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Mitochondrial activity as an indicator of fish freshness

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Declarations of interest: none

Abstract

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

Chemical compounds used in this study:

- Rhodamine 123 (PubChem CID: 9929799)
- Carbonyl cyanide 3-chlorophenylhydrazone (PubChem CID: 2603)
- Tris (hydroxymethyl)aminomethane (PubChem CID: 6503)
- Ethylene-bis(oxyethylenenitrilo)tetraacetic acid (PubChem CID: 6207)
- 4-Morpholinepropanesulfonic acid (PubChem CID: 70807)
- Malate (PubChem CID: 525)
- Succinate (PubChem CID 160419)
- Glutamate (PubChem CID: 33032)
- Adenosine 5′-diphosphate (PubChem CID: 128882)
- Carboxyatractyloside (PubChem CID: 20055804)

- Cytochrome c (PubChem CID 16057918)
- Potassium chloride (PubChem CID: 4873)
- Sucrose (PubChem CID: 5988)
- Magnesium chloride (PubChem CID: 5360315)
- Potassium dihydrogen phosphate (PubChem CID: 516951)
- Dimethyl sulfoxide (PubChem CID: 679)
- Glutaraldehyde (PubChem CID: 3485)
- Sodium cacodylate (PubChem CID: 2724247)
- Osmium tetroxide (PubChem CID: 30318)
- Uranyl acetate (PubChem CID: 10915)
- Acetonitrile (PubChem CID: 6342)

Keywords

Mitochondrial membrane potential (ΔΨm); mitochondrial respiration; fish freshness; gilthead sea bream (*Sparus aurata*)

1. Introduction

The quality of aquatic products is defined by objective criteria such as food safety, nutritional quality, origin, and traceability of the products (G Olafsdottir, Martinsdóttir, Oehlenschläger, Dalgaard, Jensen, Undeland, et al., 1997; G. Olafsdottir, Nesvadba, Di Natale, Careche, Oehlenschläger, Tryggvadóttir, et al., 2004). It also depends on less objective criteria such as the organoleptic properties and the freshness of the products. Fish freshness is "dependent on different biological and processing factors" (Gudrun Olafsdottir, Nesvadba, Di Natale, Careche, Oehlenschläger, Tryggvadottir, et al., 2004). To meet expectations in terms of freshness, the fishing industry is trying to improve information on the date of capture of the product. However, treatments and storage conditions can vary greatly, strongly affecting quality and fish freshness. Seafood products are highly perishable and processing factors determine the quality of the product (Cheng, Sun, Han, & Zeng, 2014; Mendes, 2018).

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

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

Many studies have shown *post mortem* structural changes in myofibrillar proteins (Delbarre-Ladrat, Chéret, Taylor, & Verrez-Bagnis, 2006). These changes in muscle tissue have often been correlated with the mechanical properties of the tissues (texture, elasticity, suppleness, hardness, cohesion, etc.) (Taylor, Fjaera, & Skjervold, 2002). The level of myofibrillar protein degradation can be correlated with fish alteration. In some of these studies, the structural changes in myofibrillar proteins have been associated with structural changes in *post mortem* mitochondria (María Dolores Ayala, Abdel, Santaella, Martínez, Periago, Gil, et al., 2010; Parsons & Green, 2010). Studies using electron microscopy have reported *post mortem* morphological mitochondrial changes such as swelling and disrupted cristae in muscle in cold storage from the gilthead sea bream (*Sparus aurata*) (María Dolores Ayala, et al., 2010) and the Pacific bluefin tuna (*Thunnus orientalis*) (Roy, Ando, Itoh, & Tsukamasa, 2012). We focused on mitochondria to study their structural and functional changes in sea bream fillets stored at +4°C.

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

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

To determine *post mortem* changes in mitochondrial structure and function, two approaches were used: firstly, morphological changes were analysed by transmission electron microscopy; and secondly, a functional analysis was performed to assess the oxygen consumption and membrane potential ($\Delta\Psi_m$) of mitochondria isolated from gilthead sea bream fillets stored at +4°C.

2. Materials and methods

2.1 Reagents and materials

Rhodamine 123 (Rh123), carbonyl cyanide 3-chlorophenylhydrazone (CCCP), Tris (hydroxymethyl)aminomethane (Trizma® base), ethylene-bis(oxyethylenenitrilo)tetraacetic acid (EGTA), proteinase type XXIV, bovine serum albumin (BSA), 4-morpholinepropanesulfonic acid (MOPS), malate, succinate, adenosine 5′-diphosphate (ADP), carboxyatractyloside (CAT) and cytochrome c from equine heart were purchased from Sigma-Aldrich (St. Louis, MO, USA). Potassium chloride (KCl) and glutamate were acquired from Fisher Labosi (Paris, France). Sucrose, magnesium chloride ($MgCl_2$), and potassium dihydrogen phosphate (KH_2PO_4) were purchased from Acros Organics (Morris, NJ, USA). Rh123 and CCCP were prepared in dimethyl sulfoxide (DMSO) purchased from Thermo Scientific (San Diego, CA, USA).

2.2 Fish muscle origin and storage

The gilthead sea bream (300-400 g) were sourced from Aquanord sea farm (Gravelines, France). This farmed fish model was chosen in order to obtain accurate data on living conditions, slaughter, and storage, which influence the study of freshness. Breeding conditions were: temperature 18 ± 6°C, pH 8.2, total ammonia < 30 pmol/L, and dissolved oxygen 99 % (v/v) to saturation (7 ppm). Within the Aquanord sea farm, the fish were killed by asphyxiation/hypothermia and kept on ice (0 to 2°C) in expanded polystyrene boxes for 4 hours of transport. We bought the fish from the company after their death. Upon arrival at the laboratory, the fish were immediately skinned and filleted. The fillets were stored on ice in a cold room (+4°C) for 5 days and used for experiments every 24 hours over 6 days: Day 0, Day 1, Day 2, Day 3, Day 4 and Day 6. The ice was renewed every day. Plastic wrapping was used to avoid contact between the fillets and the ice or the accumulated water.

2.3. *Sample preparation and transmission electron microscopy*

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

2.4. *Mitochondrial isolation from fish fillets*

The method for mitochondrial isolation was adapted from Pasdois, Parker, Griffiths, and Halestrap (2011). All the steps in mitochondrial isolation were performed in a cold room at +4°C. Red muscle was dissected from the fillet (10 g) and finely diced with scissors. The fine pieces obtained (2-3 mm^3) were incubated at +4°C for 7 minutes under stirring in 20 mL of isolation buffer (180 mM KCl, 80 mM sucrose, 5 mM $MgCl_2$, 10 mM Tris, 2 mM EGTA, pH 7.2 at +4°C) supplemented with 0.1 mg/mL of bacterial proteinase type XXIV. The resulting tissue suspension was poured into a 30 mL glass Potter homogeniser and homogenised for 3 min using a motorised Teflon pestle at 300 rpm. The homogenate was centrifuged at 7 500 *g* for 10 minutes. The resulting pellet was first washed and then resuspended in 20 mL isolation buffer containing 2 mg/mL of fatty acid free BSA and homogenised for 3 minutes at 150 rpm. The homogenate was then centrifuged at 700 *g* for 10 min. The supernatant was centrifuged at 1500 *g* for 10 min. The resulting supernatant was centrifuged again at 7 000 *g* for 10 min. The mitochondrial pellet obtained was resuspended with a low volume (50 µL) of isolation buffer in order to obtain a concentrated mitochondrial suspension. The protein concentration was determined using a Bio-Rad protein assay kit, derived from the Bradford method (1976), using BSA as a standard. Mitochondria were kept on ice at a final concentration of 60-100 mg/mL for not more than 4 h.

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

A Xenius XC spectrofluorometer (SAFAS, Monaco) was used to monitor the fluorescence of Rh123 (Emaus, Grunwald, & Lemasters, 1986) in order to evaluate changes in mitochondrial $\Delta\Psi_m$ of isolated mitochondria extracted at different storage times (Day 0, Day 1, Day 2, Day 3 and Day 4). Importantly, this experimental approach does not enable the user to measure $\Delta\Psi_m$ but gives a qualitative index of mitochondrial polarisation. 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, pH 7.2) at 25°C was added to a 3 mL plastic cuvette. Rh123 (50 nM final) and respiratory substrates – glutamate (5 mM), malate (2 mM) and succinate (5 mM) – were added sequentially. The baseline fluorescence of free Rh123 was recorded during 2 minutes and mitochondria (0.2 mg/mL) were then added. The generation of $\Delta\Psi_m$ leads to accumulation of Rh123 in the mitochondrial matrix and consequent fluorescence quenching (dye stacking). To evaluate mitochondrial function, the following additions were performed sequentially. ADP (1 mM), CAT (5 µM) and CCCP (2 µM). The sampling rate was 1 Hz, bandwidth 15 nm at excitation and emission, and photomultiplier tube voltage was 700 V. Samples were excited at 500 nm and fluorescence was collected at 535 nm.

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

$$100-\left(\left(\frac{X-Y}{X}\right)\mathrm{x}\,100\right)$$

Where: X = Rh123 fluorescence intensity after CCCP addition

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

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

2.6. Respiration assay

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

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

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

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

2.7. Statistical analysis

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

3. Results

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

In order to study *post mortem* (PM) cell structural changes, the ultrastructure of gilthead sea bream muscle tissue was observed by transmission electron microscopy (TEM) from Day 0 to 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 (144 h PM: Fig. 1d).

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

The disorganisation of the I-bands was observed from Day 3 of storage, and it was amplified at Day 4 (Fig. 1.c). At Day 6, I-bands were barely identifiable (Fig. 1.d). The loss of density of the Z-line was mainly observed at Day 6. The disruption of the parallel alignment of Z-lines, M-lines and I-bands was observed from Day 3 (Fig. 1.b) and amplified at Day 6 (Fig. 1.d). From Day 3, the myofilaments of actin and myosin were no longer aligned and became tight, with some gaps detectable.

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

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

3.2 Post mortem *assessment of mitochondrial function*

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

Figure 2 shows typical oxygraphic recordings of the changes in mitochondrial function from 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 respiration (substrates only) reached 13.9 nmol O_2/min/mg proteins. Following ADP addition, mitochondrial respiration was stimulated (39.95 nmol O_2/min/mg proteins), due to the consumption of the proton gradient by the ATP synthase. Addition of CAT inhibited the adenine nucleotides translocator and consequently ATP synthesis. As a consequence, oxygen consumption decreased to reach a new steady state of respiration not coupled to ATP synthesis (7 nmol O_2/min/mg proteins). The addition of cytochrome c, showing the integrity of the outer membrane of mitochondria by measuring the effect on respiration, demonstrated that the endogenous cytochrome c had no effect on respiration. The mitochondrial outer membrane was intact. Finally, the decoupling agent CCCP was added. The respiration rate strongly increased during a very short time, illustrating an increase of respiratory activity to maintain the disruption of $\Delta\Psi_m$ (Fig. 2a.b.c.d.). That was not the case for Day 4 (Fig. 2e).

The results for mitochondrial oxygen consumption at Day 0 were compared with mitochondrial oxygen consumption at Day 1 (Fig. 2.b), Day 2 (Fig. 2.c), Day 3 (Fig. 2.d), and Day 4 (Fig. 2.e).

From Day 0 (Fig. 2.a) to Day 3 (Fig. 2.d), the mean basal respiration rate was around 13.67 nmol O_2/min/mg proteins. From Day 4 (Fig. 2.e), the respiration rate increased to reach 17.75 nmol O_2/min/mg proteins. From Day 0 to Day 3 (Figs. 2.a-d), ADP, CAT and CCCP produced an effect on oxygen consumption. From Day 4 (Fig. 2.e), the effect of these compounds was very weak, which showed mitochondrial decoupling. From Day 0 (Fig. 2.a) to Day 4 (Fig. 2.e), the respiration rate at state 3 decreased gradually from 39.95 nmol O_2/min/mg proteins to 24.225 nmol O_2/min/mg proteins. From Day 0 (Fig. 2.a) to Day 3 (Fig. 2.d), the difference between the basal respiration rate and state 3 respiration rate was important. At Day 4 (Fig. 2.e), differences between the basal respiration rate (17.54 nmol O_2/min/mg proteins) and the state 3 respiration rate (24.22 nmol O_2/min/mg proteins) were very weak, demonstrating that ADP addition had barely effect.

Figure 3.a illustrates the repeatability of results from Figure 2, according to 3-5 independent experiments. Changes in respiration (basal, state 3 and after CAT addition) are illustrated in Figure 3a. From Day 0 to Day 3, no difference in basal respiration and a slight decrease of the state 3 respiration rate were observed. By Day 4 (Fig. 3a), the basal respiratory rate had increased by 45% (10.48 ± 3.10 nmol O_2/min/mg proteins to 15.93 ± 5.38 nmol O_2/min/mg proteins, $p < 0.05$). At Day 4, an increase in the respiration rate after CAT addition was observed (8.88 ± 2.36 nmol O_2/min/mg proteins to 15.66 ± 5.66 nmol O_2/min/mg proteins, $p < 0.05$). At Day 4, mitochondria were not responding to ADP addition, indicating either dysfunction of the ATP synthasome or increased permeability of the inner membrane to protons.

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

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

3.3 Post mortem *assessment of mitochondrial* $\Delta\Psi_m$

To further characterise changes in mitochondrial function *post mortem*, a qualitative assessment of mitochondrial $\Delta\Psi_m$ was performed (Fig. 4). $\Delta\Psi_m$, being generated by the electron transport chain, is correlated to mitochondrial function and integrity (Zorova, Popkov, Plotnikov, Silachev, Pevzner, Jankauskas, et al., 2018). The potentiometric dye

Rh123 was used to qualitatively assess $\Delta\Psi_m$. Rh123 accumulates in the mitochondrial compartment as a function of $\Delta\Psi_m$. The higher the potential, the more dye will enter the matrix. Following accumulation and stacking of the dye within the matrix, its fluorescence is quenched. Modulation of the electron transport chain activity by ADP, CAT or CCCP leads to changes in $\Delta\Psi_m$ and consequent movement of the dye across the mitochondrial inner membrane.

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

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

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

In order to obtain an overall view of changes in $\Delta\Psi_m$ at different storage times *post mortem*, the recorded fluorescence intensity was analysed at different states of respiration (Fig. 5). The fluorescence associated with different states of respiration (basal/ADP/CAT) was normalised by the fluorescence associated with CCCP treatment (as described in Materials and methods). From Day 0 to Day 2, the effects of ADP and CAT on Rh123 fluorescence were significant. From Day 3 to Day 4, neither ADP nor CAT affected Rh123 fluorescence, demonstrating severe mitochondrial dysfunction.

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

4. Discussion

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

Post mortem *structural changes in fish skeletal muscle*

Electron microscopy images were used to study the structural changes in mitochondria in fish fillets stored *post mortem* at +4°C over 6 days. By choosing Days 3 and 4, we aimed to check whether the observed mitochondrial activity changes were associated with major structural changes. At Day 0 (6 hours *post mortem*), fish cell muscle retained its structural integrity. The myofibril arrangement was conserved and well organised. No gaps between sarcomeres were observable and collagen was still visible. These observations were consistent with those in other studies, which described good preservation of muscle myofilaments in gilthead sea bream fillet at Day 0 (María Dolores Ayala, et al., 2010; María Dolores Ayala, Santaella, Martínez, Periago, Blanco, Vázquez, et al., 2011; Caballero, Betancor, Escrig, Montero, De Los Monteros, Castro, et al., 2009). The majority of mitochondria were intact with non-altered membranes and a network of compact and well organised cristae. Some authors who have studied changes in myofilament structure in fish muscle have also been able to observe changes in mitochondria (María Dolores Ayala, et al., 2010). For example, in gilthead sea bream fillet (kept at +4°C), these authors observed swelling of some organelles, such as mitochondria and sarcoplasmic reticulum 3 hours *post mortem*. Another study on sea bass, carried out by the same authors, showed rapid changes in mitochondrial structure (with swelling) 3 hours *post mortem* (Ma D Ayala, Albors, Blanco, Alcázar, Abellán, Zarzosa, et al., 2005). Similarly, Roy, Ando, Itoh, and Tsukamasa (2012) described altered mitochondria with swollen cristae in Pacific bluefin tuna muscle cells at Day 0. Studies specifically focused on mammalian mitochondria showed good mitochondrial structure preservation several hours *post mortem*. From a structural point of view, it appears that gilthead sea bream mitochondria (like for mammalian mitochondria: (Barksdale, Perez-Costas, Gandy, Melendez-Ferro, Roberts, & Bijur, 2010)) undergo few changes within a few hours of the animal's death. From Day 3 to Day 6 (72 h – 144 h *post mortem*), several forms of structural damage in muscle myofilaments were observed in gilthead sea bream fillet, with general disorganisation of myofibril alignment, showing several gaps. The same observations were reported from Day 5 in gilthead sea bream muscle, such as alterations of sarcomeres at the I-band level, alteration of actin filaments, and disruption of Z-lines (María Dolores Ayala, et al., 2011).

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

Mitochondrial activity: an early fish freshness indicator

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

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

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

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

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

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

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

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

5. Conclusion

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

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Figure captions

Figure 1: Longitudinal sections of gilthead sea bream muscle at different *post mortem* times: 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 band; col: collagen; dg: dense granules; H: H zone; I: I band; M: M line; mit: mitochondria; sar: sarcoplasm; sr: sarcoplasmic reticulum; Tr: triad; Z: Z line. Bars: a, b, c, d: 500 nm.

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

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

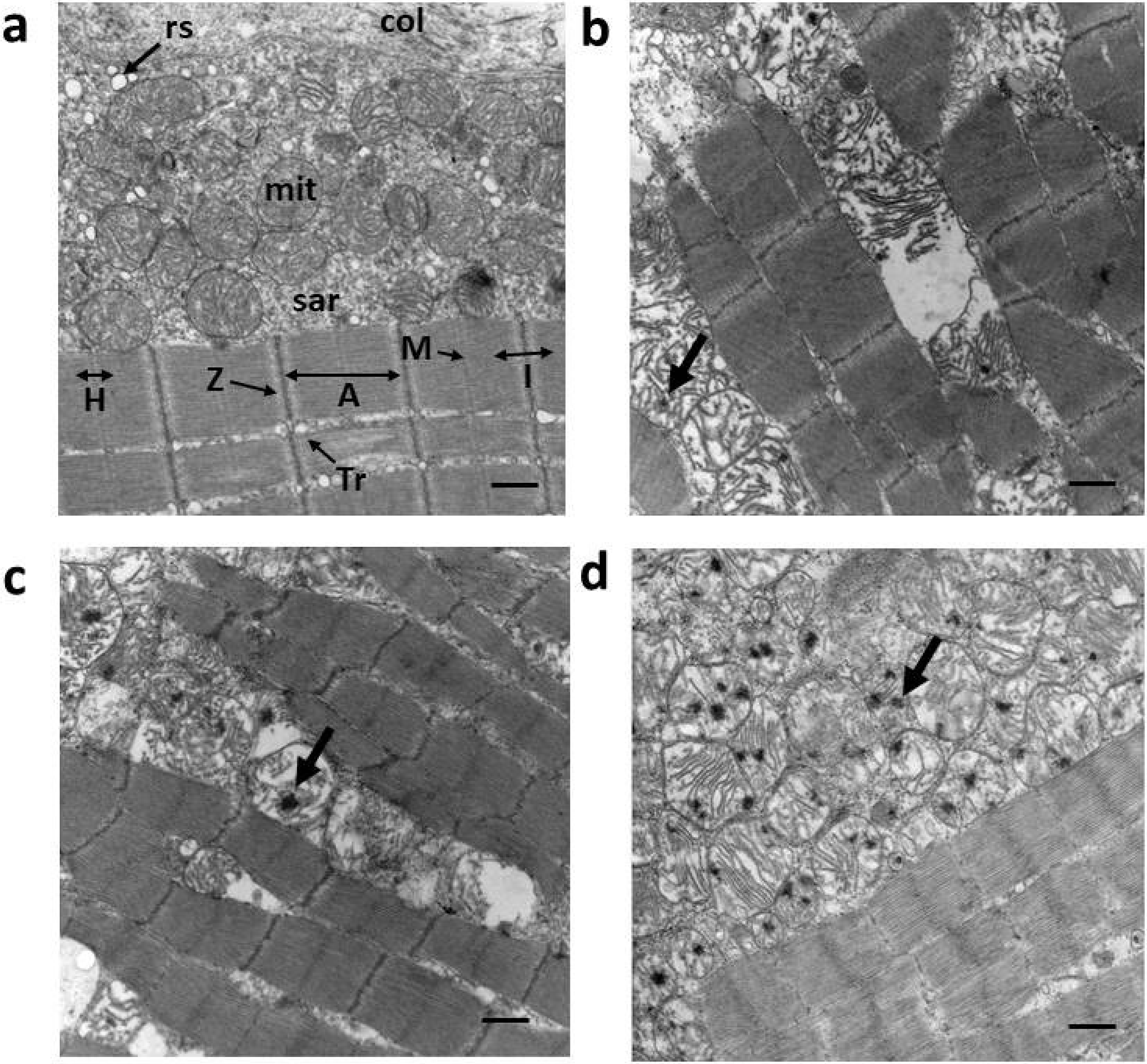
Figure 3: Evaluation of mitochondrial intactness parameters

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

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

Rhodamine 123 fluorescence traces of isolated mitochondria are represented at different storage times: Day 0 (a), Day 1 (b), Day 2 (c), Day 3 (d), and Day 4 (e). After the addition of mitochondria (Mito.) (0.2 mg/mL), the molecules ADP (2 mM), CAT (5 µM) and CCCP (2 µM) were added to a 3 mL cuvette. These graphs are representative of one experiment.

Figure 5: Summary graphs of Rh123 fluorescence levels at different states of respiration normalised to CCCP at different storage times. The different storage times were: Day 0, Day 1, Day 2, Day 3, and Day 4. Asterisks denote values that are significantly different at different storage times. The t-test was performed using the SPSS Statistic 17 programme; ($p < 0.05$; N=4-8).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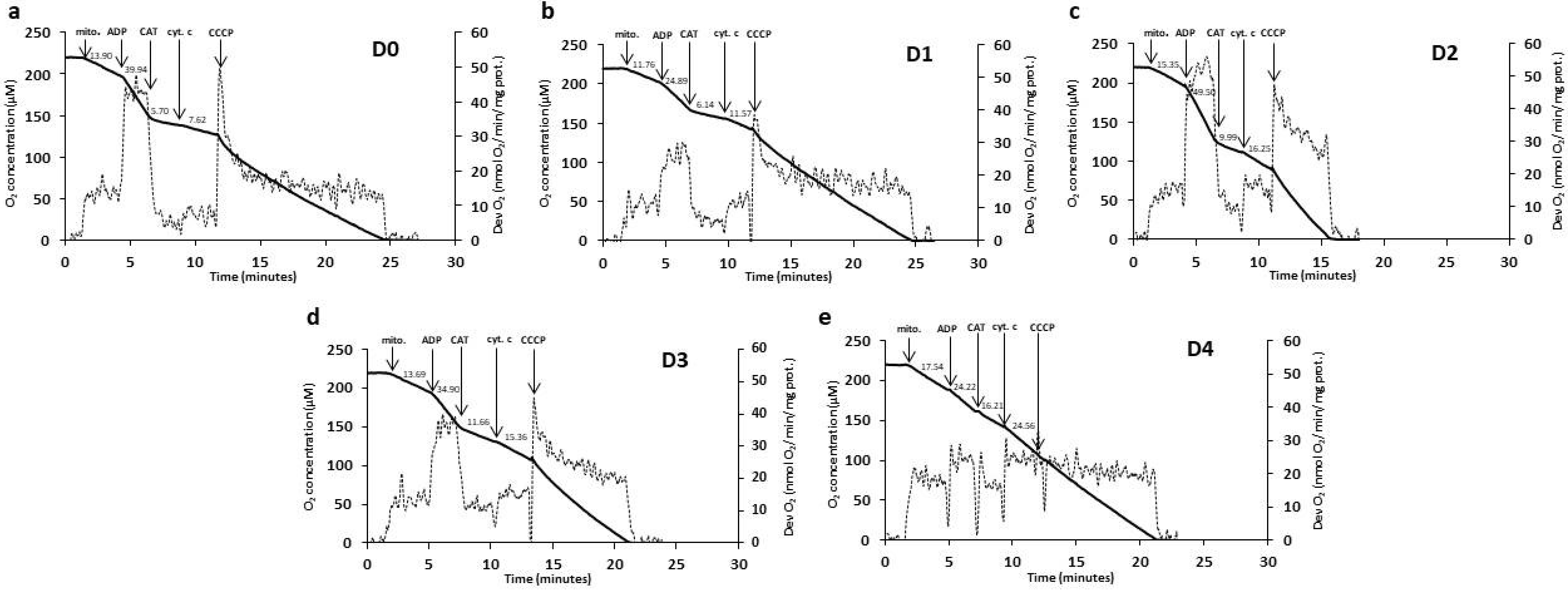
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O_2 concentration (μM)
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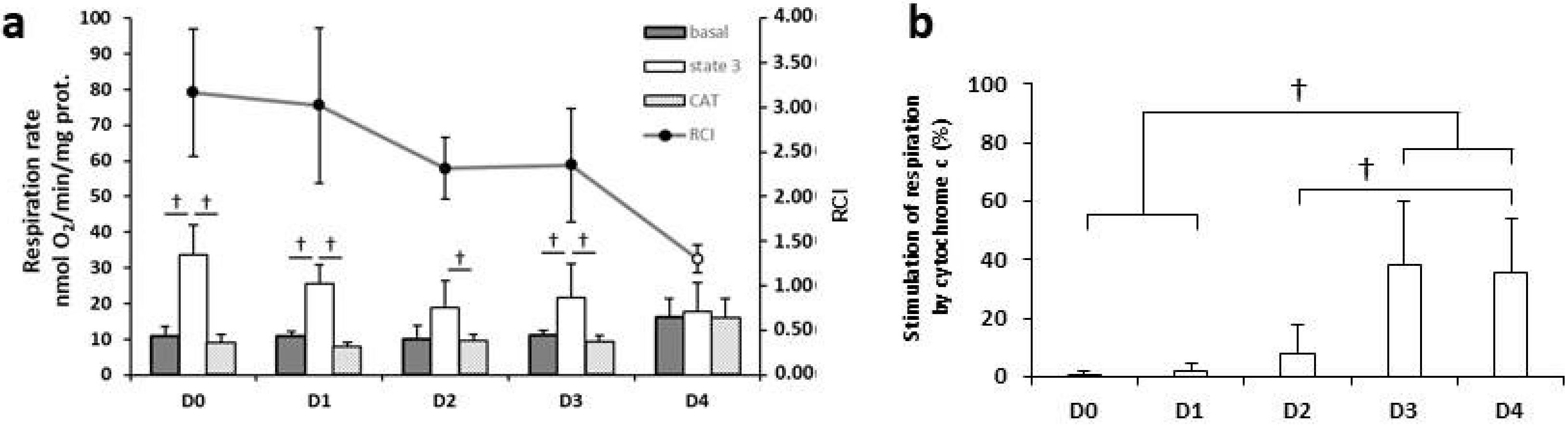
a
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state 3
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Stimulation of respiration
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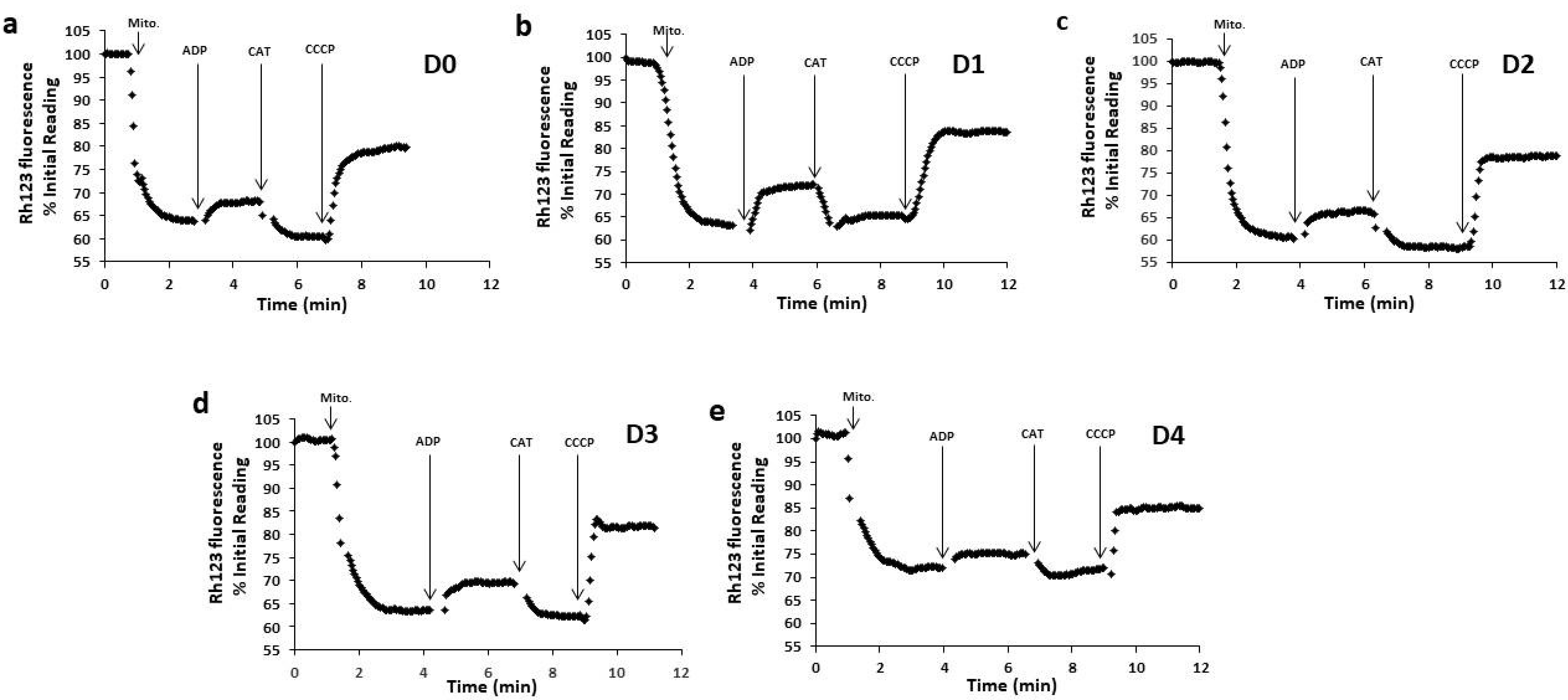
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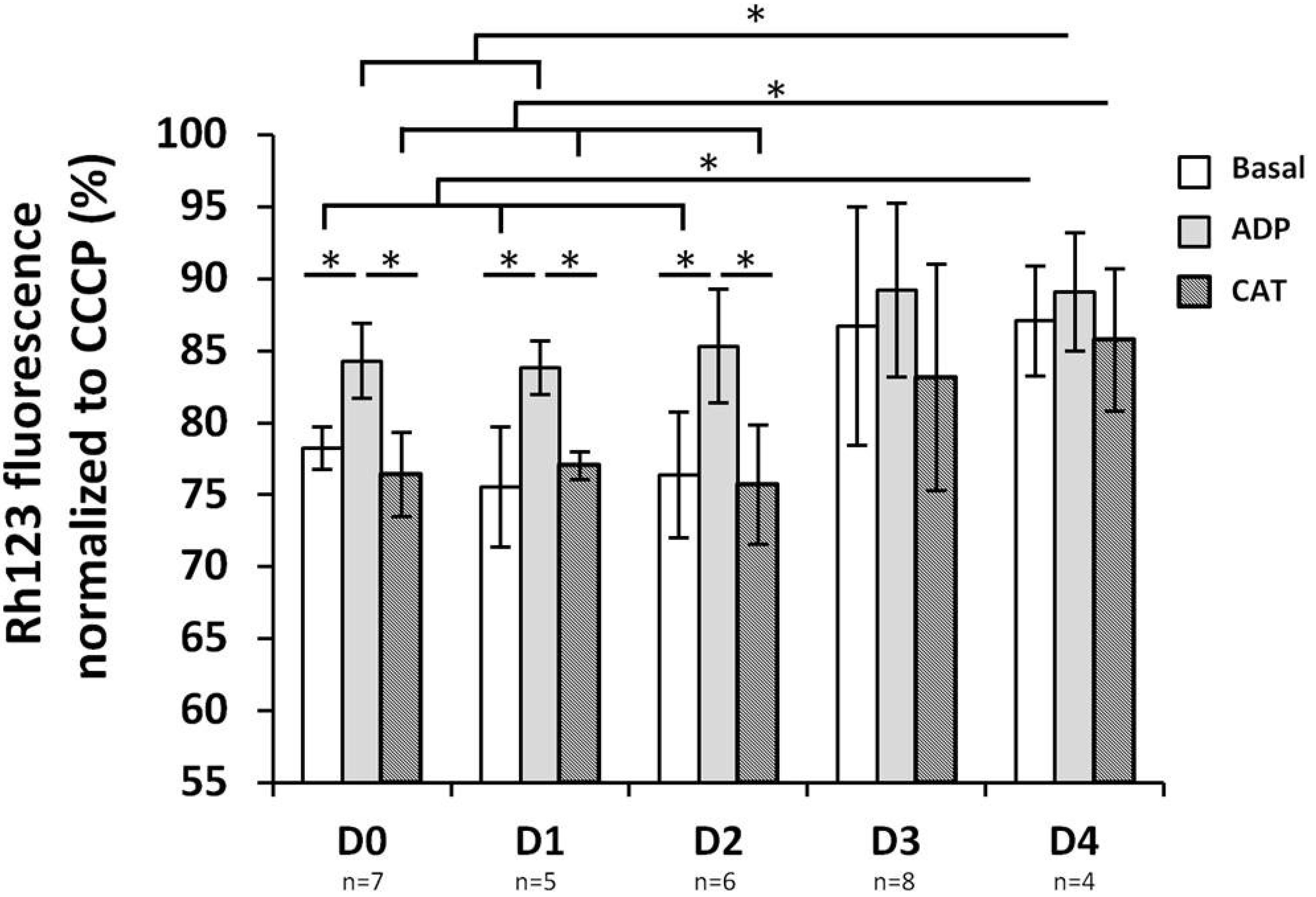
Rh123 fluorescence normalized to CCCP (%)
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*
Basal
ADP
CAT
D0
n=7
D1
n=5
D2
n=6
D3
n=8
D4
n=4