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1 ***Mitochondrial activity as an indicator of fish freshness***

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23 Declarations of interest: none

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## 25 **Abstract**

26 The current methods used to routinely assess freshness in the fishing industry reflect more a  
27 state of spoilage than a state of freshness. Mitochondria, **the seat of cellular respiration,**  
28 undergo profound changes in *post mortem* tissues. **The objective of this study was to**  
29 **demonstrate that mitochondrial activity constitutes a putative early fish freshness marker.** The  
30 structure of gilthead sea bream (*Sparus aurata*) muscle tissue was evaluated over time by  
31 transmission electron microscopy. Respiration was assessed in mitochondria isolated from sea  
32 bream fillets using oxygraphy. Membrane potential ( $\Delta\Psi_m$ ) was determined by fluorescence  
33 (Rhodamine 123). Mitochondrial activity of fillets stored at +4°C was studied for 6 days.  
34 Changes in mitochondrial cristae structure appeared from Day 3 **highlighting the presence of**  
35 **dense granules.**  $\Delta\Psi_m$  and mitochondrial activity were **significantly** disrupted in sea bream  
36 fillets after 96 hours of storage at +4°C. Mitochondrial activity constituted a reliable and early  
37 indicator of fish freshness.

### 38 Chemical compounds used in this study:

- 39 - Rhodamine 123 (PubChem CID: 9929799)
- 40 - Carbonyl cyanide 3-chlorophenylhydrazone (PubChem CID: 2603)
- 41 - Tris (hydroxymethyl)aminomethane (PubChem CID: 6503)
- 42 - Ethylene-bis(oxyethylenitrilo)tetraacetic acid (PubChem CID: 6207)
- 43 - 4-Morpholinepropanesulfonic acid (PubChem CID: 70807)
- 44 - Malate (PubChem CID: 525)
- 45 - Succinate (PubChem CID 160419)
- 46 - Glutamate (PubChem CID: 33032)
- 47 - Adenosine 5'-diphosphate (PubChem CID: 128882)
- 48 - Carboxyatractyloside (PubChem CID: 20055804)

- 49 - Cytochrome c (PubChem CID 16057918)
- 50 - Potassium chloride (PubChem CID: 4873)
- 51 - Sucrose (PubChem CID: 5988)
- 52 - Magnesium chloride (PubChem CID: 5360315)
- 53 - Potassium dihydrogen phosphate (PubChem CID: 516951)
- 54 - Dimethyl sulfoxide (PubChem CID: 679)
- 55 - Glutaraldehyde (PubChem CID: 3485)
- 56 - Sodium cacodylate (PubChem CID: 2724247)
- 57 - Osmium tetroxide (PubChem CID: 30318)
- 58 - Uranyl acetate (PubChem CID: 10915)
- 59 - Acetonitrile (PubChem CID: 6342)

## 60 **Keywords**

61 Mitochondrial membrane potential ( $\Delta\Psi_m$ ); mitochondrial respiration; fish freshness; gilthead  
62 sea bream (*Sparus aurata*)

## 63 **1. Introduction**

64 The quality of aquatic products is defined by objective criteria such as food safety,  
65 nutritional quality, origin, and traceability of the products (G Olafsdottir, Martinsdóttir,  
66 Oehlenschläger, Dalgaard, Jensen, Undeland, et al., 1997; G. Olafsdottir, Nesvadba, Di  
67 Natale, Careche, Oehlenschläger, Tryggvadóttir, et al., 2004). It also depends on less  
68 objective criteria such as the organoleptic properties and the freshness of the products.  
69 Fish freshness is “dependent on different biological and processing factors” (Gudrun  
70 Olafsdottir, Nesvadba, Di Natale, Careche, Oehlenschläger, Tryggvadottir, et al., 2004).  
71 To meet expectations in terms of freshness, the fishing industry is trying to improve  
72 information on the date of capture of the product. However, treatments and storage  
73 conditions can vary greatly, strongly affecting quality and fish freshness. Seafood

74 products are highly perishable and processing factors determine the quality of the product  
75 (Cheng, Sun, Han, & Zeng, 2014; Mendes, 2018).

76 Consumers are becoming more attentive and demanding concerning the quality of food,  
77 including seafood. By definition, a fish can be considered fresh when its organoleptic,  
78 physical and chemical characteristics are very close to those of a living fish  
79 (Oehlenschläger & Sörensen, 1997). Savvy consumers or experts are able to discriminate  
80 fresh products by a sensory approach. Sensory evaluation grids have been developed to  
81 train a less experienced audience. However, these approaches remain highly dependent on  
82 the consumer's level of perception and knowledge of the product.

83 Further research is required to develop methods to evaluate fish freshness, and there are  
84 still many challenges. Microbiological, organoleptic and chemical methods are used  
85 routinely in the fishing industry to evaluate fish freshness. As an example, the total  
86 volatile base nitrogen (TVB-N) level increases significantly during the late stages of  
87 storage (10-20 days of storage), and is therefore a limited freshness indicator (Castro,  
88 Padrón, Cansino, Velázquez, & Larriva, 2006; Parlapani, Mallouchos, Haroutounian, &  
89 Boziaris, 2014), but should rather be seen as an advanced spoilage indicator. Moreover,  
90 TVB-N/total biogenic amines (TBA) analyses are not applicable to all fish species.  
91 Therefore, research in this area and the development of new fish freshness indicators are  
92 still a challenge for scientists and industry.

93 Many studies have shown *post mortem* structural changes in myofibrillar proteins  
94 (Delbarre-Ladrat, Chéret, Taylor, & Verrez-Bagnis, 2006). These changes in muscle tissue  
95 have often been correlated with the mechanical properties of the tissues (texture,  
96 elasticity, suppleness, hardness, cohesion, etc.) (Taylor, Fjaera, & Skjervold, 2002). The  
97 level of myofibrillar protein degradation can be correlated with fish alteration. In some of  
98 these studies, the structural changes in myofibrillar proteins have been associated with

99 structural changes in *post mortem* mitochondria (María Dolores Ayala, Abdel, Santaella,  
100 Martínez, Periago, Gil, et al., 2010; Parsons & Green, 2010). Studies using electron  
101 microscopy have reported *post mortem* morphological mitochondrial changes such as  
102 swelling and disrupted cristae in muscle in cold storage from the gilthead sea bream  
103 (*Sparus aurata*) (María Dolores Ayala, et al., 2010) and the Pacific bluefin tuna (*Thunnus*  
104 *orientalis*) (Roy, Ando, Itoh, & Tsukamasa, 2012). We focused on mitochondria to study  
105 their structural and functional changes in sea bream fillets stored at +4°C.

106 Mitochondria play a central role in cell death mechanisms such as apoptosis and necrosis  
107 (Parsons & Green, 2010). Several studies have shown that mitochondrial activity was still  
108 present *post mortem* in storage conditions of +4°C in bovine, murine and human models  
109 (Barksdale, Perez-Costas, Gandy, Melendez-Ferro, Roberts, & Bijur, 2010; Cheah &  
110 Cheah, 1971; Tang, Faustman, Hoagland, Mancini, Seyfert, & Hunt, 2005). Mitochondrial  
111 activity in fish has been studied in the areas of eco-physiology (Blier & Guderley, 1993;  
112 Hilton, Clements, & Hickey, 2010; Lionetti, Mollica, Donizzetti, Gifuni, Sica, Pignalosa,  
113 et al., 2014) and eco-toxicology (Cambier, Benard, Mesmer-Dudons, Gonzalez,  
114 Rossignol, Brethes, et al., 2009; Soares, Gutierrez-Merino, & Aureliano, 2007; Van den  
115 Thillart & Modderkolk, 1978). However, to our knowledge, there are currently no studies  
116 on the impact of storage at +4°C on *post mortem* changes in fish mitochondrial structure  
117 and function.

118 **The purpose of this study was therefore to evaluate whether mitochondrial structure and**  
119 **function could be considered reliable and early markers of fish freshness.**

120 To determine *post mortem* changes in mitochondrial structure and function, two  
121 approaches were used: firstly, morphological changes were analysed by transmission  
122 electron microscopy; and secondly, a functional analysis was performed to assess the

123 oxygen consumption and membrane potential ( $\Delta\Psi_m$ ) of mitochondria isolated from  
124 gilthead sea bream fillets stored at +4°C.

## 125 **2. Materials and methods**

### 126 *2.1 Reagents and materials*

127 Rhodamine 123 (Rh123), carbonyl cyanide 3-chlorophenylhydrazone (CCCP), Tris  
128 (hydroxymethyl)aminomethane (Trizma<sup>®</sup> base), ethylene-bis(oxyethylenitrilo)tetraacetic  
129 acid (EGTA), proteinase type XXIV, bovine serum albumin (BSA), 4-  
130 morpholinepropanesulfonic acid (MOPS), malate, succinate, adenosine 5'-diphosphate  
131 (ADP), carboxyatractyloside (CAT) and cytochrome c from equine heart were purchased  
132 from Sigma-Aldrich (St. Louis, MO, USA). Potassium chloride (KCl) and glutamate were  
133 acquired from Fisher Labosi (Paris, France). Sucrose, magnesium chloride (MgCl<sub>2</sub>), and  
134 potassium dihydrogen phosphate (KH<sub>2</sub>PO<sub>4</sub>) were purchased from Acros Organics (Morris,  
135 NJ, USA). Rh123 and CCCP were prepared in dimethyl sulfoxide (DMSO) purchased from  
136 Thermo Scientific (San Diego, CA, USA).

### 137 *2.2 Fish muscle origin and storage*

138 The gilthead sea bream (300-400 g) were sourced from Aquanord sea farm (Gravelines,  
139 France). This farmed fish model was chosen in order to obtain accurate data on living  
140 conditions, slaughter, and storage, which influence the study of freshness. Breeding  
141 conditions were: temperature  $18 \pm 6^\circ\text{C}$ , pH 8.2, total ammonia < 30 pmol/L, and dissolved  
142 oxygen 99 % (v/v) to saturation (7 ppm). Within the Aquanord sea farm, the fish were killed  
143 by asphyxiation/hypothermia and kept on ice (0 to 2°C) in expanded polystyrene boxes for 4  
144 hours of transport. We bought the fish from the company after their death. Upon arrival at the  
145 laboratory, the fish were immediately skinned and filleted. The fillets were stored on ice in a  
146 cold room (+4°C) for 5 days and used for experiments every 24 hours over 6 days: Day 0,

147 Day 1, Day 2, Day 3, Day 4 and Day 6. The ice was renewed every day. Plastic wrapping was  
148 used to avoid contact between the fillets and the ice or the accumulated water.

### 149 2.3. *Sample preparation and transmission electron microscopy*

150 To prepare samples, **as previously described** (Michalec, Holzner, Barras, Lacoste, Brunet,  
151 Lee, et al., 2017), 3 mm<sup>3</sup> pieces of muscle were cut from fillets and fixed in 2.5%  
152 glutaraldehyde (**Merck KGaA, Darmstadt, Germany**) buffered with 0.1M sodium cacodylate  
153 (**Sigma-Aldrich**), postfixed in 1% osmium tetroxide (**Sigma-Aldrich**) in the same buffer and  
154 “en bloc” stained with 2% uranyl acetate (**Agar Scientific, Stansted, Essex, UK**). After  
155 acetonitrile dehydration (**Sigma-Aldrich**), samples were embedded in epon-like resin (Embed-  
156 812). Ultrathin sections (90 nm) were cut using a Leica UC7 ultra-microtome and collected  
157 on 150 mesh hexagonal barred copper grids. After staining with 2% uranyl acetate prepared in  
158 50% ethanol (**Fisher, Loughborough, Leicester, UK**) and incubation with a lead citrate  
159 solution, sections were observed on a Hitachi H-600 transmission electron microscope  
160 equipped with a W electron source (operated at 75kV) and a side mounted Hamamatsu  
161 C4742-95 digital camera.

### 162 2.4. *Mitochondrial isolation from fish fillets*

163 The method for mitochondrial isolation was adapted from Pasdois, Parker, Griffiths, and  
164 Halestrap (2011). All the steps in mitochondrial isolation were performed in a cold room at  
165 +4°C. Red muscle was dissected from the fillet (10 g) and finely diced with scissors. The fine  
166 pieces obtained (2-3 mm<sup>3</sup>) were incubated at +4°C for 7 minutes under stirring in 20 mL of  
167 isolation buffer (180 mM KCl, 80 mM sucrose, 5 mM MgCl<sub>2</sub>, 10 mM Tris, 2 mM EGTA, pH  
168 7.2 at +4°C) supplemented with 0.1 mg/mL of bacterial proteinase type XXIV. The resulting  
169 tissue suspension was poured into a 30 mL glass Potter homogeniser and homogenised for 3  
170 min using a motorised Teflon pestle at 300 rpm. The homogenate was centrifuged at 7 500 g



171 for 10 minutes. The resulting pellet was first washed and then resuspended in 20 mL isolation  
172 buffer containing 2 mg/mL of fatty acid free BSA and homogenised for 3 minutes at 150 rpm.  
173 The homogenate was then centrifuged at 700 g for 10 min. The supernatant was centrifuged at  
174 1500 g for 10 min. The resulting supernatant was centrifuged again at 7 000 g for 10 min. The  
175 mitochondrial pellet obtained was resuspended with a low volume (50  $\mu$ L) of isolation buffer  
176 in order to obtain a concentrated mitochondrial suspension. The protein concentration was  
177 determined using a Bio-Rad protein assay kit, derived from the Bradford method (1976),  
178 using BSA as a standard. Mitochondria were kept on ice at a final concentration of 60-100  
179 mg/mL for not more than 4 h.

#### 180 2.5. *Measurement of mitochondrial $\Delta\Psi_m$*

181 A Xenius XC spectrofluorometer (SAFAS, Monaco) was used to monitor the fluorescence of  
182 Rh123 (Emaus, Grunwald, & Lemasters, 1986) in order to evaluate changes in mitochondrial  
183  $\Delta\Psi_m$  of isolated mitochondria extracted at different storage times (Day 0, Day 1, Day 2, Day  
184 3 and Day 4). Importantly, this experimental approach does not enable the user to measure  
185  $\Delta\Psi_m$  but gives a qualitative index of mitochondrial polarisation. 1 mL of respiration buffer  
186 (KCl 125 mM, MOPS 20 mM, Tris 10 mM, EGTA 10  $\mu$ M,  $\text{KH}_2\text{PO}_4$  2.5 mM, fatty acid free  
187 BSA 2 mg/mL, pH 7.2) at 25°C was added to a 3 mL plastic cuvette. Rh123 (50 nM final) and  
188 respiratory substrates – glutamate (5 mM), malate (2 mM) and succinate (5 mM) – were  
189 added sequentially. The baseline fluorescence of free Rh123 was recorded during 2 minutes  
190 and mitochondria (0.2 mg/mL) were then added. The generation of  $\Delta\Psi_m$  leads to  
191 accumulation of Rh123 in the mitochondrial matrix and consequent fluorescence quenching  
192 (dye stacking). To evaluate mitochondrial function, the following additions were performed  
193 sequentially. ADP (1 mM), CAT (5  $\mu$ M) and CCCP (2  $\mu$ M). The sampling rate was 1 Hz,  
194 bandwidth 15 nm at excitation and emission, and photomultiplier tube voltage was 700 V.  
195 Samples were excited at 500 nm and fluorescence was collected at 535 nm.

196 The Rh123 fluorescence intensities recorded at different states of respiration (basal (substrates  
197 only), state 3 (ADP addition), and CAT) were normalised to the fluorescence recorded after  
198 CCCP addition, according to the following formula:

$$199 \quad 100 - \left( \left( \frac{X - Y}{X} \right) \times 100 \right)$$

200 Where: X = Rh123 fluorescence intensity after CCCP addition

201 Y = Rh123 fluorescence intensity after mitochondria or ADP or CAT addition

202 CCCP at 2  $\mu$ M completely depolarised mitochondria by consuming all the proton gradient  
203 established by the respiratory chain. Thus, the fluorescence intensity obtained after its  
204 addition corresponded only to the dissipation of  $\Delta\Psi_m$  and enabled us to take into account the  
205 non-specific binding of the dye.

## 206 2.6. *Respiration assay*

207 Oxygraphy (Rank Brothers digital model 10, Cambridge, United Kingdom) was used to  
208 monitor the oxygen consumption of isolated mitochondria at 25°C (Frezza, Cipolat, &  
209 Scorrano, 2007). Firstly, 2.1 mL of respiration buffer (composition previously described)  
210 were added to the oxygraphic chamber supplemented with a mixture of glutamate (5 mM),  
211 malate (2 mM) and succinate (5 mM). Then, mitochondria were added at a final concentration  
212 of 0.2 mg/mL. Oxygen consumption rates were assessed without and with ADP (1 mM)  
213 (basal and state 3, respectively). Then, CAT (5  $\mu$ M) was added to block the oxygen  
214 consumption linked to ATP synthesis. In order to evaluate the permeability of the  
215 mitochondrial outer membrane, 10  $\mu$ M of exogenous cytochrome c from equine heart were  
216 added. At the end of the acquisition, CCCP was added to disrupt mitochondrial  $\Delta\Psi_m$  and to  
217 uncouple the respiratory chain, leading to an increase in oxygen consumption. The medium  
218 was stirred continuously during measurement. Calibration using sodium dithionite was  
219 performed to reach zero oxygen in the oxygraphic chamber.

220 Oxygen consumption rates were determined at each day of storage in the different  
221 experimental conditions (basal, state 3, and CAT). As an index of mitochondrial coupling, the  
222 respiratory control index (RCI) was calculated according to the following formula:

$$223 \text{ RCI} = \frac{\text{state 3}}{\text{CAT}}$$

224 Where state 3 is the respiration rate during maximum ATP synthesis and CAT is the  
225 respiration rate not linked to ATP synthesis. The integrity of the outer membrane was  
226 evaluated by calculating the percentage of oxygen consumption not linked to ATP synthesis  
227 stimulated following the addition of exogenous cytochrome c.

## 228 2.7. Statistical analysis

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

## 233 3. Results

### 234 3.1 Ultrastructural analysis of post mortem fish muscle *at different times of storage*

235 In order to study *post mortem* (PM) cell structural changes, the ultrastructure of gilthead sea  
236 bream muscle tissue was observed by transmission electron microscopy (TEM) from Day 0 to  
237 Day 6: Day 0 (6 h PM: Fig. 1a), Day 3 (72 h PM: Fig. 1b), Day 4 (96 h PM: Fig. 1c), Day 6  
238 (144 h PM: Fig. 1d).

239 At Day 0, myofibrils were characterised by intact and well organised bands (I-bands and A-  
240 bands) and lines (Z-lines and M-lines). The myofilaments of actin and myosin, which  
241 constitute the sarcomere, were well defined and their alignment was parallel.

242 The disorganisation of the I-bands was observed from Day 3 of storage, and it was amplified  
243 at Day 4 (Fig. 1.c). At Day 6, I-bands were barely identifiable (Fig. 1.d). The loss of density

244 of the Z-line was mainly observed at Day 6. The disruption of the parallel alignment of Z-  
245 lines, M-lines and I-bands was observed from Day 3 (Fig. 1.b) and amplified at Day 6 (Fig.  
246 1.d). From Day 3, the myofilaments of actin and myosin were no longer aligned and became  
247 tight, with some gaps detectable.

248 Figures 1a to 1d also illustrate the effects of *post mortem* storage on mitochondrial structures.  
249 The micrograph of gilthead sea bream muscle at Day 0 showed intact mitochondria with a  
250 dense matrix. The cristae compartments were compact and well organised. For the majority of  
251 the mitochondria, the double layer membranes were visible, with an intact and regular shape.  
252 From Day 3, mitochondrial morphology began to change: mitochondria appeared swollen.  
253 The shape of the membranes was discontinuous and in some places damaged. The cristae  
254 were elongated, tubular, disorganised and had almost disappeared in some mitochondria. The  
255 matrix had a more electron lucent appearance with the presence of a few dense granules (Fig.  
256 1.b). The number of granules increased over time (few granules on Day 3, many granules on  
257 Day 6 (Fig 1.d)). At Day 6, dense granules were located in nearly all mitochondria.

258 From a general point of view, gilthead sea bream fillet muscle cells underwent several major  
259 structural changes from 3 days *post mortem* at +4°C. The mitochondria seemed to show  
260 profound structural damage from the third day. The correlation between structural damage  
261 and mitochondrial activity *post mortem* in fish fillet muscle was studied in the subsequent  
262 experiments.

### 263 3.2 Post mortem *assessment of mitochondrial function*

264 Here, we studied mitochondrial oxygen consumption in gilthead sea bream fillet muscle by  
265 oxygraphy at different times of storage at +4°C (Fig. 2.a-e).

266 Figure 2 shows typical oxygraphic recordings of the changes in mitochondrial function from  
267 Day 0 to Day 4 (a: Day 0; b: Day 1; c: Day 2; d: Day 3; e: Day 4). At Day 0 (Fig. 2a), basal  
268 respiration (substrates only) reached 13.9 nmol O<sub>2</sub>/min/mg proteins. Following ADP addition,  
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269 mitochondrial respiration was stimulated (39.95 nmol O<sub>2</sub>/min/mg proteins), due to the  
270 consumption of the proton gradient by the ATP synthase. Addition of CAT inhibited the  
271 adenine nucleotides translocator and consequently ATP synthesis. As a consequence, oxygen  
272 consumption decreased to reach a new steady state of respiration not coupled to ATP  
273 synthesis (7 nmol O<sub>2</sub>/min/mg proteins). The addition of cytochrome c, showing the integrity  
274 of the outer membrane of mitochondria by measuring the effect on respiration, demonstrated  
275 that the endogenous cytochrome c had no effect on respiration. The mitochondrial outer  
276 membrane was intact. Finally, the decoupling agent CCCP was added. The respiration rate  
277 strongly increased during a very short time, illustrating an increase of respiratory activity to  
278 maintain the disruption of  $\Delta\Psi_m$  (Fig. 2a.b.c.d.). That was not the case for Day 4 (Fig. 2e).  
279 The results for mitochondrial oxygen consumption at Day 0 were compared with  
280 mitochondrial oxygen consumption at Day 1 (Fig. 2.b), Day 2 (Fig. 2.c), Day 3 (Fig. 2.d), and  
281 Day 4 (Fig. 2.e).  
282 From Day 0 (Fig. 2.a) to Day 3 (Fig. 2.d), the mean basal respiration rate was around 13.67  
283 nmol O<sub>2</sub>/min/mg proteins. From Day 4 (Fig. 2.e), the respiration rate increased to reach 17.75  
284 nmol O<sub>2</sub>/min/mg proteins. From Day 0 to Day 3 (Figs. 2.a-d), ADP, CAT and CCCP  
285 produced an effect on oxygen consumption. From Day 4 (Fig. 2.e), the effect of these  
286 compounds was very weak, which showed mitochondrial decoupling. From Day 0 (Fig. 2.a)  
287 to Day 4 (Fig. 2.e), the respiration rate at state 3 decreased gradually from 39.95 nmol  
288 O<sub>2</sub>/min/mg proteins to 24.225 nmol O<sub>2</sub>/min/mg proteins. From Day 0 (Fig. 2.a) to Day 3 (Fig.  
289 2.d), the difference between the basal respiration rate and state 3 respiration rate was  
290 important. At Day 4 (Fig. 2.e), differences between the basal respiration rate (17.54 nmol  
291 O<sub>2</sub>/min/mg proteins) and the state 3 respiration rate (24.22 nmol O<sub>2</sub>/min/mg proteins) were  
292 very weak, demonstrating that ADP addition had barely effect.

293 Figure 3.a illustrates the repeatability of results from Figure 2, according to 3-5 independent  
294 experiments. Changes in respiration (basal, state 3 and after CAT addition) are illustrated in  
295 Figure 3a. From Day 0 to Day 3, no difference in basal respiration and a slight decrease of the  
296 state 3 respiration rate were observed. By Day 4 (Fig. 3a), the basal respiratory rate had  
297 increased by 45% ( $10.48 \pm 3.10$  nmol O<sub>2</sub>/min/mg proteins to  $15.93 \pm 5.38$  nmol O<sub>2</sub>/min/mg  
298 proteins,  $p < 0.05$ ). At Day 4, an increase in the respiration rate after CAT addition was  
299 observed ( $8.88 \pm 2.36$  nmol O<sub>2</sub>/min/mg proteins to  $15.66 \pm 5.66$  nmol O<sub>2</sub>/min/mg proteins,  $p$   
300  $< 0.05$ ). At Day 4, mitochondria were not responding to ADP addition, indicating either  
301 dysfunction of the ATP synthasome or increased permeability of the inner membrane to  
302 protons.

303 The respiratory control index (RCI = state3/CAT) was calculated for each day (Fig. 3a). At  
304 Day 0, RCI was  $3.17 \pm 0.71$ . RCI decreased from Day 0 to Day 4 to reach  $1.30 \pm 0.15$ ,  
305 illustrating a significant difference between Day 0 and Day 4 ( $p < 0.05$ ).

306 External membrane integrity was measured by the addition of exogenous cytochrome c (Fig.  
307 3.b). From Day 0 to Day 2, an elevation of the respiration rate was observed between 0.70 and  
308 8%. An increase induced by cytochrome c was considered normal and acceptable when it is  
309 between 5 and 15% (Kuznetsov, Veksler, Gellerich, Saks, Margreiter, & Kunz, 2008). From  
310 Day 3, more than 30% stimulation of the respiratory rate was observed ( $38.5\% \pm 21.19$  at Day  
311 3 and  $35\% \pm 19.06$  at Day 4), illustrating that the outer membrane of mitochondria was  
312 damaged.

### 313 3.3 Post mortem assessment of mitochondrial $\Delta\Psi_m$

314 To further characterise changes in mitochondrial function *post mortem*, a qualitative  
315 assessment of mitochondrial  $\Delta\Psi_m$  was performed (Fig. 4).  $\Delta\Psi_m$ , being generated by the  
316 electron transport chain, is correlated to mitochondrial function and integrity (Zorova,  
317 Popkov, Plotnikov, Silachev, Pevzner, Jankauskas, et al., 2018). The potentiometric dye  
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318 Rh123 was used to qualitatively assess  $\Delta\Psi_m$ . Rh123 accumulates in the mitochondrial  
319 compartment as a function of  $\Delta\Psi_m$ . The higher the potential, the more dye will enter the  
320 matrix. Following accumulation and stacking of the dye within the matrix, its fluorescence is  
321 quenched. Modulation of the electron transport chain activity by ADP, CAT or CCCP leads to  
322 changes in  $\Delta\Psi_m$  and consequent movement of the dye across the mitochondrial inner  
323 membrane.

324 At Day 0 (Fig. 4.a), the addition of mitochondria to the buffer led to a strong fluorescence  
325 intensity decrease (quenching). Then, when ADP was added (state 3), the Rh123 fluorescence  
326 increased due to the release of the probe from the matrix in the surrounding buffer. ADP  
327 addition led to a decrease in  $\Delta\Psi_m$ . In contrast, addition of CAT restored  $\Delta\Psi_m$ , and  
328 consequently led to a decrease in fluorescence intensity. At the end of the experiment (Day 0;  
329 Fig. 4.a), addition of CCCP disrupted  $\Delta\Psi_m$  and led to a rapid increase in fluorescence. The  
330 fluorescent signal did not reach the baseline value, indicating that part of the decreased  
331 fluorescence following addition of mitochondria was not associated with the modulation of  
332  $\Delta\Psi_m$ .

333 From Day 0 to Day 4 (Fig. 4.a-e), the intensity of Rh123 fluorescence in the respiratory buffer  
334 was evaluated in a same way. From Day 0 to Day 3 (Fig. 4.a-d), the addition of mitochondria  
335 caused a fall in fluorescence of about 36% to 40%, which showed significant incorporation of  
336 Rh123 and therefore high  $\Delta\Psi_m$ . At Day 4 (Fig. 4.e), the addition of mitochondria led to a  
337 lower decrease in fluorescence (27%). From Day 0 to Day 3 (Fig. 4.a-d), addition of ADP, by  
338 its decoupling action, decreased  $\Delta\Psi_m$  and led to release of the probe (increase in  
339 fluorescence). This action was less marked on Day 4 (Fig. 4.e). Conversely, by the blocking  
340 action of the nucleotide transporters, CAT increased  $\Delta\Psi_m$  and therefore the incorporation of  
341 Rh123 into the mitochondria. This action was clearly identified from Day 0 to Day 3 (Fig.  
342 4.a-d) and less marked from Day 4 (Fig. 4.e).

343 The action of CCCP (increased fluorescence due to Rh123 release) was related to the level of  
344 probe incorporation, and was more visible at Days 0, 1, 2, and 3 (Fig. 4.a-d) than at Day 4  
345 (Fig. 4.e). The results obtained at Day 4 revealed that  $\Delta\Psi_m$  is disrupted.

346 In order to obtain an overall view of changes in  $\Delta\Psi_m$  at different storage times *post mortem*,  
347 the recorded fluorescence intensity was analysed at different states of respiration (Fig. 5). The  
348 fluorescence associated with different states of respiration (basal/ADP/CAT) was normalised  
349 by the fluorescence associated with CCCP treatment (as described in Materials and methods).

350 From Day 0 to Day 2, the effects of ADP and CAT on Rh123 fluorescence were significant.  
351 From Day 3 to Day 4, neither ADP nor CAT affected Rh123 fluorescence, demonstrating  
352 severe mitochondrial dysfunction.

353 From Day 3 to Day 4, the normalised Rh123 fluorescence was significantly higher for each  
354 state of respiration in comparison with Days 0, 1, and 2. This increase reflected a lower intake  
355 of Rh123 in the mitochondria, and consequently a fall in  $\Delta\Psi_m$ . On the basis of these results,  
356 we can conclude that after 72 h of storage (Day 3) at +4°C,  $\Delta\Psi_m$  started to decline and  
357 became significantly disrupted after 96 hours (Day 4).

#### 358 4. Discussion

359 **The research of early markers of freshness is still a current challenge.** This study focused, for  
360 the first time, on **mitochondria function as reliable indicator of fish freshness.** Mitochondrial  
361 activity **was studied** in fish skeletal muscle at different time points of *post mortem* storage at  
362 +4°C in order to show structural and functional changes in *post mortem* mitochondria. The  
363 structural changes were described based on electron microscopy images. A dual approach to  
364 mitochondrial functionality (oxymetric approach and mitochondrial potential approach  
365 ( $\Delta\Psi_m$ )) enabled us to better understand the alterations affecting the mitochondria in sea bream  
366 fillet muscle. This research showed that mitochondria undergo profound *post mortem*  
367 changes, which may be a relevant finding for assessment of food product freshness.



368 **Post mortem structural changes in fish skeletal muscle**

369 Electron microscopy images were used to study the structural changes in mitochondria in fish  
370 fillets stored *post mortem* at +4°C over 6 days. By choosing Days 3 and 4, we aimed to check  
371 whether the observed mitochondrial activity changes were associated with major structural  
372 changes. At Day 0 (6 hours *post mortem*), fish cell muscle retained its structural integrity. The  
373 myofibril arrangement was conserved and well organised. No gaps between sarcomeres were  
374 observable and collagen was still visible. These observations were consistent with those in  
375 other studies, which described good preservation of muscle myofilaments in gilthead sea  
376 bream fillet at Day 0 (María Dolores Ayala, et al., 2010; María Dolores Ayala, Santaella,  
377 Martínez, Periago, Blanco, Vázquez, et al., 2011; Caballero, Betancor, Escrig, Montero, De  
378 Los Monteros, Castro, et al., 2009). The majority of mitochondria were intact with non-  
379 altered membranes and a network of compact and well organised cristae. Some authors who  
380 have studied changes in myofilament structure in fish muscle have also been able to observe  
381 changes in mitochondria (María Dolores Ayala, et al., 2010). For example, in gilthead sea  
382 bream fillet (kept at +4°C), **these authors** observed swelling of some organelles, such as  
383 mitochondria and sarcoplasmic reticulum 3 hours *post mortem*. Another study on sea bass,  
384 carried out by the same authors, showed rapid changes in mitochondrial structure (with  
385 swelling) 3 hours *post mortem* (Ma D Ayala, Albors, Blanco, Alcázar, Abellán, Zarzosa, et  
386 al., 2005). Similarly, Roy, Ando, Itoh, and Tsukamasa (2012) described altered mitochondria  
387 with swollen cristae in Pacific bluefin tuna muscle cells at Day 0. Studies specifically focused  
388 on mammalian mitochondria showed good mitochondrial structure preservation several hours  
389 *post mortem*. From a structural point of view, it appears that gilthead sea bream mitochondria  
390 (like for mammalian mitochondria: (Barksdale, Perez-Costas, Gandy, Melendez-Ferro,  
391 Roberts, & Bijur, 2010)) undergo few changes within a few hours of the animal's death. From  
392 Day 3 to Day 6 (72 h – 144 h *post mortem*), several forms of structural damage in muscle

393 myofilaments were observed in gilthead sea bream fillet, with general disorganisation of  
394 myofibril alignment, showing several gaps. **The same observations were reported** from Day 5  
395 in gilthead sea bream muscle, such as alterations of sarcomeres at the I-band level, alteration  
396 of actin filaments, and disruption of Z-lines (María Dolores Ayala, et al., 2011).

397 Mitochondria were swollen with disrupted membranes and dense granules in the  
398 mitochondrial matrix. Dense granules were also observed (María Dolores Ayala, et al., 2010)  
399 on gilthead sea bream mitochondria from 5 days *post mortem*. These granules have also been  
400 observed *post mortem* in mammalian mitochondria (Kuypers & Roomans, 1980). Importantly,  
401 an explanation **was offered** for the appearance of dense granules in mitochondria (Wolf,  
402 Mutsafi, Dadosh, Ilani, Lansky, Horowitz, et al., 2017). These authors showed that the  
403 granules resulted from the accumulation and precipitation of calcium in the mitochondrial  
404 matrix. **During** cell death processes, calcium homeostasis is profoundly disturbed, leading to  
405 massive calcium entry into the mitochondria (Dong, Saikumar, Weinberg, & Venkatachalam,  
406 2006). This flux initially caused swelling of the organelles and then precipitation of calcium  
407 as insoluble phosphate and hydroxyapatite, which participated in mitochondrial damage and  
408 cell death (Dong, Saikumar, Weinberg, & Venkatachalam, 2006). The calcium aggregation in  
409 ischaemic conditions described by these authors could be similar to that found *post mortem* in  
410 muscle cells, which were also deprived of oxygen.

#### 411 *Mitochondrial activity: an early fish freshness indicator*

412 In the second part of our study, we focused on mitochondrial activity *post mortem* in fish  
413 muscle cells at different storage time points at +4°C: Day 0 (6 h), Day 1 (24 h), Day 2 (48 h),  
414 Day 3 (72 h), and Day 4 (96 h). Two approaches based on oxygraphy and fluorescence  
415 enabled us to investigate mitochondrial activity.

416 Mitochondria maintained significant respiratory activity for the first 3 days of storage (Day 0  
417 to Day 3). From Day 4, respiratory activity declined significantly (96 hours *post mortem*):  
17/25

418 RCI ( $1.30 \pm 0.15$ ) was significantly lower than at Day 0 ( $3.17 \pm 0.71$ ). RCI is a useful  
419 measure to assess mitochondrial function in isolated mitochondria, and its decrease is  
420 associated with mitochondrial dysfunction (Brand & Nicholls, 2011). This mitochondrial  
421 dysfunction resulted from electron transport chain alteration, and this activity can be explored  
422 with different substrates (ADP, CAT, cytochrome C, and CCCP). On the fourth day (Day 4),  
423 decoupling agents such as CCCP and ADP had no action on mitochondrial respiration (no  
424 increase in respiratory activity), showing the inability of respiratory chains to adapt to the loss  
425 of mitochondrial potential (via ADP or CCCP addition). Mitochondrial decoupling at Day 4  
426 could be due to mitochondrial membrane permeabilisation. Alteration of the mitochondrial  
427 outer membrane was confirmed by the activating effect of exogenous cytochrome c on  
428 respiration activity (30% at Day 4).

429 The fluorescent probe Rh123 was used to evaluate the  $\Delta\Psi_m$  of isolated mitochondria. By  
430 comparing findings with oxygraphic results, we can clearly observe the uncoupling action of  
431 ADP and CCCP on mitochondria isolated from sea bream fillets stored at +4°C (Day 0, Day  
432 1, and Day 2). ADP and CCCP led to dissipation of mitochondrial potential, which decreased  
433 the uptake of the probe and thus increased the overall fluorescence in the surrounding buffer.  
434 From Day 3, ADP and CCCP additions had no significant effects on Rh123 fluorescence  
435 intensity, indicating mitochondrial decoupling. In addition, the intensity of Rh123  
436 fluorescence increased from Day 3 for all states of respiration. This increase was associated  
437 with a decrease in Rh123 quenching. The high values for standard deviations obtained at Day  
438 3, representative of eight independent experiments, highlighted the marked heterogeneity of  
439 the results at this precise time compared to Day 4. Overall, the results obtained with the  
440 Rh123 fluorescent probe demonstrated that  $\Delta\Psi_m$  was strongly and significantly disrupted  
441 from Day 4. This disruption was correlated to the decline in mitochondrial activity, and  
442 consequently to cell health and mitochondrial membrane integrity (Zorova, et al., 2018).

443 The two approaches (oxygraphic and fluorescence) coincided well and showed that isolated  
444 mitochondria of sea bream fillets stored at +4°C retained activity 2 to 3 days *post mortem*.  
445 From Day 3/Day 4, mitochondrial respiratory activity and  $\Delta\Psi_m$  strongly decreased. On the  
446 basis of the concept developed by Cheah and Cheah (1971), the “critical storage time” was  
447 between 72 hours (Day 3) and 96 hours (Day 4) for the *Sparus aurata* model stored on ice at  
448 +4°C. Maintenance of mitochondrial activity has been demonstrated in other studies in ox  
449 neck muscle (Cheah & Cheah, 1971, 1974) and in mouse and human brain tissue (Barksdale,  
450 Perez-Costas, Gandy, Melendez-Ferro, Roberts, & Bijur, 2010).

451 In stress conditions of anoxia present after death, calcium homeostasis is disrupted and  
452 calcium levels increase in the sarcoplasm. Mitochondria have the ability to maintain their  
453 activity *post mortem*. This maintenance occurs because mitochondrial ATP synthase can run  
454 in reverse, hydrolysing ATP generated by glycolysis in order to maintain  $\Delta\Psi_m$  (St-Pierre,  
455 Brand, & Boutilier, 2000). Sarcoplasmic calcium enters mitochondria through the maintained  
456  $\Delta\Psi_m$ . From Day 3/Day 4, marked calcium accumulation in mitochondria was found and  
457 appeared in the form of dense granules by electron microscopy. Mitochondrial calcium  
458 overload developed, leading to  $\Delta\Psi_m$  disruption and mitochondrial membrane permeabilisation  
459 (Dong, Saikumar, Weinberg, & Venkatachalam, 2006). Membrane permeabilisation may be  
460 associated with the formation of mitochondrial permeability transition pores, leading to the  
461 release of cell death-inducing factors, cytochrome c, and huge amounts of calcium in the  
462 sarcoplasm. This release probably plays a role in integrating death signals and may participate  
463 in proteolytic enzyme activation (via the release of cytochrome c and mitochondrial calpain  
464 activation) (Boudida, Becila, Gagaoua, Boudjellal, Sentandreu, & Ouali, 2015; Smith &  
465 Schnellmann, 2012).

466 Day 4 is a crucial storage time point in gilthead sea bream muscle cells, and is the starting  
467 point of marked cell alteration. From Day 4, it has been reported that insoluble collagen

468 decreased strongly, leading to a loss of firmness and an increase in the water-holding capacity  
469 (Suárez, Abad, Ruiz-Cara, Estrada, & García-Gallego, 2005). At Day 4, the molecule  
470 dystrophin, which provides a link between cytoskeletal actin and the extracellular matrix,  
471 almost disappeared, while actin and desmin were detected in fish muscle at early stages of  
472 alteration *post mortem* (14 days). Loss of dystrophin is correlated to detachment myofibres  
473 and myocommata, and a reduction in flesh hardness (Caballero, et al., 2009). From Day 4,  
474 free LDH activity released from sea bream muscle strongly increased, demonstrating an  
475 increase of fish autolysis after 4 days of storage at +4°C (Diop, Watier, Masson, Diouf,  
476 Amara, Grard, et al., 2016).

477 In future studies, it would be interesting to assess whether mitochondrial activity is  
478 maintained for a longer period of time in other experimental conditions, such as a storage  
479 temperature of +0°C/+2°C, the use of natural additives, or modified atmosphere packaging.

## 480 **5. Conclusion**

481 The mitochondrial structural changes (swelling, membrane and cristae alteration, and  
482 accumulation of dense granules) were correlated with an increase in permeability (sensitivity  
483 to cytochrome c),  $\Delta\Psi_m$  disruption, and a decrease in respiratory activity. The mitochondria of  
484 sea bream fillets stored at +4°C maintained significant respiratory activity for the first three  
485 days of storage. Therefore, mitochondria could be useful targets for evaluating the freshness  
486 of seafood, and the starting point for the development of a fish freshness kit concerning the  
487 first stages of spoilage. It would be very interesting to determine whether these structural and  
488 physiological mitochondrial changes in a fresh seafood product (such as fish fillets) have an  
489 influence on perceptions and the health of consumers.

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620



621 **Figure captions**

622 **Figure 1:** Longitudinal sections of gilthead sea bream muscle at different *post mortem* times:

623 6 h *post mortem* (Day 0) (a), 72 h (Day 3) (b), 96 h (Day 4) (c), and 144 h (Day 6) (d). A: A  
624 band; col: collagen; dg: dense granules; H: H zone; I: I band; M: M line; mit: mitochondria;  
625 sar: sarcoplasm; sr: sarcoplasmic reticulum; Tr: triad; Z: Z line. Bars: a, b, c, d: 500 nm.

626 **Figure 2:** *Post mortem* assessment of respiratory activity of gilthead sea bream isolated  
627 mitochondria by oxygraphy

628 Oxygraph traces and their first derivate (dotted line) are represented at different storage times:  
629 Day 0 (a), Day 1 (b), Day 2 (c), Day 3 (d), and Day 4 (e). After the addition of mitochondria  
630 (mito.) (0.2 mg/mL), the molecules ADP (2 mM), CAT (5  $\mu$ M), cytochrome c (Cyt. C) (10  
631  $\mu$ M) and CCCP (2  $\mu$ M) were added to the incubation chamber. These graphs are  
632 representative of one experiment.

633 **Figure 3:** Evaluation of mitochondrial intactness parameters

634 RCI, respiration rates (a) and stimulation of respiration by cytochrome c (b) at different *post*  
635 *mortem* intervals. The mitochondria were isolated from gilthead sea bream muscle at different  
636 storage times: Day 0, Day 1, Day 2, Day 3 and Day 4. White circles denote values that are  
637 significantly different from values recorded at Day 0, Day 1, or Day 2. Daggers denote values  
638 that are significantly different. The t-test was performed using the SPSS Statistic 17  
639 programme; ( $p < 0.05$ ; N=3-5).

640 **Figure 4:** *Post mortem* assessment of the mitochondrial membrane potential of gilthead sea  
641 bream isolated mitochondria by fluorimetry (Safas).

642 Rhodamine 123 fluorescence traces of isolated mitochondria are represented at different  
643 storage times: Day 0 (a), Day 1 (b), Day 2 (c), Day 3 (d), and Day 4 (e). After the addition of

644 mitochondria (Mito.) (0.2 mg/mL), the molecules ADP (2 mM), CAT (5  $\mu$ M) and CCCP (2  
645  $\mu$ M) were added to a 3 mL cuvette. These graphs are representative of one experiment.

646 **Figure 5:** Summary graphs of Rh123 fluorescence levels at different states of respiration  
647 normalised to CCCP at different storage times. The different storage times were: Day 0, Day  
648 1, Day 2, Day 3, and Day 4. Asterisks denote values that are significantly different at different  
649 storage times. The t-test was performed using the SPSS Statistic 17 programme; ( $p < 0.05$ ;  
650  $N=4-8$ ).









