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1 **PREFERENTIAL SYNTHESIS OF VERY LONG CHAIN POLYUNSATURATED**
2 **FATTY ACIDS IN *EUTREPTIELLA* SP. (EUGELNOZOA) REVEALED BY**
3 **CHROMATOGRAPHIC AND TRANSCRIPTOMIC ANALYSES[1]**
4

5 **Rita C. Kuo[2]**
6 Department of Marine Sciences, University of Connecticut, Groton, CT 06340, USA
7 **Huan Zhang**
8 Department of Marine Sciences, University of Connecticut, Groton, CT 06340, USA
9 **James D. Stuart**
10 Department of Chemistry, University of Connecticut, Storrs, CT 06269, USA
11 **Anthony A. Provatas**
12 Center of Environmental Sciences and Engineering, University of Connecticut, Storrs, CT
13 06269, USA
14 **Linda Hannick**
15 SAIC-Frederick, Inc., Frederick National Laboratory for Cancer Research, Rockville, MD
16 20852, USA
17 **Senjie Lin[3]**
18 Department of Marine Sciences, University of Connecticut, Groton, CT 06340, USA
19

20 [1]Received May 20, 2020
21 [2]Current address: Joint Genome Institute, Lawrence Berkeley National Laboratory, Walnut
22 Creek, CA 94598, USA.
23 [3]Address for correspondence: Phone, 860-405-9168; Email, senjie.lin@uconn.edu
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25 Running Title: Fatty acid and lipid synthesis in alga *Eutreptiella*
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31 **Abstract**
32

33 Algal lipids are important fuel storage molecules in algae and a currency for energy transfer in
34 the marine food chain as well as materials for biofuel production, but their production and
35 regulation are not well understood in many species including the common coastal phytoplankton
36 *Eutreptiella* spp. Here, using gas chromatography-tandem mass spectrometry (GC/MS/MS), we
37 discovered 24 types of fatty acids (FAs) in *Eutreptiella* sp. with a relatively high proportion of
38 long chain unsaturated FAs. The abundances of C16, C18 and saturated FAs decreased when
39 phosphate in the culture medium was depleted. Among the 24 FAs, docosahexaenoic acid (22:6)
40 and eicosapentaenoic acid (20:5) were the most abundant, suggesting that *Eutreptiella* sp.
41 preferentially invests in the synthesis of very long chain polyunsaturated fatty acids (VLCPFA).
42 Further transcriptomic analysis revealed that *Eutreptiella* sp. likely synthesizes VLCPFA via Δ8
43 pathway and uses type I and II fatty acid synthases. Using RT-qPCR, we found that some of the
44 lipid production genes, such as β-ketoacyl-ACP reductase, fatty acid desaturase, acetyl-CoA
45 carboxylase, acyl carrier protein, Δ8 desaturase, and Acyl-ACP thioesterase, were more actively
46 expressed during light period. Besides, two carbon-fixation genes were more highly expressed in
47 the high lipid illuminated cultures, suggesting a linkage between photosynthesis and lipid
48 production.

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50 **Keywords:** *Eutreptiella,* lipid biosynthesis, nutrient limitation, transcriptomic, unsaturated fatty
51 acids
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54 **Introduction**

55 Lipids are important fuel storage molecules in algae as well as other organisms, and as algae are
56 the base of the aquatic ecosystem, lipids are an important currency of energy transfer in the
57 aquatic food chain (Jonasdottir 2019). Lipid content also represents the quality of an alga as
58 materials for biofuel production (Sajjadi et al. 2018). Within algae, lipids are essential for
59 structural constituents of cellular membranes in all organisms and protection of delicate internal
60 organs and hormones in animals (Singh 2002). In the marine ecosystem, fatty acids are a
61 fundamental energy source for growth and cell membrane fluidity (especially in cold water) in
62 animals such as zooplankton and fish (*e.g.* Arzel et al. 1994, Cossins et al. 1977, Klein Breteler
63 2004, Rainuzzo et al. 1997, Tang and Taal 2005), which consume phytoplankton as food. To
64 gain better understanding on the energy flow in marine food chains, it is important to study fatty
65 acid and lipid biosynthesis pathways in dominant phytoplankton (prey) species. As the type and
66 abundance of lipids in the cells also determine the potential of algae as source of biofuel,
67 understanding the regulatory pathways of lipid production in algae is also important for
68 evaluating or genetically enhancing algal species for biofuel production.

69 In land plants, photosynthesis produces small precursors for lipid production in
70 chloroplasts. The small precursors are later converted into long chain fatty acids via two enzyme
71 systems, acetyl-CoA carboxylase (ACCase) and fatty acid synthase (FAS). ACCase is essential
72 for lipid metabolism as it catalyzes the first step of fatty acid synthesis and is found in both
73 bacteria and eukaryotes (Cronan and Waldrop 2002, Harwood 1988). FAS consists of 6 enzyme
74 activities and an acyl-carrier protein (CA) to catalyze a 2 carbon elongation process (Bloch and
75 Vance 1977, Nelson and Cox 2008). It produces palmitate and stearate and these fatty acids are
76 subject to elongation, desaturation or further modification by elongase or desaturase in

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77 chloroplasts (Gunstone et al. 2007). There are two major types of fatty acid synthases. Fatty acid
78 synthase I (FASI), found in vertebrates and fungi, consists of a multi-enzyme complex contained
79 in a single polypeptide chain (Nelson and Cox 2008, Schweizer and Hofmann 2004). In contrast,
80 fatty acid synthase II (FASII), found in plants and bacteria, contains discrete enzymes (Nelson
81 and Cox 2008, White et al. 2005). Among euglenoid algae, *Euglena gracilis* composes both
82 FASI and plastid FASII for *de novo* fatty acid synthesis (Delo et al. 1971, Worsham et al. 1993).
83 In addition to FASII, Goldberg and Bloch (1972) reported another ACP-dependent fatty acid
84 synthase (FASIII) that elongates acyl-CoA derivatives from C10 to C18 to longer chain ACP
85 thioesters in plastids.

86 Similar to land plants, microalgae initiate fatty acid synthesis in chloroplasts (Guschina
87 and Hardwood, 2006) and lipid biosynthesis is probably linked directly to photosynthesis.
88 Studies have shown that the activities of ACCase are related to the light reactions of
89 photosynthesis, as NADPH and ATP produced in the photochemical reaction are required for the
90 synthesis of palmitic acid (Sasaki et al. 1997). Furthermore, fatty acid synthesis in photosynthetic
91 organisms obviously relies on carbon fixation for carbon precursors for fatty acid synthesis (Bao
92 et al. 2000). The synthesized fatty acids are in turn used for triacylglycerol (TAG) synthesis
93 (Thelen and Ohlrogge 2002). In addition, dihydroxyacetone phosphate, the precursor for glycerol
94 3-phosphate needed in the synthesis of lipid TAG, can be produced from Calvin-Benson Cycle
95 (Nelson and Cox 2008).

96 It is well understood that lipid production in microalgae is influenced by nutrient and
97 other environmental conditions (e.g. Gouveia and Oliveira 2009, Griffiths and Harrison 2009, Hu
98 et al. 2008, Illman et al. 2000, Liu et al. 2008). Nutrient limitation can enhance the production of
99 lipid in algal cells. For example, the lipid content of some *Chlorella* species (*e.g. C. emersonil*

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100 *and C. pyrenoldosa*) can increase to more than 60% of the dry weight under nitrogen-deprived
101 conditions (Griffiths and Harrison 2009, Illman et al. 2000). However, our knowledge of the
102 genetic regulatory mechanisms of lipid biosynthesis in marine microalgae is limited and
103 fragmentary (Guchina and Harwood 2006, Hu et al., 2008), particularly for euglenids such as
104 *Eutreptiella* spp.

105 *Eutreptiella* is a genus of photosynthetic euglenoid, which have excellent nutritive value
106 (*i.e.* high vitamin and lipid content, Takeyama et al., 1996; Yamane et al., 2001) and are rather
107 abundant in some marine ecosystems (Henriksen et al. 2002). Some *Eutreptiella* spp. can
108 seasonally be a dominant group of phytoplankton (Álvarez-Góngora and Herrera-Silveira 2006,
109 Bates and Strain 2006, Olli et al. 1996, Rodríguez-Graña et al. 2008, Seong et al. 2006), forming
110 blooms in nutrient-rich coastal or brackish waters (Anderson et al. 2000, Lindholm 1993, Olli et
111 al. 1996, Stonik and Selina 200, Stonik 2007). In some areas, *Eutreptiella braarudii* alone can
112 make up to 46% of the phytoplankton population (Stonik, 2007). As primary producers, these
113 algae are important in energy flow and nutrient cycling in the coastal marine ecosystem.
114 Meanwhile, as solar energy converters, these algae are potentially candidates of biofuel species.

115 In this study, we used 454 high-throughput sequencing to study transcriptomic profiles
116 and gas chromatography-tandem mass spectrometry (GC/MS/MS) to identify fatty acids
117 produced under phosphate-depleted and phosphate-replete conditions, and conducted reverse-
118 transcription qPCR (RT-PCR) to quantify expression of several key enzyme coding genes to
119 investigate molecular mechanisms that regulate lipid biosynthesis in *Eutreptiella* sp.

120

121 **Materials and Methods**

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122 ***Culture preparation and RNA isolation.*** Two sets of cultures were maintained in phosphate-
123 depleted medium (to produce high-lipid culture) and phosphate-replete f/2 medium (to produce
124 low-lipid culture); cells were harvested, total RNA and mRNA were isolated as reported
125 previously (Kuo et al. 2013)

126 ***Lipid measurement.*** Nile Red (9-diethylamino-5H-benzo-α-phenoxazine-5-one, a lipid-soluble
127 fluorescent probe) staining was used to measure relative abundance of neutral lipids as reported
128 (Lee et al., 1998, Hu *et al*., 2008). Forty µl of Nile Red solution in acetone (250 mg/l) were
129 added to 10 ml of algal suspension at room temperature for 10 min. A spectrophotometer
130 (HITACHI, Tokyo, Japan) was then used to measure the fluorescence with excitation at the
131 wavelength of 490 nm and emission in the wavelength band of 580 to 590 nm, as reported in
132 Kuo and Lin (2012).

133 ***Lipid extraction and fatty acid identification by gas chromatography-tandem mass***
134 ***spectrometry (GC/MS/MS).*** A gravimetric method was applied to determine actual lipid contents
135 by using chloroform-methanol method according to Bligh and Dyer (1959). In short, lipids were
136 extracted from freeze-dried cells with chloroform/methanol (1:2, v/v) and the residue was
137 extracted one more time with 1:1 chloroform/methanol. The chloroform layer was collected and
138 evaporated under a gentle flow of nitrogen gas. Derivatization for GC/MS/MS analysis followed
139 the method summarized by (Carvalho and Malcata 2005). Briefly, samples were dissolved in 1
140 ml of a freshly prepared mixture of dry acetyl chloride and methanol, at a ratio of 5:100 (v/v),
141 and kept at 100 °C under pure nitrogen for 1 h. After cooling, 1 ml of hexane wad added and
142 mixed by vortexing. Purification of the solution was achieved by adding 1 ml of saturated
143 sodium chloride solution. The prepared solution of fatty acid methyl esters was filtered using

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144 0.45 μm Millipore filter. Analysis of the solution was performed on a Waters Quattro Micro
145 GC/MS/MS system equipped with a Rxi-5Sil MS column (30 m x 0.25 mm, 0.25 μm film
146 thickness, Restek Co., PA). Helium was used as the carrier gas with a flow rate of 1 ml/min.
147 Initial oven temperature was 70 °C for 1 min and the temperature gradient was 5 °C/min from
148 70 °C to 270 °C with no holding time. Sample was introduced in splitless injection mode.
149 Injector temperature was 270 °C and purged for 1 min with a purge flow of 25 ml/min. Total run
150 time was 41 min. The MS source temperature was set at 250 °C and the GC interface was
151 275 °C. A fatty acids methyl ester mixture (FAMQ-005 FAME Reference Standard,
152 AccuStandard Inc., CT, USA) was used as external standards. The mass spectrum and the
153 corresponding retention time of each component were exported to NIST MS Search 2.0 software
154 to identify compounds by means of library and standard comparisons.
155 ***454 sequencing of the SL-based transcriptomes.*** mRNA samples isolated as described above
156 from the four culture conditions were used to synthesize cDNA, which was subsequently used
157 for 454 sequencing as reported previously (Kuo et al. 2013). Briefly, a modified random oligo
158 named 454AT$_7$N$_9$ (5'-
159 CGTATCGCCTCCCTCGCGCCATCAGTAATACGACTCACTATAGGGAGNNNNNNNNN-
160 3', where N is any of the 4 nucleotides) was used to synthesize the 1st strand cDNAs. The
161 cDNAs were then used as templates for PCR amplification of the 5'-end of the cDNAs of the
162 *trans*-spliced transcripts using ExTaq with a *Eutreptiella* spliced leader-based primer
163 454BEutSL, 5'-
164 GAGACTATGCGCCTTGCCAGCCCGCTCAGACACTTTCTGAGTGTCTATTTCTTTTCG-
165 3'), paired with 454AT$_7$ (5'-
166 CGTATCGCCTCCCTCGCGCCATCAGTAATACGACTCACTATAGGGAG-3'). After PCR

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167 we selected the amplicons from agarose gel in the size range of 300-700 bp as the template for
168 emulsion PCR (emPCR) using GS Titanium SV emPCR Kit. Sequencing was carried out using
169 GS Titanium Sequencing Kit on the GS FLX System at the Center for Applied Genetics and
170 Technology, University of Connecticut.

171 ***Sequence processing and annotation.*** Sequence reads were processed and analyzed as recently
172 reported (Kuo et al. 2013). In short, raw sequencing reads from all of the 4 samples were pooled
173 together and trimmed using CLC Genomics Workbench (CLC Bio, Aarhus, Demark). After
174 quality and primer trimming, sequences shorter than 150 nt were discarded. In order to filter off
175 454 sequencing errors and create reduced-redundancy sequence dataset, USEARCH (Edgar
176 2010) was used for sequence clustering. The resulting unique transcripts were then annotated
177 using Blast2Go V.2.5.0 (Götz et al. 2008) against NCBI's non-redundant (nr) database using
178 BLASTx algorithm (Altschul et al. 1990), with a cut-off-E-value $\leq 10^{-3}$.

179 ***Reverse-transcription quantitative PCR (RT-qPCR).*** In order to further investigate the
180 expression patterns of genes potentially regulating lipid production in *Eutreptiella*, some of the
181 genes identified from the transcriptomic data as related to carbon fixation and lipid synthesis
182 were selected and further analyzed by RT-qPCR. First strand cDNA libraries were prepared by
183 the methods mentioned earlier. It is suggested that at least two reference genes are needed for
184 proper normalization of gene expression levels (Guo and Ki 2012, Vandesompele et al. 2002).
185 Tubulin, actin, and elongation factor 1α were selected as candidates, as they had been shown to
186 be good reference genes in some microalgae and plants (Guo and Ki 2012, Le Bail et al. 2008).
187 We used the geometric average of the expression levels of these reference genes to normalize the
188 measured expression levels of target genes as reported (Vandesompele et al. 2002). For each
189 gene investigated, two sets of primers were designed (Table 3). To prepare standards, the primer

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190 combination that specifies longer gene fragment was used to PCR amplify each target gene from
191 *Eutreptiella* cDNA. The amplicons of the target genes were checked with gel electrophoresis to
192 assure the absence of primer dimmers and then purified using DNA Clean & Concentrate™
193 column (Zymo Research, Orange, CA). The purified DNA was serially diluted to 10^2, 10^3, 10^4,
194 10^5, and 10^6 gene copies to generate standard curves to analyze the amplification efficiency and
195 primer specificity for every primer pair. qPCR was performed using the iCycler iQ™ real-time
196 PCR Detection System (Bio-Rad Laboratories, Inc., Hercules, CA) with SYBR Green supermix.
197 The qPCR reactions included a single denaturation cycle of 95 °C for 3 min, 40 cycles of 95 °C
198 for 20 sec, 58 °C for 30 sec, and 72 °C for 15 sec, followed by a melt curve analysis from 55 to
199 100 °C.

200 **Results**

201 ***Physiological conditions of the cultures.*** Two sets of cultures were grown under phosphate-
202 depleted (to produce high-lipid culture) and phosphate-replete (to produce low-lipid culture)
203 conditions. On the 11th day, the phosphate-replete cultures were in exponential growth phase
204 whereas phosphate-depleted cultures already reached stationary phase. When normalized to per
205 cell basis, the lipid contents of the cultures grown in phosphate-depleted medium were about 2-
206 fold higher than that of the cultures grown in phosphate-replete medium (Figure 1). This
207 observed difference in lipid content prompted cell harvesting from both cultures in the light and
208 dark periods, which yielded four samples referred to as high-lipid-light, high-lipid-dark, low-
209 lipid-light, and low-lipid-dark cultures.

210 ***Comparison of gene diversity among samples.*** The four samples were subjected to RNA
211 extraction, cDNA library construction, and 454 sequencing (Kuo et al. 2013). The Venn diagram
212 in Figure 2 summarizes the numbers of unique genes, total genes and shared genes among the 4

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213 cDNA libraries. The analysis revealed that each library contained different numbers of total
214 genes as well as different unique genes that were not shared by any other libraries. Between the
215 two light cultures, the high-lipid cultures had more unique genes and total genes. Between the
216 cultures in the same lipid content categories, cultures harvested under the light condition
217 displayed more unique genes (i.e. light-specific) and total genes. Overall, high-lipid-light
218 cultures expressed the highest number of unique and total genes among the 4 samples,
219 suggesting that a more complex system was involved in lipid production and light reaction in
220 *Eutreptiella* sp.

221 ***Fatty acid synthesis.*** Based on the functional annotation, we have identified genes encoding key
222 enzymes involved in the lipid biosynthesis and catabolism (Table 1). Fatty acid biosynthesis
223 from acetyl-CoA involved in two enzymes: ACCase and FAS. Eukaryotic type ACCase (72%
224 identical to ACCase of *Thalassiosira pseudonana*; GenBank accession number: XP_002296083)
225 and type I FAS (67% identical to FASI of *Caenorhabditis elegans*; NP_492417) were found in
226 our dataset. Figure 3 illustrates a potential lipid biosynthesis pathway of *Eutreptiella* sp. ACCase
227 catalyzes carboxylation of acetyl-CoA to produce malonyl-CoA, which is catalyzed to malonyl-
228 ACP by malonyl-CoA transacylase. Next, long-chain saturated fatty acids are synthesized by
229 type I fatty acid synthase, which have 7 active sites within a single large polypeptide complex
230 (Nelson and Cox, 2008). The active enzyme domains include β-ketoacyl-ACP synthase (KAS),
231 malonyl/acetyl-CoA-ACP transferase (MAT), β –hydroxyacyl-ACP dehydratase (HAD), enoyl-
232 ACP reductase (EAR), β –ketoacyl-ACP reductase (KAR), and Acyl-ACP thioesterase (TE).
233 Synthesized palmitate (C16:0) and stearate (C18:0) via the activity of FASI are further subjected
234 to elongation, desaturation, or other modifications (see next section for more details). Besides
235 FASI, individual genes encoding β –ketoacyl-ACP reductase and Acyl-ACP thioesterase related

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236 to type II fatty acid synthase (FASII) were also identified (Table 1). Malonyl-CoA transacylase,
237 which functions in the fatty acid synthesis pathway in other plants, was missing in our dataset.
238 ***Fatty acid identification and biosynthesis pathways.*** The stepwise desaturation and elongation
239 of C18 acid lead to the extension of fatty acids and synthesis of unsaturated fatty acids (Nelson
240 and Cox, 2008). The major fatty acids found in *Eutreptiella* sp. were the very long chain
241 polyunsaturated fatty acids, docosahexaenoic acid (DHA, 22:6n-3). Various desaturases and
242 elongases were found in our study (Table 1), including Δ5 desaturase, Δ6 desaturase, Δ8
243 desaturase, Δ9 desaturase, Δ12 desaturase and Δ15 desaturase. The elongases were: Δ5 elongase,
244 Δ6 elongase, and Δ9 elongase.
245 In order to link the identified genes to their expected roles in *Eutreptiella* sp., the fatty
246 acid (FA) profiles of *Eutreptiella* sp. were analyzed by screening C16-C24 fatty acids via
247 GC/MS/MS. We identified 24 fatty acids (including C16, C20, C22, and C24 fatty acids) from
248 the cultures of *Eutreptiella* sp. (Figure 4 and Table 2). In both of the phosphate-depleted and
249 phosphate-replete cultures, *Eutreptiella* sp. was relatively rich in polyunsaturated fatty acids
250 (PUFAs), especially very long chain polyunsaturated ω3 fatty acids (VLCPFA),
251 docosahexaenoic acid (DHA, 22:6) and eicosapentaenoic acid (EPA, 20:5). DHA had the highest
252 amount in both cultures (Figure 4), so we used it as a standard to estimate relative abundance of
253 other fatty acids (Table 2). Trace amount of tetracosahexaenoic acid, a C24 VLCPFA, was
254 found. Three saturated fatty acids, palmitic acid (16:0), stearic acid (18:0), and behenic acid
255 (20:0), were also identified. The abundance of saturated fatty acids was relatively low in the
256 phosphate-depleted cultures. The analysis also revealed the presence of C16 unsaturated fatty
257 acids: 7-hexadeceonoid acid (16:1), 9-hexadecenoid acid (16:1), 7,10-hexadecadienoic acid
258 (16:2), 7, 10, 13-hecadecatrienoic acid (16:3), and 4, 7, 10, 13-hexadecatetraenoic acid (16:4) in

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259 both of the cultures. In the phosphate-depleted cultures, the relative abundance of C16 and C18
260 fatty acids was lower than the fatty acids in the phosphate-replete cultures (Figure 4 and Table
261 2), suggesting that phosphate availability in the culture medium affected the lipid composition in
262 *Eutreptiella* sp.

263 Based on the transcriptome and GC/MS/MS results, *Eutreptiella* sp. is predicted to be
264 capable of producing various intermediates via ω3 and ω6 pathways, as well as producing
265 VLCPFA EPA and DHA by using the Δ8 alternative pathway, as reported in *Euglena* (Meyer et
266 al. 2003; Qi et al. 2004; Wallis and Browse 1999). Figure 5 shows the potential fatty acid
267 elongation pathways for *Eutreptiella* sp. as constructed from the transcriptome results. The first
268 desaturation for unsaturated fatty acids synthesis is catalyzed by Δ9 desaturase to introduce a
269 double bond into stearic acid resulting in 18:1 –ACP. Then a series of elongation and
270 desaturation takes place to produce the 24 types of unsaturated fatty acid mentioned before
271 (Figure 5). The elongation procedure is terminated when the acyl group is removed by acyl-ACP
272 thioesterase (TE) or oleoyl-ACP hydrolase (OAH), or when the fatty acids are transferred to the
273 biosynthesis of triacylglycerol.

274 ***Triacylglycerol (TAG) biosynthesis.*** Microalgal TAG biosynthesis is proposed to take place via
275 transfer of fatty acids from CoA to glycerol-3-phosphate using the direct glycerol pathway
276 (Ratledge 1988). However, the existing knowledge on the pathways and enzymes involved in
277 TAG biosynthesis in most of the microalgae (e.g. euglenophytes) are limited. In this study, four
278 transcripts coding for the enzymes involved in TAG biosynthesis were found in our *Eutreptiella*
279 sp. transcriptomic dataset. They were glycerol 3-phosphate dehydrogenase (GPD), glycerol 3-
280 phsphate acyltransferase (GPAT), and acylglycerophosphate acyltransferase (AGPAT) (Table 1).

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281 For TAG biosynthesis, the precursor glycerol 3-phosphate is produced during glycolysis from
282 dihydroxyacetone phosphate by the action of glycerol 3-phosphate dehydrogenase.
283 Based on the data, a potential pathway for *Eutreptiella* sp. TAG biosynthesis is proposed
284 (Figure 3). The first step is the acylation of the two hydroxyl groups of glycerol3-phosphate by
285 two fatty acyl-CoA to yield phosphatidic acid, which is hydrolyzed by phosphatidate
286 phosphatase to form 1,2 diacylglycerol. Diacylglycerols are then converted to triacylglycerols by
287 transferring a third fatty acyl-CoA. Phosphatidic acid phosphohydrolase (PAP) and
288 diacylglycerol acyltransferase (DGAT) were missing in our cDNA libraries.
289 ***Gene expression levels as measured using reverse transcription quantitative PCR.*** We further
290 confirmed the differential expression patterns of several genes using reverse transcription
291 quantitative PCR (RT-qPCR). Nine genes involved in lipid synthesis were chosen for the
292 analysis, including type I fatty acid synthase (FASI), 3 different types of β –ketoacyl-ACP
293 reductase (KAR1, KAR2, KAR3, 12-33% identity at amino acid level), acetyl-CoA carboxylase
294 (ACCase), acyl carrier protein (ACP), thioesterase (TE), Δ8 desaturase, and an unclassified fatty
295 acid desaturase (FAD). Seven of the nine genes, KAR2, KAR3, FAD, ACCase, ACP, Δ8, and
296 TE had higher expression levels in the high-lipid-light cultures (Figure 6 and Figure 7). The
297 expression of FASI showed an opposite trend between high-lipid and low-lipid cultures. In high-
298 lipid cultures, FASI was expressed at a higher level in the light period, whereas in the low-lipid
299 cultures, FASI was expressed at a lower level in the light period (Figure 6). As for genes
300 involved in triacylglycerol synthesis, glycerol 3-phosphate dehydrogenase (GPD) and 1-acyl-sn-
301 glycero3-phosphate o-acyltransferase (AGPAT) exhibited slightly higher expression (transcript
302 abundance) in high-lipid-light cultures (Figure 7).

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303 We also examined the expression of genes potentially involved in carbon fixation in
304 *Eutreptiella* sp.: phosphoenolpyruvate carboxylase (PEPCase), 2 variants of
305 phosphoenolpyruvate carboxykinase (PEPCK1 and PEPCK2, 54% identical at amino acid level),
306 pyruvate-phosphate dikinase (PPDK), and phosphoribulokinase (PRK). Three out of the five
307 genes (i.e. PPDK, PRK, and PEPCK1) showed positive correlation of transcript abundance with
308 lipid production. In particular, PEPCK1 and PRK expression levels in the high-lipid-light
309 cultures were over 6- and 4-fold higher than in the low-lipid-light cultures, respectively (Figure
310 8).
311
312 **Discussion**
313 ***Unique lipid and fatty acid production patterns.*** While the effects of depleting nitrate on lipid
314 production has been studied in many algae (Griffiths and Harrison 2009, Hu et al. 2008), effects
315 of phosphate are less documented. Our preliminary experiments showed that phosphate depletion
316 more strongly promotes lipid content in *Eutreptiella* sp. cells than nitrate depletion (data not
317 shown). The study reported here also showed that phosphate stress markedly increased
318 *Eutreptiella* lipid content. Among the cultures grown in parallel, lipid content was 2 fold as
319 much in the phosphate-depleted cultures as that in the phosphate-replete, f/2 medium-grown,
320 cultures (Figure 1). The type of main energy reserve (carbohydrates or lipids) in algae depends
321 on which carbon fixation product is utilized in the Calvin-Benson cycle. Among those products,
322 hexose phosphates are used in the synthesis of carbohydrates, while 3-phophoglycerate is used
323 for lipid synthesis (Raven 1974). In addition, the availability of inorganic nitrogen influences
324 carbon partition (Huppe and Turpin 1994, Raven 1974). The presence of inorganic nitrogen

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325 source favors 3-phosphoglycerate and increases the flux of photosynthetically fixed carbon into
326 citrate cycle (Raven 1974), which is one of the sources for acetyl CoA (Nelson and Cox 2008).
327 Interestingly, *Eutreptiella* sp. has abundant polyunsaturated fatty acids. DHA was the
328 most abundant fatty acid from both the phosphate-depleted and phosphate-replete cultures.
329 Polyunsaturated fatty acids are valuable for cold adaptation (Wallis et al. 2002 and references
330 therein). The relatively high abundance of unsaturated fatty acids in *Eutreptiella* sp. may be due
331 to the adaptation to their native environment in Long Island Sound, where the temperature
332 reaches down below in the winter although there has been a warming trend (Snyder et al. 2019).
333 All the fatty acids that we identified in this study have also been reported in *Euglena gracilis*
334 (Hulanicka et al. 1964, Korn 1964, Meyer et al. 2003), except C24:1 and C24:6 fatty acids.
335 Different from *E. gracilis*, *Eutreptiella* sp. has a very high proportion of very long chain
336 unsaturated fatty acids (C22 and longer, i.e. DHA and EPA), rendering this species to be of high
337 nutritional value for zooplankton and other heterotrophic organisms in the marine ecosystem.
338 Moreover, the proportions of saturated fatty acids (i.e. C16:0, C18:0, and C20:0) and unsaturated
339 C16 and C18 fatty acids decreased when phosphate was depleted (Figure 4 and Table 2),
340 suggesting that it is possible to enhance the production of desired components of fatty acids from
341 *Eutreptiella* sp. by manipulating nutrient composition in the culture medium. Although it is not
342 clear why the abundance of C16:0 and C16:4 fatty acids were much lower in the phosphate-
343 depleted cultures, phosphate stress might alter the composition of fatty acids in the algal cells, as
344 plants and algae can respond to stress by adjusting membrane fluidity and by releasing
345 unsaturated fatty acids from the membrane (Maréchal et al. 1997, Upchurch 2008).
346

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347 ***Expressed genes linked to lipid synthesis in* Eutreptiella *sp.*** Fatty acids are synthesized via two
348 enzyme systems, ACCase and FAS (Nelson and Cox 2008). ACCase plays a critical role in
349 regulating fatty acid synthesis and is essential for lipid metabolism as it catalyzes the first step of
350 fatty acid synthesis and is found in all kingdoms of life except archaea (Cronan Jr and Waldrop
351 2002, Harwood 1988). It comprises three functional constituents: biotin carrier protein, biotin
352 carboxylase, and transcarboxylase, and differentiates into two distinct types: one consisting of
353 three separate subunits (prokaryote-type), and the other being a single multi-domain
354 multifunctional polypeptide (eukaryote-type) (Sasaki et al. 1995). The eukaryote-type occurs in
355 mammals, fungi or yeast, while both the prokaryote- and the eukaryote- type can be found in
356 plants (Huang et al. 2002, Sasaki et al. 1995). The ACCase sequences found in our dataset shares
357 ~72% amino acid identity to an eukaryotic type in the diatom *Thalassiosira pseudonana*,
358 suggesting that *Eutreptiella* sp. ACCase is also an eukaryotic type. In the qPCR analysis,
359 ACCase showed highest expression level in the high-lipid-light cultures, indicating that this gene
360 is related to photosynthetic lipid production in *Eutreptiella* sp.
361 Our results suggest that *Eutreptiella* sp. may have both type I fatty acid synthase (FASI)
362 and type II fatty acid synthase (FASII), which are the two major types of fatty acid synthases that
363 naturally occur in different groups of organisms (Nelson and Cox 2008). FASI, found in
364 vertebrate and fungi, is a single multifunctional polypeptide with 7 active sites, whereas FASII,
365 found in plants and bacteria, compose discrete and freely diffusible enzymes. Interestingly,
366 *Euglena gracilis*, which is another euglenoid species, has different types of fatty acid synthesis
367 pathways and enzymes to synthesize lipids under different environmental conditions (Delo et al.
368 1971, Goldberg and Bloch 1972, Inui et al. 1984). ACP-dependent FASI is located in the cytosol
369 and mainly produces C16 fatty acids (Goldberg and Bloch 1972). When grown in the light, *E.*

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370 *gracilis* utilizes ACP-dependent FAS II in plastids to produce fatty acids (Ernst-Fonberg and
371 Bloch 1971). Inui et al. (1984) also reported a malonyl-CoA independent fatty acid synthesis
372 system to form wax ester from paramylon as energy storages in *E. gracilis* under anaerobic
373 conditions. We found genes coding for FASI and for β –ketoacyl-ACP reductase and Acyl-ACP
374 thioesterase of FASII in *Eutreptiella* sp. Our failure to retrieve the rest of the FASII gene
375 complex from our cDNA libraries could be due to their being plastid-coded genes, which would
376 not contain Eut-SL that we used to construct the cDNA libraries, or just simply because they
377 were not expressed. Whether *Eutreptiella* sp. contains the other type of ACP-dependent FAS
378 similar to that in *E. gracilis* remains to be further investigated in the future.

379 Based on the fatty acid composition and possession of genes coding for enzymes
380 involved in fatty acid elongation and desaturation in *Eutreptiella* sp., it is evident that the ω3, ω6
381 and alternative Δ8 pathways occur in the euglenoid algae. Different from most of plants and most
382 algae, we found that *Eutreptiella* is similar to *Euglena* in using Δ8 desaturase to introduce the
383 third double bond on 20:2 (11, 14) to form 20:3 (8, 11, 14) (Meyer et al. 2003, Qi et al. 2004).
384 The 20:3 fatty acid is then converted to 22:5 EPA via stepwise Δ5 desaturase, Δ5 elongase and
385 Δ4 desaturase (Figure 5). In order to produce DHA, *Eutreptiella* may also utilize another Δ8
386 pathway to introduce the 4th double bound on 20:3 (11,14,17), resulting in 20:4 (8,11,14,17) for
387 later stepwise process of elongation and desaturation. Although Δ4 desaturase was not identified
388 in our dataset, it is very likely that *Eutreptiella* sp. has this gene since it was identified and
389 involved in DHA synthesis in *E. gracilis* (Meyer et al. 2003). In our study, both EPA would need
390 Δ4 desaturase to form their last double bond. We also detected trace amount of 15-tetracosenoic
391 acid (24:1) and 6,9,12,15,18,21-tetracosahexaenoic acid (24:6). The 24:6 fatty acid can be found
392 in some algae (Mansour et al. 2005), but the synthesis pathway is not well studied. We proposed

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393 that the 24:6 fatty acid could potentially be synthesized by elongation from DHA or elongation
394 and desaturation from other C22 fatty acids.
395 The 16:4 (4,7,10,13) fatty acid was also abundant in phosphate-replete cultures of
396 *Eutreptiella* sp. Highly unsaturated stearidonic acid (18:4) and hexadecatetraenoic acid (16:4) are
397 produced in some macroalgae (Dembitsky et al. 1991, Ishihara et al. 2000) and microalgae
398 (Wallis and Browse 1999). In diatoms, the phytoplankton accounting for about 40% of algal
399 primary production, the most common fatty acids range from C14:0 to C22:6, with the most
400 common number of double bonds two to three and rarely more than 6 (Yi et al. 2017).
401 Unsaturated C16 fatty acids have been identified in *E. gracilis*, the close relative of *Eutreptiella*
402 sp. (Constantopoulos and Bloch 1967). Increasing light intensity can enhance the production of
403 16:4 acid and 18:3 α-linolenic acid in *E. gracilis* (Constantopoulos and Bloch 1967). Korn
404 (1964) suggested a possible C16 pathway for *E. gracilis*: 16:1 (7) → 16:2 (7, 10) → 16:3 (7, 10,
405 13) →16:4 (4, 7, 10, 13). Although we identified 16:4 (4, 7, 10, 13) in *Eutreptiella* sp., the genes
406 responsible for the synthesis of this abundant fatty acid remain uncertain.
407
408 ***Regulation of genes associated with lipid production.*** Our RT-qPCR revealed that many of the
409 genes examined had different expression levels between the dark and light periods. Genes
410 involved in high lipid biosynthesis showed higher expression levels under light condition,
411 suggesting that these genes promoted lipid production in *Eutreptiella* sp. during photosynthesis.
412 Those genes were ACCase, ACP, KAR2, KAR3, FAD, Δ8 desaturase, TE, AGPAT, and GPD.
413 The results of RT-qPCR verified our physiological observation from a 24h diel experiment,
414 showing that lipids in *Eutreptiella* sp. cells start to accumulate during the light period after cells
415 have actively divided (Kuo and Lin, 2013). Other evidence showed that the activity of ACCase is

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416 correlated to that of the Calvin-Benson Cycle enzymes, with the greatest activities under light
417 (Sasaki et al. 1997) and negligible in the dark (Bao et al. 2000).

418 Three genes encoding carbon fixation enzymes showed higher expression levels under
419 the high-lipid-light condition, indicating that those enzymes are related to the biosynthesis of
420 precursors for lipid production. Phosphoribulokinase (PRK) and one copy of
421 phosphoenolpyruvate carboxykinase (i.e. PEPCK1) exhibited the highest level of relative gene
422 expression among these genes. PEPCK activity is linked to CO_2 fixation in algae (Reiskind and
423 Bowes 1991). It converts oxaloacetate into CO_2 and phosphoenolpyruvate, bridging C4 to C3
424 pathway in providing CO_2 for photosynthesis. It is noteworthy that C4 enzymes have been
425 identified in this species (Kuo et al. 2013). The phosphoenolpyruvate can later be converted into
426 pyruvate, which is an important precursor for Acetyl-CoA. PRK catalyzes the reaction of ATP
427 and ribulose 5-phosphate to produce ADP and ribulose 1,5-bisphosphate, the substrate needed to
428 capture CO_2 in photosynthesis. The concomitant up-regulation of the fatty acid synthesis and
429 photosynthesis genes suggests the linkage of the two carbon metabolic processes.

430

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623 **Figure legends**

624 Figure 1. Growth and lipid production of *Eutreptiella* cultures under phosphate-replete and
625 phosphate-depleted conditions. (A) Growth curves of *Eutreptiella* cultures. (B) Growth rates and
626 lipid fluorescence levels at the time when cultures were harvested.

627 Figure 2. Common and unique expressed genes between different culture conditions. HL: high-
628 lipid-light cultures. HD: high-lipid-dark cultures. LL: low-lipid-light cultures. LD: low-lipid-dark
629 cultures. Numbers outside the diagram represent total number of genes found in each library.

630 Figure 3. Putative lipid biosynthesis pathway in *Eutreptiella* sp. inferred from our transcriptomic
631 data. GPD: glycerol 3-phosphate dehydrogenase (EC: 1.1.1.53); GPAT: glycerol-3-phosphate
632 acyltransferase (EC: 2.3.1.15); AGPAT: acylglycerophosphate acyltransferase(EC: 2.3.1.51);
633 PP: phosphatidate phosphatase (EC: 3.1.3.4); DGAT: diacylglycerol acyltransferase (EC:
634 2.3.1.20); FA: fatty acids; FA-CoA: fatty acyl-CoAs. FA: fatty acids. Dashed boxes indicate
635 enzymes that were not found in our dataset. The process of FA elongation and desaturation is
636 shown in Figure 5.

637 Figure 4. Chromatograms of total fatty acid esters from *Eutreptiella* sp. Note that DHA (peak 19)
638 was the most abundant fatty acid. Corresponding fatty acids (labeled by length of carbon, e.g.
639 C16) were listed in Table 3. (A) Chromatogram of fatty acid esters from the phosphate–replete
640 cultures. (B) Chromatogram of fatty acid esters from the phosphate-depleted cultures. Insets are
641 closer-ups of some of the small peaks of esters. The first number (bold-typed) by the peak is
642 peak ID, the second number retention time, and the third number peak area (only trace elements
643 are shown). Note that retention times between the two samples are slightly different.

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644 Figure 5. Putative pathways of fatty acids elongation and desaturation inferred from *Eutreptiella*
645 sp. transcriptome annotation and GC/MS/MS analyses. Boxes with dashed lines indicate the
646 enzymes or substrates that were not found in our study.

647 Figure 6. Expression levels of genes involved in fatty acids biosynthesis in *Eutreptiella* sp. HD:
648 high-lipid-dark cultures. HL: high-lipid-light cultures. LL: low-lipid-light cultures. LD: low-
649 lipid-dark cultures.

650 Figure 7. Expression levels of genes involved in fatty acids biosynthesis (FAD, Δ 8, and TE) and
651 triacylglycerol biosynthesis (GPD, GPAT, and AGPT). HD: high-lipid-dark cultures. HL: high-
652 lipid-light cultures. LL: low-lipid-light cultures. LD: low-lipid-dark cultures.

653 Figure 8. Expression levels of genes involved in carbon fixation. HD: high-lipid-dark cultures.
654 HL: high-lipid-light cultures. LL: low-lipid-light cultures. LD: low-lipid-dark cultures.

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659 Table 1. Genes potentially involved in fatty lipid biosynthesis in *Eutreptiella* sp. Sequences and
660 BLAST results were listed in the supporting file "lipid_biosynthesis_genes".

Gene	EC code	Number of transcripts
Fatty acid biosynthesis		
Acetyl-CoA carboxylase	EC 6.4.1.2	2
Fatty acid synthase (animal type)	EC 2.3.1.85	1
Acyl-ACP thioesterase	EC 3.1.2.14	1
Acyl carrier protein		1
β-ketoacyl-ACP reductase	EC 1.1.1.100	3
Fatty acid desaturation		
Δ^5 desaturase	EC 1.14.19.-	2
Δ^6 desaturase	EC 1.14.19.-	5
Δ^8 desaturase	EC 1.14.19.-	2
Δ^9 desaturase (Acyl-ACP desaturase)	EC 1.14.19.2	1
Δ^{12} desaturase	EC 1.14.19.-	1
Δ^{15} desaturase	EC 1.14.19.-	1
Fatty acid elongation		
Δ^5 elongase		1
Δ^6 elongase	EC 1.14.19.-	1
Δ^9 elongase	EC 1.14.19.2	1
Palmitoyl-CoA hydrolase	EC 3.1.2.2	1
Palmitoyl hydrolase	EC 3.1.2.22	1
acetyl-CoA C-acyltransferase	EC 2.3.1.16	1
3-hydroxyacyl-CoA dehydrogenase	EC 1.1.1.35	2
Triacylglycerol biosynthesis		
Glyerol-3-phosphate dehydrogenase	EC 1.1.5.3	1
Glycerol3-phosphate acyltransferase	EC 2.3.1.15	2
Acylglycerophosphate acyltransferase	EC 2.3.1.51	2
Fatty acid metabolism		
Acyl-CoA dehydrogenase	EC 1.3.99.3	3
Long-chain-fatty-acid-CoA ligase	EC 6.2.1.3	2
Acetyl-CoA C-acyltransferase	EC 2.3.1.16	1
3-hydroxyacyl-CoA dehydrogenase	EC 1.1.1.35	2
Carnitine o-palmitoyltransferase	EC 2.3.1.21	1
Enoyl-CoA hydratase	EC 4.2.1.17	1
Acetyl-CoA thiolase	EC 2.3.1.9	1
Aldehyde dehydrogenase (AND+)	EC 1.2.1.3	2

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661 Table 2. Types and relative abundance of fatty acids detected in *Eutreptiella* sp.

Peak ID	Retention time	Systematic name	Abbreviation	Relative abundance (%)F/2*	Relative abundance (%)F/2-P*
1	26.19	4,7,10,13-Hexadecatetraenoic acid	16:4(n-3)	98.04	11.44
2	26.37	7,10-Hexadecadienoic acid	16:2(n-6)	2.59	0.08
3	26.47	7,10,13-Hexadecatrienoic acid	16:3(n-3)	12.63	0.54
4	26.62	7-Hexadecenoid acid	16:1(n-9)	11.89	0.27
5	26.97	9-Hexadecenoid acid	16:1(n-7)	13.54	0.79
6	27.1	Hexadecanoic acid	16:0	90.45	11.28
7	29.11	6,9,12-Octadecatrienoic acid	18:3(n-6)	3.90	0.13
8	30.04	6.9.12.15-Octadecatetraenoic acid	18:4(n-3)	41.00	12.40
9	30.23	9,12-Octadecadienoic acid	18:2(n-6)	3.20	0.53
10	30.36	9,12,15-Octadecatrienoic acid	18:3(n-3)	72.63	23.58
11	30.47	9-Octadecenoic acid	18:1(n-9)	7.97	0.58
12	30.85	Octadecanoic acid	18:0	8.43	2.12
13	33.1	5,8,11,14-Eicosatetraenoic acid	20:4(n-6)	6.44	1.88
14	33.22	5,8,11,14,17-Eicosapentaenoic acid	20:5(n-3)	78.69	40.02
15	33.53	8,11,14,17-Eicosatetraenoic acid	20:4(n-3)	8.39	4.02
16	33.77	8,11-Eicosadienoic acid	20:2(n-9)	1.13	0.71
17	33.87	11,14,17-Eicosatrienoic acid	20:3(n-3)	3.16	1.02
18	36.17	4,7,10,13,16-Docosapentaenoic acid	22:5(n-6)	68.78	54.93
19	36.3	4,7,10,13,16,19-Docosahexaenoic acid	22:6(n-3)	100.00	100.00
20	36.4	7,10,13,16-Docosatetraenoic acid	22:4(n-6)	43.11	47.18
21	36.49	7,10,13,16,19-Docosapentaenoic acid	22:5(n-3)	3.56	6.03
22	37.54	Docosanoic acid	22:0	5.94	1.01
23	38.79	6,9,12,15,18,21-Tetracosahexaenoic acid	24:6 (n-3)	5.32	0.85
24	40.14	15-Tetracosenoic acid	24:1(n-9)	0.46	-

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663 *The numbers of relative abundance were obtained by dividing the peak area of the fatty acid to
664 the peak area of DHA; F/2 and F/2-P depict phosphate-replete and phosphate-depleted F/2
665 medium, respectively.
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667 Table 3. Primers used in reverse-transcription quantitative PCR (RT-qPCR) analysis in this
668 study.

Primer name	Sequences (5´-3´)	RT-qPCR for *Eutreptiella* sp. gene
ACPF1	GCAGCAAAGCAAATCAAACCA	acyl carrier protein forward
ACPF2	CAACCCAACTCTCACTTCTACAT	acyl carrier protein forward
ACPR	GCATCAGCGACCGATACAATA	acyl carrier protein reverse
AGPCF	TTACGAACTTGCGATGCTTCA	1-acyl-sn-glycerol-3-phosphate acyltransferase forward
AGPCR1	GGAGGTATGATTGGAGATGATGAG	1-acyl-sn-glycerol-3-phosphate acyltransferase reverse
AGPCR2	CGATGACTTGACACCACCAA	1-acyl-sn-glycerol-3-phosphate acyltransferase reverse
ACCASE-F	GTGTTGGCATGTTGTCTCTTG	ACCase forward
ACCASE-R1	GGATACCGCCATTGGAACC	ACCase reverse
ACCASE-R2	GAAGTCGGCTTGAATCTGTGA	ACCase reverse
DELTA8FADF	GACATCATTGAGAATTATCGGAACC	delta-8 fatty acid desaturase forward
DELTA8FADR1	GCGTCACCATCAGCATAGC	delta-8 fatty acid desaturase reverse
DELTA8FADR2	CGGAAGTCCTCAGCAACCT	delta-8 fatty acid desaturase reverse
PRKF	TTCCAAGCCAATCTACAACCAT	Phosphoribulokinase forward
PRKR1	CGAATCTGAGACGGAAGAACTT	Phosphoribulokinase reverse
PRKR2	GCAACACGGTCATCCAACA	Phosphoribulokinase reverse
ThioesteraseF	AGCAATCGGCAGGAGCAA	Thioesterase forward
ThioesteraseR1	ACAGGCAAGCGGCATCT	Thioesterase reverse
ThioesteraseR2	TGGAACAACGGCAGCAATG	Thioesterase reverse
KAR1-F	AAGCCATCGCTGTTGGATTG	β-Ketoacyl-ACP reductase1 forward
KAR1-R1	ACCGCATCCCAATCAGCAA	β-Ketoacyl-ACP reductase1 reverse
KAR1-R2	CTGATTCGTAATGTCTGCCTGAA	β-Ketoacyl-ACP reductase1 forward
KAR2-F1	TTCAACGACGCAGCCTGTA	β-Ketoacyl-ACP reductase2 forward
KAR2-F2	CCGTCAACTCGCTCATTGTT	β-Ketoacyl-ACP reductase2 forward
KAR2-R	GCCTCTTCTGTCATCTTGTCAA	β-Ketoacyl-ACP reductase2 reverse
KAR3-F1	CGGTGCTTCCAGAGGTGTT	β-Ketoacyl-ACP reductase3 forward
KAR3-F2	GGAGCCTCTGTGGTTGTCA	β-Ketoacyl-ACP reductase3 forward
KAR3-R1	TTCATCTGCTTGTCCATCATCTC	β-Ketoacyl-ACP reductase3 reverse
FAD-F	AACATTCTAACGCCACACCAT	FAD family gene forward
FAD-R1	ACTGCCACATTGAGACTCCT	FAD family gene reverse
FAD-R2	ACAGTCGGTGCGTTGTAATC	FAD family gene reverse
GPD-F	CTTGGCAGGCTTCTGTAATCG	glycerol-3-phosphate dehydrogenase forward

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GPD-R1	GCTTCGTGGATCGTGAGGA	glycerol-3-phosphate dehydrogenase reverse
GPD-R2	ATCACCACAACATCGTACTCCT	glycerol-3-phosphate dehydrogenase reverse
GPAT-F	CCAGATGAGGCTTCTTCTACAA	glycerol-3-phosphate acyltransferase forward
GPAT-R1	CAACAGCGATGGCGTTCAT	glycerol-3-phosphate acyltransferase reverse
GPAT-R2	TCACCGCATCCACGAACT	glycerol-3-phosphate acyltransferase reverse
PEPCASE-F	GCCTGTCTTGAATGACTCCTT	Phosphoenolpyruvate carboxylase forward
PEPCASE-R1	GCATACTTGACCAGCAGTGA	Phosphoenolpyruvate carboxylase reverse
PEPCASE-R2	CCGTGGTTCTGGATCAGGTT	Phosphoenolpyruvate carboxylase reverse
PEPCK1-F1	AAGGCTCTGGCGACATTAAG	Phosphoenolpyruvate carboxykinase1 forward
PEPCK1-F2	TGGCGACATTAAGAACAGCATA	Phosphoenolpyruvate carboxykinase1 forward
PEPCK1-R	TCTGGTTGAGAGGAATGAGTGT	Phosphoenolpyruvate carboxykinase1 reverse
PEPCK2-F	TGGTATTGATTGAGCACAACAATG	Phosphoenolpyruvate carboxykinase2 forward
PEPCK2-R1	GAGGTCTTGATTGTGGATTGGAAT	Phosphoenolpyruvate carboxykinase2 reverse
PEPCK2-R2	TACTTGCGGATCTCGGTGATA	Phosphoenolpyruvate carboxykinase2 reverse
PEPCK3-F	GCAACGGACGATGTGAACC	Phosphoenolpyruvate carboxykinase3 forward
PEPCK3-R1	CGCATGTGAGGAACCAACTG	Phosphoenolpyruvate carboxykinase2 reverse
PEPCK3-R2	GTCTGATTGCGGTCCAACTTAT	Phosphoenolpyruvate carboxykinase2 reverse
PPDK-F1	CTTATCTCCCACAAATGACTACCC	phosphate dikinase forward
PPDK-F2	ACTGGTTCATCTTCGCCTCAT	phosphate dikinase forward
PPDK-R	TCAGCCAAGTTCGCACCTT	phosphate dikinase reverse
EF-1aF	GCACCTCTCACCATCTTACAAT	elongation factor 1 alpha forward
EF-1aR1	GTTCCGCCTTCAGCTTGTC	elongation factor 1 alpha reverse
EF-1aR2	TTTCCTTTCGCTTTCCCTTCTT	elongation factor 1 alpha reverse
TubulinF	CCTTGGYCAGGCTGGTATCC	tubulin forward
TubulinR1	CGGCGTTGAAGCACAAGAA	tubulin reverse
TubulinR2	AGTTGTTGGCGGCATCCT	tubulin reverse
ActinF	AAGCGAAGCGTGGTATCTTG	actin forward
ActinR1	ACATAATCTGCGTCATCTTCTCC	actin reverse
ActinR2	GGTTCATAGGAGCCTCAGTGA	actin reverse

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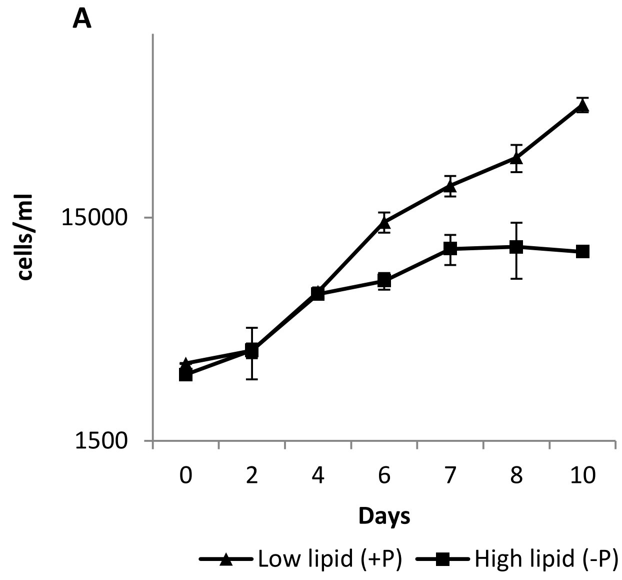

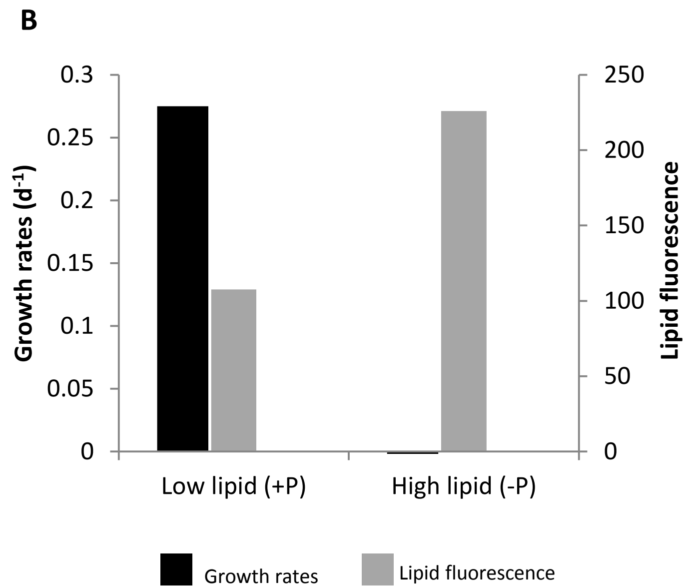

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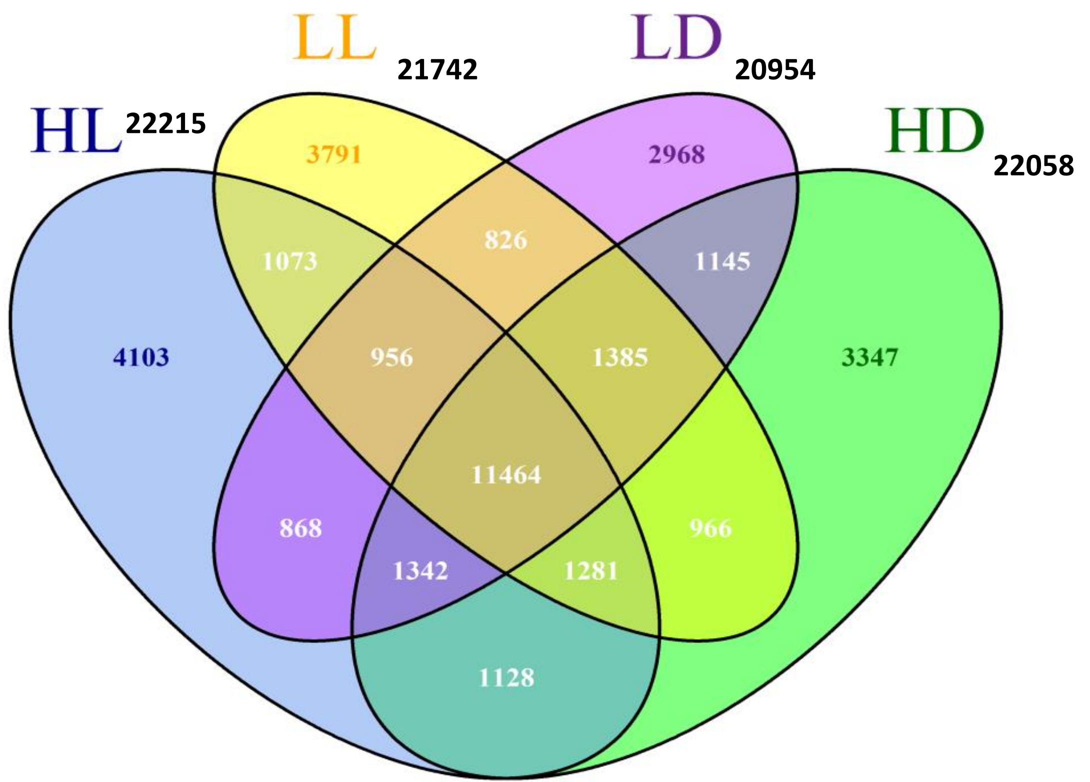

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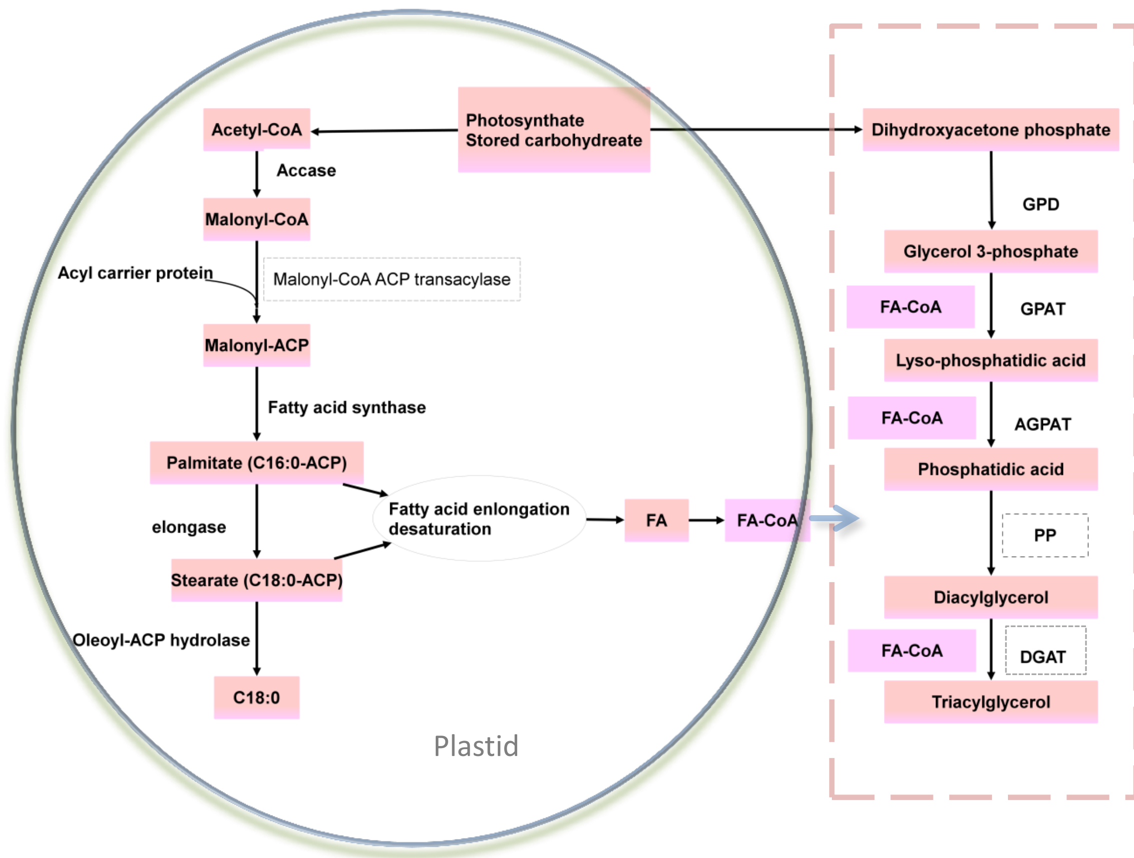

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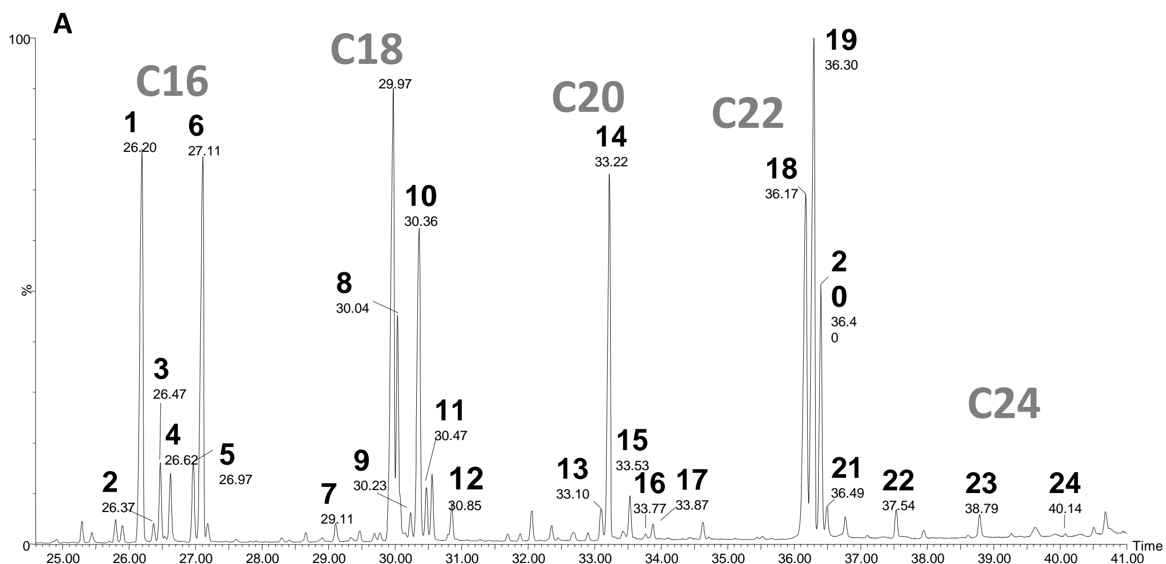

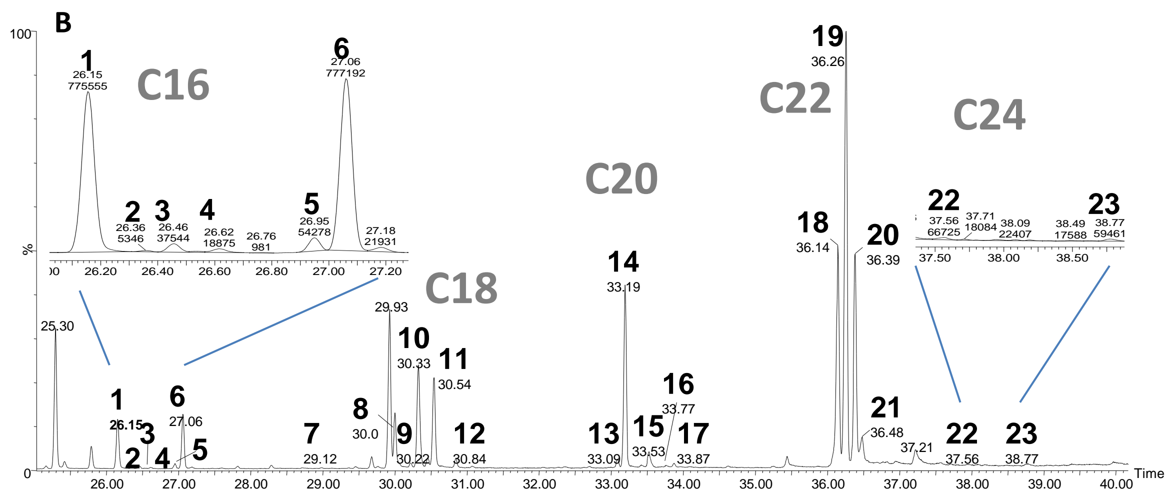

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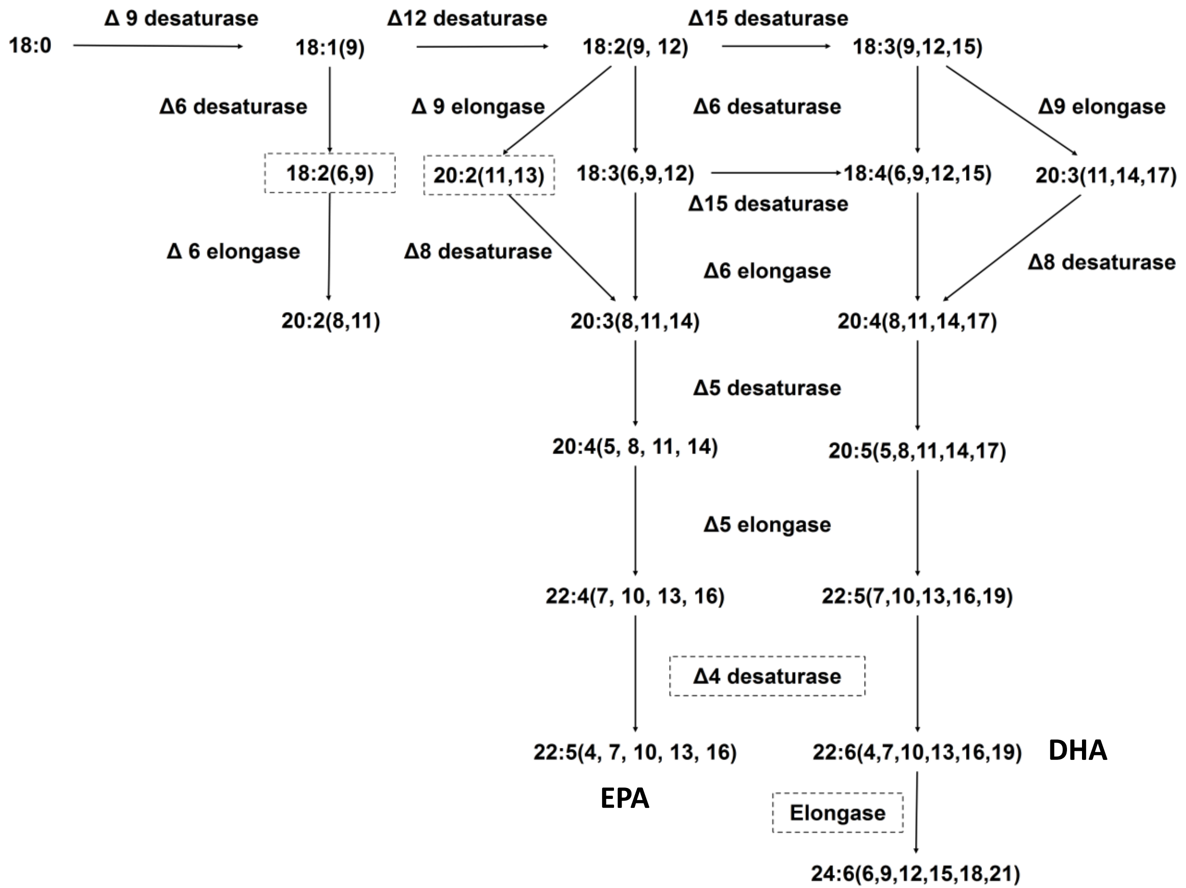

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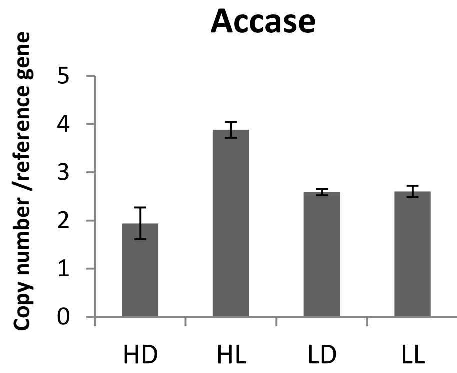

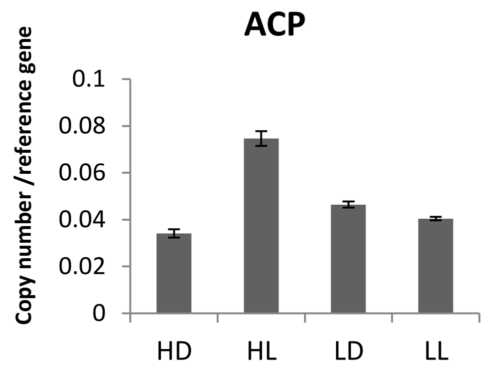

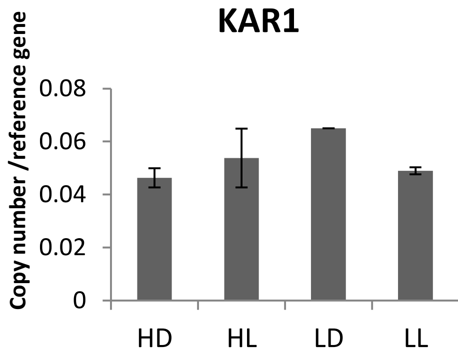

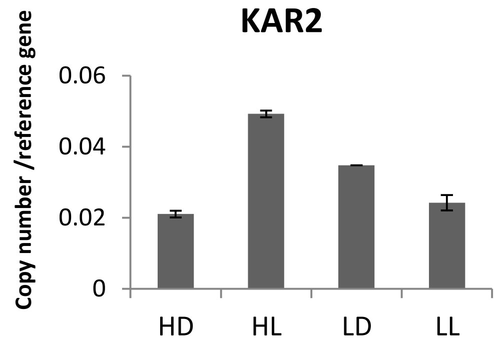

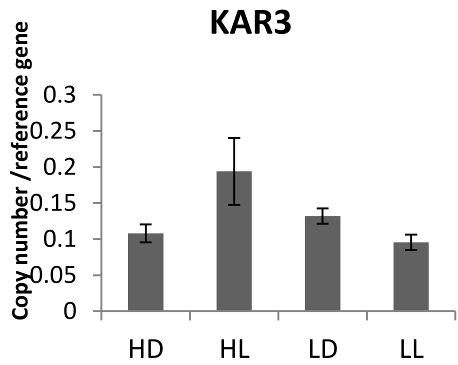

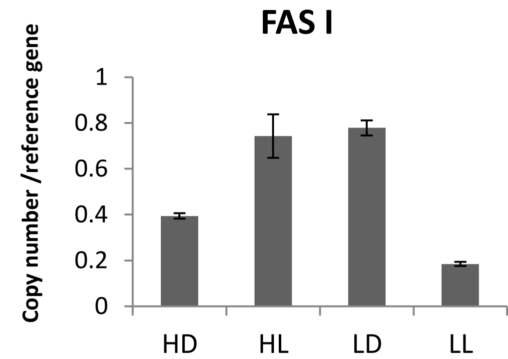

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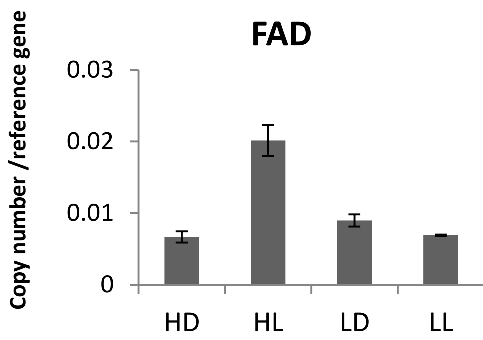

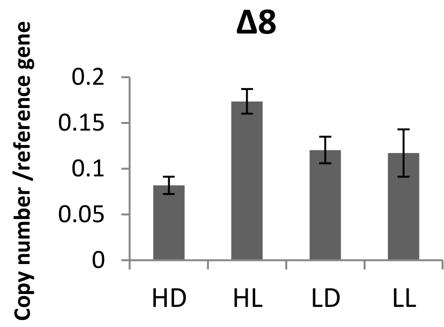

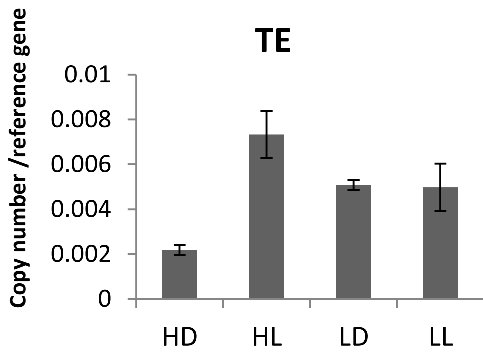

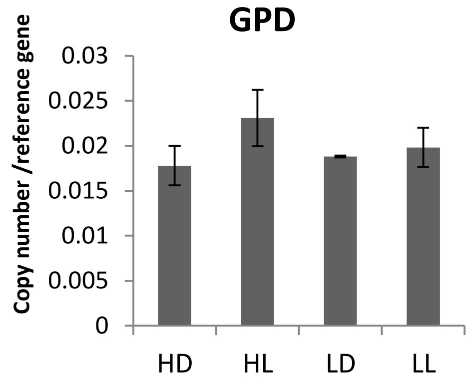

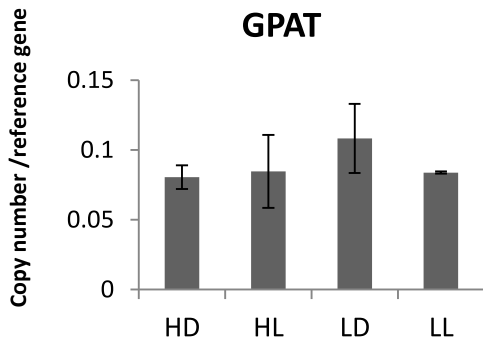

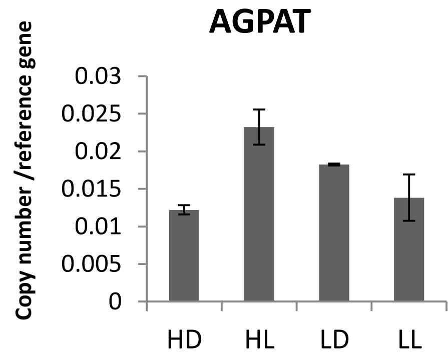

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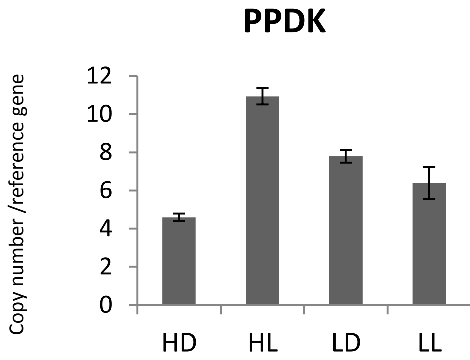

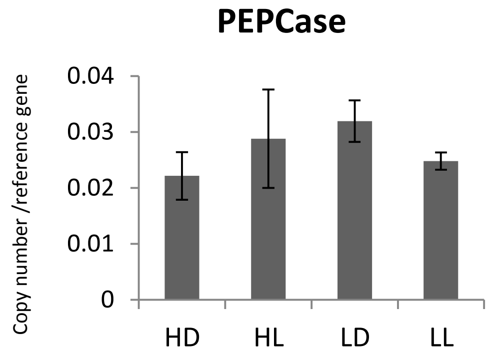

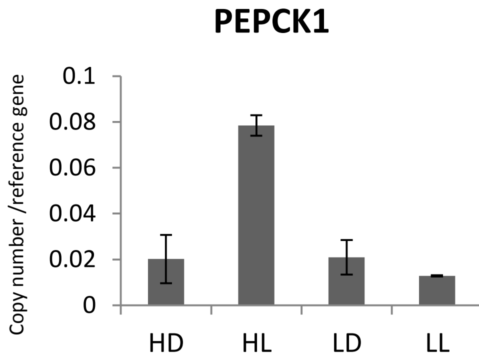

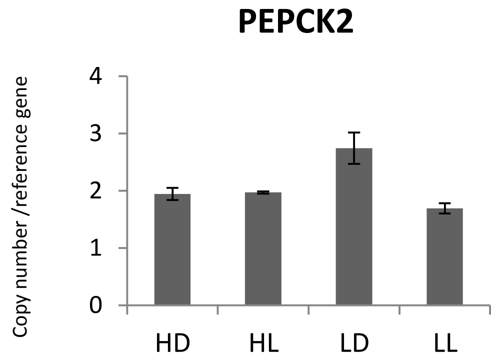

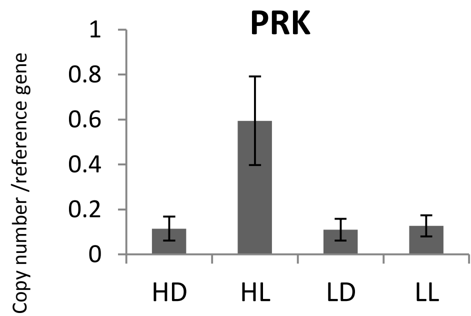

Kuo et al. Figure 8