Subcellular preservation in giant ostracod sperm from an early Miocene cave deposit in Australia

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Cypridoidean ostracods are one of a number of animal taxa that reproduce with giant sperm, up to 10,000 μm in length, but they are the only group to have aflagellate, filamentous giant sperm. The evolution and function of this highly unusual feature of reproduction with giant sperm are currently unknown. The hypothesis of long-term evolutionary persistence of this kind of reproduction has never been tested. We here report giant sperm discovered by propagation phase contrast X-ray synchrotron micro- and nanotomography, preserved in five Miocene ostracod specimens from Queensland, Australia. The specimens belong to the species Heterocypris collaris Matzke-Karasz et al. 2013 (one male and three females) and Newnhamia mckenziana Matzke-Karasz et al. 2013 (one female). The sperm are not only the oldest petrified gametes on record, but include three-dimensional subcellular preservation. We provide direct evidence that giant sperm have been a feature of this taxon for at least 16 Myr and provide an additional criterion (i.e. longevity) to test hypotheses relating to origin and function of giant sperm in the animal kingdom. We further argue that the highly resistant, most probably chitinous coats of giant ostracod sperm may play a role in delaying decay processes, favouring early mineralization of soft tissue.

1. Introduction

Ostracods of the suborder Cypridocopina (order Podocopida) are famed for having some of the longest sperm in the animal kingdom, surpassed by only a handful of insect groups [1]. Sperm in extant Cypridocopina species range from 0.44 to 10.00 mm in length and are often longer than the males that produced them [2,3]. In contrast to giant sperm of other animal groups, which have exceptionally long flagella (e.g. insects like Drosophila, Notonecta, Divales [1] and references therein), Cypridocopina sperm (figure 1d,e) are aflagellate and can be described as an extremely elongated ‘sperm-head’ with a nucleus-derivate that runs from one end to the other [4]. This organelle is partially encased by two enormous, spiralling mitochondria (figure 1a,b). Although tailless, these gargantuan gametes are motile; contractile organelles produce both ripples along the length of the posterior region and longitudinal rotation of the entire sperm (figure 1b), and all of the sperm body enters the egg during fertilization [3,5,6].

The readily fossilized calcitic carapaces of ostracods, together with their high diversity and ubiquitous presence in aquatic habitats, have resulted in them becoming the most abundantly preserved arthropod in the fossil record, albeit typically with no preserved ‘soft-parts’. The rare occasions that ‘soft-parts’,...
i.e. appendages and internal body, are fossilized can reveal exquisite detail and provide unparalleled data on the evolution of the group [7–15]. For example, preserved Zenker organs, which are muscular-chitinous pumps used to transfer giant sperm to females [16] (figure 1c), in Cretaceous ostracods have been used as indirect evidence that giant sperm are a long-lived trait in the group [9]. However, the oldest previously known occurrences of ostracod sperm are from two records of Holocene age [8,17], and fossilized sperm are generally exceptionally rare; the only other record comes from a springtail trapped in Late Eocene Baltic amber [18].

2. Material and methods

Ostracod specimens (871 carapaces and valves) were extracted from a freshwater karstic limestone from the Riversleigh’s Bitesantenary Site (D Site Plateau, Riversleigh World Heritage...
property, Lawn Hill National Park, northwestern Queensland, Australia; details of the site locality can be obtained on application to the Queensland Museum in Brisbane, Australia). The Bitesantennary Site represents a 0.7 m deep cave-fill covering an area of 5 m², surrounded by a Late Oligocene limestone that formed the original cave wall [19,20]. Unnamed Early Miocene freshwater limestone cave sediments (23–16 Ma) have infilled a Miocene cave within a more massive, Late Oligocene tufaceous freshwater limestone, commonly interpreted to be the Carl Creek Limestone [20]. Remnants of aquatic fauna preserved in the cave-fill, together with thousands of bat fossils and stalagmites, indicate that this site represents a cave, which was open to light, at least partly contained standing water, and provided a refuge for bats.

Elemental composition in the limestone was measured using an Olympus-InnovX Delta-X field portable XRF (E. Cohen 2013, personal communication). A 1-kg sample of this limestone was immersed in 3.5% acetic acid over two weeks, and the resulting ostracods were washed in freshwater and dried. All specimens are registered in the fossil collection of the Queensland Museum.

To obtain data on mineral specification of the preservation, one specimen (QMF56081) has been subjected to Raman spectroscopy (see the electronic supplementary material for technical details). The measurements have been carried out directly on the ventrally preserved soft parts.

Sixty-six specimens were used for tomographies carried out on beamline ID19 of the European Synchrotron Radiation Facility (Grenoble, France) (QMF54756, QMF56080-56102, QMF56105-56110, QMF57288-57309, QMF57362-57375; 28 specimens of *Heterocypris collaris*, 12 specimens of *Candonocypris fimbulus*, three specimens of *Newmannia mckenziana* and 23 specimens in open nomenclature). Details are compiled in the electronic supplementary material (table S1). Here, a pink beam of 19 keV energy (insertion device U17.6, gap 20) was used for scans with 1500 projections over 180° at 0.2 s per projection. A FreLoN 2K14 CCD camera was mounted on a visible light microscope coupled to an 8.8 μm LSO scintillator. Propagation distance was 20 mm, voxel size 0.56 μm. Data reconstruction was done with a single distance phase retrieval algorithm adapted from Paganin et al. [21].

Specimens QMF56080 and QMF54756 were subjected to nanotomography with 25 nm voxel size at the nano-imaging station ID22N1. The samples were put in the divergent, partially coherent X-ray beam emerging from a 60 nm focus with 29.5 keV energy. Magnified in-line holograms with a fixed focus-detector distance (705 mm) and at four different focus-sample distances were used as input for a robust and high-resolution phase retrieval algorithm [22]. Tomographic reconstruction of the density maps was performed using filtered backprojection with the ESRF software PyHST. On all reconstructed slices, ring artefacts were corrected, followed by 16 bits TIFF stack conversion and visualization using the software Amira (vsg). Movies were created using Amira (VSG) and DImage (open source).

Scanning electron microscopy was carried out on gold-coated specimens at 15 kV (Polaron Sputter Coater; Leo 1430VP). For transmission electron microscopy, whole ostracods were fixed for 24 h in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) and postfixed in 2% OsO4 (same buffer). Dehydrated animals (ascending ethanol and acetone series) were embedded in an Araldite–Poly/Bed 812 (Polysciences) mixture. Thin sections (Reichert-Jung Ultracut E) were stained (unary acetate and lead citrate), then examined using a JEOL JEM-1011 electron microscope.

### 3. Results

Synchrotron tomographic investigation at the ESRF of 66 ostracod specimens from the early Miocene Bitesantennary Site (23–16 Ma) of the Riversleigh World Heritage Area, northwestern Queensland, Australia, revealed the presence of a number of internal organs, including parts of the reproductive system, and fossilized giant sperm in two species: *H. collaris* Matzke-Karasz *et al*. 2013 and *N. mckenziana* Matzke-Karasz *et al*. 2013.

Synchrotron microtomographies optically extracted reproductive organs from the mineral matrix in eight specimens. Four males (QMF56080, QMF56093, QMF56099 and QMF57302) were identified by the presence of sperm pumps (Zenker organs) [23,24], preserved as tubular voids that are lined by an annulated wall and surrounded by radial spines typical of the group (figure 2; electronic supplementary material, videos S1 and S2). Four other specimens each have paired female seminal receptacles preserved (QMF54756, QMF56081, QMF56094 and QMF56098), including one female *N. mckenziana* (figure 3; electronic supplementary material, video S3) with almost pristinely preserved clivae of the seminal ducts leading from the vaginas to the inflated seminal receptacles (figure 3; electronic supplementary material, video S4).
Figure 3. A female specimen of *N. mckenziana* (QM54756) with preserved paired spiral ducts and seminal receptacles filled with giant sperm. (a) SEM image of the fossil from ventral, with preserved soft parts seen through the gape of the carapace. (b) Tomographic slice through the middle of the animal, showing sperm-filled seminal receptacles. (c,d) Posterior part of the fossil with seminal receptacles (highlighted by segmentation in beige in (d)). (e,f) Posterior end of the fossil with spiral ducts (highlighted by segmentation in yellow (f)). (g,h) Seminal receptacles of the left body side magnified, from external and with spiral duct (g) and semi-transparent, allowing a view on stored sperm cells (h). (i) Tomographic slice with lateral section of a spiral duct. (j) Seminal receptacle and spiral duct are clearly connected. (k,l) Different views of the spiral duct as figured in (g,j). Scale bars, 100 μm (a–f), 20 μm (g–l).
supplementary material, video S4), illustrating the extraordinary high quality of preservation. In 28 out of 66 (42.4%) of the specimens analysed by synchrotron radiation, the walls of the oesophagus could be detected. The oesophageal walls are heavily sclerotized, chitinous supports in Recent ostracods. Six out of 28 (21.4%) specimens with identifiable internal structures were preserved with valves closed.

Fossil spermatozoa of different preservation quality were found in one male (QMF56080) and three female H. collaris (QMF56081, QMF56094 and QMF56098), and one female N. mckenziana (QMF54756) (electronic supplementary material, videos S5 and S6). In the H. collaris male (QMF56080), sperm are arranged in paired clumps in the mid and posterior sections of the body (figure 4c–e); a pair of large, ring-shaped clumps approximately 0.45 mm across in the mid-dorsal area of the carapace and paired secondary clumps approximately 0.31 mm across, with convoluted looping, in the posteroventral region. Although the two types of clumps are adjacent, it is not clear whether they are directly connected. Each clump consists of numerous sperm, but exact numbers remain unclear.

The ring-shaped clumps correspond to the seminal vesicles of living species (e.g. in Mytilocypris mytiloides, as shown in figure 4d,e), chambers anterior of the Zenker organs where sperm is stored prior to copulation. The secondary clumps correspond to the position of the convoluted distal section of the vas deferens leading from the testes to the seminal vesicles (figure 4d). We therefore interpret the clumps of sperm as in situ occurrences, directly related to the anatomical structure of the male reproductive system. While mumified sperm preserved in the position of seminal vesicles have been reported for Holocene ostracods [17], we here report fossil sperm preserved also in the vas deferens. The large amounts of sperm preserved indicate that the specimen was a mature male, which died during the reproductive season of this species and perished prematurely before its stores of sperm could be expended. The sperm are arranged (sub) parallel with each other. Sperm length is difficult to determine because both ends of individual sperm are not visible. Living congeners (H. incongruens, barbara and rotundata), with Zenker organ lengths in the same range as fossil H. collaris (0.33–0.38 mm), have maximum sperm lengths between 1.0 and 1.6 mm, corresponding well to their carapace lengths.

With the investigated H. collaris fossil being 1.26 mm long, we estimate a maximum sperm length of 1.2–1.3 mm.

In three females of the same species, H. collaris, sperm were found packed loosely in the seminal receptacles, which are clearly defined as three-dimensional, paired ovoid-shaped organs in the hind body (specimens QMF56081, QMF56094 and QMF56098). The sperm’s preservation quality is low compared with the male, with less (but still clear) evidence of their filamentous character. By contrast, spermatozoa in a much better preservation state were found densely packed in the receptacles of N. mckenziana. Such dense packing suggests that the last insemination had occurred not long before the animal died.

Spermatozoa in the female N. mckenziana (QMF54756) and the male H. collaris (QMF56080) were subjected to nanotomography, revealing internal structures that also characterize spermatozoa of modern cypridoidean ostracods. Most of the spermatozoa in the N. mckenziana specimen are clearly differentiated, showing the external furrows of the
coat but lacking any internal pattern. A small number, however, are more difficult to discern from the surrounding matrix, but clearly show a dark, longitudinally spiralling band internally, representing the sperm nucleus (figure 5a).

The sperm are approximately 2.5 μm in width. In the male H. collaris, the longitudinal coiling is clearly visible from preserved furrows of the external coat (figure 5b,c; electronic supplementary material, video S7). Additionally, an internal Y-shaped monorail, an unpaired invagination of the sperm coat, can be seen. In living cypridoidaean ostracods, this monorail can have different shapes; a TEM section of a living congener’s sperm (H. barbara) shows that this organelle is also Y-shaped (figure 5f). Nanotomography resolved a nucleus preserved in every sperm covered by the field-of-view (figure 5d,e). Tracing of nuclei and monorail visualize the typical three-dimensional coiling within the interior of the filamentous sperm (figure 6). In this species, the sperm are approximately 4 μm in width.

Measurements of preserved soft parts in specimen QMF56081 using a confocal micro-Raman spectrometer resulted in a high band between 960 and 980 cm⁻¹, which is typical of phosphate in biogenic and geological apatites [25]. High abundance of bat fossils (and thus deposition of guano), in-the-field EDX measurements of the limestone, as well as direct measurements in a fossil thus unambiguously point to a phosphatic (apatitic) preservation of the described ostracod soft parts.

4. Discussion

(a) Preservation

The majority of vertebrate fossils at the Bitesantennary Site are derived from eight different bat species. Elemental analysis of limestone outcrops indicates both elevated P and a correlation between P, Zn, U and Sr where bone fragments are present (E. Cohen 2013, personal communication). Some samples with no visible bone fragments containing elevated P and Sr but low U and Zn may be attributed to guano in the matrix of the reworked carbonate deposits, including some from which ostracods were extracted. The extraordinary soft part preservation in the Bitesantennary ostracods may be related to the mineral composition of the water in the original Bitesantennary cave. Given the many thousands of bat fossils occurring in the same deposit, it is probable that the water in this cave was enriched with phosphorus produced by copious amounts of bat guano.
However, the preservation modes of the Bitesantennary ostracods are heterogeneous, suggesting a high variety in micropatial embedding scenarios. Two specimens from the Bitesantennary Site (QMF56080 and QMF56083) showed an unusual mineral precipitation on the surface of the appendages [24]. As this phenomenon was restricted to the surface of the appendages and body, it is unlikely to have played a role in the preservation of the sperm.

The *in situ* preservation of the internal sexual organs indicates that they were intact until mineralization of the sperm occurred. Thus, fossilization took place after some decay of the body and appendages, but before the internal sexual apparatus collapsed. This scenario is supported by several Bitesantennary ostracods that have their posterior soft body protruding from the carapace and/or a void in the dorsalmost area of the soft body, both features resulting from putrefaction processes involving decay gases. We posit that the sperm were preserved owing to rapid burial slowing down decay processes, followed by anaerobic bacterial activity enhancing phosphatic mineralization of soft tissue [24].

Frequently, a fine lining of internal voids and soft part surfaces can be observed (e.g. figure 4). It cannot be resolved whether these are natural encrustations from mineral saturated liquids that formed during diagenesis, or an artefact originating from the immersion of the sediment in acetic acid. However, as the lining does not interfere with the clearly detectable organs and cells, we argue that it happened after the tissue preserved.

The frequent presence of the oesophageal walls and the Zenker organs in the Bitesantennary ostracods attest to the prominent role of sclerotized chitinous structures in soft body preservation. Mature giant sperm cells of Recent freshwater ostracods possess a highly resistant, most probably chitinous coat [6], which is reflected in records of mummified sperm [8,17]. In the male *H. collaris* fossil containing sperm, none were preserved in the vicinity of the testes, which are attached to the inner surface of the outer carapace lamella in living Cypridocopia. As spermatids developing in the testes and the more proximal sperm ducts lack coats, preservation appears restricted to mature sperm with coats. We thus argue that the sperm coats (i) helped to delay the decay processes in favour of early mineralization of the sperm, and (ii) helped stabilize the sperm-filled body regions when the animals perished.

Sperm preserved within female receptacles of *H. collaris* are of lower preservational fidelity than those in the male specimen, but are still recognizable as filamentous spermatozoa. The record of reproductive organs in 100 Myr-old ostracods from the Santana Formation [9] included three-dimensionally prepared seminal receptacles; however, the loose aggregates of particles present in those receptacles [9] were not interpreted as remains of spermatozoa. Preservation quality of the spiral ducts and genital muscles in the fossil *N. mckenziana* is exceptional and can only be explained by a very early start of mineralization perhaps facilitated by locally higher levels of phosphorus.

(b) Fossil cell organelles

Fossil cell content has so far mainly been recorded for plant cells, e.g. in Eocene fruit and leaf cells [26], calcified Jurassic royal fern cells [27], Carboniferous pollen and spores [28] or Precambrian thallophytic seaweeds [29]. Plant- and algae-specific features, such as stable cell walls, chemical plant cell components (e.g. lignin and tannin) and plant-specific embedding scenarios (e.g. coalified compression) may facilitate this preservation of high fidelity [30]. By contrast, reports of animal cell organelles are rare, with nuclei of silici- fied epithelial frog cells from the Eocene Geiseltal Lagerstätte being the most famous examples [31,32]. Other records comprise phosphatized muscle cell nuclei in fish from the Cretaceous Santana Formation [33], phosphatized cell organelles in sponges [29] and presumed metazoan embryos [34,35] from the Precambrian Doushantuo Formation.

The fossil subcellular elements described here are the first reported from preserved animal male gametes and are outstanding in that they retain their original, three-dimensional positions in the cell, spiralling regularly around each other along the preserved length of the spermatozoa (e.g. [4]).

The ventral monorail detected in *H. collaris* sperm is distinctly Y-shaped, fully in accordance with the Y-shaped monorail we found in the living congener *H. barbara*, but different to the shape in other genera [4]. The widths in the sperm in cross sections are different in the two specimens, with those of *H. collaris* (ca 4 μm) being clearly wider than those of *N. mckenziana* (ca 2.5 μm). Measurements in extant species of the Notodromadidae (to which *N. mckenziana* belongs) suggest a sperm width of around 2 μm (R.M.K. and R.J.S. 2013, personal observation) while those of *H. barbara*, a living congener of *H. collaris*, measure around 4 μm in their posterior part (figure 5f). We infer from these data that even genus-specific traits of the sperm, such as sperm width and shape of cell organelles, can survive mineralization.

(c) Evolution of giant sperm

Most work concerning giant sperm has previously focused on the fruit fly genus *Drosophila*, where it is postulated to be the result of female cryptic choice. Giant sperm in *Drosophila* is considered to be a fast evolving trait of recent origin that has evolved numerous times in the genus [36]. This is clearly not the case in ostracods, as demonstrated by the Early Miocene Riversleigh specimens, which suggests that the reason for evolving giant sperm can have a far more ancient rationale. Although several hypotheses have been put forward to explain the function and evolution of giant sperm in Cypridioidea, most of these have been discounted [3] and none have taken into account the geological longevity of the feature. In contrast to other groups with giant sperm, the potential of studying their evolution from fossils is currently unique in ostracods. Only in this group is there an unparalleled fossil record combined with relatively frequent occurrences of soft body preservation and an exceptionally resistant extracellular sperm coat.

In *Drosophila melanogaster*, a coevolution of receptacle and sperm length has been experimentally identified [37], confirming the hypothesized post-copulatory selection force on the sperm. While the *Drosophila* receptacles are of well-defined size and shape, those of cypridoidean ostracods are membranous, elastic, sac-like organs that get inflated like balloons when filled with sperm. Their final length/volume cannot easily be predicted. However, the duct connecting the vagina and seminal receptacle, the spiral duct, is exceptionally long in cypridoidean ostracods, coiled tightly into a structure formed like a clew (e.g. up to 12.0 mm long in an ostracod of 3.0 mm body length with sperm of up to 4.8 mm length; [3]). It stands to reason that the length of the spiral duct is related to the length of the sperm that must pass it to reach the seminal
receptacle; however, it is not obvious why this duct reaches lengths that are several times as long as the sperm.

The unambiguous identification of the two long spiral ducts leading to the two giant seminal receptacles preserved in a female *N. mckenziana* proves the coevolution of male sperm gigantism and the morphology of the female reproductive tract. Further, this specimen gives direct fossil evidence of insemination with giant sperm. The tightly filled seminal receptacles suggest that mating had occurred not long before the animal died.

The fossil clew of the spiral duct in *N. mckenziana* reported here, with its high number of coils, is morphologically very similar to those of living species in the same group. In conjunction with the giant sperm in the typically formed, inflated receptacle, the fossil evidence suggests that this Early Miocene species reproduced in a manner very similar to living species. Consequently, the evolutionary roots of this distinctive mode of reproduction in ostracods must be older than the Early Miocene, which does not contradict earlier claims based on indirect evidence [9] that giant sperm were used by Cretaceous ostracods from the Santana Formation. The roots of this mode of reproduction must therefore be placed even deeper in time.

Cypridioidean sperm, with their aflagellate and at the same time highly filamentous morphology, are fundamentally different to other giant sperm. As here elaborated, this unique form is a very conservative feature that must have evolved a very long time ago and seems to have been irreversible at least since the Miocene. Starting from standard-type cell organelles, a massive reorganization of the sperm nucleus and mitochondria into these exceptionally long and unique sperm must have taken place, and no evolutionary alteration of this trait occurred for tens of millions of years. This stands in contrast to the sperm of other taxa, which show dramatic evolutionary divergence (so much so that species-specific diversity is unexpectedly high in some taxa [1]). Consequently, it is not just the origin and function of ostracod giant sperm that are biological enigmas, the lack of evolution of this feature is an enigma in itself.

In conclusion, we recommend making use of synchrotron X-ray tomographic methods on a broader scale in ostracod palaeontology, because it is non-invasive and allows for identification of soft part preservation even when the latter cannot be observed with standard optical techniques due to carapace closure. The high rate of preservation of soft tissues in the investigated ostracod fauna suggests that such preservation is more frequent than traditional, surface-restricted fossil investigations may indicate. Information to be extracted from preserved soft parts might fundamentally improve the understanding of ostracod evolution in general and its value cannot be overestimated. In particular, the fossil record of ostracods represents an excellent opportunity to study longevity and evolution of reproduction involving giant sperm.

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