have suggested that the Doushantuo fossils are better interpreted as giant sulphur bacteria. They argue that there is insufficient evidence to justify linking the putative developmental stages, because modern bacterial analogues such as *Achromatium*, *Beggiatoa*, *Thiomargarita* and *Thioploca* are comparable in size, possess mucous sheaths, filaments and/ or inclusions that resemble the complex 'cyst' wall of the Doushantuo fossils and because *Thiomargarita* can undergo reductive binary division reminiscent of cleavage. Furthermore, they argue that the bacterial interpretation can better explain phenomena such as the abundance of specimens in the Doushantuo and their fossilization – because *Thiomargarita* is known to concentrate phosphate from seawater and, as a result, sediments surrounding it show elevated concentrations of phosphate minerals.

While the bacterial hypothesis competes well to explain the Doushantuo fossils, it is not without its own attendant problems (Donoghue 2007) and, indeed, some of its purported benefits do not stand up to scrutiny. For instance, Bailey *et al.* 2007a, b argue that the bacterium interpretation explains the phenomena of abundance and mineralization, in a manner that the animal embryo hypothesis cannot. The Upper Grey Facies, from which a majority of embryos have been described, is widely appreciated to be a secondary, size-sorted assemblage of fossils that were originally fossilized elsewhere in a facies more akin to the Lower Black Facies where the fossils are far less abundant but more pristine (Dornbos *et al.* 2005b; Xiao *et al.* 2007a). Furthermore, while *Thiomargarita* is capable of concentrating phosphate, it affords no mechanism of mediating mineralization (Xiao *et al.* 2007a); presumably also microbes mediated mineralization in the same manner required by the animal embryo hypothesis.

The giant sulphur bacterium hypothesis additionally, albeit implicitly, requires that the organisms in question were fossilized without decay because comparisons have always been drawn to living or fixed specimens and, presumably, a recently or long-dead giant sulphur bacterium would appear somewhat different. Unfortunately, no evidence on this has yet been presented. However, the taphonomy of the Doushantuo fossils in question has been broadly considered (Bengtson 2003; Bengtson and Budd 2004; Dornbos *et al.* 2005a; Dornbos *et al.* 2005b; Xiao and Knoll 1999a) and we may consider how this compares with the decay of *Artemia* diapause cysts. The comparison is not perfect because although the Doushantuo fossils have been interpreted as diapause cysts (Yin *et al.* 2007), *Artemia* enters diapause at gastrulation (Spooner, *et al.* 1994) whereas the Doushantuo fossils have been interpreted to represent only cleavage stages; there is no evidence of gastrulation (Hagadorn *et al.* 2006). Nevertheless, it is the best comparison currently available.

Dornbos *et al.* (2005a) describe progressive taphonomic shrinkage and deformation of component blastomeres, with the spaces that opened between cells and the outer envelope filled in by coarse calcium phosphate cement. This was considered late-stage mineralization, as opposed to the earlier mineralized microcrystalline calcium phosphate that replicates features such as the cell walls or the surrounding envelope. The fossils show evidence that progressive decay led to collapse and disaggregation of the component cells and, ultimately, loss of the sphericity of the envelope. This pattern is particularly well documented in the more completely preserved examples presented by Yin *et al.* (2007) that show shrinkage of the putative embryo within its diapause cyst and it is even apparent in a great majority of specimens from the Doushantuo Formation, referred to *Megasphaera*, where an undifferentiated central body is eccentrically located within its cyst (Text-fig. 9A, B; Xiao and Knoll 2000; Yin *et al.* 2004). These patterns compare favourably with the shrinkage and collapse of the embryo and cyst described from our experiments. Initially, embryos of *Artemia* shrink away from only a portion of the cyst wall, resulting in an embryo that is located eccentrically with respect to the cyst wall (Text-fig. 3C, F), just as in *Megasphaera*. The pattern of blastomere separation and collapse recognized in fossils is also generated in experimental studies using the embryos of various bilaterians (Gostling *et al.* 2008; Raff *et al.* 2006).

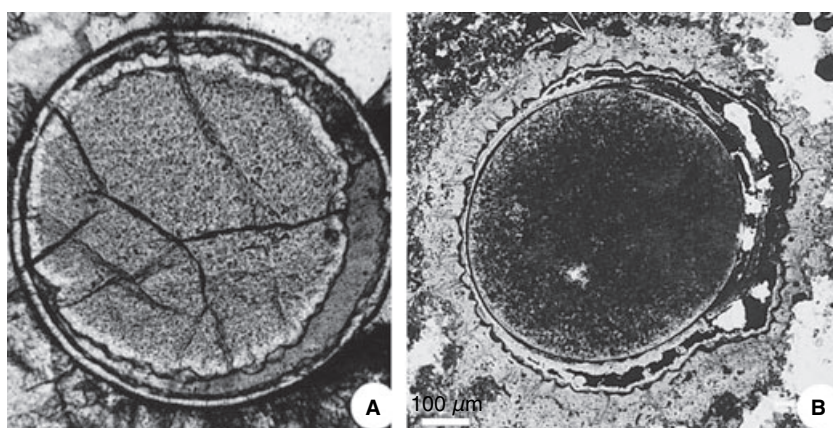
Although attempts have been made to describe the taphonomy of the Doushantuo fossils, like the analyses of the biology of the fossils, they have focused especially upon multicelled specimens. This provides a distorted impression of the fossil deposit in which the multicelled specimens represent approximately 1% of the total, the remaining 99% of specimens being simply deflated or deformed, infilled or empty, sculptured or smooth cysts. However, this mosaic of preservation quality and the relatively small proportion of fossil embryos that may be readily recognized as such, correspond well with the variation in preservation state seen in our experiments.

Of course, to a great extent, the taphonomic factors that we observed in our experiments and compared with putative fossil embryos will be common to the decay of organic spherical structures, because they are based on physical properties that are generic to geometrical structures at a given scale. Thus, we might anticipate giant bacteria to show the same phenomena. Experimental data would be very welcome. However, the most important element of the *Artemia* study is that it indicates that the physical structural integrity of a dead diapause embryo could be maintained over a timescale of months to years under conditions of simple anoxia, which is compatible with what is known of the environment of preservation of the Doushantuo assemblage that, at times, must have extended to reducing conditions (Dornbos *et al.* 2005b; Xiao and Knoll 1999a). Furthermore, the preferential preservation potential of the diapause cyst, in comparison with earlier and later developmental stages, provides a ready explanation for their abundance in the Doushantuo phosphorite fossil assemblage. These observations expand the explanatory power of the animal embryo interpretative model for the Doushantuo fossils and diminish perceived advantages of the giant bacterium model proposed by Bailey *et al.* (2007a, b).

Comparative experimental taphonomy of larvae and adults

The pattern and rate of decay of larvae and adult brine shrimp are closely comparable to previous experimental taphonomy studies on other animal systems, which have shown that although decay rate may vary with environmental variables, the broad pattern of decay remains consistent. Eumalacostracans have been the subject of many such studies (Allison 1986; Allison 1988; Briggs and Kear 1994; Hof and Briggs 1997; Plotnick 1986). Allison (1988) noted differences in both the timing and pattern of decay in the eumalacostracans *Nephrops* and *Palaemon*, based on differences in cuticular composition. However, both showed a

TEXT-FIG. 9. Putative animal embryos from the Ediacaran Doushantuo Formation, South China, showing separation of the embryo within from the cyst wall. A, Reproduced from Xiao and Knoll (1999a) with the permission of the authors and The Paleontological Society. B, Reproduced from Yin *et al.* (2004) with the permission of the authors and the *Acta Palaeontologica Polonica*. Scale bar: A, 53 μm ; B, 100 μm .



pattern of decay common to *Artemia* in which the component elements of the cuticle disarticulate after decay of the connective tissue. Notably, the cuticle of *Nephrops*, a composite of chitin and calcite, degraded rapidly; the calcite component fragmented extensively after decay of the chitin. In contrast, the cuticle of the decaying *Palaemon*, although entirely chitinous, retained its structural integrity longer, but degraded extensively later. After 25 weeks, at the end of the experiments, less of its cuticle remained than that of *Nephrops*. In comparison, *Artemia* underwent a less rapid decay than either *Nephrops* or *Palaemon* and this may be due in part to its comparatively small size. Although hatched larvae and adults of *Artemia* were maintained as intact cuticular units for a protracted period of time, subsequent degradation did not occur through disarticulation of cuticular units, but through cuticle failure as in *Nephrops* and *Palaemon*.

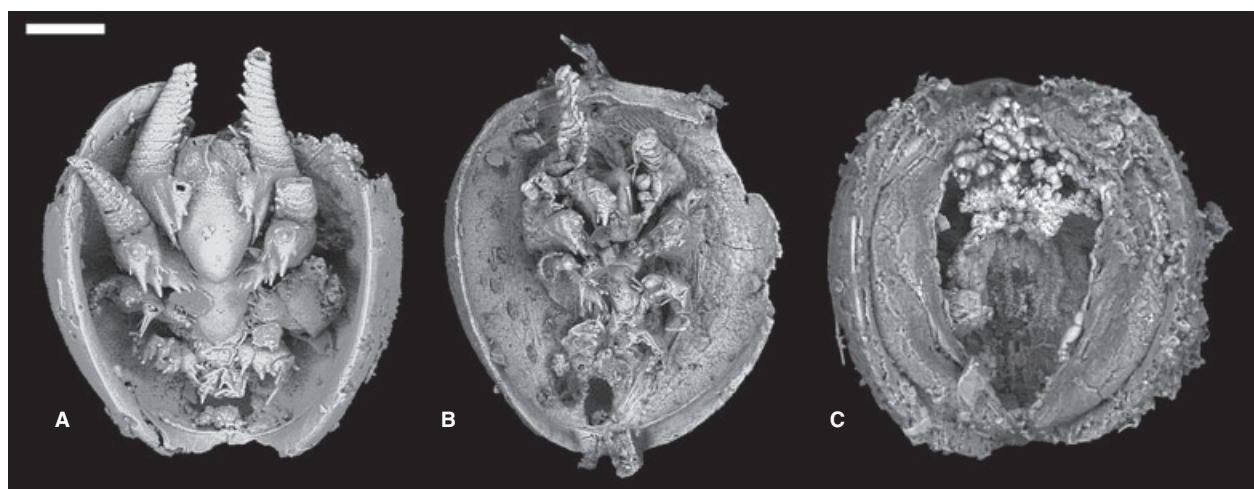
Although there are abundant crustaceans in the fossil record, it is difficult to compare the results of these experiments with compression fossils, such as those from the Granton Shrimp Bed (Briggs *et al.* 1991). Three-dimensional preservation of crustaceans is not uncommon (for a review see Maas *et al.* (2006) but, although these have been subjected to exhaustive monographic treatment, paradoxically, because only the better preserved specimens are illustrated (Maas *et al.* 2003), we have little knowledge of their decay patterns and processes. Here, we compared the results of our experiments with specimens of an as yet unnamed phosphatocopid stem-crustacean (Text-fig. 10), exhibiting Orsten fauna type preservation, from the Late Cambrian Bitiao Formation, Wangcun Section, Yongshun

County, western Hunan, China (Dong *et al.* 2005b). Preserved specimens range from those preserved in exquisite detail, to those in which the appendages show evidence of loss of the setae (Text-fig. 10A), to others in which the cuticle of the appendages has undergone progressive shrinkage (Text-fig. 10B). Eventually, the cuticular carcass is shrunk to an indeterminate mass within the carapace and, ultimately, all that remains is the carapace itself (Text-fig. 10C).

This pattern of degradation is broadly that exhibited by larvae and adults of *Artemia*. Furthermore, the observation that cuticle remains intact while the musculature and visceral tissues shrivel and decay within, concurs with the observation in Orsten-type fossils that preservation rarely extends beyond cuticle (Maas *et al.* 2006). It has argued that factors intrinsic to the cuticle might explain its preservation in preference to other tissues, both in situations of Orsten-type and Burgess Shale-type preservation (Briggs and Kear 1994; Butterfield 2003; Hof and Briggs 1997; Stankiewicz *et al.* 1998). The decay experiments presented here suggest that it is the extended timescale over which the structural integrity of cuticle is maintained, in comparison with other tissues that explains its preferential preservation.

Varying preservation potential of embryos, larvae and adults, and bias in the fossil record

Our results have shown that in *Artemia*, there is considerable variance in the preservation potential of embryos vs. hatched larvae and adults and, to a lesser extent, between



TEXT-FIG. 10. Phosphatocopids from the Upper Cambrian of Bitiao Formation of the Wangcun Section, Hunan Province, South China, showing a range of decay prior to fossil replication of the remaining soft tissues. A, Specimen showing little evidence of decay though some evidence of mechanical damage to the antennae and appendages (GMPKU2201). B, Specimen shows evidence of shrinkage and distortion of the cuticle, comparable to stages of decay of larvae and adults of *Artemia salina* after liquefaction of the viscera and muscles (GMPKU2261). C, Specimen showing evidence of extensive decay to the extent that the cuticle of the body is largely lost and the principal element that remains is the carapace, which was mineralized *in vivo* as the exoskeleton (GMPKU2262). Scale bar: A, 82 μm ; B, 81 μm ; C, 47 μm .

larvae and adults. Evidence from the fossil record would appear to suggest that the varying preservation potential of *Artemia*'s developmental stages may be representative of marine invertebrates more generally.

Developmental series, including embryological stages, are available for very few fossil organisms. The only well documented series is for *Olivoooides*, a putative scyphozoan cnidarian from the Lower Cambrian of China (Bengtson and Yue 1997; Yue and Bengtson 1999). Embryonic stages are not known for the Orsten stem- and crown-crustaceans. A number of these fossils are known only as one developmental stage, albeit as numerous individuals. For instance, the minute eucrustacean type-A larvae are known from localities around the world and yet they cannot be allied with other known developmental stages (Maas *et al.* 2006; Müller and Walossek 1986). This suggests that, as in *Artemia*, it is not simply early or later developmental stages that can be preferentially preserved, because they are protected within a cyst or because they are extensively sclerotized, respectively. Rather, and for whatever reason, any component development stage may exhibit preferential preservation and this should serve as a caution against allying otherwise exotic developmental stages recovered from fossil Lagerstätte.

Finally, the results of this study should go some way to explain the polarized preservation of developmental stages that is so characteristic of assemblages of fossil embryos (Donoghue and Dong 2005; Donoghue *et al.* 2006a). A majority of the localities at which fossil embryos have been recovered are both monospecific and essentially restricted to an individual stage of embryonic development, predominant because of its precocious development of cuticle, which is generally the only character preserved in these fossils (Donoghue *et al.* 2006a). The assemblage with the greatest variation in developmental stages has been recovered from the Kuanchuanpu Formation, Lower Cambrian of South China. However, although numerous developmental stages are preserved, other than *Olivoooides*, no convincing evidence has yet been presented that these fossilized ontogenies extend beyond the embryo.

Most perplexing of all has been the Ediacaran Doushantuo assemblage which, until recently, was been restricted not merely to embryonic stages, but to cleavage stages. Later stage larvae (Chen *et al.* 2002; Chen *et al.* 2000) and adults (Chen *et al.* 2004) that have been identified are not convincing (Bengtson and Budd 2004; Xiao *et al.* 2000). The most convincing candidates for later developmental stages are embryos (Xiao *et al.* 2007b). However, the absence of hatchlings and adults may not be evidence that the affinity of these fossils is to be found outside of Metazoa (contra Bailey *et al.* 2007a, b). Rather, we have shown that embryos and, especially, diapause

cysts, have extraordinarily high preservation potential that they can remain within the sedimentary environment for more than 12 months postmortem and yet retain their structural integrity. This is more than compatible with the timescale necessary for induction of conditions conducive to fossilization via replication of soft tissues via bacterially mediated authigenic mineralization. This preservation and, therefore, fossilization potential is not shared to the same degree with later larval and adult developmental stages and, as such, it provides an explanation for the polarized composition of the embryo fossil assemblage in the Doushantuo Formation that is both plausible and, indeed, likely valid.

CONCLUSIONS

Our experimental results show that developmental stages have differential preservation potential and, in contrast to expectation, embryonic stages can exhibit greater preservation potential even in comparison with their adult counterparts and rates of decay can vary even between subtly distinct larval stages. These data urge caution in the interpretation of fossil assemblages of developmental sequences which are likely to be incomplete and encompass a mosaic of taxa that should not be associated developmentally merely on evidence of their co-association.

Our data also provide an explanatory model for the composition of deposits such as the Ediacaran Doushantuo Formation where embryos are abundant but postembryonic stages are rare to absent. This phenomenon has been marshalled in evidence against their interpretation as animal embryos leading to their reinterpretation as fossilized giant bacteria. The differential decay resistance of the encysted diapause embryos and the adults of our model, *Artemia salina*, indicates that the phenomenal abundance of the fossils in the Doushantuo assemblage is not incompatible with their interpretation as animal embryos.

Finally, our decay experiments have confirmed a pattern of decay in excysted larvae and adults of *Artemia salina* seen previously in adults of eumalacostracans: liquefaction of muscles and viscera, resulting in an cuticle sack that eventually shrinks, distorts and ultimately undergoes complete structural failure. This is directly compatible with the extent of preservation seen in the micro-crustaceans of Orsten-type Lagerstätten where, although the cuticular details are commonly preserved to a sub micron resolution, preservation rarely extends beyond the cuticle.

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