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## Effect of thermal and nutritional conditions on fatty acid metabolism and oxidative stress response in juvenile European sea bass (*Dicentrarchus labrax*)

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1 **Effect of thermal and nutritional conditions on fatty acid metabolism and oxidative stress**  
2 **response in juvenile European sea bass (*Dicentrarchus labrax*)**

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## 19 **Abstract**

20 Coastal nursery areas are subjected to a wide range of natural and anthropogenic  
21 stressors, including global warming, which indirectly influence trophic food webs. A global  
22 rarefaction of n-3 polyunsaturated fatty acids (PUFA) in trophic networks is in progress. The  
23 aim of this study was to assess the effect of a reduction in the dietary availability of n-3 PUFA  
24 on some molecular and biochemical parameters related to lipid metabolism and oxidative stress  
25 response in juvenile European sea bass (*Dicentrarchus labrax*) raised at two temperatures  
26 (15°C and 20°C). Fish were fed for five months with a reference diet (RD; 1.65% n-3 PUFA  
27 on a dry matter basis, DM), used as a proxy of trophic networks where n-3 PUFA is plentiful,  
28 and a lower n-3 PUFA diet (LD; 0.73% n-3 PUFA DM), designed to mimic a decrease in n-3  
29 PUFA resulting from global changes (the n-3 PUFA levels tested remained above the nutritional  
30 minimum required for this species). Results showed that diet did not affect the hepatic  
31 expression of some **mRNA coding for** transcriptional factors involved in regulating the  
32 metabolic pathways related to fatty acid bioconversion. **Although our molecular analysis was**  
33 **limited to transcript expression, these data suggest** the presence of a threshold in the  
34 nutritional supply of PUFA above which the activation of these molecular pathways does not  
35 occur. However, the expression for most of the **transcripts tested was up-regulated at 20°C.**  
36 Despite the high peroxidation index in fish fed RD, very few modifications of the oxidative  
37 stress response were associated with diet. **At 20°C, an increase of** the enzymatic antioxidant  
38 response **was observed**, but there was no correlation with the peroxidation index or  
39 malondialdehyde products.

## 40 **Introduction**

41 Many fish species have life history strategies in which successive ontogenetic stages  
42 occupy different habitats. In those characterized by the production of large amounts of small  
43 pelagic eggs and the lack of parental care, the survival of eggs, larvae, and juveniles strongly  
44 depends on abiotic and biotic environmental factors acting on their habitats (Cushing, 1995;  
45 Sogard, 1997; Juanes, 2007; Houde, 2008). Coastal zones and estuaries are productive nursery  
46 grounds for a large number of marine species because they provide refuge from predators  
47 together with high food availability and enhanced survival, development, and growth of early  
48 stages (Beck et al., 2001; Able et al., 2013). However, coastal and estuarine nursery areas are  
49 subjected to a wide range of anthropogenic and natural stressors. Coastal habitats in particular  
50 face anthropogenic activities, including global warming, which indirectly influence trophic  
51 food webs (Harley et al., 2006; Lehodey et al., 2006; Brander, 2007; Rijnsdorp et al., 2009;  
52 Gattuso et al., 2015; Hixson and Arts, 2016).

53 Lipids represent an important energy supply and play a crucial role as major components  
54 of cell membranes (Sargent et al., 2002). In marine trophic networks, polyunsaturated fatty  
55 acids (PUFA) are mainly supplied by the dominant phytoplankton species, such as diatoms and  
56 dinoflagellates (Hu et al., 2008; Lang et al., 2011). The n-3 PUFA (also known as omega 3),  
57 particularly eicosapentenoic acid (EPA) and docosahexaenoic acid (DHA), are naturally  
58 abundant for primary consumers. However, it has been shown that phytoplankton growth and  
59 biochemical composition, including fatty acids (FA), are affected by temperature (e.g., Arts et  
60 al., 2009). An increase in water temperature results in a decrease in the omega 3 content of  
61 phytoplankton species (Ackman and Tocher, 1968; Thompson et al., 1992; Renaud et al., 2002;  
62 Guschina and Harwood, 2006). At least two recent studies have shown a global decline of n-3  
63 PUFA in trophic networks while n-6 tends to be more abundant (Colombo et al., 2016; Hixson  
64 and Arts, 2016). The high availability of n-3 PUFA in phytoplankton may explain the lack of

65 selection pressure on its biosynthesis pathway in several marine fish species and thus why  
66 piscivorous fish have lost the capacity for de novo synthesis of n-3 PUFA (Tocher et al., 2006).  
67 In most other fish species, it has been shown that marine species have low capacities to convert  
68 18-carbon fatty acids into n-3 PUFA (Owen et al., 1975; Mourente and Tocher, 1994; Sargent  
69 et al., 2002; Geay et al., 2010, 2012) due to apparent deficiencies in one or more steps of the  
70 biosynthesis pathway (Ghioni et al., 1999; Tocher and Ghioni, 1999; Santigosa et al., 2010).  
71 This makes these fishes highly dependent upon dietary sources of n-3 PUFA (Ghioni et al.,  
72 1999; Tocher and Ghioni, 1999). To what extent the n-3 PUFA decline induced by global  
73 climate change may impact species that depend on phytoplankton to fulfill their needs whether  
74 as primary or secondary consumers is not yet known. However, it has also been reported that  
75 most of the genes coding for the main components of this biosynthesis pathway are present **and**  
76 **that their transcription** could be up/down regulated by some nutritional or environmental  
77 factors (Dias et al., 1998; Gomez-Requeni et al, 2003; Figueiredo-Silva et al., 2009). Thus it is  
78 possible to study the regulation of lipid metabolism and the FA synthesis pathway that directly  
79 or indirectly result from climate change.

80 Environmental and nutritional factors may alter the amount of functional protein  
81 involved in lipid metabolism through a range of transcriptional (e.g., activity of transcription  
82 factors), post-transcriptional (e.g., mRNA stability), **translational**, and post-translational (e.g.,  
83 phosphorylation) mechanisms. The transcriptional regulation of genes involved in lipogenesis  
84 by PUFA is well documented. PUFA are known to bind and directly influence the activity of a  
85 variety of transcription factors including peroxisome proliferator activated receptors (**PPAR**),  
86 which in turn have been shown to **regulate the transcription of** many genes involved in lipid  
87 homeostatic processes (Jump, 2002). There appears to be several *ppar* variants expressed in  
88 fish tissues: *ppara* is mainly expressed in the liver, *pparγ* is mainly expressed in adipose tissue,  
89 and *pparβ* has been found in most tissues (Boukouvala et al., 2004). **Sterol regulatory element**

90 **binding protein-1 (*srebp-1*) is another key transcription factor known to be involved in**  
91 **fatty acid synthesis assembly (Horton et al., 2002). It is well documented that expression**  
92 **of the *ppar* and *srebp-1* genes are regulated at the transcriptional level by dietary FA in**  
93 **mammals** (Lindi et al., 2003; Juliano et al., 2004; MacLaren et al., 2006). Similar results have  
94 been reported in fish (Leaver et al., 2005, 2007; Jordal et al., 2007; Dong et al., 2015), including  
95 the European sea bass (Geay et al. 2010, 2012).

96 In mammals, *ppar* and *srebp-1* are the main transcription factors regulating the **mRNA**  
97 expression of elongase and desaturase (Nakamura and Nara, 2002, 2003), **which are key**  
98 **enzymes involved in PUFA biosynthesis. The first step of the bioconversion pathway**  
99 **requires the delta 6-desaturase gene (*fads2*; Sprecher, 2000).** Another important enzyme  
100 system is the fatty acid synthetase complex (FAS), which catalyzes the key lipogenesis pathway  
101 in fishes (Sargent et al., 2002). The main products are saturated 16:0 (palmitic acid) and 18:0  
102 (stearic acid) FA. In the carnivorous fishes blackspot seabream (*Pagellus bogaraveo*;  
103 Figueiredo-Silva et al., 2009), European sea bass (*Dicentrarchus labrax*; Dias et al., 1998), and  
104 gilthead sea bream (*Sparus aurata*; Gomez-Requeni et al., 2003), dietary fat intake has been  
105 shown to inhibit FAS activity.

106 Biological membranes rich in PUFA are highly sensitive to oxidation due to their high  
107 level of unsaturation (Pamplona et al., 2000; Hulbert, 2005); this oxidation can lead to the  
108 production of reactive oxygen species (ROS). ROS are generated as by-products of cellular  
109 metabolism, and mitochondria are a primary site for ROS production (Barja, 2007). Higher  
110 temperatures may promote ROS production, especially in organisms rich in membrane PUFA,  
111 and this could lead to various cellular and tissular dysfunctions (Kawatsu, 1969; Watanabe et  
112 al., 1970; Murai and Andrews, 1974; Sakai et al., 1998). The relative rates of peroxidation of  
113 different acyl chains have been used to convert the fatty acid composition of a particular  
114 membrane into the peroxidation index (PI; see Hulbert et al., 2007, for details). The PI

115 represents the theoretical susceptibility of membrane lipid composition to lipid peroxidation,  
116 and it appears to be homeostatically regulated with respect to dietary PI. In practice,  
117 malondialdehyde (MDA) is one lipid peroxidation product that can be measured (Frankel,  
118 2005). Under most physiological states, ROS production is closely matched by the antioxidant  
119 response. Antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), and  
120 peroxidases, form an important part of the antioxidant response (Lesser, 2006). In fish species,  
121 COX and CS activity have been monitored because they are known to be adjusted to the  
122 maximum aerobic metabolism capacity in fish (Childress and Somero, 1979; Thuesen and  
123 Childress, 1993, 1994).

124 European seabass migrate offshore to spawn, and the developing larvae drift to sheltered  
125 coastal or estuarine nurseries where they spend their first few years of life (Holden and  
126 Williams, 1974; Kelley, 1988; Vinagre et al., 2009). During that period, juvenile sea bass  
127 exhibit rapid growth and are faced with a broad spectrum of environmental constraints and  
128 feeding conditions, making it an attractive model organism to examine the regulatory response  
129 of the PUFA biosynthesis pathway. The aim of this study was to test the effects of two levels  
130 of n-3 PUFA dietary contents on different molecular (**transcript** expression) and biochemical  
131 (MDA, enzymatic activity) variables related to FA metabolism and antioxidant response,  
132 respectively, in European sea bass juveniles raised at two different temperatures. To obtain  
133 realistic responses in terms of a global change scenario, we exposed European sea bass to  
134 temperatures of 15°C (current average temperature to which the Atlantic sea bass population is  
135 exposed) and 20°C (to mimic the temperature rise that is expected over the next 80 years;  
136 Gattuso et al. 2015). We hypothesized that a diet poor in n-3 PUFA would activate lipid  
137 biosynthesis while a diet richer in n-3 PUFA would lead to oxidative stress and would worsen  
138 under higher temperature conditions.

## 139 **Materials and methods**

## 140 Feeding experiment

141 The protocols for fish maintenance, diet composition, and nutritional conditioning are  
142 detailed in Gourtay et al. (2018). They are summarized here to facilitate understanding of the  
143 experimental protocol. Adult European sea bass were captured in winter 2013 by fishermen in  
144 the Gulf of Morbihan (Plomeur, France) and brought to the Aquastream hatchery (Lorient,  
145 France). After three years in captivity, a batch of four females and 10 males were bred in the  
146 facility. At day 2 post hatching (dph-2), sea bass larvae were transferred to the Ifremer rearing  
147 facility in Brest (France), where experiments were conducted. Larvae were divided among three  
148 conical tanks (230 L, 10  $\mu$ m filtered seawater, UV, salinity 35, initial density 10,000 larvae  
149 tank<sup>-1</sup>). Water temperature in the tanks was progressively increased from 14°C to 20°C over  
150 six days. Larvae were fed with *Artemia* from mouth opening (dph-8) to dph-39 and with  
151 microparticulate feed (Marinstart, Le Gouessant, Lamballe, France) until dph-74. After dph-74,  
152 juveniles were fed with larger pellets for ornamental fish (EPA + DHA = 1.5%; Le Gouessant,  
153 Lamballe, France) until the beginning of the experiment at d-93.

154 The two diets tested were identical except for the FA source. The low n-3 PUFA diet  
155 (LD) contained only colza oil as a source of FA (essentially oleic acid [18:1n-9], linoleic acid  
156 [18:2n-6], and linolenic acid [18:3n-3]; EPA+DHA = 0.73% DM), while the reference diet  
157 (RD) contained 50% colza oil and 50% fish oil, the latter being richer in EPA and DHA (20:5n-  
158 3, 22:6n-3; EPA+DHA = 1.65% DM). Diets were isoenergetic and contained the same  
159 percentages of proteins and lipids (Table 1).

160 At d-93, juveniles ( $0.75 \pm 0.02$  g;  $3.57 \pm 0.02$  cm; mean  $\pm$  SD) were divided among 12  
161 indoor 500 L tanks supplied with filtered and aerated natural seawater, six of which were  
162 maintained at 15°C and the other six at 20°C. Each tank contained 300 fish, representing a mean  
163 biomass of  $263.93 \pm 0.28$  g. During the following 150 days, fish were fed one of the two  
164 experimental diets. Feeding took place for 7 h during daytime (08:00 to 15:00) using an



165 automatic distributor (treadmill, 2 cm h<sup>-1</sup>); daily visual control was done to ensure that the food  
166 distributed had been consumed and that fish were fed *ad libitum*. Each diet × temperature  
167 combination was replicated in three tanks.

## 168 Tissue sampling

169 To **account for** the impact of temperature alone on fish growth, sampling dates were  
170 calculated in degree-days (**dd**) relative to the first day of the experiment (**D0**) at **dph-93 and 0**  
171 **dd**. At 720 **dd** after the beginning of the experiment (**dph-129, D36**, and **dph-141, D48**, at 20°C  
172 and 15°C, respectively) and at 1660 **dd** (**dph-176, D83**, and **dph-204, D111**, at 20°C and 15°C,  
173 respectively), 32 fish were randomly sampled from each tank (three tanks per treatment, N =  
174 96 per treatment), anaesthetized (lethal concentration of MS-222, 200 mg L<sup>-1</sup>), and their livers  
175 excised. Because livers were small, the livers of eight juveniles were pooled for a total of four  
176 pools per tank and 12 per experimental condition. Pools were stored at -80°C. Two pools per  
177 tank were used for molecular biology analysis (N = 6 per experimental treatment for a total of  
178 24), one pool was used for enzyme activity, and the fourth was used for malondialdehyde  
179 (MDA) analysis (N = 3 per experimental condition).

## 180 Peroxidation index, enzyme activity, and malondialdehyde assay

181 The peroxidation index (PI) was calculated for total dietary lipids and the polar fraction  
182 of muscle lipids (see Gourtay et al. [2018] for details of fatty acid content) following the  
183 equation provided by Hulbert et al. (2007):

$$184 \text{ PI} = (0.025 \times \% \text{ monoenoics}) + (1 \times \% \text{ dienoics}) + (2 \times \% \text{ trienoics}) + (4 \times \% \text{ tetraenoics}) + (6 \\ 185 \times \% \text{ pentaenoics}) + (8 \times \% \text{ hexaenoics}).$$

186 Liver samples were reduced to powder using a mixer mill (MM 400, Retsch, 30 Hz, 30 s) with  
187 liquid nitrogen. To extract total proteins, 100 mg of liver powder was diluted in 1 mL lysis

188 buffer (150 mM NaCl, 10 mM Tris-HCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X100, 0.5%  
189 Igepal, 1% phosphatase inhibitor cocktail II, 2% NaPPi, and 1 tablet EDTA-free protease  
190 inhibitor cocktail, pH adjusted to 7). Samples were then homogenized on ice using a pro  
191 polytron® (BioBlock Scientific; Illkirch, Bas-Rhin, France). For protein extraction, samples  
192 were left on ice for 40 min and then centrifuged (3-30K Sigma, 6000 g, 1 h, 4°C). The middle  
193 phase containing the protein extract was recovered with a Pasteur pipette and centrifuged (3-  
194 30K Sigma, 10000 g, 45 min, 4°C). The resulting middle phase containing the protein extract  
195 was recovered with a Pasteur pipette, separated into aliquots, and stored at -80°C for the  
196 enzyme activity assay.

197 Total protein concentrations of the liver homogenates were assayed in triplicate  
198 according to the Bio-Rad DC Protein Assay (ref: 5000116, Bio-Rad), which is based on the  
199 Lowry assay (Lowry et al., 1951).

200 Specific CAT activity was measured in triplicate according to Aebi (1984) by observing  
201 the decomposition of H<sub>2</sub>O<sub>2</sub> into oxygen and water. The reaction mixture consisted of 20 mM  
202 phosphate (pH 7) and 10 mM H<sub>2</sub>O<sub>2</sub>. We used a Multiskan GO (Thermo Scientific) plate reader  
203 with GREINER UV-Star 96 (ref: M3812, Sigma). The kinetics of the colouring solutions were  
204 monitored at 22°C by reading absorbance at 240 nm every 10 s for 4 min.

205 The specific enzyme activity of superoxide dismutase (SOD, combined Cu, Zn-SOD,  
206 and Mn-SOD) was determined in triplicate using a commercial kit (ref: 19160, Sigma), with a  
207 NUNC F-bottom 96-well (Thermo Scientific) and Multiskan GO (Thermo Scientific) plate  
208 reader. Final absorbances were read at 450 nm after 20 min incubations at 37°C.

209 The specific enzyme activity of cytochrome c oxidase (COX) was measured in triplicate  
210 using a commercial kit (ref: CYTOCOX1, Sigma) combined with an Evolution 21 UV-Visible  
211 Spectrometer (Thermo Scientific). The kinetics of colouring solutions were monitored at 25°C  
212 by reading absorbance at 550 nm every 0.25 s for 1 min.

213 The specific enzyme activity of citrate synthase (CS) was measured in triplicate  
214 according to Srere (1969) and Bergmeyer et al. (1974). The assay medium contained 100 mM  
215 Tris-HCl (pH 8), 10 mM DTNB, and 2 mM acetyl-CoA. Liver homogenates and assay media  
216 were protected from light and incubated for 5 min in NUNC F-bottom 96-well plates (Thermo  
217 Scientific); 5 mM oxaloacetate solution was added to initiate the reaction. The kinetics of  
218 colouring solutions were monitored at 25°C by reading absorbance at 412 nm every 10 s for 4  
219 min.

220 The lipid peroxide assay was adapted from the thiobarbituric acid reactive substances  
221 (TBARS) protocol (Uchiyama and Mihara, 1978). Liver samples were ground on ice using a  
222 POLYTRON PT-MR 2100 with distilled water containing 1% BHT in methanol, with 50 mg  
223 of liver for 1 mL of grinding solution. A total of 500 µL of 1% TBA and 1.5 mL of 1%  
224 phosphoric acid were added to 500 µL of liver homogenate. This mixture was heated for 30  
225 min at 100°C and cooled for 30 min on ice, at which point 2 mL of butanol were added and the  
226 mixture vortexed. Protein precipitates were removed by centrifugation at 800 g. The absorbance  
227 of the supernatant was read in NUNC F-bottom 96-well plates (Thermo Scientific) with a  
228 Multiskan GO spectrophotometer (Thermo Scientific) at 532 nm. The results were calculated  
229 using an MDA standard curve with butanol as the solvent.

230

## 231 **mRNA** expression analysis

### 232 RNA extraction

233 Liver samples were reduced to powder using a mixer mill (MM 400, Retsch, 30 Hz,  
234 30 s) with liquid nitrogen. Samples (50–100 mg) were placed in 1 mL of Extractall reagent  
235 (Eurobio; Courtaboeuf, Essone, France) and homogenized using a pro polytron® (BioBlock  
236 Scientific; Illkirch, Bas-Rhin, France) tissue disruptor for 30 s. Potential contaminating DNA

237 was removed using an RTS DNase<sup>TM</sup> Kit (MoBio Laboratories Inc., Carlsbad, CA, USA)  
238 according to the manufacturer's recommendations. Total RNA was extracted following the  
239 manufacturer's instructions. The quantity and purity of RNA were assessed using an ND-1000  
240 NanoDrop® spectrophotometer (Thermo Scientific Inc.; Waltham, MA, USA). RNA integrity  
241 was determined by electrophoresis using an Agilent Bioanalyzer 2100 (Agilent Technologies  
242 Inc.; Santa Clara, CA, USA). All samples had an RNA integrity number (RIN) higher than  
243 seven and could thus be used for real-time quantitative polymerase chain reaction (qPCR)  
244 analysis. RNA samples were stored at -80°C until use.

#### 245 cDNA synthesis

246 After extraction, total RNA was reverse transcribed into cDNA using an iScript<sup>TM</sup>  
247 cDNA Synthesis kit (Bio-Rad Laboratories Inc., Hercules, CA, USA) following the  
248 manufacturer's instructions. Briefly, 500 ng total RNA was reverse transcribed into cDNA in a  
249 volume of 20 µL that was composed of 15 µL of sample; 4 µL 5 × iScript<sup>TM</sup> Reaction Mix  
250 containing oligo (dT), random primers, and RNaseA inhibitor; and 1 µL of iScript<sup>TM</sup> reverse  
251 transcriptase. Reverse transcription (RT) negative controls were also performed on each sample  
252 using the same reaction mix except for the reverse transcriptase (substituted by water). The  
253 cDNA synthesis reaction was incubated for 5 min at 25°C followed by 30 min at 42°C and  
254 terminated by incubation for 5 min at 85°C to inactivate the enzyme. RT was performed using  
255 a T 100 Thermal-cycler (Bio-Rad Laboratories Inc.). cDNA samples were stored at -20°C until  
256 use.

#### 257 Quantitative real-time RT-PCR analysis

258 The analysis of **mRNA** expression in liver tissue of European sea bass was carried out  
259 by qPCR using the primers listed in Table 2. Primers were designed using Primer3plus  
260 (<http://primer3plus.com/>) based on sequences available from the Genbank database

261 (<https://www.ncbi.nlm.nih.gov/genbank/>). **mRNA** expression was quantified using a C1000  
262 touch thermal cycler (CFX96 system, Bio-Rad Laboratories Inc.). For each primer pair, qPCR  
263 efficiencies were estimated by standard curves resulting from serial dilutions (from 1/10 to  
264 1/270) of a pool of cDNA. In the present study, qPCR efficiencies for each primer pair ranged  
265 from 95 to 100%, with  $R^2 > 0.99$ . Samples were then analyzed in triplicate. The final well  
266 volume was 15  $\mu$ L, containing 5  $\mu$ L cDNA (1/30 dilution) and 10  $\mu$ L of reaction mix (0.5  $\mu$ L  
267 of each primer [10  $\mu$ M]), 1.5  $\mu$ L RNase/DNase-free water, 7.5  $\mu$ L iQ<sup>TM</sup> SYBR<sup>®</sup> Green  
268 Supermix (Bio-Rad Laboratories Inc.) containing antibody-mediated hot-started iTaq DNA  
269 polymerase, dNTPs, MgCl<sub>2</sub>, SYBR<sup>®</sup> Green I dye, fluorescein, stabilizers, and enhancers. In  
270 each plate, negative controls (RT negative controls) were systematically included to ensure the  
271 absence of residual genomic DNA contamination. Non-template controls (H<sub>2</sub>O) were also  
272 analyzed. Each plate contained an inter-run calibrator in triplicate (dilutions of a pool of all  
273 samples). The qPCR thermal cycling included an initial activation step at 95°C for 2 min  
274 followed by 39 cycles of 5 s at 95°C and 20 s at 60°C. A melting curve was performed at the  
275 end of the amplification phase to confirm the amplification of a single product in each reaction.  
276 The corresponding C<sub>q</sub> (quantification cycle) value was determined automatically for each  
277 sample using the Gene Expression Module of the CFX Manager software (Bio-Rad  
278 Laboratories Inc.). C<sub>q</sub> is the number of cycles required to yield a detectable fluorescence signal.  
279 The CFX Manager Software was used to normalize the relative quantity of messenger with the  
280  $\Delta\Delta$ Ct method. The **expression level of** elongation factor 1 (*ef1a*) and ribosomal (**28S**) **mRNA**  
281 were tested to correct for loading differences or other sampling variations present in each  
282 sample. The *ef1a* **mRNA** was used **for normalization** in the liver tissue of juvenile fish since  
283 it did not show any significant variation of expression among experimental conditions ( $P >$   
284 0.05).

285 Statistical and data analysis

286 Relative **mRNA** expressions and enzyme activity data were log<sub>10</sub> transformed to obtain  
287 normal distributions. The effects of time, temperature, and diet were tested for peroxidation  
288 index, relative **mRNA** expression (*ppara*, *pparβ*, *pparγ*, *fas*, *srebp-1*, *fads2*), enzyme activity  
289 (CAT, SOD, CS, COX), and MDA content using three-way ANOVAs. Normality and  
290 homoscedasticity of data were tested using Shapiro-Wilk and Levene tests, respectively. When  
291 appropriate, Tukey mean comparison tests were done because homoscedasticity was respected.  
292 Differences were considered significant at  $\alpha = 0.05$ . Statistical analyses were conducted in R  
293 (ver. 3.3.3; R Development Core Team).

## 294 **Results**

### 295 Liver mass and protein content

296 Results showing the effect of diet, temperature, and time on liver mass and hepatic  
297 protein content are shown in Table 3A. After 720 dd of feeding, liver mass was significantly  
298 higher (+48%) in juveniles reared at 20°C than at 15°C, and diet had no significant effect (Table  
299 3A). At 1660 dd, the increase in liver mass was higher for juveniles reared at 20°C (+67%) than  
300 for those reared at 15°C (+59%), **while liver mass was still higher (+58%) in juveniles reared**  
301 **at 20°C than at 15°C**. Again, no diet effect was detected. Liver protein concentration decreased  
302 significantly over time ( $0.12 \pm 0.01$  mg protein mg liver<sup>-1</sup> at 720 dd vs.  $0.10 \pm 0.00$  mg protein  
303 mg liver<sup>-1</sup> at 1660 dd), and no significant effect of diet or of temperature was observed.

### 304 Peroxidation index and MDA

305 The PI of the RD diet was 34% higher than that of the LD diet ( $15.86 \pm 1.78$  vs.  $10.52$   
306  $\pm 0.50$ ). Accordingly, the PI in the polar lipids of juveniles fed RD was significantly higher  
307 (+18%) compared to fish fed LD ( $45.36 \pm 6.96$  vs.  $38.50 \pm 5.45$ ; Table 3A). The temperature  $\times$   
308 diet interaction was significant although no difference was detected with the post-hoc test

309 (Table 3A). Surprisingly, MDA content was not affected by diet or temperature; however, MDA  
310 content increased with time ( $2.06 \pm 0.48$  vs.  $2.77 \pm 0.45$  nmol mg protein<sup>-1</sup>; Table 3A).

### 311 Oxidative metabolism and antioxidant response

312 Results showing the effect of diet, temperature, and time on oxidative stress indicators  
313 are also shown in Table 3A. CS activity remained stable regardless of the experimental  
314 treatment, while COX activity varied depending on diet × temperature and degree-days ×  
315 temperature interactions. There was no temperature effect on COX activity at 720 dd, but fish  
316 raised at 20°C exhibited a higher COX activity at 1660 dd than fish reared at 15°C. COX activity  
317 was significantly lower in fish fed RD at 720 dd, although this diet effect was no longer present  
318 at 1660 dd. The COX/CS ratio was higher (+15%) in juveniles fed LD than in those fed RD  
319 ( $6.01 \pm 1.21$  vs.  $5.09 \pm 1.80$ ). In addition, the COX/CS ratio remained stable over time for fish  
320 raised at 15°C but increased in fish raised at 20°C. CAT activity increased slightly but  
321 significantly over time at both 15°C and 20°C ( $70 \pm 7.93$  vs.  $105 \pm 27.01$ ). At 20°C, however,  
322 juveniles fed the RD diet displayed CAT activity that was more than twice the activity measured  
323 in LD-fed fish. At 1660 dd, the diet effect was no longer present. SOD activity was significantly  
324 lower (−8%) in juveniles fed RD compared to those fed LD ( $3.94 \pm 0.36$  U mg protein<sup>-1</sup> vs.  $4.25$   
325  $\pm 0.43$  U mg protein<sup>-1</sup>). SOD activity significantly decreased (−8%) with time ( $4.25 \pm 0.31$  U  
326 mg protein<sup>-1</sup> at 1660 dd vs.  $3.95 \pm 0.47$  U mg protein<sup>-1</sup> at 720 dd). There was also a significant  
327 increase in SOD activity (+10%) at the higher temperature ( $3.91 \pm 0.43$  U mg protein<sup>-1</sup> at 15 °C  
328 vs.  $4.29 \pm 0.33$  U mg protein<sup>-1</sup> at 20°C).

### 329 Relative **mRNA** expressions

330 The relative **mRNA** expression of *fads2* increased with time. At 720 dd, the relative  
331 **level of *fads2* mRNA** was highest at 20°C in juveniles fed LD and lowest at 15°C in juveniles  
332 fed RD (Table 3, Fig. 1A). After 1660 dd of feeding, there was no significant increase of the

333 *fads2* mRNA level in juveniles fed LD at 15°C or RD at 20°C; however, the level was higher  
334 in juveniles fed RD at 15°C and LD at 20°C (Table 3B, Fig. 1A). The expressions of other  
335 mRNA showed no differences according to diet: expressions of *fas*, *ppar $\gamma$* , and *srebp-1* mRNA  
336 were significantly lower (158%, 29%, and 24%, respectively) for fish raised at 15°C than at  
337 20°C (Table 3B, Fig. 1B). These expressions were respectively 630%, 91%, and 118% lower  
338 at 720 dd than at 1660 dd (Table 3B, Fig. 1B). The highest relative mRNA expression for *ppara*  
339 was observed at 1600 dd for fish raised at 20°C (Table 3B, Fig. 1B); no treatment effect was  
340 observed for the expression of *ppar $\beta$*  mRNA (Table 3B).

## 341 Discussion

342 In a previous study, Gourtay et al. (2018) showed that the depletion of n-3 PUFA and a  
343 lower temperature (15°C) contributed to a decreased growth rate in sea bass: the depleted n-3  
344 PUFA diet resulted in lower contents of essential fatty acids (EFA, ARA, DHA, EPA) in muscle  
345 even though a higher retention of those FA was noted in fish fed this diet (Gourtay et al., 2018).  
346 The aim of the present work was to examine to what extent these diets and temperatures (15°C  
347 and 20°C) can affect the expression of mRNA coding for enzymes/transcription factors  
348 involved in lipid metabolism and biochemical variables related to antioxidant response in liver.  
349 We hypothesized that a diet poor in n-3 PUFA would activate lipid biosynthesis while a diet  
350 richer in n-3 PUFA would lead to oxidative stress.

351 **We are aware that our quantitative analyses of transcripts coding for actors**  
352 **involved in lipid metabolism do not necessarily reflect the activities of the proteins and**  
353 **thus cannot be used to infer metabolic activities. However, the relative levels of transcripts**  
354 **reflect the transcriptional regulation of metabolic constituents, which may reflect the**  
355 **sensitivity of the associated metabolic pathway to environmental or dietary factors.**

356



## 357 **mRNA expression, lipid metabolism, and antioxidant enzymes**

358 **Our results based on *fads2* mRNA level quantification only partly support the**  
359 **hypothesis of an activation of the pathway involved in lipid biosynthesis.** Indeed, the  
360 relative expression of *fads2* mRNA was affected by diet, but even though the highest mRNA  
361 level was observed in LD-fed fish at 20°C on the first sampling date, the situation was  
362 completely reversed at the end of the study, making the results difficult to interpret. We  
363 observed no effect of diet on *fas*, *ppara*, *pparβ*, *pparγ*, or *srebp-1* mRNA expression. Regulation  
364 at translational and post-translational levels certainly cannot be excluded, but data on mRNA  
365 expression suggest no diet effects on these indicators at the dietary PUFA levels tested here.  
366 This indicates that LD was probably not low enough in PUFA to exert negative effects **at the**  
367 **transcriptional level on these constituents**, even though it did result in a lower growth rate in  
368 both length and mass (Gourtay et al., 2018). Comparable conclusions were drawn by Yilmaz et  
369 al. (2016) for juvenile sea bass and by Tocher et al. (2006) for cod. Our results contrast with  
370 those obtained for sea bass by Vagner et al. (2007a, b, 2009), who showed that the *fads2* mRNA  
371 level was higher in larvae fed a low PUFA diet (0.5% or 0.7% EPA+DHA, % DM) compared  
372 to those fed a high PUFA diet (1.7% or 3.7% EPA+ DHA, % DM). González-Rovira et al.  
373 (2009) also showed a significantly higher *fads2* mRNA level in the liver of sea bass fed linseed  
374 and rapeseed oils compared to those fed fish oil, while it remained stable in individuals fed a  
375 diet based on olive oil. Because all these sea bass studies were based on different dietary  
376 formulations and different developmental stages, it is difficult to determine why our results  
377 would differ from theirs. As mentioned above, previous studies reporting modulation of the  
378 elongation and desaturation pathways used diets that were highly deficient in EPA + DHA  
379 (Vagner et al. 2007a, b, 2009). We could have used a diet that would have been more deficient.  
380 However, LD was already sufficiently low in n-3 PUFA to significantly slow the growth rate,  
381 and an effect on *fads2* mRNA level in the liver was expected. Obviously, the level of depletion

382 we used does not seem to dramatically affect regulation of the lipid metabolism pathway  
383 because the requirements are still met in this species (Skalli et al., 2006), even though this was  
384 not tested at all the possible steps from mRNA to the number of proteins.

385         There is growing evidence that inclusion of high levels of PUFA in fish diet, because of  
386 their high degree of unsaturation, can induce oxidative lipid damage (Álvarez et al., 1998; Luo  
387 et al., 2012). Thus, we were expecting lower response indicators of oxidative stress in fish fed  
388 **LD. The CS enzyme catalyzes the first step of the tricarboxylic acid cycle, while COX has**  
389 **a specific role as terminal electron acceptor in the electron transport system. They are**  
390 **both key enzymes involved in** aerobic metabolism and can be used as indicators of aerobic  
391 metabolic capacity (Marie et al. 2006). **The tricarboxylic acid cycle in liver also supports**  
392 **biosynthetic processes such as lipid biosynthesis or gluconeogenesis from malate (Owen**  
393 **et al., 2002; Windisch et al., 2011). Accordingly, the COX to CS ratio can be used to reflect**  
394 **preferred metabolic pathways and relative metabolic adjustments in response to warming**  
395 **in a tissue. Furthermore, changes in COX activity may be related to alterations in**  
396 **mitochondrial membrane structure (Wodtke, 1981; O'Brien and Mueller, 2010) and those**  
397 **in CS activity to changes in mitochondrial matrix volume (e.g., Hardewig et al., 1999;**  
398 **Guderley and St-Pierre, 2002; Guderley, 2004). In our study, COX activity was the lowest**  
399 **in fish fed RD at 720 dd; low COX activity can suggest decreased oxidative capacities,**  
400 **which may support lower metabolic costs with the RD diet.** The COX/CS ratio was higher  
401 in LD-fed fish, suggesting higher aerobic metabolism and possibly indicating a functional  
402 adaptation of mitochondria to adjust energy demand and metabolic fuel availability (i.e., lipids  
403 **biosynthesis**). PI is a theoretical index that is calculated with *in vitro* oxidation rate values  
404 (Holman, 1954; Cosgrove et al., 1987; Hulbert et al., 2007), and it does not account for all the  
405 *in vivo* processes that occur to deal with ROS production. CAT activity (degradation of  
406 hydrogen peroxide into water and oxygen) was twice as high in RD-fed juveniles compared to

407 those fed LD, and they showed the highest PI—which is consistent with our expectations—but  
408 only at 20°C. On the other hand, MDA content remained stable regardless of diet, but we are  
409 aware that the method we used was not the most reliable one, even though it is commonly used  
410 in fish studies (Mourente et al., 2002; Simonato et al., 2015; Sobjak et al., 2017). However, the  
411 activity of SOD (catalyzing the dismutation of superoxide radicals into oxygen or hydrogen  
412 peroxide) was higher in juveniles fed LD than in those fed RD, suggesting the presence of more  
413 substrate to be catalyzed.

#### 414 **Temperature and diet effects**

415 Higher temperatures in combination with poor n-3 PUFA could affect the lipid  
416 metabolism pathway. The relative mRNA expressions of *fas*, *ppary*, and *srebp-1* increased over  
417 time and with acclimation temperature, but the temperature effect was independent of the diet  
418 effect, indicating that **a positive transcriptional regulation of lipid metabolism** may occur at  
419 high temperatures. The *fads2* mRNA expression tended to increase over time, but not  
420 significantly due to the high inter-individual variability within experimental treatments at 1660  
421 dd. Such a variability in relative mRNA expression has been observed in this species (Geay et  
422 al., 2010), and more investigation is required to determine the reasons. Gourtay et al. (2018)  
423 showed that higher growth was observed in juveniles fed the same experimental diets at 20°C  
424 compared to 15°C, accompanied with higher polar DHA and ARA contents and neutral DHA  
425 content (only at 1660 dd for this parameter). **Again, it must be remembered that the quantity**  
426 **of transcripts measured by qPCR does not necessarily reflect the amount of proteins,**  
427 **especially since temperature may affect the transcription–translation relationship.**  
428 **Nevertheless, our results** support the hypothesis that acclimation temperature enhanced the  
429 expression of mRNA associated with regulation of the lipid metabolism.

430 In several organisms including fish, it has been shown that COX and CS activities reflect  
431 maximum aerobic metabolic capacity (Childress and Somero, 1979; Thuesen and Childress,  
432 1993, 1994). Elevated temperature stimulates metabolic processes in ectotherms, increasing  
433 ATP demand and, therefore, electron transfers along the mitochondrial respiratory chain,  
434 possibly explaining the higher COX activity and COX/CS ratio recorded at 20°C. **These results**  
435 **corroborate those of Seebacher et al. (2005) in *Pagothenia borchgrevinki*, which responds**  
436 **to warming by increasing muscle COX activity. The authors suggest that high COX**  
437 **activity comes along with increased oxidative capacities, and these may support elevated**  
438 **metabolic costs at warmer temperatures.** Such circumstances are also likely to enhance ROS  
439 production as a side product, **which can imply higher likelihood of oxidative stress, but only**  
440 **if the rates of ROS production exceed the system's capacity to eliminate those ROS that**  
441 result in oxidative stress. Increased environmental temperature has been associated with  
442 oxidative stress in fish (Parihar and Dubey, 1995; Heise et al., 2006a, b; Lushchak and  
443 Bagnyukova, 2006; Bagnyukova et al., 2007). Vinagre et al. (2012) studied the impact of  
444 temperature on oxidative stress in sea bass juveniles and found that lipid peroxidation and  
445 catalase activity were very sensitive to environmental temperature. Accordingly, SOD activity  
446 was higher at 20°C than at 15°C, and the highest CAT activity was observed at 720 dd in fish  
447 fed LD at 20°C.

448 Liver mass increased with time and temperature, but proteins remained relatively stable.  
449 This means that total enzymatic activity (per mg of tissue) was related to liver mass only. At  
450 20°C, total enzymatic activity should be higher and thus enable fish to cope with oxidative  
451 stress. The absence of change with temperature in the MDA content, which is one of the  
452 products of lipid peroxidation (Frankel, 2005), again did not match the original hypothesis.  
453 **Some of the variables that we measured showed differences during the experiment**  
454 **without interaction with either diet or temperature. Thus, liver protein concentration and**

455 liver SOD activity decreased over time in juveniles, while the MDA content and relative  
456 mRNA expression of *fads2* increased with time. Whether these modifications related to  
457 development or with acclimation to physical experimental conditions is hard to say, but  
458 obviously they were independent of diet or temperature conditions.

## 459 **Conclusion**

460 Our objective was to use long-term experimental conditions with treatment levels that  
461 were realistic in terms of a global change scenario to test the hypothesis that a diet poor in n-3  
462 PUFA would activate lipid biosynthesis while a diet richer in n-3 PUFA would lead to oxidative  
463 stress and would worsen under higher temperature conditions. We were unable to confirm this  
464 working hypothesis. We conclude that both levels of n-3 PUFA tested in this study were high  
465 enough so as not to impair lipid metabolism in sea bass juveniles. The combination of a decrease  
466 in dietary n-3 PUFA along with an increase in temperature conditions will modify the oxidative  
467 stress response, but at a level that juveniles can cope with.

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## 475 **Compliance with ethical standards**

476 Conflict of interest

477           The authors declare that they have no conflicts of interest.

478   Ethical approval

479           All applicable international, national, and/or institutional guidelines for the care and use  
480 of animals were followed. Experiments were performed under French national regulations and  
481 approved by the Comité d'Éthique Finistérien en Expérimentation Animale (CEFEA,  
482 registration code C2EA-74) (Authorization APAFIS 3056# 20151207173873100).

483

484 **List of symbols and abbreviations**

ARA:	arachidonic acid
CAT:	catalase enzyme
COX:	cytochrome c oxidase enzyme
CS:	citrate synthase enzyme
d-:	days post hatch
dd:	degree-days
DHA:	docosahexaenoic acid
DM:	dry matter
ef1a:	elongation factor 1
EFA:	essential fatty acid
EPA:	eicosapentaenoic acid
FA:	fatty acid
fads2:	delta 6 desaturase gene
fas:	fatty acid synthase gene
LD:	low n-3 polyunsaturated diet
MDA:	malondialdehyde
MUFA:	monounsaturated fatty acid
n-3:	omega 3
n-6:	omega 6
PI:	peroxidation index
ppar:	peroxisome proliferator activated receptors
PUFA:	polyunsaturated fatty acid
qPCR:	quantitative polymerase chain reaction
RD:	reference diet
RIN:	RNA integrity number
ROS:	reactive oxygen species
RT:	reverse transcription
SOD:	superoxide dismutase enzyme
TBARS:	thiobarbituric acid reactive substances

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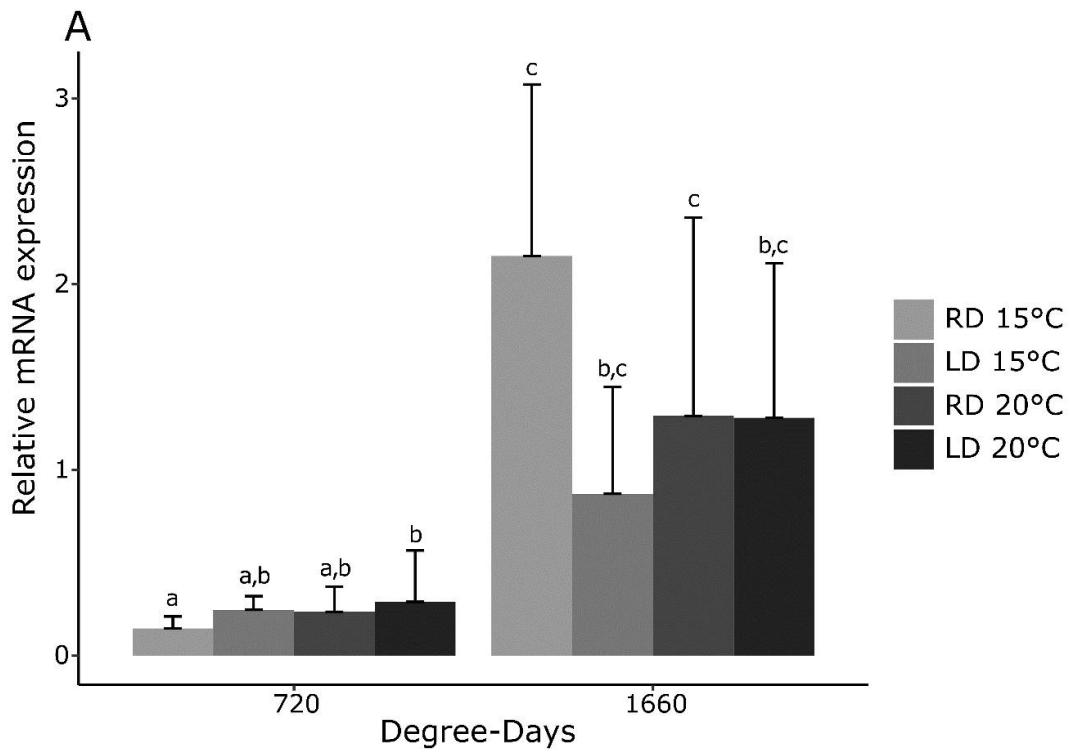
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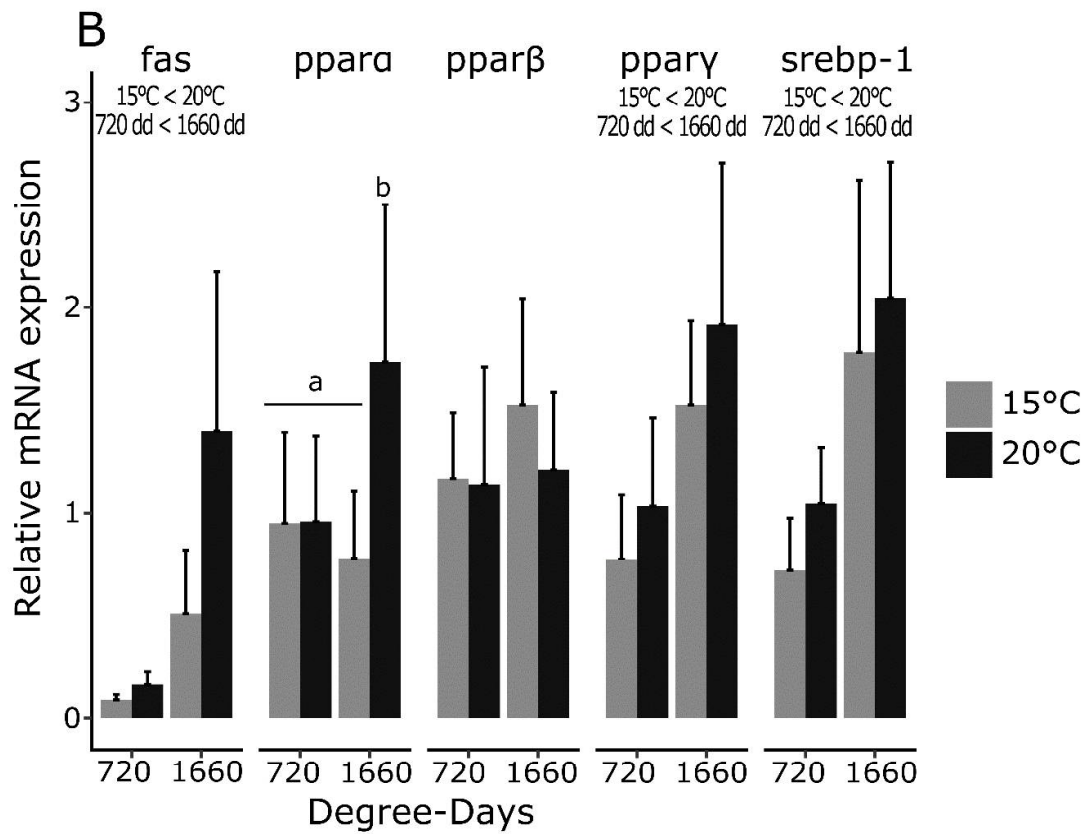
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746 **Figures**

747 **Figure 1:** Results of three-way ANOVAs on lipid metabolism. A) Interaction between diet,  
748 time (in degree-days [dd]), and temperature **on *fads2* mRNA expression level**. B) Effect of  
749 time (dd) and temperature on relative **mRNA** expression for *fas*, *ppara*, *pparβ*, *pparγ*, and  
750 *srebp-1*. When factor interactions were significant, groups were compared with Tukey post-hoc  
751 tests ( $\alpha = 0.05$ ); significantly different groups were assigned different letters. Bars represent  
752 means and error bars are standard deviations; N = 6.



753



754

755 **Tables**

756 Table 1: Composition of experimental diets. For dry matter, proteins, total lipids, triglycerides,  
 757 and phospholipids, data are presented as % of dry mass. Data for specific fatty acid (FA)  
 758 categories are presented as % of total lipids. LD: low n-3 polyunsaturated fatty acid (PUFA)  
 759 diet, RD: reference n-3 PUFA diet; SFA: saturated FA; MUFA: monounsaturated FA; ARA:  
 760 arachidonic acid; EPA: eicosapentaenoic acid; DHA: docosahexaenoic acid

	Low n-3 PUFA diet	Reference n-3 PUFA diet
	LD	RD
	Proximal composition (%DM)	
Dry matter	94.84	95.12
Ash	5.16	4.88
Proteins	50.48	50.23
Nitrogen Free Extract (NFE) <sup>a</sup>	22.38	23.26
Total lipids	21.98	21.63
Triglycerides	16.99	17.05
Phospholipids	4.70	4.71
Energy (kJ) <sup>b</sup>	2045.41	2042.73
	% of total lipids in diet	
SFA	2.18	2.97
Monosaturated FA	7.32	6.48
n-3	1.93	2.68
n-6	2.65	2.28
n-9	6.56	5.46
EPA+DHA	0.73	1.65
18:1n-9	5.69	4.65
18:2n-6	2.50	2.16
18:3n-3	0.97	0.77
18:3n-6	0.00	0.01
18:4n-3	0.08	0.14
20:4n-6	0.03	0.07
20:5n-3	0.28	0.94
22:5n-3	0.03	0.07

22:6n-3

0.45

0.71

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761 <sup>a</sup> Calculated as  $100 - (\% \text{proteins} + \% \text{total lipids} + \% \text{ash})$

762 <sup>b</sup> Calculated as  $\% \text{proteins} \times 16.7 \text{ kgJ g}^{-1} + \% \text{total lipids} \times 37.7 \text{ kgJ g}^{-1} + \text{NFE} \times 16.7 \text{ kgJ g}^{-1}$

763 Each diet (LD and RD) was manufactured in a single batch of 50 kg to supply the entire  
764 experiment. Since diets were only produced once, there was no variation during the experiment.

765

766 Table 2: Specific primers used for quantitative PCR with Genbank accession numbers

Gene name (abbreviation)	Accession number	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')
28S ribosomal RNA ( <i>28S</i> )	CBXY010007006	TTTCCCATGAGAGAGCAGGT	TCAGATGCGCTTCTTAGGATGT
Elongation factor 1 a ( <i>ef1a</i> ) *	AJ866727.1	GCTTCGAGGAAATCACCAAG	CAACCTTCCATCCCTTGAAC
Fatty acid synthase ( <i>fas</i> )	MF566098.1	CCTCTGAACCTGGTCTGGTG	ATTGGAGAGAGCCTCCACGA
Delta-6-desaturase ( <i>fads2</i> )	EU439924.1	AGCATCACGCTAAACCCAAC	CAAGCCAGATCCACCCAGTC
Peroxisome proliferator activated receptors alpha ( <i>ppara</i> )	AJ880087.1	ACCTCAGCGATCAGGTGACT	AACTTCGGCTCCATCATGTC
Peroxisome proliferator activated receptors beta ( <i>pparβ</i> )	AJ880088.1	GCTCGGATCTGAAGACCTTG	TGGCTCCATACCAAACCACT
Peroxisome proliferator activated receptors gamma ( <i>pparγ</i> )	AY590303	CAGATCTGAGGGCTCTGTCC	CCTGGGTGGGTATCTGCTTA
Sterol regulatory element binding protein 1 ( <i>srebp-1</i> )	FN677951	CTGGAGCCAAAACAGAGGAG	GACAGGAAGGAGGGAGGAAG

767 \*Used as housekeeping gene

768 Table 3: A) Effect of diet, temperature, and time on liver oxidative stress indicators (N=3) and B) lipid metabolism (N=6). When factor interactions  
769 were significant, groups were compared with Tukey post-hoc tests ( $\alpha = 0.05$ ); significantly different groups were assigned different letters. RD:  
770 reference diet, LD: low n-3 polyunsaturated fatty acid diet, dd: degree-days, Temp.: temperature.

A	720 Degree-days				1660 Degree-days				Three-way ANOVA			
	15 °C		20°C		15 °C		20°C		Time	Temperature	Diet	Interaction
	RD	LD	RD	LD	RD	LD	RD	LD				
Liver mass (g)	0.3 ± 0.02 <sup>a</sup>	0.31 ± 0.01 <sup>a</sup>	0.62 ± 0.07 <sup>bc</sup>	0.55 ± 0.14 <sup>b</sup>	0.86 ± 0.07 <sup>c</sup>	0.61 ± 0.06 <sup>bc</sup>	1.77 ± 0.4 <sup>d</sup>	1.74 ± 0.19 <sup>d</sup>	n.s.	n.s.	n.s.	Time × Temp. × Diet P < 0.05 F(1,16) = 4.89
Protein (mg prot mg liver <sup>-1</sup> )	0.13 ± 0.01	0.13 ± 0.01	0.12 ± 0	0.11 ± 0.02	0.11 ± 0	0.1 ± 0.01	0.1 ± 0.01	0.1 ± 0.01	P < 0.001 F(1,16) = 24.71 1600 dd < 720 dd	n.s.	n.s.	n.s.
CAT (mU mg prot <sup>-1</sup> )	60.05 ± 2.9 <sup>a</sup>	69.62 ± 4.59 <sup>ab</sup>	78.28 ± 1.94 <sup>bc</sup>	143.37 ± 22.97 <sup>e</sup>	78.12 ± 5.82 <sup>bc</sup>	73.62 ± 4.04 <sup>ab</sup>	103.75 ± 7.3 <sup>d</sup>	97.68 ± 8.5 <sup>cd</sup>	n.s.	n.s.	n.s.	Time × Temp. × Diet P < 0.001 F(1,16) = 11.67
SOD (U mg prot <sup>-1</sup> )	3.98 ± 0.36	4.35 ± 0.17	4.09 ± 0.08	4.56 ± 0.23	3.47 ± 0.22	3.84 ± 0.45	4.22 ± 0.18	4.27 ± 0.57	P < 0.05 F(1,16) = 5.83 1600 dd < 720 dd	P < 0.01 F(1,16) = 8.86 15°C < 20°C	P < 0.05 F(1,16) = 5.66 RD < LD	n.s.
COX (mU mg prot <sup>-1</sup> )	46.5 ± 13.16	65.3 ± 3.97	39.12 ± 8.78	71.04 ± 6.38	67.9 ± 9.52	61.84 ± 11.06	98.39 ± 2.04	88.16 ± 6.61	n.s.	n.s.	n.s.	Time × Temp. P < 0.01 F(1,16) = 16.63 15°C 20°C 720 dd ≤ 15°C 1660 dd ≤ 20°C 1660 dd
CS (mU mg prot <sup>-1</sup> )	9.75 ± 1.06	10.22 ± 0.64	12.84 ± 1.29	13.53 ± 5.24	13.6 ± 0.8	11.99 ± 1.16	13.12 ± 0.43	12.38 ± 1.41	n.s.	n.s.	n.s.	Time × Diet P < 0.001 F(1,16) = 8.26 RD 720 dd < other groups
COX/CS	4.72 ± 0.98	6.4 ± 0.48	3.11 ± 1.02	5.61 ± 1.4	5.03 ± 0.96	5.14 ± 0.64	7.51 ± 0.39	7.22 ± 1.3	n.s.	n.s.	P < 0.05 F(1,16) = 7.56 RD < LD	Time × Temp. P < 0.001 F(1,16) = 16.63 20°C 720 dd ≤ 15°C 720 dd 1660 dd ≤ 20°C 1660 dd
MDA	0.27 ± 0.04	0.3 ± 0.08	0.23 ± 0.08	0.21 ± 0.02	0.28 ± 0.06	0.28 ± 0.03	0.32 ± 0.05	0.25 ± 0.03	n.s.	n.s.	n.s.	n.s.
PI	41.88 ± 3.07	37.90 ± 1.83	46.33 ± 3.31	40.73 ± 1.05	54.07 ± 2.07	38.24 ± 10.44	39.15 ± 7.12	37.13 ± 6.25	n.s.	n.s.	P < 0.01 F(1,16) = 9.90 LD < RD	Time × Temp. P < 0.05 F(1,16) = 7.16 Tukey test not significant

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B	720 Degree-days				1660 Degree-days				Three-way ANOVA*		
	15°C		20°C		15°C		20°C		Temperature	Time	Interactions
	RD	LD	RD	LD	RD	LD	RD	LD			
<i>fads2</i>	0.15 ± 0.07 <sup>a</sup>	0.25 ± 0.07 <sup>ab</sup>	0.24 ± 0.14 <sup>ab</sup>	0.29 ± 0.28 <sup>b</sup>	2.15 ± 0.93 <sup>c</sup>	0.87 ± 0.58 <sup>bc</sup>	1.29 ± 1.07 <sup>bc</sup>	1.28 ± 0.83 <sup>c</sup>	n.s.	n.s.	Time × Temp. × Diet P < 0.05 F(1,38) = 4.47
<i>fas</i>	0.1 ± 0.02	0.09 ± 0.03	0.15 ± 0.02	0.18 ± 0.08	0.7 ± 0.31	0.35 ± 0.23	1.36 ± 0.86	1.43 ± 0.79	P < 0.001 F(1,38) = 30.37 15°C < 20°C	P < 0.001 F(1,38) = 153.77 720 dd < 1660 dd	n.s.
<i>ppara</i>	0.9 ± 0.42	1 ± 0.5	0.93 ± 0.28	0.99 ± 0.55	0.74 ± 0.22	0.82 ± 0.42	1.89 ± 1.05	1.6 ± 0.5	n.s.	n.s.	Time × Temp. P < 0.01 F(1,38) = 9.91 Other groups < 20°C 1660 dd
<i>pparβ</i>	1.09 ± 0.31	1.25 ± 0.33	1.22 ± 0.55	1.06 ± 0.63	1.82 ± 0.66	1.29 ± 0.2	1.28 ± 0.47	1.15 ± 0.32	n.s.	n.s.	n.s.
<i>pparγ</i>	0.83 ± 0.37	0.72 ± 0.27	1.22 ± 0.46	0.85 ± 0.33	1.68 ± 0.33	1.4 ± 0.45	2.13 ± 0.95	1.74 ± 0.66	P < 0.05 F(1,38) = 4.68 15°C < 20°C	P < 0.001 F(1,38) = 38.46 720 dd < 1660 dd	n.s.
<i>Srebp-1</i>	0.77 ± 0.32	0.67 ± 0.18	1.12 ± 0.28	0.98 ± 0.27	2.13 ± 0.89	1.49 ± 0.74	2.12 ± 0.66	1.99 ± 0.71	P < 0.05 F(1,38) = 6.95 15°C < 20°C	P < 0.001 F(1,38) = 43.41 720 dd < 1660 dd	n.s.

775 \*No significant effect of diet was observed; diet column has been removed from the table.



Figure1A

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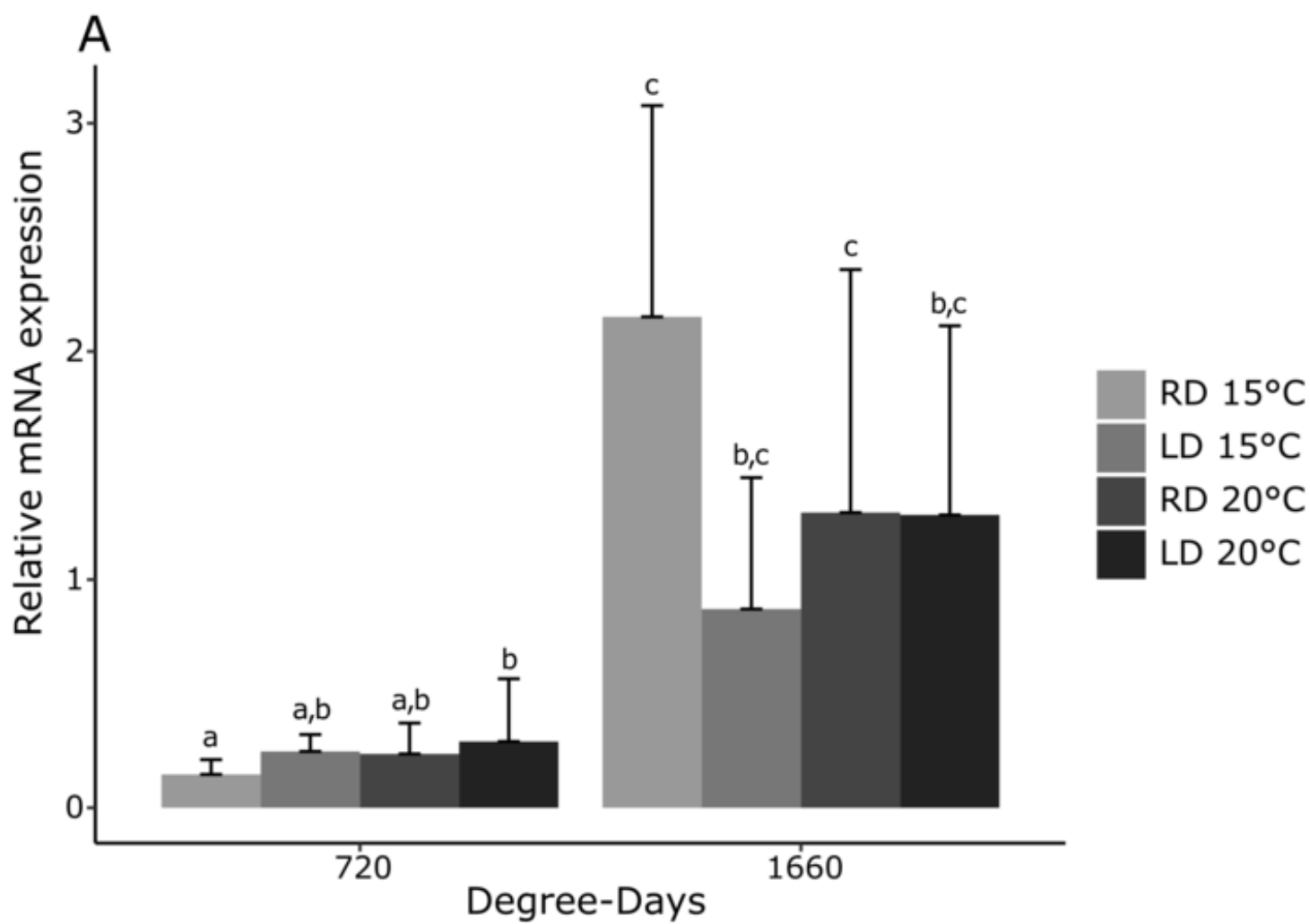


Figure1B

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