Orchestrating a Heist: Uptake and Storage of Metals by Apicomplexan Parasites

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Introduction

The acquisition and storage of metals has been a preoccupation of life for millennia. Transition metals, in particular iron, copper, and zinc, have vital roles within cells. However, metals also make dangerous cargos; inappropriate uptake or storage of transition metals leads to cell death. This paradox has led to cells developing elegant and frequently redundant mechanisms for fine-tuning local metal concentrations. In the context of infection, pathogens must overcome further hurdles, as hosts act to weaponize metal availability to prevent pathogen colonisation and spread.

The Apicomplexa are a broad family of obligate intracellular eukaryotic parasites infecting a range of hosts from marine invertebrates to mammals. The best studied Apicomplexa are; *Plasmodium* spp., cause of malaria, one of the most deadly infectious diseases; *Toxoplasma gondii*, a ubiquitous pathogen of warm-blooded animals which causes miscarriage and blindness; and *Cryptosporidium* spp., a leading cause of diarrheal mortality in children under 5. The Apicomplexa share key metabolic and structural features including; residence within membrane-bound parasitophorous vacuole in the cytoplasm of the host cell; a single mitochondrion (although this has been lost in *Cryptosporidium* spp.); an apicoplast – a relic plastid which retains a number of essential metabolic pathways, and specialised secretory organelles which make up the apical complex for which the phylum is named transmission (Harding and Frischknecht, 2020; Jacot et al., 2016; Striepen et al., 2007). Apicomplexa also share complex, multi-host lifecycles, although with large variation in hosts and methods of. *Plasmodium* is taken up from infected hosts by mosquitoes in a

blood meal where it completes its sexual lifecycle. Mosquitos then inject the parasite back into the mammalian host where it passes through the liver before returning to asexual replication and expansion within red blood cells (Aly et al., 2009; Venugopal et al., 2020). In contrast, *Toxoplasma gondii* replicates within all cell types and tissues and forms slow-growing cysts within brain and muscle tissue. Upon ingestion of cysts by cats, *T. gondii* completes its sexual lifecycle, resulting in excretion of oocysts which are orally infectious to humans and most mammals (Martorelli Di Genova and Knoll, 2020). *Cryptosporidium* is also acquired from consumption of oocysts which enter and infect the gut. Unusually for the Apicomplexa, *Cryptosporidium* completes its entire lifecycle in the gut of one host, resulting in the excretion of oocysts (Tandel et al., 2019).

Here, we summarise the existing work on iron, zinc and copper in the context of apicomplexan parasites, focusing on the transporters required. Each metal presents distinct challenges to the parasite, e.g. exchangeable iron is a potential source of dangerous reactive oxygen species and available copper is almost non-existent within mammalian hosts. However, by summarising what is known about metal transport in these organisms, we hope to provide a basis for further study of this fascinating topic.

Iron ingress and imprisonment

Iron is an essential nutrient for the vast majority of known organisms where it plays a crucial role in core processes including oxidative phosphorylation and DNA replication and repair. Iron is primarily utilized by cells as part of either haem (Kloehn et al., 2020), iron sulphur (Fe-S) clusters (Dellibovi-Ragheb et al., 2013; LaGier et al., 2003) or diiron group cofactors (Yamasaki et al., 2021).

The importance of iron to the growth of apicomplexan parasites during mammalian infection is well known. Iron supplementation in mice was shown to increase *P. yoelii* burden in the liver (Goma et al., 1996), while iron deficiency may be associated with reduced risk of malaria, though the choice of markers used (e.g. ferritin saturation) impacts risk estimates (Muriuki et al., 2019). Additionally, treatment with iron chelators has been shown to suppress parasite growth *in vitro* and *in vivo* (Ferrer et al., 2012; Pollack et al., 1987; Thipubon et al., 2015). However, a mechanism for this is not well understood, as the therapeutic effects appear variable with infection stage and host as well as the chelator and its mode of administration (Bunnag et al., 1992; Ferrer et al., 2012; Gordeuk et al., 1992; Portugal et al., 2011; Thuma et al., 1998). *Plasmodium* spp. also require iron in their mosquito host, and iron accumulation in the mosquito has been linked to mosquito infection susceptibility (Maya-Maldonado et al., 2021). In *T. gondii,* iron has been shown to be important in parasite replication and pathogenesis *in vitro* and i*n vivo* (Almeida et al., 2019; Dimier and Bout, 1998; Mahmoud, 1999; Oliveira et al., 2020) although there have been no clinical studies. There has also been little work on the importance of iron to *Cryptosporidium*, although anaemia was not associated with

Cryptosporidium prevalence in one trial (Mengist et al., 2015). Due to the lack of the respiratory chain in *Cryptosporidium*, it is likely that the parasite's iron requirements are lower than other Apicomplexa, although iron is likely still required by the parasite (Kloehn et al., 2020; LaGier et al., 2003; Miller et al., 2018).

Acquisition of iron is non-trivial. In an oxygen-containing environment, iron is readily oxidised to the ferric (Fe^{3+}) form which is poorly soluble at physiological pH and therefore not readily available for uptake. As obligate intracellular pathogens, any iron must be subverted from the host, however a conserved host defence is to limit available iron, known as nutritional immunity. These interactions are important as iron availability is often a key determinant of infection outcome (Clark et al., 2014; Dimier and Bout, 1998; Oliveira et al., 2020). There are two main options for the Apicomplexa to acquire host iron, the parasites could take up and recycle host iron-containing proteins, or they could directly access the host labile iron pool (LIP), a pool of exchangeable iron only loosely bound to small molecules in the cytoplasm. Despite its abundance in its erythrocyte hosts, *Plasmodium* do not appear to access iron from haemoglobin, or other host-haem containing proteins, as they lack functional haem oxygenase (Sigala et al., 2012). Instead, *Plasmodium* uses haemoglobin catabolism as a source of amino acids (Liu et al., 2006), and the majority of host haem is crystallised into hemozoin. *Plasmodium* appears to utilise host cell haem directly, as haem biosynthesis enzymes are dispensable in blood stage parasites (Kloehn et al., 2021; Nagaraj et al., 2013; Sigala et al., 2015). In contrast, *T. gondii* also ingests host cell material during infection (Dou et al., 2014), however does not appear able to use exogenous haem (Bergmann et al., 2020) and the digestion of iron-containing proteins has not been confirmed (Kloehn et al., 2021). The form of iron that the parasites can use is also important. Many species encode ferric reductases (Andrews et al., 2003; Arosio et al., 2017; Zaidi et al., 2017; Zhang et al., 2019) such as FRE2/3 from yeast, which can reduce ferric (Fe^{3+}) to ferrous (Fe^{2+}) iron prior to transport or use. There is no evidence of any ferric reductases in the apicomplexan genomes, suggesting that the parasites take up a source of ferrous iron directly.

The host cell LIP makes an attractive source for parasite iron as it is likely that pores, formed in the parasitophorous vacuolar membrane by parasite proteins (Garten et al., 2018; Gold et al., 2015), could permit iron from the LIP to enter the parasitophorous vacuole. From the intravacuolar space, iron could then be moved into the parasite by specific transporters. A member of the ZIP family of divalent metal iron transporters, named ZIPCO, was localised to the plasma membrane and shown to be required for growth of liver stage *P. berghei* (Sahu et al., 2014). This growth defect could be rescued by supplementation by iron and zinc, suggesting there is some redundancy in the iron acquisition strategies employed by this parasite (Sahu et al., 2014). However, ZIPCO was only expressed in liver stage parasites, and it remains unclear how other parasite stages acquire iron. ZIPCO is conserved between the apicomplexans, including in *T. gondii* where it is predicted to be essential, although has not yet been characterised.

ZIPCO remains the only characterised apicomplexan transporter with a predicted role in iron uptake. However, apicomplexan genomes contain homologues for transporters which have been well characterised in other systems, including the conserved divalent metal iron transporter 1 (DMT1). DMT1 facilitates import of ferrous iron, and other metal ions, into the cell in many systems, including other protozoan parasites (Ballesteros et al., 2018; Smyth et al., 2006), and as such may play a similar role in apicomplexans. The mechanisms of iron uptake are likely of significant importance to the parasites. Most prokaryotic and eukaryotic species encode several distinct pathways for iron uptake, e.g. the distantly related intravacuolar parasite *Leishmania mexicana* has at least three identified system for taking up iron, either directly or through haem uptake and digestion (Zaidi et al., 2017). For *T. gondii,* which can replicate in any cell type with differing levels of available iron, fine control of iron uptake would be essential to maintain growth. The limited number of metal transporters identifiable from the genome sequences (**Table 1**) suggests that these pathways differ significantly from characterised pathways from model organisms,

Intracellular transport and detoxification of iron

The redox potential of iron which makes it so useful also presents a problem. The reaction of iron with oxygen-containing molecules results in the production of damaging reactive oxygen species (Dixon and Stockwell, 2014). As such the level and distribution of iron within cells must be carefully controlled. Mammals, plants and bacteria use the iron-binding protein ferritin (or similar proteins) to store iron in the cytoplasm, however, no ferritin homologs have been found in apicomplexans. Yeast and plants have a different approach to iron storage. Iron is stored in organelles or vacuolar compartments (Li et al., 2001; Sorribes-Dauden et al., 2020; Kim et al., 2006; Roschzttardtz et al., 2009; Zhang et al., 2012). This strategy makes use of membrane transporters to facilitate ferrous iron crossing organelle membranes, likely via a proton-driven antiport mechanism (Kato et al., 2019). Apicomplexan genomes contain homologs for several of these transporters including vacuolar iron transporter (VIT) (Labarbuta et al., 2017; Sharma et al., 2021; Slavic et al., 2016). In *P. falciparum*, VIT is expressed throughout the parasite life cycle. Parasites lacking VIT exhibited reduced parasitaemia and liver stage development, contained more exchangeable iron and were more sensitive to iron stress (Slavic et al., 2016). Interestingly, *Plasmodium* VIT appears specific for ferrous iron, while VIT from other organisms are less selective (Sharma et al., 2021; Slavic et al., 2016) suggesting the need for of further metal transporters. These results were corroborated recently in *T. gondii,* where VIT was required for survival under iron stress and to maintain iron stores in the parasite. In *T. gondii,* VIT was also shown to be important *in vivo*, highlighting the role of iron storage in pathogenesis (Aghabi et al., 2021). Interestingly, the localization of VIT appears to differ between the species, in *Plasmodium* VIT localised to the ER (Slavic et al., 2016) while in *T. gondii* VIT had a highly dynamic, vesicular localization similar to that

of the vacuolar compartment (Aghabi et al., 2021). This may show differences in iron storage between the species, possibly due to the abundance of iron in haem available to *Plasmodium*. Iron also needs to be mobilised from intracellular stores, through the action of transporters. Apicomplexa encode a conserved NRAMP homologue which may play a role in iron mobilisation from vacuolar stores (Nevo and Nelson, 2006). Interestingly, this gene is dispensable in *T. gondii* but essential in *P. falciparum*, perhaps underlining the differential importance of iron mobilisation in these cell types (Sidik et al., 2016; Zhang et al., 2019). The mitochondrion, the location of haem and Fe-S biosynthesis, is one of the primary destinations for iron within the cell. Iron is likely moved into the mitochondrion using the homolog of the yeast mitochondrial iron transporter Mrs3/4, mitoferrin, which is conserved in both *Toxoplasma* and *Plasmodium* genomes (**Table 1**) and likely essential, although yet to be characterised.

There remain several open questions as to how iron is moved into other cellular spaces (outlined in **Fig. 1**). Iron transporters into the Golgi and secretory system have been identified in other organisms (Seo et al., 2012; Xiao et al., 2014) and may also exist in the Apicomplexa. Intriguingly, a ZIP (Zip– Irt- like) metal transporter, likely to transport iron and/or zinc (**Table 2**) is predicted to be localized to the rhoptries, specialized secretory organelles used by apicomplexan parasites in the process of invasion. There is no known use for iron or zinc in this compartment which underlines the potential new metal biology still to be discovered in this phylum. Further, the apicoplast contains a dedicated Fe-S biogenesis pathway, essential for organelle maintenance and parasite survival (Charan et al., 2017; Gisselberg et al., 2013). The apicoplast, an essential organelle of secondary endosymbiosis, has long been an attractive therapeutic target for apicomplexan disease. However, despite its requirement for iron, there has been no identification of the transporters required to bring iron across the four membranes of the apicoplast, and as such identification of the mechanism of iron transport would be of particular interest.

Iron regulation

Whilst the networks which regulate host iron content are well described in other organisms (see (Wang and Pantopoulos, 2011) for an excellent review), regulation of iron uptake and storage in Apicomplexa is not well understood. Mammals regulate iron uptake and storage through aconitase (Alén and Sonenshein, 1999; Marondedze et al., 2016; Tang and Guest, 1999), a dual function enzyme/RNA-binding protein which can interact with stem-loop structures called iron responsive elements (IREs), found in specific mRNAs (Hentze et al., 1987; Koeller et al., 1989). Depending on IRE position, this stabilises or destabilises the mRNA, influencing translation. There is some data to suggest that *Plasmodium* may employ a similar system. *P. falciparum* aconitase has been demonstrated to bind both host (Loyevsky et al., 2001) and parasite IREs (Hodges et al., 2005; Loyevsky et al., 2003), however the parasite IREs are highly divergent from their

mammalian counterparts and the utility of this system in the Apicomplexa is currently unknown. There are also some intriguing hints that Apicomplexa may be able to alter the iron homeostasis of their hosts, infection with *T. gondii* resulted in changes in host transferrin levels by stabilization of the IRE (Gail et al., 2004). *T. gondii* secretes a number of effector proteins into the host cell which have significant effects on host cell transcription (Hakimi et al., 2017) and it is possible that infection subverts host cell iron homeostasis to benefit the parasite, however this requires further investigation.

While iron is one of the most abundant metals within cells, its uptake, mobility and regulation have not yet been well studied in the Apicomplexa. Initial studies have demonstrated that iron is essential, however many of the molecular details remain to be discovered. *Plasmodium* presents an interesting case study, as it faces various iron stresses throughout its lifecycle and requires strategies to both detoxify the abundant iron in the bloodstream form, and acquire enough iron in the mosquito to power replication. By examining iron uptake, usage and regulation in these divergent eukaryotes, we have the opportunity to discover new biology, as well as novel vulnerabilities for future treatment.

P. falciparum					*T. gondii*		
Type	**Gene name**	**Pf ID**	**Phenotype**	**Localisation**	**Tg ID**	**Phenotype score**	**Localisation prediction**
Transporter	NRAMP	PF3D7_0523800	Essential	-	TGME49_267270	0.49	-
	VIT1	PF3D7_1223700	Dispensable	ER (Slavic et al., 2016)	TGME49_266800	-1.22	Vacuolar/ vesicular (Aghabi et al., 2021)
	ZIPCO	PF3D7_1022300	Essential in liver stage	Periphery of parasites (Sahu et al., 2014)	TGME49_225530	-2.94	-
	DMT1	PF3D7_0523800	Essential	-	TGME49_267270	0.49	-
	mitoferrin	PF3D7_0905200	Essential	-	TGME49_277090	-3.05	mitochondrial

Table 1. Genes involved in iron transport in apicomplexan parasites. *P. falciparum* phenotype data from (Zhang et al., 2018). *T. gondii* phenotype data from (Sidik et al., 2016), values > ~ - 1.5 are considered dispensable, LOPIT localization data from (Barylyuk et al., 2020).

Zinc seizure and storage

Zinc is an essential cofactor for a large number of proteins including DNA binding domains, metalloproteases and ribosomal subunits (Cassandri et al., 2017; Eide, 2006). In the Apicomplexa, many zinc-binding proteins are conserved, and-although the majority have not yet been functionally characterised-several are important throughout the parasites' life cycles. For example, zinc finger proteins have been shown to regulate life cycle transitions, and secreted zinc-bound metalloproteases are required for the parasite's lytic cycle (Gopalakrishnan et al., 2017; Hajagos et al., 2012; Semenovskaya et al., 2020; Tanveer et al., 2013). In *Plasmodium* it has been shown that parasites accumulate large amounts of zinc (approximately 400 % of that found within normal erythrocytes) and that inhibition of zinc acquisition prevents parasite replication (Marvin et al., 2012). A similar, although less dramatic, increase in zinc levels in the host cell is seen upon *T. gondii* infection (Al-Sandaqchi et al., 2018), suggesting that manipulation of host cell zinc levels is common between the Apicomplexa. Within *Plasmodium*, much of this zinc appears to be weakly bound to chaperones within the mitochondrion of the parasite (Marvin et al., 2012). The purpose of this is not clear, but may have a role in mitochondrial respiration, as was demonstrated in the intracellular parasitic protozoa *Leishmania donovani* (Kumari et al., 2017). Despite the accumulation of zinc within infected cells, zinc deficiency or supplementation, either in rodents or in humans, does not appear to alter pathogenesis of *Cryptosporidium* or *Plasmodium* respectively (Hamaguchi et al., 2006; Müller et al., 2001; Veenemans et al., 2011). It is possible that zinc accumulation does not change the availability of zinc to the parasite. Zinc sequestration and relocalization are important facets of nutritional immunity (Vignesh and Deepe, 2016), and it is likely that these successful pathogens have developed highly effective mechanisms for zinc uptake in the face of host efforts.

A number of transporters required for zinc uptake and mobilisation in model organisms have been identified (Eide, 2006). Within mammalian cells, zinc is transported into the cell by a several high affinity transporters. Proteins of the ZIP (Zrt-, Irt-like Protein) family move zinc into the cytoplasm while cation diffusion facilitator (CDF) proteins move zinc from the cytoplasm to the lumen of membrane-bound compartments. Within a cell, almost all exchangeable zinc is bound to chaperones which move it to where it is required, e.g. into the ER and Golgi where it can be inserted into newly synthesized proteins. Excess zinc is toxic through a number of mechanisms including by displacing metal cofactors, disrupting protein folding and inducing apoptosis, and so cytosolic levels of exchangeable zinc are maintained at a very low level (Maret, 2009; Maret and Krężel, 2007; Plum et al., 2010).

In mammalian and bacterial cells, zinc is removed from the cytosol by zinc efflux transporters, however, in a similar manner to iron, yeast stores zinc within a vacuole (Eide, 2006). Recently the first apicomplexan zinc transporter, named ZnT, was characterised in *T. gondii* (**Fig. 1 and Table 2**). ZnT is localised to dynamic, vesicular compartments (Chasen et al., 2019), which, in concert with X-ray microanalysis, suggest zinc is

stored within acidocalcisomes (Luo et al., 2001; Rohloff et al., 2011). Confirming its suspected role, ZnT was found to complement a yeast zinc storage mutant and to be essential for maintaining zinc tolerance within *T. gondii* (Chasen et al., 2019). Interestingly, although the ZnT transporter is conserved in *Plasmodium* and highly expressed in late blood stages, it is not required for parasite replication (Aurrecoechea et al., 2009). Instead, Znt was required for male gamete exflagellation and ookinete formation (Kenthirapalan et al., 2016). ZIP1, a predicted Zn or Fe permease, was shown to have a role in blood stage replication, however was also required for gamete production (Kenthirapalan et al., 2016; Sayers et al., 2018). A possible *T. gondii* ZIP1 homolog (TGME49_261720) was predicted to localise to the plasma membrane and is likely essential *in vitro.* These results suggest that zinc has increased importance in the differentiation of *Plasmodium* to sexual stages, perhaps the rational for the accumulation in the asexual bloodstream form.

The mechanism of zinc acquisition remains an open question. Although *Plasmodium* encodes only two ZIP-domain containing proteins, *T. gondii* encodes four (**Table 2**) which could perform this role, however these have not yet been characterised. Further, due to its numerous roles in essential proteins, zinc is likely to be required in other organelles including the mitochondrion, ER and Golgi (**Fig. 1**). There is currently no direct evidence for a zinc requirement in the apicoplast, although it is possible as the potential zinc transporter ZIP1 contains an apicoplast targeting sequence in *Plasmodium* (Sayers et al., 2018). Mammalian and yeast cells encode transporters to move zinc between organelles (Bafaro et al., 2017; Eide, 2006), however functional homologs of these have not yet been identified in apicomplexan parasites. Given the lack of homologues to known zinc transporters, this may suggest that the Apicomplexa utilize divergent mechanisms for zinc uptake and transport within the cell. This, combined with the importance of zinc to parasite biology, makes parasite zinc pathways an attractive target for intervention.

Plasmodium					*T. gondii*		
Type	**Gene name**	**Pf ID**	**Phenotype**	**Localization**	**Tg ID**	**Phenotype score**	**Localisation prediction**
Transporter	ZnT /CDF	PF3D7_0715900	Dispensable	-	TGME49_251630	-3.06	Plant-like vacuole Vesicular (Chasen et al., 2019)
Zrt-, Irt-like	ZIP1	PF3D7_0609100	Essential	-	TGME49_261720	-4.31	Plasma membrane
					TGME49_254080	1.41	Secretory organelles, rhoptries
					TGME49_260300	-1.4	-

Table 2. Transporters expected to be involved in zinc transport in apicomplexan parasites. *P. falciparum* phenotype data from (Zhang et al., 2018). *T. gondii* phenotype data from (Sidik et al., 2016), values > ~ - 1.5 are considered dispensable, localization prediction data from (Barylyuk et al., 2020).

Capture and cloistering of Copper

Although only required in very small amounts, copper plays an key role in cellular processes. It acts as a cofactor in a number of essential enzymes including Zn/Cu superoxide dismutase (Cu/Zn-SOD), cytochrome *c* oxidase and other enzymes involved in diverse pathways such as pigmentation and peptide processing (Balamurugan and Schaffner, 2006). Of these, cytochrome *c* oxidase is conserved in apicomplexans and is essential for energy production. *Plasmodium*-infected erythrocytes accumulate copper (Marvin et al., 2012). The reason for this is not known, but is possibly related to parasite cytochrome *c* production. However, this accumulation is likely important as copper chelation has been shown to block *Plasmodium* replication in erythrocytes (Asahi et al., 2014; Rasoloson et al., 2004). Blocking replication this effect appears more pronounced when using Cu^{+}, rather than Cu^{2+}, chelators (Asahi et al., 2014), suggesting that Cu^{+} is the form required by the parasite. Copper is also required by *Plasmodium* in the mosquito definitive host (Maya-Maldonado et al., 2021) although it was not clear if the various *Plasmodium* life cycle stages in the mosquito have different copper requirements. No studies have yet looked specifically at the copper requirements of *T. gondii* or *Cryptosporidium*. However, although copper is likely to be required by *T. gondii,* several *Cryptosporidium* species have lost cytochrome *c* oxidase, replacing it with alternative oxidase (AOX), and so may not require for copper (Liu et al., 2016). However, even in the absence of cytochrome *c* oxidase, *Cryptosporidium* spp. have maintained a likely homolog of CTR and a Cu-binding P-ATPase (see below) (LaGier et al., 2001), providing circumstantial evidence for a requirement for copper beyond energy production in these cells. The effects of *Plasmodium* infection on host cell copper are unclear, previously it was shown copper levels either decrease (Rasoloson et al., 2004) or increase (Marvin et al., 2012) in trophozoite-infected red blood cells. *T. gondii* infection appeared to increase the copper content (Al-Sandaqchi et al., 2018). Within cells, very little exchangeable copper is present with the large majority bound tightly to host cell proteins making copper acquisition a challenge for intracellular pathogens (Li et al., 2019).

In mammalian and yeast cells, copper uptake occurs through a high affinity copper transporter CTR1, copper is then bound to acceptors such as GSH and transported to chaperones (Kaplan and Maryon, 2016). Copper efflux occurs though Golgi-localised P-ATPases which traffic to the plasma membrane upon copper overload, removing copper from the cell (Kaplan and Maryon, 2016). Two putative copper transporters have been investigated in *Plasmodium* (**Table 3**), a copper transporter CTR1 and a Cu P-ATPase named CuTP (Choveaux et al., 2012; Kenthirapalan et al., 2014; Rasoloson et al., 2004). PfCTR1 was localised to the erythrocyte membrane and is predicted to be essential during asexual replication in Plasmodium (Choveaux et al., 2012).

In *T. gondii,* a CTR1 homologue is likely essential and is predicted to localise to the Golgi, although no characterisation has yet been carried out. The P-ATPase CuTP is conserved in *Cryptosporidium* where it was shown to specifically bind copper (LaGier et al., 2001). Work in both *P. berghei* and *T. gondii* showed that CuTP was localised to vesicular structures at all life cycle stages, and showed some overlap with the vacuolar marker VP1 in *T. gondii,* suggesting a role of CuTP in copper storage (Kenthirapalan et al., 2014). CuTP was not shown to be essential for blood stage growth or gametocyte production in *P. berghei,* however is essential in gametocyte fertility (Kenthirapalan et al., 2014), suggesting that this life cycle stage has an altered requirement for copper. The Apicomplexa also encode a homolog of the mitochondrial phosphate/copper transporter PIC2 (Zhu et al., 2021) which is predicted to be essential and mitochondrially-localised in *T. gondii,* however has not yet been characterised.

There are a number of fairly well-defined pathways in plants, fungi and mammalian cells to sense and respond to changes in copper levels (Blaby-Haas and Merchant, 2012, Ehrensberger and Bird, 2011) however no homologues of known response elements can be found in the Apicomplexa. It is possible that as obligate intracellular parasites, they no longer need to respond to changes in copper levels as their hosts will contain sufficient copper for replication. Alternatively, the parasites can sense and respond, however the components are divergent from known pathways and so have not yet been identified.

In summary, the role of copper in apicomplexan development deserves further investigation. There is evidence that maintenance of copper homeostasis is required by the parasites, and some of the genes involved in this process have been identified and initially characterised (**Table 3** and **Fig.1**). However, the topic merits systematic investigation as copper homeostasis has been shown to be vital in the virulence of a number of pathogens (Blaby-Haas and Merchant, 2012; Ehrensberger and Bird, 2011).

Plasmodium					*T. gondii*		
Type	Gene name	Pf ID	Phenotype	Localisation	Tg ID	Phenotype score	Localisation prediction
Transporter	CTR1	PF3D7_1439000	Essential	Translocates from the erythrocyte plasma membrane in early ring stage to a parasite membrane as the parasites developed to schizonts (Choveaux et al., 2012)	TGME49_262710	-2.62	Golgi
Channel	CTR2	PF3D7_1421900	Essential	-	TGME49_249200	2.31	-

ATPase	CuTP	PF3D7_0904900	Dispensable	Expressed in all Plasmodium life cycle stages. Localizes to vesicle-like structures (Kenthirapalan et al., 2014)	TGME49_201150	-1.57	Endomembrane vesicles, co-localises with the VAC (Kenthirapalan et al., 2016)
Metallochaperone	Cox17	PF3D7_1025600	?	Cytoplasmic localisation in asexuals (Choveaux et al., 2015)	TGME49_240550	-2.7	Mitochondrial
Mitochondrial copper/phosphate transporter	PIC2	PF3D7_1202200	Dispensable	-	TGME49_278990	-2.7	Mitochondrial

Table 3. Genes involved in copper homeostasis in apicomplexan parasites. *P. falciparum* phenotype data from (Zhang et al., 2018). *T. gondii* phenotype data from (Sidik et al., 2016), values > ~ - 1.5 are considered dispensable, LOPIT data from (Barylyuk et al., 2020).

Summary

Despite the importance of Apicomplexa, both clinically and in veterinary practice, the study of the uptake, use and storage of essential metals lags behind work done in other organisms. Transporter characterisation has improved in recent years, and the identification of several metal transporters has underlined the importance of metals to the parasites, however a number of predicted transporters remain unknown. Further, the role of transcriptional and post-transcriptional regulation in metal uptake and storage has not been addressed, despite these processes being tightly controlled in other systems. This limits our ability to understand how these pathogens interact with their hosts, and how they respond to and overcome host nutritional immunity.

As highly divergent parasitic eukaryotes which often cycle between mammalian and insect hosts, there may well be important biological differences in how metals are handled which could be exploited therapeutically. One interesting area for future investigation is the apicoplast, which depends on iron import (Gisselberg et al., 2013), but has no identified iron transporters. By identifying the strategies employed to transport metals around the cell we have the opportunity to learn more about how the common problem of metal acquisition and storage has been addressed across the broader tree of life.

Figure 1. Summary of expected iron, zinc and copper transporters in apicomplexan parasites

A schematic showing the major organelles of *T. gondii* as a model apicomplexan and the expected localisation of transporters involved in metal transport throughout the cell. As the parasitophorous vacuolar membrane (PVM) is permeable, it is not known if transporters would be required to move metals into the PV space. Apicomplexa appear to be able to endocytose material from the host which may provide a source of metal-containing proteins Iron is required in the mitochondria and apicoplast (see text for details) and may be required in the ER and is likely stored in a vacuolar compartment (VAC). A single apicoplast-localised transporter has been indicated, but it is likely that more than one would be required to cross the four membranes of the apicoplast. Zinc is likely required in the mitochondria, ER and Golgi and the zinc transporter ZnT has been localised to multiple compartments, potentially acidocalcisomes and the vacuolar compartment. Copper is required in the mitochondria and copper transporters have been putatively localised to the VAC and Golgi. Transparent transporters (marked with ?) show the likely location of transporters that have not yet been identified. Iron/iron transporters- red, copper/copper transporters-blue and zinc/zinc transporters - grey.

Funding information

C.R.H and M.A.S are funded by a Sir Henry Dale fellowship to C.R.H (213455/Z/18/Z) from the Wellcome Trust and Royal Society. C.R.H and D.A are funded by a Lord Kelvin/Adam Smith fellowship from the University of Glasgow.

Conflict of Interest

The authors declare that there are no conflicts of interest

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