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Highlights

- Mitochondrial functions of European seabass fillets were disrupted from 96h storage (D4) at +4°C
- Spectral properties of Rh123 and TMRM were correlated to mitochondrial health
- The evaluation of mitochondrial bioenergetics represents an early indicator of fish freshness
- A micro-volume fluorimeter could be considered for rapid evaluation of fish freshness

Key words

Mitochondrial potential; fish freshness; micro-volume fluorimetry

Abstract

Research and development of methods to assess fish freshness continues to be a major challenge for the fishing industry. At the same time, consumers are now increasingly attentive to food quality, including fish freshness and product history. Here, we propose a reliable,

rapid and easy-to-apply fluorimetric approach to assess fish freshness using a micro-volume fluorimeter. Mitochondrial functions were assessed in European seabass (*Dicentrarchus labrax*) fillets at different durations of storage at +4°C: from Day 0 to Day 8. We found that mitochondrial respiration and mitochondrial membrane potential ($\Delta\Psi_m$) were significantly disrupted after 4 days of storage at +4°C. The spectral properties (emission peak and fluorescence intensity) of mitochondrial membrane potential probes rhodamine 123 (Rh123) and tetramethylrhodamine methyl ester (TMRM) were strongly affected by the $\Delta\Psi_m$ integrity of the fish fillets. We highlighted two categories of fish quality as a function of $\Delta\Psi_m$: Day 0 to Day 3, $\Delta\Psi_m$ was preserved; and D4 to D7, $\Delta\Psi_m$ was disrupted. Thus, evaluation of $\Delta\Psi_m$ constitutes an early and reliable predictive indicator of fish freshness.

1. Introduction

Freshness is an important factor that contributes to fish quality (Olafsdottir, et al., 1997), and its evaluation is still a major challenge in the fishing industry. Numerous methods and technologies have been developed to evaluate fish freshness ((Mendes, 2018); (Wu, Pu, & Sun, 2019) (Cheng, Sun, Han, & Zeng, 2014), (Hassoun & Karoui, 2015)). Current traditional methods for assessing fish quality are based on physical, chemical, microbiological and sensory parameters (Rehbein & Oehlenschlager, 2009). However, these methods present numerous limits. They do not apply to all species, are time consuming, and require special skills. Consequently, there is still a need in the food industry to develop rapid, reliable and simple methods to assess fish freshness.

Understanding *post mortem* mechanisms in fish muscle cells is essential to identify new markers, and to develop methods for the evaluation of fish freshness. In a previous study, we assessed *post mortem* changes in mitochondrial functions and integrity in gilthead seabream (*Sparus aurata*) fish muscle cells. We demonstrated that mitochondrial respiration and mitochondrial membrane potential ($\Delta\Psi_m$) were significantly altered after 4 days of storage at

+4°C (Jérôme Cléach, et al., 2019). Our data showed, for the first time, that mitochondrial activity constituted a putative reliable indicator to assess fish freshness at different early *post mortem* time points.

In *post mortem* conditions, fish muscle is deprived of oxygen and nutrients, and is consequently in an ischemic state. In this context, mitochondrial activity is disrupted. Many methods are available to investigate mitochondrial functions and integrity, such as the analysis of the mitochondrial respiratory chain by oxygraphy and the evaluation of mitochondrial membrane potential ($\Delta\Psi_m$) by fluorimetry (Kuznetsov, et al., 2008). Mitochondrial membrane potential ($\Delta\Psi_m$) and the proton gradient (ΔpH) constitute the components of the proton motive force (Mitchell, 2011). $\Delta\Psi_m$ is essential for ADP phosphorylation and ATP synthase activity. $\Delta\Psi_m$ is also involved in many less well studied functions such as: homeostasis, mitochondrial function regulation, and transport of ions, proteins, and nucleic acids (Zorova, et al., 2018). Therefore, monitoring oxygen consumption by oxygraphy and evaluation of $\Delta\Psi_m$ are useful approaches to assess mitochondrial health.

The purpose of this study was to correlate *post mortem* mitochondrial bioenergetics with fish freshness. This study focused on the assessment of $\Delta\Psi_m$ as a reliable method to determine fish quality at early *post mortem* time points. We studied the $\Delta\Psi_m$ values of mitochondria isolated from European seabass (*Dicentrarchus labrax*) skeletal muscle at different durations of storage at +4°C to comply with the storage temperatures widely used in seafood processing companies: from 6 hours after slaughtering (D0) to Day 7. First, in order to validate mitochondrial extraction and assess mitochondrial respiration, we calculated the respiratory control index (RCI) at different *post mortem* time points. Then, $\Delta\Psi_m$ was assessed using a Nanodrop 3300 micro-volume fluorimeter (MVF). This device has many advantages; for example, it is small, portable, easy-to-use and to maintain. These features make it ideal for effective rapid evaluation of fish freshness in the food industry, without the need for special

technical skills. In a previous study, we demonstrated the strong capabilities of the MVF to assess membrane potential with cationic fluorescent probes (J Cléach, et al., 2018). In this study, we compared the action of two cationic fluorescent probes, traditionally used in the study of mitochondrial potential: rhodamine 123 (Rh123) and tetramethylrhodamine methyl ester (TMRM).

2. Materials and methods

2.1 Reagents

4-morpholinepropanesulfonic acid (MOPS), bovine serum albumin (BSA), carbonyl cyanide 3-chlorophenylhydrazone (CCCP), ethylene-bis(oxyethylenenitrilo)tetraacetic acid (EGTA), malate, proteinase type XXIV, rhodamine 123 (Rh123), succinate, tetramethylrhodamine methyl ester perchlorate (TMRM), and Tris (hydroxymethyl)aminomethane (Trizma® base) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Potassium chloride (KCl) and glutamate were acquired from Fisher Labosi (Paris, France). Magnesium chloride ($MgCl_2$), potassium dihydrogen phosphate (KH_2PO_4) and sucrose were purchased from Acros Organics (Morris, NJ, USA). Rh123, TMRM and CCCP were prepared in dimethyl sulfoxide (DMSO) purchased from Thermo Scientific (San Diego, CA, USA).

2.2 Fish origin and storage

The European seabass (*Dicentrarchus labrax*) (300–450 g) used in this study were sourced from Aquanord sea farm (Gravelines, France), as previously described (Jérôme Cléach, et al., 2019). This farmed fish model was chosen in order to obtain accurate data on living conditions, slaughtering, and storage, which can potentially influence the study of freshness. Upon arrival at the laboratory, the fish were immediately filleted. The fillets were stored on ice in a cold room (+4°C) for 8 days and used for experiments every 24 h over 8 days: Day 0, Day 1, Day 2, Day 3, Day 4, Day 5, Day 6, Day 7 and Day 8. The ice was renewed every day.

99 Plastic wrapping was used to avoid contact between the fillets and the ice or the accumulated
100 water.

101 *2.3 Isolation of mitochondria from fish fillets*

102 The method for mitochondria isolation was adapted from Pasdois, Parker, Griffiths, and
103 Halestrap (2011). It was also previously used for extraction of gilthead seabream (*Sparus*
104 *aurata*) skeletal muscle cell mitochondria (Jérôme Cléach, et al., 2019). All the steps in
105 mitochondrial isolation were performed in a cold room at +4 °C. Fish muscle was dissected
106 from the fillet (3 g) and finely diced with scissors. The fine pieces obtained (2–3 mm^3) were
107 incubated at +4°C for 7 min under stirring in 20 mL of isolation buffer (180 mM KCl, 80 mM
108 sucrose, 5 mM $MgCl_2$, 10 mM Tris, 2 mM EGTA, pH 7.2 at +4°C), supplemented with 0.1
109 $mg.mL^{-1}$ of bacterial proteinase type XXIV. The resulting tissue suspension was poured into a
110 30 mL glass Potter homogenizer and homogenized for 3 min using a motorized Teflon pestle
111 at 300 rpm. The homogenate was centrifuged at 7,500 *g* for 10 min. The resulting pellet was
112 first washed and then resuspended in 20 mL isolation buffer containing 2 $mg.mL^{-1}$ of fatty
113 acid-free BSA, and homogenized for 3 min at 150 rpm. The homogenate was then centrifuged
114 at 700 *g* for 10 min. The supernatant was centrifuged at 1,500 *g* for 10 min. The resulting
115 supernatant was centrifuged again at 7,000 *g* for 10 min. The mitochondrial pellet obtained
116 was then suspended with a low volume (40 µL) of isolation buffer in order to obtain a
117 concentrated mitochondrial suspension. The protein concentration was determined using a
118 Bio-Rad protein assay kit, derived from the method developed by Bradford (1976), with BSA
119 as a standard. Mitochondria were kept on ice at a final concentration of 60–100 $mg.mL^{-1}$ for
120 not more than 4 h.

121 *2.4 Respiratory control index (RCI)*

122 Oxygraphy (Rank Brothers Digital Model 10, Cambridge, United Kingdom) was used to
123 monitor mitochondrial functions after isolation by measuring the respiratory control index

(RCI) at different days of storage at +4°C (Day 0, Day 1, Day 2, Day 3, Day 4, Day 5, Day 6 and Day 7), as previously described (Jérôme Cléach, et al., 2019). First, 2.1 mL of respiration buffer (KCl 125 mM, MOPS 20 mM, Tris 10 mM, EGTA 10 µM, KH_2PO_4 2.5 mM, fatty acid-free BSA 2 $mg.mL^{-1}$, pH 7.2) was added to the oxygraph chamber supplemented with glutamate (5 mM), malate (2 mM), and succinate (5 mM). Then, mitochondria were added at a final concentration of 0.2 $mg.mL^{-1}$. Oxygen consumption rates were assessed without and with ADP (1 mM) (basal and state 3, respectively). Then, carboxyatractyloside (CAT) (5 µM) was added to block the oxygen consumption linked to ATP synthesis. The experiment was performed at +25°C. Saturating dithionite (Sigma-Aldrich) was added to the oxygraph chamber to calibrate the device, and to achieve the zero oxygen calibration.

RCI was calculated using the following formula:

$$RCI = \frac{ADP\ (state\ 3)}{CAT}$$

Where state 3 is the respiration rate during maximum ATP synthesis, and CAT is the respiration rate not linked to ATP synthesis.

2.5 Evaluation of mitochondrial membrane potential

A NanoDrop 3300 MVF (ThermoFisher Scientific, distributed by Ozyme, Saint-Cyr-l'École, France) was used to monitor the fluorescence of Rh123 and TMRM in order to evaluate changes in mitochondrial $\Delta\Psi_m$ of isolated mitochondria extracted at different storage durations at +4°C (Day 0, Day 1, Day 2, Day 3, Day 4, Day 5, Day 6 and Day 7). 1 mL of respiration buffer (KCl 125 mM, MOPS 20 mM, Tris 10 mM, EGTA 10 µM, KH_2PO_4 2.5 mM, fatty acid-free BSA 2 $mg.mL^{-1}$, pH 7.2) at +25°C was added to a 5 mL round-bottom polypropylene tube, originally designed for flow cytometry analysis. Rh123 (50 nM final) or TMRM (50 nM) were added to the respiratory buffer without respiratory substrates (as a negative control) or with respiratory substrates: glutamate (5 mM), malate (2 mM), and succinate (5 mM). Just prior to acquisition, isolated mitochondria were added at a

concentration of 0.2 mg.mL^{-1}. The decoupling agent CCCP (2 µM final) was then added to the preparation to disrupt the $\Delta\Psi_m$, leading to Rh123 and TMRM output from the mitochondria matrix.

Rh123 and TMRM were used in quenching mode. At high concentrations (50 – 100 nM), cationic probes stack inside the mitochondrial matrix to form aggregates leading to the quenching of the fluorescent emissions of the aggregated molecules (Perry, Norman, Barbieri, Brown, & Gelbard, 2011).

2.6 Micro-volume fluorimeter settings

The MVF was originally designed to quantify nucleic acids and proteins. However, it is possible to create and edit methods with the software to study a wide range of fluorophores. In this way, we created a method to analyze the fluorescence of probes Rh123 and TMRM. The excitation sources available include: UV LED (excitation maximum 365 nm), Blue LED (excitation maximum 470 nm), and White LED (460-650 nm excitation). White LED was selected to study Rh123 and TMRM fluorescence. The virtual emission filter interval ($\Delta\lambda$) was set at ± 20 nm. 2 µL of sample were used to measure the fluorescence intensity. The assay blank was carried out with the respiratory buffer. For each sample, 5 measures were acquired, as recommended by the manufacturer. Importantly, this experimental approach did not enable the user to measure $\Delta\Psi_m$, but gave a degree of alteration of mitochondrial polarization. The measurement of fluorescence was reported in relative (non-absolute) fluorescent units (RFU).

Rh123 and TMRM fluorescence intensities recorded at the two states of respiration (with or without substrates) were normalized to the fluorescence recorded after CCCP addition, according to the following formula:

$$100 - \left(\left(\frac{X - Y}{X}\right) * 100\right)$$

Where: X = Rh123 or TMRM fluorescence intensity after CCCP addition

174 Y = Rh123 or TMRM fluorescence intensity after mitochondria addition with or
175 without substrates.

176 The calculated normalized fluorescence values using the above formula were based on the
177 maximum emission value. CCCP at 2 µM completely depolarizes mitochondria by consuming
178 all the proton gradient established by the respiratory chain. As such, the fluorescence intensity
179 obtained after its addition corresponded only to the dissipation of $\Delta\Psi_m$, and made it possible
180 to take into account the non-specific binding of the dye.

181 *2.7 Statistical analysis*

182 The statistical analysis and graphs were generated with SPSS 17 software. Each experiment
183 was performed at least in triplicate. Data are expressed as mean ± standard deviation.
184 Unpaired two-sample *t*-tests were used to express the significance of difference ($p < 0.05$)
185 between means, and Levene's test to determine the homogeneity of variance.

186 # 3. Results

187 *3.1 Changes in the respiratory control index*

188 First, mitochondrial functions in European seabass muscle cells were studied by assessing
189 mitochondrial respiration. To do this, the respiratory control index (RCI = ADP (state 3) /
190 CAT) was measured at different *post mortem* storage time points at +4°C (D0 to D8) (**Fig. 1**).
191 At D0, the RCI value was 7.3 ± 1.25. At D2, RCI significantly decreased to reach 4.03 ± 1.15.
192 From Day 4, the RCI value was near 1: D4 = 1.34 ± 0.33 and D8 = 1.08 ± 0.15. In short, from
193 D0 to D4, RCI values gradually decreased to reach the minimum value at D4. From D4, *post*
194 *mortem* conditions led to mitochondrial respiration disruption.

195 *3.2 Changes in membrane potential of isolated mitochondria (ΔΨm) assessed with cationic*
196 *fluorescent probes*

197 The mitochondrial membrane potential of mitochondria isolated from European seabass
198 muscle cells was assessed at different storage time points: D0 to D7. The two cationic

fluorescent probes Rh123 and TMRM were used to evaluate $\Delta\Psi_m$. An MVF was required to record dye fluorescence intensities. The fluorescence intensity of the cationic probes enabled determination of the $\Delta\Psi_m$. When the mitochondrial functions and integrity were preserved, the generation of $\Delta\Psi_m$ led to dye stacking inside the mitochondrial matrix. As a consequence, the fluorescence properties of the cationic probes were affected: fluorescence intensity decreased (quenching) and emission spectra shifted. In reverse, when mitochondria were damaged, probe accumulation was weak and fluorescence quenching was low. Therefore, the level of fluorescence of cationic probes was informative concerning mitochondrial health.

Mitochondria isolated from fish muscle at different *post mortem* time points were incubated with Rh123 in the presence of respiratory substrates (glutamate, malate and succinate) (**Fig. 2A: black lines**). As a negative control, the protonophore CCCP was added after mitochondria staining to disrupt $\Delta\Psi_m$ (**Fig. 2A: red lines**). From D0 to D3, fluorescence intensities were between 600 and 650 RFU. Then, from D4 to D5, they were higher and reached 700 RFU. From D6 to D7, fluorescence was recorded at 800 RFU. Thus, from D0 to D7, fluorescence gradually increased from 600 RFU to 800 RFU, highlighting *post mortem* disruption of $\Delta\Psi_m$. From D0 to D7, the fluorescence intensity recorded after CCCP addition was always around 900 RFU. This value constituted a reference corresponding to mitochondria with disrupted $\Delta\Psi_m$. From D6 to D7, the values recorded without CCCP (black curve) were close to those recorded after CCCP addition (red line), showing the strong disruption of $\Delta\Psi_m$. As a negative control, we also stained isolated mitochondria without respiratory substrates, to confirm that the fluorescence quenching was specifically due to the energization of mitochondria (data not shown). A blue line has been drawn at 600 RFU to visualize the significant increasing change in fluorescence intensity from D0-D3 to D4-D7. These data show $\Delta\Psi_m$ disruption in the European seabass fish muscle mitochondria after 96 hours of storage at +4°C

In order to confirm our results with the Rh123 probe, we used a second cationic fluorescent probe sensitive to $\Delta\Psi_m$. Mitochondria isolated from European seabass muscle cells were stained with TMRM, following the same protocol used with the Rh123 probe. A typical fluorescence profile of TMRM after isolated mitochondria labelling was found at different *post mortem* time points (**Fig. 2B: black lines)**. As a negative control, the protonophore CCCP was added after mitochondria staining to disrupt $\Delta\Psi_m$ (**Fig. 2B: red lines**). From D0 to D3, fluorescence intensities were between 700 and 750 RFU. Then, from D4 to D5, fluorescence was between 1,000 and 1,100 RFU. From D6 to D7, fluorescence reached 1,200 RFU. As such, from D0 to D7, fluorescence gradually increased from 700 RFU to 1,200 RFU, highlighting *post mortem* disruption of $\Delta\Psi_m$. From D0 to D7, the fluorescence intensities recorded after CCCP addition were between 1,200 and 1,400 RFU. This range of values constituted a reference corresponding to mitochondria with disrupted $\Delta\Psi_m$. From D6 to D7, the values recorded without CCCP (black curve) were close to those recorded after CCCP addition (red line), showing the strong disruption of $\Delta\Psi_m$. As a negative control, we also stained isolated mitochondria without respiratory substrates to confirm that the fluorescence quenching was specifically due to the energization of mitochondria (data not shown). A blue line has been drawn at 700 RFU to visualize the significant decreasing change in the quenching fluorescence from D0-D3 to D4-D7. As for Rh123 staining, the data recorded with the TMRM probe demonstrated $\Delta\Psi_m$ disruption after 96 hours of storage at +4°C. Therefore, the results obtained with the TMRM probe were correlated with those recorded with the Rh123 probe.

3.3 Statistical analysis of changes in the fluorescence of cationic probes Rh123 and TMRM at different post mortem *time points*

The experiments illustrated in Figures 2A and 2B were performed at least three times in order to acquire a statistical overall view of changes in $\Delta\Psi_m$ at different durations of fish fillet

storage (**Fig. 3**). For both probes, fluorescence intensity recorded for each day was normalized to CCCP. Importantly, the cationic probes Rh123 and TMRM are sensitive to the $\Delta\Psi_m$. However, they can also interact non-specifically with other organelles such as the endoplasmic reticulum, lysosomes, and other cell fragments (Cottet-Rousselle, Ronot, Leverve, & Mayol, 2011). This addition of CCCP allowed us to record a fluorescence intensity in mitochondria with a disrupted mitochondrial membrane. This approach of rationalization with CCCP therefore enabled us to take into account the non-specific binding of the dyes.

Fig. 3A illustrates changes in Rh123 fluorescence normalized to CCCP at different *post mortem* time points. From D0 to D3, normalized Rh123 fluorescence was between 65 and 75 %. From D4 to D7, fluorescence was around 85 %. Two zones delimited by a dotted line at 75 % of fluorescence were identified. This dotted line highlighted a significant change in $\Delta\Psi_m$ between the two periods: "D0-D3" and "D4-D7".

Changes in TMRM fluorescence were also normalized with CCCP at the same *post mortem* time points as for the Rh123 probe (**Fig. 3B**). From D0 to D3, normalized TMRM fluorescence was between 60 and 70 %. From D4 to D7, fluorescence was between 80 and 95 %. Like for the statistical data obtained with the Rh123 probe, a dotted line traced at 75 % delimited two zones, and highlighted a significant change in $\Delta\Psi_m$ between the two periods: "D0-D3" and "D4-D7".

3.4 Evolution of the Rh123 and TMRM fluorescence emission peak wavelengths at different post mortem *time points*

Cationic probe characteristics such as the emission peak wavelength depend on mitochondrial functions and integrity ((Emaus, Grunwald, & Lemasters, 1986); (Scaduto Jr & Grotyohann, 1999)). For each condition and both probes, emission peak wavelengths were studied with the MVF (**Fig. 4**).

For a given typical experiment with energized mitochondria stained with Rh123, the fluorescence emission peaks were recorded for each day of storage (**Fig. 4A**). From D0 to D2, the emission peak wavelength (EPW) was 528 nm. From D3 to D4, the EPW was 526 nm. Then, from D5 to D7, the EPW was 524 nm. Consequently, from D0 to D7, the emission peak shifted from 528 nm to 524 nm. As a control, emission fluorescence was measured after addition of CCCP (524 nm).

The emission peak was also recorded at different days of storage after TMRM staining (**Fig. 4B**). From D0 to D3, the EPW was 579 nm. From D4 to D5, the EPW was 577 nm. Then, from D6 to D7, the EPW was 576 nm. Consequently, for a given experiment with energized mitochondria, the TMRM emission profile changed, with a shift from 579 nm to 576 nm from D0 to D7. As a negative control, when mitochondria were decoupled after CCCP addition, the EPW was 576 nm.

For both probes, the EPWs were measured for each duration of storage and compared by statistical analysis (**Fig. 4C, 4D**). For Rh123 staining (**Fig. 4C)**, when mitochondria were energized, the EPW was higher than 526 nm (527-529 nm) from D0 to D3, and less than 526 nm after 4 days of storage (D4 to D7). A dotted red line on the graph delimited and highlighted the significant difference between the two zones. When energized mitochondria were stained with CCCP, emission fluorescence peaks were measured between 524 and 525 nm. As a control, peak emission fluorescence was also recorded between 524 and 525 nm in the absence of substrates (data not shown). These data demonstrated the specific action of energized mitochondria on the characteristic emission peak. As a result, the phenomena of red-shift was only visible with energized mitochondria with respiratory substrates. The same observations were made for TMRM staining (**Fig. 4D)**, except that the wavelength significantly discriminating D0-D3 from D4-D7 was located at approximately 577.5 nm (**red dotted line: Fig. 4D**).

4. Discussion

The development of methods to evaluate fish freshness and thereby its quality is currently still a challenge and an issue in the fishing industry. In a previous study (Jérôme Cléach, et al., 2019), we demonstrated that mitochondrial functions and integrity constitute reliable and early indicators to evaluate gilthead seabream freshness. In the present study, we focused our experiments on the evaluation of mitochondrial membrane potential ($\Delta\Psi_m$). To this end, we followed a new approach using an MVF to evaluate $\Delta\Psi_m$ with cationic fluorescent probes. In order to study the universality of this method, we performed our experiments on another farmed species: the European seabass (*Dicentrarchus labrax*). Our results provided knowledge on *post mortem* mitochondrial bioenergetics in skeletal muscle cells.

4.1 Post mortem *respiration of mitochondria isolated from fish fillets*

As a first approach, we studied mitochondrial functions of European seabass fillet muscle cells using oxygraphy. The RCI value was calculated to evaluate mitochondrial bioenergetics. Under given conditions, high RCI indicated good mitochondrial functions, and on the contrary low RCI usually reflected mitochondrial dysfunction (Brand & Nicholls, 2011). RCI was therefore a good indicator of mitochondrial health. RCI values were calculated at different *post mortem* durations of storage: D0, D2, D3, D4 and D8. From D0 to D4, RCI values gradually decreased. From D4 to D8, the minimum value of RCI was reached, demonstrating mitochondrial decoupling. In our previous study (Jérôme Cléach, et al., 2019), the RCI was evaluated using mitochondria isolated from farmed gilthead seabream *(Sparus aurata*) at different durations of storage at +4°C. Comparing the results obtained for both species, we observed that RCI values were higher in the European seabass model than in the gilthead seabream model during the initial *post mortem* days. This can be explained by the fact that RCI values depend on and vary as a function of species and tissues (Hulbert, Turner, Hinde, Else, & Guderley, 2006). However, for both species, day 4 constituted the critical

storage day where mitochondrial functions were significantly disrupted (respiration and $\Delta\Psi_m$).

On the one hand, RCI measurement allowed us to validate the high quality of our mitochondrial extraction protocol. On the other, it made it possible to establish a correlation between mitochondrial oxygen consumption and $\Delta\Psi_m$ at different *post mortem* time points.

4.2 Post mortem *membrane potential of mitochondria isolated from fish fillets*

As a second approach, $\Delta\Psi_m$ was assessed at different days *post mortem* using a fluorimetric method, like for oxygraphy. From D0 to D3, isolated mitochondria conserved their $\Delta\Psi_m$ in the presence of respiratory substrates. After 96 h of storage (D4), $\Delta\Psi_m$ was significantly disrupted. As a result, there was correlation between changes in mitochondrial respiration and $\Delta\Psi_m$ in fish muscle cells in *post mortem* conditions. In our previous study, with the gilthead seabream model, we also demonstrated disruption of $\Delta\Psi_m$ at D4. Thus, for both species, mitochondrial bioenergetics were significantly altered at the same *post mortem* time points. Therefore, D4 represented a "critical" storage time where mitochondrial respiration and $\Delta\Psi_m$ were significantly and strongly disrupted in both species. This similarity of results can be explained by the fact that these two species present numerous common points. Importantly, they have the same lifestyle. Moreover, the tested fish originated from the same aquaculture farm and consequently had the same nutrition, the same living environment, and were slaughtered by the same method. All these parameters could probably have a direct impact on *post mortem* changes in mitochondrial functions and integrity.

4.3 The post mortem *mitochondrial survival strategy*

RCI decreased significantly during the first 3 days, with maintenance of the mitochondrial membrane potential over the same period. On the basis of these results, from D0 to D3, mitochondria isolated from European seabass fillet muscle cells retained their membrane potential and the integrity of the respiratory chain. Over the first 3 days, the RCI decreased,

illustrating that ADP could no longer accelerate respiratory chains. These results correlate with those previously obtained on gilthead sea bream (Jérôme Cléach, et al., 2019). From D4 to D8, the RCI decreased significantly and was accompanied by a reduction in membrane potential to reach its minimum value.

The maintenance of $\Delta\Psi_m$ in *post mortem* conditions (8.5 hours and 24 hours, respectively) was previously reported by Barksdale, et al. (2010) in other species. In *post mortem* conditions, muscle cells are deprived of oxygen and nutrients. Similarly, it was reported that 25% of oxygen was still present in beef *longissimus thoracis et lumborum* a few minutes after slaughtering. After 48 hours of storage at +4°C, 10% of the oxygen was still present in beef muscle (England, et al., 2018). As mentioned in this study, even a low amount of oxygen and a low number of viable mitochondria can participate in *post mortem* metabolism. We can suppose that a certain percentage of oxygen is also present in fish fillets after slaughtering, and that this allows for survival of mitochondria. Previous studies have clearly shown the key role of mitochondria in *post mortem* metabolism ((St-Pierre, Brand, & Boutilier, 2000), (Nicholas J. Hudson, 2012), (England, et al., 2018)). Therefore, mitochondria are selfish and prioritize their own survival by consuming ATP reserves in order to maintain their homeostasis and functionality ((St-Pierre, et al., 2000), (Lehmann, Segal, Muradian, & Fraifeld, 2008), (Nicholas J. Hudson, 2012)). This process of mitochondrial survival in anoxia is called “mitochondrial treason” (N. J. Hudson, et al., 2017).

4.4 Spectral properties of Rh123 and TMRM as indicators of fish freshness

The originality of this study was based on a new approach to assess mitochondria with an MVF. This device made it possible to acquire fluorescence emission spectra. We observed that Rh123 and TMRM fluorescence emissions were strongly impacted as a function of *post mortem* time points. Two characteristics of Rh123 and TMRM are to be considered to evaluate fish freshness: the level of quenching fluorescence and the emission peak

wavelength. From D0 to D3, for both probes, the levels of quenching were higher at the D0-D3 interval than at the D4-D7 interval. Moreover, the emission fluorescence peak shifts were only observable at D0-D3, also for both probes. Therefore, mitochondrial probe fluorescence and emission peaks were linked to the "energized state" of mitochondria. Emaus, et al., (1986) were the first to highlight the fluorescence properties of Rh123 using uncoupled and coupled rat isolated mitochondria. They considered spectral red shifts and fluorescence quenching to be a consequence of electrophoretic dye uptake, followed by high capacity binding in the mitochondrial matrix in coupled mitochondria, but not in uncoupled mitochondria. The same observation was also reported in another study with TMRM ((Scaduto Jr & Grotyohann, 1999)). These authors showed that TMRM excitation and emission spectra change, as shown by quenching and peak emission shift, after incubations of heart rat mitochondria with dye once energized with glutamate, malate and succinate. As proposed by Diop, et al. (2016), we can also classify in this study a fish fillet in the D0-D3 zone or in the D4-D7 zone, on the basis of high quenching and shift of the fluorescence peak or high quenching and no shift of the fluorescence peak, respectively. In this way, we highlighted new markers to evaluate fish freshness. In the future, it may be useful to investigate whether the fluorescence quenching and the emission peak wavelength shifts occur in the case of labeling of cells or tissues, as for isolated mitochondria.

5. Conclusions

In this study, the respiratory activity and $\Delta\Psi_m$ of mitochondria isolated from European seabass fillet were assessed at different *post mortem* time points. Mitochondria were stained with the cationic probes Rh123 and TMRM, and fluorescence was recorded with an MVF. We found dysfunction of mitochondrial bioenergetics after 96 hours of storage at +4°C (D4). Moreover, the spectral properties of the probes, such as emission peak wavelength and fluorescence intensity, were significantly affected from D4. This study therefore confirmed

399 that evaluation of mitochondrial bioenergetics represents a reliable and early indicator of fish
400 freshness among the various existing indicators. It would be interesting in the future to
401 establish a "specific freshness profile" for different fish species, in particular for fish with
402 high commercial value such as Atlantic salmon (*Salmo salar*) or rainbow trout
403 (*Oncorhynchus mykiss*).

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Figures caption

Fig. 1: Changes in the respiratory control index (RCI) of mitochondria isolated from European seabass filet muscle cells at different *post mortem* storage times at +4°C

Letters denote values that are significantly different at different storage times. The *t*-test was performed using the SPSS Statistics 17 system. The different letters (a, b, c, d) above each bar represent significant differences; ($p < 0.05$; n=3).

Fig. 2: Changes in $\Delta\Psi_m$ of European seabass fillet muscle cell-isolated mitochondria assessed with the Rh123 (A) or TMRM (B) probes

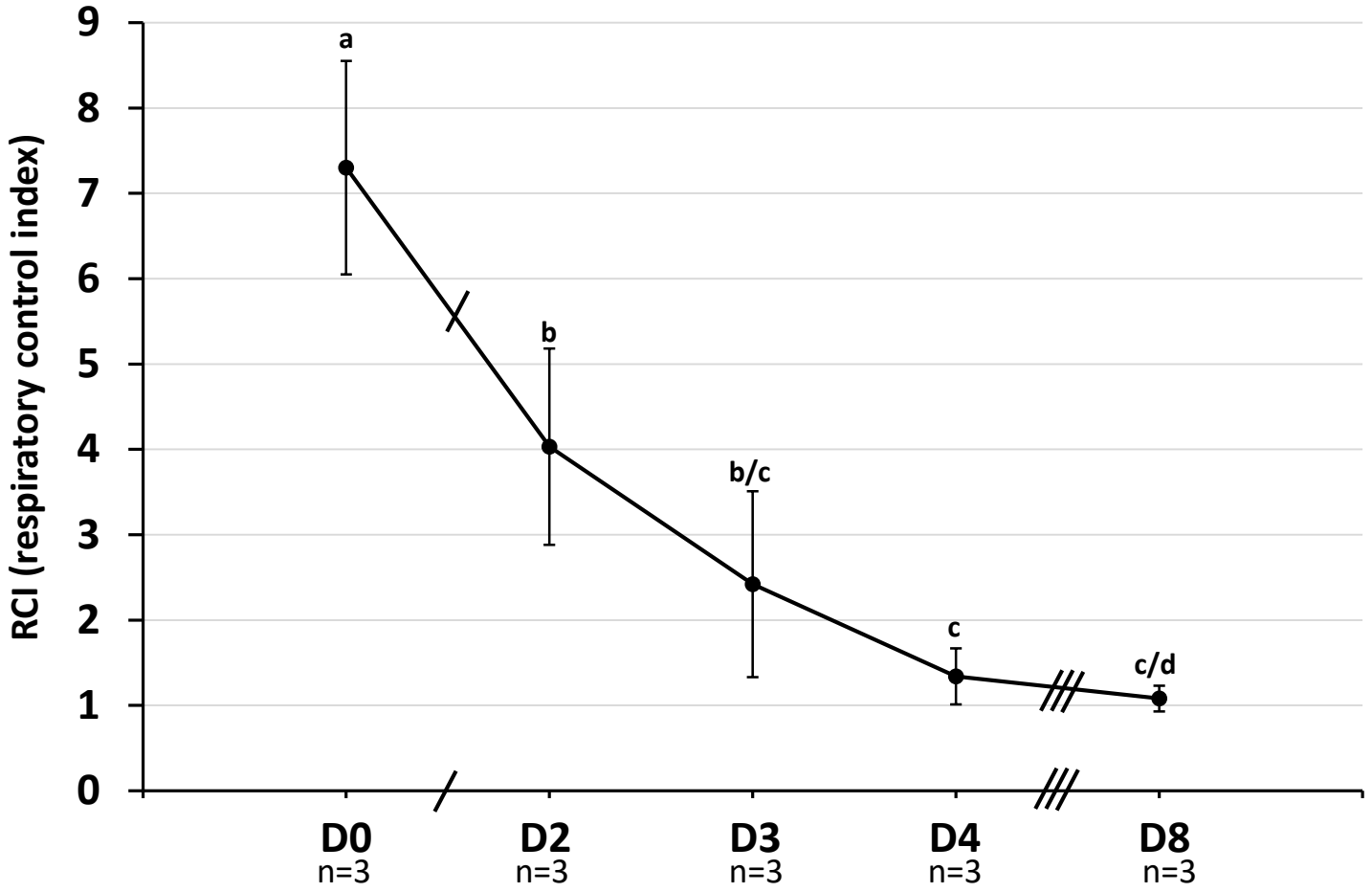
A: Rh123 probe: relative fluorescence units were measured at different time points of *post mortem* storage at +4°C (from Day 0 to Day 7). $\Delta\Psi_m$ was evaluated in the presence of substrates without (dark lines) or with (red lines) CCCP. The blue line highlights the significant change in membrane potential between the periods D0 to D3 and D4 to D7

B: TMRM probe: relative fluorescence units were measured at different time points of *post mortem* storage at +4°C (from Day 0 to Day 7). $\Delta\Psi_m$ was evaluated in the presence of substrates without (dark lines) or with (red lines) CCCP. The blue line highlights the significant change in membrane potential between the periods D0 to D3 and D4 to D7.

Fig. 3: Summary graphs of Rh123 (A) or TMRM (B) fluorescence levels normalized to CCCP at different storage times

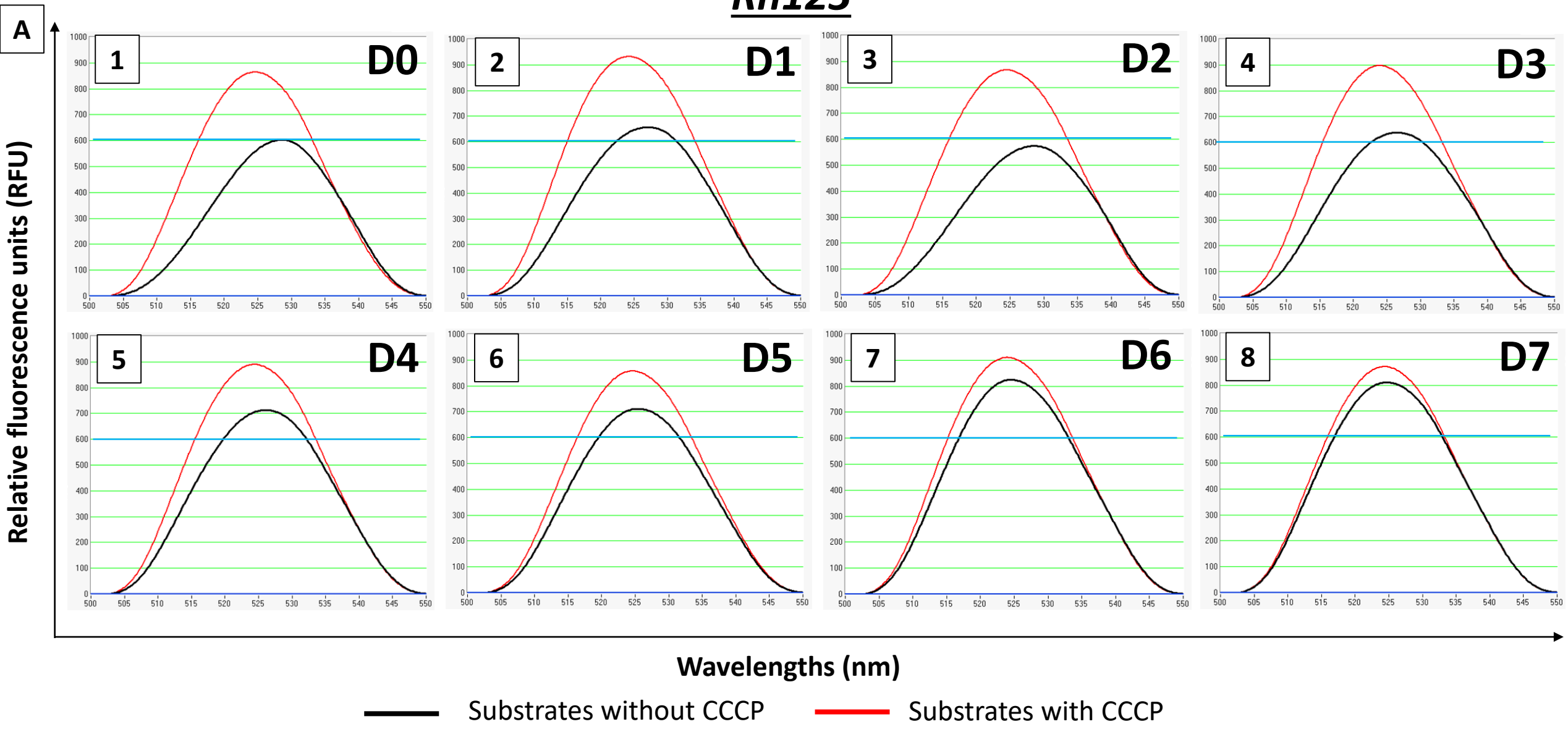
The different letters (a, b, c) represent significant differences; ($p < 0.05$; n=3–6). The dotted line highlights the significant change in membrane potential between the periods D0 to D3 and D4 to D7.

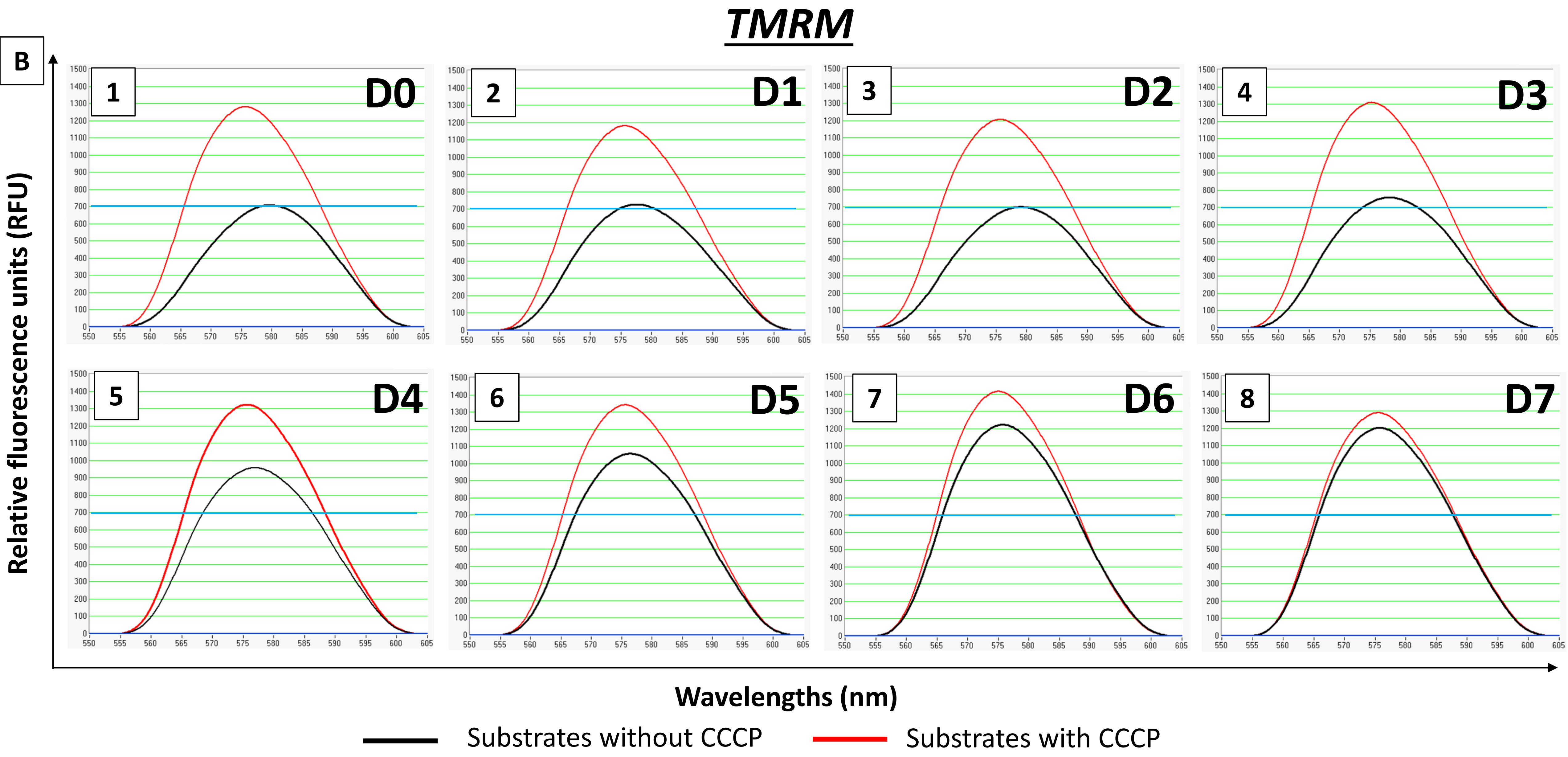
503 **Fig. 4**: Changes in the Rh123 (A) and TMRM (B) fluorescence emission peak wavelengths as
504 a function of different storage times of seabass fillets and their statistical representations (C
505 and D)
506 Figures A and B are representative of a typical experiment.
507 The different letters (a, b, c, d) above each bar (C and D) represent significant differences; (p
508 < 0.05; n=3).
509 The blue vertical lines indicate the wavelengths 528 nm (A) or 579 nm (B), corresponding to
510 the fluorescence peak after staining of mitochondria isolated from fish fillet muscle cells
511 between D0 and D3.
512 The grey vertical lines indicate the wavelengths 524 nm (A) or 576 nm (B), corresponding to
513 the fluorescence peak after staining of mitochondria isolated from fish fillet muscle cells
514 between D4 and D7.
515 The red vertical dotted lines (A and B) highlight the limit between two mitochondrial
516 functional states.
517 The blue curves (C and D) illustrate changes in the fluorescence emission peak wavelengths
518 in the presence of stained mitochondria from D0 to D7.
519 The grey curves (C and D) illustrate changes in the fluorescence emission peak wavelengths
520 in the presence of stained mitochondria and CCCP from D0 to D7.
521 The red horizontal dotted lines (C and D) highlight the limit between two mitochondrial
522 functional states.



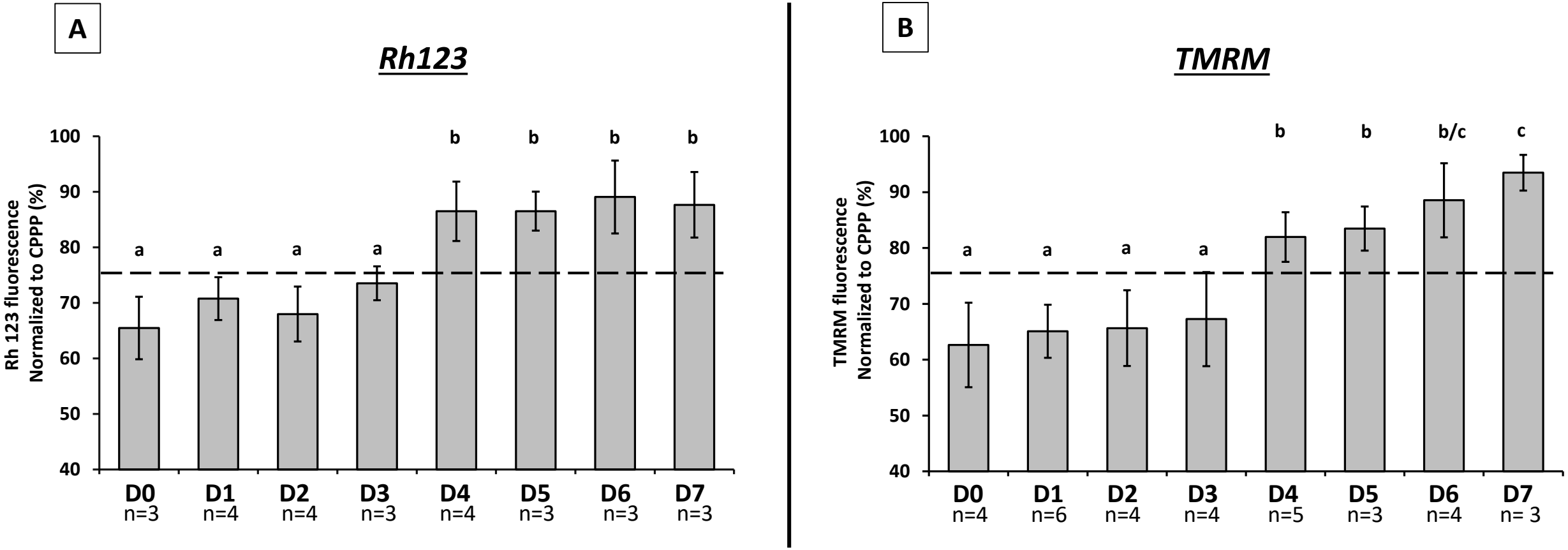
	Day of storage				
	D0	**D2**	**D3**	**D4**	**D8**
RCI value	7.3 ± 1.25	4.03 ± 1.15	2.42 ± 1.09	1.34 ± 0.33	1.08 ± 0.15

Rh123


A
1
D0
2
D1
3
D2
4
D3
5
D4
6
D5
7
D6
8
D7
Relative fluorescence units (RFU)
Wavelengths (nm)
Substrates without CCCP
Substrates with CCCP

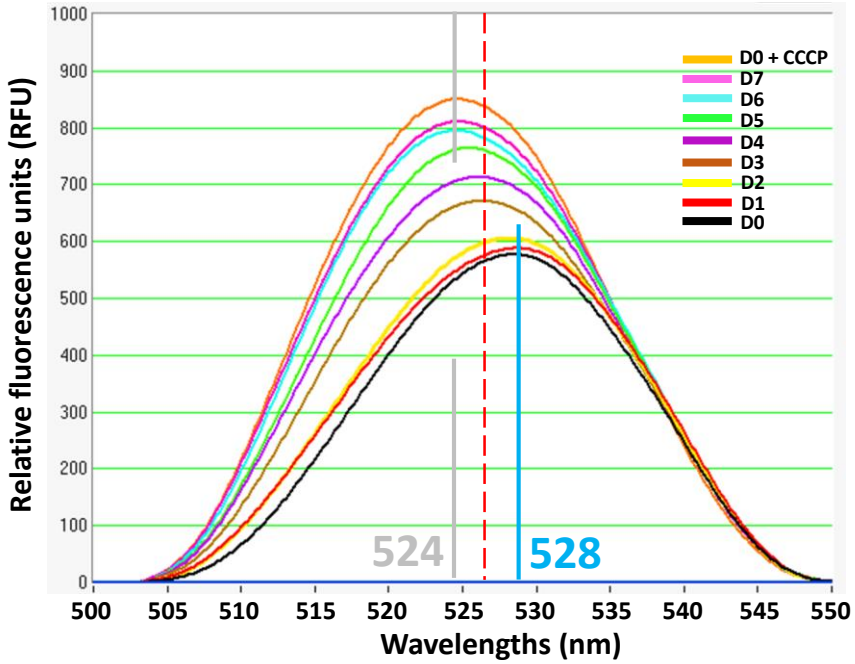


TMRM
B
1
D0
2
D1
3
D2
4
D3
5
D4
6
D5
7
D6
8
D7
Relative fluorescence units (RFU)
Wavelengths (nm)
Substrates without CCCP
Substrates with CCCP

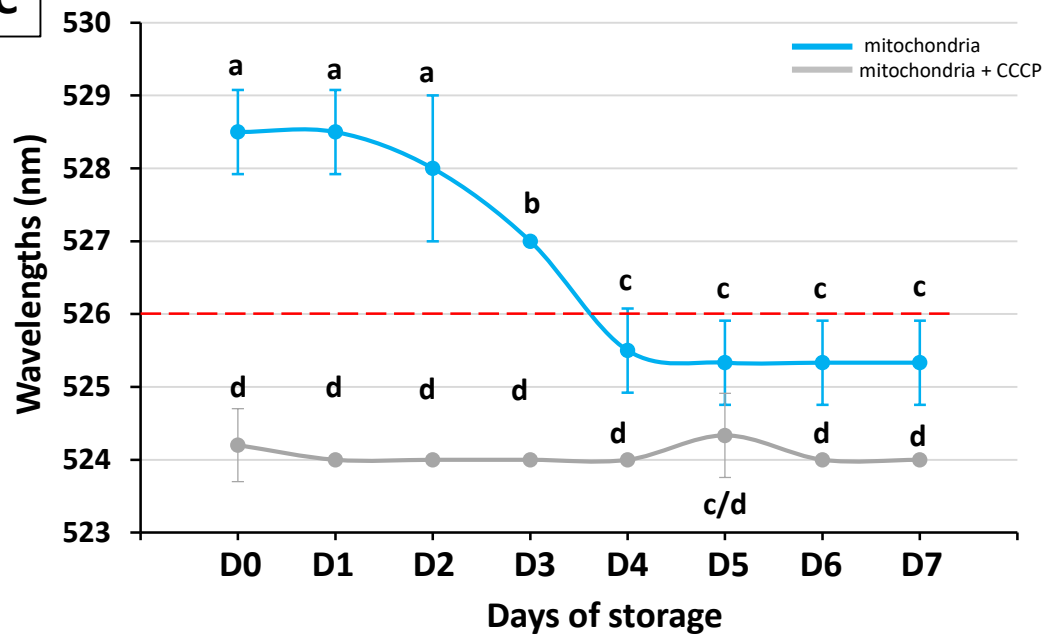

A
Rh123
Rh 123 fluorescence
Normalized to CPPP (%)
100
90
80
70
60
50
40
a
a
a
a
b
b
b
b
D0 n=3
D1 n=4
D2 n=4
D3 n=3
D4 n=4
D5 n=3
D6 n=3
D7 n=3
B
TMRM
TMRM fluorescence
Normalized to CPPP (%)
100
90
80
70
60
50
40
a
a
a
a
b
b
b/c
c
D0 n=4
D1 n=6
D2 n=4
D3 n=4
D4 n=5
D5 n=3
D6 n=4
D7 n= 3

A

Rh123

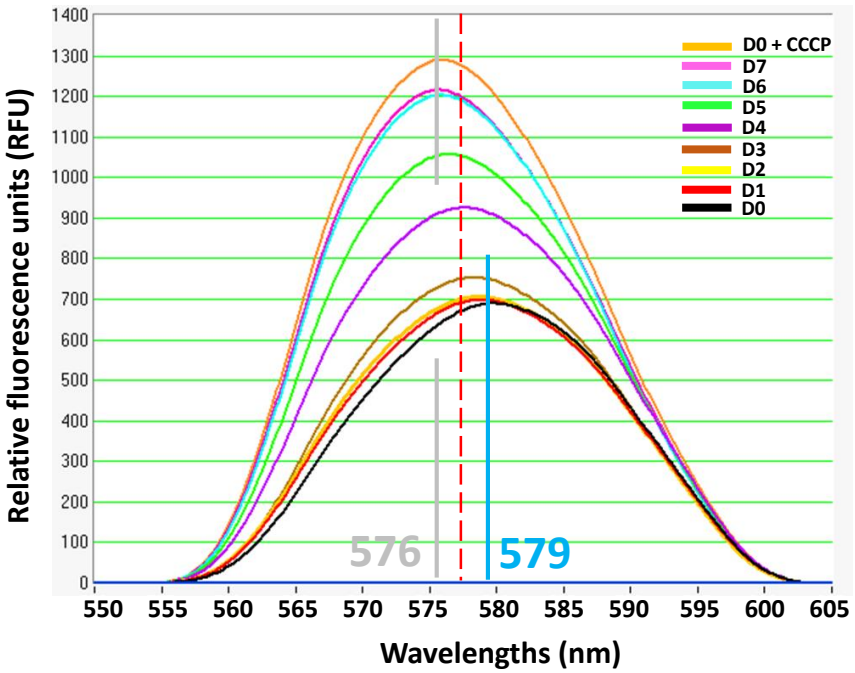


C



B

TMRM



D