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SHORT COMMUNICATION

Exceptionally well-preserved giant spermatozoa in male and female specimens of an ostracod *Cypria ophtalmica* (Crustacea: Ostracoda) from Late Glacial lacustrine sediments of Southern Carpathians, Romania

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Abstract Exceptionally well-preserved giant spermatozoa observed between abundant decalcified carapace valves of ostracods (Crustacea: Ostracoda) were found in Late Glacial to Holocene (14,400 to 10,000 cal years BP) lacustrine sediments in the southern Romanian Carpathians. Analysis by scanning electron microscopy and laser scanning confocal microscopy revealed good preservation of the appendages enabling specific identification as *Cypria ophtalmica* (Candonidae) and indication of the presence of both female and male specimens based on the sexual dimorphism of the second antenna. This record represents the oldest and richest direct evidence of virtually morphologically unaltered animal spermatozoa preserved in females after mating.

Keywords Giant spermatozoa in females · Exceptional preservation · Ostracoda · Romanian Carpathians · Late Glacial · Holocene

Introduction

Ostracoda are small aquatic crustaceans with spermatozoa that are the second longest (after some species of *Drosophila*) in the animal kingdom. Species belonging to the suborder Cypridocopina, which are approximately 1-mm-long, can have 1-cm-long sperm, i.e., ten times of their body length (Matzke-Karasz 2005). It is probable that these giant sperm evolved only once in ostracods and have been present for at least the past 100 million years (Matzke-Karasz et al. 2009).

In contrast to calcified bivalved carapaces, which fossilize easily and have an outstanding fossil record that dates back to the Early Ordovician (480 million years ago), ostracod limbs and internal organs have a low preservation potential (Wilkinson et al. 2010). Instances of preservation of sclerotized parts of the ostracod soft body are rare and known for only approximately 30 extinct and extant species

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(see review in Wilkinson et al. 2010, and more recent records by Olempska et al. 2012 and Tanaka et al. 2012).

To date, direct evidence of fossil giant ostracod spermatozoa is known only for 16 specimens of five species found at two sites (Germany and the UK). The oldest record dates back to 5,000 years and was obtained from lake marls in eastern England (Matzke-Karasz et al. 2001). At that time, this finding also represented the oldest record of fossil animal spermatozoa, with the exception of the 40-million-year-old sperm included in two stalked spermatophores of a springtail preserved in amber (Poinar 2000). More recently, indirect evidence of giant ostracod sperm was furnished by the presence of male Zenker organs (sperm pump) and inflated female seminal receptacles revealed by the holotomography technique applied to five specimens of 100-million-year-old *Harbinia micropapillosa* (Bate) from Brazil, a nonmarine species of the family Cyprididae (Matzke-Karasz et al. 2009). Here, we report on the oldest giant ostracod spermatozoa, preserved in large numbers in both males and females from lacustrine sediments in Southern Carpathians (Romania) over the past 14 millennia.

Material and methods

The ostracod material was obtained from the 90-cm-long bottom section of a 4.9-m-long sediment sequence (core TDB-1: 45°23′47″N, 22°54′06″E) recovered in 2007 with a Livingstone piston corer from the 1.1-m-deep bottom of a small (0.5 ha) and shallow fishless lake/pond Tăul dintre Brazi, surrounded by a floating carpet of peat moss and acid bog-habitat plants. The lake is situated in the subalpine belt (1,740 m AMSL) of a Norway spruce and stone pine mixed forest in the Retezat Mts. National Park in the Romanian Carpathians, an area covered by brown acid soil underlaid by granodiorite bedrock (Magyari et al. 2009).

The chronology of the TDB-1 core was established from accelerator mass spectrometric ^{14}C dating of terrestrial plant macrofossils. Based on the age-depth model, the lake, of a kettle-hole origin, became free from ice ca. 15,700 calibrated years before the present (cal years BP), and sediment accumulation was continuous throughout the Late Glacial and Holocene (Magyari et al. 2009).

For the ostracods, 7 to 12 cm^3 of fresh sediment was processed using standard methods (Danielopol et al. 2002) continuously, primarily at 2-cm intervals, throughout the recovered profile. All of the ostracod remains were hand-picked in wet conditions without preliminary treatment from each of the 39 fresh sediment samples. The recovered carapaces and valves were then counted, identified, and screened for chitinous remains under reflected and transmitted light microscopes either in distilled water, ethanol, or glycerine. Ostracods appeared at the beginning of the Late Glacial in a sample from 14,400 cal years BP, when first afforestation and warming occurred in the area (Magyari et al. 2011), and appeared in all of the subsequent samples of the studied profile with the mean abundance of 34 valves per 1 cm^3 of fresh sediment (Iepure et al. 2011; Korponai et al. 2011).

For comparison with the ancient material, a sample of live specimens was collected in December 2010 with a hand net (mesh size of 120 μm) from the shallow bottom of the lake and further processed with methods identical to those used for the sediment samples from the core. Both the ancient specimens with the well-preserved spermatozoa and the specimens collected live were analyzed and imaged using their natural autofluorescence with a Leica SPE laser scanning confocal microscope (LSCM) at 10× 0.30 N.A, 40× oil 1.25 N.A., and 63×gli 1.30 N.A. on temporary glycerin slides. The image stacks were preliminarily viewed with FIJI (http://fiji.sc/wiki/index.php/Fiji), and then rendered with Amira ver. 5.3.3. and Voltex algorithm. To study the details of the finest structures of spermatozoa and limb remains, scanning electron microscopy (SEM) was also used with a dehydrating method (critical-point-drying protocol). The study material is deposited in the Institute of Speleology "Emil Racoviță" in Cluj, Romania.

Results and discussion

The sediment sequence that was studied, corresponding to the period of 15,200–10,000 cal years BP, yielded a total of approximately 12,000 decalcified valves of a single ca. 0.6-mm-long eurytopic species *Cypria ophtalmica* (Jurine). However, the chitinous remains of some limbs, Zenker organ, and spermatozoa appeared in exceptionally well-preserved form between the soft valves (Figs. 1a and 2a) of ~5–10 % of the specimens of both sexes in almost all of the studied sediment sequence. The age of the oldest sediment sample from which the ancient spermatozoa were retrieved was 14,400 cal years BP, whereas the youngest one was 10,000 cal years BP (Iepure et al. 2011).

Most of the recorded spermatozoa formed pairs of circular bundles positioned dorsally in mid-length of the carapace (Fig. 1a), suggesting that they were stored in the male seminal vesicles in front of the entrance of the Zenker organ on both sides of the body (Fig. 1b). However, some carapaces contained less regular spermatozoa bunches situated more posteriorly and often dislocated from each other (Fig. 2a), which, based on the living material, we interpret as females that had their seminal receptacles filled with spermatozoa after insemination. Identification of female specimens with spermatozoa was also proved based on the sexually dimorphic

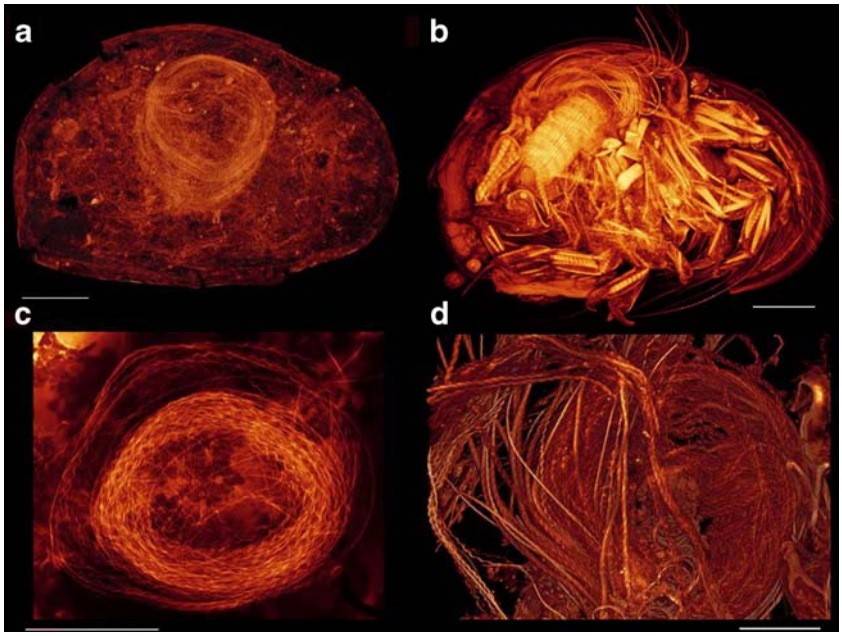

Fig. 1 Giant spermatozoa of 13,300-year-old (**a**, **c**) and recent (**b**, **d**) males of *C. ophtalmica* viewed using the LSCM technique. Lateral view of entire specimens with bundles of spermatozoa (**a**, **b**) and details of the external morphology of spermatozoa (**c**, **d**) (*Scale bars*: **a**, **b**=100 μm; **c**, **d**=60 μm)

second antenna with the penultimate segment undivided and the male t-setae absent (Fig. 2a, b). Applying both the LSCM and SEM techniques allowed us to confirm that overall shape and size estimate (diameter, 0.5–0.8 μm) were in good agreement with the standard spermatozoon type of the superfamily Cypridoidea, described as a long nonflagellate coiled filament (Figs. 1c and 2c–f). Compared with the modern material (Fig. 1b, d), portions of the ancient sperm resemble the corkscrew-like anterior region (Fig. 2e–f), whereas other portions have the appearance of an undulating filament (Fig. 2c–d). This appearance may be a result of the removal of an extracellular sheath after insemination (if observed in females)

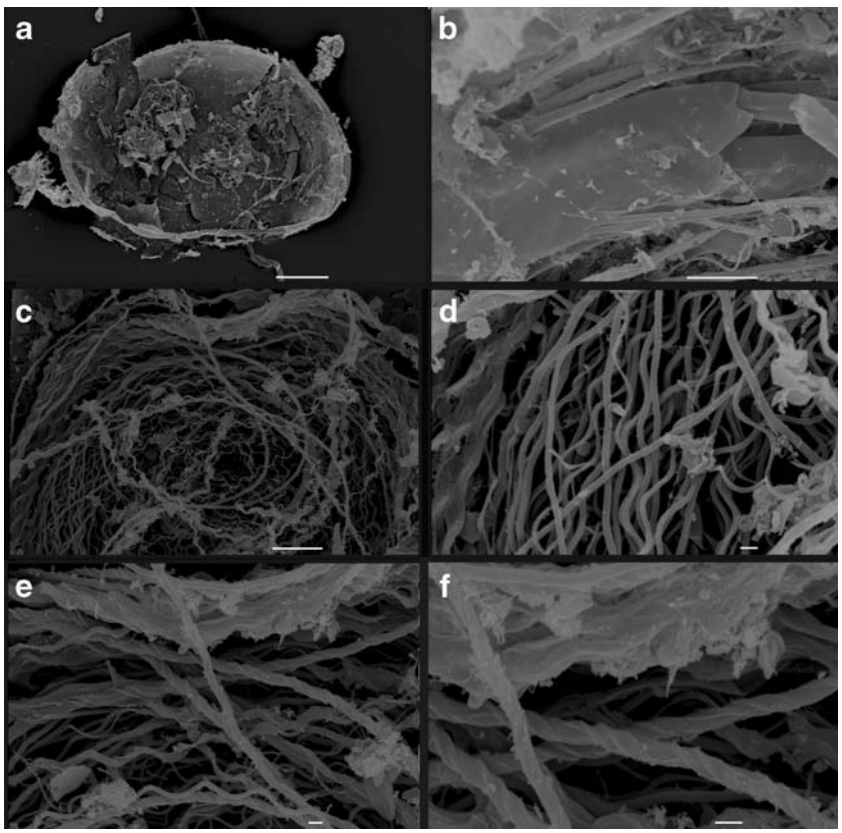

Fig. 2 Spermatozoa preserved in a 13,300-year-old female of *C. ophtalmica* viewed using SEM. Lateral view of a specimen (interior of left valve) with bundles of spermatozoa and appendage remains (**a**), details of the second antenna (**b**), and details of the finest external morphology of spermatozoa (**c**–**f**) (*Scale bars*: **a**=100 μm; **b**, **c**=10 μm; **d**–**f**=1 μm)

or of the disintegration of this sheath after burial or the treatment of the ancient material of both sexes (Matzke-Karasz et al. 2001).

It is probable that such well-preserved material resulted from the relatively acidic nature of the sediment, a consequence of the decomposition of organic plant matter combined with relatively low temperatures and the substantial presence of acid soil in the forest situated on the intrusive igneous rocks of the basin of Tăul dintre Brazi. Based on our finding of the oldest record of animal spermatozoa preserved in both sexes, it is likely that ancient sperm may be more commonly mummified in Quaternary sediments than the few existing ostracod records would indicate. Rare occurrences of soft body preservation in the fossil record are extremely valuable to test morphologies and evolutionary hypotheses based on hard body parts. We believe that our finding of the ancient animal spermatozoa preserved within the space of ca. 4.5 thousand years as morphologically unaltered material, retaining submicrometer scale details, and exceeding the age and number of other records raises the possibility of DNA extraction and amplification, and potentially allowing new insights into genetic consequences of long-term environmental changes on the reproductive modes in ostracods.

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