Conserved fatty acid profiles and lipid metabolic pathways in a tropical reef fish exposed to ocean warming – an adaptation mechanism of tolerant species?

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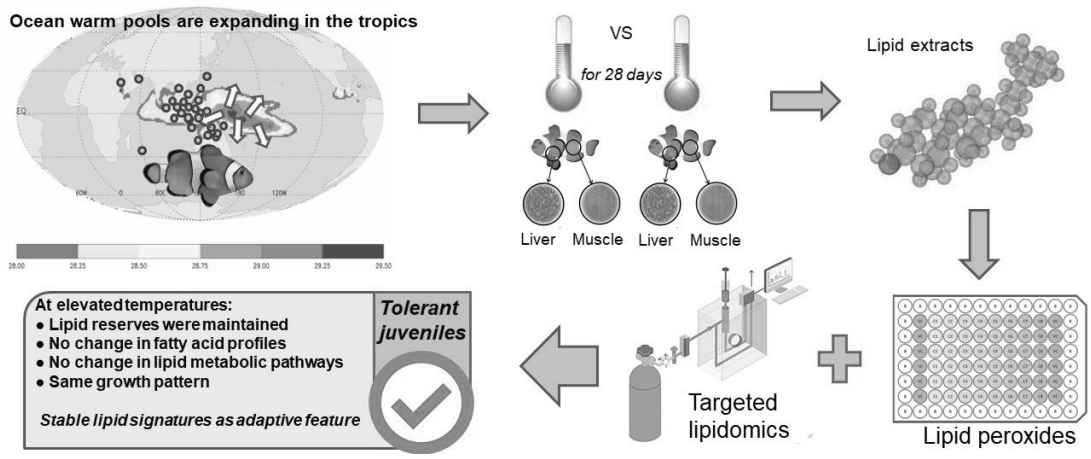
ABSTRACT

Climate warming is causing rapid spatial expansion of ocean warm pools from equatorial latitudes towards the subtropics. Sedentary coral reef inhabitants in affected areas will thus be trapped in high temperature regimes, which may become the “new normal”. In this study, we used clownfish *Amphiprion ocellaris* as model organism to study reef fish mechanisms of thermal adaptation and determine how high temperature affects multiple lipid aspects that influence physiology and thermal tolerance. We exposed juvenile fish to two different experimental conditions, implemented over 28 days: average tropical water temperatures (26 ˚C, control) or average warm pool temperatures (30 ˚C). We then performed several analyses on fish muscle and liver tissues: i) total lipid content (%), ii) lipid peroxides, iii) fatty acid profiles, iv) lipid metabolic pathways, and v) weight as body condition metric. Results showed that lipid storage capacity in *A. ocellaris* was not affected by elevated temperature, even in the presence of lipid peroxides in both tissues assessed. Additionally, fatty acid profiles were unresponsive to elevated temperature, and lipid metabolic networks were consequently well conserved. Consistent with these results, we did not observe changes in fish weight at elevated temperature. There were, however, differences in fatty acid profiles between tissue types and over time. Liver showed enhanced α-linolenic and linoleic acid metabolism, which is an important pathway in stress response signaling and modulation on environmental changes. Temporal oscillations in fatty acid profiles are most likely related to intrinsic factors such as growth, which leads to the mobilization of energetic reserves between different tissues throughout time according to organism needs. Based on these results, we propose that the stability of fatty acid profiles and lipid metabolic pathways may be an important thermal adaptation feature of fish exposed to warming environments.

Keywords: clownfish; warming environments; coral reefs; fatty acid profiling; lipid metabolic networks



Highlights

- Effects of warm temperature on lipid profiles and pathways were tested in clownfish
- Muscle and liver were unresponsive to temperature change
- Lipid storage capacity was maintained, even with lipid peroxidation
- No change was detected in metabolic pathways at warm temperature
- Growth remained unchanged, suggesting ability to acclimate

140 1. Introduction

141 Oceans are a major heat sink for Earth's climate system (Herbert et al., 2010). Global
142 warming has caused tropical oceans to warm by 0.8 ˚C to 1.6 ˚C in the past decades
143 (IPCC, 2019). As a consequence, large fluctuations of ocean isotherms are also occurring,
144 causing a rapid expansion of ocean warm pools (Arora et al., 2016). Areas with sea surface
145 temperatures (SST) between 29 ˚C – 30 ˚C in tropical oceans and seas have increased at
146 a rate of 2.29×10^6 km^2 per decade since the 1980s, whereas areas with SST between 26
147 ˚C and 28 ˚C have been contracting, and subtropical areas with SST below 25 ˚C have
148 changed little (Lin et al., 2011). The distribution of tropical ocean temperatures is thus being
149 skewed towards the warmer edge. Additional thermal pressures are expected to be exerted
150 on marine ecosystems, with predicted future anomalies for the tropical regions in the range
151 of + 0.9 ˚C to + 3.3 ˚C across the different climate warming scenarios for the late 21st
152 century (2081-2100; IPCC, 2014, 2013).

153

154 Previous studies of marine organisms' responses to a warming environment have
155 highlighted the importance of reorganizing biodiversity in space and time (Groom et al.,
156 2012), with shifts in ecosystem structure and function (Bijma et al., 2013; Graham et al.,
157 2008). For instance, tropical reef fish have been found to be "on the move", especially at
158 the latitudinal limits of their distribution (S. Bejarano, pers. com.). This ongoing process
159 depends on fish environmental tolerance limits as well as their capacity for adaptation and
160 rates of molecular and phenotypic trait evolution (Bruneel et al., 2018; Payne et al., 2016;
161 Rummer et al., 2014). However, ongoing expansion of ocean warm pools beyond areas of
162 regular occurrence means that many tropical and subtropical fish populations and species,
163 especially slow swimmers, or sedentary ones, may find themselves trapped and living
164 permanently at or beyond the limits of their optimum tolerance range. Despite the high
165 thermal tolerance of these ectotherms, it is not clear if such an environment will lead to
166 increased thermal specialization and novel adaptations or to deleterious effects for already
167 warm-adapted species. This knowledge is especially relevant given that species with low

capacity for movement and/or limited ability for further acclimation to heat may be disproportionally affected (Madeira et al., 2018; Roessig and Woodley, 2004).

The deleterious effects of high temperatures are usually a consequence of energetic tradeoffs at the organism level, theorized by Sokolova (2013) as energy-limited tolerance to stress. During stress, a higher energetic investment in physiological compensatory mechanisms occurs, at the expense of growth and reproductive output (Angilletta et al., 2003; Munday et al., 2012). This is explained by the loss of bioenergetics balance, in which an exhaustion of cellular energetic fuels occurs, with consequent changes in availability, structure and composition of the main energy reserves, namely lipids (Balogh et al., 2013; Valles-Regino et al., 2015; Wen et al., 2017). These effects are expected to cascade through the food web, with severe impacts for marine populations (Anacleto et al., 2014; Colombo et al., 2020). In fact, lipids provide the densest form of energy in marine ecosystems, but they are also very susceptible to oxidative damage arising from stressful conditions (Parrish, 2013). Higher trophic levels are expected to be the most impacted by a decrease in lipid quality and/or availability, because organisms will be left with no option but to feed on less nutritious food. For instance, Hixson and Arts (2016) suggested that ocean warming could lead to a worldwide reduction of omega-3 long-chain polyunsaturated fatty acids (FA) availability in marine organisms. These essential FA are mostly obtained through diet and have vital roles in cellular processes such as membrane remodeling and homeoviscous adaptation, determinants of thermo-sensitivity (Csoboz et al., 2013; and see also Ernst et al., 2016; Eghtesadi-Araghi and Bastami, 2011 and Sinensky, 1974 for a review). Additionally, essential FA play important roles in nutrient transport and enzyme activity (Neidleman, 1987). The decreased bioavailability of omega-3 FA under current warming conditions is already affecting the fitness of top predators (e.g. decreasing spawning and egg quality, fertilization and hatching rates, Rainuzzo et al., 1997) and consequently ecosystem stability (Colombo et al., 2020; Parrish, 2013).

In this context, the utility of systems-biology approaches, such as the large-scale study of lipids and their interactions, pathways and networks, coupled to computational methods, is useful for identifying metabolic state and organism health (Checa et al., 2015; Török et al., 2014). Specifically, targeted investigations of alterations in the composition and content of lipid classes like fatty acids provide information on physiological and pathological condition and facilitate mechanistic insights into changes in cellular function, biochemical mechanisms of stress and impaired health processes under climate warming.

Past studies have shown that both freshwater and marine fish exposed to higher temperatures usually i) decrease their overall body lipid content, e.g. Atlantic salmon *Salmo salar* (Ruyter et al., 2006; Todd et al., 2008); ii) decrease diversity in FA composition, e.g. duskytail grouper *Epinephelus bleekeri* (Dey et al., 1993); iii) decrease levels of polyunsaturated and long-chain polyunsaturated fatty acids (PUFA and HUFA) over time, e.g. seabass *Dicentrarchus labrax*, (Barbosa et al., 2017) ; iv) decrease levels of omega-3 FA, e.g. tuna *Thunnus alalunga,* (Pethybridge et al., 2015), v) increase overall levels of saturated fatty acids (SFA), e.g. golden mahseer *Tor putitora*, (Akhtar et al., 2014) and vi) have higher levels of polar lipids and decreased levels of neutral lipids (especially triglycerides) (e.g., seabass *D. labrax*, Bouaziz et al., 2017). These studies ultimately suggest that environmental variation leads to altered physiological phenotypes, with significant internal stoichiometry, metabolic and bioenergetic consequences. However, crucial knowledge gaps persist, especially with regards to the comprehension of the mechanisms and layers of molecular networks that underlie these changes. Advances are therefore needed to provide insights into the cellular regulatory pathways that determine phenotypic outcomes of marine organisms under climate change.

In this study, we profiled fatty acids of the model tropical reef fish *Amphiprion ocellaris* to understand how fatty acid metabolism shapes thermal acclimation and adaptation potential of shallow water, sedentary tropical fish to a warmer environment. We have previously shown that *A. ocellaris* responds to temperature increase by upregulating the cellular stress

response in muscle, gills and liver (Madeira et al. 2016). As a follow-up, fatty acid profiles, pathways and networks, as well as lipid contents and lipid peroxides, anchored in body condition metrics (weight) were assessed in fish exposed to control (26 ˚C) and warmer conditions (30 ˚C) in order to test the following hypotheses: H1) different tissues show variable susceptibility to variation in lipid composition and metabolism under high temperature; H2) tissues show reduced lipid contents as consequence of temperature-induced lipid peroxidation damage and higher bioenergetic costs to maintain homeostasis; H3) FA profiles display changes in saturated and unsaturated fatty acids as temperature increases; H4) omega-3 FA concentrations decrease, whereas omega-6 FA concentrations increase at high temperature, with cascading consequences for metabolic pathways.

2. Materials and methods

2.1. Animal housing

We selected the Pomacentridae fish *Amphiprion ocellaris* as study species since it has proven to be a suitable model for climate change studies (Madeira et al., 2016; Madeira et al., 2017). It is also a well-established ornamental aquaculture species, easy to obtain, with simple handling and care (Khoo et al., 2018; Vargas et al., 2016). Ocellaris clownfish have a bi-phasic life cycle with an oceanic larval phase (2 weeks) and a reef benthic phase in juvenile (post-recruitment), adult and embryo stages, in which the fish live symbiotically with their host sea anemones (Roux et al., 2019). Clownfish live in groups, in a dominance-based social hierarchy composed of a breeding pair and several smaller non-breeding male fish. The adults have an average life-span of 6-10 years in the wild (Fautin and Allen, 1992). Three-month old juvenile fish (N = 48, weight 1.05 ± 0.28 g, length 3.54 ± 0.36 cm) were purchased at the Tropical Marine Centre Iberia hatchery (Loures, Portugal) and placed in a marine life-support system upon arrival at experimental facilities, where they were acclimated for two weeks at a constant temperature of 26 ± 0.5 ˚C.

2.2. Experimental setup

During experiment trials, 26 ± 0.5 ˚C was set as the control temperature, which represents the average water temperature at which *A. ocellaris* lives (Allen, 2009; Madeira et al., 2017). Additionally, this temperature also reflects the thermal history conditions of our specimens – clownfish from the hatchery were reared at this temperature, as well as the original brood stock. The high temperature treatment was set at 30 ± 0.5 ˚C, which corresponds to warm pool temperatures, at the limit of *A. ocellaris'* native thermal range (22 ˚C – 30 ˚C, Allen, 2009; Madeira et al., 2017). The aquaria system consisted of an indoor re-circulating volume of 2,000 L, with filtered and aerated seawater. It comprised six polyvinyl tanks (dimensions 35 x 35 x 55 cm), one common sump, an external skimmer and a UV filter. Tanks were the experimental units, with three replicates for each temperature condition (N = 8 animals.tank^{-1}, mirroring density of fish in nature in a single host anemone). Fish were fed twice a day with a mixture of frozen and dry food (shrimp, mussels and *Spirulina* pellets in proportion 1:1:1 weight). Salinity was kept at 35 ppt (measured once a day); pH at 8 ± 0.01, ammonia below < 0.1 mg.l^{-1} and nitrites below 0.3 mg.l^{-1} (measured once a week). The light-dark cycle was kept at 12L:12D. Animal sampling was performed once *per* week for 28 days. Five specimens were randomly sampled on day 0 from both treatments as an overall baseline control (before any change in temperature), euthanized by cervical dissection, measured, weighed, and muscle and liver tissues dissected and frozen at -80 ˚C. Water temperature in the high temperature treatment tanks was then increased at a rate of 0.10 ˚C.hr^{-1}, maintained with thermostat heaters (ELITE 200W) and continuously monitored with Petco thermometers (UK). On days 7, 14, 21 and 28, three to five specimens from each temperature (control and high temperature) were sampled as previously described (N_{total} = 3-5 specimens × 5 timepoints × 2 treatments = 38 specimens sampled in total).

2.3. Total lipid extraction and quantification

All glassware used in this protocol was combusted prior to use at 450 ˚C for 5 h. Tissue samples (N_{total} = 38 specimens × 2 tissues = 76 samples) were freeze-dried and ground to

a fine homogeneous powder. Lipids from 2-5 mg and 10-30 mg of tissue (liver and muscle, respectively) were extracted with 1 ml of 2:1 dichloromethane:methanol (DCM:MeOH), vortexed for 5 s and sonicated for 10 min in an ice-cold water bath. Samples were then frozen at -20 ˚C overnight to allow the settlement of tissue particles in the solvent. The supernatant from each sample was then transferred to new glass vials with glass pipettes and the resulting total lipid extracts (TL) were evaporated to dryness under a stream of N_2. All the previous extraction steps were repeated at least three times for each sample. Lipid content (LC) in each sample was then calculated as follows:

$$\%\mathrm{LC} = \left(\mathrm{Weight}_{\mathrm{TL}} \div \mathrm{Weight}_{\mathrm{tissue\ sample}}\right) \times 100$$

2.4. Lipid peroxidation levels

Tissue samples (N_{total} = 38 specimens × 2 tissues = 76 samples) were homogenized in 1 ml of phosphate buffer saline (140 mM NaCl, 3 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4) using a Tissue Master 125 homogenizer on ice. The crude homogenates were then centrifuged at 4 ˚C for 15 min at 10,000 × g. The supernatants were collected, transferred to new microtubes and frozen immediately (-80 ˚C). The lipid peroxides assay was adapted from the thiobarbituric acid reactive substances (TBARS) protocol (Uchiyama and Mihara, 1978). In brief, five µl of each sample were diluted in 45 µl of 50 mM NaH_2PO_4 buffer. Then, 12.5 µl of SDS 8.1 %, 93.5 µl of trichloroacetic acid 20 % and 93.5 µl of thiobarbituric acid 1 % were added, together with 50.5 µl of ultrapure water before vortexing the microtubes for 30 s, followed by an incubation in boiling water for 10 min. Samples were afterwards placed on ice for a few minutes to cool, 62.5 µl of Milli-Q grade ultrapure water were added and absorbance was read at 530 nm in a microplate reader (Benchmark, Bio-Rad). To quantify the lipid peroxides, an eight-point calibration curve (0 to 0.3 µM TBARS) was calculated using malondialdehyde bis (dimethylacetal) standards (Merck). Lipid peroxides results were then normalized per mg of TL.

2.5. Clownfish fatty acids profiling

311 For fatty acid profiling, 50 µl of a recovery standard with *n*-$C_{19:0}$ alkanoic acid in ethyl
312 acetate were quantitatively added to the TL of each sample (N_{total} = 38 specimens × 2
313 tissues = 76 samples). TL were then methylated to produce fatty acid methyl esters
314 (FAMEs) using 1 ml of BF_3 in MeOH (14% by volume, Sigma Aldrich), sonicated for 5 min
315 and placed in an oven for 2 h at 100 °C. After adding 2 ml of nanopure water, FAMEs were
316 extracted with 1 ml hexane, vortexed and then centrifuged for 3 min at 1500 rpm to
317 accelerate the separation process. The hexane phase of each sample was collected into
318 new vials. Extraction of FAMEs with hexane rinses was repeated at least three times.
319 Samples were then blown down to dryness with a N_2 stream and dissolved in 300 µl of
320 ethyl acetate prior to quantification of FAMEs by gas chromatography with a flame
321 ionization detector (GC-FID) (GC-2010 Plus, Shimadzu, Japan). An AOC-20i autosampler
322 (Shimadzu) injected the samples through a split/splitless injector operated in splitless mode
323 at 280°C. The GC column was an InertCap 5MS/NP column (30 m; 0.25 mm; 0.25 µm; GL
324 Sciences, Japan). The GC oven program heated from 70 ˚C to 130 ˚C at 20 ˚C.min^{-1} and
325 then from 130 ˚C to 320 ˚C at 4˚C.min^{-1} and held for 20 min. The GC-FID limit of detection
326 (LOD) was 20 ng.$µl^{-1}$ for all FA. All compounds were quantified relative to the *n*-$C_{19:0}$ fatty
327 acid recovery standard and the final concentration expressed as µg.mg^{-1} of dry weight.
328 Compounds were identified by comparing their retention times to those of laboratory
329 standards (Supelco 37 Component FAME Mix, ref. CRM47885). Samples with unidentified
330 compounds were further analyzed under identical chromatographic conditions by gas
331 chromatography–mass spectrometry (GC–MS) with a QP2020 mass
332 spectrometer (Shimazdu) and comparing the resulting mass spectra to reference spectra in
333 the National Institute of Standards and Technology (NIST) library.
334
335 2.6. Data analysis
336
337 2.6.1. Fish body condition: weight
338 Body mass (g) was analyzed for outliers (box-whiskers plot) and tested for normality
339 (Shapiro's Wilk test) and homoscedasticity (Levene's test). Following assumptions, the

effects of acclimating temperature on weight were tested with a Student's t-test ($p < 0.05$ for significance).

2.6.2. Lipid contents and lipid peroxides

For lipid contents and lipid peroxides, data were first tested for outliers (box-whiskers plot), normality (Shapiro's Wilk test) and homoscedasticity (Levene's test). To test if there was a relationship between the extent of lipid peroxidation and a change in capacity for lipid storage (as consequence of changes in metabolism with acclimating temperature), an ANCOVA with a separate slope design was used, as lipid peroxides levels and temperature are not independent. Analyses were done in STATISTICA v8 (Statsoft, USA).

2.6.3. Fatty acid profiles and data mining

Data matrices were pre-processed before statistical analysis. Datasets were assessed and treated for missing data (MD) in MetImp v1.2 (free available web tool, https://metabolomics.cc.hawaii.edu/software/MetImp/). A group-wise missing filter was initially applied, eliminating all FAs with over 50 % MD. Then, MD imputation, which is a standard procedure in GC data, as coherent and complete data matrices are a pre-requisite for accurate and reliable statistical analysis (Kokla et al., 2019), was performed. To input missing values to the remaining FA variables (16.45 % MD in the total dataset), we followed the protocol established by Wei et al., (2018) for GC-MS data, and the MCAR/MAR (Missing Completely at Random/Missing at Random) algorithm, based on random-forests (with seed 1234) was used for this purpose.

The final data matrix was composed of 26 FA (Table 1) plus 9 added variables, consisting of FA summed into different classes and 3 variables consisting of FA ratios, yielding 38 variables in total. The two latter included (i) SFA (0 double bonds: 14:0, 15:0, 16:0, 17:0, 18:0, 20:0 and 22:0), (ii) MUFA (monounsaturated fatty acids, 1 double bond: 16:1n-7, 17:1n-7, 18:1n-9 *cis* and *trans*, 19:1n-9, 20:1n-9, 24:1n-9), (iii) PUFA (2-3 double bonds: 18:2n-6 *cis* and *trans*, 20:3n-3, 20:3n-6, 22:2n-6, 26:2n-6, 27:2), (iv) HUFA (highly

unsaturated fatty acids, ≥ 4 double bonds: 20:5n-3, 20:4n-6, 22:6n-3, 22:5n-3, 22:4n-6), (v) omega-3 fatty acids (double bond three atoms away from the terminal methyl group: 20:3n-3, 20:5n-3, 22:6n-3, 22:5n-3), (vi) omega-6 fatty acids (double bond six atoms away from the terminal methyl group: 18:2n-6 *cis* and *trans*, 20:3n-6, 20:4n-6, 22:2n-6, 22:4n-6, 26:2n-6), (vii) omega-7 fatty acids (double bond seven atoms away from the terminal methyl group: 16:1n-7 and 17:1n7); (viii) omega-9 fatty acids (double bond nine atoms away from the terminal methyl group: 18:1n-9 *cis* and *trans*, 19:1n-9, 20:1n-9, 24:1n-9), (ix) total sum of all FA, and lastly fatty acid ratios (x) ARA/EPA (arachidonic acid 20:4n-6/eicosapentanoic acid 20:5n-3), (xi) EPA/DHA (eicosapentanoic acid 20:5n-3/docosahexaenoic acid 22:6n-3) and (xii) ∑n-6/∑n-3. The complete dataset was log-transformed and auto-scaled (mean centered and divided by the standard deviation of each variable) prior to statistical analyses.

With the aim of identifying the main and interactive effects of (i) temperature, (ii) tissue and (iii) time in FA profiles and classes, as well as the FA variables that contribute to the differences found, several multivariate analyses were performed. An unsupervised principal components analysis (PCA) was applied to explore FA data structure, followed by heat maps with hierarchical clustering of FA classes, constructed based on the following metrics (i) distance measure: Pearson correlation (similarity of expression profiles), (ii) clustering algorithm: complete linkage (forms compact clusters), (iii) feature auto-scale. These analyses were run in Metaboanalyst v4.0 (https://www.metaboanalyst.ca/) (Chong et al., 2019). A PERMANOVA analysis was then run using PERMANOVA+ v1.0.6 as implemented in PRIMER v6.1.16. A resemblance matrix was calculated based on Euclidean distances, and the analysis was based on Type III SS for unbalanced designs, with 9999 permutations of residuals under a reduced model. For factors and interaction of factors (with more than two factor categories) significantly affecting FA profiles, pairwise tests were employed to test which groups were different. A SIMPER analysis was also run to identify contributions from each FA and FA classes to the significant differences found for main factors in the PERMANOVA.

398

399 2.6.4. Fatty acid quantitative enrichment and pathway analysis

400 Functional enrichment and pathway analysis were performed only for relevant factors that
401 displayed significant effects on overall FA profiles of *A. ocellaris*, as detected by
402 PERMANOVA analysis. The objective was to gain biological insights into the functional
403 roles of pre-defined subsets of FA analytes in animal metabolism and physiology (Ma et al.,
404 2019). To define the set of individual FA variables to be enriched, a threshold was defined
405 based on the following criterion: within a significant factor effect, fatty acids identified in the
406 SIMPER analysis as contributing up to a cumulative percentage of 90% for the observed
407 differences were selected for analysis. Lipid annotation was then performed, based on
408 LIPID MAPS for chemical structure and KEGG (Kyoto Encyclopedia of Genes and
409 Genomes) as well as HMBD (Human Metabolome Database) for function (Fahy et al.,
410 2007; Kanehisa et al., 2017; Züllig et al., 2020). Only well annotated FA were mapped.
411 Functional enrichment and pathway analysis were then performed in Metaboanalyst v4.0
412 (Chong et al., 2019).

413

414 Quantitative enrichment analysis (QEA) was performed by setting the '*normal metabolic*
415 *pathways*' based on SMPDB (Small Molecule Pathway Database) metabolite library
416 (Frolkis et al., 2009; Jewison et al., 2014). The analysis was done based on FA
417 concentration and annotation, using the global test algorithm, in which a generalized linear
418 model is used to estimate a Q-statistic for the FA set (with FDR corrected p-values), to
419 describe the correlation between compound concentration profiles and phenotypes, as well
420 as list the different metabolic pathways in which each FA takes part. Pathway topology
421 analysis was also performed by selecting the pathway libraries from SMPDB (Howe et al.,
422 2013) and was based on two features (i) quantitative functional enrichment (described
423 previously, significant if FDR < 0.05) and (ii) pathway topology analysis to estimate node
424 importance. This analysis was implemented using the relative betweeness centrality, which
425 takes into account the role of the FA, position and direction of the interaction, measuring
426 the centrality of a given FA in the metabolic network (and ultimately gives information about

427 the relative importance or role of the FA in the organization of the metabolic network, see
428 Xia and Wishart, 2010 for further details). The pathway impact was considered relevant if >
429 0.1.
430
431
432 3. Results
433
434 3.1. Body condition
435 Animals' weight (mean ± SD) was similar between temperature treatments after 28 days of
436 the experiment. Individuals raised at 26 ˚C had an average body mass of 1.09 ± 0.26 g,
437 whereas individuals raised at 30 ˚C had an average body mass of 1.06 ± 0.30 g (t-test =
438 0.404, p-value = 0.688).
439
440 3.2. Total lipids and lipid peroxidation levels
441 Results of the ANCOVA model with separate slopes suggest that there are no significant
442 changes in lipid contents in response to temperature in covariation with lipid peroxide
443 (LPO) levels, both in muscle (test of whole model multiple R = 0.270, F = 0.870, p-value =
444 0.466) and liver (test of whole model multiple R = 0.398, F = 1.949, p-value = 0.142).
445 Results indicate that both intercepts (temperature, F = 2.424, p-value = 0.129 for muscle,
446 and F = 0.811, p-value = 0.375 for liver) and slopes (temperature × LPO, F = 1.035, p-value
447 = 0.336 for muscle and F = 2.694, p-value = 0.083 for liver) are statistically equal, showing
448 that there is the same amount of lipid contents when there are no lipid peroxides at both
449 temperatures and that the rate of lipid content change per unit of lipid peroxides is also the
450 same at 26 ˚C and at 30 ˚C.
451
452 3.3. Fatty acid profiles
453 The list of FA analyzed in this study is presented in Table 1 (for complete data matrices
454 with mean ± SD fatty acid masses at different temperatures, tissues and times see
455 supplemental material, Tables S1 and S2). The most abundant FA in *A. ocellaris* liver

456 tissue were palmitic acid, stearic acid, linoleic acid and oleic acid (16:0, 18:0, 18:2n-6c and
457 18:1n-9c, respectively), whereas palmitic acid, linoleic acid, oleic acid and
458 docosahexaenoic acid (16:0, 18:2n-6t, 18:1n-9c and 22:6n-3 respectively) were the most
459 abundant in muscle tissue (see Tables S1 and S2). Results from PCA analyses, in which
460 FA were plotted grouped by factors (cumulative explained variance of 69.5 % for PC1 and
461 PC2) showed i) no specific patterns for temperature groups (Fig. 1), ii) two clear clusters
462 separating muscle from liver (Fig. 2), and also iii) no specific patterns for time groups (Fig.
463 3). Additionally, FA 14:0, 16:1n-7, 18:2n-6, 18:1n-9, 20:5n-3, 20:3n-6, 20:2n-3 and 20:1n-9
464 were all positively correlated, which in turn were negatively correlated to 18:0, 17:0, 22:5n-
465 3, 22:0, 24:1n-9, 26:2n-6 and 27:2. The collective behavior of these FA is mainly
466 represented by PC1. PC2 is influenced most by FA 15:0, 19:1n9, and 24:0, which are
467 positively correlated, and 20:4n-6, 22:5n6, 22:4n6, 22:2n-6, which were negatively
468 correlated to the previous ones (see Fig. S3 of PCA biplot in supplemental material).
469
470 In the heat map analyses of FA classes in response to temperature, results showed an
471 increasing trend of omega-3, -6, -7 and -9, as well as SFA, PUFA and HUFA at elevated
472 temperature, but a decreasing trend of MUFA and EPA/DHA as well as ARA/EPA ratios
473 (Fig. 1). Trends in FA classes between different tissues (Fig. 2) showed that liver displayed
474 higher concentrations of all classes and ratios when compared to muscle, except for
475 ARA/EPA ratio, which was higher in muscle. Finally, trends in FA classes between time
476 groups (Fig. 3) suggest oscillations in FA concentration along time, with higher
477 concentrations at T0, T14 and T28, and lower overall concentrations at T7 and T21.
478
479 Multi-factorial PERMANOVA testing the effects of temperature, tissue and time on fatty
480 acids and fatty acid classes and ratios showed significant differences for tissue (Pseudo-F
481 = 61.091, P(perm) < 0.001), time (Pseudo-F = 2.394, P(perm) = 0.004) and the interaction
482 of both (Pseudo-F = 1.966, P(perm) = 0.017) (Table 2). No significant differences were
483 found for temperature effects, or interactions of time and tissue with temperature. Pairwise
484 tests for time groups indicated that T14 was different from the rest of time levels except for

T0 (T7 vs. T14, P(perm) = 0.004; T14 vs. T21, P(perm) = 0.001; T14 vs. T28, P(perm) = 0.028). No other differences were found between pairwise combinations of T0, T7, T21 and T28. Moreover, pairwise tests for the interaction of tissue with time showed that liver was different from muscle at all time levels (T0, T7, T14, T21 and T28 all had P(perm) < 0.001) and that within liver, T0 differed from T7 (P(perm) = 0.045) and that T14 was also different from the remaining time levels (T0 vs. T14, P(perm) = 0.005; T7 vs. T14, P(perm) = 0.001; T14 vs. T21, P(perm) = 0.007; T14 vs. T28, P(perm) = 0.002). Within muscle, no differences were detected along time, from T0 to T28.

Results from SIMPER analysis are presented in Table 3 for the effects of independent factors tissue and time on FA profiles. Up to 90 % of cumulative FA contribution to significant differences observed among tissues and among time levels was homogenously distributed between 25 and 31 fatty acid variables, out of the 38 analyzed. The top five variables contributing to tissue differences were 27:2, 18:0, 26:2n-6; 17:0 and ∑SFA, whereas the top five variables contributing to multiple significant time comparisons were: 14:0, 20:3n-3+20:3n-6, 16:1n-7, 19:1n-9 and 20:5n-3 (Table 3).

3.4. Quantitative functional enrichment and pathway analysis

Based on PERMANOVA results, enrichment and pathway analysis were performed for significant effects of tissue on FA profiles. Since no significant effects of temperature were detected in FA concentrations, this implies that there are also no significant changes in FA pathway topology or function under elevated temperature, when compared to the control temperature. Effects of time, although significant on FA profiles, were not used in these analyses, as oscillation of FA concentration along time is a regular occurrence of minor interest to the objectives of this research.

Functional enrichment and pathway analysis were based on 20 FA for differences between tissues. QEA identified 9 relevant metabolic pathways that showed significant differences between liver and muscle tissues (FDR < 1.0E-12, Fig. 4). Among these pathways, the

most relevant was α-linolenic and linoleic acid metabolism with an impact of 0.25 for topology, indicating that the involved FA (C18:2n-6c, C20:5n-3, C20:3n-6, C22:6n-3, C22:5n-3, see Table S3) are central metabolites in this pathway.

4. Discussion

As modulators of biological membrane properties, lipids play a vital role in physiological stress management and capacity for adaptive responses in the face of climate change (Eghtesadi-Araghi and Bastami, 2011; Ernst et al., 2016). Here, we tested whether a tropical shallow-water reef fish, *A. ocellaris*, displays thermal adaptations at the lipid level that enable it to sustain elevated environmental temperatures. Specifically, we investigated temperature induced changes in total lipids, lipid peroxidation, fatty acid profiles and metabolic pathways in fish tissues that may signal either acclimation and tolerance or impaired health processes (see Balogh et al., 2013; Neidleman, 1987), coupled to weight assessments. Our main purpose was to evaluate warm-adapted species heat coping strategies under ocean warm pool expansion scenarios.

Stability of lipid signatures as a thermal adaptation mechanism of juvenile clownfish

Overall, our results showed that bioenergetic balance, as well as fatty acid concentrations, chain lengths and lipid metabolic pathways remained mostly unchanged under elevated temperature in the studied tropical reef fish. Additionally, even with increasing levels of lipid peroxides under elevated temperature, overall lipid contents in *A. ocellaris* were unchanged and lipid accumulation rates also remained the same at 26 ˚C and 30 ˚C. This suggests that lipid peroxide levels measured in muscle and liver tissues at elevated temperature were merely a reflection of faster metabolism and higher respiration rates that occur during acclimation to high temperature, given that no energy tradeoff was observed. In fact, several authors have suggested that lipid peroxides, which occur mostly on phospholipids, PUFA, glycolipids and cholesterol (Ayala et al., 2014; Parrish, 2013), may act as crucial signaling molecules that initiate stress protein responses and membrane remodeling to

543 control the extent of cellular thermo-sensitivity (Csoboz et al., 2013; Liu et al., 2020), which
544 was described by Török et al. (2014) as the 'membrane sensor hypothesis'. When stress
545 levels remain below the threshold level of deleterious effects or when animals display
546 increased resistance to peroxidation (Munro and Blier, 2012), an acclimation process is
547 therefore induced until a new stage of physiological homeostasis is attained. This process
548 is temporary and usually lasts between 1 to 3 weeks (Lesser, 2006; Okazaki and Saito,
549 2014), a duration similar to our experiment trials. According to previous studies with
550 clownfish (see Madeira et al. 2016, 2017), a time-dependent induction of the heat stress
551 response did occur in the presence of lipid peroxides at 30 ˚C (e.g. induction of Hsp70 and
552 antioxidant enzymes such as catalase), which eventually resulted in full acclimation of this
553 species. Interestingly, previous studies have also suggested that peroxidation index and
554 longevity are negatively correlated in many vertebrate animals (Christen et al., 2020; Munro
555 and Blier, 2012), and ocellaris clownfish are known to live up to 10 years in the wild. This is
556 a considerable lifespan in comparison with other coral reef fish species and may be
557 partially related to generally low peroxidation levels for this species.
558
559 Moreover, FA profiles remained similar between temperature treatments and no
560 interactions of temperature with tissue type were detected. We also observed that
561 concentrations of unsaturated fatty acids as well as of saturated fatty acids between
562 temperature treatments remained stable, contrary to what would be expected during
563 adjustments of lipid properties under temperature change. These adjustments in fatty acid
564 saturation levels are crucial for plastic changes in cell membrane rigidity, thickness and
565 relative hydrophobicity (Neidleman, 1987). Nevertheless, it is worth noting that Hernando et
566 al. (2018) found that the maintenance of a stable unsaturated to saturated FA ratio actually
567 seemed to be important to mitigate lipid damage under environmental stress, but there is
568 still no clear consensus on the underlying biological pathways.
569
570 The assessed tissue of ocellaris clownfish contained low overall levels of HUFA (~ 13 % of
571 total FA), which are the most susceptible to warming as they lead to higher production of

572 reactive alkyls that induce denaturation of functionally important biomolecules (Christen et
573 al., 2020; Valles-regino et al., 2015). This characteristic coupled to i) low overall EPA levels
574 (< 1 % of total FA in both tissues) which have been found to increase thermal resistance
575 (Christen et al., 2020) (for instance in contrast to Atlantic salmon which has ~ 5 % EPA in
576 total muscle FA and is considered rich in n-3 EPA contents, Horn et al., 2020), and to ii)
577 high overall MUFA levels (~ 17 % of total FA), which are preferential fuels for energy
578 metabolism in fish (Sidell et al., 1995) and play a significant role in maintaining ATP levels
579 can explain juvenile clownfish tolerance to elevated temperature. Indeed, we found no
580 further evidence of alteration in lipid metabolic pathways in clownfish at 30 ˚C in the
581 assessed life-stage.
582
583 The maintenance of FA ratios among temperature treatments corroborates our previous
584 results, given that altered ratios of different omega fatty acids can signal physiological
585 imbalance. For instance, maintaining high levels of n-7 fatty acids is thought to improve
586 heart function in vertebrates (Vannice and Rasmussen, 2014). This is an important feature
587 for ectothermic animals, given that heart function relies on tissue aerobic capacity (Christen
588 et al., 2020), and high temperatures lead to increased heart rate and oxygen demand
589 (Pörtner et al., 2004). Moreover, n-9, n-6 and n-3 fatty acids are important mediators in pro-
590 and anti-inflammatory pathways, which are usually triggered by higher availability and/or
591 oxidation of fatty acids (Calder, 2011, 2010). Therefore, these FA play significant roles in
592 stress responses. For instance, a balanced EPA/DHA ratio is thought to be efficient at
593 reducing changes in ROS and for curtailing systemic inflammation, as the metabolization of
594 n-3 FA gives rise to resolvins, which have powerful anti-inflammatory action (Calder, 2012,
595 2011). On the other hand, an increase in ARA/EPA ratio leads to a cascade of pro-
596 inflammatory effects (e.g. ARA is a precursor of inflammatory prostaglandins and
597 leukotrienes as well as proapoptotic metabolites) (Christen et al., 2020; Innes and Calder,
598 2018). Therefore, the stability of such FA ratios suggests that a healthy and balanced
599 metabolism was maintained in fish at 30 ˚C. Similarly, Coleman et al., (2019) found that
600 health, tissue biochemistry and nutritional properties of the yellowfin bream (*Acanthopagrus*

australis) were unaffected under near-future temperature conditions. The authors suggest that this may be an evolutionary consequence of species inhabiting environmentally challenging conditions, as may be the case for species inhabiting shallow coral reefs.

Additionally, this fish species showed no signs of weight changes after 28 days of exposure to 30 ˚C. The combination of these results suggests that juvenile *A. ocellaris* has the ability to persist in an elevated temperature regime. The thermal tolerance of this species' life-stage had also been observed in a previous study that evaluated juvenile *A. ocellaris* thermal preference, thermal limits and aerobic scope. Indeed, Velasco-Blanco et al., (2019), showed a thermal window width of 301.5 $^{\circ}C^2$ and a critical thermal maximum of 34 ˚C to 40 ˚C for juvenile clownfish (depending on acclimation temperature). This clearly evidences eurythermal characteristics for this life-stage. As Christen et al., (2020) noted, it may well be that upper thermal limits are potentially related to cell and organelle membranes robustness against oxidation and maintenance of lipid peroxide levels below deleterious thresholds, as observed in this study. Nevertheless, other authors have observed that juveniles' growth rate and condition at high temperature in related *Amphiprion* spp. seem to vary widely (Rushworth et al., 2011).

It should be noted that this life stage is known to be more tolerant to heat than larvae or adults (e.g., as shown for *Sparus aurata*, Madeira et al., 2020), as juveniles generally have wider thermal windows (Dahlke et al., 2020). This is relevant considering that *A. ocellaris* is likely more exposed to high temperatures during the reef phase of its life cycle (that includes juveniles, adults and embryos) where it inhabits shallow waters, when compared to the oceanic larval phase. Within the reef phase, we expect that embryos and spawning adults may possibly display higher vulnerabilities than juveniles, as the latter seem to be well-equipped to deal with changing environmental conditions, as would be expected from a life-stage that is ready to settle in new reef environments. Interestingly, other studies with species from the *Amphiprion* genus have shown that adults exposed to 30 ˚C had reduced reproductive output (*A. melanopus,* Miller et al., 2015) and that adult dominant behavior

630 and aggression towards conspecifics seems to be accentuated with increasing temperature
631 (*A. latezonatus*, Rushworth et al., 2011), which may result in higher mortality. To
632 corroborate this, a recent meta-analysis by Dahlke et al., (2020) of 694 marine and
633 freshwater fish species from all climatic zones showed that the adult mature stage is often
634 the most sensitive to environmental challenges in the life cycle of a fish, as temperature
635 requirements for reproduction and energetic trade-offs represent a critical bottleneck at this
636 stage. Additionally, recent research has shown that (i) temperature effects on reproduction
637 are modulated by nutritional status in coral reef fish (Donelson et al. 2010), and (ii) long-
638 term thermal stress may cause decreased egg and sperm quality and viability in reef fish,
639 including *Amphiprion* species (Miller et al., 2015). This is partially associated with reduced
640 lipid reserves and yolk volume and/or lack of adequate fish nutrition (Bobe, 2015). There
641 may even be a complete inhibition of reproduction in species that are constrained in their
642 capacity to shift geographic range (Pankhurst and Munday, 2011), as is the case of coral-
643 reef dependent species. Ultimately, thermal stress in the most severe cases can lead to a
644 disarrangement of compensatory restructuring of FA in fish tissues, causing health
645 impairments, organ failure and death (Liu et al., 2020). Given that our experiments only
646 lasted for 28 days, it is unknown whether longer exposures could have different outcomes.
647 We emphasize that other life-stages would have to be evaluated before conclusions can be
648 drawn on the overall species tolerance level, warranting further studies in *A. ocellaris*.
649
650 Finally, the increase in temperature tested in this study is still within the thermal range of
651 the species as a whole (22 ˚C – 30 ˚C) and can be experienced by fish populations in
652 specific locations. Thus, a 4 ˚C increase in temperature may be an abiotic change that
653 clownfish can broadly tolerate, except possibly at its lowest latitude limit. If 30 ˚C were to
654 become the “new normal” for this fish species, it would be relevant to investigate whether
655 population thermal history and local adaptation comparing the center to the edge of the
656 distribution range populations leads to distinct response patterns and thermal adaptation
657 ability. Moreover, if temperature further increases up to 32 ˚C during El Niño events,
658 organisms may switch from a physiological compensation strategy (i.e., allocation of energy

659 to cellular defenses, repair mechanisms and molecular adjustments) to a metabolic
660 conservation strategy (i.e., blocking stress responses, with severe deleterious effects) (see
661 Petitjean et al., 2019). In the second option, severe thermal stress would likely lead to a
662 depletion of lipid reserves with a loss of lipid storage capacity due to bioenergetic
663 imbalance and lipid damage (Klepsatel et al., 2016), causing sublethal stress and animal
664 mortality.
665
666 *Enhanced lipid metabolic pathways: muscle vs. liver*
667 The overall amount of lipid contents was higher in liver in comparison to muscle, as were
668 the overall concentrations of each FA per mg of dry tissue, demonstrating that this organ is
669 an important source of these biomolecules, similarly to other marine and freshwater fish
670 (e.g. *Pagellus acarne*, *Sardina pilchardus*, *Trachinus draco,* Guil-Guerrero et al., 2011 and
671 *Salmo trutta macrostigma,* Haliloğlu et al., 2011). However, this natural richness in lipids
672 did not seem to increase the thermo-sensitivity of liver tissue, contrary to what most
673 literature suggests (e.g. see study by Sun et al., 2019 with freshwater fish *Cyprinus carpio*).
674
675 The differences found in fatty acid profiles between muscle and liver tissues were mainly
676 related to the abundance of long chain fatty acids, which had higher concentrations in liver.
677 Most FA in animal tissues is usually composed of 16 to 18 carbons (Sargent et al., 1997),
678 which are dietary FA, and the way in which these and the other FA produced by the
679 organism are then used by the various organs is reflected in their structure and function. In
680 liver, which is the main organ where FA are synthesized, stored and metabolized (Boglino
681 et al., 2012; Guil-Guerrero et al., 2011; Haliloğlu et al., 2011), we found higher relative
682 proportions of SFA and PUFA, although absolute concentrations of all FA classes were
683 higher per mg of liver tissue. In contrast, muscle had higher relative proportions of MUFA,
684 PUFA and HUFA, when compared to other FA classes. This may be related to the fact that
685 muscle metabolism presents a considerable degree of flexibility. Although glycolysis is the
686 main pathway for energy production in this tissue, thermogenic functions in muscle may
687 lead to lipid uptake, with FA mobilization from storage to active sites under mild exercise, or

688 during starvation when glycogen reserves are not readily available, or even during
689 overfeeding, as lipid oversupply inhibits glucose oxidation (Hocquette et al., 2010; Morales
690 et al., 2017; Sheridan, 1988). These differences in organ function and metabolic pathways
691 explain the differences we found between their FA profiles.
692
693 Our results from pathway analysis with gene enrichment tools between tissues confirm
694 variability in metabolic pathways, as the differences found were mainly related to fatty acid
695 biosynthesis (which occurs mostly in liver) and β-oxidation (which occurs in muscle).
696 Nevertheless, the most impactful pathway was α-linolenic and linoleic acid metabolism,
697 which was enhanced in liver. This pathway leads to the formation of omega-3 and omega-6
698 fatty acids through a series of conversions of the precursors α-linolenic and linoleic acid
699 (respectively), which are then metabolized into a series of short-lived metabolites
700 (eicosanoids) that act as signaling molecules in cells (Salway, 2013). These eicosanoids
701 play important roles in stress responses (autocrine, paracrine and endocrine functions, e.g.
702 mounting or inhibiting inflammation and other immune responses, changes in ion transport,
703 regulation of cell growth and blood flow to tissues, see Calder, 2011; Calder, 1998). The
704 enhancement of this pathway in liver when compared to muscle of *A. ocellaris* therefore
705 suggests that liver tissue may be significantly involved in acclimation and maintenance of
706 homeostasis during environmental stress.
707
708 *Temporal oscillations in FA profiles*
709 Our results showed significant temporal variations in FA profile throughout the experiment
710 (with 28 FA contributing to 90 % of differences found), and interactions between time and
711 tissue indicated that these occurred mainly in liver during the first two weeks of the
712 experiment. Arguably, liver serves a major role in lipid storage especially in slow swimming
713 teleost fish (Braekkan, 1959), as is the case of ocellaris clownfish. More importantly, liver is
714 the interface between exogenous and endogenous fatty acid interorgan transport loops in
715 fish (Sheridan, 1988). For instance, liver repackages dietary lipids and combines it with *de*
716 *novo* synthesized lipids and carrier proteins for transport and delivery to other organs and

717 storage sites (e.g. see studies by Black & Skinner, 1986, and by Salmerón, 2018, in
718 rainbow trout *Oncorhynchus mykiss* and a review of several fish species – including
719 zebrafish *Danio rerio*, Salmoniformes and Perciformes, respectively). Therefore, it is
720 expected that as lipids are mobilized from liver to supply energetic needs of different
721 tissues as juvenile fish grow, the FA profile in liver will vary with time. Moreover, the
722 regulation of lipid mobilization throughout time depends on various hormones that act as
723 adipokinetic agents (Sheridan, 1988) and modulate endogenous lipogenesis and lipolysis
724 (e.g. insulin, growth factors, cortisol and sex hormones, Weil et al., 2013). In turn,
725 hormones can be affected by several internal or external factors. Given that water
726 parameters (except for temperature in this study, which did not affect FA profiles), as well
727 as feed supplied to animals during the experiments were the same for all animals at all
728 times, it is likely that other factors affecting hormones were at play, including intrinsic ones
729 such as developmental stage and sexual maturity (Leaf et al., 2018; Li et al., 2018). In our
730 experimental trials, each tank contained several fish in typical dominance-based social
731 groups. Dominance is a key determinant of development and maturation in this fish species
732 (Khoo et al., 2018; Mitchell and Dill, 2005), as there is usually a dominant fish pair that
733 reaches sexual maturity and smaller non-breeding fish within each social group. Although
734 we did not assess developmental status throughout the experiment, we suggest that
735 temporal lipid dynamics may have been affected as social relationships between fish
736 changed throughout the experiment. For instance, after each sampling time point, when
737 some fish were removed from tanks and euthanized for analyses, rearrangements in social
738 dominance may have occurred. Social dominance not only affects growth and maturation of
739 fish through hormonal changes, but could also influence the food intake of the smaller fish
740 which are lower in hierarchy, leading to oscillations in lipid accumulation and mobilization
741 and, consequently, FA profile over time.
742
743
744 5. Conclusions

745 Overall, current increases in tropical ocean temperatures do not affect *A. ocellaris* lipid
746 contents, fatty acid profile and lipid metabolism or weight, suggesting that stable lipid
747 signatures may be an adaptive feature of thermal tolerant juvenile fish stages, as also
748 recently suggested for marine invertebrate species (e.g. marine annelids, Madeira et al.
749 2021). By maintaining overall energetic balance at higher temperature, these organisms
750 may persist during the expansion of ocean warm pools, at least in the short term. Whether
751 the thermal tolerance found in this study will allow long-term persistence of the species in
752 the environment under temperature regime changes will however ultimately depend on
753 other interactive factors, such as i) *A. ocellaris* life-stage specific vulnerability and capacity
754 for adaption on evolutionary timeframes; ii) habitat suitability, as coral reefs have already
755 been severely affected at 30 ˚C in past years, see Hoegh-Guldberg et al., 2017 for a
756 review); iii) clownfish host anemone survival under climate change, as there have reports of
757 anemone mortality or reduction in size during bleaching events, with detrimental effects on
758 anemonefish populations, including reduced social group sizes, decreased dominant fish
759 length, and reduced fecundity and recruitment, namely in *Amphiprion* spp. (Mitchell and
760 Dill, 2005; Saenz-Agudelo et al., 2011; Beldade et al., 2017); and iv) plankton nutrition
761 quality and availability, coupled to foraging efficiency of ocellaris clownfish in warming
762 oceans (Nowicki et al., 2012).
763
764 We also conclude that FA profile differences between muscles and liver coupled to specific
765 enhanced lipid metabolic pathways in liver, namely α-linolenic and linoleic acid metabolism,
766 indicate that liver plays an important role in maintaining homeostasis during
767 environmentally challenging situations, through the production of lipid-derived metabolites
768 that drive stress responses and/or acclimation. Additionally, temporal changes detected in
769 FA profiles, mainly in liver, may be a result of growth processes that modulate organism
770 lipid metabolism through time.
771
772 Finally, we conclude that the influence of future oceans in organisms' lipid signatures may
773 be difficult to predict, as species-, population- or even life-stage-specific mechanisms seem

774 to regulate energy and lipid homeostasis. Particularly, we highlight the need for research
775 comparing climate change effects on lipid metabolic networks and plasticity mechanisms
776 between wild populations from different areas of the distribution range, or among species
777 along natural thermal gradients, to infer selection gradients that influence energy-limited
778 tolerance to stress. Translocation experiments would also be useful to test the influence of
779 local adaption on lipid responses to environmental change.
780
781 For now, it seems plausible to expect that changes in individuals' FA profiles and
782 composition may be less affected when compared to overall changes in lipid availability in
783 ecosystems as consequence of shifts in taxonomic structure of communities and
784 biodiversity reorganization with climate change.
785
786
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FIGURE CAPTIONS

Fig. 1 Fatty acid profiles of clownfish *Amphiprion ocellaris* subjected to 26 ˚C (n = 21 fish) and 30 ˚C (n = 17 fish). **(A)** PCA 2D score plot of FA profiles **(B)** heatmap of clustered FA classes; the colour scale ranges from red (higher than mean concentration) to blue (lower than mean concentration). Rows are the fatty acid classes and ratios, and columns are the mean of sample values. No significant differences were found in FA profiles between temperatures (PERMANOVA Pseudo-F = 0.910, p-value = 0.435).

Fig. 2 Fatty acid differences between muscle vs liver tissue, analysed in n = 38 clownfish. **(A)** PCA 2D score plot of FA profiles **(B)** heatmap of clustered FA classes; the colour scale ranges from red (higher than mean concentration) to blue (lower than mean concentration). Rows are the fatty acid classes and ratios, and columns are the mean of sample values. Significant differences were found in FA profiles between tissues (PERMANOVA Pseudo-F = 61.091, p-value < 0.001).

Fig. 3 Fatty acid differences between T0 vs T7 vs T14 vs T21 vs T28 sampling timepoints assessed in the clownfish *Amphiprion ocellaris* (n = 3-5 fish per time-point). **(A)** PCA 2D score plot of FA profiles **(B)** heatmap of clustered FA classes; the colour scale ranges from red (higher than mean concentration) to blue (lower than mean concentration). Rows are the fatty acid classes and ratios, and columns are the mean of sample values. Significant

differences were found in FA profiles between times (PERMANOVA Pseudo-F = 2.394, p-value = 0.004).

Fig. 4 Quantitative enrichment and pathway analysis of clownfish (*Amphiprion ocellaris*) fatty acid concentration comparing muscle vs liver tissues. **(A)** Metabolite set enrichment analysis showing significantly enriched pathways based on the group of 20 functionally related FA used as input data **(B)** pathway topology analysis, higher pathway relevance if impact > 0.1 and -log(p) > 8 (for FDR < 0.05). All the matched pathways are displayed as circles and the colour and size of each circle are based on p-value and pathway impact value, respectively.

TABLES

Table 1. List of twenty-six fatty acids analyzed in this study. Abb: FA – fatty acids; SFA – saturated fatty acids; MUFA – mono-unsaturated fatty acids; PUFA – polyunsaturated fatty acids; HUFA – highly unsaturated fatty acids; NA – Not available; LMSD – Lipid Maps Structure Database; KEGG – Kyoto Encyclopedia of Genes and Genomes; HMBD – Human Metabolome Database.

Analytes (FA)	Common name	Formula	Class	Omega	LMSD ID	KEGG ID	HMDB ID	Retention time (min)
14:0	Myristic acid	$C_{14}H_{28}O_2$	SFA	-	LMFA01010014	C06424	HMDB0000806	14.91
15:0	Pentadecylic acid	$C_{15}H_{30}O_2$	SFA	-	LMFA01010015	C16537	HMDB0000826	17.29
16:1n-7	Cis-9-Palmitoleic acid	$C_{16}H_{30}O_2$	MUFA	n-7	LMFA01030056	C08362	HMDB0003229	19.17
16:0	Palmitic acid	$C_{16}H_{32}O_2$	SFA	-	LMFA01010001	C00249	HMDB0000220	19.71
17:1n-7	10Z-Heptadecenoic acid	$C_{17}H_{32}O_2$	MUFA	n-7	LMFA01030283	C16536	HMDB0060038	21.52
17:0	Margaric acid	$C_{17}H_{34}O_2$	SFA	-	LMFA01010017	NA	HMDB0002259	22.01
18:2n-6c	Linoleic acid	$C_{18}H_{32}O_2$	PUFA	n-6	LMFA01030120	C01595	HMDB0000673	23.54
18:2n-6t	Linoelaidic acid	$C_{18}H_{32}O_2$	PUFA	n-6	LMFA01030123	NA	HMDB0006270	23.72
18:1n-9c	Cis-Oleic acid	$C_{18}H_{34}O_2$	MUFA	n-9	LMFA01030002	C00712	HMDB0000207	23.72
18:1n-9t	9-elaidic acid	$C_{18}H_{34}O_2$	MUFA	n-9	LMFA01030073	C01712	HMDB0000573	23.85
18:0	Stearic acid	$C_{18}H_{36}O_2$	SFA	-	LMFA01010018	C01530	HMDB0000827	24.30
19:1n-9	10Z-nonadecenoic acid	$C_{19}H_{36}O_2$	MUFA	n-9	LMFA01030362	C00174	HMDB0013622	25.92
20:5n-3	Eicosapentaenoic acid (EPA)	$C_{20}H_{30}O_2$	HUFA	n-3	LMFA01030759	C06428	HMDB0001999	27.11
20:4n-6	Arachidonic acid (ARA)	$C_{20}H_{32}O_2$	HUFA	n-6	LMFA01030001	C00219	HMDB0001043	27.25
20:3n-3	11,14,17-eicosatrienoic acid	$C_{20}H_{34}O_2$	PUFA	n-3	LMFA01030159	C16522	HMDB0060039	27.50
20:3n-6	8Z,12E,14Z-eicosatrienoic acid	$C_{20}H_{34}O_2$	PUFA	n-6	LMFA01030388	C03442	HMDB0002925	27.50
20:1n-9	Cis-gondoic acid	$C_{20}H_{38}O_2$	MUFA	n-9	LMFA01030085	C16526	HMDB0002231	28.05
20:0	Arachidic acid	$C_{20}H_{40}O_2$	SFA	-	LMFA01010020	C06425	HMDB0002212	28.60
22:6n-3	Docosahexaenoic acid (DHA)	$C_{22}H_{32}O_2$	HUFA	n-3	LMFA01030185	C06429	HMDB0002183	31.06
22:5n-3	Docosapentaenoic acid (DPA)	$C_{22}H_{34}O_2$	HUFA	n-3	LMFA04000044	C16513	HMDB0006528	31.22

22:4n-6	Adrenic acid	$C_{22}H_{36}O_2$	HUFA	n-6	LMFA01030178	C16527	HMDB0002226	31.35
22:2n-6	13Z,16Z-docosadienoic acid	$C_{22}H_{40}O_2$	PUFA	n-6	LMFA01030405	C16533	HMDB0062219	32.00
22:0	Behenic acid	$C_{22}H_{44}O_2$	SFA	-	LMFA01010022	C08281	HMDB0000944	32.60
24:1n-9	Nervonic acid	$C_{24}H_{46}O_2$	MUFA	n-9	LMFA01030092	C08323	HMDB0002368	35.86
26:2n-6	17,20-hexacosadienoic acid	$C_{26}H_{48}O_2$	PUFA	n-6	LMFA01030133	NA	NA	39.11
27:2	5Z,9Z-heptacosadienoic acid	$C_{27}H_{50}O_2$	PUFA	NA	LMFA01020363	NA	NA	41.91

Table 2. Multivariate PERMANOVA results, testing the main effects of Temperature (26 °C vs 30 °C), Tissue (muscle vs liver) and Time (T0, T7, T14, T21, T28) as well as factor interactions on fatty acids concentration. Significant results are presented with an asterisk.

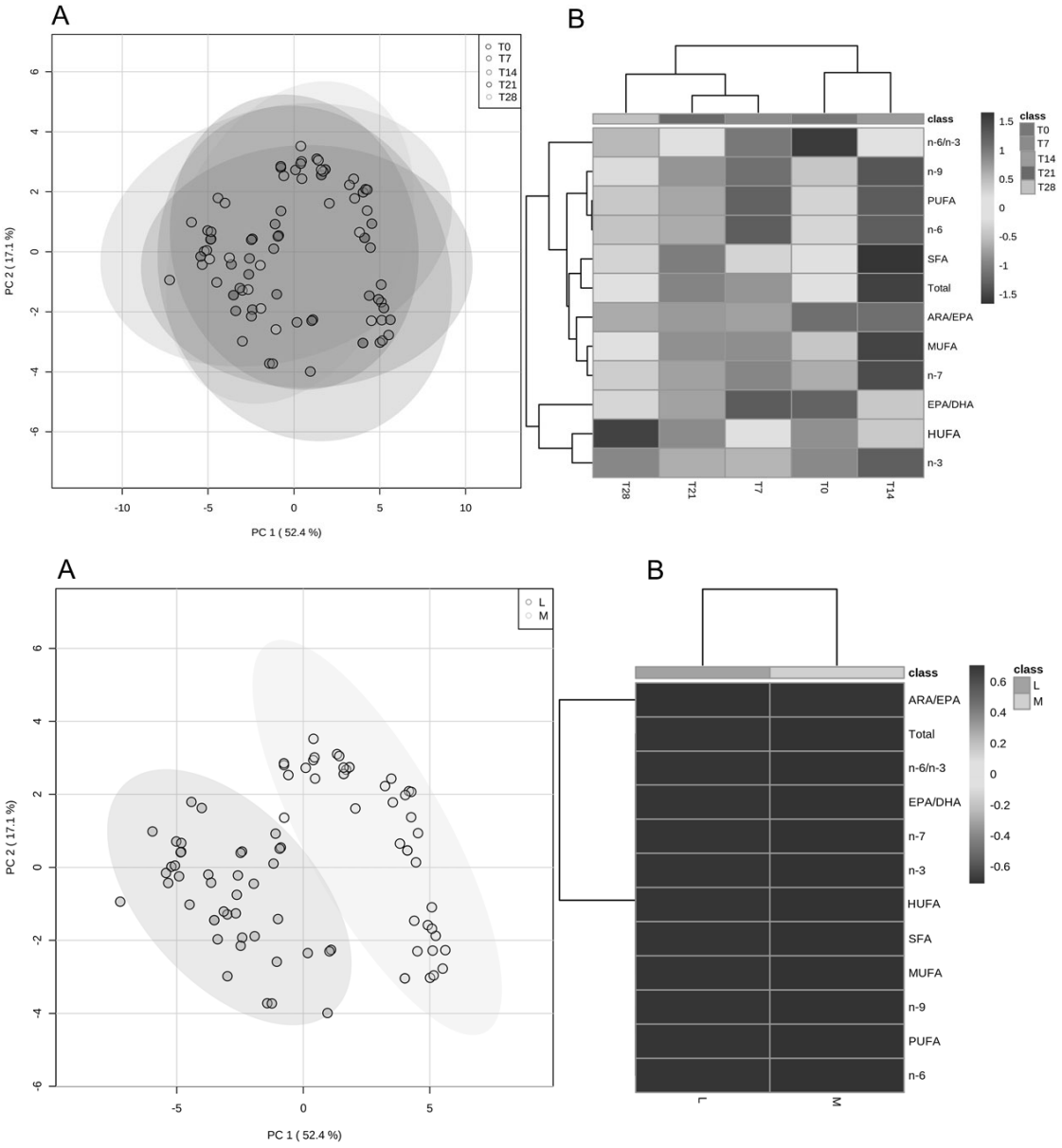
	df	***MS***	***Pseudo-F***	***P(perm)***	***perms***
Temperature	1	17.595	0.910	0.435	9935
Tissue	1	1181.300	61.091	<0.001*	9929
Time	4	46.287	2.394	0.004*	9916
Temperature × Tissue	1	19.527	1.010	0.372	9929
Temperature ×Time	4	20.910	1.081	0.351	9921
Tissue × Time	4	38.008	1.966	0.017*	9919
Temperature × Tissue × Time	4	17.211	0.890	0.569	9903

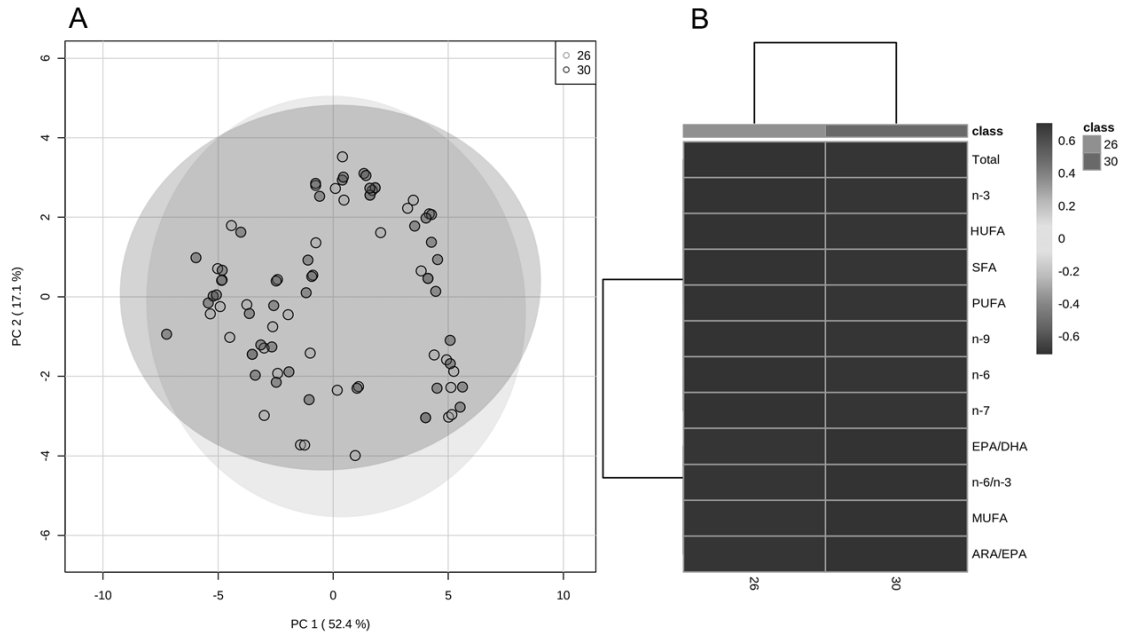
Table 3. Similarity of percentage analysis (SIMPER) of FA relative contribution to significant differences found among tissues and time groups (cutoff threshold at 90 % cumulative contribution).

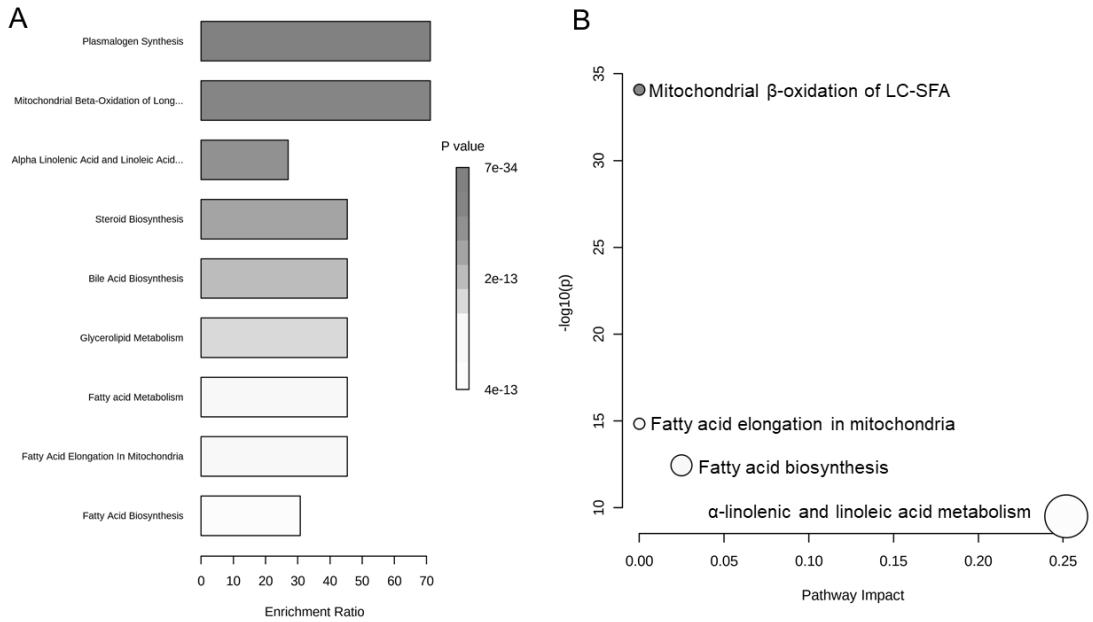
Tissue			**Time**								
M vs. L			**T7 vs. T14**			**T14 vs. T21**			**T14 vs. T28**		
Fatty acid	*Contrib%*	*Cum.%*	*Fatty acid*	*Contrib%*	*Cum.%*	*Fatty acid*	*Contrib%*	*Cum.%*	*Fatty acid*	*Contrib%*	*Cum.%*
27:2	3.65	3.65	*14:0*	6.05	6.05	*14:0*	5.91	5.91	*14:0*	7.72	7.72
18:0	3.6	7.25	*20:3n-3 + 20:3n-6*	5.5	11.55	*20:3n-3 + 20:3n-6*	5.64	11.55	*20:3n-3 + 20:3n-6*	6.51	14.23
26:2n-6	3.5	10.75	*16:1n-7*	4.68	16.23	*20:5n-3*	4.98	16.52	*16:1n-7*	5.55	19.78
17:0	3.43	14.17	*19:1n-9*	4.62	20.85	*18:2n-6t + 18:1n-9c*	4.7	21.22	*∑n-7*	4.97	24.75
∑SFA	3.39	17.56	*∑n-3*	4.57	25.41	*19:1n-9*	4.17	25.39	*20:5n-3*	4.25	29.01
∑Total	3.35	20.91	*22:6n-3 (DHA)*	4.47	29.89	*20:4n-6 (ARA)*	4.09	29.48	*22:2n-6*	3.98	32.99
18:1n-9t	3.31	24.22	*∑n-7*	4.28	34.17	*15:0*	4.09	33.57	*19:1n-9*	3.91	36.9
24:1n-9	3.3	27.52	*∑HUFA*	4.21	38.38	*22:6n-3 (DHA)*	4.08	37.66	*EPA/DHA*	3.89	40.78
∑PUFA	3.21	30.74	*EPA/DHA*	4.01	42.38	*∑n-3*	4.04	41.7	*15:0*	3.85	44.64
∑MUFA	3.16	33.9	*22:4n-6*	3.9	46.28	*16:1n-7*	3.87	45.57	*22:6n-3 (DHA)*	3.83	48.47
ARA/EPA	3.13	37.03	*20:5n-3*	3.7	49.98	*EPA/DHA*	3.76	49.33	*20:4n-6 (ARA)*	3.73	52.2
∑n-9	3.05	40.08	*20:4n-6 (ARA)*	3.51	53.48	*∑HUFA*	3.68	53.01	*17:1n-7*	3.6	55.81
17:1n-7	3.03	43.12	*18:2n-6t + 18:1n-9c*	3.43	56.91	*∑n-7*	3.54	56.55	*20:0*	3.55	59.36
16:0	2.99	46.11	*17:1n-7*	3.26	60.17	*20:0*	3.22	59.77	*∑n-3*	3.51	62.87
22:5n-3 (EPA)	2.87	48.98	*22:2n-6*	3.11	63.28	*17:1n-7*	3.03	62.8	*∑HUFA*	3.46	66.32
∑n-6	2.86	51.84	*22:0*	3.02	66.3	*22:4n-6*	2.98	65.77	*22:4n-6*	3.41	69.74
∑n-6/∑n-3	2.84	54.68	*15:0*	3.01	69.31	*22:0*	2.62	68.39	*18:2n-6t + 18:1n-9c*	2.87	72.61
EPA/DHA	2.81	57.49	*∑n-6*	2.8	72.1	*22:2n-6*	2.44	70.83	*∑MUFA*	2.35	74.96
20:1n-9	2.81	60.3	*20:0*	2.78	74.88	*∑n-6*	2.3	73.13	*∑n-6*	2.24	77.19
18:2n-6c	2.73	63.03	*18:2n-6c*	2.66	77.55	*∑n-9*	2.18	75.31	*18:2n-6*	2.14	79.33
∑n-7	2.69	65.72	*22:5n-3 (EPA)*	2.5	80.05	*∑n-6/∑n-3*	2.09	77.4	*∑n-9*	2.06	81.38
16:1n-7	2.59	68.31	*∑MUFA*	2.23	82.27	*20:1n-9*	2.04	79.44	*20:1n-9*	2.05	83.43
22:0	2.54	70.85	*20:1n-9*	2.17	84.44	*18:2n-6*	2	81.44	*∑n-6/∑n-3*	1.98	85.42
18:2n-6t + 18:1n-9c	2.52	73.37	*∑n-9*	2.11	86.55	*∑MUFA*	1.93	83.37	*22:0*	1.95	87.37
20:3n-3 + 20:3n-6	2.5	75.87	*∑n-6/∑n-3*	2.02	88.57	*ARA/EPA*	1.92	85.29	*ARA/EPA*	1.71	89.09

22:6n-3 (DHA)	2.39	78.26			*16:0*	1.87	87.17
20:5n-3	2.35	80.61			*∑PUFA*	1.62	88.79
14:0	2.34	82.95					
15:0	2.26	85.21					
22:2n-6	2.23	87.44					
∑n-3	2.2	89.64					

FIGURES


A
B
T0
T7
T14
T21
T28
PC 2 (17.1 %)
PC 1 (52.4 %)
class
n-6/n-3
n-9
PUFA
n-6
SFA
Total
ARA/EPA
MUFA
n-7
EPA/DHA
HUFA
n-3
L
M


A
B
26
30
PC 2 (17.1 %)
PC 1 (52.4 %)
class
Total
n-3
HUFA
SFA
PUFA
n-9
n-6
n-7
EPA/DHA
n-6/n-3
MUFA
ARA/EPA
0.6
0.4
0.2
0
-0.2
-0.4
-0.6


A
Plasmalogen Synthesis
Mitochondrial Beta-Oxidation of Long...
Alpha Linolenic Acid and Linoleic Acid...
Steroid Biosynthesis
Bile Acid Biosynthesis
Glycerolipid Metabolism
Fatty acid Metabolism
Fatty Acid Elongation In Mitochondria
Fatty Acid Biosynthesis
P value
7e-34
2e-13
4e-13
Enrichment Ratio
B
Mitochondrial β-oxidation of LC-SFA
Fatty acid elongation in mitochondria
Fatty acid biosynthesis
α-linolenic and linoleic acid metabolism
-log10(p)
Pathway Impact

SUPPLEMENTAL MATERIAL

Table S1. Fatty acid profile (µg.mg^{-1} dry tissue weight, mean±SD) of *A. ocellaris* muscle tissue at different temperature conditions (26 ˚C vs 30 ˚C) and timepoints (T0, T7, T14, T21, T28), N_{total} = 38 specimens.

MUSCLE	Overall control T0	26 °C					30 °C					*Overall muscle*
		T7	T14	T21	T28	*Overall 26 ºC*	T7	T14	T21	T28	*Overall 30 ºC*	
C14:0	0.96±0.79	0.96±0.77	0.60±0.42	0.24±0.02	0.74±0.43	0.74±0.65	0.62±0.87	1.11±1.16	0.30±0.13	0.85±0.36	0.78±0.75	0.76±0.66
C15:0	0.48±0.31	0.42±0.18	0.28±0.19	0.58±0.04	0.25±0.06	0.40±0.23	0.47±0.15	0.38±0.13	0.36±0.19	0.32±0.14	0.41±0.21	0.40±0.22
C16:1n7	1.69±1.16	1.55±1.37	1.42±0.61	0.27±0.08	1.42±0.72	1.35±1.07	0.93±0.87	1.95±1.38	0.87±0.75	1.47±0.77	1.40±1.13	1.38±1.10
C16:0	7.54±5.78	8.35±5.30	7.90±2.42	3.39±0.37	8.63±2.86	7.38±4.47	6.45±3.63	7.00±4.26	6.26±3.37	2.42±1.05	6.24±4.41	6.80±4.48
C17:1n7	0.30±0.15	0.43±0.38	0.27±0.09	0.14±0.01	0.28±0.12	0.30±0.23	0.21±0.11	0.38±0.17	0.19±0.09	0.29±0.10	0.28±0.15	0.29±0.19
C17:0	0.24±0.07	0.27±0.16	0.27±0.08	0.17±0.03	0.23±0.05	0.24±0.10	0.19±0.05	0.28±0.08	0.21±0.07	0.26±0.07	0.23±0.08	0.24±0.09
C18:2n6c	3.05±1.68	2.54±1.85	2.37±0.76	1.51±0.17	2.85±0.87	2.52±1.41	1.64±1.31	2.67±1.75	1.76±0.64	2.47±0.57	2.33±1.47	2.42±1.44
C18:2n6t + C18:1n9c[1]	5.93±3.06	3.38±2.75	4.81±1.89	2.82±0.69	5.61±1.67	4.57±2.59	3.99±2.30	6.25±3.70	4.07±1.82	5.75±2.27	5.20±2.95	4.89±2.80
C18:1n9t	1.94±0.98	1.61±1.16	1.41±0.59	0.61±0.06	1.74±0.67	1.52±0.93	1.18±0.73	1.94±1.22	1.33±0.95	1.44±0.58	1.59±1.00	1.55±0.97
C18:0	2.95±0.99	2.42±1.11	2.56±0.61	1.72±0.21	2.97±0.61	2.56±0.92	2.33±0.71	2.95±0.96	2.28±0.50	2.57±0.53	2.64±0.85	2.60±0.88
C19:1n9	0.27±0.14	0.41±0.17	0.22±0.14	0.41±0.08	0.23±0.02	0.30±0.15	0.29±0.14	0.23±0.10	0.28±0.09	0.28±0.19	0.27±0.13	0.28±0.15
C20:5n3	0.79±0.13	0.81±0.28	0.81±0.10	0.78±0.05	1.17±0.17	0.85±0.22	0.73±0.10	0.85±0.10	1.06±0.54	1.16±0.49	0.89±0.34	0.87±0.29
C20:4n6 (ARA)	1.48±0.28	1.58±0.48	1.21±0.31	1.01±0.11	1.76±0.51	1.41±0.44	1.25±0.33	1.31±0.30	1.56±0.51	1.23±0.38	1.37±0.38	1.39±0.41
C20:3n3 + C20:3n6[2]	0.41±0.20	0.45±0.35	0.44±0.16	0.23±0.01	0.57±0.18	0.42±0.24	0.25±0.16	0.72±0.42	0.38±0.20	0.55±0.19	0.46±0.31	0.44±0.28
C20:1n9	1.71±1.02	1.78±1.37	1.57±0.80	0.67±0.14	1.50±0.29	1.52±1.00	1.26±0.51	1.43±0.78	1.14±0.60	1.89±1.00	1.47±0.84	1.49±0.92
C20:0	0.36±0.32	0.46±0.25	0.26±0.18	0.37±0.17	0.17±0.03	0.33±0.25	0.25±0.20	0.24±0.13	0.23±0.14	0.41±0.32	0.29±0.24	0.31±0.24
C22:6n3 (DHA)	3.59±0.73	3.46±1.76	2.92±0.48	2.14±0.53	4.29±0.85	3.29±1.21	2.87±0.80	3.25±0.69	3.13±0.79	3.18±0.75	3.21±0.79	3.25±1.02

[1,2] These FA have the same retention time and are eluted in a single merged peak in the GC.

C22:5n3 (EPA)	0.23±0.07	0.36±0.23	0.20±0.04	0.18±0.01	0.23±0.09	0.25±0.14	0.20±0.07	0.20±0.07	0.21±0.04	0.22±0.04	0.21±0.06	0.23±0.11
C22:4n6	1.17±0.45	1.16±0.78	0.77±0.29	0.45±0.05	1.63±0.82	1.03±0.66	0.75±0.35	1.03±0.46	0.82±0.38	1.11±0.45	0.97±0.45	1.00±0.56
C22:2n6	2.20±1.52	1.56±1.18	1.36±0.88	0.29±0.01	1.64±0.83	1.49±1.23	1.16±1.04	1.53±1.22	0.75±0.33	1.51±1.00	1.45±1.23	1.47±1.23
C22:0	0.28±0.23	0.39±0.30	0.18±0.06	0.20±0.05	0.17±0.00	0.26±0.21	0.15±0.02	0.16±0.04	0.15±0.03	0.16±0.02	0.18±0.13	0.22±0.17
C24:1n9	0.90±0.27	0.72±0.14	0.67±0.15	0.83±0.23	0.81±0.09	0.78±0.21	0.71±0.17	0.65±0.17	0.60±0.03	0.74±0.11	0.73±0.20	0.75±0.21
C26:2n6	1.44±0.13	1.42±0.14	1.47±0.20	1.91±0.51	1.65±0.39	1.54±0.32	1.63±0.25	1.36±0.10	1.50±0.19	1.61±0.10	1.50±0.20	1.52±0.27
C27:2	1.00±0.33	0.89±0.08	1.02±0.25	1.24±0.37	1.09±0.24	1.03±0.28	1.12±0.33	0.83±0.12	0.83±0.10	1.08±0.25	0.97±0.28	1.00±0.28
∑SFA	12.81±6.94	13.27±6.95	12.05±3.19	6.68±0.41	13.16±3.93	11.91±5.69	10.46±5.03	12.12±4.80	9.79±3.87	6.98±0.78	10.78±5.32	11.33±5.53
∑MUFA	6.82±3.19	6.50±4.23	5.56±1.84	2.93±0.41	5.98±1.73	5.77±3.08	4.59±2.08	6.58±3.61	4.41±2.14	6.11±2.25	5.72±2.97	5.74±3.03
∑PUFA	4.02±6.23	10.24±4.99	11.47±3.89	7.99±1.37	13.42±2.92	11.57±4.94	9.80±4.52	13.35±7.11	9.29±2.65	12.97±3.61	11.91±5.65	11.74±5.32
∑HUFA	7.26±1.43	7.36±3.18	5.91±1.13	4.56±0.72	9.08±2.44	6.83±2.43	5.79±1.54	6.64±1.52	6.78±2.05	6.89±1.88	6.65±1.74	6.74±2.11
∑n-3	5.02±1.02	5.07±2.32	4.36±0.71	3.34±0.57	6.26±1.28	4.81±1.62	4.05±1.00	5.02±1.12	4.78±1.48	5.10±1.23	4.77±1.23	4.79±1.43
∑n-6	19.26±7.49	15.54±7.48	15.35±4.74	10.35±0.73	20.00±5.33	16.28±6.76	13.55±5.98	18.11±8.22	13.97±4.23	17.41±5.34	16.49±7.00	16.38±6.88
∑n-7	1.99±1.29	1.98±1.70	1.69±0.67	0.41±0.08	1.70±0.84	1.65±1.25	1.13±0.97	2.33±1.54	1.06±0.83	1.76±0.85	1.67±1.27	1.66±1.26
∑n-9	10.76±4.95	7.90±4.85	8.67±2.99	5.33±1.02	9.89±2.55	8.68±4.20	7.45±3.41	10.50±5.78	7.42±3.11	10.10±3.69	9.25±4.66	8.97±4.45
EPA/DHA	0.06±0.02	0.15±0.15	0.07±0.02	0.09±0.03	0.05±0.01	0.09±0.08	0.08±0.05	0.06±0.02	0.07±0.03	0.07±0.01	0.07±0.03	0.08±0.06
ARA/EPA	6.79±1.18	6.27±3.73	6.53±2.19	5.50±0.60	7.84±0.83	6.57±2.32	6.81±2.17	6.79±1.38	8.02±3.70	5.71±1.76	6.87±2.27	6.72±2.30
∑n-6/∑n-3	3.72±0.78	3.06±0.67	3.44±0.51	3.21±0.68	3.15±0.21	3.34±0.67	3.24±0.60	3.44±0.90	2.93±0.10	3.36±0.37	3.35±0.70	3.35±0.69
∑Total FA	40.91±16.79	37.37±18.72	34.98±9.80	22.17±1.67	41.63±11.01	36.08±15.15	30.64±13.03	38.69±14.98	30.28±10.50	32.96±6.81	35.05±14.14	35.56±14.65

Table S2. Fatty acid profile (µg.mg^{-1} dry tissue weight, mean±SD) of *A. ocellaris* liver tissue at different temperature conditions (26 ˚C vs 30 ˚C) and timepoints (T0, T7, T14, T21, T28), N_{total} = 38 specimens.

LIVER	**Overall control**	**26 °C**					**30 °C**					***Overall liver***
	T0	**T7**	**T14**	**T21**	**T28**	***Overall 26 °C***	**T7**	**T14**	**T21**	**T28**	***Overall 30 °C***	
C14:0	1.68±0.80	0.64±0.47	3.34±2.79	0.90±0.25	0.96±0.43	1.61±1.58	0.75±0.42	4.99±1.88	1.07±0.20	0.42±0.12	1.94±1.90	1.78±1.69
C15:0	0.51±0.19	0.51±0.17	0.51±0.10	0.53±0.02	0.51±0.16	0.51±0.15	0.44±0.10	0.75±0.28	0.48±0.17	0.35±0.07	0.52±0.23	0.52±0.19
C16:1n7	5.56±2.69	2.25±1.74	13.79±13.14	2.93±1.45	3.07±1.05	6.00±5.02	1.96±1.02	14.00±8.91	5.34±0.76	1.43±0.33	6.06±5.42	6.03±5.25
C16:0	32.31±15.32	26.25±7.20	38.94±13.81	20.03±12.37	32.77±7.01	30.76±13.45	26.26±14.10	62.23±33.18	39.76±4.41	25.83±1.42	38.21±23.36	34.57±19.53
C17:1n7	1.14±0.46	0.89±0.47	1.60±0.56	0.78±0.33	1.04±0.45	1.12±0.55	0.62±0.41	2.21±0.74	0.98±0.30	0.58±0.17	1.16±0.77	1.14±0.68
C17:0	0.95±0.33	1.15±0.38	1.20±0.34	1.49±0.08	1.49±0.04	1.21±0.36	1.31±0.30	1.41±0.40	1.30±0.47	1.46±0.20	1.27±0.40	1.24±0.38
C18:2n6c	4.66±2.10	3.78±2.56	5.13±1.06	5.61±2.32	5.37±1.27	4.80±2.07	5.33±3.25	7.97±3.48	8.36±2.65	3.01±0.42	6.01±3.33	5.42±2.85
C18:2n6t + C18:1n9c[3]	12.79±4.60	7.45±5.72	15.39±8.38	4.34±3.54	13.29±4.47	11.00±7.04	10.61±5.63	26.49±10.38	17.98±4.83	7.86±0.95	15.68±9.19	13.39±8.54
C18:1n9t	5.29±2.30	5.23±2.02	7.73±2.15	5.45±0.56	7.00±1.83	6.12±2.23	5.69±2.22	9.49±3.96	6.48±1.55	4.30±0.44	6.42±3.09	6.27±2.71
C18:0	12.02±3.68	13.74±2.54	15.07±2.14	10.08±6.10	15.66±1.01	13.40±3.84	14.44±0.92	14.67±1.97	13.58±2.34	13.41±1.15	13.65±2.51	13.52±3.23
C19:1n9	0.18±0.05	0.36±0.18	0.35±0.19	0.35±0.05	0.22±0.04	0.29±0.15	0.29±0.09	0.27±0.08	0.30±0.09	0.35±0.01	0.27±0.09	0.28±0.13
C20:5n3	1.10±0.60	0.94±0.85	1.63±1.00	1.14±0.37	1.71±0.04	1.28±0.78	1.72±0.48	2.18±0.74	0.87±0.27	0.93±0.33	1.42±0.74	1.35±0.76
C20:4n6 (ARA)	1.29±0.55	1.14±0.76	1.55±0.49	1.37±0.37	1.67±0.17	1.38±0.57	2.11±0.35	1.89±0.34	1.38±0.63	1.21±0.38	1.62±0.59	1.50±0.59
C20:3n3 + C20:3n6[4]	0.84±0.43	0.63±0.33	2.00±1.14	0.63±0.24	0.85±0.06	1.04±0.83	0.80±0.08	4.04±1.24	1.35±0.25	0.71±0.21	1.64±1.47	1.34±1.23
C20:1n9	3.58±2.01	3.14±1.57	4.72±1.00	3.76±0.69	5.24±1.53	4.01±1.66	3.41±2.24	4.38±1.83	4.24±2.19	2.32±0.26	3.67±2.03	3.84±1.87
C20:0	0.34±0.07	0.44±0.06	0.43±0.12	0.38±0.06	0.36±0.00	0.39±0.09	0.45±0.06	0.46±0.14	0.32±0.11	0.39±0.02	0.40±0.11	0.39±0.10
C22:6n3 (DHA)	1.94±0.87	1.98±1.92	2.47±2.35	2.39±0.49	2.75±0.16	2.26±1.58	3.33±0.83	3.03±0.67	2.52±0.90	2.61±0.33	2.70±0.92	2.48±1.30
C22:5n3 (EPA)	0.52±0.24	0.63±0.27	0.74±0.27	0.92±0.33	0.95±0.02	0.72±0.30	1.90±1.13	0.86±0.59	1.19±0.31	1.28±0.38	1.15±0.80	0.94±0.65
C22:4n6	0.77±0.52	0.67±0.47	0.93±0.43	0.92±0.42	1.21±0.04	0.87±0.46	1.80±1.55	1.56±0.25	0.99±0.44	1.11±0.25	1.27±0.91	1.07±0.75

[3,4] These FA have the same retention time and are eluted in a single merged peak in the GC.

C22:2n6	2.37±1.58	1.31±0.94	2.82±0.51	2.01±0.44	2.27±1.44	2.16±1.22	1.80±0.91	3.22±1.25	2.87±0.57	1.13±0.22	2.36±1.29	2.26±1.26
C22:0	0.27±0.07	0.42±0.09	0.35±0.11	0.35±0.10	0.34±0.06	0.35±0.10	0.56±0.21	0.53±0.34	0.45±0.20	0.52±0.04	0.46±0.24	0.40±0.19
C24:1n9	1.57±0.74	1.68±0.21	2.04±0.73	1.79±0.16	1.98±0.35	1.80±0.57	1.77±0.21	1.90±0.65	1.90±0.54	1.80±0.06	1.78±0.55	1.79±0.56
C26:2n6	5.72±1.33	6.36±2.72	6.69±1.74	7.95±0.87	7.03±0.27	6.61±1.87	5.77±2.38	7.90±2.76	6.87±1.61	9.54±0.31	6.96±2.38	6.79±2.16
C27:2	3.58±1.00	4.68±0.26	4.43±1.33	3.80±0.92	4.22±0.05	4.17±0.99	4.12±0.83	5.05±1.59	2.80±1.40	4.19±0.17	3.98±1.37	4.07±1.20
∑SFA	48.07±18.85	43.15±9.96	59.85±11.60	33.74±12.72	52.10±8.72	48.23±15.64	44.20±15.04	85.06±31.44	56.95±6.69	42.37±2.28	56.44±25.08	52.43±21.40
∑MUFA	17.31±5.65	13.55±5.84	30.22±15.23	15.06±2.53	18.55±5.23	19.35±10.75	13.74±5.87	32.25±11.92	19.26±3.86	10.78±1.11	19.36±10.30	19.35±10.53
∑PUFA	29.96±9.77	24.22±9.63	36.46±7.51	24.34±2.84	33.03±7.46	29.78±9.54	28.43±9.30	54.66±17.94	40.23±10.23	26.45±2.01	36.62±15.81	33.28±13.56
∑HUFA	5.61±2.71	5.37±4.16	7.32±4.34	6.74±1.87	8.28±0.10	6.50±3.46	10.86±1.65	9.53±1.97	6.94±2.46	7.13±1.58	8.16±2.92	7.35±3.30
∑n-3	4.39±2.07	4.19±3.25	6.83±3.76	5.09±1.36	6.25±0.06	5.29±2.89	7.76±0.97	10.11±1.44	5.92±1.42	5.53±1.19	6.91±2.55	6.12±2.84
∑n-6	30.38±9.97	23.33±12.81	36.98±8.98	25.22±4.58	34.44±7.56	30.12±11.02	31.55±9.38	56.10±16.65	42.32±11.29	27.18±2.38	38.22±15.68	34.26±14.20
∑n-7	6.69±2.85	3.14±2.19	15.38±13.69	3.71±1.75	4.11±1.50	7.12±6.46	2.58±1.42	16.21±9.25	6.33±0.70	2.02±0.47	7.22±7.00	7.17±7.75
∑n-9	23.41±9.27	17.86±8.71	30.23±9.86	15.69±2.95	27.73±8.21	23.22±10.10	21.77±10.03	42.53±13.77	30.92±8.50	16.62±1.55	27.82±13.41	25.58±12.13
EPA/DHA	0.27±0.04	0.52±0.27	1.13±1.37	0.37±0.08	0.35±0.02	0.56±0.76	0.73±0.73	0.26±0.16	0.49±0.06	0.48±0.11	0.45±0.40	0.50±0.60
ARA/EPA	2.60±0.62	1.83±0.71	2.27±0.66	1.57±0.23	1.76±0.19	2.07±0.68	1.37±0.51	3.36±1.79	1.14±0.32	0.95±0.13	1.93±1.31	2.00±1.06
∑n-6/∑n-3	7.84±3.67	6.39±1.81	7.15±4.01	5.26±1.49	5.52±1.27	6.63±3.04	4.14±1.34	5.49±1.03	7.14±0.79	5.16±1.18	5.94±2.44	6.28±2.77
∑Total	100.95±33.97	86.29±25.54	133.85±18.50	79.88±14.44	111.96±21.52	103.86±31.44	97.24±28.41	181.50±30.70	123.38±22.19	86.73±5.12	120.57±44.32	112.41±39.47

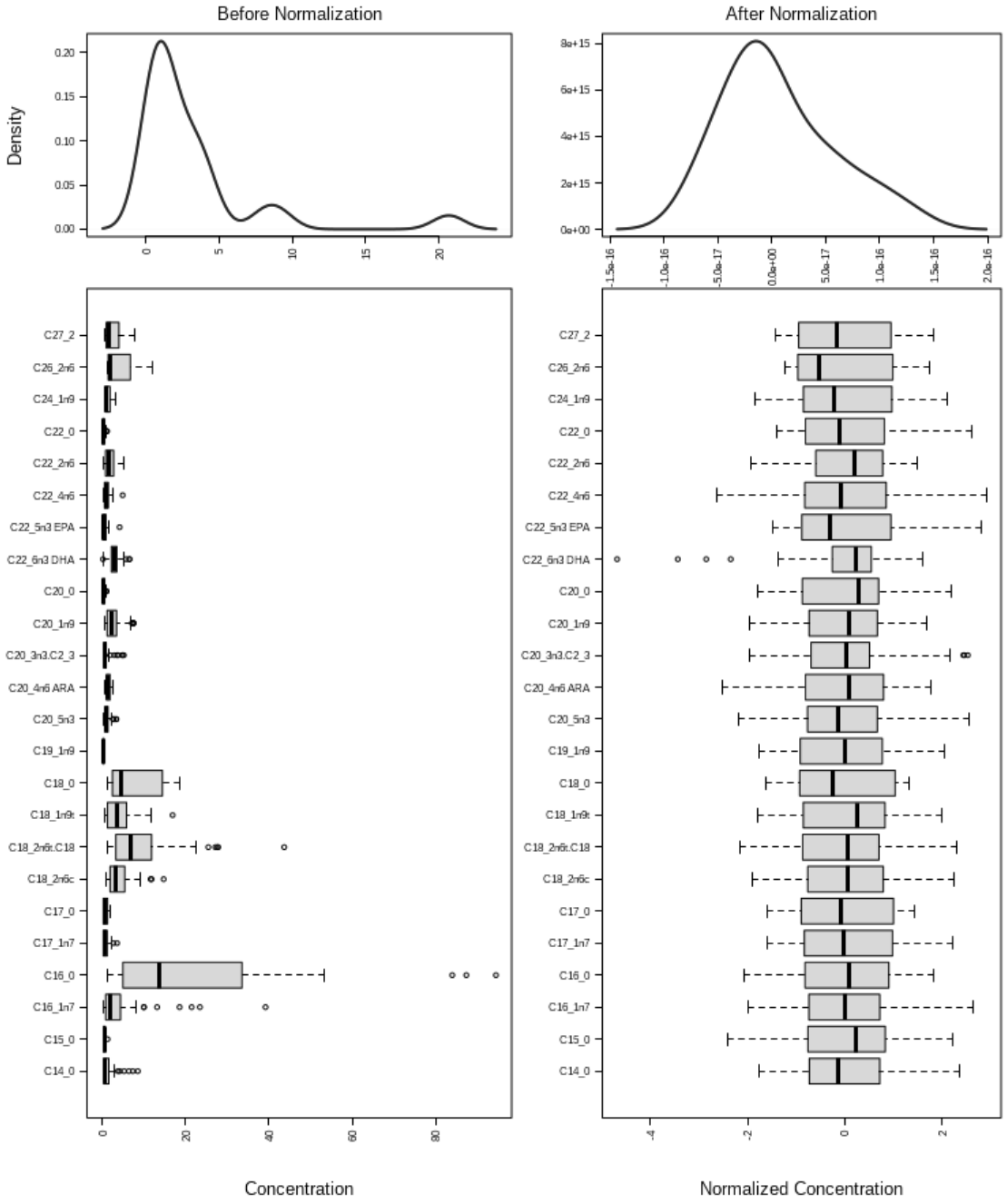


Figure S1. Fatty acid concentration (µg.mg^{-1} dry weight, distribution, and variance) before and after normalization procedure (log transformed and auto-scaled – mean centered and divided by the standard deviation of each variable).

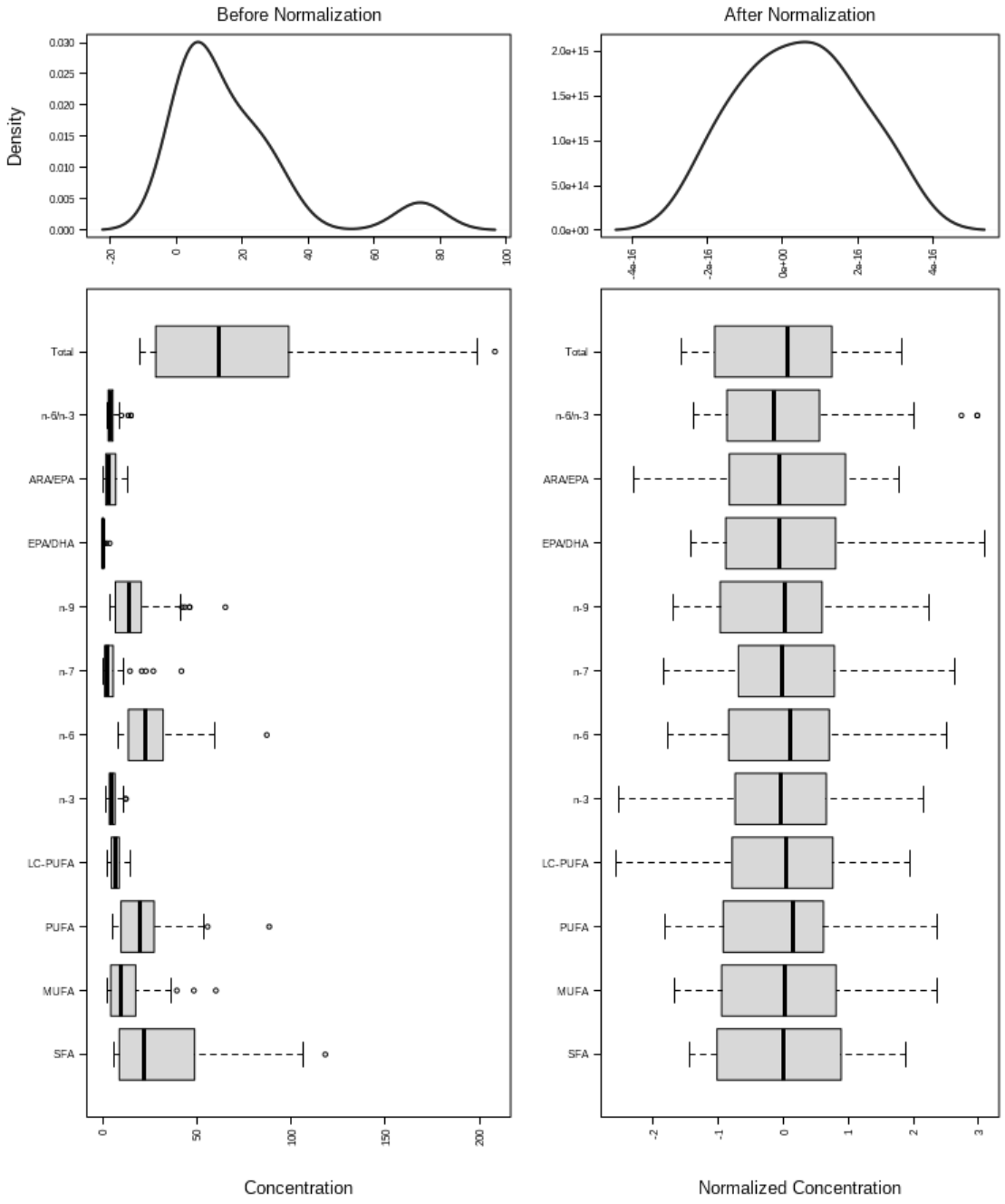


Figure S2. Fatty acid classes concentration (μg.mg^{-1} dry weight) and fatty acid ratios (distribution and variance) before and after normalization procedure (log transformed and auto-scaled – mean centered and divided by the standard deviation of each variable).

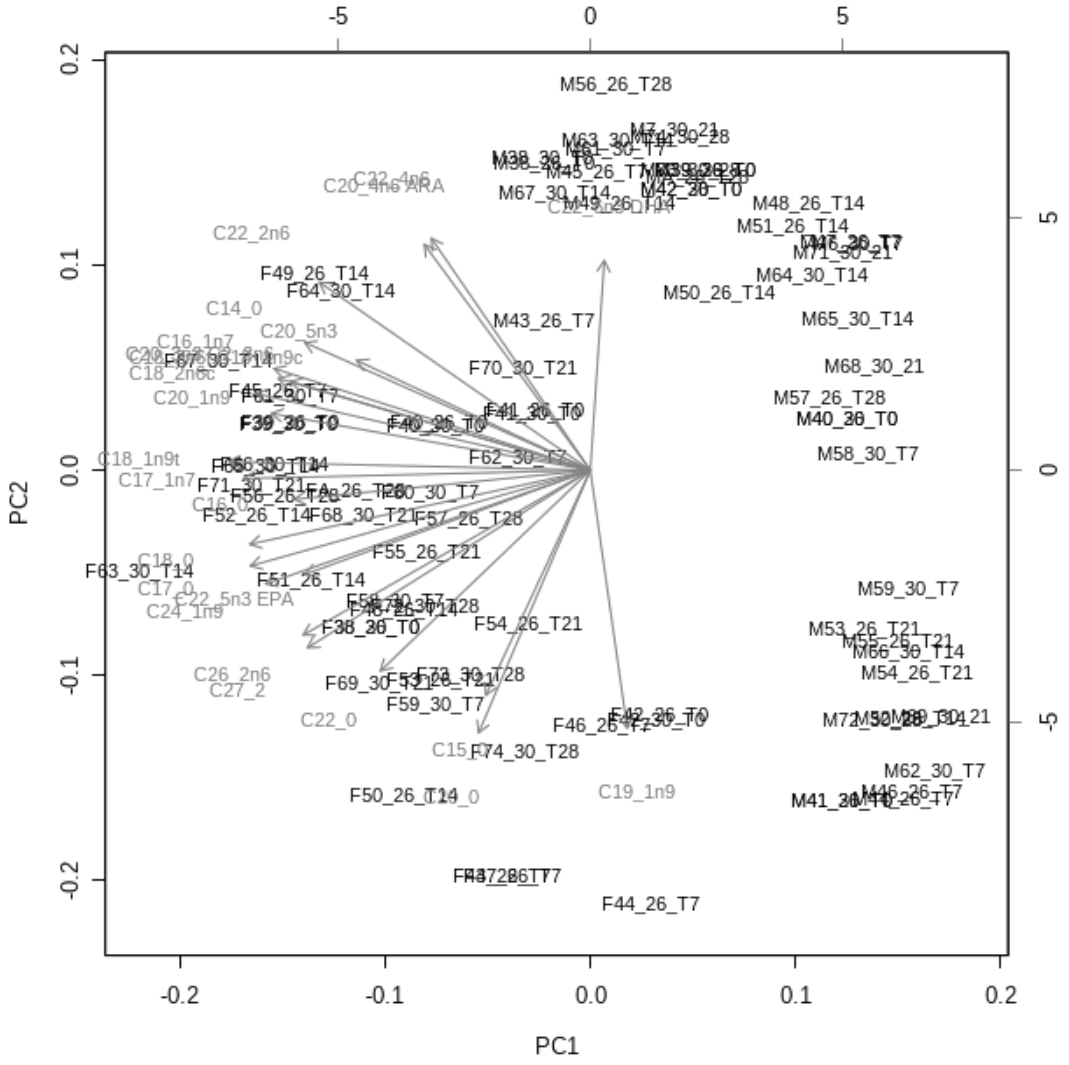


Figure S3. PCA biplot of samples scores (black) and fatty acids' loadings (vectors in red). The further away the vectors are from a PC origin, the more influence they have on that PC. Small angles between vectors imply positive correlation between them, whereas large angles suggest negative correlation and a 90° angle indicates no correlation between two fatty acids.

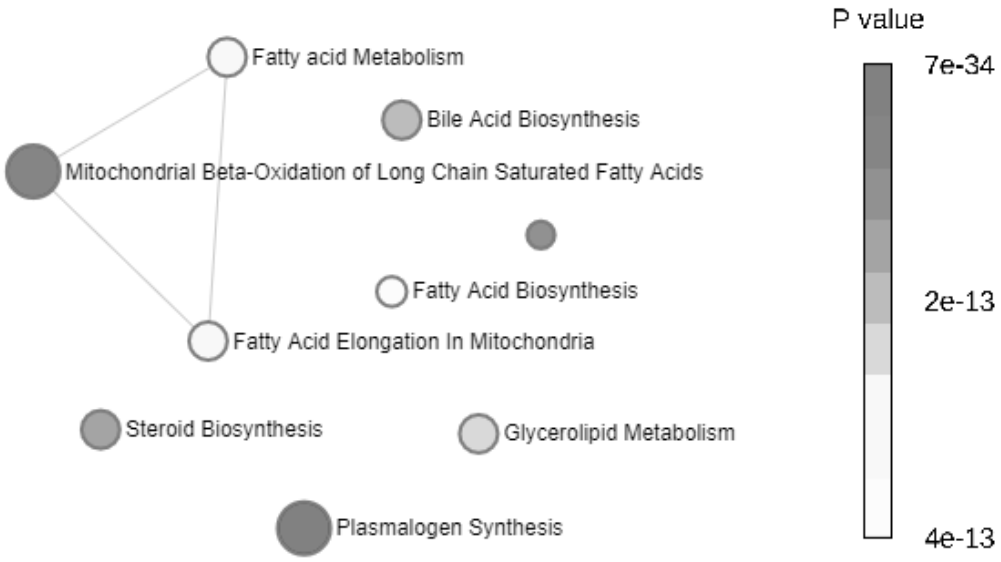


Figure S4. Functional network for enriched FA set for differences between muscle vs liver. Each node represents a metabolite set with its colour based on its p-value (FDR corrected) and its size is based on the number of hits (FA). Two metabolite sets are connected by an edge if the number of their shared metabolites is over 25 % of the total number of their combined metabolite sets.

Table S3. Results from quantitative enrichment analysis. The Q-statistic for a metabolite set is the average of the Q-statistics for each metabolite in the set. This table is complementary to Fig. 4A in the main text. Relative abundance of the relevant listed FA was significantly higher in liver when compared to muscle, except for C18:2n-6c (linoleic acid).

Metabolic pathway	**Total compounds**	**Hits**	**FA**	**Q-stat**	**Raw p-value**	**FDR**	**SMPD Library Reference**
Plasmalogen synthesis	26	1	C18:0	83.67	8.30E-35	3.73E-34	https://smpdb.ca/view/SMP0000479
Mitochondrial β-oxidation of LC-SFA	28	1	C18:0	83.67	8.30E-35	3.73E-34	http://www.smpdb.ca/view/SMP00482
α-Linolenic acid and linoleic acid metabolism	19	5	C18:2n-6c C20:5n-3 C20:3n-6 C22:6n-3 C22:5n-3	31.76	3.84E-16	1.15E-15	http://www.smpdb.ca/view/SMP00018
Steroid biosynthesis	48	1	C16:0	53.35	1.45E-15	1.63E-15	http://www.smpdb.ca/view/SMP00023
Bile acid biosynthesis	65	1	C16:0	53.35	1.45E-15	1.63E-15	http://www.smpdb.ca/view/SMP00035
Glycerolipid metabolism	25	1	C16:0	53.35	1.45E-15	1.63E-15	http://www.smpdb.ca/view/SMP00039
Fatty acid metabolism	43	1	C16:0	53.35	1.45E-15	1.63E-15	http://www.smpdb.ca/view/SMP00051
Fatty acid elongation in mitochondria	35	1	C16:0	53.35	1.45E-15	1.63E-15	http://www.smpdb.ca/view/SMP00054
Fatty acid biosynthesis	35	2	C14:0 C16:0	36.21	3.60E-13	3.60E-13	https://smpdb.ca/view/SMP0000456

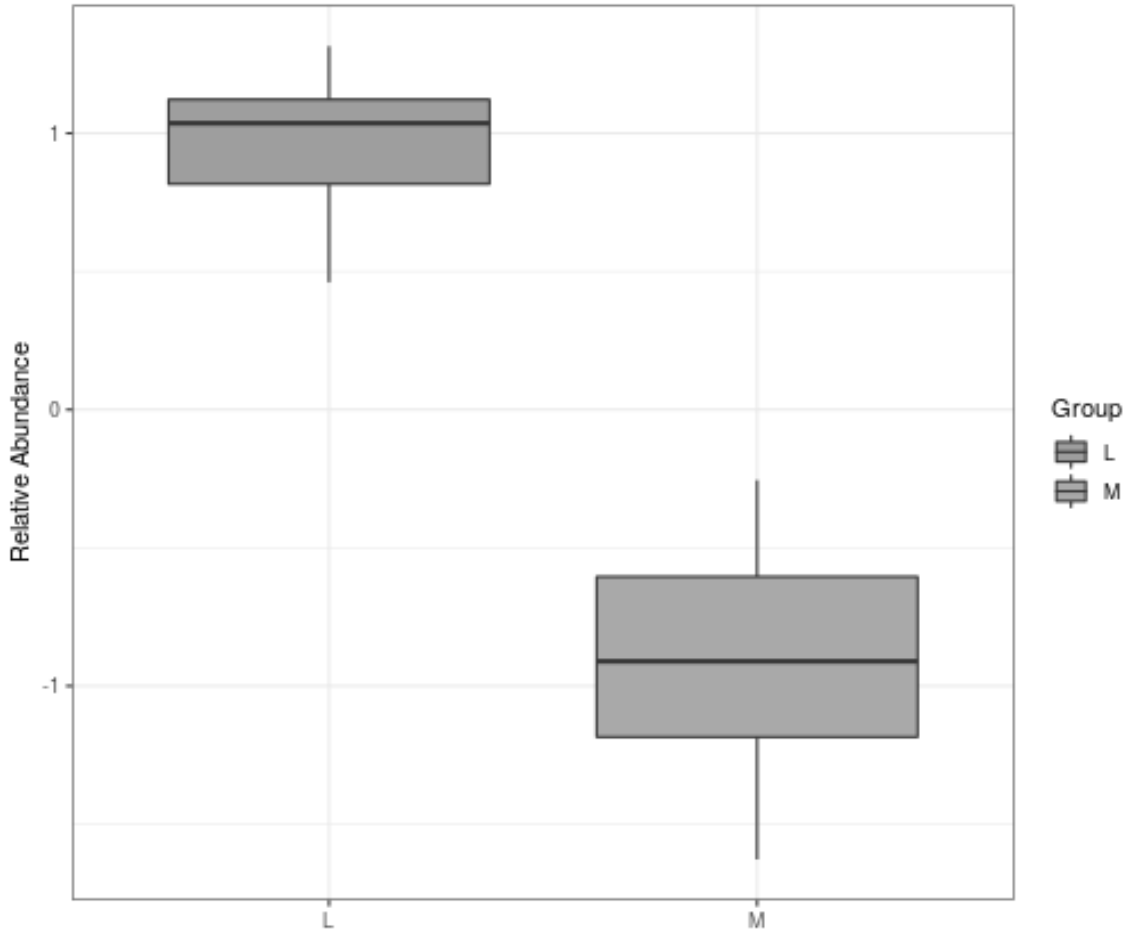


Figure S5. Relative abundance of C18:0 (stearic acid) in liver and muscle. C18:0 is involved in two key biological pathways: (i) plasmalogen synthesis and (ii) mitochondrial β-oxidation of long chain saturated fatty acids.

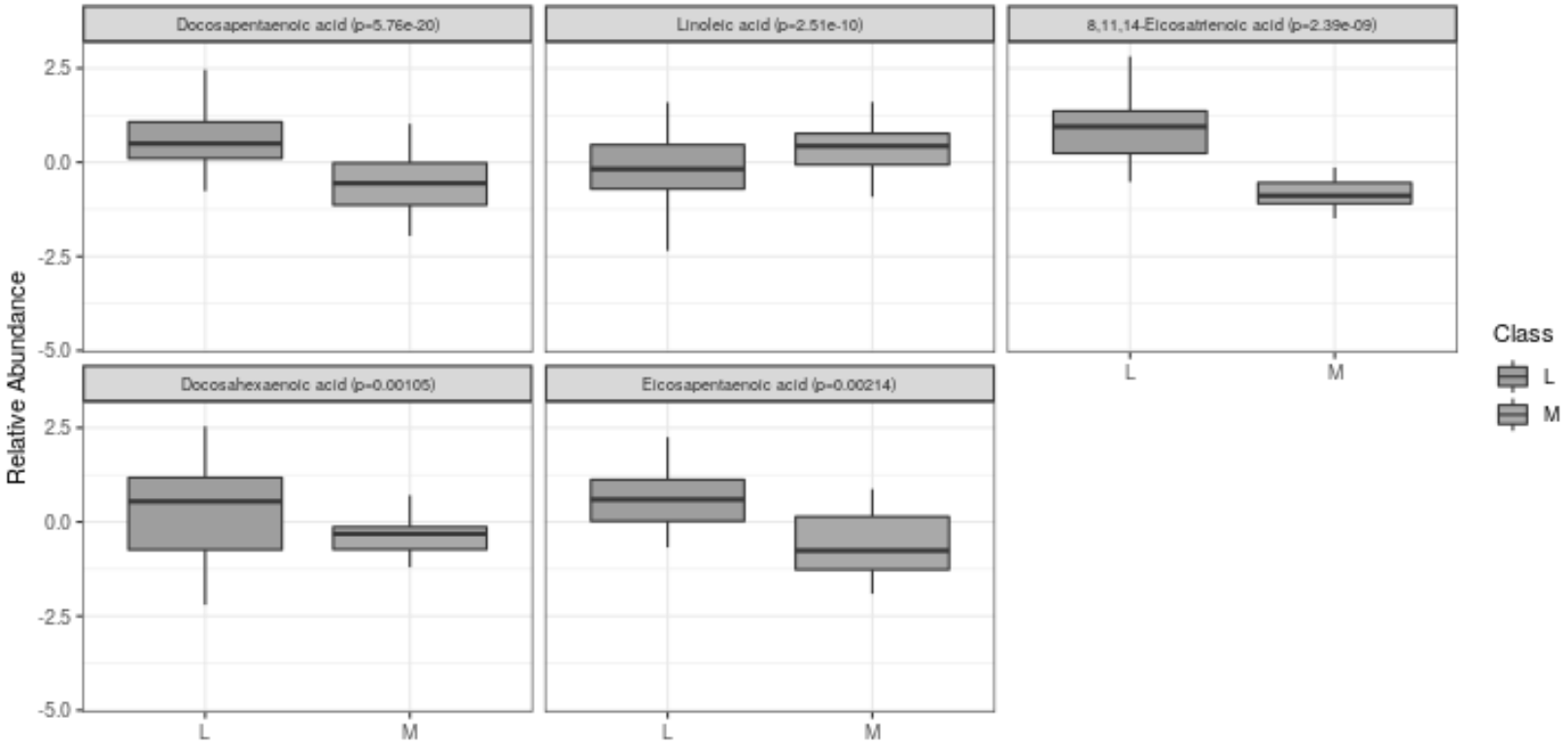


Figure S6. Relative abundance of C22:5n-3 (Docosapentaenoic acid), C18:2n-6c (linoleic acid), C20:3n-6 (8Z,12E,14Z-eicosatrienoic acid), C22:6n-3 (Docosahexaenoic acid) and C20:5n-3 (eicosapentaenoic acid) in liver and muscle. These FA are involved in one key biological pathway: (i) α-Linolenic acid and linoleic acid metabolism. P-values of Q-statistic for each individual metabolite.

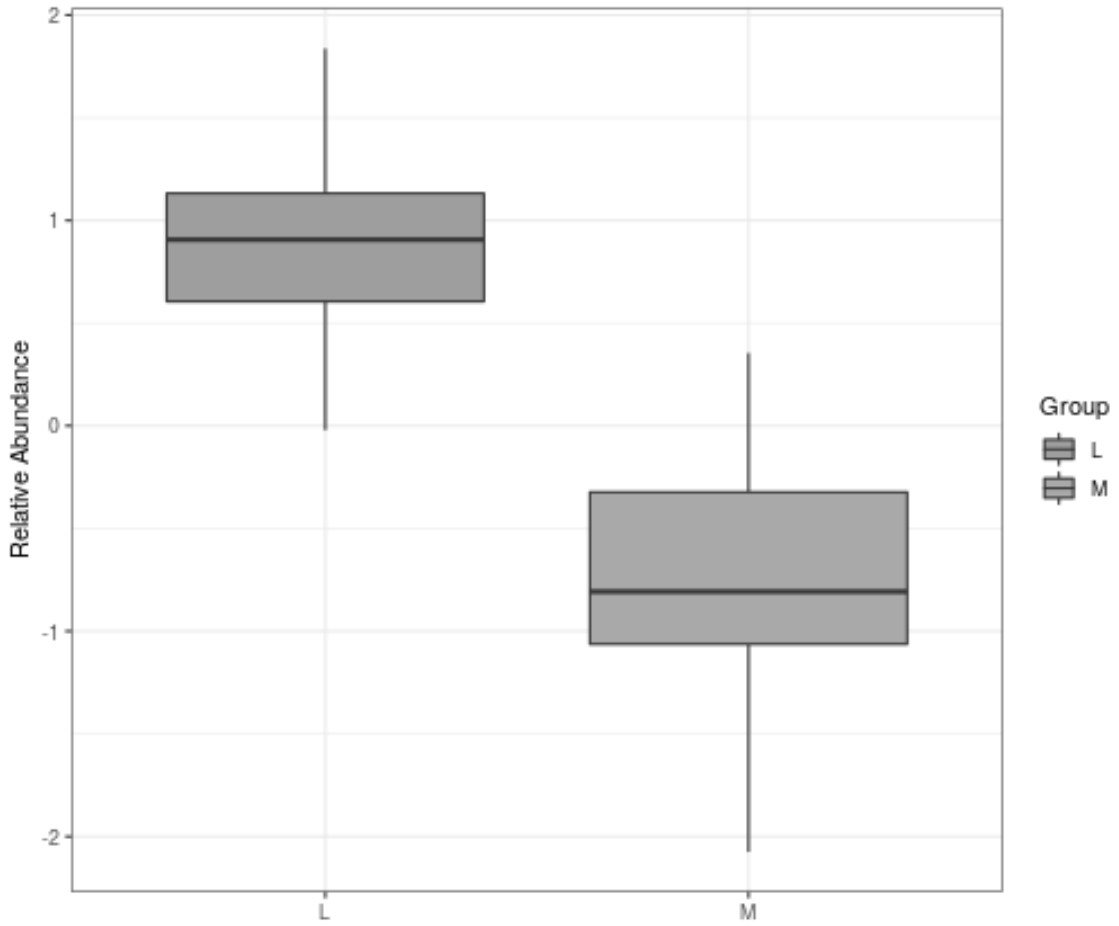


Figure S7. Relative abundance of C16:0 (palmitic acid) in liver and muscle. C16:0 is involved in several key biological pathways: (i) steroid biosynthesis, (ii) bile acid biosynthesis, (iii) glycerolipid metabolism, (iv) fatty acid metabolism, (v) fatty acid elongation in mitochondria

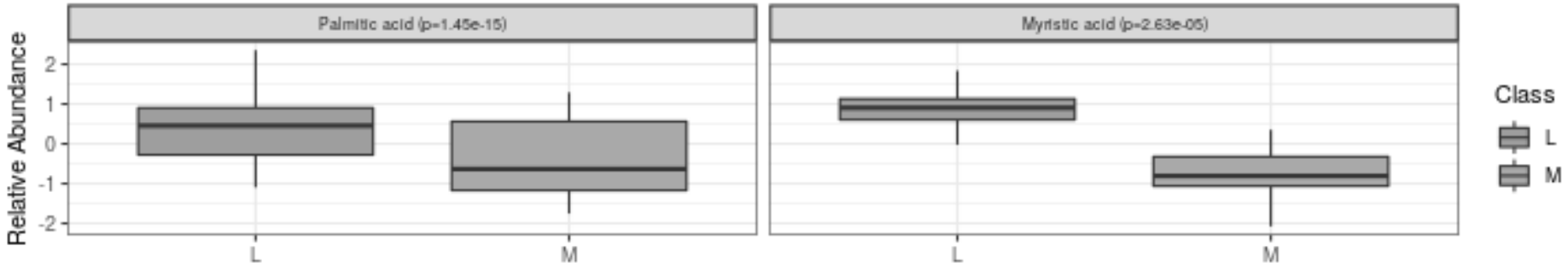


Figure S7. Relative abundance of C16:0 (palmitic acid) and C14:0 (myristic acid) in liver and muscle. These two FA are involved in a key biological pathway: (i) fatty acid biosynthesis. P-values of Q-statistic for each individual metabolite.