Iron, Climate change and the ‘brain eating amoeba 'Naegleria fowleri'

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Running title: "Iron, Climate change and *Naegleria fowleri* "

Abstract

Naegleria fowleri is the causative agent of primary amoebic meningoencephalitis (PAM), a rare but devastating infection with a 96.4% mortality rate. Iron is a limiting factor for *N. fowleri* growth, and its presence during growth activates its human pathogenic potential. Analysis of the *Naegleria* genome reveals the presence of genes encoding proteins present in anaerobic organisms, many of which are iron-containing enzymes. Here we propose a pathway involving ferroproteins that facilitates the anaerobic growth of *Naegleria*. Climate change is modelled (by others) to increase soil erosion and to lead to a more reducing regime in some areas, both of which are expected to result in increased iron availability in soils and waterways. We posit that an increased iron supply will permit *N. fowleri* to survive in low-oxygen environments through the increased expression of several iron-binding proteins involved with ATP production and pathogenic potential. Together with general warming, increased iron availability may increase the incidence of PAM, causing the geographic range of *N. fowleri* to spread poleward. Competition with other free-living amoebae, including other *Naegleria* species, is likely to limit the distribution and abundance of *N. fowleri* and more details of these interactions are required to predict and intervene.

Key words: Primary Amoebic Meningoencephalitis, Anaerobic respiration; denitrification; nitrite reductase; Nitric oxide reductase; Nitrite/formate transporter

Contents

Introduction

Naegleria fowleri is an amoeboflagellate in the class Heterolobosea. It is distributed world-wide (De Jonckheere, 2011), but it is particularly abundant in water bodies, either natural or man-influenced, that remain above 30ºC. It is the only member of the genus known to infect humans, causing Primary Amoebic Meningoencephalitis (PAM) (Mungroo et al., 2019; Siddiqui et al., 2016). This rare infection has a mortality rate of 96.4% and the incidence of reported cases is increasing (Maciver et al., 2020). PAM is usually initiated by *N. fowleri* amoebae entering the nares, invading the nasal epithelia and gaining access to the brain by tracking along the olfactory nerve, typically to the frontal and parietal lobes (Ong et al., 2017). The presence of *N. fowleri* induces a severe inflammatory response possibly mediated by interleukin 1β and interleukin 6 (Kim et al 2016). Tissue damage results from the actions of proteases, phospholipases and pore-forming peptides released from the amoebae (Marciano-Cabral and Cabral, 2007; Martínez-Castillo et al., 2015) and death usually results from oedema and increased intracranial pressure (Lopez et al, 2012).

Iron stimulates growth and activates *N. fowleri* pathogenicity

Iron (Fe) ions serve both as electron donors and acceptors and is essential for almost all organisms and the availability of Fe limits the growth of invading bacteria and parasites. To prevent bacterial growth, vertebrates lock up Fe in specific Fe-binding proteins such as transferrins and lactoferrin, which prevents the bound Fe from creating toxic free radicals. In

74 the virtual absence of free Fe in the host, parasites have evolved strategies to obtain Fe. Just
75 as parasites must secure enough iron to sustain them, too much iron is toxic to parasites as it
76 participates in Fenton-type reactions (Winterbourn, 1995) in the presence of oxygen, producing
77 reactive oxygen species that are harmful to the parasite.

78 The demands on amoebae of a bacteriovorous lifestyle and those of a human parasite are likely
79 to be very different and so it is expected that a change in lifestyle will require the activation of
80 different genes (Tiewcharoen et al., 2012). It is known that the passage of *N. fowleri* through
81 mice activates its pathogenic potential (Lee et al., 1983), and it is suspected that Fe also plays
82 a role here. Fe is a limiting factor in the growth of *N. fowleri* in culture (Band and Balamuth,
83 1974; Newsome and Wilhelm, 1983), which may explain why the amoebae are concentrated
84 within the Fe-rich layers in lakes (Kyle and Noblet, 1985). Not only has the availability of Fe
85 in the growth media been found to correlate strongly with the growth and motility of *N. fowleri,*
86 but it is also linked to its pathogenicity (Bradley et al., 1996; Newsome and Wilhelm, 1981).
87 Amoebae in culture with a higher availability of Fe were found to be more resistant to
88 complement lysis and were more pathogenic in mouse models (Bradley et al., 1996). Iron
89 availability (Figure 1) is therefore a crucially important factor in the risk of PAM for example
90 it is suggested that extracellular secretion of *Naegleria* cysteine proteases may facilitate the
91 release of Fe from host Fe-binding proteins such as transferrin and haemoglobin (Martínez-
92 Castillo et al., 2015). Iron may also be important in *Naegleria*'s tolerance of stresses, such as
93 reduced availability of oxygen.
94
95 **Iron-containing proteins may facilitate anaerobic respiration in *Naegleria***
96 Whereas some Heterolobosean genera such as *Psalteriomonas* (Broers et al., 1990) and
97 *Creneis* (Pánek et al., 2014) are anaerobes, *Naegleria* are generally regarded as aerobes (Weik
98 and John, 1977). The axenic *N. gruberi* NEG could not be grown anaerobically, and neither
99 could a fresh *N. gruberi* isolate (Bexkens et al., 2018). However, there is evidence that some
100 species of *Naegleria* may well differ in their ability to grow anaerobically, as *N. fowleri* has
101 been found more than *N. gruberi* in anoxic regions of lakes, and has a lower oxygen
102 requirement in culture (Weik and John, 1977). That *N. fowleri* requires temperatures above
103 30^{o}C to thrive is also consistent with its tolerance of low oxygen levels, as oxygen solubility is
104 reduced at higher temperatures. Other uncharacterised *Naegleria* strains are reported to persist
105 (Priya et al., 2008) and even to grow (Priya et al., 2019) under anaerobic conditions in the
106 presence of specific bacteria. It is possible that a period of adjustment is necessary for

Naegleria to adapt to an anaerobic mode from an aerobic mode, and perhaps the nutritional requirements are also different. We suspect that iron may have a role in the adaptation to an anaerobic state. Published *Naegleria* genomes (Fritz-Laylin et al., 2010) (Zysset-Burri et al., 2014) (Liechti et al., 2018) indicate that in addition to the normal repertoire of mitochondrial genes, there is a series of genes usually expressed by anaerobic protists, including a pyrophosphate-dependent phosphofructokinase (Wessberg et al., 1995), acetate:succinate CoA transferase (Van Hellemond et al., 1998), bacterial-type hemerythrin (French et al., 2008), acetyl-CoA synthetase and [FeFe] hydrogenase genes ((Ginger et al., 2010; Koonin, 2010) (Nývltová, 2015; Opperdoes et al., 2011). However, [FeFe] hydrogenase is reported to be cytoplasmic in *N. gruberi*. Three so- called maturases, HydE, HydF and HydG, are required for the assembly and insertion of the catalytic iron-sulphur clusters into [FeFe]-hydrogenase, and homologs of all these maturases are found in the genome of *Naegleria* (Fritz-Laylin et al., 2010; Zysset-Burri et al., 2014). FeFe hydrogenase is reported to produce H_2 gas (Tsaousis et al., 2014) in *Naegleria* and this protein is down-regulated under low-Fe conditions (Mach et al., 2018). The [FeFe] hydrogenase and its maturases are predicted to contain mitochondrial import signals (Ginger et al., 2010), suggesting that these proteins are imported into mitochondria under specific conditions. Hemerythrin was found to be the most down-regulated protein in *Naegleria* under low-iron conditions (Mach et al., 2018) and this non-heme iron containing protein is thought to act as an oxygen store in some organisms, its expression increasing the fitness of *Pseudomonas aeruginosa* during microoxic, but not anoxic, respiration (Clay et al., 2019). Although the details are not yet clear, the *Naegleria* hemerythrin homolog Nfa1 is an immunogen associated with pathogenicity (Shin et al., 2001). Antibodies to this protein inhibit the cytopathogenicity of *Naegleria* toward cultured Chinese hamster ovary cells (Jeong et al., 2004). Expression of Nfa1 protein was strongly increased in the presence of these target cells (Kang et al., 2005) and cytopathogenicity is reduced by silencing of the Nfa1 gene (Jung et al., 2009). Furthermore, when the Nfa1 gene from *N. fowleri* is expressed in *N. gruberi*, its cytotoxicity toward Chinese hamster ovary cells increases (Jeong et al., 2005). Together with actin (Sohn et al., 2010), the Nfa1 protein is localised to the amoebastomes, or food cups, (Kang et al., 2005) known to be involved in the trogocytosis (piecemeal ingestion) of target cells (John et al., 1984). Recent work has shown that in low-Fe conditions, *N. fowleri* prioritizes Fe for essential Fe-binding proteins involved in mitochondrial respiration but does not induce the reductive uptake of iron as other parasites do (Arbon et al., 2020); and these authors therefore suggest that this represents a weakness in the pathogen that could be exploited to treat PAM.

Lipids have been shown to be the preferred energy source for *Naegleria gruberi* (Bexkens et al., 2018). The *N. fowleri* genome indicates that it too prefers lipids and fatty acid oxidation seems to be essential for its growth (Sarink et al., 2020). This agrees with the observation that both the cyst and trophozoite contain vesicles that stain with lipophilic dyes (Carter, 1970), but do not stain with glycol dyes (Pittam, 1963). Recent data (Herman et al, 2020) indicate that genes involved in the metabolism of long chain fatty acids, such as those found in the brain, are upregulated in highly pathogenic *N. fowleri*. Although *Naegleria* have fully-functioning aerobic mitochondria (Fritz-Laylin et al., 2010), Figure 2 shows that there are possible pathways encoded by the *Naegleria* genome to produce ATP under anaerobic conditions. Glycolysis produces pyruvate as usual, and pyruvate is then oxidized by pyruvate dehydrogenase to acetyl-CoA and NADH. Acetyl-CoA can then generate ATP either directly, through the action of either acetyl-CoA synthetase or by acetate:succinate CoA transferase and succinyl-CoA synthetase. Acetyl-CoA synthetase is upregulated in *N. fowleri* challenged with human cells (Tiewcharoen et al., 2012), and acetate is also produced. Acetyl-CoA may be produced though the β-oxidation of fatty acids and while this does not require oxygen directly, for each C2-unit removed, one NADH and one UQH_2 is produced and these have to be re-oxidized, normally by the mitochondrial electron-transport chain, which obligatorily reduces oxygen. Overall, therefore, one O_2 is reduced per acetyl-CoA generated. During anaerobic respiration, NADH produced by β-oxidation may be removed by the *Naegleria* [FeFe] hydrogenase, an enzyme associated with anaerobic organisms, but this still leaves the re-oxidation of reduced ubiquinone to be explained.

A limited dissimilatory denitrification pathway in *Naegleria*

Many prokaryotes couple ATP production to the facultative reduction of electron acceptors other than O_2, such as NO_3^- or SO_4^{2-} (Richardson, 2000), and the ciliated protist *Loxodes* (Finlay et al., 1983) and fungi such as *Fusarium oxysporum* are known to use denitrification as a terminal reduction process when respiring anaerobically, instead of reducing O_2 (Zhou et al., 2010). We suggest that *Naegleria* has homologues of genes involved in the denitrification of nitrite, namely nitrite reductase and a nitrite/formate transporter (Table 1; Figure 2; Table S1 Appendix A, Supplementary data) and speculate that these too may operate as terminal electron-acceptors in *Naegleria* under anaerobic conditions. Although the *N. gruberi* gene XP_002681617.1 is annotated as a nitrate reductase in Genbank and elsewhere (Opperdoes et al, 2011), we argue that it is not. This sequence makes a convincing alignment to the structures

of cytochrome b5 reductase domains, for example in *Ulva prolifera* nitrate reductase, but it crucially lacks the Mo-pterin domain found in true nitrate reductases. We considered that this domain might exist as a separate protein that could interact non-covalently, but the Mo-pterin domains in the *N. gruberi* genome, while similar to those in nitrate reductases, had clear sequence motifs that identify them as members of the closely related but distinct sulphite oxidase family, and not as *bona fide* nitrate reductases. In agreement with this we find evidence for a nitrite transporter but not a nitrate transporter in *Naegleria* genomes. Using the Phyre 2 server (Kelly et al, 2015), the putative nitrite/formate transporter (XP_002683227.1) can be threaded convincingly onto the *V. cholerae* sequence of structure 3klz structure (Figure 5c) and the active ligand positions are preserved in the putative *Naegleria* transporter.

The *Naegleria* nitrite reductase gene (XP_002674759) is homologous to *nirK* genes, which encodes a copper-containing protein (Kim et al., 2009) (Figure 3c). This putative nitrite reductase (XP_002674759) has 50 % sequence identity to the *N. gonorrhoeae* enzyme (Table 1; Figure 3b) for which there is a crystal structure (pdb code 1kbw) (Boulanger and Murphy 2002). We were able to fit the putative nitrite reductase sequence to the *N. gonorrhoeae* enzyme structure using the Phyre2 server. The catalytic site of the enzyme showed the presence of the copper ions and their ligands with conservation of the amino-acids that bind copper ions and ligands (3c). In *N. gonorrhoeae* this enzyme is induced under anaerobic conditions and is an outer-membrane lipoprotein (Boulanger and Murphy 2002). The *N. gruberi* enzyme, however, does not have the characteristic palmitoylation motif or the lipoprotein characteristics of the *N. gonorrhoeae* enzyme, and moreover it has as high sequence identities to typical bacterial nitrate reductases, which are cytoplasmic. Thus, while the structure of the *N. gruberi* enzyme may be similar to that of *N. gonorrhoeae* nitrite reductase, it is likely to be cytoplasmic, like other bacterial enzymes that use nitrite as an electron acceptor.

N. fowleri is reported to be able to produce NO (Rojas-Hernández et al., 2007) but it is assumed that it does so through the oxidation of arginine by nitric oxide synthase since antibodies to human NO synthase cross-react with *N. fowleri* proteins (Rojas-Hernández et al., 2007) and the *Naegleria* genome contains genes with a nitric oxide synthase domain (*e.g.* XP_002679491).

Naegleria also have a homolog of the prokaryotic iron-sulphur cluster repair protein, which is similar to but distinct from the hemerythrin family. The *Escherichia coli* homolog YtfE is reported to catalyse the reduction of nitric oxide to nitrous oxide (Lo et al., 2016), so this

enzyme is another possible participant in the final denitrification step in *Naegleria*. We have constructed a 3D-model (4c) of the *N. gruberi* putative nitric oxide reductase (XP_002669995) based on the X-ray crystallographic structure of the *E coli* YtfE enzyme (protein data bank code 5FNP) using the Phyre2 server.

Thus, we have found evidence that *Naegleria* genomes potentially encode proteins that could transport nitrite, reduce it to nitric oxide, and further reduce this to nitrous oxide. Both reactions have reduction potentials that make them suitable replacements for O_2 as the electron acceptor under anaerobic conditions.

The scheme shown in Fig.2 shows ATP production from two substrate-level phosphorylation reactions, catalysed by acetyl-CoA synthetase and succinyl-CoA synthetase. NAD^+, reduced during oxidation of glucose or fatty acids, is reoxidized by hydrogenase, whereas ubiquinone, reduced in the β-oxidation of fatty acids, is reoxidized by reduction of nitrite to nitric oxide and of nitric oxide to nitrous oxide. Although the reductases themselves appear to be cytoplasmic, the latter steps might also be coupled to mitochondrial ATP synthase, if electron-transfer from ubiquinol to the reductases takes place through mitochondrial complex III.

Barriers to the anaerobic growth of *Naegleria*

We have argued that Fe is needed for *Naegleria* to produce many of the gene products necessary for anaerobic growth as they are Fe-dependent enzymes (table S1 Appendix A, Supplementary data). The postulated requirement for nitrite for anaerobic energy production (Figure 2) may explain why anaerobic growth is not readily seen in *Naegleria* when switched to anaerobic conditions (Bexkens et al., 2018). In anaerobic soils and water bodies nitrite may be available through associated nitrite-producing bacteria, explaining the requirement for the presence of specific bacteria for the anaerobic growth of *Naegleria* reported by others (Priya et al., 2019). Large accumulations of nitrite in river systems, for example, have been explained by the action of bacteria in anaerobic sediments (Kelso et al., 1997). It is unlikely that genes involved in the aerobic and anaerobic pathways are actively transcribed simultaneously so the switch between modes will require time to replace one set of proteins with another. *Naegleria* are well-known for their ability to transform from amoebae to flagellates within 90 minutes through the activation of hundreds of genes (Fulton 1983). We speculate that the switching between aerobic and anaerobic metabolism (or vice versa) will require a similar time frame.

The proteins encoded by some of the genes involved in Fe transport and denitrification (Figure 1 and 2) require Fe. In summary, there would seem to be three separate barriers to anaerobic growth, Fe availability, nitrite availability, and time.

Climate change and Iron availability

The importance of ground-water as a niche for *N. fowleri* has been discussed (Maciver et al., 2020) and there is evidence that increases in surface temperature can cause an increase in ground-water temperature also, although the relationship is not yet understood (Taylor and Stefan, 2009). The phenomenon of surface-water warming is likely to increase *N. fowleri* numbers, (Baig, 2019; Diaz, 2012; Mahmood, 2015), but the need for people to cool down by immersing themselves is another factor that is likely to increase, bringing more people into contact with the pathogen. Although most victims of PAM probably contact *N. fowleri* through contaminated water entering their nostrils, it seems that 'dry infections' also occur when *N. fowleri* cysts enter the nostrils in dust (Lawande et al., 1979; Maciver et al., 2020). These dry infections are thought to occur primarily in arid regions such as West Africa and India and such conditions may become more prevalent in other areas as a result of climate change. Where shallow, warm surface water evaporates slowly, any *N. fowleri* trophozoites therein could form cysts that eventually become airborne in dust clouds.

Rainfall run-off has been found to increase the abundance of *N. fowleri* in ponds, presumably through soil washing into them (John and Howard, 1995; Kyle and Noblet, 1985). In addition to providing bacteria with nutrients that would then increase the numbers of *N. fowleri*, iron from the soil may directly support increased growth. As we have seen, several studies have highlighted the rate-limiting effect of iron availability on the growth of *N. fowleri* and the increased erosion may synergise with higher temperatures to favour *N. fowleri* growth.

Since the 1980s there has been a large increase in Fe levels in many European rivers and surface-water (Ekström et al., 2016; Neal et al., 2008). Fe levels have risen in the UK, Germany and Finland and by 470% in one Swedish river (Kritzberg and Ekström, 2012). It is suggested that reducing conditions in river catchment soils are an important factor in the increasing Fe concentrations in these surface waters (Gotoh and Patrick, 1974; Kritzberg and Ekström, 2012). Many catchment areas have seen increased rainfall, and climate models predict that this will increase further (Ekström et al., 2016). Therefore, increased moisture would produce reducing conditions under which iron is more soluble, so accounting for the large increase in iron in

surface-water. Under the anoxic conditions produced by waterlogging, microbial activity reduces Fe^{3+} to the more soluble Fe^{2+} (Figure 6). This increased solubility of Fe is likely to synergise with the increase in erosion caused by increased rainfall (Nearing et al., 2004) so that in addition to the strong warming seen in Northern Europe, conditions favour the growth of *N. fowleri* and these trends in temperature and rainfall are predicted to increase further. The same changes are likely to occur in many other regions of the world. Temperature, too, is an important determinant of redox balance, as bacterial reduction of Fe^{3+} increases strongly with temperature (Schilling et al., 2019).

Monochloramine disinfectant loss through ferrous iron in water supplies

Chlorination has been shown to be vital in removing *N. fowleri* from municipal water supplies and failures in chlorination have been associated with PAM infections in Pakistan (Karim et al., 2020) in the USA (Cope et al., 2015 and in Australia (Dorsch et al., 1983). Ferrous iron has been found to remove chloramine (NH_2Cl) by an autocatalytic mechanism (scheme **1**, below) under conditions that are likely to prevail and at a rate fast enough to be expected to lower disinfection efficiency in water supplies (Vikesland and Valentine, 2000).

$$2Fe^{2+} + NH_2Cl + 6H_2O \rightarrow 2Fe(OH)_3 + NH_3 + Cl^- + 5H^+ \qquad (\mathbf{1})$$

It is therefore likely that in addition to stimulating the growth and pathogenicity of *N. fowleri* the increased iron availability may further favour this pathogen by lowering disinfection efficiency.

CO_2-induced acidification of surface-water

Although there has been much attention on oceans becoming acidified as a result of anthropomorphic CO_2 accumulation, the same also holds for freshwater. For instance, there is evidence of significant acidification of reservoirs in Germany by between 0.2 and 0.3 pH units in only 35 years, which is correlated with the increase in atmospheric CO_2 (Weiss et al., 2018). This change in pH is not expected to impact *N. fowleri* growth directly since it survives between pH 4.6 and 9.5 (Carter, 1970; Lam et al., 2019). However above pH 4 the solubility of iron under oxidizing conditions is very low (Kraemer, 2004), so acidification may synergise further with reducing conditions and temperature in increasing Fe solubility (Figure 6).

The Flagellate-Empty Habitat hypothesis and the distribution of *Naegleria fowleri*

Griffin's flagellate-empty habitat hypothesis (FEHH) postulates that *N. fowleri* numbers are limited by competition with related amoeboflagellates and other amoebae, and that as a consequence either natural or man-made situations that selectively remove these competitors allow *N. fowleri* to flourish (Griffin, 1983). The *N. fowleri* motile flagellate form is seen as an important advantage in this process, since not all competing *Naegleria* produce flagellates, at least in culture conditions (De Jonckheere et al, 2000). In accordance with the FEHH, *N. fowleri* is often detected only in low numbers relative to other amoebae, and only under certain conditions (among which is an elevated temperature) does *N. fowleri* dominate. Evidence for competition amongst *Naegleria* species has been suggested to explain low numbers of *N. fowleri* compared to other thermophilic *Naegleria* (Kilvington et al., 1991), and it is reported from biofilm experiments (Miller et al., 2018) in which *N. fowleri* competed with other free-living amoebae and with *N. lovaniensis,* in support of Griffin's hypothesis. For this approach to be fully tested so that it can be used to predict *N. fowleri* blooms, much more information, such as prey preference (Puzon et al., 2017), Fe availability, salinity, oxygen, and temperature tolerances for all possible competitors, must be obtained, which would be a huge task. However, in restricted or simplified situations such as cooling towers this approach might be more feasible.

There are at least two cases in which *N. fowleri* has appeared against expectations and which may be accounted for by the FEHH. On the 20th August 2010, a 7-year-old girl was admitted to hospital with PAM symptoms to which she later succumbed (Kemble et al., 2012). She had been swimming for extensive periods in a lake in Minnesota, for which the surface temperature was measured to be 22.1^{o}C some days after the girl's exposure. This is a low temperature for the survival or maintenance of large numbers of *N. fowleri.* On the18th of May 1980, Mount St Helens, (Washington State, USA) erupted, effectively sterilizing Spirit Lake. Against all expectations Spirit Lake was found to contain *N. fowleri* (Detterline and Wilhelm, 1991) consistent with FEHH (Frady, 2013). In both situations it is possible that disturbed local conditions briefly allowed *N. fowleri* to flourish through the absence of competitors and that in the Minnesota case, factors other than elevated temperature (such as Fe availability) were responsible.

Conclusions

Rapid and extensive changes in Fe availability have been observed in several regions and these have been connected to climate change. A fuller understanding of Fe availability and the role

of Fe in both the pathogenicity and the distribution of *Naegleria fowleri* is clearly required. That Fe may be even more important than temperature in the distribution and pathology of this famously thermophilic amoeba is a small irony.

Acknowledgements

This work was funded by Amoebics Ltd, Edinburgh and by grant PI18/01380 from Instituto de Salud Carlos III, Spain and RICET [RD16/0027/0001 project, from Programa Redes Temáticas de Investigación Cooperativa, FIS (Ministerio Español de Salud, Madrid, Spain) and FEDER.

Table 1. Translated *N. gruberi* genome sequences that have similarities with nitrogen transport/ reduction enzymes.

Putative Activity	**Accession Code *N. gruberi***	**Pdb code similar protein (species)**	**Identity (%)**	**Coverage (%)**
Nitrite/formate transporter	XP_002683227.1	3klz (*V. cholerae*)	32	76
Nitrite Reductase $NO_2^- \rightarrow$ NO	XP_002674759	1kbw *(N. gonorrhoeae)*	50	77
Nitric Oxide Reductase NO$\rightarrow$ N_2O	XP_002669995	5fnp (*E. coli*)	31	95

Figure Legends

Figure 1. Putative iron acquisition and utilization by *Naegleria* trophozoites. Ferric reductase activity on the surface of *Naegleria* reduces Fe^{3+} to Fe^{2+} (Arbon et al., 2020), which may then enter through a ZIP14 transporter, then taken into mitochondria via mitoferrin, bound by ferritin or exported by ferroportin. Non-heme Fe-containing *Naegleria* hemerythrin localised in the pseudopod and amoebastome may act as an oxygen store, effectively buying the cell time to upregulate anaerobic genes when it encounters low oxygen conditions.

Figure 2. Possible metabolic pathway of energy metabolism in *Naegleria* under low-oxygen situations. The β-oxidation of fatty acids delivers acetyl-CoA which, together with acetyl-CoA produced from pyruvate by pyruvate dehydrogenase (PDH), drives ATP formation, either directly through acetyl-CoA synthetase (ACS) as previously proposed (Fritz-Laylin et al., 2010; Nývltová 2015), or indirectly through acetate:succinate CoA transferase (ASCT) and succinyl-CoA synthetase (SCS) as previously suggested (Fritz-Laylin et al., 2010). UQH_2 produced by β-oxidation is re-oxidised to UQ, and the denitrification pathway is proposed to act as the terminal electron acceptor in this process. This pathway involves nitrite reductase (Nir), and nitric oxide reductase / FeS cluster repair di-iron protein (NOr) (Lo et al., 2016). NADH is re-oxidized by a [FeFe]-hydrogenase (2FeHyd) to produce H_2 gas (Tsaousis et al., 2014). Genes encoding proteins for the enzymes (oblong shapes) are grey where homologs have been identified (Table S1) in *Naegleria* genomes. Enzymes that incorporate Fe ions and so are potential candidates for increased expression under high-Fe conditions are labelled with green sphere.

Figure 3a.

An unrooted phylogenetic analysis of copper-containing nitrite reductase gene products. Maximum-likelihood analysis of the protein sequences showing branch support. The tree was created with PhyML (Guindon et al, 2003) using the GTR model with 200 bootstrap pseudo-replicates implemented using Seaview 4 (Gouy et al, 2010). The possession of this gene is limited to a few phyla that do not include mammals. The *Naegleria* Nir homologs (blue) group both closely with the fungal members (orange), but also close to plants (green). The red algae (purple) and amoebozoa group (red) more distantly, and prokaryotes (black) more distantly still.

Figure 3b. Sequence alignment of *N. gruber*i putative nitrite reductase with the *N. gonorrhoeae* sequence and a model based on the *N. gonorrhoeae* structure (pdb code 1kbw). Structural features were identified using the ENDscript server (Robert and Gouet, 2014).

Figure 3c. Left figure. The ligands of the two Cu^{2+} ions (CuII, CuI, cyan) are shown, along with the position for nitrite in site CuII. Histidine 349 is shown with carbon atoms in orange to denote that it comes from another subunit in the trimer of polypeptide chains that form nitrite

reductase. **Middle figure**, The 3D-model for the full *N. gruberi* nitrite reductase sequence of one polypeptide chain. The two Cu^{2+} ion site are labelled. The position of insertions and deletions are denoted as in Figure 3a. All insertions and deletions are at surface loops and far from the active site and hydrophobic core. **Right figure**, A view rotated 90° from (middle). The subunit contributing residue Histidine 349 to bind Cu^{2+} in site CuII is shown in orange trace.

Figure 4a.

An unrooted phylogenetic analysis of putative nitric oxide reductase / FeS cluster repair gene products (*E. coli YtfE* gene family*)*. Maximum-likelihood analysis of the protein sequences showing branch support. The tree was created with PhyML (Guindon et al, 2003) using the GTR model with 200 bootstrap pseudo-replicates implemented using Seaview 4 (Gouy et al, 2010). The protein is mainly limited to bacteria (black) and archaea (blue) and a distinct version of this protein is expressed in fungi (orange) as noted by (Overton et al, 2008). Four eukaryotes have been found to possess the bacterial gene (red) but we suspect that both *Trichonephila clavipes* (Golden silk orb spider) and *Lucilia cuprina* (Sheep blowfly) are bacterial contaminates since no other spider or dipteran appear to harbour these genes. On the other hand, both *Trichomonas vaginalis* and *Naegleria* are represented by more than one gene both from axenic cultures making it highly likely that these parasitic eukaryotes express these proteins. It seems that both *Trichomonas* and *Naegleria* have independently received putative nitric oxide reductase / FeS cluster repair genes from a different bacterial source through two horizontal gene transfer events.

Figure 4b.

Sequence alignment of *N. gruber*i putative nitric oxide reductase with *E. coli* sequence and a model based on the *E. coli* structure (pdb code 5fnp). Sequence alignment of *N. gruberi* putative nitric oxide reductase (XP_002669995) with *E. coli* sequence of structure 5fnp (protein data bank). Residues ligating ions in the active site are denoted by an "L" under the sequence. VCM denotes the 'vicinal cysteine motif' (DYCCGG) which is conserved is many members of the nitric oxide reductase family (for technical reasons these were mutated to DYAAGG for the crystal structure determination of 5FNP).

Figure 4c. A 3D-Model for *N. gruberi* nitric oxide reductase based on the X-ray crystallographic structure of the *E. coli* enzyme (protein data bank code 5FNP).

Figure 5a. An unrooted phylogenetic analysis of the putative nitrite/formate transporter Maximum-likelihood analysis of the protein sequences showing branch support. The tree was created with PhyML (Guindon et al, 2003) using the GTR model with 100 bootstrap pseudo-replicates implemented using Seaview 4 (Gouy et al, 2010). The Excavata homologs are highlighted in blue, fungal members orange, plants (green), red algae purple, amoebozoa group red, SAR group brown, alveola light purple, prokaryotes (black).

Figure 5b.

Sequence alignment of *N. gruberi* putative nitrite transported (XP_002683227_1) with *V. cholerae* sequence of structure 3klz (protein data bank).Identities are shown as open boxes, and those involved in ligand binding are shown in black boxes with a letter "L" below.

Figure 5c.

Shows the structural alignment onto 3klz (Waigh et al., 2010). The sequence numbers are for the *N. gruberi* protein. Formate molecules are shown forming hydrogen bonds and close contacts with the residues highlighted as "L" in part (a). The start sequence position for an insertion (*N. gruberi* with respect to the *V. cholerae* sequence) is shown in a green sphere. The start position for a deletion is shown in a red sphere. The single insertion is into a cytoplasmic loop. The single deletion encompasses two helices on the periplasmic side of the transporter. Both are far from the binding site for formate and both are structurally feasible, in that they do not disrupt the overall fold of the transporter.

Figure 6.

Clay particles (grey hexagons) occur as stacked sheets with a space between each sheet, so they have a large surface area. Under oxidizing conditions these sheets carry a negative charge and bind Fe^{3+}. Under reducing or acidic conditions, iron exists as Fe^{2+}, and is no longer bound to the clay particles but free in solution (Gotoh and Patrick, 1974; McBride, 1994).

458
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460 **Appendix A. Supplementary Data**
461 Supplementary material related to this article can be found, in the online version at DOI…
462
463
464 **References**
465

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701 **Appendix A. Supplementary Data**

702

703 **Table S1. Genes involved in Fe and anaerobic respiration in *Naegleria*. Data from**
704 **annotated genomes, the literature, and this study. * Many other candidate genes.**
705

Gene / protein	Genbank/AmoebaDB	Protein's putative function
Acetate:succinate CoA transferase	**N. gruberi* EFC47705.1 **N. fowleri* NF0074120	Conversion of acetyl-CoA to acetate and succinate from succinyl CoA
Acetyl-CoA synthetase	**N. gruberi* XP_002680510.1 **N. gruberi* XP_002678527.1 **N. fowleri* NF0020240 **N. fowleri* NF0126460	Conversion of acetyl-CoA to ATP and acetate.
[FeFe]-Hydrogenase	*N. gruberi* XP_002674266.1 *N. fowleri* NF0008540	Reoxidizes NADH directly by the formation of H_2
Ferredoxin	*N. gruberi* XP_002680107.1 *N. fowleri* NF0126490	An electron transferring protein
Ferric reductase	**N. gruberi* XP_002681613.1 **N. fowleri* NF0021390	Reduces Fe^{3+} to Fe^{2+} outside the cell to allow it to be taken up possibly through ZIP14 type channels
Ferritin	*N. gruberi* XP_002676421.1 *N. fowleri* NF0106200	An Fe^{2+}-binding protein
Ferrochelatase	*N. gruberi* XP_002681413.1 *N. fowleri* NF0059730	Heme biosynthesis
Ferroportin	*N. gruberi* XP_002672655.1	Exports excess Fe^{2+} from cells

	N. fowleri NF0014540	
Frataxin	*N. gruberi* XP_002681651.1 *N. fowleri* NF0118220	Thought to facilitate assembly of iron-sulphur clusters
Heme lyase	*N. gruberi* NP_066539.1 *N. fowleri* YP_007890060.1	Catalyses the addition of the heme group to cytochrome c
Hemerythrin	**N. gruberi* XP_002680302.1 **N. fowleri* NF0119700, NF0127030, AAF35899.1	Possibly oxygen storage? These proteins do not contain a heme group despite the name.
Phosphofructokinase (pyrophosphate-dependent)	*N. gruberi* EFC47701.1 *N. fowleri* AAA85791.1	Key rate limiting enzyme in glycolysis
Pyruvate dehydrogenase Alpha Beta	 *N. gruberi* XP_002673628.1 *N. fowleri* NF0058380 *N. gruberi* XP_002676847.1 *N. fowleri* NF0038020	The complex catalyses the conversion of pyruvate to acetyl - CoA
Mitoferrin	*N. gruberi* XP_002671468.1 *N. fowleri* NF0079420	Regulates mitochondrial iron levels by transporting across membranes
Nitrite/formate transporter	*N. gruberi* XP_002683227.1 *N. gruberi* XP_002678435.1 *N. fowleri* KAF0982384.1	Transports nitrite across membranes
(Nitrate reductase)	*N. gruberi* XP_002681617 *N. fowleri* NF0084590	A Fe-containing enzyme that reduces nitrate to nitrite. These genes lack the crucial Mo domain and so are <u>not</u> believed to function as nitrate reductases in *Naegleria*
Nitric oxide reductase	*N. gruberi* XP_002669995.1 *N. fowleri* NF0055500	Produces N_2O from NO. The *Naegleria* enzyme is homologous to the *E. coli* YtfE/FeS repair enzyme.

Nitrite reductase	*N. gruberi* XP_002674759.1 *N. fowleri* NF0120230	A copper-containing nitrite reductase that reduces nitrite to nitric oxide during denitrification.
Succinyl-CoA synthetase alpha Succinyl-CoA synthetase beta	*N. gruberi* EFC50706.1 *N. gruberi* XP_002683450.1 *N. fowleri* NF0048280 (mRNA10) *N. gruberi* EFC41294.1 *N. gruberi* XP_002674038.1 *N. fowleri* NF0063530 (mRNA1)	Catalyses the reversible reaction of succinyl-CoA to succinate and produces ATP
ZIP14	*N. gruberi* XP_002674492.1 *N. fowleri* NF0119990	An Fe^{2+} channel through which *Naegleria* may obtain free iron from the extracellular space.

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