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Experiment Report Form

The double page inside this form is to be filled in by all users or groups of users who have had access to beam time for measurements at the ESRF.

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Deadlines for submission of Experimental Reports

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Experiment Report supporting a new proposal ("relevant report")

If you are submitting a proposal for a new project, or to continue a project for which you have previously been allocated beam time, you must submit a report on each of your previous measurement(s):

- even on those carried out close to the proposal submission deadline (it can be a *"preliminary report"),*
- even for experiments whose scientific area is different form the scientific area of the new proposal,
- carried out on CRG beamlines.

You must then register the report(s) as "relevant report(s)" in the new application form for beam time.

Deadlines for submitting a report supporting a new proposal

- 1st March Proposal Round - **5th March**
- 10th September Proposal Round - **13th September**

The Review Committees reserve the right to reject new proposals from groups who have not reported on the use of beam time allocated previously.

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Should you wish to make more general comments on the experiment, please note them on the User Evaluation Form, and send both the Report and the Evaluation Form to the User Office.

Instructions for preparing your Report

- fill in a separate form for each project or series of measurements.
- type your report in English.
- include the experiment number to which the report refers.
- make sure that the text, tables and figures fit into the space available.
- if your work is published or is in press, you may prefer to paste in the abstract, and add full reference details. If the abstract is in a language other than English, please include an English translation.

ESRF	**Experiment title:** Iron localisation and speciation in the parasite *Toxoplasma gondii*	**Experiment number**: LS-3200
Beamline: ID21	**Date of experiment**: from: 28/03/2023 to: 01/04/2023	**Date of report**: 05/09/2023
Shifts: 12	**Local contact(s)**: Hiram Castillo hiram.castillo_michel@esrf.fr	*Received at ESRF:*

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Report:

1. Introduction and Aims

Toxoplasma gondii is an obligate intracellular apicomplexan parasite that can infect all warm-blooded species. Infection is of significant clinical and veterinary relevance worldwide, having both economic and public health consequences. *Toxoplasma* is arguably the most successful pathogen in the world, infecting 30% of the population worldwide. In immunocompetent individuals, infection is asymptomatic, however, the disease is dangerous in immunocompromised individuals and to the developing foetus, resulting in congenital defects or miscarriages.

Iron is an essential nutrient for almost all organisms. Iron acquisition systems of many bacteria are important for pathogenesis[8,17]. Moreover, the virulence of several parasites is modulated by host iron levels. For example, low iron due to anaemia can reduce the severity of malaria[3]. Iron is an essential nutrient for the survival of *T. gondii*, as haem and iron-sulphur biosynthesis pathways are essential[4], strongly suggesting the importance of iron in *T. gondii's* metabolism. Moreover, depletion of iron using iron chelators has been shown to restrict parasite replication of both *Toxoplasma in vitro* and *in vivo*[5,6,7,11,12].

Toxoplasma must acquire iron from the host cell it is infecting. However, the mechanisms of iron uptake, as well as subcellular transport of iron have not been previously studied in *Toxoplasma gondii*. We have recently shown that an iron transporter (VIT) is required for iron storage in *Toxoplasma,* disruption of this transporter makes the cells hypersensitive to excess iron and prevents virulence of the parasite[1].

However, although iron uptake and storage are required for *Toxoplasma*, the mechanism of iron uptake remains unknown. Previous studies in the related eukaryotic parasite, *Plasmodium,* identified a transporter, named ZIPCO which is believed to be involved in the uptake of iron[14]. ZIPCO contains an eight transmembrane

domains and belongs to the ZIP protein family, which has been linked to the transport of iron, and possibly zinc. *Toxoplasma gondii* has a homologue of this gene, and is predicted to be essential[16] and to localise to the plasma membrane of the parasites[19]. However, the role of ZIPCO has not yet been investigated *T. gondii.*

As ZIPCO is predicted to be essential for parasite survival, we have generated a cell line which allows for inducible knockdown of this major iron transporter. We also generated a parasite cell line which overexpresses this transporter (unpublished results). However, much remains unknown about how the parasite acquires iron and transports it. The proposal therefore aimed at mapping the localisation of iron in wildtype parasites as well as our transporter mutants at a subcellular resolution. We also aimed at determining the speciation of iron within the wildtype parasite as well as the effect of the mutant iron transporter on the distribution of iron.

Although parasite host modulation in the context of iron is not well studied in *T. gondii*, infection of macrophages by the intravacuolar parasite *Leishmania* impairs macrophage iron homeostasis. Intracellular *Leishmania donovani* manipulate iron uptake mechanisms by macrophages by directly scavenge iron from the labile iron pool (LIP) of macrophages, enabling the parasite to utilize it for it's intracellular growth[18]. *Leishmania amazonesis* has been shown to increase the LIP by downregulating ferroporti[18]. Studies have also previously shown that host cell LIP is upregulated following *Plasmodium* infection[2]. In contrast, infection of macrophages with the intervacuolar bacterium *Salmonella typhimurium*, led to upregulation of ferroportin[11], reducing the LIP and preventing bacterial growth[9]. Many bacteria and eukaryotic pathogens acquire iron from the host in the form of transferrin[13]. Overexpressing transferrin receptor (TfR1) has been shown to increase the LIP[15]. Studies have previously shown that TfR1 expression is upregulated in cells infected with *Toxoplasma gondii*. Moreover, host cell LIP has also been shown to increase following *Plasmodium* infection[2]. Furthermore, the bacterial pathogen *Francisella tularenis* causes an upregulation of TFR1, which increases the macrophage LIP and is critical for its survival and intracellular proliferation. In contrast, *Salmonella typhimurium* does not require TFR1 expression for successful intracellular survival and Salmonella-infected macrophages maintain their LIP at normal levels[10]. These data suggest that intracellular pathogens modulate host iron in distinct ways. Our work in the lab has found that infection leads to changes in iron acquisition proteins in host macrophages (unpublished results). However, little is known about how *Toxoplasma* modulates iron in the host cell. The proposal therefore also aimed at mapping iron in infected and uninfected macrophages in order to examine changes in iron quantity and/or distribution.

2. Experimental Techniques

We requested 12 shifts on ID21 to analyse 10 samples. The samples were the following:

- Uninfected Bone Marrow Derived Macrophage's (BMDMs)
- BMDMs infected with wildtype *T. gondii*
- BMDMs infected with *T.gondii* overexpressing the major iron transporter ZIPCO
- Uninfected RAW murine macrophages 24h
- RAW murine macrophages infected with wildtype *T. gondii*
- Extracellular wildtype *T. gondii*
- Extracellular wildtype *T. gondii* pretreated with ferric ammonium citrate
- Extracellular *T. gondii* overexpressing the major iron transporter ZIPCO
- *T. gondii* where the major iron transporter has not been knocked down
- *T. gondii* where the major iron transporte, ZIPCO, has been knocked down

For each sample we aimed to obtain one coarse map at 2 x 2 μm, 1 fine map at 300 nm resolution (approx. 3 h) and then about 100 scans for XANES (approx. 2 h) on selected samples. In infected cells, XANES were taken from the background, intracellular parasites, extracellular parasites as well as the host cell. This aimed to give us the resolution needed to map our element of interest (primarily iron) at a subcellular resolution, and to determine the valence of iron, in both infected host cells and in the parasite.

3. Results

We successfully obtained approx. 800 XANES spectra, across all samples (including background scans). We also obtained 213 MAPS (at various resolutions). At the highest resolution we are able to localise iron within the parasite (**Fig. 1A**) and to distinguish parasites inside host cells (**Fig. 1B**) and see the localisation of iron within these cells.

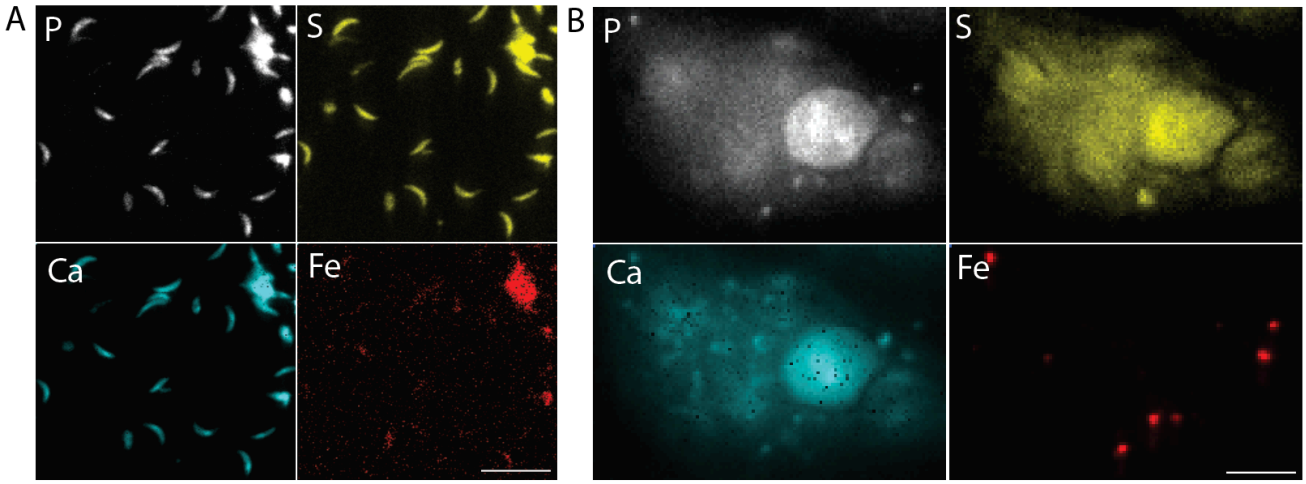

Figure 1 .Iron localisation in extracellular parasites (A) and infected macrophage (B). Scale bar 5 uM

Using our transporter mutant, we were able to quantify iron from parasites and demonstrate for the first time that depletion of ZIPCO lead to undetectable levels of iron within the parasites (**Fig. 2A**). We quantified this, and found that parasites which lack the iron transporter have no detectable iron levels, while wildtype parasites contain approximately 200 pM iron **(Fig. 2B)**.

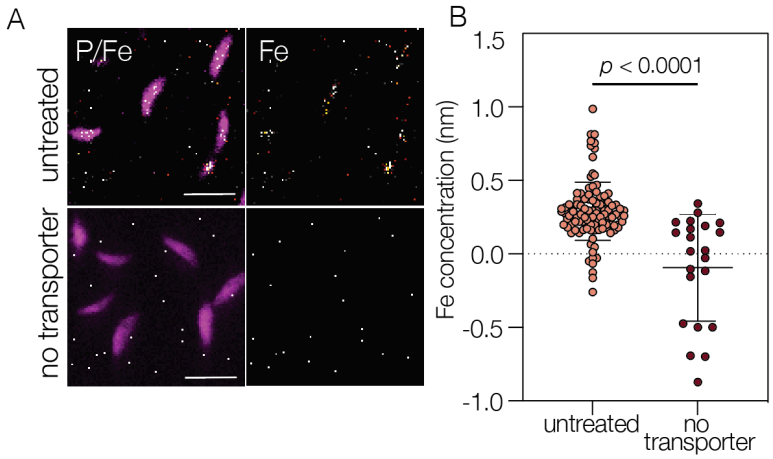

Figure 2. Depletion of the proposed iron transporter leads to undetectable levels of iron within the parasites. Each points represents a single parasite. Scale bar 5 uM.

We were also successful in examining the valence of iron within the parasite. Although more detailed analysis will be performed, we can see that parasite spectra cluster more closely with Fe-oxalate than the Fe-foil standard on a PCA plot (**Fig. 3**).

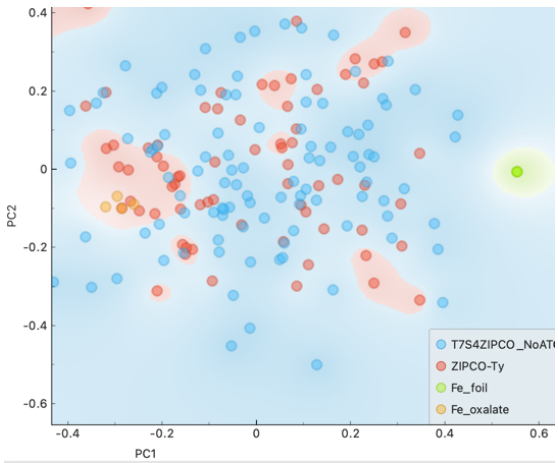

Figure 3. PCA plot of various parasite samples and Fe-foil and Fe-oxalate standard controls showing parasite spectra (in blue and red) clustering more closely with Fe-oxalate (orange) than the Fe-foil standard (green).

We can see more detail that parasites lacking iron cluster apart from wildtype parasites and parasites treated with excess iron. (**Fig. 4**).

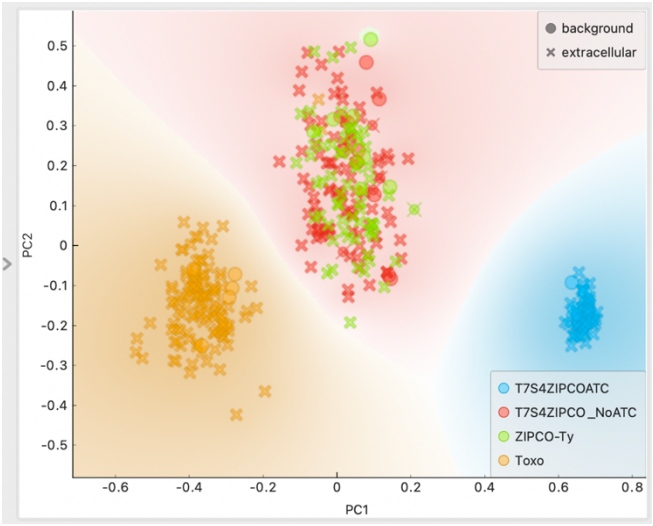

Figure 4. PCA plot of various parasite samples showing clear distinction between parasites without iron (blue), normal parasites (red and green) and parasites treated with excess iron (orange).

The valence of parasite iron is also clearly distinct from host cell iron stores (**Fig. 5**). This matches with predictions made from the parasite genomes, including the lack of ability of parasites to store iron within Fe^{3+} ferritin cages.

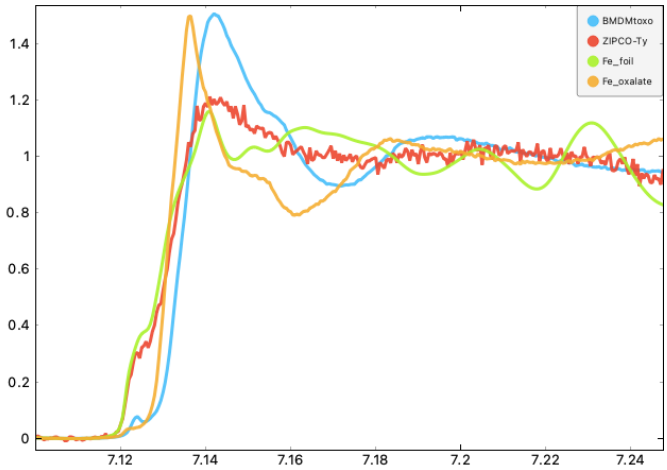

Figure 5: Normalised XANES spectra of host (blue), parasite (red) and control spectra (green and orange).

We also find that although BMDMs infected with wildtype *T. gondii* (red) cluster close together with uninfected BMDMs (green), BMDMs infected with parasites that overexpress the major iron transporter ZIPCO (blue), cluster distinctly to BMDMs uninfected/infected with wildtype *Toxoplasma* (**Fig. 6**)

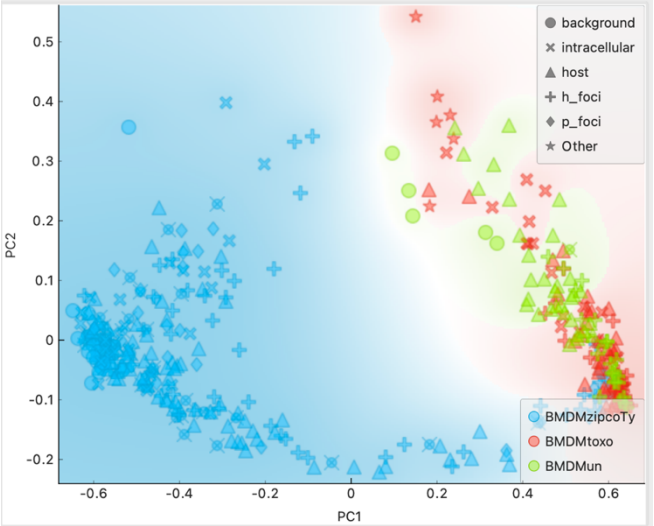

Figure 6: Principal component analysis (PCA) plot demonstrating that BMDMs infected with parasites that overexpress the major iron transporter ZIPCO (blue), cluster distinctly from uninfected BMDMs (green) and BMDMs infected with wildtype *Toxoplasma* (red), who overlap.

4. Conclusions and Significance

In conclusion, the main findings of this study supports that *T. gondii* acquires iron through the action of the ZIPCO transporter. For the first time, we show that in the absence of ZIPCO, *Toxoplasma* have significantly less iron compared to wildtype parasites. We also find that parasites that were pretreated with iron, cluster distinctly from wildtype parasites. There was also a clear difference in clustering between BMDMs that were infected with wildtype parasites and BMDMs that were infected with *T. gondii* that overexpressed ZIPCO, suggesting that parasites that overexpress this iron transporter may be playing a role in modulating the host cell iron following infection. Further analysis on this data is required to understand the mechanisms of parasite host modulation in the context of iron in more detail.

The results obtained from this proposed study are very important and central to apicomplexan metal biology. It is currently not known how the parasite takes up iron and how it modules the host cell iron following infection. The results obtained from our first visit to the ESRF in 2022 allow us, for the first time, to start to understand the mechanisms by which *T. gondii* acquires iron from the host, and how the parasite modulates iron in the host cell at a subcellular resolution. As iron is critical for both the parasite as well as the immune response, it is important to understanding this battle for this essential metal. Further understanding this has the potential to suggest novel strategies for the control of these parasites.

5. References

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