Aquaculture 582 (2024) 740541

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aquaculture

Stunning/slaughtering by cold shock in saline water: Effects on fish stress, post-mortem changes, and product quality in rainbow trout

Francesco Bordignon [a], Martina Bortoletti [b], Angela Trocino [a,b,*], Gerolamo Xiccato [a], Marco Birolo [a], Eleonora Fiocchi [c], Amedeo Manfrin [c], Giuseppe Radaelli [b], Daniela Bertotto [b]

[a] Department of Agronomy, Food, Natural resources, Animals and Environment (DAFNAE), University of Padova, Viale dell'Università 16, 35020 Legnaro, Padova, Italy
[b] Department of Comparative Biomedicine and Food Science (BCA), University of Padova, Viale dell'Università 16, 35020 Legnaro, Padova, Italy
[c] Istituto Zooprofilattico Sperimentale delle Venezie, Viale dell'Università 10, 35020 Legnaro, Padova, Italy

ARTICLE INFO

Keywords:
Fish welfare
Rigor mortis
Cortisol
Shelf life

ABSTRACT

This study evaluated the effects of two stunning/slaughtering methods, percussion vs. immersion in cold saline (−6 °C, 5% NaCl) water, on stress status and freshness evolution in rainbow trout (*Oncorhynchus mykiss*). Stress indicators (cortisol, malondialdehyde-MDA) were measured in plasma, muscle, fin and skin mucus of 30 fish (15 per treatment), while freshness evolution and quality (pH, *rigor mortis* index, fillet and skin colour, and muscle texture) were assessed in 70 fish (whole fish and fillets; 35 per treatment) at 3, 48, 168, and 264 h of storage.

Compared with percussion, cold immersion resulted in lower cortisol levels in skin mucus (−58%; $P < 0.001$), muscle (−53%; $P < 0.01$), and fin (−51%; $P < 0.01$), and higher muscle MDA (+90%; $P < 0.001$), while cortisol (184 ng/ml, on average) and MDA (13.7 nmol/mg protein) of plasma were not affected. Skin colour was darker in fish subjected to cold immersion compared with percussion. As for fillet, no differences were found in pH (6.46), L* (47.3), a* (−0.89), b* (10.1), TVB-N (17.6 mg/100 g of fillet), fatty acid profile, or texture, where only chewiness was higher (+28% and + 32% for whole fish and fillet, respectively; $P < 0.05$) when cold immersion was used compared with percussion. Over time, TVB-N showed the highest value at 264 h of storage (26.5 mg/100 g of fillet), and pH decreased from 6.57 at 3 h of storage to 6.39 at 48 h and 6.37 at 168 h, before increasing to 6.50 at 264 h ($P < 0.001$). Immersion in cold saline water was proved to be a viable alternative to percussion for stunning/slaughtering rainbow trout, being an ease technique, cost-effective, and with reduced labour, while producing a similar stress response amplitude and no relevant changes on product quality.

1. Introduction

The rapid expansion of aquaculture has increased the public awareness on the welfare of farmed fish (Robb and Kestin, 2002) where, according to the resolution of the European Parliament about the Strategy for the Sustainable Development of European Aquaculture (2009/2107(INI) (European Parliament, 2011), new knowledge is needed regarding the welfare of farmed fish, on farm, at stunning and slaughtering, and during transport and corresponding welfare indicators (Papaharisis et al., 2019). To date, although several recommendations have been proposed to reduce stress before and during fish slaughtering (EFSA, 2004, 2009a, 2009b; WOAH, 2022), no specific Regulations are available about the procedure to be adopted (Bermejo-Poza et al., 2021). Thus, further studies are needed to identify suitable stunning/slaughtering methods that reduce animal stress and suffering, maintain an ease operation and low costs, measuring the effects on product quality (Van De Vis et al., 2003).

In 2021, the aquaculture production in Europe (EU-27 Countries) averaged at 1.13 million of tons, out of which farmed fish were 51% of the total production, with rainbow trout (*Oncorhynchus mykiss*) as the main species (17% of total aquaculture) followed by sea bass (9%) and sea bream (9%) (EUMOFA, 2023). More than half of the European trout volume is produced in Italy (37,200 ton), France (34,424 ton), and Denmark (29,479 ton) (EUMOFA, 2023). As for on farm welfare of trout, first EFSA (2008) identified different factors that can affect its welfare, and thus, its production, which conclusions and recommendations have been integrated more recently by the specific guidelines about water quality and fish handling published by the European Platform for

* Corresponding author at: Department of Agronomy, Food, Natural resources, Animals and Environment (DAFNAE), University of Padova, Viale dell'Università 16, 35020 Legnaro, Padova, Italy.
E-mail address: angela.trocino@unipd.it (A. Trocino).

https://doi.org/10.1016/j.aquaculture.2024.740541
Received 30 August 2023; Received in revised form 23 December 2023; Accepted 2 January 2024
Available online 3 January 2024

Animal Welfare (EPAW, 2022). As for slaughtering and stunning, in trout as in other fish, acute stress before slaughtering caused by crowding and handling often implies high muscular activity, lactic acid accumulation, quick muscle pH fall, and early onset of rigor and rigor resolution besides nucleotide breakdown, resulting in adverse effects on flesh quality (Lefevre et al., 2016; Daskalova, 2019). In salmonids, the main consequences on organoleptic properties of the final product include changes in the colour (Robb et al., 2000; Lefèvre et al., 2008), texture (Gomez-Guillen et al., 2000; Wills et al., 2004; Lefevre et al., 2016), and lipid oxidation and metabolism (Secci et al., 2016a, 2016b). In the last years, various stunning and slaughtering methods have been investigated in rainbow trout, including air asphyxia (Giuffrida et al., 2007; Duran et al., 2008; Concollato et al., 2016, 2019), carbon monoxide asphyxia (Concollato et al., 2016, 2020; Dalle Zotte et al., 2020), percussion (Duran et al., 2008; Isık et al., 2019), electroshock (Giuffrida et al., 2007; Concollato et al., 2019, 2020; Dalle Zotte et al., 2020; Bermejo-Poza et al., 2021) and live chilling (Bermejo-Poza et al., 2021; Saraiva et al., 2024).

Live chilling/cold stunning is widely used in warm-water species like European sea bass (*Dicentrarchus labrax*) and gilthead seabream (*Sparus aurata*), which do not tolerate low temperatures (Zampacavallo et al., 2015). On the other hand, although immersion in ice water is used in salmonids like rainbow trout (Bermejo-Poza et al., 2021) and Atlantic salmon (*Salmo salar*) (Skjervold et al., 2001), these species are tolerant to temperatures around 0 °C (Fletcher et al., 1988). Therefore, the period to reach unconsciousness is longer (Ashley, 2007) and more stressful, resulting in increased heart rate (Van De Vis et al., 2003) and plasma cortisol levels (Skjervold et al., 2001; Bermejo-Poza et al., 2021). An effective stunning procedure for salmonids might require a temperature drop below 0 °C which could be achieved by salt addition in the water (Ntzimani et al., 2023). This allows to reduce the freezing point below 0 °C and provokes a stronger thermal shock in fish (Reid et al., 2022). Besides, during this procedure, fish are left in the water, reducing manipulation-associated stress, and potentially leading to a less stressful loss of consciousness.

The present study tested the hypothesis that a cold shock would allow a low-suffering loss of consciousness for trout compared to classical percussion which would result in a longer fish shelf-life. Therefore, we compared the effects on animal stress and fish freshness evolution in rainbow trout of two stunning/slaughtering methods, percussion vs. cold immersion, this latter consisting of an immersion of fish in cold salted water (5% NaCl) at temperature around −6 °C.

2. Materials and methods

2.1. Ethic statement

This study was conducted in compliance with Council Directive 98/58/EC concerning the protection of animals kept for farming purposes, enacted in Italy through the Legislative Decree No. 146/2001 and approved by the Ethics Committee of the Istituto Zooprofilattico Sperimentale delle Venezie (OPBA DA23/2018 del 24/04/2018).

2.2. Slaughtering and fish sampling

The sampling was performed in a commercial farm located in Friuli (North-East of Italy). Fish were reared in concrete tanks within a common flow-through aquaculture system (water temperature: 14 °C; dissolved oxygen: 8.5 mg/l) during 6 months until the day of slaughtering (30 kg/m^3 of stocking density) and fed during the same period with a commercial diet for rainbow trout at a feeding rate of 1% live weight.

Before slaughtering, the fish were subjected to three days of fasting according to standard commercial practices. Then, at the day of slaughtering, a total of 100 rainbow trout (286 ± 51 g of weight) were submitted to two different stunning/slaughtering procedures: 1) percussion, i.e., fish were removed from the rearing tank and a sharp blow was delivered to the head to render the brain unconscious; and 2) immersion in cold saline water, i.e., fish were removed from the rearing tank and immersed in cold (−5.8 ± 0.5 °C) saline (5% NaCl) water during 5 min until loss of consciousness and subsequently death. Loss of consciousness was assessed through the evaluation of the vestibulo-ocular reflex (Kestin et al., 2002). Trout were caught from the tank in 10 sequential rounds with 10 fish each that were immediately subjected to percussion (5 fish) or immersed in cold saline water (5 fish). A total of 30 trout (15 per treatment) were used to obtain samples for stress indicators. The remaining 70 trout (35 per treatment) were used for freshness and quality analyses: 10 fish (5 per treatment) were immediately sampled and processed (Set 1); 20 fish (10 per treatment) were used to evaluate pH and *rigor mortis* evolution at 3, 6, 9, 12, 20, 24, 28, 32, 36 and 44 h post mortem and then sampled at 48 h also for rheological and chemical analysis (Set 2); 20 fish (10 per treatment) were sampled at 168 h of storage (Set 3), and the remaining 20 fish (10 per treatment) were sampled at 264 h of storage (Set 4) (Fig. 1).

2.3. Fish stress evaluation

As detailed above, immediately after unconsciousness assessment, 30 rainbow trout (15 per treatment) were weighed and underwent tissue and organ sampling. Firstly, the skin mucus was sampled by scraping the left side of the fish with a small plastic rod. Blood was collected from caudal vein and placed in lithium-heparinised tubes to obtain the plasma, by means of a 1200 ×*g* centrifugation for 10 min at 4 °C. After blood sampling, two portions (1 cm × 1 cm) of muscle (from the caudal peduncle) and a sample of caudal fin were taken to measure cortisol and malondialdehyde (MDA). Samples were stored at −20 °C.

Cortisol levels were measured using a specific microtiter radioimmunoassay (RIA), validated for usage in rainbow trout, as described by Bertotto et al. (2010). Muscle and fin samples (100 mg) were thawed out and pulverized in liquid nitrogen, and 100 mg of the resulting powders were suspended in 500 µl RIA buffer (Na_2HPO_4-$12H_2O$ 61 mM, NaH_2PO_4-H_2O 40 mM, NaCl 154 mM, pH 7.2). The resulting powders and plasma samples (100 µl) were then extracted with 8 ml of diethyl ether. Briefly, a 96-well microtiter plate (Optiplate, PerkinElmer Life Sciences, Waltham, MA, USA) was coated with anti-rabbit c-globulin serum raised in goat (dilution 1:1000 in 0.15 mM sodium acetate buffer, pH 9, at 4 °C) and, after phosphate-buffered saline (PBS) double washing, incubated overnight at 4 °C with the specific antiserum solution. Standards, quality controls, unknown extracts and 3H tracers were then added and, after overnight incubation at 4 °C, the plate was washed with PBS, added with 200 µl scintillation cocktail (Microscint 20, PerkinElmer Life Sciences, Waltham, MA, USA) and counted on a beta-counter (Top-Count, PerkinElmer Life Sciences).

The anti-cortisol serum showed the following cross-reactions: cortisol 100%, prednisolone 44.3%, 11-deoxycortisol 13.9%, cortisone 4.95%, corticosterone 3.5%, prednisone 2.7%, 17-hydroxyprogesterone 1.0%, 11-deoxycorticosterone 0.3%, dexamethasone 0.1%, progesterone <0.01%, 17-hydroxypregnenolone <0.01%, pregnenolone <0.01%.

Lipid peroxidation was assayed in plasma and muscle by measuring thiobarbituric acid-reactive substances (TBARS) (Yoshida et al., 2005). Briefly, thiobarbituric acid reaction was carried out by mixing 25 µl sodium dodecyl sulfate solution (8.1%, *w/v*), 187.5 µl acetic acid buffer (20%, *v/v*, pH 3.5), 187.5 µl thiobarbituric acid (1%, *v/v*), 75 µl water, and 6.25 µl ethanol containing butylated hydroxytoluene (0.8%, *w/v*) with 100 µl of supernatant. The reaction mixture was incubated at 100 °C for 10 min and then cooled on ice followed by mixing vigorously with 125 µl water and 625 µl of n-butyl alcohol and pyridine (15/1, *v/v*). Then, the mixture was centrifuged (0 °C, 1400 ×*g*) for 10 min and the supernatant was measured spectrophotometrically at 532 nm. Tetramethoxypropane was used as a standard to estimate TBARS formation as nanomoles of MDA equivalents per mg of total protein, determined using the bicinchoninic acid (BCA) kit (Thermo Fisher, Waltham, MA, USA).

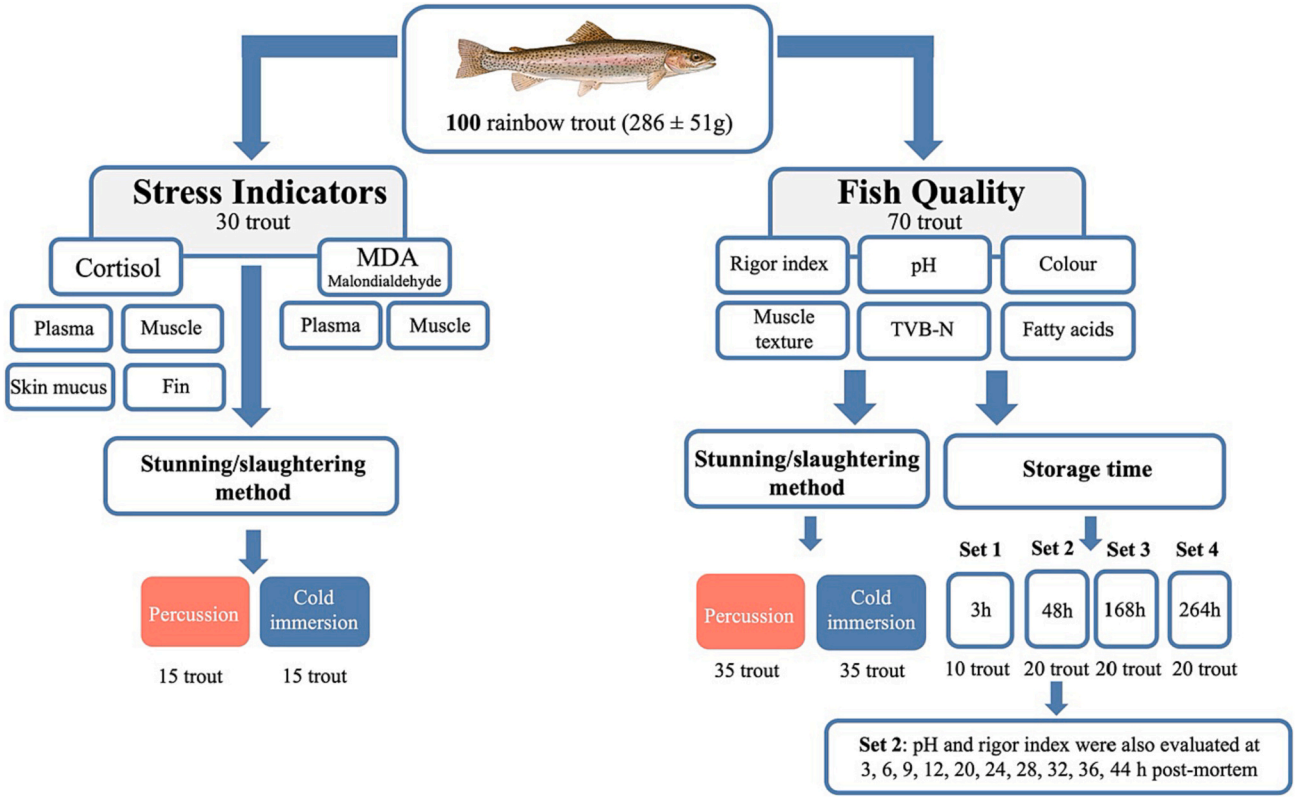

Fig. 1. Scheme of the sampling plan and analysis. Percussion: sharp blow to the head. Cold immersion: trout immersed in cold (−5.8 ± 0.5 °C) saline water (5% of NaCl) for 5 min.

2.4. Rigor mortis index evaluation

We studied *rigor mortis* and pH changes during the first 48 h after death to assess the impact of stunning/slaughtering method on the early *post-mortem* period when *rigor mortis* initiates, as these factors can influence the subsequent evolution of freshness and shelf life.

After slaughtering, each trout was measured for total length (from the anterior margin of the upper lip to the distal part of the caudal fin). To obtain the *rigor mortis* index (RI), the following formula from Bito et al. (1983) was used:

$RI = (D_1 - D_0) / D_0 \times 100$.

where D_0 (cm) is the vertical distance between the base of the caudal fin and the table surface (used as a support base for the fish, with cranial part laying on the table, and caudal part left free without support), measured immediately after death; D_1 (cm) is the vertical distance between the base of the caudal fin and the table surface (same as previous) at the selected time interval. On intact fish, D_1 was measured at different times in lab during the first 48 h after slaughtering (at 3, 6, 9, 12, 20, 24, 28, 32, 36, 44 and 48 h; Set 2) and then at 3 h (Set 1), 168 h (Set 3) and 264 h after slaughtering (Set 4) on the different sets of fish, as described above and reported in Fig. 1.

2.5. Carcass dissection and rheological analyses

For all sets, each specimen was weighed before dissection. Then, after the removal of viscera, the carcasses were filleted. The two fillets with skin obtained for each specimen were weighed and the fillet yield calculated (fillet weight / slaughter weight × 100).

After dissection, the colour indices L*a*b* (Commission International de l'Eclairage, 1976) of the skin of the intact fish and then of the fish fillets were measured at three points (anterior area, medial - under dorsal fin - area and posterior area) on the trout and fillet dorsal side using a Minolta CM–508C spectrophotometer (Minolta Corp., Ramsey, NJ, USA). Then, the hue and chroma indexes were calculated (Commission International de l'Eclairage, 1976).

As mentioned above, the pH measurement was repeatedly performed within the first 48 h (at 3, 6, 9, 12, 20, 24, 28, 32, 36 and 44 h) on 20 fish (10 per treatment) (Set 2). On intact fish, the pH was measured on three different points (cranial, medial, and caudal area) using the portable Sension+ pH-meter (Hach, Loveland, CO, USA), specifically designed for flesh and featured with automatic temperature control. When the fillets were obtained, the flesh pH was measured as described above (three points in all fish (70 fish, 35 per treatment).

On intact fish first and then on fillets, a Texture Profile Analysis (TPA) was performed, using a TA.HDI dynamometer (Stable Micro System Ltd., Goldaming, UK). The measurement was performed using a 20 mm-diameter cylindrical probe, moving with 5 mm compression at a constant speed of 2 mm/s for two consecutive cycles, separated by a 5-s interval. The Texture Export software was used to obtain and elaborate the measurements. Hardness, springiness, and cohesiveness were computed. Hardness measures the highest force applied at the first compressive cycle. Cohesiveness measures the reaction of the tissue to the second compressive cycle (ratio between the second work area and the first work area). Springiness measures the tissue response to the first compression to return to the pre-compressive status (ratio between the first work area and the second work area).

2.6. Chemical analysis of fillets

After rheological analysis, fillets of all sets were separated from the skin and minced, using a Grindomix grinder (Retsch, Haan, Germany). A first amount of the minced fillet was used to evaluate the total volatile basic nitrogen (TVB-N), a freshness index in accordance to European Commission 95/149/CE (EEC, 1995). The volatile nitrogenous bases were extracted from a 10-g sample of minced fillet by a solution of 0.6 M perchloric acid. After alkalization, the extract was submitted to steam

distillation and the volatile base components were absorbed by an acid receiver. The TVB-N content was determined by tritiation of the absorbed bases using a KT 200 Kjeltec™ (FOSS, Hilleroed, Denmark).

A 10-g sample of minced fillet was stored under vacuum at −18 °C to undergo a fatty acid (FA) profile analysis. After defrosting, fat was extracted with accelerated solvent extraction (ASE®, Dionex 350, Sunnyvale, CA, USA, Application Note 334). The procedure consisted of two extraction cycles at 100 °C, with a hexane/isopropanol (3:2) solution as solvent (5-min heating phase and 1-min extraction phase).

After ASE extraction, vials containing extracted solution underwent evaporation using Genevac EZ-2 evaporator (Genevac Ltd., Ipswich, UK). The lipid content was weighed and esterified at 50 °C in heater. Esterification to evaluate fatty acids esters was performed using an acid esterification (H_2SO_4) according to Christie procedure (Christie, 1982). After that, the sample was injected into a split/splitless gas chromatograph (Agilent Technologies 7820 A, Santa Clara, CA, USA), featured with an Omegawax (Supelco SP 2560, Sigma-Aldrich, St. Louis, MO, USA) capillary column (30 m × 0.25 mm internal diameter, 0.25 μm film thickness), with a 0.25 ml/min flow, and featured with a JeW 19091S 431 HPS ms second capillary column (3.8 m × 0.25 mm internal diameter, 0.25 μm film thickness), with a 1.4 ml/ min flow. Hydrogen was used as the carrier.

An oven temperature of 50 °C was held 2 min, raised to 220 °C at the rate of 4 °C/ min, and then held 17 min at 220 °C. Injector and detector temperatures were both set at 250 °C. Fatty acids were identified by comparing their retention times with a standard mixture of 37 fatty acids methyl esters (FAMEs; standard 37-Component FAME Mix, 47,885-U Supelco; PUFA-3, Menhaden Oil, 47,085-U). The concentration of individual FAMEs was expressed as a percentage of the total area of eluted FAMEs (known plus unknown). Data elaboration was performed with the Chemstation software (Agilent Technologies). The index of atherogenicity (IA) and the index of thrombogenicity (IT) were calculated according to Ulbricht and Southgate (1991).

2.7. Statistical analyses

Data regarding pH and *rigor mortis* obtained during the first 48 h of storage on Set 2 were submitted to ANOVA with stunning/slaughtering method, storage time, and their interaction as the main effects, and each specimen as a repeated measure, using the PROC MIXED of SAS (SAS (Statistical Analysis System Institute, Inc.), 2013). Data of rheological and chemical analyses (all sets) and data of cortisol in different matrices and MDA levels in the muscle were submitted to ANOVA, considering stunning/slaughtering method, storage time (for quality analyses; 3 h, 48 h, 164 h, and 268 h), and their interaction as the main effects, using the PROC GLM of SAS. A $P \leq 0.05$ level was accepted as representing statistically significant differences.

3. Results

3.1. Fish stress indicators

Cortisol levels did not differ in plasma (184 ng/ml), but lower levels were found in muscle (−53%, $P < 0.01$), fin (−51%, $P < 0.01$), and skin mucus (−58%, $P < 0.001$) of trout subjected to cold immersion compared with those subjected to percussion (Fig. 2). Similarly, MDA did not differ in plasma but in muscle was higher in the former compared to the latter trout (+91%, $P < 0.01$) (Fig. 3).

3.2. Rigor mortis and pH evolution during the first 48 h post mortem

As for the effect of the stunning/slaughtering method, RI was higher in trout subjected to percussion than cold immersion (on average 13.2% vs. 11.5%; $P < 0.001$) (Table 1), but a significant interaction between

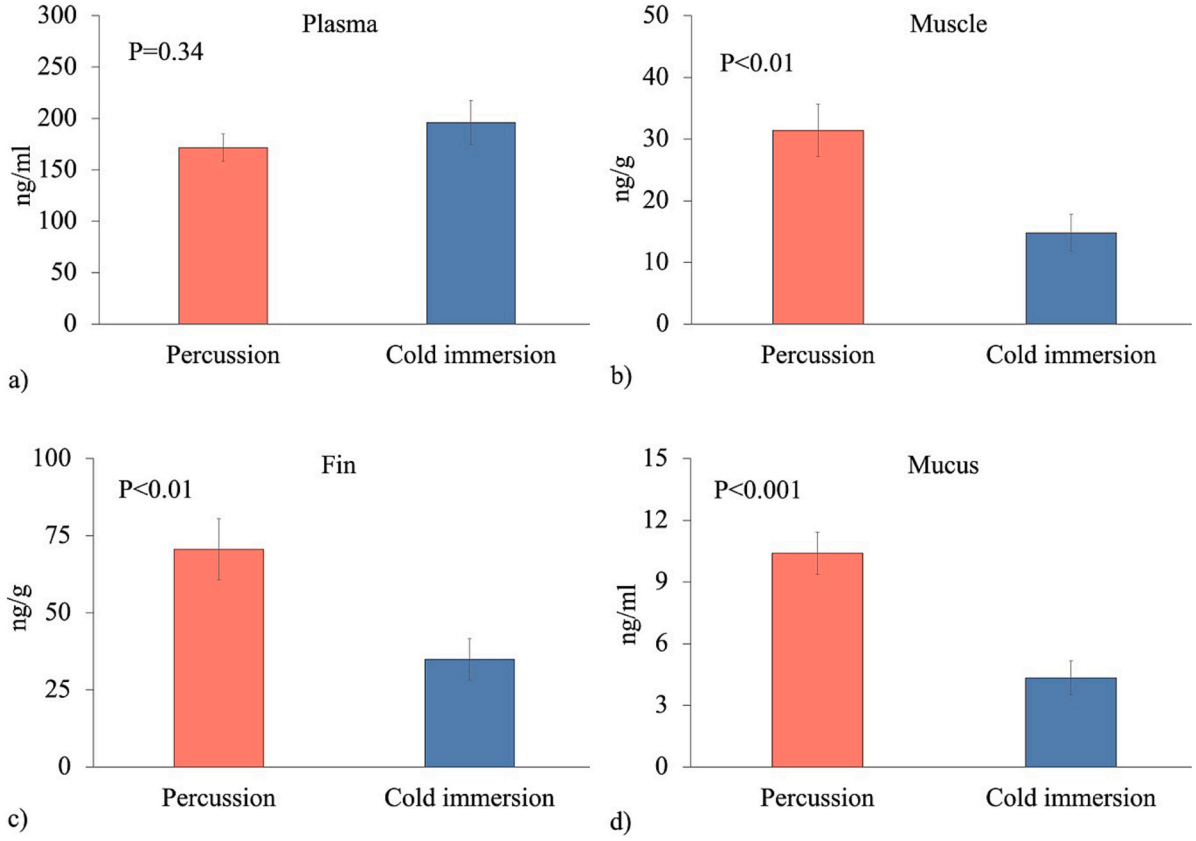

Fig. 2. Effect of stunning/slaughtering method (percussion vs. cold immersion) on cortisol levels (ng/ml, ng/g) measured in trout plasma (a), muscle (b), fin (c) and mucus (d). Percussion: sharp blow to the head. Cold immersion: trout immersed in cold (−5.8 ± 0.5 °C) saline water (5% of NaCl) for 5 min.

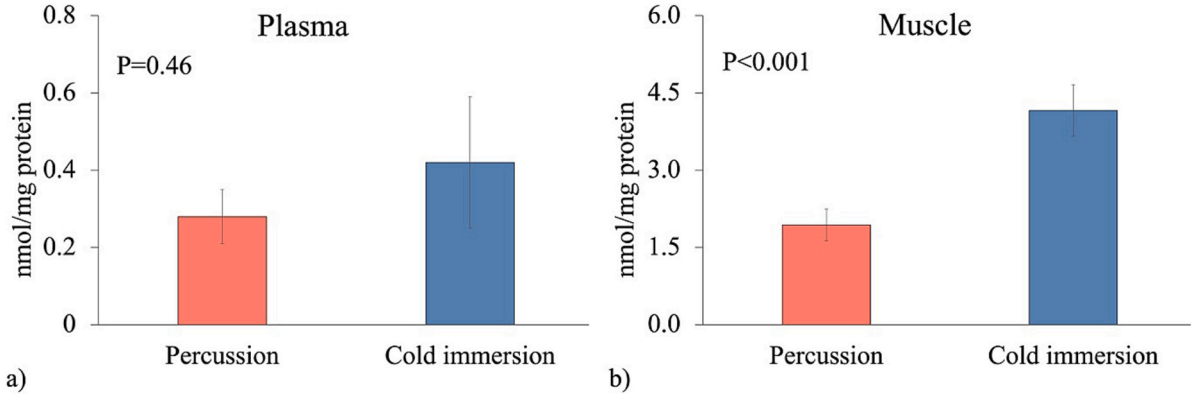

Fig. 3. Effect of stunning/slaughtering method (percussion vs. cold immersion) on malondialdehyde (MDA) measured on trout plasma (a) and muscle (b). Percussion: sharp blow to the head. Cold immersion: trout immersed in cold (−5.8 ± 0.5 °C) saline water (5% of NaCl) for 5 min.

Table 1
Effects of stunning/slaughtering methods (percussion vs. cold immersion) and storage time (3 h, 48 h, 168 h, and 264 h) on fish traits at slaughter, rigor index and pH of fillets of rainbow trout.

	Stunning/slaughtering method (M)[1]		Storage time (T)				Probability			RMSE
	Percussion	Cold immersion	3 h	48 h	168 h	264 h	M	T	M × T	
Measures, n	45	45	30[2]	20	20	20				
Slaughter weight, g	288	283	292	280	293	277	0.63	0.69	0.99	53
Fish total length, cm	27.7	27.4	27.8	27.4	27.8	27.2	0.37	0.62	0.89	1.70
Fillet yield, %	49.1	46.9	48.7	50.4	47.1	45.7	<0.01	<0.01	0.98	3.89
Rigor index, %	13.2	11.5	41.6	0.03	2.42	5.59	0.69	<0.001	0.93	17.4
Fillet pH	6.47	6.44	6.57	6.39	6.37	6.50	0.14	<0.001	0.38	0.07

RMSE: Root mean square error.
[1] Percussion: sharp blow to the head; cold immersion: trout immersed in cold (−5.8 ± 0.5 °C) saline water (5% of NaCl) for 5 min.
[2] Includes measures taken after 3 h on Set 3 h (10 trout, 5 per experimental group) and on Set 48 h (20 trout, 10 per experimental group).

the stunning/slaughtering method × hours *post-mortem* was observed ($P < 0.001$). In details, RI was similar at the first sampling (3 h after slaughter); then, it sharply increased (from 31% to 75%) at 6 h *post-mortem* and remained high (76%, on average) up to 12 h *post-mortem* in trout subjected to percussion to reach zero (which indicates the resolution of *rigor mortis*) at 36 h after slaughter (Fig. 4a). In trout subjected to cold immersion, RI remained around 41% on average between 3 and 12 h *post-mortem* and then decreased to 14% and 3% at 20 h and 24 h *post-mortem*, respectively (Fig. 4a).

On the other hand, average pH (6.46) during the first 48 h post mortem was not significantly affected by the stunning/slaughtering method (Table 1). As for the evolution, after slaughtering, the muscle pH decreased until reaching the minimum values (6.52 on average) at 9 h post mortem, and then increased at 12 h (6.54) and 28 h (6.60) post mortem following a similar trend in all trout regardless from the stunning/slaughtering method (Fig. 4b).

3.3. Trout rheological traits during storage

Rheological traits were measured on the whole fish and the fillets after dissection to evaluate quality changes according to the storage time, at 3 h, 48 h, 168 h and until 264 h of storage (11 d after slaughtering), in trout subjected to the two stunning/slaughtering methods.

The whole fish RI and the fillet pH measured from 3 h to 264 h post

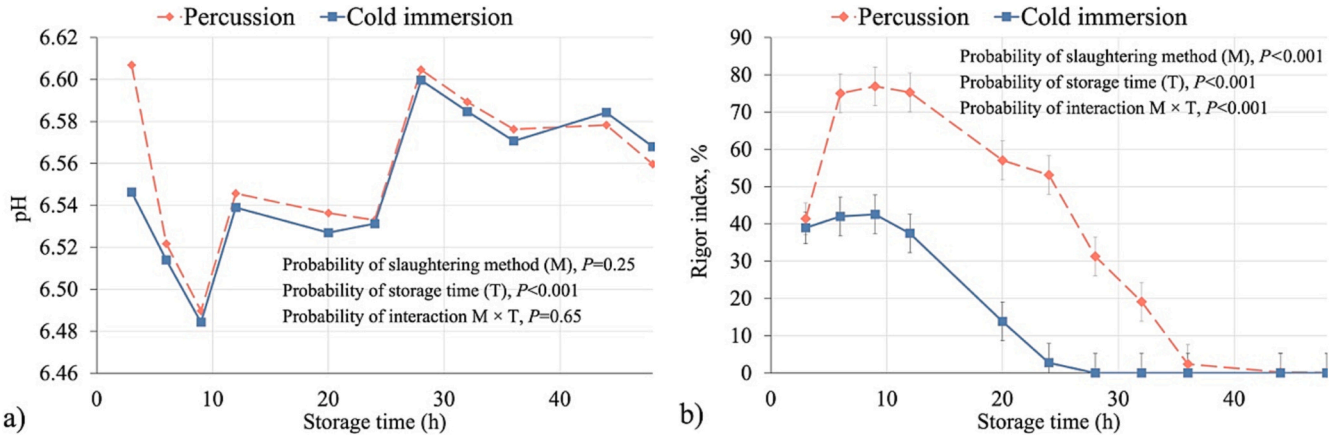

Fig. 4. Effect of stunning/slaughtering method (percussion vs. cold immersion) on the evolution of fish muscle pH (a) and rigor index (b) during the first 48 h post-mortem. Percussion: sharp blow to the head. Cold immersion: trout immersed in cold (−5.8 ± 0.5 °C) saline water (5% of NaCl) for 5 min.

mortem were not affected by the stunning/slaughtering method while significantly decreasing (from 42% to 2.70% and from 6.57 to 6.39, respectively; $P < 0.001$) from 3 h to 48 h post mortem (Table 2).

At the skin (Table 2), L* (−5%; $P < 0.001$), a* (−64%; $P < 0.001$), and b* (−1.22 vs. 6.08; $P < 0.001$) were lower in fish subjected to cold immersion compared to those subjected to percussion. As the storage time increased until 264 h, skin a* (+42%; $P < 0.001$) increased while b* (−0.76 vs. 4.02; $P < 0.001$) decreased compared with 48 h and 168 h of storage; L* decreased (−19%; $P < 0.001$) from 48 h to 168 h of storage and then increased (+18%; $P < 0.001$) from 168 h to 264 h of storage (Table 3). A significant ($P < 0.001$) interaction between the stunning/slaughtering method and storage time was found in the colour of fish skin (Table 2; Fig. 4). Regarding L*, the lowest value (37.0) was found at 168 h of storage in fish subjected to percussion, whereas the highest values (50.3 and 48.1) were found at 48 h and 264 h of storage in the same experimental group. Then, a lower a* was found at 48 h and 168 h of storage in fish subjected to cold immersion compared with the percussion group (−2.58 vs. −0.76; $P < 0.001$). As for b*, the highest values (7.64 and 8.73) were found at 48 h and 168 h of storage in fish subjected to percussion, whereas the lowest value (−3.38) was found at 264 h of storage in fish subjected to cold immersion (Fig. 5).

As for fillet, no differences for colour indexes were found according to the stunning/slaughtering method, whereas colour indexes showed changes from 3 h to 48 h and 168 h and then returned to the initial values (3 h of storage) after 264 h of storage (Table 2).

The results of TPA analysis on the whole fish and fillets were not affected by the stunning/slaughtering method, except for chewiness which was higher (+28% and + 32% for whole fish and fillet, respectively; $P < 0.05$) in fish subjected to cold immersion compared with percussion (Table 3).

On the other hand, some changes were observed with storage time. As for the whole fish, hardness decreased from 3 h to 48 h of storage (−66%; $P < 0.001$), cohesiveness from 168 h to 264 h of storage (−13%; $P < 0.01$), and chewiness from 48 h to 168 h of storage (−52%; $P < 0.001$), whereas gumminess and resilience gradually decreased ($P < 0.001$) with storage time (Table 3). As for fish fillets, cohesiveness decreased from 48 h to 168 h of storage (−14%; $P < 0.01$), and the elasticity index from 3 h to 48 h of storage (−11%; $P < 0.01$), whereas resilience constantly decreased ($P < 0.01$) with storage time (Table 3).

3.4. Fillet TVB-N content and fatty acid profile

On average, the TVB-N content of fillet resulted slightly higher (+5%; $P < 0.01$) in trout subjected to percussion than in those to cold immersion (Fig. 6a). Then, TVB-N showed the lowest value (13.8 mg/100 g of fillet) at 48 h of storage and reached the highest ($P < 0.001$) value (26.5 mg/100 g of fillet) at 264 h of storage (Fig. 6b).

As for the fatty acid profile of fish fillets, the rate of total saturated fatty acids (SFA) averaged 22.2%, monounsaturated fatty acids (MUFA) 44.8%, polyunsaturated fatty acids (PUFA) 33.1% and total n-3 PUFA 7.67%, with an average n-3/n-6 PUFA ratio of 0.31 (Table 4). While the stunning/slaughtering method had no effect, SFA and MUFA, and MUFA/PUFA ratio changed with storage, showing the lowest values at 168 h of storage ($P < 0.001$). An opposite trend was found for PUFA, n-3 PUFA, and PUFA/SFA ratio (Table 4).

4. Discussion

We studied a new stunning/slaughtering method for rainbow trout based on a cold shock generated by the immersion of fish, previously reared in water at 14 °C, in cold (−5.8 ± 0.5 °C) and saline (5% NaCl) water for 5 min compared with percussion. This latter method is the most commonly used to stun/kill rainbow trout both in France (EC, 2018) and in the small commercial farms of Germany (Jung-Schroers et al., 2020), whereas in Italy (Clemente et al., 2023) and Denmark (EC, 2018) percussion stands on the second place after electric water bath.

The RI and muscle pH during the first 48 h after slaughtering were measured at different intervals to evaluate if the methods could have differently affected the *post-mortem* evolution, and then the quality of the trout during a storage for 11 d, and how this could be related to slaughtering stress and/or other issues. Indeed, in fish subjected to cold immersion, the muscular activity during stunning was likely reduced by the very low temperature and fish did not struggle, as it could happen with slaughtering in ice and air or in ice/water slurry. Thus, the pre-slaughter depletion of glycogen storage and the changes in lactic acid did not substantially differ from trout subjected to percussion. In fact, soon after slaughtering, differences in pH were not significant between the two groups and, importantly pH was similar in the two groups of animals at the different time points during the 48 h after killing, which implies a similar post mortem evolution as for the anaerobic respiration of glycogen and the corresponding production of lactic acid. Thus, the final pH after 48 h was similar in the two groups, as already observed in other studies evaluating different stunning and slaughtering methods in rainbow trout (Concollato et al., 2016, 2020).

On the other hand, the different evolution of *rigor mortis* during the first 48 h in trout subjected to cold immersion compared to those subjected to percussion stand for some effects at the muscle fibres level and their metabolisms. Based on literature, in rainbow trout stored in ice, the time from death to the onset of *rigor mortis* ranges from 45 h to 54 h (Roth et al., 2006), but could be reached even after 2 h when a high pre-slaughter stress occurs as observed in catfish (Le et al., 2020). In fact, several pre-slaughtering and slaughtering factors can play an important

Table 2
Effects of stunning/slaughtering methods (percussion vs. cold immersion) and storage time (3 h, 48 h, 168 h, and 264 h) on the colour of skin and fillet of rainbow trout.

	Stunning/slaughtering method (M)[1]		Storage time (T)				Probability			RMSE
	Percussion	Cold immersion	3 h	48 h	168 h	264 h	M	T	M × T	
Skin										
Fish (n)	30	30	–	20	20	20				
L*	45.1	42.7	–	47.7^{b}	38.5^{a}	45.5^{b}	0.09	<0.001	0.03	5.47
a*	−0.72	−2.01	–	-1.54^{a}	-1.64^{a}	-0.92^{b}	<0.001	<0.001	<0.001	0.58
b*	6.08	−1.22	–	3.55^{b}	4.48^{b}	-0.76^{a}	<0.001	<0.001	0.02	1.94
Chroma	6.55	3.50	–	5.23^{b}	6.26^{b}	3.58^{a}	<0.001	<0.001	<0.001	1.45
Hue	114	204	–	143^{a}	135^{a}	199^{b}	<0.001	<0.001	0.79	29.7
Fillet										
Fish (n)	35	35	10	20	20	20				
L*	47.3	47.3	42.9^{a}	48.7^{b}	48.8^{b}	48.8^{b}	0.95	<0.001	0.28	1.78
a*	−0.93	−0.84	-0.52^{b}	-1.20^{a}	-1.19^{a}	-0.63^{b}	0.64	0.03	0.38	0.80
b*	9.91	10.03	11.0^{b}	9.49^{a}	9.09^{a}	10.3^{b}	0.57	<0.001	0.87	1.05
Chroma	10.0	10.2	11.1^{b}	9.62^{a}	9.25^{a}	10.4^{b}	0.59	<0.001	0.90	1.03
Hue	96.0	95.3	93.2^{a}	97.5^{b}	97.9^{b}	94.1^{a}	0.81	<0.01	0.70	4.50

RMSE: root mean square error; L*: lightness; a*: redness; b*: yellowness.
[1] Percussion: sharp blow to the head; cold immersion: trout immersed in cold (−5.8 ± 0.5 °C) saline water (5% of NaCl) for 5 min.

Table 3
Effects of stunning/slaughtering methods (percussion vs. cold immersion) and storage time (3 h, 48 h, 168 h, and 264 h) on the texture profile analysis of whole fish and fillet of rainbow trout.

	Stunning/slaughtering method (M)[1]		Storage time (T)				Probability			RMSE
	Percussion	Cold immersion	3 h	48 h	168 h	264 h	M	H	M × T	
Fish (n)	35	35	10	20	20	20				
Whole fish										
Hardness (g)	1037	1161	2177^{b}	871^{a}	723^{a}	623^{a}	0.07	<0.001	0.56	273
Cohesiveness	0.76	0.76	0.79^{b}	0.78^{b}	0.78^{b}	0.68^{a}	0.99	0.003	0.81	0.10
Springiness (mm)	0.64	0.69	0.39	0.85	0.75	0.67	0.72	0.20	0.99	0.55
Elasticity index	0.87	0.86	0.92	0.83	0.85	0.87	0.72	0.20	0.99	0.11
Gumminess (g)	787	865	1647^{c}	673^{b}	564^{ab}	420^{a}	0.16	<0.001	0.65	212
Chewiness (g mm^{-1})	403	514	672^{b}	568^{b}	294^{a}	301^{a}	0.04	<0.001	0.37	214
Resilience	0.48	0.50	0.58^{c}	0.49^{b}	0.47^{ab}	0.41^{a}	0.31	<0.001	0.73	0.08
Fillet										
Hardness (g)	85.4	102.8	85.7	107.2	80.2	103.7	0.15	0.24	0.58	46.0
Cohesiveness	0.58	0.60	0.65^{b}	0.61^{b}	0.54^{a}	0.55^{a}	0.46	<0.01	0.34	0.09
Springiness (mm)	0.85	0.89	0.73^{a}	0.85^{ab}	0.99^{b}	0.92^{ab}	0.44	<0.01	0.78	0.21
Elasticity index	0.66	0.64	0.71^{b}	0.66^{a}	0.60^{a}	0.63^{a}	0.44	<0.01	0.79	0.08
Gumminess (g)	50.5	63.8	56.5	66.7	47.6	57.8	0.10	0.31	0.37	31.3
Chewiness (g mm^{-1})	40.4	53.2	41.6	53.5	39.9	52.2	0.03	0.16	0.37	21.8
Resilience	0.29	0.30	0.33^{b}	0.30^{ab}	0.29^{ab}	0.26^{a}	0.32	<0.01	0.40	0.06

RMSE: root mean square error.
[1] Percussion: sharp blow to the head. Cold immersion: trout immersed in cold (−5.8 ± 0.5 °C) saline water (5% of NaCl) for 5 min.

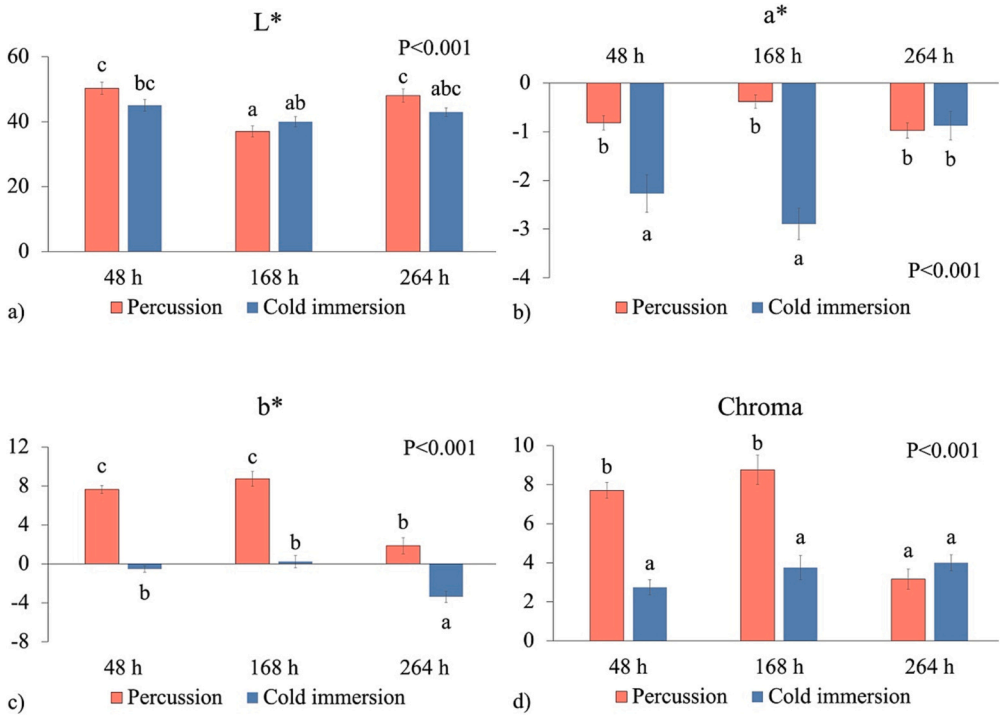

Fig. 5. Effect of the interaction between stunning/slaughtering method and storage time on the colour of fish skin: lightness (a), red index (b), yellow index (c) and chroma (d). Percussion: sharp blow to the head. Cold immersion: trout immersed in cold (−5.8 ± 0.5 °C) saline water (5% of NaCl) for 5 min. Means with different superscript letters significantly differ ($P < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

role on the state of muscle energy reserves and, consequently, the evolution of *rigor mortis* (Concollato et al., 2016). Under our conditions, while rigor index was similar in trout slaughtered with the two methods at 3 h after slaughtering (about 40%), it did not increase in trout subjected to cold immersion (on average 43% from 3 to 9 h after slaughtering) differently from what happened in trout subjected to percussion where RI sharply increased until 75% after 6 h to remain high until 12 h post mortem (on average 76% from 6 to 12 h). After 12 h, the resolution of *rigor mortis* occurred in both groups even if it was completed earlier in trout subjected to cold immersion compared to those subjected to percussion, which can be directly related to the lower initial value of the rigor index. Previous authors have found that the low muscle temperature at death might alter the patterns of rigor development in trout subjected to cold immersion for stunning/slaughtering (Skjervold et al., 2001).

In fact, under our conditions, differences in the development of *rigor mortis* were not due to differences in the accumulation of lactic acid produced by a different muscular activity before death (Amlacher, 1961) or by stress during the stunning procedure (Skjervold et al., 1999, 2001) since pH evolution during the first 48 h post mortem was the same in the fish subjected to the two stunning/slaughtering methods. Rather, it can be hypothesized that the low temperatures (about −6 °C) applied

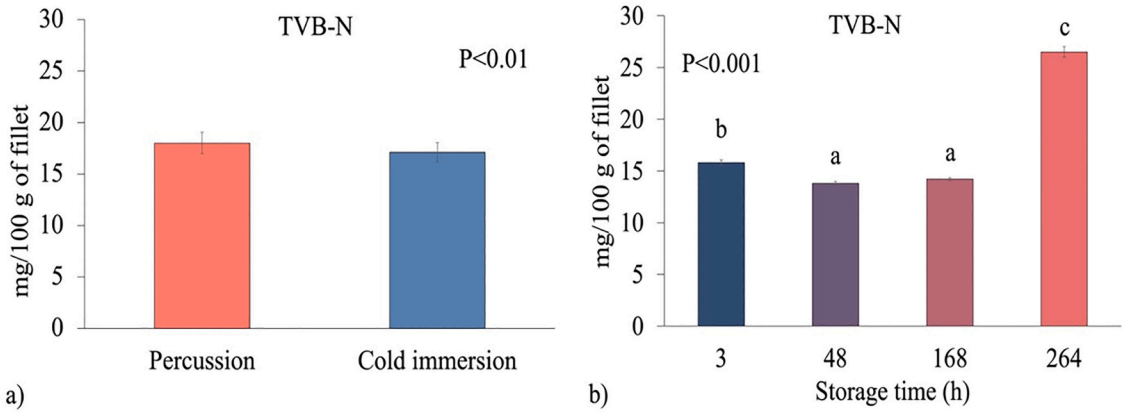

Fig. 6. Effects of stunning/slaughtering method (a) and storage time (b) on total volatile base nitrogen (TVB-N) (mg/100 g of fillet) of rainbow trout. Percussion: sharp blow to the head. Cold immersion: trout immersed in cold (−5.8 ± 0.5 °C) saline water (5% of NaCl) for 5 min. Means with different superscript letters significantly differ ($P < 0.05$).

Table 4
Effects of stunning/slaughtering methods (percussion vs. cold immersion) and storage time (3 h, 48 h, 168 h, and 264 h) on the fatty acid profile of rainbow trout fillets.

	Stunning/slaughtering method (M)[1]		Storage time (T)				Probability			RMSE
	Percussion	Cold immersion	3 h	48 h	168 h	264 h	M	T	M × T	
Fillets (n)	35	35	10	20	20	20				
C14:0	1.37	1.36	1.40^{b}	1.35^{b}	1.28^{a}	1.42^{bc}	0.72	<0.001	0.46	0.07
C16:0	14.9	15.2	15.1^{ab}	15.1^{ab}	14.4^{a}	15.6^{b}	0.25	<0.001	0.81	0.75
C18:0	4.09	4.20	4.13^{b}	4.25^{b}	3.93^{a}	4.29^{b}	0.05	<0.001	0.59	0.22
C16:1n-7	2.12	2.11	2.19^{b}	2.07^{a}	2.04^{a}	2.15^{b}	0.67	<0.001	0.52	0.11
C18:1n-9	36.1	36.6	36.8^{b}	36.9^{b}	34.7^{a}	37.2^{b}	0.15	<0.001	0.77	1.34
C18:1n-7	2.65	2.70	2.70^{ab}	2.70^{ab}	2.59^{a}	2.72^{b}	0.17	<0.01	0.32	1.23
C20:1n-9	1.42	1.43	1.44^{b}	1.44^{b}	1.35^{a}	1.46^{b}	0.55	<0.01	0.47	0.09
C18:2n-6	22.0	21.7	21.9^{ab}	21.8^{ab}	22.5^{b}	21.3^{a}	0.23	<0.001	0.66	0.89
C18:3n-3	3.01	2.89	2.92^{a}	2.86^{a}	3.27^{b}	2.76^{a}	0.10	<0.001	0.68	0.30
C20:4n-6	0.56	0.54	0.50^{a}	0.54^{a}	0.68^{b}	0.48^{a}	0.48	<0.001	0.91	0.11
C20:5n-3	0.77	0.69	0.65^{a}	0.69^{a}	0.95^{b}	0.63^{a}	0.11	<0.001	0.72	0.19
C22:6n-3	2.78	2.53	2.33^{a}	2.46^{a}	3.90^{b}	1.92^{a}	0.31	<0.001	0.81	1.00
SFA	22.0	22.3	22.2^{b}	22.2^{b}	21.1^{a}	23.1^{b}	0.21	<0.001	0.70	1.07
MUFA	44.5	45.1	45.4^{b}	45.2^{b}	42.8^{a}	45.8^{b}	0.18	<0.001	0.68	1.66
PUFA	33.5	32.6	32.5^{a}	32.6^{a}	36.0^{b}	31.1^{a}	0.16	<0.001	0.68	2.55
∑n-3	7.96	7.38	7.17^{a}	7.28^{a}	9.73^{b}	6.50^{a}	0.16	<0.001	0.72	1.63
∑n-6	25.0	24.6	24.7^{ab}	24.7^{ab}	25.8^{b}	24.0^{a}	0.23	<0.001	0.58	1.06
∑n-3/∑n-6	0.32	0.30	0.29^{a}	0.29^{a}	0.38^{b}	0.27^{a}	0.17	<0.001	0.76	0.05
PUFA/SFA	1.54	1.47	1.48^{a}	1.47^{a}	1.71^{b}	1.35^{a}	0.16	<0.001	0.63	0.19
MUFA/PUFA	1.35	1.40	1.41^{b}	1.40^{b}	1.20^{a}	1.48^{b}	0.17	<0.001	0.68	0.15
Nutritional indices										
IA	0.26	0.27	0.27^{b}	0.27^{b}	0.25^{a}	0.28^{b}	0.35	<0.001	0.69	0.02
IT	0.35	0.37	0.37^{b}	0.36^{b}	0.31^{a}	0.39^{b}	0.16	<0.001	0.75	0.04

RMSE: root mean square error. SFA: Saturated Fatty Acids; MUFA: Monounsaturated Fatty Acids; PUFA: Polyunsaturated Fatty Acids. IA: Index of atherogenicity; IT: Index of thrombogenicity.

[1] Percussion: sharp blow to the head. Cold immersion: trout immersed in cold (water temperature at −5.8 ± 0.5 °C) saline water (5% of NaCl) for 5 min.

to fish subjected to cold immersion in our trial could have strongly reduced the intensity of muscle contractions (Curran et al., 1986) or have damaged muscle fibres and actin/myosin filaments to a certain extent which prevented the occurrence of *rigor mortis*, as it happens when fish is frozen (Sotelo et al., 1995; Reid et al., 2022). In fact, any freezing due to the cold immersion could have produced ice crystals in the muscle cells, which in turn have the potential of destroying their structure with a consequent release of proteinase and pro-oxidants, ultimately impairing muscle quality (Nakazawa and Okazaki, 2020). On the other hand, under our conditions, all other *post-mortem* process remained unchanged, as for the anaerobic metabolism after death and acid lactic production in the first stages, as measured by pH, and later protein denaturation associated to *post-mortem* processes, as measured by the similar RI decrease in cold-immersed and in percussed trout.

Additionally, and noteworthy, the use of cold immersion did not likely result in a significant increase in the stress response and oxidative stress as measured by cortisol and MDA levels respectively, since their content either remained unchanged in the plasma.

Cortisol levels were even lower than those observed with percussion in the alternative matrices (muscle, fin, and mucus). The absence of significant differences in plasma cortisol levels between the two methods suggests that cold immersion may not induce a higher stress response than percussion, which is acknowledged as one of the least stressful methods (EFSA, 2009b). On the other hand, the lower cortisol levels observed in the alternative matrices of fish subjected to cold immersion may be attributed to the slower diffusion of cortisol caused by the low temperature (Barton and Iwama, 1991; Jaxion-Harm and Ladich, 2014). Indeed, the cortisol content (184 ng/ml) we recorded in trout plasma largely exceeded the basal levels reported for rainbow trout (5 ng/ml) (Bertotto et al., 2010), indicating that both methods finally induced stress in the fish or, more likely, that collecting procedures with short-term crowding and air exposure before stunning might have the

major role on increasing trout plasma cortisol, which can reach 187 ng/ml after a short period of crowding (30–50 min) (Sloman et al., 2001; Merkin et al., 2010). Nevertheless, this procedure is the standard under the commercial and field conditions in which trout are managed and that we wanted to investigate.

On the other hand, a comprehensive physiological evaluation of the trout stress response related to the two slaughtering/stunning methods should include more indicators other than cortisol and MDA, such as plasma osmolality (Saraiva et al., 2024), antioxidant enzymatic activity (Isık et al., 2019), heat-shock proteins 70 (Zahedi et al., 2019) and, based on a multi-level approach, behaviour and brain proteomics (Saraiva et al., 2024), besides product quality.

Pre-slaughter stress often induces changes in flesh colour, producing darker fillets in salmon (Roth et al., 2006) and rainbow trout (Lefèvre et al., 2008; Lefevre et al., 2016). We found that fillet colour did not differ between stunning/slaughtering methods, whereas cold immersion produced a darker skin, as already observed in rainbow trout when coping with stressors (Iger et al., 1995; Khan et al., 2016; Gesto et al., 2017). Although no statistical significance was found between the two methods, the low temperature in cold immersion may have stimulated a greater melanocyte contraction and subsequently resulted in increased melanin release (Iger et al., 1995), causing a darker fish skin. In sparids, initial alterations in skin colour due to slaughtering procedures are usually offset after one day of ice storage, with no further changes until 18 days (Pavlidis et al., 2006; Vardanis et al., 2011). Otherwise, in our study, the differences in trout skin colour remained until 7 days of storage and then disappeared after 11 days. These results should be carefully considered in view of the implementation of cold-immersion as a stunning/slaughtering method, as skin colour is an important quality indicator and darker fish could be discarded by consumers, especially in the case of table-size fish (Luo et al., 2021).

The effect of slaughtering method on fillet shelf-life, including TVB-N content, has been poorly investigated in rainbow trout. No differences were found in the TVB-N of fish slaughtered either by percussion or air asphyxia (Duran et al., 2008), similarly to what we observed when comparing percussion with cold immersion. In other species, air asphyxia produced a higher TVB-N compared with ice bath in the flesh of tambaqui (*Colossoma macropomum*) (Mendes et al., 2017), whereas no variations were found between the fillets of grass carp (*Ctenopharyngodon idella*) slaughtered with electricity or ice bath (Scherer et al., 2006). Indeed, storage time rather than slaughtering method is the crucial factor in decreasing freshness in fish, as confirmed by our study. In fact, TVB-N content remained rather similar from 3 h to 7 days but then doubled from 7 to 11 days, exceeding the threshold limit of acceptability (20 mg/100 g) for rainbow trout (Moosavi-Nasab et al., 2021).

We confirmed the limited impact of the stunning/slaughtering method on trout fillet quality, including colour, texture, and fatty acid profile, as observed in previous works (Concollato et al., 2019; Dalle Zotte et al., 2020). Cold immersion resulted in a firmer texture in both fillet and whole fish where short-term stress may lead to muscle softening, while the hardness of the muscle may increase as a result of crowding stress or prolonged muscular activity in trout (Skjervold et al., 2001; Dalle Zotte et al., 2020), which mechanisms has to be clarified in the case of cold immersion.

Although handling, transportation, and environmental conditions can affect the activity of lipogenic and lipolytic enzymes and ultimately result in changes in the fatty acid composition of the fish muscle as previously proved in fish other than salmonids (Goes et al., 2019; Bolzan et al., 2021), we did not find any differences in our fillets, consistently with previous reports that compared different stunning/slaughtering methods in rainbow trout (Secci et al., 2016a) and Atlantic salmon (Secci et al., 2016b). Nonetheless, pre-slaughter handling and stress have been shown to increase the lipid oxidation of fish fillets (Secci and Parisi, 2016). In our study, the higher MDA content found in the muscle of fish subjected to cold immersion could be the consequence of a pro-oxidant effect of salt in the cool water, as reported by Morzel and van de Vis (2003) in eels, and a prolonged time before death under aerobic conditions, even if at a decreased metabolic rate, compared with percussion (Duran et al., 2008) that might have promoted muscle physical damage that favours catalytic enzymes (Morzel and van de Vis, 2003).

5. Conclusions

A cold shock by an immersion in cold saline water could be considered a viable alternative to percussion, which is a commonly used method, for stunning and slaughtering rainbow trout. This method is easy to perform, cost-effective and requires less labour, while also producing a stress response comparable to percussion. Regarding product quality, cold immersion resulted in darker fish skin and, likely, prevented the onset or decreased the degree of *rigor mortis* which was associated to an earlier resolution, but without any relevant effects on fillet colour, texture, or fatty acid profile. The effects of cold water immersion at the muscle level as for the evolution of *rigor mortis* are worth of further investigations.

Authors' contributions

DB, AM, and AT conceived, designed and supervised the experiment. AM acquired the financial support for the project. EF and DB collected and prepared samples for analyses. Martina Bortoletti and DB performed analyses for cortisol. AT, GX, and Marco Birolo organised and performed analyses for fish quality and freshness evolution. FB performed the statistical analyses. FB, AT, GR, and DB analysed and interpreted the data, and wrote the first draft of the manuscript. All authors critically reviewed the manuscript for intellectual content and gave final approval of the version to be published.

CRediT authorship contribution statement

Francesco Bordignon: Writing – original draft, Writing – review & editing, Data curation, Formal analysis. **Martina Bortoletti:** Investigation, Writing – review & editing. **Angela Trocino:** Conceptualization, Data curation, Investigation, Methodology, Supervision, Writing – original draft, Writing – review & editing. **Gerolamo Xiccato:** Methodology, Writing – review & editing. **Marco Birolo:** Investigation, Writing – review & editing. **Eleonora Fiocchi:** Investigation, Writing – review & editing. **Amedeo Manfrin:** Conceptualization, Funding acquisition, Writing – review & editing, Supervision. **Giuseppe Radaelli:** Data curation, Writing – review & editing, Formal analysis. **Daniela Bertotto:** Conceptualization, Investigation, Methodology, Writing – original draft, Writing – review & editing, Data curation, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgements

The research was funded by the Italian Ministry of Health to Amedeo Manfrin (RC 07/13 Tecniche innovative per lo stordimento della trota iridea (*Oncorhynchus mykiss*) prima dell'uccisione e della lavorazione). The Authors wish to thank Fabio Del Tedesco of Agroittica Friulana Porcia (PN, Italy) for providing trout and technical and logistic support.

References

Amlacher, E., 1961. Rigor mortis in fish. In: Fish As Food. Elsevier, pp. 385–409. https://doi.org/10.1016/B978-0-12-395569-2.50018-2.

Ashley, P.J., 2007. Fish welfare: current issues in aquaculture. Appl. Anim. Behav. Sci. 104, 199–235. https://doi.org/10.1016/j.applanim.2006.09.001.

Barton, B.A., Iwama, G.K., 1991. Physiological changes in fish from stress in aquaculture with emphasis on the response and effects of corticosteroids. Annu. Rev. Fish Dis. 1, 3–26. https://doi.org/10.1016/0959-8030(91)90019-G.

Bermejo-Poza, R., Fernández-Muela, M., De la Fuente, J., Pérez, C., González de Chavarri, E., Díaz, M.T., Torrent, F., Villarroel, M., 2021. Effect of ice stunning versus electronarcosis on stress response and flesh quality of rainbow trout. Aquaculture 538, 736586. https://doi.org/10.1016/j.aquaculture.2021.736586.

Bertotto, D., Poltronieri, C., Negrato, E., Majolini, D., Radaelli, G., Simontacchi, C., 2010. Alternative matrices for cortisol measurement in fish. Aquac. Res. 41, 1261–1267. https://doi.org/10.1111/j.1365-2109.2009.02417.x.

Bito, M., Yamada, K., Mikumo, Y., Amano, K., 1983. Studies on rigor mortis of fish. I. Difference in the mode of rigor mortis among some varieties of fish by modified Cutting's method. Bull. Tokai Reg. Fish. Res. Lab. No. 109, 89–96.

Bolzan, L.P., Barroso, D.C., Souza, C.F., Oliveira, F.C., Wagner, R., Baldisserotto, B., Val, A.L., Baldissera, M.D., 2021. Dietary supplementation with nerolidol improves the antioxidant capacity and muscle fatty acid profile of *Brycon amazonicus* exposed to acute heat stress. J. Therm. Biol. 99, 103003 https://doi.org/10.1016/j.jtherbio.2021.103003.

Christie, W.W., 1982. A simple procedure for rapid transmethylation of glycerolipids and cholesteryl esters. J. Lipid Res. 23, 1072–1075. https://doi.org/10.1016/S0022-2275(20)38081-0.

Clemente, G.A., Tolini, C., Boscarino, A., Lorenzi, V., Dal Lago, T.L., Benedetti, D., Bellucci, F., Manfrin, A., Trocino, A., Rota Nodari, S., 2023. Farmed fish welfare during slaughter in Italy: survey on stunning and killing methods and indicators of unconsciousness. Front. Vet. Sci. 10, 1253151. https://doi.org/10.3389/fvets.2023.1253151.

Commission International de l''Eclairage, 1976. ISO/CIE 11664–4: 2019—Colorimetry—Part 4: CIE 1976 L*a*b* Colour Space. Available at: https://www.techstreet.com/cie/standards/iso-cie-11664-4-2019?gateway_code=cie&product_id=2078733#document.

Concollato, A., Olsen, R.E., Vargas, S.C., Bonelli, A., Cullere, M., Parisi, G., 2016. Effects of stunning/slaughtering methods in rainbow trout (*Oncorhynchus mykiss*) from death until rigor mortis resolution. Aquaculture 464, 74–79. https://doi.org/10.1016/j.aquaculture.2016.06.009.

Concollato, A., Dalle Zotte, A., Vargas, S.C., Cullere, M., Secci, G., Parisi, G., 2019. Effects of three different stunning/slaughtering methods on physical, chemical, and sensory changes in rainbow trout (*Oncorhynchus mykiss*): stunning/slaughtering and rainbow trout quality. J. Sci. Food Agric. 99, 613–619. https://doi.org/10.1002/jsfa.9222.

Concollato, A., Secci, G., Dalle Zotte, A., Vargas, S.C., Olsen, R.E., Lira de Medeiros, A.C., Parisi, G., 2020. Effects of stunning methods on *pre rigor* changes in rainbow trout (*Oncorhynchus mykiss*) reared at two different temperatures. Ital. J. Anim. Sci. 19, 477–486. https://doi.org/10.1080/1828051X.2020.1759465.

Curran, C.A., Poulten, R.G., Brueton, A., Jones, N.S.D., 1986. Cold shock reactions in iced tropical fish. Int. J. Food Sci. Technol. 21, 289–299. https://doi.org/10.1111/j.1365-2621.1986.tb00408.x.

Dalle Zotte, A., Concollato, A., Secci, G., Cullere, M., Parisi, G., 2020. Rainbow trout (*Oncorhynchus mykiss*) farmed at two different temperatures: post rigor mortis changes in function of the stunning method. Czech J. Anim. Sci. 65, 354–364. https://doi.org/10.17221/144/2020-CJAS.

Daskalova, A., 2019. Farmed fish welfare: stress, post-mortem muscle metabolism, and stress-related meat quality changes. Int. Aquat. Res. 11, 113–124. https://doi.org/10.1007/s40071-019-0230-0.

Duran, A., Erdemli, U., Karakaya, M., Yilmaz, M., 2008. Effects of slaughter methods on physical, biochemical and microbiological quality of rainbow trout *Oncorhynchus mykiss* and mirror carp *Cyprinus carpio* filleted in pre-, in- or post-rigor periods. Fish. Sci. 74, 1146–1156. https://doi.org/10.1111/j.1444-2906.2008.01634.x.

EC, 2018. Report from the commission to the European Parliament and the Council on the possibility of introducing certain requirements regarding the protection of fish at the time of killing, 2018. https://eur-lex.europa.eu/legal-content/en/TXT/?uri=CELEX%3A52018DC0087 (Accessed 18 August 2023).

EEC, 1995. Total volatile basic nitrogen (TVB-N) limit values for certain categories of fishery products and specifying the analysis methods to be used. Commission Decision 95/149/EEC of 8 March 1995. Off. J. Europ. Commun. L 97, 84–87. Available at: http://www.legislation.gov.uk/eudn/1995/149/adopted (Accessed 9 May 2023).

EFSA, 2004. Opinion of the Scientific Panel on Animal Health and Welfare on a request from the Commission related to welfare aspects of the main systems of stunning and killing the main commercial species of animals. EFSA J. 45, 1–29. Available at: https://www.efsa.europa.eu/en/efsajournal/pub/45 (Accessed 9 May 2023).

EFSA, 2008. Scientific Opinion of the Panel on Animal Health and Animal Welfare on a request from the European Commission on the Animal welfare aspects of husbandry systems for farmed trout. EFSA J 796, 1–22. https://doi.org/10.2903/j.efsa.2008.796.

EFSA, 2009a. Scientific opinion of the panel on animal health and welfare on a request from the European Commission on species-specific welfare aspects of the main systems of stunning and killing of farmed rainbow trout. EFSA J. 1013, 1–55. Available at: https://www.efsa.europa.eu/en/efsajournal/pub/1012 (Accessed 9 May 2023).

EFSA, 2009b. Scientific opinion of the panel on biological hazards on a request from the European Commission on food safety considerations concerning the species-specific welfare aspects of the main systems of stunning and killing of farmed fish. EFSA J. 1190, 1–16. https://doi.org/10.2903/j.efsa.2009.1190 (Accessed 9 May 2023).

EPAW, 2022. EU Platform on Animal Welfare - Own Initiative Group on Fish, 2022. Guidelines on Water Quality and Handling for the Welfare of Farmed Vertebrate Fish. Available at: https://food.ec.europa.eu/system/files/2022-07/aw_platform_plat-conc_guide_farmed-fish_en.pdf.

EUMOFA, 2023. The EU Fish Market, 2023 Edition. https://doi.org/10.2771/38507 (Accessed 22 December 2023).

European Parliament, 2011. European Parliament resolution of 17 June 2010 on a new impetus for the strategy for the sustainable development of European aquaculture (2009/2107(INI)). Off. J. Eur. Union CE 236/132. Available at: https://eur-lex.europa.eu/legal-content/EN/ALL/?uri=CELEX:52010IP0243 (Accessed 9 May 2023).

Fletcher, G.L., Kao, M.H., Dempson, J.B., 1988. Lethal freezing temperatures of Arctic char and other salmonids in the presence of ice. Aquaculture 71, 369–378. https://doi.org/10.1016/0044-8486(88)90206-2.

Gesto, M., Skov, P.V., Jokumsen, A., 2017. Emergence time and skin melanin spot patterns do not correlate with growth performance, social competitive ability or stress response in farmed rainbow trout. Front. Neurosci. 11, 319. https://doi.org/10.3389/fnins.2017.00319.

Giuffrida, A., Pennisi, L., Ziino, G., Fortino, L., Valvo, G., Marino, S., Panebianco, A., 2007. Influence of slaughtering method on some aspects of quality of gilthead seabream and smoked rainbow trout. Vet. Res. Commun. 31, 437–446. https://doi.org/10.1007/s11259-007-3431-8.

Goes, E.S.D.R., Goes, M.D., Castro, P.L.D., Lara, J.A.F.D., Vital, A.C.P., Ribeiro, R.P., 2019. Imbalance of the redox system and quality of tilapia fillets subjected to pre-slaughter stress. PLoS One 14, e0210742. https://doi.org/10.1371/journal.pone.0210742.

Gomez-Guillen, M.C., Montero, P., Hurtado, O., Borderias, A.J., 2000. Biological characteristics affect the quality of farmed Atlantic salmon and smoked muscle. J. Food Sci. 65, 53–60. https://doi.org/10.1111/j.1365-2621.2000.tb15955.x.

Iger, Y., Balm, P.H.M., Jenner, H.A., Bonga, S.E.W., 1995. Cortisol induces stress-related changes in the skin of rainbow trout (*Oncorhynchus mykiss*). Gen. Comp. Endocrinol. 97, 188–198. https://doi.org/10.1006/gcen.1995.1018.

Isık, A., Atamanalp, M., Alak, G., 2019. Evaluation of antioxidant level and protein oxidation of rainbow trout (*Oncorhynchus mykiss*) fillets during rigor and post-rigor. J. Food Saf. 1, e12746 https://doi.org/10.1111/jfs.12746.

Jaxion-Harm, J., Ladich, F., 2014. Effects of temperature change on cortisol release by common carp *Cyprinus carpio*: temperature and cortisol release of *Cyprinus carpio*. J. Fish Biol. 84, 1221–1227. https://doi.org/10.1111/jfb.12331.

Jung-Schroers, V., Hildebrandt, U., Retter, K., Esser, K.H., Hellmann, J., Kleingeld, D.W., Rohn, K., Steinhagen, D., 2020. Is humane slaughtering of rainbow trout achieved in conventional production chains in Germany? Results of a pilot field and laboratory study. BMC Vet. Res. 16, 197. https://doi.org/10.1186/s12917-020-02412-5.

Kestin, S.C., Robb, D.H., Van De Vis, J.W., 2002. Protocol for assessing brain function in fish and the effectiveness of methods used to stun and kill them. Vet. Rec. 150, 302–307. https://doi.org/10.1136/vr.150.10.302.

Khan, U.W., Øverli, Ø., Hinkle, P.M., Pasha, F.A., Johansen, I.B., Berget, I., Silva, P.I.M., Kittilsen, S., Höglund, E., Omholt, S.W., Våge, D.I., 2016. A novel role for pigment genes in the stress response in rainbow trout (*Oncorhynchus mykiss*). Sci. Rep. 6, 28969. https://doi.org/10.1038/srep28969.

Le, T.T., Nguyen, H.T., Pham, M.A., 2020. Rigor mortis development and effects of filleting conditions on the quality of Tra catfish (*Pangasius hypophthalmus*) fillets. J. Food Sci. Technol. 57, 1320–1330. https://doi.org/10.1007/s13197-019-04166-x.

Lefèvre, F., Bugeon, J., Aupérin, B., Aubin, J., 2008. Rearing oxygen level and slaughter stress effects on rainbow trout flesh quality. Aquaculture 284, 81–89. https://doi.org/10.1016/j.aquaculture.2008.07.017.

Lefevre, F., Cos, I., Pottinger, T.G., Bugeon, J., 2016. Selection for stress responsiveness and slaughter stress affect flesh quality in pan-size rainbow trout, *Oncorhynchus mykiss*. Aquaculture 464, 654–664. https://doi.org/10.1016/j.aquaculture.2016.07.039.

Luo, M., Lu, G., Yin, H., Wang, L., Atuganile, M., Dong, Z., 2021. Fish pigmentation and coloration: molecular mechanisms and aquaculture perspectives. Rev. Aquac. 13, 2395–2412. https://doi.org/10.1111/raq.12583.

Mendes, J.M., Dairiki, J.K., Inoue, L.A.K.A., de Jesus, R.S., 2017. Advantages of recovery from pre-slaughter stress in tambaqui *Colossoma macropomum* (Cuvier 1816) agroindustry in the Amazon. Food Sci. Technol. 37, 383–388. https://doi.org/10.1590/1678-457x.14316.

Merkin, G.V., Roth, B., Gjerstad, C., Dahl-Paulsen, E., Nortvedt, R., 2010. Effect of pre-slaughter procedures on stress responses and some quality parameters in sea-farmed rainbow trout (*Oncorhynchus mykiss*). Aquaculture 309, 231–235. https://doi.org/10.1016/j.aquaculture.2010.08.025.

Moosavi-Nasab, M., Khoshnoudi-Nia, S., Azimifar, Z., Kamyab, S., 2021. Evaluation of the total volatile basic nitrogen (TVB-N) content in fish fillets using hyperspectral imaging coupled with deep learning neural network and meta-analysis. Sci. Rep. 11, 5094. https://doi.org/10.1038/s41598-021-84659-y.

Morzel, M., van de Vis, H., 2003. Effect of the slaughter method on the quality of raw and smoked eels (*Anguilla anguilla* L.): slaughter and flesh quality of eel. Aquac. Res. 34, 1–11. https://doi.org/10.1046/j.1365-2109.2003.00754.x.

Nakazawa, N., Okazaki, E., 2020. Recent research on factors influencing the quality of frozen seafood. Fish. Sci. 86, 231–244. https://doi.org/10.1007/s12562-020-01402-8.

Ntzimani, A., Angelakopoulos, R., Semenoglou, I., Dermesonlouoglou, E., Tsironi, T., Moutou, K., Taoukis, P., 2023. Slurry ice as an alternative cooling medium for fish harvesting and transportation: study of the effect on seabass flesh quality and shelf life. Fish. Aquac. J. 8, 385–392. https://doi.org/10.1016/j.aaf.2021.01.006.

Papaharisis, L., Tsironi, T., Dimitroglou, A., Taoukis, P., Pavlidis, M., 2019. Stress assessment, quality indicators and shelf life of three aquaculture important marine fish, in relation to harvest practices, water temperature and slaughter method. Aquac. Res. 50, 2608–2620. https://doi.org/10.1111/are.14217.

Pavlidis, M., Papandroulakis, N., Divanach, P., 2006. A method for the comparison of chromaticity parameters in fish skin: preliminary results for coloration pattern of red skin Sparidae. Aquaculture 258, 211–219. https://doi.org/10.1016/j.aquaculture.2006.05.028.

Reid, C.H., Patrick, P.H., Rytwinski, T., Taylor, J.J., Willmore, W.G., Reesor, B., Cooke, S. J., 2022. An updated review of cold shock and cold stress in fish. Fish Biol. 100, 1102–1137. https://doi.org/10.1111/jfb.15037.

Robb, D.H.F., Kestin, S.C., 2002. Methods used to kill fish: field observations and literature reviewed. Anim. Welf. 11, 269–282. https://doi.org/10.1017/S0962728600024854.

Robb, D.H.F., Kestin, S.C., Warriss, P.D., 2000. Muscle activity at slaughter: I. Changes in flesh colour and gaping in rainbow trout. Aquaculture 182, 261–269. https://doi.org/10.1016/S0044-8486(99)00273-2.

Roth, B., Slinde, E., Arildsen, J., 2006. Pre or post mortem muscle activity in Atlantic salmon (*Salmo salar*). The effect on rigor mortis and the physical properties of flesh. Aquaculture 257, 504–510. https://doi.org/10.1016/j.aquaculture.2005.10.021.

Saraiva, J.L., Faccenda, F., Cabrera-Álvarez, M.J., Povinelli, M., Hubbard, P.C., Cerqueira, M., Farinha, A.P., Secci, G., Tignani, M.V., Pulido Rodriguez, L.P., Parisi, G., 2024. Welfare of rainbow trout at slaughter: integrating behavioural, physiological, proteomic and quality indicators and testing a novel fast-chill stunning method. Aquaculture 581, 740443. https://doi.org/10.1016/j.aquaculture.2023.740443.

SAS (Statistical Analysis System Institute, Inc.), 2013. SAS/STAT(R) 9.2 User's Guide, second ed. SAS Institute Inc., Cary, NC, USA. Available at: http://support.sas.com/documentation/cdl/en/statug/63033/HTML/default/viewer.htm#glm_toc.htm.

Scherer, R., Augusti, P.R., Bochi, V.C., Steffens, C., Fries, L.L.M., Daniel, A.P., Kubota, E. H., Neto, J.R., Emanuelli, T., 2006. Chemical and microbiological quality of grass carp (*Ctenopharyngodon idella*) slaughtered by different methods. Food Chem. 99, 136–142. https://doi.org/10.1016/j.foodchem.2005.06.048.

Secci, G., Parisi, G., 2016. From farm to fork: lipid oxidation in fish products. A review. Ital. J. Anim. Sci. 15, 124–136. https://doi.org/10.1080/1828051X.2015.1128687.

Secci, G., Parisi, G., Dasilva, G., Medina, I., 2016a. Stress during slaughter increases lipid metabolites and decreases oxidative stability of farmed rainbow trout (*Oncorhynchus mykiss*) during frozen storage. Food Chem. 190, 5–11. https://doi.org/10.1016/j.foodchem.2015.05.051.

Secci, G., Serra, A., Concollato, A., Conte, G., Mele, G., Olsen, R.E., Parisi, G., 2016b. Carbon monoxide as stunning/killing method on farmed Atlantic salmon (*Salmo salar*): effects on lipid and cholesterol oxidation. J. Sci. Food Agric. 96, 2426–2432. https://doi.org/10.1002/jsfa.7362.

Skjervold, P.O., Fjæra, S.O., Østby, P.B., 1999. Rigor in Atlantic salmon as affected by crowding stress prior to chilling before slaughter. Aquaculture 175, 93–101. https://doi.org/10.1016/S0044-8486(99)00037-X.

Skjervold, P.O., Fjæra, S.O., Østby, P.B., Einen, O., 2001. Live-chilling and crowding stress before slaughter of Atlantic salmon (*Salmo salar*). Aquaculture 192, 265–280. https://doi.org/10.1016/S0044-8486(00)00447-6.

Sloman, K.A., Taylor, A.C., Metcalfe, N.B., Gilmour, K.M., 2001. Stress from air emersion fails to alter chloride cell numbers in the gills of rainbow trout. J. Fish Biol. 59, 186–190. https://doi.org/10.1111/j.1095-8649.2001.tb02351.x.

Sotelo, C.G., Piñeiro, C., Pérez-Martín, R.I., 1995. Denaturation of fish proteins during frozen storage: role of formaldehyde. Eur. Food Res. Technol. 200, 14–23. https://doi.org/10.1007/BF01192902.

Ulbricht, T.L.V., Southgate, D.A.T., 1991. Coronary heart disease: seven dietary factors. Lancet 338, 985–992. https://doi.org/10.1016/0140-6736(91)91846-m.

Van De Vis, H., Kestin, S., Robb, D., Oehlenschläger, J., Lambooij, B., Münkner, W., Kuhlmann, H., Kloosterboer, K., Tejada, M., Huidobro, A., Otterå, H., Roth, B., Sørensen, N.K., Akse, L., Byrne, H., Nesvadba, P., 2003. Is humane slaughter of fish possible for industry? Aquac. Res. 34, 211–220. https://doi.org/10.1046/j.1365-2109.2003.00804.x.

Vardanis, G., Sfichi-Duke, L., Tort, L., Divanach, P., Kotzabasis, K., Pavlidis, M., 2011. The use of biochemical, sensorial and chromaticity attributes as indicators of postmortem changes in commercial-size, cultured red porgy *Pagrus pagrus*, stored on ice: freshness indicators in red porgy. Aquac. Res. 42, 341–350. https://doi.org/10.1111/j.1365-2109.2010.02628.x.

Wills, C.C., Proctor, M.R.M., McLoughlin, J.V., 2004. Integrated studies on the freshness of rainbow trout (*Oncorhynchus mykiss* Walbaum) postmortem during chilled and frozen storage. J. Food Biochem. 28, 213–244. https://doi.org/10.1111/j.1745-4514.2004.tb00067.x.

WOAH, 2022. Welfare aspects of stunning and killing of farmed fish for human consumption. In: Aquatic Animal Health Code. https://www.woah.org/fileadmin/Home/eng/Health_standards/aahc/current/chapitre_welfare_stunning_killing.pdf.

Yoshida, Y., Itoh, N., Hayakawa, M., Piga, R., Cynshi, O., Jishage, K., Niki, E., 2005. Lipid peroxidation induced by carbon tetrachloride and its inhibition by antioxidant as evaluated by an oxidative stress marker, HODE. Toxicol. Appl. Pharmacol. 208, 87–97. https://doi.org/10.1016/j.taap.2005.01.015.

Zahedi, S., Akbarzadeh, A., Mehrzad, J., Noori, A., Harsij, M., 2019. Effect of stocking density on growth performance, plasma biochemistry and muscle gene expression in rainbow trout (*Oncorhynchus mykiss*). Aquaculture 498, 271–278. https://doi.org/10.1016/j.aquaculture.2018.07.044.

Zampacavallo, G., Parisi, G., Mecatti, M., Lupi, P., Giorgi, G., Poli, B.M., 2015. Evaluation of different methods of stunning/killing sea bass (*Dicentrarchus labrax*) by tissue stress/quality indicators. J. Food Sci. Technol. 52, 2585–2597. https://doi.org/10.1007/s13197-014-1324-8.