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THE FEASIBILITY OF USING GAS MIXTURE TO STUN SEABREAM (Sparus aurata) BEFORE SLAUGHTERING IN AQUACULTURE PRODUCTION

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Abstract:	Current European Union regulation explicitly states that farmed fish should be spared any avoidable pain, distress or suffering at the time of slaughter. It has been shown that fish suffer when they are killed in an ice slurry, the most common method of killing farmed fish in the Mediterranean. Thus, it is necessary to find a method of slaughtering Mediterranean fish that is, (1) efficient in inducing unconsciousness with minimal pain and distress, (2) practical to be applied to a large group of animals at the same time, and (3) feasible to be used at sea. The present study assesses the welfare of Gilthead seabream (Sparus aurata) stunned by two different gas mixtures authorised for stunning other farmed species. To achieve this objective, commercial sized seabream were stunned and /or sacrificed under different protocols: a) killed directly in ice slurry, b) exposed to a mixture of 30% CO2 + 70% N2, and then moved to ice slurry and c) exposed to a mixture of 40% CO2 + 30% N2 + 30% O2 and then moved to ice slurry. Electroencephalograms (EEG) were recorded to evaluate the state of consciousness of seabream during stunning, while blood and brains were sampled to obtain acute stress indicators and relative gene expression, respectively. Additionally, dead fish were kept for in situ meat quality evaluation. When exposed to the gas mixtures, fish lost balance at 1min 23s ± 31s with CO2 + N2 and 1min 12s ± 32s, with CO2 + N2 + O2, respectively. Cortisol, lactate and glucose levels were significantly lower in all fish exposed to gas prior to ice slurry than in fish slaughtered directly in ice slurry ($p < 0.05$). Electroencephalogram records indicated that fish started to lose consciousness when they lost balance and sank to the bottom of the tank. No differences were found in the meat quality (pH and rigor mortis) among the three treatments. Altogether, the study concludes that the use of carbon dioxide together with nitrogen prior to immersion in ice slurry is more humane than ice slurry alone.
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THE FEASIBILITY OF USING GAS MIXTURE TO STUN SEABREAM (*Sparus aurata*) BEFORE SLAUGHTERING IN AQUACULTURE PRODUCTION

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16 ABSTRACT

17 Current European Union regulation explicitly states that farmed fish should be spared
18 any avoidable pain, distress or suffering at the time of slaughter. It has been shown that
19 fish suffer when they are killed in an ice slurry, the most common method of killing
20 farmed fish in the Mediterranean. Thus, it is necessary to find a method of slaughtering
21 Mediterranean fish that is, (1) efficient in inducing unconsciousness with minimal pain
22 and distress, (2) practical to be applied to a large group of animals at the same time, and
23 (3) feasible to be used at sea. The present study assesses the welfare of Gilthead
24 seabream (*Sparus aurata*) stunned by two different gas mixtures authorised for stunning
25 other farmed species.

26

27 To achieve this objective, commercial sized seabream were stunned and /or sacrificed
28 under different protocols: a) killed directly in ice slurry, b) exposed to a mixture of 30%
29 CO_2 + 70% N_2, and then moved to ice slurry and c) exposed to a mixture of 40% CO_2 +
30 30% N_2 + 30% O_2 and then moved to ice slurry. Electroencephalograms (EEG) were
31 recorded to evaluate the state of consciousness of seabream during stunning, while
32 blood and brains were sampled to obtain acute stress indicators and relative gene
33 expression, respectively. Additionally, dead fish were kept for *in situ* meat quality
34 evaluation.

35

36 When exposed to the gas mixtures, fish lost balance at 1min 23s ± 31s with CO_2 + N_2
37 and 1min 12s ± 32s, with CO_2 + N_2 + O_2, respectively. Cortisol, lactate and glucose
38 levels were significantly lower in all fish exposed to gas prior to ice slurry than in fish
39 slaughtered directly in ice slurry ($p < 0.05$). Electroencephalogram records indicated

that fish started to lose consciousness when they lost balance and sank to the bottom of the tank. No differences were found in the meat quality (pH and *rigor mortis*) among the three treatments.

Altogether, the study concludes that the use of carbon dioxide together with nitrogen prior to immersion in ice slurry is more humane than ice slurry alone.

Keywords: Stunning, stress indicators, electroencephalogram, unconsciousness, *Sparus aurata*

1. INTRODUCTION

Aquaculture currently provides almost 50% of all aquatic products consumed by the world's population (FAO 2018), and this proportion is rising due to an increase in the demand for fishery products when the catches obtained from extractive fishing stagnant or decline. The Mediterranean species, gilthead seabream (*Sparus aurata*) is one of the five most cultured species in Europe and the total aquaculture production of sea bream in 2018 was 89,523 tonnes (FAO 2005-2020). This production is mainly from floating sea cages.

At present, there is no legislation in the European Union (EU) to specifically protect the welfare of farmed fish at slaughter. However, the European Regulation on the protection of animal welfare during slaughter and killing (Council Regulation EC 1099/2009) states that animals, including fish, should be spared any avoidable pain, or suffering during stunning and slaughter. An effective stunning leads to a brain state that is incompatible with this capacity and persistence of consciousness (EFSA, 2004). If insensibility is gradually induced, then it should be insured that fish do not the above mentioned negative states during the induction phase. To date and in accordance with the scientific literature and EFSA report (2009), there are two alternative methods that induce immediate loss of consciousness and meet the requirements of the Regulation 1099/2009: (1) stunning by mechanical percussion method followed by bleeding (Van der Vis et al. 2003) and (2) electrical stunning followed by killing (Van der Vis et al. 2003, Lambooij et al. 2008). The main problem for automated percussive stunning is variation in the size of fish within the population, which can cause a mis- stun in fish, especially those weighing less than 1 kg (EFSA, 2009). Electrical stunning is the method commonly used in trout farms throughout the United Kingdom (HSA, 2018)

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and, which has been evaluated as safe for workers on these land-based trout farms (Morzel et al. 2003, Knowles et al. 2007).

Mediterranean fish farmers, working on the deck of a boat where the harvest is collected favour a method that requires reduced space and simplicity to safely perform on thousands of individuals at a time with minimum handling. EFSA (2009) mentioned that the most common practice of slaughtering sea bream is in ice water ("ice slurry") and indicated that this method is associated with a long period (minutes) during which the animal is conscious before unconsciousness and death are achieved. During this period until unconsciousness, the fish suffers from suffocation, inferred through physiological and behavioural responses (Kestin et al. 2002, Robb and Kestin 2002, Van der Vis et al. 2003, Acerete et al. 2009). An alternative stunning method with potential to be used on boats would be the exposure to water saturated with gas mixtures such as carbon dioxide (CO_2) or nitrogen (N_2). Gas mixtures containing CO_2 induce hypercapnic hypoxia and inhibit neurones through acidosis. However, CO_2 narcosis is aversive to fish, which react with violently to high concentrations of with quick accelerated swimming, thrashing and attempts to escape (Marx et al. 1997, Robb and Kestin 2002, van de Vis et al. 2003, Sanderson and Hubert 2007). Immobility is reached within 2-4 minutes, however, fish would experience pain and distress even if unable to demonstrate it behaviourally (Kiessling et al. 2004). Sea bass (*Dicentrarchus labrax*) exposed to CO_2 remain conscious for 7-10 min and after this period, unconsciousness was demonstrated by complete cessation of rhythmic opercular respiratory movements and heartbeat, absence of VOR (vestibulo-ocular reflex) and pin-prick response (EFSA, 2009). No information was found for seabream exposed to CO_2, nevertheless an adverse reaction would be expected and has been observed (personal observation by the

100 authors). The gases argon (Ar), oxygen (O_2) and nitrogen (N_2) have been experimentally
101 used in mixtures with CO_2 in animals almost always terrestrial, such as pigs, broilers or
102 rats, in an attempt to reduce the stress caused by hypercapnia (Gerritzen et al. 2000,
103 McKeegan et al. 2007, Kirkden et al. 2008, Coenen et al. 2009, Dalmau et al. 2010, Xu
104 et al. 2011). These studies concluded that these gas mixtures could be used as stunning
105 methods which induced fewer signs of aversion and breathlessness than only CO_2 where
106 gas mixtures are already accepted for poultry and pigs (Council Regulation
107 EC1099/2009). In land animals, it is known that stunning with CO_2 –based gas mixture
108 has some advantages: meat quality is better than with using electrical stunning (Dich-
109 Jørgensen et al. 2016), it is cheaper and readily available and it is compatible with the
110 speed of operation in large slaughterhouses as animals are stunned in groups
111 (Europgroup for animals, 2019). Nevertheless, it is necessary to evaluate and validate
112 whether the use of these gas mixtures represents an alternative and more humane
113 method for fish.
114
115 Currently, there is very little information on the assessment of welfare and stress during
116 the slaughter of Mediterranean species and its impact on meat quality (Van der Vis et al.
117 2003, Knowles et al. 2007, Acerete et al. 2009, Matos et al. 2010), as there are no
118 feasible and scientifically validated measures. Conscious animals have the capacity to
119 receive, process and respond to information from internal and external environments
120 (EFSA, 2004). Therefore, in general, consciousness is associated with the awake state
121 and the ability to perceive, interact and communicate with the environment and others
122 (Zeman, 2001). The opposite state, that is, unconsciousness, is defined as: “a state of
123 unawareness (loss of consciousness) in which there is temporary or permanent
124 disruption to brain function”. As a consequence of this disruption, the unconscious

125 animal is unable to respond to normal stimuli (EFSA, 2006). Disruption of brain
126 function can occur as a result of brain concussion, administration of anaesthetics, anoxia
127 or an electroconvulsive shock (Lopes da Silva, 1982). To establish whether the
128 application of gas mixtures can be considered humane, a range of behavioural indicators
129 (e.g. coordinated swimming and escape behaviours, ability to maintain equilibrium,
130 "eye roll" reflex, and ventilatory reflexes) can be implemented to evaluate the degree of
131 consciousness/sensibility in fish (Kestin 2002). However, it has become increasingly
132 clear that behavioural measures alone are not sufficient to assess insensibility, as some
133 commercially used methods may induce sedation and/or paralysis without analgesia or
134 anaesthesia prior to insensibility. Therefore, it is necessary to obtain neurophysiological
135 or neurochemical evidence of insensibility to ascertain the impact of various
136 commercial slaughter procedures. One of the most reliable methods of assessing the
137 state of consciousness is monitoring the brain activity by recording of the
138 electroencephalogram or EEG (Raj et al. 1997, Rodriguez et al. 2008, Bowman et 2019,
139 2020, Brijs et al. 2021).
140
141 Measurement of indices of stress can indicate the welfare status of fish (Pickering,
142 1992). A typical stress response includes plasma glucose and lactate increase (Lowe-
143 Linde and Niimi 1984, Rotllant and Tort 1997). High levels of cortisol have often been
144 associated with increases in glycemia and plasma lactate, therefore, blood glucose and
145 lactate are considered reliable markers of stress in fishes (Pickering et al. 1982,
146 Simontacchi et al. 2008, Roque et al. 2010). Cortisol is the most informative and
147 accessible marker of stress in fish (Reddy and Leatherland 1998). Elevated cortisol
148 levels are thought to have knock-on effects on blood cells and plasma glucose and
149 lactate; therefore, these variables are also considered representative of the stress status

150 of fish (Rottlant and Tort 1997). Plasma electrolytes are the most commonly measured
151 indicators of the secondary phase stress response in fish and may provide indirect
152 measurement of altered cortisol (Reddy and Leatherland, 1998).
153
154 In addition to optimising fish welfare, it is also necessary to evaluate the impacts of the
155 stunning/slaughter methods on meat quality. From the time of slaughter, the fish carcass
156 starts a process of deterioration that will condition its commercial possibilities.
157 Considering that the loss of quality related to the perception of freshness attributes will
158 be inevitable, efforts should be aimed at delaying the process as much as possible.
159 Minimizing peri-mortem stress will reduce the degradation of ATP-related products
160 (Erikson et al. 1997) and delay the time of occurrence of *rigor mortis* (Erikson 2001) to
161 improve the characteristics of fillets (Robb et al. 2000) and texture (Roth et al. 2002).
162 Therefore, a stunning method that induces loss of consciousness quickly and minimizes
163 adverse reactions by fish will be favourable not only from the point of view of fish
164 welfare, but also on the quality of the final product (Marx et al. 1997). The effects on
165 the quality of the fish according to the method of stunning and slaughter have been
166 studied in several species, mainly salmonids (Skjervold et al. 1999, 2001, Bahuaud et al.
167 2010), although there are also studies on gilthead seabream (Panebianco et al. 2006,
168 Giuffrida et al. 2007, Campus et al. 2010, Matos et al. 2010).
169
170 The present study assesses the welfare of seabream stunned with gas mixes that have
171 been used in other species (chickens, pigs and trout) for the slaughtering of animals in
172 group. It responds to the legislative requirements as well as a demand from a productive
173 sector. The effects of exposing seabream to CO_2+O_2+N_2 and to CO_2+N_2 were evaluated
174 by recording behaviour (loss of equilibrium) and EEG (to assess consciousness) and by

175 measuring acute stress indicators (cortisol, glucose and lactate). A final evaluation was
176 made on the meat quality to validate the slaughter protocol verifying if the fillet quality
177 was maintained or improved.
178
179 ## 2. MATERIAL AND METHODS
180 ### 2.1. Ethics statement
181 The housing, husbandry and use of animals for the procedures described in this
182 manuscript were carried out according to Spanish and European legislation. The project,
183 including this experimental procedure, was approved by IRTA's (Institute of Agrifood
184 Research and Technology, Caldes de Montbui, Spain) Ethics Committee and the
185 Catalan government (approval number: 6722).
186
187 ### 2.2. Experimental fish:
188 Seabream came to IRTA from a commercial facility at nursery size (2-5 g wet weight)
189 and were grown for 18 to 24 months in a recirculation aquaculture system (RAS)
190 (IRTAmar®) at 20-21 ºC with 100% saturation of dissolved oxygen and full-strength
191 seawater. Fish were fed daily with a Skretting diet for their size and species. A total of
192 72 fish were used for the different experiments which weighed a minimum of 250 g wet
193 weight and the average size was 303 ± 58 g.
194
195 ### 2.3. Experimental procedure:
196 #### 2.3.1. Baseline study (Control fish)
197 Ten seabream were directly chilled in ice slurry to have a baseline control, mimicking
198 commercial conditions. Time to unconsciousness was not monitored as we considered
199 that this procedure did not adequately stun and kill the fish. Blood was collected from

200 the caudal vein five minutes after the cessation of breathing, loss of body movements
201 and absence of reaction during handling. Brains from eight fish were extracted
202 immediately after blood sampling and kept in -80 ºC for further molecular analysis.
203 After sampling, dead fish were kept inside a 4 ºC chamber in ice in perforated recipients
204 to drain water and used for the *in situ* meat quality analysis. EEG was also performed on
205 three fish (see below).
206
207 2.3.2. Experimental procedure 1: exposure to CO_2+N_2+O_2
208 The experiment was performed with 15 fish exposed to a gas mixture of 40% CO_2 +
209 30% N_2 + 30% O_2 (Freshline 3 Mix 50/20, Carburos Metalicos, Spain). Gas mixtures
210 were selected in these proportions because the mixture was commercially available and
211 had previously been used in land animals (Llonch et al. 2013). In order to define the
212 concentrations of gas to be used, we measured the level of CO_2 in the water when the O_2
213 was <2 mg /L when using only CO_2+N_2. Then the concentration of gas to be used with
214 the cylinder of CO_2+N_2+O_2 was defined by using the same level of CO_2.
215 A 60 L container with 35-40 L of seawater was used. The gas mixture was bubbled in
216 the seawater from a gas cylinder attached to a manometer with an airline and an air
217 stone until the required concentration of gas mixture in the water was achieved. and
218 Temperature, dissolved oxygen (WTW Oxi 3210) and CO_2 (Handheld OxyGuard CO_2 l)
219 were continuously measured throughout the experiment. The conditions in the water
220 were in the range of 36 - 50 ppm CO_2, 3.8 - 6.1 mg / L O_2. Temperature of the water in
221 all experimental procedures was maintained at 21 - 22ºC. Fish were exposed to this gas
222 mixture individually and were left an additional 5 min after having lost balance and
223 turned belly up. Blood was then collected. A further group of 10 fish was exposed to the

gas mixture at the same time for meat quality analysis (more details below) following the same procedure. Conditions in this case were 38 ppm CO_2 and 7.7 mg / L O_2.

2.3.3. Experimental procedure 2: exposure to CO_2+N_2

For this experiment a combination of 30% CO_2 + 70% N_2 (Freshline 30 Alimentacion; Carburos Metalicos, Spain) was used. The same containers as in experiment 1 were used and gas was dissolved in the water as previously described. The experiment was performed in two groups of 15 and 16 fish. First group (N = 15) was used in a similar exposure as experimental procedure 1 and the second group was used to record the EEG. The conditions in the water for both groups were in the range of 41 - 57 ppm CO_2, 0.6 - 1.2 mg / L O_2, and 69 ppm CO_2, 2.2 mg / L O_2 respectively for the first and second group. All the fish were left in the gas mixture 5 min after having rotated belly up and blood samples were then collected. A further group of eight fish was used *in situ* meat quality analysis. Conditions in this case were 32 ppm CO_2 and 1.7 mg / L O_2.

All treatments, number of fish and samples taken are specified in Table 2 (see Results section).

2.4. Behavioural responses

For the screening experiments, behaviour was the response used to assess whether a fish was unconscious using the following criteria:

- The fish lost balance and turned belly up (onset of unconsciousness) (Raj and Gregory 1996, Dalmau et al. 2016).
- The fish did not react when strongly grabbed by the caudal fin (Schoettger and Julin 1967)

From when fish lost balance, we waited between 3 and 10 min before transferring the fish to ice slurry where it died. The exposure to anaesthetic gas was initially 10 min from the moment when the fish turned belly up. Afterwards, we observed that 5 min exposure did not change the result, i.e., no fish would react being moved from the water supplemented with gas to the ice slurry indicating they were in a non-return condition. Finally, we observed that 3 min was the minimum period of exposure after loss of equilibrium and turning belly-up to observe no return.

2.5. Electroencephalogram (EEG)

To ensure the behavioural responses assessed were synchronised with the electroencephalographic record, we first evaluated that fish behaved similarly when exposed to the same conditions. It was verified that the degree of variation in behaviour and time to perform these behaviours (loss of balance and duration of aversion) among individuals were not different. For this purpose, groups of three fish, which were not previously used in any experimental procedure, were exposed to a mixture of CO_2+N_2 at the same time, and the latency to turn belly up was measured. The timing when fish turned belly up within each group was similar and within a 1-2 s period.

Once we verified the response times were not different between fish exposed to the same gas mixture, a single water mixture with CO_2+N_2 was prepared and divided into two equal tanks for the exposure. Two fish were exposed to the gas mixture at the same time. One immobilised fish with the EEG record already started (see below) was placed in one tank at the same time the other fish was liberated in the water of the other tank. Both fish were treated similarly before being introduced into the tanks with the gas mixture, netting and time of air exposure were the same, but only the EEG fish was

attached to the EEG (see below). The fish liberated to swim freely in the tank was filmed, therefore obtaining in parallel a behaviour video and an EEG record to correlate the EEG with the screening behaviour (lose balance and turning belly up). This experimental design had previously been used and loss of posture was established as the onset of unconsciousness (Dalmau et al. 2016). The water conditions were: 17.7 ºC; 69 ppm CO_2, 2.2 mg / L O_2. The experiment was repeated 8 times (8 fish in EEG and 8 fish free in a tank, N = 16).

The QCON Monitor® (Quantum Medical, Spain) is a cerebral consciousness monitor based on wireless technology that assesses brain activity. From the QCON Monitor® (QCON Manual version 6, Valencia et al. 2012), the Index of Consciousness (IoC) and the burst suppression index (BS%) can be estimated to assess unconsciousness during states of anaesthesia (Litvan et al. 2002). The IoC is an algorithm that analyses the raw EEG with a unitless scale from 0 (isoelectric EEG, coma) to 99 (awake) (Revuelta et al. 2008). The BS% indicates the percentage of isoelectric activity during the preceding 30 s and also ranges from 0 to 100 (Litvan et al. 2002). The QCON® monitor is currently used in human patients (Valencia et al. 2012), rabbits (Silva et al. 2011) and pigs (Llonch et al. 2011).

In order to record brain activity through EEG, fish were restrained individually by tying or strapping the fish to a division that was placed in the exposure tank. Two electrodes (Contell Asset Support, Netherlands) were placed on the animal's skull either side of the middle line at the point where the brain is located and separated 5 mm from each other for a transhemispherical electroencephalography (EEG) recording. The reference electrode was placed in the muscle 2-3 cm below the dorsal fin on the right-hand side of the fish. Subsequently, the 3 electrodes were connected to a computer by means of a

150 cm coaxial cable (QCON monitor; Quantum Medical; Barcelona, Spain) to record brain activity using EEG as described in EFSA (2013) and Llonch et al. (2015). The QCON® monitor was then fitted to the electrodes to record EEG data. The data was transferred to a Personal Computer (Acer, Aspire One) for data to be analysed. The moment when the fish became unconscious was identified by plotting the log readings of the brain suppression rate (BS%) and the index of consciousness (IoC) in the same graph and finding the exact moment where the two lines crossed, which indicated the point the fish became unconscious. Baseline EEG activity of the animals was recorded for 1 min, before the animals were placed into the tank and exposed to the gas treatment and the record was maintained 5 min after the free fish lost balance and turned belly up. The fish tied to the division were immersed in the exposure tank once the baseline EEG record was verified to be of good quality. The fish were immersed in the exposure tank leaving only the top of the head out, where the electrodes entered the skull. As previously mentioned, the other fish was released in a second exposure tank at the exact same time EEG fish was placed into an exposure tank, after having been air exposed for the same amount of time as the EEG fish. EEG is a painful and stressful method for fish that for ethical reasons should be used on as few fish as possible. Therefore, it was decided to only perform EEG for the CO_2+N_2 group that was clearly demonstrated to induce loss of consciousness and because the results showed no significant difference between the two gas treatments (see results section). EEG fish were manipulated in the same manner and after the basal EEG was recorded, they were carefully placed under the ice slurry leaving the top of the head out.

2.6. Blood analysis

At the end of the two experiments (procedures 1 and 2) and baseline study, blood was collected (≈ 1 ml) from the caudal vein with 5 mL heparinised syringes with a needle 21Gx 1 ½". Once the blood samples were collected, the haematocrit was measured and the plasma was obtained by centrifugation, and subsequently frozen at -80 ºC until further analysis. The parameters analysed were cortisol (meditec kit, ELISA method), lactate (Abcam kit), glucose (Cromotest kit), magnesium (Cromotest kit) and total protein (Bradford microplate method). All the kits were used according to the manufacturer's instructions and if the reaction was to be developed in volumes higher than 300 µL, at the end processed samples were loaded in microplates to facilitate the reading of the optical density in a plate reader (Tecan, Infinite M200 Series). Each fish was sampled 5 min after the fish turned belly up.

2.7. Molecular analysis

In the end of the two experiments (procedures 1 and 2) and baseline study, whole brains were extracted from the dead fish and immersed in RNA later and placed for 48h at 4 ºC. Brains were then frozen at -80 ºC for further analysis. The RNA was extracted from 100 mg of the preserved brains using TRI Reagent RNA Isolation Reagent (SigmaAldrich, Germany) following manufacturer's instructions. The cDNA was synthetized using 1 µg of total RNA and oligo dT (20) in 20 µL reactions and the SuperScript1 III First-Strand Synthesis SuperMix 50 rxn kit (Invitrogen, Life technologies, USA) following the manufacturer's protocol. Before performing the rt-qPCR, primers (Table 1) were validated by conventional PCR using a cDNA pool from all the samples.

Table 1. Primers used in this study specific for seabream species.

348

Gene name	Amplicon size	Primer sequence (5'→3')	Accession number	Reference
18s rRNA	134 bp	F: GCA TTT ATC AGA CCC AAA ACC R: AGT TGA TAG GGC AGA CAT TCG	AY993930	Perez Sanchez et al. 2011
ef1α	134 bp	F: CCC GCC TCT GTT GCC TTC G R: CAG CAG TGT GGT TCC GTT AGC	AF184170	Perez Sanchez et al. 2011
gapdh	111 bp	F: ATCAAGAAGGTCGTCAAGGC R: AGATGGAGGAGTGGCTGTC	DQ641630	Malandrakis et al. 2014
hsp70	174 bp	F: ATT GTT CTG CGC ATC ATC AA R: GCC TCC ACC AAG ATC AAA GA	EU805481	Benhamed et al. 2016
COX2	192 bp	F: GAG TAC TGG AAG CCG AGC AC R: GAT ATC ACT GCC GCC TGA GT	AM296029	Sepulcre et al. 2007

349

350 MyTaq™ HS Mix (Bioline) was used to run the conventional PCR with the following
351 conditions: initial activation step at 95˚C for 3 min, followed by 40 cycles: denaturation
352 at 95 ˚C for 5 s, annealing at Tm (58–60˚C) 95 ˚C for 15 s and extension at 60˚C for 1
353 min and 95 ˚C 15 s, hold 50 ˚C 10 min. Primer efficiency was evaluated by serial
354 dilutions to ensure that it was close to 100% performing real time PCR. Target
355 transcripts (*gapdh, ef1a* and *hsp*70) were analysed by real-time quantitative PCR (rt-
356 qPCR) (see primers in Table 1). The qPCR was run using a Biometra Optical
357 Thermocycler (Analytik Jena, Goettingen, Germany) in 96-well plates in duplicate 20
358 μL reaction volumes containing 10 μL of Luminaris Color HiGreen qPCR Master Mix

(Thermo Scientific), 1 μL of the primer corresponding to the analysed gene (10 pmol), 3 μL of RNA/DNA water free and 5 μL of cDNA in its corresponding dilution. Furthermore, amplifications were carried out with a systematic negative control (NTC; no template control) containing no cDNA. Standard amplification conditions contained an UDG pre-treatment at 50˚C for 2 min, an initial activation step at 95˚C for 10 min, followed by 35 cycles: 15 s at 95˚C, 30 s at the annealing Tm and 30 s at 72˚C. Elongation 1 min 95 ˚C, 30 s 55 ˚C and 30s 95 ˚C. Results were normalised using the housekeeping gene *18S*. The mRNA abundance for each gene was determined using the Pfaffl method (Pfaffl, 2001) on relative quantification.

2.8. *In situ* meat quality analysis

In situ meat quality analysis data was collected from 8 fish per treatment where two parameters were assessed:

a) pH. Measurements were made with a pH meter (pHmeter Crison pH25+) attached to a probe which was inserted in a cut made in the muscle with a scalpel. The side of the fish where the cut was made was changed at each measurement. Measurements were taken at 0, 2, 6, 10, 24, 48 and 72h *postmortem.*

b) *Rigor mortis* (RM). Measurements were made at the same time as pH measurements. Rigor development was monitored by carefully placing the fish on a plane surface with two thirds of its length beyond the edge of the surface, i.e. without support provided by the surface. The sag of the tail from the horizontal plane was recorded after five seconds and the rigor index calculated: Rigor index (%) = 100 (current height − height before entering rigor) / height

before entering rigor. The fish was then carefully replaced back inside their box until the next measurement.

For the *in situ* meat analysis fish were kept in polystyrene boxes and placed inside a 4ºC camera, but the actual measurements were performed at room temperature

2.9. Statistical analysis

The data obtained from the QCON monitor (IoC and BS%) were analysed using linear general models, with Proc Mixed proceeding for repeated measures of SAS (SAS 9.4). In both cases, the variables were submitted to symmetrical composition covariance structure (CS). When the variance analysis showed significant differences ($p < 0.05$), the comparison of least square mean values (LSMEANS) was adjusted to Tukey multiple comparison test (Rodriguez et al. 2008, Dalmau et al. 2016).

One-way ANOVA on ranks was used to detect differences among treatments for the blood parameters analysed. Multiple comparisons were made with the Dunn's method. Although time to loss of balance could be assessed as an independent variable for the gas treatments, this was not possible for the control group thus a *t-test* was made to see if the time take to lose balance was significantly different between the two gas treatments. Another *t –test* was also performed for the moment when the values of the IoC and the BS% in the EEG crossed versus the moment when the free fish lost balance. Alle these variables fulfilled the requisites for the use of a parametric test.

A repeated measures (RM) two-way ANOVA was performed to verify the meat quality indexes did not change with the stunning with gases. The two factors were treatment and time. Meat quality values were not normally distributed and the homoscedasticity test was not passed for either parameter, we still preferred to this test instead of ranked

one way ANOVA for each point in time. All ANOVAs and *t-tests* were performed using Sigmaplot (version 12.0).

Level of significance in all statistical tests was considered lower than 0.05 (*P-value* < 0.05).

3. RESULTS

Fish were successfully stunned and killed by both gas treatments. Average times per treatment for fish to lose balance and turn belly up varied from 1 to 3 minutes with gases to 52 minutes in ice slurry (Table 2). The longest and shortest periods of time passed between exposure to treatment and loss of posture are shown in table 2. This table also lists the type of samples taken from each group of fish. In the case of ice slurry, it was difficult to evaluate when fish were unconscious and therefore, we decided to present the results of when fish were dead. In the case of exposure to gas mixtures, fish started to swim calmly around the tank and between 30 to 80 seconds later, all fish became aware of their situation displaying signs of aversion for periods of around 10 to 12 seconds just immediately before losing balance and turning upside down. Signs of aversion were a very strong acceleration of swimming and raising the head out of the water, but not jumping. Once fish had turned belly up and did not move, tail grabbing was applied without any reaction from the fish. Time elapsed from entering the tank until loss of posture and balance was 01:12 ± 00:32 for fish exposed to CO_2+N_2+O_2 and 01:23 ± 0:31 for fish exposed to CO_2+N_2. A t-test indicated no differences ($P > 0.1$) between the time elapsed until loss of balance in both gas treatments.

Table 2: Time elapsed for fish to turn belly up according to the experimental procedure.

Treatment	**Ice slurry (N=10)**	**CO_2+N_2+O_2 (N= 15)**	**CO_2+N_2+O_2 (N=8) (a)**	**CO_2+N_2 (N=15)**	**CO_2+N_2 (N=8) (a)**	**CO_2+N_2 (N=16)**
Concentration (ppm)	__	36 - 50 CO_2,3.8 - 6.1 O_2	38 CO_2, 7.7 O_2	41 – 57 CO_2, 0.6 - 1.2 O_2	32 CO_2, 1.7 O_2	69 CO_2, 2.2 O_2
Mean± SD (mm:ss)	52:00 ± 10:00*	01:12 ± 00:32	01:23	01:23 ± 0:31	01:29	03:03± 0:38
Max (hh:mm:ss)	01:13:00	02:15	01:23	02:45	01:29	04:12
min (mm:ss)	36:00	00:25	00:59	00:40	00:50	02:15
Other samples	Meat, Blood, RNA	Blood and RNA	Meat	Blood and RNA	Meat	EEG

(a) This group was exposed at the same time and this value corresponds to the last fish that moved.

* Time until death.

Max = the longest period recorded in a fish to turn belly up in a particular treatment.

min = the shortest period recorded in a fish to turn belly up in a particular treatment.

SD = standard deviation.

328 The IoC-view® recordings were successful in 7 animals assessed out of 8 exposed to
329 CO_2+N_2. One pair of fish from the exposure to CO_2+N_2 was discarded due to bad
330 reading. The mean (± SD) basal IoC was 90.2 (± 11). The IoC started to decrease
331 significantly ($P < 0.05$) at 63 (± 20.2) s after placing the fish in the water saturated with
332 the gas mixture (IoC = 89 [± 3.7]). It continued decreasing and reached its lowest value
333 on average (IoC = 2) at 343 (± 203.99) s after the start of the exposure to the saturated
334 water (Table 3).
335
336 The mean (± SD) basal BS% was 0. The BS% started to increase significantly ($P <$
337 0.05) at 63 (± 20.2) s after placing the fish in the water saturated with the gas mixture. It
338 continued increasing and reached its highest value on average (BS% = 94) at 379 (±
339 182) s after the start of the exposure to the saturated water.
340 All basal EEG were significantly different from the final readings for both IoC and
341 BS% ($p < 0.05$).
342
343 Time of unconsciousness by EEG was 3:07 ± 1:17 and to loss of balance (turning belly
344 up) was 3:03 ± 0:38 which was statistically the same with P = 0.89.
345
346 Table 3 Time elapsed to loss of consciousness (EEG) of seabream exposed to CO_2+N_2.
347

Fish	IoC value at start	1) IoC decrease (mm:ss)	2) Loss of consciousness (mm:ss)	3) Loss of balance (mm:ss)
1	67	02:57	05:00	04:12
2	95	01:10	02:45	02:15

3	88	00:01	02:30	03:04
4	91	00:11	03:00	03:04
5	99	00:20	02:38	02:52
6	98	00:50	01:20	02:30
7	97	01:57	04:42	03:25
8	99	00:50	07:50	NA
9	91	00:10	02:50	NA
10	99	01:01	08:11	NA

348

349 The moment when fish lost consciousness was estimated by plotting the log of the
350 readings as shown in Figure 1. Values of BS% would raise sharply from 0 to close more
351 than 80 in seconds as the IoC started decreasing (see Figure 1).

352 1) Moment when IoC starts to decrease significantly (mm:ss)

353 2) Moment when fish lost consciousness according to EEG signal (mm:ss)

354 3) Moment when the free fish lost balance and turned belly- up (mm:ss)

355 NA- Fish were under a layer of ice slurry and could not be observed.

356 For the 7 pairs of fish exposed in parallel, a *t-test* indicated the moment where the
357 values of IoC crossed the BS% values was not significantly different from the moment
358 when fish lost posture and balance ($p > 0.1$).

359

360 Blood parameters mean values exhibited variation between treatments. Haematocrit
361 varied from 71.4 % (CO_2+O_2+N_2) to 40.3 % (ice slurry); Glucose from 14.09 (CO_2+N_2)
362 to 247.3 g/dL (Ice slurry); Cortisol from 2.48 (CO_2+N_2) to 474.1 nmol/uL (Ice slurry);
363 Lactate from 0.464 (CO_2+O_2+N_2) to 12.44 nmol/mL (ice slurry); Protein from 5.29
364 (CO_2+N_2) to 18.26 mg/mL (CO_2+O_2+N_2); Magnesium from non-detected (ND, CO_2+N_2

and $CO_2+O_2+N_2$) to 9.86 mg/dL ($CO_2+O_2+N_2$). Statistical analysis showed that in general treatment with ice slurry was significantly different from exposure to gas mixtures for the following parameters: cortisol, glucose, lactate and magnesium ($P < 0.05$, Table 4).

Table 4 Biochemical plasmatic parameters measured in fish from the different treatments and control.

Treatment	Haematocrit %	Glucose g/dL	Cortisol nmol/uL	Lactate nmol/mL	Protein mg/mL	Magnesium mg/dL
Ice slurry	51.0±1.7	145.8±16.5	109.5±38.9	521±28.6	14.6±0.31	2.7±0.7
$CO_2+O_2+N_2$	52.4±6.8	135.3±41.9	41.9±41.1*	60.6±34.1*	15.5±1.67	4.36±2.11*
CO_2+N_2	50.8±4.9	79.1±14.1*	35.7±32.2*	132.5±31.5*	13.7±3.2	5.02±1.76*

* indicates the treatment is significantly different from the direct exposure to ice slurry (control).

Relative gene expressions were estimated using *18S* gene as the housekeeping gene. Relative gene expression had a very high variation both intra and inter groups and no differences were obtained between treatments. The gene expression results have been included as supplementary material.

The pH started descending as soon as the fish were dead although in the fish killed with ice slurry this decrease was slower. Initial values of pH were 7.21 ± 0.14, 6.83 ± 0.16 and 6.74 ± 0.15, and they decreased until 72h where pH values were 6.41 ± 0.07, 6.38 ± 0.12, 6.35 ± 0.05 for slaughtering in ice slurry, exposure to $CO_2+N_2+O_2$ and exposure to CO_2+N_2, respectively. Meat will be of better quality when the pH is lower (Love, 1980),

385 after 72h there were no differences among treatments (Figure 2A). Again from 8 hours
386 onwards all treatments showed a parallel pH progress.
387
388 The experimental gas mixture treatments used induced a faster instauration of *rigor*
389 *mortis* (RM) when compared to killing directly in ice slurry. The later the instauration
390 of RM the better, so that there is time to process fish (Figure 2B). At 2 hours, RM was
391 the following 37.94 ± 20.12, 79.22 ± 20.41, 81.87 ± 5.66 for killing in ice slurry,
392 exposure to CO_2+N_2+O_2 and exposure to CO_2+N_2, respectively. This meant that, after
393 2h the group of fish placed directly in ice slurry was the only group that had not entered
394 RM phase, however from 8h onwards all groups showed a parallel RM evolution. At
395 72h, RM values were 68.03 ± 8.91, 67.67 ± 17.74, 71.63 ± 7.22 for slaughtering in ice
396 slurry, exposure to CO_2+N_2+O_2 and exposure to CO_2+N_2, respectively.
397
398 4. DISCUSSION
399 The aim of this study was to evaluate the effectiveness of two gas mixtures to be used as
400 a method to stun Mediterranean fish from aquaculture production using seabream as a
401 model species. Most fish took less than 1 minute and 30 seconds to initiate loss of
402 equilibrium, irrelevant of the gas mixture. In the exposure to CO_2+N_2, an EEG
403 demonstrated that fish start losing consciousness at the point they lose balance and turn
404 upside down. Thus, fish losing balance and turning upside down might be defined as the
405 moment when fish start losing consciousness. In practice, these behavioural responses
406 can be used an operational indicator for stunning and killing fish. Nevertheless, fish can
407 only be considered properly stunned 3 minutes after having lost balance, since it is
408 important to ensure they will not recover when moved to the ice slurry, in order to
409 ensure the welfare of all fish. However, this suggestion must be taken with caution due

410 to the reduced number of fish used in the experiment. At this point, soon (5 minutes)
411 after losing balance and consciousness some fish were blood sampled for primary
412 indicators of stress (glucose, cortisol and lactate) and significant differences were found
413 between fish killed directly in ice slurry *versus* fish exposed to the gas mixture. In
414 addition, *in situ* meat analysis was not different among treatments leading us to
415 conclude that flesh quality is not affected by introducing this stunning method. Both
416 mixtures seemed to induce similar reactions and no differences between treatments were
417 perceived.
418
419 To our knowledge, there is no data available on stunning seabream with gas mixtures.
420 However, Zampacavallo and collaborators (2003, 2015) stun-killed seabass in ice water
421 saturated with 60% CO_2 + 40% N_2 and with 30% CO_2 + 70% N_2, respectively. In their
422 studies, the authors confirmed a significant reduction in the time take to achieve death
423 from 20 minutes to 6 and 10 minutes respectively.
424
425 In the present study, no treatment rendered the fish unconscious in an immediate
426 manner. Nevertheless, fish only displayed aversion to their situation for 10-12 seconds
427 immediately before turning belly up and showing signs of losing consciousness. This
428 aversion moment has been observed in other species where gas mixtures were used for
429 stunning (Llonch et al. 2013, Dalmau et al. 2016, Verhoeven et al. 2016). The time
430 which fish were, most likely, in a situation that impaired their welfare were those 10
431 seconds before the fish started to lose consciousness. In the beginning of the exposure,
432 fish swam calmly around the tank. Exposure to CO_2 alone is problematic since fish
433 display several signs of aversion (Van der Vis et al. 2003, Erikson 2011, Roque
434 personal observation), however, adding N_2 and / or O_2 has been suggested to mitigate

this aversion (Gerritzen et al. 2000, McKeegan et al. 2007, Kirkden et al. 2008, Coenen et al. 2009, Dalmau et al. 2010, Xu et al. 2011). All these studies showed that mixing gases with N_2 worked better than CO_2 alone for the species concerned (EFSA, 2009). The addition of oxygen (O_2) for Artic char did not increase time to loss of balance showing that O_2 does not antagonise the anaesthetic capacity of CO_2 (Sandblom et al. 2013).

Rodríguez et al. (2008) and Llonch et al. (2011) concluded that a significant decrease in the electrical activity of the brain is considered a sign of the onset of unconsciousness in pigs. This is also the case for rabbits (Dalmau et al. 2016). Moreover, EFSA's review (2013) clearly states that changes in EEG power are considered a good indicator of brain activity in studies where animals were stunned with gas. In the present study, an IoC significantly lower than basal values occurred from a few seconds to nearly 3 minutes after the immersion in a bath containing a mixture of CO_2 and N_2. The experiment showed that fish lose consciousness, with the IoC decreasing as the BS% increased. According to the manufacturer's manual a IoC between 0 and 40 corresponds to deep anaesthesia in humans, but as the device was not developed for fish species, we never finished the record with a IoC < 5 to ensure the fish was at a point of no return. Van der Vis et al. (2003) observed a difference of approximately 5 minutes between a salmon exposed to CO_2 losing balance and being declared unconscious by losing the VER. In the present study, the mean time difference between loss of balance and IoC value being lower than BS% was 4.87 s. Nevertheless, we still used loss of balance as the operational indicator of unconsciousness as it is very easy to appreciate even when observing a group of fish instead of individual fish, where other indicators such as VER would be difficult to appreciate. Still, as stated in material and methods section, fish

were left in the exposure tanks for a minimum period after having lost balance, in order to ensure they could not react or recover. Timings in this experiment were longer than those when just observing fish and this is most likely explained by a longer handling procedures. Both fish (free swimming and EEG fish) were outside the water for more than one minute (setting up of the EEG and one-minute record) and this increases the level of stress in the fish making it more difficult for them to anesthetise (Zahl et al. 2013).

Acute stress parameters (glucose, cortisol and lactate) were significantly higher in fish sacrificed directly in ice slurry implying that this treatment was more stressful for fish. A typical stress response includes plasma glucose and lactate increase (Lowe-Linde and Niimi, 1984; Rotllant and Tort 1997). High levels of cortisol have often been associated with increases in glycemia and plasma lactate; therefore, blood glucose and lactate are considered reliable markers of stress in fishes (Pickering et al., 1982; Simontacchi et al. 2008; Roque et al. 2010). Cortisol response peaks after 2.5 to 60 minutes (Pankhurst 2011) and present experimental design follows previous literature demonstrating significant differences among treatments (Zampacavallo et al. 2003, 2015, Daskalova et al. 2016, Gräns et al. 2016). In Artic char exposed to a mixture of CO_2 + O_2 (50-50), cortisol increased significantly from basal levels only 30 minutes post exposure (Sandblom et al. 2013), which would not be a problem in the present study since fish would be dead by then. This delayed cortisol response could be related to long deep anaesthesia (Sandblom et al. 2013) which makes these mixes fit for the purpose of this study. In the present study, no recovery investigation was made since the purpose was an irreversible stunning method and personal observations established that seabream weighing between 250-500 g (commercial size) do not recover if exposed to the gas

485 mixture for at least 3 minutes after they lost the balance. In the present study, stress
486 parameters were measured 5 minutes after fish had lost balance and consciousness and
487 demonstrated that fish in ice slurry had higher levels of stress at this point. However, the
488 time period to this point was different in different treatments and show to be much
489 longer in ice slurry (52:00 ± 10:00 m). It cannot be discounted that the stress response
490 was similar across treatments but had more time to develop in the ice slurry treatment.
491 This also seems to be the case with seabass where fish stunned in ice water saturated
492 with CO_2+N_2 and sampled 30 to 60 minutes later, presented much higher values than in
493 present case (Zampavallo et al. 2003, 2015). However, clearly at the point of loss of
494 consciousness ice slurry fish had higher levels that can indicate higher stress and these
495 ice slurry fish had a considerably longer period (52:00 ± 10:00 m) in this stressful state
496 before losing consciousness compared to the < 4 minutes registered in gas treatments.
497 Magnesium was significantly higher in the fish exposed to gases, and this is most likely
498 due to acidification of water and blood by the CO_2 (Shrivastava et al. 2019) and fish
499 must compensate for the blood acidosis. The significant alteration in plasma ions as
500 magnesium in fish exposed to gas mixture might represent disturbances in acid-base
501 balance, oxygen and carbon dioxide transport (Roque et al. 2010). This result was in
502 accordance with the findings of Tort et al. (2003).
503
504 Longer awareness of the fish killed in ice slurry is probably the explanation of the
505 increase in glucose, since this results as a response to the release of stress-induced
506 hormones in the blood circulation, which trigger muscle or liver glycogenolysis,
507 releasing glucose for the increased energy requirement during stress (Eslamloo et al.
508 2014). This increased energy demand also leads to the increment of blood lactate,
509 caused by the anaerobic activity of muscles (Wang and Richards 2011, Zampacavallo et

al. 2003) and hypoxic stress (Eslamloo et al. 2014) in the case where the gas mix did not contain oxygen.

The gene expression (supplementary data) in relation to the treatments was very variable and no conclusions could be drawn. On reflexion, we realise the very short-term exposure to the experimental treatments (stunning lasting less than 2 minutes) most likely did not induce a marked response on the synthesis of mRNA and thus we would be measuring more than anything the pre-stunning *antemortem* period which was common to all the fish. The differences would then be explained by the individuality of the fish (Jolles et al. 2020) where even though the fish had been exposed to the same circumstances, the individual fish varied either the response or at least the abundance of the response. The mRNA had high quality when measured by spectrophotometry and visualised in a gel, however, it must be pointed that some mRNA are very short lived, 5 to 10 minutes (Guaniyogi and Brewer 2001), and the fish were kept in the water five minutes before sampling. Another aspect to potentially contribute to this situation, was that the fish were stunned in a very hypoxic environment which leads to an increase of ATP and consequently to cellular degradation, including the mRNA. Many studies use sacrifice in anaesthesia as the negative control, a decision was taken that for the present study such control would not be used because there is a vast amount of literature demonstrating the use of anaesthesia is not without stress for the fish (Toni et al. 2015, Bodur et al. 2018, Freitas Souza et al. 2019, Teles et al. 2019). Slaughtering the fish by hitting them on the head would be a solution to this, unfortunately this is not feasible when you need to sample the brain and cutting of the spinal cord leaves the heart and the brain in most cases on the same half of the fish which is not a good situation from a consciousness perspective.

In the present study, the treatment did not seem to affect the meat quality. This was slightly surprising because the fish in the ice slurry treatment were more stressed than the ones submitted to gas exposure according to the blood parameters. In fact, at the beginning (2 hours), both meat quality parameters had better values for the fish killed directly in ice slurry (pH: 7.01 versus 6.75 and 6.79, *rigor mortis*: 37.94 versus 79.22 and 81.87 for ice slurry, $CO_{2+}N_{2+}O_2$ and $CO_{2+}N_2$, respectively). For both parameters, these values are similar to those previously reported in the literature for seabream (Tejada and Huidobro 2002) and similar to seabass (Zampacavallo et al. 2003, 2015). When fish are stressed during crowding, they deplete their energy reserves prior to slaughter and *rigor mortis* occurs sooner than when the fish have been crowded carefully. A delayed *rigor mortis* allows processing to take place before rigor occurs (Sigholt et al. 1997). With early *rigor mortis* the flesh can be difficult to process, reducing both the yield and flesh quality and resulting in a shorter shelf-life (Morzel et al. 2003). *Rigor mortis* is characterised by a progressive rigidity of the body due to a reduction of ATP levels in the muscle. Thus, an intense stress *antemortem* will increase the anaerobic metabolism which will consume energy reserves and will accelerate both the start and the resolution of the *rigor mortis* (Nakayama et al. 1992, Eriksson et al. 1997, Sigholt et al. 1997, Robb 2001). Under stressful conditions, there will be an accumulation of lactic acid in the muscle that will induce a reduction of the pH during sacrifice. This pH reduction will contribute to a faster *postmortem* drop of the muscle pH in the stressed fish. However, this was not the case in the present study. Even though, the muscle pH in ice slurry treatment was higher for the first 2 hours *postmortem*, from 8 hours onwards there were no differences among treatments indicating that if stunning was not improving the quality of the meat, it did not

significantly deteriorate it, which was also in accordance with studies in other fish species, such as Atlantic salmon (Sigholt et al. 1997) and eels (Morzel and Van der Vis, 2003). Flesh pH is considered a good indicator of the muscle texture (Love, 1980) and of the shelf-life of the fish (Foegeding et al. 1996, Zampacavallo et al. 2003, 2015). *Postmortem* muscle pH is around 7 and then it decreases to 6.5 or less due to lactic acid accumulation.

Altogether, the results obtained in the present study suggested that both gas mixture treatments, CO_2+N_2+O_2 and CO_2+N_2, have a high feasibility to be used as stunning method by the aquaculture industry to preserve the welfare of a Mediterranean fish species like gilthead seabream.

5. CONCLUSIONS

Although stunning with gas mixtures is not an immediate stunning method for seabream, the exposure to either 30% CO_2 and 70% N_2, or 40% CO_2, 30% N_2 and 30% O_2 induce less suffering than ice slurry treatment alone (EFSA 2009) which is a clear advantage to the seabream production.

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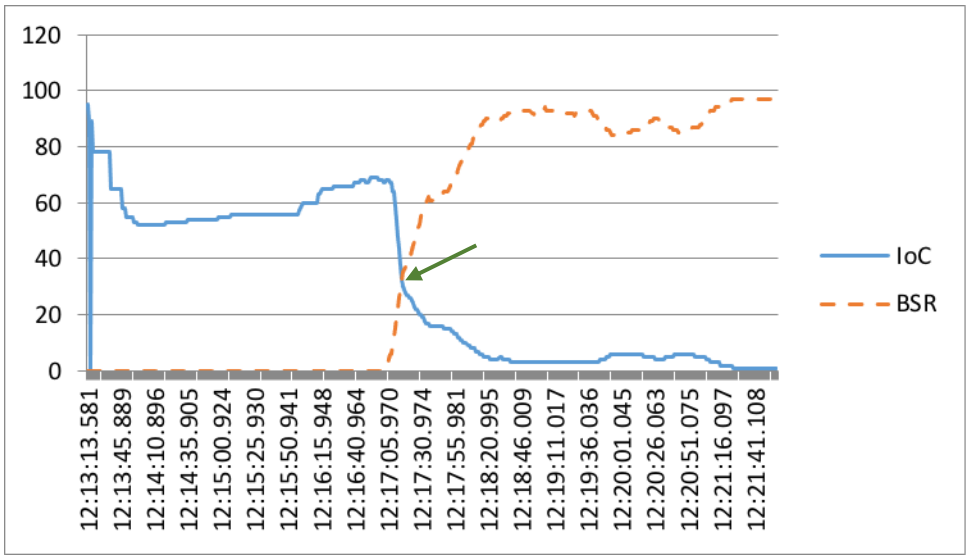
List of figures

Figure 1A



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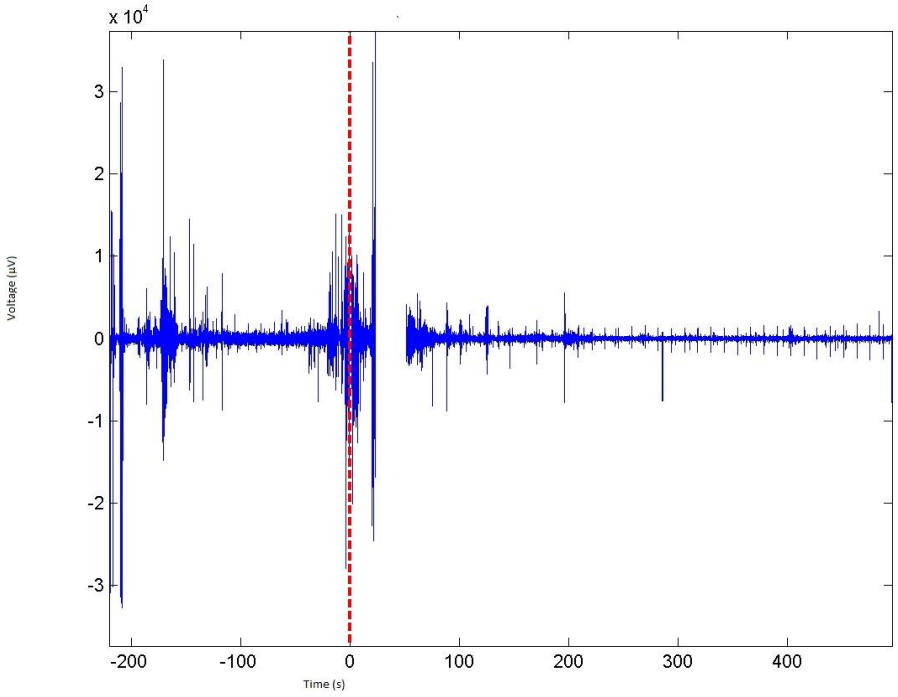

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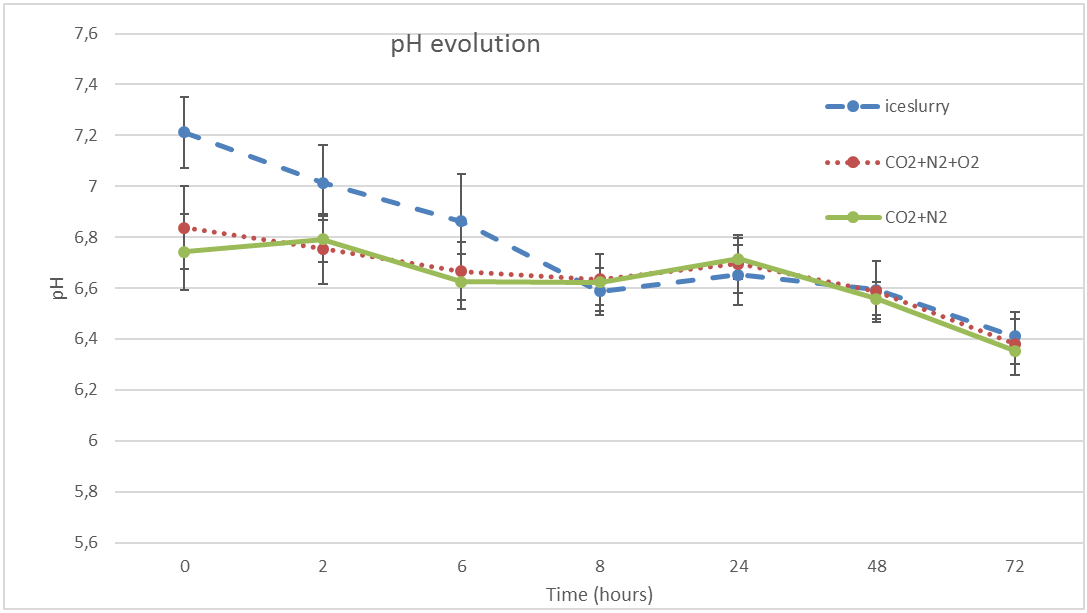

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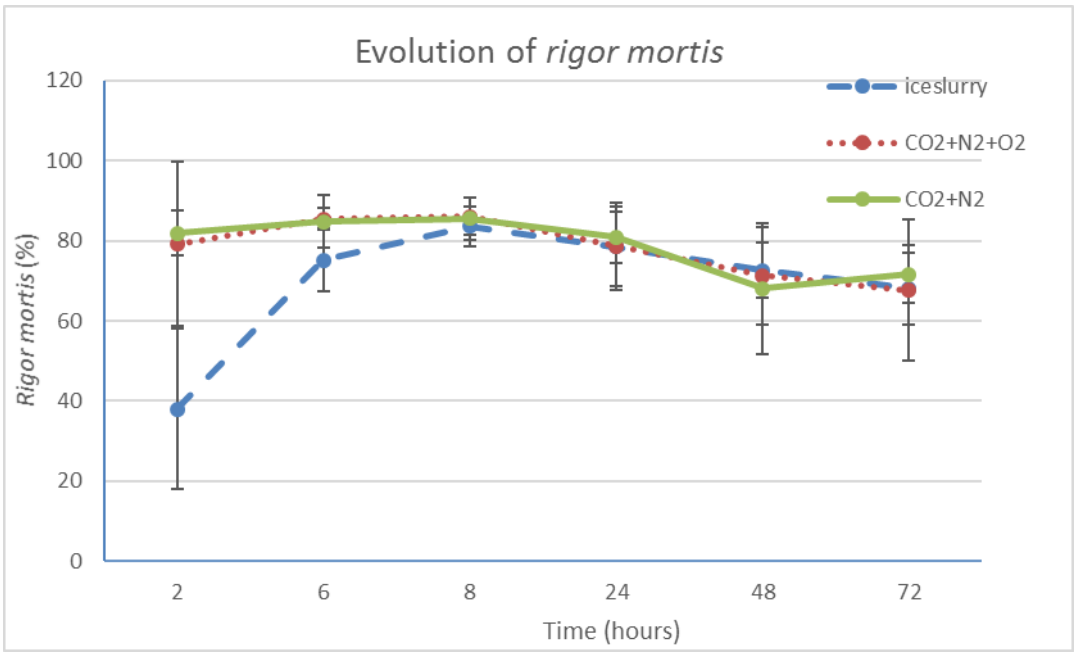

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1000 Supplementary material

1001 Relative gene expressions were estimated using *18S* gene as the house keeping gene. A

1002 one way ranked ANOVA to search for differences among treatments showed that the

1003 expression was different for *gapdh, hsp70, cox2* and *ef2α*, where:

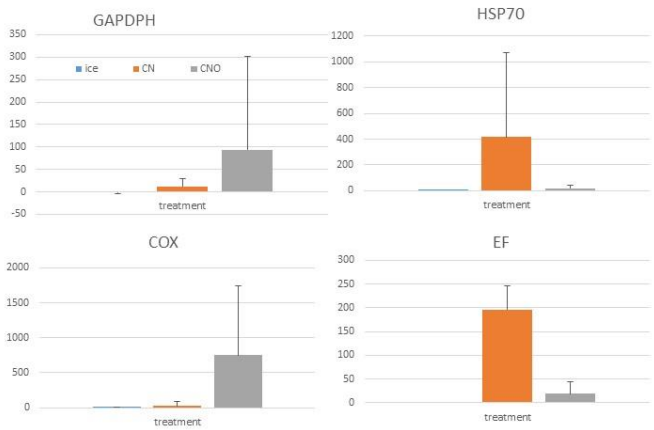
1004 Fish exposed to CO_2+O_2+N_2 differentially expressed *gapdh* (P = 0.014) from fish

1005 exposed directly to ice slurry; fish exposed to CO_2+N_2 differentially express *hsp70* (P =

1006 0.002) and *ef2α* (P = 0.005) from fish exposed directly to ice slurry (Figure S1).

1007

1008 Figure S1:



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