

NMR-based metabolic profiling and discrimination of wild tropical tunas by species, size category, geographic origin, and on-board storage condition

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1	NMR-based metabolic profiling and discrimination of wild tropical tunas by species, size				
2	category, geographic origin, and on-board storage condition				
3					
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14					
15	Abstract				
16	Tunas are among the most traded and valued fish species, and good traceability of tuna products				
17	in the world market is needed to protect both consumers and tuna stocks. To that purpose, high-				
18	resolution proton nuclear magnetic resonance (¹ H NMR) spectroscopy combined with				
19	multivariate data analysis was used to investigate the molecular components of the aqueous				
20	extract of white and red muscles in three species of wild tropical tuna species, namely yellowfin				
21	tuna (Thunnus albacares), skipjack tuna (Katsuwonus pelamis) and bigeye tuna (T. obesus).				
22	Principal component analysis (PCA) and partial least squares-discriminant analysis (PLS-DA)				
23	applied to the processed ¹ H NMR spectra showed significant separation according to the species				
24	and size category (i.e., small tunas < 80 cm fork length vs large tunas >80 cm fork length), the				
25	storage conditions on-board the purse-seine vessels (i.e., brine- vs deep-freezing), and the				
26	geographical origin, i.e., where the tuna was caught (Mozambique Channel vs western-central				
27	Indian Ocean). The major groups of metabolites responsible for differentiation in PLS-DA score				
28	plots were the dipeptides (anserine, carnosine) and organic acids (lactate,				
29	creatine/phosphocreatine) in the white muscle, and the free amino acids, essential nutrients				
30	(choline and its derivatives, phosphatidylethanolamine), dipeptides and organic acids in the red				
31	muscle. Our results show that NMR-based metabolomics is a powerful tool to efficiently				
32	discriminate specific profiles among wild tuna species, raw muscle tissues, fish storage				
33	conditions and tuna geographical origin.				

- *Keywords:* Metabolomics, High-Resolution ¹H NMR; Highly migratory large pelagic fish; Purse
- 36 seine tuna fisheries; western Indian Ocean; Raw white and red muscles.

37 1 Introduction

Sustainable fisheries management relies on the availability of accurate species-specific catch data 38 and may suffer when reported data of species or fishing area are erroneous or inaccurate (Watson 39 & Pauly, 2001). Moreover, ensuring traceability of fishery products "from ocean to plate" is a 40 major concern for instances and food markets worldwide. For example, with the objectives to 41 achieve a high level of consumers' health protection and to guarantee their right to information, 42 43 the European Regulation (No 1169/2011) imposes labelling of fishery products with the commercial and scientific name of the species, the area where the organism was caught, the 44 45 production methods, and the product's nutritional characteristics. Nevertheless, differences in quality and price according to species, geographical origin, production/fishing modes, or the life 46 47 history of the live species and product may lead to mislabelling. Efficient methods to assess fishery products authenticity and origin are thus required to help improving fisheries traceability 48 and sustainability, guaranteeing market transparency, and protecting consumers' safety and rights 49 (Nielsen et al., 2012). 50

51 The quality and/or origin of fishery product is mostly determined by its biochemical composition, i.e., metabolite profile. Over the past twenty years, Nuclear Magnetic Resonance (NMR) 52 53 spectroscopy coupled with multivariate analysis has been applied for determining the metabolite 54 profiles of various kinds of food including fish (Martinez et al., 2005; Picone et al., 2011; Wei et al., 2020). The metabolite content of a given sample constitutes a unique profile for the product 55 56 of all low molecular weight molecules present in, or excreted by, cells or bacteria. Low molecular weight metabolites in fish tissues include free amino acids, nucleotides and related compounds, 57 58 peptides, organic bases, sugars and inorganic constituents (Piñeiro et al., 2003). In general, the levels of these metabolites vary according to species, environmental conditions, diet, fish tissue, 59 60 freshness, stress, and post-mortem storage, handling and processing (Piñeiro et al., 2003; 61 Samuelsson & Larsson, 2008). Moreover, compared to other techniques widely used for 62 metabolic profiling such as mass spectrometry, the use of NMR allows a simpler sample preparation and a higher analysis throughput (Jung et al., 2010). Because NMR analyses produce 63 highly complex datasets, multivariate analyses such as Principal Component Analysis (PCA) and 64 Partial Least Squares-Discriminant Analysis (PLS-DA) have been designed specifically to 65 analyse NMR derived data (Hatzakis, 2019). ¹H NMR spectroscopy is therefore a powerful non-66 specific high-throughput analytical technique to produce a metabolic profile and identify potential 67 compounds useful in characterizing, discriminating and authenticating the metabolic 68 specifications of a particular fishery product and its quality (Hatzakis, 2019). 69

Tunas are among the most traded and valued fish species, with annual global catch of around 6 70 71 million tonnes, i.e. 10% of the world's international seafood trade (FAO, 2020). While bluefin tunas are the most highly valued species although representing about 1.2% of total tuna catch, the 72 73 global tuna fisheries are dominated by three tropical tuna species, i.e. skipjack tuna (Katsuwonus pelamis -SKJ), yellowfin tuna (Thunnus albacares -YFT), and bigeye tuna (T. obesus -BET) with 74 75 51%, 26%, and 8% of the total tuna catch, respectively (FAO, 2020). From the different fishing 76 gears targeting tropical tunas, industrial purse-seine contributes most to global catch, i.e. 69% 77 compared to 12% for pole-and-line, 10% for longline, and 9% for other gears during the period 78 2014 to 2018 (FAO, 2020). Purse seine is the less selective gear both in terms of size and species composition. Indeed, purse-seiners catch a mix of free-swimming schools and schools associated 79 with drifting floating objects leading to the simultaneous catch of the three co-occurring tropical 80 tunas, from early juveniles (<30 cm) to the largest and oldest individuals (>150 cm for YFT and 81 BET) (Fonteneau et al., 2013). While tunas are mixed in the wells in the case of brine-freezing 82 (i.e., salted water refrigerated at around -18°C), they are sorted according to species, size 83 category and quality appearance in the case of deep-freezing (i.e., -40°C dry well) storage. The 84 duration of fish storage follows the length of the purse seiner's trips, which is 6 to 9 weeks in the 85 Indian Ocean. Purse seine tropical tunas are supplied to the factories (i) in deep-frozen form for 86 large YFT and BET (i.e., > 10 kg) classified on board with "high quality grade" and sold mainly 87 in pre-cooked loins, or (ii) in brined form for SKJ, small tunas (i.e., < 10 kg), and the remaining 88 large YFT and BET that are being processed and sold as cans (Table S1). Misidentification of 89 tuna can occur when fish are sorted onboard purse-seine vessels, at unloading and/or at the 90 factories, especially in the case of small YFT and BET with similar morphological features 91 92 (Pecoraro et al., 2016). This raises the possibility of accidental mislabelling, intentional species 93 substitution, or mixing of valuable fish by less valuable ones in the commercial tuna market, motivated by the difference of market prices between species and/or when catch restrictions on 94 overfished specific tuna species exist such as YFT in the Indian Ocean (IOTC Secretariat, 2019). 95 96 Moreover, the purse-seine fishery catching tropical tunas throughout the year, the meat quality is 97 likely to be subject to changes depending on the geographical area where the tuna was caught, the 98 related diet available and consumed by the tuna in the area, as well as its age (i.e., size) and reproductive status, all these factors known to influence tuna's muscle biochemical composition 99 (Chassot et al., 2019; Pecoraro et al., 2016; Sardenne et al., 2016a; Sardenne et al., 2017). The 100 changes in tuna meat quality may thus affect the efficiency of processing and/or the nutritional 101 102 benefits for consumers.

Although NMR spectrometry was applied on tuna as early as the 90's, only few studies remain 103 104 available in the literature, especially dealing with tuna muscles (Jääskeläinen et al., 2019; Medina et al., 2000; Sacchi et al., 1993). In this context, the present study aimed at characterizing 105 106 and discriminating the ¹H NMR low molecular weight metabolic profiles of the muscles of three co-occurring tropical tuna species, namely skipjack, yellowfin and bigeye tunas, targeted by the 107 purse-seine fishery in the western Indian Ocean. The ¹H NMR spectra of tuna muscles' aqueous 108 109 extracts were then further analysed using PCA and PLS-DA to test for metabolic differences 110 according to tuna size, on-board freezing storage conditions (brine- vs deep-freezing) and geographical origin. Tuna muscle consists of white (or light) and red (or dark) muscles, the latter 111 representing higher proportions in tuna species compared to other fish (e.g., 6-8% of total body 112 mass) (Dickson, 1995); this is because of the high migratory abilities of tunas and their related 113 high needs for fat, glycogen and myoglobin. Both tuna muscles were thus taken into account in 114 the present study: their ¹H NMR metabolic profiles were characterised and compared, and the 115 influence of each of the factors listed above was tested. 116

117

118 2 Material and methods

119 2.1 Chemicals

Methanol, dichloromethane, ethylene glycol tetra-acetic acid (EgTA) and potassium chloride (KCl) were purchased from Sigma-Aldrich (St Quentin Fallavier, France). Deuterium oxide (D₂O) and sodium 3-trimethylsilyl-2,2,3,3-tetradeuteriopropionate (TMSP) were obtained from Eurisotop (Saint-Aubin, France).

124

125 2.2 Tuna sampling

126 A one-year multi-species sampling was carried out throughout 2013 in the western Indian Ocean. A total of 194 tropical tunas including 60 YFT, 62 BET and 72 SKJ were collected during the 127 unloading of purse seiners at Victoria, Seychelles (Table S1). Fishing date and accurate GPS 128 129 fishing position from purse seiners' logbooks allowed for the distinction of two main geographical origin areas that differ according to their oceanographic specificities (Chassot et al., 130 131 2019): Mozambique Channel (MOZ, n=52) and western-central Indian Ocean (WCIO, n=142) (Fig.1). The onboard storage condition was also recorded during sampling, allowing for the 132 133 separation between fish stored in brine freezing wells (-18°C) versus those stored in dry deepfreezing wells (-40°C) (Table S1). All sampled fish were weighed (kg to the nearest 0.1 kg), and 134 135 measured in fork length, which refers to the length from the tip of the snout to the fork of the tail

- 136 (FL, cm to the nearest 0.5 cm). Then, one sample of around 2 g on a wet weight (ww) basis was
- taken from the dorsal white muscle (under the dorsal spine on the left side) and a second one
- 138 from the red muscle (under the pectoral fin on the left side) of each tuna. All samples were stored
- 139 at -80 °C until further analyses.



Fig. 1. Location of yellowfin (YFT, n=60), bigeye (BET, n=62) and skipjack (SKJ, n=72) tuna caught by
purse seine vessels in the western Indian Ocean throughout 2013. The limit of the two geographical origin
areas, Mozambique Channel (MOZ) and western-central Indian Ocean (WCIO) is indicated with dashed
line. Star indicates Port Victoria, Seychelles.

146 2.3 Sample preparation

A total of 388 samples of tunas' white and red muscles (from 194 tunas) were processed for the 147 analysis of ¹H NMR Spectroscopy. Approximately 100±0.1 mg ww of each tissue was weighed, 148 crushed with 1 mL of methanol:ethylene glycol tetraacetic acid (EgTA) (5 mM in water) (2/1, 149 v/v) using a FastPrep System[®] (MP Biomedicals, Illkirch, France), and extracted with 2 mL of 150 dichloromethane:methanol (2:1, v/v). After addition of 0.2 volume of aqueous potassium chloride 151 (KCl) (0.9% w/v), the extracts were further vortexed and centrifuged at 5000g for 10 min, then 152 the lipid supernatants were removed, while the aqueous extracts were freeze-dried. The resulting 153 powders were reconstituted in 700 μ L of deuteriumoxide (D₂O) in which sodium 3-trimethylsilyl-154 155 2,2,3,3-tetradeuteriopropionate (TMSP; as a chemical shift reference at 0 ppm; 1 mM) was added. Each sample was vortexed and centrifuged for 10 min, 4°C at 5000g to remove any 156 157 precipitate. Then, 600 µL aliquots were transferred to standard 5 mm - NMR tubes (Norell ST 158 500, Landisville, NJ) for analysis.

160 2.4 ¹H NMR Analysis

The ¹H-NMR spectra were obtained on a Bruker DRX-600-Avance NMR spectrometer (Bruker, 161 Wissembourg, France) operating at 600.13 MHz for ¹H resonance frequency using an inverse 162 detection 5mm ¹H-¹³C-¹⁵N cryoprobe attached to a CryoPlatform (the preamplifier cooling unit). 163 164 The ¹H-NMR spectra were acquired at 300K using the Carr-Purcell-Meiboom-Gill (CPMG) spin-165 echo pulse sequence with pre-saturation, with a total spin echo delay $(2n\tau)$ of 240ms to attenuate broad signals from proteins and lipoproteins. A total of 128 transients were collected in 32,000 166 data points using a spectral width of 20 ppm, a relaxation delay of 2 sec, and an acquisition time 167 168 of 1.36 sec. The spectra were Fourier transformed by multiplication of the FIDs by an exponential weighting function corresponding to a line-broadening of 0.3Hz. All spectra were 169 170 manually phased and baseline corrected and referenced to TMSP using Bruker TopSpin 2.1 software (Bruker, GMBH, Karlsruhe, Germany). To confirm the chemical structure of 171 metabolites of interest, 2D ¹H-¹H COSY (Correlation Spectroscopy) and 2D ¹H-¹³C-HSQC 172 (Heteronuclear Single Quantum Coherence Spectroscopy) NMR experiments were performed on 173 selected samples. Spectral assignment was based on matching 1D and 2D data to reference 174 with 175 spectra in a home-made reference database, as well as other databases (http://www.brmb.wisc.edu and http://www.hmdb.ca), and reports in the literature. Absolute 176 177 concentrations of the metabolites were calculated from the integrals of the TMSP signal (known concentration standard) and, the metabolite signal (unknown concentration) as described in 178 179 (Amiel et al., 2020).

180

181 2.5 Data reduction and multivariate statistical analyses

182 Data were reduced using the AMIX software (version 3.9, Bruker, Rheinstetten, Germany) to 183 integrate variable wide regions corresponding to the δ 9.0-0.90 ppm region for aqueous white and 184 red muscle extracts. A total of 64 and 78 NMR buckets were selected for white muscle and red 185 muscle respectively, according to resonance signals and multiplicity between 9.0 and 0.9 ppm. 186 Regions corresponding to noise or residual water, methanol and dichloromethane were not 187 selected. To account for differences in sample amount, each integrated region was normalized to 188 the total spectral area.

Multivariate analyses were used to study the effect of *Species* on the metabolome of white muscle and red muscle separately. The effects of *Storage condition* on-board purse-seiners (i.e., brine-*vs* deep-freezing) and *Geographical origin* (i.e., MOZ *vs* WCIO) were also investigated for each species and muscle type. Finally, the effect of *Size category* was considered for YFT and BET

only: two distinct size categories were taken into account, based on YFT and BET life history 193 194 traits (i.e., small tunas < 80 cm FL vs large tunas > 80 cm FL, with FL=80 cm corresponding to the size at which YFT and BET reach their size-at-first maturity, change their aggregative 195 196 behaviour and habitat from surface to deeper waters and thus their diet; (Chassot et al., 2019, 2015; Sardenne, Bodin, et al., 2016)). SKJ is a smaller tuna species than YFT and BET 197 198 (maximum length of 110 cm for SKJ compared to 230 cm for YFT and BET), and does not show specific behavioural changes with size being aggregated in schools near the surface through their 199 200 lifespan. For this reason, the factor Size category was not considered for SKJ. A PCA was first 201 performed to reveal intrinsic clusters and detect potential outliers. PLS-DA was then used to 202 model the relationship between groups and spectral data. Before analysis, the orthogonal signal correction filtering was applied to remove variation not linked to the factors of interest 203 (physiological, experimental, or instrumental variation). Filtered data were mean-centred and 204 scaled (unit variance or Pareto scaling). For the figures, we used Hotelling's T2 statistics to 205 construct 95% confidence ellipses. The goodness-of-fit parameter R²Y parameter represents the 206 explained variance. Seven-fold cross-validation was used to determine the number of latent 207 variables to include in the PLS-DA model and to estimate the predictive ability (Q² parameter) of 208 the adjusted model. The robustness of the PLS-DA regression models was assessed by comparing 209 $R^{2}Y$ and Q^{2} : $R^{2}Y$ varies between 0 and 1, where 1 indicates a perfect fit between the model and 210 the data; When Q^2 is greater than 0.5, the model is considered to have good predictability, and if 211 Q^2 is greater than 0.9, then the model is considered to have excellent predictability. 212

In addition, the robustness of the PLS-DA model was assessed using a permutation test (200 213 permutations). Discriminant variables were determined using the VIP (variable importance in the 214 215 projection) value, a global measure of the influence of each variable on the PLS components. Variables with VIP > 0.8 were considered discriminant. A non-parametric Kruskal–Wallis test 216 was then used to determine which metabolites were significantly different between groups. NMR 217 variables with a p-value <0.05 were considered significantly different. SIMCA-P software (V12; 218 Umetrics AB, Umea, Sweden) was used to perform the multivariate analyses and R software 219 220 (version 2.12, http://www.r-project.org) was used to perform univariate Kruskal-Wallis test.

221

222 **3** Results and discussion

223 3.1 Identification of tropical tunas' white and red muscle metabolite profiles

Both muscles (388 samples) displayed similar metabolites, although changes in the relative intensities of some resonances were observed. Figures 2A and 2B show representative ¹H NMR spectra from white and red muscles of tuna respectively. The assignment of the peaks to specific metabolites was achieved based on the literature and confirmed by 2D COSY and HSQC spectroscopy. The ¹H NMR spectra of tuna muscle samples contained a number of assignable amino acids, organic acids, glucose and lipids, as observed in fish muscle in general (Konosu & Yamaguchi, 1982; Piñeiro et al., 2003). Twenty-five metabolites were identified based on their 1D and 2D spectra, and Table 1 lists the compounds assigned. The comparison of tuna muscles spectra allowed for the identification of four groups of signals and related metabolites:

233 Group I – degradation biomarkers: The low-field region (8.6-4.4 ppm) of the NMR spectra 234 displays signals belonging to adenosine triphosphate (ATP) degradation products, i.e., inosine, 235 inosine-5-monophosphate (IMP), and hypoxanthine. ATP predominates the nucleotides in muscle of live animals under normal conditions, but after death a series of enzymatic reactions leads to 236 the decomposition of ATP into adenosine diphosphate (ADP), adenosine monophosphate (AMP), 237 inosine monophosphate (IMP), inosine, hypoxanthine, xanthine and ultimately uric acid. When 238 the level of ATP has dropped under a critical level, the muscle enters rigor mortis. Moreover, 239 hypoxanthine is a known contributor to the bitter off-flavour of spoiled fish while IMP is usually 240 associated to the desirable taste of fresh fish. The degree of freshness is thus often expressed as 241 the K-value, defined as the ratio of the sum of inosine and hypoxanthine concentrations to the 242 243 total concentration of ATP metabolites (Karube et al., 1984).

Group II – essential metabolites: In the mid-field region (5.2-3.2 ppm) of the NMR spectra, the 244 signals of choline and its derivative metabolites, namely phosphoethanolamine (PE) and 245 glycerophosphorylcholine (GPC) were detected. The spectral region is also characterized by the 246 presence of sugar signals (glucose). Choline and its derivatives result from the hydrolysis of 247 phospholipids, in particular of phosphatidylcholine, which are found in high proportions in the 248 249 lipid fraction of SKJ, YFT and BET (Sardenne et al., 2016b). Involvement of these compounds in 250 aging of samples due to storage conditions has already been pointed out (Scano et al., 2012). Hydrolysis of the ester bond that links the glycerol backbone of lipid molecules to fatty acids 251 results in the release of the latter in their free form, making them more prone to oxidation 252 (Refsgaard et al., 2000) which can be very fast in highly unsaturated fatty acids such as 253 254 docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) present in tuna muscle in high 255 quantity. Therefore, hydrolysis of the lipid molecules makes the food matrix more exposed to oxidation. 256

Group III – energy expenditure: The signals with the highest intensity were observed in the lowfield and mid-field NMR spectra regions, and assigned to anserine, lactate, creatine and phosphocreatine. Low-intensity signals belonging to carnosine, succinate, and trimethylamine (TMA) were also identified. Creatine and phosphocreatine represent an important energy deposit

in skeletal muscle. In fact, most of the creatine is phosphorylated in resting muscle and supplies 261 262 energy in the form of high energy phosphate for muscular contraction. Fish exposed to stress prior to death have lower values of phosphocreatine and ATP than unstressed fish (Erikson et al., 263 264 1997). Moreover, degradation of creatine/phosphocreatine into creatinine/phospho-creatinine 265 through intramolecular cyclization has been reported to be favoured by different chemicalphysical conditions including temperature (Chan et al., 1994). Distinct peaks from anserine (β-266 267 alanyl-1-methylhistidine) and carnosine (β -alanyl-histidine), the most frequently dipeptides 268 occurring in fish (Abe, 1983; Konosu & Yamaguchi, 1982) were visible in all samples. The high 269 level of anserine observed in tuna muscle is typical for migratory pelagic species (Abe et al., 1986; Konosu & Yamaguchi, 1982). These dipeptides are believed to act as a buffer during 270 anaerobic metabolism, a fact reflected by the high levels in muscles used for burst activity 271 (Boldyrev & Severin, 1990). In addition, anserine and carnosine have been proposed to play 272 273 additional roles in controlling enzyme activity, inhibiting oxidative reactions, and as 274 neurotransmitter (Boldyrev, 2001; Boldyrev & Severin, 1990). Anserine decomposes into its 275 constituents β -alanine and 1-methylhistidine by hydrolysis. This permitted to estimate the loss of quality during ice storage by measuring the levels of 1-methylhistidine, β -alanine, anserine and 276 277 tryptophan (Ruiz-Capillas & Moral, 2001). Lactate is typically found in high abundance in tuna muscle as it is involved in burst swimming activity: glucose is broken down and oxidized to yield 278 279 pyruvate, and lactate is then produced from the pyruvate faster than the body can process it, 280 causing lactate concentrations to rise by a factor 7-20 in tuna muscle after fast swimming/hunting 281 (Guppy et al., 1979). Lactate also plays an important role both in the evolution of the freshness degree and in the formation of an agreeable odour or not (Bramstedt, 1962). Its concentration 282 283 reflects the initial glycogen stores before death, the fish handling (e.g., duration of the fishing 284 operation), and the extraction procedure.

Group IV – muscle structure and its degradation: The remaining signals in the high-field (3.0-0.9 285 ppm), mid-field (4.2-3.2 ppm) and low-field (6.8-7.5 ppm) NMR spectra regions belongs to the 286 aliphatic groups, with 12 amino acids identified. Tropical tuna muscles are characterized by a 287 288 relatively low lipid concentration, and a high concentration of proteins and thus their constituents, 289 amino acids (Sardenne et al., 2016b). Indeed, the ¹H-NMR allowed to detect leucine, isoleucine, valine, lysine, alanine, methionine, glycine, taurine, proline, tyrosine, tryptophan, and 290 291 phenylalanine. Most of these amino acids are free although some might be part of peptides. Large proteins were not visible because their slow tumbling leads to broad signals. Proteins play 292 293 important roles in physiological functions including osmoregulation and buffering capacity (Van Waarde, 1988); in addition, they contribute to the aroma and flavour of the fish (Konosu & 294

- Yamaguchi, 1982), and increase its antioxidant capacity (Chan et al., 1994). During storage and
 processing, proteins are degraded, and the level of free amino acids and peptides in muscle
 changes.
- It is important to underline a low presence of trimetylamine (TMA, peak at 2.89 ppm) in spectra
- acquired in this study which is in accordance with previous study on Indian Ocean YFT 299 (Jääskeläinen et al., 2019). TMA is a microbial metabolite and it can only be used as an index of 300 spoilage and not as an index of freshness (Jääskeläinen et al., 2019). Development of TMA in 301 seafood depends primarily on the content of the substrate trimethylamine-oxide (TMAO, peak at 302 303 3.27 ppm) in the fish raw material, which is reduced into TMA by some species in the 304 bacteriological flora of spoiling fish as Shewanella putrefaciens, Photobacterium phosforeum, and Vibrionaceae (Gram & Huss, 1996). In frozen fish, this reaction can be replaced by a slow 305 enzymatic transformation to dimethylamine (DMA) and formaldehyde. TMA can also result from 306 the breakdown of choline from phospholipids. 307



Fig. 2. Typical 600 MHz ¹H NMR CPMG spectra from (A) white muscle and (B) red muscle of wild
 tropical tuna. GPC: Glycerophosphorylcholine; IMP: Inosine-5-monophosphate; Phe: Phenylalanine; PE:
 Phosphatidylethanolamine.

Table 1. Representative ¹H-NMR assignments for white and red muscles of wild tropical tuna at 600 MHz. Group indicates the four groups of signals and related

315 metabolites identified. I – degradation biomarkers; II – essential metabolites; III – energy expenditure; IV – muscle structure and its degradation. Concentration

316 ranges of metabolites were indicated in blue for white muscle, and in red for red muscle. Concentrations were calculated from proton NMR spectra. NQ means 317 "non quantifiable"

Metabolite	Concentration range	δ_H ppm (multiplicity, coupling constant, assignment)/ δ_C ppm	Group	Type of
	(mM/g muscle)			metabolite
Inosine (Ino)	0.1-32; 0.1-25	4.29 (m, CH)/88.2; 4.44 (m, CH)/73.1; 6.10 (d, J=5.7 Hz, CH)/90.9;	Ι	Nucleoside
		8.24 (s, CH)/148.9; 8.35 (s, CH)/142.7		
Hypoxanthine (Hyp)	NQ	8.21 (s, CH)/135.0; 8.24 (s, CH)/146.2	I	Purine
Inosine-5-monophosphate	0.1-34; 0.1-10	8.21 (s, CH)/148.9; 8.54 (s, CH)/142.6; 6.15 (d, J=5.7 Hz, CH)/89.9;	Ι	Enzyme
(IMP)		4.38 (m,CH)/87.1		
Lactate (Lac)	47-778 ; 32-608	1.32 (d, J=6.9 Hz, CH ₃)/22.9; 4.11 (q, J=6.9 Hz, CH)/71.4	II	Organic Acid
Glycerophosphorylcholine	0.1-15; 1-47	3.21 (s, N(CH ₃) ₃)/56.6; 3.64 (m, CH ₂)/64.6; 4.30(m, CH ₂)/62.1	II	Essential nutrient
(GPC)				
Choline (Cho)	2-32; 2-38	3.22 (s, N(CH ₃) ₃)/56.7; 3.52 (m, CH ₂)/70.1; 4.08 (m, CH ₂)/58.5	II	Essential nutrient
Glucose (Glu)	0.3-35; 0.2-58	3.24 (m, CH) /76.9; 3.40 (m, CH)/72.3; 3.50 (m, CH)/74.2; 3.73 (m,	II	Monosaccharide
		CH)/63.3; 3.91 (m, CH ₂)/63.4; 4.65 (d, J=7.9 Hz, CH)/98.7; 5.24 (d,		
		J=3.7 Hz, CH)/94.9		
Phosphatidylethanolamine (PE)	5-165 ; 3-90	4.02 (m)	II	Phospholipids
Succinate (Suc)	0.1-16; 0.7-21	2.42 (s, CH ₂)/36.8	III	Organic Acid
Anserine (Ans)	4-235; 4-106	2.67 (m, CH ₂)/34.8; 3.08 (dd, CH ₂)/28.3; 3.24 (m, CH ₂)/38.3; 3.84 (s,	III	Dipeptide
		CH ₃)/35.2; 4.51 (dd, CH)/56.1; 7.16 (s, CH)/122.8; 8.35 (s, CH)/138.4		
Carnosine (Car)	0-60; 0-33	2.68 (m, CH ₂)/34.8; 3.08 (dd, CH ₂)/30.8; 3.24 (m, CH ₂)/38.6; 4.51 (m,	III	Dipeptide
		CH, ring)/57.6; 7.06 (s, CH, ring)/119.9; 8.17(s CH, ring)/137.4		
Trimethylamine (TMA)	0-1; 0.5-14	2.91 (s, N(CH ₃) ₃)/47.5	III	Amine
Creatine/phosphocreatine	13-213; 10-137	3.04 (s, CH ₃)/39.5; 3.93 (s, CH ₂)/66.4	III	Organic Acid
(Crt/P-crt)				
Isoleucine (Ile)	0.1-21; 0.4-18	0.94 (t, J=7.4 Hz, CH ₃)/13.9	IV	Amino acid
Leucine (Leu)	0.1-42; 0.8-37	0.96 (t, J=5.9 Hz, CH ₃)/23.6	IV	Amino acid
Valine (Val)	0.2-34; 0.7-23	0.99 (d, J=7.0 Hz, CH ₃)/19.4; 1.05 (d, J=7.0 Hz, CH ₃)/20.8; 2.28 (m,	IV	Amino acid
		CH)/31.9		
Alanine (Ala)	0.5-47;3-57	1.48 (d, J=7.1 Hz, CH ₃)/18.9; 3.78 (q, J=7.2 Hz, CH)/53.1	IV	Amino acid
Lysine (Lys)	0.1-17; 0.8-28	1.73 (m, CH ₂)/29.2; 1.92 (m, CH ₂)/32.7	IV	Amino acid
Proline (Pro)	NQ	2.04 (m, CH ₂)/32.1; 2.36 (m, CH ₂)/31.7; 3.35 (m, CH ₂)/48.9; 4.13 (m,	IV	Amino acid
	· ·		1	I

		CH)/64.0		
Glutamate (Glu)	0.2-31; 2-34	2.07 (m, CH ₂)/29.6; 2.37 (m, CH ₂)/36.1; 3.78 (m, CH)/56.7	IV	Amino acid
Methionine (Met)	NQ	2.13 (m, CH ₃ / CH ₂)/32.7	IV	Amino acid
Taurine (Tau)	0.7-34;22-337	3.26 (t, J=7.3 Hz, CH ₂)/50; 3.44 (t, J=7.3 Hz, CH ₂)/38.1	IV	Amino acid
Glycine (Gly)	0.8-27; 5-88	3.57 (s, CH ₂)/44.3	IV	Amino Acid
Tyrosine (Tyr)	0-9; 0.2-7	6.91 (m, CH ring)/118.9; 7.20 (m, CH ring)/133.5	IV	Amino acid
Phenylalanine (Phe)	0-12; 0-12	7.43 (m, CH ring)/131.8; 7.34 (d, J=7 Hz, CH ring)/132.1 7.39 (m, CH,	IV	Amino acid
-		ring)/130.4		

318 3.2 Discrimination between tropical tuna species

319 3.2.1. Effect of Species on ¹H NMR profiles of tuna muscles.

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The PLS-DA model derived from tunas' OSC-filtered white muscle spectra revealed R^2Y and Q^2 321 322 values of 0.70 and 0.70 for two latent components, respectively, indicating good fit and prediction abilities for the PLS regression model. The three tropical tuna species were separately 323 324 grouped on the PLS-DA score plot with six compounds or types of molecules, responsible for the inter-species discrimination (Fig. S1A, Table S2). Dipeptides (anserine and carnosine) were the 325 326 highest in BET followed by SKJ and finally YFT. Phospholipids (PE) had the highest levels in SKJ and the lowest levels in BET. Finally, organic acids (creatine, phosphocreatine and 327 succinate) were predominant in YFT, excepted for lactate that showed the highest and lowest 328 levels in SKJ and BET, respectively. 329

Better fits and prediction abilities were obtained with the PLS-DA model derived from tunas' 330 OSC-filtered red muscle spectra ($R^2Y = 0.84$ and $Q^2 = 0.79$ for four latent components) showing 331 a clear separation along the first latent component (t[1] axis) between the three tropical tuna 332 species (Fig. S1B). 27 NMR variables were identified, corresponding to 16 compounds or classes 333 334 of molecules (Table S2), and three NMR variables were not identified (i.e., "unknown"). 335 Metabolites from group III (anserine, carnosine, creatine, phosphocreatine, lactate and succinate) and phospholipids (PE) showed similar inter-species differences in the red muscle as previously 336 337 observed in the white muscle. Additionally, six amino acids contributed to the discrimination of the tropical tuna species: SKJ had the highest levels of isoleucine, leucine, and lysine and the 338 339 lowest levels of glutamate and valine; BET had the highest levels of glutamate and valine and the 340 lowest levels of taurine; and finally, YFT had the highest levels of taurine and the lowest levels of 341 isoleucine, leucine and lysine. The essential nutrient GPC (group II) was also discriminant with 342 higher levels observed in SKJ than in BET and YFT red muscle. Lastly, fish quality biomarkers 343 (group I: inosine, IMP and hypoxanthine) were predominant in SKJ red muscle followed by YFT and BET. 344

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346 *3.2.2. Effect of Species and Size category on ¹H NMR profiles derived from tuna muscles*

The PCA score plots derived from the white and red muscle profiles were investigated to differentiate the three tropical tuna species, in particular YFT and BET, taking into account the size category (i.e., small tunas ≤ 80 cm FL versus large tunas ≥ 100 cm FL) of the studied fish (data not shown). Supervised PLS-DA models applied on OSC-filtered revealed R²Y of 0.39 and 0.59, Q² values of 0.36 and 0.50 with three and four latent components for the tuna's white and red muscle spectra, respectively, indicating poor fit but good prediction abilities for these PLS regression models. The PLS-DA score plot derived from the OSC-filtered white muscle allowed for the discrimination of SKJ and small YFT only (Fig. 3A). The PLS-DA score plot derived from the red muscle was more discriminant with a clear separation of SKJ, small YFT and large BET, while small BET and large YFT remained confounded (Fig. 3B).

357 Supervised PLS-DA models applied on OSC-filtered data were then applied on the white and red muscle datasets after excluding SKJ data. The derived PLS-DA score plot did not give any valid 358 model ($Q^2 < 0.30$), whereas it allowed for the separation of small and large YFT and BET ($R^2Y =$ 359 0.43 and $Q^2 = 0.41$ for two latent components; Fig. 3C). A total of 15 compounds or classes of 360 361 molecules were identified and one remained unknown, were responsible for the differences between Species and Size category in the red muscle (Table S3). From the 15 identified chemical 362 species, 13 were common with those responsible for the discrimination of the three tropical 363 species only (Table S2). Differences between SKJ and the two other species were specified when 364 taking into account YFT and BET size categories. Anserine, lactate, succinate, glutamate, and 365 methionine showed similar levels between SKJ and the small YFT and BET but different ones 366 with large tunas; the opposite trends were observed for TMA. Regarding BET versus YFT, 367 valine, anserine and carnosine were predominant in BET and in large individuals compared to 368 small ones for both species. Creatine / phosphocreatine, taurine and lactate were higher in SKJ 369 and in small individuals compared to large ones for YFT, BET and both species respectively. 370 Finally, although no difference was observed between YFT and BET, methionine, TMA and GPC 371 were affected by Size category with the large tunas having the highest levels of methionine and 372 TMA and the lowest levels of GPC (for YFT only). 373



377 Fig. 3 Two-dimensional Partial Least Squares-Discriminant Analysis (PLS-DA) score plots based on the metabolic profile from white and red muscle aqueous extracts of the three tropical tuna species 378 categorized by size, i.e., small yellowfin tuna (YFT_S, n=25), large yellowfin tuna (YFT_L, n=35), small 379 380 bigeye tuna (BET S, n=34), large bigeye tuna (BET L, n=28) and skipjack tuna (SKJ, n=72). (A) PLS-DA score plot of OSC-filtered integrated ¹H NMR spectra of white muscle extracts for all species/size 381 categories, using three components ($R^2Y = 0.39$ and $Q^2 = 0.36$); (**B**) PLS-DA score plot of OSC-filtered 382 integrated ¹H NMR spectra of red muscle extracts for all species/size categories, using five components 383 $(R^2Y = 0.59 \text{ and } Q^2 = 0.50);$ (C) PLS-DA score plot of OSC-filtered integrated ¹H NMR spectra of red 384 385 muscle extracts for four species/size categories only (excluding skipjack), using two components ($R^2Y =$ 0.43 and $Q^2 = 0.41$). 386

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388 3.3 Discrimination of tropical tunas according to storage condition on-board purse seiners

The traceability of the tunas' storage conditions on-board purse seiners allowed for the PLS-DA analysis of the differences in NMR metabolomic profiles from tropical tunas white and red muscle extracts.

The PLS-DA score plot derived from the SKJ and large tuna's OSC-filtered white muscle spectra 392 as well as from the large tuna's red OSC-filtered muscle spectra did not give any valid model (Q² 393 < 0.30), suggesting that both freezing modes affect similarly these size class and species. Only 394 the white muscle of small tunas (YFT and BET) showed differences between brine-freezing and 395 deep-freezing storage conditions ($R^2Y = 0.69$ and $Q^2 = 0.46$ for two latent components; Fig. 4A), 396 with 14 metabolites identified (Table S4). Among them, nine metabolites corresponded to amino 397 acids that exhibited higher levels in deep-frozen small tunas, most likely reflecting a lower level 398 399 of protein degradation during this storage mode. Deep-frozen small tunas were also characterised

- by lower IMP and higher creatine/phosphocreatine levels compared to brine-frozen ones, which
 could reflect a slower process of ATP and creatine/phosphocreatine degradation (Chan et al.,
 1994).
- In the red muscle, the freezing storage condition was a discriminant factor for small YFT and 403 BET ($R^2Y = 0.91$ and $Q^2 = 0.82$ for three latent components; Fig. 4B), and SKJ ($R^2Y = 0.79$ and 404 $Q^2 = 0.73$ for one latent component; Fig. 4C). Deep-frozen small tuna's red muscle had higher 405 levels of four amino acids, glycogen and PE, as a result of lower process of degradation; also, 406 407 from group III, anserine/carnosine and creatine/phosphocreatine were higher in deep-frozen small 408 tunas compared to brine-frozen tunas, while opposite trends were observed for lactate and 409 succinate. Finally, a lower number of metabolites was identified as responsible for the differences 410 between freezing modes (nine against 12 compounds in small tunas' muscles), with PE, anserine, 411 carnosine and glutamate showing higher levels in deep-frozen but lower levels of succinate and 412 taurine compared to brine-frozen SKJ red muscle.



Fig. 4. Two-dimensional Partial Least Squares-Discriminant Analysis (PLS-DA) score plots based on the metabolic profile from white and red muscle aqueous extracts of the tropical tuna species depending on the storage conditions onboard the purse-seine vessels, i.e., brine-freezing and deep-freezing. (A) PLS-DA score plot of OSC-filtered integrated ¹H NMR spectra of white muscle extracts for small tunas (n = 83 and 48 stored in brine- and deep-freezing wells, respectively), using two components ($R^2Y = 0.69$ and $Q^2 = 0.46$); (B) PLS-DA score plot of OSC-filtered integrated ¹H NMR spectra of red muscle extracts for small tunas (n = 83 and 48 stored in brine- and deep-freezing wells, respectively), using three components ($R^2Y = 0.91$ and $Q^2 = 0.82$); (C) PLS-DA score plot of OSC-filtered integrated ¹H NMR spectra of red muscle extracts for SKJ (n = 42 and 17 stored in brine- and deep-freezing wells, respectively), using one component ($R^2Y =$ 0.79 and $Q^2 = 0.73$).

423 3.4 Discrimination of the purse seine tropical tunas according to their geographical origin

- The information on the fishing locations retrieved from the purse-seine logbooks was merged with the NMR metabolomic profiles from tropical tunas white and red muscle extracts and analysed with PLS-DA.
- Fig. 5A, 5B and 5C represent the PLS-DA score plots derived from the white muscle of small 427 YFT and BET ($R^2Y = 0.82$ and $Q^2 = 0.73$ for two latent components), SKJ ($R^2Y = 0.74$ and $Q^2 =$ 428 0.70 for two latent component) and large YFT and BET ($R^2Y = 0.79$ and $Q^2 = 0.77$ for one latent 429 component), respectively. Fig. 5D, 5E and 5F represent the PLS-DA score plots derived from the 430 red muscle of small YFT and BET ($R^2Y = 0.84$ and $Q^2 = 0.73$ for two latent components), SKJ 431 $(R^2Y = 0.89 \text{ and } Q^2 = 0.83 \text{ for one latent component})$ and large YFT and BET $(R^2Y = 0.85 \text{ and } Q^2 = 0.83 \text{ for one latent component})$ 432 $Q^2 = 0.77$ for three latent component), respectively. All PLS regression models gave good fit and 433 prediction abilities. 434
- Nine metabolites were identified in the white muscle of small and large tunas as responsible for 435 the differences between Geographical origin, against only six in the white muscle of SKJ. For all 436 species and size categories, most metabolites were pertaining to the groups II and III. Based on 437 438 the identified and level changes in metabolites, a greater resemblance of NMR profiles was however observed between SKJ and small tunas compared to large tunas. For instance, the 439 geographical origin of the small tunas and SKJ was well distinguished with small tunas from 440 441 WCIO having higher levels of essential nutrients (choline and PE), lactate, hypoxanthine, IMP and carnosine in white muscle, but lower levels of anserine compared to fish from MOZ (Table 442 443 S5). Regarding large tunas, the white muscle of WCIO fish was characterised by lower anserine, 444 PE, creatine/phosphocreatine, IMP and carnosine.
- 445 In the red muscle, 18, 16 and 10 metabolites were contributing to the discrimination of large 446 tunas, small tunas and SKJ respectively, according to the Geographical origin. Metabolites 447 belong to groups II, III, and IV for small tunas and SKJ, and to all groups for large tunas. In particular, WCIO large tunas had higher GPC, creatine/phosphocreatine, IMP and inosine but 448 lower TMA, methionine and alanine, and WCIO SKJ had higher choline, isoleucine and leucine, 449 hypoxanthine+IMP+carnosine than their congeners from MOZ. Furthermore, large and small 450 tunas exhibited similar variations for 10 metabolites corresponding to higher taurine and 451 anserine+PE+carnosine, and lower glucose, glycogen, glutamate, isoleucine, leucine, lysine, 452 valine and hypoxanthine+IMP+carnosine in WCIO fish. Fewer similitudes were observed 453 454 between small tunas and SKJ and between large tunas and SKJ with only four (PE, anserine,

455 carnosine and creatine/phosphocreatine) and one (lactate) similar metabolites variations,456 respectively.

The discrimination of WCIO and MOZ tunas observed in this study may result from differences 457 458 of trophic and behavioural ecology. Indeed, while tropical tunas feed mainly on fish and squids when inhabiting WCIO waters, crustaceans predominate their diet in MOZ (Chassot et al., 2019). 459 460 Such "red feed" is often related to fish tissue softening in pelagic species (Huss et al., 1995) and may favour proteolytic breakdown producing low molecular weight peptides and free amino 461 462 acids. The high occurrence of such metabolites may reduce the commercial acceptability of fish, 463 but also accelerate bacterial spoilage and produce biogenic amines (Biji et al., 2016). Moreover, 464 the lower levels of burst activity markers in MOZ tuna noted in this study could be related to 465 differences of habitat size and schooling behaviour between the two studied areas. In MOZ, 466 tropical tunas (all species/size categories) are mainly associated with fish aggregating devices (FADs) and present a relatively restricted habitat, whereas they are found both in free-swimming 467 468 schools and aggregated under FADs in WCIO (especially large tunas) where they are able to cover large distances to search for potential preys (Chassot et al., 2019; Fonteneau et al., 2013). 469



470 Fig. 5. Two-dimensional Partial Least Squares-Discriminant Analysis (PLS-DA) score plots based on the metabolic profile from white and red muscle aqueous 471 extracts of the tropical tuna species per geographical origin, i.e., Mozambique Channel (MOZ) and western-central Indian Ocean (WCIO). (A) PLS-DA score plot 472 of OSC-filtered integrated ¹H NMR spectra of white muscle extracts for small yellowfin and bigeye tunas (n = 18 and 40 in MOZ and WCIO, respectively), using 473 two components ($R^2Y = 0.82$ and $Q^2 = 0.73$); (**B**) PLS-DA score plot of OSC-filtered integrated ¹H NMR spectra of white muscle extracts for skipjack (n = 23 and 474 48 in MOZ and WCIO, respectively), using two components ($R^2Y = 0.74$ and $Q^2 = 0.70$); (C) PLS-DA score plot of OSC-filtered integrated ¹H NMR spectra of 475 476 white muscle extracts for large vellowfin and bigeve tunas (n = 11 and 54 in MOZ and WCIO, respectively), using one component ($R^2Y = 0.79$ and $Q^2 = 0.77$); (**D**) PLS-DA score plot of OSC-filtered integrated ¹H NMR spectra of red muscle extracts for small yellowfin and bigeye tunas (n = 18 and 40 in MOZ and 477 478 WCIO, respectively), using two components ($R^2Y = 0.84$ and $Q^2 = 0.73$); (E) PLS-DA score plot of OSC-filtered integrated ¹H NMR spectra of red muscle extracts for skipjack tuna (n = 23 and 48 in MOZ and WCIO, respectively), using one component ($R^2Y = 0.89$ and $Q^2 = 0.83$); (F) PLS-DA score plot of OSC-479 filtered integrated ¹H NMR spectra of red muscle extracts for large yellowfin and bigeye tunas (n = 11 and 54 in MOZ and WCIO, respectively), using three 480 components ($R^2Y = 0.85$ and $Q^2 = 0.77$). 481

482 **4** Conclusion

In conclusion, high-resolution ¹H NMR spectroscopy of aqueous extracts from white and red 483 muscles of tropical tunas proved to be very efficient for evidencing rich metabolic information 484 (amino acids, dipeptides, organic acids, and other essential nutrients) allowing to discriminate 485 species and size categories, and in a lesser extent on-board storage modes. Discrimination with 486 respect to geographical origin for small tropical tunas was also possible (mainly based on burst 487 activity markers and essential nutrients), which could improve traceability of tuna products in 488 world market. Considering the global scale of this market, geographical comparisons should be 489 490 extended to the global ocean.

491 492

493 Authors' contribution

Conceptualization, NB and HG; Funding Acquisition, NB and EC; Project Administration, NB,
HG, LD; Sampling and Data Acquisition, AA, CC, EF, FS and NB; Data Curation and Analysis,
AA, CC and MT-F; Writing – Original Draft Preparation, AA and NB; Writing – Review &
Editing, All co-authors.

498

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