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1 **Lipid remodelling and an altered membrane associated proteome may drive the**  
2 **differential effects of EPA and DHA treatment on skeletal muscle glucose uptake and**  
3 **protein accretion**

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22 **Running Title:** Skeletal muscle lipid remodelling by EPA and DHA.

23

24 **Abstract**

25 In striated muscle, EPA and DHA have differential effects on the metabolism of glucose and  
26 differential effects on the metabolism of protein. We have shown that, despite similar incorporation,  
27 treatment of C<sub>2</sub>C<sub>12</sub> myotubes (CM) with EPA but not DHA improves glucose uptake and protein  
28 accretion. We hypothesized that these differential effects of EPA and DHA may be due to divergent  
29 shifts in lipidomic profiles leading to altered proteomic profiles. We therefore carried out an  
30 assessment on the impact of treating CM with EPA and DHA on lipidomic and proteomic profiles.  
31 FAME analysis revealed that both EPA and DHA led to similar but substantial changes in fatty acid  
32 profiles with the exception of arachidonic acid, which was decreased only by DHA, and DPA, which  
33 was increased only by EPA treatment. Global lipidomic analysis showed that EPA and DHA induced  
34 large alterations in the cellular lipid profiles and in particular, the phospholipid classes. Subsequent  
35 targeted analysis confirmed that the most differentially regulated species were phosphatidylcholines  
36 and phosphatidylethanolamines containing long chain fatty acids with 5 (EPA treatment) or 6 (DHA  
37 treatment) double bonds. As these are typically membrane associated lipid species we hypothesized  
38 that these treatments differentially altered the membrane-associated proteome. SILAC based  
39 proteomics of the membrane fraction revealed significant divergence in the effects of EPA and DHA  
40 on the membrane associated proteome. We conclude that the EPA specific increase in polyunsaturated  
41 long chain fatty acids in the phospholipid fraction is associated with an altered membrane associated  
42 proteome and these may be critical events in the metabolic remodelling induced by EPA treatment.

43 **Keywords:** cell signalling, fish oil, fatty acid, insulin, lipidomics, lipids

44

45 **Introduction**

46 Fatty acids play an important role in skeletal muscle metabolism, not only as substrates for oxidative  
47 phosphorylation or vital structural components of membranes but also as regulators of enzyme  
48 activities and signalling molecules (8). Furthermore, dysfunctions in the control of fatty acid  
49 metabolism can be an important factor in the aetiology of conditions such as insulin resistance and  
50 muscle atrophy (8). The lipid composition of skeletal muscle undergoes constant fluctuations and is  
51 reflective of dietary fat intake (6). There is strong evidence to suggest that the enrichment of skeletal  
52 muscle with omega-3 (n-3) fatty acids may have therapeutic benefits on muscle metabolism and  
53 function (14). Oral n-3 supplementation above the Reference Dietary Intake is known to result in  
54 significant incorporation of n-3 fatty acids into the skeletal muscle lipid pool (6, 13, 30).

55 Several n-3 supplementation studies in humans have observed beneficial effects ranging from an  
56 increased sensitivity to anabolic stimuli (43, 44) and muscle function (40, 45). Eight weeks n-3  
57 supplementation improved the muscle protein synthetic response (MPS) to a hyperinsulinaemic amino  
58 acid infusion in both young (44) and elderly individuals (43). Furthermore, when taken alongside a  
59 resistance based exercise programme (12, 40) or in the absence of any strength training (45) n-3 fatty  
60 acids enhance strength and or physical function in the elderly. Concurrent with anabolic properties, n-  
61 3 FAs also display anti-catabolic effects. n-3 supplementation prevented muscle mass losses in burned  
62 guinea pigs (5) and protected against muscle mass loss during anti-neoplastic therapy in cancer  
63 patients (34). However, when combined in systematic reviews do not always show a beneficial effect  
64 on muscle mass during cancer treatment (39). Moreover n-3 FAs attenuated soleus atrophy in rodents  
65 that underwent 10 days of hind limb immobilization (55).

66 As well as effects on muscle protein metabolism there is building evidence to suggest that n-3 fatty  
67 acids may also modulate glucose metabolism. Current meta-analyses detect a neutral or small effect of  
68 n-3 FAs on measures of insulin sensitivity (4). However, there are several studies across a range of  
69 models utilizing higher doses of n-3s which support a role for n-3s in improving muscle mitochondrial  
70 function and glucose metabolism (23, 28, 35, 37, 47, 48). For instance, replacing 3.4% of the Kcals

71 with n-3 fatty acids on a high fat diet protects mice against declines in glucose tolerance during a 10-  
72 week high fat diet despite similar increases in body weight compared to high fat alone (28). Whereas  
73 supplementation studies in humans have not demonstrated a consensus (4), an interesting lipid  
74 infusion trial has shown n-3 fatty acids to be bioactive in humans with respect to glucose metabolism  
75 (46). More specifically, the addition of n-3 fatty acids to a lipid infusion of n-6 fatty acids attenuated  
76 the decline in insulin stimulated glucose disposal caused by n-6 infusion alone suggesting that the n-3  
77 fatty acids have a protective effect on glucose metabolism in the presence of an n-6 overload (46).

78 Fish oil and n-3 supplements are a heterogeneous mixture of fatty acids of which eicosapentaenoic  
79 acid (EPA, 20:5) and docosahexaenoic acid (DHA 22:6) are thought to be the most biologically  
80 active. Due to many studies using a combination of EPA and DHA and varying ratios of each fatty  
81 acid it is difficult to assert whether EPA or DHA alone is causing the observed effects or if EPA and  
82 DHA work synergistically or antagonistically for that matter. The molecular mechanisms of n-3  
83 action are still poorly understood. Work from Olefsky's lab suggests that GPR120 acts as a general n-  
84 3 receptor in macrophages and adipocytes that, when activated by n-3 fatty acids leads to increases in  
85 whole body insulin sensitivity by reducing inflammation (37). EPA has also been shown to  
86 antagonise the action of TNF $\alpha$  on C<sub>2</sub>C<sub>12</sub> myotube formation in a manner partially dependent on  
87 PPAR $\gamma$  (29). Additionally, EPA reduces the activation of Nuclear Factor-kappa Beta (NF-Kb) leading  
88 to a reduction in Muscle RING finger protein-1 (Murf-1) signalling, an important mediator of muscle  
89 atrophy in cultured myotubes (22). Furthermore, a follow up study employing both EPA and DHA  
90 demonstrated that DHA was more efficient in this mechanism than EPA (52). However, EPA has  
91 been shown to improve metabolic flexibility in response to changing substrate availabilities (21). EPA  
92 has also previously been shown to improve both basal and insulin stimulated glucose uptake in  
93 cultured myotubes (1). However, it remains to be seen whether DHA similarly improves glucose  
94 uptake in skeletal muscle. Despite the numerous similar intracellular effects and similar structure,  
95 EPA and DHA may have divergent physiological effects in skeletal muscle. EPA improves skeletal  
96 muscle protein metabolism while DHA has a non-significant effect (24). In other striated muscle  
97 models such as cardiomyocytes, EPA but not DHA increases glucose and fatty acid uptake despite

98 similar effects on cell signalling (18). In plasma both EPA and DHA reduced triacylglycerol (TAG)  
99 but only DHA modulates HDL (high density lipoprotein) and LDL (low density lipoprotein) particle  
100 size (53). Collectively these data suggest that in certain contexts, EPA and DHA can have differential  
101 biological effects.

102 The molecular mechanisms underpinning the divergent physiological effects of EPA vs DHA are  
103 currently under explored. However, differential remodelling of the lipid profile may partially explain  
104 the divergent physiological response observed between EPA and DHA. Currently studies attempting  
105 to address how n-3 fatty acids affect the lipidomic profile of skeletal muscle are limited. As expected  
106 n-3 intake leads to incorporation into the lipid pool of multiple tissues i.e. plasma, muscle, adipose  
107 tissue and liver with a significant proportion being directed towards phospholipid pools (7, 27, 30, 42-  
108 44). This incorporation is not limited to the plasma membrane and is also incorporated into  
109 subcellular organelles such as mitochondria (20). It is hypothesized that a primary driver of the effects  
110 of EPA and DHA is the displacement of arachidonic acid (AA) from membranes with studies from a  
111 range of models supporting this (27, 32, 42) while some *in vivo* human studies observe no change in  
112 total AA in the skeletal muscle lipid pool (30). However, this does not discount the possibility that  
113 AA might be displaced from specific lipid fractions. Multiple human studies have assessed the impact  
114 of n-3 supplementation on skeletal muscle phospholipid pool however the use of a heterogeneous mix  
115 of n-3 fatty acids precludes the ability to detect the isolated effects of EPA vs DHA on lipid profiles  
116 (6, 13, 30). Furthermore, into which skeletal muscle phospholipid fractions EPA and DHA are  
117 incorporated are poorly understood. In plasma phospholipids EPA and DHA induce a similar lipid  
118 profile yet EPA increased docosapentanoic acid (DPA 22:5 n-3) and a differential but non-significant  
119 increase in stearate acid (SA 18:0) (31). In smooth muscle cell phospholipids both EPA and DHA are  
120 heavily incorporated into the phosphatidylcholine (PC) fraction but EPA is divergently incorporated  
121 into the phosphatidylinositol (PI) and phosphatidylserine (PS) fraction while DHA is incorporated  
122 into the phosphatidylethanolamine (PE) fraction (33).

123 One of the main cellular fates for fatty acids is incorporation into complex lipid species and so it  
124 seems logical to hypothesize that the differential action of EPA and DHA may be due to differential

125 effects on the cellular lipidome. To date no study has characterised the impact of EPA and DHA  
126 individually on lipidomic profiles in skeletal muscle. In this manuscript we demonstrate that the C<sub>2</sub>C<sub>12</sub>  
127 cell line acts as a model in which EPA and DHA have differential effects on metabolism. We  
128 followed these experiments by an extensive assessment of lipid changes hypothesizing that the  
129 divergent effects of EPA and DHA are associated with differential regulation of the skeletal muscle  
130 lipidome. The lipidomic profiling indicated that multiple membrane associated lipid species were  
131 differentially altered by EPA and DHA treatments. We therefore hypothesized that the lipidomic  
132 remodelling would be associated with remodelling of the membrane-associated proteome. SILAC  
133 based proteomics of the membrane fraction indicated EPA and DHA differentially regulate the  
134 membrane associated proteome. Therefore, the effects of EPA may be due to membrane associated  
135 proteomic remodelling secondary to lipidomic remodelling of the membrane-associated lipids.

136

137 **Materials and methods**

138 **Materials**

139 All plasticware for tissue culture was purchased from fisher scientific (UK). Tissue culture media and  
140 sera were purchased from Invitrogen. Fatty acids EPA and DHA (>99%, liquid form) were purchased  
141 from Sigma Aldrich (Dorset, UK). H<sup>3</sup>2-DG was purchased from Hartman Analytic. All solvents were  
142 LC-MS grade (Fisher Scientific, Loughborough, UK).

143 **Cell culture**

144 C<sub>2</sub>C<sub>12</sub> myoblasts were grown in Dulbecco's Modified Eagle Medium (DMEM) containing 20% fetal  
145 bovine serum and 1% penicillin/streptomycin and incubated at 37°C and 5% CO<sub>2</sub>. Myoblasts were  
146 maintained at ~60% confluence. Differentiation was induced once confluence reached 80-90% by  
147 changing the media to differentiation media (DMEM supplemented with 2% horse serum and 1%  
148 Penicillin/Streptomycin) for 72 hours. Following 72 hours differentiation cells were treated with 50  
149 µM EPA or 50 µM DHA pre-bound to 2% fatty acid free BSA for 72 hours before collection. As a  
150 control cells were treated with 2% fatty acid free (FAF) BSA for 72 hours before collection. Fatty  
151 acids were conjugated to 2% fatty acid free BSA in DM by constant agitation for 1 hour at 37°C.  
152 Following treatment cell pellets were collected following 3 washes in 2% FAF BSA in PBS and  
153 centrifuged at 800 rpm for 4 minutes, excess liquid was removed and pellets were frozen in liquid  
154 nitrogen and stored at -80°C until further analysis.

155 **FAME analysis**

156 Total lipids were extracted by homogenising in 20 volumes of chloroform/methanol (2:1 v/v). Total  
157 lipids were prepared according to the method of Folch et al. (17) and non-lipid impurities were  
158 removed by washing with 0.88% (w/v) KCl. The weight of lipids was determined gravimetrically  
159 after evaporation of solvent and overnight desiccation under vacuum. Fatty acid methyl esters  
160 (FAME) were prepared by acid-catalysed transesterification of total lipids according to the method of  
161 Christie et al. (35). Extraction and purification of FAME was performed as described by Ghioni et al.  
162 (36). FAME were separated by gas-liquid chromatography using a ThermoFisher Trace GC 2000



163 (ThermoFisher, Hemel Hempstead, UK) equipped with a fused silica capillary column (ZBWax, 60m  
164 x 0.25  $\mu\text{m}$  x 0.25 mm i.d.; Phenomenex, Macclesfield, UK) with hydrogen as carrier gas and using  
165 on-column injection. The temperature gradient was form 50 to 150°C at 40°C/min and then to 195°C  
166 at 1.5°C/min and finally to 220°C at 2°C/min. Individual methyl esters were identified by reference to  
167 published data (Ackman, 1980). Data were collected and processed using the Chromcard for  
168 Windows (version 2.00) computer package (Thermoquest Italia S.p.A., Milan, Italy). All experiments  
169 were carried in duplicate from 4 independent experiments. Data were represented as fold change from  
170 the respective BSA control condition, logged to  $\log_2$  and significance was determined by t-test and  
171 corrected for false discovery rate.

### 172 **Global lipidomic analysis of C<sub>2</sub>C<sub>12</sub> myotubes**

173 Lipid extraction was performed according to the method described above. The lipids were analysed by  
174 liquid chromatography-mass spectrometry (LC-MS) using a Thermo Exactive Orbitrap mass  
175 spectrometer (Thermo Scientific, Hemel Hempstead, UK), equipped with a heated electrospray  
176 ionization probe and coupled to a Thermo Accela 1250 UHPLC system. All samples were analysed in  
177 both positive and negative ion mode over the mass to charge (m/z) range 200-2000. The lipids were  
178 separated on to a Thermo Hypersil Gold C18 column (1.9  $\mu\text{m}$ , 2.1mm x 100 mm,). Mobile phase A  
179 consisted of water containing 10 mM ammonium formate and 0.1% (v/v) formic acid. Mobile phase B  
180 consisted of 90:10 isopropanol/acetonitrile containing 10 mM ammonium formate and 0.1% (v/v)  
181 formic acid. The initial conditions for analysis were 65%A/35%B. The percentage of mobile phase B  
182 was increased to 100% over 10 min and held for 7 minutes before re-equilibration with the starting  
183 conditions for 4 minutes. The raw LC-MS data were processed with Progenesis QI v2.0 software  
184 (Non-linear Dynamics, Newcastle, UK) and searched against LIPID MAPS ([www.lipidmaps.org](http://www.lipidmaps.org)) and  
185 the Human Metabolome Database (<http://www.hmdb.ca/>) for identification. All experiments were  
186 carried out in duplicate from three independent experiments.

187

### 188 **Phospholipid profiling of C<sub>2</sub>C<sub>12</sub> myotubes**

189 In order to assess the incorporation of EPA and DHA into cellular phospholipids the lipid extracts  
190 from C<sub>2</sub>C<sub>12</sub> myotubes were analysed by electrospray ionisation-tandem mass spectrometry (ESI-  
191 MS/MS). All analyses were performed using a Thermo TSQ Quantum Ultra triple quadrupole mass  
192 spectrometer equipped with a heated electrospray ionization probe. Samples were directly infused into  
193 the ion source at a flow rate of 5 µL/min. Phosphatidylcholine, lysophosphatidylcholine and  
194 sphingomyelin species were identified by precursor scanning for mass to charge ratio (m/z) 184 in  
195 positive ion mode. Phosphatidylethanolamine and lysophosphatidylethanolamine species were  
196 identified by neutral loss scanning for m/z 141 in positive ion mode. Phosphatidylserine species were  
197 identified by neutral loss scanning for m/z 87 in negative ion mode. Phosphatidylinositol species were  
198 identified by precursor scanning for m/z 241 in negative ion mode. The data were expressed as a  
199 percentage composition of the relevant phospholipid fraction.

200

#### 201 **Glucose uptake**

202 C2C12 myotubes, were exposed to 50µM EPA or 50µM DHA pre-bound to 2% fatty acid free BSA  
203 or 2% fatty acid free BSA as a control for 48 h before a 2 h serum-starve. Following the 2 h serum  
204 starve cells were exposed to insulin (100 nmol/l) or vehicle control for 30 mins. Myotubes were  
205 incubated (12 min) with 10 µmol/l 2-deoxy-D-[3 H]glucose (2DG; 24.4 kBq/ml; Hartman Analytic) at  
206 20°C. Non-specific uptake was determined using 10 µmol/l cytochalasin B (Sigma–Aldrich). After  
207 lysis, cell-associated radioactivity was measured (Beckman, High Wycombe, UK; LS 6000IC  
208 scintillation counter), and protein was quantified using the Bradford reagent. Data represented are the  
209 average of 6 independent experiments carried out in duplicate.

#### 210 **Mitochondrial function**

211 C2C12 myotubes, were exposed to 50µM EPA or 50µM DHA pre-bound to 2% fatty acid free BSA  
212 or 2% fatty acid free BSA as a control for 48 h. Following 48 h in the respective treatments cells were  
213 degassed and exposed to a mito-stress test in the Seahorse cellular respiration analyser as previously  
214 described (16).

215 **Muscle protein synthesis and muscle protein breakdown**

216 Protein degradation was assessed by the quantification of the released L-[2,4,<sup>3</sup>H] phenylalanine into  
217 the culture media. Following 4 days of differentiation myotubes were incubated with medium  
218 containing 2.5 μCi L-[2,4,<sup>3</sup>H] phenylalanine/ml and the label was maintained for 24 hours in order to  
219 label long lived proteins. Following the pulse the cells were washed 2x in PBS and incubated in cold  
220 chase media (DMEM + 2mM L-phenylalanine) for 3 hours to allow for degradation of short lived  
221 proteins. Myotubes were then treated with either 50 μM EPA/DHA bound to 2% FAF-BSA or 2% FAF-  
222 BSA alone for 24 hrs. Following treatment an aliquot of the media was removed and radioactivity  
223 released was assessed by scintillation counting. The remaining myotubes were then thoroughly  
224 washed with ice cold saline (0.9%) and lysed with 50mM NaOH + 1% SDS for a minimum of 30 mins  
225 at room temperature. Residual radioactivity in cell lysates was then assessed by scintillation  
226 counting. Total radioactivity was calculated as the sum of the L-[2,4,<sup>3</sup>H] phenylalanine released into  
227 the media and the residual cell retained L-[2,4,<sup>3</sup>H] phenylalanine. Protein breakdown is presented as  
228 the fraction of the total incorporated L-[2,4,<sup>3</sup>H] phenylalanine released into the media.

229

230 Basal protein synthesis was assessed by the incorporation of L-[2,4,<sup>3</sup>H] phenylalanine into peptide  
231 chains. Following differentiation, myotubes were treated with either 50 μM EPA/DHA bound to 2%  
232 FAF-BSA or 2% FAF-BSA alone for 24 hrs. At the end of the treatment period the media was removed  
233 and DMEM containing 1μCi L-[2,4,<sup>3</sup>H] phenylalanine (0.5 μCi/ml) was added for 180 mins. The  
234 reaction was stopped by 2x washes in ice cold saline (0.9%) before 3x washes with Trichloroacetic  
235 acid (TCA) (10%) to remove any unincorporated tracer. Residual TCA was then removed by rinsing  
236 cells with methanol and plates left to dry. Myotubes were then lysed in 50mM NaOH + 1% SDS for a  
237 minimum of 30 minutes. An aliquot was collected for liquid scintillation counting to assess <sup>3</sup>H  
238 incorporation into proteins and the remaining lysate was used to determine protein content by the  
239 DC protein assay. Protein synthesis is presented as counts per minute/μg of protein.

240

241 **Protein content**

242 Protein content following 72 h treatment with 50 $\mu$ M EPA or 50 $\mu$ M DHA pre-bound to 2% fatty acid  
243 free BSA or 2% fatty acid free BSA as a control was determined by multiplying the concentration of  
244 the supernatant [as determined using the bicinchoninic acid protein assay according to the  
245 manufacturer's instructions (Sigma Aldrich, UK)] by the total volume of supernatant collected from a  
246 6 well plate. Data are representative of 5 independent experiments carried out in triplicate.

247

248 **Cell processing**

249 Cell lysates were collected from 6 well plates by scraping on ice in RIPA buffer [50 mmol/l Tris·HCl  
250 pH 7.5, 50 mmol/l NaF, 500 mmol/l NaCl, 1 mmol/l sodium vanadate, 1 mmol/l EDTA, 1% (vol/vol)  
251 Triton X-100, 5 mmol/l sodium pyrophosphate, 0.27 mmol/l sucrose, and 0.1% (vol/vol) 2-  
252 mercaptoethanol and Complete protease inhibitor cocktail (Roche)] followed by snap freezing on  
253 liquid nitrogen. For preparation for western blotting samples were thawed and debris was removed by  
254 centrifugation at 4°C for 15 min at 13,000 g. The supernatant was then removed, and protein  
255 concentration was determined using the bicinchoninic acid protein assay according to the  
256 manufacturer's instructions (Sigma Aldrich, UK).

257

258 **Western blotting**

259 For WB, 100 $\mu$ g of supernatant was made up in Lamelli sample buffer, and 15  $\mu$ g of total protein was  
260 loaded per well and run at 150 V for 1 h 15 min. Proteins were then transferred onto Whatman  
261 Immobilon Nitrocellulose membranes (Fisher Scientific, Loughborough, UK) at 30 V overnight on  
262 ice. Membranes were blocked in 3% BSA-Tris-buffered saline (containing vol/vol 0.1% Tween 20)  
263 for 1 h at room temperature, followed by incubation in primary antibodies [PKBthr308 (#2965) or  
264 total PKB (#4691) GLUT 1(Santa cruz, sc-7903), GLUT4 (#2213S), Hexokinase 1 (#2204S),  
265 Hexokinase 2 (#2867S), Mito profile (abcam, #ab110413) (New England Biolabs unless stated)] at  
266 4°C overnight. Membranes underwent three 5 min washes in TBST followed by incubation in the  
267 appropriate secondary antibodies [secondary horseradish peroxidase conjugated antibody was  
268 purchased from ABCAM (#6721)] for 1 h at room temperature. Membranes were again washed three

269 times for 5 min followed by incubation in enhanced chemiluminescence reagent (BioRad, Herts, UK).  
270 A BioRad ChemiDoc (Herts, UK) was used to visualize and quantify protein expression. Phospho-  
271 PKB was normalized to the corresponding total protein. Data are representative of 3 independent  
272 experiments carried out in duplicate.

### 273 **Membrane proteome**

274 Proteins associated with membranes were assessed using the SILAC proteomic method (38). C<sub>2</sub>C<sub>12</sub>  
275 myoblasts were grown in Dulbeccos' modified eagles medium (DMEM) supplemented with 20%  
276 dialysed (10 kKDa) foetal bovine serum plus labelled amino acids lysine and arginine in a humidified  
277 atmosphere of 37°C and 5% CO<sub>2</sub>. Cells intended to act as the control group were grown in with  
278 unlabelled lysine and arginine (light), the EPA treatment group were grown in R6K4 media (l-  
279 arginine-13C6 hydrochloride, l-lysine-4,4,5,5-d4 hydrochloride[medium]) while the DHA treatment  
280 group were grown with R10K8 containing media (l-arginine-13C6, 15N4 hydrochloride, l-lysine-  
281 13C6,15N2 hydrochloride [Heavy]). The use of combined labelled arginine and lysine ensures that  
282 nearly all peptides will contain a label after tryptic digestion. Cells were allowed to grow for at least 6  
283 population doublings to ensure full incorporation of labelled amino acids. We observed that use of  
284 dialysed sera and labelled media did not affect doubling time, cell morphology or differentiation  
285 capacity. Upon reaching 90-100% confluence the media was replaced with DMEM containing 2%  
286 dialysed donor horse serum (10 kDa) to induce differentiation. After 3-4 days of differentiation,  
287 myotubes were treated with either control, 50µM EPA or 50 µM DHA for 72 hours. For membrane  
288 proteome analysis, membranes were isolated using the Thermo scientific Mem per plus protein  
289 membrane extraction kit. The membrane proteome was assessed by LC-MS/MS.

### 290 **Mass spectrometry**

291 The resulting peptides were fractionated using an Ultimate 3000 nanoHPLC system in line with an  
292 Orbitrap Fusion Tribrid mass spectrometer (Thermo Scientific). In brief, peptides in 1% (vol/vol)  
293 formic acid were injected onto an Acclaim PepMap C18 nano-trap column (Thermo Scientific). After  
294 washing with 0.5% (vol/vol) acetonitrile 0.1% (vol/vol) formic acid peptides were resolved on a 250  
295 mm × 75 µm Acclaim PepMap C18 reverse phase analytical column (Thermo Scientific) over a 150

296 min organic gradient, using 7 gradient segments (1-6% solvent B over 1min., 6-15% B over 58min.,  
297 15-32%B over 58min., 32-40%B over 5min., 40-90%B over 1min., held at 90%B for 6min and then  
298 reduced to 1%B over 1min.) with a flow rate of 300 nl min<sup>-1</sup>. Solvent A was 0.1% formic acid and  
299 Solvent B was aqueous 80% acetonitrile in 0.1% formic acid. Peptides were ionized by nano-  
300 electrospray ionization at 2.0 kV using a stainless steel emitter with an internal diameter of 30 µm  
301 (Thermo Scientific) and a capillary temperature of 275°C.

302 All spectra were acquired using an Orbitrap Fusion Tribrid mass spectrometer controlled by Xcalibur  
303 2.1 software (Thermo Scientific) and operated in data-dependent acquisition mode. FTMS1 spectra  
304 were collected at a resolution of 120 000 over a scan range (m/z) of 350-1550, with an automatic gain  
305 control (AGC) target of 300 000 and a max injection time of 100ms. Precursors were filtered using an  
306 Intensity Range of 1E4 to 1E20 and according to charge state (to include charge states 2-6) and with  
307 monoisotopic precursor selection. Previously interrogated precursors were excluded using a dynamic  
308 window (40s +/-10ppm). The MS2 precursors were isolated with a quadrupole mass filter set to a  
309 width of 1.4m/z. ITMS2 spectra were collected with an AGC target of 20 000, max injection time of  
310 40ms and CID collision energy of 35%.

### 311 **Quantification and bioinformatics analysis**

312 The raw mass spectrometric data files obtained for each experiment were collated into a single  
313 quantitated data set using MaxQuant (version 1.2.2.5) (10) and the Andromeda search engine software  
314 (11). Enzyme specificity was set to that of trypsin, allowing for cleavage N-terminal to proline  
315 residues and between aspartic acid and proline residues. Other parameters used were: (i) variable  
316 modifications, methionine oxidation, protein N-acetylation, gln → pyro-glu, Phospho(STY); (ii) fixed  
317 modifications, cysteine carbamidomethylation; (iii) database: target-decoy human MaxQuant  
318 (ipi.HUMAN.v3.68); (iv) heavy labels: R6K4 and R10K8; (v) MS/MS tolerance: FTMS- 10ppm ,  
319 ITMS- 0.6 Da; (vi) maximum peptide length, 6; (vii) maximum missed cleavages, 2; (viii) maximum  
320 of labeled amino acids, 3; and (ix) false discovery rate, 1%. Peptide ratios were calculated for each  
321 arginine- and/or lysine-containing peptide as the peak area of labeled arginine/lysine divided by the  
322 peak area of nonlabeled arginine/lysine for each single-scan mass spectrum. Peptide ratios for all

323 arginine- and lysine-containing peptides sequenced for each protein were averaged. Data is  
324 normalised using 1/median ratio value for each identified protein group per labelled sample.

### 325 **Statistics**

326 Statistical analyses were carried out in Graphpad Prism with ANOVA followed by Tukey's HSD test.  
327 For FAME analyses data were assessed in R statistical packages and tested by t-test and corrected by  
328 false discovery rate. Statistical significance was determined with a  $p < 0.05$ . The global lipidomic data  
329 sets were subjected to principal component analysis (PCA) and orthogonal projection latent structure-  
330 discriminant analysis (OPLS-DA) with Pareto scaling using SIMCA-P v13.0 software (Umetrics,  
331 Umea, Sweden). The OPLS-DA models were validated by using the internal cross-validation  
332 function. Membrane protein abundance was considered altered if fold change was lower than 0.75 or  
333 greater than 1.25. Enrichment of biological processes was determined using gene ontology, using the  
334 whole genome of *Mus musculus* as a background reference list.

335

336 **Results**

337 **EPA and DHA substantially increase the abundance of omega-3 species but have differential**  
338 **effects on individual omega-3 fatty acids.**

339 After exposure to 50uM EPA or DHA for 72 hrs C<sub>2</sub>C<sub>12</sub> myotubes were collected for FAME analysis  
340 to determine lipid profiles. EPA and DHA significantly increased total omega-3 fatty acid content  
341 from baseline values (EPA; 951 ± 81% p=0.0014, DHA; 750 ± 56% p=0.0009) with no significant  
342 difference detected between EPA and DHA treatment (p=0.115) (**Figure 1**). The changes in omega-3  
343 abundance are a result of differential shifts in specific omega-3 fatty acids caused by EPA or DHA  
344 treatment. The increase in omega-3 content by DHA is a result of accumulation of mainly DHA [22:6  
345 n-3, 3050% ± 310% (**Figure 5**)]. While, incubation of myotubes with EPA results in the accumulation  
346 of EPA [(20:5 n-3), 1630% ± 23.38] and DPA [(22:5 n-3), 1318% ± 199.8%] (**Figure 5**). These data  
347 suggest that EPA is elongated to DPA whilst DHA remains largely unmodified.

348 **EPA and DHA have differential effects on skeletal muscle glucose uptake.**

349 Insulin stimulated 2-deoxy-glucose (2DG) uptake was determined after 48 h in either EPA or DHA.  
350 EPA treatment significantly increased both basal and insulin stimulated 2DG uptake indicating that  
351 EPA treatment increases the capacity for glucose uptake (**Figure 2A**). DHA did not have any  
352 significant effects on 2DG uptake (**Figure 2A**). The observed changes in 2DG uptake did not appear  
353 to be related to any change in insulin stimulated PKB phosphorylation as phospho-blot analysis  
354 revealed that insulin stimulated PKB phosphorylation was the same between treatments and controls  
355 (**Figure 2B**). In addition EPA/DHA treatment did not appear to affect GLUT1 or GLUT4 expression  
356 (**Figure 1C**), nor did they appear to affect the expression of Hexokinase 1 or Hexokinase 2 (**Figure**  
357 **1D**).

358 **EPA and DHA treatment does not alter mitochondrial respiration.**

359 As no changes in glucose transporters were detected we next assessed whether changes in  
360 mitochondrial oxygen consumption may explain the increase in glucose uptake following EPA  
361 treatment. C<sub>2</sub>C<sub>12</sub> myotubes were treated with 50uM EPA or DHA for 24 hours. Following treatment



362 multiple inhibitors/uncouplers (Oligomycin, FCCP, rotenone/anitmycin A) were used to probe various  
363 parameters of mitochondrial function using the seahorse XF mito stress test. A two way ANOVA  
364 found a significant interaction between oxygen consumption and inhibitor compound, indicating the  
365 successful manipulation of mitochondrial function (**Figure 3A**). However, fatty acid treatment did not  
366 lead to changes in cellular oxygen consumption on any parameter measured (**Figure 3A**). Consistent  
367 with the lack of changes in mitochondrial function, there were no alterations in the abundance of ATP  
368 synthase and UCQR2 (Complex V and Complex III respectively) (**Figure 3B/C**).

369 **EPA enhances protein accretion through a reduction in protein breakdown while DHA has a**  
370 **neutral effect**

371 After a 72 hour incubation with EPA or DHA, myotubes were collected in order to determine total  
372 protein content. When myotubes were incubated with EPA total protein content was enhanced while  
373 incubation with DHA had a neutral effect (**Figure 4C**). Protein balance is determined by the balance  
374 between synthesis and breakdown of proteins. Thus, the observed protein accretion may be reflected  
375 in changes in either protein synthesis or breakdown. In order to understand the mechanisms  
376 underlying the changes in protein content we directly assessed protein synthesis and breakdown and  
377 related signalling processes. Neither fatty acid had any effect on basal protein synthesis after a 24-  
378 hour incubation (**Figure 4A**). Anabolic signalling assessed by the phosphorylation status of mTOR,  
379 P70S6K1 and 4E-BP1 were not different between groups, corresponding with lack of changes in  
380 protein synthesis (**Figure 4E/F**). EPA reduced protein breakdown compared to both the vehicle and  
381 DHA treatments (**Figure 4B**). No changes were detected in the level of ubiquitin tagged proteins  
382 (**Figure 4D/F**).

383 **EPA treatment differentially regulates DPA levels whilst DHA treatment differentially regulates**

384 **arachidonic acid levels.** As previously mentioned, incubation of C2C12 with 50  $\mu$ M EPA or 50  $\mu$ M  
385 DHA led to substantial cellular incorporation of total omega-3 fatty acids (**Figure 1A**). This was  
386 associated with the above mentioned physiological changes. In order to determine the potential  
387 mechanisms by which these effects occur we determined via FAME analysis the fatty acid changes  
388 responsible for the increase in total n-3 levels in the cells treated with EPA/DHA. In order to clearly

389 distinguish differential fatty acid shifts we presented the complete fatty acid profiles as fold change  
390 [(log<sub>2</sub>) **Figure 5**]. The most clearly differentiated fatty acid is DPA (22:5 n3) which demonstrates a  
391 significant 1318 ± 200% increase with EPA treatment whilst DHA treatment induces a 17.83 ±  
392 17.37% decrease in DPA content. Additionally there is a trend (p=0.06) for EPA treatment to increase  
393 the DHA content of the cells (37.11 ± 12.62%) suggesting that only a small proportion of the EPA is  
394 converted to DHA. Surprisingly arachidonic acid (20:4 n6) was only significantly decreased by DHA  
395 treatment (-22.35 ± 3.174%) and remained unaffected by EPA treatment. Intriguingly we also  
396 observed that both EPA and DHA increased the content of the saturated fatty acid palmitate (PA 16:0)  
397 (**Figure 5**). In order to build a more complete picture of the impact of EPA and DHA on the lipidome  
398 we proceeded with a global lipidomics assessment.

399 **Global lipidomics reveals that EPA and DHA treatments induce substantial divergence in the**  
400 **lipidome.** Lipid extracts of cells treated with BSA (control), EPA or DHA were analysed by LC-MS  
401 in positive and negative ion modes, processed and subjected to multivariate data analysis. PCA  
402 highlights any natural clustering or separation within a data set and thereby enables similarities or  
403 differences between study groups to be explored. The PCA scores plots of both the positive and  
404 negative ion data sets revealed that EPA and DHA supplementation caused a substantial divergence in  
405 the lipidome, effectively segregating control and treated cells (**Fig. 6A and 6B**). Having established  
406 the existence of clustering behaviour between the sample cohorts more powerful multivariate methods  
407 were used to characterise the specific lipid changes responsible for the observed shift in the lipidome  
408 of the EPA and DHA treated myotubes. The OPLS-DA scores plots and associated 'S' plots of the  
409 positive ion data are shown in **Fig. 7A and 7B**. The results indicated that many of the key  
410 discriminating lipids associated with EPA and DHA treatment were phospholipids and in particular  
411 molecular species of PC and PE.. In EPA treated cells elevations in PC and PE species containing  
412 both 20:5 and 22:5 fatty acids (in agreement with FAME analysis) were observed, whereas there were  
413 relative increases in the abundance of phospholipid species with a 22:6 fatty acid in DHA treated  
414 cells. The analysis also revealed that both EPA and DHA treatments also resulted in an elevation of  
415 PC 32:0, a saturated species. DHA was also found to be incorporated into a number of triglyceride

416 species (see supplemental). In order to fully understand the impact of EPA and DHA treatment we  
417 followed up these experiments through the targeted analysis of the myotube phospholipids.

418 **Targeted phospholipid analysis reveals that EPA and DHA increase the fraction of lipid species**  
419 **containing long chains and 5 or more double bonds at the expense of shorter chain, less**  
420 **saturated species.** ESI-MS/MS was utilised to characterise the profiles of myocyte phospholipid  
421 classes. Representative mass spectra of PC and PE are shown in **Figure 8A and 8B**. The general  
422 trends (**Figure 9**) in both EPA and DHA were similar with the displacement of shorter chain in  
423 apparent preference for longer chain highly unsaturated fatty acids. However, the magnitude of  
424 change for these displacements was often higher with DHA. There was no evidence of alterations in  
425 the profile of sphingomyelin species in response to fatty acid treatments. ESI-MS/MS analysis of PS  
426 and PI was also performed however the low signal intensities of these lipids did not permit a robust  
427 quantification. Interestingly EPA and DHA supplementation led to a higher abundance of  
428 phospholipid species containing saturated fatty acids. For instance DHA induced an increase in PC  
429 (32:0) and lysophosphatidylethanolamine (LPE) (16:0), whilst EPA induced an increase in PC (32:0),  
430 LPC (16:0), (18:0) and LPE (18:0). It is therefore evident that in spite of significant increases in  
431 saturated fatty acids in these EPA and DHA treatment still improve or maintain glucose uptake  
432 respectively.

433 **Altered composition of phospholipids is associated with altered membrane associated proteomic**  
434 **profiles.**

435 As the lipidomic remodelling indicated substantial changes induced by n-3 treatment in the membrane  
436 associated lipid species we carried out SILAC based proteomics profiling of the membrane fraction to  
437 assess if the lipid remodelling altered the proteins in the membrane fraction. Over 3000 proteins  
438 associated with the membrane compartment were identified in the SILAC screen. These results were  
439 filtered down to 625 proteins (see supplemental) with a coefficient of variance  $\leq 5\%$  to describe  
440 consistent changes in membrane abundance. Proteins were considered enriched or reduced in the  
441 membrane with a fold change cut off of 0.25. Proteins similarly affected by EPA and DHA were  
442 removed from analysis. Proteins with altered abundance were then subject to gene ontology analysis

443 for biological processes (see supplemental). Membrane proteins altered by EPA were associated with  
444 protein folding ( $p = 4.76E-03$ ). Additionally, these proteins were subjected to String analysis which  
445 revealed that these proteins were highly likely to interact with each other (Figure 10B). EPA also  
446 increased Calumenin in the membrane fraction (1.34 fold). Interestingly, Calumenin plays a role in  
447 calcium sensitive protein folding (49). DHA altered proteins associated with a number of processes,  
448 primarily related to oxidative metabolism and ribosomal formation (see table 1). Further examination  
449 of ribosomal proteins revealed that DHA induced the significant reduction in ribosomal proteins  
450 associated with both small and large subunits at the membrane (Figure 10C). Conversely, EPA  
451 induced a small increase in ribosomal proteins (**Figure 10C**).

452

453 **Discussion**

454 This study is the first to carry out a comprehensive analysis of the lipidomic profiles of a skeletal  
455 muscle cell line in response to two differentially bioactive n-3 fatty acids. Furthermore, it is the first  
456 study, to our knowledge, to combine this with a profile of the membrane associated proteome. We  
457 clearly demonstrate the differential metabolic activities of EPA vs DHA in the C<sub>2</sub>C<sub>12</sub> skeletal muscle  
458 cell line and provide data demonstrating the differential impact that EPA and DHA have on the  
459 skeletal muscle lipidome. Our data suggests that the bioactivity of EPA may be due to its preferential  
460 incorporation (and possibly elongation to DPA) into the phospholipid fraction where it substantially  
461 alters the long chain polyunsaturated fatty acid composition of major phospholipid classes. Likely  
462 secondary to the alterations in membrane associated phospholipids we see an altered membrane  
463 associated proteome. These changes in the membrane lipid-protein composition may be a key driver  
464 for the metabolic effects of n3 fatty acids.

465 Similar to previous cell culture based studies (1, 21), we show that EPA has a positive effect on  
466 glucose uptake. Both basal and insulin stimulated muscle glucose uptake were improved by EPA but  
467 not DHA. The increase in glucose uptake appears to be independent of changes in PKB signalling as  
468 measured by phosphorylation status which suggests that enhanced proximal insulin and possibly PKB  
469 signalling are not part of the mechanism of action. Furthermore, there was no significant change to the  
470 expression of the glucose transporters GLUT1/4, hexokinase1/2 or the mitochondrial enzymes  
471 UQCRC2 and ATP-synthase. Nor was there any significant change in mitochondrial function as  
472 assessed by the mitochondrial stress test. These data are difficult to consolidate, however, we  
473 hypothesize that the mechanism of action of EPA on glucose uptake may be dependent less upon  
474 changes in protein expression and more dependent upon protein localisation perhaps improving the  
475 functional coupling of glucose metabolism enzymes.

476 In addition to the EPA induced improvements in glucose uptake we also noted a significant  
477 improvement in protein accretion with EPA treatment, while DHA showed no significant effect. To  
478 determine the mechanism by which cells treated with EPA accumulate more protein we assessed

479 muscle protein synthesis and muscle protein breakdown. While Kamolrat et al (24) observed  
480 enhanced leucine stimulated MPS following EPA treatment, we detected no significant changes in  
481 basal muscle protein synthesis or the phosphorylation of anabolic signalling markers. Instead, we  
482 determined that the effect of EPA on protein accretion was likely driven by an ~10% reduction in  
483 muscle protein breakdown. Analysis of global ubiquitination via western blotting indicates that the  
484 reduction in muscle protein breakdown may not be driven by a change in the activity of the ubiquitin  
485 system. Rather, it may be driven by reduced lysosomal degradation (9). In saying that we must  
486 concede however, that a 10% reduction in ubiquitin driven protein breakdown would be challenging  
487 to detect via western blotting.

488 In an effort to determine the molecular mechanism of action of EPA on skeletal muscle glucose  
489 uptake and protein accretion we tested the hypothesis that EPA and DHA treatments would induce  
490 significant lipid remodelling leading to remodelling of the cellular proteome. We found that while the  
491 total n-3 content was similar between treatments, EPA resulted in a larger variation in lipid species  
492 accumulating mainly as EPA and DPA and to a lesser extent DHA while DHA treatment mainly  
493 resulted in DHA accumulation with a decrease in DPA and a limited retro-conversion to EPA. The  
494 main differentially regulated fatty acids were DPA, increased in EPA and decreased in DHA, while  
495 only DHA decreased AA. Interestingly DPA accumulated to a similar extent as EPA [1630% (EPA)  
496 vs 1318% (DPA)], findings consistent with previous literature in other tissues (2, 26). Given that DPA  
497 increased to a similar extent to EPA we are unable to determine whether it is EPA or DPA that is the  
498 main driver behind the metabolic effects observed. These data suggest that upon intake into the cell  
499 EPA is elongated to DPA and to a lesser extent DHA. The elongation of EPA to DPA but not DHA  
500 may be explained by the differential affinities of the desaturases and elongases involved in fatty acid  
501 metabolism. In the n-3 pathway elovl2 catalyzes the conversion of EPA → DPA → 24:5 n-3, the  
502 precursor to DHA. However, increasing EPA concentrations is known to lower the saturation point in  
503 the conversion of DPA → 24:5 n-3 which may play a role the accumulation of DPA without being  
504 further metabolised to DHA (19). Our data indicate that one of the primary fates of EPA and DHA  
505 was incorporation into the phospholipid fraction. In the global lipidomics screen we found EPA or

506 DPA containing lipid species associated with the phospholipid pool whilst DHA containing species  
507 were often associated with the TAG pool whilst EPA containing species were rarely associated with  
508 the TAG pool (see supplemental spread sheet). Fatty acids in the TAG pool are stored in discrete lipid  
509 droplets and therefore may be less metabolically active than the phospholipids associated with the  
510 membranes. This differential incorporation into the various lipid pools may partially explain the  
511 beneficial metabolic effects of EPA.

512 The potential relevance of DPA as a mediator of many of the physiological effects of n-3  
513 supplementation is beginning to be further understood. DPA more potently inhibits platelet  
514 aggregation than EPA or DHA (3) as well as more potently stimulating endothelial cell migration than  
515 EPA or DHA (25). In macrophages EPA is a known inhibitor of the cyclooxygenase pathway and  
516 elongation to DPA is an important factor in this inhibition (36). We would suggest that the elongation  
517 of EPA to DPA seen in our study may also have important physiological roles in the increase in  
518 skeletal muscle glucose uptake by EPA. As with the global lipidomic analysis we observed the  
519 incorporation of long chain PUFAs into phospholipid species mainly at the expense of specific SFAs  
520 and MUFAs, however some specific SFAs were increased by both EPA and DHA. We identified  
521 multiple differentially regulated phospholipid species across PE, PC, LPE and LPC classes. In the  
522 EPA treated group a number of species were enriched by long chain PUFAs with 5 or more double  
523 bonds in addition to a number of EPA or DPA containing phospholipids in the PC and PE, fractions In  
524 comparison, the DHA treated group increased the long chain PUFA containing phospholipids but did  
525 not increase or increase as much as EPA the content of species containing 22:5 or 40:5. Interestingly,  
526 with DHA treatment there was a trend for an increase in the palmitate containing lyso-PE content  
527 above that of control and EPA. Additionally, while it appears that many phospholipids containing  
528 saturated fatty acids are displaced in favour of polyunsaturated containing phospholipids we observe a  
529 rise in PC (32:0) with both EPA and DHA, which may reflect the rise in palmitate observed with  
530 FAME analysis. Incorporation of PUFAs into phospholipids is known to increase membrane fluidity  
531 and we speculate that this increase may be a compensatory mechanism to maintain a base level of  
532 membrane rigidity.

533 An attractive mechanism for the differential effects of EPA and DHA may lie in the reduced  
534 production of different inflammatory eicosanoids. However, only DHA reduced the total amount of  
535 AA. Additionally, the displacement of phospholipids alongside a lack of inflammatory stimulus  
536 suggests it is unlikely that this would mediate the metabolic differences seen in this model. The G  
537 protein coupled receptor GPR120 has previously been identified as a general n-3 sensor in a number  
538 of tissues except skeletal muscle that elicits potent anti-inflammatory and consequently insulin  
539 sensitizing effects (37). To our knowledge no such receptor exists in skeletal muscle that can  
540 discriminate between EPA and DHA deeming it unlikely that the observed differential effects are  
541 mediated by EPA or DHA through specific receptor activated signalling.

542 Phospholipid species are not merely inert structural components of cellular membranes and their  
543 various roles in intracellular processes are beginning to be further understood. PS and PE are related  
544 phospholipid species found predominantly in the inner membrane and contribute to the membrane  
545 targeting and activation and modification of protein kinases [as reviewed (51)]. It has also previously  
546 been seen that the lyso-PC, a hydrolyzed form of phospholipid, stimulated adipocyte glucose uptake  
547 in a manner dependent upon chain length and saturation of the acyl group (54). We observed an  
548 incorporation of long chain PUFAs (possibly EPA and DPA) into the PC, PS, PE and certain lyso-PL  
549 species and therefore cannot discount that the change in acyl chain length and unsaturation level alters  
550 the function of these PL species and leads to an increase in glucose uptake through a currently  
551 unknown mechanism. Because, the lipid composition of the membrane can alter the targeting of  
552 various proteins to the membrane (50, 51) we speculated that part of the mechanism of action of EPA  
553 might be via a change in the composition of the membrane associated proteome. To test this theory  
554 we carried out a 3 way SILAC experiment on the membrane fractions of cells treated with vehicle,  
555 EPA or DHA.

556 Our SILAC experiment illustrated the proof of concept that the incorporation of EPA and DHA into  
557 phospholipid species was associated with the alteration of proteins interacting with the membrane  
558 compartment. There are a number of mechanisms by which proteins can bind to the membrane which  
559 are influenced by the fatty acid composition of the lipid bilayers (50, 51). Gene ontology analysis



560 indicated that proteins associated with protein folding at the membrane were overrepresented  
561 following EPA incorporation. There is experimental evidence that a number of the proteins identified  
562 with this process interact, as probed by the STRING database. Furthermore, analysis of ribosomal  
563 proteins indicated a small but significant shift of ribosomal proteins towards the membrane fraction in  
564 EPA treated cells. Since the endoplasmic reticulum is a membranous structure these data suggest that  
565 EPA is increasing the content of ribosomes at the ER. This shift in ribosomes towards the ER could  
566 lead to improved fidelity of protein production as the ER is key to protein quality control (15). We  
567 propose a mechanism in which protein folding is enhanced, thereby enhancing the fidelity with which  
568 proteins are synthesised thereby reducing protein breakdown leading to increased protein accretion.  
569 Further experimental work is needed to confirm this hypothesis since mis-folded proteins tend to be  
570 degraded by the ubiquitin-proteasome system (9) and our data indicated that global ubiquitination was  
571 not reduced by EPA. By comparison, DHA caused a striking reduction in the abundance of a number  
572 of ribosomal proteins with the membrane fraction. If future work confirms that total ribosomal content  
573 is unchanged with these conditions then these data would indicate an increase in cytosolic ribosomes.  
574 This differential shift in ribosomal compartmentalisation may indicate a shift in protein expression  
575 profiles. Proteins that enter the secretory pathways or integral membrane proteins are synthesised in  
576 the ER while other proteins are translated in the cytosolic ribosome pool (41).

577 DHA also altered the abundance of proteins involved in ATP coupled proton transport and acetyl-  
578 CoA metabolism. The proteins identified with ATP synthesis coupled transport were mainly  
579 downregulated proteins in the ATP synthase complex. This would be expected to manifest as a  
580 reduced ability to generate ATP yet no changes were observed in ATP synthase dependent oxygen  
581 consumption. The effects of DHA may not have been severe enough to observe at basal levels.  
582 Although maximal respiration is measured during the mito stress test, it is induced by uncoupling so  
583 may not be indicative of changes in ATP synthesis. Previous studies have observed that omega-3 fatty  
584 acids can alter mitochondrial function, altering ADP kinetics without altering maximal respiration  
585 (20). It remains to be seen if this reduction in ATP synthase proteins would alter the mitochondrial  
586 response to cellular stress or changing substrate availability.

587 In summary, we demonstrate that EPA and DHA display divergent metabolic activities in a skeletal  
588 muscle cell line, which may be partially mediated by differential remodelling of the lipidome. We  
589 speculate that the remodelling of the membrane-associated proteome is secondary to the changes  
590 observed in the saturation profile of the membrane-associated phospholipid species. Whilst the  
591 proteomic data did not reveal a mechanism for the effects of EPA on glucose uptake, our data support  
592 the proof of concept that a redistribution of the proteome may be responsible. Gene ontology analysis  
593 of the proteomic data indicate that the mechanism of action of EPA on protein metabolism may be  
594 driven by an improved fidelity with which proteins are synthesised. Based on the shifts in ribosomal  
595 proteins found in the membrane fractions future work should determine if EPA and DHA alter the  
596 transcript profiles in various ribosomal fractions.

597

- 599 1. **Aas V, Rokling-Andersen MH, Kase ET, Thoresen GH, and Rustan AC.** Eicosapentaenoic acid  
600 (20:5 n-3) increases fatty acid and glucose uptake in cultured human skeletal muscle cells. *J Lipid Res*  
601 47: 366-374, 2006.
- 602 2. **Achard F, Benistant C, and Lagarde M.** Interconversions and distinct metabolic fate of  
603 eicosapentaenoic, docosapentaenoic and docosahexaenoic acids in bovine aortic endothelial cells.  
604 *Biochim Biophys Acta* 1255: 260-266, 1995.
- 605 3. **Akiba S, Murata T, Kitatani K, and Sato T.** Involvement of lipoxygenase pathway in  
606 docosapentaenoic acid-induced inhibition of platelet aggregation. *Biol Pharm Bull* 23: 1293-1297,  
607 2000.
- 608 4. **Akinkuolie AO, Ngwa JS, Meigs JB, and Djousse L.** Omega-3 polyunsaturated fatty acid and  
609 insulin sensitivity: a meta-analysis of randomized controlled trials. *Clin Nutr* 30: 702-707, 2011.
- 610 5. **Alexander JW, Saito H, Trocki O, and Ogle CK.** The importance of lipid type in the diet after  
611 burn injury. *Ann Surg* 204: 1-8, 1986.
- 612 6. **Andersson A, Nalsen C, Tengblad S, and Vessby B.** Fatty acid composition of skeletal muscle  
613 reflects dietary fat composition in humans. *Am J Clin Nutr* 76: 1222-1229, 2002.
- 614 7. **Browning LM, Walker CG, Mander AP, West AL, Gambell J, Madden J, Calder PC, and Jebb**  
615 **SA.** Compared with daily, weekly n-3 PUFA intake affects the incorporation of eicosapentaenoic acid  
616 and docosahexaenoic acid into platelets and mononuclear cells in humans. *J Nutr* 144: 667-672,  
617 2014.
- 618 8. **Burdge GC, and Calder PC.** Introduction to fatty acids and lipids. *World Rev Nutr Diet* 112: 1-  
619 16, 2015.
- 620 9. **Ciechanover A.** Intracellular protein degradation: from a vague idea thru the lysosome and  
621 the ubiquitin-proteasome system and onto human diseases and drug targeting. *Cell Death Differ* 12:  
622 1178-1190, 2005.
- 623 10. **Cox J, and Mann M.** MaxQuant enables high peptide identification rates, individualized  
624 p.p.b.-range mass accuracies and proteome-wide protein quantification. *Nat Biotechnol* 26: 1367-  
625 1372, 2008.
- 626 11. **Cox J, Neuhauser N, Michalski A, Scheltema RA, Olsen JV, and Mann M.** Andromeda: a  
627 peptide search engine integrated into the MaxQuant environment. *J Proteome Res* 10: 1794-1805,  
628 2011.
- 629 12. **Da Boit M, Sibson R, Sivasubramaniam S, Meakin JR, Greig CA, Aspden RM, Thies F,**  
630 **Jeromson S, Hamilton DL, Speakman JR, Hambly C, Mangoni AA, Preston T, and Gray SR.** Sex  
631 differences in the effect of fish oil supplementation on the adaptive response to resistance exercise  
632 training in older people: a randomized control trial. *Am J Clin Nutr* 2016.
- 633 13. **Dangardt F, Chen Y, Gronowitz E, Dahlgren J, Friberg P, and Strandvik B.** High physiological  
634 omega-3 Fatty Acid supplementation affects muscle Fatty Acid composition and glucose and insulin  
635 homeostasis in obese adolescents. *J Nutr Metab* 2012: 395757, 2012.
- 636 14. **Di Girolamo FG, Situlin R, Mazzucco S, Valentini R, Toigo G, and Biolo G.** Omega-3 fatty  
637 acids and protein metabolism: enhancement of anabolic interventions for sarcopenia. *Curr Opin Clin*  
638 *Nutr Metab Care* 17: 145-150, 2014.
- 639 15. **Ellgaard L, and Helenius A.** Quality control in the endoplasmic reticulum. *Nat Rev Mol Cell*  
640 *Biol* 4: 181-191, 2003.
- 641 16. **Findlay JA, Hamilton DL, and Ashford ML.** BACE1 activity impairs neuronal glucose  
642 oxidation: rescue by beta-hydroxybutyrate and lipoic acid. *Front Cell Neurosci* 9: 382, 2015.
- 643 17. **Folch J, Lees M, and Sloane Stanley GH.** A simple method for the isolation and purification  
644 of total lipides from animal tissues. *J Biol Chem* 226: 497-509, 1957.
- 645 18. **Franekova V, Angin Y, Hoebbers NT, Coumans WA, Simons PJ, Glatz JF, Luiken JJ, and Larsen**  
646 **TS.** Marine omega-3 fatty acids prevent myocardial insulin resistance and metabolic remodeling as  
647 induced experimentally by high insulin exposure. *Am J Physiol Cell Physiol* 308: C297-307, 2015.

- 648 19. **Gregory MK, Gibson RA, Cook-Johnson RJ, Cleland LG, and James MJ.** Elongase reactions as  
649 control points in long-chain polyunsaturated fatty acid synthesis. *PLoS One* 6: e29662, 2011.
- 650 20. **Herbst EA, Paglialunga S, Gerling C, Whitfield J, Mukai K, Chabowski A, Heigenhauser GJ,**  
651 **Spriet LL, and Holloway GP.** Omega-3 supplementation alters mitochondrial membrane composition  
652 and respiration kinetics in human skeletal muscle. *J Physiol* 592: 1341-1352, 2014.
- 653 21. **Hessvik NP, Bakke SS, Fredriksson K, Boekschoten MV, Fjorckenstad A, Koster G, Hesselink**  
654 **MK, Kersten S, Kase ET, Rustan AC, and Thoresen GH.** Metabolic switching of human myotubes is  
655 improved by n-3 fatty acids. *J Lipid Res* 51: 2090-2104, 2010.
- 656 22. **Huang F, Wei H, Luo H, Jiang S, and Peng J.** EPA inhibits the inhibitor of kappaBalpha  
657 (IkappaBalpha)/NF-kappaB/muscle RING finger 1 pathway in C2C12 myotubes in a PPARgamma-  
658 dependent manner. *Br J Nutr* 105: 348-356, 2011.
- 659 23. **Jucker BM, Cline GW, Barucci N, and Shulman GI.** Differential effects of safflower oil versus  
660 fish oil feeding on insulin-stimulated glycogen synthesis, glycolysis, and pyruvate dehydrogenase flux  
661 in skeletal muscle: a <sup>13</sup>C nuclear magnetic resonance study. *Diabetes* 48: 134-140, 1999.
- 662 24. **Kamolrat T, and Gray SR.** The effect of eicosapentaenoic and docosahexaenoic acid on  
663 protein synthesis and breakdown in murine C2C12 myotubes. *Biochem Biophys Res Commun* 432:  
664 593-598, 2013.
- 665 25. **Kanayasu-Toyoda T, Morita I, and Murota S.** Docosapentaenoic acid (22:5, n-3), an  
666 elongation metabolite of eicosapentaenoic acid (20:5, n-3), is a potent stimulator of endothelial cell  
667 migration on pretreatment in vitro. *Prostaglandins Leukot Essent Fatty Acids* 54: 319-325, 1996.
- 668 26. **Kaur G, Sinclair AJ, Cameron-Smith D, Barr DP, Molero-Navajas JC, and Konstantopoulos N.**  
669 Docosapentaenoic acid (22:5n-3) down-regulates the expression of genes involved in fat synthesis in  
670 liver cells. *Prostaglandins Leukot Essent Fatty Acids* 85: 155-161, 2011.
- 671 27. **Lamaziere A, Wolf C, Barbe U, Bausero P, and Visioli F.** Lipidomics of hepatic lipogenesis  
672 inhibition by omega 3 fatty acids. *Prostaglandins Leukot Essent Fatty Acids* 88: 149-154, 2013.
- 673 28. **Lanza IR, Blachnio-Zabielska A, Johnson ML, Schimke JM, Jakaitis DR, Lebrasseur NK,**  
674 **Jensen MD, Sreekumaran Nair K, and Zabielski P.** Influence of fish oil on skeletal muscle  
675 mitochondrial energetics and lipid metabolites during high-fat diet. *Am J Physiol Endocrinol Metab*  
676 304: E1391-1403, 2013.
- 677 29. **Magee P, Pearson S, Whittingham-Dowd J, and Allen J.** PPARgamma as a molecular target  
678 of EPA anti-inflammatory activity during TNF-alpha-impaired skeletal muscle cell differentiation. *J*  
679 *Nutr Biochem* 23: 1440-1448, 2012.
- 680 30. **McGlory C, Galloway SD, Hamilton DL, McClintock C, Breen L, Dick JR, Bell JG, and Tipton**  
681 **KD.** Temporal changes in human skeletal muscle and blood lipid composition with fish oil  
682 supplementation. *Prostaglandins Leukot Essent Fatty Acids* 90: 199-206, 2014.
- 683 31. **Mori TA, Burke V, Puddey IB, Watts GF, O'Neal DN, Best JD, and Beilin LJ.** Purified  
684 eicosapentaenoic and docosahexaenoic acids have differential effects on serum lipids and  
685 lipoproteins, LDL particle size, glucose, and insulin in mildly hyperlipidemic men. *Am J Clin Nutr* 71:  
686 1085-1094, 2000.
- 687 32. **Mori TA, Codde JP, Vandongen R, and Beilin LJ.** New findings in the fatty acid composition  
688 of individual platelet phospholipids in man after dietary fish oil supplementation. *Lipids* 22: 744-750,  
689 1987.
- 690 33. **Morisaki N, Kanzaki T, Fujiyama Y, Osawa I, Shirai K, Matsuoka N, Saito Y, and Yoshida S.**  
691 Metabolism of n-3 polyunsaturated fatty acids and modification of phospholipids in cultured rabbit  
692 aortic smooth muscle cells. *J Lipid Res* 26: 930-939, 1985.
- 693 34. **Murphy RA, Mourtzakis M, and Mazurak VC.** n-3 polyunsaturated fatty acids: the potential  
694 role for supplementation in cancer. *Curr Opin Clin Nutr Metab Care* 15: 246-251, 2012.
- 695 35. **Neschen S, Morino K, Dong J, Wang-Fischer Y, Cline GW, Romanelli AJ, Rossbacher JC,**  
696 **Moore IK, Regittng W, Munoz DS, Kim JH, and Shulman GI.** n-3 Fatty acids preserve insulin  
697 sensitivity in vivo in a peroxisome proliferator-activated receptor-alpha-dependent manner.  
698 *Diabetes* 56: 1034-1041, 2007.

- 699 36. **Norris PC, and Dennis EA.** Omega-3 fatty acids cause dramatic changes in TLR4 and  
700 purinergic eicosanoid signaling. *Proc Natl Acad Sci U S A* 109: 8517-8522, 2012.
- 701 37. **Oh DY, Talukdar S, Bae EJ, Imamura T, Morinaga H, Fan W, Li P, Lu WJ, Watkins SM, and**  
702 **Olefsky JM.** GPR120 is an omega-3 fatty acid receptor mediating potent anti-inflammatory and  
703 insulin-sensitizing effects. *Cell* 142: 687-698, 2010.
- 704 38. **Ong SE, Blagoev B, Kratchmarova I, Kristensen DB, Steen H, Pandey A, and Mann M.** Stable  
705 isotope labeling by amino acids in cell culture, SILAC, as a simple and accurate approach to  
706 expression proteomics. *Mol Cell Proteomics* 1: 376-386, 2002.
- 707 39. **Ries A, Trottenberg P, Elsner F, Stiel S, Haugen D, Kaasa S, and Radbruch L.** A systematic  
708 review on the role of fish oil for the treatment of cachexia in advanced cancer: an EPCRC cachexia  
709 guidelines project. *Palliative medicine* 26: 294-304, 2012.
- 710 40. **Rodacki CL, Rodacki AL, Pereira G, Naliwaiko K, Coelho I, Pequito D, and Fernandes LC.**  
711 Fish-oil supplementation enhances the effects of strength training in elderly women. *Am J Clin Nutr*  
712 95: 428-436, 2012.
- 713 41. **Ron D.** Translational control in the endoplasmic reticulum stress response. *The Journal of*  
714 *Clinical Investigation* 110: 1383-1388, 2002.
- 715 42. **Sjovall P, Rossmeisl M, Hanrieder J, Kuda O, Kopecky J, and Bryhn M.** Dietary uptake of  
716 omega-3 fatty acids in mouse tissue studied by time-of-flight secondary ion mass spectrometry (TOF-  
717 SIMS). *Anal Bioanal Chem* 407: 5101-5111, 2015.
- 718 43. **Smith GI, Atherton P, Reeds DN, Mohammed BS, Rankin D, Rennie MJ, and Mittendorfer B.**  
719 Dietary omega-3 fatty acid supplementation increases the rate of muscle protein synthesis in older  
720 adults: a randomized controlled trial. *Am J Clin Nutr* 93: 402-412, 2011.
- 721 44. **Smith GI, Atherton P, Reeds DN, Mohammed BS, Rankin D, Rennie MJ, and Mittendorfer B.**  
722 Omega-3 polyunsaturated fatty acids augment the muscle protein anabolic response to  
723 hyperinsulinaemia-hyperaminoacidaemia in healthy young and middle-aged men and women. *Clin*  
724 *Sci (Lond)* 121: 267-278, 2011.
- 725 45. **Smith GI, Julliard S, Reeds DN, Sinacore DR, Klein S, and Mittendorfer B.** Fish oil-derived n-  
726 3 PUFA therapy increases muscle mass and function in healthy older adults. *Am J Clin Nutr* 102: 115-  
727 122, 2015.
- 728 46. **Stephens FB, Mendis B, Shannon CE, Cooper S, Ortori CA, Barrett DA, Mansell P, and**  
729 **Tsintzas K.** Fish oil omega-3 fatty acids partially prevent lipid-induced insulin resistance in human  
730 skeletal muscle without limiting acylcarnitine accumulation. *Clin Sci (Lond)* 127: 315-322, 2014.
- 731 47. **Storlien LH, Jenkins AB, Chisholm DJ, Pascoe WS, Khouri S, and Kraegen EW.** Influence of  
732 dietary fat composition on development of insulin resistance in rats. Relationship to muscle  
733 triglyceride and omega-3 fatty acids in muscle phospholipid. *Diabetes* 40: 280-289, 1991.
- 734 48. **Storlien LH, Kraegen EW, Chisholm DJ, Ford GL, Bruce DG, and Pascoe WS.** Fish oil prevents  
735 insulin resistance induced by high-fat feeding in rats. *Science* 237: 885-888, 1987.
- 736 49. **Tripathi R, Benz N, Culetton B, Trouve P, and Ferec C.** Biophysical characterisation of  
737 calumenin as a charged F508del-CFTR folding modulator. *PLoS One* 9: e104970, 2014.
- 738 50. **Vance JE.** Phospholipid synthesis and transport in mammalian cells. *Traffic* 16: 1-18, 2015.
- 739 51. **Vance JE, and Tasseva G.** Formation and function of phosphatidylserine and  
740 phosphatidylethanolamine in mammalian cells. *Biochim Biophys Acta* 1831: 543-554, 2013.
- 741 52. **Wang Y, Lin QW, Zheng PP, Zhang JS, and Huang FR.** DHA inhibits protein degradation more  
742 efficiently than EPA by regulating the PPARgamma/NFkappaB pathway in C2C12 myotubes. *Biomed*  
743 *Res Int* 2013: 318981, 2013.
- 744 53. **Woodman RJ, Mori TA, Burke V, Puddey IB, Watts GF, Best JD, and Beilin LJ.**  
745 Docosahexaenoic acid but not eicosapentaenoic acid increases LDL particle size in treated  
746 hypertensive type 2 diabetic patients. *Diabetes Care* 26: 253, 2003.
- 747 54. **Yea K, Kim J, Yoon JH, Kwon T, Kim JH, Lee BD, Lee HJ, Lee SJ, Kim JI, Lee TG, Baek MC, Park**  
748 **HS, Park KS, Ohba M, Suh PG, and Ryu SH.** Lysophosphatidylcholine activates adipocyte glucose

749 uptake and lowers blood glucose levels in murine models of diabetes. *J Biol Chem* 284: 33833-33840,  
750 2009.  
751 55. **You JS, Park MN, Song W, and Lee YS.** Dietary fish oil alleviates soleus atrophy during  
752 immobilization in association with Akt signaling to p70s6k and E3 ubiquitin ligases in rats. *Appl*  
753 *Physiol Nutr Metab* 35: 310-318, 2010.

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760 Mackinlay for assistance with the FAME analysis.

761

762 **Figure Legends**

763 **Figure 1. EPA and DHA enhance n-3 content to a similar degree.** C<sub>2</sub>C<sub>12</sub> myotubes were incubated  
764 in either fatty acid free 2% BSA or fatty acid free 2% BSA pre-conjugated to 50 μM EPA or 50 μM  
765 DHA for 72hrs. Fatty acid analysis was carried out by FAME analysis (n=4 in duplicate) and data are  
766 presented as % change from BSA control grouped by fatty acid species; SFA – saturated fatty acids,  
767 MUFA – monounsaturated fatty acids, n-9 PUFA – omega-9 polyunsaturated fatty acids, n-6 PUFA –  
768 omega-6 polyunsaturated fatty acids, n-3 PUFA – omega-3 polyunsaturated fatty acids.

769 **Figure 2. EPA and DHA have differential effects on C<sub>2</sub>C<sub>12</sub> glucose uptake independently of**  
770 **changes in PKB Thr308 phosphorylation or GLUT1/4 and Hexokinase1/2 expression.** Glucose  
771 uptake was determined using a radiolabelled 2 Deoxy-glucose uptake assay (n=6 in duplicate) (A) and  
772 PKB Thr308 phosphorylation (n=4 in duplicate) (B) was determined using SDS-PAGE and phospho-  
773 specific antibodies, the signal for which was normalised to t-PKB expression (representative blots are  
774 inset). GLUT1/4 expression (C) were normalised to t-eEF2 whilst Hexokinase1/2 expression (D) were  
775 normalised to α-tubulin. Bars not connected by the same letter are significantly different from each  
776 other (p<0.05).

777 **Figure 3. EPA and DHA treatment do not modulate mitochondrial function or mitochondrial**  
778 **protein expression.** C<sub>2</sub>C<sub>12</sub> myotubes were incubated in either fatty acid free 2% BSA or fatty acid  
779 free 2% BSA pre-conjugated to 50 μM EPA or 50 μM DHA for 48hrs. One set of myotubes was  
780 exposed to a mitochondrial stress test in the SeaHorse XFA and oxygen consumption was assessed  
781 (A). A parallel set of myotubes were collected for western blot analysis of mitochondrial protein  
782 expression with the mito-blot panel (B). Representative blots are shown in (C).

783 **Figure 4. EPA enhances protein accretion through a reduction in protein breakdown.** A) Protein  
784 synthesis was measured by a L-[2,4,<sup>3</sup>H] phenylalanine incorporation assay following a 24 hr  
785 incubation with n-3 fatty acids (n=5 in duplicate) B) protein breakdown was assessed by the release of  
786 L-[2,4,<sup>3</sup>H] phenylalanine into culture media after 24hr treatment with n-3 fatty acids (n=10, in  
787 duplicate) C) total protein content was assessed after a 72hr incubation with EPA or DHA (n=5, in

788 duplicate) D) Phosphorylation of proteins within the mTOR signalling pathway were assessed by  
789 western blotting after a 24 hr incubation with n-3 fatty acids (n=3 in duplicate) E) ubiquitination of  
790 proteins were assessed by western blotting after a 24hr incubation with fatty acids (n=3 in duplicate).  
791 # indicates significantly different from Veh and DHA, \* indicates significantly different from  
792 corresponding control condition (p<0.05).

793 **Figure 5. Complete fatty acid profiles reveal a number of fatty acids differentially regulated by**  
794 **EPA and DHA.** C<sub>2</sub>C<sub>12</sub> myotubes were incubated in either fatty acid free 2% BSA or fatty acid free  
795 2% BSA pre-conjugated to 50 μM EPA or 50 μM DHA for 72hrs (n=4 in duplicate). Cells were  
796 pelleted and washed 3x using PBS with 2% fatty acid free BSA. Fatty acid analysis was carried out by  
797 FAME analysis. The fold change was determined from the BSA control condition and logged (log<sub>2</sub>).  
798 ND indicates non detectable, \* indicates significant difference between EPA vs DHA (p<0.05).

799 **Figure 6. Global lipidomic analysis characterises shifts in lipid composition of C<sub>2</sub>C<sub>12</sub> myotubes.**  
800 Principal component analysis (PCA) scores plots of lipid profiles generated by LC-MS in (A) positive  
801 ion and (B) negative ion modes. Cultured cells were incubated with either fatty acid free 2% BSA  
802 (green circles), fatty acid free 2% BSA pre-conjugated to 50 μM EPA (red triangles) or 50 μM DHA  
803 (blue squares) for 72hrs. Global lipidomic analysis was performed on the cells and the data sets were  
804 subjected to PCA with Pareto scaling. Each point represents a single cell sample (n=6). The PCA  
805 revealed that the control, EPA and DHA groups could be discriminated on the basis of their lipid  
806 profiles. The control cells were found to cluster in one area of the scores plot whilst cells treated with  
807 EPA or DHA appeared in regions away from the controls indicating that were alterations in their lipid  
808 composition as a result of the fatty acid treatments.

809 **Figure 7. Lipidomic profiling reveals cellular lipid species containing EPA, DPA and DHA.**  
810 Positive ion orthogonal partial least-squares discriminant analysis (OPLS-DA) for (A) BSA vs DHA  
811 and (B) BSA vs EPA (n=6). Green circles indicate BSA treated C<sub>2</sub>C<sub>12</sub> myotubes; blue squares indicate  
812 DHA treated C<sub>2</sub>C<sub>12</sub> myotubes and red triangles indicate EPA treated C<sub>2</sub>C<sub>12</sub> myotubes. The OPLS-DA  
813 scores plots indicated that EPA and DHA groups had distinct lipid profiles compared to the controls.  
814 In order to determine the lipids responsible for the inter-class differences associated S-plots of



815 covariance versus the correlation were generated. Each point in the S-plots represents a lipid detected  
816 in the LC-MS analysis with the lipids at the top and bottom of the plots showing the greatest changes.  
817 The analysis revealed that there was a relative increase in the abundance of lipid species containing  
818 20:5, 22:5 or 22:6 fatty acids in EPA and DHA treated cells respectively.

819

820 **Figure 8. Targeted phospholipid analysis indicates preferential incorporation of long chain**  
821 **polyunsaturated fatty acids into specific phospholipid classes.** C<sub>2</sub>C<sub>12</sub> myotubes were solvent  
822 extracted and molecular species of (A) phosphatidylcholine and (B) phosphatidylethanolamines were  
823 detected by ESI-MS/MS in positive-ion mode by means of a precursor ion scan for m/z 184 and a  
824 neutral loss scan of m/z 141 respectively.

825 **Figure 9. Targeted phospholipid analysis presented as % abundance for PC, PE, LPC and LPE**  
826 **species.** C<sub>2</sub>C<sub>12</sub> myotubes were solvent extracted and molecular species of (A) lyso-  
827 phosphatidylcholine, (B) phosphatidylcholine, (C) lyso-phosphatidylethanolamine and (D)  
828 phosphatidylethanolamine were detected by ESI-MS/MS in positive-ion mode by means of a  
829 precursor ion scan for m/z 184 and a neutral loss scan of m/z 141 respectively. Data are presented as  
830 % abundance ± SEM. Bars not connected by the same letter are significantly different from one  
831 another (p<0.05).

832 **Figure 10. Membrane associated proteomics reveals protein folding machinery and ribosomal**  
833 **proteins differentially shift to the membrane in response to EPA treatment.** C<sub>2</sub>C<sub>12</sub> myotubes were  
834 incubated in SILAC media for 7 doublings followed by plating and differentiation for 72hrs in SILAC  
835 differentiation media. Following differentiation myotubes were incubated in either fatty acid free 2%  
836 BSA or fatty acid free 2% BSA pre-conjugated to 50 μM EPA or 50 μM DHA for 72hrs (n=3 in  
837 duplicate). Cells were collected and fractionated, the membrane fraction was then submitted to  
838 proteomics analysis. (A) Venne diagram illustrating the number of proteins found to be changed in the  
839 membrane fraction in response to EPA/DHA. (B) String diagram illustrating the interactions between  
840 the proteins identified from the GO analysis as being involved in 'protein folding.' (C) Fold change in

841 ribosomal proteins in the membrane fraction in response to EPA/DHA. \* indicates a significant fold  
842 change from control ( $p < 0.05$ ). All differences between EPA and DHA were significant.

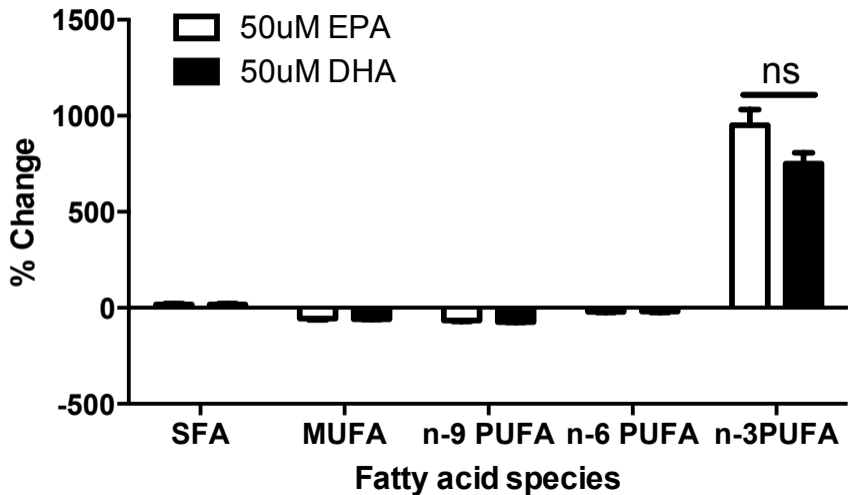
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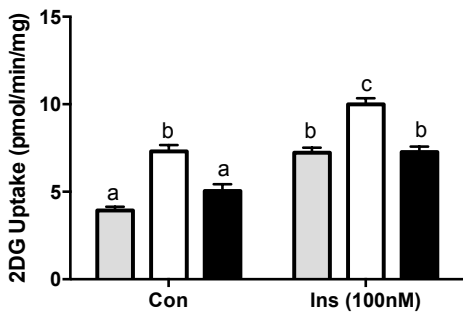
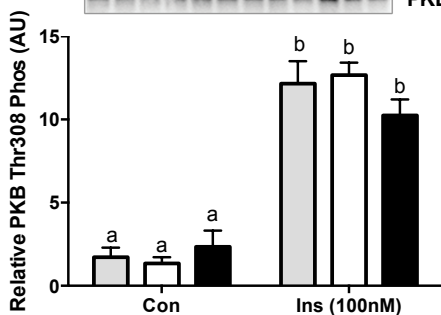
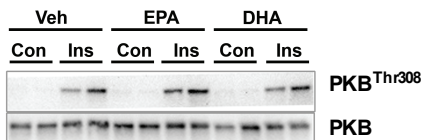
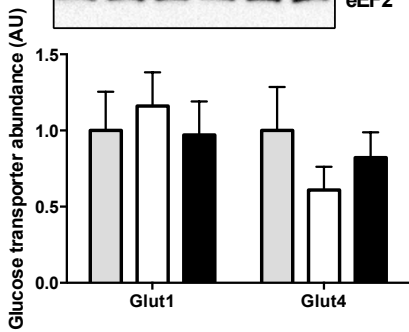
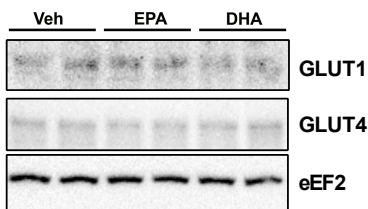
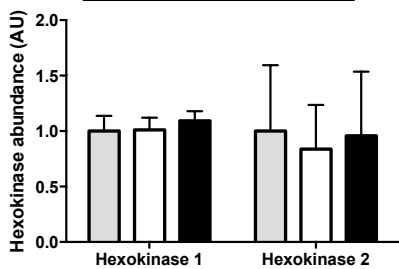
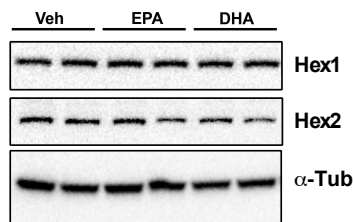
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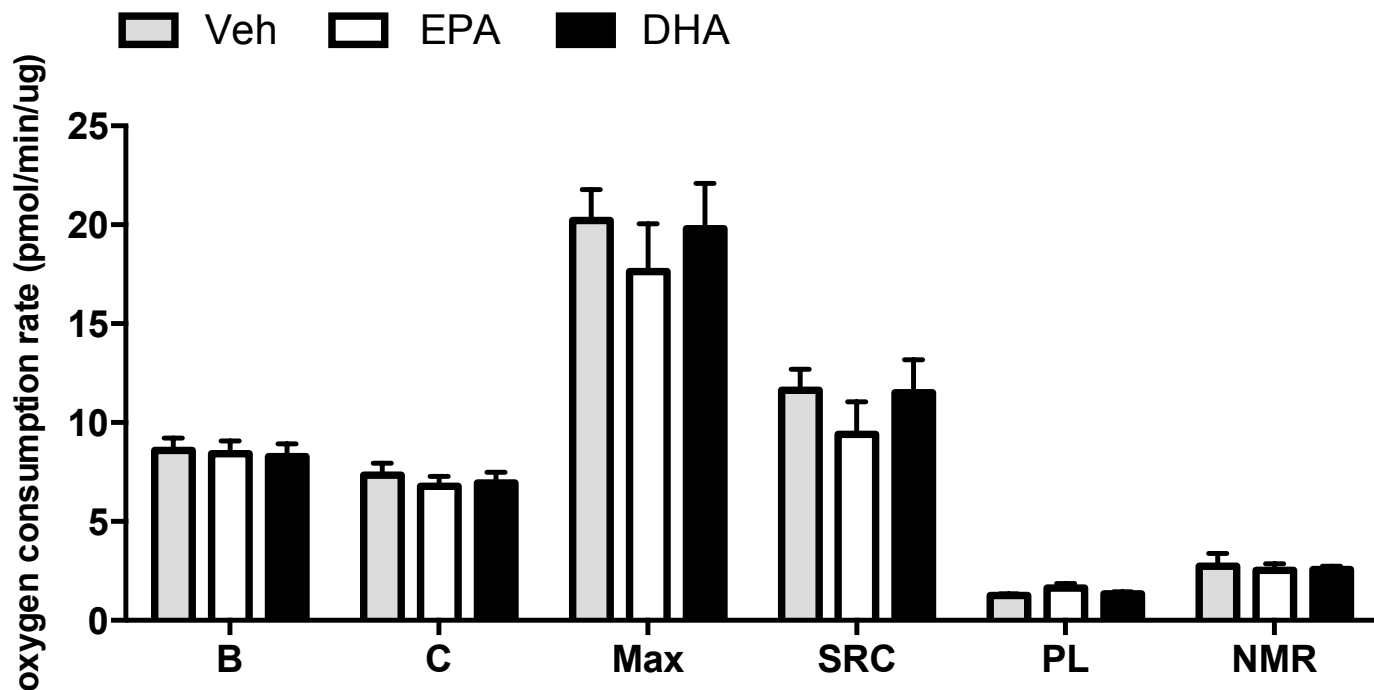
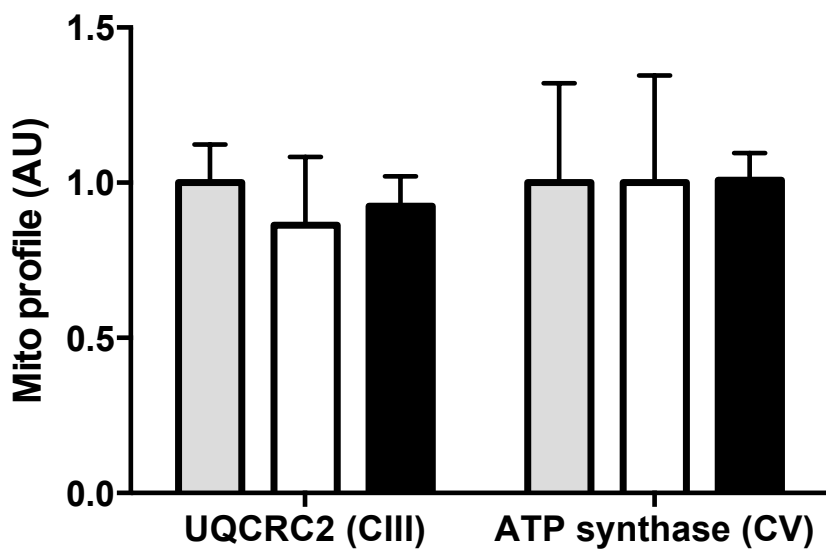
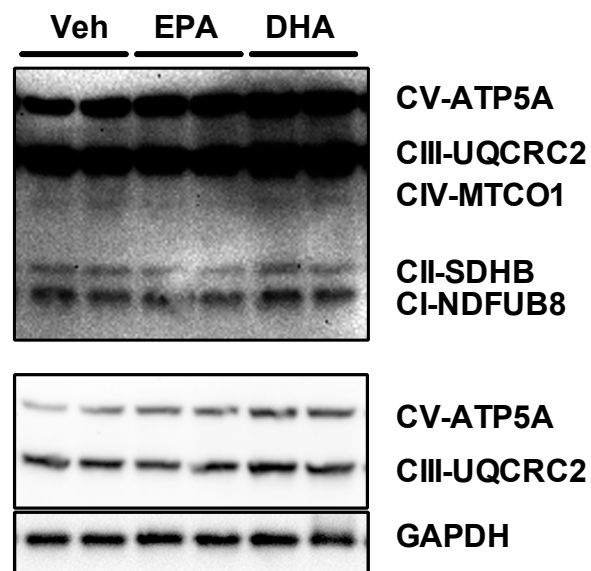
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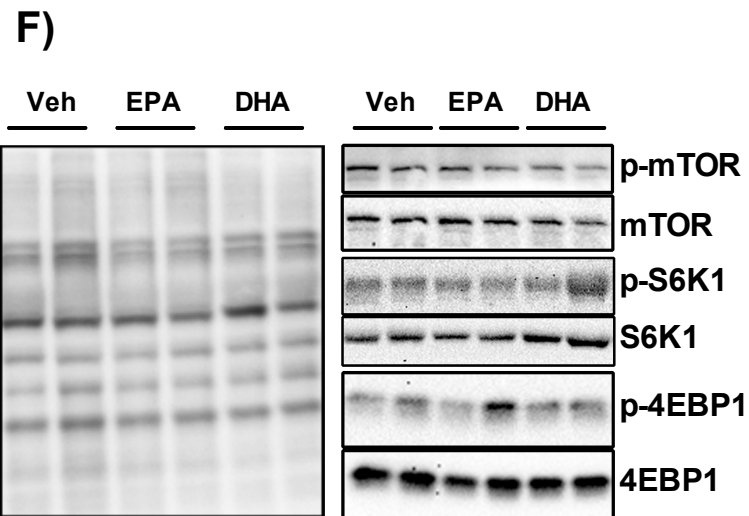
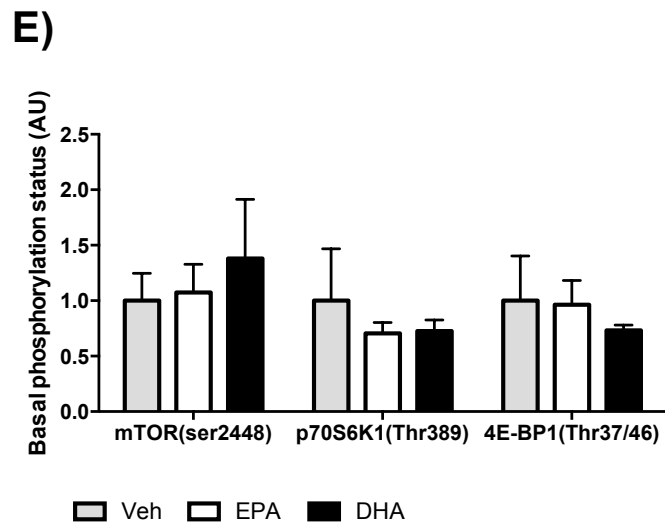
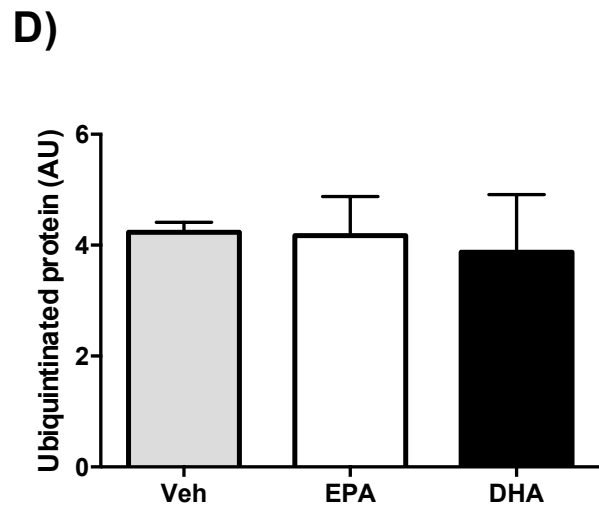
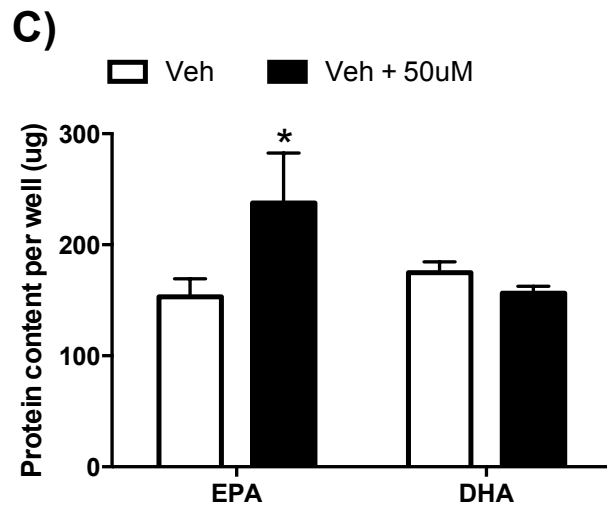
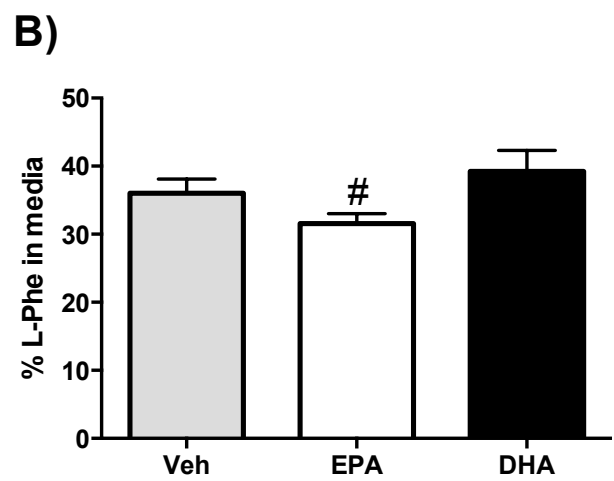
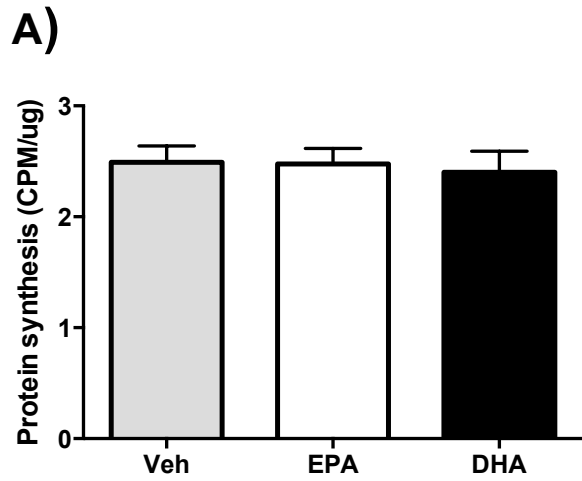


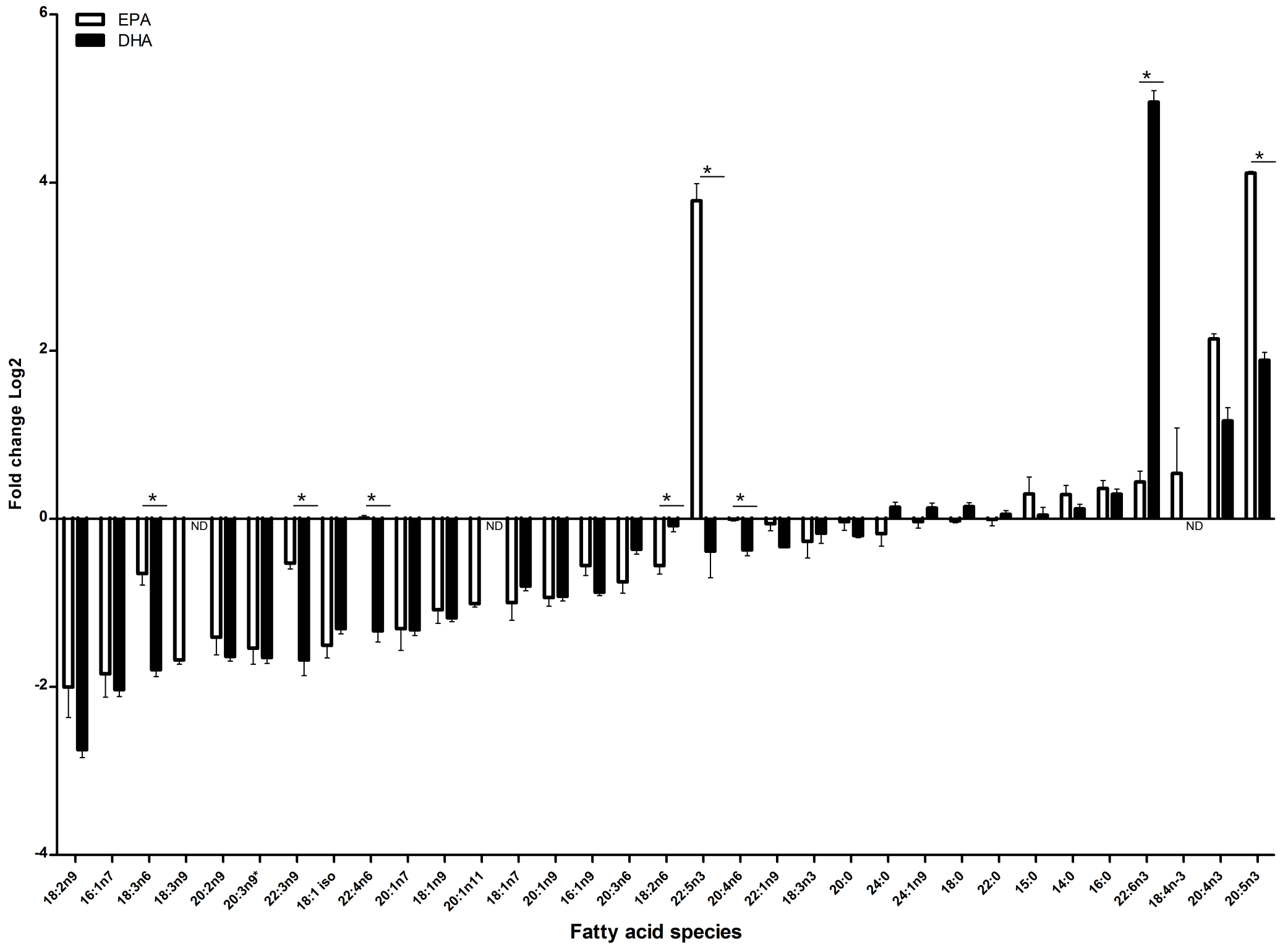
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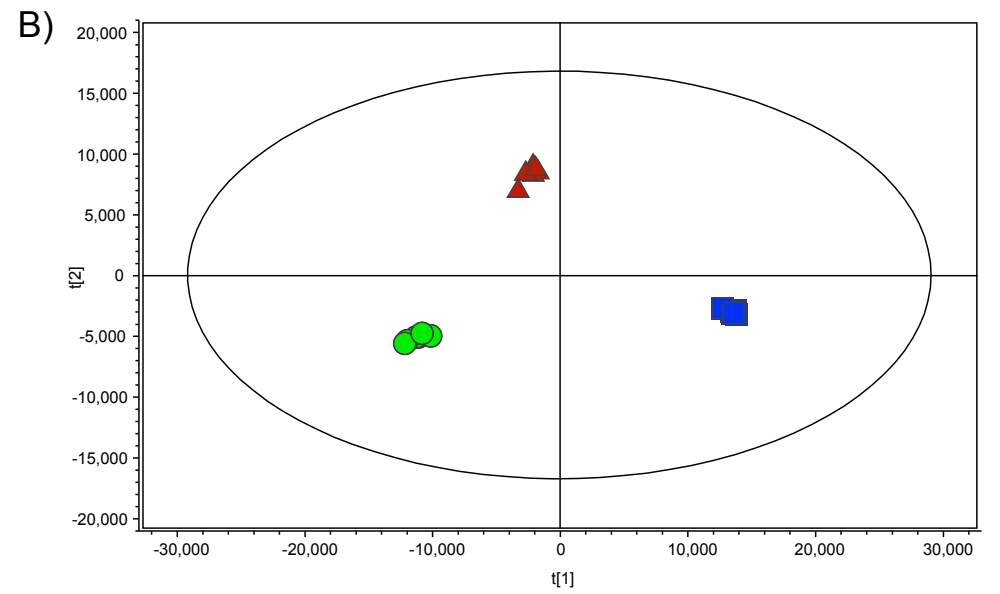
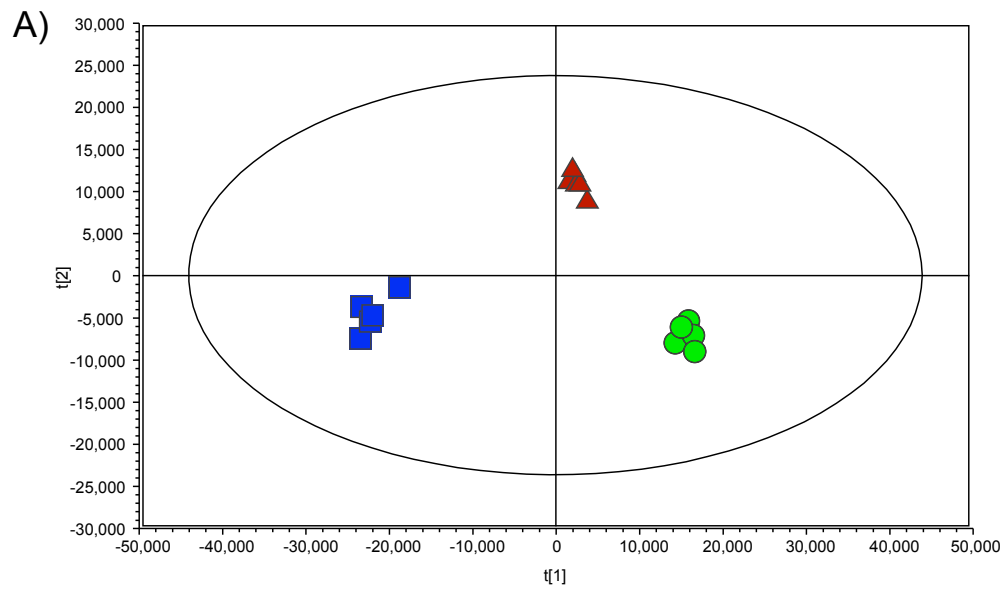
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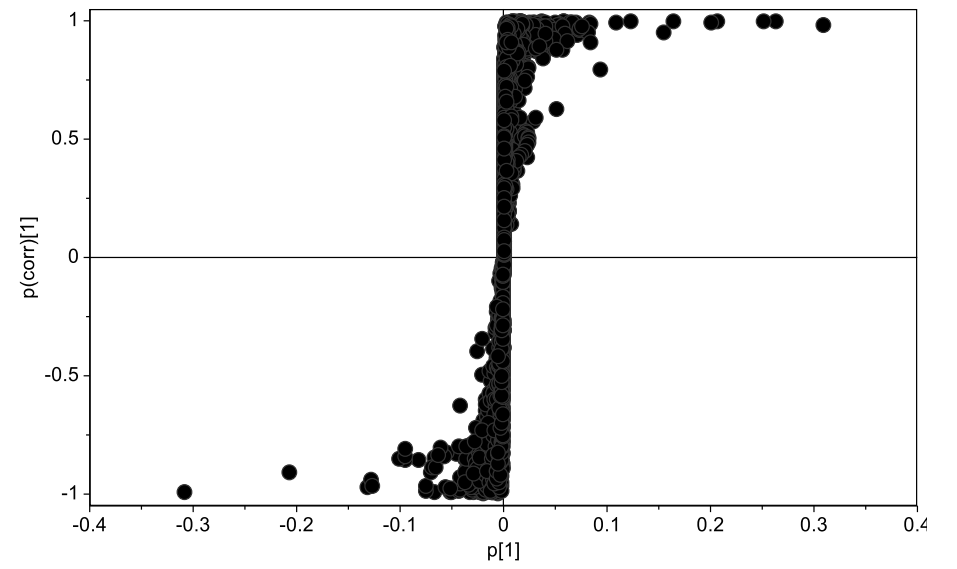
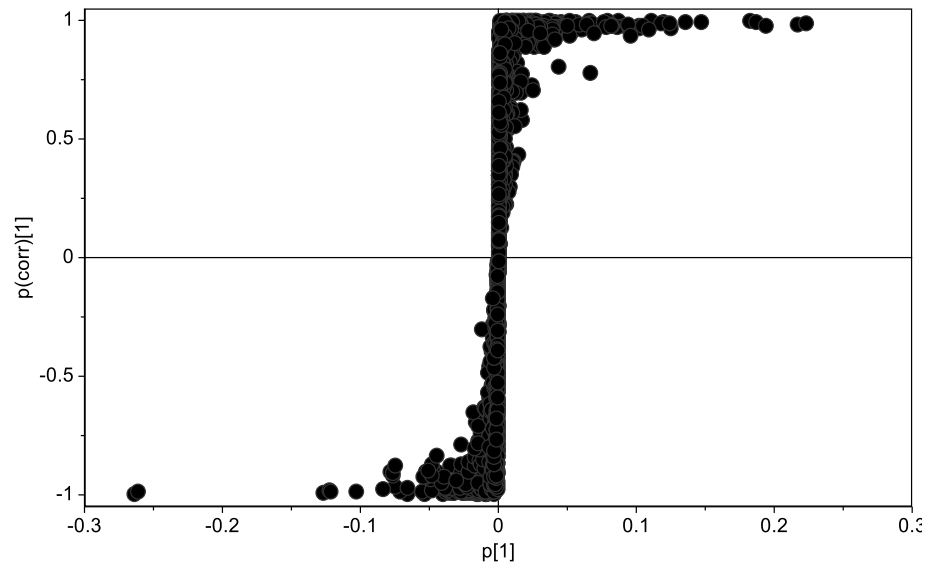
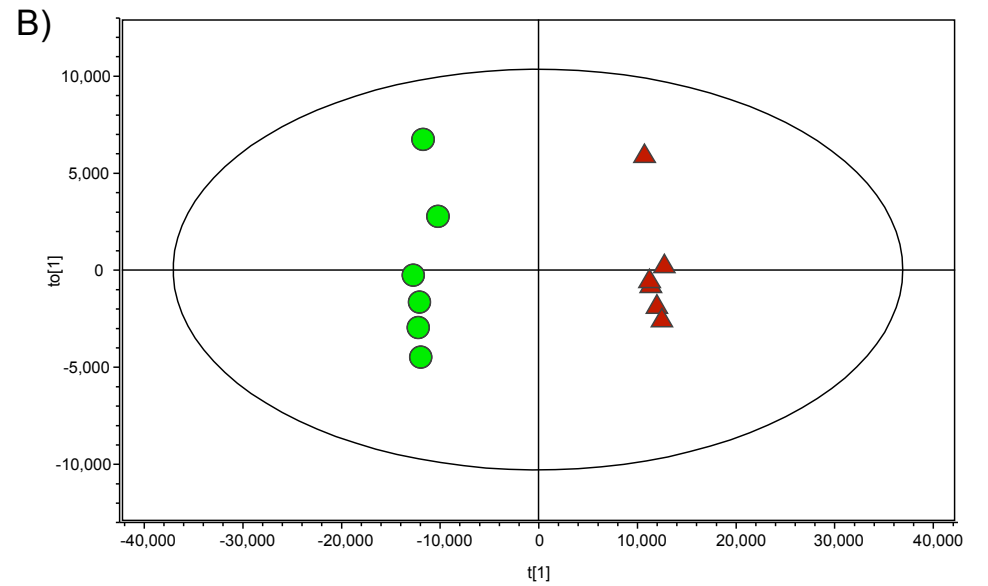
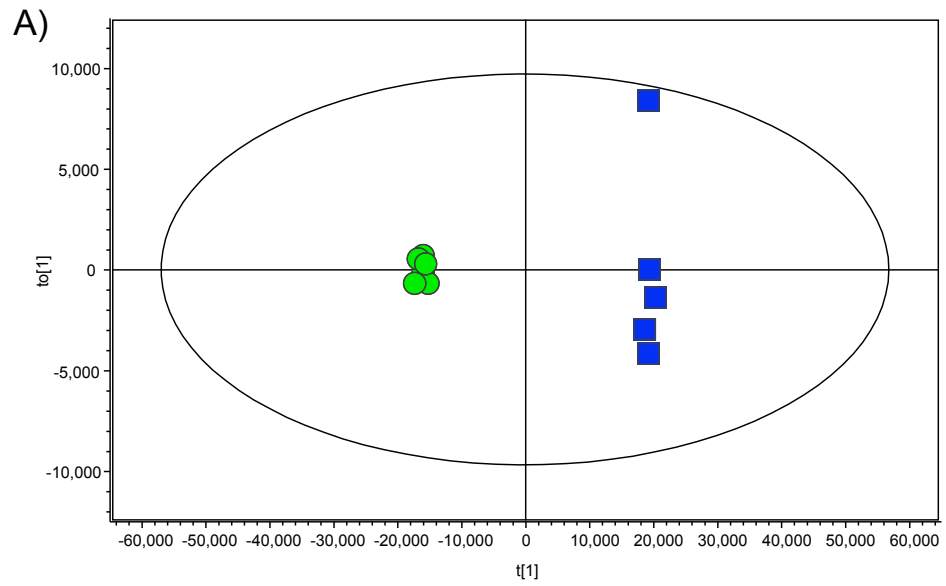
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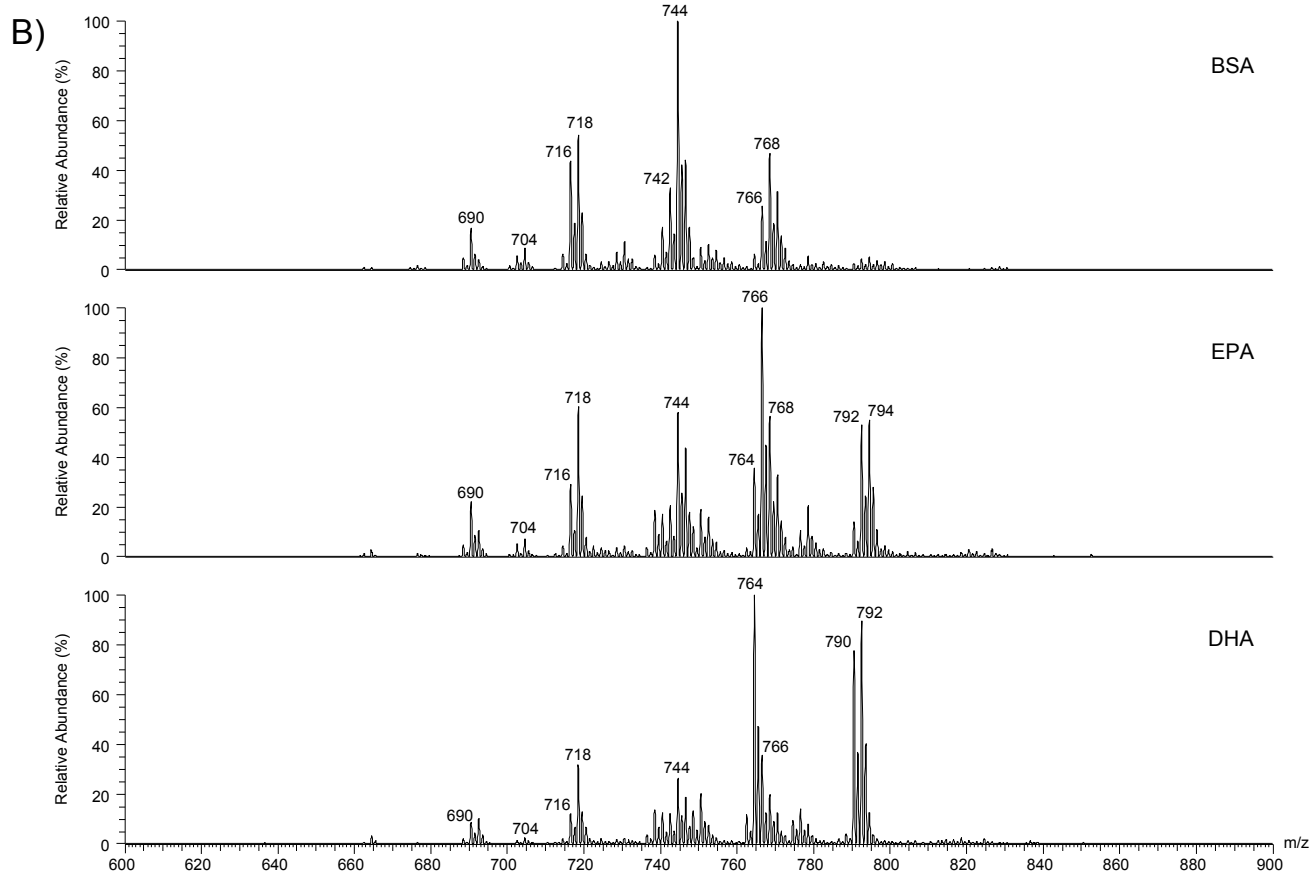
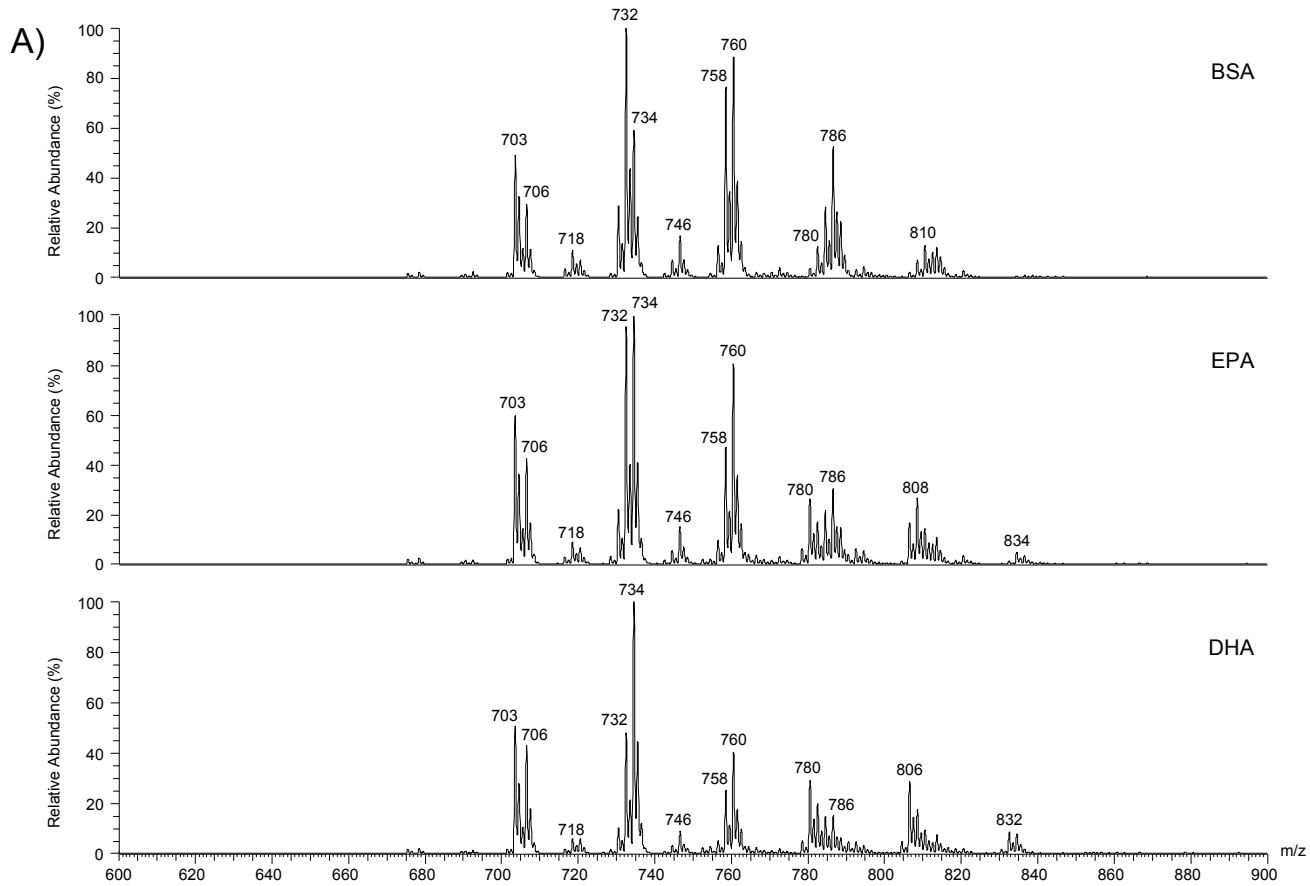


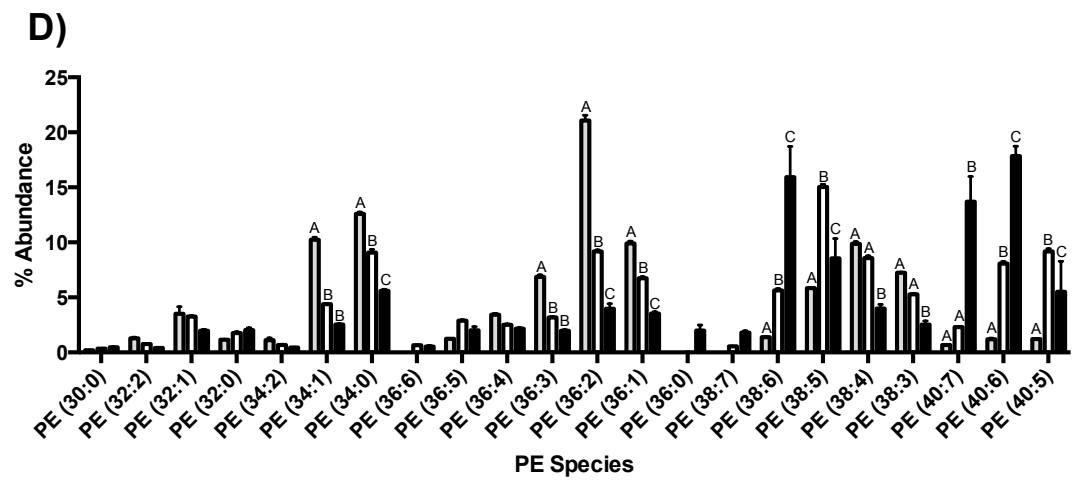
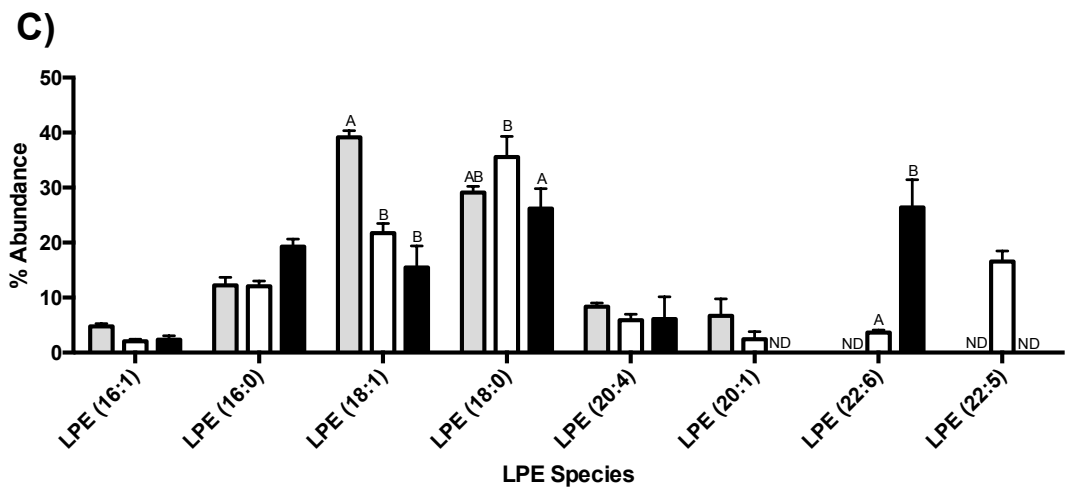
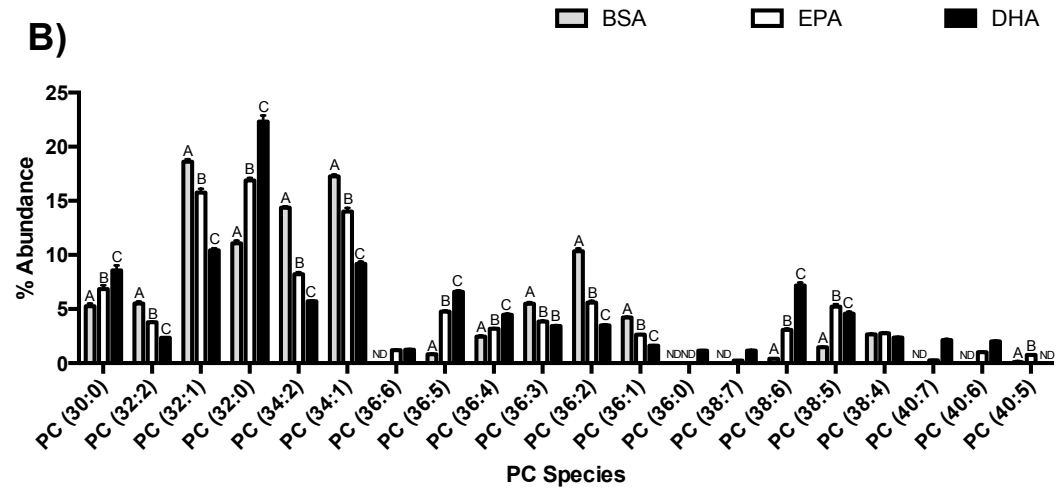
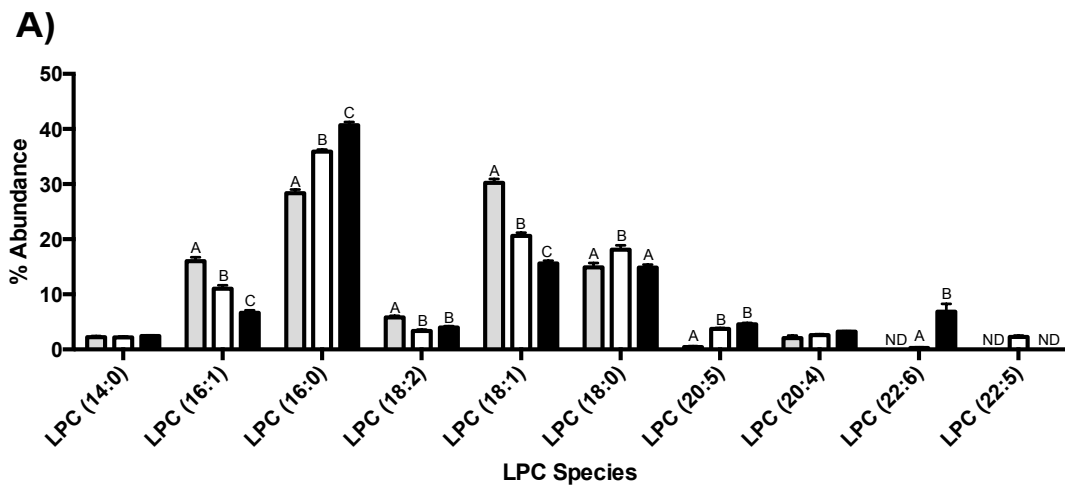








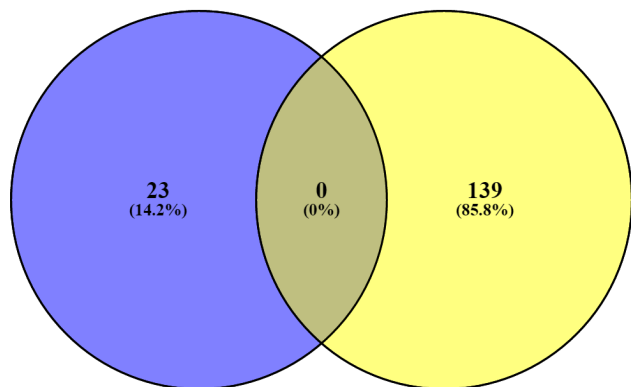




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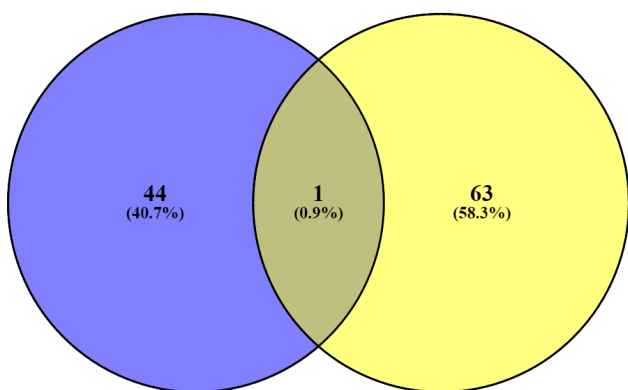
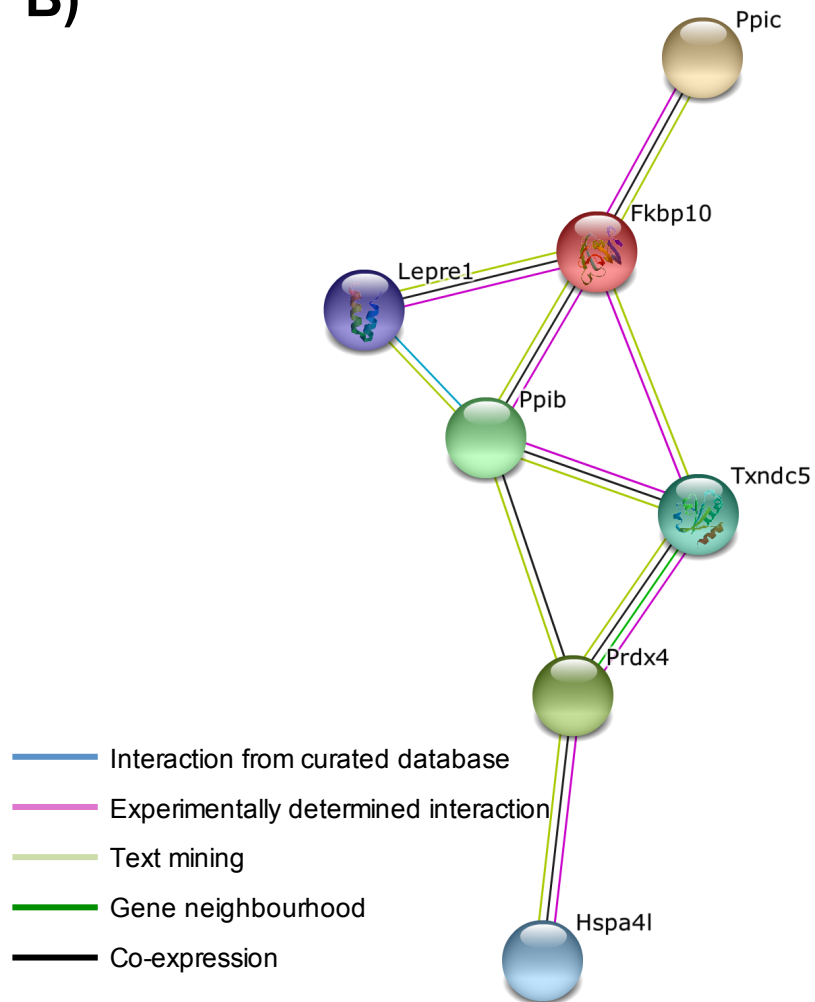
Down EPA

Down DHA



Up EPA

Up DHA

**B)****C)**

□ EPA ■ DHA

