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

***Detailed Response to Reviewers**

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 µm² please verify the calculation this area correspond to a square image of 24µm 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 seroproteinacesous 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

Histological discrimination of fresh and frozen/thawed fish meat: European hake (Merluccius merluccius) as a possible model for white meat fish species ~~Morphological and morphometrical 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 ~~sero~~proteinaceous 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.

Keywords

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

1. Introduction

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

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

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

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

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

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

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

An alternative method for discriminating fresh and frozen/thawed products is based on the recognition of histological structural changes (empty vacuolar spaces) induced by freezing (Love, 1958; Simeonidou, Govaris & Vareltzis, 1997; Sigurgisladóttir S, Ingvarsdóttir H, Torrissen OJ, Cardinal M, Hafsteinsson, 2000; Alizadeh, Chapleau, De Lamballerie, & Le-Bail, 2007; Alizadeh, Chapleau, De Lamballerie, & Le-Bail, 2009). This approach, firstly proposed as a discriminating method for the common carp (*Cyprinus carpio* L.) (Pavlov, Dimitrov, Penchev & Georgiev, 2008), has been recently applied to other species characterized by different muscular composition and fat content: gilthead (*Sparus aurata*), red mullet (*Mullus barbatus*), swordfish (*Xiphias gladius*), bonito (*Sarda sarda*), salmon (*Salmo salar*), turbot (*Psetta maxima*), albacore (*Thunnus alalunga*), little tunny (*Euthynnus alletteratus*), rainbow trout (*Oncorhyncus mykiss*) and anchovy (*Engraulis encrasicolus*) (Bozzetta *et al.*, 2012; Richelmi *et al.*, 2013; Popelka, Nagy, Pipová, Marcinčák, & Lenhardt, L., 2014, Meistro *et al.*, 2016). Despite the high accuracy of these methods in the detection of the freezing process, the presence of unspecific 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 aim of the present study was to contribute in setting up a standard operating histological procedure that enables to discriminate fresh from frozen-thawed fish products for *M. merluccius* (European hake). Preliminary objectives of this study were to investigate the histological pattern of fresh *M. merluccius* muscle tissue for selecting the most appropriate sampling site and to assess possible time dependent tissue modifications during shelf-life. Secondly, histological parameters were recorded for the differentiation of fresh and frozen muscle tissue of *M. merluccius*. Finally, an

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

2. Materials and Methods

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

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

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

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

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

2.3 Histological evaluation of fresh and frozen M. merluccius

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

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

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

3. Results

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

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

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

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

3.3 Histological evaluation of fresh and frozen M. merluccius

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

1. Different degree of muscle destructuration;

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

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

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

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

IQF_MM (mean 2.0), with z= -5.05, p<0.001. On the contrary, no significant differences were found on structural scores between F_MM and IQF_MM groups. Chi-squared test showed a significant effect of freezing for frequency of vacuoles ($\chi2$=69.1 p<0.001) and interstitial proteinaceous material ($\chi2$=80.3 p<0.001) when all groups were included in the statistical analysis. When pairwise comparisons were made, CF_MM was not statistically different from IQF_MM. By aggregating data from frozen samples (CF_MM + IQF_MM) and comparing them against data of fresh tissues (F_MM) results were again statistically significant for both the parameters (presence of vacuoles: $\chi2$=64.9, p<0.001; presence of ~~sero~~proteinaceous material: $\chi2$=77.0, p<0.001).

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

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

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

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

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

4.3 Histological evaluation of fresh and frozen M. merluccius

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

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

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

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

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

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

5. Conclusions

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

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

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Figures captions

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

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

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

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

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

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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

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 preoteinaceus 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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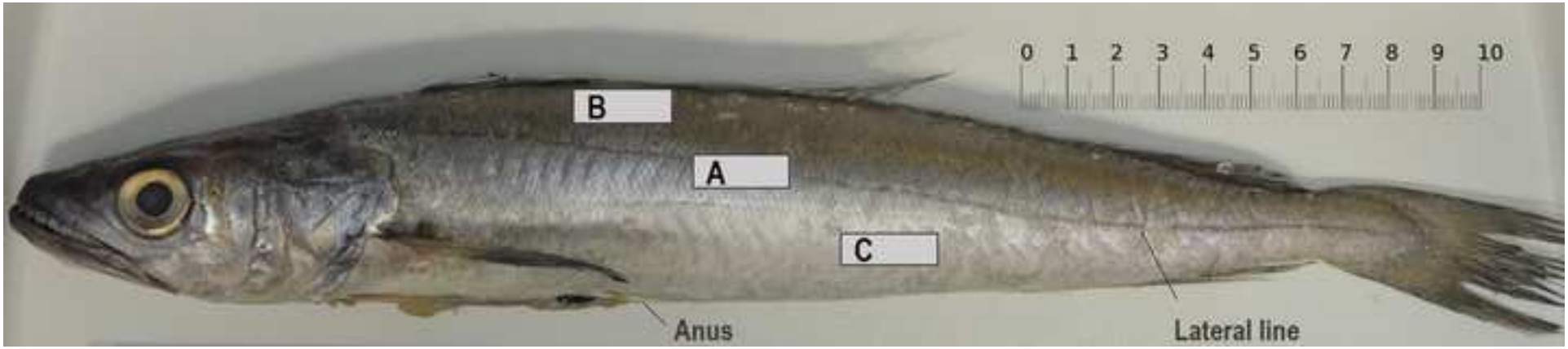

Figure

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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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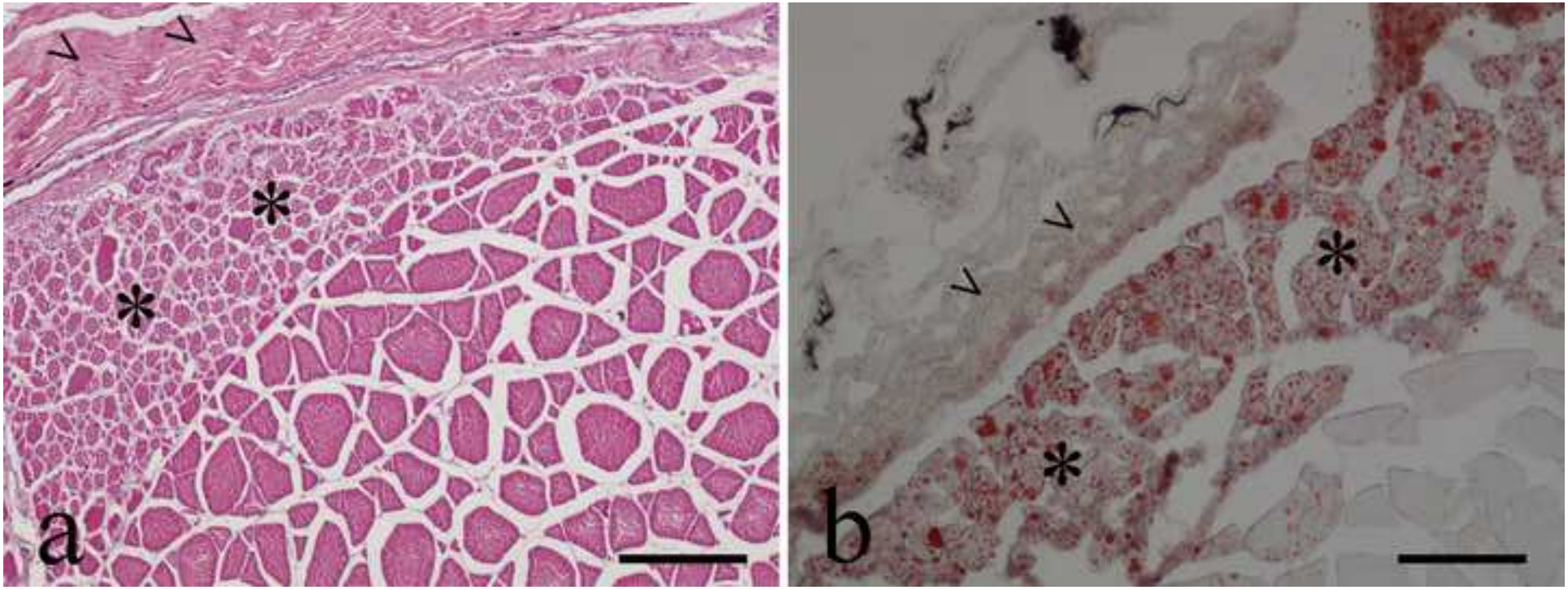

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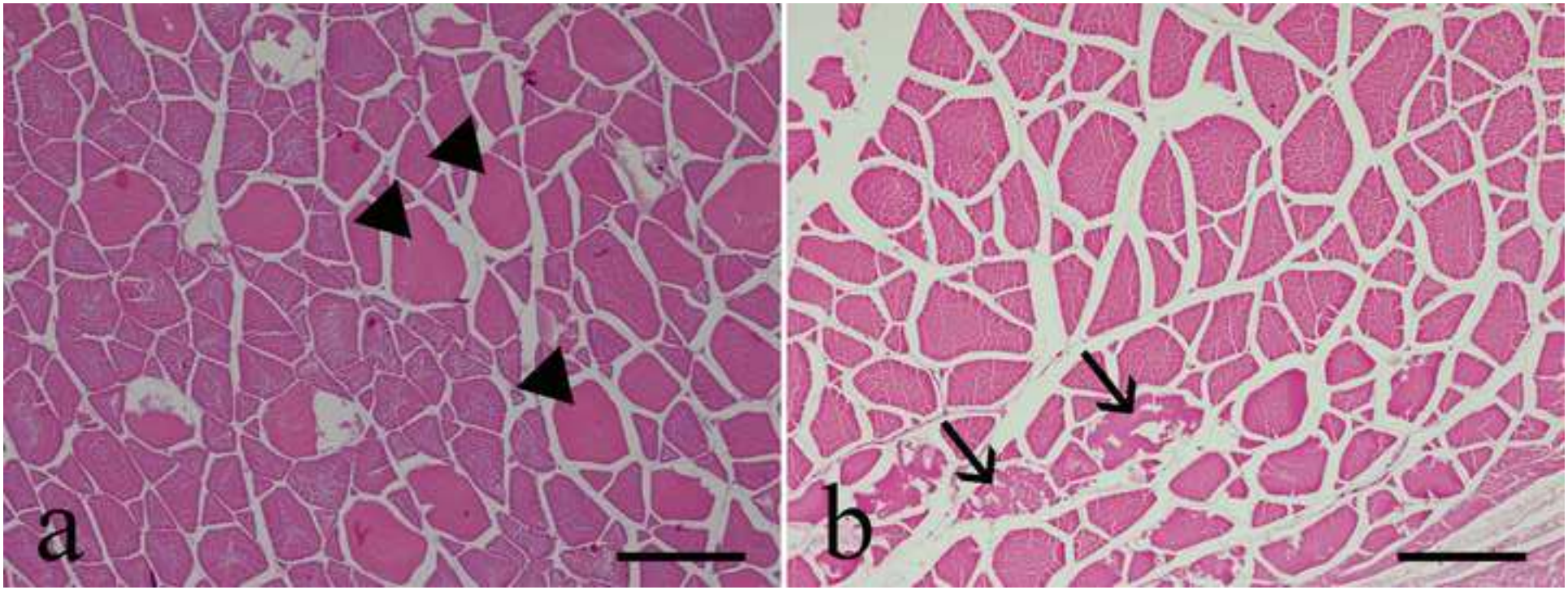

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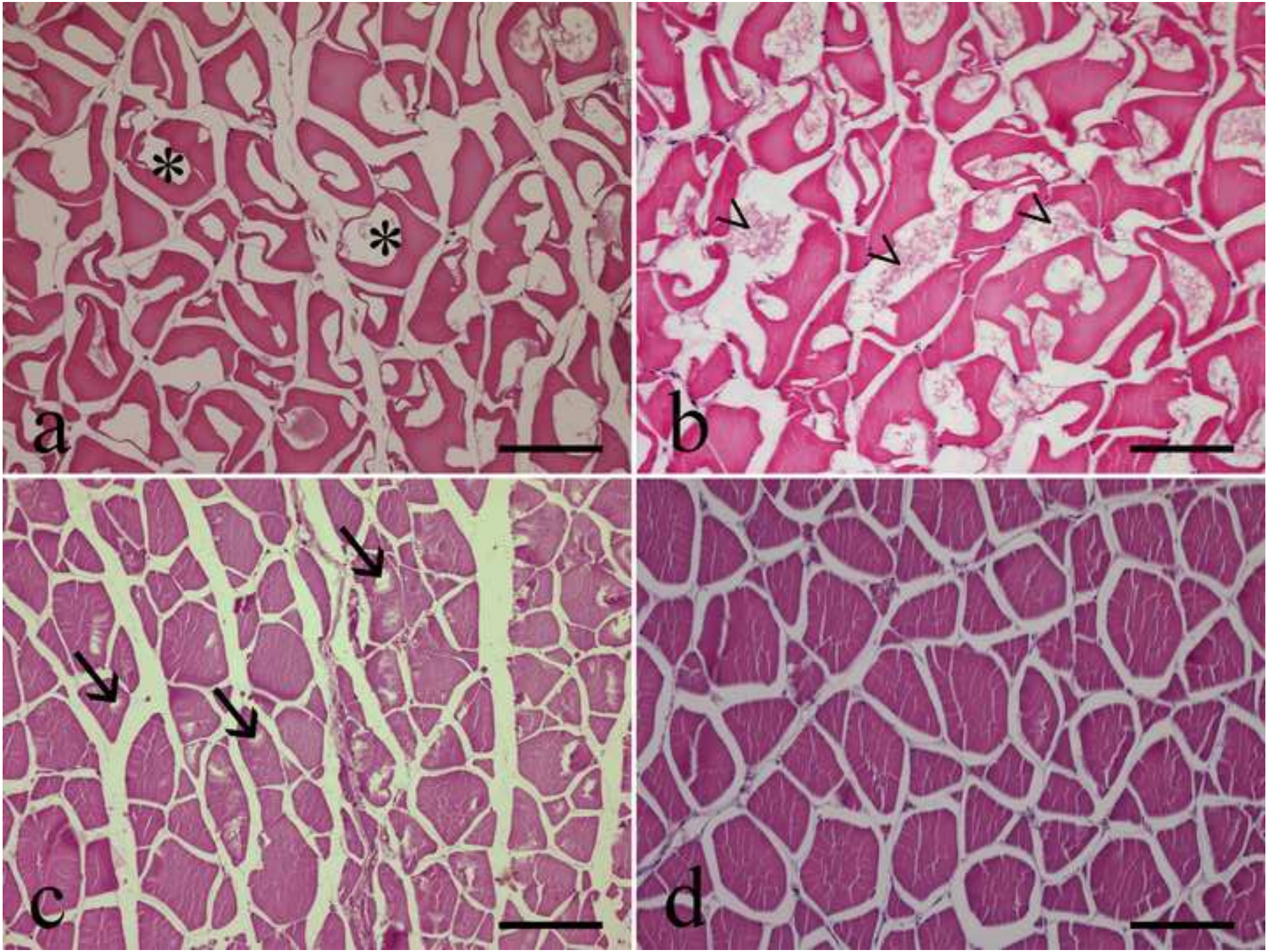

e-component

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