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Highlights

Mitochondrial properties were used to discriminate fresh and frozen-thawed fillets

Permeability of muscle fibres and mitochondrial membranes to NADH was measured

Two approaches were used to measure permeability: spectrophotometry and oxygraphy

Frozen-thawed fibres were more permeable to NADH and less sensitive to alamethicin

Abstract

There is no comprehensive method for differentiating between fresh and frozen-thawed fish fillets. This is an ongoing problem, particularly in relation to regulations. In this study, we showed the relevance of using the properties of mitochondria to discriminate fresh fish fillets from frozen-thawed fish fillets. The use of red muscle fibres of Gilthead sea bream allowed us to leave mitochondria in their physiological environment and to avoid possible alterations of mitochondrial membranes during isolation steps. The impact of freezing on fillets was evaluated by measuring the permeability of fibres and mitochondrial membranes to nicotinamide adenine dinucleotide + hydrogen (NADH). NADH permeability of fresh fillet fibres stored at 4°C was compared to the permeability of fibres extracted from frozen-thawed fillets. Two approaches were used to measure permeability changes: a spectrophotometric method that measured consumption of NADH by complex I, and an oxygraphic approach that measured stimulation of O_2 consumption by NADH. Fibres from frozen-thawed fillets were more permeable to NADH and were less sensitive to the permeabilizer alamethicin. The

sensitivity of this method allowed us to clearly detect red muscle fibres from frozen-thawed fish versus fresh fish fillets.

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

1. Introduction

Many species of fish are caught at considerable distances from consumption sites. As a result, storage of aquatic products can include a freezing stage. These products are generally intended to supply the frozen food market (Costello & Ovando, 2019). Consumption of frozen products has tended to decrease in recent years, although it increased again in 2020 with the COVID-19 pandemic. Detecting possible supply of frozen-thawed seafood to the fresh fish market is a real problem. The development of molecular tools to detect fraud within the aquatic products industry is becoming increasingly relevant (Sone et al., 2019).

Given that freezing leads to the formation of ice crystals that cause irreversible damage to plasma and intracytoplasmic membranes (Li, 2018), we built on this finding in previous studies. We measured the impact of fish fillet freezing on the permeabilization of mitochondria isolated from red muscle fibres (Bouchendhomme et al., 2022). Freezing of aquatic products leads to changes in mitochondrial membranes which become far more permeable. This permeability can be measured in different ways: via the release of matrix proteins or metabolites, or via permeability to certain substrates (Sileikyte et al., 2010). Increased permeability to nicotinamide adenine dinucleotide + hydrogen (NADH) is associated with mitochondrial membrane alteration related to freezing-thaw shock (Kroemer et al., 2007).

Isolation of mitochondria requires a series of extraction steps. In this study, our objective was to reduce analysis time by using muscle fibres directly, without a mitochondria extraction step. This technique has been used for many years to explore mitochondrial functions for medical purposes, including the detection of myopathies, neurodegenerative diseases and cancer (Kuznetsov et al., 2022). Muscle fibres are usually mechanically dissociated and permeabilized with saponin or digitonin to make substrates and inhibitors accessible to mitochondria (Kuznetsov et al., 2008). This treatment is used to enable exploration of mitochondrial functions while preserving their state and their interactions with other organelles (Picard et al., 2010, 2011). The use of muscle fibres is particularly well adapted to the discrimination of frozen-thawed aquatic products from fresh ones. In this context, freezing shock induces permeabilization of plasma and intracytoplasmic membranes making the fibres

and mitochondria more sensitive to substrates and to certain inhibitors present in the extracellular medium (Stéphenne et al., 2007). Due to the high concentration of mitochondria in red muscle, the amount of fibres required for the analysis is low.

Evaluation of mitochondrial function in muscle fibres has been used to measure differences in meat quality in pork and beef products (Ramos et al., 2020; Werner et al., 2010). Use of muscle fibres allows for the measurement of differences in post-mortem respiratory activities in various breeds. Permeabilized fibres have also been used to link post-mortem mitochondrial activities to meat quality (England et al., 2018; Ramos et al., 2021). In this study, we aimed to use isolated red muscle fibres to explore mitochondrial functions that might be affected by freezing processes (Tolstorebrov et al., 2016). The chosen model allowed us to measure the sensitivity of muscle fibres to NADH with or without permeabilization (Mayevsky & Barbiro-Michaely, 2009; Mayevsky & Rogatsky, 2007). Our study focused on the action of freezing on plasma and mitochondrial membranes by measuring the sensitivity of mitochondria to NADH (Batandier et al., 2004). Two techniques were used: the first was to measure consumption of NADH on fresh and frozen-thawed fibres by a spectrophotometric method. The second was to measure O_2 consumption by oxygraphy on fresh and frozen-thawed fibres, in the presence of NADH. The permeabilization level of the membranes was normalized by using a permeabilizing agent, alamethicin (Matic et al., 2005). In this way, it was possible to assess levels of membrane permeabilization by measuring the level of NADH consumption and the level of O_2 respiration. The objective was to study whether permeabilization induced by freezing remained significant compared to permeabilization induced by autolysis processes (Pegg, 2010), linked to the storage of products at low temperatures (2–4°C) (Diop et al., 2016).

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). Filets were stored in a cold room (+4°C) on ice. The core temperature of fishes was 2°C. Ice renewal was carried out every day. Fillets intended for freezing were frozen at -40°C at D0 in order to minimize damage to cell structures (Bao et al., 2021). Analyses were performed at day 1, day 4 and day 8 for fresh fillets and for frozen-thawed fish after 9 days in the freezer. The thawing phase was carried out in a cold room at 4°C. To avoid contact between fillets and ice, plastic wrapping was used.

2.2 Reagents

Bovine serum albumin (BSA), ethylene glycol-bis (2 amino-ethylether)-N,N,N',N'-tetraacetic acid (EGTA), sucrose, potassium chloride (KCl), Tris(hydroxymethyl)aminomethane (Trizma® base), 4-morpholinepropanesulfonic acid (MOPS), alamethicin, β-nicotinamide adenine dinucleotide (NADH), decylubiquinone and cytochrome c were purchased from Sigma-Aldrich (St. Louis, MO, USA). 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 Fibre preparation

Fibres originated from red muscle of Gilthead seabream. In brief, 10 mg of fibres were taken from the dorsal part of the red muscle. Fibres were dilacerated using scalpels and with the help of very fine tweezers under a binocular magnifying glass. Under these conditions, muscle fibres were separated from conjunctive tissue. Fibres were then rinsed with respiratory buffer. The respiratory buffer was composed of 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 $mg.mL^{-1}$. Fibres were stored in respiratory buffer until analysis. Measurements on the fibres were carried out in a time of 90 min or less. This handling time included isolation and analysis time.

2.4 NADH consumption by spectrophotometry

NADH consumption was measured by spectrophotometry (UV vis spectrophotometer, UV-1280, Shimadzu Europa GmbH, Duisburg, Germany). The spectrophotometric tank contained 1 mL of the respiratory medium, 5 mg of fibres, cytochrome c (10 µM) and decylubiquinone (0.1 mM). Rotenone (2.5 µM) was used as a control. Alamethicin (5 µM) was added to permeabilize the fibres. NADH was used at 0.1 mM final concentration in all tanks. The zero calibration was done without NADH and reading was performed at 340 nm. Measurements were taken at t=0s, t=100s, t=200s and t=300s. Between each measurement, tanks were placed on a vibrating stirrer at 150 rpm (Titramax 100, Heidolph instruments GmbH, Schwabach, Germany).

The specific activity (SA) was calculated from the law of Beer-Lambert:

$$SA = \left(\frac{(\frac{\Delta A}{\varepsilon . l})/\Delta T}{\text{quantities of fibres}}\right)/1000 \text{ nmol of NADH.min}^{-1}\text{.mg}^{-1} \text{ of wet mass.}$$

129 $\varepsilon = 6220\ M^{-1}.cm^{-1}$

130 *2.5 O_2 consumption by oxygraphy*

131 O_2 consumption was measured using an oxygraph O-2k (Oroboros Instruments, Innsbruck,
132 Austria). The 100 % calibration was done when the chamber was open in the presence of a
133 maximum amount of O_2. The 0 % calibration was done in the closed chamber and with
134 addition of dithionite. Before addition of the fibres, cytochrome c (10 µM) and
135 decylubiquinone (0.1 mM) were added. After 3 minutes, the fibres were added and the
136 chamber was closed. NADH (1 mM) was added, then alamethicin (5 µM). The experiment
137 was performed at +25°C.

138 O_2 consumption was calculated using this formula:

$$O_2 \text{ consumption} = \frac{(O_2\ consumption\ with\ substrate - O_2\ consumption\ without\ substrate)}{quantities\ of\ fibres}\ x\ 60$$

139 O_2 consumption was expressed in pmol of $O_2.min^{-1}.mg^{-1}$ of wet mass.

140 *2.6 Discrimination threshold*

141 A ratio can be calculated using this formula:

$$ratio = \frac{consumption\ with\ alamethin}{consumption\ without\ alamethicin}$$

142 This ratio was used to determine a discrimination threshold value. Below a certain value,
143 frozen-thawed fillets were found.

144 *2.7 Statistical analysis*

145 Statistical analyses and graphs were generated with XLSTAT 2022.1.1. Each experiment was
146 repeated 4 times. Data were expressed as means ± standard deviation. A *t*-test was used to
147 express the significance of difference ($p < 0.05$) between the different means.

148 **3. Results**

149 *3.1 Measurement of NADH consumption by spectrophotometry*

150 Rates of NADH consumption (in nmol of $NADH.min^{-1}.mg^{-1}$ of wet mass) on isolated fresh
151 fillet fibres kept at 4°C (F) and on isolated frozen-thawed fillet fibres (T) and in the presence
152 or absence of the permeabilizer alamethicin (+/-) were studied (Fig. 1).

153 NADH consumption on fresh fibres was low. It was 779.96 nmol of $NADH.min^{-1}.mg^{-1}$ of wet
154 mass. The addition of alamethicin (FD+) led to a considerable permeabilization effect, which

resulted in a significant increase in NADH consumption by fresh fibres. Consumption reached 2161.27 nmol of NADH.min^{-1}.mg^{-1} of wet mass. Consumption of NADH by frozen-thawed fibres without the addition of alamethicin was high and was 3152.86 nmol of NADH.min^{-1}.mg^{-1} of wet mass, whereas the addition of alamethicin did not lead to any increased consumption of NADH. Frozen-thawed fibres had a higher NADH consumption rate and were not sensitive to the action of the permeabilizer (alamethicin), unlike fresh fibres.

3.2 Effect of fillet storage time on NADH consumption

Rates of NADH consumption (in nmol of NADH.min^{-1}.mg^{-1} of wet mass) on isolated fresh fillet fibres stored at 4°C and for different storage times (1 day, 4 days and 8 days) were studied (Fig. 2).

Storage time had no significant effects on NADH consumption by fresh fibres. NADH consumption decreased from 779.86 nmol of NADH.min^{-1}.mg^{-1} of wet mass at D1 to 476.53 nmol at D4, and to 967.68 nmol at D8. The storage time at 4°C did not seem to have any influence on the permeabilization of fibres by alamethicin. At D1, the consumption of NADH in the presence of alamethicin was 2161.27 nmol of NADH.min^{-1}.mg^{-1} of wet mass. It was 2271.98 nmol and 2276.8 nmol of NADH.min^{-1}.mg^{-1} of wet mass for the times D4 and D8, respectively. Regardless of the storage time, alamethicin had the same effect on the permeabilization of fibres and therefore on the consumption of NADH.

3.3 Effect of freezing on O_2 consumption by oxygraphy

O_2 concentrations in the oxygen chamber containing 10 mg of isolated fresh fillet fibres and the rates of O_2 consumption by these fibres were monitored over time (Fig. 3A). Addition of isolated fibres from a fresh fillet resulted in low consumption of O_2 (less than 25 pmol.s^{-1}.mL^{-1}). After addition of NADH, a slight increase in O_2 consumption was observed; the plateau indicated a value of 135 pmol.s^{-1}.mL^{-1} which demonstrated slight permeabilization of the mitochondria. After addition of the permeabilizer (alamethicin), a significant increase in O_2 consumption was observed, which was 355 pmol.s^{-1}.mL^{-1}. This increase showed that fresh fibres were sensitive to permeabilization; NADH was therefore able to significantly activate respiration (i.e., O_2 consumption).

O_2 concentrations in the oxygen chamber containing 10 mg of isolated frozen-thawed fillet fibres and the rates of O_2 consumption by these fibres were monitored over time (Fig. 3B). Addition of isolated fibres from a frozen-thawed fillet resulted in low consumption of O_2 (less than 20 pmol.s^{-1}.mL^{-1}). After addition of NADH, a significant increase in O_2 consumption was observed. The rate of NADH consumption increased without any real plateau effect. It

reached about 180 pmol.s^{-1}.mL^{-1} before the addition of alamethicin which induced greater permeabilization of mitochondria. After addition of the permeabilizer (alamethicin), we did not observe a significant increase in O_2 consumption. The lack of alamethicin effect showed that fibres from frozen-thawed fillet were not very sensitive to permeabilization. The rate of O_2 consumption on fibres isolated from frozen-thawed fillet was about two times lower than for fibres isolated from fresh fillet.

3.4 Effect of fillet storage time on O_2 consumption

O_2 consumption by the fibres as a function of storage time of fish fillets and type of packaging (fresh or frozen-thawed) is shown in Fig. 4. The rate of O_2 consumption was expressed in pmol of O_2.min^{-1}.mg^{-1} of wet mass and 4 oxygraphs were generated for each condition. O_2 consumption by isolated fresh fish fibres decreased over time from 1373.79 pmol of O_2.min^{-1}.mg^{-1} of wet mass at D1 to 243.72 pmol of O_2.min^{-1}.mg^{-1} of wet mass at D8. This showed an alteration of the respiratory chain complexes which resulted in decreased O_2 consumption for an identical stimulation with NADH. Contrary to the spectrophotometric method, O_2 consumption by the fibres isolated from frozen-thawed fish was not higher than the fibres isolated from fresh fillets. It was 1373.79 pmol of O_2.min^{-1}.mg^{-1} of wet mass for the fresh fibres at D1 versus 1234.08 pmol of O_2.min^{-1}.mg^{-1} of wet mass for the frozen-thawed fibres. Measuring O_2 consumption of isolated fibres exposed to NADH therefore did not appear to be sufficient to discriminate between fresh from frozen-thawed fish.

For fibres of fresh fillet, we observed an alamethicin effect at all 3 storage times (D1, D4 and D8). Alamethicin increased NADH permeability of the isolated fresh fillet fibres, which resulted in increased O_2 consumption. O_2 consumption of the fibres in the presence of alamethicin decreased over time from 4608.75 pmol of O_2.min^{-1}.mg^{-1} of wet mass at D1 to 891.06 pmol of O_2.min^{-1}.mg^{-1} of wet mass at D8. This represents a decrease of 80.66 % and reflects the fact that respiratory chain efficiency decreased with storage time of the fillets at 4°C. For fibres of frozen-thawed fillet, there was no significant alamethicin effect. O_2 consumption was 1234.08 pmol of O_2.min^{-1}.mg^{-1} of wet mass on fibres without alamethicin, and 1848.09 pmol of O_2.min^{-1}.mg^{-1} of wet mass in the presence of alamethicin. On the 4 trials performed, this difference was not significant. Freezing led to sufficient permeabilization not to be amplified by a permeabilizer (alamethicin). We can also note lower O_2 consumption of frozen-thawed fillet fibres compared to fresh fillet fibres in the presence of alamethicin. Consumption decreased from 4608.75 pmol of O_2.min^{-1}.mg^{-1} of wet mass for FD1+ to

1848.09 pmol of $O_2.min^{-1}.mg^{-1}$ of wet mass for T+. Freezing destabilized the activity of the respiratory chain complexes.

3.5 Ratio determination to discriminate fish quality (frozen-thawed fillets)

An alamethicin effect was present in both methods, spectrophotometric and oxygraphic. This is why we calculated in this part a ratio R = (Activity in presence of alamethicin / Activity in absence of alamethicin).

In Figure 5A, we present the ratios obtained for the spectrophotometric method as a function of the storage time of fillets at 4°C and for different packaging modes (fresh and frozen-thawed). The R ratio for fresh aquatic products was around 2.5–3. The ratio calculated for frozen-thawed aquatic products was around 1. Using the statistical data, a ratio of 1.5 emerged for this method. Fresh products could be defined as products with an R ratio > 1.5, frozen-thawed products as products with an R ratio < 1.5.

In Figure 5B, we present the ratios obtained for the oxygraphic method as a function of the storage time of fillets at 4°C and for different packaging modes (fresh and frozen-thawed). Ratios were calculated as R = (O_2 consumption rate in the presence of alamethicin expressed in pmol of $O_2.min^{-1}.mg^{-1}$ / O_2 consumption rate in the absence of alamethicin). The ratio for fresh products was higher than 3. The calculated ratio for frozen-thawed products was around 1.5. Using the statistical data, a ratio of 2 emerged for this method. Fresh products could be defined as products with an R ratio > 2, frozen-thawed products as products with an R ratio < 2.

4. Discussion

Changes in membrane permeability associated or not with increased NADH consumption have been demonstrated by different methodological approaches. These studies based on fluorescence spectroscopic approaches (Karoui et al., 2021), infrared spectroscopy (Chang et al., 2020; Cheng et al., 2013; Kamruzzaman et al., 2015), Raman spectroscopy (Herrero, 2008), nuclear magnetic resonance spectroscopy (Cheng et al., 2013), or hyperspectral imaging (Xu & Sun, 2017) allow the measurement of the profound changes induced during the frozen/thawed step. Freezing causes profound membrane alterations that lead to permeabilization of plasma and intracytoplasmic membranes. Plasma and mitochondrial membranes are naturally impermeable to NADH (Ying, 2006), but freezing physically alters the membranes making the respiratory enzyme complexes accessible to NADH. In a previous study, we showed that measuring the NADH permeabilization of isolated mitochondria makes

it possible to discriminate between fresh and frozen-thawed seafood products. The use of muscle fibres allows us to avoid all the extraction steps, but brings an additional level of complexity insofar as conditions are more heterogeneous (diffusion of NADH in the fibres) and NADH can be associated with numerous sarcoplasmic enzymatic reactions (Picard et al., 2012).

4.1 Frozen-thawed fish fillet detection by the spectrophotometric approach

In this study, we showed the effect of freezing at -40°C on the rupture of plasma and mitochondrial membranes. Consumption of NADH by the fibres was measured by spectrophotometry at 340 nm. On fibres isolated from fresh fillets (from D1 to D8), NADH consumption was low. Consumption of NADH was not attributable to consumption by the mitochondria because it was insensitive to rotenone (data not shown). In the fresh state, it has been reported that plasma and mitochondrial membranes remain intact and consumption of NADH is therefore low and not attributable to the mitochondria (Schantz & Henriksson, 1987). Additionally, consumption of NADH by isolated fresh fish fillet fibres did not increase with the storage time of the fillets at 4°C. This showed that the autolysis that could occur on the fibres and that could make NADH accessible to the cytosol did not increase its consumption. In a previous study, we demonstrated autolysis on a fish fillet by release of lactate dehydrogenase over several days of storage at 4°C (Diop et al., 2016). We can hypothesize that this autolysis could make the fibres more permeable to NADH but not the mitochondria. Artificial permeabilization by alamethicin leads to a significant increase in NADH consumption by the fibres and thus to permeabilization of the plasma and mitochondrial membranes (Matic et al., 2005). Here, consumption of NADH was indeed attributable to the mitochondria because it was able to be blocked by rotenone. Therefore, the basal consumption by mitochondria on isolated fresh fillet fibres remained low due to the low level of permeabilization of plasma and mitochondrial membranes. NADH consumption on fresh fillet fibres was able to be stimulated by alamethicin. This permeabilizer acted on plasma and mitochondrial membranes, making mitochondria sensitive to NADH. Contrary to what we had observed on isolated mitochondria, consumption of NADH by isolated fibres from fresh fillet and in the presence of alamethicin, was not affected by the storage time at 4°C. This was an argument in favour of using fibres to avoid possible denaturation during extraction processes, especially for mitochondria isolated from fillets that have been stored longer at 4°C (Zamzami et al., 2007).

The relevance of using fibre permeabilization with NADH as a freezing marker was justified by two important experimental elements. The first was that freezing shock induced a very significant increase in NADH consumption by the fibres. Freezing leads to a rupture of plasma and mitochondrial membranes, making complex I accessible to NADH (Yamada et al., 2020). Blocking of complex I by rotenone leads to a very significant decrease in NADH consumption by the fibres, suggesting that this consumption is indeed associated with mitochondria (N. Li et al., 2003). Freezing shock thus permeabilized the plasma and mitochondrial membranes. The second experimental argument was the absence of a permeabilizing effect of alamethicin on NADH consumption by isolated frozen-thawed fillet fibres. The permeabilizing action of freezing on plasma and mitochondrial membranes was sufficiently strong that no permeabilizing effect of alamethicin on isolated frozen-thawed fillet fibres was able to be observed (Carraro & Bernardi, 2020). The action of freezing resulted in maximum NADH consumption by the fibres.

The spectrophotometric approach was based on the consumption of NADH by complex I of mitochondria. The storage of aquatic products at low temperatures (freezing) over relatively long periods of time could lead to oxidation phenomena. The main site of production of superoxide compounds, involved in oxidation reactions, is complex I (Mazat et al., 2020). This production could lead to the oxidation of lipids and peripheral proteins and contribute to a loss of activity of complex I and meat quality. Therefore, measuring the rate of NADH consumption by the fibres cannot be an objective factor of alteration. This rate should be normalized to the rate of NADH consumption by the fibres after addition of alamethicin. This ratio makes it possible to take into account possible losses of activity of complex I linked to the storage time at 4°C or to the storage time in frozen state. This ratio allowed us to determine whether the fibres were sensitive or insensitive to permeabilization (alamethicin). Fresh aquatic products were defined as products with an R ratio >1.5 and frozen-thawed aquatic products were defined as products with an R ratio < 1.5.

4.2 Frozen-thawed fish fillets detection by the oxygraphic approach

The oxygraphic method can be considered more specific to mitochondria since O_2 consumption is directly proportional to NADH consumption by the respiratory chains. O_2 consumption by fibres isolated from fresh fillet and exposed to NADH tended to decrease during the time the fillet was kept at 4°C. This result was somewhat surprising but was not attributable to the change in permeability of the fibres to NADH, but rather related to a loss of activity of the respiratory chains (Larosa & Remacle, 2018). By permeabilizing the fibres

(using alamethicin), we observed a significant decrease in O_2 consumption with the storage time of the fillets at 4°C. This result showed that the activity of enzyme complexes within the respiratory chain tended to decrease with storage time. The same result was also observed in a previous study on mitochondria isolated from fresh fillets (Bouchendhomme et al., 2022). These results show that measuring increased plasma and mitochondrial membrane permeability by NADH may be partially biased by a loss of respiratory chain activity.

O_2 consumption by isolated fresh fillet fibres was able to be stimulated by alamethicin. As with the spectrophotometric method, alamethicin acted on the plasma and mitochondrial membranes and allowed the diffusion of NADH into the mitochondria, activating O_2 consumption. The activity of the respiratory chains strongly decreased with the storage time, but the alamethicin effect was observed for all times (D1, D4 and D8).

For the oxygraphic method, freezing did not result in a significant increase in O_2 consumption of the fibres, whereas it induced an alteration of the plasma and mitochondrial membranes. Why did this happen? Part of the explanation came from the observation of O_2 consumption of the fibres in the presence of alamethicin. This consumption also decreased substantially and was close to the O_2 consumption without alamethicin. Therefore, like for fresh fibres, O_2 consumption by fibres isolated from frozen-thawed fillets stimulated by the increased permeability of fibres to NADH was partly compensated for by a loss of activity of the respiratory chains. The respiratory chains seem to be sensitive to the freezing process (Stéphenne et al., 2007). Therefore, direct O_2 consumption on NADH cannot be an indicator of freezing. On the contrary, on fibres from frozen-thawed fillets, no alamethicin effect was observed. It has been shown that freezing causes maximum permeabilization of the fibres which become insensitive to the effect of the permeabilizer (D. Li et al., 2018; Rasmusson et al., 2022).

As for the spectrophotometric approach, we noted that the direct effect of NADH on O_2 consumption by the fibres cannot be used as a marker of membrane alteration and therefore of freezing. It was necessary to reduce this activity to the maximum activity that was obtained in the presence of alamethicin. Ratio R was defined as the ratio between O_2 consumption without alamethicin / O_2 consumption in the presence of alamethicin. Calculating this ratio also allowed us to avoid losses of respiratory chain activity linked to storage at 4°C or to the storage time in frozen state. Determining the state of the product (fresh or frozen-thawed) is linked to the sensitivity of the fibres to the permeabilizer alamethicin. If the R ratio is > 2, the product can be defined as fresh, and if the R ratio is < 2, the product can be defined as frozen-thawed.

5. Conclusion

This study confirmed the relevance of using mitochondria as a frozen-thawed marker. The use of fish muscle fibres allowed us to avoid the isolation steps of mitochondria and this makes it possible to obtain results in a much shorter time. Freezing-thawing led to permeabilization of the plasma and mitochondrial membranes. NADH was then able to access complex I and the respiratory chains to activate O_2 consumption. Fibres isolated from fresh fillets were sensitive to permeabilization with alamethicin, resulting in increased permeability to NADH. Fibres isolated from frozen-thawed fillets were insensitive to the action of alamethicin. This approach yielded a rapid result on the preservation state of the Gilthead seabream. To generalize this test to other aquatic products, it would be useful to extend this study to the white muscle of fish fillets.

Declaration of interest

None

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Figure legends

Figure 1: Relative specific activity of red muscle fibres from fresh and frozen-thawed Gilthead seabream fillet.

F-: Red muscle fibres from fresh fillet without alamethicin

F+: Red muscle fibres from fresh fillet with alamethicin

T-: Red muscle fibres from frozen-thawed fillet kept at -40°C without alamethicin

T+: Red muscle fibres from frozen-thawed fillet kept at -40°C with alamethicin

Figure 2: Measurement of NADH consumption by spectrophotometry in red muscle fibres from fresh Gilthead seabream fillet storage at 4°C.

FD1-: Red muscle fibres from fresh fillet Day 1 without alamethicin

FD1+: Red muscle fibres from fresh fillet Day 1 with alamethicin

FD4-: Red muscle fibres from fresh fillet Day 4 without alamethicin

FD4+: Red muscle fibres from fresh fillet Day 4 with alamethicin
FD8-: Red muscle fibres from fresh fillet Day 8 without alamethicin
FD8+: Red muscle fibres from fresh fillet Day 8 with alamethicin
Red muscle fibres were incubated with alamethicin (+) or without alamethicin (-)
Asterisks denote values that are statistically significant
A *t*-test was performed ($p < 0.05$, n = 4)

Figure 3: O_2 consumption of Gilthead seabream red muscle fibres by oxygraphy
Oxygraph traces (blue curve) and first derivate (red curve) are represented at different storage conditions.
A. Oxygraph of red muscle fibres from D1 fresh fillet
B. Oxygraph of red muscle fibres from 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 red muscle fibres (10 mg), NADH (0.1 mM) and alamethicin (2.5 µM) were added to the incubation chamber.

Figure 4: Relative O_2 consumption of red muscle fibres from fresh and frozen-thawed Gilthead seabream fillets.
FD1-: Red muscle fibres from fresh fillet Day 1 without alamethicin
FD1+: Red muscle fibres from fresh fillet Day 1 with alamethicin
FD4-: Red muscle fibres from fresh fillet Day 4 without alamethicin
FD4+: Red muscle fibres from fresh fillet Day 4 with alamethicin
FD8-: Red muscle fibres from fresh fillet Day 8 without alamethicin
FD8+: Red muscle fibres from fresh fillet Day 8 with alamethicin
TD8-: Red muscle fibres from frozen-thawed fillets kept for 8 days at -40°C without alamethicin
TD8+: Red muscle fibres from frozen-thawed fillets kept for 8 days at -40°C with alamethicin
Red muscle fibres were incubated with alamethicin (+) or without alamethicin (-)
Asterisks denote values that are statistically significant
A *t*-test was performed ($p < 0.05$, n = 4)

Figure 5: Relative R ratio from fresh and frozen-thawed Gilthead seabream fillets.
For both methods, the R ratio is determined by the ratio between the activity in the presence of alamethicin and the activity in the absence of alamethicin (R = (activity with alamethicin) / (activity without alamethicin)).

A. R ratio of red muscle fibres by the spectrophotometric method

B. R ratio of red muscle fibres by the oxygraphic method

The red line defines the ratio that appears to be significant to distinguish fresh from frozen-thawed fillets.

A *t*-test was performed ($p < 0.05$, n = 4)

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