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Effects of a carbon monoxide stunning method on rigor mortis development, fillet quality and oxidative stability of tench (Tinca tinca)

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(Article begins on next page)

1 **Effects of a carbon monoxide stunning method on rigor mortis development, fillet**
2 **quality and the oxidative stability of tench (*Tinca tinca*)**

3

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22

23 **ABSTRACT**

24 The growing economic interest in tench has led to the need for further information on
25 the best slaughter methodologies for this species in order to respect animal welfare and
26 preserve fillet quality. Sixty farmed tench (*Tinca tinca*) were randomly divided into
27 three groups of killing: carbon monoxide (CO), electrical (ES) and percussive (PS)
28 stunning. The behaviour of the fish, the onset of *rigor mortis* and the gill cortisol
29 concentration were determined as stress indexes, while after rigor resolution, the quality
30 of the fillets were followed over a period of 10 days of refrigerated storage (+2.5 °C) by
31 determining physical and chemical properties of the fillets. The observations indicated
32 that the fish did not seem to perceive CO negatively, and normal swimming activity was
33 recorded. No external or internal damage was recorded for any of the killed fish. The
34 evolution of the rigor mortis index indicated that CO reached full rigor at about 15
35 hours *post mortem*, that is, an intermediate value was reached between ES (9 h) and PS
36 (19 h). The fish stunned with CO showed significantly lower gill cortisol level than the
37 ES and PS ones, that is, 0.408, 0.453 and 0.455 ng/mg protein, respectively. As far as
38 the quality parameters are concerned, the slaughter method significantly affected pH,
39 redness (a*) and yellowness (b*). No differences in pH were recorded for the killing
40 procedures at the end of rigor resolution, whereas CO derived flesh presented the lowest
41 pH from the second day till the end of the storage, when values of 6.44, 6.51, and 6.60
42 pH were found for CO, ES, and PS, respectively. The CO flesh presented the most
43 stable redness index over the whole trial and the lowest b* value from the beginning.
44 The drip loss values were unaffected by the killing method, but increased significantly
45 with the storage time. Neither the slaughter method nor the storage negatively impacted
46 on the fatty acid composition, which resulted to be equally rich in PUFA n3 and n6
47 (around 40 g/100 g total fatty acids). A numerical difference in the secondary lipid
48 oxidation products emerged for the different killing groups at the end of the refrigerated

49 storage. The CO flesh was the least oxidised, with the samples showing 1.33 mg MDA-
50 equivalents/100 g muscle against 1.60 and 1.55 found for ES and PS, respectively.

51

52 Keywords: Tench, stress, carbon monoxide, rigor mortis, cortisol, lipid oxidation,

53

54 **1. Introduction**

55 Tench (*Tinca tinca*, Linnaeus 1758) is a benthos-eating omnivorous cyprinid species
56 widely distributed throughout Europe, which shows a great potential for the aquaculture
57 sector. Many regions in Europe have a continental aquatic medium, which is
58 particularly appropriate for the growth of autochthonous tench. Thus, it is frequently
59 sold in local markets. In addition, the growing economic interest in tench is underlined
60 by the presence of niche productions, such as the Golden hump tench of the Poirino
61 highland, which has been awarded a European Protected Designation of Origin. In order
62 to develop a profitable tench farm industry, many technical aspects pertaining to the
63 culture of this species have been investigated over the last few decades, such as larval
64 and juvenile feeding trials (Sáez-Royuela et al., 2015; Wolnicki et al., 2017), the
65 determination of its lipid composition (Gasco et al., 2010; Ljubojević et al., 2014;
66 Łuczńska et al., 2012), the quality of its flesh (Gai et al., 2014), the effect of the rearing
67 system on the fish texture (Vácha et al., 2013) and the effect of slaughtering methods on
68 *rigor mortis* (Gasco et al., 2014).

69 The effect of the killing method on both animal welfare (EFSA, 2009; van de Vis et al.,
70 2003) and fish quality (Duran et al., 2008; Simitzis et al., 2013) is one of the most
71 critical and interesting topics of recent years. In many fish species, slaughtering
72 represents a moment of high stress with grave consequences on quality properties of the
73 fish, and the onset of *rigor mortis* being altered (Gasco et al., 2014), on the K freshness
74 index value (Tejada, 2009), as well as on the pH, colour and water holding capacity
75 (Robb et al., 2000). As a result of a request of the European Commission (EFSA, 2009),
76 welfare aspects pertaining to the main stunning and killing systems should be species-
77 specific. Currently, no official suggestions are available concerning the best slaughter
78 methodologies for tench. In Italy, tench are generally killed by asphyxia in air, a very

79 painful practice, which, as suggested by EFSA, should generally be avoided (EFSA,
80 2009).

81 In recent years, the use of carbon monoxide (CO) has emerged as a new stunning/killing
82 procedure for Atlantic salmon (*Salmo salar*, Linnaeus 1758) (Bjørlykke et al., 2013;
83 Secci et al., 2016) and rainbow trout (*Oncorhynchus mykiss*, Walbaum 1792)
84 (Concollato et al., 2014). These studies have shown that CO can be considered a good
85 stunning/killing method due to its sedative, low stressing response, and it can lead to
86 death if properly applied (Concollato et al., 2014). In addition, CO does not seem to
87 affect the oxidative stability of lipid or the cholesterol fraction of Atlantic salmon (Secci
88 et al., 2016).

89 The present work has had the aim of investigating the effect of euthanizing tench by
90 dissolving CO directly in water and comparing the result with no gas treatments, such as
91 percussion and electrical stunning. The effects on *rigor mortis* development and of
92 stress indices, such as cortisol, were investigated immediately after death. In addition,
93 drip loss, colour, pH, fatty acid composition and lipid oxidation were evaluated both
94 immediately after death and during a short term period (10 days) of refrigerated storage
95 (2.5 °C).

96

97 **2. Materials and Methods**

98 The experimental protocol was designed according to the guidelines of the current
99 European and Italian laws on the care and use of experimental animals (Directive
100 2010/63/UE, put into law in Italy with D. Lgs. 26/2014). The trial was conducted by
101 certified personnel.

102

103 **2.1 Killing procedure and fish behaviour analysis**

104 Sixty tench (mean weight 156.5 ± 9.8 g) collected from a tench farm (Cascina Italia,
105 Ceresole d'Alba, Italy) during the autumn period, were kept in concrete aerated tanks
106 for 4 days and fed the same commercial diet as that of carp used on the farm. The fish
107 were then made to fast for 24 h before killing. At the moment of slaughtering, the fish
108 were randomly divided into three groups for killing (20 fish/group): carbon monoxide
109 stunning (CO), electrical stunning (ES) and percussive stunning (PS). The fish
110 designated to be killed by CO were then placed into a plastic tank (20 litre, $T = 17 \pm$
111 0.5 °C) and 100% food grade CO (SIAD, Rosta, Italy) was bubbled directly into the
112 water using three ceramic diffusers, until the fish were confirmed dead upon visual
113 inspection (57 min). On the basis of the swimming activity, reactivity to visual and
114 tactile stimuli, equilibrium and ability to ventilate, the fish behaviour was assessed
115 according to Roth et al. (2003) using a GoPro digital camera (GoPro Hero 4 Silver
116 Edition, GoPro Inc., San Mateo, CA, USA).

117 Trained staff applied percussion to the head of fish restraining manually using a wooden
118 knob. Finally, for the ES slaughter method, fish were simultaneously subjected to a 22
119 V current (30 s) using a dry electro-stunner, manufactured according the local public
120 health service indications. Briefly, the fish were placed, without overlapping each other,
121 onto a wider stainless steel grid, which was connected to the electrodes of the stunner
122 electric panel, where the setting parameters (voltage) and time could be selected.

123

124 **2.2 Rigor mortis (RM) index**

125 The tench were tagged individually with a plastic label. The curvature of the tail (Bito et
126 al., 1983) was used as a basis for the RM measurement. After death (0), each fish was
127 placed on a flat surface for the next 27 h, its image was recorded by means of a digital
128 camera (Nokia D3100) and the obtained images were analysed using the ImageJ 1.37v
129 freeware software (National Institutes of Health, Bethesda, MD, USA). Measurement

130 Calibration of the measurements was made to the nearest 0.01 mm. The rigor index (I_R)
131 was then calculated by means of the $I_R = 100 \cdot (L_0 - L_t) / L_0$ formula, where L is the
132 vertical distance between the base of the caudal fin and the table surface, measured
133 immediately after death (L_0) and during storage (L_t , with t from 1 to 27 hours). The fish
134 were stored between layers of ice in plastic boxes placed in a refrigerated room (+
135 2.5 °C) between each measurement.

136

137 **2.3 Cortisol quantification**

138 Immediately after death, some gill filaments (approximately 10–20 mg of tissue) were
139 collected from all the fish to quantify the cortisol levels; the gill filaments were placed
140 in plastic tubes, immediately frozen to -80 °C and kept at this temperature until they
141 were analysed.

142 The cortisol levels in the gill filaments were analysed using a multi-species cortisol
143 enzyme immunoassay kit (L003; Arbor Assays[®], Ann Arbor, MI, USA), designed to
144 quantitatively measure the cortisol present in the different biological substrates (i.e.
145 dried fecal extracts, saliva, serum and tissue culture media samples) according to the
146 manufacturer's instructions.

147 According to Gesto et al. (2015), the gill tissue was first homogenized with an
148 ultrasonic homogenizer in 120 µL of phosphate-buffered saline (pH = 7.33). After
149 centrifugation, the supernatant was diluted (1/5, with ELISA buffer) and used for the
150 ELISA assay. No extraction was required as the linearity performed with multiple
151 dilutions (1:4, 1:8, 1:16 and 1:32) was good ($R^2 = 0.984$) and the inter- and intra-assay
152 coefficients of variation were less than 15%. All of the analyses were repeated twice.

153 As proposed by Gesto et al. (2015), the gill cortisol data were normalized according to
154 the tissue protein content, and the gill cortisol concentration was then expressed as
155 ng/mg protein. The protein concentration was assayed with the bicinchoninic acid

156 method (Smith et al., 1985).

157

158 **2.4 Physical parameters**

159 Immediately after RM resolution, 10 fish were frozen per slaughter method (-20 °C),
160 while the remaining 10 fish per group were degutted and filleted. The right hand side
161 fillets were immediately vacuum packed and frozen (-20 °C) and subsequently used for
162 fatty acid and lipid oxidation product (see sections 2.5 and 2.6) quantification as T0
163 samples.

164 Each left hand side fillet was weighed, labelled and kept on blotting paper in open bags
165 in a refrigerated room (+2.5 °C). The pH value was recorded on the cranial part of the
166 epaxial neck region, using a Crison portable pH-meter (Crison Instruments, S.A., Alella,
167 Spain) fitted with a combined electrode and an automatic temperature compensator,
168 over a period of up to 10 days at 5 sampling points (T0, T2, T4, T6, and T10). For the
169 drip loss (DL) measurements, each fillet was delicately blotted to remove any loose
170 liquid on its surface, and its weight was recorded at the same sampling points. DL was
171 determined by calculating the difference in weight for the different storage days. At the
172 same time as the DL measurements, the flesh colour was assessed by means of a
173 Chroma Meter CR-400 Konica Minolta Sensing bench colorimeter (Minolta Sensing
174 Inc., Osaka, Japan) (Ø 25 mm measuring area, 45° circumferential illumination/0°
175 viewing angle geometry). The colour measurements were reported in terms of lightness
176 (L*), redness (a*) and yellowness (b*) in the CIELab colour space model (Commission
177 Internationale de l'Éclairage, 1976). The values were recorded for CIE standard
178 illuminant D65 and the CIE 2° standard observer. The colour values were obtained
179 considering the average of three readings per sample. The colour differences between
180 the experimental groups were determined by considering ΔE_{2000} , calculated with the
181 Mokrzycki and Tatol (2011) formula: $\Delta E_{(\beta-\alpha)} = [(L^*_{\beta} - L^*_{\alpha})^2 + (a^*_{\beta} - a^*_{\alpha})^2 + (b^*_{\beta} - b^*_{\alpha})^2]^{0.5}$

182 where α and β represent the colour parameter values (L^* , a^* , and b^*) measured for CO,
183 ES and PS. At T10, the left hand side fillets were vacuum packed, frozen at $-20\text{ }^{\circ}\text{C}$ and
184 subsequently used for the fatty acid and lipid oxidation product quantification (section
185 2.6).

186

187 **2.5 Fatty acid determination**

188 The total lipid content of the samples was determined according to the Folch et al.
189 (1957) method and the fatty acids (FA) in the lipid extract were determined after trans-
190 esterification to methyl esters (FAME) using a base-catalyzed trans-esterification
191 followed by boron trifluoride catalyzed esterification (Morrison & Smith, 1964). The
192 FA composition was determined by means of gas chromatography (GC) using a Varian
193 GC 430 gas chromatograph (Varian Inc., Palo Alto, CA, USA), equipped with a flame
194 ionization detector (FID), and a Supelco Omegawax™ 320 capillary column (30 m \times
195 0.32 mm i.d., 0.25 μm film and a polyethylene glycol bonded phase; Sigma-Aldrich,
196 Saint Louis, MO, USA). The oven temperature was held at $100\text{ }^{\circ}\text{C}$ for 2 min, increased
197 to $160\text{ }^{\circ}\text{C}$ over 4 min, then increased to $220\text{ }^{\circ}\text{C}$ over 14 min, and finally kept at $220\text{ }^{\circ}\text{C}$
198 for 25 min. The injector and the detector temperatures were set at $220\text{ }^{\circ}\text{C}$ and $300\text{ }^{\circ}\text{C}$,
199 respectively. One μL of sample in hexane was injected into the column, with helium as
200 the carrier gas, which was kept at a constant flow of 1.5 mL/min. The split ratio was
201 1:20. Chromatograms were recorded using Galaxie Chromatography Data System
202 1.9.302.952 (Varian Inc., Palo Alto, CA, USA) computing integrator software. The fatty
203 acids were identified by comparing the FAME retention time with the standard Supelco
204 37 component FAME mix (Sigma-Aldrich, Saint Louis, MO, USA). The fatty acids
205 were quantified, through calibration curves, using tricosanoic acid (C23:0) (Sigma-
206 Aldrich, Saint Louis, MO, USA) as the internal standard. The Polyene index (PI) was
207 calculated from the fatty acid profile as $(\text{C}20:5\text{ n}3 + \text{C}22:6\text{ n}3)/\text{C}16:0$.

208

209 **2.6 Lipid oxidation products**

210 The 2-thiobarbituric acid reactive substances (TBARS) were measured, according to
211 Vyncke (1970), on the T0 and T10 samples, and the results were expressed as mg of
212 malondialdehyde (MDA)-equivalents/kg muscle (mg MDA-eq/ kg muscle).

213 The conjugated dienes (CD) were measured according to Srinivasan et al. (1996). The
214 absorbance of 100 μ L of lipid extract, suspended in 3 mL of hexane, was measured at
215 232 nm to determine the conjugated dienes. The concentration of conjugated dienes was
216 obtained using the molar extinction coefficient of 29000 mL mmol⁻¹ cm⁻¹. The results
217 were expressed as mmol hydroperoxides/kg lipid.

218

219 **2.7 Statistical analysis**

220 The obtained data were evaluated by means of the GLM procedure of the SAS statistical
221 software (SAS, 2004), and considering the slaughter method (treatment, T), storage (S)
222 and their interaction (T×S) as the main effects. The data were presented as the means of
223 each group and the residual standard deviation (RSD), together with the significance
224 levels of the main effects and interactions. Significance was established at $p < 0.05$.

225 **3. Results**

226 **3.1 Swimming and behaviour analyses**

227 Once CO had been bubbled into the tank, the fish exhibited normal behaviour for about
228 840 s (841±33 s), and then started to have equilibrium problems and to show a reduced
229 swimming activity that corresponded to Stage 1 (Roth et al., 2003). Stage 2 (light
230 narcosis) was reached after about 480 more seconds (1200-1440 s total; 1340±91 s),
231 while deep Stage 3 narcosis (no swimming activity, problems with operculum
232 ventilation and total loss of equilibrium) was reached in all the fish after 2100 s

233 (2111±75 s) of contact with CO in the water. Once all the fish showed no operculum
234 ventilation (2812±99 s), they were considered to be in Stage 4. The fish were kept 10
235 minutes more in the tanks until death was confirmed for all the fish.

236 **3.2 Rigor mortis (RM) index**

237 The rigor mortis index evolution indicated that the ES fish had a faster onset of rigor
238 mortis than the CO and PS tench (Figure 1). Full rigor mortis was reached in the three
239 slaughter methods at about 9, 15 and 19 hours *post mortem* for ES, CO and PS,
240 respectively.

241 **3.3 Cortisol quantification**

242 The gill cortisol levels of the experimentally stunned tench are reported in Figure 2. The
243 fish stunned with CO showed significantly ($p < 0.001$; RSD: 0.034) lower levels of
244 cortisol than those detected in the ES and PS groups. In fact, the cortisol levels
245 measured in the ES fish (0.453 ng/mg protein) and in the PS fish (0.445 ng/mg protein)
246 were approximately 9-10% higher than the cortisol levels detected in the CO fish (0.408
247 ng/mg protein).

248 **3.4 Physical parameters**

249 No fillet defects, such as gaping, bruising or blood spots, were observed in the fillets of
250 the differently slaughtered fish. The pH, drip loss (DL) and colour parameter values of
251 the tench are reported in Table 1. Both the slaughter methods and the storage affected
252 the pH value. Significantly lower pH values were found in the CO group than in the
253 others from the second day of storage onwards. The pH value tended to increase slightly
254 but significantly as the effect of storage increased, irrespective of the killing method.
255 DL was unaffected by the slaughter methods, but was altered by storage. As expected,
256 DL increased considerably over the 10 days at 2.5 °C.

257 The adopted slaughter method also affected the fillet colour (Table 1), in terms of a*
258 and b* values. No effect on lightness emerged, even though the CO group showed the
259 most lightness at the end of the storage. Interestingly, CO and PS were the least yellow
260 ones, right from the beginning of the trial, as indicated by their b* values. ES instead
261 presented the highest ($p < 0.05$) a*, and b* parameters. All the colour parameters
262 changed significantly during storage. The ES and PS lightness values decreased
263 significantly during storage, whereas a* and b* tended to rise over all the storage days.
264 As far as the a* values are concerned, a significant interaction between treatment and
265 storage was also found. The CO group maintained the lowest a* value among the
266 experimental groups for the entire duration of the test. In addition, the redness of the CO
267 fillets was not significantly altered, and was almost unchanged between T0 and T10. On
268 the contrary, a* was observed to increase significantly in the ES fish between 2 (T2) and
269 6 (T6) days of chilled storage, then dropped significantly at T10 (Table 1). Finally,
270 intermediate a* values were shown for the PS tench between ES and CO, from T0 till
271 the end of the trial, and they remained almost unchanged. However, in order to clarify
272 the differences in colour, ΔE_{2000} was calculated by comparing in pairs the colour values
273 of the fillets derived from the tench differently killed. ΔE_{CO-ES} , ΔE_{CO-PS} , and ΔE_{ES-PS}
274 were 2.65, 0.91, and 1.91, respectively. Interestingly, from these results, it can be
275 deduced that no difference in colour was perceived between PS and CO ($\Delta E_{2000} < 1$),
276 whereas ES was observed to be different from both CO and PS, with ΔE_{2000} being
277 calculated between 1 and 3.5 (Mokrzycki & Tatol, 2011).

278 **3.5 Fatty acid determination**

279 The average lipid content was around 3.12 g/100 g fillet, irrespective of the slaughter
280 method.

281 The FA composition of the total lipids is given in Table 2. The most representative fatty
282 acids were C18:1, C18:2 n6, C16 and C22:6 n3 (docosahexaenoic acid, DHA). C16:1
283 n7, C20:4 n6, and C20:5 n3 (eicosapentaenoic acid, EPA) also amounted to more than 3
284 g/100 g of the total fatty acids.

285 Overall, PUFA was the most represented lipid fraction (around 42%), and was
286 composed of an n3/n6 ratio of 1, and this was followed by the monounsaturated
287 (MUFA) and saturated (SFA) fractions. Neither the slaughter method nor the storage
288 affected the fatty acid composition to any great extent.

289 The polyene index did not significantly change as a result of killing or storage. Its
290 values remained around almost 1.

291 **3.6 Lipid oxidation products**

292 The primary and secondary oxidation product, CD TBARS, values are reported in Table
293 3. The slaughter method did not affect these parameters. However, the storage time
294 increased both the CD ($p > 0.05$) and TBARS ($p < 0.05$) in the fish fillets after 10 days
295 of storage. Furthermore, even though no statistical differences were found, the CO tench
296 were the least oxidized group at T10, and were followed by the PS and ES groups.

297

298 **4. Discussion**

299 The use of the correct stunning methodologies to process fish is of pivotal importance to
300 ensure both the welfare and the *post mortem* product quality of fish as well as their shelf
301 life.

302 In this trial, no fish recovered at the end of the treatments, and neither external nor
303 internal damage was recorded. These results differ from those reported by Lamboojij et
304 al. (2006), who observed fish that recovered after the direct use of ES in water, but
305 confirm the observations of Gasco et al. (2014) who reported that no fish recovered

306 after dry electro-stunning. In a previous research (Gasco et al., 2014), the same authors
307 concluded that a too long (30 s) or a too high voltage exposure (51 V) caused skin burns
308 and bloodspots on the fillets. In the present trial, both parameters (30 s and 22 V)
309 seemed to be appropriate, as none of the problems recorded before were observed. ES is
310 considered easy to apply to a large number of fish, but a too short time of electricity
311 exposure does not guarantee the effective death of fish, but only induces
312 unconsciousness (Lines & Spence, 2012). Therefore, a further process is often required
313 (gill cutting) to kill fish. In our research, no fish recovered after the electricity treatment,
314 thus indicating that death was immediate.

315 CO is not used widely for fish, but recent publications indicate how it could be used
316 without any visible stress response (Bjørlykke et al., 2011, 2012; Concollato et al.,
317 2014; Mantilla et al., 2008). The behavioural observation indicated that fish did not
318 seem to perceive the CO negatively, and a normal swimming activity was recorded in
319 the first minutes after contact with the substance, as already reported in other researches
320 (Bjørlykke et al., 2013; Concollato et al., 2014). No erratic swimming was recorded,
321 and no fish attempted to escape from the tank. The absence of attempting to escape
322 behaviour and the considerably long time that the fish needed to reach Stage 4 could be
323 ascribed to the ability of tench (and cyprinids) to tolerate critical water conditions, e.g.,
324 a low dissolved oxygen concentration, compared to salmonids (Jensen et al., 1993;
325 Podhorec et al., 2017). This seems to confirm that gradually increasing CO levels in
326 water can exert a calming effect on fish and can be considered positive, from a welfare
327 point of view.

328 The I_R evolution (Figure 1) showed clear differences among the experimental groups,
329 with an earlier onset of RM in the tench treated with ES. This confirms previous
330 observations (Gasco et al., 2014) where ES treated tench showed an earlier RM onset
331 than PS fish. CO fish showed an RM onset that was intermediate between the ES and

332 PS fish. When I_R was considered as a stress indicator, a lower level of stress was
333 observed in the CO fish than in the ES ones. The slaughter method where the RM onset
334 and resolution were the slowest was Ps, thus confirming that this slaughtering
335 methodology is optimal for the welfare of tench, especially considering their high value
336 on specific markets. Nevertheless, well trained personnel are needed to perform the
337 procedure (Gasco et al., 2010; Gasco et al., 2014).

338 Barton and Iwama (1991) categorized the stress response of fish into primary,
339 secondary, and tertiary responses. The primary response includes endocrine changes,
340 such as in the measurable levels of corticosteroids. Cortisol activates the secondary
341 response: it modulates some metabolic pathways that alter the blood chemistry, such as
342 the carbohydrate metabolism, thus stimulating glucose production through
343 gluconeogenesis (Barton & Iwama., 2002). When stressors persist, the primary and
344 secondary responses may affect the fish at an overall-animal level, and this leads to the
345 tertiary response. Consequently, the use of corticosteroids to assess stress in fish has
346 been studied extensively (Barton, 2002; Martínez-Porchas et al., 2009; Ellis et al.,
347 2012). Plasma cortisol has also been measured in cyprinids, including tench, as an
348 indicator of stress, but contrasting results are available in the literature. Gallardo et al.
349 (2010) found no statistical differences in cortisol levels between tench confined in
350 keepnets and a control group. On the contrary, juvenile tench under a white light
351 treatment had a significantly higher plasma cortisol content than fish under a dark
352 treatment (Owen et al., 2010). The *ante-mortem* phases, including the slaughtering
353 techniques, represent stressing moments for fish that can affect their welfare and
354 quality. For these reasons, CO has been proposed over the last few years as a new
355 stunning technique for fish. Several studies have shown that a CO killing treatment does
356 not cause any stress response, since it does not affect the swimming behaviour of fish,
357 but in some researches, plasma cortisol (and other stress related blood parameters) did

358 not clearly highlight these effects. In fact, no significant differences emerged in the
359 cortisol values between CO-treated Atlantic salmon and a control group (Bjørlykke et
360 al., 2011). However, the authors suggested that the detected cortisol values were high in
361 both groups, and the results were probably affected by experimental procedures (e.g,
362 hauling, transport, handling) that could have stressed the fish. Concollato et al. (2016)
363 have recently found lower plasma cortisol levels in rainbow trout stunned with CO, than
364 for other stunning methods (electroshock and asphyxia in the air). However, the
365 difference was not significant, due to the wide variability of the measured cortisol
366 values. In the present trial, the lower level of gill cortisol found in CO tench supports
367 the hypothesis that CO reduces the impact of stress and acts as a calming and/or a
368 sedative agent during slaughter procedures (Bjørlykke et al., 2013). Gesto et al. (2015)
369 were the first to use gills as a matrix to quantify the cortisol concentration in rainbow
370 trout and zebrafish (*Danio rerio* Hamilton, 1882). Furthermore, to the best of our
371 knowledge, this is the first time that the cortisol concentration has been measured in
372 CO-stunned tench and gill has been used as the matrix. Therefore, no reference values
373 are available in the existing literature. Having said this, the results obtained in the
374 present study appear to be comparable (although slightly lower) with the values detected
375 by Gesto et al. (2015): rainbow trout and zebrafish exposed to an acute stress showed
376 average gill cortisol concentrations that were approximately equal to 0.7 ng/mg protein
377 and 0.5 ng/mg protein, respectively.

378 The adopted killing method could have a negative impact on the final pH of the carcass
379 and consequently on its water holding capacity (Poli et al., 2005). Stress may in fact
380 cause a rapid glycogen and ATP depletion, anaerobic catabolism occurs quickly and
381 lactic acid is produced. A muscular pH reduction is a consequence of this catabolism.
382 The results of the present study have indicated that, immediately after rigor resolution,
383 the pH values did not differ significantly in the differently killed tench groups.

384 However, CO was found to have lower pH values than at all the other sampling points,
385 thus leading to the speculation of a protective effect of CO against microbiological
386 growth (Pivarnik et al., 2013), the main agent responsible for increases in pH during
387 storage. In this sense, other studies are suggested in order to clarify the potential use of
388 CO as both a killing and a preservative method.

389 It is widely accepted that the lower the muscle pH is, the higher the protein muscle
390 denaturation. In addition, as a consequence of a protein modification, a reduction in the
391 water retention capacity and an increase in drip loss have been observed (Poli et al.,
392 2005). It has already been shown that stress increases the drip loss in tench slaughtered
393 by percussion, electrically or by CO₂ (Gasco et al., 2014). On the contrary, data from the
394 present research have indicated that the differences in pH apparently did not affect the
395 loss of water from the fillets, in agreement with data found by Mantilla et al. (2008) on
396 CO-euthanizing tilapia. Thus, the stress caused by the utilization of CO may not be so
397 critical for the compromising of protein modifications and for the water retention
398 capacity.

399 Robb (2000) suggested that hyperactivity at slaughtering caused the flesh to be less red
400 and more yellow. High b* values were observed for the ES fish. Moreover, the increase
401 in this index during storage could be due to both lipid and protein oxidation. A previous
402 study has in fact shown that some oxidized forms of protein could give a brown-
403 yellowish appearance to the muscle (Kristinsson & Demir, 2003). Interestingly, the a*
404 index seemed to be influenced a great deal by both slaughtering and storage. CO
405 showed similar redness values to the PS ones for the entire duration of the trial, thus
406 contradicting previous data on the effectiveness of CO in increasing the redness colour
407 of white and red muscles when live fish were exposed to it (Mantilla et al., 2008).
408 Nevertheless, from the study of Mantilla et al. (2008) it also emerged that not only was
409 CO delivered scarcely to the white muscle during the CO euthanasia of tilapia, but its

410 concentration dropped during the first two days of chilled storage (4 °C). In addition,
411 the lengths of the CO treatment period have been proved to affect the a* value of
412 Atlantic salmon immediately after death (Bjørlykke et al., 2011). Hence, both the length
413 of CO slaughtering and the prevalence of white muscle in tench fillets might explain the
414 absence of a significant effect of the slaughter method on the redness index of the PS
415 and CO groups. On the other hand, the effect of the killing method on the flesh colour
416 has often been observed in fish (Bjørlykke et al., 2011; Lefèvre et al., 2008; Simitzis et
417 al., 2013), but this effect depends to a great extent on the slaughter method that is
418 adopted and on the fish species. A more intense a* value was measured, for example,
419 after the electrical stunning of rainbow trout than catfish (*Silurus glanis*, Linnaeus 1758)
420 (Marx et al., 1999), hence confirming the data obtained for tench.

421 The fatty acid composition is known to undergo a great variability, as it is influenced by
422 numerous intrinsic and extrinsic factors, such as species and diet. For this reason, it is
423 difficult to make reasonable comparisons. However, the present results are in agreement
424 with a previous study on tench in which the prevalence of the PUFA fraction was
425 reported, followed by MUFA and SFA (Łuczńska et al., 2012). Even the
426 representativeness of the single fatty acids was in agreement with previously reported
427 data (Łuczńska et al., 2012; Ljubojević et al., 2014). A similar n3/n6 ratio was also
428 observed in the case of tench reared on natural food in a pond (1.0) and tench fed
429 supplementary wheat in a pond (1.1) (Steffens & Wirth, 2007). Interestingly, the PUFA
430 fraction, especially n3, did not decrease with storage.

431 CO has been added to food for decades, because of its ability to improve oxidative
432 stability (Cornforth & Hunt, 2008) due to the affinity of CO to binding heme protein (at
433 least 240 times higher than O₂). Because of oxygen depletion from the tissue, an
434 increase in oxidative stability should be noted. The present finding instead revealed no
435 effect of stunning on either the primary or secondary oxidation products. The lipid

436 oxidation results agree with data on oxidative damage on Atlantic salmon subdued by a
437 CO stunning/killing method (Secci et al., 2016). In both researches, CO did not seem to
438 improve the oxidative stability of lipids during refrigerated storage. As previously
439 discussed for colour, it is possible that the CO concentration in the muscle was not
440 sufficient to efficiently prevent lipid oxidation. Nevertheless, the tendency of the CO
441 group to be the least oxidised group at the end of the trial should be underlined, and it is
442 thus possible to hypothesise that the CO action against oxidative damage might be
443 studied better during long-term frozen storage.

444

445 **5. Conclusions**

446 Carbon monoxide appears to be a suitable technique for killing tench. Fish exposed to
447 CO have shown very low stress responses in both behaviour and stress index (for
448 example, cortisol) analyses. The behavioural observation indicated that the fish did not
449 seem to perceive CO negatively, and a normal swimming activity was recorded. CO in
450 water can in fact exert a calming effect on fish and can be considered positive, from a
451 welfare point of view. Even the *rigor mortis* onset has indicated a lower level of stress
452 in the CO fish than in the ES ones. A high animal welfare ensures, as in this case, a
453 good flesh quality both immediately after rigor resolution and at the end of a
454 refrigerated storage period. The flesh from the fish treated with CO has shown a more
455 stable colour as well as the lowest secondary oxidation products. The antimicrobial
456 effect of CO suggested by the lowest pH values deserves further investigations.

457

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461

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609

610 **Table 1.** Physical parameters (pH, colour, drip loss – DL, %) of tench stunned by
 611 carbon monoxide (CO), electrical stunning (ES) and percussion (PS), immediately after
 612 death and during 10 days of refrigerated storage (2.5 °C).

		pH	DL	L*	a*	b*
T0	CO	6.31 ^b	-	54.09	-2.47 ^f	5.36 ^{Bd}
	ES	6.33 ^b	-	54.54 ^a	-1.97 ^{def}	6.84 ^{Ad}
	PS	6.37 ^c	-	52.96 ^{ab}	-2.26 ^{ef}	6.04 ^{ABd}
T2	CO	6.43 ^{Ba}	5.84 ^d	52.75	-1.27 ^{cdef}	6.56 ^{Bc}
	ES	6.50 ^{ABa}	6.34 ^d	52.54 ^{ab}	1.72 ^a	8.82 ^{Abc}
	PS	6.52 ^{Ab}	5.80 ^d	54.18 ^a	-1.47 ^{cdef}	7.36 ^{Bc}
T4	CO	6.44 ^{Ba}	8.23 ^c	53.27	-1.40 ^{cdef}	7.08 ^{bc}
	ES	6.51 ^{ABa}	8.81 ^c	51.53 ^b	0.66 ^{ab}	7.90 ^{cd}
	PS	6.52 ^{Ab}	8.31 ^c	50.67 ^c	-0.38 ^{bc}	7.19 ^c
T6	CO	6.47 ^{Ba}	10.87 ^b	52.61	-0.95 ^{cde}	8.10 ^{Bb}
	ES	6.57 ^{Aa}	11.33 ^b	52.11 ^b	1.52 ^a	9.42 ^{Ab}
	PS	6.55 ^{Aab}	10.99 ^b	52.27 ^{abc}	-0.31 ^{bc}	8.73 ^{ABb}
T10	CO	6.44 ^{Ca}	14.13 ^a	52.07	-1.23 ^{cdef}	9.95 ^a
	ES	6.51 ^{Ba}	14.36 ^a	50.77 ^b	-0.74 ^{cd}	10.71 ^a
	PS	6.60 ^{Aa}	14.46 ^a	51.56 ^{bc}	-0.86 ^{cde}	9.87 ^a
Treatment		<0.05	NS	NS	<0.05	<0.05
Storage		<0.05	<0.05	<0.05	<0.05	<0.05
T×S		NS	NS	NS	<0.05	NS
RSD ¹		0.112	1.53	3.43	2.26	1.80

613 NS. Not Significant ($p > 0.05$). A, B, C: as superscript letters mean significant differences ($p <$
 614 0.05) among treatments; a, b, c: as superscript letters mean significant differences ($p < 0.05$)
 615 among storage time. If significant ($p < 0.05$) interaction (T×S) emerged, a, b, c mean
 616 significantly different samples.

617 ¹ RSD: Residual Standard Deviation.

618

619 **Table 2.** Fatty acid composition (g/100 g total fatty acids) of tench stunned by carbon
 620 monoxide (CO), electrical stunning (ES) and percussion (PS), immediately after death
 621 (T0) and after 10 days (T10) of refrigerated storage (2.5 °C).

	Treatment (T)			Storage (S)		Significance			RSD ¹
	CO	ES	PS	T0	T10	T	S	T×S	
C16	15.24	14.52	14.55	14.70	14.85	NS	NS	NS	0.88
C16:1 n7	6.59	6.79	7.00	6.80	6.79	NS	NS	NS	1.64
C18	3.30	3.10	3.21	3.16	3.24	NS	NS	NS	0.42
C18:1 n9	24.23	24.12	25.27	24.72	24.36	NS	NS	NS	2.79
C18:2 n6	15.81	15.74	15.23	15.68	15.50	NS	NS	NS	1.58
C20:4 n6	3.04	3.32	2.96	3.13	3.08	NS	NS	NS	1.13
C20:5 n3	3.21	3.35	2.93	3.16	3.17	NS	NS	NS	0.61
C22:6 n3	12.42	11.93	12.42	12.08	12.43	NS	NS	NS	2.82
ΣSFA	20.72	19.86	19.84	20.01	20.27	NS	NS	NS	1.08
ΣMUFA	35.83	36.30	37.66	36.84	36.39	NS	NS	NS	3.84
ΣPUFA						NS	NS		
n6	21.82	22.12	21.25	21.84	21.62			NS	1.24
ΣPUFA						NS	NS		
n3	21.05	21.14	20.70	20.78	21.14			NS	3.39
PI	1.03	1.05	1.05	1.04	1.05	NS	NS	NS	0.03

622 The fatty acids C12:0, C13:0, C14:1 n5, C15:0, C15:1, C16:1 n9; C16:2 n4, C16:3 n4, C16:4
 623 n1, C17:0, C17:1, C18:1 n7, C18:3 n6, C18:3 n4, C18:4 n1, C20:0, C20:1 n11, C20:1 n7, C20:2
 624 n6, C20:3 n6, C20:3 n3, C20:4n6, C20:4 n3, C21:0, C21:5 n3, C22:0, C22:1 n9, C22:1 n7,
 625 C22:2 n6, C22:4 n6, C22:5 n6, C24:0, and C24:1 n9 were also detected but not reported because
 626 in percentage <3%. They were utilised to calculate Σ.

627 NS. Not Significant ($p > 0.05$), A, B, C,: as superscript letters mean significant different values
 628 ($p < 0.05$).

629 ¹ RSD: Residual Standard Deviation.

630

631 **Table 3.** Lipid oxidation expressed as conjugated dienes (CD, mmol hydroperoxides/kg
 632 lipid) and TBARS (mg MDA-eq/kg muscle) of tench stunned by carbon monoxide
 633 (CO), electrical stunning (ES) and percussion (PS), immediately after death (T0) and
 634 after 10 days (T10) of refrigerated storage (2.5 °C).

		CD	TBARS
T0	CO	5.067	0.293
	ES	5.905	0.293
	PS	5.616	0.256
T10	CO	5.941	1.330
	ES	5.929	1.603
	PS	5.608	1.553
Treatment		NS	NS
Storage		NS	<0.001
T×S		NS	NS
RSD ¹		0.935	0.448

635 NS. Not Significant ($p > 0.05$).

636 ¹ RSD: Residual Standard Deviation.

637

638 **Caption of the Figures**

639

640 **Figure 1.** Rigor index (IR) evolution during 27 hours of tench killed by carbon
641 monoxide (CO), electrical stunning (ES) and percussive stunning (PS). Bar represents
642 the mean \pm standard error of the values reported for each sampling point (hours)
643 measurements recorded on twenty fish for each stunning procedure.

644

645 **Figure 2.** Gill cortisol levels (ng/mg protein) of tench stunned by carbon monoxide
646 (CO), electrical stunning (ES), and percussion (PS). Bar represents the least square
647 mean \pm standard error of the mean. Different letters (a, b) indicate significant ($p <$
648 0.001) difference within treatments.