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1 **Localization and Functional Characterization of the Alternative**
2 **Oxidase in *Naegleria***

3
4 Running title: *Naegleria gruberi*'s AOX

5
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31
32
33 **Keywords**

34 *Naegleria*, Respiration; mitochondria; confocal microscopy; evolution; adaptation

47 **ABSTRACT**

48
49 The Alternative oxidase (AOX) is a protein involved in maintaining the Krebs cycle in instances
50 where the respiratory chain has been inhibited, while allowing for the maintenance of cell growth
51 and necessary metabolic processes for survival. Among eukaryotes, alternative oxidases have
52 disperse distribution and are found in plants, fungi and a few protists, including *Naegleria* ssp.
53 *Naegleria* species are free-living unicellular amoeboflagellates, and include the pathogenic species
54 of *N. fowleri*, the so-called brain eating amoeba. Using a multidisciplinary approach, we aimed to
55 understand the evolution, localization and function of AOX and the role that plays in *Naegleria*'s
56 biology. Our analyses suggest that the protein was present in last common ancestor of the genus and
57 structure prediction showed that all functional residues are also present in *Naegleria* species. Using
58 a combination of cellular and biochemical techniques, we also functionally characterize *N. gruberi*'s
59 AOX in its mitochondria and we demonstrate that its inactivation affects its proliferation.
60 Consequently, we discuss the benefits of the presence of this protein in *Naegleria* species, along
61 with its potential pathogenicity role in *N. fowleri*. We predict that our findings will spearhead new
62 explorations to understand the cell biology, metabolism and evolution of *Naegleria* and other free-
63 living relatives.

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94 INTRODUCTION

95
96 *Naegleria gruberi* is a free-living, heterotrophic, microbial eukaryote and a close relative of
97 *N. fowleri*, the so-called “brain-eating amoeba”. It is a non-pathogenic member of the excavate
98 supergroup, which contains key pathogens such as Kinetoplastids (*Trypanosoma*, *Leishmania*),
99 *Giardia* and *Trichomonas*, and is evolutionarily distant from animals, fungi and plants (Adl *et al.*,
100 2019). *Naegleria* resides primarily as an amoebic (trophozoite) form, but upon environmental
101 stimuli can transform into a flagellate, synthesizing basal bodies and flagella *de novo*, or encyst to
102 allow for dispersion (De Jonckheere *et al.*, 2001). It possesses all the major organelles deemed to be
103 canonical for eukaryotes, including nucleus, mitochondria, peroxisomes (Fritz-Laylin *et al.*, 2010)
104 and a Golgi (Herman *et al.*, 2018). This cellular complexity is reflected in the *N. gruberi* genome
105 sequence (Fritz-Laylin *et al.*, 2010) found to encode an extensive complement of cellular machinery
106 and was argued to be reflective of the ancient sophistication present in the last eukaryotic common
107 ancestor (Koonin, 2010).

108
109 Found in soils and freshwater worldwide, *N. gruberi* can thrive in a wide range of osmotic
110 and oxygenic conditions (De Jonckheere, 1979, 2014; Tymł *et al.*, 2016). It has the capacity for full
111 aerobic and anaerobic metabolism, and was predicted to have assimilated unique biochemical
112 adaptations both within and outside the mitochondria (Fritz-Laylin *et al.*, 2010, 2011; Ginger *et al.*,
113 2010). Among those, only a handful of pathways have been localized and characterized, in the
114 trophozoite stage of this microbial eukaryote. Some examples include the cytosolic localization and
115 functional characterization of the [FeFe]-hydrogenase (Tsaousis *et al.*, 2014), the mitochondrial
116 localization and functional characterization of ferritin (Mach *et al.*, 2018), and the oxygen-dependent
117 metabolism of lipids (Bexkens *et al.*, 2018). While the last report provided a hint on the function of
118 *Naegleria*’s alternative oxidase (AOX), a thorough investigation on this important oxygen-dependent
119 enzyme is lacking.

120
121 The alternative oxidase (AOX) is a terminal oxidase typically involved in bypassing the
122 electron transport chain in plant mitochondria, even though it has also been identified and localized
123 in the mitochondria and related organelles of many non-related microbial eukaryotes including
124 trypanosomes (Clarkson *et al.*, 1989), *Candida albicans* (Yan *et al.*, 2009), *Cryptosporidium*
125 (Roberts *et al.*, 2004) and *Blastocystis* (Stechmann *et al.*, 2008; Tsaousis *et al.*, 2018). Due to its
126 absence in humans, the protein is considered a potential drug target, and has been well studied in
127 some of these pathogenic species (Shiba *et al.*, 2013; Tsaousis *et al.*, 2018; Duvenage, Munro and
128 Gourlay, 2019). Despite the wide distribution and extensive research on this group of proteins, their
129 overall physiological roles are still unclear (Moore and Albury, 2008). Intriguingly, it has been
130 suggested that AOX may be involved in maintaining tricarboxylic acid cycle turnover under high
131 cytosolic phosphorylation potential, stress tolerance (oxygen), and thermogenesis (Finnegan, Soole
132 and Umbach, 2004; Moore *et al.*, 2013).

133
134 Herein, we employed a multiphasic approach to characterize the AOX of *N. gruberi*, while
135 comparing it with both the *N. fowleri* and *N. lovaniensis* homologues and examining their origins.
136 Upon structural characterization of all the counterparts, we generated a specific polyclonal antibody
137 against *N. gruberi* AOX, with which we localized the protein in *N. gruberi*’s mitochondria using a
138 combination of assorted cellular approaches. Experiments with high-resolution respirometry
139 demonstrated that the *N. gruberi* homologue confers cyanide resistance. This study represents the
140 first thorough characterization of AOX in *Naegleria* species, which could provide further

141 understanding into the biochemical adaptations of this peculiar and highly adaptable microbial
142 eukaryote.

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145

146 **MATERIALS AND METHODS**

147

148 **Bioinformatic Analysis**

149 The predicted amino acid sequences of the alternative oxidase homologues of *N. fowleri* and
150 *N. lovaniensis* were obtained from Genbank (NCBI) using the following accession numbers;
151 GCA_008403515.1 (*N. fowleri*), GCA_003324165.1 (*N. lovaniensis*). The amino acid sequence of
152 the AOX from *N. gruberi* was obtained from uniprot (D2V4B2). The Phyre2 web portal for protein
153 modelling, prediction and analysis was used in intensive mode (Kelley *et al.*, 2015) to derive the
154 structure for NgAOX. Structures were then downloaded and analyzed using PyMol. For sequence
155 alignment Clustal Omega (Sievers *et al.*, 2011) was used, output was downloaded as fasta file and
156 analyzed using Jalview 2.11.0 (Waterhouse *et al.*, 2009). Sequence identifiers for AOX alignments
157 were: *Trypanosoma brucei brucei* (Q26710), *Cryptosporidium parvum* (Q6W3R4), yeast; *Candida*
158 *albicans* (A0A1D8PEM4), plant; *Arabidopsis thaliana* (Q39219), and, fungi; *Neurospora crassa*
159 (Q01355).

160

161 **Phylogenetic analysis**

162 Three local databanks of proteomes, representative of all diversity from eukaryotes, bacteria
163 and proteobacteria were assembled from the National Center for Biotechnology Information
164 (NCBI): 193 proteomes from Eukaryotes (one per genus); 1017 proteomes from Bacteria (3 per
165 family) and 1082 proteomes from Proteobacteria (1 per genus). Homology searches were performed
166 using HMMSEARCH, from the HMMER-3.1b2 package (Johnson, Eddy and Portugaly, 2010), with
167 the option --cut_ga to screen all the proteomes in the three databanks for the presence of AOX pfam
168 domain (PF01786.17). All the hits were then manually curated in order to discard false positives.
169 The remaining hits were aligned using MAFFT-v7.407 (Katoh and Standley, 2013) with the linsi
170 option and trimmed with BMGE-1.1 (Criscuolo and Gribaldo, 2010) using the BLOSUM30
171 substitution matrix to select unambiguously aligned positions. A maximum likelihood tree was then
172 generated using IQTREE-1.6.12 (Nguyen *et al.*, 2015) under the TEST option with 1000 ultrafast
173 bootstrap replicates.

174

175 **Cell Culture**

176 *N. gruberi* strain NEG-M (kindly provided by Lillian Fritz-Laylin, Biology Department,
177 University of Massachusetts, Amherst, USA) was cultured in M7 media at 28 °C (Tsaousis *et al.*,
178 2014). Cells were passaged every 3 to 5 days to prevent overconfluency.

179

180 **Antibody generation**

181 A 16 amino acids peptide (nh2- CMHRDYNHDMSDKHRA –conh2) was designed by
182 Eurogentec based on the provide sequence of AOX of *Naegleria gruberi* (XP_002672918) (see
183 **Suppl Figure S2**). 5 mg of the peptide was coupled with the carrier protein KHL (Keyhole Limpet
184 Hemocyanin) and was subsequently used to inoculate a rabbit (through the Eurogentec's speedy
185 program) for antibody production. The antibody's affinity (Eurogentec; Peptide: 1911009, Rabbit
186 237) was confirmed through ELISA.

187

188 **Cell Fractionation and western blots**

189 To separate organelles from cytosol, cell fractionation by centrifugation was carried out as
190 previously described (Herman *et al.*, 2018). Lysates were mixed with 4x sample buffer and heated to
191 95 °C for 10 minutes. Samples were then loaded in two tris-glycine gels for gel electrophoresis. One
192 gel was subjected to Coomassie staining overnight and destained the following day to assess equal
193 loading. The other gel was transferred to PVDF membrane using a trans-blot turbo transfer system
194 according to manufacturer's protocol (Bio-rad). Membranes were blocked with 5% milk in TBS
195 buffer containing 0.5% tween-20 for 1 hour at room temperature. Primary antibody staining was
196 carried out overnight at 4 °C with the following antibody dilutions: anti-AOX 1:1000, and
197 previously published antibodies anti-HydE 1:1000 and, SdhB 1:1000 (Tsaousis *et al.*, 2014).
198 Membranes were washed four times with TBS-T for 5 minutes prior to secondary staining with
199 HRP-conjugated goat anti-mouse and goat anti-rabbit antibodies (Invitrogen). For detection,
200 membranes were incubated with ECL reagent (Bio-rad) for 30 seconds and imaged using syngene
201 G:BOX imager. Membranes were stripped with mild stripping buffer composed of 13 mM glycine,
202 3.5 mM sodium dodecyl sulfate, 1% tween-20, pH 2.2. PVDF membranes were washed twice for 10
203 minutes in mild stripping buffer, followed by two washes in PBS for 5 minutes, and lastly two
204 washes for 5 minutes with TBS-T, prior to blocking for the next immunoprobe.
205

206 **Immunofluorescence Microscopy**

207 *N. gruberi* was seeded onto sterile poly-L-lysine treated glass coverslips in a 6-well plate and
208 incubated overnight at 25 °C. The following day the cells were treated with 250 nM MitoTracker
209 Red for 30 minutes. The media was then aspirated and cells were washed with 1x PBS, followed by
210 fixation using 2% formaldehyde for 20 minutes. After fixation, 2% formaldehyde was removed and
211 cells were permeabilised with 0.1% triton-X 100 for 10 minutes. After permeabilization cells were
212 washed three times with 1x PBS and blocked using 3% bovine serum albumin in PBS for 1 hour.
213 Primary antibody staining was carried out at room temperature for 1 hour with custom made AOX
214 antibodies (Eurogentec; Peptide: 1911009, Rabbit 237), diluted to 1:1000. Secondary antibody
215 staining was carried out for 1 hour in the dark using anti-Rabbit-IgG-Alexa 488. Slides were washed
216 and mounted using Prolong Gold Antifade with DAPI. Laser Confocal Microscopy was carried out
217 using the LSM 880 Laser Confocal with Airyscan by Zeiss. Laser sets used were 405, 488 and 594,
218 with airyscan detector plate imaging for high resolution. Images were captured and analyzed using
219 Zen software suite by Zeiss.
220

221 **Immunoelectron Microscopy**

222 Samples were prepared as previously described (Herman *et al.*, 2018). Blocking of the
223 samples was achieved via a 1 hour incubation in 2% BSA in PBS with 0.05% Tween 20. Primary
224 antibody staining was performed by incubating AOX antibodies at 1:10, 1:50 and 1:100 dilutions for
225 15 hours at 8 °C. The sample grids were then incubated for 30 minutes at room temperature, with
226 the corresponding gold-conjugated secondary antibodies. Counter-staining was achieved by
227 incubation with 4.5% uranyl acetate in PBS for 15 minutes and a 2-minute incubation in Reynold's
228 lead citrate. The sample grids were imaged with a Jeol 1230 Transmission Electron Microscope
229 operated at 80kV and images were captured with a Gatan One view digital camera.
230

231 **High Resolution Respirometry**

232 Real time respirometry was monitored using an OROBOROS Oxygraph-2k with Clark
233 polarographic oxygen electrodes and automatic titration-injection micropump. The chambers of the
234 oxygraph were calibrated using 2 ml of M7 media without glucose for 20 minutes. *N. gruberi* cells

235 were seeded in the chamber at a density of 100,000 cells per ml. Respiration was monitored before
236 and during drug additions. The drugs used to assess respiration were added in the following order; 1
237 mM potassium cyanide, 5 μ M antimycin A and 1.5 mM salicylhydroxamic acid (SHAM). The
238 experiment was then repeated with the drug additions in reverse order. Student-t test was used to
239 determine significance.

240

241 **Cell Proliferation Assay**

242 *Naegleria* cells were seeded at a density of 2000 cells per well of a clear F-bottom 96 well
243 plate and incubated in identical conditions as stated above. The plate was placed on a Juli™Stage
244 live cell monitoring system, programmed to capture an image of each well every hour for 5 days. On
245 day-2, wells were inoculated with concentrations of SHAM at 1 mM, 0.1 mM, 0.001 mM and, 0.001
246 mM. Plates were then incubated for a further 3 days. At the end of the experiment the images were
247 used to count cell numbers using ImageJ. Using an image with a scale bar, we used the grid function
248 with a known area per square that was applied to all images taken at 0, 24, 48, 72, 96 and 120 hours.
249 Using the cell counter function, we counted the number of cells in a grid square, and multiplied it to
250 the surface area size of a F-bottom 96-well plate in order to estimate the total cell number per well.
251 The experiment was completed with three biological replicates, whereby each biological replicate
252 consisted of three technical replicates. Cell counts were graphed using GraphPad Prism 8.

253

254

255

256 **RESULTS**

257

258 **Phylogenetic analysis of alternative oxidase proteins**

259 We carried out an exhaustive search for AOX homologs in updated eukaryotic and bacterial
260 databanks. As bacterial hits were mainly from proteobacteria, we also searched for AOX homologs
261 in a proteobacteria databank with more diversity within this phylum. We identified 323 AOX
262 homologs: 265 from 193 eukaryotic taxa, 56 from 1,082 proteobacterial taxa, and 2 from 896
263 bacterial taxa (other than proteobacteria) (**Suppl table S1**).

264 Regarding the eukaryotic distribution, in addition to previously described groups Alveolata,
265 Euglenozoa, Metazoa, Choanoflagellates, Stramenopiles, Fungi, Rhodophyta, Heterolobosea and
266 Viridiplantae (Pennisi *et al.*, 2016), we identified AOX homologs in some eukaryotes that were not
267 reported: Apusozoa, Amoebozoa, Filasterea, Haptophyceae and Rhizaria. No homologs were found
268 in Kipferlia, Metamonada and Hexamitida (**Suppl Figure S1** and **Suppl table S1**). Bacterial hits
269 were much less diversified, as they are restricted to alphaproteobacteria, betaproteobacteria and
270 gammaproteobacteria. The other two hits belong to Bacteroidetes and the CPR (Candidate Phyla
271 Radiation) and they probably correspond to transfers from proteobacterial taxa (**Suppl Figure S1**).
272 Our phylogenetic analysis shows that AOX homologs of Metazoans (UFB=86%), Haptophyceae,
273 Choanoflagellata (UFB=100%) and Heterolobosea (UFB= 100%; inset **Figure 1**) form distinct and
274 monophyletic groups pointing toward the presence of an AOX in the ancestors of each group.
275 Regarding bacterial sequences, although they branch together and form a monophyletic clade
276 (UFB= 97%), it seems that proteobacterial taxa transferred AOX sequences to other bacteria
277 (Bacteroidetes and CPR) and to some Euglenozoa. This bacterial clade forms a sister group of
278 Streptophyta nad Rhodophyta (UFB= 97%), however, it is not clear which eukaryotic group
279 transferred AOX sequences to proteobacteria.

280 The presence of the two separate and monophyletic groups Streptophyta (UFB= 100%),
281 Chlorophyta (UFB=82%) shows that, although the presence of AOX could not be inferred in the

282 ancestor of Archaeplastida, it can be inferred in the ancestors of both groups. Finally, regarding
283 Fungi, the presence of three separate and monophyletic groups containing Ascomycota and
284 Basidiomycota (UFB= 80%), Chytridiomycota (UFB= 100%) and a mix of Zoopagomycota,
285 Chytridiomycota, Cryptomycota, Mucoromycota, Blastocladiomycota and Microsporidia (UFB=
286 74%), suggests that AOX was acquired at least three times independently in this group.
287

288

289 **Amino acid alignments reveal conserved residues in *Naegleria gruberi*, *N. fowleri* and *N.***

290 ***lovaniensis***

291 The amino acid sequences of AOX in *N. gruberi*, *N. fowleri* and *N. lovaniensis*, were aligned
292 against the AOX found in protists; *Trypanosoma brucei brucei*, *Cryptosporidium parvum*, yeast;
293 *Candida albicans*, plant; *Arabidopsis thaliana* and, fungus; *Neurospora crassa*. The N-termini
294 displayed little conservation. However, key conserved residues were detected across all species from
295 the middle of the sequences, continuing towards the C-terminus (**Suppl. Figure S2**). Most of the
296 conserved residues localized in the alpha helical arms, including the residues responsible for
297 membrane interaction, diiron binding domain and, three universally conserved tyrosines (**Suppl**
298 **Table S2**). The presence of these key features strongly suggests that *Naegleria*'s predicted AOX
299 would retain functional activity.
300

301 **Structure modelling of AOX from *Naegleria gruberi***

302 Using Phyre2, we were able to generate models of the NgAOX monomer. The resulting
303 model reveals a monomer containing the characteristic four-bundle helix, in which resides the active
304 site (**Figure 2**). The active site is composed of the characteristic four glutamate residues and two
305 histidine residues which are responsible for diiron binding. In addition, the universally conserved
306 tyrosine residue necessary for activity is present at residue number 175. Furthermore, helices $\alpha 1$ and
307 $\alpha 4$ contains a strong hydrophobic region, also a key feature of AOX, as these helices are involved in
308 membrane insertion. Amino acid conservation of glycine 85 and glycine 165 between all *Naegleria*
309 species and *Trypanosoma* contribute to the structural kink of both $\alpha 2$ and $\alpha 5$ helices. We also
310 observed conservation between amino acids responsible for membrane-binding regions and for
311 dimerization (**Suppl Table S2**). These amino acid conservations highlight the conserved structure of
312 AOX, its characteristic helices and hydrophobicity patch for membrane anchoring.
313

314 **Localization of AOX in *N. gruberi***

315 To verify the presence and localization of AOX, fractionation by centrifugation was carried
316 out to isolate the mitochondria and cytosol. Using a custom-made antibody raised against *N. gruberi*
317 AOX, our western blot analysis reveals the presence of AOX in the mitochondrial fraction and not
318 in the cytosolic fraction (**Figure 3C**) Successful fractionation was confirmed by immunoblotting
319 Hydrogenase E protein, which localizes exclusively in the cytosol and succinate dehydrogenase, a
320 previously confirmed mitochondrial marker (Tsaousis *et al.*, 2014). The localization of AOX was
321 also carried out by immunofluorescent microscopy, whereby *N. gruberi* cells were stained with
322 mitotracker red before fixation, and subsequently fixed and immunostained for detecting AOX. Our
323 imaging reveals a high degree of colocalization between the mitotracker signal and the AOX signal
324 derived from the secondary antibodies. This indicates that AOX is localized in the mitochondria
325 (**Figure 3A & B & Suppl. Figure S3**). To further confirm these observations, we have subsequently
326 carried out immunoelectron microscopy (IEM) on resin fixed *N. gruberi* sample grids. As a result,
327 we detected positive signal inside derived from the immunogold labelling, strongly suggesting its
328 localization is within the inner membrane of the mitochondria (**Figure 4**).

329

330 **Real time respirometry reveals AOX confers cyanide resistance**

331 In order to assess whether *N. gruberi* respire from either the classical respiratory chain or
332 through AOX, real-time respirometry data was collected. Cells were cultured for three days and
333 washed with M7 media without the addition of glucose. Afterwards, 2 ml of cell resuspension was
334 seeded into the respirometer chambers at a density of 10^5 cells per ml. After establishing the routine
335 respiration, a series of drugs were added sequentially to each chamber and oxygen flux was
336 monitored (**Figure 5**). Initially, 1 mM of complex IV inhibitor potassium cyanide was injected into
337 the chambers, revealing an increase in oxygen flux and decrease in oxygen concentration, which
338 would suggest that respiration via alternative oxidase pathway is active, as the presence of cyanide
339 has no effect on *N. gruberi*. The addition of complex III inhibitor, Antimycin A, had no effect on
340 respiration. Lastly, addition of 1.5 mM of AOX inhibitor SHAM resulted in a significant decrease in
341 respiration (**Figure 5A, B**). When the experiment was repeated with drug additions in the reverse
342 order, the SHAM treatment resulted in a significant decrease in respiration, whereas Antimycin A
343 and KCN had no further effects on respiration (**Figure 5B, C**). This would suggest that *N. gruberi*
344 respire predominantly via the AOX pathway.

345

346 **The presence of SHAM in culture media reduces cellular proliferation**

347 Due to the decrease respiration in *N. gruberi* in the presence of SHAM, we opted to evaluate
348 cell growth under varying concentrations of SHAM using a Juli™Stage live cell monitoring system
349 (**Figure 6**). Cells were grown in 96-well culture plates for 48 hours to allow proliferation. At the 48
350 hour mark, we pipetted varying amounts of SHAM in culture media to reach the following final
351 concentrations; 1 mM, 0.1mM, 0.01 mM and, 0.001 mM. The plate was then returned to the
352 incubator for another 72 hours. By counting the number of cells under each condition, we were able
353 to verify the negative effect of SHAM on proliferation. Concentrations of SHAM at 1 mM to 0.1
354 mM were effective at stopping cell proliferation altogether, whereas 0.01 mM SHAM greatly
355 reduced cell proliferation. SHAM concentration of 0.001 mM had no effect on cell proliferation, as
356 the cell counts followed a similar pattern to the negative control.

357

358

359 **DISCUSSION**

360

361 The genome of *Naegleria gruberi* (Fritz-Laylin *et al.*, 2010) encodes several homologues of
362 alternative oxidases, but until now, there was no thorough report to investigate the origin and
363 distribution of these proteins within microbial eukaryotes. Our sophisticated search and analyses
364 suggest that the AOX gene was present in the ancestors of multiple eukaryotic groups, suggesting
365 potential acquisition at earlier stages in the eukaryotic evolution. Specifically, for the *Naegleria*
366 species, our phylogenetic analysis indicates that AOX gene duplication took place prior to
367 speciation events, which further suggests the earlier requirement of two homologues as an
368 adaptation to the unique lifestyles of these organisms. In *N. gruberi*, these homologues have been
369 previously shown to be differentially expressed during the organism's two major stages, trophozoite
370 and flagellate (<https://phycocosm.jgi.doe.gov/pages/blast-query.jsf?db=Naegr1>) (Fritz-Laylin *et al.*,
371 2010). The reason behind this is currently unknown and no conclusions can be extracted from the
372 primary amino acid sequences of the two homologues. Our sequence alignment analysis reveals that
373 the key residues required for AOX to be functional are present across all homologues and all three
374 *Naegleria* species. One of the key domains presented in the sequences is the diiron domain,
375 characterized by four glutamic acid residues and two histidine residues involved in forming

376 hydrogen bonds with Fe²⁺ and Fe¹⁺ ions, of which the latter are considered to be universally
377 conserved (Moore *et al.*, 2013). In addition, our sequence alignment reveals the presence of the three
378 universally conserved tyrosine residues (Moore *et al.*, 2013) in the electron transport chain (Moore
379 *et al.*, 2013; Shiba *et al.*, 2013). Interestingly, our sequence alignment revealed a deviation in
380 conservation of the residue trp-151 (*TbAOX*), including between the pathogenic *N. fowleri*, non-
381 pathogenic *N. lovaniensis* and, *N. gruberi* species. It has previously been reported that a mutation
382 from trp-206 (trp-151 on *tbAOX*) to ala-206 resulted in a decrease in respiration of 95% (Crichton *et*
383 *al.*, 2010). However, wet lab experiments would be required to ascertain the effect of the difference
384 in respiration found in *Naegleria* species.

385 Following up from the *in silico* analysis, we aimed to biochemically characterize the *N.*
386 *gruberi* AOX homolog. It had been previously experimentally demonstrated that *N. gruberi*'s
387 preferred food substrate are lipids over glucose, which was subsequently associated with the unique
388 abundance of metabolites in the brain (Bexkens *et al.*, 2018). The authors demonstrated that *N.*
389 *gruberi* has a biochemically active AOX, but they did not show any localization. We have designed
390 a peptide and generated an anti-*Naegleria* polyclonal antibody that could cross-react with all
391 *Naegleria* species and be used for localization studies. Our immunoblotting results show a strong
392 band appearing at 28 kDa, which was the predicted mass of *N. gruberi*'s AOX. This signal only
393 appeared in the whole cell lysate and mitochondrial fraction, with no signal in the cytosolic fraction.
394 This data was strengthened by our immunofluorescent microscopy experiments, which revealed a
395 high degree of colocalization between the AOX staining and a commercial mitochondrial stain
396 (Tsaousis *et al.*, 2014). Lastly, an experiment using immunogold labelling showed staining signal
397 from inside the mitochondria, which we find encouraging as AOX is located on the inner
398 mitochondrial membrane (Berthold, Andersson and Nordlund, 2000).

399 Next, real-time respirometry data showed strong evidence of AOX being an active
400 component in *N. gruberi*, since the addition of KCN did not lower the oxygen flux. Interestingly,
401 previous work explored the potential cyanide resistance of *N. gruberi* as part of a larger
402 metabolomic study (Bexkens *et al.*, 2018). The authors reported an 80% decrease in respiration,
403 which contrasts with our results. The authors also reported that addition of SHAM further decreased
404 respiration by 14%. The differences between the results reported here and the aforementioned study
405 is likely to be attributed to the differing growth conditions or prolonged differential adaptations of
406 the laboratory strains (Schuster, 2002). Nonetheless, our cellular and biochemical data confirm their
407 hypothesis regarding the presence of AOX in *N. gruberi* mitochondria. This however leads to
408 significant questions in the pursuit of elucidating the complex metabolic mechanisms in *N. gruberi*,
409 in particular, which environmental conditions favor expression and utilization of AOX, there is no
410 report demonstrating the endurance of *Naegleria* species under, for example, hypoxic conditions.
411 *Naegleria* species favor warm and moist environments and have been isolated from lakes, rivers,
412 geothermal springs along with man-made bodies of water such as swimming pools, thermal effluent,
413 sewage sludge and water-cooling circuits from power station [for review see (Chalmers, 2014)]. As
414 a result, *Naegleria* species seem to acclimatize in various temperatures, turbidities and metal
415 concentrations, which subsequently affect the mitochondrial functions. In other organisms, mainly
416 plants, AOX has been implicated in metabolic and signaling homeostasis and was demonstrated to be
417 particularly important during a variety of stresses, including alterations in temperature, nutrient
418 deficiency, oxygen levels and metal toxicity (Vanlerberghe, 2013a; Saha, Borovskii and Panda,
419 2016). Such observations may be true for *Naegleria* as well, and further investigations using these
420 different parameters are required to understand the true role of the various AOX homologs in
421 *Naegleria*'s survival. For example, a previous report has shown differences between metabolic
422 activities of iron-saturated and iron restricted trophozoites of *N. gruberi*, which could subsequently

423 be attributed to potential iron homeostasis centrally regulated by the mitochondria (Mach *et al.*,
424 2018). Again, it will be interesting to further investigate the role and function of the AOX in such
425 mechanisms.

426 While reviewing the adaptations of *Naegleria* in the various environments, we cannot avoid
427 discussing the potential role of AOX in the pathogenesis of *N. fowleri*. A recently published ‘omics
428 approach investigating the potential genes that could be driving the pathogenicity of *N. fowleri*
429 demonstrated up-regulation of mitochondrial energy conversion genes including those involved in
430 ubiquinone biosynthesis, isocitrate dehydrogenase (TCA cycle), complex I and complex III of
431 oxidative phosphorylation (Herman *et al.*, 2020). While the authors have demonstrated up-
432 regulation of enzymes indirectly involved in oxidative stress pathway (e.g. agmatine deiminase),
433 they were not able to demonstrate any significant change in the expression levels of any of the *N.*
434 *fowleri* AOX homologs (Herman *et al.*, 2020). Based on these observations, it would be interesting
435 to investigate the expression levels of AOX from the amoebas collected directly from the brain,
436 either through transcriptomics and /or proteomics, as well as investigate whether this protein is
437 essential for the survival of *N. fowleri* in such a complicated and variable environment.

438 It has been observed that there are significant differences in concentration levels of
439 metabolites between various brain regions (Cichocka and Bereś, 2018). *N. fowleri* is typically found
440 in olfactory bulb of the brain (Moseman, 2020), which it has a unique metabolic network signature
441 and is highly abundant in various salts (e.g. sodium, potassium, calcium), metals (e.g. iron, copper
442 and magnesium) (Gardner *et al.*, 2017), metabolites (histidine-containing dipeptides such as
443 anserine, carnosine, b-alanine), cholesterol, poly-unsaturated fatty acids and prostaglandins (Choi
444 *et al.*, 2018), as well as featuring variable concentrations of oxygen (Özugur, Kunz and Straka, 2020).
445 Differences in the concentrations of these factors have been previously shown to provide stimuli for
446 alterations in the expression of AOX in plants (Vanlerberghe, 2013) and trypanosomes (Vassella *et*
447 *al.*, 2004). As previously discussed, AOX was also shown to be implicated in metabolic and
448 signaling hemostasis and was demonstrated to be particularly important during a variety of stresses,
449 including alterations in temperature, nutrient deficiency, oxygen levels and metal toxicity (Saha,
450 Borovskii and Panda, 2016). Additionally, it has been demonstrated that salt stress negatively
451 impacts mitochondria function, resulting in decreased electron transport activities, with increased
452 mitochondrial ROS and lipid peroxidation, followed by subsequent induction of mitochondrial
453 ROS-scavenging systems, including increase activity of AOX (Ferreira *et al.*, 2008; Mhadhbi *et al.*,
454 2013; Saha, Borovskii and Panda, 2016). We speculate that similar implications could be associated
455 with the function of AOX in *N. fowleri* populating the brain. Demonstrating that anti-AOX drugs are
456 effective against *Naegleria* growth, is of great importance to investigate whether these proteins are
457 essential for the survival of *N. fowleri* in the brain, and determine if it is possible to efficiently
458 utilize these compounds (Murphy and Lang-Unnasch, 1999; Ebiloma *et al.*, 2019; Barsottini *et al.*,
459 2020) against the “brain-eating amoeba”.

460 Herein, we provide the first thorough investigation of the localization and functional
461 characterization of the alternative oxidase proteins in *Naegleria* species. These single-protein-
462 focused studies are essential in contributing to our understanding of the biochemical and cellular
463 adaptations of this exceptional microbial eukaryote as well as provide another piece in *Naegleria*’s
464 evolutionary puzzle. As a result, our investigation on the function of the AOX provides an
465 additional step towards developing this organism as a model to understand various pan-eukaryotic
466 adaptations and more importantly how metabolism could affect its opportunistic nature.

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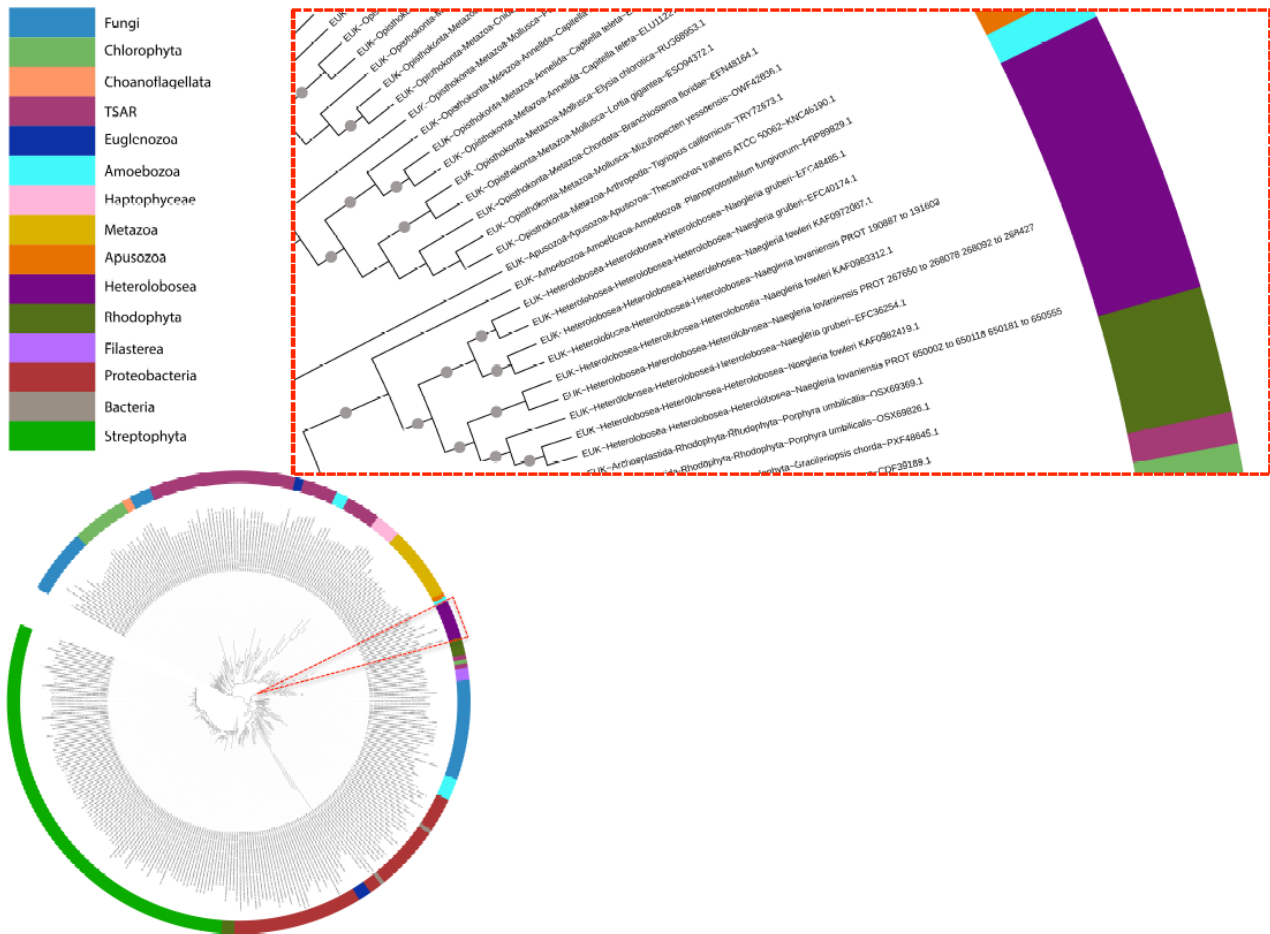
ACKNOWLEDGEMENTS

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AUTHORS CONTRIBUTIONS

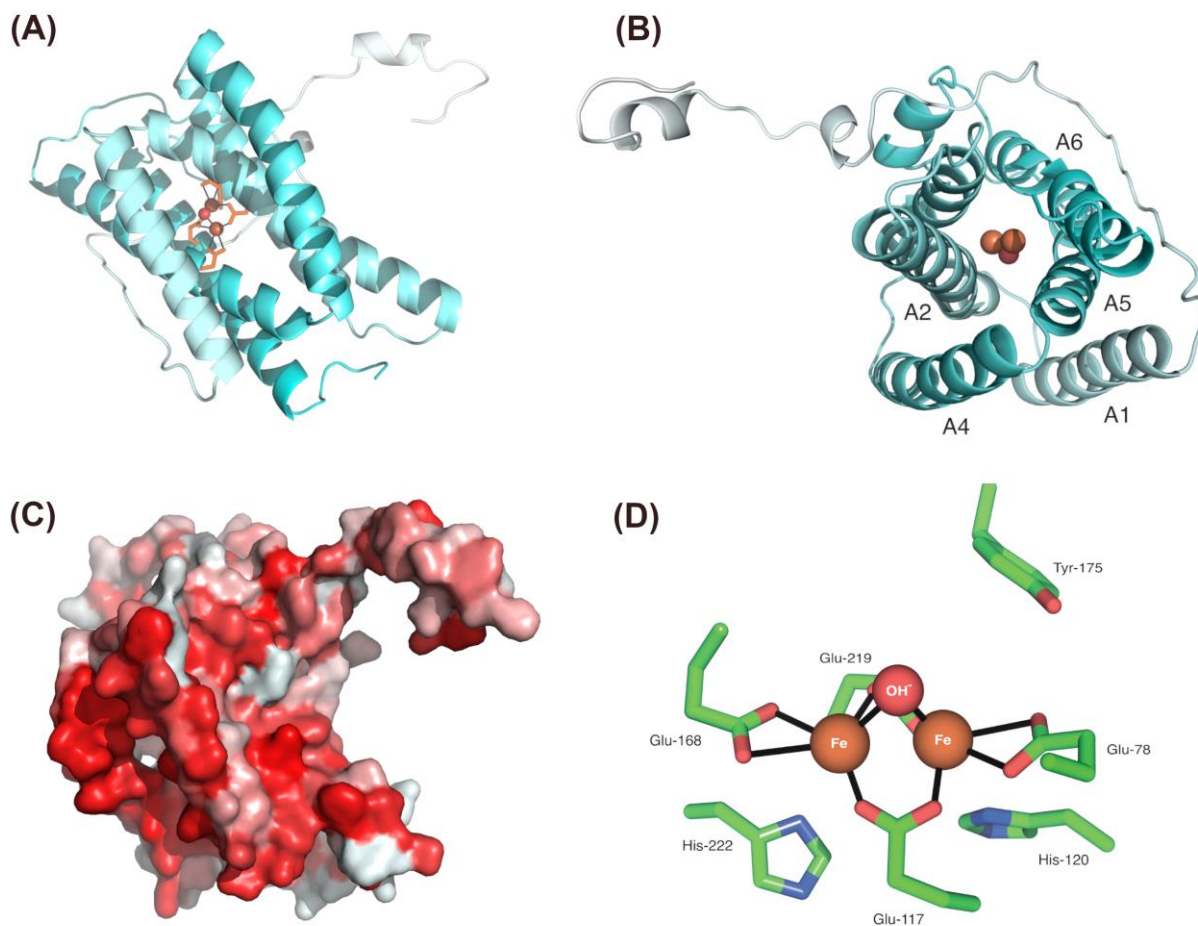
D.C., A.O., S.G., C.W.G., and A.D.T. designed the experiments. D.C., A.O., N.T., E.K., I.R.B., and E.E. conducted the experiments. D.C., N.T. and G.T. performed the data analysis. D.C. and A.D.T. wrote the manuscript and all co-authors reviewed and approved it.

FIGURE LEGENDS



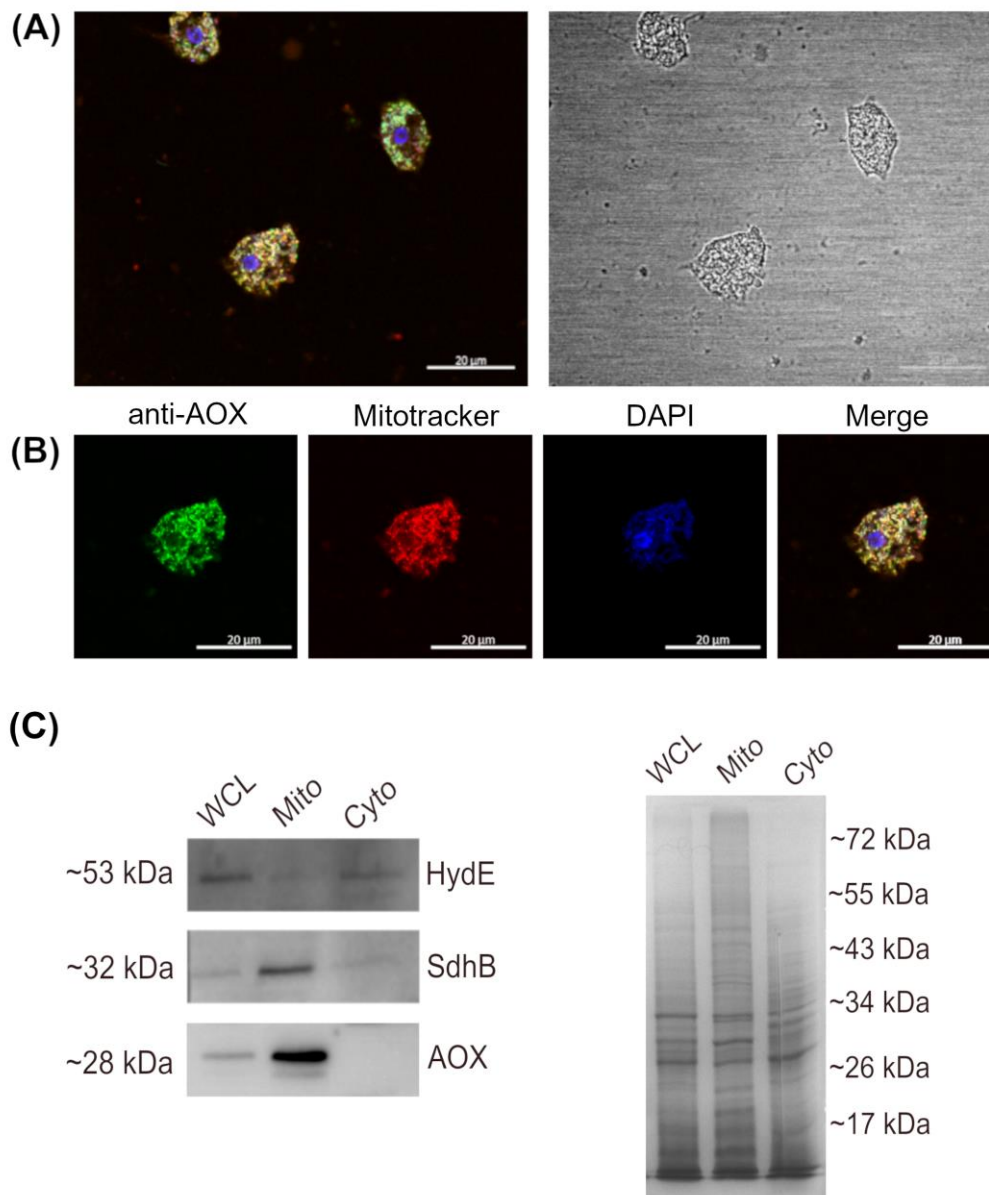
491 **Figure 1. Phylogenetic trees demonstrating the origin of alternative oxidases in heterobolosea.**
 492 Maximum likelihood tree of AOX homologs in eukaryotes and bacteria (323 sequences, 148 amino
 493 acid positions). The tree was inferred with IQTREE using the LG+I+G4 model selected under the
 494 BIC criterion. Grey dots correspond to supports higher than 80%. The scale bar corresponds to the
 495 average number of substitutions per site. Inset focus on the heterobolosean section of the tree,
 496 demonstrating the evolution and duplications events through the *Naegleria* genus. Full phylogenetic
 497 tree can be found in **Suppl. Figure S1**.

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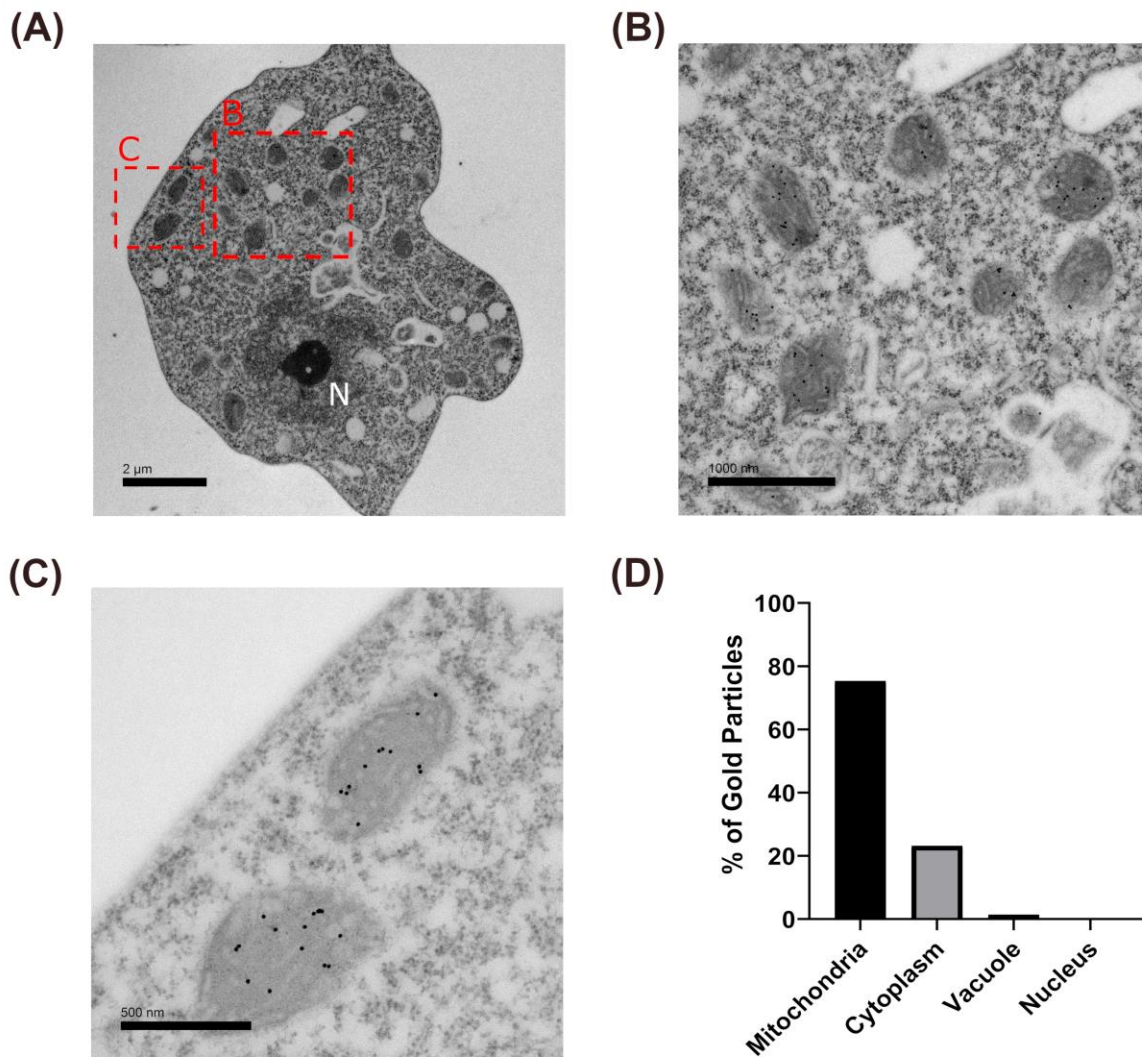
508 **Figure 2. Structural modelling of *N. gruberi* AOX reveals canonical features.**
 509 Using Phyre2 we modelled the structure of ngAOX. We observed the presence of the di-iron domain
 510 and alpha-helical bundles, as viewed from the side (A) and top-down (B). Rendering the structure by
 511 hydrophobicity shows the typical hydrophobic patch required for membrane anchoring (C).

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527 **Figure 3. Confocal microscopy and western blot reveals mitochondrial localization of AOX.**
 528 (A & B) *N. gruberi* cells were treated with mitotracker red prior to fixation and then probed with
 529 AOX antibodies (green). Nuclear marker and mtDNA staining is shown in blue (DAPI staining) Our
 530 confocal imaging reveals a high degree of co-localization between the mitotracker red signal and the
 531 green signal derived from immunoprobng AOX. These results provide visual confirmation of the
 532 expression of AOX and their localization in the mitochondria. Localization figures of more *N.*
 533 *gruberi* cells can be found in **Suppl. Figure S3**. For western blotting lysates were fractionated by
 534 centrifugation to yield samples containing pelleted mitochondrial fraction and a clarified cytosolic
 535 fraction. Successful fractionation from the whole cell lysate was confirmed by immunblotting (left-
 536 hand side) for hydrogenase maturase E (HydE) that localizes in the cytosol, and succinate
 537 dehydrogenase B (SdhB), which localizes exclusively in the mitochondria (C). Immunostaining
 538 against AOX revealed a band around ~28 kDa in the whole cell lysate and mitochondrial fraction
 539 only. A Coomassie stain was carried out to the parallel SDS-PAGE gel parallel to assess equal
 540 loading between samples (right-hand side).

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545 **Figure 4. Immunoelectron microscopy reveals AOX localization within the inner**
546 **mitochondrial membrane.**

547 Fixed *N. gruberi* samples were probed for IEM to assess their localization in the mitochondria. The
548 AOX signal derived by the immunogold secondary antibodies bound against AOX primary
549 antibodies localizes predominantly inside the mitochondria.

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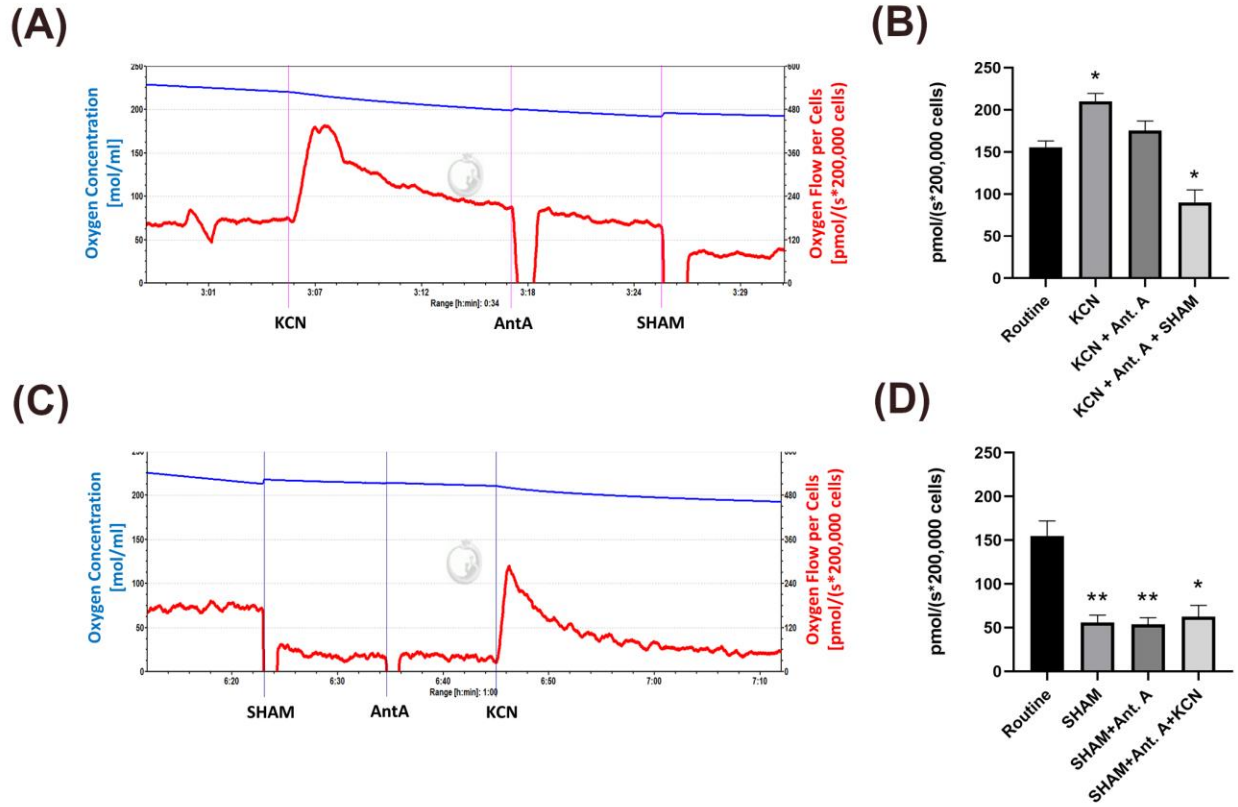
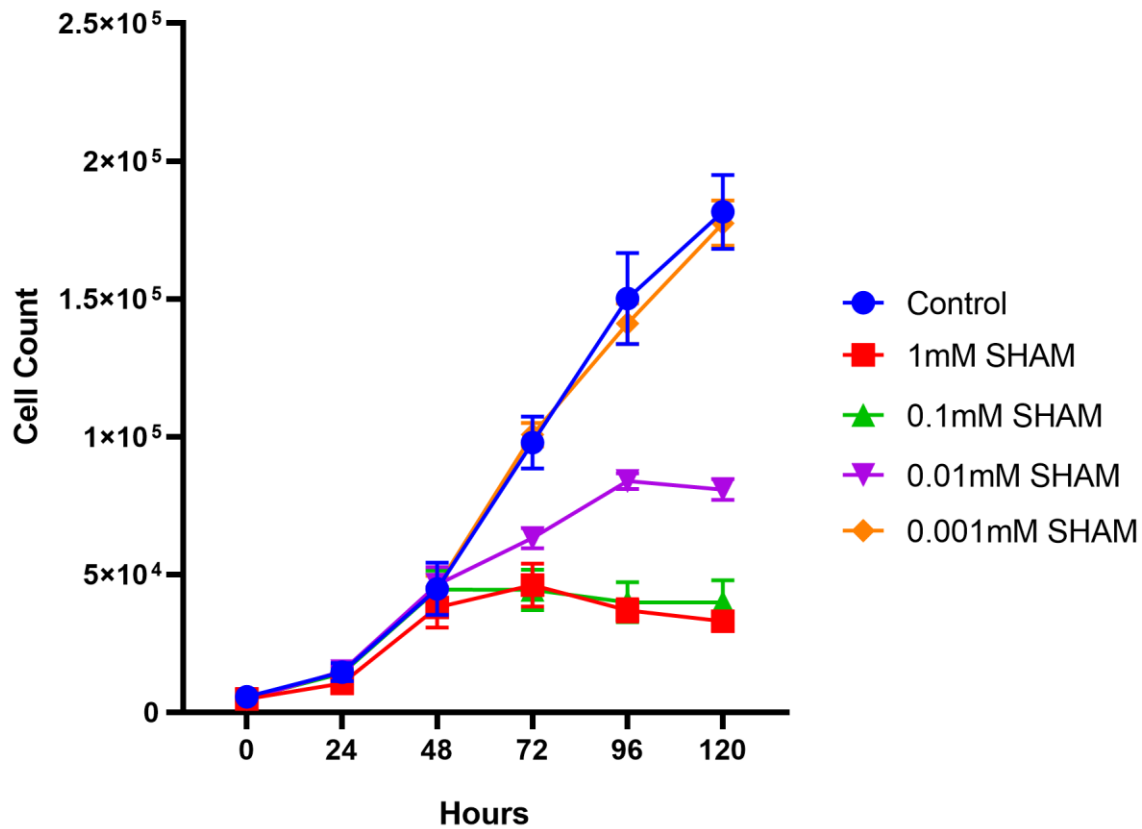


Figure 5. High resolution real-time respirometry reveals cyanide resistant respiration.

N. gruberi cells were subjected to real-time respirometry in the presence of metabolic inhibitors to assess respiration. Cells were added to the chambers without the presence of inhibitors to assess normal respiration, followed by the addition of a mitochondrial complex inhibitor. The order of inhibitors were KCN (complex IV inhibitor) followed by Antamycin A (complex III inhibitor) and SHAM (AOX inhibitor) (A). The experiments were then completed in reverse order (B). KCN did not display decreases in respiration, whereas SHAM significantly reduced respiration. P values; * <0.05, **<0.01

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Figure 6. Growth of *N. gruberi* is reduced in increasing concentrations of SHAM.

Using the JuLI™Stage Live cell monitoring system, we were able to monitor the growth rates of *N. gruberi* cells in varying concentrations of SHAM. Cells were seeded in wells of a 96-well plate and left to grow for 48 hours prior to the addition of SHAM at 1mM, 0.1mM, 0.01mM and 0.001mM concentration. By counting cells, we noticed a significant decrease in cell numbers when challenged with 1 mM and 0.1mM SHAM. In the presence of 0.01mM SHAM we saw a decrease in proliferation rate, peaking at 96 hours. We observed no effect using SHAM at 0.001mM. Error bars are standard error of the mean.

602 **Supplementary Figures**

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604 **Supplementary Figure S1. Sequence alignments of AOX reveals conserved domains**

605 Maximum likelihood tree of AOX homologs in eukaryotes and bacteria (323 sequences, 148 amino
606 acid positions). The tree was inferred with IQTREE using the LG+I+G4 model selected under the
607 BIC criterion. Grey dots correspond to supports higher than 80%. The scale bar corresponds to the
608 average number of substitutions per site.

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610 **Supplementary Figure S2. Sequence alignments of AOX reveals conserved domains**

611 To assess level of conservation between AOXs we aligned ngAOX, nfAOX and nlAOX amino acid
612 sequences against other well characterized AOXs; *Trypanosoma brucei*, *Candida albicans*,
613 *Arabidopsis thaliana*, *Cryptosporidium parvum* and, *Neurospora crassa*. We observed a
614 considerable amount of conservation between all AOXs towards the middle and C-terminal end,
615 where the presence of the alpha helical bundles and di-iron binding domains reside. * denotes key
616 amino acids presented in Table below. † denotes a deviation in conserved amino acids of AOX

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618 **Supplementary Figure S3. Confocal microscopy demonstrating mitochondrial localization of**
619 **AOX.**

620 Additional *N. gruberi* cells demonstrating localization of AOX in their mitochondria. *N. gruberi*
621 cells were treated with mitotracker red prior to fixation and then probed with AOX antibodies
622 (green). Nuclear marker and mtDNA staining is shown in blue (DAPI staining) Our confocal
623 imaging reveals a high degree of co-localization between the mitotracker red signal and the green
624 signal derived from immunoprobng AOX.

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627 **LITERATURE CITED**

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