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

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

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ABSTRACT

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

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

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

1. Introduction

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

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

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

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

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

2. Materials and Methods

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

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 ±
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 $I_R = 100*(L0-Lt)/L0$ formula, where L is the
132 vertical distance between the base of the caudal fin and the table surface, measured
133 immediately after death (L0) and during storage (Lt, 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: $\Delta E_{(\beta-\alpha)}=[(L^*_\beta-L^*_\alpha)^2+(a^*_\beta-a^*_\alpha)^2+(b^*_\beta-b^*_\alpha)^2]^{0.5}$

where α and β represent the colour parameter values (L*, a*, and b*) measured for CO, ES and PS. At T10, the left hand side fillets were vacuum packed, frozen at -20 °C and subsequently used for the fatty acid and lipid oxidation product quantification (section 2.6).

2.5 Fatty acid determination

The total lipid content of the samples was determined according to the Folch et al. (1957) method and the fatty acids (FA) in the lipid extract were determined after trans-esterification to methyl esters (FAME) using a base-catalyzed trans-esterification followed by boron trifluoride catalyzed esterification (Morrison & Smith, 1964). The FA composition was determined by means of gas chromatography (GC) using a Varian GC 430 gas chromatograph (Varian Inc., Palo Alto, CA, USA), equipped with a flame ionization detector (FID), and a Supelco Omegawax™ 320 capillary column (30 m × 0.32 mm i.d., 0.25 μm film and a polyethylene glycol bonded phase; Sigma-Aldrich, Saint Louis, MO, USA). The oven temperature was held at 100 °C for 2 min, increased to 160 °C over 4 min, then increased to 220 °C over 14 min, and finally kept at 220 °C for 25 min. The injector and the detector temperatures were set at 220 °C and 300 °C, respectively. One μL of sample in hexane was injected into the column, with helium as the carrier gas, which was kept at a constant flow of 1.5 mL/min. The split ratio was 1:20. Chromatograms were recorded using Galaxie Chromatography Data System 1.9.302.952 (Varian Inc., Palo Alto, CA, USA) computing integrator software. The fatty acids were identified by comparing the FAME retention time with the standard Supelco 37 component FAME mix (Sigma-Aldrich, Saint Louis, MO, USA). The fatty acids were quantified, through calibration curves, using tricosanoic acid (C23:0) (Sigma-Aldrich, Saint Louis, MO, USA) as the internal standard. The Polyene index (PI) was calculated from the fatty acid profile as (C20:5 n3 + C22:6 n3)/C16:0.

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 µL 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

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

3.2 Rigor mortis (RM) index

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

3.3 Cortisol quantification

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

3.4 Physical parameters

No fillet defects, such as gaping, bruising or blood spots, were observed in the fillets of the differently slaughtered fish. The pH, drip loss (DL) and colour parameter values of the tench are reported in Table 1. Both the slaughter methods and the storage affected the pH value. Significantly lower pH values were found in the CO group than in the others from the second day of storage onwards. The pH value tended to increase slightly but significantly as the effect of storage increased, irrespective of the killing method. DL was unaffected by the slaughter methods, but was altered by storage. As expected, 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. $\Delta E_{CO\text{-}ES}$, $\Delta E_{CO\text{-}PS}$, and $\Delta E_{ES\text{-}PS}$
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 Lambooij 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

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

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

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

The adopted killing method could have a negative impact on the final pH of the carcass and consequently on its water holding capacity (Poli et al., 2005). Stress may in fact cause a rapid glycogen and ATP depletion, anaerobic catabolism occurs quickly and lactic acid is produced. A muscular pH reduction is a consequence of this catabolism. The results of the present study have indicated that, immediately after rigor resolution, 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 CO_2 (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 O_2). 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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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

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

	Treatment (T)			Storage (S)		Significance			
	CO	ES	PS	T0	T10	T	S	T×S	RSD[1]
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 n6	21.82	22.12	21.25	21.84	21.62	NS	NS	NS	1.24
ΣPUFA n3	21.05	21.14	20.70	20.78	21.14	NS	NS	NS	3.39
PI	1.03	1.05	1.05	1.04	1.05	NS	NS	NS	0.03

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 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 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, C22:2 n6, C22:4 n6, C22:5 n6, C24:0, and C24:1 n9 were also detected but not reported because in percentage <3%. They were utilised to calculate Σ.

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

[1] RSD: Residual Standard Deviation.

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 ± 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 ± standard error of the mean. Different letters (a, b) indicate significant ($p <$
648 0.001) difference within treatments.