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Highlights

- The use of metal(loid)s to kill microbial prey is central to the predatory lifestyle of phagocytic protists.
- Due to high energy requirements, this phagocytic feeding mechanism most likely originated only after the endosymbiotic acquisition of the high-energy yielding mitochondrion.
- This intense energy and metal bioavailability need imply the resultant recruitment of metals by the phagosome for microbial prey killing most likely coincided with widespread oxygenation of the ocean-atmosphere system.
- The late evolution of the phagotrophic eukaryotes suggests the potential origin of the prey killing phagosome originated closer to the emergence of the multicellular metazoans and the immune systems.

• **A brief history of metal recruitment in protozoan predation**

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Abstract

Metals and metalloids are used as weapons for predatory feeding by unicellular eukaryotes on prokaryotes. This review emphasizes the role of metal(loid) bioavailability over the course of Earth's history, coupled with eukaryogenesis and the evolution of the mitochondrion to trace the emergence and use of the metal(loid) prey-killing phagosome as a feeding strategy. Members of the genera *Acanthamoeba* and *Dictyostelium*, use metals such as Zn and Cu, and possibly metalloids to kill their bacterial prey after phagocytosis. We provide a potential timeline on when these capacities first evolved and how they correlate with perceived changes in metal(loid) bioavailability through Earth history. The origin of phagotrophic eukaryotes must have post-dated the GOE in agreement with redox-dependent modification of metal(loid) bioavailability for phagotrophic poisoning. However, this predatory mechanism is predicted to have evolved much later — closer to the origin of the multicellular metazoans and the evolutionary development of the immune systems.

Metals and Life

Life evolved in close intimacy with changing paleo-seawater chemistry, reflected by the diverse assemblage of elements utilized for the synthesis of biomolecules and as structural support in modern living things. Particularly, the adoption and use of trace metals in fundamental biological processes is thought to correlate with their historical seawater bioavailability through Earth history [1-8]. For example, iron (Fe) comprises 5.5% of the Earth's crust and 84% of the total core [9]. It is therefore unsurprising that

all cells use Fe in many highly conserved processes including gene expression, intermediate metabolism and DNA replication [10]. During the Archean, ~2.5-3.0 billion years ago (Ga), Fe was highly bioavailable in the anoxic seawater that dominated the oceans, but began to decline dramatically following the Paleoproterozoic Great Oxidation Event (GOE) at the Archean-Paleoproterozoic boundary, when the first permanent rise of oxygen in the ocean-atmosphere system [11] triggered large scale oxidative removal of Fe from seawater. This eventually led to the present-day oxygen-rich Fe-depleted ocean [9]. Equally, the nickel (Ni) content of Archean seawater is thought to have been significantly much higher, followed by a steep redox-dependent decline during the GOE [12]. Taken together, this suggested abundance of both Fe and Ni in Archean seawater correlates well with recent theories on their importance in the origin of the earliest life forms, as they are both important constituents of ancient metabolic pathways and enzymes like (NiFe)-hydrogenase and carbon monoxide dehydrogenase [13].

Similarly, various heavy metals (also known as transition metals) such as copper (Cu), manganese (Mn) and zinc (Zn), critical for the functioning of many modern oxygen-dependent metabolic pathways and enzymatic reactions, are thought to have become more important post-GOE, especially in connection with the rise of the oxygen-respiring metazoans [1-8]. However, because life first emerged in the predominantly anaerobic Fe-rich Archean seawater conditions, the biosphere was dominated by microbes containing ancient metabolic pathways with Fe as a cofactor (e.g., the iron-

sulfur clusters), resulting in them being vulnerable to excess intracellular concentrations of bioessential and non-essential heavy metals that became more bioavailable after the Archean interval [14]. We propose that this vulnerability was exploited by an evolved lineage of microbes capable of efficiently using post-Archean ocean metal(loid) content to poison vulnerable microbial competitors. This prey-predator interplay became particularly significant as a survival mechanism with the emergence of the eukaryotes, when they acquired the ability to use phagocytosis to engulf other organisms and kill them as their principal food source. The evolution of the mitochondrion supported this new feeding strategy by providing the required energy. Therefore, protist-dependent metal(loid) predation had to have started after eukaryogenesis, here defined as being able to carry out phagocytosis and being able to generate sufficient energy via the mitochondria [15]. Thus in this review we attempt to provide a broad timeline for eukaryogenesis, linked to the environmental and molecular processes that led to certain metal(loid)s being chosen to aid in protist predation. In principle, a number of metals could play a role but experimental evidence only exists for Cu, Zn and As [16-18]. Therefore, we focus on these metals and analyze how unicellular eukaryotes have been able to acquire them and how amoeba (and probably other protists and later macrophages) use them in predation.

Metal recruitment by eukaryotic microorganisms

Metal uptake, particularly in the case of Zn and Cu, into unicellular eukaryotes is better studied in diatoms than in protozoa. However, similar processes should be employed.

95 Zinc is an essential micronutrient for the growth of phototrophic protists such as
96 diatoms. To cope with Zn biolimitation in oceans, marine diatoms evolved multiple
97 adaptive mechanisms in response to Zn deficiency, for instance, increasing expression
98 of the high-affinity uptake systems, and substituting cobalt II (Co(II)) and cadmium II
99 (Cd(II)) in place of Zn(II) as the cofactor within certain subclasses of algal carbonic
100 anhydrase [19, 20]. A recent study identified two Zn/Co responsive proteins, ZCRP-
101 A/B, involved in the metabolic response to Zn/Co limitation in marine diatoms and
102 found the widespread ZCRP-A/B homologs in marine protists [21]. ZCRP-A is a
103 putative Zn(II) metallochaperone responsible for the allocation and reallocation of Zn
104 under low Zn conditions, and ZCRP-B is a component of a membrane-bound transport
105 complex.

106

107 Copper transporter (CTRs) [22] involved in Cu(I) uptake in marine diatom
108 *Thalassiosira oceanica* [23], and their homologs are identified in many other diatoms
109 [24, 25]. Before Cu uptake by CTRs, reduction of inorganic and organic complexes of
110 Cu(II) at the cell surface is required. The reduction rates were enhanced under Cu
111 limitation, and in proportion to Cu(II)-ethylenediaminetetraacetic acid reduction
112 potential but independent of Cu(II) concentration. This reduction-dependent uptake
113 system makes it possible for diatoms utilizing Cu(II) organic complexes in oceans. It
114 also reflects the recent evolution of diatoms in fully oxidized Phanerozoic oceans where
115 Cu(II) organic complexes are more stable, compared to the predominant anoxic
116 Precambrian marine realm. This increased stability and bioavailability of dissolved

oxidized Cu(II) organic complexes in fully oxidized Phanerozoic seawater conditions [11], compared to strong removal of Cu from seawater in association with sulfides and Fe oxides in the Fe-sulfide-rich Precambrian oceans [26], would have enabled efficient use of Cu for phagocytic prey poisoning in recently evolved protists.

Phagosome acidification, lysosomal enzymes and oxidative burst

Unicellular eukaryotes feeding phagocytotically on bacteria are extremely abundant and ecologically important in virtually all aquatic and terrestrial ecosystems [27]. Mechanisms of killing phagocytosed bacteria prior to digestion include acidification of the phagosome, oxidative bursting, antimicrobial peptides and poisoning by metal(loid)s such as Cu(I), As(III) and Zn(II) [28-30, 16-17].

The first step in maturation of the phagosome is the recruitment of H⁺-vacuolar ATPase (V-ATPase) to acidify the phagosome, achieved by fusion with lysosomal vesicles [31]. The created proton gradient drives the functioning of the phagosome while acidification kills and digests the living bacteria prey. In addition, the proton gradient enables the functioning of a number of ion transporters involved in metal poisoning and nutritional immunity. Following acidification of the phagosome, a pH of about 4.5-5 is attained in macrophages and 3.5-4 in *D. discoideum* [32, 33]. In these acidic conditions, a variety of distinct vesicular enzymes, including proteases, hydrolases and lysozymes, are sequentially recruited to the phagosome to breakdown different bacterial components [34].

139

140 In the mononuclear phagocyte system (MPS), including monocytes, macrophages, and
141 dendritic cells, the major source of reactive oxygen species (ROS) is NADPH oxidase
142 (NOX) which generates superoxide by transferring electrons from NADPH to O₂ in
143 the phagosome. Superoxide can then be converted into additional ROS in subsequent
144 reactions [35]. Free Fe(II) generated by destruction of FeS clusters can generate more
145 ROS by the Fenton reaction. Cu(I) accumulated in phagosomes to poison microbes can
146 also catalyze a Fenton-like reaction further generating more deleterious ROS. ROS
147 production in the phagosome by homologs of NOX₂ is potentially possible in *D.*
148 *discoideum* as the genome contains three genes encoding three catalytic NOX₂ subunits,
149 NoxA, NoxB and NoxC. However, a double mutant lacking *noxA* and *noxB* did not
150 exhibit any defect in growth or killing of *Klebsiella*, indicating ROS production might
151 not be necessary for killing of bacterial prey [36]. However, a triple mutant with neither
152 *noxA*, *noxB* nor *noxC* displayed increasing bacterial contamination of fruiting bodies
153 after growth on *Klebsiella* compared to the wild type, indicating a role in immunity [37].
154

155 **Metal(loid)-mediated bacterial killing mechanisms in protozoa**

156 The predation of bacterial prey has been shown to involve Zn, Cu and As in *D.*
157 *discoideum* and we predict in other protists. Here we explore the molecular mechanisms
158 underlying predation by Zn, Cu and As. Poisoning by excess Zn has been shown in *D.*
159 *discoideum* [18]. Zn became visible quickly after uptake of beads and peaked after
160 around 15-20 min. Of the four ZnT –type transporters, only ZntB localized with both

the V-ATPase in the membrane of the phagosome and also in the membranes of Zn-containing endosomes, also referred to as zincosomes. Zincosomes were shown to merge with phagosomes thereby delivering excess Zn to the phagosomes and contributing to Zn poisoning in *Dictyostelium* [18]. Whether ZntB also transports Zn directly into the phagosomes is not known at this point. Similarly, in macrophages, Zn²⁺ was also shown to be delivered to phagosomes via fusion with Zn²⁺-containing endosomes (zincosomes) [38]. Whether metal-containing vesicles, zincosomes, only deliver Zn or together with other metals like Cu, remains to be determined. Regardless, *zntB* deletion did not affect bacterial killing efficiency by amoebae, indicating that they use a variety of methods to kill ingested bacteria. Further, ZntA was shown to be mainly located in the contractive vacuole as part of the Zn detoxification pathway. Both ZntC and ZntD were shown to be localized in the juxtanuclear region [18]. Nonetheless, bacteria triggered upregulation of ZntC in *D. discoideum* (unpublished data), while *Escherichia coli* strains deficient in *zntA* or *zitB* were found to be more vulnerable to predation.

In addition to Zn, *D. discoideum* was shown to also use Cu to poison ingested prey, the “brass dagger”. For instance, Cu was elevated in phagosomes of *D. discoideum* fed on *E. coli* [16]. Moreover, all of the three putative Cu-transporting P_{1B}-ATPases were upregulated to different degrees in the presence of living bacteria. Significant upregulation of ATP1 following the addition of Cu indicate a role in Cu efflux and resistance. Interestingly, significant upregulation of ATP7A and ATP3 in the presence

183 of bacteria, suggest they are involved in Cu trafficking and delivery of Cu into the
184 bacteria containing phagosomes (Fig. 1). Similarly, *Dictyostelium* P80, a homolog of
185 Ctr1 — the high-affinity plasma membrane Cu(I) permease in mammalian
186 macrophages — was also upregulated in the presence of bacteria. P80 is localized on
187 the cell surface and endocytic compartments including the phagosomal membrane [18,
188 39]. This indicates potential involvement of P80 in the uptake of Cu and direct transport
189 into Cu-containing vesicles which are then fused with the phagosome (Fig. 1). As an
190 alternative, p80 could take up Cu and then deliver it to either of the three P_{IB}-ATPases,
191 ATP1, ATP7A or ATP3 via a metallochaperone. Both the expression of the
192 *Dictyostelium* homologue of the phagosomal Cu(I) transporter AP7A and ATP3 are
193 upregulated in the presence of ingested bacteria, indicating a role in elevating the Cu
194 concentration in the phagosome (Fig. 1). However, the exact pathway of either direct
195 Cu transport into the phagosome or first into Cu-containing vesicles that then merge
196 with the phagosome, remain unknown. Selection for bacterial Cu resistance by protist
197 predation has recently been demonstrated in the natural environment [40]. Based on a
198 field survey, we found predation by soil protists positively contributed to the enrichment
199 of the *copA* gene in the bacterial community, through influencing Cu resistant and
200 sensitive ecological clusters. Protistan members in Amoebozoa, Ciliophora and
201 Cercozoa were important predictors of Cu resistant ecological clusters. Moreover,
202 genomic survey of the putative Cu-trafficking proteins identified a wide distribution of
203 proteins that are homologous to Ctr/P80 and Cu(I)-translocating P-type ATPases in
204 phagotrophic protists, especially in Conosa, Lobosa and Ciliophora. These Cu-

trafficking proteins have been predicted to be involved in Cu uptake and delivery to kill the engulfed bacteria, leading to a post-ingestional selection of bacterial Cu resistance.

There is also experimental evidence that arsenic (As) is used for poisoning as the As sensitive strain *E. coli* AW3110 was killed more rapidly than the wild type *E. coli* strain W3110 [17]. Perhaps *Dictyostelium* and possibly other protists use every metal they can handle to poison ingested prey throwing “everything but the kitchen sink” into the phagosome. These mechanisms have so far only been confirmed in *Dictyostelium discoideum* and *Acanthamoeba castellanii*. It remains to be determined whether other lineages of eukaryotes also use metal(loid)s as poison to kill their prey. We predict that this is a widespread ancient mechanism.

Synergistic depletion of Fe and Mn in predation

Depletion of essential metals such as Fe or Mn is another strategy employed by both macrophages and amoebae such as *D. discoideum* to make their bacterial prey more vulnerable. Poisoning by soft metals such as Cu(I) or highly elevated reactive oxygen species will destroy Fe-S clusters in many essential enzymes. The free Fe thus generated is detrimental to the cells as it can damage DNA and create more ROS. Consequently, free Fe is often quickly pumped out of the cytoplasm to avoid damage [41], and out of the phagosome, together with Mn, which is able to protect cells from ROS generated toxicity by itself, and other metals like Zn using NRAMP (Natural Resistance Associated Macrophage Protein) transporters. Thus to survive, bacterial Fe-S clusters

need to be rebuilt, implying they need to take up Fe, which is impossible in the Fe-extruding phagosome. In addition, Mn is a cofactor in Mn-superoxide dismutase (Mn-SOD), which provides a critical protective function as one of the most important antioxidants in the cell. In *Dictyostelium*, NRAMP1 was shown to be responsible for pumping Fe out of the phagosome into the cytosol and needs acidification of the phagosome through the V-ATPase to catalyze Fe efflux (Fig. 1) [42, 43]. Since *E. coli* strains defective in Fe but also Mn uptake displayed a reduced survival rate, efflux of Fe and Mn out of the phagosome by NRAMP1 seem to be a likely contributor [16]. NRAMPB (formerly NRAMP2) appears to be involved in phagosome Fe depletion of Fe and a mutant NRAMPB was shown to be more susceptible to *Legionella pneumophila* (Fig. 1) [43].

Hence, bacteria could partly resist predation by evolving resistance towards these metals and we have previously proposed that the abundance of bacterial heavy metal resistance determinants in the environment is not only dependent on environmental concentrations of heavy metals but also selected for by protozoan predation (Fig. 2) [16, 40]. Not to forget, the acquisition of bacterial resistance to metal(loid)s may have major implications for human and animal health as it may lead to the co-selection of antibiotic- (multi-) resistant bacteria due to the possession of both resistance mechanisms on the same mobile genetic element [44, 45].

Emergence of eukaryotes accelerated bacterial prey predation

Predation in protozoa depends on phagocytosis and requires a lot of energy hence the need for mitochondria. Since these processes are intrinsically tied to eukaryogenesis, we therefore we give a short overview of eukaryogenesis. Phagotrophic “protozoa” or “protists” do not represent a monophyletic group (that is, a clade), and the ability to feed phagocytotically on bacteria has been independently lost, and perhaps even gained, across the eukaryote tree [46, 47, 15]. Moreover, over the past decade, it has become increasingly clear that the host cell that internalized the ancestral mitochondrion during eukaryogenesis was an archaeon [48] – a prediction [49] bolstered by the recent discovery and cultivation of the Asgard archaea [50-52]. In this scenario, the microbial eukaryotic host cell belonged to an archaeal lineage that either branched within or was sister to the Asgard superphylum, the closest known prokaryotic relatives of eukaryotes [53]. While it was originally predicted that this archaeal host may have been capable of phagocytosis [50], which it used to internalize the bacterial ancestor of the mitochondrion, it now appears that the mitochondrion was acquired via phagocytosis-independent means [51, 52, 54, 55]. As of now, there remains no evidence for phagocytosis, or endocytosis more generally, in the Asgard archaea or any other archaeal lineage [56]. Overall, it appears that phagocytosis evolved after – not before – the origin of mitochondria.

Timing the origin of mitochondria is difficult. Mitochondria must have originated prior to the last eukaryote common ancestor (LECA), which definitively possessed mitochondria [57]. According to the most recent molecular clock estimates, LECA

emerged somewhere between 1.9 and 1.0 Ga [58, 59].

Molecular clocks have also estimated when the bacterial lineage that the free-living ancestor of the mitochondrion belonged to diverged from its closest Alphaproteobacterial relatives [59, 60]. According to these estimates, the mitochondrial first eukaryote common ancestor, or mito-FECA, emerged between 2.1 and 0.91 Ga [61]. While it is permissible, according to these estimates, for mitochondria to have originated by 1.9 Ga – already 200 million years after the GOE – there is no definitive geologic evidence for eukaryotes at this time. Indeed, the oldest putative total-group eukaryote fossils (possibly representing extinct eukaryote lineages that diverged prior to LECA) are dated to 1.67-1.63 Ga [62], while the oldest definitive crown-group (mitochondria-bearing) eukaryote fossil dates to 1.05 Ga [63]. Overall, if the mitochondrion evolved hundreds of millions of years after the GOE, then the origin of phagocytosis must have evolved hundreds of millions of years after the GOE, as well [64]. This would also mean that the use of specific metal(loids) as a predation tool to kill prey by phagocytes also post-dates the GOE.

Phagocytosis is not confidently reconstructed back to LECA [46], which implies that LECA was an osmotroph [15]. Indeed, a recent ancestral state reconstruction analysis predicted that LECA was indeed non-phagocytic, and that phagocytosis evolved multiple times in different eukaryote lineages [47]. In this scenario, phagocytosis not only post-date mitochondria, but also eukaryogenesis, which ultimately culminated with

LECA. If LECA evolved relatively late (closer to 1.2 Ga than to 1.9 Ga) [65], then phagocytosis could post-date the Great Oxidation Event (GOE), when oxygen first rose permanently in the atmosphere, by more than a billion years [15]. Indeed, the oldest direct evidence for modern phagotrophic protists in the rock record comes from fossil testate amoebae, likely belonging to the arcellinids [66], from 0.789-0.759 Ga [67]. Additional 0.770-0.742 Ga evidence for ciliates comes from preserved gammacerane [68] – the geologically stable form of tetrahymenol, which is synthesized by the Oligohymenophorea (ciliate protozoa), which are known phagotrophs [69]. As an important caveat, however, non-mineralizing and bacteria-eating phagotrophs are less likely to enter the fossil record than cell-wall bearing photosynthetic and osmotrophic eukaryotes, meaning that bacterivorous protozoa could be more ancient than the microfossil record alone suggests [70]. Indeed, recent ecosystem models linking eukaryote trophic strategies with eukaryote cell size predict that phagocytosis was an active eukaryote feeding strategy as far back 1.7 Ga [71]. More direct fossil and geochemical evidence for eukaryotes capable of phagotrophy comes from the very late Mesoproterozoic (1.6-1.0 Ga) to the early Neoproterozoic (1.0-0.538 Ga) [15], meaning that phagocytosis must have evolved by this time (Fig. 3B)

The Great Oxidation Event (GOE) – metal(loid) bioavailability

Although debated, the emergence of complex oxygen-respiring eukaryotes after the rise of atmospheric oxygen, implies some level of causal dependence. Similarly, and as already mentioned, the permanent rise of oxygen in the atmosphere changed the

behaviour of redox sensitive metals and therefore their bioavailability. Moreover, as demonstrated above, the bioavailability of Cu, Zn, Fe, Mn and As is important for presently known metal-dependent phagocytic predation and function of immune systems. Particularly, metal poisoning by phagotrophs, as observed in the modern oxygenated biosphere, could not have evolved until after the GOE which started at ~2.45 Ga near the Archean-Paleoproterozoic boundary, and climaxed at ~2.2 Ga [72, 73]. The GOE was intimately tied to changes in the content and redox state of trace metals in Proterozoic oceans [1, 11]. Indeed, the global-scale oxidation of reduced elements abundant on the early Earth surface to more oxidized forms and minerals by free molecular oxygen (O₂) changed marine trace metal(loid) bioavailability [1, 2, 74, 3, 75]. As a consequence, the GOE transformed the course of the subsequent use of metals in enzymes such as cytochrome *c* oxidase [4], the development of adaptive detoxification mechanisms and selection and use as biological weapons.

Hydrothermal activity is proposed as the main source of Cu, Zn, Fe, Mn and As to Archean seawater, while riverine supply, consistent with the GOE rise in oxidative weathering of the continents, became more influential during the Proterozoic [75]. According to this model, oxygenation of the atmosphere promoted the dissolution of an untapped massive continental sulfide reservoir [76]. For instance, the breakdown of sulfide minerals such as chalcocite (Cu₂S) and chalcopyrite (CuFeS₂), might have elevated the content of Cu²⁺ dissolved in the oceans [26, 77]. While a systematic reduction in dissolved marine Fe content and limited marine euxinia (i.e., anoxic and

sulfidic) [78], meant that Cu and Zn might have also become more bioavailable in the oceans [26, 77, 5]. As an example, a dramatic rise in the size of marine sedimentary Zn deposits from 2.02-1.45 Ga, has been linked to increased oxygenic weathering of reduced sulfur compounds following the permanent oxygenation of Earth's surface [78]. However, a reconstruction of associated Zn concentrations preserved in marine euxinic black shales (Fig. 3A), suggest that Proterozoic seawater Zn content was likely similar to the modern [1, 3, 5]. This model supports the idea that sulfidic conditions were not widespread in the post-GOE oceans, otherwise seawater Zn depletion would be expected because of efficient removal with sulfide minerals to sediments, creating seawater Zn deficiency.

It is worth noting that in addition to seawater concentrations, bioavailability and selective use of redox sensitive metals for physiological and biochemical pathways on the Earth surface, would have been influenced by the radical redox change that accompanied the GOE. Moreover, estimated global marine trace metal and metalloid budgets in these oceans may not always account for important variations promoted by local processes like hydrothermal activity and intensity, source provenance and weathering dynamics. For instance, recently it was proposed that localized increase in post-GOE dissolved seawater Zn concentrations may have, for example, enabled the emergence of problematic post-GOE eukaryotic-like colonial organisms of unknown affinity in the Francevillian Basin at ~2.1 Ga [6, 7]. The link between the latter and Zn, speaks to the importance of trace metals in discussions relevant to biological evolution, especially for eukaryotic organisms because of their higher need for Zn compared to

prokaryotes. Nonetheless, it is generally thought that increasing use of Zn by eukaryotes was driven mainly by increasing cellular need than temporal changes in seawater Zn concentration, as it is considered that marine Zn concentrations have been mostly non-biolimiting throughout most of Earth history [1, 3, 5].

In the case of Cu, amplification of terrestrial Cu supply to the oceans by oxidative weathering implies that Cu became abundantly available in the ocean surface as Cu^{2+} instead of Cu^+ , compared to the preceding anoxic Archean world. This change in the redox state of Cu, similar to the case of As discussed below, we believe was more relevant for biological evolution and adaptation after the GOE, than mere increase in global seawater content. Yet when combined, the change in the redox state and rise or fall in the bioavailable concentrations of various metals and metalloids in seawater, would have exerted an incredible evolutionary bottleneck on life to adapt to this new oxidized ecosystem state.

Marine sedimentary As levels were shown to be much lower before the GOE [79]. However, these lower sedimentary concentrations possibly reflect the fact that in the highly reduced Archean Ocean, most As would have been present in the highly mobile As(III) form, resulting in limited sequestration in Archean marine sediments. Much lower sedimentary As concentrations coincide with the onset and duration of the Paleoproterozoic and Neoproterozoic Snowball Earth glaciations, interpreted to reflect obstruction of oxidative continental supply by ice sheets [79]. The subsequent transition

to higher marine sedimentary As concentrations in parallel with the Paleoproterozoic GOE and the Neoproterozoic Oxygenation Event (NOE), following the end of the glaciations, suggest increase in oxidative weathering and the oxidation of abundant reduced As species on land and in the ocean to As(V) [79, 80]. Thus, the global change to a more oxidized ocean, did not only promote the eventual evolution of complex life, it exerted evolutionary pressure and control on emergent unicellular eukaryotes in the recruitment of specific metals and metalloids, as biological weapons for prey neutralization.

Metal(loid) bioavailability, eukaryogenesis and phagosome development

The sedimentary Zn record potentially reflects an increase in eukaryote biomass -- including the biomass of red algae, as suggested by the organic biomarker record [81] -- around 800 million years ago (Ma) [8], Zn concentrations and their stable isotopic distribution [6, 7]. While the timing of LECA's emergence is still uncertain (ca. 1.8-1.2 Ga) [59], it clearly post-dated the GOE [61], making it likely that at least Cu, Zn and As would be available in sufficient amounts to allow poisoning by the earliest phagotrophic protists. However, as the concentrations of metal(loids) in any given environment vary greatly, it seems likely that protozoa recruit the metals and metalloids they can and use these for poisoning. *Dictyostelium* was shown to not be significantly affected by very low concentrations of Zn or Cu, indicating robust mechanisms of homeostasis, including pathways for sufficient recruitment of metals for predation [82].

Moreover, the eukaryotic ancestral Asgard archaea are obligate anaerobes [83], implying their evolutionary trajectory was not constrained by oxygen availability or the GOE, and hence their associated metal(loid)s acquisition mechanisms. They are capable of efficient As detoxification via reductive transformation of As(V) to As(III), using broadly similar eukaryotic pathways, as well as being resistant to toxicity. Similarly, they carry out effective Zn and Cu uptake and efflux. The recent correlation of dissolved seawater Zn abundance and oxygen availability to the emergence of the 2.1 Ga Francevillian biota [6, 7], stresses the important role metals and oxidized environments may have broadly played on the appearance and disappearance of biological assemblages throughout Earth history. If correct, the origin of the mitochondrion could ultimately extend back to 2.1 Ga, with eventual calibration of molecular clocks against the purported Francevillian eukaryotic biota [6, 7]. This assumption is dependent on the conjecture that their unique appearance and disappearance was not a localized experiment that failed to spread.

Regardless, we infer that the already existing metal(loid) uptake pathways inherent in the ancestral eukaryotic archaeal lineage were subsequently integrated with the later development of the phagosome. This ability, when coupled with the increasing availability of relevant metal(loid)s post-GOE, together with the exceptional adaptation of the emergent eukaryotic cell to oxidizing surface conditions, would have resulted in the development of the efficient prey-killing phagosome.

Concluding Remarks

This review summarizes and highlights the link between biological metal(loid) acquisition, eukaryogenesis and evolution of the mitochondrion, coupled to the origin of phagotrophic bacterial prey poisoning in unicellular eukaryotes as a predatory feeding mechanism. The protists that have been studied in the most detail so far, poison their prey with heavy metals or metalloids. The increasing use of metal(loid)s by phagotrophic protists for predation was probably dependent to a large extent on the concentration of various metal(oid)s in seawater and their redox-dependent aqueous solubility properties, after the GOE. We propose that the ability to perform phagocytosis and the coupling to predatory prey killing with metal(loids) in the phagosome of unicellular protists, was only possible after invention of the oxygen-respiring mitochondrion. However, the exact timing of eukaryogenesis and evolution of the mitochondrion is still debated. Consequently, we suggest that a more holistic integration of the fossil record for better calibration of molecular clock dates of non-mitochondrial and mitochondrial genomes is required to establish a firmer timeline. Judging from available data the evolution of the metal(loid) prey-killing phagosome likely evolved among the earliest phagotrophic protists and we predict that most extant protists use a similar feeding strategy. The acquisition of this feeding mechanism is of major ecological and environmental implications since protist grazing employing heavy metal(loid)s to kill prey has been shown to select for the presence of heavy metal and metalloid resistance determinants in prokaryotes and other prey.

447 **Declaration of Competing Interest**

448 The authors declare no competing financial interest.

449

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455

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Figure legends

Fig. 1 Metal intoxication in bacteria-host interactions. Words in purple indicate transporters, NRAMP (Natural Resistance Associated Macrophage Protein) transporters. Zinc containing vesicles have been detected. Whether these vesicles also contain Cu(II), As(III) or other metals is not known at this time. Copper and zinc are present at elevated concentrations in the phagosome to aid in killing prey regardless of their respective pathways in both amoeba and macrophages.

Fig. 2 Metal mediated bacterial killing by protists selects metal resistant bacteria. Metals such as Zn and copper are recruited during protozoan predation. Zn could be transported into bacteria-containing phagosomes by ZntB, or alternatively, stored in zincosomes which later fuse with phagosomes. ATP7A and other ATPases may be involved in Cu(I) trafficking into phagosome. Whether Cu(I) will be stored in vesicles and merged with phagosome is unknown. Bacteria with metal resistance (M^R) show fitness advantages compared to metal sensitive bacteria (M^S) in phagosomes filling with toxic metals. After predation, the remaining M^R bacteria will be released through the emptying process of vacuoles, or lysis of vacuoles and protozoan cells.

Fig. 3 Temporal marine sedimentary Zn, Cu and As dynamics and a model for eukaryogenesis and mitochondrion and phagocytosis evolution. Proposed changes in marine sedimentary (A) Zn, (B) Cu and (C) As concentrations preserved in black shales deposited through Earth history adapted from [26, 5, 79]. (D) A model for evolution of atmospheric oxygen according to [11] and the estimated evolutionary trajectory for

671 eukaryotes, the mitochondrion and phagocytosis through Earth history [15, 59, 61, 63,
672 84, 85] GOE=Great Oxidation Event; PAL=Present day atmospheric oxygen levels;
673 Ga=billions of years; LECA=Last Eukaryote Common Ancestor; mito=mitochondria.
674

Outstanding Questions

Is it possible that a wider variety of metals are used for phagocytic prey poisoning than is presently understood?

Are biogeochemical changes in metal bioavailability in the immediate environment linked to their selective evolutionary use for phagocytic bacterial prey poisoning as a food source?

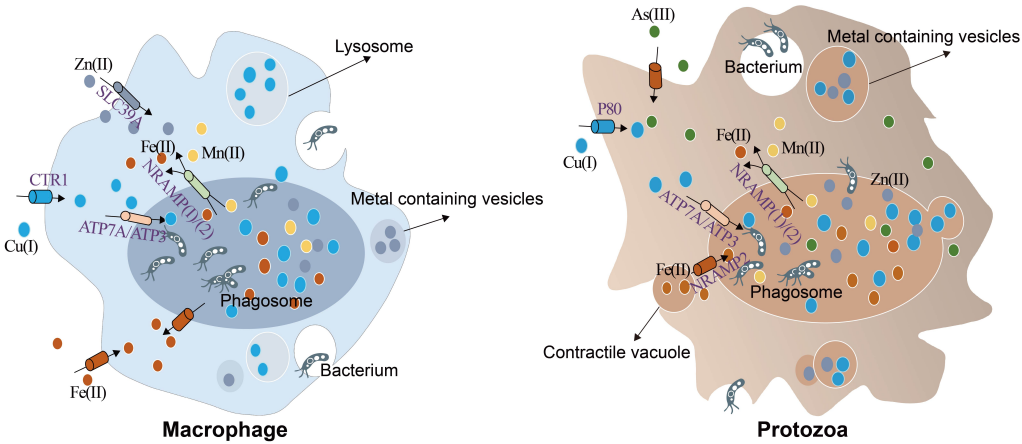
Does the evolutionary history of genes used in phagocytic predation by protists reflect marine metal bioavailability dynamics through Earth History and what is the distribution of these genes on the tree of life?

What is the particular role of metal bioavailability in the historical development of the phagosome as a prey killing apparatus?

Did phagocytosis evolve in the Proterozoic Eon and was this before or after the endosymbiotic acquisition of the mitochondrion?



Figure 1



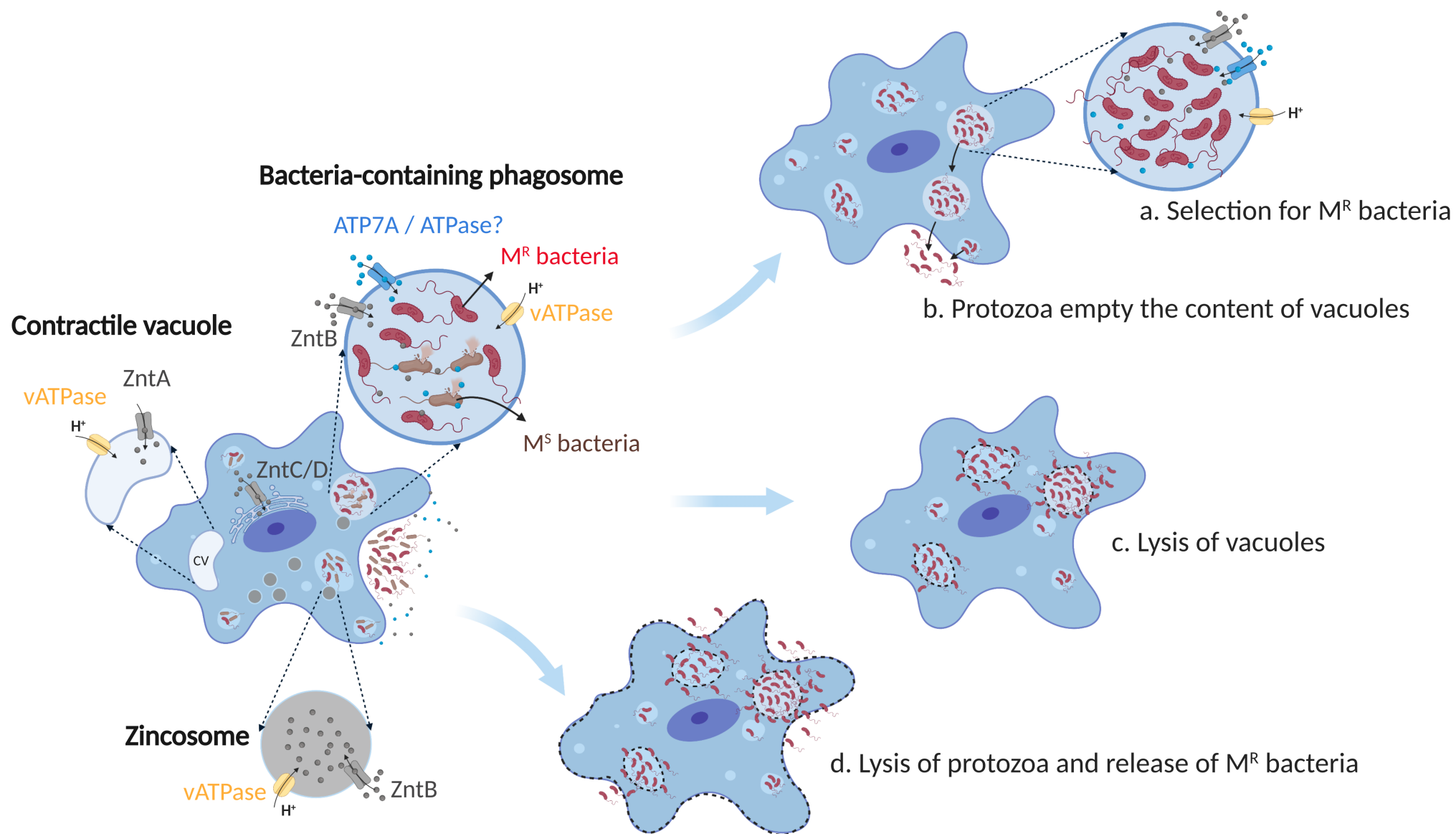


Figure 3

