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Tiny vampires in ancient seas: evidence for predation via perforation in fossils from the 780–740 million-year-old Chuar Group, Grand Canyon, USA

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One explanation for the Early Neoproterozoic expansion of eukaryotes is the appearance of eukaryovorous predators—i.e. protists that preyed on other protists. Evidence for eukaryovory at this time, however, is indirect, based on inferences from character state reconstructions and molecular clocks, and on the presence of possible defensive structures in some protistan fossils. Here I describe 0.1–3.4 μm circular holes in seven species of organic-walled microfossils from the 780–740 million-year-old Chuar Group, Grand Canyon, Arizona, USA, that are similar to those formed today by predatory protists that perforate the walls of their prey to consume the contents inside. Although best known in the vampyrellid amoebae, this ‘vampire-like’ behaviour is widespread among eukaryotes, making it difficult to infer confidently the identity of the predator. Nonetheless, the identity of the prey is clear: some—and perhaps all—of the fossils are eukaryotes. These holes thus provide the oldest direct evidence for predation on eukaryotes. Larger circular and half-moon-shaped holes in vase-shaped microfossils from the upper part of the unit may also be the work of ‘tiny vampires’, suggesting a diversity of eukaryovorous predators lived in the ancient Chuar sea.

1. Introduction

Predators exert a strong influence on the evolution of their prey, driving both innovation and diversification [1,2]. Although most attention has been paid to the role of metazoan predators in shaping the Phanerozoic history of life [3], predatory behaviour—killing for nutritional purposes—is much more ancient and widespread. Bacteria were probably preyed on by other bacteria in Earth’s earliest ecosystems [4], and were almost certainly preyed on by eukaryotes by Late Mesoproterozoic time, when we first see evidence for red algae and therefore primary plastids, acquired via phagocytosis of a cyanobacterium [5]. It is not clear, however, when eukaryotes themselves became prey. A recent study suggests that the earliest eukaryotes were bacterivorous, and that eukaryovory—the ability of protists to eat other protists—evolved much later, arising independently in several eukaryotic clades that diversified *ca* 800 million years ago (Ma) [6,7]. This is consistent with other indirect evidence for the rise of eukaryovory around this time [6,8,9], including the first appearance of mineralized skeletons and organic tests in protists, possibly used for defence [10,11]; bio-marker evidence for the eukaryotic production of lytic toxins that functioned either in defence or as a weapon for killing eukaryotic prey [12]; and fossil evidence for arcellinid testate amoebae, protists whose modern relatives consume both bacteria and eukaryotes [10,13].

Here I report the presence of circular holes in the walls of protistan fossils from the 780–740 Ma Chuar Group, Grand Canyon, Arizona, USA, similar to holes made today by predatory protists that perforate the walls of their prey to consume the cell contents inside. These holes provide the earliest direct fossil evidence for predation on eukaryotes and support the view that by 740 Ma, predation was an important agent shaping eukaryote ecology and evolution [6,8,9].

Figure 1. Sub-micrometre circular perforations in the walls of several organic-walled microfossils from the 780–740 Ma Chuar Group, Grand Canyon, Arizona. (*a,b,d,f*) '*Trachysphaeridium*' *laufeldii*. (*b*) TEM image showing a perforation in cross-section; note the bevelled walls. Only the outer wall of the fossil is perforated; the inner, ornamented wall is not. Specimen is same as that shown in (*a*); hole is shown in the lower of the two close-up views. Note that the lighter areas in the image are the organic material of the fossil; the darker areas are the Pt coating. (*c*) A specimen of *Leiosphaeridia* sp. (*e*) New species 2, interpreted to be a scale-bearing protist. (*g*) A specimen of *Valeria lophostriata*. Scale bar is 10 µm for microfossils shown in (*a,c–f*); 30 µm for (*g*); and 1 µm for all close-up views of perforations. Additional perforations are indicated by small orange circles on microfossil specimens; note their irregular distribution. (Online version in colour.)

2. Background and methods

The 1600 m-thick Chuar Group comprises mostly shales and siltstones exposed over a 15 km² area in the eastern Grand Canyon [14]. Chuar sediments were deposited in a shallow marine restricted seaway [14] located within 18° of the palaeoequator [15]. A U–Pb detrital zircon age of *ca* 782 Ma from the underlying Nankoweap Formation [16] and a U–Pb zircon age of 742 ± 6 Ma from an ash at the top of the Chuar Group [17] place these strata in the late Tonian Period of the Neoproterozoic Era, about 20 Myr prior to the 'snowball Earth' glaciations [18], during the first major diversification of eukaryotes [9].

The holes described here occur in organic-walled microfossils found throughout the Chuar Group. Although originally spheroidal vesicles, the fossils now occur as 1–2 µm-thick discs, a few tens to hundreds of micrometres in diameter. Twenty-three species of organic-walled microfossils have been described from the Chuar Group, including the 1–3 mm compression *Chuaria circularis* [19,20]. In addition, 17 species of vase-shaped

microfossils (VSMs), interpreted as the remains of arcellinid and possibly euglyphid testate amoebae occur in the upper part of the unit [10,13].

Fossils were released from their host rocks via HCl and HF acid maceration and viewed using scanning electron microscopy (SEM) and transmission electron microscopy (TEM) systems housed at UCSB's Earth Science Department and Materials Research Laboratory (see [20] for more details).

3. Description of holes

The holes occur in seven species and an unnamed form. Five of these species and the unnamed form are interpreted to be the remains of vegetative cells or resting cysts (figures 1*a,c,d,f,g* and 2*b*; [20]); a sixth species is interpreted to be a possible scale-bearing protist (figure 1*e*; [20]) and a seventh species, represented by 30–100 µm sized smooth-walled fragments, is assumed to be *C. circularis* (figure 2*a*). The holes are circular in

Figure 2. Micrometre-sized circular perforations in fragments of (a) a smooth-walled fossil, probably *Chuaria circularis* and (b) *Cerebrospira globosa*. Note their bevelled appearance. Scale bar is 10 μm for (a), 20 μm for (b) and 2 μm for all close-up views. (Online version in colour.)

shape and occur in specimens that may otherwise show no signs of pitting or degradation (e.g. figure 1c). In some cases, they are bevelled (e.g. figures 1b and 2), with the diameter narrowing towards the interior of the fossil (figure 1b). The holes do not always perforate the wall completely; in some cases, they extend only through the outer wall of a double-walled fossil (figure 1b,f). They range in size from 0.1 to 3.4 μm in diameter, but exhibit much narrower size ranges within species and within specimens (figure 3). Within '*Trachysphaeridium*' *laufeldii*, for example, hole diameters range from 0.1 to 0.6 μm (mean = 0.3 μm , $n = 34$); within individual specimens the range narrows to 0.2 μm (figure 3). Similarly-sized holes are found in *Leiosphaeridia* sp. and *Valeria lophostriata*, as well as in two new species and an indeterminate form (described in [20]; figure 3). By contrast, larger holes are found in fragments of *Cerebrospira globosa* (= *C. buickii*; see [20]) (figure 2b; 1.2–2.1 μm in diameter; mean = 1.3 μm ; $n = 8$) and *C. circularis* (figure 2a; 0.6–3.4 μm in diameter; mean = 2.0 μm , $n = 42$). Holes of different sizes may occur in fossils from the same rock sample, but always in different species (figure 3). The difference in hole sizes among species broadly corresponds to size differences among the species: species with vesicles typically tens of micrometres in diameter have smaller holes (0.1–0.6 μm); those with vesicles hundreds to thousands of micrometres in diameter have larger holes (0.6–3.4 μm).

Holes typically number between 1 and 10 in a single specimen (median = 5 observed per specimen), although holes in *C. circularis* may be more densely distributed, in some cases, overlapping (figure 2a; overlapping holes have not been observed in other species). In specimens with multiple holes, their spatial distribution is scattered rather than regular (e.g. figure 1a,d,f). For most species, only a few (one to six) specimens were examined under SEM, making it difficult to estimate the frequency of those with holes. However, in '*T. laufeldii*', a species that was both abundant and a focus of close study [20], about a tenth of the specimens examined (9 of 108) exhibited holes, although this is probably an underestimate given that the holes can easily escape notice.

4. Origin of holes

Both the irregular spatial distribution of the holes within individual specimens and the fact that they occur in a variety of disparate species but are not widespread in any single one,

Figure 3. Distribution of hole sizes (indicated markers) within specimens (represented by vertical lines), from nine samples from the Chuar Group. Samples are in stratigraphic order from left to right, oldest to youngest. The species of each specimen is indicated by colour and marker shape. Note that while there is a broad range of hole sizes within the Chuar Group assemblage, the range of sizes within species and within specimens is much narrower. See the electronic supplementary material, table S1 for more information. (Online version in colour.)

suggest the holes are not an original character of the organism, such as pores in the cell wall. Similarly, while minerals can perforate fossils during sediment compaction and diagenesis, the pits and holes they leave are polygonal in shape and irregular in size, and may extend through both walls of a flattened fossil (S. M. Porter 2015, personal observation; [21])—characteristics never observed in the holes described here. Post-mortem degradation of the wall material itself (i.e. scavenging by

Figure 4. Perforations in spores of the fungus *Cochliobolus sativus* made by vampyrellid amoebae [32,33]. (a) Overview of spores, several showing circular perforations. (b) Close-up view of spore, showing several circular perforations, each approximately $0.2\ \mu\text{m}$ in diameter. Scale bar is $20\ \mu\text{m}$ in (a), $2\ \mu\text{m}$ in (b). Reprinted with permission from [32], Copyright © Canadian Science Publishing or its licensors.

microbes) seems unlikely, as there is no evidence for a range of decomposition among specimens in the assemblage: they either have one to several holes or they have none at all; no specimens are, for example, half eaten or mostly eaten, and none show the wide zones of erosion, or irregularly shaped, irregularly sized pits and perforations associated with modern bacterial scavenging [22,23]. Indeed, if these holes were interpreted to reflect post-mortem degradation, it would be necessary to invoke an ad hoc scenario in which microbial decomposition began and almost immediately stopped at precisely the same stage repeatedly throughout Chuar Group deposition, such that specimens from samples spanning approximately 1400 m of stratigraphy show holes—if they show them at all—that are consistently the same size.

Instead, it seems that the best interpretation for the holes is that they were formed as a means to get through the wall, not to eat it. In support of this view, the holes are similar to those made by modern predatory protists that perforate the resistant walls of their prey to consume the cell contents inside. This ‘vampire-like’ behaviour is widespread among eukaryotes, occurring in, for example, the amoeboid trophic phases of some amoebozoan slime moulds [24]; several close relatives of the Fungi [25,26]; the foraminiferan *Floresina amphiphaga* [27]; and several different cercozoan clades [28–31]. (Note that the term parasite has been applied to some of these organisms because they insert themselves fully inside the wall of the ‘host’ cell, consuming it from within [26]. However, as this behaviour still results in the death of the ‘host’, it falls under the broad definition of predation used here: killing for nutritional purposes.) The behaviour is best known from the aptly named ‘vampire amoebae’ or Vampyrellida (Cercozoa, Rhizaria), a group of naked, heterotrophic, filose and reticulose amoebae that are widespread and diverse in freshwater, soil and marine environments, preying on fungi, algae, protozoa and small metazoans [30,31]. A single vampire amoeba typically produces multiple, irregularly distributed, consistently sized circular holes in the walls of its prey (figure 4; [32,33]), with different species forming holes of different sizes: e.g. $2\text{--}6\ \mu\text{m}$ diameter holes in *Arachnula impatiens* [34], $6\text{--}8\ \mu\text{m}$ in *Lateromyxa gallica* [35], $0.2\text{--}0.5\ \mu\text{m}$ in *Theratromyxa* sp. [34,36] and $0.2\text{--}0.6\ \mu\text{m}$ in *Vampyrella vorax* [36]. In addition to specificity of hole size, there is evidence for specificity in prey: Hess *et al.* [30] observed that *Vampyrella lateritia* prefers zygnematalean green algae and *V. pendula* prefers oedogonialean green algae, and that neither will eat the other’s favoured food.

Some species form holes via an annular incision, lifting out and ingesting the resulting disc, producing holes with a bevelled edge [35,37]; other species that make very tiny holes (e.g. $0.2\text{--}0.6\ \mu\text{m}$ holes formed by *Theratromyxa* sp.) are thought to macerate the entire area, rather than cutting out a disc [36].

In summary, several observations support the view that the Chuar holes were made by predatory protists: their similarity in shape and size to holes made by modern predators (e.g. figure 4); the fact that a single microfossil may have multiple similarly-sized holes, consistent with the fact that a single predatory protist may make numerous holes in its prey ([26,36]); and apparent specificity in hole size and microfossil species (figure 3), consistent with species-specificity in hole size and prey preference observed in vampyrellids [30,34].

Given that the ability to perforate cell walls is apparently widely convergent (occurring even in some bacteria [38]), it is not possible to identify with confidence the predators that made the holes in the Chuar fossils; they may be early members of one of the modern vampire-like clades, or they may represent an extinct clade that independently evolved the ability to perforate resistant organic walls. What can be identified with confidence, however, is their prey: at least four of the species are eukaryotes, as indicated by their size, wall ornamentation and excystment structures; the most commonly perforated of these, ‘*T. laufeldii*’, is characterized by networks of thin filaments arising from the vesicle wall, rounded excystment structures and bud-like protuberances, all hallmarks of eukaryotic organization [20,39]. (A prokaryotic affinity cannot be ruled out for the other three prey species—*C. circularis*, *Leiosphaeridia* sp. and new species 2.) Thus, the Chuar holes provide direct evidence for predation on eukaryotes *ca* 780–740 Ma, corroborating the view that predators were an important influence on protistan ecology and evolution by mid-Neoproterozoic time [6,8,9].

5. Other vampires in the Chuar Group?

An earlier study of VSMs from the Chuar Group revealed the presence of half-moon-shaped holes in several specimens, interpreted to reflect predation (figure 5*a–d*; [13]). The half-moon holes are $15\text{--}35\ \mu\text{m}$ in length and are consistently oriented with their flat edge parallel to the long axis of the VSM test, suggesting stereotypy in prey manipulation. In addition, a few VSM specimens exhibit circular holes, $13\text{--}30\ \mu\text{m}$ in diameter, in one case occurring in a test that also exhibits a half-moon hole (figure 5*c,e–g*; not previously reported). Like the much smaller holes that are the focus of this paper, these holes seem to be best explained by the action of vampire-like predators, though their much larger size would indicate different species of predators at work. Together with the VSMs themselves, interpreted to be the remains of arcellinid testate amoebae, and the biomarker cryostane—thought to be derived from eukaryotic sterols that protected their producer from lytic toxins [12]—these holes suggest that a diversity of eukaryovorous predators lived in the Chuar sea.

6. Other ancient vampires?

The very small size of many of the Chuar holes (e.g. $0.1\text{--}0.6\ \mu\text{m}$) means that they are only visible via SEM, a technique not routinely used in the study of Proterozoic microfossils.

Figure 5. Half-moon and circular holes in vase-shaped microfossils from the Chuar Group. (a) *Bonniea pytiniaia*; (b,d) possible *Bonniea pytiniaia*; (c) *Cycliocyrrillium torquata*; (e) *Bonniea dacruchares*; (f) *Cycliocyrrillium simplex*; (g) *Trigonocyrrillium horodyskii*. White arrows in (c,e,f,g) point to circular holes; black arrows in (c,d) point to half-moon holes.

Nonetheless, a cursory review of the literature indicates the presence of similar holes in a few other microfossils of the same age. Butterfield *et al.* ([40], fig. 12H) documented 1–3 µm circular holes in a specimen of *C. globosa* (= *C. buickii*) from the ca 750 Ma Svanbergfjellet Formation, Akademikerbreen Group, Svalbard (cf. figure 2b), and suggested they were formed by predators or scavengers (other specimens from that same unit also have similarly-sized circular holes; L. A. Riedman 2015, personal communication). A fragment of *C. globosa* with what appears to be a small circular hole was also illustrated in Nagovitsin *et al.* ([41], fig. 9e) as part of a Tonian-aged assemblage from the Siberian Platform. Numerous, scattered 4 µm holes in organic-walled tubes from the Palaeo-Mesoproterozoic Ruyang Group, China, may hint at a much older presence of predatory microbes ([42], fig. 2e,f), although more detailed examination via SEM is required to rule out other explanations.

Vampyrellid and other predatory protists might be useful models to explain minute circular holes also observed in younger fossils. For example, Wrona [43] reported the presence of numerous, irregularly scattered, sometimes bevelled, circular holes in Ordovician chitinozoans, organic, vase-like microfossils thought to be metazoan egg cases [44]. Hole sizes range from 0.7 µm to 12 µm in diameter, but individual specimens exhibit holes with much narrower size ranges. The presence of an incomplete perforation in one specimen ([43], pl. 23, fig. 7b) shows that the holes were made via an annular incision, similar to the mechanism used by some vampyrellid amoebae [35,37]. Similarly, the numerous micrometre-scale borings found in some living and fossil foraminifera may be the result of vampyrellids or other predatory protists, rather than metazoans [45].

7. Conclusion

Body fossils and biomarkers from approximately 1200 to 740 Ma rocks record taxonomic and ecological expansion of

eukaryotes, evidenced by an increase in eukaryotic micro- and macrofossil diversity; the appearance of morphologically disparate forms, including scale- and test-bearing taxa; increases in the relative contribution of eukaryotic algae to primary productivity; and the appearance of multicellularity in several eukaryotic clades [6,9]. It has been suggested that eukaryovorous predators played an important role in this expansion by driving diversification, increased productivity and morphological innovation in their prey [6,8,9]. It has been difficult to test these hypotheses, however, because unlike the Phanerozoic fossil record, in which the widespread occurrence of drill holes and shell repair scars has allowed predation intensity to be quantified through time (e.g. [46]), the Precambrian record of predation is virtually non-existent. The recognition that Neoproterozoic microfossils preserve minute ‘drill’ holes made by perforating predators, and that these holes may be widespread in Neoproterozoic assemblages, offers the prospect that a record of predation intensity can be extended much further back in time, making it possible to test the extent to which predator–prey interactions shaped the early evolution of life.

Data accessibility. All data associated with this manuscript are available as the electronic supplementary material. Additional information on specimen locations and accession numbers can be found at Dryad digital repository: <http://dx.doi.org/10.5061/dryad.6pr6c>.

Competing interests. I declare I have no competing interests.

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