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**Histological discrimination of fresh and frozen/thawed fish meat:
European hake (*Merluccius merluccius*) as a possible model for white meat
fish species**

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Abstract: The present study aimed at setting up a standard operating histological procedure to discriminate fresh from frozen-thawed fish products of the species *Merluccius merluccius* (European hake). A preliminary histological analysis of fresh *M. merluccius* muscle was performed to select the sampling site and highlight possible time-dependent tissue alterations during shelf-life. To set a suitable operational grid for discriminating the freezing process, morphological and morphometrical parameters were assessed on 90 muscle tissue samples collected from 30 fresh, 30 experimentally frozen at -20°C and 30 Individual Quick Frozen (IQF) specimens of *M. merluccius*. Structural score, presence of freezing vacuoles, a number of vacuoles per field higher than 1.12 and the presence of interstitial seroproteinaceous material, which had achieved statistical significance in group comparisons were chosen as freezing markers. Accuracy and repeatability, assessed on the analysis of two independent operators (on-training and expert), showed high analytical specificity and sensitivity and a concordant diagnostic performance regardless the operators expertise. The grid was finally validated by a single blind test on 30 additional *M. merluccius* commercial products and allowed the allocation of all the samples to fresh or frozen status without inconclusive results. The method could be profitably applied against fraudulent adulteration practices.

Dear Editor,

please find enclosed the manuscript entitled **“Morphological and morphometrical discrimination of fresh and frozen/thawed fish meat: European hake (*Merluccius merluccius*) as a possible model for white meat fish species”** to be considered for publication in LWT - Food Science and Technology.

Freezing is the commonest technology applied to prolong fish preservation, although it may produce muscle physical-chemical modifications altering the product's quality and induce a higher spoilage rate of the frozen-thawed product. Thus, clear information is needed for guaranteeing consumer's safety and fair commercial practices. In this respect, the European legislator imposed the obligation to declare the process of freezing and thawing occurred before the sale by indicating the designation 'defrosted' on the product's label. Nevertheless, deliberate substitutions of fresh with frozen-thawed fish are still common fraudulent incidents.

The recognition of histological structural alterations represents a tool for discriminating freezing treatments. This approach, firstly proposed for the common carp meat has been recently applied to other species (gilthead, red mullet, swordfish, bonito, salmon, turbot, albacore, little tunny, rainbow trout and anchovy). Even though the method was confirmed as a highly sensitive and specific, the presence of unspecified microscopic alterations reduced the assay accuracy and precision leading to “non-conclusive results”. In addition, possible microscopic alterations eventually occurring within the product's shelf-life were not assessed.

The present study aimed at providing a standard operating histological procedure to discriminate fresh and frozen-thawed fish. The procedure, set and validated on *M. merluccius* (European hake), a species never analyzed until today, was thought to be extended to the analysis of the white meat fish category. A preliminary analysis of the muscular tissue histology of 15 whole fresh specimens sampled at different shelf life time was conducted to highlight possible time dependent modifications and to select the tissue sampling site. Then, the operative procedure was set by the analysis of both morphological and morphometrical parameters on a total of 90 muscular tissue samples belonging to fresh and frozen exemplars for the selection of objective indices of freezing process. Four parameters (structural score, presence of vacuoles, presence of extracellular and intracellular seroproteinaceous material, number of vacuole per field) were included in the final operative grid after a statistical analysis. The histological grid accuracy and repeatability assessed on the analysis of two distinct operators confirmed high specificity and sensitivity of the method and a high diagnostic concordance irrespective of the previous operators' skill. The method, validated by a single-blind test on 30 additional commercial products, was confirmed as a reliable check tool to be applied against the occurrence of fraudulent incidents and for the monitoring of the

quality of the freezing process both in seafood business operator self-check monitoring and official controls.

The manuscript has not been published elsewhere nor is it being considered for publication elsewhere. All authors contributed to the intellectual or technical content of the study and to the drafting of the article. Finally, each of the listed authors approved this manuscript, agree to the order in which their names are listed, declare that no conflict of interests exists and disclose any commercial affiliation.

Best regards
Andrea Armani

Dear Editor,

We are sending back the revised version of the paper FOODCONT-S-18-00800. The title has been changed according to the reviewer's request. New title: **"Histological discrimination of fresh and frozen/thawed fish meat: European hake (*Merluccius merluccius*) as a possible model for white meat fish species"**

Here you can find our answers to reviewer's comments.

Reviewers' comments:

Reviewer #1: This work presents a characterization of freezing effect on muscle structure using histological technique on Hake and a methodology to discriminate fresh and freeze/thaw products in order to propose a method to avoid fraudulent sales.

The paper is well written and the study very well conducted from the sampling protocol to the statistical analysis. The results are well discussed with a relevant bibliography.

In conclusion only minor revisions would be needed before this manuscript could be considered for publication.

We really thank the reviewer for appreciating the paper.

Minor revision

The title can be a little bit confusing, especially the term morphology is more appropriate to overall morphology of the fish, that was not be studied here. As only histological analysis have been performed I propose : "Histological discrimination of fresh and frozen/thawed fish meat: European hake (*Merluccius merluccius*) as a possible model for white meat fish species".

The title has been changed as suggested

In the Material and methods explain how the IQF fish are processed (degree, speed of freezing)

A brief description about the processing parameters has been included accordingly (Material and methods section lines 163-164)

Line 152: 567.073 μm^2 please verify the calculation this area correspond to a square image of 24 μm side that not correspond to a 20X magnification.

It was a typing error. We now amended the manuscript erasing the dot.

The term Proteinaceous and moreover Seroproteinaceous seems not to be the right term, generally speaking muscle fiber comprise myofibrillar and sarcoplasmic protein, the soluble sarcoplasmic proteins corresponds probably to the proteinaceous material observed bit not the seroproteinaceous that comes from the blood sera, so use either sarcoplasmic or proteinaceous but not seroproteinaceous.

The term seroproteinaceous has been amended throughout the manuscript.

Line 426 "for the analysis of the" instead of "for the analysis of
to the"

Done

1 **Histological discrimination of fresh and frozen/thawed fish meat: European hake**
2 **(Merluccius merluccius) as a possible model for white meat fish species** ~~Morphological and~~
3 ~~morphometrical discrimination of fresh and frozen/thawed fish meat: European hake~~
4 ~~(Merluccius merluccius) as a possible model for white meat fish species~~
5

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27 **Abstract**

28 The present study aimed at setting up a standard operating histological procedure to discriminate
29 fresh from frozen-thawed fish products of the species *Merluccius merluccius* (European hake). A
30 preliminary histological analysis of fresh *M. merluccius* muscle was performed to select the
31 sampling site and highlight possible time-dependent tissue alterations during shelf-life. To set a
32 suitable operational grid for discriminating the freezing process, morphological and
33 morphometrical parameters were assessed on 90 muscle tissue samples collected from 30 fresh, 30
34 experimentally frozen at -20° C and 30 Individual Quick Frozen (IQF) specimens of *M.*
35 *merluccius*. Structural score, presence of freezing vacuoles, a number of vacuoles per field higher
36 than 1.12 and the presence of interstitial ~~sero~~proteinaceous material, which had achieved statistical
37 significance in group comparisons were chosen as freezing markers. Accuracy and repeatability,
38 assessed on the analysis of two independent operators (on-training and expert), showed high
39 analytical specificity and sensitivity and a concordant diagnostic performance regardless the
40 operators expertise. The grid was finally validated by a single blind test on 30 additional *M.*
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42 status without inconclusive results. The method could be profitably applied against fraudulent
43 adulteration practices.

44 **Keywords**

45 Seafood fraud, Freezing-thawing, histology, *Merluccius merluccius*, white meat fish species

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54 **1. Introduction**

55 Fish and seafood are widely accepted as essential components of a balanced diet thanks to their
56 nutritional properties, in particular to their fatty acid composition (Domingo, Bocio, Falcó & Llobet
57 2007). Based on lipid content and meat type, fish are commercially classified as fatty (i.e., salmon,
58 herring, anchovy, sardine, and mackerel) or lean fish and as red or white meat fish
59 (<http://www.fao.org/wairdocs/tan/x5916e/x5916e01.htm>); the latter includes both freshwater and
60 marine, wild or farmed species characterized by low-medium fat content and the absence of dark
61 fiber muscles (Ackman, 1989).

62 To date the whitefish market is one of the largest segment in the global seafood supply chain and
63 at European level major products within this category are represented by cod (*Gadus morhua*),
64 Alaska pollock (*Gadus chalcogrammus*) and hake species (*Merluccius* spp.) (EUMOFA, 2017).
65 Hake demand has slightly increased over the last ten years compared to other species, reaching the
66 highest value in 2014 and alone representing the 15% of the total ground fish originating from
67 extra-EU countries. According to the report of the European Market Observatory for Fisheries and
68 Aquaculture Products (EUMOFA, 2017), hake, that is reported as one of the highest ranked in
69 terms of commercial value, is generally sold on the market as fresh or frozen. However, the
70 peculiar chemical composition of fresh hake makes its shelf life limited due to enzymatic autolysis,
71 lipid oxidation and microbial activities, which directly depends on *post-mortem* processing and
72 storage (Ghaly, Dave, Budge & Brooks, 2010).

73 Despite freezing is one of the most common method for seafood shelf-life extension and long-
74 term preservation, the process is known to induce muscle structure changes and chemical
75 modifications (protein denaturation, lipid oxidation, cell osmotic shrinkage and mechanical tissue
76 damage caused by the intracellular and extracellular water crystallization and cellular dehydration),
77 thus interfering with the overall organoleptic quality of the fish product (Zhu, Ramaswamy &
78 Simpson 2004; Venugopal., 2006; Burgaard, 2010; Gökoğlu & Yerlikaya, 2015; Uddin, 2010). In

79 addition, thawed fish is characterized by a higher perishability than chilled fish primarily due to
80 microbiological spoilage. The microbial flora, only partially inactivated by the freezing process, is
81 indeed positively affected by the increasing thawed tissue water activity (Pan & Chow, 2004;
82 Kolbe & Kramer, 2007). All these aspects are main drivers for the European consumers' preference
83 of fresh fish (Claret et al., 2012; Vanhonacker, Pieniak & Verbeke, 2013; Reis et al., 2017). This
84 preference greatly influence the market value of fresh and frozen fish. On the Italian market, the
85 price of fresh hake is about 14.75€/kg while the frozen product is sold at 2.10€/kg ([http://www.asa-
86 press.com/r-spesa/borsa170.html](http://www.asa-press.com/r-spesa/borsa170.html)).

87 Information about the storage method involved in fish preservation represents one of the key
88 issues to guarantee the consumer's safety and awareness. Therefore, at the European level,
89 Regulation EU No 1169/2011 and No 1379/2013 established that "*information on the physical
90 condition of the food or the specific treatment which it has undergone*" must be reported on
91 seafood labels. In the case of foods that have been frozen before sale and which are sold defrosted,
92 the name of the food shall be accompanied by the designation 'defrosted'. However, for a
93 consumer it is very hard to differentiate a fresh from a frozen-thawed fish on the basis of the
94 organoleptic characteristics (Karoui, Thomas, & Dufour, 2006), and deliberate substitution of
95 frozen/thawed fish in place of fresh fish are recorded as common finding of fraudulent incidents
96 (Uddin et al., 2005; Fasolato et al., 2008; Upton, 2015).

97 The occurrence of fraudulent substitutions leads to the setting of analytical methods for the
98 discrimination of frozen-thawed and fresh fish. This has been accomplished by means of
99 morphological, physiological, chemical and physical parameters (Duflos, Le Fur, Mulak, Becel &
100 Malle., 2002; Uddin, 2010). However, the reliability of these methods is limited in case of fish with
101 a long shelf life (Duflos et al., 2002) and in presence of processed products such as skinned and
102 filleted fish (Hassoun & Karoui, 2017). More recently, alternative physical methods based on front-
103 face fluorescence, near infrared spectroscopy, solid-phase gas chromatography and mass
104 spectrometry, have been proposed as non-destructive methods for fresh-frozen product

105 discrimination (Karoui et al., 2006; Uddin 2010, Fasolato et al., 2012, Leduc et al., 2012, Zhu et al.
106 2013, Ottavian, Fasolato, Facco, & Barolo, 2013). Although all methods have been shown to be
107 effective for the analysis of whole and filleted products they require a large set of reference
108 samples for the assay validation and the development of calibration models for each species
109 (Ottavian et al., 2013; Hassoun & Karoui, 2017).

110 An alternative method for discriminating fresh and frozen/thawed products is based on the
111 recognition of histological structural changes (empty vacuolar spaces) induced by freezing (Love,
112 1958; Simeonidou, Govaris & Vareltzis, 1997; Sigurgisladóttir S, Ingvarsdóttir H, Torrissen OJ,
113 Cardinal M, Hafsteinsson, 2000; Alizadeh, Chapleau, De Lamballerie, & Le-Bail, 2007; Alizadeh,
114 Chapleau, De Lamballerie, & Le-Bail, 2009). This approach, firstly proposed as a discriminating
115 method for the common carp (*Cyprinus carpio* L.) (Pavlov, Dimitrov, Penchev & Georgiev, 2008),
116 has been recently applied to other species characterized by different muscular composition and fat
117 content: gilthead (*Sparus aurata*), red mullet (*Mullus barbatus*), swordfish (*Xiphias gladius*),
118 bonito (*Sarda sarda*), salmon (*Salmo salar*), turbot (*Psetta maxima*), albacore (*Thunnus alalunga*),
119 little tunny (*Euthynnus alletteratus*), rainbow trout (*Oncorhynchus mykiss*) and anchovy (*Engraulis*
120 *encrasicolus*) (Bozzetta et al., 2012; Richelmi et al., 2013; Popelka, Nagy, Pipová, Marcinčák, &
121 Lenhardt, L., 2014, Meistro et al., 2016). Despite the high accuracy of these methods in the
122 detection of the freezing process, the presence of unspecific microscopic alterations reduced the
123 assay accuracy and precision leading to “non-conclusive results”. In addition, possible microscopic
124 alterations eventually occurring within the product’s shelf-life were not assessed.

125 The aim of the present study was to contribute in setting up a standard operating histological
126 procedure that enables to discriminate fresh from frozen-thawed fish products for *M. merluccius*
127 (European hake). Preliminary objectives of this study were to investigate the histological pattern of
128 fresh *M. merluccius* muscle tissue for selecting the most appropriate sampling site and to assess
129 possible time dependent tissue modifications during shelf-life. Secondly, histological parameters
130 were recorded for the differentiation of fresh and frozen muscle tissue of *M. merluccius*. Finally, an

131 operational protocol was set up and tested on a subset of randomly selected previously examined
132 samples and then validated by a single-blind control procedure on commercial samples.

133 **2. Materials and Methods**

134 ***2.1 Tissue histology of fresh *M. merluccius* muscle for sampling site selection***

135 *2.1.1 Specimens collection and processing.* Eight fresh medium size (200-300 g) whole *M.*
136 *merluccius* (caught within the previous 24 hours) were collected at a local fish market. Two cm
137 long fresh muscle samples were obtained from the left side of each fish, from three different
138 anatomical sites: a) the lateral line, b) dorsal muscle next to the column and distant from the lateral
139 line and c) ventral muscle posterior to the anal opening; (Fig. 1). Samples were either promptly
140 fixed in a 10% buffered formalin solution (pH 7.4) for paraffin embedding or cryo-protected with
141 30% sucrose for cryo-sectioning. Tissue processing of formalin fixed samples was performed in a
142 controlled automatic processor (Shandon TP 1020; Leika, Milan, Italy) and paraffin embedding
143 was accomplished to obtain transversal sections of the muscle fibers. Five μm thick sections were
144 stained with hematoxylin and eosin (H&E) under standard protocol. Cryo-sectioned samples were
145 stained with Oil Red O to evaluate the lipid distribution within samples.

146 ***2.2 Assessment of histological time-dependent tissue modification on fresh *M. merluccius****

147 To avoid the misinterpretation of hypothetical shelf-life time-dependent alterations as thawing
148 modifications, other 15 whole fresh specimens of medium size (200-300 g; caught within the
149 previous 24 hours) obtained at a local fish market, were included in this study. Of these, 5 were
150 sampled within 24 hours (24H group); 5 were sampled after 48 hours of conservation at 4 °C (72H
151 group) and the last 5 specimens, after an additional conservation at 4 °C for further 48 hours (120H
152 group). The samples, all collected from the dorsal muscle, were processed as previously described
153 and all alterations observed were recorded. The observations were conducted within 10 consecutive
154 fields at 20x magnification, each field corresponding to $567.073 \mu\text{m}^2$. Areas occupied by time-
155 dependent tissue modifications were recorded on H&E stained sections using a light microscope

156 (Nikon, Eclipse 80i) connected to a personal computer via a Nikon digital camera (Digital Sight
157 DS-U1) and measurements were carried out with the NIS-Elements Br accompanying software.

158 **2.3 Histological evaluation of fresh and frozen *M. merluccius***

159 **2.3.1 Sampling.** Histological evaluation was performed on a total of 90 samples as follows.
160 Thirty fresh *M. merluccius* (caught within the previous 24 hours) of about 200-300 g weight, were
161 collected at a local fish market. A muscle punch from the dorsal muscle was promptly fixed in a
162 10% formalin solution (fresh *M. merluccius*, F_MM). The remaining fish were frozen in a
163 conventional laboratory freezer at -20 °C for 15 days; then, after controlled thawing (4 °C for 12
164 hours), 30 new tissue samples were collected in the contralateral area, symmetrically to the first
165 sampling site (CF_MM, conventionally frozen *M. merluccius*). Moreover, other 30 *M. merluccius*
166 fish that had undergone an Individual Quick Freezing (IQF) process, which is usually performed in
167 an air-blast tunnel at -35°C to -45°C for 1h to 3h and a different speed according to the fish size
168 (200g to 400g(Venugopal, 2006)), were purchased and sampled after controlled thawing (IQF_MM,
169 commercially frozen *M. merluccius*). After processing, sectioning and H&E staining (see section
170 2.1.1) morphological and morphometric parameters were recorded for the differentiation of fresh
171 and frozen muscle tissue of *M. merluccius*.

172 **2.3.2 Morphology.** After a preliminary screening of histological slides, the following parameters
173 were selected for morphological assessment: a) the overall muscle structural organization, b) the
174 presence of freezing vacuoles defined as polygonal spaces with smooth angles within the muscle
175 myofiber and c) the presence of interstitial proteinaceous material, defined as a slightly granular
176 basophilic material accumulated in the *interstitium* between myofibers. These parameters were
177 scored on four randomly selected areas and: a) overall structural organization (assessed at a 10x
178 magnification) was scored as 0= fully destructured muscle, as 1 = partially (<50%) destructured
179 muscle , 2= well preserved muscle ; b) myofiber vacuolization (assessed at a 20x magnification)
180 was recorded as 0 = absence and 1= presence; c) interstitial proteinaceous material, (observed at a
181 10x magnification) was scored as 0 = absence and 1= presence.

182 2.3.3 *Morphometry*. Four hot spot areas from samples that scored either 1 or 2 at the above
183 mentioned parameter “a)” were selected at low power and measurements performed at 20x
184 magnification within a predetermined field. Total number of vacuoles in the four selected fields,
185 number of vacuoles per fields, mean vacuole size, mean size of myofibers containing vacuoles and
186 percentage of the myofiber occupied by vacuoles were recorded. Analyzes were performed on
187 H&E stained sections as mentioned above (2.2).

188 2.4 *Statistical analysis*

189 2.4.1 *Shelf life test*. A paired-sample t-test was used to assess the difference in lysis surface
190 between samples kept for 24 hours and those kept for 72 and 120 hours. Results were considered
191 significant when $p < 0.05$.

192 2.4.2 *Selection of the parameters for discrimination of fresh and frozen-thawed fish muscle*.

193 Different statistical tests were applied as follows. The organization of the muscle structure (score)
194 was analysed by comparing the score distribution (from 0 to 2) within the three groups using the
195 Kruskal-Wallis test; if overall significance was observed, further differences among groups were
196 assessed using the Mann-Whitney U test with k-1 comparisons (k is the total number of examined
197 groups). The presence (1) or absence (0) of vacuoles and ~~sero~~proteinaceous material was evaluated
198 by the chi-squared test to compare differences of frequency of positive samples (presence of
199 vacuoles and interstitial ~~sero~~proteinaceous material) within the three groups (F_MM, CF_MM,
200 IQF_MM). The same test was used to compare the effect of freezing (presence of vacuoles and
201 ~~sero~~proteinaceous material) by the comparison of both CF_MM and IQF_MM, evaluated together,
202 against group F_MM. Morphometrical parameters (number of vacuoles per fields, mean vacuole
203 size, mean size of myofibers containing the vacuoles and percentage of myofiber occupied by
204 vacuoles) were investigated using the ANOVA test. When a significant result was obtained a post-
205 hoc Dunnet test was performed. For all the analyses, significant results were those associated with
206 $p < 0.05$. The parameters that were confirmed as significant were used to set up the final protocol. In
207 case of morphometric parameters, a cut-off value was established. In particular, the number of

208 vacuoles per field parameter was selected to define a cut-off value for the discrimination of fresh
209 and frozen products. The cut-off limit for the discrimination of freezing was determined using the
210 95% Confidence Interval (95% C.I.) calculated on the mean number of vacuoles per area in the
211 F_MM group (95% C.I. = 0 - 1.12) and using its upper level, considering that the two other groups
212 were characterized by means of 2.24 – 4.47 (CF_MM) and 6.77-9.95 (IQF_MM) with 95% C.I.
213 Thus, all those samples showing a number of vacuoles per field equal or below 1.12 were
214 considered fresh, conversely, all the samples showing a higher value were classified as frozen.

215 ***2.5 Operational protocol and assessment of the role of the operators***

216 *2.5.1. Operational protocol.* An evaluation grid was designed using morphological and
217 morphometric parameters and the cut-off level established by statistical analyses (Fig. 2). Then, it
218 was presented to the operators concurrently to the histological sections to issue a judgment of
219 freezing expressed as Positive (frozen) and Negative (fresh).

220 *2.5.2. Assessment of the role of the operators.* Two independent operators, a student (Operator 1:
221 on-training) and a pathologist (Operator 2: expert), were asked to use the operational protocol to
222 reclassify 50 out of the 90 samples selected by the Stat Trek random number generator
223 (<http://stattrek.com/statistics/random-number-generator.aspx>). Randomly selected samples were 17
224 fresh and 33 frozen (18 CF_MM, and 15 IQF_MM). Sensitivity and specificity achieved by the two
225 operators were calculated using contingency table analysis. The level of concordance between
226 Operator 1 and 2 was evaluated with the Cohen statistical index k. With a k Cohen index >80%
227 satisfactory concordance was achieved, while full concordance was defined as a 100% k Cohen
228 index. For these analyses EPI6 software for windows was used (Dean et al., 1994).

229 ***2.6 Final Validation***

230 Thirty additional commercial fish, belonging to both fresh (caught within the 24hours; 13
231 specimens) and IQF frozen (17 specimens), were collected and processed as described in section
232 2.1.1. These samples were presented to the operators without any indication about their origin
233 (single-blind control procedure). The judgment (fresh or frozen) was issued through the analysis of

234 three histological sections for each sample. In particular, the operators were asked to issue the final
235 judgment on the basis of the result obtained on at least two out of the three sections analyzed for
236 each sample according to the evaluation grid developed in this study (Fig. 2).

237 **3. Results**

238 ***3.1 Tissue histology of fresh *M. merluccius* muscle for sampling site selection***

239 Myofibers of fresh *M. merluccius* were always arranged in fascicles surrounded by connective
240 tissue. Two different myofiber types were identified at the H&E staining: large polygonal fibers
241 whose cytoplasm was packed with myofibrils and small myofibers whose cytoplasm often showed
242 several small round empty spaces (Fig. 3a). Oil Red O staining showed that the empty spaces found
243 in H&E stained sections were lipid droplets (Fig. 3b). Fascicles containing lipid droplets were
244 found lying between the skin and the underneath muscle in all samples collected from the lateral
245 line (100% of the specimen), in 25% of samples collected from the dorsal muscle and in half of the
246 samples obtained from the ventral muscle (50%). In samples from the lateral line and the ventral
247 area they were also found within the deep muscle tissue. Thus, the dorsal area was selected as
248 sampling site.

249 ***3.2 Assessment of histological time-dependent tissue modifications on fresh *M. Merluccius****

250 The assessment of fresh *M. merluccius* at different shelf-life time points revealed histological
251 focal areas of either swollen (Fig. 4a) and shrunken-fragmented (Fig. 4b) eosinophilic lytic
252 myofibers. On the ten total fields observed at 20X of magnification, shrunken-fragmented
253 myofibers were observed in 2 to 6 fields in the samples at 24h of storage (mean =3.2); 2 to 4 at 72h
254 (mean=3) and 0 to 6 at 120h (mean=3.4). Significant differences were not found when comparing
255 the size of lytic areas over different shelf life samples (data not shown).

256 ***3.3 Histological evaluation of fresh and frozen *M. merluccius****

257 ***3.3.1. Morphological assessment.*** The preliminary screening of histological slides showed the
258 presence of:

259 1. Different degree of muscle destructuretion;

- 260 2. Freezing vacuoles (Fig. 5a) recognized for their squared or polygonal shape, smooth margin
261 and empty space or space filled with a slightly basophilic material;
- 262 3. ~~Seroproteinaceous~~Proteinaceous material in the interstitial space (Fig. 5b).
- 263 4. Myofiber empty spaces of irregular angular shape (Fig. 5c) or as thin short empty fractures
264 (Fig. 5d). These alterations were observed occasionally in both fresh (F_MM) and frozen
265 (CF_MM and IQF_MM) tissues and were considered as artefactual findings produced by tissue
266 processing;
- 267 5. Either swollen and shrunken-fragmented eosinophilic myofibers (lytic fibers) were seen,
268 without significance differences, in all groups.

269 Considering that our goal was to select “changes” related to the freezing process, artefactual
270 findings (point 4) and lytic myofibers (point 5) were not included as parameters to be used for the
271 discrimination of fresh and frozen-thawed muscle tissue.

272 As regards the other selected parameters results are summarized in Table 1 and reported in detail
273 in Table 1SM. The structural organization of the muscular component was generally well
274 maintained in F_MM samples. No vacuolar alterations similar to freezing vacuoles were recorded
275 except for three samples in which only 1 intracellular vacuole in 3 out of 4 fields of observation
276 was observed. In CF_MM partial or full muscle tissue destructuration prevailed. In the presence of
277 fully destructured score (0) the freezing vacuoles were broken and uncomplete (not delimited) and
278 surrounded by released ~~sero~~proteinaceous material; thus the samples were not morphometrically
279 evaluable. In IQF_MM the structural organization of the muscular component was generally well
280 maintained. Noteworthy, freezing vacuoles of regular shape were homogeneously distributed
281 within the single myofibers, while vacuoles in the CF_MM were randomly and not homogeneously
282 scattered throughout the muscle fibers.

283 Kruskal-Wallis test for overall structural organization showed high statistically significant
284 differences ($\chi^2=44.68$ $p<0.001$) between the tree groups. When Mann-Whitney test was performed
285 CF_MM (mean 0.9) was different from either F_MM (mean 1.9) showing $z= - 4.83$, $p<0.001$ or

286 IQF_MM (mean 2.0), with $z = -5.05$, $p < 0.001$. On the contrary, no significant differences were
287 found on structural scores between F_MM and IQF_MM groups. Chi-squared test showed a
288 significant effect of freezing for frequency of vacuoles ($\chi^2 = 69.1$, $p < 0.001$) and interstitial
289 proteinaceous material ($\chi^2 = 80.3$, $p < 0.001$) when all groups were included in the statistical analysis.
290 When pairwise comparisons were made, CF_MM was not statistically different from IQF_MM. By
291 aggregating data from frozen samples (CF_MM + IQF_MM) and comparing them against data of
292 fresh tissues (F_MM) results were again statistically significant for both the parameters (presence
293 of vacuoles: $\chi^2 = 64.9$, $p < 0.001$; presence of ~~sero~~proteinaceous material: $\chi^2 = 77.0$, $p < 0.001$).

294 *3.3.2. Morphometrical assessment.* As reported in section 2.3.3, the assessment of
295 morphometrical parameters (number of vacuoles per fields, mean vacuole size, mean size of
296 myofibers containing the vacuoles and percentage of the myofiber occupied by vacuoles) was
297 performed only on samples that scored either 1 or 2 as regards muscle organization (Table 1). In
298 particular, 3 F_MM samples, 18 CF_MM and all the IQF_MM samples were assessed.

299 Mean number of vacuoles per field progressively increased from F_MM (0.5 per field, ranging
300 from 0.25 to 0.75) to CF_MM (3.50 per field, ranging from 1 to 8.75) and IQF_MM (8.3 per field,
301 ranging from 3.75 to 19). Similarly, mean percentage of the myofiber occupied by vacuoles was
302 11% in F_MM, 22.1% in CF_MM and 31% in IQF_MM (Table 1SM).

303 The mean values of the two quantitative parameters (number of vacuoles per field and
304 percentage of the myofiber occupied by vacuoles), investigated using the ANOVA test, were
305 confirmed significantly different across groups ($F = 64.3$, $p < 0.001$ for vacuoles per field and
306 $F = 257.8$, $p < 0.001$ for percentage of myofiber occupied). When Dunnett test was performed, the
307 difference was statistically significant between F_MM and CF_MM ($p < 0.001$) as well as F_MM
308 and IQF_MM ($p < 0.001$) for both parameters. Thus, they were confirmed as applicable indices for
309 the discrimination between fresh and frozen products.

310 *3.4 Operational protocol and assessment of the role of the operators*

311 *3.4.1. Operational protocol.* Above mentioned parameters that achieved statistical significance
312 in group comparisons were included in the final operational grid for the discrimination of fresh and
313 frozen-thawed *M. Merluccius* (Fig. 2). The operators were asked to use the grid to reclassify a
314 randomly selected blind set of previously examined samples (n=50); samples that achieved a cut-
315 off value > 1.12 (number of vacuoles per field) were automatically assigned to the frozen category
316 in the provided ms excel worksheet. However, sample reaching a cut off value = 1.12 was assigned
317 to frozen status only in presence of interstitial ~~sero~~proteinaceous material.

318 *3.4.2. Assessment of the role of the operators: reliability assessment.* Both operators assigned all
319 but one sample to the correct category (Table 2). Operator 1 (on-training) showed 100% sensitivity
320 (95% C.I.: 85%-100%) and 94% specificity (95% C.I.: 71%-100%) while Operator 2 (expert)
321 showed 97% sensitivity (95% C.I.: 84%-100%) and 100% specificity (95% C.I.: 80%-100%). The
322 Cohen index used to evaluate the degree of agreement between the two classifications was $k=91%$,
323 $p<0.01$ (95% C.I.:79%-100%). This confirmed a significant analytical concordance between the
324 operators.

325 ***3.6 Final validation of the operational procedure***

326 Both the operators assigned all the 30 additional commercial fish samples (13 fresh and 17 IQF)
327 to the correct category and the scores attributed to the three sections were analogous, confirming a
328 substantial structural homogeneity between the different portions of the tissue punch collected from
329 each sample.

330 **4. Discussion**

331 ***4.1 Tissue histology of fresh *M. merluccius* muscle for sampling site selection***

332 To evaluate the anatomical distribution of muscle fibers and to obtain homogeneous data for
333 statistical comparisons, a specific anatomical sampling site was identified by a preliminary analysis
334 of *M. merluccius* tissue histology. This preliminary step also aimed at recognizing the presence of
335 any vacuolar shaped intracellular space that would resemble the myofiber vacuolization reported as
336 the main change associated with freezing (Ayala et al., 2005; Bozzetta et al., 2012; Meistro et al.,

337 2016). Previous studies, conducted on 84 marine species, have shown different distribution and
338 variable percentages of white and red muscle fibers (Greek-Walker & Pull, 1975). Regardless of
339 the species, the dorsal muscles are predominantly composed of white fibers while the red ones, if
340 present, are exclusively localized in the most superficial portion (Johnston, 1981; Greek-Walker &
341 Pull, 1975). This pattern was confirmed in this study also for the specie *M. merluccius* for which a
342 precise anatomical distribution of the two muscle fiber types had not been described before.
343 Therefore, even though lipid vacuoles can be easily differentiated from freezing vacuoles for their
344 shape and size (small, perfectly round, optically empty on H&E stained sections) the dorsal area
345 was chosen as the elective site in this study as more homogeneous by a structural point of view.
346 Thus, this area might in fact represent a “species-independent” reference sampling site for the
347 analysis of white fish species even by not specifically trained operators.

348 ***4.2 Assessment of histological time-dependent tissue modifications on fresh M. merluccius***

349 The possible onset of tissue modifications related to fish spoilage within the expected shelf life
350 of the product, that are generally due to the combination of enzymatic autolysis oxidation and
351 microbial growth (Ghaly et al., 2010; George, Van Wettere, Michaels, Crain, & Lewbart, 2016),
352 was also considered in the preliminary assessment. Since myofiber vacuoles are reported as the
353 main change associated with freezing, the analysis was focused on the detection of the possible
354 presence of vacuoles in fresh samples at different time of conservation, since this aspect was not
355 considered in the previous studies (Bozzetta et al., 2012; Popelka et al., 2014; Meistro et al., 2016).
356 In this respect, George et al. (2016), in a study about histopathologic evaluation of *post mortem*
357 changes in fresh water fish species preserved in several storage conditions (room temperature,
358 refrigeration, freezing) and at different sampling intervals (4, 24, 48 hours), reported the onset of
359 mild to evident tissue alterations (cellular oedema/swelling) subsequent to autolytic phenomena in
360 all storage conditions. Conversely, the vacuolar lesions recorded were all exclusively associated to
361 freezing-thawing processes. Accordingly, in the present study no vacuolar changes were found in
362 samples at 24, 72 and 120 hours (kept at 4 °C) analyzed in the study. On the contrary scattered

363 areas of swollen and shrunken-fragmented eosinophilic fibers were recorded. These alterations
364 were similar to those described by Sigurgisladottir et al. (2000) in frozen salmon (*Salmo salar*) and
365 by Popelka et al. (2014) in rainbow trout (*Oncorhynchus mykiss*), likely due to autolytic enzymes
366 which are known to be the main responsible for the post mortem tissue softening (Ahmed, Donkor,
367 Street, & Vasiljevic, 2015).

368 ***4.3 Histological evaluation of fresh and frozen M. merluccius***

369 Once determined that small round vacuoles present in fresh samples were lipid filled and that
370 either swollen and shrunken-fragmented eosinophilic fibers were likely a consequence of autolysis,
371 the further assessment of fresh vs frozen-thawed samples aimed at identifying peculiar parameters
372 related to freezing for being included in the final operative analytical protocol.

373 Tissue de-structuring was the first parameter included in the list of the recorded alterations: this
374 finding was primarily observed in CF_MM and not in IQF_MM samples in which muscle
375 organization was generally well maintained. In this respect, the use of a slow freezing technique
376 might have led to the onset of osmotic phenomena, as already reviewed by Pham (2008) and Kiani
377 and Sun (2011), leading to morphological alterations such as dehydration and shrinkage. Moreover,
378 it is known that the slow rate of temperature decrease enhances the formation of large extracellular
379 ice crystals (Kiani & Sun, 2011). All these phenomena predispose to the breaking of the cell
380 membranes during the thawing procedure (Pham, 2008). On the contrary, a fast-freezing technique,
381 as the IQF technology, generally produces a more uniform intracellular and extracellular water
382 crystallization (Pham, 2008). This aspect was further confirmed in this study by the absence of
383 significant differences between the structural scores of F_MM and IQF_MM. Therefore, a tissue
384 structure score might be proposed as parameter to estimate the quality of the freezing process
385 during product' shelf life. In fact, the high quality of frozen seafood may be lost either by
386 interruption of the cold chain during transportation (Gormley, Walshe, Hussey, & Butler, 2002) or
387 by non-industrial freezing improperly applied by wholesalers to slow down the tissue spoilage
388 mechanisms of unsold fresh products (Bozzetta et al., 2012).

389 | The second and third listed parameters (freezing vacuoles and ~~sero~~proteinaceous material) were
390 found to be significantly freezing-dependent. Non-homogeneous distribution of freezing vacuoles
391 in CF_MM samples compared to the homogeneous pattern observed in IQF_MM, was consistent
392 with findings previously described by Ayala et al. (2005). Also the presence of interstitial
393 proteinaceous material in the *interstitium* between myofibers in CF_MM samples, was plausibly
394 due to the formation of ice macrocrystals induced by slow freezing process for the tissue
395 deformation and the impairment of cell membrane integrity (Pham, 2008; Alizadeh et al., 2007).

396 ***4.4 Operational protocol and assessment of the role of the operators and final validation***

397 The high specificity and sensitivity of the operational protocol in this study was confirmed by the
398 reliability assessment of the two operators. There was a low probability of false positive and false
399 negative occurrence. Albeit minimal, the difference in diagnostic performance revealed by the
400 contingency table was related to the operator's experience since it was hypothesized that an expert
401 operator may consider a minimal vacuolar change as a freezing vacuole while these may be
402 overlooked by the trainee but not experienced operator.

403 The application of the cut-off threshold obtained from the statistical analysis on the quantitative
404 parameter vacuole per field, allows the allocation of all the samples to the fresh or frozen status
405 thus avoiding inconclusive results.

406 **5. Conclusions**

407 Even though several analytical methods can help in the identification of frozen products sold as
408 fresh, these techniques are often cost, and reagent demanding and require highly skilled operators.
409 Therefore, industry and official authorities are interested in convenience, non-destructive, non-
410 invasive and cost-effective methods. In the present study the use of histology as suitable analytical
411 tool to prevent fraudulent substitutions of fresh with frozen–thawed fish, was confirmed. The
412 selected histological parameters and the final operative protocol applied to the European hake (*M.*
413 *merluccius*), may represent a reliable and cost-effective procedure to be proposed for the analysis of
414 white fish category. Further experiments are however needed to confirm the possibility of applying

415 the protocol to different species. The method can be also applied to verify both the quality of the
416 freezing process and the correct maintenance of the cold chain of frozen products during transport
417 and storage phases before sale. Besides the scientific evidences offered by the study possible
418 expected outcomes are linked to the increasing reliance, transparency and trust between diverse
419 actors along the chain that may enhance market competitiveness as well as consumers wellness.

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423 overall quality of seafood products.

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426 **Figures captions**

427 **Fig. 1.** Anatomical position of the three sampling sites of muscular tissue (tissue punch length =
428 2cm) evaluated in the study on *Merluccius merluccius* exemplar (size = 250 g). a) lateral line, b)
429 dorsal muscle, c) ventral muscle.

430 **Fig. 2.** Evaluation grid proposed to the operators for the analysis of ~~te~~the histological sections.
431 *Score structure: 2 = well preserved muscle, 1 = partially destructured muscle, 0 = fully
432 destructured muscle. Final judgement: N = negative (fresh product), P = positive (frozen product).

433 **Fig. 3.** Detail of a histological section of *Merluccius merluccius* dorsal muscle. a) Small myofibers
434 with several small round empty spaces within the cytoplasm that are grouped in a fascicle (asterisk)
435 laying between the superficial connective tissue (arrowhead) and underlying large polygonal muscle
436 fibers (H&E staining, bar 200µm). b) Small round spaces within the cytoplasm of the small
437 myofibers laying beneath the connective tissue (arrowhead) red stained with Oil Red O for lipids
438 (asterisk) (bar 100 µm).

439 **Fig. 4.** Histology of *Merluccius merluccius* muscle at different shelf-life time. a) Scattered
440 multifocal homogeneously swollen deep eosinophilic myofibers (arrowheads) at 72h shelf-life and
441 b) grouped shrunken and fragmented lytic myofibers (arrows) at 120h shelf-life (H&E stain, bar
442 100µm).

443 **Fig. 5.** Histology of *Merluccius merluccius* muscle with different myofiber alterations. a) Squared
444 and round freezing vacuoles (asterisk) with empty spaces or spaces containing slightly basophilic
445 material, b) ~~sero~~proteinaceous material (arrowheads) in the interstitial space among myofibers
446 containing freezing vacuoles, c) myofiber empty spaces of irregular angular shape (arrows) not
447 related to freezing and d) thin short empty fractures within myofibers not related to freezing (H&E
448 stain, bar 100µm).

449

450 **References**

451 Ackmann RG (1989) Nutritional composition of fats in seafoods. *Progress in Food & Nutrition*
452 *Science*, 13(3-4),161-289

453 Ahmed, Z., Donkor, O., Street, W. A., & Vasiljevic, T. (2015). Calpains-and cathepsins-induced
454 myofibrillar changes in post-mortem fish: Impact on structural softening and release of bioactive
455 peptides. *Trends in Food Science & Technology*, 45(1), 130-146.

456 Alizadeh, E., Chapleau, N., De Lamballerie, M., & Le-Bail, A. (2007). Effect of different freezing
457 processes on the microstructure of Atlantic salmon (*Salmo salar*) fillets. *Innovative Food Science &*
458 *Emerging Technologies*, 8(4), 493-499.

459 Alizadeh, E., Chapleau, N., de-Lamballerie, M., & Le-Bail, A. (2009). Impact of freezing process on
460 salt diffusivity of seafood: application to salmon (*Salmo salar*) using conventional and pressure shift
461 freezing. *Food and bioprocess technology*, 2(3), 257-262.

462 Ayala, M. D., Albors, O. L., Blanco, A., Alcázar, A. G., Abellán, E., Zarzosa, G. R., & Gil, F. (2005).
463 Structural and ultrastructural changes on muscle tissue of sea bass, *Dicentrarchus labrax* L., after
464 cooking and freezing. *Aquaculture*, 250(1), 215-231.

465 Bozzetta, E., Pezzolato, M., Cencetti, E., Varello, K., Abramo, F., Mutinelli, F., Ingravalle, F., &
466 Teneggi, E. (2012). Histology as a valid and reliable tool to differentiate fresh from frozen-thawed fish.
467 *Journal of Food Protection*, 75(8), 1536-1541.

468 Burggaard, M. G. (2010). Effect of frozen storage temperature on quality-related changes in fish
469 muscle: Changes in physical, chemical and biochemical quality indicators during short- and long-term
470 storage. Kgs. Lyngby, Denmark: Technical University of Denmark (DTU). Available at
471 www.food.dtu.dk

472 Claret, A., Guerrero, L., Aguirre, E., Rincón, L., Hernández, M. D., Martínez, I., Peleteiro J. B., Grau
473 A., Rodríguez-Rodríguez, C. (2012). Consumer preferences for sea fish using conjoint analysis:
474 Exploratory study of the importance of country of origin, obtaining method, storage conditions and
475 purchasing price. *Food Quality and Preference*, 26(2), 259-266.

476 Dean AG, Dean JA, Coulombier D, Brendel KA, Smith DC, Burton AH, et al. (1994). Epi info Ver 6:
477 A word processing, database and statistics programme for epidemiology on microcomputers. Atlanta,
478 Georgia, USA: Centre for Disease Control and Prevention. Available at:
479 <http://www.cdc.gov/epiinfo/Epi6/ei6.htm>.

480 Domingo, J. L., Bocio, A., Falcó, G., & Llobet, J. M. (2007). Benefits and risks of fish consumption:
481 Part I. A quantitative analysis of the intake of omega-3 fatty acids and chemical contaminants.
482 *Toxicology*, 230(2), 219-226.

483 Duflos, G., Le Fur, B., Mulak, V., Becel, P., & Malle, P. (2002). Comparison of methods of
484 differentiating between fresh and frozen–thawed fish or fillets. *Journal of the Science of Food and*
485 *Agriculture*, 82(12), 1341-1

486 EUMOFA, 2017. <http://www.eumofa.eu/documents/20178/108446/The+EU+fish+market+2017.pdf>

487 Fasolato, L., Balzan, S., Riovanto, R., Berzaghi, P., Mirisola, M., Ferlito, J. C., Serva, L., Benozzo,
488 F., Passera, R., Tepedino, V., & Novelli, E. (2012). Comparison of visible and near-infrared reflectance
489 spectroscopy to authenticate fresh and frozen-thawed swordfish (*Xiphias gladius* L). *Journal of Aquatic*
490 *Food product Technology*, 21(5), 493-507.

491 Fasolato, L., Mirisola, M., Tepedino, G., Balzan, S., Arcangeli, G., Rosteghin, M., Corrain, C.,
492 Manfrin, A., & Berzaghi, P. (2008). Mai più decongelato per fresco. Dossier Eurofishmarket, Available
493 at <https://www.eurofishmarket.it/b2b/decongelato.pdf>

494 George, J., Van Wettere, A. J., Michaels, B. B., Crain, D., & Lewbart, G. A. (2016). Histopathologic
495 evaluation of postmortem autolytic changes in bluegill (*Lepomis macrochirus*) and crappie (*Pomoxis*
496 *anularis*) at varied time intervals and storage temperatures. *PeerJ*, 4, e1943.

497 Ghaly, A. E., Dave, D., Budge, S., & Brooks, M. S. (2010). Fish spoilage mechanisms and
498 preservation techniques. *American Journal of Applied Sciences*, 7(7), 859.

499 Gökoğlu, N., & Yerlikaya, P. (2015) Chapter 7: Freezing technology In: Gökoğlu, N & Yerlikaya P.
500 (Eds.). *Seafood chilling, refrigeration and freezing: Science and Technology*. Wiley & sons, 2015 (1st ed)
501 (pp.163-185).

502 Gormley, R., Walshe, T., Hussey, K., & Butler, F. (2002). The effect of fluctuating vs. constant
503 frozen storage temperature regimes on some quality parameters of selected food products. *Lebensmittel-*
504 *wissenschaft Und-technologie-food Science and Technology*, 35(2), 190e200.

505 Greek- Walker, M., & Pull, G. A. (1975). A survey of red and white muscle in marine fish. *Journal*
506 *of Fish Biology*, 7(3), 295-300.

507 Hassoun, A., & Karoui, R. (2017). Quality evaluation of fish and other seafood by traditional and
508 nondestructive instrumental methods: Advantages and limitations, *Critical Reviews in Food Science and*
509 *Nutrition*, 57(9), 1976-1998, DOI: 10.1080/10408398.2015.1047926.

510 Johnston IA. (1981). Structure and function of fish muscles. *Symp. zaal. Sac. Land.* 48. 71-113

511 Karoui, R., Thomas, E., & Dufour, E. (2006). Utilisation of a rapid technique based on front-face
512 fluorescence spectroscopy for differentiating between fresh and frozen–thawed fish fillets. *Food*
513 *Research International*, 39, 349–355.

514 Kiani, H., & Sun, D. W. (2011). Water crystallization and its importance to freezing of foods: A
515 review. *Trends in Food Science & Technology*, 22(8), 407-426.

516 Kolbe, E., & Kramer, D. (2007). Planning for seafood freezing. Alaska sea Grant College Program,
517 Cooper Publishing, Alaska. pp.112

518 Leduc, F., Krzewinski, F., Le Fur, B., N’Guessan, A., Malle, P., Kol, O., & Duflos, G. (2012).
519 Differentiation of fresh and frozen/thawed fish, European sea bass (*Dicentrarchus labrax*), gilthead

520 seabream (*Sparus aurata*), cod (*Gadus morhua*) and salmon (*Salmo salar*), using volatile compounds by
521 SPME/GC/MS. *Journal of the Science of Food and Agriculture*, 92, 2560–2568

522 Love R.M. (1958). The expressible fluid of fish fillets IX. Other types of cell damage caused by
523 freezing. 3. *Sci. Food Agric.* 9:262-268, 1958.

524 Meistro, S., Pezzolato, M., Muscolino, D., Giarratana, F., Baioni, E., Panebianco, A., & Bozzetta, E.
525 (2016). Histology as a Valid Tool to Differentiate Fresh from Frozen-Thawed Marinated Fish. *Journal of*
526 *Food Protection*, 79(8), 1457-1459.

527 Ottavian, M., Fasolato, L., Facco, P., & Barolo, M. (2013). Foodstuff authentication from spectral
528 data: toward a species-independent discrimination between fresh and frozen-thawed fish samples.
529 *Journal of Food Engineering*, 119, 765-775.

530 Pan, B., & Chow, C.J. (2004). Freezing secondary seafood products. In: Nollet, L.M.L, & Toldra F.
531 (Eds.) *Handbook of Frozen products*. CRC Press, Boca Raton (pp. 325-339)

532 Pavlov, A., Dimitrov, D., Penchev, G., & Georgiev, L. (2008). Structural changes in common carp
533 (*Cyprinus carpio* L.) fish meat during freezing. *Bulgarian Journal of Veterinary Medicine*, 11(2), 131-
534 136.

535 Pham, Q. T. (2008). Advances in food freezing/thawing/freeze concentration modelling and
536 techniques. *Japan Journal of Food Engineering*, 9(1), 21-32.

537 Popelka, P., Nagy, J., Pipová, M., Marcinčák, S., & Lenhardt, L. (2014). Comparison of chemical,
538 microbiological and histological changes in fresh, frozen and double frozen rainbow trout
539 (*Oncorhynchus mykiss*). *Acta Veterinaria Brno*, 83(2), 157-161.

540 Regulation (EU) No 1169/2011 of the European Parliament and of the Council of 25 October 2011 on
541 the provision of food information to consumers, amending Regulations (EC) No 1924/2006 and (EC) No
542 1925/2006 of the European Parliament and of the Council, and repealing Commission Directive
543 87/250/EEC, Council Directive 90/496/EEC, Commission Directive 1999/10/EC, Directive 2000/13/EC
544 of the European Parliament and of the Council, Commission Directives 2002/67/EC and 2008/5/EC and
545 Commission Regulation (EC) No 608/ 2004. *Official Journal of the European Union*, L 304.

546 Regulation (EU) No 1379/2013 of the European Parliament and of the Council of 11 December 2013
547 on the common organisation of the markets in fishery and aquaculture products, amending Council
548 Regulations (EC) No 1184/2006 and (EC) No 1224/2009 and repealing Council Regulation (EC) No
549 104/2000. *Official Journal of the European Union*, L 354.

550 Reis, M. M., Martinez, E., Saitua, E., Rodriguez, R., Perez, I., & Olabarrieta, I. (2017). Non-invasive
551 differentiation between fresh and frozen/thawed tuna fillets using near infrared spectroscopy (Vis-NIRS).
552 *LWT-Food Science and Technology*, 78, 129-137.

553 Richelmi, G. B., Pezzolato, M., Gili, S., Gallina, S., Decastelli, L., Tarasco, R., Abete, M.C.,
554 Ingravalle, F., Serracca, L., Pavino, D., Vivaldi, B., Riina, M.V., Acutis, P.L., Prearo, M., Caramelli, M.,
555 & Bozzetta E. (2013). Pilot project to set up a control programme on fishery products. *Italian Journal of*
556 *Food Safety*, 2(2), 25.

557 Sigurgisladóttir S, Ingvarsdóttir, H., Torrissen, O.J., Cardinal, M., & Hafsteinsson, H. (2000) Effects
558 of freezing/thawing on the microstructure and the texture of smoked Atlantic salmon (*Salmo salar*).
559 *Food Research International*, 33, 857-865

560 Simeonidou S, Govaris A, Vareltzis K. (1997). Effect of frozen storage on the quality of whole fish
561 and fillets of horse mackerel (*Trachurus trachurus*) and Mediterranean hake (*Merluccius*
562 *mediterraneus*). *Z Lebensm Unters Forsch A*, 204, 405-410

563 Uddin, M. (2010). Differentiation of fresh and frozen-thawed fish. In: Leo, L. M. L. Nollet, & F.
564 Toldrá (Eds.), *Handbook of seafood and seafood products analysis*. CRC Press. (Part V safety, Chapter
565 37, pp.735-750)

566 Uddin, M., Okazaki, E., Turza, S., Yumiko, Y., Tanaka, M., & Fukuda, Y. (2005). Nondestructive
567 visible/NIR spectroscopy for differentiation of fresh and frozen-thawed fish. *Journal of Food Science*,
568 70, 506-510

569 Upton HF. (2015). Seafood fraud. Congressional Research Service, www.crs.gov Document available
570 at: <http://nationalaglawcenter.org/wp-content/uploads/assets/crs/RL34124.pdf>

571 Vanhonacker, F., Pieniak, Z., & Verbeke, W. (2013). European consumer perceptions and barriers for
572 fresh, frozen, preserved and ready-meal fish products. *British Food Journal*, 115(4), 508-525.

573 Venugopal, V. (2006). Quick freezing and individually quick frozen products. In: Venugopal (Ed.)
574 *Seafood processing*. (pp.446) Taylor and Francis, CRC Press, New York. (Chapter 4; pp 95-140)

575 Zhu, F., Zhang, D., He, Y., Liu, F., & Sun, D. W. (2013). Application of visible and near infrared
576 hyperspectral imaging to differentiate between fresh and frozen–thawed fish fillets. *Food and Bioprocess*
577 *Technology*, 6(10), 2931-2937.

578 Zhu, S., Ramaswamy, H.S., & Simpson, B.K. (2004). Effect of high-pressure versus conventional
579 thawing on color, drip loss and texture of Atlantic salmon frozen by different methods. *LWT – Food*
580 *Science and Technology*, 37 (3), 291-299

Highlights

- A histological procedure to discriminate fresh/frozen-thawed *M. merluccius* was set up
- Morphological and morphometrical parameters were evaluated on fish muscle tissue
- An operational grid based on four histological parameters was proposed
- The validated procedure is applicable by both specialist analysts and trained operators

Table 1. Results of the assessment of the morphological parameters on histological slides of fresh (F_MM), conventionally frozen (CF_MM) and Individual Quick Frozen (IQF_MM) *M. merluccius* specimens. 0= muscle organization fully destructured, 1= muscle organization partially (<50%) destructured, 2 = muscle organization well preserved. V= vacuols; IPM: interstitial preteinaceous material. * only a vacuole was detected

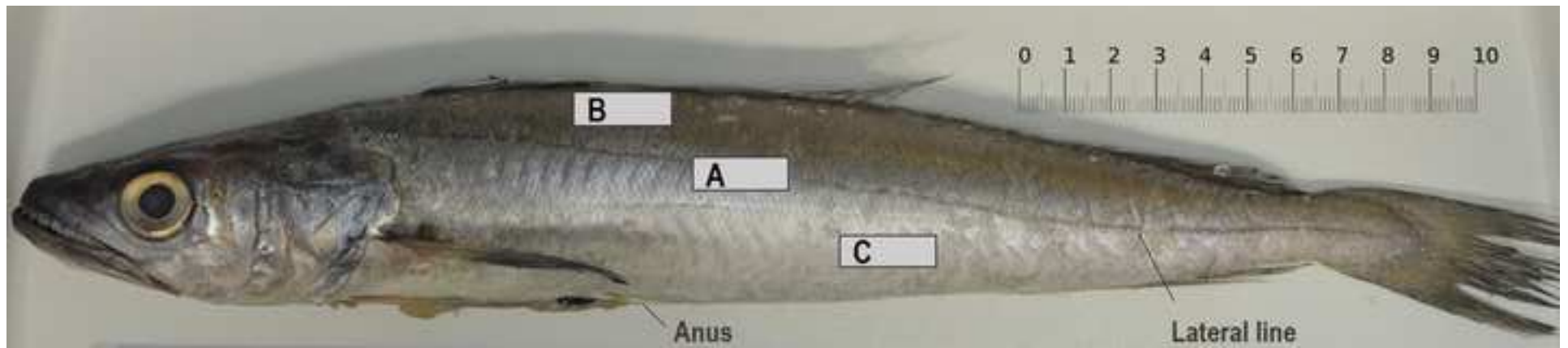
Sample category	Total number Total %		Organization of muscle structure			Presence of V		Presence of IPM	
			0	1	2	0 (A)	1 (P)	0 (A)	1 (P)
F_MM	N.	30	0	3	27	27	3*	29	1
	%	100	0	10.0	90.0	90.0	10.0	97.0	3.0
CF_MM	N.	30	9	11	10	2	28	1	29
	%	100	30.0	36.7	33.3	6.7	93.3	3.3	96.7
IQF_MM	N.	30	0	0	30	0	30	0	30
	%	100	0	0	100	0	100	0	100

Table 2. Contingency table results of Operator 1 (Op1) and Operator 2 (Op 2).

Fish category				Fish category			
Op1	Fresh	Frozen	Total	Op 2	Fresh	Frozen	Total
Fresh	1	33	34	Fresh	0	32	32
Frozen	16	0	16	Frozen	17	1	18
Total	17	33	50	Total	17	33	50

Figure

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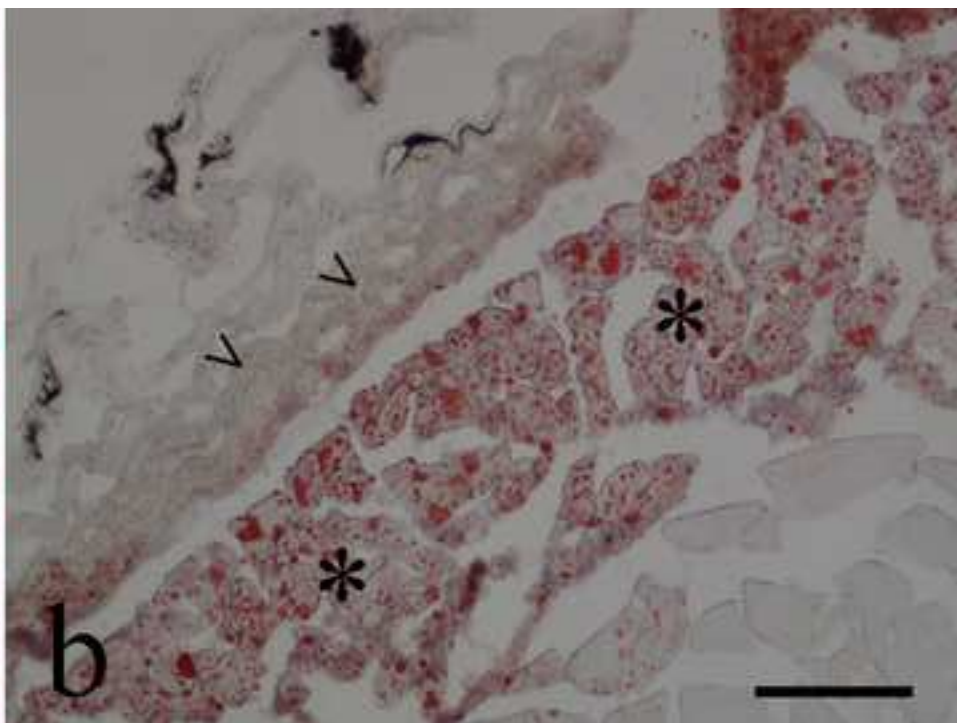
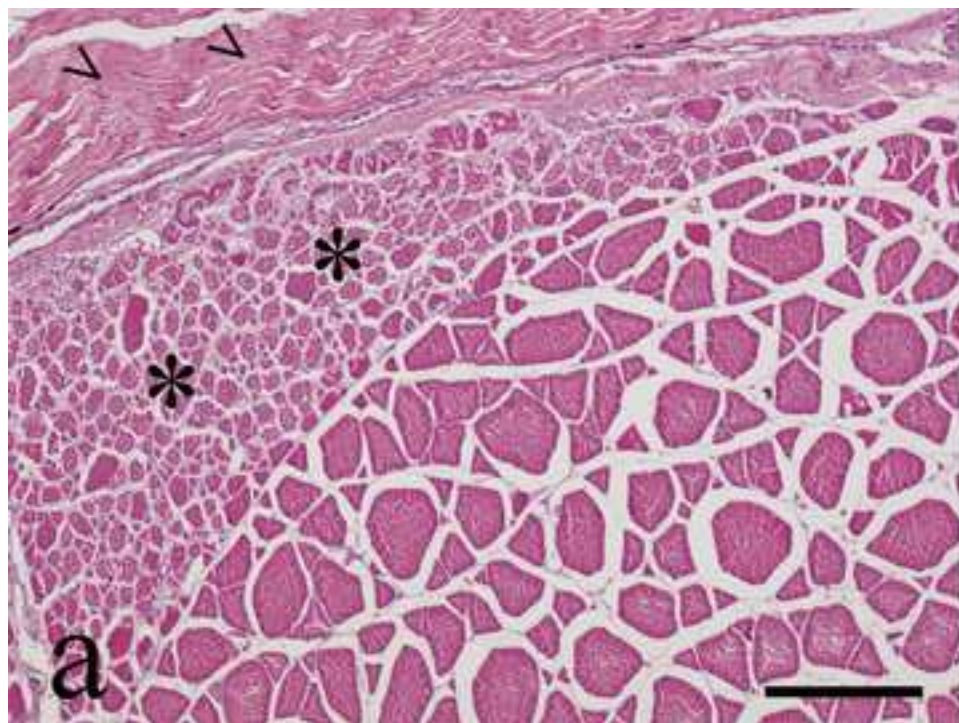


Figure

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Sample code	Structural score at 10X magnification (2, 1, 0)*	Presence of freezing vacuoles at 10x magnification (0=no; 1=y)	Number of vacuoles per field at 20x magnification (4 subsequent non-contiguous fields)				Presence of seroproteinaceous material at 10x magnification (0=no, 1=yes)	Final judgement (N, P)
1								
2								
N								

Figure
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Figure

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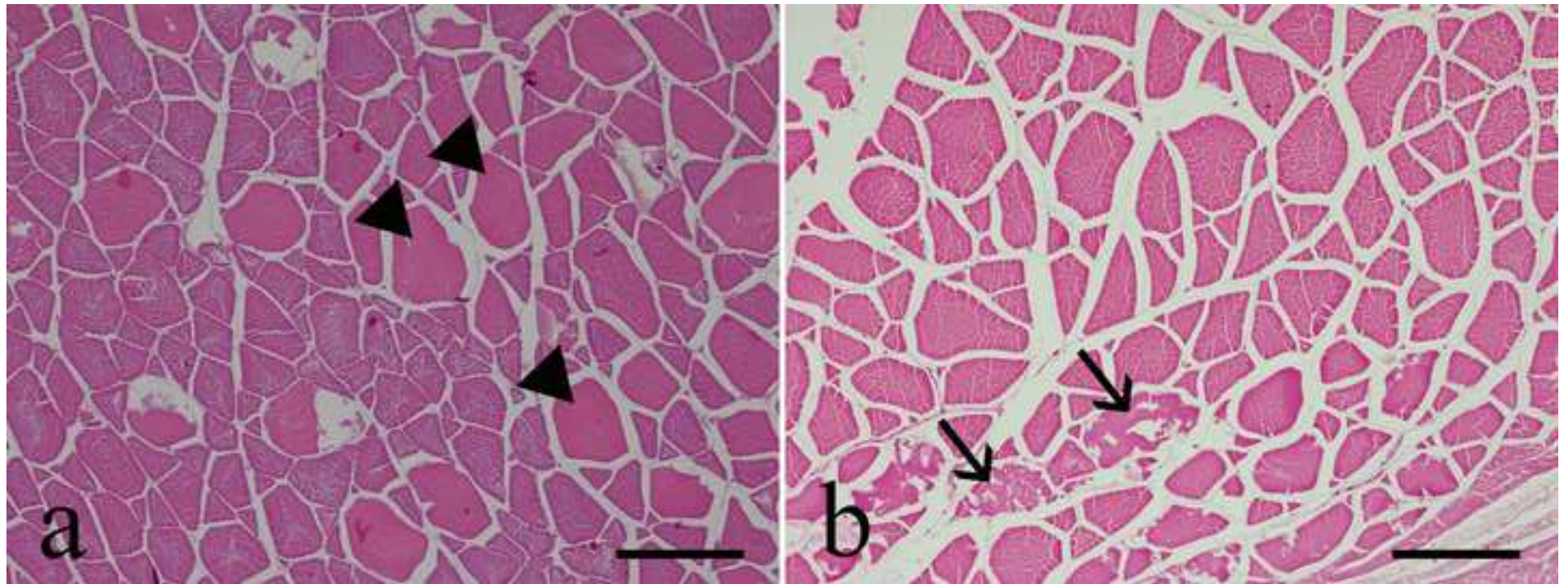
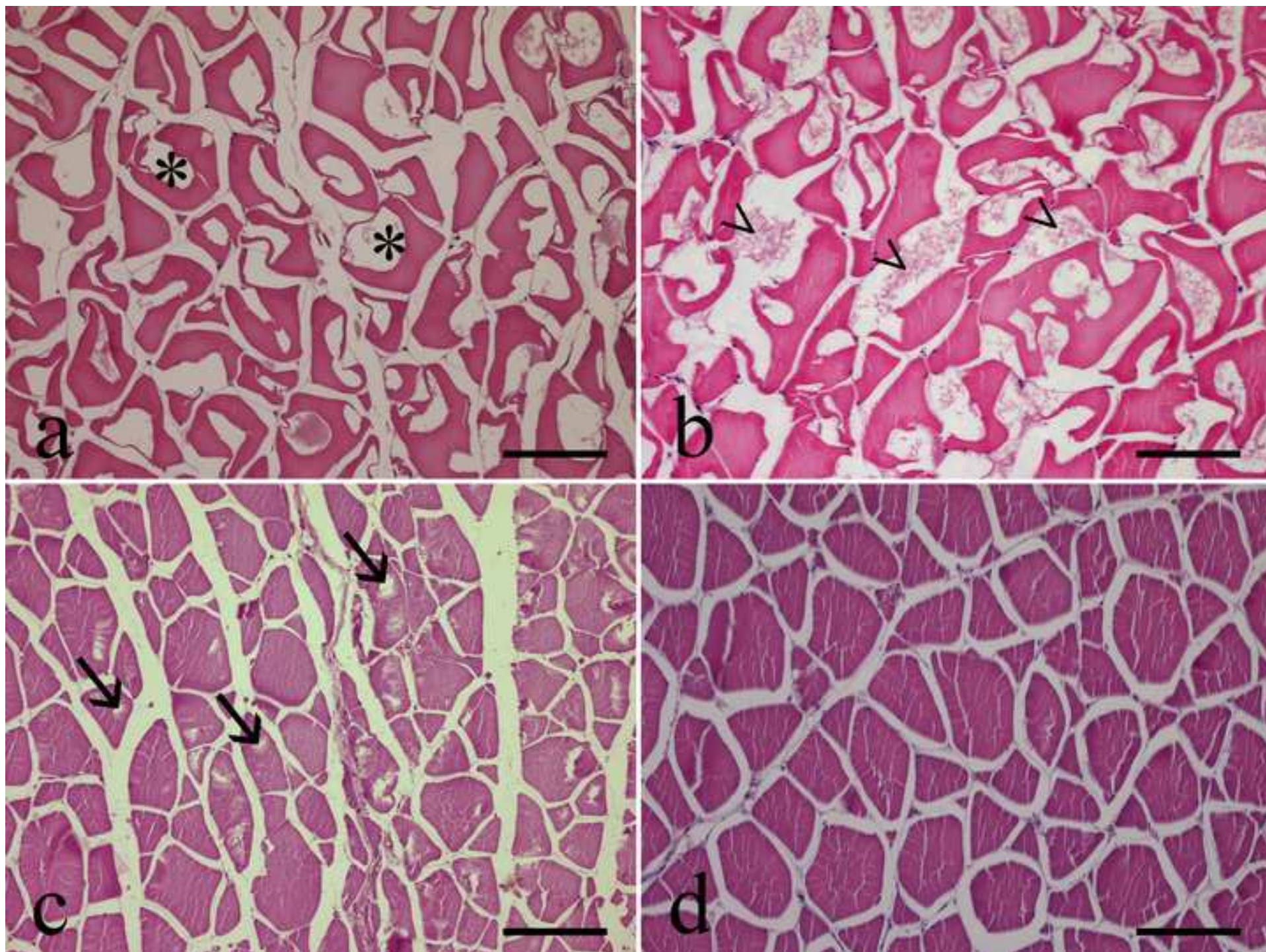


Figure
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e-component

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