The relationship between membrane fatty acid content and mitochondrial efficiency differs within- and between-omega-3 dietary treatments

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ABSTRACT

An important, but underappreciated, consequence of climate change is the reduction in crucial nutrient production at the base of the marine food chain: the long-chain omega-3 highly unsaturated fatty acids (n-3 HUFA). This can have dramatic consequences on consumers, such as fish as they have limited capacity to synthesise n-3 HUFA *de novo*. The n-3 HUFA, such as docosahexaenoic acid (DHA, 22:6n-3) and eicosapentaenoic acid (EPA, 20:5n-3), are critical for the structure and function of all biological membranes. There is increasing evidence that fish will be badly affected by reductions in n-3 HUFA dietary availability, however the underlying mechanisms remain obscure. Hypotheses for how mitochondrial function should change with dietary n-3 HUFA availability have generally ignored ATP production, despite its importance to a cell's total energetics capacity, and in turn, whole-animal performance. Here we (i) quantified individual variation in mitochondrial efficiency (ATP/O ratio) of muscle and (ii) examined its relationship with content in EPA and DHA in muscle membrane of a primary consumer fish, the golden grey mullet *Chelon auratus*, receiving either a high or low n-3 HUFA diet. Mitochondria of fish fed on the low n-3 HUFA diet had higher ATP/O ratio than those of fish maintained on the high n-3 HUFA diet. Yet, mitochondrial efficiency varied up about 2-fold among individuals on the same dietary treatment, resulting in some fish consuming half the oxygen and energy substrate to produce the similar amount of ATP than conspecific on similar diet. This variation in mitochondrial efficiency among individuals from the same diet treatment was related to individual differences in fatty acid composition of the membranes: a high ATP/O ratio was associated with a high content in EPA and DHA in biological membranes. Our results highlight the existence of interindividual differences in mitochondrial efficiency and its potential importance in explaining intraspecific variation in response to food chain changes.

1. Introduction

Fish provide critical sustenance for millions of people worldwide and have far reaching impacts on the productivity of ecosystems (McIntyre et al., 2016). Yet, ongoing and future climate change threatens the persistence of fish populations globally (Pörtner and Knust, 2007). An important, but underappreciated, consequence of climate change is the reduction in production at the base of the food chain of essential nutrient: the long-chain omega-3 highly unsaturated fatty acids (n-3 HUFA) (da Motta Pacheco et al., 2014; Galloway and Winder, 2015; Hixson and Arts, 2016). Water warming (Hixson and Arts, 2016), acidification (Bermudez et al., 2015), or UV irradiation (Kang, 2011), all affect n-3 HUFA primary producers physiology and community assemblages, leading to a dominance of n-3 HUFA-impoverished taxa (Galloway and Winder, 2015). The n-3 HUFA such as eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) are essential to the structure and function of all biological membranes and are thus considered to be important drivers of organism performance (Hulbert et al., 2005; Ishizaki et al., 2001; Mazorra et al., 2003; Vagner et al., 2015). Endogenous biosynthesis of n-3 HUFA from precursors is limited in most vertebrates, including marine fish (Alimuddin et al., 2005; Arts and Kohler, 2009; Oboh et al., 2017). Changes in the n-3 HUFA availability in the fish diet causes strongly correlated changes in the fatty acid composition of their biological membranes (Guderley et al., 2008; Nogueira et al., 2001; Ramsey et al., 2005). Fish on n-3 HUFA-deficient diets can perform badly: reductions in EPA and DHA dietary content reduced growth (Norambuena et al., 2015; Vagner et al., 2015) and thermal tolerance (Vagner et al., 2014), and altered whole-organism metabolic traits (Vagner et al., 2015; Vagner et al., 2014). Determining what causes animal performance to vary with n-3 HUFA diet constitutes a fundamental step in predicting the impacts of projected oceanic changes on fish resilience (Kang, 2011).

Consideration of mitochondrial capacity to make ATP may improve our understanding of the links between dietary n-3 HUFA availability and whole-animal performance. Mitochondrial ATP is produced via oxidative phosphorylation, a process through which energy substrates are oxidized to generate a protonmotive force that drives the phosphorylation of ADP to ATP. Hypotheses for how mitochondrial function should change with dietary n-3 HUFA availability have focused on variation in respiratory capacities and have generally ignored variation in ATP production (Kraffe et al., 2007; Ramsey et al., 2005; but see Herbst, 2014; Vagner et al., 2015; Vagner et al., 2014). Although ATP production depends on the rate of substrate oxidation, the number of ATP molecules produced for each atom of oxygen consumed by the mitochondria during substrate oxidation (ATP/O ratio) can vary (Brand, 2005; Salin et al., 2015). A fraction of the protonmotive force that is generated from substrate oxidation is dissipated through proton leak across mitochondrial inner membranes and this leakage can decrease the protonmotive force available to produce ATP (Brand, 2005; Kadenbach, 2003). Thus, the greater the mitochondria leak, the less efficiently an animal converts its metabolic

78 substrates into ATP, and the lower the ATP/O ratio. The proportion of energy dissipated in the
79 proton leak and the efficiency to make ATP vary among conspecific (Bottje and Carstens, 2009;
80 Robert and Bronikowski, 2010; Salin et al., 2016b) and can be influenced by environmental factors
81 including diet (Fontaine et al., 1996; Salin et al., 2018). A number of studies have found positive links
82 between intraspecific heterogeneity in efficiency to produce mitochondrial ATP and the whole-
83 organism performance, such as locomotory performance (Coen et al., 2012; Distefano et al., 2018;
84 but see Jahn and Seebacher, 2019), developmental rate (Salin et al., 2012), growth efficiency (Bottje
85 and Carstens, 2009; Salin et al., 2019) and reproductive output (Robert and Bronikowski, 2010),
86 suggesting that it might be a trait of ecological relevance.
87 Recent research has recognized the importance of accounting for individual heterogeneity in
88 predicting responses to global changes (Hamel et al., 2018). Because some individuals perform much
89 better than others within the same environment, individual heterogeneity is likely to directly
90 influence the potential for species to evolve adaptations for a reduced n-3 HUFA availability. A
91 number of studies have found positive links between n-3 HUFA content in membranes and
92 mitochondrial proton leak when comparing among species (Brand et al., 1994; Brookes et al., 1998),
93 but contradictory results were found when comparing treatment groups with studies reporting
94 positive (Martin et al., 2013), negative (Fontaine et al., 1996; Guderley et al., 2008) or no
95 relationships (Guderley et al., 2008) between content in n-3 HUFA in mitochondrial membranes and
96 leak respiration. N-3 HUFA are thought to have important effects on the mitochondrial capacity to
97 make ATP; but until now, there has, to our knowledge, been no assessment of whether membrane n-
98 3 HUFA content could explain variation in mitochondrial metabolism among individuals.
99 The present experiment integrates measurements of mitochondrial efficiency and
100 mitochondrial proton leak to determine whether reductions in EPA and DHA availability in food led to
101 changes in energy metabolism of a primary consumer fish. We first examined the effect of dietary n-
102 3 HUFA content on mitochondrial function, in particular on the efficiency to produce ATP (ATP/O
103 ratio) and the respiratory capacities to offset the proton leak (LEAK respiration). Secondly, we tested
104 whether differences between individuals in mitochondrial efficiency and mitochondrial LEAK
105 respiration vary with membrane n-3 HUFA content. To address this, we experimentally manipulated
106 the quantity of n-3 HUFA in food for wild-caught juvenile golden grey mullet (*Chelon auratus*). Fish
107 were fed either a high n-3 HUFA or low n-3 HUFA diet, and their membrane fatty acid composition
108 and mitochondrial functioning were determined in skeletal muscle. We choose juvenile golden grey
109 mullet as our study organism because they are likely to be the first levels of the food chain to face a
110 decline in availability of dietary n-3 HUFA, as this fish fed mainly on primary producers (Lebreton et
111 al., 2011; Mourente and Tocher, 1993). We analysed mitochondrial properties in the skeletal muscle
112 because the mitochondrial function of this tissue is known to influence whole-animal performance

(Coen et al., 2012; Salin et al., 2016a), and that fatty acid dietary content influences muscle membrane fatty acid composition (McKenzie et al., 1998; Vagner et al., 2015).

2. Materiel and methods

2.1. Fish origin and care

Wild juvenile golden grey mullets (n = 23) were netted from the marshes of L'Houmeau France (46°12'14.4''N 1°11'43.7''W) in November 2017 and transported to the laboratory Littoral Environment Society (LIENSs), France, where all the experiments were conducted. Fish were kept in a common thermoregulated 300 L-tank supplied with aerated recirculated sand-filtered natural seawater and equipped with an external biological filter (Eheim, Deizisau, Germany). Fish were maintained under a 12 L : 12 D photoperiod, and fed daily with commercial pellets (Le Gouessant®, Lamballe, France). Temperature (13.8 ± 0.2°C) and salinity (28.7 ± 0.1) were monitored daily (TetraCon® 325, Laboratoires Humeau, La Chapelle-sur-Erdre, France) and were kept similar to that of the sampling site. Oxygen (87.9 ± 2.6% air saturation) was monitored once a week. Fish were acclimated for seven weeks to these conditions. The collection and handling of the animals were carried out under the jurisdiction of the Departmental Service of Fisheries and the Animal Care Committee of France (# 12886), respectively.

In January 2018, fish were anesthetized in 0.1 g L^{-1} MS-222 (Ethyl 3-aminobenzoate methanesulfonate) in seawater, measured for body mass (34.3 ± 6.5 g), individually pit-tagged (Biolog-id, Bernay, France), and randomly assigned to one of the four replicate 300-L holding tanks (n=5-6 fish per tank). All fish were fasted for 24h beforehand to ensure their guts were empty. Fish were acclimated for 4 weeks in their new tanks, during which time they were fed commercial pellets twice daily to a total of 2% of their biomass (Le Gouessant ®, Lamballe, France).

In February 2018, fish were gradually acclimated to 20°C by means of a sequence of 3 step increases of 2°C over a two-week period. This temperature was chosen because it is the temperature at which whole-organism performance declined in mullet fed on a low n-3 HUFA diet, but is within their natural thermal range as 20°C reflects the mean summer temperature in mullet natural environment (Vagner et al., 2015; Vagner et al., 2014). Fish were left another 2 weeks period of acclimation to initiate physiological adjustment to the change in temperature (Bouchard and Guderley, 2003). Temperature (19.9 ± 1°C) and salinity (29.6 ± 1.1) were daily measured in the four experimental tanks.

2.2. Diet treatment and tissue sampling

Differences in dietary n-3 HUFA content were achieved by replacing fish oil (rich in n-3 HUFA) of the High n-3 HUFA diet with rapeseed oil (poor in n-3 HUFA) in the Low n-3 HUFA diet (See ingredients in

148 Table S1 *(a)*). Experimental diets were isocaloric and isolipidic. High and Low n-3 HUFA diets
149 contained 17.5 % and 1.2 % EPA+DHA, respectively, per total fatty acid mass of the diets, which
150 represents about 15-fold difference in EPA and DHA content between diets (See Table S1 *(a)* for fatty
151 acid composition of the diets). The EPA and DHA content in High n-3 HUFA diet cover the needs of
152 several fish species (Robin and Skalli, 2007), although the needs of the golden grey mullet are not
153 known. The Low n-3 HUFA diet was estimated to significantly reduce n-3 HUFA content in biological
154 membranes and has been shown to impair aerobic performance of golden grey mullet at 20°C
155 (Vagner et al., 2015).
156 Following the period of acclimation to water temperature of 20°C, fish were switched to the
157 experimental diets for about two months (mean ± SEM = 61 ± 2 days, range: 45 – 73 days). This
158 duration was chosen because it is sufficient to detect differences in the membrane fatty acid
159 composition between diet treatments (Robin and Skalli, 2007). Because only two fish per day could
160 be analysed for their mitochondrial function at the end of the experiment, the duration of the diet
161 treatment differed between processing batches. Fish were randomly allocated to the treatment: fish
162 had their food progressively switched to either the High n-3 HUFA diet (n = 11) or the Low n-3 HUFA
163 diet (n = 12). The experimental diet to commercial diet ratio was increased every second day from
164 25%:75% to 50%:50%, 75%:25% and finally to 100%:0%. Body mass did not differ between fish
165 groups subsequently assigned to the two food treatments (High n-3 HUFA diet: 31.3 ± 1.8 g; Low n-3
166 HUFA diet: 34.9 ± 1.5 g, *t*-test: t_{21} = -1.510, p = 0.146). Body mass was re-measured (as above) every
167 2-3 weeks and rations were recalculated to adjust for growth.
168 At the end of the food treatment period, fish were fasted for 24 h before being anesthetized
169 and culled. Fish were weighed, measured and two samples of skeletal muscle were immediately
170 dissected, taken dorsally from the lateral line (to have both red and white muscle) and just behind
171 the head. One aliquot was collected from one side of the fish and kept in ice-cold isolation buffer
172 (sucrose 250 mM, EGTA 1mM, Tris-HCl 20mM, pH 7.4 at 4°C) for immediate mitochondrial assay,
173 while the other aliquot (≈1 g) was collected from the other side and immediately flash-frozen for
174 subsequent fatty acid analysis.
175
176 *2.3. Determination of fatty acid composition*
177 *2.3.1. Lipid extraction*
178 Lipids from muscle of fish were extracted following the method used in Mathieu-Resuge et al. (2019).
179 Muscle aliquots were ground into a fine homogeneous powder in liquid nitrogen. Lipids from 200-
180 250 mg of muscle powder were then extracted in 6 ml of mixture chloroform/methanol (2:1; v/v). To
181 ensure complete lipid extraction, samples were sonicated at 4 °C during 2 x 5 min. Lipids from
182 experimental diets were extracted following the same method. Pellets were ground in a mortar and

183 powder was transferred in chloroform/methanol (2:1; v/v). All lipid extracts were then stored at −20
184 °C under nitrogen atmosphere until further analysis.
185
186 *2.3.2. Purification of polar lipids*
187 Fatty acids of biological membranes are connected to a polar head group, altogether named polar
188 lipid (PL), unlike fatty acids of energy stores that constitute triglyceride, a neutral lipid. To analyse the
189 FA composition of biological membrane, PL were separated from neutral FA and FA from only PL
190 were considered. PL were separated from neutral one by solid-phase extraction following the
191 method described in (Martin et al., 2013). Briefly, an aliquot of muscle total lipid extract (1/6) was
192 evaporated to dryness under nitrogen, recovered with 3 washings of 0.5 ml of chloroform/methanol
193 (98:2; v/v) and deposited at the top of a silica gel column (40 mm × 4 mm, silica gel 60A 63–200 µm)
194 previously heated at 450°C and deactivated with 6 weight % H_2O. After elution of neutral lipids with
195 10 mL of chloroform/methanol (98:2; v/v), PL were eluted with 20 mL of methanol, transferred into a
196 vial containing 2.3 µg of internal standard (tricosanoic acid C23:0) and stored at -20°C under nitrogen
197 atmosphere.
198
199 *2.3.3. Polar lipid transesterification*
200 Polar lipid transesterification was realized as described in Mathieu-Resuge et al. (2019). PL fractions
201 were evaporated to dryness under nitrogen. Fatty acid methyl esters (FAME) were obtained by
202 addition of 800 µL of H_2SO_4 / methanol (3.4 %; v/v) and heating at 100°C for 10 min. FAME were then
203 extracted in 800 µL of hexane and washed three times with 1.5 ml of distilled water saturated in
204 hexane.
205
206 *2.3.4. Gas chromatography analysis of FAME*
207 FA composition was analysed by GC coupled with Flame-Ionization Detector (GC-FID) as described by
208 Mathieu-Resuge et al. (2019) with a Varian CP8400 gas chromatograph. After splitless-mode
209 injection, FAME were separated in parallel on two different columns (DBWAX 30 m × 0.25 mm ID x
210 0.2 µm and DB5 30 m × 0.25 mm ID x 0.2 µm, Agilent). FAME were identified by comparisons of their
211 retention time with those of commercial standards (Supelco 37 Component FAME Mix, PUFA No.1
212 and No.3, and Bacterial Acid Methyl Ester Mix, Sigma) and lab-made standard mixtures. The internal
213 standard (C23:0) allowed to calculate the FA content (µg mg^{-1} muscle wet mass). Fatty acid content
214 was then determined as the percentage of fatty acid mass per total mass of fatty acids in membrane
215 lipids from the polar lipids fraction. The sum of EPA and DHA content in membrane lipids was
216 calculated as:

$$\Sigma \text{ EPA + DHA content} = \frac{\text{mass of EPA} + \text{ mass of DHA}}{\text{total mass of fatty acids}} \times 100$$

2.4. Mitochondrial function

2.4.1. Isolation of mitochondria

Isolation of muscle mitochondria and measurement of mitochondrial efficiency were adjusted from published protocols (Ghanizadeh Kazerouni et al., 2016; Salin et al., 2016c). Briefly, mitochondria were isolated from 1 g (mean ± SEM: 1.07 ± 0.03 g) of muscle. Tissue was finely chopped on ice and homogenized in 10 mL of isolation buffer by four gentle passes in a Potter-Elvehjem homogenizer and centrifuged at 500 g for 5 min at 4°C. The supernatant was transferred in a clean tube and centrifuged at 1000 g for 5 min at 4°C and then centrifuged at 9000 g for 10 min at 4°C. The pellet containing mitochondria was gently resuspended in 500 µL assay buffer at 4°C (20mM Taurine, 10mM KH_2PO_4, 20 mM HEPES, 110 mM D-sucrose, 60 mM K-lactobionate, 1g L^{-1} BSA fatty acid free, pH 7.2 at 20˚C).

2.4.2 Mitochondrial ATP/O ratio and LEAK respiration measurements

Mitochondrial efficiency has been quantified through the measurement of the ATP/O ratio, which is the amount of ATP generated per unit of oxygen consumed. Mitochondrial LEAK respiration was determined as the rate of oxygen consumption when ATP synthesis was inhibited. We used a protocol that simultaneously measures both mitochondrial ATP production and the oxygen consumption related to that ATP production, as in Salin et al. (2016c). Assays were conducted at 20°C.

Oxygen and magnesium green fluorescence signals were detected simultaneously using two respirometry chambers equipped with fluorescent sensors and recorded using DatLab software (Oroboros Instruments, Innsbruck Austria). To estimate ATP production we used the magnesium-sensitive fluorescent probe, Magnesium Green, to determine changes in [Mg^{2+}] (Szmacinski and Lakowicz, 1996). ATP production was then calculated from the rate of change in [Mg^{2+}] and is based on the unequal affinities of ATP and ADP for Mg^{2+} (Chinopoulos et al., 2009).

The oxygen electrodes were calibrated at two points: air-saturated assay buffer (daily) and zero oxygen after sodium dithionite addition (fortnightly). Stepwise additions of $MgCl_2$ at each run were performed for calibration of the fluorescent signal into Mg^{2+}. The two binding affinity (K_d) values of ATP and ADP for Mg^{2+} were determined in presence of isolated mitochondria and calculated as in (Chinopoulos et al., 2014) ; the values were $K_{d\text{-ATP}}$ = 0.197 mM and $K_{d\text{-ADP}}$ = 1.609 mM.

Isolated mitochondria from each fish were added to one of the two measurement chambers of the oxygraph immediately following isolation; fish from a processing pair (i.e. a Low and a High n-3 HUFA) were measured in parallel. The remaining part of the isolated mitochondria was preserved on

ice for use in a replicate trial. Assays contained 200 µl of isolated mitochondria with 1.8 mL of assay buffer in the presence of complex I substrates (5 mM pyruvate and 0.5 mM malate) and complex II substrate (10 mM succinate). Magnesium green (2.2 µM), EGTA (0.1 mM), EDTA (5 µM) and $MgCl_2$ (1 mM) were added to determine changes in [Mg^{2+}] and so to calculate the rate of ATP production as in (Chinopoulos et al., 2014).

The rate of oxygen consumption to support ATP production was assessed by adding a saturating concentration of ADP (2 mM, Mg^{2+} free) to the chamber. The raw rate of ATP production was also measured in this condition. The rate of oxygen consumption to offset the leakage of proton – LEAK respiration - was then measured after inhibition of mitochondrial ATP synthesis (with 4 µM carboxyatractyloside). The rate of ATP hydrolysis was also measured in this condition. The rate of ATP hydrolysis was then added to the raw rate of ATP production to obtain the corrected rate of ATP production. Addition of complex I inhibitor (0.5 µM rotenone) and complex III inhibitor (2.5 µM antimycin A) allowed determination of residual oxygen consumption, which was then subtracted from all other oxygen consumption values. The second replicated trial was used to control for repeatability of the assay. It was identical to the first one, but started an hour and half later, using the remaining isolated mitochondrial and the same measurement chamber. Every second day, the measurement chamber associated with a treatment group was reversed to control for any inter-respirometry chamber difference in readings. No effect of the choice of measurement chamber on mitochondrial function was detected.

2.4.3. Analysis of Mitochondrial ATP/O ratio and LEAK respiration

We expressed respiration rate as pmoles of O_2 sec^{-1} ml^{-1} of assay buffer and ATP production as pmoles of ATP sec^{-1} ml^{-1} of assay buffer for each replicate. Finally, the ATP/O ratio was calculated as the ratio of corrected ATP production to two-fold respiration that supported ATP production; the rate of respiration is doubled since each molecule of oxygen is comprised of two oxygen atoms. Replicated ATP/O ratios were highly correlated (Pearson's $r = 0.749$, $P < 0.001$). However, we found a consistent shift in the values of the measurements between the first and the second trials of ATP/O ratio (drift between trials: Paired t = -6.194, $P < 0.001$). The data from the second trial of muscle assay were excluded because the mitochondrial integrity may have been impaired with time post-isolation, as previously found in liver mitochondria (Salin et al., 2016c). Only data of the first LEAK respiration and ATP/O ratio trial were kept for the main analyses. Protein concentration was measured at 595 nm using the Bradford method with bovine serum albumin as a standard. We expressed LEAK respiration as pmoles of O_2 sec^{-1} mg^{-1} of mitochondrial protein. See descriptive statistics of the mitochondrial function in Table S2.

287 *2.5. Statistical analysis*

288 We first examined the effect of the treatments (High and Low dietary n-3 HUFA content) on
289 the membrane fatty acid composition (Σ EPA + DHA content). Normality (Shapiro-Wilk test) and
290 homoscedasticity (Bartlett-test) were tested. When normality and homoscedasticity were not
291 satisfied, non-parametric analyses were carried out. We used Wilcoxon tests to determine whether
292 muscle membrane content of EPA and DHA, and their sum (Σ EPA + DHA content) differed between
293 dietary treatment groups. To examine potential differences in final body mass between treatments
294 that could explain differences in mitochondrial function, a *t*-test was employed. The effect of the
295 dietary treatment on mitochondrial function (ATP/O ratio and LEAK respiration) were also analysed
296 using *t*-tests.

297 We then used linear model analyses to test whether ATP/O ratio and LEAK respiration were
298 correlated with membrane lipid content in Σ EPA + DHA. The models included mitochondrial function
299 (ATP/O or LEAK respiration) as the dependant variable, dietary treatments as a categorical effect, and
300 Σ EPA + DHA content as continuous predictor. The normality of the linear model residuals was
301 validated for all models using Shapiro–Wilk tests. We also tested the interactions between dietary
302 treatment and membrane lipid content in Σ EPA + DHA. All interactions were non-significant, so they
303 were removed from the models.

304 The duration of the dietary treatment was included as potential covariate, the processing
305 batch and fish tanks were included as potential random factor in initial analysis. Each of these
306 variable were not significant, so were removed from all the final models. All statistical analyses were
307 performed with the free software R (R Core Team, 2017), with R Version 3.6.1, with significance level
308 set to $p < 0.05$. Data are presented as means ± standard error of the mean (SEM).

309

310 **3. Results**

311 *3.1. Effect of the dietary treatment on membrane fatty acid composition*

312 Muscle of mullets fed on Low n-3 HUFA diet showed significant differences in their membrane
313 fatty acid composition compared to those of fish fed on High n-3 HUFA diet (Table S3). EPA and DHA
314 content in muscle membranes were significantly lower in Low n-3 HUFA fish compared to High n-3
315 HUFA fish (EPA: $W = 119$, $p < 0.001$; DHA: $W = 132$, $p < 0.001$; Table 1). Not surprisingly, fish on
316 average had a lower content of Σ EPA+ DHA in their muscle when fed the Low n-3 HUFA diet ($W =$
317 130, $p < 0.001$, Table 1). Final body mass did not differ between High and Low n-3 HUFA fish ($t = -$
318 1.668; $p = 0.112$), excluding body mass as confounding sources of differences between dietary
319 treatments.

320

321 *3.2. Effect of the dietary treatment on mitochondrial function*

ATP/O ratio was significantly higher in the mitochondria of Low compared to High n-3 HUFA mullet (t = -3.107, p = 0.005; Fig. 1A). However, there was no effect of dietary n-3 HUFA content on LEAK respiration in muscle mitochondria (t = -0.423, p = 0.677; Fig. 1B).

3.3. Relationships across individuals between mitochondrial function and membrane fatty acid composition

The ATP/O ratio ranged from 1.46 to 3.33 for mitochondria of mullet eating the High n-3 HUFA diet and from 2.05 to 3.67 for mitochondria of fish on Low n-3 HUFA diet (Fig. 2A). Variation in mitochondrial function between individuals was mainly explained by differences in n-3 HUFA content in muscle (Figure S1). Regardless of the food treatment, the muscle ATP/O ratio of a fish was strongly and positively related to its Σ EPA + DHA content: individuals that had the higher mitochondrial efficiency under either diet had the highest content in EPA and DHA in their membranes (t = 2.513, p = 0.021; Fig. 2A and Table 2). There was no relationship between a fish's mitochondrial LEAK respiration and its Σ EPA + DHA content among individuals with the same dietary treatment (t = -0.104, p = 0.919, Fig. 2B and Table 2). We found no correlation between ATP/O ratio and LEAK respiration from the same mitochondria (High n-3 HUFA diet: Pearson's r = 0.229, p = 0.499; Low n-3 HUFA diet: Pearson's r = -0.157, p = 0.627).

4. Discussion

We asked whether a decline in dietary n-3 HUFA content leads to changes in mitochondrial metabolic phenotype for a model of primary consumer fish, the golden grey mullet. We manipulated dietary content in n-3 HUFA and assessed membrane FA composition and mitochondrial function in mullet. We found that diet strongly influenced membrane fatty acid composition: mullets on a Low n-3 HUFA diet had lower levels of n-3 HUFA in muscle biological membranes, which suggested that the mitochondrial membranes contained less n-3 HUFA. Previous studies have indeed demonstrated that modification of the fatty acid composition of the diet causes strongly correlated changes in the membrane fatty acid composition of mitochondria (Herbst et al., 2014; Jeromson and Hunter, 2014; Ramsey et al., 2005), including those of fish species (Guderley et al., 2008; Morash et al., 2009). Our findings reveal that ATP/O ratio increased significantly in mitochondria of fish fed a low n-3 HUFA diet, so that a decline in dietary content in n-3 HUFA elicits greater mitochondrial efficiency to produce ATP. Surprisingly, the differences in membrane n-3 HUFA content between individuals had an opposite effect on mitochondrial efficiency: under the same dietary treatment, individuals that displayed higher EPA and DHA membrane content had mitochondria with the highest ATP/O ratio.

Aerobic performance of marine fish will not only be challenged in a near future by increased oxygen needs and low oxygen availability at high temperatures but probably also by a shift in ocean

productivity (Behrenfeld et al., 2006), where EPA and DHA availability might decline considerably (Hixson and Arts, 2016). Mitochondrial aerobic capacity has traditionally been determined in terms of oxygen consumption, enzymatic activities or density of mitochondria (Brookes et al., 1998; Guderley et al., 2008; Martin et al., 2013; Morash et al., 2009; Ramsey et al., 2005; Yu et al., 2014). However, oxygen consumption provides only an indirect measure of ATP production, and the relationship between oxygen consumption and ATP production can vary both among and within individuals. Our results clearly show that when evaluating mitochondrial adjustment in response to a reduction in n-3 HUFA availability in diet, variation in the amount of ATP generated per molecule of oxygen consumed by mitochondria can have major importance in explaining associated changes in whole-animal performance. Past work on mullets found that fish fed on a low n-3 HUFA diet consumed less oxygen compared to the fish fed a high n-3 diet to reach a similar swimming speed (Vagner et al., 2015; Vagner et al., 2014). Our results suggest that higher mitochondrial efficiency may result in lower oxygen requirement for fish to produce ATP and sustain muscle contraction for locomotory performance, as previously demonstrated in human (Coen et al., 2012). Mitochondria with high efficiency can be beneficial for energy-demanding cellular processes (Salin et al., 2015), yet there could also be a cost. Mitochondria are a major producer of reactive oxygen species (ROS) and mitochondrial efficiency can be positively related to ROS production (Brand, 2000; Salin et al., 2015), an area which would require further study.

Here, we found no significant differences in LEAK respiration of mullet muscle across dietary treatments. In contrast, LEAK respiration in trout muscle were higher for mitochondria from fish fed a low n-3 HUFA diet than for mitochondria from fish fed a high n-3 HUFA diet (Guderley et al., 2008). Another experiment in trout has shown the opposite effect: animals fed a lower n-3 HUFA diet displayed lower LEAK respiration in muscle mitochondria (Martin et al., 2013). We stress that it is entirely possible for the mitochondrial LEAK response to dietary content in n-3 HUFA to vary among individuals, species and other environmental factors, but also that the consequence on the proton leakage might depend on the magnitude and duration of the dietary treatment. Equivocal support may indicate that the responses of LEAK to changes in membrane fatty acid composition does not fall along a linear response curve, as typically assumed. Instead, the effect of dietary content in n-3 HUFA on membrane composition, and in turn proton permeability might be biphasic (Abbott et al., 2010) and might promote non-linear response of LEAK. While testing a large range of n-3 HUFA dietary content was beyond the scope of the present study, it may only be possible to explain contrasted pattern of LEAK response if many dietary levels of n-3 HUFA are considered.

In our study, mullets from the same food treatment displayed important individual variation in mitochondrial efficiency that covary with membrane fatty acid composition: individual that had higher EPA and DHA content in their membranes had mitochondria that synthesized ATP more

efficiently than those of individuals with lower EPA and DHA content. Previous studies suggested that variation in FA composition of the mitochondrial inner membrane can influence the mitochondrial membrane conductance of protons, and in turn the proportion of oxygen used to offset the proton leakage (Hulbert et al., 2007), leading to variation in the efficiency of ATP production (Brand, 2005). However, this does not appear to be the case in the present study, as LEAK did not covary with ATP/O ratio and membrane fatty acid composition. An explanation for this discrepancy might lie in the fact that mitochondrial efficiency also depends on protein abundance and activity of the mitochondrial inner membrane-bound complexes involved in oxidative phosphorylation (i.e. electron transport complexes and ATP synthase). This alternative explanation is based on the fact that fatty acid composition of membrane lipids may affect lipid–protein interactions and therefore the function of embedded proteins (Brenner, 1984). As well as varying with membrane FA composition and protein function, mitochondrial ATP/O ratio can shift in response to energy substrate use (Brand, 2005). Previous work in Atlantic salmon has demonstrated that individual variation in DHA and EPA in muscle content was associated with differences in the expression of genes involved in lipid catabolism and carbohydrate metabolism (Horn et al., 2019). However, further research is needed to determine whether differences in energy substrates utilization determine individual variation in mitochondrial efficiency in mullet.

Some mullets on the low n-3 HUFA dietary treatment had actually higher EPA and DHA contents than others on the high n-3 HUFA dietary treatment that were eating almost 15 times as much EPA and DHA. Individual variation in membrane fatty acid composition is likely to be a complex, integrative characteristics influenced by several metabolic pathways. If individual differences in fatty acid membrane composition covary with the rates of lipid assimilation, biosynthesis and degradation, it is important to recognize that fatty acid composition may vary with pathways that are generally neglected. For example, ability to biosynthesis EPA and DHA in marine fish is very limited, as it is generally insufficient to compensate dietary deficiency (Tocher, 2003), but the link between individual variation in n-3 HUFA content and their rate of biosynthesis might only appear across individuals eating the same amount of n-3 HUFA. Another explanation might be an individual heterogeneity in the rate of degradation of EPA and DHA, while also considered minor in fish (McKenzie, 2001). Individual variation in the rate of assimilation may also be significant for n-3 HUFA content in membrane. Regardless of their food intake, individual fish that have higher levels of EPA and DHA in membrane lipids may have preferentially retained these fatty acids from their diet. Our observations illustrate that an understanding of individual variability in membrane fatty acid composition can be gained only through consideration of differential metabolic pathways of fatty acids.

The individual covariation between mitochondrial efficiency and membrane fatty acid composition we observed might be because higher EPA and DHA contents in the membrane promote higher efficiency of the mitochondria, as explained above. However, given that our study is correlative, it is perhaps high mitochondrial efficiency that may promote membranes with relatively high levels of EPA and DHA, because more ATP is available to fuel metabolic pathways that retain dietary n-3 HUFA. Assimilation of fatty acids in the intestine can be energetically costly (Mansbach and Gorelick, 2007), and for example, fish that have higher efficiency to make ATP may actually have higher assimilation rate of EPA and DHA compared with fish at lower mitochondrial efficiency. The variation in mitochondrial efficiency of muscle tissue studied here might be representative of the mitochondrial efficiency in other tissues, including intestine, although previous studies looking at correlation of mitochondrial efficiency across tissues in the same individual have shown equivocal results (Salin et al., 2019). Further study looking at mitochondrial metabolism in different tissues will be necessary to determine if golden grey mullets are able to improve their mitochondrial efficiency across multiple tissues.

Flexibility in mitochondrial efficiency may be particularly important since the capacity of the mitochondria to produce ATP can set limits on the capacity of an organism to respond to environmental changes (Blier et al., 2013; Sokolova et al., 2012). Our data imply that the greater mitochondrial efficiency induced by the Low n-3 HUFA diet might benefit for the ability of the mitochondrial to make ATP for energy-demanding cellular processes when reduction in EPA and DHA availability in food web. Information on the consequences of individual heterogeneity in mitochondrial efficiency on fish performance would allow a better understanding of the effect of decline in EPA and DHA availability in marine food web. Further research should focus on identifying the individual variation in metabolic pathway of EPA and DHA. This type of variation may be very important in an evolutionary context as well, not only generating phenotypic variation among individuals, but also allowing animals to reduce the energy costs of making ATP by increasing the mitochondrial efficiency.

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Table 1: Mean ± SEM eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) content and sum of EPA and DHA content (Σ EPA + DHA) in muscle membrane fatty acids (percentage of the EPA and DHA mass per total fatty acids mass in membrane, %), of juvenile golden grey mullet given High (n=11) or Low (n=12) omega-3 highly unsaturated fatty acids (n-3 HUFA) diet. Different letters within a row indicate significant differences between diet treatments (Wilcoxon tests, at the significant level $p < 0.05$). More details on FA composition of muscle membranes are provided Table S3.

Muscle membrane FA composition (%)	***High n-3 HUFA fish***	***Low n-3 HUFA fish***
EPA	9.8 ± 0.3[a]	5.7 ± 1.0[b]
DHA	20.8 ± 0.7[a]	10.1 ± 2.0[b]
Σ EPA + DHA	30.6 ± 0.8[a]	15.8 ± 3.0[b]

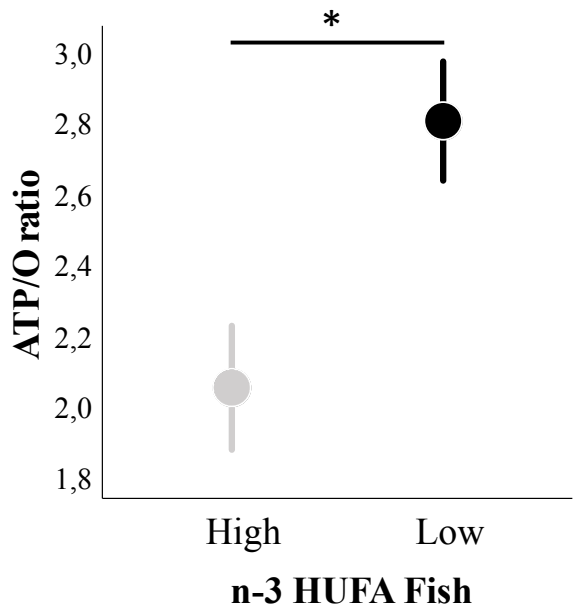
Table 2: Results from linear model analyses of muscle mitochondrial efficiency (ATP/O ratio) and respiratory capacities to offset the proton leak (LEAK respiration) in juvenile golden grey mullet as a function of the sum of eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) content - Σ EPA + DHA in muscle (percentage of the EPA and DHA mass per total fatty acid mass in membrane). Bold denotes significant results.

Dependant variable	*Source of variation*	*Parameter estimate ± SEM*	*t-value*	*p-value*
ATP/O ratio	Intercept	2.21 ± 0.23	7.978	**< 0.001**
	Σ EPA + DHA	**0.04 ± 0.01**	**2.513**	**0.021**
	diet	**1.30 ± 0.31**	**4.232**	**< 0.001**
LEAK respiration	Intercept	56.59 ± 6.45	8.769	<0.001
	Σ EPA + DHA	0.04 ± 0.34	-0.104	0.919
	diet	2.61 ± 7.17	0.364	0.720

FIGURES

Figure 1. Effect of omega-3 highly unsaturated fatty acids (n-3 HUFA) dietary content on (*a*) mitochondrial efficiency estimated as ATP/O ratio (t_{21} = -3.107, p = 0.005) and (*b*) mitochondrial LEAK respiration (t_{21} = -0.423, p = 0.677) of muscle of golden grey mullet that were kept either on High (n=11) or Low (n=12) n-3 HUFA diet. Data are plotted as mean ± SEM. * denotes significant effect.

(a)



(b)

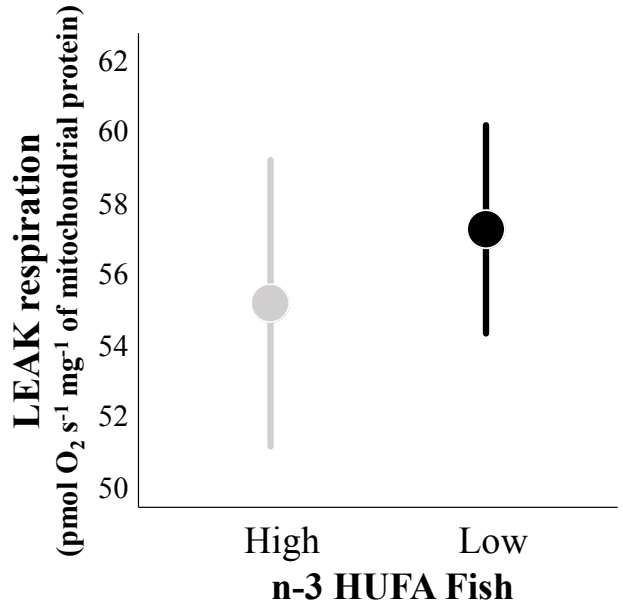
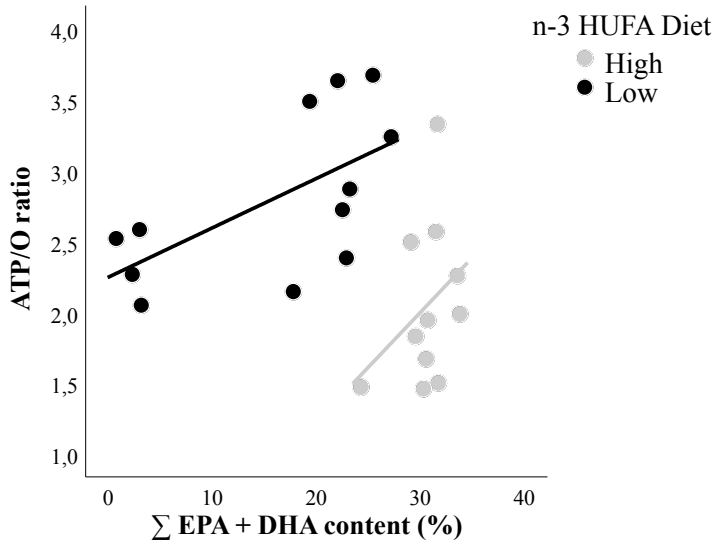
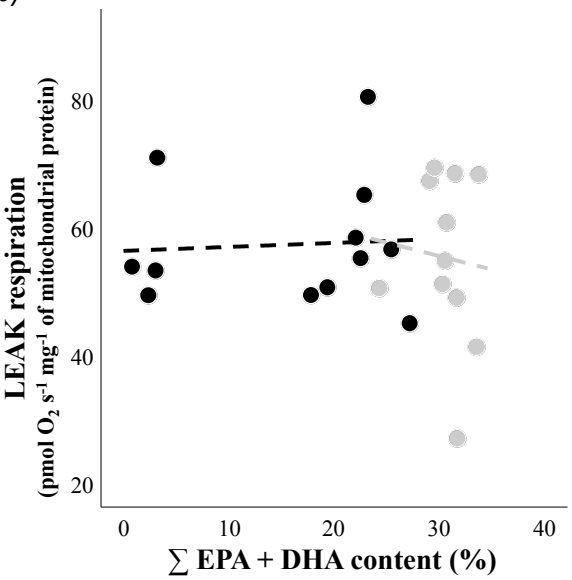


Figure 2. Relationships between the mitochondrial function and membrane lipid content in eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) content (expressed as Σ EPA + DHA content, percentage of the sum of EPA and DHA mass per total fatty acid mass in membranes) of muscle of juvenile golden grey mullet fed on a Low (n=12) versus High (n=11) omega-3 highly unsaturated fatty acid (n-3 HUFA) diet. Mitochondrial efficiency (ATP/O ratio) in relation to *(a)* Σ EPA + DHA content, *(b)* LEAK respiration in relation to Σ EPA + DHA content. Continuous lines show significant effects, dashed lines show non-significant effects (see Table 2 for details).

(a)



(b)



ELECTRONIC SUPPLEMENTARY MATERIAL

Table S1: *(a)* Ingredient (in g 100 g^{-1}) and *(b)* fatty acid (FA) composition expressed as a percentage of FA mass per total FA mass (% mean ± standard error of the mean; *n* = 3) in the High and Low omega-3 highly unsaturated fatty acid (n-3 HUFA) diets (made at INRA, Donzag, France). Only FA that occur above 1 % in at least a treatment group are represented. Bold denotes significant results of one-way ANOVA. Assumptions of normal distribution and homoscedasticity of residuals were met.

(a)

Ingredients [a]	*High n-3 HUFA diet*	*Low n-3 HUFA diet*
Fish meal LT 94	17	17
Casein	30	30
Rapeseed oil	2	10
Fish oil	8	0
Precooked starch	30	30
Vitamin mixture [b]	8	8
Mineral mixture [c]	4	4
Betaine	1	1

[a] *Sources*: fish meal LT 94: Norse (Fyllingsdalen, Norway); casein: Sigma-Aldrich (Germany); rapeseed oil: Système U (Créteil, France); fish oil: pure cod oil Cooper (Melun, France); precooked starch: Prégéflo Roquette Frères (Lestrem, France); vitamin mixture (INRA Jouy-en-Josas, France)

[b] Vitamin mixture (g kg^{-1} vitamin mix): retinyl acetate 1; cholecalciferol 2.5; Dl-α- tocopheryl acetate 5; menadione 1; thiamine-HCl 0.1; riboflavin 0.4; D-calcium pantothenate 2; pyridoxine–HCl 0.3; cyanocobalamin 1; niacin 1; choline 200; ascorbic acid (ascorbyl polyphosphate) 5; folic acid 0.1; D-biotin 1; mesoinositol 30.

[c] Mineral mixture (g kg^{-1} mineral mix): KCl 90; KI 0,04; $CaHPO_4 \cdot 2H_2O$ 500; NaCl 40; $CuSO_4 \cdot 5H_2O$ 3; $ZnSO_4 \cdot 7H_2O$ 4; $CoSO_4$ 0.02; $FeSO_4 \cdot 7H_2O$ 20; $MnSO_4 \cdot H_2O$ 3; $CaCo_3$ 215; MgOH 124; Na_2SeO_3 0 03; NaF 1.

(b)

Diet FA composition (%)	*High n-3 HUFA*	*Low n-3 HUFA*	F	Df	p-value
14 :0	**4.30 ± 0.20**	**0.41 ± 0.02**	**362.2**	**1**	**< 0.001**
16 :0	**18.67 ± 0.80**	**6.10 ± 0.26**	**223.6**	**1**	**< 0.001**
18 :0	**4.59 ± 0.21**	**1.91 ± 0.09**	**142.7**	**1**	**< 0.001**
16 :1n-7	**4.83 ± 0.11**	**0.44 ± 0.01**	**1630**	**1**	**< 0.001**
18 :1n-9	**25.04 ± 0.23**	**56.74 ± 0.30**	**7004**	**1**	**< 0.001**
18 :1n-7	**2.56 ± 0.36**	**0.05 ± 0.05**	**46.2**	**1**	**< 0.01**
20 :1n-9	1.82 ± 0.08	4.94 ± 2.09	2.2	1	0.21
18 :2n-6	**5.06 ± 0.36**	**15.13 ± 1.82**	**29.6**	**1**	**< 0.01**
18 :3n-3	**2.42 ± 0.25**	**6.49 ± 1.03**	**14.7**	**1**	**0.02**
20 :4n-6	**1.38 ± 0.07**	**0.27 ± 0.04**	**205.1**	**1**	**< 0.001**
20 :5n-3	**6.63 ± 0.60**	**0.39 ± 0.07**	**106.3**	**1**	**< 0.001**
22 :5n-3	1.64 ± 0.09	0.88 ± 0.31	5.5	1	0.07
22 :6n-3	**10.95 ± 0.63**	**0.78 ± 0.14**	**246.3**	**1**	**< 0.001**

Table S2: Mean ± SEM mitochondrial function of muscle of golden grey mullet fed with High (n=11) and Low omega-3 highly unsaturated fatty acid (n-3 HUFA) diet. Rate of ATP production, rate of oxygen consumption to support ATP production (OXPHOS respiration), and to offset the proton leakage (LEAK respiration). Fluxes are expressed in pmol ATP produced and oxygen consumed s^{-1} mg^{-1} of mitochondrial protein. The respiratory control ratio (RCR), an index of mitochondrial coupling (Brand and Nicholls, 2011) was calculated as the ratio of OXPHOS respiration to the LEAK respiration.

Muscle mitochondrial function	***High n-3 HUFA fish***	***Low n-3 HUFA fish***
ATP production	3267.6 ± 445.8	3869.7 ± 237.4
OXPHOS respiration	836.8 ± 112.0	733.6 ± 60.8
LEAK respiration	55.1 ± 4.0	57.1 ± 2.9
RCR	14.6 ± 1.5	13.0 ± 1.0

Table S3: Results from Kruskal-Wallis tests comparing fatty acid contents (percent of FA mass per total FA mass) in membrane lipids of the muscle of golden grey mullet (*Chelon auratus*) that were either fed a High or Low omega-3 highly unsaturated fatty acid (n-3 HUFA) diet. Only FA that occur above 1 % in at least a treatment group are represented. Means are expressed ± standard error of the mean. Bold denotes significant results. N = 11-12 per treatment group.

Muscle membrane FA composition (%)	*High n-3 HUFA*	*Low n-3 HUFA*	*Chi^2*	*p-value*
15 :0	**0.97 ± 0.08**	**1.68 ± 0.29**	**4.125**	**0.04**
16 :0	18.49 ± 0.75	21.15 ± 2.20	0.015	0.902
18 :0	9.78 ± 0.39	9.91 ± 1.44	0.379	0.54
17 :1n-7	1.29 ± 0.09	1.66 ± 0.17	1.83	0.18
18 :1n-9	**11.42 ± 0.46**	**19.32 ± 1.98**	**15.51**	**< 0.001**
18 :1n-7	2.95 ± 0.07	2.81 ± 0.08	1.67	0.20
20 :1n-9	**1.03 ± 0.06**	**1.41 ± 0.09**	**8.72**	**0.003**
22 :1n-7	1.42 ± 0.10	1.40 ± 0.19	0.034	0.85
18 :2n-6	**1.70 ± 0.24**	**4.95 ± 0.72**	**11.05**	**< 0.001**
20 :4n-6	**4.16 ± 0.08**	**2.31 ± 0.42**	**16.5**	**< 0.001**
20 :5n-3	**9.82 ± 0.32**	**5.70 ± 1.03**	**10.64**	**< 0.001**
22 :5n-6	**1.35 ± 0.08**	**0.89 ± 0.14**	**8.37**	**< 0.01**
22 :5n-3	5.58 ± 0.15	3.97 ± 0.62	2.76	0.09
22 :6n-3	**20.78 ± 0.66**	**10.08 ± 1.96**	**16.5**	**< 0.001**

Figure S1: Spearman's correlation analyses between the membrane lipid fatty acids and the mitochondrial function of muscle of golden grey mullet fed with (a) High (n=11) and (b) Low omega-3 highly unsaturated fatty acid (n-3 HUFA) diet. FA composition were calculated as percent of FA mass per total FA mass. Only FA that occur above 1 % in at least a treatment group are represented. Mitochondrial function were expressed as the mitochondrial efficiency (ATP/O) and the LEAK respiration. N = 11-12 per treatment group. The coloured circles represent significant correlations between two variables ($p < 0.05$). The numbers inside cells are the associated Spearman's correlation (r_S).