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1 NON-LETHAL SAMPLING METHOD FOR THE ANALYSIS OF WHITE MUSCLE FATTY ACID
2 PROFILES IN EUROPEAN SEA BASS (*Dicentrarchus labrax*)

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5 Abstract :

6 This study presents a novel non-lethal sampling method for assessing fatty acid (FA) composition in juvenile
7 European sea bass (*Dicentrarchus labrax*) using subcutaneous white muscle biopsies. This research aimed to
8 evaluate the suitability of the biopsy for FA analysis using two lipid extraction protocols and comparing them to
9 a lethal routine method. The results showed that a mass of fresh tissue as low as 1.4 mg provided good quality FA
10 chromatograms for both reserve and membrane lipids. Although the biopsy method displayed high variability in
11 terms of FA quantity among intra-individual replicates, it showed good FA profile repeatability in both reserve
12 and membrane lipids. The study highlights the potential of this non-lethal approach for studying FA dynamics in
13 fish, with its application particularly promising for ecological and experimental studies. However, careful biopsy
14 implementation is recommended to account for potential lipid droplet and lipid distribution variability within the
15 tissue.

16 Key words: Fatty acids, European sea bass, Muscle biopsy, Lipid composition, Method comparison

17 1. Introduction

18 Lipids are essential molecules in animals that serve both as energy sources and reserves (non-polar lipids; NL), as
19 well as key structural components of cell membranes (polar lipids; PL) (Sargent et al. 2003). Due to their structural
20 function, lipids modulate cell membrane functionality (Singer and Nicholson 1972; Hulbert and Else 1999; Tocher
21 2003), which in turn influences various biological processes in cells, tissues, organs, and ultimately affects the
22 overall performance of an individual (Tocher 2010). Fatty acids are also involved in the modulation of gene
23 expression (réf) and influences the function of membrane proteins thus affecting cellular signaling and
24 communication (Murphy 1990). Both NL and PL are composed of fatty acids (FA), among which polyunsaturated
25 fatty acids (PUFA) are the main component of cell membranes and thus play a key role in maintaining vital
26 functions of most animals (Sargent et al. 2003). Among PUFA, eicosapentaenoic acid (20:5n-3, EPA) and
27 docosahexaenoic acid (22:6n-3, DHA) have especially been demonstrated to be essential for most marine fish to
28 ensure growth, reproduction, development (Tocher 2010). However, as for most animals, these FA are weakly
29 synthesized *de novo* by fish, and must be obtained through the diet. In natural environments, EPA and DHA are
30 primarily synthesized at the base of the aquatic food web by primary producers like marine microalgae (Arts and
31 Kohler 2009) and then accumulate through the food chain, ultimately reaching higher consumers (Sargent et al.
32 1993; Tocher et al. 2006; Li et al. 2010).

33 Fish fatty acid composition analysis is mandatory to meet several objectives, for example, to understand the link
34 between dietary FA and physiological performance of an individual. Experimental research has explored for
35 instance the links between dietary FA, fish muscle membrane FA composition, and individual performances, such
36 as growth, locomotion or metabolism (Tocher 2003; Calder 2012). The effects of dietary FA on fish physiology
37 have also been studied in the context of global change, in combination with other environmental stressors like

38 hypoxia (McKenzie et al. 2000; McKenzie et al. 2008), warming (Gourtay et al. 2018; Vagner et al. 2019) or
39 acidification (Hurst et al. 2019). Examining fish FA composition is also valuable for understanding the structure
40 of trophic food webs in natural ecosystems, as some fatty acids serve as trophic biomarkers that can be used to
41 identify consumer prey and elucidate trophic interactions within an ecosystem (Dalsgaard et al. 2003; El-Sabaawi
42 et al. 2010; Mathieu-Resuge et al. 2019; Bertrand et al. 2022).

43 Typically in fish, FA analysis requires the sacrifice of the animal to obtain sufficient amount of tissue from specific
44 organs (e.g, 50-200 mg, wet weight WW, Meyer et al. 2017), such as the liver, brain or muscle (Couturier et al.
45 2020; Betancor et al. 2021), to meet the minimal biomass requirements according to the analytical protocol . Aside
46 from being a lethal method, another drawback of this approach is that it only allows us for the determination of an
47 individual's FA composition at a single point in time. As a result, monitoring changes in an individual's FA
48 composition over time becomes challenging, which could otherwise offer valuable insights into the dynamics of
49 FA incorporation. However, this is only the case for small animals like juvenile fish and larger animals can be
50 sampled without risking killing the individual.

51 Non-lethal sampling methods have already been used in fish biology to address trophic ecology and ecotoxicology,
52 using fins and scales (Cano-Rocabayera et al. 2015; Jeffries et al. 2021) or blood (Lawrence et al. 2020). Non-
53 lethal sampling of muscle has also been realized to study mercury concentrations, with a tissue WW ranging from
54 47 to 220 mg from fishes of a minimal size of 36 cm and 600 g (Baker et al. 2004). In 2013, Olsen et al. studied
55 muscle FA composition using biopsy on adult salmon *Salmo salar* with a mean size and weight of 84 cm and 6.6
56 kg, respectively, and with biopsy samples weighing a minimum of 40 mg WW. However, one of the main
57 challenges of these non-lethal sampling methods is the necessity to take a small quantity of sampled tissue to
58 guarantee minimal harm to fish, especially when working with small species or young individuals (Meyer et al.
59 2017). Given that lipid distribution within a tissue can exhibit heterogeneity (Nanton et al. 2007; Barbosa et al.
60 2018), the small amount of tissue collected through biopsies may lead to an inaccurate representation of the tissue's
61 lipid content. This could result in a lack of repeatability, as the variability in FA composition between replicates
62 obtained from different areas of the same tissue is likely to be high.

63 The objective of our research was to evaluate a novel nonlethal sampling technique for determining the FA
64 composition of juvenile European sea bass (*Dicentrarchus labrax*; Linnaeus 1758) using subcutaneous white
65 muscle biopsies. We anticipate that this approach will: i) yield adequate tissue sample quantities for generating
66 interpretable FA profiles ii) would be repeatable within an individual, with a low intra-individual variability, also
67 compared to the inter-individual variability in terms of FA quantity and proportions, and iii) produce results
68 comparable to a traditional but lethal method of sampling using higher tissue biomass. As working with dry
69 samples is of interest in some particular cases, such as field studies, thanks to its convenience for transport and
70 storage (Sardenne et al. 2019), we wanted to test whether the conditioning (freeze-drying or fresh) of biopsied
71 tissues had an effect on FA quantities and proportions and could be compared to our routine tissue analysis.

72 2. Material and methods

73 2.1. Ethics statement

74 All fish manipulations were performed according to the Animal Care Committee of France (ACCF) and performed
75 at the Ifremer facilities in Plouzané (France, Agreement number: B29-212-05). All fish manipulations were

76 performed under anesthesia using tricaine (200 mg l⁻¹, ethyl 3-aminobenzoate methane sulfonate), and all efforts
77 were made to minimize animal suffering during manipulation.

78 2.2. Animal origin

79 Juvenile European sea bass originating from aquaculture farm have been raised from January 2020 to April 2021
80 in a single tank. They were maintained at the natural seasonal temperature of the bay of Brest (min-max: 10-19°C)
81 and were fed ad-libitum with a commercial diet thrice a week (NeoStart, Le Gouessant, Lamballe, France).

82 2.3. Muscle sampling and lipid extraction

83 In April 2021, six individuals randomly chosen (15 ± 2 cm; 50 ± 10 g) were euthanized using tricaine (400 mg l⁻¹
84 ¹) in seawater. On each individual, six subcutaneous white muscle biopsies were immediately performed on the
85 left dorsal side of the fish, just behind the head, using a biopsy punch (2 mm LCH-PUK-20, Kai Medical, Solingen,
86 Germany) (Fig. S1).

87 Three out of the six biopsies (further named “Fresh”, 8.8 ± 1.0 mg WW) were directly dived into 6 mL of a mix
88 of chloroform/methanol (2:1, v/v), following a modified method from (Folch, 1957) for lipid extraction. The three
89 other biopsies (further named “FD” for Freeze-Dried, 9.5 ± 0.8 mg WW) were first flash frozen in liquid nitrogen,
90 and then freeze-dried for 36 hours before being dived into 6 mL of a mix of chloroform/ methanol (2:1, v/v).

91 After having performed biopsies, a large muscle sample (further named “MC” for Muscle control, 80.0 ± 24.4 mg
92 WW) was sampled on the same area as where biopsies were performed, in each individual (Fig. S1). MC were
93 treated following our routine protocol for FA analyses. It was flash frozen, then grounded in liquid nitrogen into a
94 homogenous powder and finally, 40 to 100 mg WW of muscle powder was dived in a 6 mL mix of
95 chloroform/methanol (2:1, v/v). To ensure a total lipid extraction, all the lipid extracts (Fresh, FD, and MC) were
96 sonicated for 10 min and agitated for 20 min before being stored at -20°C under nitrogenous atmosphere prior
97 further FA analysis.

98 2.4. Analysis of the muscle fatty acid composition

99 2.4.1. Lipid separation

100 For all samples, lipids were separated into neutral lipids (NL) and polar lipids (PL) fractions following the method
101 described by Le Grand et al. (2014). An aliquot (one sixth) of the total lipid extract was used for Muscle control
102 samples, whereas the whole lipid extract was used for biopsies (both Fresh and FD), as the mass of biopsy sampled
103 tissue ranged from 1.4 to 14.4 mg for Fresh and from 5.5 to 13.6 mg for FD samples. The lipid extract aliquot was
104 evaporated to dryness under nitrogen, recovered with three washings of 0.5 mL of chloroform/methanol (98:2,
105 v/v), and deposited at the top of a silica gel column (40 mm × 4 mm, silica gel 60 A 63–200 µm rehydrated with
106 6% H₂O (70–230 mesh)). NL were eluted with 10 mL of chloroform/methanol (98:2, v/v) and collected in a glass
107 vial containing 2.3 µg of internal standard (tricosanoic acid, C23:0). PL were then eluted with 20 mL of methanol
108 and collected in the same way as NL.

109 2.4.2. Neutral lipid and polar lipid transesterification

110 NL and PL fractions were processed following a modified version of Mathieu-Resuge et al. (2019). Briefly, NL
111 and PL fractions were evaporated to dryness under nitrogen. Fatty Acid Methyl Esters (FAME) were then formed
112 by adding 800 µL of H₂SO₄/methanol (3.4%, v/v) and heating at 100°C for 10 min in a dry bath. After cooling,

113 800 μ L of hexane and 1.5 mL of hexane-saturated distilled water were added to the mixture. After removal of the
114 aqueous phase, the hexane phase, containing FAME, was washed two more times with hexane-saturated distilled
115 water.

116 2.4.3. FAME analysis

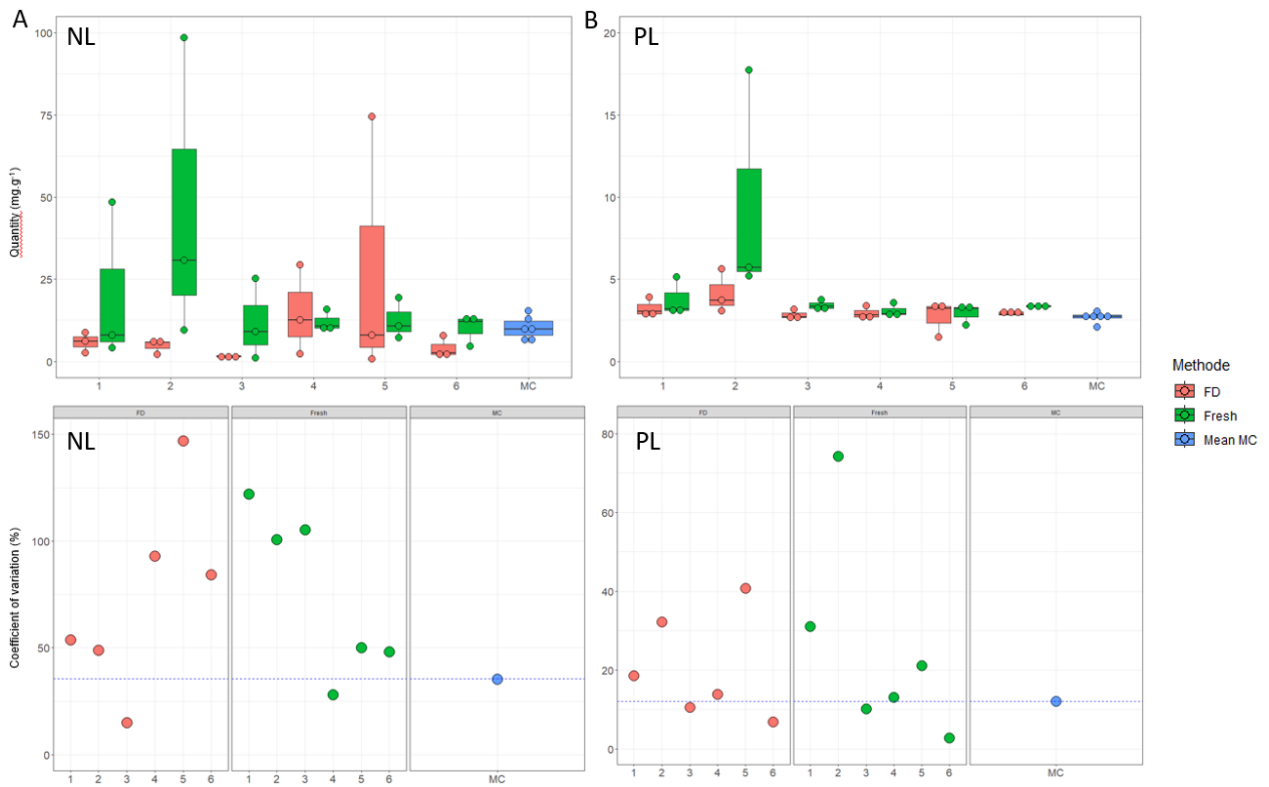
117 Analyses of FAME was performed on a Varian CP8400 gas chromatograph using simultaneously two separations
118 on a polar column (DBWAX: 30 mm \times 0.25 mm ID \times 0.2 μ m, Agilent) and an apolar column (DB5: 30 m \times 0.25
119 mm ID \times 0.2 μ m, Agilent) using hydrogen as carrier gas with a flow rate set at 2 mL.min⁻¹, in splitless mode. The
120 oven temperature was programmed as follows: first, initial heating from 60°C to 150 °C at 50 °C.min⁻¹, then to
121 170 °C at 3.5 °C.min⁻¹, to 185 °C at 1.5 °C.min⁻¹, to 225 at 2.4 °C.min⁻¹ and finally to 250 °C at 5.5 °C.min⁻¹
122 and maintained for 25 min. Injector temperature was set at 250°C, detector temperature at 280°C. Identification of
123 FAME was realized by comparison of their retention time based on those of commercial standards (Supelco 37
124 Component FAME Mix, PUFA No.1 and No.3, and Bacterial Acid Methyl Ester Mix, Sigma, MO, USA). Internal
125 standard allowed to calculate FA content (μ g.mg⁻¹ WW). Fatty acid proportion was defined as the mass percentage
126 of each fatty acid to the total fatty acid content.

127 2.5. Statistical analysis

128 All statistical analyses were conducted using R Studio (v4.2.1). In order to determine if the biopsy method would
129 be spatially repeatable within one individual, we calculated the intra-individual variation as the CV (standard
130 deviation/mean) of the studied parameter (FA quantities and proportion), for each individual and for each biopsy
131 method (n=3 Fresh and n=3 FD for each individual). This was performed on both quantity and proportions of EPA,
132 DHA, PUFA, monounsaturated fatty acid (MUFA) and saturated fatty acid (SFA) in both NL and PL fractions. In
133 order to be able to characterize the range of intra-individual variability, we compared these intra-individual CV to
134 the corresponding inter-individual CV performed on MC samples (n=6).

135 We hypothesized that if the intra-individual CV exceeded either 10% (Sardenne et al. 2019) or the corresponding
136 inter-individual CV, the biopsy method would not be repeatable enough to be validated and used with confidence.
137 On the contrary, if the intra-individual variability was lower than 10% or than the inter-individual variability, we
138 hypothesized that the biopsy method would be repeatable. It is the necessary condition allowing us to calculate the
139 mean of the biopsy triplicates and to conduct a PERMANOVA test to compare the effect of the method (Fresh,
140 FD, MC) on the FA proportions. Only the ten main FA were considered in this analysis. To evaluate if there were
141 any differences in specific FA proportions between the methods, each FA was analyzed using a one-way ANOVA
142 test. Normality and homoscedasticity were assessed on residuals with the Shapiro-Wilk and Bartlett tests,
143 respectively. If ANOVA assumptions were not met, a Kruskal-Wallis test was employed instead. When ANOVA
144 or Kruskal-Wallis tests indicated significant differences, a post-hoc Tukey HSD test was conducted to differentiate
145 the conditions. Data are presented as mean \pm SEM.

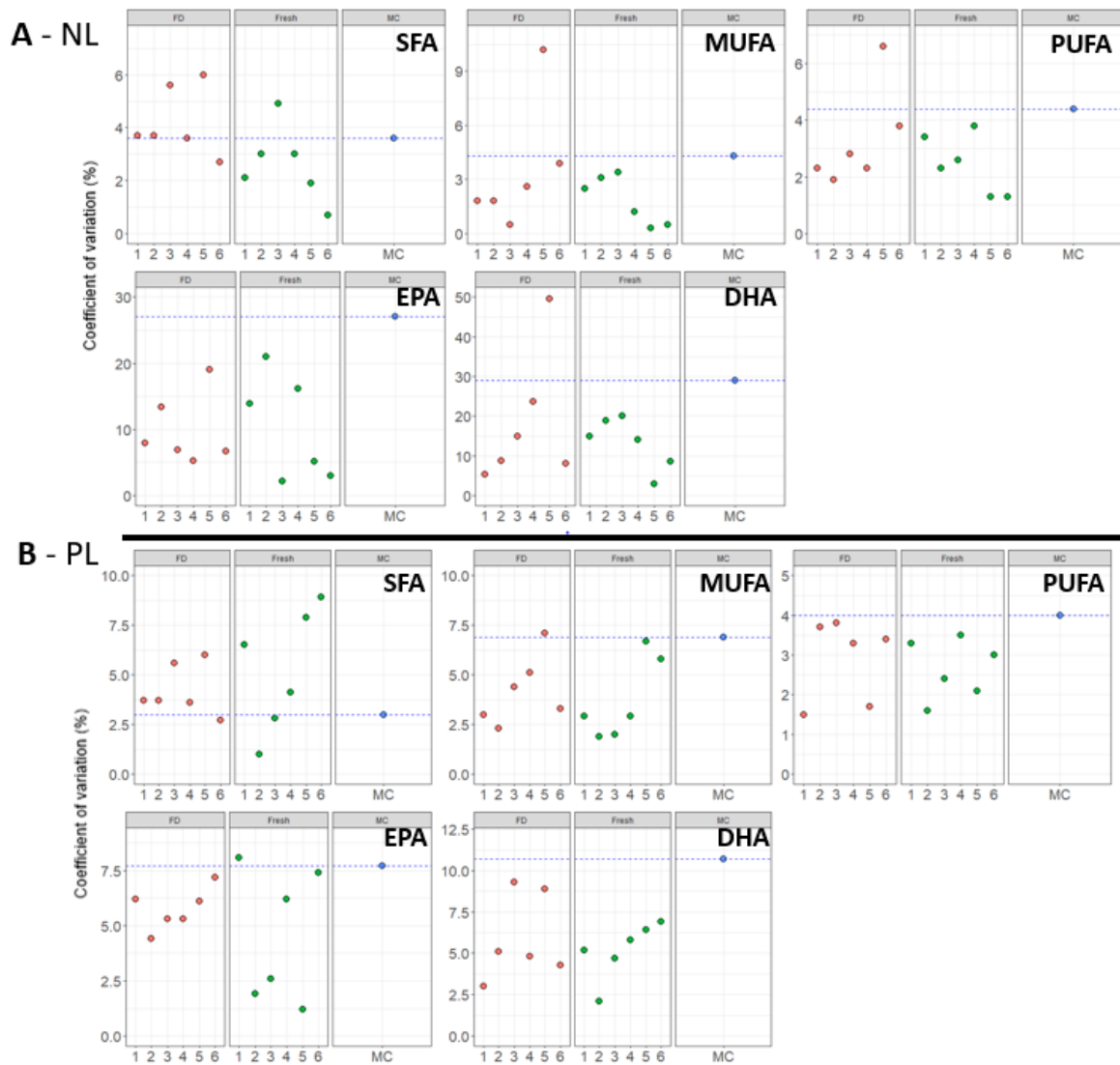
146 3. Results



148 **Fig. 1: Fatty acid quantities (mg.g⁻¹ WW upper panels) and coefficients of variation (percentage, lower**
 149 **panels) of FA quantities in neutral lipids (A, C) and polar lipids (B, D) of different method.** The different
 150 methods are presented as followed: Freeze-dried protocol (FD, red, n=3 intra-individual replicates), fresh protocol
 151 (Fresh, green, n=3 intra-individual replicates) and muscle control (MC, blue, n=6 inter-individual replicates) in
 152 white muscle of juvenile European sea bass, *Dicentrarchus labrax*. On the boxplots, each dot represent a replicate.
 153 On the dot plot, each dot represents the CV of the 3 (for biopsies) or 6 replicates (for MC) and the blue dotted line
 154 represent the value of MC CV.

155 3.1. Adequacy of biopsied tissues for FA analysis.

156 White muscle biopsy samples ranging from 1.5 to 14.4 mg were sufficient to obtain good quality chromatograms.
 157 In addition, the biopsy method allowed us to quantify over 90% of the FA identified in the Muscle control method.
 158 The biopsy total lipid extracts contained 86.1 ± 13.5 µg (min-max: 5.5-409.0 µg) of FA for NL and 28.8 ± 1.7 µg
 159 (mix-max: 10.8-45.6 µg) of FA for PL (not shown). These values were similar to those contained in the 1/6 aliquots



160 of MC total lipid extracts used in the routine method: $86.1 \pm 13.5 \mu\text{g}$ (min-max: 5.5-409.0 μg) of FA for NL and
 161 $28.8 \pm 1.7 \mu\text{g}$ (mix-max: 10.8-45.6 μg) of FA for PL (not shown).

162 **Fig. 2: Coefficient of variation (percentage) of FA proportions in NL (A) and PL (B) for the different**
 163 **methods.** Main family and FA are presented: Saturated fatty acids (SFA), monounsaturated fatty acids (MUFA),
 164 polyunsaturated fatty acids (PUFA), ecosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) for freeze-
 165 dried protocol (FD, red, n=3 intra-individual replicates), fresh protocol (Fresh, green, n=3 intra-individual
 166 replicates) and muscle control (MC, blue, n=6, inter-individual replicates) in white muscle of juvenile European
 167 sea bass, *Dicentrarchus labrax*. Each dot represents the CV of the 3 (for biopsies) or 6 replicates (for MC) and the
 168 blue dotted line represent the value of MC CV.

169 3.2. Repeatability of the biopsy method within an individual

170 Our results indicate that both biopsy methods resulted in high intra-individual variability in total FA quantity in
 171 NL (Fig. 1 NL A) (Fresh: min-max: 1.0-98.4 mg g^{-1} , FD: min-max: 0.7-74.4 mg g^{-1}) this variability, illustrated by
 172 the coefficient of variation of the three replicates per individual and biopsy method (CV mean = $75 \pm 40\%$, min-
 173 max = 15-147%) was always above 10% (Fig. 1C). The CV values were higher than those of MC for 5 out of 6
 174 individuals for both methods. In PL, the intra-individual variability of total FA quantity was lower than in NL (Fig.

175 1B) but still displayed considerable differences between extreme values (Fresh: min-max: 2.2-17.7 mg.g⁻¹, FD:
 176 min-max: 1.4-5.6 mg.g⁻¹). The CV was higher than 10% for most individuals and ranged from 3% to 74% for both
 177 Fresh and FD methods (CV mean = 23 ± 19.8%, Fig. 1D). The CV values were higher than those of MC for 4 out
 178 of 6 individuals for both methods. In general, no biopsy method distinguished itself from the other in terms of
 179 variability.

180 Regarding the NL fraction, SFA, MUFA and PUFA proportions (Fig. 2A) varied less within individuals (CV
 181 <10%), than EPA and DHA (Fig. 2A, E-F, EPA min-max: 3-21.1%; DHA min-max: 2.9-49.6%), whatever the
 182 sampling method employed, either biopsy or routine. For EPA, the biopsy CV was always lower than the MC CV.
 183 For DHA, 11 out of 12 CV were lower than the MC CV. Regarding PL, even if the CV varied among individuals,
 184 they remained under 10% for all individuals and for both methods (Fig. 2B).

185 3.3. Comparison between biopsy methods and a traditional but lethal method of sampling

186 Concerning FA quantities, the biopsy results showed a high intra-individual variability for both methods. We then
 187 considered that biopsy replicates would not be repeatable enough and then could not be pooled to obtain a mean per
 188 individual, and thus, that testing the effect of method on FA quantities would not be relevant.

189 Regarding biopsy methods, most of the FA proportions in NL and PL had a CV <10% and had lower CV than the
 190 inter-individual one (Fig. 2). We thus hypothesized that the biopsy triplicates performed within individuals could
 191 be pooled, and that the effect of method can be tested on FA proportions. When considering the 10 major fatty
 192 acids (Fig. S2), no significant effect of the method was observed for both NL and PL (PERMANOVA, p > 0.05).
 193 However, when considering the FA individually, 16:0 and 18:0 FA exhibited a significantly higher proportion in
 194 the NL fraction when analyzed with the FD method than with the two others (Table 1), and consequently, SFA
 195 proportions followed this trend. No statistical differences were observed among the three methods for PL FA
 196 proportions (Table 1).

197 **Table 1: Fatty acid proportion (mass percentage of each FA over total fatty acids) of the 10 major fatty**
 198 **acids measured in neutral (NL) and polar (PL) lipids with the three methods: Fresh biopsy, freeze-dried**
 199 **biopsy (FD) and muscle control (MC) in white muscle of juvenile European sea bass, *Dicentrarchus labrax*.**

200 Data are presented as inter-individual means (± SEM, n=6), after pooling intra-individual biopsy triplicates. The
 201 effect of the method on the FA proportions was tested using a one-way ANOVA or Kruskal-Wallis test, followed
 202 by Tukey's post hoc test or pairwise Wilcoxon test. Significance was accepted at p < 0.05. Different letters read in
 203 line indicate significant differences between methods. *** p < 0.001, ** p < 0.01, * p < 0.05, - NS (not significant).
 204 SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids.

| NL Fatty Acid | Muscle Biopsies | | Muscle Control | Statistics |
|---------------|-------------------------|-------------------------|-------------------------|------------|
| | Fresh | Freeze-Dried | | |
| 14:0 | 2.9 ± 0.2 | 3.1 ± 0.2 | 3.1 ± 0.2 | - |
| 16:0 | 18.1 ± 0.3 ^a | 18.2 ± 0.1 ^a | 18.2 ± 0.1 ^a | ** |
| 18:0 | 3.7 ± 0.2 ^a | 3.6 ± 0.1 ^a | 3.6 ± 0.1 ^a | ** |
| 16:1n-7 | 5.7 ± 0.3 | 5.9 ± 0.3 | 5.9 ± 0.3 | - |
| 18:1n-7 | 3.0 ± 0.1 | 3.1 ± 0.1 | 3.1 ± 0.1 | - |

| | | | | | |
|----------------------|-------------------------|-------------------------|-------------------------|-----------------------|-------------------|
| 18:1n-9 | 29.0 ± 1.5 | 28.8 ± 1.2 | 28.8 ± 1.2 | - | 205 |
| 18:2n-6 | 13.4 ± 1.1 | 13.1 ± 0.7 | 13.1 ± 0.7 | - | |
| 18:3n-3 | 2.5 ± 0.2 | 2.4 ± 0.1 | 2.4 ± 0.1 | - | |
| 20:5n-3 | 5.5 ± 0.7 | 5.5 ± 0.6 | 5.5 ± 0.6 | - | |
| 22:6n-3 | 2.8 ± 0.4 | 2.8 ± 0.3 | 2.8 ± 0.3 | - | |
| SFA | 25.6 ± 0.6 ^a | 26.0 ± 0.3 ^a | 26.0 ± 0.3 ^a | ** | |
| MUFA | 42.8 ± 0.9 | 43.1 ± 0.8 | 43.1 ± 0.8 | - | |
| PUFA | 31.3 ± 0.5 | 30.7 ± 0.5 | 30.7 ± 0.5 | - | |
| PL Fatty Acid | Muscle Biopsies | | | Muscle control | Statistics |
| | Fresh | Freeze-Dried | | | |
| 16:0 | 23.2 ± 0.3 | 23.7 ± 0.5 | 22.6 ± 0.3 | - | |
| 18:0 | 5.9 ± 0.1 | 5.8 ± 0.1 | 6.1 ± 0.2 | - | |
| 16:1n-7 | 2.5 ± 0.1 | 2.4 ± 0.1 | 2.7 ± 0.2 | - | |
| 18:1n-7 | 2.1 ± 0.0 | 2.1 ± 0.0 | 2.1 ± 0.1 | - | |
| 18:1n-9 | 13.1 ± 0.4 | 13.6 ± 0.4 | 14.0 ± 0.5 | - | |
| 18:2n-6 | 5.8 ± 0.4 | 5.9 ± 0.4 | 6.2 ± 0.4 | - | |
| 20:4n-6 | 1.7 ± 0.0 | 1.7 ± 0.0 | 1.7 ± 0.1 | - | |
| 20:5n-3 | 14.7 ± 0.2 | 14.5 ± 0.2 | 14.2 ± 0.4 | - | |
| 22:5n-3 | 2.4 ± 0.1 | 2.2 ± 0.2 | 2.3 ± 0.1 | - | |
| 22:6n-3 | 17.0 ± 0.5 | 16.8 ± 0.5 | 16.3 ± 0.7 | - | |
| SFA | 31.1 ± 0.4 | 31.5 ± 0.5 | 30.6 ± 0.4 | - | |
| MUFA | 19.8 ± 0.5 | 20.2 ± 0.5 | 21.1 ± 0.6 | - | |
| PUFA | 46.8 ± 0.5 | 46.2 ± 0.5 | 45.8 ± 0.7 | - | |

206

4. Discussion

207

4.1. Adequacy of biopsied tissue for FA analysis

208

Our results show that white muscle biopsy using punch of 2 mm on sea bass juvenile of about 15 cm and 50 g is adequate to perform fatty acid analysis. The sampled biopsied tissues were sufficient to yield at least 5.5 µg of FA for NL and 10.8 µg for PL, which was enough to obtain qualitative chromatograms with FA having a peak area reaching the minimal threshold. The average FA quantity extracted was even much higher than these minimums, which supports that white muscle biopsy method provides an adequate quantity of tissue for FA analysis. Olsen et al. (2013) stated that 5 µg of FA is the minimum amount required to obtain interpretable chromatograms. In the same study, the authors suggested to use a minimum of 60 mg WW of muscle but this value should consider the type of tissue, life stage of the fish, the species, as well as the analytical equipment used. The fish size and weight are crucial considerations when sampling tissue to ensure minimal impact on the animal. In our study, we did not tested the effect of biopsy on fish growth and health condition, as they were sacrificed. But in a related study, Quéméneur et al. (2022) conducted red muscle biopsies on European sea bass of a similar size (around 76.7g in weight) using the same biopsy punch as ours. They observed that the biopsy did not affect the fish's immune system, and the fish continued to gain weight post-procedure. Henderson et al. (2016) used a larger biopsy punch (4mm compared to our 2mm) on Moses perch *Lutjanus russelli* and Grass emperor *Lethrinus laticaudis*. These

221

222 fish had a total length close to those used in our study (approximately 15.8 and 14.0 cm, respectively), and it was
223 found that the biopsy procedure led to only temporary stress and did not result in any fish mortality.

224 4.2. How repeatable is the FA analysis within an individual using the biopsy method?

225 Quantities of FA obtained with the biopsy methods were highly variable within individuals for both NL (almost
226 100-time fold between min and max) and PL (almost 5-time fold between min and max). One hypothesis could be
227 that these variations might be caused by the presence of lipid droplets in the tissue. Lipid droplets are intracellular
228 structures mainly composed of NL, serving as lipid storage in various tissues, including muscle (Favé et al. 2004;
229 Granneman et al. 2017; Kiessling et al. 2006), that would hence induce higher FA content variability in NL rather
230 than in PL. However, lipid droplets are less abundant in fish white muscle compared to red muscle (Granneman et
231 al. 2017; Kiessling et al. 2006) and may only be present in few samples, leading to FA quantities significantly
232 higher in samples containing lipid droplets. In addition, the distribution of lipids in white muscle is known to vary
233 depending on the sampling location (Charette et al. 2021), which may cause FA quantities to differ based on the
234 sampling location of the biopsy.

235 4.3. Comparison between methods and traditional lethal sampling

236 Contrary to quantities, the analysis of FA proportions using biopsy was repeatable. The low variability for SFA,
237 MUFA, and PUFA proportions in NL measured within individuals (CV<10%) suggests relative homogeneity of
238 these proportions within individuals, while higher variability in EPA and DHA proportions could indicate dietary
239 or metabolic impacts leading to specific heterogeneity in FA profile. Is the biopsy method comparable to a
240 traditional but lethal method?

241 For NL, the variability measured within individuals and illustrated by the high CV values that exceeded the
242 targeted 10% threshold as well as the inter-individual variability (Sardenne et al. 2019), makes it difficult to
243 compare the two-biopsy methods with the control methods. A higher quantity of tissue would have supported less
244 variability between methods, as shown by Sardenne et al. (2019) who compared the effect of extraction method
245 from wet and dry muscle tissue (around 100 mg) from gilthead seabream *Sparus aurata* and did not find any
246 differences between methods when considering total lipids (NL + PL).

247 In terms of proportions, the biopsy method seemed to yield results comparable to the MC for both extraction
248 protocols. As the intra-individual variability did not reach the threshold of our study, we considered the biopsy
249 method repeatable and comparable for both extraction protocols. Indeed, no differences between biopsy and
250 control methods were observed when considering the whole profile of 10 major FA for NL and PL. However,
251 differences were observed when considering individually certain SFA (16:0 and 18:0) for the FD method despite
252 being repeatable with our criteria. Nevertheless, we acknowledge that applying a uniform threshold to both
253 proportions and quantities may not be wholly pertinent, as it involves two distinct aspects. It might be necessary
254 to calibrate this threshold more appropriately, depending on whether we are investigating proportions or quantities
255 due to their different nature and analytical variabilities.

256 5. Conclusions

257 This study assessed the feasibility of a novel approach for measuring FA composition in European sea bass using
258 subcutaneous white muscle biopsies. The method allowed successfully obtaining adequate sample biomass and

259 produced high-quality chromatograms for both analytical protocols. FA quantity obtained with both biopsy
260 methods was highly variable within individuals, potentially due to factors such as lipid droplets and heterogeneous
261 lipid distribution in the sampled tissue. We thus recommend to take a special care when realizing the biopsy, and
262 to ensure that a sufficiently deep piece of tissue is removed, avoiding the muscle directly in contact with the skin
263 which has different FA composition. The analysis of FA proportions appeared to be achievable with biopsy with
264 both protocols. Although it is challenging to determine the optimal biopsy method for FA analysis in fish, this
265 study demonstrates the potential of using subcutaneous white muscle biopsies in European sea bass for examining
266 FA composition, with a particular focus on FA proportions. The fish species, its fat content and its size must also
267 be considered to optimize lipid extraction and minimize the effect of the procedure on the animal.

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270 7. Competing interests

271 The authors declare that they have no known competing financial interests or personal relationships that could
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275 9. References

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