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Tiffanie Bouchendhomme: Conceptualization, Methodology, Investigation, Writing - Original Draft

Méline Soret: Investigation, Resources

Anne Devin: Validation, Writing - Review & Editing

Philippe Pasdois: Validation, Writing - Review & Editing

Thierry Grard: Project administration, Validation, Supervision, Funding acquisition, Writing - Review & Editing

Philippe Lencel: Conceptualization, Investigation, Validation, Visualization, Investigation, Writing - Review & Editing

Differentiating between fresh and frozen-thawed fish fillets by mitochondrial permeability measurement

Tiffanie Bouchendhomme[a], Méline Soret[a], Anne Devin[b], Philippe Pasdois[c], Thierry Grard[a]* and Philippe Lencel[a]

[a]*Univ. Littoral Côte d'Opale, UMRt 1158 BioEcoAgro, USC ANSES, INRAe, Univ. Artois, Univ. Lille, Univ. Picardie Jules Verne, Univ. Liège, Junia, F-62200 Boulogne-sur-Mer, France*

[b]*UMR CNRS 5095 Institut de biochimie et génétique cellulaires (IBGC), F-33077 Bordeaux, France*

[c]*Univ. Bordeaux, INSERM U1045, IHU-LIRYC, F-33600 Pessac, France*

* corresponding author: Tel: +33 3 21 99 25 08

E-mail address: Thierry.grard@univ-littoral.fr (T. Grard)

Email addresses:

Tiffanie Bouchendhomme: tiffanie.bouchendhomme@univ-littoral.fr

Méline Soret: meline.soret@univ-littoral.fr

Anne Devin: anne.devin@ibgc.cnrs.fr

Philippe Pasdois: philippe.pasdois@ihu-liryc.fr

Philippe Lencel: philippe.lencel@univ-littoral.fr

Abstract

In this study, we investigated the properties of mitochondria to discriminate fresh from frozen-thawed fish fillets. Mitochondria were isolated from gilthead seabream fillets and the impact of freezing was evaluated by measuring the permeability of mitochondrial membranes. Freezing led to permeabilization of mitochondrial inner membranes to reduced nicotinamide adenine dinucleotide (NADH). The increase in permeability related to freezing shock was compared to the physiological permeabilization of mitochondria isolated from gilthead seabream fillets stored at 4°C. Two approaches were chosen to measure the increase in permeability: a spectrophotometric method to measure the consumption of NADH by complex I, and an oxygraphic method to measure O_2 consumption by respiratory chains after exposure of mitochondria to NADH. Mitochondria isolated from frozen-thawed fillets were highly permeable to NADH and were no longer sensitive to a membrane permeabilizing agent: alamethicin. Altogether, our scientific approach allowed us to discriminate mitochondria isolated from fillets that have been exposed or not to a freezing shock (-80°C) and thus to discriminate between fresh and frozen-thawed fish fillets.

Key words: oxygraphy, spectrophotometry, mitochondria, NADH, permeabilization

1. Introduction

Fish are increasingly being caught far from the place of distribution and consumption. New chilling and freezing processes are also making it possible to better preserve fish products during transport (Erikson, Misimi, & Gallart-Jornet, 2011; Tolstorebrov, Eikevik, & Bantle, 2016). New chilling processes called "super chilling" that allow low core temperatures and that help to preserve products for longer are now being used. Freezing processes have also evolved considerably. Products can now be frozen at ultra-low temperatures (ULT -50°C), which helps to preserve their quality (Nakazawa, *et al.*, 2020).

At sale, different types of fraud may be observed, including substitution of species or sale of frozen-thawed fish as fresh fish (Chiesa, *et al.*, 2020). In these conditions, there is a need to develop techniques that allow fresh fish to be differentiated from frozen-thawed products.

The freezing process induces certain disruptions in fish, with changes in muscle structure and biochemical parameters. Freezing of seafood products is associated with rupture of the plasma and intracytoplasmic membranes (Uemura, *et al.*, 2006), which leads to enrichment of exudates in proteins and intracytoplasmic enzymes (Diop, *et al.*, 2016; Ethuin, *et al.*, 2015).

The rupture of intracellular compartments has been studied through numerous biochemical and enzymatic approaches. The nuclear magnetic resonance (NMR) technique makes it possible to distinguish fresh fish from frozen-thawed fish by measuring differences in concentrations of certain metabolites in tissues (Shumilina, Møller, & Dikiy, 2020).

Physical and spectral approaches have also been developed. They have the advantage of being fast and non-intrusive. They detect the physio-chemical changes that occur in frozen products (Velioğlu, Temiz, & Boyaci, 2015) (Fernández-S., *et al.*, 2012). Certain spectroscopic methods measure changes in compounds like NADH and are able to discriminate between fresh and frozen fish (Hassoun, 2021) (Ottavian, Fasolato, Facco, & Barolo, 2013).Membrane alteration is highly dependent on the nucleation of ice crystals and therefore on the rate of freezing. Freezing leads to localized increases in solutes, with variations in denaturing osmotic pressure (Dalvi-Isfahan, *et al.*, 2019). On the other hand, the freezing temperature affects the proportion of free water and the size of the crystals within the tissue. The size of crystals is largely responsible for the alteration of cells within the tissue. Ice crystals are formed and an alteration of osmotic pressure is also observed, inducing denaturation of proteins (Li, Zhu, & Sun, 2018; Strateva & Penchev, 2020). A faster speed of freezing reduces the formation of ice crystals and indirectly significantly increases the quality of the fish (Sone, Skåra, & Olsen, 2019).

It appears that intracellular ice formation is more deleterious to tissues. Intracellular crystals seem to form at relatively fast freezing rates (2°C/min) versus 0.3°C/min for slow freezing (Pegg, 2010). In optimized cryopreservation processes, the quality of vitrification (rapid freezing in the presence of high concentrations of a cryoprotective agent) can be assessed by measuring mitochondrial integrity (Restrepo, Varela, Duque, Gómez, & Rojas, 2019), although these methods are entirely incompatible with the food industry.

In previous studies, we have shown that mitochondria can be used as a marker of freshness. Once isolated from fresh fillets, they gradually lose their potential, which can be measured by mitotrackers such as rhodamine 123 or tetramethylrhodamine, methyl ester (TMRM) in fluorimetry and flow cytometry (Cléach, Soret, Grard, & Lencel, 2020; Soret, *et al.*, 2022).

Damage to mitochondrial membranes from frozen-thawed fillets is accompanied by a loss of mitochondrial potential. This loss of potential is, however, not sufficient to justify whether the mitochondria were altered by a freezing process. Therefore, we studied the extent of NADH diffusion and consumption within the mitochondria to have an index of the degree of inner mitochondrial membrane (IMM) permeabilization (Batandier, Leverve, & Fontaine, 2004) to discriminate fresh from frozen-thawed fish.

Two approaches, one enzymatic and the other using oxygen consumption measurement, were taken to measure the degree of NADH permeability of mitochondria. The loss of mitochondrial integrity was measured by the level of IMM permeability to NADH. The first step was to measure the rate of NADH consumption by mitochondria isolated from fresh and frozen-thawed fillets. The second step was to study the level of activation of O_2 consumption by NADH in the presence of mitochondria isolated from fresh fillets or from frozen-thawed fillets.

2. Materials and methods

2.1 Biological material

Gilthead seabream (*Sparus aurata*) (300–450 g) were sourced from Aquanord-Ichtus sea farm (Gravelines, France), as previously described (Cléach, *et al.*, 2019). The fish were kept in isothermal polystyrene boxes, with ice. Upon arrival at the laboratory, two hours after death, the fish were immediately filleted. The fillets had a size of 16 cm long, 7 cm wide and an average weight of 75 g. The fillets were stored on ice in a cold room (+4°C) for eight days. The ice was renewed every day. Some fillets were frozen at day 0 at -80°C. These fillets were individually placed in freezer bags. They were stored in a freezer at -80°C (Model U725 innova, New Brunswick Scientific, New Jersey USA). Analyses were performed at day 1 and day 8 for fresh fish, and after 8 days of freezing and two hours of thawing at room temperature for frozen-

thawed fish. The fillets were then kept at 4°C. Plastic wrapping was used between fillets and ice to avoid contact.

2.2 Reagents

Bovine serum albumin (BSA), 4-morpholinepropanesulfonic acid (MOPS), ethylene glycol-bis (2 amino-ethylether)-N,N,N',N'-tetraacetic acid (EGTA), sucrose, potassium chloride (KCl), Tris(hydroxymethyl)aminomethane (Trizma® base), bacterial proteinase type XXIV, alamethicin, β-nicotinamide adenine dinucleotide (NADH), rotenone, decylubiquinone and cytochrome c were purchased from Sigma-Aldrich (St. Louis, MO, USA). Bio-Rad protein assay dye reagent concentrate was purchased from Bio-Rad Laboratories (Munich, Germany). Magnesium chloride ($MgCl_2$) and potassium phosphate (KH_2PO_4) were purchased from Acros Organics (Morris Plains, NJ, USA). Alamethicin was prepared in methanol purchased from Fisher Scientific (Loughborough, UK). Cytochrome C was prepared in distilled water. Decylubiquinone was prepared in dimethyl sulfoxide (DMSO) purchased from Thermo Scientific (San Diego, CA, USA).

2.3 Mitochondrial isolation from fresh and frozen-thawed fish fillets

Mitochondrial isolation was based on the method developed by Cléach, *et al.* (2019), using red muscle. Red muscle was dissected from the fillet and diced with scissors to obtain a finely cut tissue. This tissue was incubated with 25 mL of isolation buffer (180 mM KCl, 80 mM sucrose, 5 mM $MgCl_2$, 10 mM Tris, 2 mM EGTA, pH 7.2 at +4°C) for 8 min under stirring at +4°C supplemented with bacterial proteinase type XXIV. The tissue suspension was poured into a 30 mL glass Potter homogenizer and homogenized for 3 min using a motorized Teflon pestle at 300 rpm (Hei-TORQUE 400, Heidolph instruments, Schwabach, Germany). Initial centrifugation was performed at 7,000 *g* for 10 min (Megafuge 16R Heraeus, Thermo Scientific, San Diego, CA, USA). The resulting pellet was then washed with isolation buffer and 25 mL of this buffer was added to resuspend the pellet. The homogenate was homogenized at 150 rpm for 3 min and then centrifuged at 700 *g* for 10 min. The supernatant was recovered and centrifuged at 1,500 *g* for 10 min. This step was repeated once. The supernatant was recovered and centrifuged one last time at 7,000 *g* for 10 minutes. All steps were performed at +4°C. The mitochondrial pellet was then diluted with 60 µL of isolation buffer. To determine the final concentration of the pellet, a Bio-Rad protein assay kit was used. BSA was used as a standard. Mitochondria were kept on ice at a final concentration of 50–60 $mg.mL^{-1}$.

The same protocol was used for the frozen-thawed fillets. In this case, the final concentration of mitochondria was about 20–30 $mg.mL^{-1}$.

2.4 NADH consumption by spectrophotometry

NADH consumption was measured by spectrophotometry (UV-vis spectrophotometer, UV-1280, Shimadzu Europa GmbH, Germany) as previously described by Venard, *et al.* (2003). In the spectrometric cuvette, 1 mL of the respiratory buffer was added, then 0.2 $mg.mL^{-1}$ of mitochondria. In all cuvettes, cytochrome C (10 µM) and decylubiquinone (0.1 mM) were included. A control cuvette was carried out with addition of rotenone (2.5 µM). Alamethicin (2.5 µM) was added to another cuvette, to observe membrane permeabilization. To all cuvettes, NADH with final concentration of 0.1 mM was added. Zero calibration was performed before addition of NADH and the reading was performed at 340 nm.

Specific activity (SA) was calculated from the Beer-Lambert law.

$$SA = \frac{(\Delta A/(\varepsilon.l))/\Delta T}{[proteins]}$$ µmol of $NADH.min^{-1}.mg^{-1}$ of proteins

$\varepsilon = 6220\ M^{-1}.cm^{-1}$

Relative specific activity was obtained with this formula:

$$\%\ SA = \frac{SA}{SAmax} \times 100\ \%$$

SA max was represented by fresh fillets at D1 in the presence of alamethicin.

2.5 O_2 consumption by oxygraphy

Consumption monitoring was done using an oxygraph O-2k (Oroboros Instruments, Innsbruck, Austria). 100 % calibration was performed in the presence of the maximum amount of O_2 when the chamber was open. Similarly, 0 % calibration was performed using dithionite. A respiratory buffer was added to the chamber. An amount of 2.5 mL of the respiratory buffer was added (KCl 125 mM, MOPS 20 mM, Tris 10 mM, KH_2PO_4 2.5 mM, $MgCl_2$ 2.5 mM, EGTA 10 µM and BSA 2 $mg.mL^{-1}$). Before addition of the mitochondria, cytochrome C (10 µM) and decylubiquinone (0.1 mM) were added. The final concentration of mitochondria in each chamber was 0.2 $mg.mL^{-1}$. NADH (1 mM) was added, then alamethicin (2.5 µM). The experiment was performed at +25°C.

The rate of O_2 consumption was calculated with this formula:

$$O_2\ consumption = \frac{\left(\frac{O_2}{[proteins]}\right)*60}{1000}$$ nmol of $O_2.min^{-1}.mg^{-1}$ of proteins

Relative O_2 consumption was obtained with this formula:

$$\%\ O_2\ consumption = \frac{O_2}{O_2max} \times 100\ \%$$

O_2max was represented by fresh fillets at D1 in the presence of alamethicin.

2.6 Microscopy

Samples were prepared according to the protocol of Michalec, *et al.* (2017). In short, 3 mm^3 pieces of red muscle were cut from fillets and fixed in 2.5 % glutaraldehyde (Merck KGaA, Darmstadt, Germany) buffered with 0.1 M sodium cacodylate (Sigma-Aldrich), post-fixed in 1 % osmium tetroxide (Sigma-Aldrich). The pieces were incubated in 2 % uranyl acetate (Agar Scientific, Stansted, UK). After dehydration with acetonitrile, the samples were cast in an epon-like resin (EMbed-812). Ultrathin sections (90 nm) were made with a Leica UC7 ultramicrotome and collected on 150-mesh hexagonal copper grids. The sections were stained with 2 % uranyl acetate in 50 % ethanol and lead citrate. The sections were observed under a Hitachi H-600 transmission electron microscope equipped with a W electron source (operating at 75 kV) and a Hamamatsu C4742-95 digital camera mounted on the side.

2.7 Statistical analysis

Statistical analyses were performed and graphs generated with the PAST (free software for scientific data analysis, univariate and multivariate statistics) program version n°4.03. Each experiment was performed in triplicate. Data are expressed as mean ± standard deviation. A Wilcoxon test was used to express the significance of difference ($p < 0.05$) between means.

3. Results

3.1 Morphological modification of mitochondria by freezing

The ultrastructure of gilthead seabream muscle tissue from fresh fillets (D1) and frozen (D1)-thawed (D2) fillets was observed by transmission electron microscopy (TEM). On the fresh tissue (Fig. 1A), the different bands were clearly organized. The actin and myosin filaments were well defined and aligned in parallel. The I and A bands were distinguished as well as the Z and M lines (Fig. 1A1). The numerous mitochondria appeared compact and well organized. The mitochondrial cristae were well structured and did not show any vacuolization. The inner membrane invaginations were well visible and the outer membrane was dense and marked, reflecting good integrity (Fig. 1A2).

On the frozen-thawed tissue (Fig. 1B), a deep destructing of the tissue was observed. The sarcomeres were very disorganized. The size of the sarcomeres was considerably reduced. The I and A bands were much less visible. The A-band was considerably reduced, which translated into the disappearance of the H-band and the I-band. The Z-lines remained identifiable and made it possible to appreciate the reduced size of the sarcomeres (Fig. 1B1). The mitochondria were deeply altered. Disappearance of a large proportion of the mitochondrial cristae was observed, showing vacuolization (large spaces between cristae) (Fig. 1B2). The peripheral

membrane seemed to be much less dense in some places, which suggested the presence of breaches.

3.2 Measurement of mitochondrial NADH consumption by spectrophotometry

Discrimination between intact and altered mitochondria was made by measuring the permeability of the inner mitochondrial membrane. If the IMM is unaltered, NADH is unable to cross it. Thus, activation of respiratory chain enzymes by NADH is a good indicator of membrane permeabilization. The increase in permeability of the mitochondrial membranes to NADH was measured by a spectrophotometric method (consumption of NADH by complex I).

3.2.1 Measurement of the impact of freezing on NADH consumption by mitochondria

NADH has a peak absorbance at 340 nm. The consumption of NADH by mitochondria isolated from fresh (Day 1) and frozen (Day 0)-thawed (Day 8) fillets was therefore followed at 340 nm over a period of 300 seconds (Fig. 2). NADH is the substrate of a large number of cytoplasmic and mitochondrial enzymes. Consequently, a negative control was obtained by adding rotenone. Rotenone specifically blocks complex I and thus the consumption of NADH by mitochondrial complex I. This result allowed us to verify that the consumption of NADH (outside the respiratory chain) was negligible (FR). Similarly, a positive control was obtained by adding alamethicin. This is a permeabilizing agent that makes mitochondria fully permeable to NADH. Figure 2 shows that the addition of alamethicin led to a maximum consumption rate of NADH, both on mitochondria isolated from a fresh fillet (F+ curve) and on mitochondria isolated from a frozen-thawed fillet (T+ curve). The effect of alamethicin on mitochondria from a frozen-thawed fillet was very little, because the rate of consumption of NADH by these mitochondria was already very high. This indicated that permeabilization of inner mitochondrial membrane from a frozen-thawed fillet to NADH was high. The effect of alamethicin on mitochondria from frozen-thawed fillets was weak, because the level of permeabilization of mitochondria was already very high (T+). On the other hand, the effect of alamethicin was strong on mitochondria from fresh fillets (F+). Alamethicin strongly increased the rate of consumption of NADH by mitochondria. The consumption of NADH without alamethicin was much higher on mitochondria isolated from frozen-thawed fillets (T- curve) than on mitochondria isolated from fresh fillets (F- curve). However, alamethicin had a much greater permeabilizing effect on mitochondria isolated from fresh fillets than on mitochondria isolated from frozen-thawed fillets.

3.2.2 Impact of fillet storage time on NADH consumption

Storage of fresh fillets at 4°C increased membrane permeability and decreased the enzymatic activity of complex I, which affected the consumption of NADH by the mitochondria. 100 % activity (Fig. 3) corresponded to the consumption rate of mitochondria isolated from a fresh fillet and permeabilized with alamethicin (maximum activity of complex I: FD1+). The other results were normalized to FD1+. Storage time at 4°C affected the rate of NADH consumption after permeabilization with alamethicin. Total activity seemed to decrease from 100 % at D1 (FD1+) to 47.64 % at D8 (FD8+). This marked decrease probably resulted from the inhibition or alteration of complex I. However, the alamethicin effect was still present and substantial on fresh tissue at the D1 and D8 timepoints. However, we did not observe any significant effect of alamethicin on mitochondria isolated from frozen-thawed fillets (TD8- and TD8+).

Therefore, mitochondria isolated from fresh fillets had relatively low basal NADH consumption (29.35 % of total activity: FD1-), while mitochondria isolated from frozen-thawed fillets presented much higher consumption (78.06 % of total activity: TD8+). On the other hand, freezing induced alteration of the mitochondrial membranes. The membranes became permeable and allowed consumption of NADH. On mitochondria from frozen-thawed fillets, the level of permeabilization was sufficiently high not to observe any alamethicin effect.

3.3 Measurement of mitochondrial O_2 consumption by oxygraphy

Increased inner mitochondrial membrane permeability to NADH was measured by the oxygraphic method. The supply of NADH to the respiratory chain induced consumption of O_2. O_2 concentrations were followed in real time in the oxygen chamber (Fig. 4: left axis and blue curve). These concentration measurements allowed us to calculate continuous O_2 consumption rate (right axis and red curve expressed in pmol O_2 $s^{-1}.mL^{-1}$).

3.3.1 Measurement of the impact of freezing on O_2 consumption by mitochondria

For mitochondria isolated from fresh fillets stored at 4°C for one day (Fig. 4A), simple addition of mitochondria to mitochondrial buffer did not result in O_2 consumption. The absence of substrates did not allow for the activation of respiratory chains and thus O_2 consumption. The addition of NADH resulted in O_2 consumption of 65.44 nmol $O_2.min^{-1}.mg^{-1}$ of proteins, which reflected low permeabilization of mitochondria to NADH. The addition of alamethicin resulted in very high consumption of O_2, rising to 310.84 nmol $O_2.min^{-1}.mg^{-1}$ of proteins. This increase by a factor of 4.7 showed that mitochondria were permeabilized by alamethicin. Mitochondrial membranes isolated from fresh fillets are permeable by alamethicin. For mitochondria isolated from frozen-thawed fillets (Fig 4B), the addition of mitochondria to mitochondrial buffer did not lead to increased O_2 consumption, but the addition of NADH led to a significant increase

in O_2 consumption, of the order of 173.32 nmol $O_2.min^{-1}.mg^{-1}$ of proteins, i.e., about 2.6 times greater than for mitochondria isolated from fresh fillets. Further, alamethicin did not increase NADH induced oxygen consumption rate, which showed that mitochondria inner membranes were already permeabilized after freezing.

3.3.2 Impact of fillet storage time on O_2 consumption by mitochondria

Relative O_2 consumption of mitochondria isolated from fresh and frozen-thawed seabream fillets was studied (Fig. 5). The oxygraphic method confirmed the results obtained in spectrophotometry. At D1, the permeability of mitochondria to NADH was low (19.82 %: FD1-). Respiratory activity (O_2 consumption) was maximal when mitochondria were permeabilized by alamethicin (FD1+). At D8, O_2 consumption of mitochondria isolated from fresh fillets was lower in the presence of NADH (15.36 %: FD8-) than O_2 consumption of mitochondria isolated from frozen-thawed fillets (55.75 %: TD8-). Total respiratory activity (after addition of alamethicin) at D8 was much lower (32.34 %: FD8+), but we still observed an alamethicin effect. On mitochondria isolated from frozen-thawed fillets, the difference in O_2 consumption with and without alamethicin was not significant and seemed to show that freezing strongly altered mitochondrial membranes (55.75 %: TD8-; 65.26 %: TD8+). The diffusion of NADH was then maximal and resulted in high O_2 consumption. O_2 consumption on mitochondria isolated from frozen-thawed fillets (65.26 %: TD8+) was significantly lower than for fresh fillets (100 %: FD1+). Observation of the alamethicin effect on the O_2 consumption of mitochondria allowed us to discriminate fresh from frozen-thawed fillets.

4. Discussion

4.1 Mitochondrial membrane alteration by freezing

In this study, we showed that freezing led to profound cellular and tissue alterations. Muscle fibers and the outer and inner membranes of mitochondria are deeply altered by freezing at -80°C. Mitochondria are an excellent marker of differentiation for fresh-thawed fish because they are easily isolated and have an outer membrane and an inner membrane. Freezing directly affects mitochondrial membrane permeability. It is also necessary to understand the differences in permeabilization due to freezing or to physiological processes of permeabilization (Galluzzi, *et al.*, 2007). The outer membrane of mitochondria is naturally permeable to small molecules (less than 5 kDa). The permeability of the outer membrane can increase after translocation of proteins (Bax, Bid) to the outer membrane. These proteins form pores and increase the permeability of the outer membrane. The permeability of the inner membrane may also change, in particular with the formation of transition pores (Bonora, Giorgi, & Pinton, 2021). Storage

of the fillets at 4°C activates the processes of mitochondrial membrane permeabilization. The inner membrane is normally impermeable to NADH. Activation of the respiratory chains by NADH is therefore a marker of inner membrane permeabilization (Batandier, *et al.*, 2004). We also need to distinguish between this physiological permeabilization and permeabilization due to freezing.

In this study, we did not choose to use the external membrane to measure the effects of freezing. Some molecules such as cytochrome C could be a candidate indicator of outer membrane alteration (Gouveia, Bajwa, & Klegeris, 2017). Mitochondrial extraction processes respect the integrity of the mitochondria (Valenti, de Bari, De Filippis, Ricceri, & Vacca, 2014). However, storing the fillets at 4°C weakens the mitochondria that become more fragile upon extraction. Examination of the outer membrane can be a good indicator of the quality of the extraction. Cytochrome C is also a good indicator of the integrity of the outer membrane. Cytochrome C is localized in the intermembrane space and is released as soon as the integrity of the outer membrane is affected. However, to measure the impact of freezing, the permeability of the outer mitochondrial membrane was not retained because it changes too substantially with the storage time and the quality of the extraction. To compensate for cytochrome C leakage, we added a non-negligible amount of exogenous cytochrome C (10 µM). The added cytochrome C contributes to electron transfer in the respiratory chain and allowed us to assess the permeability of the inner membrane (Waterhouse, *et al.*, 2001).

In a previous study, we showed that storage at 4°C led to membrane alterations characterized by a loss of mitochondrial potential in mitochondria isolated from fresh fillets (Soret, *et al.*, 2022). Not surprisingly, the loss of mitochondrial potential was also observed in mitochondria isolated from frozen-thawed fillets (data not shown). Frozen-thawed mitochondria became permeable to H^+ protons and were unable to maintain their membrane potential.

In this study, we showed that freezing at -80°C led to a disruption of the inner membranes. This was associated with an increase in inner mitochondrial membrane permeability to NADH. The consumption of NADH by complex I was followed by spectrophotometry and also by monitoring of O_2 consumption by oxygraphy. We showed that on mitochondria isolated from frozen-thawed fillets, NADH permeability was higher than on mitochondria isolated from fresh fillets. Thus, freezing resulted in very high permeabilization of mitochondria. Storing fresh fillets at 4°C still resulted in proteolysis, shown by damage to enzymes and mitochondrial membranes (Javadov & Karmazyn, 2007). Permeability testing on mitochondria isolated from fillets stored for 8 days at 4°C confirmed that the permeability remained significantly lower than the permeability induced by freezing. This shows that freezing strongly affected the

permeability of mitochondria. Additionally, alamethicin (permeabilizing agent) had a significant effect on mitochondria isolated from fresh fillets, but no action on mitochondria isolated from frozen-thawed fillets, regardless of the storage time. This reflected significant permeabilization of mitochondria isolated from frozen-thawed fillets.

NADH is a substrate for many enzymatic reactions (Ying, 2006). Isolation of mitochondria has been found to eliminate spurious reactions. The use of rotenone made it possible to inhibit complex I and thus to measure the consumption of NADH that was not attributable to the respiratory chain. In this study, rotenone blocked all NADH consumption flux, which meant that NADH consumption was clearly associated with the respiratory chains. Freezing increased the membrane permeability of mitochondria to NADH, which discriminated fresh from frozen-thawed fillets.

4.2 Mitochondrial enzymatic alteration

Storage of fillets at 4°C maintains mitochondrial activity over several days. However, the respiratory chains quickly produce super-reactive oxygenated compounds. These highly reactive compounds contribute to the oxidation of mitochondrial lipids and proteins (Lenaz, 1998). Complex I is particularly affected by oxidation reactions (Batandier, *et al.*, 2004). In this study, we observed that at D1, the rate of NADH consumption and O_2 consumption on mitochondria isolated from fresh fillets and in the presence of alamethicin was maximal. At D8, the NADH consumption and O_2 consumption of mitochondria isolated in the presence of alamethicin was strongly reduced, which showed a loss of activity of the complex I of the respiratory chain at least.

The consumption of NADH by isolated mitochondria cannot be an indicator of freezing by itself. Importantly, we noticed that NADH consumption was higher on mitochondria isolated from frozen-thawed fillets than on mitochondria isolated from fresh fillets. This difference was not significant because the loss of complex I activity during freezing tends to decrease the difference in consumption due to increased permeability. This difference could be further decreased if the storage time at 4°C of the fillets (post-freezing) was increased.

O_2 consumption in the presence of NADH remains relevant to discriminate fresh from frozen-thawed fillets, but we observed very high loss of activity when the fillet was kept at 4°C for a long period of time. The oxidation of certain compounds of the respiratory chain must participate in this loss of activity. To compensate for some of this oxidation and storage loss, we used decylubiquinone (0.1 mM). As for cytochrome C, the exogenous contribution of

decylubiquinone compensated in part for the oxidation reactions produced during storage at 4°C or during freezing (Sharpley, Shannon, Draghi, & Hirst, 2006).

To consider the loss of activity of the complexes linked to the storage time at 4°C or freezing, we used a permeabilizer: alamethicin. Alamethicin allowed us to discriminate mitochondria isolated from fresh fillets from mitochondria isolated from frozen-thawed fillets. It still had an effect on mitochondria isolated from fillets stored for 8 days at 4°C. Alamethicin no longer acted on mitochondria isolated from frozen-thawed fillets, which had a high level of permeabilization. The use of alamethicin normalized the results and made it possible to measure the maximum activity (NADH consumption and O_2 consumption) for each condition. The activity measured in the presence of alamethicin gave an idea of the level of alteration of the respiratory chain activity related to storage at 4°C or related to freezing.

Freezing was performed at -80°C for a short storage time of 8 days. Using alamethicin, the maximum activity of NADH consumption and O_2 consumption by mitochondria decreased with freezing and with storage time at 4°C. The decrease in O_2 consumption was slightly greater than the decrease in NADH consumption (complex I activity). The accumulated alterations in the respiratory chain amplify the inhibition of O_2 consumption (Peterson, Johannsen, & Ravussin, 2012). Also, the freezing time at -80°C did not seem to affect these decreases in activity. Longer freezing times (1 month) did not show amplification of the inhibition phenomena (data not shown). Freezing at -80°C seemed to stabilize the oxidation reactions. Interestingly, these losses of activity seemed rather to be attributable to the freezing shock. Freezing disrupts membrane fluidity, which causes dysfunction of the interactions between complexes of the respiratory chain and thus of the complexes (Valenti, *et al.*, 2014).

In short, freeze-thaw shock altered mitochondrial membranes sufficiently to make them insensitive to the permeabilizing effect of alamethicin.

5. Conclusion

Isolation of mitochondria from seabream fillets was used to explore mitochondrial functions and consequently their alterations. Freezing of fish fillets is associated with substantial destruction of mitochondrial membranes, leading to permeabilization of the inner membrane to NADH. The degree of permeabilization of isolated mitochondria to NADH can be measured by the activity of complex I and O_2 consumption in the presence of NADH. Moreover, the enzymatic activities were normalized by the addition of alamethicin, which is a permeabilizing agent. Mitochondria isolated from red tissue of fillets that are insensitive to alamethicin are highly permeabilized mitochondria, and are therefore indicators of frozen-thawed fish products.

These results could be generalized to many species and therefore meet the expectations of the seafood industry concerning the differentiation of fresh fillets from frozen-thawed fillets from the point of view of compliance with regulations.

Declaration of interest

None

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Figure legends

Figure 1: Electron microscopy of red muscle sections of gilthead seabream fillets stored at 4°C or frozen storage

Fig A1/A2: fresh (Day 1) muscle section

Fig B1/B2: frozen (Day 1)-thawed (Day 2) muscle section

A: A band; H: H zone; I: I band; M: M line; mit: mitochondria; Z: Z line; Scale: Bars = 500 nm

Figure 2: Measure of NADH consumption by spectrophotometry in isolated seabream mitochondria

FR: Mitochondria isolated from fresh fillet (Day 1) with rotenone

F-: Mitochondria isolated from fresh fillet (Day 1) without alamethicin

F+: Mitochondria isolated from fresh fillet (Day 1) with alamethicin

T-: Mitochondria isolated from frozen (Day 0)-thawed (Day 8) fillet kept at -80°C without alamethicin

T+: Mitochondria isolated from frozen (Day 0)-thawed (Day 8) fillet kept at -80°C with alamethicin

Figure 3: Relative specific activity of mitochondria isolated from fresh and frozen-thawed seabream fillet

FD1: Mitochondria isolated from fresh fillet Day 1

FD8: Mitochondria isolated from fresh fillet Day 8

TD8: Mitochondria isolated from frozen-thawed fillet kept for 8 days at -80°C

Mitochondria were incubated with alamethicin (+) or without alamethicin (-)

The relative activity was reduced to FD1+ (with alamethicin) activity

Asterisks denote values that are statistically significant

A Wilcoxon test was performed ($p < 0.05$, n = 3)

Figure 4: O_2 consumption by oxygraphic assay in mitochondria isolated from seabream

Oxygraph traces (blue curve) and first derivate (red curve) are represented at different storage conditions

A. Oxygraph of mitochondria from D1 fresh fillet

B. Oxygraph of mitochondria from D8 frozen-thawed fillet

The run was carried out in the presence of cytochrome C (10 µM) and decylubiquinone (0.1 mM). After the addition of mitochondria (0.2 $mg.mL^{-1}$), NADH (0.1 mM) and alamethicin (2.5 µM) were added to the incubation chamber

Figure 5: Relative O_2 consumption of mitochondria isolated from fresh and frozen-thawed seabream fillets

FD1: Mitochondria isolated from fresh fillets Day 1

FD8: Mitochondria isolated from Fresh fillets Day 8

TD8: Mitochondria isolated from frozen-thawed fillets kept for 8 days at -80°C

Mitochondria were incubated with alamethicin (+) or without alamethicin (-)

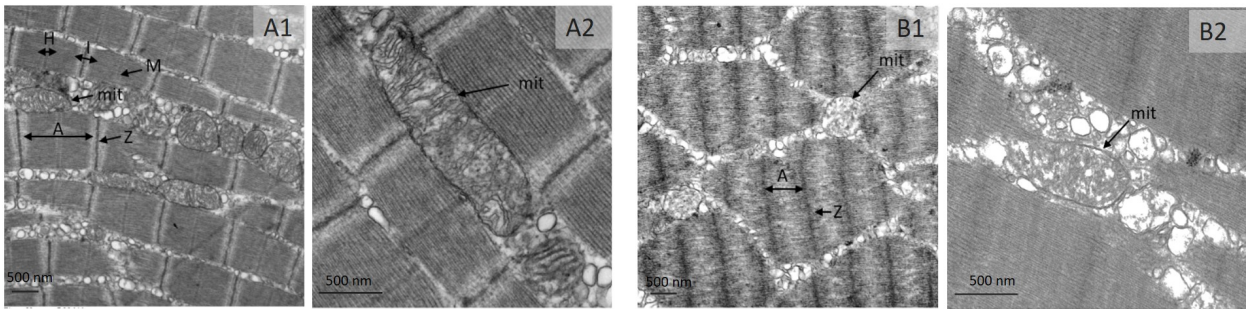
The relative O_2 consumption was reduced to FD1+ (with alamethicin) activity

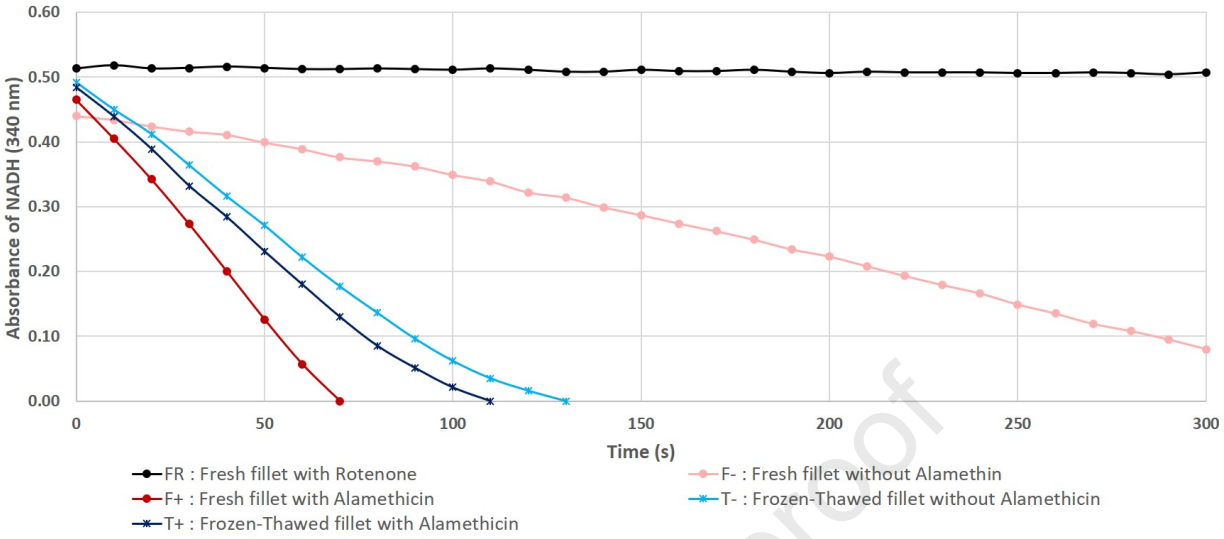
Asterisks denote values that are statistically significant

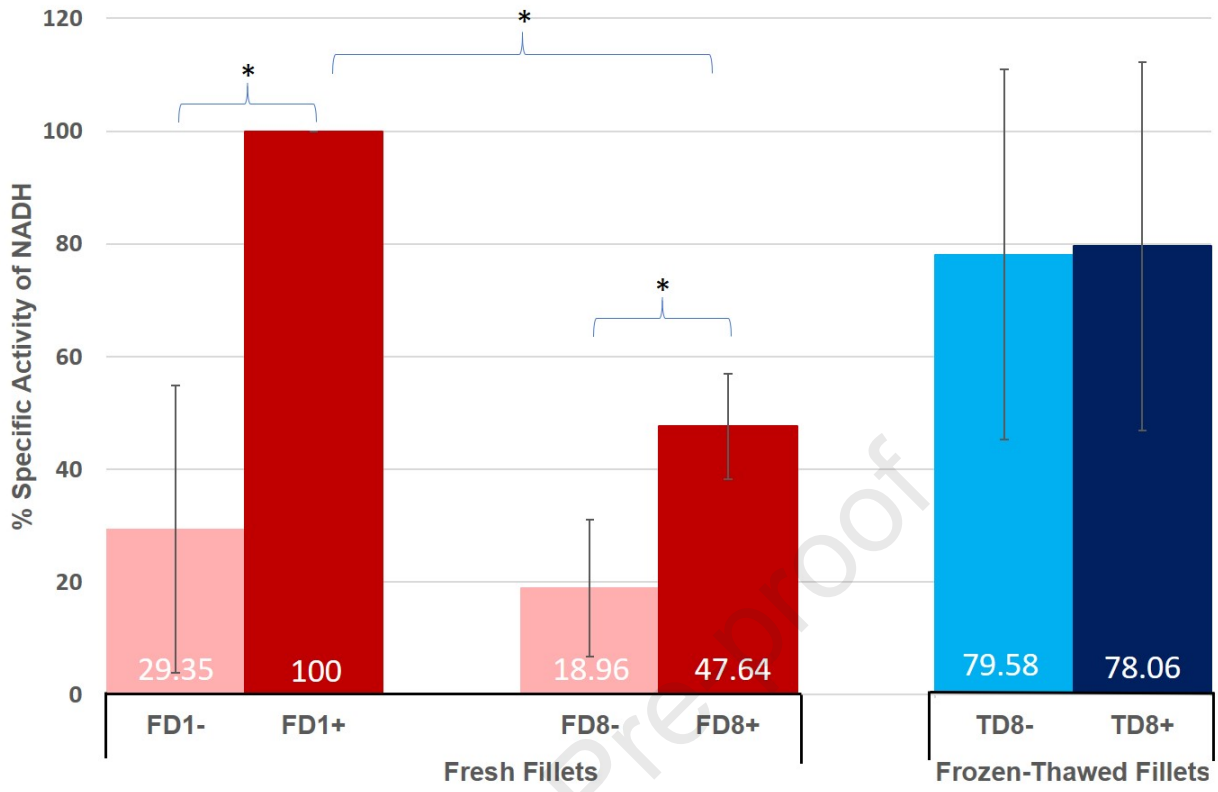
A Wilcoxon test was performed ($p < 0.05$, n = 3)

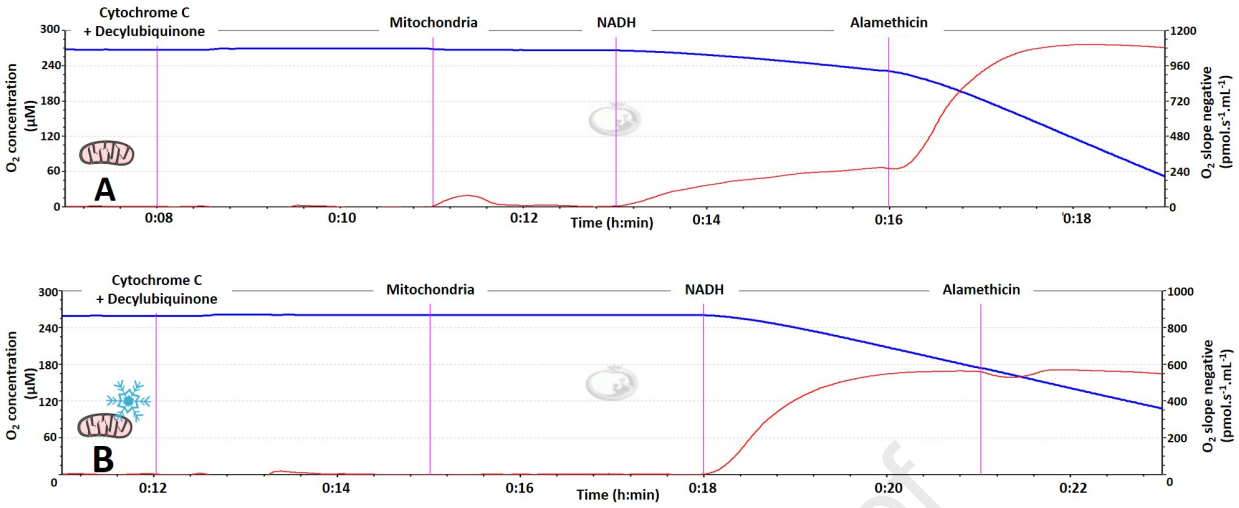
A: Fresh fillets

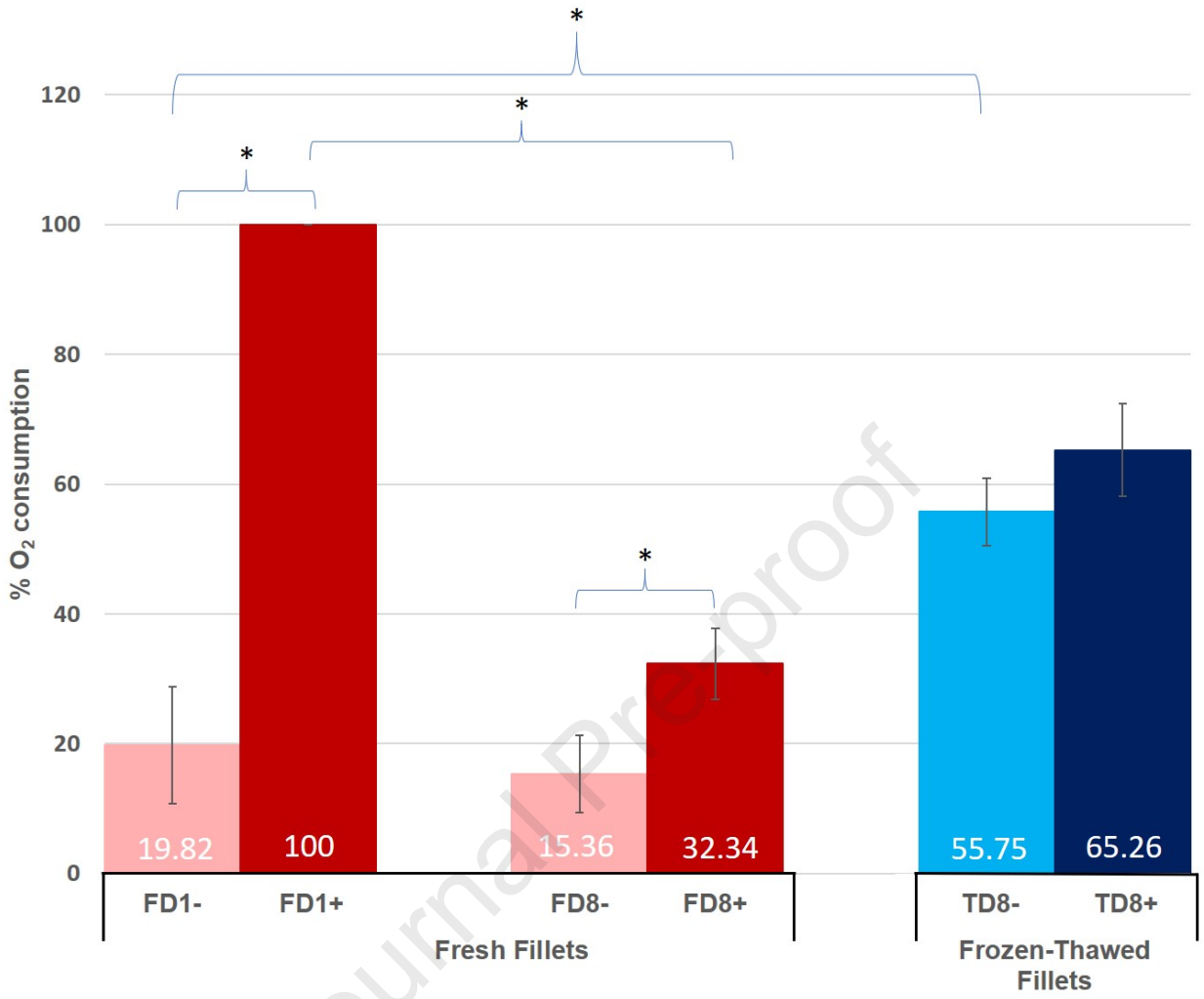
B: Frozen-thawed fillets











It is difficult to differentiate between fresh and frozen-thawed fish fillets.

Mitochondria were extracted from fish fillets and effects of freezing were assessed.

Freezing caused permeabilization of mitochondria to NADH.

Two methods were used to assess permeability: spectrophotometry and oxygraphy.

Both techniques identified mitochondria isolated from fish exposed to freezing shock.