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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Abstract

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

Introduction

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

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

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

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

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

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

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

Feeding experiment

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

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

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

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

Tissue sampling

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

Peroxidation index, enzyme activity, and malondialdehyde assay

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

PI = (0.025 × % monoenoics) + (1 × % dienoics) + (2 × % trienoics) + (4 × % tetraenoics) + (6 × % pentaenoics) + (8 × % hexaenoics).

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

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

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

Specific CAT activity was measured in triplicate according to Aebi (1984) by observing the decomposition of H_2O_2 into oxygen and water. The reaction mixture consisted of 20 mM phosphate (pH 7) and 10 mM H_2O_2. We used a Multiskan GO (Thermo Scientific) plate reader with GREINER UV-Star 96 (ref: M3812, Sigma). The kinetics of the colouring solutions were monitored at 22°C by reading absorbance at 240 nm every 10 s for 4 min.

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

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

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

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

mRNA expression analysis

RNA extraction

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

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

cDNA synthesis

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

Quantitative real-time RT-PCR analysis

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

(https://www.ncbi.nlm.nih.gov/genbank/). **mRNA** expression was quantified using a C1000 touch thermal cycler (CFX96 system, Bio-Rad Laboratories Inc.). For each primer pair, qPCR efficiencies were estimated by standard curves resulting from serial dilutions (from 1/10 to 1/270) of a pool of cDNA. In the present study, qPCR efficiencies for each primer pair ranged from 95 to 100%, with $R^2 > 0.99$. Samples were then analyzed in triplicate. The final well volume was 15 µL, containing 5 µL cDNA (1/30 dilution) and 10 µL of reaction mix (0.5 µL of each primer [10 µM]), 1.5 µL RNase/DNase-free water, 7.5 µL iQ™ SYBR® Green Supermix (Bio-Rad Laboratories Inc.) containing antibody-mediated hot-started iTaq DNA polymerase, dNTPs, MgCl2, SYBR® Green I dye, fluorescein, stabilizers, and enhancers. In each plate, negative controls (RT negative controls) were systematically included to ensure the absence of residual genomic DNA contamination. Non-template controls (H_2O) were also analyzed. Each plate contained an inter-run calibrator in triplicate (dilutions of a pool of all samples). The qPCR thermal cycling included an initial activation step at 95°C for 2 min followed by 39 cycles of 5 s at 95°C and 20 s at 60°C. A melting curve was performed at the end of the amplification phase to confirm the amplification of a single product in each reaction. The corresponding Cq (quantification cycle) value was determined automatically for each sample using the Gene Expression Module of the CFX Manager software (Bio-Rad Laboratories Inc.). Cq is the number of cycles required to yield a detectable fluorescence signal. The CFX Manager Software was used to normalize the relative quantity of messenger with the ΔΔCt method. The **expression level of** elongation factor 1 (***ef1a***) and ribosomal (***28S***) **mRNA** were tested to correct for loading differences or other sampling variations present in each sample. The *ef1a* **mRNA** was used **for normalization** in the liver tissue of juvenile fish since it did not show any significant variation of expression among experimental conditions ($P > 0.05$).

Statistical and data analysis

Relative **mRNA** expressions and enzyme activity data were log10 transformed to obtain normal distributions. The effects of time, temperature, and diet were tested for peroxidation index, relative **mRNA** expression (*pparα*, *pparβ*, *pparγ*, *fas*, *srebp-1*, *fads2*), enzyme activity (CAT, SOD, CS, COX), and MDA content using three-way ANOVAs. Normality and homoscedasticity of data were tested using Shapiro-Wilk and Levene tests, respectively. When appropriate, Tukey mean comparison tests were done because homoscedasticity was respected. Differences were considered significant at $\alpha = 0.05$. Statistical analyses were conducted in R (ver. 3.3.3; R Development Core Team).

Results

Liver mass and protein content

Results showing the effect of diet, temperature, and time on liver mass and hepatic protein content are shown in Table 3A. After 720 dd of feeding, liver mass was significantly higher (+48%) in juveniles reared at 20°C than at 15°C, and diet had no significant effect (Table 3A). At 1660 dd, the increase in liver mass was higher for juveniles reared at 20°C (+67%) than for those reared at 15°C (+59%**), while liver mass was still higher (+58%) in juveniles reared at 20°C than at 15°C.** Again, no diet effect was detected. Liver protein concentration decreased significantly over time (0.12 ± 0.01 mg protein mg liver^{-1} at 720 dd vs. 0.10 ± 0.00 mg protein mg liver^{-1} at 1660 dd), and no significant effect of diet or of temperature was observed.

Peroxidation index and MDA

The PI of the RD diet was 34% higher than that of the LD diet (15.86 ± 1.78 vs. 10.52 ± 0.50). Accordingly, the PI in the polar lipids of juveniles fed RD was significantly higher (+18%) compared to fish fed LD (45.36 ± 6.96 vs. 38.50 ± 5.45; Table 3A). The temperature × 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 ± 0.48 vs. 2.77 ± 0.45 nmol mg protein^{-1}; 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 ± 1.21 vs. 5.09 ± 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 ± 7.93 vs. 105 ± 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 ± 0.36 U mg protein^{-1} vs. 4.25
325 ± 0.43 U mg protein^{-1}**)**. SOD activity significantly decreased (–8%) with time (4.25 ± 0.31 U
326 mg protein^{-1} at 1660 dd vs. 3.95 ± 0.47 U mg protein^{-1} at 720 dd). There was also a significant
327 increase in SOD activity (+10%) at the higher temperature (3.91 ± 0.43 U mg protein^{-1} at 15 °C
328 vs. 4.29 ± 0.33 U mg protein^{-1} 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*, *pparγ*, 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 *pparα*
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 *pparβ* **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

mRNA expression, lipid metabolism, and antioxidant enzymes

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

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

Temperature and diet effects

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

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

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

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

Conclusion

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

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Compliance with ethical standards

Conflict of interest

The authors declare that they have no conflicts of interest.

Ethical approval

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

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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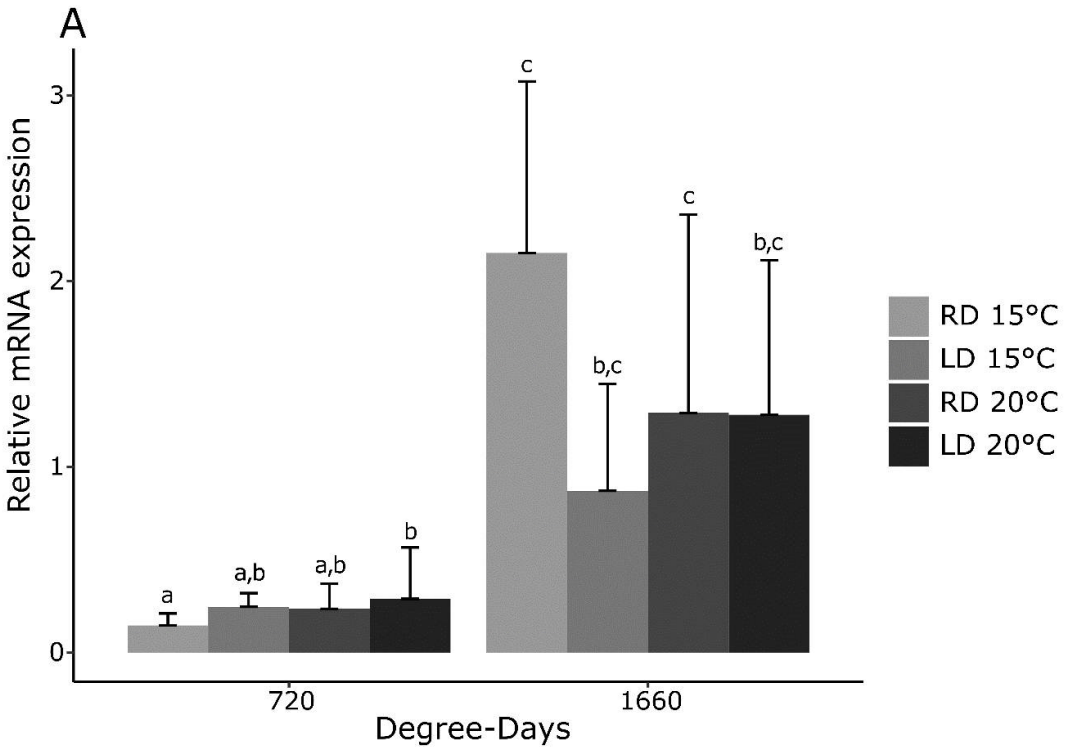
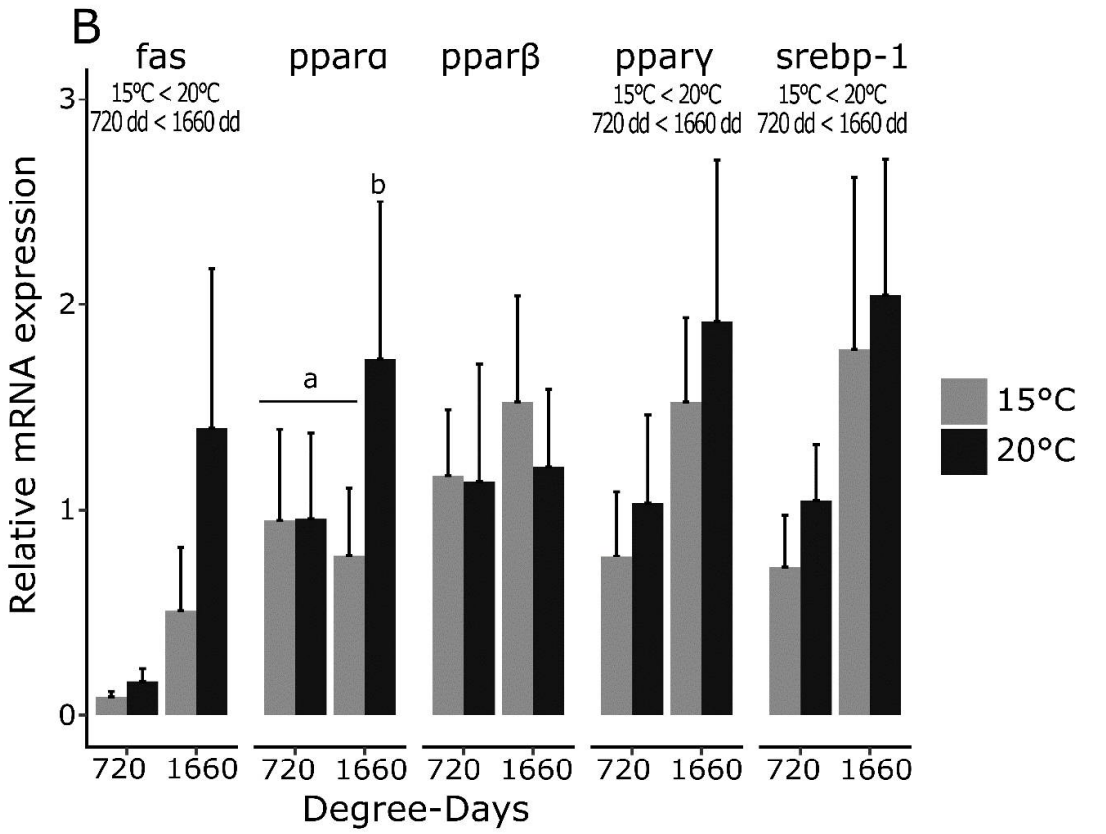
Figures

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


A
Relative mRNA expression
0
1
2
3
a
a,b
a,b
b
c
b,c
c
b,c
720
1660
Degree-Days
RD 15°C
LD 15°C
RD 20°C
LD 20°C

753


B
fas
15°C < 20°C
720 dd < 1660 dd
pparα
pparβ
pparγ
15°C < 20°C
720 dd < 1660 dd
srebp-1
15°C < 20°C
720 dd < 1660 dd
Relative mRNA expression
0
1
2
3
a
b
720
1660
Degree-Days
15°C
20°C

754

Tables

Table 1: Composition of experimental diets. For dry matter, proteins, total lipids, triglycerides, and phospholipids, data are presented as % of dry mass. Data for specific fatty acid (FA) categories are presented as % of total lipids. LD: low n-3 polyunsaturated fatty acid (PUFA) diet, RD: reference n-3 PUFA diet; SFA: saturated FA; MUFA: monounsaturated FA; ARA: 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)[a]	22.38	23.26
Total lipids	21.98	21.63
Triglycerides	16.99	17.05
Phospholipids	4.70	4.71
Energy (kJ)[b]	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

761 [a] Calculated as 100 – (%proteins + %total lipids + %ash)

762 [b] Calculated as %proteins × 16.7 kgJ g^{-1} + %total lipids × 37.7 kgJ g^{-1} + NFE × 16.7 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 (*28S*)	CBXY010007006	TTTCCCATGAGAGAGCAGGT	TCAGATGCGCTTCTTAGGATGT
Elongation factor 1 a (*ef1a*) *	AJ866727.1	GCTTCGAGGAAATCACCAAG	CAACCTTCCATCCCTTGAAC
Fatty acid synthase (*fas*)	MF566098.1	CCTCTGAACCTGGTCTGGTG	ATTGGAGAGAGCCTCCACGA
Delta-6-desaturase (*fads2*)	EU439924.1	AGCATCACGCTAAACCCAAC	CAAGCCAGATCCACCCAGTC
Peroxisome proliferator activated receptors alpha (*pparα*)	AJ880087.1	ACCTCAGCGATCAGGTGACT	AACTTCGGCTCCATCATGTC
Peroxisome proliferator activated receptors beta (*pparβ*)	AJ880088.1	GCTCGGATCTGAAGACCTTG	TGGCTCCATACCAAACCACT
Peroxisome proliferator activated receptors gamma (*pparγ*)	AY590303	CAGATCTGAGGGCTCTGTCC	CCTGGGTGGGTATCTGCTTA
Sterol regulatory element binding protein 1 (*srebp-1*)	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					
	RD	LD	RD	LD	RD	LD	RD	LD	Time	Temperature	Diet	Interaction
Liver mass (g)	0.3 ± 0.02^{a}	0.31 ± 0.01^{a}	0.62 ± 0.07^{bc}	0.55 ± 0.14^{b}	0.86 ± 0.07^{c}	0.61 ± 0.06^{bc}	1.77 ± 0.4^{d}	1.74 ± 0.19^{d}	**n.s.**	**n.s.**	**n.s.**	Time × Temp. × Diet $P < 0.05$ $F(1,16) = 4.89$
Protein (mg prot mg liver $^{-1}$)	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^{-1})	60.05 ± 2.9^{a}	69.62 ± 4.59^{ab}	78.28 ± 1.94^{bc}	143.37 ± 22.97^{e}	78.12 ± 5.82^{bc}	73.62 ± 4.04^{ab}	103.75 ± 7.3^{d}	97.68 ± 8.5^{cd}	**n.s.**	**n.s.**	**n.s.**	Time × Temp. × Diet $P < 0.001$ $F(1,16) = 11.67$
SOD (U mg prot^{-1})	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^{-1})	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^{-1})	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				
	RD	LD	RD	LD	RD	LD	RD	LD	Temperature	Time	Interactions
fads2	0.15 ± 0.07^{a}	0.25 ± 0.07^{ab}	0.24 ± 0.14^{ab}	0.29 ± 0.28^{b}	2.15 ± 0.93^{c}	0.87 ± 0.58^{bc}	1.29 ± 1.07^{bc}	1.28 ± 0.83^{c}	n.s.	n.s.	Time × Temp. × Diet $P < 0.05$ $F(1,38) = 4.47$
fas	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.
pparα	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
pparβ	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.
pparγ	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.
Srebp-1	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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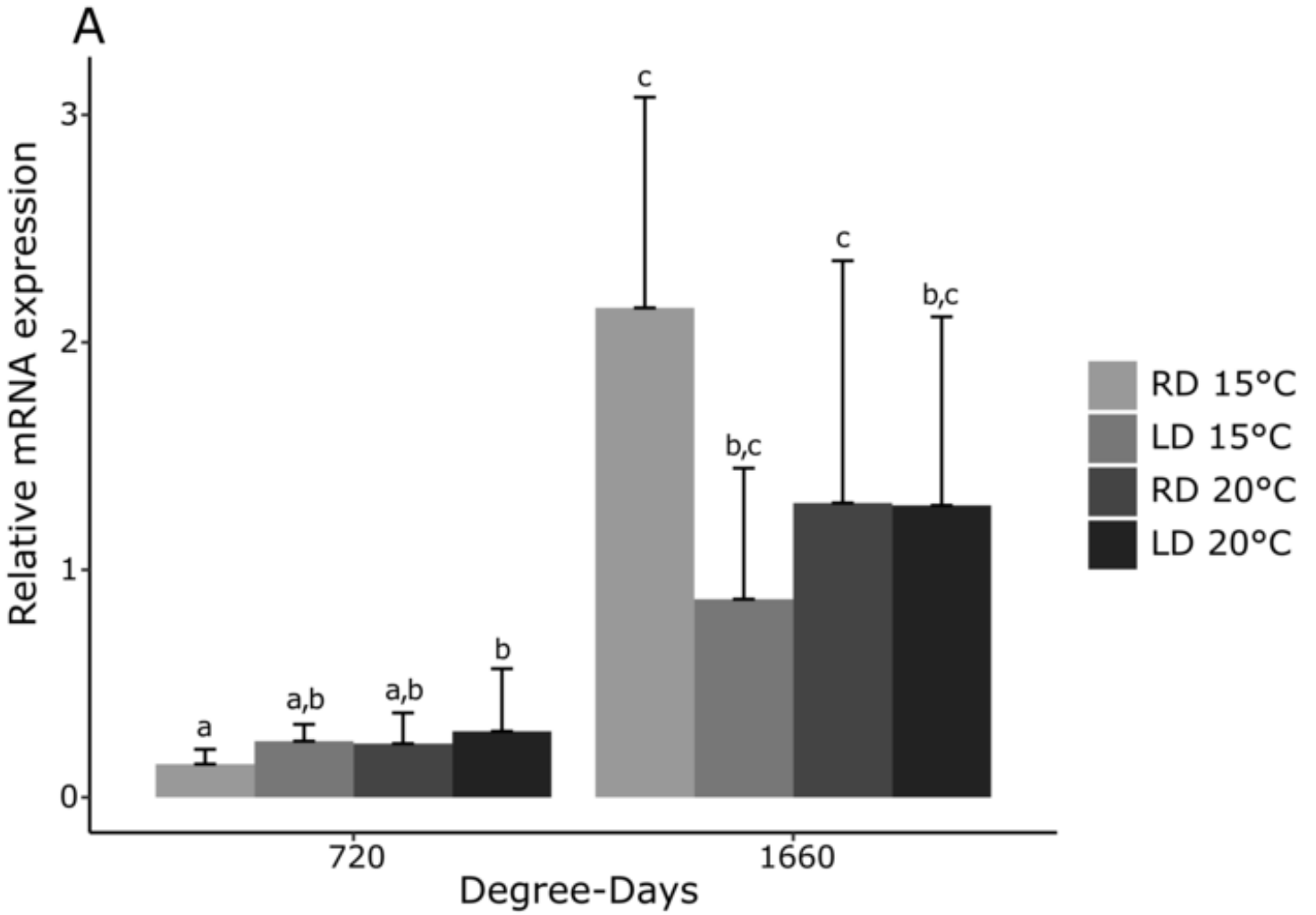

Figure1B

Click here to access/download;Figure;fig B mRNA.png