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***Magnetic susceptibility of iron in malaria-infected red  
blood cells***

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Abbreviations footnote

PBS: Phosphate Buffered Saline

Key Words: haemozoin, haemoglobin, magnetic susceptibility, malaria

## Summary

During intra-erythrocytic maturation, malaria parasites catabolize up to 80% of cellular haemoglobin. Haem is liberated inside the parasite and converted to haemozoin, preventing haem iron from participating in cell-damaging reactions. Several experimental techniques exploit the relatively large paramagnetic susceptibility of malaria-infected cells as a means of sorting cells or investigating haemoglobin degradation, but the source of the dramatic increase in cellular magnetic susceptibility during parasite growth has not been unequivocally determined. *Plasmodium falciparum* cultures were enriched using high-gradient magnetic fractionation columns and the magnetic susceptibility of cell contents was directly measured. The forms of haem iron in the erythrocytes were quantified spectroscopically. In the 3D7 laboratory strain, the parasites converted approximately 60% of host cell haemoglobin to haemozoin and this product was the primary source of the increase in cell magnetic susceptibility. Haemozoin iron was found to have a magnetic susceptibility of  $(11.0 \pm 0.9) \times 10^{-3} \text{ mL.mol}^{-1}$ . The calculated volumetric magnetic susceptibility (SI units) of the magnetically enriched cells was  $(1.88 \pm 0.60) \times 10^{-6}$  relative to water while that of uninfected cells was not significantly different from water. Magnetic enrichment of parasitised cells can therefore be considered dependent primarily on the magnetic susceptibility of the parasitised cells.

## ***Introduction***

Measurements of the magnetic susceptibility of blood were performed first by Faraday in 1845 and later, with greater precision and care for sample preparation, by Pauling and Coryell [1]. A diamagnetic susceptibility measured for the iron in oxyhaemoglobin molecules was interpreted as evidence for no unpaired electrons in oxyhaemoglobin, but Pauling and Coryell also noted a paramagnetic contribution to the total magnetic susceptibility of deoxyhaemoglobin. The possibility of interaction between the haem groups in each globin molecule was rejected on principle and instead, an effective magnetic moment of  $5.4 \mu_B$  for each iron atom in deoxyhaemoglobin was calculated, consistent with a model of a ferrous iron atom at the centre of each porphyrin ring, bonded to the globin molecule and four surrounding nitrogen atoms through ionic rather than covalent bonds. Further studies of the magnetic properties and structure of methaemoglobin found evidence for five unpaired electrons in each molecule, giving rise to an effective moment of  $5.8 \mu_B$  per iron atom [2]. Although these results were, and to some extent remain controversial [3-5], many of the studies detecting net paramagnetic susceptibilities in samples of oxyhaemoglobin were later considered to be flawed [6] as small but significant amounts of deoxyhaemoglobin were present in solutions of concentrated haemoglobin, even after exposure to air.

Savicki et al also determined the magnetic susceptibility of the polypeptide constituent of haemoglobin [6]. Globin is more dense and thus more diamagnetic than water and so completely oxygenated erythrocytes are diamagnetic relative to water [7]. However, cells containing entirely deoxygenated or oxidised haemoglobin are paramagnetic relative to

water. A model derived by Spees and co-workers [7] predicts the magnetic susceptibility of cells containing varying fractions of oxygenated and deoxygenated haemoglobin. The model was later extended by Moore and co-workers, first to describe cells containing methaemoglobin and later for cells infected with malaria parasites [8, 9].

Owen and co-workers first demonstrated the use of high-gradient magnetic columns to extract deoxygenated erythrocytes from a suspension of whole blood [10]. The experiment was later repeated with asynchronous cultures of *P. falciparum* [11], extracting cells containing mature stage parasites. Other experimental techniques use the relative motion of erythrocytes in an applied magnetic field as a surrogate measure of the magnetic susceptibility of the haem iron [8, 9, 12-14]. Moore and co-workers demonstrated that cells containing sufficient methaemoglobin concentrations [8] or containing mature parasites [9] will migrate in the direction of increasing field strength, indicating that these cells contain paramagnetic iron species.

Malaria parasites penetrate erythrocytes and use host cell haemoglobin as a principal source of amino acids [15]. As the polypeptide component of haemoglobin is digested, large quantities of free haem are liberated and immediately oxidised to haematin (ferriprotoporphyrin IX hydroxide). The ferric iron in haematin is potentially toxic to the parasites as the iron may catalyse cell-damaging reactions such as the Fenton reaction [16, 17], so the haematin is detoxified by conversion to particulate haemozoin, the characteristic malaria pigment. The distinctive brown crystals, clearly visible in the

midgut of the mosquito and within infected erythrocytes [18], led to the identification of the *Anopheles gambiae* mosquito as the malaria vector [19].

The structure of haemozoin was elucidated by Slater and co-workers [20], and later refined by Bohle and co-workers [21] and Pagola and co-workers [22]. The current model shows bonds between the central ferric iron of one haem group and a side-chain carboxylate group of another. These dimers are then formed into crystals by hydrogen bonds. The pathways of haemoglobin degradation and haemozoin synthesis are not yet established and reports of the fraction of cellular haemoglobin converted to haemozoin differ, with values ranging from 25% to 80% [23-26].

The intrinsic relationship between the chemical and magnetic properties of iron provides a means of elucidating the form and structure of iron compounds. The paramagnetic susceptibilities of some chemical forms of iron are several orders of magnitude greater than the diamagnetic susceptibilities of organic materials [27], so magnetic methodologies are potentially a highly sensitive and specific means of studying the forms of iron in biological systems. Previous experiments using magnetic fractionation [11] and magnetophoresis [9] indicate a change of the chemical state of haem iron during parasite growth. However, the form(s) of iron responsible for the increase in cellular magnetic susceptibility have not been unequivocally determined.

To address this question, *P. falciparum* parasites were magnetically separated and, rather than isolating the different iron-containing compounds, magnetic measurements were

performed on cell contents as a whole. The forms of iron present in the cells were identified using a combination of spectrophotometry and magnetometry. Standard samples of these forms were prepared and characterised both magnetically and spectroscopically. The physical processes underlying the enrichment of mature stage parasites in the magnetic fractionation columns were explored and several potential uses of magnetic fractionation processes in malaria research were considered.

### **Methods and Materials**

Twenty-four unsynchronised cultures of *P. falciparum* 3D7 clones were maintained according to the method of Trager and Jensen [28]. Thin blood films, stained with 10% v/v Giemsa, were examined using a Zeiss optical microscope before and after magnetic fractionation. At least 1 000 cells per film were counted and cells were categorised as unparasitised, immature (ring and early trophozoite stages, i.e. the first 30 hours of parasite life cycle) and mature (late trophozoite and schizont stages i.e. 30 – 48 hours of the parasite life cycle). The parasitaemia (defined as percentage of erythrocytes infected with parasites) and the mature parasite density (defined as percentage of erythrocytes infected with late trophozoites and schizonts) were measured. Cultures were selected for magnetic fractionation when parasitaemia ranged between 6 – 10% and the mature parasite density ranged between 2.1 – 5.4%.



### Magnetic Fractionation

Prior to fractionation, each culture was centrifuged at 2000 rpm for 10 minutes and the culture medium was removed. The pellet, containing approximately 1mL packed cells, was then resuspended in 5 mL phosphate-buffered saline (PBS) with foetal calf serum [29]. The cells were exposed to ambient oxygen to ensure complete oxygenation of functional haemoglobin.

Commercially available midiMACS columns and magnet were used to separate the cultures [29]. The magnetic field strength between the poles of the magnet was measured with a Hall probe. A sample of the magnetisable spheres forming the column matrix was taken and the magnetisation as a function of field strength was measured with a SQUID magnetometer. Sphere radii were measured with an optical microscope and 112 spheres were weighed to determine the mean sphere mass.

One fractionation column was used for each culture. The column was held between the poles of the magnet and the flow rate of cells through the column was regulated with an adjustable tap connected to the column with a length of silicone tubing. The average fluid velocity within the magnetisable length of the column was calculated from the flow rate, the length of the column containing the magnetisable spheres and the void volume. Flow rate was maintained at approximately  $0.2 \text{ mL}\cdot\text{min}^{-1}$  so that the average fluid velocity was less than  $340 \text{ }\mu\text{m}\cdot\text{s}^{-1}$ . After the sample had passed through, the column was washed twice with 1 mL of fractionation buffer to flush out the nominally non-magnetic erythrocytes trapped within the matrix. The fraction of cells that passed through the column whilst it

was exposed to the magnetic field was classed as the non-magnetic fraction. The column was then removed from the magnet and flushed through with fractionation buffer, and the cells collected were classed as the magnetic fraction. Fractionation of each culture typically yielded 50 to 100  $\mu\text{L}$  of packed cells in the magnetic fraction, and so magnetic fractions from three or four fractionations were pooled. The total volume of cells in each pooled magnetic fraction was at least 200  $\mu\text{L}$  as this was the minimum volume suitable for magnetometry experiments. The non-magnetic and the pooled magnetic fractions were centrifuged and the pelleted cells were smeared for microscopy. The samples were then freeze-thawed to lyse the cells and ultra-centrifuged at 19 000 g for 30 minutes to remove the supernatant, containing PBS and water. Cell membranes formed a thin layer between the haemoglobin and the supernatant, and when possible were removed. Magnetometry subsamples of at least 200  $\mu\text{L}$  and spectrophotometry subsamples of 50  $\mu\text{L}$  were taken from each individual non-magnetic fraction and each pooled magnetic fraction.

A magnetic fraction was diluted with uninfected red blood cells to create a sample with a mature parasite density intermediate to the non-magnetic and the remaining magnetic fractions. The final mature parasite density of the diluted sample was 13%. Owing to the small sample volume, haemozoin extraction and quantification were not performed.

### **Elemental Analysis**

Following magnetic examination the magnetometry samples of a known volume were digested in 10 mL of 1:1 ratio of 10 M AR grade nitric / sulphuric acid to remove the

organic materials. The resulting aqueous solutions were then diluted up to a known volume for inductively coupled plasma atomic emission spectrometry and the iron and chlorine concentrations were measured.

### **Spectrophotometry**

A standard solution of oxyhaemoglobin was prepared by bubbling pure oxygen gas through fresh red cells. Standard solutions of methaemoglobin were prepared by exposing 5 mL packed cells to a 5 mM sodium nitrite solution and incubating at 37°C for 2 hours [8]. The solutions were frozen and thawed to lyse the cells, then centrifuged at 13 000 g and the cell membranes removed. A standard sample of haematin was prepared by dissolving 1 g of porcine haematin (commercially available from Sigma-Aldrich) in 1 M sodium hydroxide solution [30], then diluting with 2.5% SDS buffered with 25mM Tris to pH 7.8, to a final concentration of 1.6 mM.

Dilutions of each standard sample were prepared in PBS and the absorption spectrum between 350 and 700 nm was recorded using a DU640 spectrometer (Beckman Coulter). Dilutions of the haematin solution were used to form a standard curve for absorption versus concentration.

It is not possible to measure haemozoin concentration *in situ* with spectrophotometric techniques as biological matrices and free haem alter the absorption spectrum of haemozoin [31, 32]. Likewise, the oxyhaemoglobin-to-methaemoglobin ratio cannot be measured in solutions containing significant concentrations of haemozoin. A 1:100 v/v

dilution of each non-magnetic sample with less than 5% parasitaemia was prepared in PBS in triplicate and the absorption coefficients at 400, 577 and 630 nm were measured. Winterbourn's formula was used to determine the relative concentrations of oxyhaemoglobin and methaemoglobin, and thus the ratio of oxygenated haemoglobin to methaemoglobin [17], as follows:

$$\begin{aligned} [\text{Methaemoglobin}] &= 279A_{630} - 3.0A_{577} \\ [\text{Oxyhaemoglobin}] &= 66A_{577} - 80A_{630} \end{aligned} \tag{1}$$

where  $A_{630}$  and  $A_{577}$  are the absorption coefficients at 630 nm and 577 nm respectively.

Haemozoin was isolated by freeze-thawing cells to induce lysis, then centrifuging the sample and removing the supernatant. The pellets, containing haemozoin and other forms of non-protein bound haem, were treated with SDS and centrifuged. The SDS-insoluble haemozoin pellets were then solubilized with 0.1 M NaOH. Solutions were diluted with 2.5% SDS buffered with 25 mM Tris to pH 7.8 and the absorbance at 400 nm was measured [30]. Haemozoin could not be isolated in sufficient quantities from the non-magnetic fractions for accurate quantification and so the best estimate for the haemozoin concentration of these samples is zero with an uncertainty of approximately 5%.

### **Magnetometry**

Magnetometry measurements were made using a Quantum Design MPMS-7 magnetic susceptometer with a superconducting quantum interference device based sensor.

Samples for magnetometry consisted of approximately 200  $\mu\text{L}$  of cell lysate. Each sample was suspended in a plastic tube and held in place by a polycarbonate plug. After freezing the plug was removed and the sample was supported with ice made from de-ionised water. The plastic tubes and surrounding ice were much longer than the dimensions of the magnetometer sense coils, and so did not contribute to the measured signal. However, as the cell lysate was surrounded by ice, the magnetic susceptibility of the samples was measured relative to the volumetric susceptibility of ice. All cell lysate samples were less than 1 cm in length and hence could be approximated as point dipoles for the purpose of analysis of the MPMS-7 signal output.

Measurements of sample magnetic moment were corrected for changes in the density of water and of the sample constituents with temperature [5]. Molar magnetic susceptibility values are quoted in the cgs units system to facilitate comparisons with literature data. Conversion of susceptibility values to the SI unit system requires multiplication by a factor of  $4\pi$ .

Magnetic susceptibility, measured at 265 K and 10 K, was determined from the measurement of the magnetisation of the sample, relative to pure ice, in an applied field. The field was swept from 30 000 to 0 Oersteds (3 to 0 Tesla) and the magnetic susceptibility determined from the gradient of the magnetisation of the sample as a function of field. The volumetric magnetic susceptibility relative to air,  $\chi_{\text{sample}}$ , was determined by subtracting the volumetric magnetic susceptibility of ice, i.e.  $\chi_{\text{sample}} = \Delta\chi_{\text{sample}} + \chi_{\text{H}_2\text{O}}$ , where  $\Delta\chi_{\text{sample}}$  is the measured volumetric susceptibility of the

sample relative to ice and  $\chi_{\text{H}_2\text{O}}$  is the volumetric susceptibility of ice ( $\chi_{\text{H}_2\text{O}} = -0.72 \times 10^{-6}$  at 265 K and  $-0.75 \times 10^{-6}$  at 10 K) [33]. Some samples were found to contain small volumes of the magnetic fractionation buffer, as indicated by detectable concentrations of chlorine in the samples. At room temperature, the volumetric susceptibility of the buffer is similar to that of water, but the relative changes in density necessitated corrections for the diamagnetic volumetric susceptibility of the buffer at 265 K and 10 K. The globin contribution to the magnetic susceptibility of the sample,  $\chi_{\text{globin}}$ , was calculated as  $\chi_{\text{globin}} = n_{\text{globin}} M_{\text{globin}} \chi_{\rho(\text{globin})}$ , where  $\chi_{\rho(\text{globin})}$  is the mass susceptibility of globin in the haemoglobin molecule, measured by Savicki and co-workers as  $-0.58 \times 10^{-6} \text{ mL.g}^{-1}$  [6],  $M_{\text{globin}} = 64\,230 \text{ g.mol}^{-1}$  is the molecular mass of globin and  $n_{\text{globin}}$ , the concentration of globin, was calculated as  $n_{\text{globin}} = 1/4$  the molar concentration ( $\text{mol.mL}^{-1}$ ) of iron in the sample. The molar magnetic susceptibility of the iron,  $\chi_{\text{Fe}}$ , was then calculated as  $\chi_{\text{Fe}} = \frac{\chi_{\text{sample}} - \chi_{\text{globin}}}{n_{\text{Fe}}}$ , where  $n_{\text{Fe}}$  is the molar concentration ( $\text{mol.mL}^{-1}$ ) of iron in the sample. Curie law-type dependence of magnetic susceptibility with temperature was assumed and so the value of the molar magnetic susceptibility of the iron at room temperature was calculated from each measurement using

$$\chi_{\text{Fe}}(298\text{K}) = \chi_{\text{Fe}}(\text{measured}) \frac{T(\text{measured})}{298}.$$

The molar magnetic susceptibility of the iron at room temperature was taken to be the mean of the values obtained from the 10 K and the 265 K measurements. The standard deviation of the molar magnetic susceptibility at room temperature was calculated from the standard deviations of the 10 K and the 265 K measurements. It should be noted that

the molar magnetic susceptibility of the iron is expressed in units of mL per mole of iron. (To compare the  $\chi_{\text{Fe}}$  values in this work with previous work measuring the magnetic susceptibility of iron per mole of haemoglobin, multiply the  $\chi_{\text{Fe}}$  values by four.)

## **Results**

### **Optical Microscopy**

Mature parasite densities in the unfractionated cultures ranged from 2.1 to 5.4%. After magnetic fractionation, the mature parasite density ranged from 34 to 84% in the magnetic fractions, and from 0.8 to 6.3% in the non-magnetic fractions. The process of magnetic fractionation increased the density of mature parasites between 6.3 and 31 fold, with a mean enrichment of 22 fold.

A sample with an initial mature parasite density of 4.2% was passed through a fractionation column, depleting the mature parasite density to 3.9% in the non-magnetic fraction. The non-magnetic fraction was then passed through a second fractionation column, further reducing the mature parasite density in the final non-magnetic fraction to 3.4%. The magnetic fractions extracted from the first and second fractionation process had mature parasite densities of 75% and 83% respectively.

### **Spectrophotometry**

In the magnetic fractions the percentage of iron as haemozoin ranged from 25 to 52%, with a mean (and standard deviation) of  $41 \pm 11\%$ . Haemozoin concentration versus the mature parasite density is illustrated in Figure 1. As expected, the haemozoin

concentration correlates with mature parasite density. A linear model, constrained to pass through the origin, was fitted to the data and showed that the mature parasites converted on average  $63 \pm 2\%$  of cellular iron to haemozoin (Pearson's coefficient  $r = 0.99$ ,  $p < 0.01$ ).

The non-magnetic fractions appeared to contain little or no haemozoin and so the percentage of haemoglobin converted to methaemoglobin was measured using the Winterbourne formula. The methaemoglobin concentration ranged from 5.3 to 49% of cellular haemoglobin, with a mean (and standard deviation) concentration of  $34 \pm 10\%$ . The absorption spectra of the oxygenated haemoglobin and methaemoglobin standard samples indicated 3% and 100% methaemoglobin concentrations respectively.

### **Magnetometry**

The median molar magnetic susceptibility of the iron in the magnetic fractions was  $5\,600 \times 10^{-6} \text{ mL.mol}^{-1}$  (range  $2\,200 \times 10^{-6}$  to  $6\,700 \times 10^{-6} \text{ mL.mol}^{-1}$ ), almost six times greater than the median magnetic susceptibility of the iron in the non-magnetic fractions (median and range of  $1\,100 \times 10^{-6} \text{ mL.mol}^{-1}$  and  $670 \times 10^{-6}$  to  $1\,500 \times 10^{-6} \text{ mL.mol}^{-1}$  respectively). The magnetic susceptibilities of the iron in the standard samples of methaemoglobin, haematin and oxyhaemoglobin were measured as  $(14\,000 \pm 800) \times 10^{-6}$ ,  $(13\,000 \pm 650) \times 10^{-6}$  and  $(25 \pm 500) \times 10^{-6} \text{ mL.mol}^{-1}$  respectively. The susceptibilities corresponded to magnetic moments of  $5.8 \pm 0.1$ ,  $5.6 \pm 0.1$  and  $0.2 \pm 0.1$  Bohr magnetons per haem group for the methaemoglobin, haematin and oxyhaemoglobin samples respectively, in close



agreement with recent literature values [7, 8, 34]. After correcting the measurement of oxyhaemoglobin for a contribution from a 3% methaemoglobin component as measured via spectrophotometry, the magnetic susceptibility of the iron in oxyhaemoglobin was calculated to be  $-(350 \pm 500) \times 10^{-6} \text{ mL.mol}^{-1}$ .

Distributions of the magnetic susceptibilities of the iron in the magnetic and non-magnetic fractions and the standard samples are displayed in Figure 2. It is worth noting that even a relatively large error on both the concentration and the magnetic susceptibility of globin and / or other protein constituents will have little effect on the magnetic susceptibility values calculated for cellular iron. Repeated measurements of magnetic susceptibility and iron concentration of methaemoglobin samples indicate that the final error of the magnetic susceptibility of the iron is approximately 5%.

A statistically significant correlation was identified between the magnetic susceptibility of the iron in the cell lysates and the mature parasite density. A linear model was fitted to the data, shown in Figure 3 (Pearson's coefficient  $r = 0.99$ ,  $p < 0.01$ ), and the parameters from the model are shown in the following equation:

$$\chi_{Fe} (\times 10^{-6} \text{ mL.mol}^{-1}) = (830 \pm 60) + (67 \pm 21) \times \text{mature}(\%), \quad (2)$$

where *mature*(%) is the mature parasite density. The model indicates a net paramagnetic susceptibility of cellular iron in uninfected cells and / or cells infected with immature parasites. The magnetic susceptibility of  $(830 \pm 60) \times 10^{-6} \text{ mL.mol}^{-1}$  corresponds to approximately 5 - 7% of iron atoms in the high-spin ferric state. Extrapolation of the model predicts a mean magnetic susceptibility of iron of  $(7\,600 \pm 2\,200) \times 10^{-6} \text{ mL.mol}^{-1}$

for mature parasitised cells, indicating that a further  $59 \pm 16$  % of cellular iron has been converted to high spin ferric iron by the parasites.

The magnetic susceptibility of iron versus spectrophotometric measurements of the percentage of cellular iron as haemozoin are shown in Figure 4 with a linear model fitted to the data (Pearson's coefficient  $r = 0.92$ ,  $p < 0.01$ ). The model yields the following equation:

$$\chi_{Fe} (\times 10^{-6} \text{ mL.mol}^{-1}) = (1000 \pm 100) + \frac{[Hmz]}{100} \times (110 \pm 9) \quad (3)$$

where  $[Hmz]$  is the percentage of cellular iron as haemozoin. The gradient of the model corresponds to a magnetic susceptibility of haemozoin iron of  $(11\ 000 \pm 900) \times 10^{-6}$  mL.mol<sup>-1</sup> for haemozoin, which is not significantly different from the magnetic susceptibility of  $(13\ 000 \pm 600) \times 10^{-6}$  mL.mol<sup>-1</sup> evaluated for the iron in the haematin sample. The model also predicts a net paramagnetic susceptibility of  $(1\ 000 \pm 100) \times 10^{-6}$  mL.mol<sup>-1</sup> of cellular iron prior to haemozoin synthesis, implying that unparasitised cells and / or cells infected with immature parasites contained other forms of paramagnetic iron. The magnetic susceptibility of non-haemozoin paramagnetic iron,  $\chi_{Fe}(\text{non-Hmz})$ , was calculated for each sample using the following equation,

$$\chi_{Fe}(\text{non-Hmz}) (\times 10^{-6} \text{ mL.mol}^{-1}) = \frac{\chi_{Fe}(\text{sample}) - [Hmz] \bullet \chi_{Fe}(\text{Hmz})}{(100 - [Hmz])} \quad (4)$$

where  $\chi_{\text{Fe}}(\text{sample})$  is the magnetic susceptibility of the iron in the sample,  $\chi_{\text{Fe}}(\text{Hmz}) = 11\,000 \times 10^{-6} \text{ mL}\cdot\text{mol}^{-1}$  and  $[\text{Hmz}]$  is the percentage of cellular iron as haemozoin as measured via spectrophotometry and therefore  $(100-[\text{Hmz}])$  is the percentage of non-haemozoin cellular iron. No significant correlation between the magnetic susceptibility of the non-haemozoin iron and the mature parasite density was detected. A mean ( $\pm$  standard deviation) magnetic susceptibility of the iron of  $(710 \pm 200) \times 10^{-6} \text{ mL}\cdot\text{mol}^{-1}$  was calculated for non-haemozoin iron.

#### Cell volumetric magnetic susceptibilities

Erythrocyte volumetric magnetic susceptibilities,  $\chi_{\text{RBC}}$ , were evaluated using the measured molar magnetic susceptibilities of cellular iron together with a model based on that developed by Spees and co-workers [7]. The red cell volumetric magnetic susceptibility is given by:

$$\chi_{\text{RBC}} = (1 - n_{\text{Hb}} \cdot v_{\text{Hb}}) \chi_{\text{H}_2\text{O}} + n_{\text{Hb}} (\chi_{\text{mol}(\text{globin})} + 4\chi_{\text{Fe}})$$

where  $v_{\text{Hb}} = 48\,000 \text{ mL}\cdot\text{mol}^{-1}$  is the molar volume of haemoglobin [4],  $\chi_{\text{mol}(\text{globin})} = -37\,800 \times 10^{-6} \text{ mL}\cdot\text{mol}^{-1}$  is the molar magnetic susceptibility of the globin [6],  $\chi_{\text{H}_2\text{O}} = -0.72 \times 10^{-6}$  [33] and  $n_{\text{Hb}} = 5.5 \times 10^{-6} \text{ mol}\cdot\text{mL}^{-1}$  is the erythrocytic haemoglobin concentration [35]. The erythrocyte magnetic susceptibilities for cells with different chemical species of iron are given in Table 1. To facilitate comparisons with literature values, all erythrocyte volumetric magnetic susceptibilities are quoted in SI units. Conversion to the cgs units system requires division by  $4\pi$ .

Interestingly, the small paramagnetic susceptibility of cellular iron in the uninfected erythrocytes, or cells containing immature parasites, increases the erythrocyte susceptibility to coincide with the volumetric susceptibility of water. As (predominantly diamagnetic) haemoglobin is ingested and haemozoin produced, the relative susceptibility of the erythrocyte increases to approximately half the relative susceptibility of cells containing fully oxidised haemoglobin.

## ***Discussion***

The enrichment of mature stage parasites by magnetic fractionation techniques implies that, during the parasite development process, the magnetic properties of the cellular iron are altered and the magnetic susceptibility of the iron increases. The correlation between the average magnetic susceptibility of the cellular iron and the mature parasite density confirms that the ten-fold increase of the magnetic susceptibility of cellular iron is the primary reason for the magnetic enrichment of mature parasites. The magnetometry measurements of methaemoglobin and haematin confirm that both substances contain high-spin ferric iron, and therefore the conversion of diamagnetic oxyhaemoglobin to either paramagnetic form would increase the net magnetic susceptibility of the cellular iron. It is likely that multiple chemical species of iron coexist within the food vacuole of the parasite. The process of transforming globin-bound haem to haemozoin has intermediate steps and the presence of paramagnetic haemozoin precursors may explain

why the value of the magnetic susceptibility of haemozoin iron extrapolated from Equation 3 was slightly lower than the value measured for synthetic haematin.

Haemoglobin is oxidised to methaemoglobin immediately following ingestion into the food vacuole, converting the iron from the ferrous to the ferric form [15]. Therefore, the magnetic susceptibility of each haem iron increases as soon as the bond between the iron and the globin is denatured, rather than when the haem molecule is incorporated into haemozoin. Thus, the magnetic susceptibility of mature cells increases most likely because of digestion of haemoglobin rather than the detoxification of haem per se. The spectrophotometry measurements indicated that haemozoin was the predominant form of iron in the mature parasites studied here, accounting for  $63 \pm 2\%$  of cellular iron, and so haemozoin formation was the primary means of haem detoxification in the 3D7 parasite strain. If, as it has been hypothesised, alternative means of haem detoxification [36] also exist, the net magnetic susceptibility of mature parasites will depend on the relative fractions of the final products of the detoxification pathways.

Spectrophotometry and magnetometry results indicate the presence of non-haemozoin paramagnetic iron, and the spectrophotometry measurements of the non-magnetic fractions show approximately 34% of cellular iron was oxidised to methaemoglobin. Haemoglobin is oxidised during normal cell aging *in vitro* and so significant levels of methaemoglobin are expected in malaria cultures. However, this value is inconsistent with the magnetometry data, which indicates that less than 10% of cellular iron in uninfected cells or cells containing immature parasites is in the ferric state. Even

relatively low concentrations of haemozoin and / or haemozoin precursors may interfere with the absorption spectra of haemoglobin, causing an overestimation of the methaemoglobin concentration via spectrophotometry.

The magnetometry results also show no correlation between the net magnetic susceptibility of non-haemozoin iron and mature parasite density. However, it is possible that the intracellular concentrations of chemical species of paramagnetic iron are continuously altered during parasite invasion and development. The parasites may preferentially ingest methaemoglobin, as it has been hypothesised that the net positive charge of the methaemoglobin complex may aid uptake into the food vacuole [37]. There is likely a relatively small, transitory pool of non-haemozoin oxidised haem within the food vacuole as haem release in the food vacuole is carefully regulated to maintain supersaturated levels of haem [38].

The findings of this study indicate that caution should be exercised when interpreting magnetic susceptibility measurements performed on malarial-infected cells as significant levels of non-haemozoin high-spin iron(III), arising through natural cell aging or parasite metabolic activity, increase the mean magnetic susceptibility of cellular iron. Ursos and Roepe [39] discuss haem chemistry and emphasise the implications of non-haemozoin haem molecules within the food vacuole. Haem may exist, in unknown concentrations, as free haem or in dimeric forms. However, as both Egan and co-workers [26] and Moore and co-workers [9] point out, the fraction of non-haemozoin iron in the food-vacuole accounts for less than 5% of cellular iron, consistent with the results of this study

showing no correlation of the non-haemozoin high spin iron(III) concentration with mature parasite density.

Oxidation of haem iron is associated with haemoglobin digestion and therefore with parasite growth, so as the parasite matures the volumetric susceptibility of the infected erythrocyte increases. Cells containing mature parasites are paramagnetic relative to the suspension buffer used in fractionations, whereas the magnetic susceptibility of unparasitised cells is indistinguishable from the buffer. The volumetric susceptibility of  $1.89 \pm 0.63 \times 10^{-6}$  (SI units) of mature parasitised cells relative to water calculated here is consistent with the value measured experimentally by Moore et al [9] of  $1.8 \times 10^{-6}$ . The magnetic fractionation procedure used in the present experiment was sufficiently sensitive to discriminate between cells with a volumetric susceptibility difference of  $1.89 \pm 0.04 \times 10^{-6}$  (SI units). For a given set of fractionation conditions, i.e. flow rate, applied magnetic field environment and suspension buffer, a critical volumetric susceptibility exists that determines whether a cell will be retained in the 'magnetic fraction' of a fractionation. Adjusting the flow rate or changing the volumetric susceptibility of the suspension medium will change the critical volumetric susceptibility of erythrocytes, so that magnetic fractionation of cultures could be targeted to extract only parasites at specific stages of development. Alternatively, fractionation of erythrocytes under specific conditions could provide a surrogate measure of the magnetic susceptibility, and potentially the ferric iron content, of the cells.

Magnetic fractionation has been shown to yield mature parasite densities of up to 98% [40], comparable to other methods such as Percoll density gradient centrifugation [41, 42]. Magnetic fractionation can also be a means of synchronising cultures as extracted parasites can be reintroduced into culture. However, the potential to target very specific stages of development through ‘fine-tuning’ of the fractionation conditions may allow for a greater degree of synchrony than can be achieved with conventional methods such as sorbitol lysis [43]. Interestingly, mature parasites were detected in all non-magnetic fractions. Repeated fractionation of non-magnetic fractions did not change the enrichment of mature parasites, indicating that the magnetic susceptibility of mature parasites retained in the column is not necessarily greater than the magnetic susceptibility of the mature parasites that pass through into the non-magnetic fraction. The fractionation process is highly specific but only moderately sensitive and so repeating magnetic fractionations on cultures will increase the mature parasite yield.



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### **Figure Captions**

FIGURE 1 Percentage of cellular iron converted to haemozoin versus mature parasite density.

FIGURE 2 Scatter plot of the molar magnetic susceptibility of iron in standard samples of oxyhaemoglobin (Oxy -  $\nu$ ), haematin (h -  $\blacktriangle$ ), methaemoglobin (Met -  $\nu$ ), and for magnetic (Mag -  $\lambda$ ) and non-magnetic (Non-mag -  $\sigma$ ) fractions of malaria infected red cell cultures.

FIGURE 3 Molar magnetic susceptibility of iron versus mature parasite density.

FIGURE 4 Molar magnetic susceptibility of iron versus percentage of cellular iron converted to haemozoin.

	$\chi_{\text{Fe}}$ ( $\times 10^{-6}$ $\text{mL}\cdot\text{mol}^{-1}$ )	Volumetric erythrocyte susceptibility $\chi_{\text{RBC}}$ ( $\times 10^{-6}$ ) (SI units)	Volumetric erythrocyte susceptibility relative to water $\Delta\chi$ ( $\times 10^{-6}$ ) (SI units)
Oxyhaemoglobin	$-350 \pm 500$	$-9.42 \pm 0.13$	$-0.38 \pm 0.13$
Methaemoglobin	$14\,000 \pm 800$	$-5.40 \pm 0.25$	$-0.29 \pm 0.25$
Mature parasitised cell	$7\,600 \pm 2200$	$-7.16 \pm 0.63$	$-1.89 \pm 0.63$
Uninfected cell	$830 \pm 60$	$-9.02 \pm 0.05$	$-0.01 \pm 0.05$

TABLE 1 Molar magnetic susceptibilities ( $\pm$  standard error) of cellular iron and of volumetric magnetic susceptibilities ( $\pm$  standard error) of unparasitised cells (or cells with little to no haemozoin), cells infected with mature parasites and cells containing fully oxygenated and fully oxidised haemoglobin. Also shown are the cell volumetric susceptibilities relative to water. The volumetric susceptibilities of the cells have been evaluated using a model adapted from model developed by Spees and co-workers [7] together with the molar magnetic susceptibilities of cellular iron measured in this study. Values for oxyhaemoglobin and methaemoglobin are calculated from the standard samples.



Figure

Percentage of cellular iron as haemozoin (%)

60

50

40

30

20

10

0

0

20

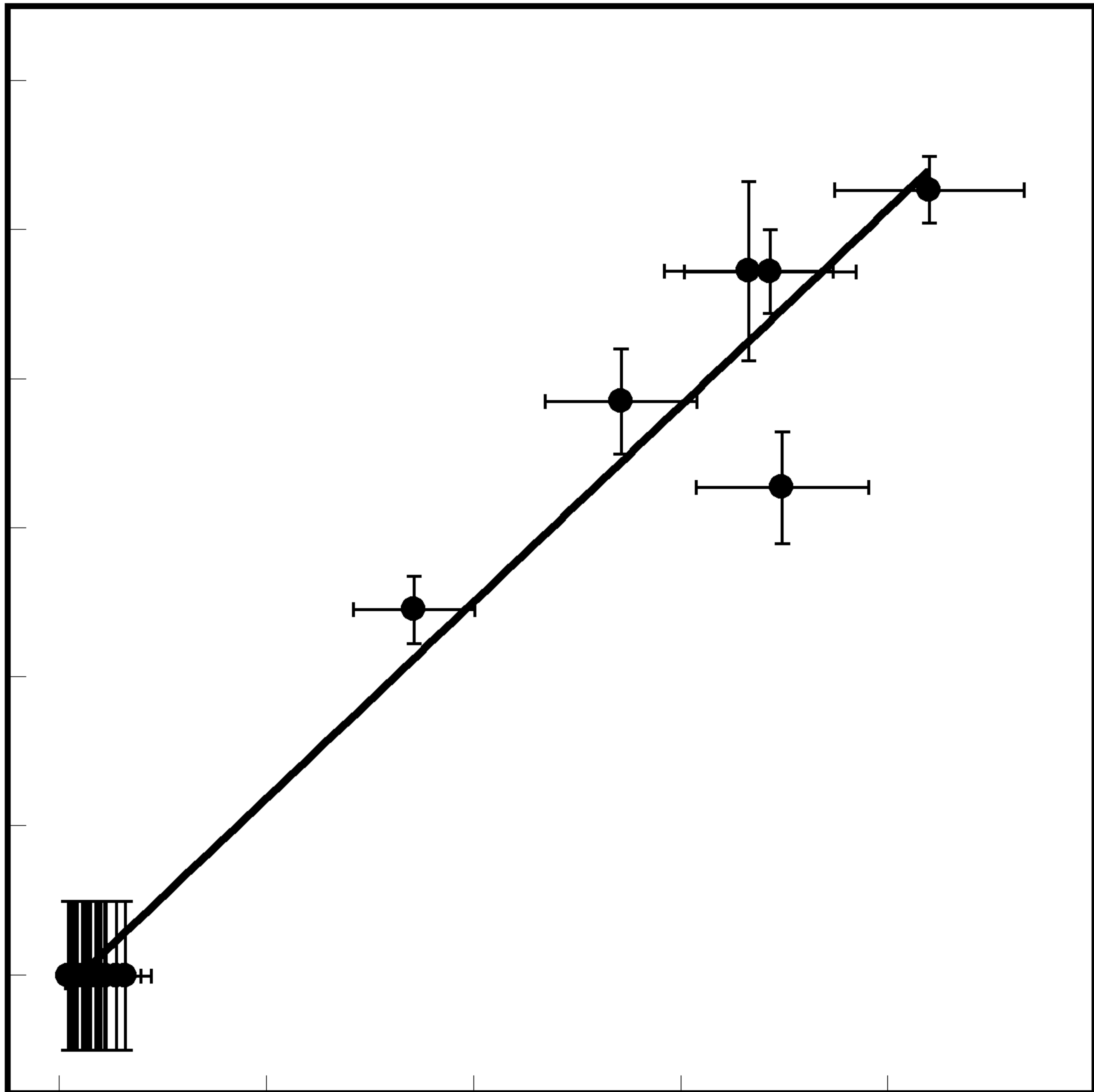
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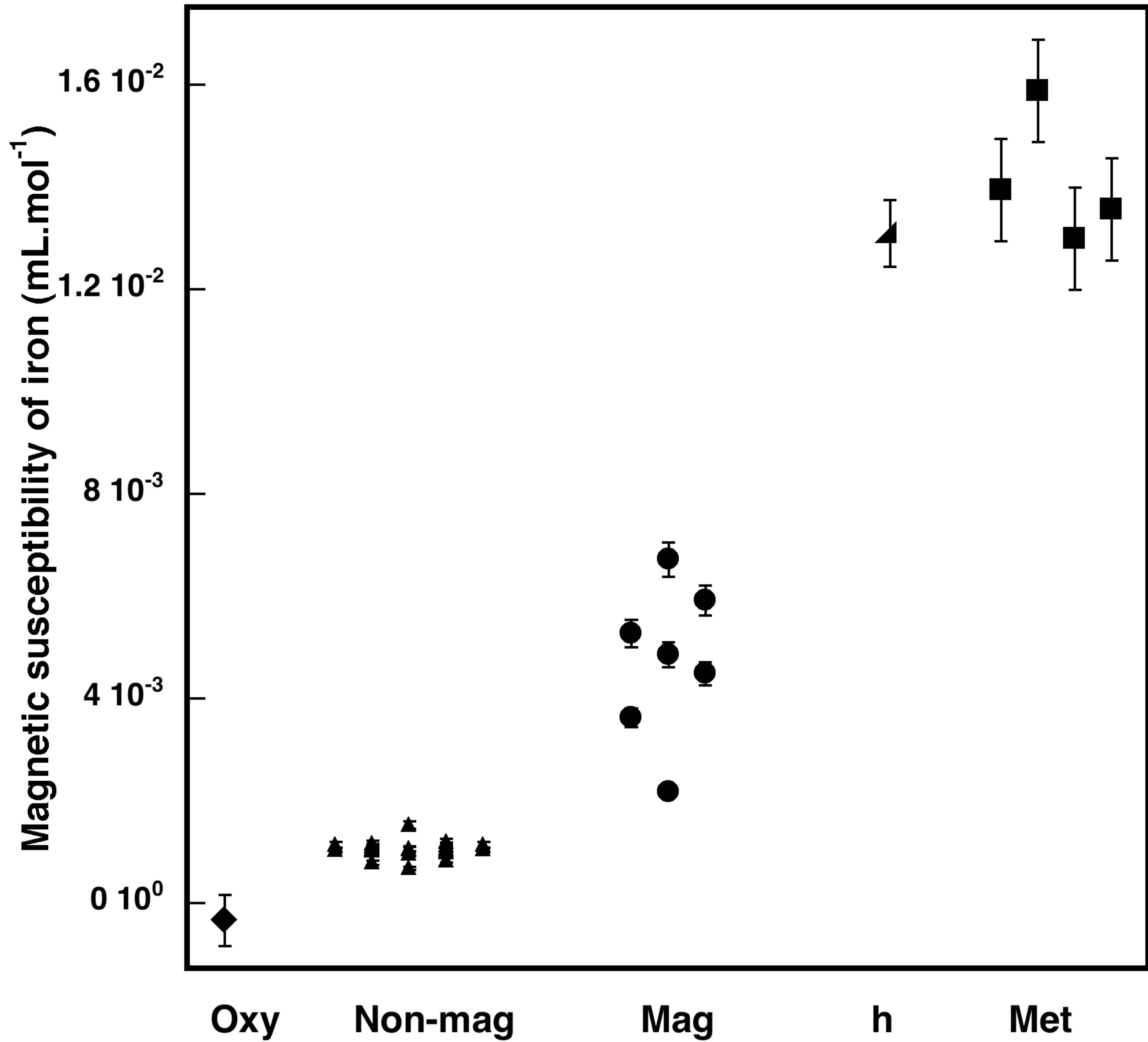
60

80

100

Mature parasite density (%)





Magnetic susceptibility of iron ( $\text{mL}\cdot\text{mol}^{-1}$ )

$7.0 \cdot 10^{-3}$   
 $6.0 \cdot 10^{-3}$   
 $5.0 \cdot 10^{-3}$   
 $4.0 \cdot 10^{-3}$   
 $3.0 \cdot 10^{-3}$   
 $2.0 \cdot 10^{-3}$   
 $1.0 \cdot 10^{-3}$   
 $0.0 \cdot 10^0$

0

20

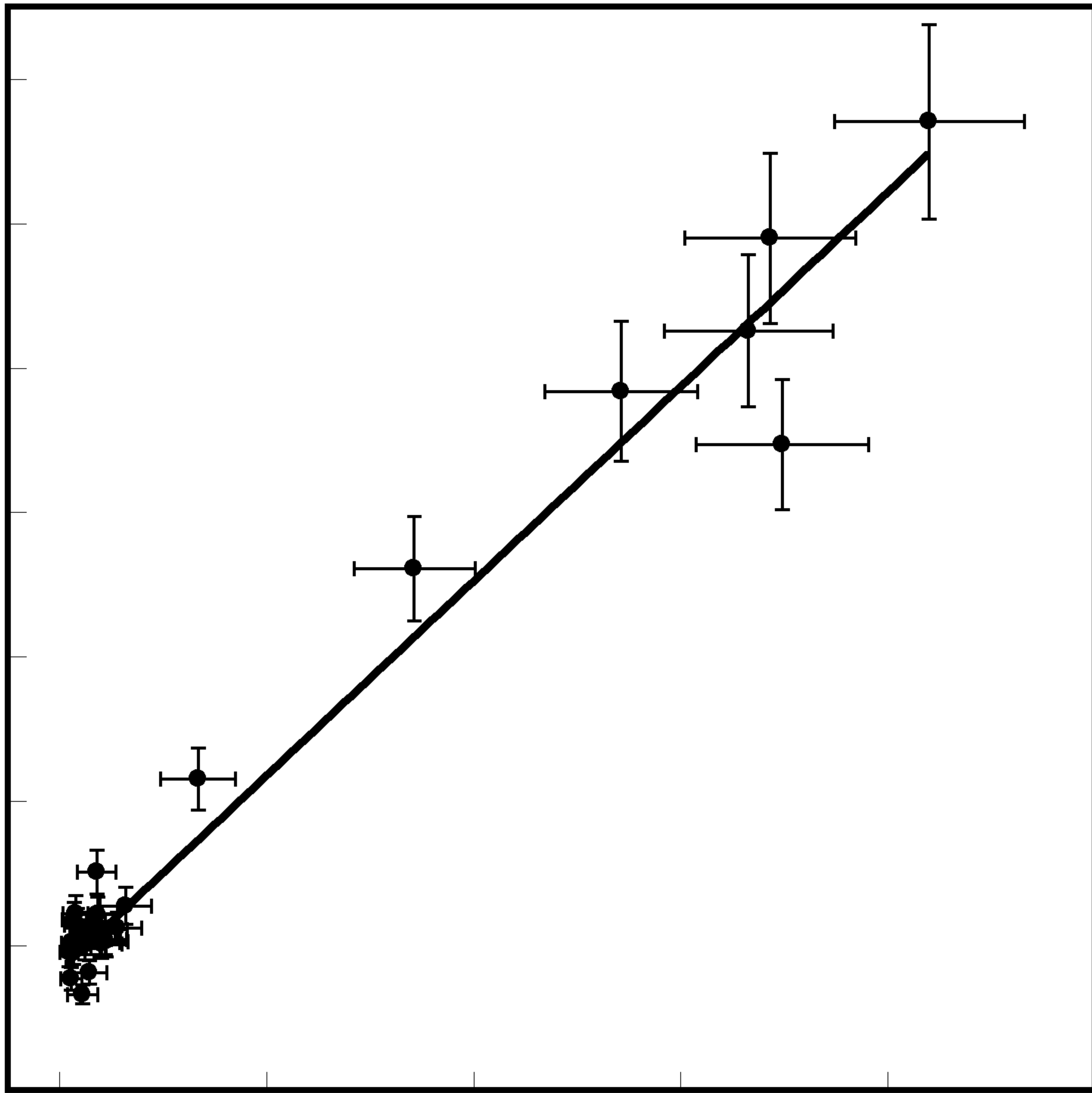
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60

80

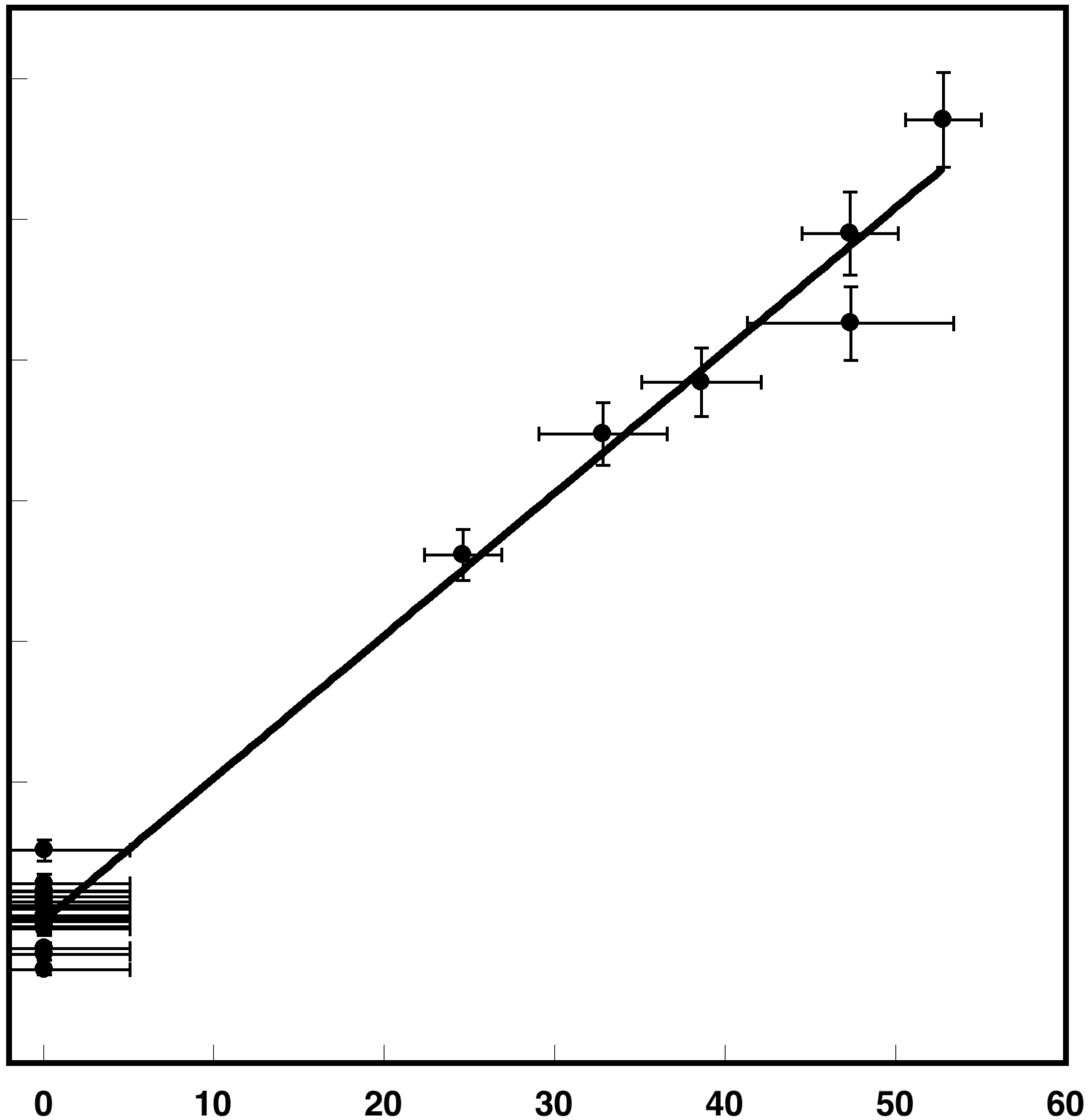
100

Mature parasite density (%)



Magnetic susceptibility of iron ( $\text{mL}\cdot\text{mol}^{-1}$ )

$7.0 \cdot 10^{-3}$   
 $6.0 \cdot 10^{-3}$   
 $5.0 \cdot 10^{-3}$   
 $4.0 \cdot 10^{-3}$   
 $3.0 \cdot 10^{-3}$   
 $2.0 \cdot 10^{-3}$   
 $1.0 \cdot 10^{-3}$   
 $0.0 \cdot 10^0$



Percentage of cellular iron as haemozoin