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Rainbow trout (*Oncorhynchus mykiss*) farmed at two different temperatures: *Post rigor mortis* changes in function of the stunning method

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Abstract: *Post rigor mortis* changes of texture, chemical and sensory properties in rainbow trout (*Oncorhynchus mykiss*) reared at two different temperature conditions (8 and 12 °C) were examined to better understand how different stunning methods, *i.e.* electroshock (E) and asphyxia with carbon monoxide (CO), can influence their evolution during refrigerated storage. Seven days after *rigor* resolution ($T_{RR}7$), considering ATP catabolites (K- and K1-values), the freshness remained well preserved regardless of the stunning method applied and water temperature. During refrigerated storage fillets from fish reared at 8 °C maintained significantly higher ($P < 0.001$) pH at the day of *rigor* resolution ($T_{RR}0$), whereas at the end of the storage time ($T_{RR}7$), 8 °C-reared fish showed a significantly lower pH value ($P < 0.05$). CO treatment was effective in ensuring a more intense red colour of the fillet and high chroma, whereas E treatment exhibited the lowest a*, b* and chroma values. The texture profile analysis showed a significant effect of the stunning method (S), water temperature (T) and S × T interaction on fillet cohesiveness. TBARS values were significantly lower ($P < 0.05$) in fish stunned by CO when compared to E group in the first 76 h *post mortem* ($T_{RR}0$). At the end of the storage period ($T_{RR}7$), no TBARS value difference was detected between treatments. The stunning method had a relevant impact on fillet sensory traits, revealing that CO fillets were the juiciest ($P < 0.05$) and presented the lowest saltiness ($P < 0.05$), aroma ($P < 0.05$) and odour ($P < 0.01$) intensity. Rearing temperature, instead, had a moderate effect on fillet sensory traits and indicated that the water temperature of 12 °C enhanced juiciness ($P < 0.05$) and tenderness ($P < 0.05$) attributes. Overall results suggested that CO is a suitable stunning method for trout that, coupled with 12 °C water temperature, are able to preserve fillet freshness, enhance colorimetric characteristics which are maintained during refrigerated storage, and provide desirable sensory traits.

Keywords: electricity; carbon monoxide; animal welfare; product quality; lipid oxidation; sensory analysis; fish

Animal welfare and product quality are linked aspects of the total quality of fish. To maintain the best original quality, fish should be stunned until death and killed by avoiding any kind of stress (Poli et al. 2005). The most relevant fish stunning methods are mechanical percussion, CO_2 narcosis

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and electrical stunning (Robb and Kestin 2002). Percussive stunning is mainly used for salmon and other large fish: the fish are hit in the frontal part of the brain which makes them instantly insensible. As the fish recovery is still possible if the destruction of the brain is partial, it is important that the method will be followed by bleeding or by another slaughtering practice (Wall 2001). Carbon dioxide narcosis is used on some salmonid farms: fish are placed in a bath with CO_2 gas saturated water (> 400 mg/l with pH of 5.0–5.5). The CO_2 dissolves in water to form an acid, the pH of fish blood is lowered and consequently the fall causes the destruction of brain activity, narcosis and eventually death (Kestin et al. 1995; Robb 2001) in about 3–4 minutes (in salmonids). Subsequently, fish are slaughtered by cutting the gills and bled. Researches have shown that several species of fish exhibit aversive behaviour towards CO_2 narcosis and loss of sensation may occur in a few minutes, depending on the species, resulting in the total exhaustion of the fish at the time of death (Robb 2001). Fish reach the condition of *rigor mortis* approximately two hours after death, when fish is still in the processing line. Due to the above-mentioned aversive behaviour, the use of CO_2 to stun fish has been banned in Norway since 2008.

The use of carbon monoxide (CO) presents an attractive alternative to the use of CO_2 for fish stunning, as it does not produce any aversive effects by animals, as happens with CO_2 (Poli et al. 2005). CO forms bonds with haem proteins (haemoglobin, myoglobin – Mb, and neuroglobin) for which it presents a much greater affinity than O_2, thus replacing it (Tsai et al. 2012); therefore, substantial absorption of CO can be lethal. When fish are exposed to CO, microbial growth, lipid oxidation and browning may possibly be reduced, therefore the shelf-life of the product is prolonged (Cornforth and Hunt 2008). This would be preferable in fatty fish like salmon and trout, which are highly vulnerable to lipid oxidation due to the high level of unsaturated fatty acids and the haem containing proteins (Secci and Parisi 2016). CO acts as a reducing agent when it forms complexes with iron or copper in enzymes, and therefore the haem-catalysed lipid oxidation is reduced when CO is bound to the haem. Meat exposed to CO at low levels showed desirable bright red, stable colour of the muscle (Cornforth and Hunt 2008). Similarly, the colour characteristics of fish fillets from Atlantic salmon and tilapia also showed to be positively affected when live fish were exposed to CO (Mantilla et al. 2008; Concollato et al. 2014).

Electronarcosis (typically 50–70 V) is used as a routine practice for laboratory purposes or on some farms, especially in the case of salmonids: it is considered "humane" because, if properly applied, the animal is rendered immediately insensible as the electric current stops the brain activity (Kestin et al. 1995). Electrical stunning is immediate, easy to control, efficient (Wall 2001), and it makes possible to anaesthetise many fish at the same time (Roth et al. 2003). On the other hand, however, the strong contraction of the muscles causes tetany rather than anaesthesia and intense electrical currents can damage the carcass (Kestin et al. 1995), causing hematoma, blood clots, spinal and vertebrae fractures (Kestin et al. 1995; Wall 2001).

Therefore, it is clear that the stunning method can greatly affect the process of *rigor mortis* and *post mortem* fillet quality changes (Robb 2001). Moreover, it also emerged that CO seems to be a promising stunning/killing technique for salmonids, alternative to the conventional electrical stunning (Secci and Parisi 2016). However, more research efforts are requested to fully understand the impact that this technique has on fish *pre* and *post rigor mortis* changes, including qualitative aspects of the fillet. Furthermore, another factor which has not been considered yet, and which could represent a possible source of variation in the observed results, is the water temperature. Based on these premises, the present research investigated the effects of two different stunning methods, *i.e.* electroshock (E) and carbon monoxide asphyxia (CO), on rainbow trout (*Oncorhynchus mykiss*) farmed at two different water temperatures (12 and 8 °C). Results of the first part of the study on the *pre rigor* changes were recently published by Concollato et al. (2020), while the present research focused on *post rigor mortis* fillet quality characteristics.

MATERIAL AND METHODS

Experimental design

The study was performed on the Edmund Mach Foundation's experimental farm, which is located in San Michele all'Adige, Trento province (Italy). The experimental protocol was designed according

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to the current guidelines on the care and use of experimental animals (European Directive 2010/63/EU, put into law in Italy with D. Lgs. 26/2014).

For the experiment, 400 rainbow trouts (*Oncorhynchus mykiss*) were equally distributed into four tanks, each containing 3 600 l of freshwater, whose level of dissolved oxygen was 9.0 ± 0.5 mg O_2/l. Water temperature in the first two tanks was 12 °C, while in tanks 3 and 4 water temperature was 8 °C. All trout were fed the same standard commercial diet. Overall, trout mean weight was 729 ± 105 g: 740 ± 105 g for tank 1, 737 ± 120 g for tank 2, 773 ± 101 g for tank 3 and 667 ± 97 g for tank 4.

After fasting (24 h), trout were subjected to the following stunning methods: electroshock (E) was applied to fish of tanks 1 (12 °C) and 4 (8 °C), while treatment with carbon monoxide (CO) was applied to fish of tanks 2 (12 °C) and 3 (8 °C). Treatment E was performed by using the electronic teaser GOZLIN TEQ002 (GOZLIN, Modena, Italy) for 30 s at 180 V: trout were captured, hauled out of water, and immediately treated with electricity. For the CO (asphyxia) treatment, trout were flushed with 100% food grade CO (SIAD, Bergamo, Italy) until stunning, which occurred within 49 minutes. The fish stunning status was visually assessed according to Roth et al. (2003). During the stunning procedures the safety of each person involved in the experiment was guaranteed by the presence of the firemen. In the case of CO treatment, they monitored the concentration of CO in the air by using portable gas detectors (GasBadge Pro, Oakdale, USA). After applying the stunning treatments, all fish were percussively slaughtered by a wooden knob. Afterwards, n = 18 trout were sampled from each experimental treatment and used for subsequent analyses: they were selected in order to represent the corresponding experimental group in terms of mean live weight and variability.

Energy metabolism, freshness indices, drip losses, pH, colour and texture profile analysis

All trout (n = 72: n = 18/treatment) were individually tagged and weighed. Afterwards, n = 3 trout/treatment were manually filleted in *pre rigor* condition, put in polystyrene containers, and stored for 10 days in a cold chamber set at 1 ± 1 °C.

On the left fillets, drip loss was calculated by weighing the left fillets immediately after death (T0), at the time of *rigor* resolution ($T_{RR}0$, *i.e.* 76 h *post mortem*), and seven days after *rigor* resolution (corresponding to the end of the trial; $T_{RR}7$). Drip loss was subsequently calculated by applying the following formula:

$$DL = [(D0 - D_{RR}0 \text{ or } D_{RR}7)/D0] \times 100 \quad (1)$$

where:

DL (t) – drip loss;

D0 – the fillet weight immediately after death;

$D_{RR}0$ – the fillet weight at the time of *rigor* resolution;

$D_{RR}7$ – the fillet weight seven days after *rigor* resolution.

A total of n = 3 values of drip loss were then calculated: from T0 till $T_{RR}0$, from $T_{RR}0$ till $T_{RR}7$, and the cumulative value from T0 till $T_{RR}7$.

On the right fillet (cranial side of the epaxial part), 1.5 g of muscle was sampled at *rigor* resolution (76 h *post mortem*) and seven days after *rigor* resolution ($T_{RR}0$ and $T_{RR}7$, respectively). They were used to detect K- and K1-values in the muscle at the end of the considered storage period ($T_{RR}7$), which are involved in an advanced period of freshness evolution.

To this purpose, the concentrations of adenosine 5'-triphosphate (ATP) and related catabolites, *i.e.* adenosine 5'-diphosphate (ADP), adenosine 5'-monophosphate (AMP), inosine 5'-monophosphate (IMP), inosine (Ino) and hypoxanthine (Hx) were determined by HPLC. In detail, 1.5 g of muscle was homogenised with 10 ml of 6% perchloric acid (PCA; Sigma-Aldrich, St. Louis, USA). After centrifugation at 3 200 × g at 4 °C for 10 minutes (Allegra®X-12R, Beckman Coulter Inc, Brea, USA) and resting at –20 °C for 20 min, the samples were filtered using 100 mm filter paper. The extract was adjusted to pH 6.8–7 using 0.6 and 0.1 M potassium hydroxide (Sigma-Aldrich, St. Louis, USA), filtered and finally it was brought to 25 ml volume with 50 mM tampon phosphate (pH 7). Before HPLC injection, 100 µl of sample were filtered using a 13 mm GHP 0.2 µm filter (Waters, Milford, USA). The HPLC apparatus comprised a pump system (Beckman mod. 125-S) equipped with a UV detector (Beckman mod. 166; Beckman Coulter Inc., Brea, USA) with absorbance fixed at 254 nm, analogic interface (Beckman mod. 406), Ultrasphere ODS Reversed-Phase column (Beckman, length

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250 mm, internal diameter 4.6 mm; particle size 5 µm; pore size 80 Å), Ultrasphere ODS pre-column (4.6 mm ID, 45 mm length), and 20-µl fixed loop. The mobile phase was KH_2PO_4, 0.5 M, pH 7.0. Standards were purchased from Sigma-Aldrich (St. Louis, USA). The injection volume was 10 µl and detection was monitored at 254 nm. The total separation time was 12 min with a flux of 1 ml/minute. Results were expressed as mM nucleotides/g muscle, and they were used to calculate K- and K1-values in the muscle as follows:

$$K = [(Ino + Hx)/(ATP + ADP + AMP + IMP + Ino + Hx)] \times 100 \quad (2)$$

$$K1 = [(Hx + Ino)/(Hx + Ino + IMP)] \times 100 \quad (3)$$

where:
K (%) – K-value;
Ino – inosine;
Hx – hypoxanthine;
ATP – adenosine 5'-triphosphate;
ADP – adenosine 5'-diphosphate;
AMP – adenosine 5'-monophosphate;
IMP – inosine 5'-monophosphate;
K1 (%) – K1-value.

The other n = 15 trout/treatment were put into polystyrene boxes covered with crushed ice and allocated in a cold room where temperature was kept in the range 0–2 °C. Trout remained in such storage conditions until *rigor mortis* resolution ($T_{RR}0$) occurred, *i.e.* 76 h *post mortem*.

After *rigor mortis* resolution (76 h *post mortem*, time $T_{RR}0$), all the 60 fishes were transferred to a processing plant (ASTRO; San Michele all'Adige, Trento, Italy), where they were mechanically filleted and weighed. Afterwards, right fillets were vacuum sealed and stored at –80 °C until sensory analysis was performed ($T_{RR}0$ samples).

Besides, left fillets were placed in polyester trays with absorbent pads on the bottom and stored for seven days ($T_{RR}7$ samples) in a cold room (2.5 °C) for the analyses scheduled during storage. At days $T_{RR}0$ and $T_{RR}7$, CIE L*a*b* colour values (Commission Internationale de l'Eclairage 1976) and pH were measured twice on each fillet. Colorimetric characteristics were recorded using a spectrocolorimeter (X-Rite, RM200QC; X-Rite, Incorporated, Neu-Isenburg, Germany), while pH was measured with a Mettler Toledo FiveEasy™/FiveGo™ pH meter (Mettler-Toledo Ltd, Leicester, UK). Furthermore, colour saturation [chroma = $\sqrt{(a^{*2} + b^{*2})}$] and hue angle [arctan (b*/a*)] were computed.

A texture profile analysis (TPA) was carried out seven days after *rigor* resolution ($T_{RR}7$), using a Zwick Roell® 109 texturometer (software: Text Expert II, v3; Zwick/Roell, Ulm, Germany) which was equipped with a 1 kN load cell. Kramer cell and Warner-Bratzler shear force tests were performed in the caudal region of each fillet. All measurements were conducted at room temperature. The Warner-Bratzler shear force test was performed using a straight blade that moved down at a constant speed of 15 mm/s to 100% of the total deformation. Maximum shear force, defined as the maximum resistance of the sample to shearing, was determined. The Kramer cell test was performed on an 80 mm × 80 mm sample; the cell was composed of five linear blades moving down at a constant crosshead speed of 10 mm/s and withdrawing at a speed of 15 mm/s. The force vs deformation curve was registered until 50% of the total deformation was reached. The test was repeated for five cycles to simulate chewing. Subsequently, five texture parameters were calculated: hardness (peak force of the first compression cycle), shear energy (the sum of the area of the first upstroke and the area of the first downstroke), cohesiveness (the ratio of the positive force area during the second compression compared to that obtained during the first compression), resilience (the ratio of the area of the upstroke compared to the area of the first downstroke during the first compression cycle) and gumminess (hardness multiplied by cohesiveness).

Lipid oxidation

The extent of lipid oxidation was assessed on n = 60 left fillets (n = 15/treatment) at the beginning (at *rigor* resolution, $T_{RR}0$) and at the end ($T_{RR}7$) of the refrigerated storage. To this purpose, the thiobarbituric acid reactive substances (TBARS) method was applied. Briefly, 2.5 g of minced fillet was added into 12.5 ml of distilled water and homogenised at 9 500 rpm (Ultra Turrax T25; Ika Werke, Staufen im Breisgau, Germany). During this process, samples were kept in a water bath containing crushed ice. To precipitate proteins, 12.5 ml

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of 10% (w/v) trichloroacetic acid were added and samples were vortexed and then filtered through Whatman No.1 filter paper. The clear filtrate (4 ml) was mixed with 1 ml of 0.06 M aqueous thiobarbituric acid in 15-ml screw cap glass tubes. Samples were then incubated for 90 min in a water bath set at 80 °C. Sample absorbance was measured using a spectrophotometer (UV VIS Spectrophotometer LAMBA 25; PerkinElmer, Waltham, USA) set at 532 nm. The calibration was performed using solutions of TEP (1,1,3,3-tetraethoxypropane) in 5% (w/v) trichloroacetic acid at concentrations ranging from 5 to 65 nmoles/4 ml. The oxidation products of each sample were quantified as malondialdehyde (MDA) equivalents (mg MDA/kg fillet).

Sensory evaluation

Trout fillets were subjected to a descriptive sensory analysis to assess potential differences between experimental groups as a result of the different stunning methods. To this purpose, panellists (n = 12) with experience in determination of the sensory profile of different food matrices were subjected to training sessions with the purpose to familiarise with the matrix of interest, to select the appropriate descriptors, and to define the relative perceived intensity on numerical and continuous scales of measurement. For each sensory attribute, the scale ranged from 0, corresponding to the lowest score, to 10 which corresponded to the highest score. In the sensory analysis, olfactory, tactile, gustative and textural features were evaluated: global odour and aroma intensity (olfactory descriptors), tactile tenderness and crispiness (tactile descriptors), saltiness and sourness (gustative descriptors), adhesiveness, juiciness and tenderness (textural descriptors).

The sensory evaluation was conducted in duplicate on 60 right fillets (n = 15/treatment) at the time of *rigor* resolution ($T_{RR}0$) on two consecutive days. After one month of frozen storage at –80 °C, fillets were thawed for 16 h at +4 °C. Each fillet was then placed into an aluminium box which had previously been drilled on the bottom, to avoid cooking the fillets in their own liquids. An aluminium foil was also placed on the top of each box. The cooking process was carried out in an electric oven preheated to 200 °C and the cooking time was set up to reach a fillet core temperature of 75 °C.

Subsequently, samples were identified by a random three-digit code and served to panellists in random order to prevent first order and carry-over effects. Each panellist received a 50-g fillet sample and evaluated, one at a time, four samples corresponding to the four treatments. Data acquisition was performed by FIZZ software (Biosystemes France; St-Ouen l'Aumône, France) installed in 12 terminals provided in laboratory's tasting booths. In each sensory session, still water at room temperature and unsalted crackers were available to panellists.

Statistical analysis

Data were analysed using the General Linear Model procedures of the statistical analysis software SAS v9.4 for Windows 2008. A two-way ANOVA considered the stunning method (two levels: CO and E) and the water temperature (two levels: 8 and 12 °C) as fixed effects. The stunning method (S) × water temperature (T) interaction was also tested. Fish weight was considered as covariate. Least square means were obtained using Bonferroni test and the significance was calculated at a 5% confidence level.

RESULTS AND DISCUSSION

Energy metabolism, freshness indices, drip loss, pH, colour and texture profile analysis

Table 1 reports the results about the K- and K1-values at the end of the storage period ($T_{RR}7$), from rainbow trout subjected to two different stunning methods. The mean K-value of rainbow trout slaughtered by asphyxia with CO was significantly lower than that of trout slaughtered by electroshock (49.95 vs 57.31%; $P < 0.05$). Moreover, the fish reared at 12 °C showed a significantly higher K-value compared to that at 8 °C (60.18 vs 47.09%; $P < 0.01$). Conversely, the K1-value was not affected either by the stunning methods applied or by the water temperature.

The rapid depletion of ATP (T0) (Concollato et al. 2020) and faster loss of freshness (higher K-value) presented by E group at $T_{RR}7$ (day 7 after *rigor* resolution) are probably associated with the tetanus and higher level of muscle activity during exposure to electrical current. K-values similar to those of E and CO groups were found by Erikson

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Table 1. K and K1 content at the end of the storage time in rainbow trout farmed at 8 °C or 12 °C and stunned by asphyxia with carbon monoxide (CO) or electricity (E)

Parameters	Stunning (S)		Temperature (T)		*P*-values			RSD
	CO	E	8 °C	12 °C	S	T	S × T	
K (%)	49.95	57.31	47.09	60.18	< 0.05	< 0.01	ns	4.51
K1 (%)	59.68	63.49	58.04	64.93	ns	ns	ns	4.79

ns = not significant; RSD = residual standard deviation

et al. (1997) in salmons evaluated after 10-day *post mortem* storage in ice. Ozogul and Ozogul (2004) reported the K-value of ~50% and ~60% (like CO and E groups of the present study, respectively) after 10 days of MAP or ice storage in rainbow trout slaughtered by a blow to the head, respectively.

Considering the K-value information, the stunning method with electroshock resulted in lower effectiveness of preserving fillet freshness compared to asphyxia with CO. Taking into account some other parameters such as plasma, ATP and AEC values immediately *post mortem* (T0) and fillet shape changes of the two groups of treatments (Concollato et al. 2020), E was likely to be also the most stressing treatment. In salmonids, the K-value seems to increase sharply during the first days of storage before levelling off at about day 7 *post mortem*. The initial K-value in rainbow trout fillets killed by a blow to the head was reported to be 23% (Liu et al. 2015). However, the variation in values reported for salmonids seems to be large, with K-value after seven days of storage ranging between 40 and 80% (Erikson et al. 1997), which is likely to be dependent on different *ante mortem* handling conditions and killing methods (Olafsdottir et al. 1997). Also considering K-value limits proposed by Erikson et al. (1997), the trout of this study presented an excellent quality range, independently of the considered treatment. Regarding the significant effect of water temperature on K-value, this could hypothetically be explained by a reduced activity of 5'-nucleotidase due to low temperature and consequent inhibition of ATP degradation (Liu et al. 2015).

K1-values were not affected by the treatments: as expected, these values showed a global higher value compared to the K-value because of an increase in ATP degradation to IMP over time (Karube et al. 1984). Despite this, fillet freshness remained well preserved up to the end of the storage time ($T_{RR}7$ = day 7 after *rigor* resolution) irrespective of the applied stunning method and water temperature.

Results of the cumulative drip loss (%) of trout fillets calculated at different time points *post mortem* (Table 2) highlighted that treatments were not significant factors in affecting this trait, which is coherent with previous findings (Bjorlykke et al. 2011). Independently of the considered group, a generalised increase in drip loss was observed during the refrigerated storage due to the natural breakdown of proteins, partly explained by the effect of cathepsin B + L activity, which decrease the ability of muscle proteins to bind and hold water (Puolanne and Halonen 2010).

Results of the effects of the stunning treatment and farming temperature on fillet pH and colour values at $T_{RR}0$ and $T_{RR}7$ of the storage time period are shown in Table 3. Stunning methods did not influence fillet

Table 2. Cumulative drip losses (DL, %) during refrigerated storage (2.5 °C) of fillets belonging to rainbow trout farmed at 8 °C or 12 °C and stunned by asphyxia with carbon monoxide (CO) or electricity (E)

Parameters	Stunning (S)		Temperature (T)		*P*-values			RSD
	CO	E	8 °C	12 °C	S	T	S × T	
DL T0–$T_{RR}0$	0.68	0.56	0.46	0.78	ns	ns	ns	0.34
DL $T_{RR}0$–$T_{RR}7$	3.16	2.78	2.90	3.05	ns	ns	ns	0.54
DL T0–$T_{RR}7$	3.81	3.33	3.34	3.81	ns	ns	ns	0.61

ns = not significant; RSD = residual standard deviation; T0 = day of slaughter; $T_{RR}0$ = day of *rigor mortis* resolution; $T_{RR}7$ = last day of storage

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pH. Differently, colour values were affected as follows: at $T_{RR}0$, lightness (L*) was significantly higher ($P < 0.05$) for E group, whereas redness (a*) and chroma exhibited significantly greater ($P < 0.01$) values in CO compared to E group (16.38 vs 14.35 for a*; 22.22 vs 20.07 for chroma). The effect of electrical stunning on fish flesh colour was expected, as previous research on rainbow trout showed that electrostimulation within 1 min from killing by anaesthesia resulted in lighter flesh (higher L*) and larger hue angle of flesh than in the unstimulated ones (Robb et al. 2000). A similar pattern was observed at $T_{RR}7$, with a* and chroma values being significantly higher ($P < 0.01$) for CO compared to E (18.21 vs 16.55 for a*; 25.62 vs 23.37 for chroma); furthermore, CO showed the significantly higher ($P < 0.05$) yellowness (b*) value compared to E (17.96 vs 16.47, respectively). It is known that CO easily binds to oxymyoglobin/oxyhaemoglobin (OMb/OHb), displacing oxygen, producing carboxymyoglobin/carboxyhaemoglobin (COMb/COHb) that has a cherry red colour. The latter are stable compounds and the degradation to meth forms MMb/MHb takes a longer time and will thus prevent discoloration. The significant difference in redness at $T_{RR}0$ (76 h *post mortem*) and $T_{RR}7$ is thus mainly due to COMb/COHb production. In Atlantic salmon, herring and mackerel anaesthetised by injecting CO in seawater, the a* value was more persistent than in the control group even after six days of cold storage (Concollato et al. 2014). A slight increase of a* value was also detected by Bjorlykke et al. (2011) both on the fillets and gills of Atlantic salmon stunned by CO compared to the control group (slaughtered by percussion).

Overall, the water temperature affected pH of fillets, but it had only a moderate effect on their colorimetric characteristics. Moreover, differently from what was previously presented for stunning methods, different water temperatures resulted in less constant differences in fillet characteristics. Indeed, whereas pH values at $T_{RR}0$ for trout reared at 8 °C were significantly higher ($P < 0.001$) than those of trout reared at 12 °C, at $T_{RR}7$ they were significantly lower ($P < 0.05$) than those at 12 °C. The low water temperature (8 °C) maintained significantly higher fillet pH at the day of *rigor* resolution ($T_{RR}0$), probably because it contributed to the reduction in the activity of the enzymes taking part in glycogenolysis and further breakdown of glucose (Skjervold et al. 2001). This is probably the reason why only at the end of the storage 8 °C-reared fish showed the lowest fillet pH. In rainbow trout it was shown that a decrease in pH resulted in significantly brighter and yellower fillets (Robb 2001). This only partly fitted with the results of the present study as at $T_{RR}0$ lower fillet lightness ($P < 0.05$) corresponded to a lower pH ($P < 0.001$) whereas at $T_{RR}7$ the pH decrease ($P < 0.05$) augmented fillet lightness ($P < 0.001$). Regarding yellowness, it was never affected by the water temperature either at $T_{RR}0$ or at $T_{RR}7$. The

Table 3. pH and colorimetric characteristics during refrigerated storage (2.5 °C) of fillets belonging to rainbow trout farmed at 8 °C or 12 °C and stunned by asphyxia with carbon monoxide (CO) or electricity (E)

	Parameters	Stunning (S)		Temperature (T)		*P*-values			RSD
		CO	E	8 °C	12 °C	S	T	S × T	
$T_{RR}0$	pH	6.79	6.76	7.06	6.49	ns	< 0.001	< 0.05	0.100
	L*	41.97	42.95	42.98	41.94	< 0.05	< 0.05	ns	1.75
	a*	16.38	14.35	15.38	15.35	< 0.001	ns	< 0.05	1.94
	b*	15.04	13.95	14.41	14.58	ns	ns	ns	2.29
	chroma	22.22	20.07	21.12	21.17	< 0.01	ns	< 0.05	2.84
	hue	42.55	44.16	43.18	43.53	ns	ns	ns	3.61
$T_{RR}7$	pH	6.57	6.60	6.56	6.61	ns	< 0.05	ns	0.071
	L*	40.37	40.88	41.65	39.61	ns	< 0.001	< 0.05	1.68
	a*	18.21	16.55	17.29	17.46	< 0.01	ns	< 0.001	1.88
	b*	17.96	16.47	16.99	17.45	< 0.05	ns	ns	2.30
	chroma	25.62	23.37	24.28	24.71	< 0.01	ns	< 0.001	2.78
	hue	44.57	44.77	44.33	45.00	ns	ns	ns	2.90

a* = redness; b* = yellowness; L* = lightness; ns = not significant; RSD = residual standard deviation; TRR0 = day of rigor mortis resolution; TRR7 = last day of storage

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Table 4. Texture profile analysis parameters of fillets belonging to rainbow trout farmed at 8 °C or 12 °C and stunned by asphyxia with carbon monoxide (CO) or electricity (E), measured at the end of the refrigerated storage (2.5 °C), *i.e.* on day 7 after *rigor* resolution

Parameters	Stunning (S)		Temperature (T)		*P*-values			RSD
	CO	E	8 °C	12 °C	S	T	S × T	
Shear stress (N)	62.82	55.89	55.50	63.21	ns	ns	ns	13.36
Hardness (N)	286.28	270.52	256.46	300.34	ns	< 0.05	ns	40.13
Cohesiveness	0.43	0.54	0.53	0.43	< 0.01	< 0.01	< 0.05	0.08
Resilience	0.09	0.14	0.13	0.09	< 0.05	ns	ns	0.05
Gumminess	118.15	140.20	133.51	124.84	ns	ns	ns	25.87

ns = not significant; RSD = residual standard deviation

significant S × T interaction observed both at $T_{RR}0$ and at $T_{RR}7$ for the a* colour value highlighted that CO stunning ensured a higher efficacy in preserving fillet redness when water temperature was 8 °C. In fact, the a* value was higher in CO_8 fillets (16.81 at $T_{RR}0$ and 18.96 at $T_{RR}7$) compared to E_8 ones (13.97 at $T_{RR}0$ and 15.64 at $T_{RR}7$), with the other groups showing intermediate results. The above-mentioned interaction was directly responsible for the observed interaction regarding the chroma value, which showed the same trend presented for redness both at $T_{RR}0$ and at $T_{RR}7$.

Results of the TPA (Table 4) showed that the stunning method affected cohesiveness ($P < 0.01$) and resilience ($P < 0.05$), which both presented significantly higher values in E fillets compared to CO ones (0.54 vs 0.43 for cohesiveness; 0.14 vs 0.09 for resilience). CO fillets were less able to fully recover the original structure during the break between two successive compressions (cohesiveness), explaining the lower resilience. This behaviour was supported also by the higher shear stress and hardness, and the lower gumminess, even if the latter was not corroborated by a statistical significance. The results revealed that pre-slaughter stress affected trout fillet firmness depending on the severity and duration of stress: short-term stress (electricity) seemed to lead to muscle softening, while long-term exhaustion (asphyxia with CO) increased fillet firmness, which agrees with Skjervold et al. (2001) and with the known stress patterns affecting also mammal meat quality.

The water temperature significantly affected fillet hardness ($P < 0.05$) and cohesiveness ($P < 0.01$): at 12 °C, fillets were harder and less cohesive than at 8 °C. This finding thus explains the lower, even if not significantly, resilience (which gives a measure of the springiness) and gumminess, and higher shear stress, resulting in overall worse TPA values. Findings of the present study supported previous literature (Gines et al. 2004) as it was found that Arctic char reared at 15 °C had significantly higher hardness and lower, but not significantly, cohesiveness than those of fish reared at 10 °C. A significant ($P < 0.05$) interaction was detected only for cohesiveness, where the combination of 8 °C and E stunning resulted in trout fillets with the highest value (Figure 1).

Lipid oxidation

Results obtained from this study only confirmed CO capability in reducing/delaying lipid oxidation of the product, when compared to E stunning method (Table 5). Fillets of fish slaughtered by CO showed lower MDA values ($P < 0.05$) than E group

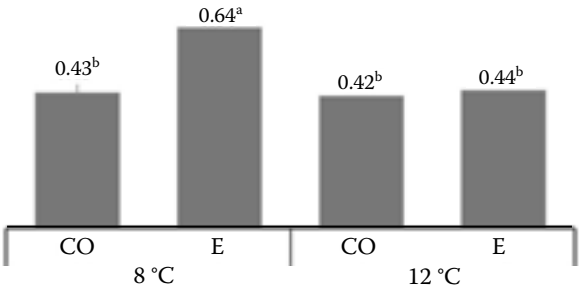

Figure 1. Texture profile analysis parameters of fillets belonging to rainbow trout farmed at 8 °C or 12 °C and stunned by asphyxia with carbon monoxide (CO) or electricity (E), measured at the end of the refrigerated storage (2.5 °C), *i.e.* on day 7 after *rigor* resolution: temperature × stunning method interaction

[a,b]Means with different superscript letters differ for $P < 0.05$

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Table 5. Lipid oxidation of fillets belonging to rainbow trout farmed at 8 °C or 12 °C and stunned by asphyxia with carbon monoxide (CO) or electricity (E), stored in a cold room (2.5 °C) and analysed at $T_{RR}0$ (day of *rigor* resolution, 76 h *post mortem*) and $T_{RR}7$ (last day of cold storage, 7 days after *rigor* resolution)

Parameters	Stunning (S)		Temperature (T)		*P*-values			RSD
	CO	E	8 °C	12 °C	S	T	S × T	
$T_{RR}0$, MDA (mg/kg)	0.66	1.10	0.91	0.85	< 0.05	ns	ns	0.71
$T_{RR}7$, MDA (mg/kg)	0.91	0.74	0.83	0.83	ns	ns	ns	0.46

MDA = malondialdehyde content; ns = not significant; RSD = residual standard deviation

(0.66 vs 1.10 mg/kg) at $T_{RR}0$. It is known that lipid oxidation is affected by many factors like oxygen, temperature, Mb content, metal catalysts and enzymes, pH, NaCl (Dominguez et al. 2019). In fact, slaughtering methods and *pre mortem* stress had no effects on lipid oxidation in the study of Huidobro et al. (2014) on gilthead sea bream (*Sparus aurata*) slaughtered with ice plus water or with liquid ice. Morzel and Van De Vis (2003) found that eel (*Anguilla anguilla*) lipids were significantly more susceptible to oxidation in fish slaughtered by the commercial method (salt baths) in comparison with those killed by gas combined with electricity. The strong affinity of CO towards Mb prevents O_2 binding to Mb making its oxidation and consequent production of superoxide radicals difficult, the latter being responsible for the initiation of lipid peroxidation (Cornforth and Hunt 2008). This would explain why the group stunned by CO showed a significantly lower MDA content than E group even after only 76 h *post mortem*. For the latter, instead, the process of lipid oxidation seemed to be more intense and rapid. In agreement with Mantilla et al. (2008) on tilapia fillets, also in the present study no difference due to the CO treatment was detected in the MDA content in trout fillets at the end of storage ($T_{RR}7$: day 7 after *rigor* resolution). When the fish are treated with CO, the CO content in their flesh is expected to decline over time and this could explain a similar oxidative status of lipids in the two treatment groups at this time point. In fact, Ishiwata et al. (1996) reported that the increase in CO concentration on extended storage is one of the indicators used by the Japanese health authorities to discriminate fish treated or not with CO to prolong the product shelf-life.

Sensory analysis

Among all the considered sensory traits of trout fillets (Table 6), odour ($P < 0.01$) and aroma ($P < 0.05$) intensity, saltiness ($P < 0.05$) and juiciness ($P < 0.05$) were significantly affected by the stunning

Table 6. Sensory traits of fillets belonging to rainbow trout farmed at 8 °C or 12 °C and stunned by asphyxia with carbon monoxide (CO) or electricity (E), stored in a cold chamber (2.5 °C) and analysed at $T_{RR}0$ (day of *rigor* resolution, 76 h *post mortem*)

Parameters	Stunning (S)		Temperature (T)		*P*-values			RSD
	CO	E	8 °C	12 °C	S	T	S × T	
Odour intensity	4.64	5.37	5.16	4.85	< 0.01	ns	ns	0.75
Aroma intensity	5.43	5.95	5.49	5.89	< 0.05	ns	ns	0.70
Tactile tenderness	6.43	6.14	6.09	6.47	ns	ns	ns	1.24
Crispiness	6.02	6.11	6.0	6.13	ns	ns	ns	1.09
Saltiness	2.89	3.45	2.94	3.40	< 0.05	ns	ns	0.68
Sourness	2.25	2.53	2.28	2.50	ns	ns	ns	0.61
Adhesiveness	3.37	3.43	3.47	3.33	ns	ns	ns	0.81
Juiciness	5.33	4.47	4.54	5.25	< 0.05	< 0.05	ns	0.94
Tenderness	6.52	5.82	5.81	6.53	ns	< 0.05	ns	1.04

ns = not significant; RSD = residual standard deviation

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method. Specifically, CO stunning decreased all the above-mentioned attributes compared to E treatment, except for juiciness whose highest score was recorded for CO trout fillets. The result of odour and aroma intensity was expected as E fillets displayed also the highest oxidative degree at the day of *rigor* resolution ($T_{RR}0$). The higher saltiness detected in E fillets could possibly be associated with a higher stress response of E fish compared to CO ones (Concollato et al. 2020): stress showed to result in conversion products from ATP metabolism possessing very specific flavours like salty-acid and bitter. At a low fillet pH, cooking loss and concentration of myofibrillar proteins increase, which determines tough and dry flesh (Rasmussen 2001); however, this was not the case of the present study as pH values of E and CO fillets at $T_{RR}0$ were similar and close to neutrality (average pH = 6.78). In general, the extent to what the salmonid flesh texture is affected by stress seems mainly dependent on its magnitude, being detectable only after a certain threshold level (Rasmussen 2001): this would explain the result heterogeneity depicted in literature about this topic.

The water temperature affected only fillet juiciness ($P < 0.05$) and tenderness ($P < 0.05$), which were improved when the trout were farmed at a higher water temperature (12 °C). Even if studies on this topic are scarce, research on the Arctic char (*Salvelinus alpinus*) showed that cooked fillet of fish reared at 15 °C had higher stiffness (defined as "degree of force to break the sample with a fork") compared to those reared at 10 °C (Gines et al. 2004), which is somewhat opposite to the findings of the present study, where 12 °C fillets were characterised by the highest tenderness. Interestingly, in the present experiment instrumental hardness was found to be higher in fillets of 12 °C trout compared to 8 °C ones, which also opposed to the sensory result reported for tenderness. This condition could hypothetically be due to the observed lower cohesiveness of 12 °C fillets compared to 8 °C ones. Being harder but less cohesive, trout fillets could have been perceived as more tender by the sensory panel.

CONCLUSION

The present experiment showed that the stunning method can affect *post rigor mortis* changes of trout fillet, being a relevant factor in influencing physicochemical quality traits. Carbon monoxide treatment is a valid stunning method for rainbow trout, alternative to the conventional electroshock, ensuring the highest fillet freshness, the lowest oxidative status and the best colorimetric characteristics, texture and sensory profile. The water temperature showed to play a certain role in the observed outcomes, with higher water temperature (12 °C) providing the best fillet freshness and sensory characteristics, with negligible effects on fillet colour and no effect on the oxidative stability of lipids. Based on the present findings, it seems that CO stunning of trout reared at 12 °C is the best combination to ensure the highest fillet quality.

Conflict of interest

The authors declare no conflict of interest.

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