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The zoospores of the thraustochytrid *Aurantiochytrium limacinum*: transcriptional reprogramming and lipid metabolism associated to their specific functions.

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2 and lipid metabolism associated to their specific functions.

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4 Younès Dello ^{1,3*}, Cécile Maës ^{1*}, Christian Morabito ^{1*}, Martin Schuler ¹, Caroline Bournaud ¹, Riccardo
5 Aiese Cigliano ², Eric Maréchal ¹, Alberto Amato ^{1#} and Fabrice Rébeillé ^{1#}

6 ¹ Laboratoire de Physiologie Cellulaire Végétale, Université Grenoble Alpes, CEA, CNRS, INRA; IRIG-
7 LPCV 38054, Grenoble Cedex 9, France

8 ² Sequentia Biotech Campus UAB, Edifici Eureka Av. de Can Domènech s/n 08193 Bellaterra
9 (Cerdanyola del Vallès), Spain

10 ³ Present address: Institute of Genetic, Environment and Plant Protection, UMR 1349 IGEPP
11 INRA/Agrocampus Ouest Rennes/Université Rennes 1, Domaine de la Motte, BP35327, 35653 Le
12 Rheu cedex, France

13

14 #Corresponding authors

15 *These authors contributed equally to this work

16 Email addresses: fabrice.rebeille@cea.fr (F. Rébeillé), alberto.amato@cea.fr (A. Amato)

17

18 Running title: Transcriptional footprint of *A. limacinum* zoospores

19

20 **Abstract**

21 *Aurantiochytrium limacinum* (Thraustochytriaceae, class Labyrinthulomycetes) is a marine
22 Stramenopile and a pioneering mangrove decomposer. Its life cycle involves a non-motile stage and
23 zoospore production. We observed that the composition of the medium, the presence of amino acids
24 in particular, affects the release of zoospores. Two opposite conditions were defined, one with a cell
25 population mainly composed of zoospores and another one with almost only non-motile cells. *In*
26 *silico* allelic frequency analysis and flow cytometry suggest that zoospores and non-motile cells share
27 the same ploidy level and are diploid. Through a RNA-seq approach, the transcriptional
28 reprogramming accompanying the formation of zoospores was investigated, with a particular focus
29 on their lipid metabolism. Based on a differential expression analysis, zoospores are characterized by
30 high motility, very active signal transduction, an arrest of the cell division, a low amino acid
31 metabolism and low glycolysis. Focusing on lipid metabolism, genes involved in lipase activities and
32 peroxisomal β -oxidation are up-regulated. qRT-PCR of selected lipid genes and lipid analyses during
33 the life span of zoospores confirmed our observations. These results highlight the importance of the
34 lipid dynamics in zoospores and show the metabolic processes required to use these energy-dense
35 molecules as fuel for zoospore survival during their quest of new territories.

36

37 Key words: Fatty acids; GPCR pathway; Lipid metabolism; PUFA synthase; RNA-sequencing; Signal
38 transduction; Thraustochytrids; Zoospores; ω 3-docosahexaenoic acid (DHA).

39 Introduction

40 Mangrove forest environments sustain a composite ecosystem (Isa *et al.*, 2017), with a food web
41 primed by decomposers (Demopoulos *et al.*, 2007). Thraustochytrids are osmo-saprotrophic protists
42 belonging to the phylum Heterokonta, class Labyrinthulomycetes (Cavalier-Smith *et al.*, 1995) and are
43 among the first organisms that colonize and decompose a fallen mangrove leaf (Raghukumar *et al.*,
44 1994). The life cycle of thraustochytrids is characterized by vegetative reproduction and a motile
45 stage, the zoospores (Honda *et al.*, 1998, Iwata *et al.*, 2017, Morabito *et al.*, 2019). The biflagellated
46 zoospores released from sporangia are essential to colonize new environments and propagate the
47 population. Thraustochytrids are nutritionally valuable preys for microzoo- and zoo-plankton,
48 especially because of their high content in very long chain polyunsaturated fatty acids (VLC-PUFAs)
49 (Kimura and Naganuma, 2001 and references therein, Dellerio *et al.*, 2018a). Furthermore, the coastal
50 zone they inhabit is the interface between the land-fixed and the ocean captured carbon, the so-
51 called 'Blue Carbon' (Nelleman *et al.*, 2009). Therefore, thraustochytrids may play a pivotal role in the
52 'mycoloop', a recently described energy pathway based on chytrid zoospores (Kagami *et al.*, 2014).
53 Through the mycoloop refractory or inaccessible carbon can be transferred to the zooplankton
54 (Kagami *et al.*, 2007).

55 Although several organisms living in aquatic environments, such as algae, fungi, oomycetes and
56 bacteria are able to produce zoospores (Agrawal, 2012), the literature concerning their metabolism
57 and physiology is quite scarce. Most of the available studies concerning eukaryotic zoospores were
58 carried out on oomycetes because several oomycetes, such as *Phytophthora infestans*, are plant
59 pathogens and zoospores initiate the infectious interaction with the hosts (Hardham, 2007). This
60 feature has fostered research to decipher the physiology of infective zoospores, with the aim to
61 prevent infection, improve cultivation and reduce crop lost. The release of zoospores from sporangia
62 may depend on several parameters such as light, nutrients, temperature shifts or intracellular pH
63 variations (Hyde *et al.*, 1991, Suzaki *et al.*, 1996, Agrawal, 2012). Once produced, zoospores sense
64 and reach new territories via phototaxis, electrotaxis or chemotaxis (Agrawal, 2012, Swafford and

65 Oakley, 2017). In the last decade, several studies on oomycetes focused on the identification of
66 infection-related factors using transcriptomics approaches (Judelson *et al.*, 2008; Chen *et al.*, 2013,
67 2014; Sun *et al.*, 2017). A panel of predicted genes, including transcription factors, protein kinases,
68 putative signaling proteins, ion channel, or calcium binding proteins were suggested to be involved in
69 the formation, survival and physiology of zoospores (Tani *et al.*, 2004; Ah-Fong *et al.*, 2017).

70 In contrast with oomycetes, little information is available about the zoospore physiology and
71 metabolism of non-infective thraustochytrids. Recently, thraustochytrids have attracted industrial
72 interest for their ability to synthesize high levels of VLC-PUFAs, in particular docosahexaenoic acid
73 (DHA, 22:6), an essential compound for human health (Simopoulo, 2008). Indeed, thraustochytrids
74 have the unique feature to synthesize FA via two enzymatic systems; the classical fatty acid synthase
75 (FAS) involved in the synthesis of relatively short saturated FA chains (16 to 18 carbons) and the PUFA
76 synthase involved in the synthesis of longer unsaturated chains, such as DHA (Morabito *et al.*, 2019).
77 The biotechnological interest towards thraustochytrids has directed efforts to unravel their
78 physiology and metabolism, with the main aim to enhance lipid production (Raghukumar, 2008;
79 Taoka *et al.*, 2009; Aasen *et al.*, 2016). This has prompted genomic and transcriptomic studies (Ma *et*
80 *al.*, 2015; Liu *et al.*, 2016; Bi Z.-Q. *et al.*, 2018; Iwasaka *et al.*, 2018; Liang *et al.*, 2018; Seddiki *et al.*,
81 2018) which provide today new databases for metabolic engineering purposes and for
82 ecophysiological understanding as well.

83 In a recent work, we investigated the ecophysiology of *Aurantiochytrium limacinum* zoospores and
84 the triggers for their formation, settlement and maturation. Zoospores are released upon a transfer
85 to a fresh nutrient-poor medium. They can swim for several days, relying on their energy storage for
86 cell survival and motility (Dellero *et al.*, 2018a). These observations raise questions about the
87 nutritional factors that trigger a shift from a sedentary to a motile state, and about the associated
88 transcriptional reprogramming and metabolic changes. Here we further describe the role of the
89 external medium in the release of zoospores and depict the metabolic and transcriptional signatures

90 associated with the vegetative-to-zoospore transition, with an emphasis on lipid metabolism. Our
91 results reveal that such a transition implies profound transcriptional and metabolic rearrangements.

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92 **Results**

93 *Zoospore release*

94 As previously shown (Dellero *et al.*, 2018a), in a rich medium (R) containing glucose and yeast extract
95 (YE) less than 3 % zoospores were observed at best (D1), whereas in a poor medium (P), in which the
96 glucose and YE concentrations were reduced to 1/40 (Supplementary Table 1), zoospores
97 represented the main cell type (Table 1). A different medium, named R2, was produced in order to
98 rule out the possibility that one or several components of the YE might repress zoosporulation. In the
99 R2 medium YE was replaced by nitrate and phosphate solutions at concentrations comparable to
100 those found in YE (Supplementary Table 1). A six-day-old R-grown culture, i.e. which did not contain
101 any zoospores, was used as inoculum. During the first 24 hours in R2, zoospores were massively
102 produced and represented 92% of the population (Table 1). A high zoospores/non-motile cells ratio
103 was maintained until day 4 (D4). Thereafter, however, zoospore fraction decreased to nil and non-
104 motile cells dominated the population. It can be concluded that one of the YE components may
105 impair the release of zoospores.

106 In order to discriminate whether amino acids (the main source of nitrogen in R) hamper
107 zoosporulation, nitrate was removed from R2 and replaced by a cocktail of amino acids in
108 concentrations that mimic YE (R3 medium, Supplementary Table 1). The zoospore/non-motile cells
109 ratio in R3 was comparable to that recorded in R, i.e. very few zoospores were observed. Inversely,
110 when amino acids were added in a P culture containing almost exclusively zoospores, the non-motile
111 cell occurrence increased at D4 (i.e. 48 hours after the addition of amino acids). At D6, a 3.4 %
112 zoospore/non-motile cells ratio was recorded. Altogether, these results highlight a potential role of
113 amino acids in controlling the initiation/maturation of the motile stage of *A. limacinum*.

114 *Ploidy level of the zoospores and non-motile cells*

115 A variant calling analysis was performed by mapping the transcriptomes of non-motile cells and
116 zoospores produced in the present work against the *Aurantiochytrium limacinum* ATCC® MYA1381™
117 reference genome. 38110 (Supplementary file 1) and 53181 (Supplementary file 2) variants were

118 identified in non-motile cells and zoospores, respectively. Single Nucleotide Polymorphisms (SNPs)
119 represented about 94 % of the variants in both groups. A vast majority of the variants (64 % and 63 %
120 of total variants for non-motile and zoospores, respectively; Supplementary Files 1 and 2) were
121 identified as alternative genotypes, i.e. all the reads in the transcriptome of non-motile cells and
122 zoospores presented the same sequence, different from that in the reference genome. Such sites can
123 be classified as intraspecific polymorphisms between the *Aurantiochytrium limacinum* ATCC®
124 MYA1381™ used as reference and *A. limacinum* CCAP 4062/1 strain. In order to estimate the ploidy
125 level of zoospores and non-motile cells, allele frequency distributions were calculated (Fig. 1A). The
126 peak of 'homozygous variants' (allele frequency = 100) on the right-hand side of the plots (Fig. 1A)
127 actually identifies the intraspecific polymorphic sites described above. Noteworthy, 13829 (36 %) and
128 19854 (37 %) variants were classified as heterozygous (0/1 or 1/0 genotype, Supplementary Files 1
129 and 2) in non-motile cells and zoospores, respectively. The allele density (y-axis in Fig. 1A) is an
130 indication of the number of reads bearing one or the other allele. A Mann Whitney Test was
131 performed to compare the distribution of the allele frequencies of non-motile cells and zoospores
132 and the difference resulted not significant ($p = 0.35$). The portion of the allele frequency curve of the
133 heterozygous variants only (Fig. 1A) is centered on 50 in both samples, therefore, it can be stated
134 that zoospores and non-motile cells share the same ploidy level and that they are both diploid. In
135 order to validate the latter result, flow cytometry was used to measure the quantity of DNA in
136 zoospores vs. non-motile cells.

137 In flow cytometry, light scatter analysis of a fast growing population in R showed a large panel of cell
138 sizes and internal complexity (granulometry) (Fig. 1B). The fluorescence pattern of propidium iodide
139 (PI) of R-grown cells displayed a main peak at an arbitrary value of 280 and a second one
140 representing cells containing about twice as much DNA as the cells forming the first peak (Fig. 1C).
141 The right-hand side of the figure indicates the presence of cells containing even more DNA. Assuming
142 that *A. limacinum* is diploid (as suggested by allele frequency analysis), the first peak would represent
143 diploid mononucleated cells and the second either mononucleated cells in G2 phase or binucleated

144 cells. Because of the presence of multinucleated cells, the interpretation of the following peaks is
145 cumbersome. Each peak could represent either a cell population with n nuclei in G2 phase or cells
146 with $2n$ nuclei in G1 phase. On the contrary, zoospore size and granulometry was more uniform (Fig.
147 1D). PI fluorescence (Fig. 1E) showed a main and relatively sharp peak and a second small peak
148 representing cells with twice as much DNA. By comparing Figures 1C and 1E, it is clear that zoospores
149 did not contain half the DNA than non-motile cells. These results strongly suggest that zoospores are
150 diploid, corroborating the allele frequency analysis (Fig. 1A).

151 *Genome wide expression and transcriptional footprints in zoospores*

152 A differential expression analysis based on RNA-seq data showed that in zoospores 4280 and 3330
153 genes were up- and down-regulated (cut-off arbitrarily set at $\log_2FC = 1$), respectively
154 (Supplementary Table 2). 7610 genes represent about half of the whole genome. RNA-seq differential
155 gene expression analysis was validated by qRT-PCR of 22 selected genes (Supplementary Table 3) on
156 RNA extracted from a completely independent experiment carried out exactly as described for RNA-
157 seq. The Pearson's correlation coefficient ($r = 0.93$) showed a strong positive linear correlation (p -
158 value $< 10^{-5}$) between qRT-PCR and RNA-seq analyses. The strong linear correlation was coupled with
159 a strong monotonic relation between the two sets of results by Spearman's Rho ($r_s = 0.88$, p -value =
160 0.05).

161 From a Gene Ontology Enrichment Analysis (GOEA) (Supplementary Fig. 1), most of the significantly
162 enriched up-regulated GO terms were 'signal transduction' (GO:0007165) and several descendants
163 like 'G-protein coupled receptor signaling pathway' (GO:0007186), or 'signal transducer activity'
164 (GO:0097677) which groups mainly transcription factors. This is indicative of an increased ability of
165 the zoospores to sense and react to their external environment. Zoospores are biflagellated cells and
166 some of the highly enriched GO terms among the up-regulated genes are, not surprisingly,
167 'microtubule associated complex' (GO:0005875), 'dynein complex' (GO:0030286), 'motor activity'
168 (GO:0003774). Among the down-regulated genes, 'translation' (GO:0006412), 'aminoacyl-tRNA ligase

169 activity' (GO:0004812), 'structural constituent of ribosome' (GO:0003735) were the most enriched
170 GO terms, suggesting an arrest of the cell division and a lower turnover of transcripts.

171 *Signal transduction*

172 Genes involved in signal transduction and signaling cascades were strongly expressed in zoospores
173 (Fig. 2A). They included GPCRs (G protein-coupled receptor), with 30 highly up-regulated transcripts
174 encoding for different GPCRs. Interestingly, seven out of these transcripts showed almost no
175 expression in the non-motile cells (fgenes1_pg.9_421, estExt_fgenes1_pg.C_6_t10488,
176 gm1.13383_g, fgenes1_pg.8_348, fgenes1_pg.36_3, fgenes1_pg.3_318, gm1.10294_g,
177 gm1.4442_g), with levels below 20 Transcripts Per Kilobase Million (TPM) compared to values ranging
178 from 696 to 3702 in the zoospores (Supplementary Table 2). In addition, many transcripts encoding
179 the signaling transduction route were up-regulated, e.g. 14 Ras GTPases as well as several proteins
180 linked to the cAMP signaling pathway (present in the G protein item in Fig. 2A; Supplementary Table
181 2).

182 *Cytoskeleton and molecular motors*

183 Thirty transcripts encoding for dynein (present in the intraflagellar transport and flagellar movement
184 items in Fig. 2A), 24 for myosin and more than 35 for kinesin were up-regulated in zoospores (Fig. 2A
185 and Supplementary Table 2). Genes involved in the tubulin-dependent cytoskeleton as well were
186 largely up-regulated, as expected for flagellated cells. These findings, together with the up-regulation
187 of transcripts encoding for proteins involved in the regulation of cellular motors (putative CAP-GLY
188 domain containing linker protein, CLIP (estExt_fgenes1_kg.C_140042)) and actin cytoskeleton
189 (formins), reveal a highly dynamic cytoplasm. Actin-Myosin transportation are important for the
190 perception of external stimuli in higher plants as well as in microalgae (Duan and Tominaga, 2018).

191 *DNA replication and transcription*

192 DNA replication was reduced in zoospores (Fig. 2B), with eight down-regulated transcripts encoding
193 for DNA polymerase, five transcripts encoding for DNA topoisomerase and 11 transcripts encoding
194 for histones (Supplementary Table 2). Genes encoding for RNA polymerases, helicases or involved in

195 chromatin remodeling were also significantly down-regulated (Fig. 2C), suggesting that DNA
196 transcription was lowered. Transcripts involved in the translation process were down-regulated as
197 well. These results suggest that zoospores do not divide, as already suggested by the flow-cytometry
198 data.

199 *Nitrogen and amino acid metabolism*

200 Genes involved in nitrogen and amino acid metabolisms were highly differentially expressed (Fig. 2D).
201 About 20 genes involved in nitrate and ammonium transport and nitrogen assimilation (such as
202 nitrate (fgenes1_pm.38_1) and nitrite reductases (e_gw1.38.18.1) or glutamate
203 (estExt_Genewise1.C_17_t10014) and glutamine synthetases (fgenes1_pm.1_555,
204 fgenes1_kg.1_553_isotig07286, estExt_Genewise1.C_17_t10014e_gw1.5.701.1)) were strongly up-
205 regulated (Supplementary Table 2), possibly denoting the nitrogen deficient medium in which
206 zoospores were produced. In contrast, most of the genes related to the metabolism and turnover of
207 amino acids (amino acid biosynthesis and degradation, urea cycle) were down-regulated. This
208 presumably indicates a reduced need of protein synthesis because of the arrest of the cell division.
209 Genes identified as 'amino acid transport' were predominantly down-regulated, but a few of them
210 were significantly up-regulated with \log_2FC ranging from 4.4 (fgenes1_pg.18_302) to 1.6
211 (e_gw1.22.185.1) (Supplementary Table 2). Their function and localization were not further
212 investigated here.

213 *Carbohydrate metabolism*

214 Genes involved in key steps of the glycolysis (Fig. 2E) were down-regulated, such as triosephosphate
215 isomerase (fgenes1_kg.10_22_isotig07893), 3-phosphoglycerate kinase enolase
216 (estExt_fgenes1_kg.C_30041), and pyruvate kinase (estExt_fgenes1_kg.C_90258) (Supplementary
217 Table 2). The same pattern was observed in several genes coding for the Krebs cycle enzymes,
218 including components of the pyruvate dehydrogenase complex, succinate dehydrogenase
219 (fgenes1_pm.8_94), citrate synthase (fgenes1_pg.13_25), isocitrate dehydrogenase
220 (estExt_fgenes1_kg.C_210136), the T protein of the glycine decarboxylase complex, as well as

221 oxidases such as cytochrome c oxidase (fgenes1_pm.10_34) and alternative oxidase (gw1.27.100.1).
222 Taken as a whole, these results suggest that carbohydrate catabolism and respiratory activities were
223 down-regulated in zoospores compared with non-motile cells. This assumption was confirmed by the
224 direct measurement of the respiratory activities (Table 2). In R-grown cells, oxygen consumption at
225 D1 considerably augmented, in correspondence to the resuming of cell divisions. Conversely,
226 zoospore respiration slowly declined with time (Table 2).

227 *Fatty acid synthesis and degradation*

228 FAS is involved in the synthesis of 16:0 which can be thereafter elongated and desaturated to
229 produce VLC-PUFAs; PUFA synthase directly produces VLC-PUFAs. Only a few genes involved in the
230 biosynthesis of FAs were differentially expressed in zoospores, at least at D1. The interpretation of
231 the results to decipher how the biosynthetic pathway to VLC-PUFAs is regulated in zoospores is
232 challenging (Fig. 2F). Two desaturases (estExt_fgenes1_kg.C_1_t10086; gw1.29.176.1) were up-
233 regulated whereas e_gw1.11.243.1 was down-regulated, and the only elongases (e_gw1.5.731.1;
234 estExt_fgenes1_kg.C_60194) found in *A. limacinum* were down-regulated (Supplementary Table 4).
235 Desaturases and elongases are likely involved in the FAS pathway (for a review, see Morabito *et al.*,
236 2019), but *FAS-1* (e_gw1.21.366.1) was not differentially expressed. Likewise, subunits A and B of the
237 PUFA synthase (*PUFA-A* fgenes1_pg.14_#_251, *PUFA-B* estExt_fgenes1_kg.C_140136) were not
238 differentially expressed, and subunit C (*PUFA-C* estExt_fgenes1_kg.C_190026) was slightly down-
239 regulated (Supplementary Table 4).

240 FA degradation takes place in both mitochondria and peroxisomes via β -oxidation. The hydrolysis of
241 FA esterified to glycerolipids is mediated by lipases. About 30 genes were identified as lipase or
242 lipase-like, out of which 17 were up-regulated and 11 down-regulated. Their expression levels
243 showed that lipases were globally upregulated, suggesting that glycerolipid degradation was
244 potentially increased as a whole (Fig. 2F and Supplementary Table 4). Most of the up-regulated
245 lipases belong to class 3 lipases, i.e. involved in the hydrolysis of ester bonds of TAGs or DAGs.
246 However, phospholipases A (*Pla1* e_gw1.1.605.1, *Pla2* e_gw1.24.47.1), B (*Plb* e_gw1.6.53.1) and D

247 (*Pld2* estExt_Genewise1Plus.C_1_t20024, *Pld3* gm1.46_g), involved in the degradation of
 248 phospholipids, were either non differentially expressed or down-regulated. This suggests that only
 249 storage lipids were part of the catabolic processes, whereas membrane-bound glycerolipids are not.
 250 FAs released from triacylglycerids (TAGs) are activated as acyl-CoA by acyl-CoA ligases before
 251 entering β -oxidation. An overview of the transcripts involved in FA degradation (Fig. 2F) suggests a
 252 global down-regulation. However, a closer inspection indicates that the peroxisomal pathway was
 253 up-regulated. Indeed, out of the three genes encoding for Long chain Acyl-CoA ligase, *Acs/2*
 254 (*fgenes1_kg.4_#_23_#_isotig03564*) was the only presenting a peroxisomal localization and the only
 255 up-regulated one. In addition, most of the transcripts involved in the peroxisomal β -oxidation (acyl-
 256 CoA ABC transporter; acyl-CoA oxidase *Acox1*, *Acox3*; bifunctional enzyme *Ehhadh1*, *Ehhadh3*;
 257 ketoacyl-CoA thiolase *Acaa1*, *Acaa2*, *Acaa4*) were globally up-regulated (Supplementary Table 4). The
 258 mitochondrial β -oxidation did not show a clear pattern: out of the five genes encoding the acyl-CoA
 259 dehydrogenase (*Acad*), three were down-regulated, one was up-regulated and one did not
 260 significantly vary (Supplementary Table 4). Altogether, these results suggest that TAGs are more
 261 actively degraded in the zoospores, liberating FAs that are conveyed preferentially to the peroxisome
 262 to be oxidized.

263 *Lipid dynamics in zoospores and non-motile cells*

264 The expression levels of five genes involved in lipid metabolism (*Tagl3* e_gw1.7.228.1, *Acox1*
 265 *fgenes1_kg.9_#_301_#_isotig03404*, *Acad1* estExt_fgenes1_kg.C_330018, *FAS-1*, *PUFA-A*) were
 266 followed for six days in zoospores and non-motile cells (Fig. 3). A $\Delta\Delta C_T$ analysis was performed by
 267 comparing the expression level of each transcript at day 1 (D1), day 2 (D2), day 4 (D4), and day 6 (D6)
 268 with the expression level of the same transcript at D1 in R medium.

269 The TAG lipase 3 (*Tagl3*) was strongly up-regulated at D1 in zoospores and represented at this time
 270 point the most expressed TAG lipase (Supplementary Table 4). Its expression level did not vary
 271 sensibly thereafter (Fig. 3A), suggesting that TAGs were constantly degraded throughout the whole
 272 zoospore life. *Tagl3* expression in non-motile cells increased progressively to reach a maximal value

273 at D4 and D6 (Fig. 3B), i.e. when TAGs accumulate (see below). Highly likely, TAG synthesis and
274 degradation may occur simultaneously in non-motile cells.
275 β -oxidation is initiated in peroxisomes and mitochondria through the activities of *Acox* and *Acad*,
276 respectively. The peroxisomal *Acox1* (Dellero *et al.*, 2018b) and mitochondrial *Acad1* (Dellero *et al.*,
277 2018b) transcripts were the most expressed *Acox* and *Acad* in both cell types (Supplementary Table
278 4). *Acox1* was slightly up-regulated in zoospores at D1 and increased thenceforward. Similarly, in
279 non-motile cells, *Acox1* expression increased with time, but at a much lower extent than in
280 zoospores. In contrast, the *Acad1* expression was down-regulated in zoospores, especially at D6,
281 whereas it did not significantly change in non-motile cells. These results emphasize again the role of
282 peroxisomes vs. mitochondria for the oxidation of FAs in zoospores.

283 In thraustochytrids, the synthesis of FAs is driven by two enzymatic systems, FAS and PUFA synthase
284 (see Introduction). In zoospores, *FAS-1* and *PUFA-A* genes showed no significant regulation at D1 and
285 D2 compared to non-motile cells (Fig. 3). In the following days, however, their expression decreased
286 in zoospores, especially *PUFA-A*. In contrast, *FAS-1* and *PUFA-A* expression increased over time in
287 non-motile cells. In both cell types, *PUFA-A* expression varied significantly more than *FAS-1*, possibly
288 indicating the higher relevance of the PUFA synthase synthetic pathway in the production of PUFAs.

289 *Fatty acid analyses*

290 To correlate gene expression with metabolic changes, the FA content was analyzed during a six-day
291 growth in both P and R media (Fig. 4). The major FA species (16:0, 22:5, 22:6) in zoospores increased
292 at D1 (the transfer in P medium occurred on D0) (Fig. 4A), then declined thereafter. In R-grown cells
293 all FA species declined the first day (Fig. 4B) then constantly raised thenceforth to get almost to the
294 initial value at D6. This illustrates the strong relationship between nitrogen availability and lipid
295 metabolism described earlier (Dellero *et al.*, 2018a). In R, cells first consumed their TAGs, then, upon
296 nitrogen limitation, TAGs were synthesized again. In P, nitrogen was limiting right at the beginning of
297 the experiment and TAGs were immediately synthesized until glucose was exhausted, then were
298 consumed to sustain the energy demand. The distribution (%) of main FAs did not vary much (Figs.

299 4C, 4D), although slight differences could be still observed between the two cell populations. Indeed,
300 zoospores showed lower 15:0 and higher 16:0 contents. Odd-numbered FAs (mainly 15:0) are
301 produced from branched-chain amino acids because their degradation leads to propionyl-CoA
302 (Crown *et al.*, 2016) that can replace acetyl-CoA for the synthesis of FAs. In R, the presence of YE, rich
303 in amino acids, corroborates the hypothesis that 15:0 synthesis is driven by the propionyl-CoA
304 derived from branched-chain amino acids. The lack of 15:0 in zoospores might be due to the reduced
305 YE concentration in P medium. Another intriguing difference is the accumulation in zoospores of 18:0
306 at D1 and 18:1 at D6. The accumulation along the zoospore life span of 18:1 could be due to a
307 residual desaturation activity of 18:0, although no $\Delta 9$ -desaturase was found in the genome of *A.*
308 *limacinum* (Dellero *et al.*, 2018b).
309 The increase of lipids in P-grown cells at D1 raises the question whether the sporangium or the
310 zoospore is responsible for the recorded lipid accumulation. The release of zoospores and lipid
311 accumulation during the first 24 hours were compared. The release of zoospores started about 8
312 hours after the transfer in P, and rapidly raised to almost 100 % of the population at 24 hours (Fig.
313 4E). The FA content in P-grown cells did not vary during the first 12 hours (Fig. 4F), then it nearly
314 doubled at 24 hours. Thus, FAs accumulated after the release of zoospores was initiated, suggesting
315 that they could originate from *de novo* synthesis.

316 Discussion

317 Zoospores were observed mostly in culture media deprived of amino acids. A nitrogen deficiency
318 cannot explain alone the production of zoospores because replacement of amino acids by NO_3^-
319 triggered the release of zoospores during the first days of culture and high biomass accumulation.
320 Amino acids could play a specific role in the life cycle of thraustochytrids. Indeed, thraustochytrids
321 feed on decaying mangrove leaves (Raghukumar *et al.*, 1995), and protein degradation in decaying
322 leaves likely releases amino acids in the close vicinity of the leaf itself. Because nitrogen availability is
323 one of the prime factor limiting cell growth (Alongi, 2009; Rao *et al.*, 1994), the presence or absence
324 of nitrogen-rich organic compounds might be one of the switches controlling the release of

325 zoospores from zoosporangia. Oomycete zoospores reach and settle in new territories via
326 chemotaxis, following attractants such as amino acids (Hardham, 2007; Swafford and Oakley, 2017).
327 Similarly, it was demonstrated that zoospores of different thraustochytrid species show a positive
328 chemotactic response to glutamic acid and pectin (Fan *et al.*, 2002). This suggests a specific ability of
329 *Aurantiochytrium* zoospores to detect the presence of amino acids in the external environment
330 (Dellero *et al.*, 2018a). Indeed, eleven genes involved in amino acid transport were down-regulated
331 and four were up-regulated with fgenesh1_pg.18_302 showing a very low expression level in non-
332 motile cells. In zoospores its expression strongly increased with a log₂FC of 4.4.
333 The ploidy of *A. limacinum* zoospores and non-motile cells was investigated via two different
334 approaches, namely the distribution of SNPs through the whole exome and flow cytometry. Both
335 methods suggest a diploid status. Flow cytometry experiments showed that the main peak of
336 fluorescence was much sharper in zoospores (Fig. 1E) than in non-motile cells (Fig. 1C), suggesting
337 that zoospores were in G1 phase and that division/DNA replication was blocked. The latter
338 hypothesis is in agreement with transcriptomic data. Diploidy of *A. limacinum* zoospores
339 corroborates the results reported for *A. acetophilum* for which sexual reproduction has been
340 described (Ganuza *et al.*, 2019). In *A. acetophilum* biflagellated cells produced by specialized
341 sporangia (Type II sporangia) can act as gametes. The physico-chemical as well as physiological
342 conditions triggering sexual reproduction were not elucidated and the ploidy level of such 'putative
343 gametes' was not tested (Ganuza *et al.*, 2019).
344 Differential gene expression analyses indicate a profound reprogramming of in zoospores. The
345 transcriptomic data were summarized into a simplified scheme representing some aspects of the
346 metabolism (Fig. 5). Zoospores are potentially characterized by their motility, highly active signal
347 transduction, a low DNA replication activity, a high nitrogen transport activity but a low amino acid
348 metabolism, a low carbohydrate catabolism, high lipase activities and a high β -oxidation of FAs in
349 peroxisomes.

350 G proteins were markedly up-regulated. They are often associated with membrane-spanning
351 receptors (Weis and Kobilka, 2018), and it is possible that the increase of expression level connotes
352 the pioneering activity of zoospores. Indeed, zoospores are likely released to explore new territories,
353 implying a high sensitivity to probe their environment in order to find new substrates or leaves. G
354 proteins also regulate, among others, transcription, cellular differentiation, secretion and motility
355 (Neves *et al.*, 2002). As expected for flagellated cells, most of the genes involved in the cytoskeleton
356 dynamics were strongly up-regulated. The signaling pathway of GPCRs is spread through the whole
357 living kingdom (de Mendoza *et al.*, 2014), e.g. it has been demonstrated that diatoms sense the
358 presence of predators via the GPCR signal transduction pathway (Amato *et al.*, 2018) and that in the
359 rhizarian protist *Plasmodiophora brassicae* GPCR signal transduction pathways underwent a robust
360 expansion and plays a pivotal role in the germination of resting spores (Bi K. *et al.*, 2019).
361 Furthermore, in oomycetes a very peculiar GPCR system evolved (Meijer and Govers, 2006).
362 Conversely, genes involved in DNA replication (such as polymerase, topoisomerase, replication factor
363 etc.) were markedly down-regulated, suggesting that zoospores were blocked in the G1 phase. The
364 arrest of the cell division was presumably associated with a decrease of several metabolic activities.
365 Noteworthy, the amino acid metabolism was largely down-regulated, indicating a potential decline of
366 protein synthesis and turnover. This is true for carbohydrate catabolism as well, since many genes
367 involved in the glycolysis and the Krebs cycle showed a decreased expression in zoospores.
368 Carbohydrate catabolism is strongly connected to the energy metabolism. Thus, results presented
369 here indicate that zoospores have a lower energy demand than dividing cells, despite their swimming
370 activity, an assumption supported by the direct recording of their respiratory activities.
371 The differential expression of genes involved in lipid metabolism did not show a clear tendency at D1,
372 except for lipase activities, which was up-regulated. In zoospores, the time point D1 corresponded to
373 a metabolic turn, where the FA metabolism shifted from synthesis to catabolism. This may explain
374 the difficulty to correlate gene expressions and FA analyses. Nevertheless, following the expressions
375 of key genes for either FA synthesis or degradation during the whole growth experiment, gene

376 expressions and FA analyses correlated well. The gene expression of *PUFA-A* always varied more
377 strongly than *FAS-1* although 22:6 and 16:0, their respective products, did not. A very fast turnover of
378 either the transcripts or the proteins may be reason of such observation. Indeed, the level of
379 transcripts of the *PUFA-A* (fgenes1_pg.14_251) in non-motile cells at D1 (about 56000 TPM) was
380 one of the highest compared to other genes involved in lipid synthesis. A 32-fold increase in
381 expression ($\log_2FC = 5$) at D6 in non-motile cells was observed, strongly suggesting that a very high
382 number of transcripts is required to sustain a high enzymatic activity. In zoospores, FA catabolism
383 was associated with an increased expression of the the peroxisomal β -oxidation genes, whilst the
384 mitochondrial β -oxidation did not follow the same pattern. In many (but not all) organisms,
385 peroxisomes oxidize very long FA chains down to a certain chain length (Reddy and Hashimoto,
386 2001). The products of the peroxisomal β -oxidation are then shuttled to mitochondria for a complete
387 oxidation to CO_2 and H_2O (Wanders *et al.*, 2016). In thraustochytrids, 16:0 β -oxidation would be
388 initiated in peroxisomes because the 16:0 and 22:6 content (in percentage, Fig. 4C) in zoospores
389 declined at comparable pace.

390 *Potential differences between oomycete and thraustochytrid zoospores*

391 One of the critical points in the transition from the sedentary to the motile state is the synthesis of
392 flagella. In oomycetes, zoosporogenesis is often induced by a cold shock, and flagella are synthesized
393 from basal bodies at the apex of the nucleus (Hardham, 2007). Zoospores are released upon
394 formation, and the whole process takes about one hour (Hyde *et al.*, 1991). *A. limacinum* zoospores
395 appear eight hours after transfer in P medium (Fig. 4E). Assuming that zoosporogenesis is induced
396 soon after the transfer, zoospore formation and release appear slower in thraustochytrids than in
397 oomycetes, suggesting that different processes could be involved.

398 Oomycete zoospores can swim for hours or even days (Hardham, 2007; Kagda *et al.*, 2018), like *A.*
399 *limacinum*'s. Thus, in both organisms, zoospore survival may depend on the endogenous energy
400 stores. Oomycetes supposedly store energy in mycolaminarin or lipids. Mycolaminarin is a (1,3,1,6)-
401 β -glucan polysaccharide that may represent up to 13% of the dry weight in oomycetes (Du and

402 Mullins, 1998; Lee and Mullins, 1994). The lipid content in the oomycete *Phytophthora infestans* is
403 rather reduced, representing at best 5% of the dry weight, with TAGs accounting for half of the total
404 (Griffiths *et al.*, 2003). This is a striking difference with *A. limacinum* where TAGs may represent up to
405 30% of the dry weight (Dellero *et al.*, 2018b), suggesting that the energetic metabolisms in these two
406 organisms rely on different pathways. Interestingly, in *P. infestans*, a taurocyamine kinase
407 (phosphagen kinase of the creatine kinase family) was found to be highly expressed and targeted
408 towards flagellar axonemes during zoosporogenesis, possibly to fulfill the ATP demand associated
409 with the flagellar movement (Kagda *et al.*, 2018). A BLAST search on *A. limacinum* genome identified
410 three genes of the creatine kinase family homologous to the *P. infestans* gene
411 (estExt_fgenes1_kg.C_140197, fgenes1_kg.3_231_isotig05435, fgenes1_kg.12_175_isotig10864)
412 and up-regulated in zoospores, suggesting that a similar function may be hypothesized in *A.*
413 *limacinum*. Recently, a differential expression analysis of the of *P. infestans* zoospores vs. mycelia,
414 the vegetative dividing stage (Ah-Fong *et al.*, 2017) revealed that, like in *A. limacinum*, signal
415 transduction and flagellar proteins were up-regulated, while many metabolic pathways, including
416 glycolysis, TCA cycle and amino acid metabolism were down-regulated. In contrast with results
417 presented here, oomycete zoospores displayed a down-regulation of the β -oxidation and higher
418 levels of transcripts involved in DNA replication. Although the pathways that generate energy are
419 probably different, it is surprising that DNA replication could increase in non-dividing cells. It was
420 postulated that the developmental program of *P. infestans* zoospores anticipates the need to resume
421 replication after encystment and maturation (Ah-Fong *et al.*, 2017). This was obviously not the case
422 for *A. limacinum* zoospores.

423 Taken as a whole, the present work shows for the first time that *A. limacinum* zoospores are mainly
424 diploid and that zoosporulation is a complex event depending on external factors, among which the
425 lack of amino acids could play an important role. Here we highlight how the transcriptional
426 reprogramming that occurs in *A. limacinum* zoospores, contributes to the metabolic changes
427 required to migrate, probe their environment and survive; three essential skills to find new territories

428 and propagate the population. The transcriptional signature of the zoospore reveals the main role
429 played by the G-protein signal transduction pathway in perceiving the external environment,
430 although the actual mediators are still unknown. Lipid metabolism plays an essential role in providing
431 the energy required to swimming for several days and the exploration of new areas.
432

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433 **Experimental procedures**

434 *Strain and media*

435 CCAP 4062/1 strain was collected in Mayotte island (Indian Ocean, 12°48'51.8''S, 45°14'21.7''E) and
436 routinely cultivated at 20 °C in 250 ml Pyrex® Erlen-Meyer flasks filled with 50 mL of R medium
437 (Supplementary Table 1) (Dellero *et al.*, 2018b) with 100 rpm orbital shaking. For all experiments, six
438 day-old axenic cultures grown on R were transferred to fresh culture media at an initial cell
439 concentration of 5×10^5 cells·mL⁻¹.

440 The R and P media used in the experiments presented here were prepared as described in Dellero *et*
441 *al.* (2018a, b); the R2 and R3 recipes are reported in Supplementary Table 1, along with the recipes of
442 R and P. All the culture media were prepared using autoclaved MilliQ water.

443 Cell enumeration was performed with a Malassez hemocytometer (ca. 100 to 200 cells counted per
444 sample) under a Zeiss AxioScopeA1 (Carl Zeiss SAS, Oberkochen, Germany) microscope. For zoospore
445 enumeration, a sample of 2 mL was fixed with a drop of 2.5 % glutaraldehyde to immobilize
446 zoospores. All the experiments were run in triplicate.

447 *RNA extraction and sequencing*

448 For RNA-seq experiments, a six-day-old culture was inoculated in triplicate in 50 mL of either R or P
449 media, at an initial cell concentration of 5×10^5 cells·mL⁻¹. After 24 hours 1.5×10^7 cells were harvested
450 by centrifugation, snap frozen in liquid nitrogen and then stored at -80 °C until use. RNA was
451 extracted using the TRI Reagent (Sigma Aldrich) as described by Amato *et al.* (2017). RNA extracted
452 from biological triplicates of R- and P-grown cultures were sent out for Illumina sequencing. Libraries
453 were produced and processed following the manufacturer's instructions and sequenced on single-
454 end 75 bp mode on NextSeq500 (Illumina, San Diego, CA). The CASAVA 1.8.2 version of the Illumina
455 pipeline was used to process raw data for both format conversion and de-multiplexing.

456 *Bioinformatics analyses of RNA-seq data*

457 Raw reads were processed with FASTQC
458 (<https://www.bioinformatics.babraham.ac.uk/projects/fastqc/>) and BBDuk (<https://jgi.doe.gov/data->

459 and-tools/bbtools/) in order to check quality and remove low quality bases and adapters. A minimum
460 quality of 25 and a minimum read length of 35 are required. High quality reads were then mapped
461 against the *Aurantiochytrium limacinum* ATCC® MYA1381™ reference genome
462 (<https://genome.jgi.doe.gov/Aurli1/Aurli1.home.html>) with STAR (Dobin *et al.*, 2012). FeatureCounts
463 (Liao *et al.*, 2013) was used to perform read summarization at gene level, only reads with quality
464 higher than 30 were used. In addition, strand-specific and paired-end mode are included. Statistical
465 analyses and plots were generated with R software. Lowly expressed genes were filtered out with the
466 HTSFilter package (Rau *et al.*, 2013), then a differential expression analysis was performed using
467 edgeR (Robinson *et al.*, 2009). Genes with an FDR less than or equal to 0.05 were considered
468 significantly differentially expressed. The lists of up- and down-regulated genes were used to perform
469 Gene Ontology Enrichment Analysis (Du *et al.*, 2010) using in-house scripts. The assembled and
470 annotated transcriptome was submitted to NCBI SRA with the accession number PRJNA590015.

471 *Estimation of the heterozygosity level*

472 The mapping files (BAM) obtained by aligning the trimmed RNA-seq reads from the six samples were
473 processed with the Opossum pipeline (Oikkonena and Lise, 2017) in order to remove reads with low
474 quality mapping (MAPQ < 40) and duplicates and to split reads mapping across introns. The three
475 replicates of the R-grown cultures were pooled and the same was done with the three replicates of
476 the P-grown cultures in order to obtain two final BAMs. The final mapping files were then analyzed
477 with Platypus (Rimmer *et al.*, 2014) in order to perform variant calling. Only variants with a depth of
478 at least 10 reads, minimum base quality of 30, a minimum posterior probability of 30 and classified as
479 PASS were considered. Allele frequencies were calculated as the ratio between the NV and NR fields
480 of the VCF file.

481 *Validation of RNA-seq data and differential gene expression analyses*

482 qRT-PCR validation of RNA-seq data was carried out on a completely independent experiment as
483 described above for RNA-seq. RNA samples were reverse transcribed using the SuperScript IV VILO
484 Mastermix with ezDNase kit (ThermoFisher) according to the manufacturer's instructions. Reactions

485 were run in a final volume of 10 μ L containing 10 ng of cDNA, 5 μ L of Power SYBR[®] Green PCR Master
486 Mix (Applied Biosystems, ThermoFisher) and 600 nM of each primer. Reactions were performed in a
487 CFX Connect[™] Real-Time System (BioRad[®]) with the following program: initial denaturation step at
488 95 °C for 10 minutes; 40 denaturation-amplification-elongation cycles (95 °C, 10 s; 55 °C, 10 s; 72 °C,
489 30 s), followed by melting curve assessment (65 °C to 95 °C, with a 0.5 °C increment). All primer
490 sequences are available in Supplementary Table 3. Transcript levels were normalized against the
491 geometric mean of three reference genes, with similar expression in both conditions: Pacifastin
492 (estExt_fgensch1_kg.C_160075), Cystein desulfurase NFS1 (estExt_fgensch1_kg.C_30063), Protein
493 involved in Snf1 protein kinase complex assembly (gw1.10.847.1) (Supplementary Table 5).
494 For the gene differential expression experiment along a six day-growth, a six-day-old culture grown
495 on R was inoculated in 50 mL of either R or P fresh culture media at an initial cell concentration of
496 5×10^5 cells·mL⁻¹. 1.5 mL were gathered by centrifugation at the following time points day 1 (D1), day
497 2 (D2), day 4 (D4), and day 6 (D6). RNA was extracted and reverse transcribed as described above.
498 qRT-PCR was performed as depicted above. Five genes were analyzed; *Tagl3*, *Acox1*, *Acad1*, *FAS-1*,
499 *PUFA-A*. The list of primer sequences is reported in Supplementary Table 5.
500 Gene abbreviations follow Dellerio *et al.* (2018b).
501 The differential expression analyses and statistics were performed using the Pair Wise Fixed
502 Reallocation Randomisation Test method developed in the Relative Expression Software Tool REST[®]
503 (Pfaffl *et al.*, 2002). Biological triplicates and technical triplicates of each reaction were performed.
504 *Flow cytometry, respiratory activities, lipid extraction and fatty acids analyses*
505 To reduce variability, a six-day old R-grown culture was inoculated at 5×10^5 cells·mL⁻¹ in 50 mL of P
506 medium to induce zoospore formation. After 24 hours, 200 μ L of the culture were spread onto a 1 %
507 agar-R plate. One of the single colonies was picked and cultivated in R medium for six days. Cells
508 were inoculated in triplicate into 50 mL of either fresh R or fresh P media at a concentration of 5×10^5
509 cells·mL⁻¹. R and P cultures were harvested at time points 15 hours and 24 hours, corresponding to
510 the production peaks of mononucleated non-motile cells and zoospores, respectively (Dellerio *et al.*,

511 2018a). Three million cells were collected from each sample by centrifugation (5 minutes at 3500 ×g
512 for R cultivated cells and 7000 ×g for P cultivated cells). Pellets were washed with 1 mL of PBS-EDTA 2
513 mM, and then fixed with 500 µL of 70 % ethanol for at least 30 minutes at room temperature. After
514 fixation, cells were washed twice with 1 mL of PBS-EDTA 2 mM and resuspended in 500 µL of PBS-
515 EDTA 2 mM. Five microlitres of propidium iodide (PI) and 5 µL of RNase I were added and cells were
516 incubated 30 minutes in the dark. All the stained samples were analyzed with a BD FACS Calibur
517 (Benton Dickinson) flow cytometer (excitation 488 nm, 585/42 emission filter).

518 Respiration was measured using a Clark-type oxygen electrode (Hansatech, Oxygraph) at 20°C.
519 Electrode was first calibrated using media saturated with either air or argon for the 100 % and the 0
520 %, respectively. Oxygen consumption was directly monitored in 1 mL of culture medium.

521 Lipids were extracted according to Folch *et al.* (1957). FAs were converted into methyl esters (FAME),
522 then analyzed by gas chromatography (GC-MS/FID) on a BPX70 (SGE) column as previously described
523 (Dellero *et al.*, 2018a, b), using 21:0 as internal standard.

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528

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710 **Figure legends**

711 **Figure 1:** The ploidy of *Aurantiochytrium limacinum* cells. (A) Density plot of allele frequencies
 712 calculated in non-motile cells and zoospores. Flow cytometry of non-motile cells (B and C) and
 713 zoospores (D and E). Light scatter analysis of cell populations showing cell size and granulometry (B
 714 and D). Propidium iodide fluorescence (C and E).

715 **Figure 2:** Differentially expressed genes (cut-off $\text{Log}_2\text{FC} = |1|$) for selected items. (A) signal
 716 transduction and cell motility; (B) DNA replication; (C) DNA transcription; (D) nitrogen and amino acid
 717 metabolisms; (E) carbohydrate metabolism; (F) lipid metabolism. GPCR: G protein-coupled receptors;
 718 RGS: regulator of G protein signaling.

719 **Figure 3:** Relative expression of key genes involved in FA synthesis and FA degradation in zoospores
 720 (A) and non-motile cells (B). *Tagl3* (TAG lipase 3) was chosen as a representative of TAG degradation.
 721 *FAS-1* and *PUFA-A* were chosen as representatives of FA synthesis. *Acad1* (acyl-CoA dehydrogenase,
 722 β -oxidation in mitochondria) and *Acox1* (acyl-CoA oxidase, β -oxidation in peroxisomes) were chosen
 723 for FA degradation. Dotted lines indicate significance threshold. Error bars indicate standard
 724 deviation of three independent biological repeats.

725 **Figure 4:** FA content in P-grown (red) and R-grown (green) cells. A and B) FA content, expressed as
 726 nmoles FA per mg dry weight. C and D) FA distribution, expressed as % of total FAs. E) Number of
 727 zoospores during the first 24 hours expressed as % of the total cell number. F) FA content evolution
 728 during the first 24 hours. Error bars indicate standard deviation of three independent biological
 729 repeats.

730 **Figure 5:** A schematic representation of *Aurantiochytrium limacinum* zoospore differential expression
 731 analysis based on RNA-seq. Metabolic pathways are illustrated with different colors: pink for DNA
 732 replication; purple for FA synthesis; turquoise for elongation and desaturation of FAs; yellow for
 733 lipase activities; light orange for signal transduction and G protein pathways; brown for
 734 mitochondrial β -oxidation; grey for peroxisomal β -oxidation; bright orange for glycolysis and the
 735 Krebs cycle; green for nitrogen and ammonium uptake; blue for amino acid metabolism; and dark

736 blue for flagellar activity and the cytoskeleton dynamics. The colors of full arrows indicate the \log_2FC
737 value (color scale on the right bottom of the figure) for each gene involved in lipid metabolism. The
738 empty arrows indicate the trend of the metabolic pathway. Abbreviations: SFA, saturated FAs; PUFA,
739 polyunsaturated FAs; TAG, triacylglycerol; DAG, diacylglycerol; MAG, monoacylglycerol. Gene
740 abbreviations (in alphabetic order): abcd, peroxisomal ABC transporter; ACC, acetyl-CoA carboxylase;
741 Acaa, 3-ketoacyl-CoA thiolase; Acad, acyl-CoA dehydrogenase; Acox, acyl-CoA oxidase; Acsl, long
742 chain acyl-CoA ligase; CACT, carnitine acylcarnitine transferase; CPT, carnitine palmitoyl transferase;
743 Δ elo, fatty acid Δ elongase; Δ FAD, Δ fatty acid desaturase; Dagl, diacylglycerol lipase; Ech, enoyl-CoA
744 hydratase; Ehhadh, bifunctional enzyme; Fas, fatty acid synthase; Hadh, 3-hydroxyacyl-CoA
745 dehydrogenase; HADH, trifunctional enzyme; Magl, monoacylglycerol lipase; Ω 3FAD, Ω 3 fatty acid
746 desaturase; Tagl, triacylglycerol lipase.

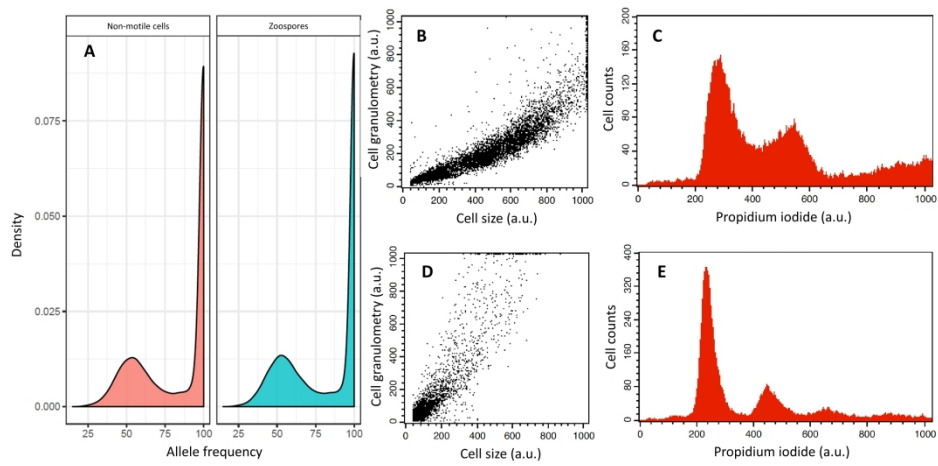
Table 1: Effect of the medium composition on the production of zoospores. The initial cell concentration was set at 5×10^5 cells mL^{-1} in all conditions. P* = after 48 hours growth, amino acids were added to the culture at the same concentration as R3 (Supplementary Table 1). The number of zoospores is expressed as percentage of total cells at day 1 (D1), day 2 (D2), day 4 (D4) and day 6 (D6). Experiments were run in triplicate.

	Media				
	R	P	R2	R3	P*
C source	6% Glucose	0.15 % Glucose	6% Glucose	6% Glucose	0.15 % Glucose
N and P sources	2% YE	0.05% YE	NaNO_3 NaH_2PO_4	AA NaH_2PO_4	0.05% YE +AA (D2)
Zoospores at D1	<3%	85%	92%	<4%	90%
Zoospores at D2	<1.5%	98%	83%	<2%	98% +AA
Zoospores at D4	<1.5%	96%	80%	0	4.8%
Zoospores at D6	0	88%	25%	0	3.4%
Total number of cells at D6 ($\times 10^7$ cells.mL^{-1})	7.2	0.45	10	8.9	0.36

Table 2: Respiratory activity in R- and P-grown cells along a six-day growth. Oxygen consumption rates are expressed as nmoles O₂ min⁻¹ per million cells ± SD. Experiments were run in triplicate.

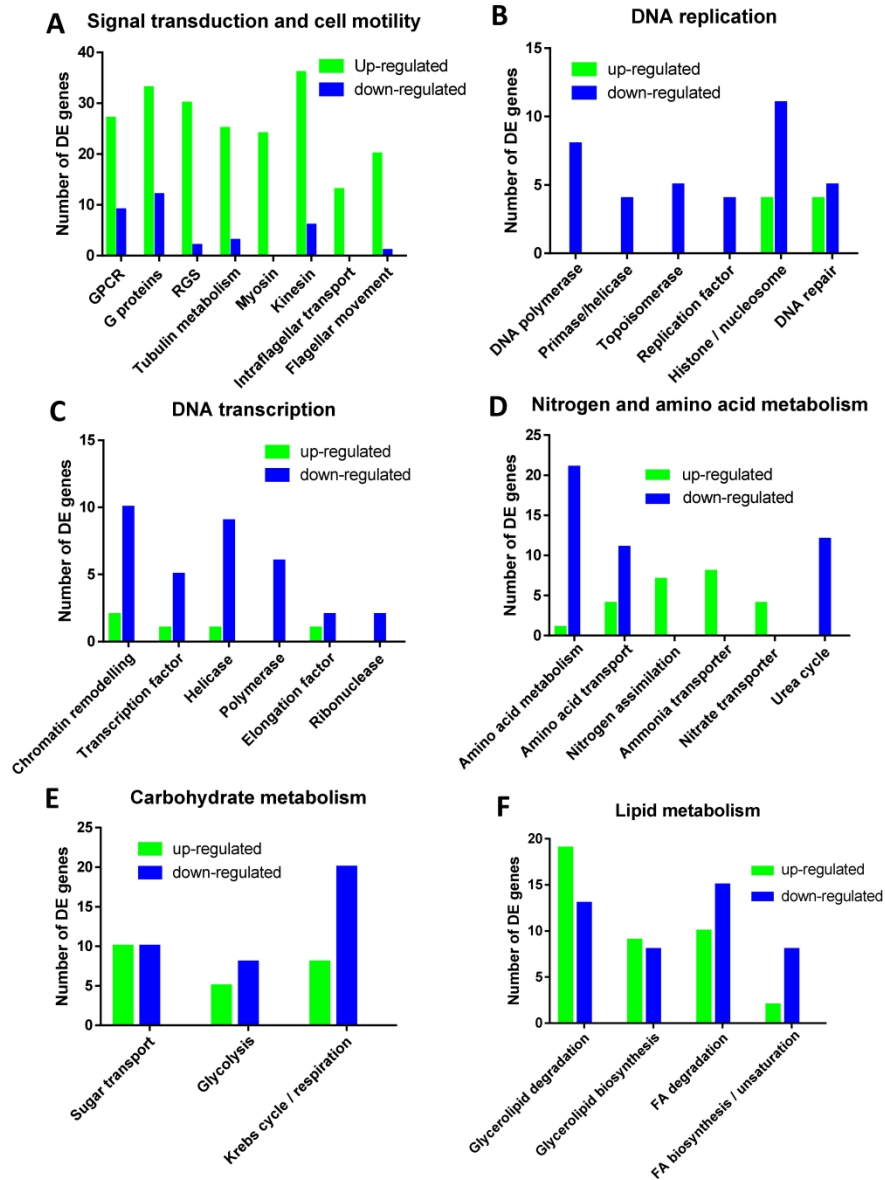
Respiratory activity after transfer to new media					
Days	0	1	2	4	6
R-grown	1.71 ± 0.16	6.16 ± 1.27	2.75 ± 0.62	3.06 ± 0.52	2.96 ± 0.24
P-grown	1.71 ± 0.16	1.76 ± 0.22	0.53 ± 0.1	0.44 ± 0.05	0.16 ± 0.05

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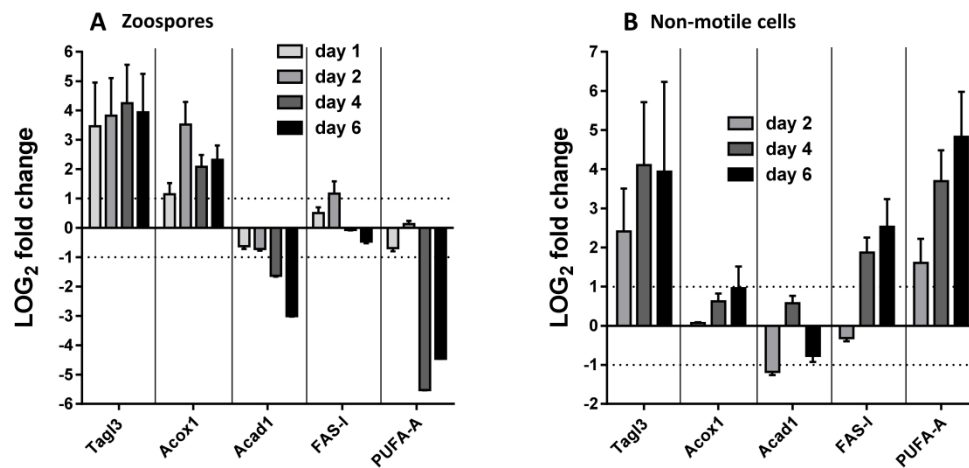
The ploidy of *Aurantiochytrium limacinum* cells. (A) Density plot of allele frequencies calculated in non-motile cells and zoospores. Flow cytometry of non-motile cells (B and C) and zoospores (D and E). Light scatter analysis of cell populations showing cell size and granulometry (B and D). Propidium iodide fluorescence (C and E).

338x190mm (300 x 300 DPI)

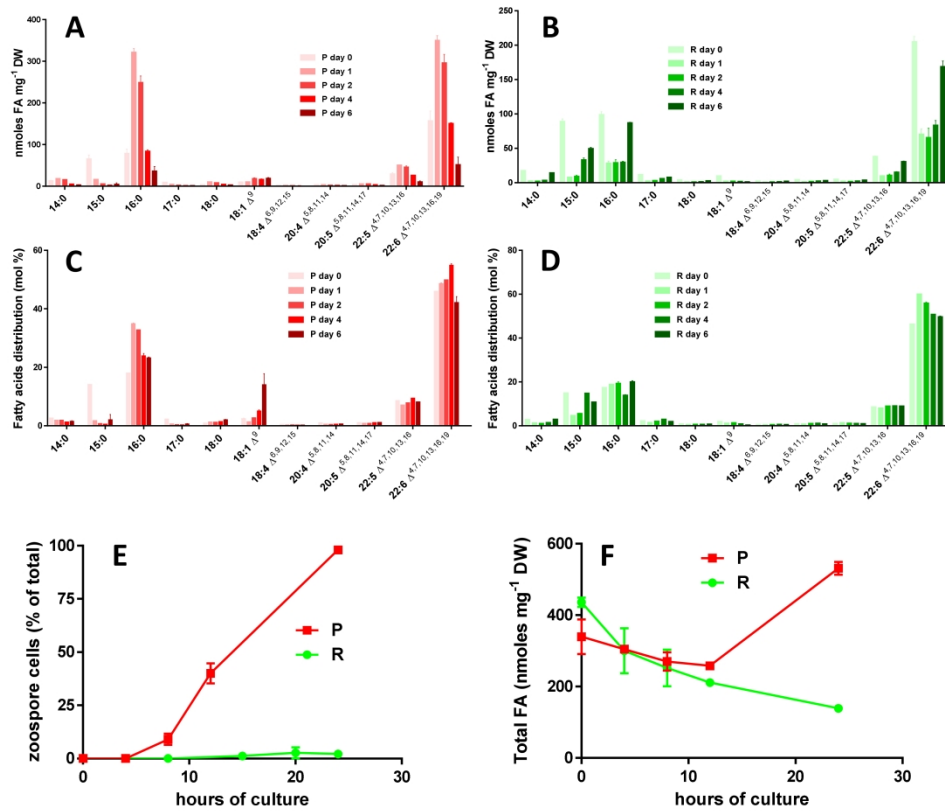


Differentially expressed genes (cut-off $\text{Log}_2\text{FC} = |1|$) for selected items. (A) signal transduction and cell motility; (B) DNA replication; (C) DNA transcription; (D) nitrogen and amino acid metabolisms; (E) carbohydrate metabolism; (F) lipid metabolism. GPCR: G protein-coupled receptors; RGS: regulator of G protein signaling.

183x241mm (600 x 600 DPI)

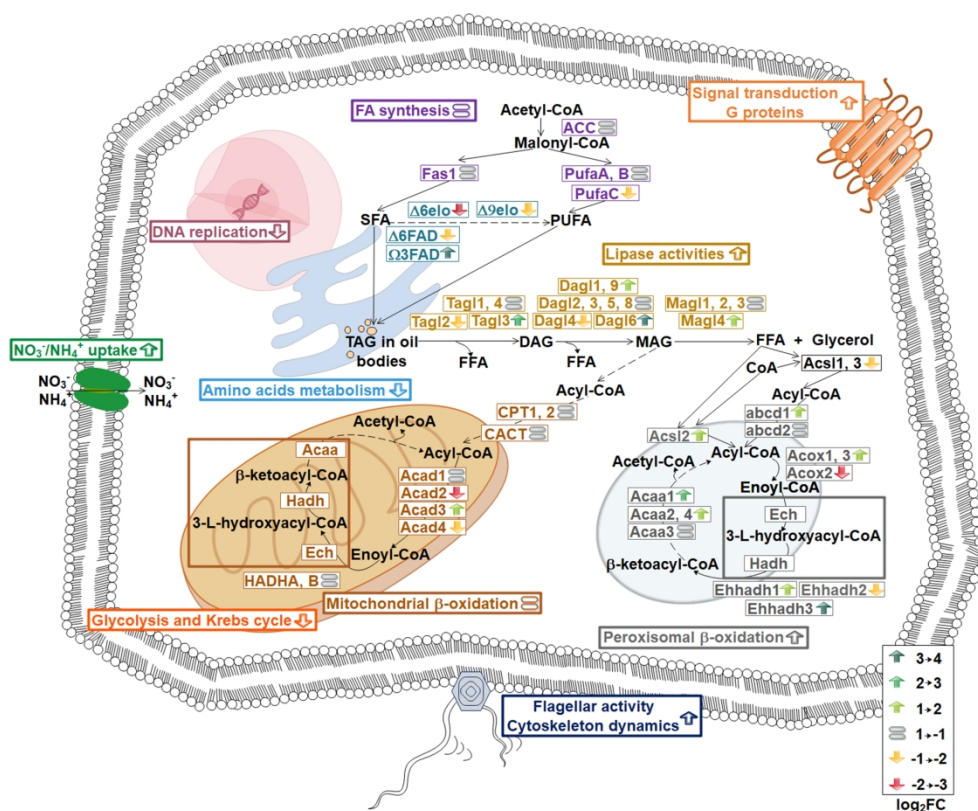


Relative expression of key genes involved in FA synthesis and FA degradation in zoospores (A) and non-motile cells (B). *Tagl3* (TAG lipase 3) was chosen as a representative of TAG degradation. *FAS-1* and *PUFA-A* were chosen as representatives of FA synthesis. *Acad1* (acyl-CoA dehydrogenase, β -oxidation in mitochondria) and *Acox1* (acyl-CoA oxidase, β -oxidation in peroxisomes) were chosen for FA degradation. Dotted lines indicate significance threshold. Error bars indicate standard deviation of three independent biological repeats.



FA content in P-grown (red) and R-grown (green) cells. A and B) FA content, expressed as nmoles FA per mg dry weight. C and D) FA distribution, expressed as % of total FAs. E) Number of zoospores during the first 24 hours expressed as % of the total cell number. F) FA content evolution during the first 24 hours. Error bars indicate standard deviation of three independent biological repeats.

192x161mm (600 x 600 DPI)



A schematic representation of *Aurantiochytrium limacinum* zoospore differential expression analysis based on RNA-seq. Metabolic pathways are illustrated with different colors: pink for DNA replication; purple for FA synthesis; turquoise for elongation and desaturation of FAs; yellow for lipase activities; light orange for signal transduction and G protein pathways; brown for mitochondrial β -oxidation; grey for peroxisomal β -oxidation; bright orange for glycolysis and the Krebs cycle; green for nitrogen and ammonium uptake; blue for amino acid metabolism; and dark blue for flagellar activity and the cytoskeleton dynamics. The colors of full arrows indicate the log₂FC value (color scale on the right bottom of the figure) for each gene involved in lipid metabolism. The empty arrows indicate the trend of the metabolic pathway. Abbreviations: SFA, saturated FAs; PUFA, polyunsaturated FAs; TAG, triacylglycerol; DAG, diacylglycerol; MAG, monoacylglycerol. Gene abbreviations (in alphabetic order): abcd, peroxisomal ABC transporter; ACC, acetyl-CoA carboxylase; Acaa, 3-ketoacyl-CoA thiolase; Acad, acyl-CoA dehydrogenase; Acox, acyl-CoA oxidase; Acsl, long chain acyl-CoA ligase; CACT, carnitine acylcarnitine transferase; CPT, carnitine palmitoyl transferase; Δ elo, fatty acid Δ elongase; Δ FAD, Δ fatty acid desaturase; Dagl, diacylglycerol lipase; Ech, enoyl-CoA hydratase; Ehhadh, bifunctional enzyme; Fas, fatty acid synthase; Hadh, 3-hydroxyacyl-CoA dehydrogenase; HADH, trifunctional enzyme; Magl, monoacylglycerol lipase; Ω 3FAD, Ω 3 fatty acid desaturase; Tagl, triacylglycerol lipase.

224x189mm (300 x 300 DPI)