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Parallel functional reduction in the mitochondria of apicomplexan parasites

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ABSTRACT

Extreme functional reduction of mitochondria has taken place in parallel in many distantly related lineages of eukaryotes, leading to a number of recurring metabolic states with variously lost electron transport chain (ETC) complexes, loss of the tricarboxylic acid (TCA) cycle, and/or loss of the mitochondrial genome. The resulting mitochondria-related organelles (MROs) are generally structurally reduced and in the most extreme cases barely recognizable features of the cell with no role in energy metabolism whatsoever (e.g., mitosomes, which generally only make iron-sulfur clusters). Recently, a wide diversity of MROs were discovered to be hiding in plain sight: in gregarine apicomplexans. This diverse group of invertebrate parasites has been known and observed for centuries, but until recent applications of culture-free genomics, their mitochondria were unremarkable. The genomics, however, showed that mitochondrial function has reduced in parallel in multiple gregarine lineages to several different endpoints, including the most reduced mitosomes. Here we review this remarkable case of parallel evolution of MROs, and some of the interesting questions this work raises.

1. Introduction: functional reduction in mitochondria

As every undergraduate learns, mitochondria are the "powerhouses of the cell". But this emphasis on ATP synthesis through oxidative phosphorylation and electron transport chains (ETCs) could also be argued to be an animal-centric bias. Even in animals, mitochondrial function extends beyond ATP synthesis, however, the functional diversity of mitochondria is most obvious when oxidative phosphorylation and ETCs are reduced or lost altogether. The first instances of reduced mitochondrial function were in protists initially thought to be "amitochondriate", or to lack mitochondria because no mitochondria were apparent in electron microscopy, and the organisms were anaerobic or microaerophilic (Müller, 1992; Müller et al., 2012). The most famous of these were collected in the so-called Archezoa, a lineage including microsporidia, metamonads, archaemoebae, and parabasalians, which were proposed to have diverged from other eukaryotes prior to the mitochondrial endosymbiosis (Cavalier-Smith, 1983). This hypothesis was eventually disproven, but resulted in a great deal of attention directed towards these groups, their metabolism, phylogenetic position, and their unusual mitochondria (Bui et al., 1996; Clark and Roger, 1995; Dolezal et al., 2005; Germot et al., 1997; Keeling and Doolittle, 1996; Tovar et al., 2003).

Indeed, the ultimate evidence against the Archezoa hypothesis was the discovery of highly-reduced, relict mitochondria in (most of) these "amitochondriates". The form these organelles took was also somewhat variable. Parabasalians were long known to contain a double membrane-bounded metabolic organelle called the hydrogenosome (Lindmark and Müller, 1973), but its metabolism was so unlike mitochondrial metabolism that its origin was unclear. It was eventually shown to have evolved from a mitochondrion mostly by reduction (Bui et al., 1996; Müller, 1993), but also in combination with a few key enzymes that are absent in many mitochondria, but present in a smattering of diverse eukaryotic groups, and which collectively allow for its unique metabolism. In microsporidia, diplomonads, and archaemoebae, the organelle was reduced much further, having little or no role in energy metabolism and instead mostly retained to make Fe-S clusters (Müller et al., 2012). These organelles are called "mitosomes" (Tovar et al., 1999), and collectively all the reduced forms of mitochondria (including hydrogenosomes and mitosomes) are called "mitochondria-related organelles", or MROs (Shiflett and Johnson, 2010). There is a lot of diversity of MROs between different lineages, but there is also some functional diversity of MROs within some lineages. For example, some microsporidia retained the glycercol-3-phosphate shuttle with alternative oxidase to dissipate excess reducing potential (Williams et al.,

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2010), while in other members of the group their mitosomes do not contribute to energy metabolism at all (Keeling et al., 2010). The oxymonads (which were initially included in the metamonad group of Archezoa) have proven the strangest case, being the only eukaryotic group to have completely lost the mitochondria, which was only possible due to their acquisition of a bacterial Fe-S cluster assembly pathway by horizontal gene transfer (Karnkowska et al., 2016).

The fall of the Archezoa hypothesis also focused more attention on other eukaryotes lacking "normal" aerobic metabolism. Many such lineages were already known but were not part of the Archezoa because they branched within some lineage whose other members generally contained aerobic mitochondria: for example, several ciliates and fungi with hydrogenosomes (Akhmanova et al., 1999; Boxma et al., 2005; Hjort et al., 2010; Williams and Keeling, 2003). Others were only discovered more recently, and include members of the stramenopiles (e.g., *Blastocystis* (Arisue et al., 2002)), rhizarians (e.g., *Mikrocytos* or *Brevimastigomonas* (Burki et al., 2013; Gawryluk et al., 2016)), or apicomplexans (e.g., *Cryptosporidium* (Keithly et al., 2005; Riordan et al., 2003)). Many other new lineages around former archezoans have also been discovered, in particular those branching with diplomonads (Kolisko et al., 2010; Takishita et al., 2012) and amoebozoans (Záhonová et al., 2023). Overall, the reduction of mitochondrial metabolism across the tree of eukaryotes is a disjointed continuum, from what we might call canonical oxidative phosphorylation all the way to the complete lack of energy metabolism we associate with MROs like mitosomes.

Throughout these discoveries, one common thread is that as new lineages with MROs were discovered and described, these lineages were generally expected to have some functionally reduced mitochondria based on their structure and/or metabolism and/or habitat. That is to say, the organisms were often discovered as anaerobes or microaerophiles and were also sometimes already known from electron microscopy to lack canonical mitochondria. New MROs are not typically "discovered" in already well-studied organisms with a history of microscopy and genomics. At least that was the case until recently, when an unexpected diversity of MROs was discovered in a diverse lineage of apicomplexans.

2. Mitochondria of model apicomplexans

Apicomplexans are a large and specious lineage of protists that are all obligate symbionts of other eukaryotes, generally animals (Perkins et al., 2000). The group is frequently described as obligate intracellular parasites, which captures some important essence of the group, but a small number of apicomplexans do not have an obligate intracellular stage of their life cycle, and most have never been formally demonstrated to be deleterious to their host or to decrease their fitness (Rueckert et al., 2019). These specifics notwithstanding, the group as a whole are obligately associated with host cells, most do live inside those cells for part or all of their life cycles, and the most famous apicomplexans certainly are parasitic. In particular, the apicomplexan *Plasmodium* causes one of the deadliest parasitic diseases of humans (malaria), and the model species *Toxoplasma* is the causative agent of a widespread infection in vertebrates. Most apicomplexan research focused on these two organisms (Meissner et al., 2007), which are members of two different subgroups, the hemosporidians and coccidia, and can as a result be mistakenly seen as representing the diversity of the entire group. In fact, hemosporidians and coccidia are actually closely related in the tree of apicomplexans (Fig. 1).

Like most of apicomplexan biology, our view of their mitochondria comes mostly from *Plasmodium* and *Toxoplasma* (Hayward and van Dooren, 2019; Lamb et al., 2023; Maclean et al., 2022; Sheiner et al., 2013). They do contain some unusual features, as well as a few strange characteristics inherited from the myzozoan ancestor shared between apicomplexans and dinoflagellates. In particular, this ancestor had massively reduced the size and coding capacity of its mitochondrial genome to include only three protein-coding genes and a handful of fragments of rRNAs (Feagin et al., 1991; Norman and Gray, 1997, 2001). This small genome subsequently underwent a lot of strange evolution in various myzozoan lineages, including fragmentation, duplication, RNA editing, programmed frame-shifts, trans-splicing, gene fusions, gene loss, and loss of stop codons (Berna et al., 2021; Jackson et al., 2012; Namasivayam et al., 2021; Slamovits et al., 2007; Waller and Jackson, 2009).

In *Plasmodium* and *Toxoplasma,* most canonical mitochondrial metabolism is retained and is similar to that of model organisms (such as animals or fungi), including the metabolism of pyruvate to acetyl-CoA, the tricarboxylic acid (TCA) cycle, the electron transport chains (ETCs), and oxidative phosphorylation. But there are also a few differences (Hayward and van Dooren, 2019; Lamb et al., 2023; Oppenheim et al., 2014; Painter et al., 2007; Seeber et al., 2008). Mitochondrial pyruvate dehydrogenase (PDH) is absent, and instead, pyruvate is imported by the mitochondrial pyruvate carrier (MPC), where it is converted to acetyl-CoA by branched chain ketoacid dehydrogenase (BCKDH) and fed into the TCA cycle. ETC complex I is also absent and replaced by an NADH dehydrogenase (NDH2), which fulfills its role as an electron donor, but not a proton pump. In addition, many of the other ETC complexes differ substantially in size and subunit composition compared to those of other well studied systems such as metazoans (Hayward and van Dooren, 2019; Maclean et al., 2022).

These variations are interesting, but do not add up to a substantially different metabolic outcome in *Plasmodium* and *Toxoplasma* mitochondria compared with the overall diversity of "normal" mitochondria across the tree of eukaryotes. The one exceptional apicomplexan has historically been *Cryptosporidium*, which lacks canonical mitochondria and has long been known to possess highly reduced MROs (Keithly et al., 2005; Riordan et al., 2003). *Cryptosporidium* MROs are also variable: while *C. muris* retains complexes I, II and V, in *C. hominis* and *C. parvum* all ETC have been lost (Abrahamsen et al., 2004; Tsaousis and Keithly, 2019; Xu et al., 2004).

3. Gregarines: unanticipated mitochondrial reduction

Gregarines are the apicomplexans that defy most things we 'know' about apicomplexans. The gregarines have been studied by light microscopy and their life cycles and infection examined for over 100 years. They encompass an enormous diversity of species that are all associated with invertebrate animal hosts (Perkins et al., 2000; Rueckert et al., 2019). Some are likely pathogenic, but this has not been examined in much detail for the vast majority of gregarines, and for some species the frequency of infection in hosts is so high they are most likely commensal (Rueckert et al., 2019). Most have intracellular stages of their life cycle, but their most obvious and long-lived stages are predominantly extracellular. They were classically considered to be the "earliest" apicomplexans, and molecular phylogeny has borne this out. The first trees based on ribosomal RNAs showed them branching deeply, but also questioned whether gregarines formed a single lineage or many paraphyletic lineages (Leander et al., 2003; Rueckert et al., 2015; Rueckert et al., 2013; Wakeman et al., 2014). More recent large-scale phylogenomic analyses have resolved this, showing all sampled gregarines form a single, basal group (Janouškovec et al., 2019; Mathur et al., 2019; Mathur et al., 2021; Salomaki et al., 2021), with an intriguing exception. One group of parasites with morphology very much like the archaegregarines was found to form a completely distinct lineage, now called squirmids, that branches outside the apicomplexans altogether, as the sister to apicomplexans plus the chrompodellids (Janouškovec et al., 2019; Mathur et al., 2019). When the first squirmid was initially characterized, it was so similar to archaegregarines that it was included in the existing (though also admittedly preposterously diverse) genus *Selenidium* based on light microscopy, electron microscopy, and rRNA phylogenetics, and yet all these similarities are due to convergence.

Early molecular phylogenies also raised the possibility that

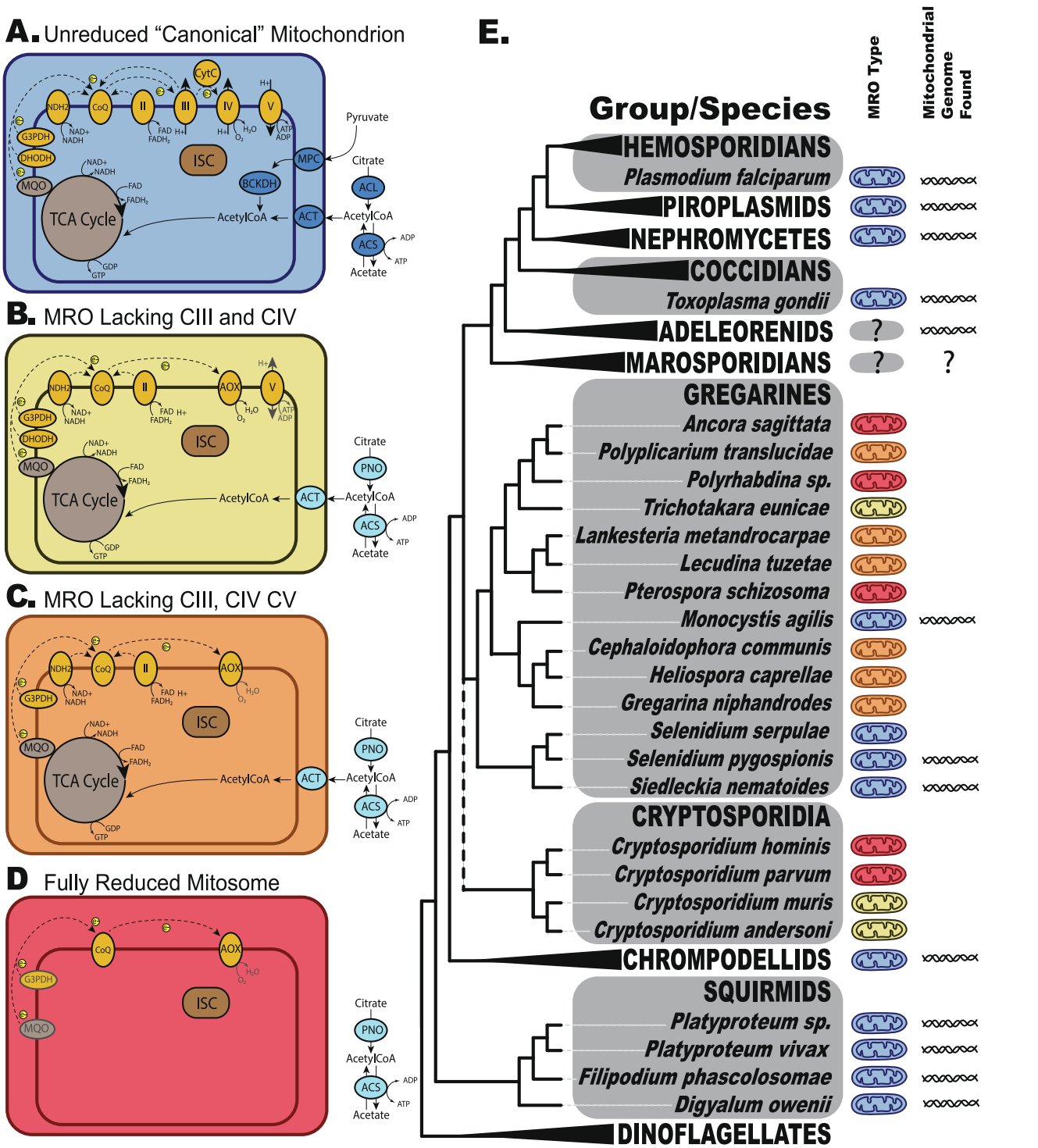

Fig. 1. The evolution of apicomplexan MRO metabolism inferred primarily from gene expression. Four increasingly reduced functional classes of MROs are indicated in parts A–D and colour coded blue, yellow, orange, or red. The distribution of these four functional classes on the tree of apicomplexans is shown in part E using the same colours, where the presence of a mitochondrial genome is also indicated. (**A**) Canonical apicomplexan mitochondrial metabolism with complete systems for pyruvate metabolism and acetyl-CoA synthesis, the TCA cycle, electron transport chain, and ISC. (**B**) Reduced MRO lacking BCKDH and matrix acetyl-CoA synthesis, complex III and complex IV, and possessing AOX. (**C**) Further reduced MRO also lacking DHODH and complex V. (**D**) The most reduced apicomplexan MROs are functionally equivalent to mitosomes, and only retain a complete pathway for ISC. G3PDH is also reported in *A. sagittata*, *Polyrhabdina sp.*, and *P. schizosoma*. MQO is also reported in *Cryptosporidium parvum* and *C. hominis*. Other symbols denoted with grey text (A–D) are assumed/hypothesized functions. Abbreviations: II, electron transport chain complex II or succinate dehydrogenase; III, electron transport chain complex III or cytochrome bc_1; IV, electron transport chain complex IV or cytochrome *c* oxidase; V, electron transport chain complex V or ATP synthase; ACL, ATP citrate lyase; ACS, acetyl-CoA synthetase; ACT, acetyl-carnitine transferase; AOX, alternative oxidase; BCKDH, branched-chain alpha-keto acid dehydrogenase; CoQ, coenzyme Q; CytC, cytochrome *c*; DHODH, dihyrdroorotate dehydrogenase; ETC, electron transport chain; G3PDH, glycerol 3-phosphate dehydrogenase; ISC, iron-sulphur cluster biosynthesis; MPC, mitochondrial pyruvate carrier; MQO, malate:quinone oxidoreductase; MRO, mitochondrial related organelle; NDH2, NADH dehydrogenase; PNO, pyruvate:NADP oxidoreductase.

cryptosporidia are somehow related to gregarines (Carreno et al., 1999; Leander et al., 2003; Zhu et al., 2000), and this remains one of the major unsolved questions in the phylogeny of apicomplexans (Mathur et al., 2023). Despite the availability now of dozens of good transcriptomes from gregarines and several complete genomes from cryptosporidia, the branch uniting or separating them remains unsupported in most analyses, but it has become clear they are both deep-branching apicomplexans, potentially in the same lineage, but it is also clear cryptosporidia do not branch within the gregarines.

Because gregarines are sisters to virtually all other apicomplexans combined, we might expect them to be very diverse (nominally they might be as diverse as all other apicomplexans combined). And indeed, at the level they are best known (gross body plan and life cycles), they show a remarkable level of diversity (Perkins et al., 2000). We are now getting to know more about their molecular biology and molecular evolution, and here too they have been shown to be highly diverse, probably the best studied aspect being their plastids (Mathur et al., 2023). But until very recently gregarine mitochondria were not considered to be of much interest. Unlike *Cryptosporidium*, gregarines were observed in electron microscopy to have mitochondria, tubular cristae were observed as expected for an apicomplexan, and although most species have feeding stages in the gut of their host, they were not discussed as being anaerobes. It may not be fair to say gregarines were considered to have normal aerobic mitochondria, but it would be fair to say that the idea they did not was never seriously discussed. They were simply assumed to be normal or never thought about very much. Recently, however, two studies using single-cell transcriptome data have unraveled that "absence of a view" by showing gregarine mitochondria are functionally very diverse, with a range that sweeps all the way from metabolically fully-functional to highly reduced mitosomes.

One study focused on terrestrial gregarines infecting cockroaches (Salomaki et al., 2021), while the other focused on gregarines from a variety of marine invertebrates (Mathur et al., 2021), and both uncovered a range of reduced MROs throughout the clade. These include the loss of BCKDH and a corresponding transition to anaerobic pyruvate metabolism, several losses of ETC complexes, and even the complete loss of the TCA cycle and ATP generation in the most extreme cases, resulting in mitosomes with only Fe-S cluster biosynthesis via the ISC complex. Based on the profiles described in these studies, mitochondrial function in gregarines can be simplified into four general categories (Fig. 1). First, some gregarine mitochondria are functionally more or less identical to those of the model systems *Plasmodium* and *Toxoplasma*. A more reduced type has lost aerobic pyruvate metabolism and instead metabolizes it anaerobically using pyruvate: NADP oxidoreductase (PNO), which converts pyruvate to acetyl-CoA in the cytosol. Acetyl-CoA is then imported to the mitochondrion by the acetyl-CoA transporter (ACT), and is fed into the TCA cycle. The ETC is also modified by the loss of complexes III and IV, cytochrome *c*, and by the addition of an alternative oxidase, which becomes an acceptor for electrons from coenzyme Q (CoQ), but does not perform a role as a proton pump. This would at face value also presumably lead to the loss of a proton gradient across the inner mitochondrial membrane, but this is a complex issue for a few reasons. First, complex V is still retained in lineages with this level of reduction; its function in ATP-generation is dependent on a proton gradient, but it could also function in reverse to create a proton gradient by using ATP, perhaps to enable the many mitochondrial transporters that require a proton gradient to function (Cunningham and Rutter, 2020). However in the next level of reduction, complex V is also lost, as is dihyroorotate dehydrogenase (DODH) as an electron donor to CoQ in the ETC. How this affects transporters or whether there is an alternative way to generate a protein gradient is an interesting question more broadly across a range of MROs in different eukaryotic lineages.

The most extreme level of reduction in gregarines has completely lost all ESCs, acetyl-CoA import, and the TCA cycle, and retains only the ISC pathway for biosynthesis of Fe-S clusters. This level of functional reduction is equivalent to mitosomes in other eukaryotic lineages, and has evolved four times independently in the apicomplexans: three times in gregarines (Fig. 1E) represented in by *Ancora sagittata*, *Polyrhabdina* sp., and *Pterospora schizosoma*, and once in a subgroup of cryptosporidians represented by *Cryptosporidium parvum* and *C. hominis*. There is some variation between these instances, including the retention of malate:quinone oxidoreductase (MQO) in the two cryptosporidian species, and the retention of the coenzyme Q electron donor glycerol 3-phosphate dehydrogenase (G3PDH) in *Ancora sagittata* and *Polyrhabdina* sp. (Mathur et al., 2021; Salomaki et al., 2021).

Cox1 and Cox3 proteins are both part of complex IV, and Cob is part of complex III. As these are the only protein-coding genes present in apicomplexan mitochondrial genomes, it follows that if complex III and IV are lost, which occurs frequently across the tree (Fig. 1E), then the mitochondrial genome can also easily be lost. Indeed, Cox1, Cox3, and Cob transcripts were found in squirmid lineages as well as in all gregarines that contain other complex III and IV proteins, but were never found in lineages otherwise lacking these complexes (Boisard et al., 2022; Mathur et al., 2021; Salomaki et al., 2021). Additional analysis of ribosomal proteins required for the translation of mitochondrial genes confirms this pattern, supporting the loss of the mitochondrial genome not just in cryptosporidians, but also in many gregarines (Fig. 1E).

4. Complex V and cristae morphology

As we noted earlier, one of the unusual things about the discovery of MROs in gregarines is that they were not anticipated based on either an obvious anaerobic lifestyle or absence of mitochondria in previous work. Electron microscopy of gregarine mitochondria existed, but were never really the focus of much attention and did not suggest atypical mitochondrial structure or function. With the benefit of hindsight, however, the structure of gregarine mitochondria requires more attention. Not surprisingly, the gregarines we now know to have retained a normal complement of ETCs also have normal looking mitochondria with the expected tubular cristae, similar in appearance to mitochondria of other apicomplexans (Fig. 2). For those species with highly reduced MROs, however, the data are scarce but intriguing. Since they were not anticipated to be functionally reduced, few TEMs have been reported, and reviewing those that are available suggest that perhaps some structural reduction has taken place, but not to the extent that might have been predicted. Specifically, it has now been shown that cristae formation is mediated by a few factors (Cogliati et al., 2016), including ETCs and complex V in particular, which are though to intrinsically bend membranes (Davies et al., 2011). In some gregarines lacking ETCs, including complex V, internal membrane structures resembling tubular cristae can nevertheless be observed (Mathur et al., 2021; Fig. 2). This is an intriguing observation, but since mitochondria have never been a primary focus of ultrastructural studies, it remains somewhat preliminary and needs more direct attention.

5. Concluding remarks: gregarine mitochondria as a natural experiment in parallel reduction

For most of the known subgroups of gregarines, genomic data are only available for a small number of representatives, and in some cases none at all. Accordingly, the functional diversity of MROs might be even greater than is presently known, or known states might have arisen more times than we currently see. Even with this limited sample, however, the diversity of mitochondrial functional reduction in the group stands out. In at least some other well-sampled lineages with MROs, reduction appears to have evolved once to an evolutionary stable state, so all sampled members of the group maintain the same or similar mitochondrial functions. For many groups we simply cannot say, since so few members of the group have been studied. But within the gregarines almost the whole known range of MRO functional diversity can be found within a single monophyletic lineage (the obvious exception is that no hydrogenosome has been found), many of these states have evolved multiple

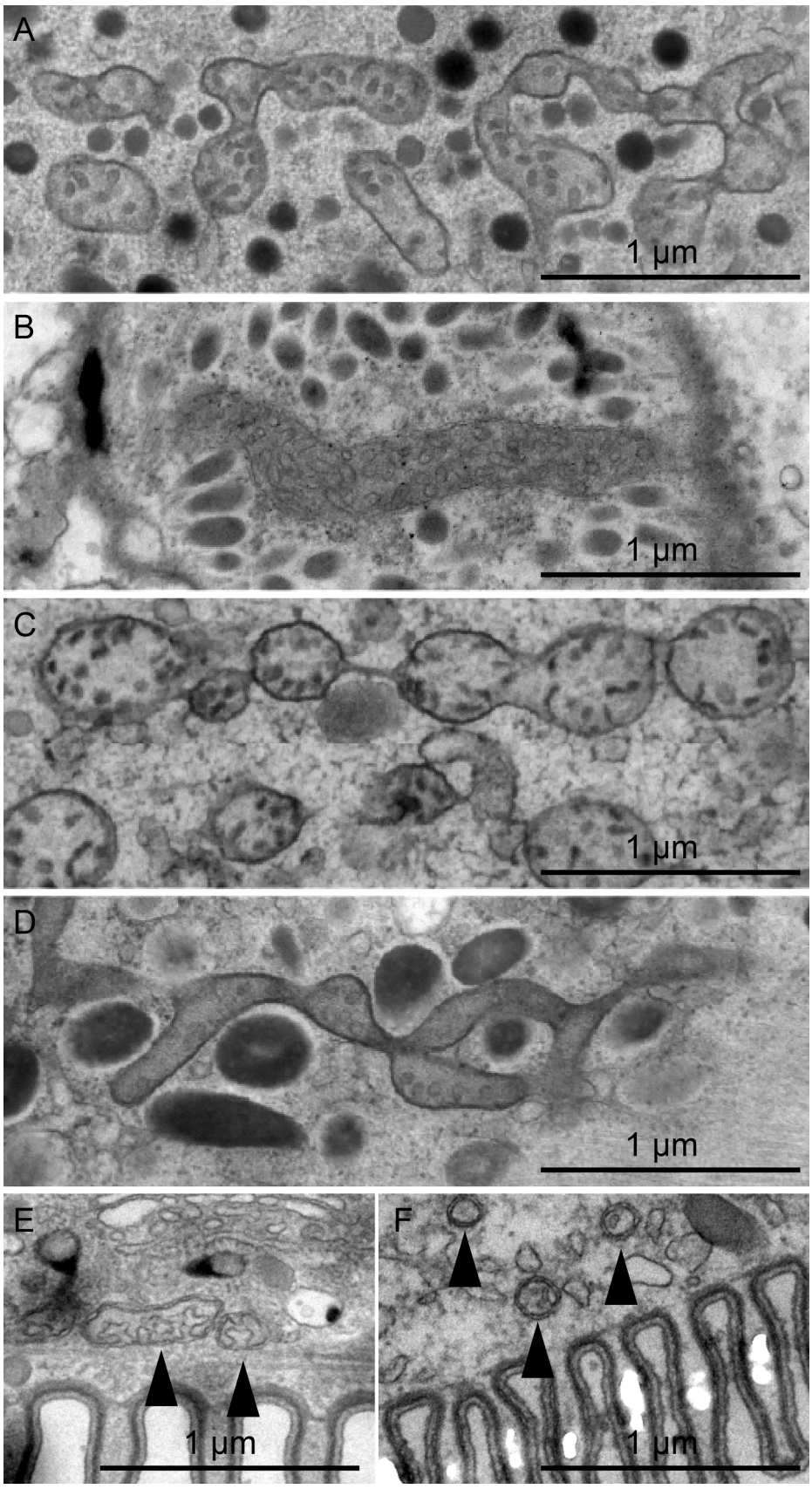

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Fig. 2. Transmission electron micrographs of mitochondria from apicomplexans with a wide range of functional reduction. (**A**) A squirmid parasite (either *Filopodium* or *Platyproteum*, which both infect the same peanut worm host), showing canonical tubular mitochondrial cristae expected for a myzozoan. (**B**) A corallicolid apicomplexan (infecting *Parazoanthis*), showing canonical mitochondrial morphology from non-gregarine apicomplexans. (**C**) *Selenium terebellae*, with canonical tubular cristae in a gregarine with a complete complement of ETC proteins. (**D**) *Selenidium serpulae*, with canonical mitochondrial structure, and a complete complement of ETC proteins, but possibly lacking a mitochondrial genome. (**E**) *Trichotokara*, with mitochondria lacking complexes III and IV. (**F**) *Lecudina*, with mitochondria lacking complexes III, IV, and V. The latter two both have internal membrane folding (arrowheads), but structurally different from canonical apicomplexan mitochondria.

times within the group, and two have also evolved in the closely-related cryptosporidia. The limited number of outcomes of functional reduction is also obviously non-random, since several states have emerged multiple times in gregarines and other eukaryotes, likely underscoring the few states that are functionally stable.

Parallelisms have emerged as a common theme in the evolution of apicomplexans and their parasitic lifestyle. This is best studied in the plastid, where photosynthesis has been lost many times independently, as have a variety of other plastid biochemical pathways, and even the loss of the actual plastid itself (Janouškovec et al., 2019; Mathur et al., 2019; Mathur et al., 2023). More dramatically, the origin of parasitism has also occurred many times independently, and in the case of the squirmids the outcome is so similar to particular subgroups of apicomplexans that they were long mistaken for members of those groups when we now know they are not even apicomplexans.

The currently available data are a major step forward for understanding gregarine MROs and their biology and evolution more generally, but as always also raise a few outstanding questions. Noteworthy of these are the functional relationship between ETCs and cristae formation, or rather what cristae look like and how do they form in the absence of ETCs like complex V. The depth of parallelisms in gregarine MROs is in part due to the presence of species with apparently normal ETC functions surrounded in the tree by relatives with reduced MROs, and it would also be of interest to probe this pattern in more detail, while at the same time look harder at these taxa to determine why they have resisted whatever factors led to the functional reduction in their kin. None of these is necessarily a simple task in a lineage with no cultivated members, but culture-free methods for both genomics and microscopy open the door to so many new ways to study such organisms, and we are optimistic that the diversity of gregarine MROs will finally have their moment in the light.

CRediT authorship contribution statement

Patrick J. Keeling: Writing – original draft. **Mahara Mtawali:** Writing – review & editing. **Morelia Trznadel:** Writing – review & editing. **Samuel J. Livingston:** Writing – review & editing. **Kevin C. Wakeman:** Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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