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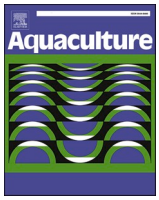

Welfare of rainbow trout at slaughter: Integrating behavioural, physiological, proteomic and quality indicators and testing a novel fast-chill stunning method

João L. Saraiva [a,b], Filippo Faccenda [c], María J. Cabrera-Álvarez [a,b,*], Michele Povinelli [c], Peter C. Hubbard [b], Marco Cerqueira [b], Ana Paula Farinha [b,d], Giulia Secci [e], Maria Vittoria Tignani [e], Lina F. Pulido Rodriguez [e], Giuliana Parisi [e]

[a] Fish Etho Group Association, Olhão, Portugal
[b] CCMAR, Centro de Ciências do Mar, Faro, Portugal
[c] Fondazione Edmund Mach, S. Michele all'Adige, Italy
[d] Escola Superior Agrária de Santarém, Portugal
[e] Dipartimento di Scienze e Tecnologie Agrarie, Alimentari, Ambientali e Forestali, Università degli Studi di Firenze, Italy

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ABSTRACT

A critical point in the life of a captive fish is the final stages of production, not only in welfare terms but also due to effects on meat quality, carcass appearance and derived economic impacts. The most common method to slaughter fish is by asphyxia either in ice-water or in the open air. In humane slaughter procedures, however, a stunning method needs to be implemented to render the fish immediately unconscious (within one second) until death. The objective of this research was to evaluate and compare the effectiveness and welfare effects of four types of stunning methods in rainbow trout (*O. mykiss*): cold shock by fast-chilling as a novel method, where the fish were immersed in liquid water at −8 °C, asphyxia (as the currently used method), electrical stunning, and anaesthesia with MS-222. We used a total of 176 trout (mean weight 524 ± 138 g), combining behavioural (individual swimming activity, equilibrium, opercular movement and eye-roll), physiological (heart rate and electrocardiogram amplitude) and circulating (plasma cortisol and osmolality) indicators with brain proteomic signatures. We also analysed the effects on fillet shelf-life and quality in each method (rigor mortis, water content, fillet colour, pH and ATP degradation).

Anaesthesia effectively induced unconsciousness, with regular and strong heartbeat and low cortisol. Quality indicators were the best among all the methods assessed. Electric shock was found to be an effective and irreversible method for inducing unconsciousness, with strong heartbeat and large variation in cortisol response and quality indicators similar to anaesthesia. On the contrary, asphyxia presented indicators of poor welfare (*e.g.,* long-lasting consciousness throughout the slaughter process, high cortisol levels), with very low flesh quality parameters. Fast-chilling also resulted in extreme signs of stress (intense mucus release, haemorrhage and no loss of consciousness), low ATP content and the worst proteomic signatures, along with an early onset and resolution of *rigor mortis* (6 and 48 h, respectively). Our results reinforce the idea that electric stunning is a promising humane method to stun farmed trout. In contrast, the fast-chilling method showed very poor results both in welfare and in quality, indicating that it is not a viable humane alternative to asphyxia. Moreover, the proteome analysis provided valuable insights into the brain mechanisms of rainbow trout at slaughter, offering potential fine-scale biomarkers of welfare.

1. Introduction

The welfare of farmed fish is now an inescapable ethical concern and has received abundant attention in recent years (*e.g.,* Saraiva et al., 2022; Tschirren et al., 2021; Turnbull, 2022). The overwhelming scientific evidence demonstrating that fish possess the neural,

* Corresponding author at: Fish Etho Group Association, Olhão, Portugal.
E-mail address: maria@fishethogroup.net (M.J. Cabrera-Álvarez).

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physiological, behavioural, and cognitive traits to be sentient beings (Braithwaite et al., 2013; Brown, 2015, 2016; Cerqueira et al., 2017; Sneddon, 2018; Sneddon and Brown, 2020) provides the background that supports the implementation of measures improving fish welfare. In fact, these should be already in effect after the Lisbon treaty (European Union, 2007; Saraiva et al., 2018). These findings and measures have stimulated research to find ways to improve the quality of life for captive fish, including not only methods and techniques to avoid negative experiences, but also ways to provide positive experiences (Arechavala-Lopez et al., 2022; dos Gauy et al., 2021). Good welfare practices are not only ethical, they also improve flesh quality (Poli et al., 2005; Poli, 2009) and drive consumer choice. A recent report interviewing 9047 adults in nine European countries (UK, Germany, France, Italy, Spain, Poland, Sweden, the Netherlands, and the Czech Republic) found that 79% of consumers think that fish welfare should be protected to the same extent as farm animals, 61% think that fish welfare has an impact in their choice of fish product and 61% would be willing to pay up to 30% more if it means fish experience better welfare (Eurogroup for Animals et al., 2018).

A critical point in the life of a captive fish is the final stages of production. This moment not only has the risk of dramatically affecting welfare (Lines and Spencer, 2012), but it can also cause serious economic harm to the farm, since the slaughter process can affect meat quality and carcass appearance (Robb and Kestin, 2002). A still widespread method to slaughter fish (including trout) is by asphyxia either in ice-water or in the open air (European Food Safety Authority (EFSA), 2009; Robb and Kestin, 2002). In fact, a recent review on farmed fish welfare during slaughter in Italy shows that asphyxia is the most common method to kill trout in this country (Clemente et al., 2023). Italy is the second largest producer of trout in EU (FEAP, 2023) and the Trentino region (where this trial was conducted, see Section 2) accounts for nearly 80% of Italian production (Maiolo et al., 2021). Therefore, asphyxia remains a relevant problem to tackle, independently of EU recommendations which leave the decision to adopt/or enforce humane stunning methods for fish entirely up to member states (Council Regulation (EC) No 1099/2009, 2009). This method induces prolonged and intense suffering, which is a poor practice in ethical, commercial, and regulatory terms (Giménez-Candela et al., 2020). In order to achieve humane slaughter, a stunning method needs to be implemented before slaughter, and it must render the fish immediately unconscious until death (Boyland and Brooke, 2017; European Food Safety Authority (EFSA), 2009). In this regard, electrical stunning prior to slaughter has been proposed as a humane method for trout (European Food Safety Authority (EFSA), 2009; Lines et al., 2003). However, a recent study showed that the application of electric current must be of higher field strength, current density, and duration than originally thought to induce a loss of consciousness of at least 15 min (Hjelmstedt et al., 2022). Additionally, trout farmers report carcass and fillet damages, with consequent decrease in value (personal communications to authors).

It is essential to highlight that welfare must be defined so it can be measured: welfare is the state of the animal as it copes with the environment (Broom, 1986, 1991). This definition of welfare has important implications, among which: (i) welfare is a characteristic of an animal, not something that is given to it; (ii) welfare will vary along a continuum, from negative to positive; (iii) welfare can be measured through indicators, independently of ethical considerations; (iv) measures of difficulty in coping with the environment give information about the welfare of the animal concerned (Broom, 1991; Saraiva et al., 2018).

Behavioural indicators are an essential tool for farmers and veterinarians to assess the internal status of the fish, and many of them have been previously studied to assess the level of consciousness during anaesthesia, stunning, and slaughter. For example, simple reflex indicators, such as eye roll and the ability to flip upright, can easily be used as direct indicators of consciousness, and can be evaluated individually or as an index (Davis, 2010). The animal is classified as insensible if responses to these indicators are lacking (Robb et al., 2000). The vestibulo-ocular reflex (VOR; the "eye roll") is an ocular movement that occurs when the body of the fish is tilted to the side on the sagittal plane as an instinctive act to maintain the equilibrium in the visual field. It is the last reflex the fish loses during anaesthesia, and is the first reflex to reappear during recovery (Kestin et al., 2002). Rhythmical opercula movements are also absent in insensitive fish, although this behavioural indicator varies among species and levels of anaesthesia (Kestin et al., 2002). Another reflex that is used to measure the level of consciousness is the "tail-grab reflex" (*i.e.*, grabbing the fish's tail and observing if it attempts to escape; Davis, 2010).

Physiological parameters, such as plasma cortisol, glucose and lactate, are well described stress signatures in fish, including rainbow trout (Barton, 2000; Flos et al., 1988). Although they have limitations, and should be considered in context (Saraiva et al., 2018; Vijayan et al., 2010), they continue to be relevant indicators of stress. Haematocrit levels are often elevated during short-term stress and increase oxygen supply in response to higher metabolic demand (Cnaani et al., 2004; Poli et al., 2005). Osmolality has also been shown to be elevated due to acute stressors in fish (Barton and Grosh, 1996; Caldwell and Hinshaw, 1994), and it could be elevated in fish exposed to electrical stunning and fast-chilling due to open cellular membrane ion channels and to induce osmotic stress due to increased external salinity, respectively. Heart rate is also a good measurement to assess the effectiveness of stunning methods, since irregular heart rate, lack of activity, or fibrillation indicate defective heart function (Lambooij et al., 2010). Additionally, internal and external injuries can help estimate the major physical effects of each method.

The increase of stress during slaughter also causes fast energy expenditure, which is typically accompanied by a low adenosine 5′-triphosphate to inosine monophosphate (ATP/IMP) ratio in muscle, as well as early *rigor mortis* onset and resolution because of the rise in lactic acid production and the consequently fall in pH (Poli et al., 2005). Moreover, a decrease in pH may interfere with the ability of protein to bind water, which will alter the water holding capacity (WHC) of fish flesh and the fillet surface's ability to scatter light (Poli et al., 2005). Robb et al. (2000) and Robb (2001) also highlighted that the cascade of events brought on by stress during slaughter can have negative impacts on fish quality, particularly on primary qualitative traits, such as colour and WHC. Yet, the stress reaction appears to be quite species-specific and connected to the practices of stunning and killing. For instance, *some authors claim that stress had no impact on rigor mortis* (Concollato et al., 2016a; Roth et al., 2002) *while others noted that stress brought forward rigor onset* (Misimi et al., 2008; Parisi et al., 2014; Poli et al., 2005; Roque et al., 2021; Secci et al., 2018) *and resolution* (Acerete et al., 2009; Parisi et al., 2014). Lefevre et al. (2016) also noted that, although the cortisol levels increased and pH decreased by pre-slaughter stress (due to confinement), fillet colour was unaffected. Moreover, Pulcini et al. (2022) found no significant effects on the WHC of *Salmo carpio* stunned with different methods despite the ATP depletion, pH drop and *rigor* onset, all these parameters being good indicators of stress.

Proteomics can be used to distinguish different fish slaughtering practices by analysing the whole proteome of a given tissue such as muscle or brain. Unlike traditional methods that focus on individual proteins, proteomics provides a holistic view of the studied tissue and offers valuable insights into the physiological state of the organism at a given moment (Rodrigues et al., 2012; Tripathy et al., 2021). This means that, when fish are subjected to different slaughtering methods or conditions, the stress suffered can induce changes in their physiological responses, stress levels, and overall well-being, which can be reflected in the protein profiles of the sampled tissue. By separating the complex mixture of proteins expressed under those conditions, expression levels of proteins can be compared qualitatively and quantitatively, following the identification and characterization of specific proteins of interest (Bassols et al., 2014). In the context of fish welfare assessment, this possibility plays a crucial role for the discovery of candidate molecular signatures that reflect the physiological alterations due to the stress

exposure. These signatures may serve as complementary tools to behavioural observations and immunochemical tests that assess secondary stress effects, such as cortisol release. Numerous studies have unequivocally demonstrated the immense potential of proteomics in the field of identifying stress signatures in various organisms. These studies, including those by Alves et al. (2010), Cordeiro et al. (2012), Metzger et al. (2016), Raposo de Magalhães et al. (2020), and Sanahuja and Ibarz (2015), have shed light on the significant contributions of proteomic techniques.

We tested a newly devised low-temperature stunner, which has been previously tested in *Salmo carpio* (Pulcini et al., 2022) and in which salt is added to water so that it remains liquid at temperatures below 0 °C. The objective was to evaluate and compare the effectiveness and welfare effects of four stunning methods in rainbow trout:

- Cold shock by fast-chilling (FC) as a novel method;
- Asphyxia (ASP) as the current method used in the majority of farms (European Commission. Directorate General for Health and Food Safety, 2017);
- Electrical stunning (ES) as a humane method (European Food Safety Authority (EFSA), 2009; Lines et al., 2003), and
- Anaesthesia with MS-222 (AN) as a positive control, since this would be in theory the most non-invasive, gentle and humane way to induce unconsciousness (Bowman et al., 2019; Sloman et al., 2019).

We used a multi-level approach to address fish welfare, combining behaviour, stress signatures, proteomics, and quality of the final product. The identification of robust behavioural indicators and signatures of fish welfare holds paramount importance in advancing our understanding of the physiological adaptive responses to stress and the effective management of fish welfare. Furthermore, it plays a crucial role in the development of sustainable fish rearing protocols with a strong emphasis on ethical responsibility. To address these critical objectives, this study endeavours to provide a comprehensive assessment of rainbow trout welfare during the slaughter process, employing an integrative and, to the best of our knowledge, unprecedented approach. Additionally, we investigated the efficacy of a novel stunning method. Through this multifaceted investigation, we aim to contribute significantly to the scientific community's knowledge and inform practical applications for enhancing fish welfare in the aquaculture industry.

2. Materials and methods

2.1. Animal housing and experimental procedures

We selected 176 rainbow trout (*Oncorhynchus mykiss*) of 524 ± 138 g and total length: 34.5 ± 3.3 cm from a resident all-female strain (Fondazione Edmund Mach, San Michele all'Adige, Italy). Prior to the experiment, all animals were housed for one week in twelve 700 L indoor freshwater tanks in groups of 14 or 15 individuals, at 13.4 °C and 8.5 mg/L dissolved oxygen (DO) levels on the day of the experiment. The fish were exposed to a 10 L:14D and were fed Veronesi commercial feed (Ecofish-Veronesi, Verona, Italy) by hand at 1.3% (*w*/w) on a daily basis. The fish were subjected to three days of fasting (40 degree-days) before the experiments to ensure clearing of the gut while complying with good welfare practices (European Food Safety Authority (EFSA), 2009; López-Luna et al., 2016).

For the stunning procedures, we caught the fish in each tank in four sequential catching rounds of up to four fish each, until the tank was empty. The first catch was used for *rigor* assessment while in all subsequent catches we used fish to observe behaviour, record the post-stun heart rate by electrocardiogram (ECG) and take samples of blood, muscle, and brain. The sampling order of tanks was randomised. In each round, fish were carried swiftly to one of the stunning procedures. We tested the stunning methods in the following order: 1) Electrical stunning, 2) Asphyxia, 3) Anaesthesia and 4) Fast-chilling. Electrical Stunning and Asphyxia trials took place on December 1st while Anaesthesia and Fast-Chilling trials took place in December 2nd, 2021.

In total, over the two days of the experiment, we assessed the behaviour of 102 fish (24 per treatment except Asphyxia that had 28 fish), recorded ECG in 32 fish (eight per treatment; note that four of these were also used for behaviour observations during the Asphyxia trial), we used 48 fish for fillet quality analyses, physiological parameters, and proteomic analysis (12 per treatment).

The details of each stunning method are described below.

2.1.1. Electrical stunning (ES)

An electrical stunner TEQ002 (Gozlin, Modena, Italy) with two mobile electrodes placed 55 cm apart in a plastic tank (40 × 60 cm) filled with 30 L of freshwater (extracted from the main system; conductivity 442 μS/cm) was used. Stunning parameters were set at 340 V and 400 mAmp, and applied for 20 s (based on Concollato et al. (2016b) using the same equipment, and our own preliminary tests) to achieve immediate and long-lasting cessation of activity. Four fish were stunned simultaneously in each round, in a total of six rounds and an observer was present during the stunning process. All fish lost all indicators of consciousness in the first second (see Section 2.2). Animals allocated for recovery assessment were observed in a tank with water from the system and maximum recovery time was fixed to 20 min, after which the test was terminated and the fish euthanised by an overdose of anaesthesia (MS-222 at 400 ppm for >5 min).

2.1.2. Asphyxia (ASP)

Four fish at a time were netted out from their home tank and held in a covered plastic tank (air temperature: 10 °C) and observed until the loss of all indicators of consciousness. The catch was repeated seven times. Maximum induction time (*i.e.*, time in open air) was fixed to 20 min, after which the test was terminated and the fish euthanised by a blow to the head if still presented signs of consciousness.

2.1.3. Anaesthesia with tricaine methanesulfonate (AN)

A 400 ppm solution of tricaine methanesulfonate (MS-222; Finquel®, Argent Laboratories, Redmont-VI, USA), strong enough to induce effective, time-efficient and long lasting unconsciousness (according to Beckman, 2016; Topic Popovic et al., 2012 and our own preliminary tests) was prepared in a plastic tank containing 30 L of water from the system. Four fish were anaesthetised simultaneously in each round, and the solution was renewed every four rounds. Anaesthesia was considered complete when fish lost all indicators of consciousness (see 2.2). Animals allocated for recovery assessment were observed in a tank with water from the housing system and maximum recovery time was fixed to 20 min, after which the test was terminated and the fish euthanised by an overdose of anaesthesia (MS-222 at 400 ppm for >5 min).

2.1.4. Fast-chilling method (FC)

An experimental stunning system composed of a chiller (maximum power 3.8 kW) and stunning tank (volume 220 L) was employed to decrease and maintain the temperature of a brine (salt concentration 10.8% *w*/w) at −8 °C. The induction time (*i.e.*, time of immersion) ranged from 1.5 to 2.0 min approximatively. Animals allocated for recovery assessment were observed in water from the housing system and maximum recovery time was fixed to 20 min, after which the test was terminated and the fish euthanised by an overdose of anaesthesia (MS-222 at 400 ppm for >5 min).

2.2. Behavioural indicators

Following all stunning methods, except asphyxia, fish were transferred to a 30 L tank containing system water so that the observer could register in detail the following parameters, their timing to cease, and eventual recovery: free-swimming (*i.e.*, whether the fish showed any locomotion without stimulation), equilibrium, escape behaviour or

voluntary movement, opercular movement, and eye roll (VOR). The loss of each of these indicators usually follows that sequence, and the cessation of consciousness was assessed *via* the presence/absence of VOR. All measurements were taken *in loco* in 24 fish for the ES, 28 fish for ASP, 26 fish for AN, and 24 fish for FC treatments. Some data points were missing in some individuals due to the difficulty of assessing the exact moment of the cessation of certain indicators.

2.3. Physiology

A different subsample of 8 fish from each stunning method was not allowed to recover and were euthanised by percussive blow to the head immediately after being stunned or after all activity had ceased in the case of ASP. Afterwards, we collected blood and brain tissue (no apparent damage from the percussive blow was found when inspected). The following analyses were performed *post-mortem*: endocrine response (plasma cortisol levels), and plasma osmolality. We also annotated any apparent internal and external injuries.

Blood was extracted by caudal vein puncture. Plasma was extracted *in loco*, following centrifugation of blood for 3 min at 5000 rpm and pipetted into a plastic tube, followed by storing at −20 °C until shipping to Centro de Ciências do Mar (Faro, Portugal) and stored at −80 °C until processed. Cortisol was quantified by an Enzyme-Linked Immunosorbent Assay (ELISA RE52611 kit, IBL International GmbH, Germany) and osmolality was measured in 10 μL samples with a vapour pressure osmometer (Wescor 5520).

2.4. Electrophysiology

We performed an electrocardiogram (ECG) test to a subsample of 8 fish for each method, immediately after being stunned, after all visible activity ceased in the case of ASP, and after a blow to the head in the case of FC (because 92% of the fish that went through FC were still moving). Due to technical issues or undetected heart rate in some fish, the final sample size was six fish for ES, six for ASP, eight for AN, and seven for FC.

After each stunning procedure, we stabilized the fish wrapping it with damp cloth. Once ready, we used three cup-shaped electrodes to record ECGs. Best results were obtained in previous tests with the right-side electrode midway and on the extreme lateral aspect of the isthmus. The left electrode was placed approximately 1 cm posterior to the right electrode but on the opposite side. A third ground electrode was placed further down the ventral body, as suggested by Cotter and Rodnick (2007). We used an A.C./D.C. strain gauge amplifier (Grass CP122, Astro-Med, West Warwick, RI; no longer in existence) connected *via* a data logger (PicoLog 1012, Pico Technology, www.picotech.com) to a laptop with PicoScope 6 software (Pico Technology) to collect and register the signal (×2000 amplification, high-pass 0.01 Hz, low-pass 100 Hz). At the completion of the trial (which took <1 min per fish), fish were sampled for blood and physiological parameters (see above).

2.5. Fish and fillet quality

All animals used for these analyses were euthanised by a blow to the head after stunning took place.

Sampling for *rigor mortis* and fillet quality evaluations.

Twenty fish were used for *rigor mortis* evolution, fillet physical (colour, pH, texture, WHC) and chemical analyses (adenosine 5′-triphosphate and its catabolites, proximate composition, total lipids, fatty acid profile, and lipid oxidative products) described in the later sections. Total lengths and weights of all fish were recorded immediately after death.

2.5.1. Rigor evolution assessment and fish sampling for quality parameter assessment

Twelve fish for each group were observed until *rigor* resolution and *rigor* index (RI) was calculated as Bito et al. (1983):

$$RI = [(L_0 - L_t)/L_0] \times 100$$

where L is the vertical distance between the base of the caudal fin and the table surface on which the fish was resting by the front half of its body, measured immediately after death (L_0) and during storage (L_t, where t ranged from 3 to 96 h after death, with measurements performed at 3, 6, 9, 12, 15, 24, 48, 72 and finally 96 h after death). Throughout the observation period, fish were stored in polystyrene boxes, covered with ice and placed in a refrigerated room (+2 °C).

Another eight fish for each group were immediately filleted. The left-side fillets were allocated to physical and chemical analyses described below (T0 samples). The right-side fillets were stored in polystyrene boxes covered by ice (at +2 °C) and sampled after *rigor* resolution ($T0_{RR}$ equivalent to 96 h *post-mortem*) to analyse physical characteristics and obtain their chemical properties.

2.5.2. Physical analyses

The colour of skin and fillets was measured on triplicate positions (cranial, medial, and caudal) of the dorsal and epaxial portion of the fish or fillet (Supplemental Fig. S1), respectively, with a CHROMA METER CR-200 (Konica Minolta, Chiyoda, Japan) according to the CIELab system (CIELAB, 1976) expressed as L^* (lightness), a^* (redness index) and b^* (yellowness index) colour parameters. For the colour of the whole fish skin, only the left side was analysed, while on the fillet muscle the colour measurements were carried out on both fillets (right and left at different storage times).

The values of pH and of maximum shear force (texture) parameters were registered. The muscle pH value was measured on triplicate fillet positions (cranial, medial, and caudal) by a pH-meter SevenGo SG2™ (Mettler-Toledo, Schwerzenbach, Switzerland). Texture was assessed as the maximum shear force value obtained utilising the Warner-Bratzler shear blade (width of 7 cm) by a Zwick Roell® 109 texturometer (Zwick Roell, Ulm, Germany), equipped with a 1 kN load cell, setting the crosshead speed at 30 mm min^{-1}. The blade was pressed through the muscle vertically to the muscle fibres on a 3 × 3 cm section obtained from the cranial-dorsal region of the fish fillet. The force value was expressed in Newtons (N). Afterwards, fillets were skinned, homogenized, and used to determine WHC by centrifugation according to Eide et al. (1982), modified by Hultmann and Rustad (2002), and to be chemically characterized.

2.5.3. ATP extraction and nucleotides degradation

One gram of muscle was sampled from the cranial epaxial portion of fillets ($n = 8$) and homogenized with 10 mL of 0.6 M perchloric acid for nucleotides extraction, as previously described by Burns (1985). The concentrations of adenosine 5′-triphosphate (ATP), adenosine 5′-diphosphate (ADP), adenosine 5′-monophosphate (AMP), inosine 5′-monophosphate (IMP), inosine (Ino) and hypoxanthine (Hx) were determined by High-Performance Liquid Chromatography (HPLC) using a Beckman Coulter Gold HPLC (Cassina De' Pecchi, MI, Italy) device, equipped with a pump system (Beckman System Gold 125-S), UV detector (Beckman System Gold mod. 166), with absorbance fixed at 254 nm, and coupled with an analogic interface (Beckman System Gold mod. 406). A pre-column Ascentis® (2 cm length, 4 mm ID; Supelco, Bellefonte, PA, USA) was added to a reverse-phase Column Ascentis® C18 (25 cm × 4.6 mm, particle size 5 μm, pore size 100 Å). A 20 μL fixed loop was utilised. The separation process was performed with the settings proposed by Pulcini et al. (2022). The results were expressed as μmol catabolites/g muscle, using a mix of standard nucleotides (Sigma-Aldrich, St. Louis, MO, USA) each at 50 μM. The Adenylate Energetic Charge (AEC; Atkinson, 1968) was calculated as:

$$AEC = (0.5\ ADP + ATP)/(AMP + ADP + ATP)$$

2.5.4. Fillet oxidative status

Primary and secondary oxidative products were quantified in homogenized fillets as conjugated dienes (CD) and thiobarbituric acid reactive substances (TBARS), following the methods proposed by Srinivasan et al. (1996) and Vyncke (1975), respectively. Results of CD were expressed as mmol hydroperoxides on 100 g of sample, while the TBARS content results were expressed as malondialdehyde equivalent in 100 g of flesh.

2.6. Proteomics

All animals used for these analyses were euthanised by a blow to the head after stunning took place.

2.6.1. Label free shotgun proteomics

2.6.1.1. Protein sample preparation. For total protein extraction, brain tissue samples ($n = 6$) were solubilized in 1500 μL extraction buffer (7 M urea, 2 M thiourea, 4% CHAPS, 30 mM Tris pH 8.5), plus 15 μL protease inhibitor cocktail (Merck KGaA) and 6 μL 250 mM EDTA. Samples were homogenized in a tissue lyser (VWR, Radnor, PA, USA) with 5 mm metal beads for 3 cycles of 30 s, at a frequency of 25 Hz with 1 min in ice in between runs. Homogenates were incubated at 4 °C for 30 min, in constant rotation, then centrifuged at 13,000 *g*, 4 °C for 30 min to remove insoluble material. Protein content was measured using the BioRad Quick Start Bradford Dye Reagent and BSA Standard Set (Bio-Rad Laboratories, Hercules, CA, USA). Total protein extracts were thereafter depleted of non-protein contaminants using the ReadyPrep™ 2D Clean-up kit (Bio-Rad), following the manufacturer's instructions. The cleaned protein pellet was resuspended in 100 mM Tris pH 8.5, 1% sodium deoxycholate, 10 mM TCEP, 40 mM chloroacetamide and protease inhibitors for 10 min at 95 °C at 1000 rpm (Thermomixer, Eppendorf, Hamburg, Germany) for further mass spectrometry (MS) analysis. Subsequently, samples were prepared according to the solid-phase-enhanced sample-preparation (SP3) protocol (Hughes et al., 2019). Enzymatic digestion was performed with 2 μg Trypsin/LysC overnight at 37 °C at 1000 rpm.

2.6.1.2. nanoLC-MS/MS analysis. Peptides (500 ng) were analysed through online nanoLC using an UltiMate™ 3000 system coupled to a Q-Exactive Hybrid Quadrupole-Orbitrap mass spectrometer (Thermo Scientific, Bremen, Germany). Samples were loaded onto a trapping cartridge (Acclaim PepMap C18 100 Å, 5 mm × 300 μm i.d., 160,454; Thermo Scientific) in a mobile phase of 2% ACN, 0.1% FA at 10 μL/min. After 3 min loading, the trap column was switched in-line to a 50 cm by 75 μm inner diameter EASY-Spray column (ES803, PepMap RSLC, C18, 2 μm; Thermo Scientific) at 250 nL/min. Separation was achieved by mixing A: 0.1% FA, and B: 80% ACN, 0.1% FA, with the following gradient: 5 min (2.5% B to 10% B), 120 min (10% B to 30% B), 20 min (30% B to 50% B), 5 min (50% B to 99% B) and 10 min (hold 99% B). Subsequently, the column was equilibrated with 2.5% B for 17 min. Data acquisition was controlled by Xcalibur 4.0 and Tune 2.9 software (Thermo Scientific).

The mass spectrometer was operated in data-dependent acquisition (DDA) positive mode alternating between a full scan (m/z 380–1580) and subsequent HCD MS/MS of the 10 most intense peaks from full scan (normalized collision energy of 27%). ESI spray voltage was 1.9 kV, and the capillary temperature was 275 °C. The global settings were as follows: use lock masses best (m/z 445.12003), lock mass injection Full MS and chromatography peak width (FWHM) of 15 s. Full scan settings: 70 k resolution (m/z 200), AGC target 3e6, maximum injection time 120 ms. DDA settings: minimum AGC target 8e3, intensity threshold 7.3e4, charge exclusion: unassigned, 1, 8, > 8, peptide match preferred, exclude isotopes on, dynamic exclusion 45 s. MS2 settings: microscans 1, resolution 35 k (m/z 200), AGC target 2e5, maximum injection time 110 ms, isolation window 2.0 m/z, isolation offset 0.0 m/z, dynamic first mass and spectrum data type profile. MS analyses were performed at the Proteomics Scientific Platform of i3S, Porto, Portugal.

2.6.2. Protein identification

Raw MS data were processed using SEQUEST® on Proteome Discoverer™ software 2.5.0.400 (Thermo Scientific) and searched against the UniProtKB Protacanthopterygii database (taxon ID 41705; Release 2022_04; 803,964 sequences) plus contaminants, for protein identification. The SEQUEST HT search engine was used to identify tryptic peptides. The ion mass tolerance was 10 ppm for precursor ions and 0.02 Da for fragmented ions. A maximum of two missed cleavage sites was allowed, with a minimum peptide length of six amino acids and 144 as the maximum. Cysteine carbamidomethylation was defined as a constant modification. Methionine oxidation, protein N-terminus acetylation, and loss of methionine and Met-loss+Acetyl were defined as variable modifications. Peptide confidence was set to high. The Inferys rescoring node was considered for this analysis. The processing node Percolator was enabled with the following settings: maximum delta Cn 0.05; decoy database search target false discovery rate (FDR) ≤ 1%, validation based on *q*-value. Protein label-free quantitation was performed with the Minora feature detector node at the processing step. Precursor ions quantification (TOP3) was performed at the processing step with the following parameters: peptides to use unique plus razor, precursor abundance was based on intensity, normalization mode was based on total peptide amount, the pairwise protein ratio calculation and hypothesis test were based on *t*-test (background based). Processing workflow results, *i.e.*, .msf files (magellan storage files), were imported into Scaffold (v.5.0.1, Proteome Software Inc., Portland, OR, USA) to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at a probability higher than 95% to achieve an FDR lower than 0.1% by the Peptide Prophet algorithm (Keller et al., 2002) with Scaffold delta-mass correction. Protein identifications were accepted if they could be established at >99% probability (protein Decoy FDR < 0.1%) by the Protein Prophet algorithm (Nesvizhskii et al., 2003) and contained at least two identified peptides. Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. Protein abundance was estimated based on TOP3 Ion Precursor Intensity and normalized against the sum of all ion intensities in each sample replicate. The MS proteomics data have been deposited on the ProteomeXchange Consortium (Deutsch et al., 2020) *via* the PRIDE (Perez-Riverol et al., 2022) partner repository with the dataset identifier PXD044301 and doi:https://doi.org/10.6019/PXD044301.

2.7. Statistical analysis

For behavioural indicators, we calculated the frequency of individuals that lost the behavioural parameters described above, excluding those individuals from which data were not collected. We then calculated the mean, standard deviation, and coefficient of variation of the time to cease the measured behavioural parameters for those individuals in which that behaviour was registered. We repeated the same calculations for the time to recover the behavioural parameters.

For the physiology parameters, the cortisol data were not normally distributed, and the osmolality data were normally distributed but were not homoscedastic, therefore we ran a Kruskal-Wallis test to compare the means in both cases, followed by pairwise comparisons with Bonferroni corrections.

For the electrophysiology parameters, the heart rate was measured in beats per minute (bpm) and the amplitude of the QRS wave (*i.e.*, the amplitude of the signal) in V. The heart rate data were not normally distributed, and the amplitude data were normally distributed but not homoscedastic; therefore, we ran a Kruskal-Wallis test to compare the means in both cases, followed by pairwise comparisons with Bonferroni

corrections.

For the quality parameters, nucleotides and physicochemical data were subjected to one-way analysis of variances (ANOVAs) setting as significant a p-value <0.05. The Tukey's multiple-comparison test was used as post-hoc test to determine the differences among the groups.

For proteomics statistical and functional analyses, we carried out an analysis of differentially abundant proteins (DAPs) and protein functional annotations.

2.7.1. Analysis of differentially abundant proteins (DAPs)

MS/MS data of rainbow trout brain samples ($n = 6$) submitted to four different treatments were merged into one single Scaffold file for further comparisons, summing a total of 24 MS/MS sample runs. The whole dataset was then filtered based on valid values (proteins quantified in at least 4 out of 6 replicates per treatment) and log10 transformed prior to statistical analysis to ensure a Gaussian distribution of the residuals (Farinha et al., 2021). After assessing the residuals' normality and homoscedasticity, differences in protein abundance across different treatments were assessed by one-way ANOVA followed by Tukey's HSD *post-hoc* test ($p < 0.05$) on the Perseus software (Stefka et al., 2016).

2.7.2. Protein functional annotation

DAPs were first annotated on the STRING database v.11.5 (https://string-db.org/) (Szklarczyk et al., 2021) using FASTA sequences as queries to search *Danio rerio* and *Homo sapiens* orthologous proteins. Proteins were then mapped on the REACTOME knowledgebase v. 83 (reactome.org; Gillespie et al., 2022). The R package *ggplot2* (Wickham, 2016) was used for visualization of pathway enrichment analysis (Benjamini-Hochberg, FDR ≤ 0.05) depicted on bubble plots.

Additionally, a Principal Component Analysis was carried out with a selection of welfare and quality indicators. For this analysis, missing values were replaced by group means. Unless stated otherwise, all tests were carried out in IBM SPSS Statistics software (29.0). Principal Component Analysis was carried out in R statistics factoextra package version 1.0.7.

3. Ethical note

All experiments were carried out in strict accordance with EU legal frameworks relating to the protection of animals used for scientific purposes (Directive 2010/63/EU), approved by the Ethics Committee of the Edmund Mach Foundation and authorized by the Italian Ministry of Health (n. 574/2020-PR).

4. Results

4.1. Behaviour

Regarding induction of unconsciousness (Fig. 1), ES induced unconsciousness immediately within the 20 s of electric shock in all fish ($n = 24$); AN within 1.5 min in all fish ($n = 26$; mean ± SD: 46 ± 20.2 s); FC failed to induce unconsciousness in 87% of the fish, with only 3 fish out of 23 loosing VOR within 2 min (mean ± SD of fish that lost VOR: 118 ± 3.3 s), with all the fish that engaged in intense escape behaviour and presented an extremely high production of mucus; ASP did not induce unconsciousness within 20 min for 44% of the fish, and took on average 17.6 min to induce unconsciousness in the remaining 56% (mean ± SD: 1058 ± 119 s). In other words, 44% of the fish died while they were conscious and took longer than 20 min to die, also showing intense movements during the process. Frequencies, means of time to lose all other behavioural parameters, and their coefficients of variation are summarised in Table 1.

Frequencies, means of time to recover all the measured behavioural parameters, and their coefficients of variance are summarised in Table 2. Regarding recovery, stunning with AN did not allow full recovery in any fish. We observed recovery of VOR in 25% of the fish stunned with ES (Table 2). Only 16.7% of the fish ($N = 3$) subjected to FC lost VOR during the induction period, and those recovered VOR between 48 s and 5 min. In all fish subjected to FC, there were apparent signs of severe brain damage (erratic swimming, abnormal movements, no reaction to stimuli) and/or gill haemorrhage, while VOR was on and off in these fish throughout the duration of the recovery period. The rest of the fish (83%) subjected to FC were conscious while dying and presented the same severe signs of neural damage. ASP at 10 °C took 20 min or longer to kill the fish, with 17.6 min on average to lose consciousness in only 56% of the fish (Table 1).

4.2. Physiology

4.2.1. Plasma osmolality

Osmolality was significantly different among at least two groups (Fig. 2; $H(3) = 27.267$, $p < 0.001$). Pairwise comparisons revealed that

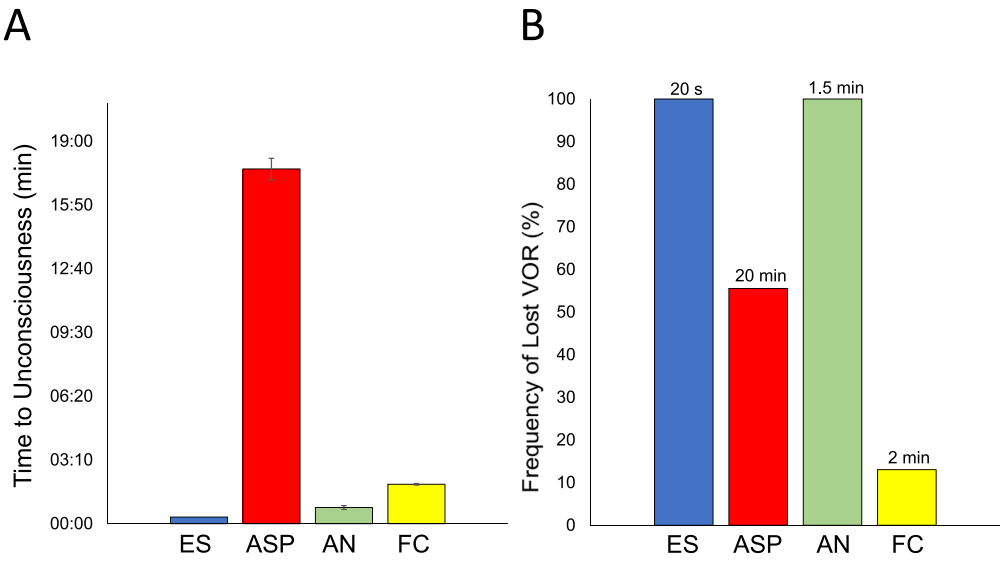

Fig. 1. A) Mean time to reach unconsciousness (*i.e.*, loss of VOR) by individuals for each treatment. Error bars show S.E.M. B) Percentage of individuals that reached unconsciousness in the different treatment. Average latency to reach unconsciousness is noted on top of each column. Studied treatments: ES: electrical stunning; ASP: asphyxia at 10 °C; AN: anaesthesia with MS-222; FC: fast-chilling method.

Table 1
Frequency, mean time, and coefficient of variance (CV) of measured behavioural parameters related to losing consciousness. N: total sample size; n: sample size for specific behavioural parameter after removing missing data points. ES: electrical stunning; ASP: asphyxia at 10 °C; AN: anaesthesia with MS-222; FC: fast-chilling method.

		ES	ASP	AN	FC
	N	24	28	26	24
	n	24	NA	8	24
	Frequency	100%	NA	100%	79%
	Mean Time (s)	20.0	NA	53.5	23.3
Loss of Free Swimming	CV	0.00	NA	0.07	0.13
	n	24	NA	26	24
	Frequency	100%	NA	100%	79%
	Mean Time (s)	20.0	NA	20.3	50.9
Loss of Equilibrium	CV	0.00	NA	0.19	0.33
	n	24	22	15	24
	Frequency	100%	100%	100%	8%
	Mean Time (s)	20.0	718.7	139.7	90.0
Loss of Voluntary Movement	CV	0.00	0.21	0.19	0.00
	n	24	22	26	24
	Frequency	100%	64%	100%	38%
	Mean Time (s)	20.0	969.5	57.9	60.3
Loss of Opercular Movement	CV	0.00	0.11	0.30	0.20
	n	24	27	26	23
	Frequency	100%	56%	100%	13%
	Mean Time (s)	20.0	1057.9	46.2	117.7
Loss of VOR*	CV	0.00	0.11	0.44	0.03

* Vestibulo-ocular reflex or "eye roll".

Table 2
Frequency, mean time, and coefficient of variance (CV) of measured behavioural parameters related to recovering consciousness. N: total sample size; n: sample size for specific behavioural parameter after removing missing data points. ES: electrical stunning; ASP: asphyxia at 10 °C; AN: anaesthesia with MS-222; FC: fast-chilling method.

		ES	ASP	AN	FC
	N	24	28	26	24
	n	24	28	26	24
	Frequency	13%	0%	0%	4%
	Mean Time (s)	736.3			195.0
Recovery of Free Swimming	CV	0.05			NA
	n	24	28	26	24
	Frequency	17%	0%	0%	8%
	Mean Time (s)	766.3			517.0
Recovery of Equilibrium	CV	0.11			0.65
	n	24	28	26	24
	Frequency	17%	0%	0%	25%
	Mean Time (s)	364.0			234.5
Recovery of Voluntary Movement	CV	0.50			1.20
	n	24	28	26	24
	Frequency	29%	0%	0%	38%
	Mean Time (s)	230.0			190.5
Recovery of Opercular Movement	CV	0.47			0.52
	n	24	28	26	4*
	Frequency	25%	0%	0%	100%
	Mean Time (s)	325.5			165.8
Recovery of VOR	CV	0.57			0.71

* All other individuals did not lose VOR.

the group treated with ES had significantly higher osmolality than the group treated with AN ($p = 0.007$), and that the group treated with ASP had higher osmolality than the group treated with AN ($p < 0.001$) and the group treated with FC ($p = 0.002$). All other comparisons were non-significant (ES *vs.* ASP: $p = 0.53$; ES *vs.* FC: $p = 0.32$; AN *vs.* FC: $p = 1.0$).

4.2.2. Cortisol

Cortisol in plasma was significantly different among at least two groups (Fig. 2; H(3) = 10.049, $p = 0.018$). Pairwise comparisons revealed that the ASP fish had significantly higher cortisol than the fish treated with AN ($p = 0.023$). All other comparisons were non-significant (ES *vs.* ASP: $p = 1.0$; ES *vs.* AN: $p = 0.11$; ES *vs.* FC: p = 1.0; ASP *vs.* FC: $p = 0.43$; AN *vs.* FC: p = 1.0).

4.3. Electrophysiology

There was no significant difference in heart rate among treatments (Fig. 3; H(3) = 3.642, $p = 0.30$). However, the amplitude of the QRS wave (*i.e.,* the amplitude of the signal) was significantly different among treatments (Figs. 3 and 4; H(3) = 19.763, p < 0.001), and a *post-hoc* test revealed that the amplitude of the signal was significantly higher in the fish treated with ES compared to the fish killed with ASP ($p = 0.015$) and to the fish exposed to FC ($p = 0.040$). Similarly, the fish on AN had higher amplitude signal than the fish killed with ASP ($p = 0.003$) and the fish exposed to FC ($p = 0.008$). There was no significant difference in the amplitude of the signal between the fish treated with ES and AN (p = 1.0), and between the fish killed with ASP and those exposed to FC (p = 1.0).

4.4. Internal and external injuries

All fish subjected to the fast-chilling method (FC) presented immediate frozen eyes and subsequent thawing during the recovery phase, as well as abundant mucus production. Five FC individuals also presented gill and/or mouth haemorrhages. No apparent injuries were observed in any fish subjected to the other treatments.

4.5. Fish and fillet quality

The group of fish analysed for quality parameters (weight: 523.58 ± 14.23 g; length: 34.54 ± 0.50 cm; fillet weight: 132.63 ± 6.53 g) showed a different evolution of *rigor* index (RI; Fig. 5), with FC and ASP fish that arrived at the values of 81 and 98%, respectively, already after 3 h from death. Full *rigor* was observed at 9 h from death in the case of ES fish while, at the same time, AN fish showed a RI value slightly above 20%. Forty-eight hours after death, the RI values were 62.34, 49.64, 32.69 and 19.69 for AN, FC, ES and ASP, respectively. The evolution of this index matches with values registered for pH (Table 3), with the ASP group of fish showing the lowest value (6.37) and the AN group showing the highest (7.24) ($p < 0.0001$).

The different stunning methods did not affect skin colour, as measured immediately after death (data not shown) whilst differences were found for fillet colour (Table 3), with AN fish showing the highest values for lightness (L^*) and for yellowness index (b^*) in comparison to the values registered for the other groups, that resulted very similar among them for these colour parameters.

AN fish had the highest WHC (99.61%; p < 0.0001) while the other groups showed similar values, ranging from 95.71 for ES to 94.32% for ASP group. No differences among fish from different treatments were found for texture.

The difference found among fish submitted to different treatments for *rigor* evolution and supported also by pH values and WHC are also corroborated by the values found for the level of ATP and that of its catabolites, as shown in Fig. 6. AN fish had the highest level of ATP and ASP the highest of IMP, while the other groups showed intermediate values (p < 0.0001); however, ES trout had significant higher level of ATP and, numerically, lower of IMP in comparison to FC group. The differences regarding the effect of the stunning method are clearly expressed by the values registered for AEC, according to the sequence AN (0.936) = ES (0.878) > FC (0.799) > ASP (0.664), while ATP/IMP ratio clearly discriminated AN (19.78) from the others, whose ratio values ranged from 0.17 to 0.35 (Table 3).

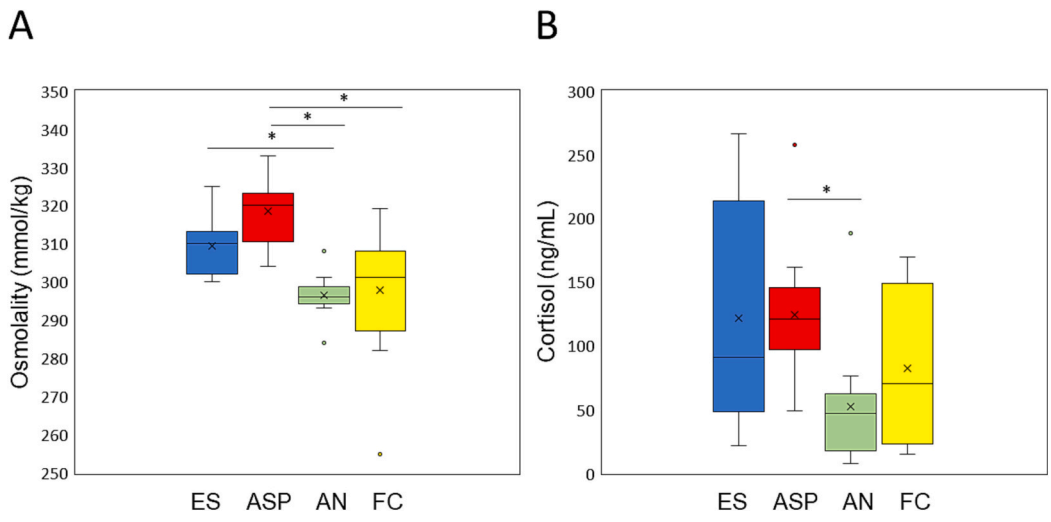

Fig. 2. Box and whiskers representation of the data, with the box containing the lower and upper quartile, the horizontal line representing the median, the × representing the mean, and the end of the whiskers marking the minimum and maximum values, with outliers represented by dots. A) Osmolality in plasma, in mmol/kg among treatments; B) Cortisol in plasma, in ng/mL. *: significant difference among treatments. Treatments: ES: electrical stunning; ASP: asphyxia 10 °C; AN: anaesthesia with MS-222; FC: fast-chilling method.

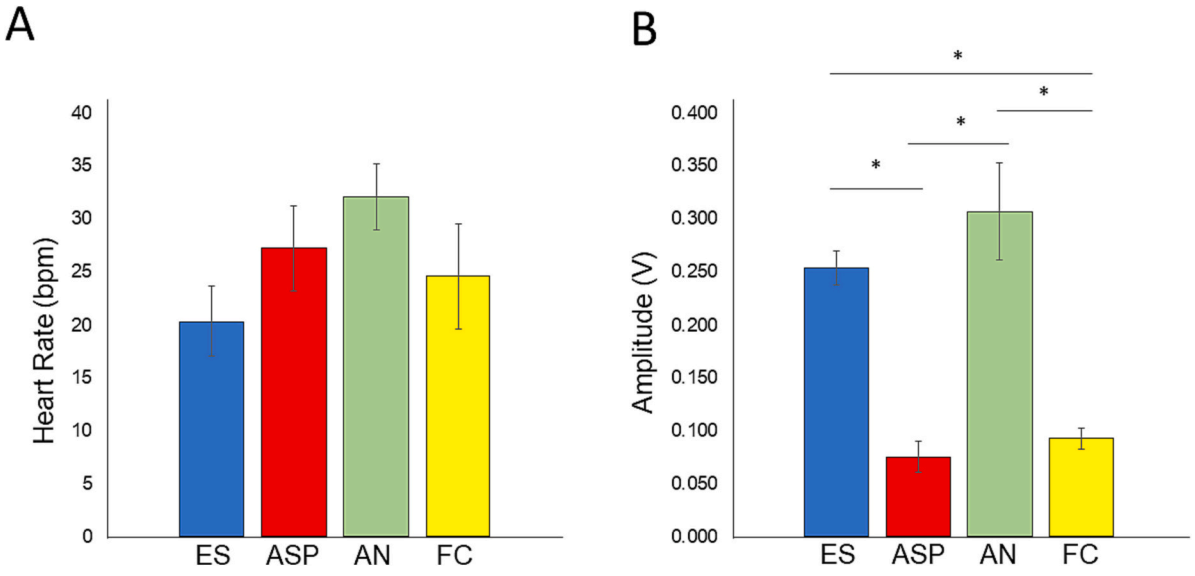

Fig. 3. A) Heart rate for each studied treatment, in beats per minute (bpm). Error bars show S.E.M. B) Amplitude of the signal for each studied treatment, in V. Error bars show S.E.M. *: significant difference among treatments. Treatments: ES: electrical stunning; ASP: asphyxia 10 °C; AN: anaesthesia with MS-222; FC: fast-chilling method.

In the case of the AN group, the slower evolution of the *rigor* justified the differences for the weight loss of fillet found at the time of *rigor* resolution, but ASP fish remain those that lost the most weight even at this time of the *post-mortem* phase (Table 3).

Slight but significant differences were shown for primary and secondary products of lipid oxidation (Table 4) both immediately after death and after *rigor* resolution. The CD values were lower ($p < 0.05$) in the ASP group than in the others at both T0 and $T0_{RR}$. Noteworthily, the AN fillets were the most oxidised immediately after death (TBARS; $p < 0.05$), but their secondary lipid oxidation product level did not differ from those of ES, and ASP fillets after *rigor* resolution, *i.e.*, 96 h *post mortem*, when the FC fillets showed the lowest TBARS value.

4.6. Proteomics

4.6.1. Brain proteome response

To study the molecular response of fish undergoing different stunning methods, changes in the brain proteome of rainbow trout exposed to the different challenges were assessed, using label-free shotgun proteomics. A total of 4458 proteins were identified across the 24 samples (6 replicates; 4 conditions) with a probability higher than 99% to achieve a FDR < 0.1% assigned by the Protein Prophet algorithm (Nesvizhskii et al., 2003), with at least two peptides, on the Scaffold software v.4.11.1 (Proteome Software Inc.). From these, 2996 reproducible proteins present in at least 4 out of 6 replicates across all treatments were selected for further statistical analyses. Differential analysis of the reproducible proteins revealed a total of 143 DAPs across all treatments in the brain samples (Supplemental Table S1). These data can be visualized in the Venn diagram in Fig. 7, discriminating the number of DAPs

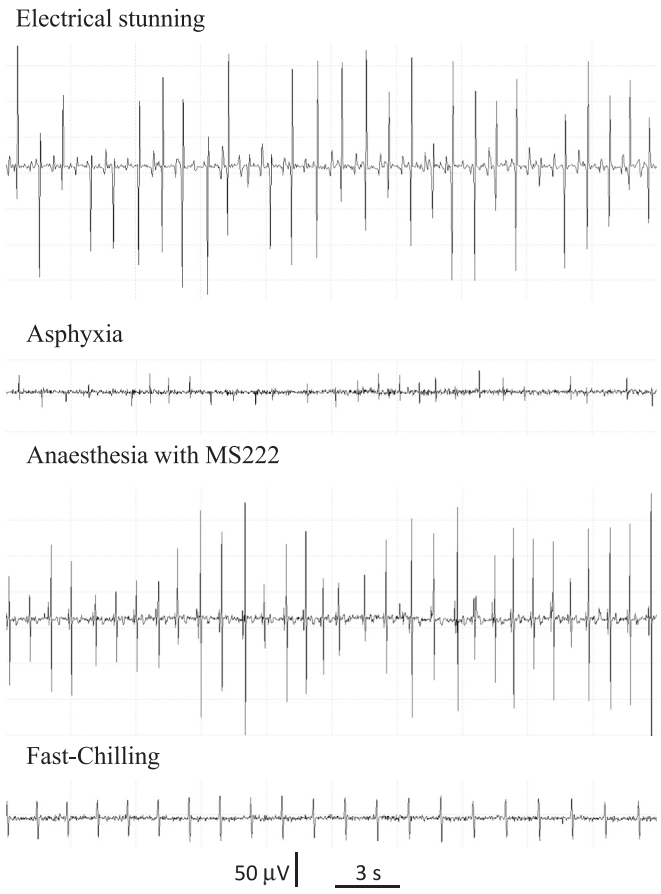

Fig. 4. ECG recording examples for the treatments considered in the trial.

per treatment and those commonly found between treatments. The major changes in the brain proteome of rainbow trout indicate that FC was the most impactful treatment for rainbow trout. FC registered the highest number of DAPs with 119 proteins, whereas ASP registered the lowest, *i.e.*, 65 proteins (Supplemental Table S1). About 96 proteins were found in the anaesthesia-exposed (AN) rainbow trout brains, whereas the ES treatment registered 78 DAPs. The most significant signalling pathways associated to the differentially abundant proteins in each treatment were further analysed according to REACTOME knowledgebase for further comparisons between treatments.

4.6.2. *Functional analysis of DAPs in the brain*

Protein functional analyses were performed according to their annotation in the REACTOME pathway knowledgebase, determining most significantly over-represented biological processes (Binomial test, $p < 0.05$; Benjamini-Hochberg, FDR ≤ 0.05) for each treatment. A similar number of proteins was annotated across all stunning methods (Supplemental Table S2), corresponding to a mean of 74% mapping of all DAPs. FC registered the highest number of significantly enriched pathways ($p < 0.05$; FDR ≤ 0.05), followed by the ES treatment, while the ASP registered the lowest number (Supplemental Table S2).

Table 3
Fillet characteristics, immediately after death and after *rigor* resolution. *L**, lightness; *a**, redness index; *b**, yellowness index; WHC, water holding capacity; AEC, Adenylate Energetic Charge.

	ES	ASP	AN	FC	*p*	RMSE
Immediately after death						
pH	6.63^{b}	6.37^{c}	7.24^{a}	6.66^{b}	<0.0001	0.148
*L**	42.47^{b}	42.54^{b}	44.87^{a}	42.59^{b}	0.051	1.928
*a**	9.45	8.96	10.33	9.24	ns	1.676
*b**	8.01^{b}	6.93^{b}	10.49^{a}	7.83^{b}	0.001	1.625
Texture, N	47.76	63.31	55.29	55.88	ns	16.487
WHC, %	95.71^{b}	94.32^{b}	99.61^{a}	95.51^{b}	<0.0001	1.457
AEC	0.878^{a}	0.664^{c}	0.936^{a}	0.799^{b}	<0.0001	0.069
ATP/IMP	0.72^{b}	0.17^{b}	19.78^{a}	0.35^{b}	<0.0001	3.196
After *rigor* resolution						
Weight loss, %	2.54^{b}	5.97^{a}	3.50^{b}	4.02^{ab}	0.013	1.889
pH	6.57	6.52	6.52	6.58	ns	0.093
*L**	45.45	44.67	45.61	44.50	ns	2.519
*a**	8.07	7.85	7.89	9.24	ns	2.350
*b**	9.03	8.25	8.68	9.82	ns	2.101
Texture, N	42.36	48.05	49.28	55.50	ns	13.046
WHC, %	96.47	96.83	96.32	97.18	ns	1.847

a,b,c: means with different letters are significantly different ($p < 0.05$); n.s.: not significant ($p > 0.05$).
RMSE: Root Mean Square Error.

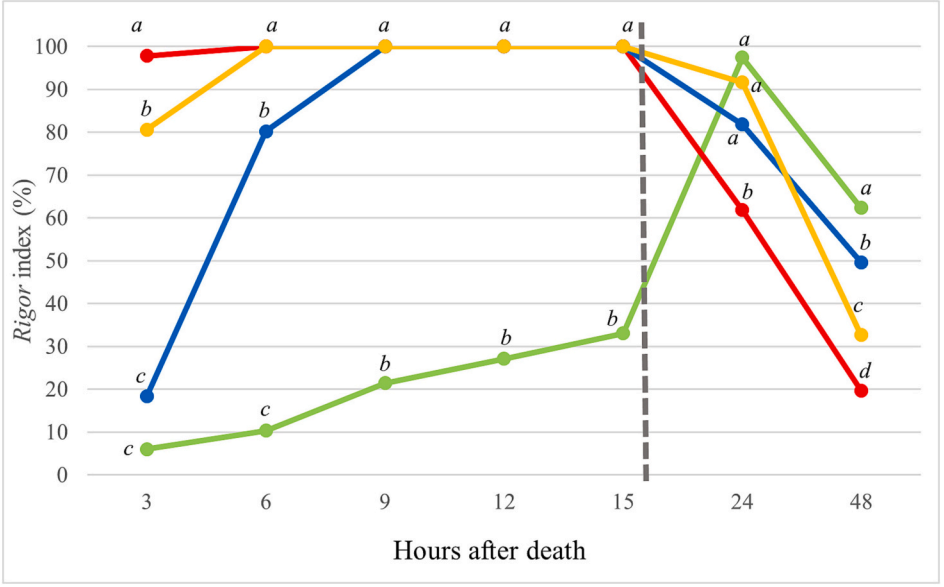

Fig. 5. *Rigor* index evolution in the groups of rainbow trout stunned with the four methods (ES, blue line; ASP, red line; AN, green line; FC, yellow line). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

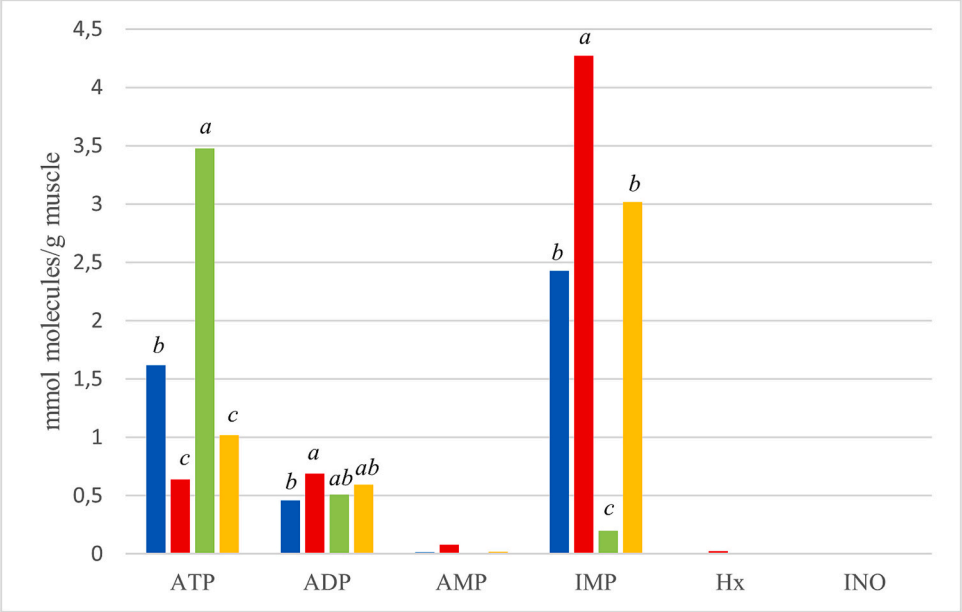

Fig. 6. Levels of ATP and its catabolites (ADP, AMP, IMP, Hx, Ino) in the groups of rainbow trout stunned with the four methods (ES, blue line; ASP, red line; AN, green line; FC, yellow line). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 4
Conjugated dienes (CD, mmolHp/100 g muscle) and TBARS (mg MDAeq/100 g muscle) in fillets analysed immediately after death (T0) and after *rigor* resolution ($T0_{RR}$).

	ES	ASP	AN	FC	*p*	RMSE
CD T0	0.108^{a}	0.094^{b}	0.114^{a}	0.109^{a}	*0.028*	0.013
CD T0RR	0.114^{a}	0.101^{b}	0.121^{a}	0.120^{a}	*0.012*	0.012
TBARS T0	0.013^{b}	0.014^{b}	0.018^{a}	0.013^{b}	*0.012*	0.003
TBARS T0RR	0.016^{a}	0.017^{a}	0.016^{a}	0.014^{b}	*0.050*	0.002

a,b: means with different letters are significantly different ($p < 0.05$).
RMSE: Root Mean Square Error.

The 25 most over-represented pathways (TOP25) triggered in the brain proteome of rainbow trout in response to the different treatments are depicted in Fig. 8. Pathways directly implicated in brain functions *e.g.*, "Neurotransmitter receptors and postsynaptic signal transmission", "Nervous system development", "Axon guidance", "Transmission across Chemical Synapses" were found to be most significant in FC (FDR $\leq$ 0.001) and AN (FDR $\leq$ 0.006) as compared with ES (FDR $\leq$ 0.033) and ASP (FDR $\leq$ 0.037) (Fig. 8; Supplemental Table S2). Interestingly, in the case of ES stunned fish, the pathway "Neuronal system" was not significantly enriched, opposed to the rest of treatments, being also observed in ES fish a less significant impact on other brain related pathways, as indicated by higher FDR values (Fig. 8; Supplemental Table S2). Pathways associated with "Cellular responses to stress" and "Cellular responses to stimuli" were highly over-represented in FC (FDR $\leq$ 0.001) and ES stunned fish (FDR $\leq$ 0.03) and despite that it was also significantly enriched in AN treated fish (FDR $\leq$ 0.02), it was not found among the TOP25 (Fig. 8; Supplemental Table S2). The significant impact on cellular stress responses in FC, ES and AN when compared to ASP is also depicted in the Voronoi diagrams in Supplemental Fig. S2 and supported by the significant involvement of "Dopamine Neurotransmitter Release Cycle" across all stunning methods except in ASP (Supplemental Table S1). Other significantly enriched pathways ($p <$ 0.01, FDR $\leq$ 0.1) can be compared in the Voronoi diagrams, like those involved in the innate (*e.g.,* "Neutrophil degranulation") and adaptive immune system response ("MHC class II antigen presentation", "Rap1 signalling"), and cytokine signalling ("noncanonical NF-kB signalling"), suggesting a lower involvement of the innate response in AN treatment, as compared with the other stunning methods (Supplemental Fig. S2). Interestingly, in ASP stunned fish there was an absence of significant cytokine signalling despite an adaptive ("MHC class II antigen presentation", "Rap1 signalling") and innate immune response ("Neutrophil degranulation") (Supplemental Fig. S2). Other important and significant differences were also evident across the various treatments *e.g.*, related to sensory reactions (over-represented in AN and FC), autophagy (lower in ES) or programmed cell death events *e.g.*, "Caspase mediated cleavage of cytoskeletal proteins" that were among the TOP25 in AN and ASP (indicated by green arrows on Fig. 8) and over-represented in FC, as well as "Apoptosis" that was over-represented in AN, ES and FC (Supplemental Fig. S2 and Table S2).

4.7. Principal component analysis

We selected the most relevant behavioural, physiological and quality indicators for each stunning method (percentage of fish losing VOR, circulating cortisol, *Rigor* Index at 3 h (RI3), fillet pH and Water Holding Capacity immediately after slaughter (pH T0 and WHC T0, respectively) and weight loss after *rigor* resolution), and to perform a Principal Component Analysis (Fig. 9). It revealed two components that explained 65% of the variability. The resulting plot shows a clear separation between treatments along both axes: AN and ASP are far apart along PC1 (the axis that explains most of the variability, *i.e.*, 45.53%) whereas ES and FC are apart along PC2, suggesting different stunning outcomes and, therefore, different aspects of fish welfare.

5. Discussion

This study aimed to compare the effectiveness of four fish stunning methods, including a novel fast-chilling (FC) technique, using an unprecedented integrative approach that encompassed behavioural observations to proteomic analyses. Rainbow trout (*Oncorhynchus mykiss*) were subjected to electrical stunning (ES), asphyxia (ASP), anaesthesia (AN), and the FC method. The study assessed the impact of each method on fish welfare and the associated molecular responses. The results obtained from behavioural observations and proteomic analyses provided valuable insights into the comparative effectiveness of the stunning methods. Electrical stunning emerged as a viable and humane method

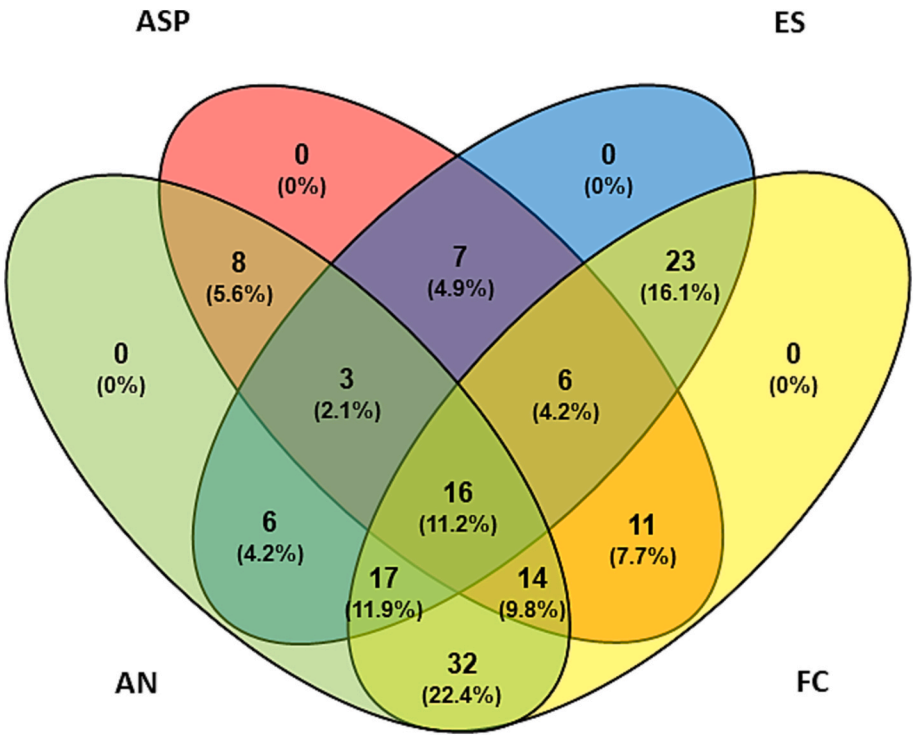

Fig. 7. Venn diagram showing the number of DAPs found across the different treatments, *i.e.*, electrical stunning (ES), asphyxia (ASP), anaesthesia (AN) and fast-chilling (FC).

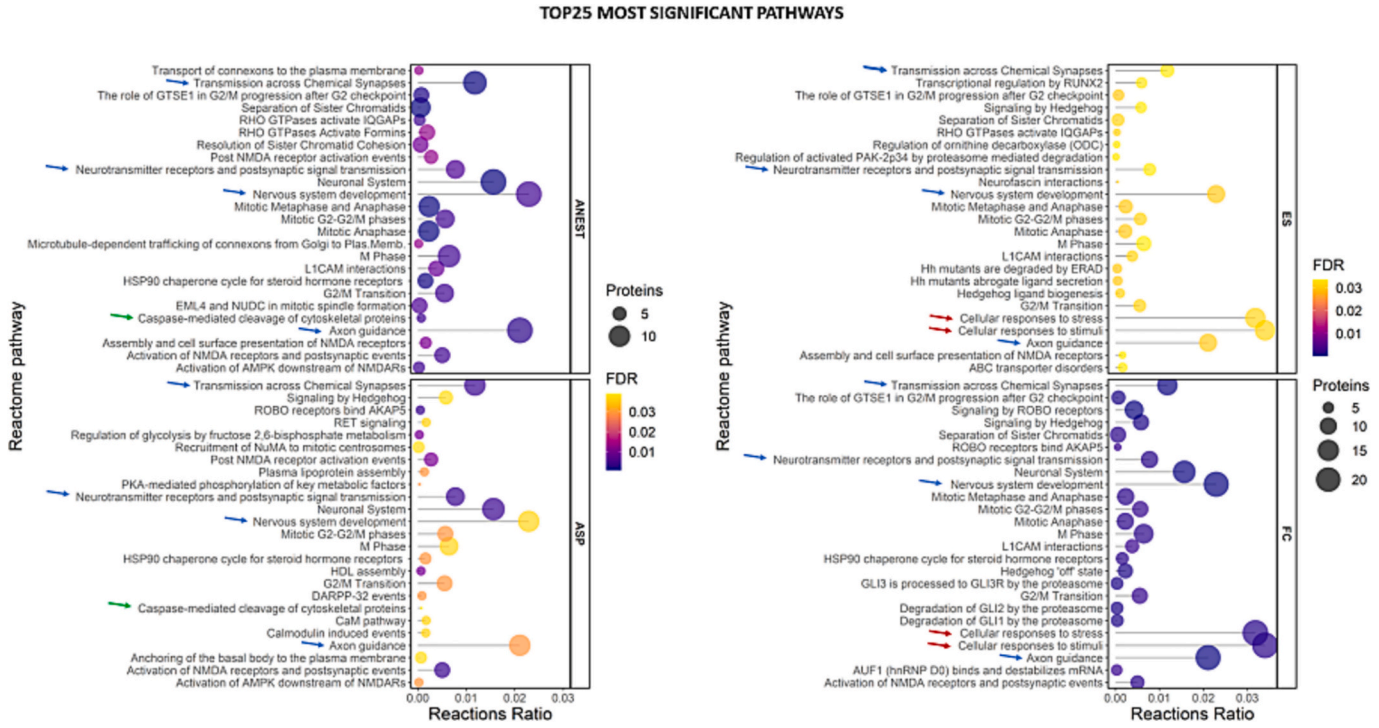

Fig. 8. Bubble-plot representing the TOP 25 most over-represented pathways triggered by different stunning methods in the brain of rainbow trout. The false discovery rate (FDR) (Benjamini-Hochberg, FDR $\leq$ 0.05) is indicated by a gradient colour on the right of the panels. The size of the bubble indicates the number of proteins found in that specific pathway; the larger the bubble, the higher the number of proteins involved. The reactions/signalling ratio scale reflects the expected representation of a given pathway in the reference genome. For the complete list of enriched pathways, please see Supplemental Table S2.

for stunning rainbow trout at a commercial scale, as it induced minimal adverse behavioural responses and showed favourable molecular indicators. However, the novel fast-chill stunning method exhibited limitations, as it led to abnormal fish behaviour and triggered molecular responses that deviated from the expected physiological patterns.

Anaesthesia with MS-222 induced smooth (yet not immediate)

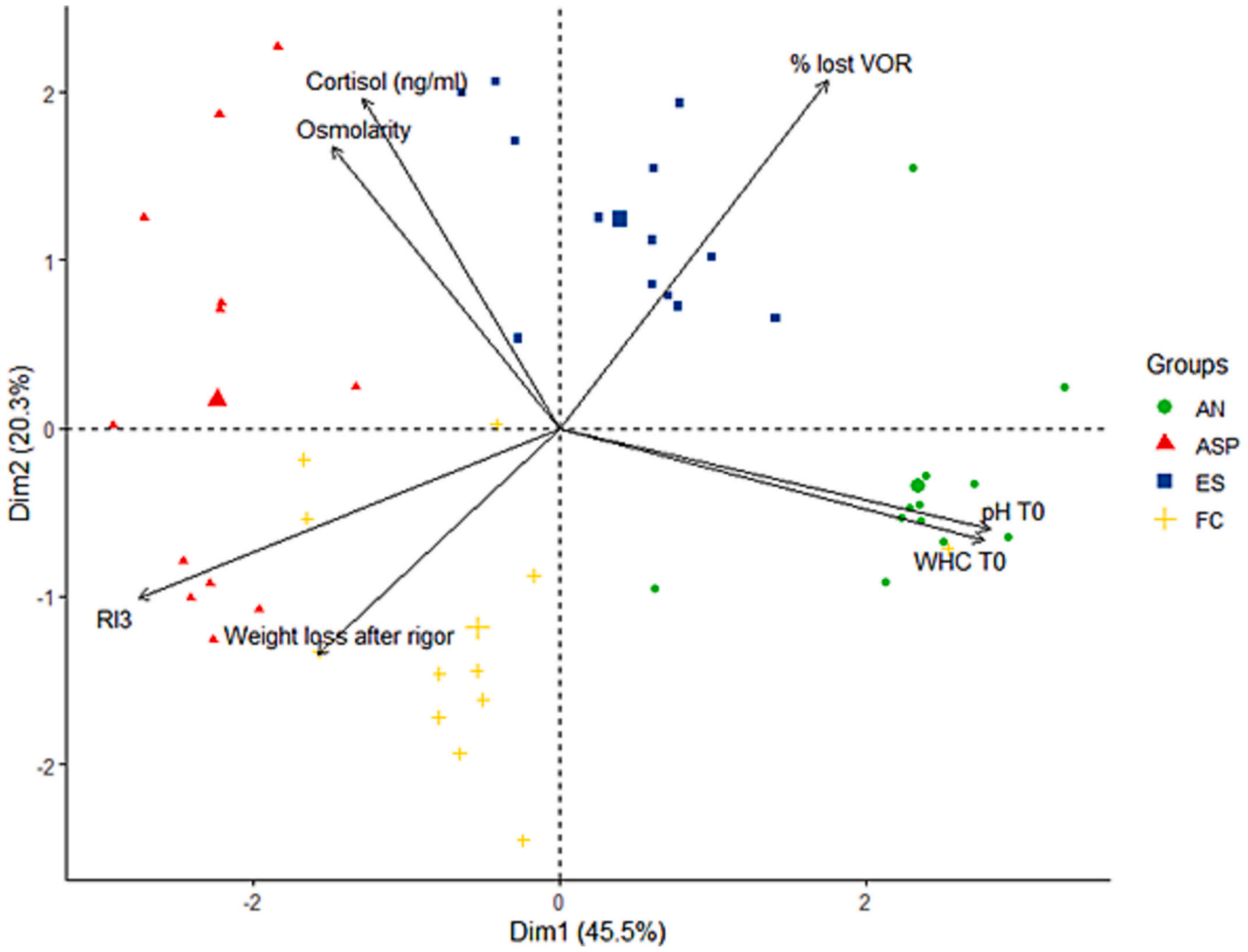

Fig. 9. Principal component analysis combining a selection of welfare and quality indicators upon stunning with electricity (ES), asphyxia (ASP), anaesthesia (AN) and fast-chilling (FC). See sections above for details on these indicators.

unconsciousness in all fish and none showed signs of recovery. The physiological functioning of the heart remained unaffected, with regular and strong heartbeat. This suggests a complete drug-induced shutdown of conscious activity in the brain, decoupling consciousness from the autonomous parts of the nervous system that control the basic life support functions such as cardiac function. Interestingly, the proteomic analysis revealed markers of intense apoptosis activity in the brain of anesthetised fish. MS-222 works by blocking cellular voltage-gated sodium channels and interrupts neuronal signals between brain areas and deactivating most sensory neurons (Leyden et al., 2022). The same apoptotic effect was demonstrated to occur in zebrafish embryos (Félix et al., 2020), raising questions about the extended and/or repeated use of this drug on experimental fish, or if other commonly-used fish anaesthetics (such as 2-phenoxyethanol or eugenol) would produce similar results at brain proteome level. The consistently lower values of physiological stress (measured through plasma cortisol, osmolality, and ATP) reliably indicate that overall stress was reduced at slaughter in anaesthetised rainbow trout when compared to the other methods. These results are supported by the proteomics data, since the pathways related to cellular stress responses despite being significantly over-represented in AN, it was not among the TOP25 most significant processes. It is important to note that our study provides only preliminary insights into the effects of anaesthesia on rainbow trout brain proteome; further research is needed to fully understand the underlying mechanisms and potential long-term consequences in terms of both fish welfare and fillet quality. Indeed, it is also interesting to note that this method (arguably the best in welfare terms in our study) also yielded the best results in terms of flesh quality and fillet appearance, but a slight increase of lipid oxidation product was found immediately after death. This is not the first time that side-effects of MS-222 have been highlighted (Topic Popovic et al., 2012); indeed, a long exposure (24 h) of rainbow trout to MS-222 (100 ppm) increased reactive oxygen species formation, whereas a 10 min exposure resulted in a significant inhibition of antioxidant capacity, evaluated in terms of superoxide dismutase, glutathione reductase, glutathione peroxidase in different fish tissues (Velisek et al., 2011).

Although chemically induced unconsciousness through anaesthesia with MS-222 presented the best results in all indicators, its use at slaughter in commercial aquaculture is prohibited in the EU and USA for food safety reasons. A viable method to induce unconsciousness quickly and effectively at commercial scale seems to be electrical stunning. In fact, according to our data on loss of VOR, unconsciousness induction was fast and irreversible. Here we also see a decoupling between brain and cardiac function, similar to AN. In fact, many other variables described in this study follow the same pattern associating higher welfare with higher quality (Fig. 9). These results align with those found by Bermejo-Poza et al. (2021), who also tested electrical stunning with immersion in ice-slurry in trout. In their study, however, although

cortisol followed the same pattern (*i.e.*, lower in electrical stunning), the absolute levels were tenfold lower than ours. This may have four main non-exclusive (or even cumulative) explanations: 1) the kits used to measure cortisol were different and it is known that differences between manufacturers may account for large discrepancies in results (Kinn Rød et al., 2017); 2) We used wet stunning in groups of 4 fish, whereas the study by Bermejo-Poza et al. (2021) used dry stunning individually; 3) the rearing and handling procedures prior to stunning might have been different; and 4) the blood sampling was performed at different times: immediately in our case, after 20 min in the other study. It is well established that pre-slaughter practices can have dramatic effects on stress markers (see Poli et al., 2005 for a review), yet we believe that cortisol clearance after 20 min may also have had an important effect in the study from Bermejo-Poza et al. (2021). Regardless, the cellular responses to electrical stimulation regarding stress are largely unknown, but there is evidence that mild electric shocks performed *in vitro* actually inhibit cortisol release in adrenal gland tissue of rats (Samidurai et al., 2018). In our study, the brain pathways involved in cellular stress responses were significantly activated in ES (in the TOP25) besides being observed a significant involvement of the pathway related to "Dopamine Neurotransmitter Release Cycle" (Fig. 8 and Supplemental Table S2), so the stress response to electrical stimulation may be evoked in higher brain areas rather than in tissues downstream. Cortisol levels after electrical stunning of fish may vary widely (Bermejo-Poza et al., 2021; Gräns et al., 2016), yet the causes and mechanisms underpinning these responses remain unknown.

Asphyxia in air is commonly used to slaughter farmed trout, especially in the Trentino region (Concollato et al., 2016a). However, our results show that not only do the fish experience a high degree of suffering, often for >20 min, but also, they are often fully conscious throughout the whole process and present indicators of extremely poor welfare in behaviour and physiology. Their meat quality is also severely affected; *rigor* sets in quickly, muscle pH is low and its appearance is affected, WHC decreases and so does fillet weight. WHC is both linked to nutritional and technological quality, since it can influence weight loss during transportation, preservation, thawing, and cooking. Indeed, low WHC value increases the loss of fluids thereby impairing fish nutritional value; on the other hand, producers of processed fish (*i.e.*, smoked fillets, minced meat, *etc.*) might desire fillet with a high WHC to avoid loss in product yield during the chain (Chan et al., 2022). This is a pattern reported in all studies testing this method (see for example Concollato et al., 2016b; Poli, 2009) and, therefore, asphyxia in air should be discontinued.

Furthermore, the low number of differential abundant proteins (DAPs) and the associated limited identification of significant biological processes at the proteome level strongly suggest a substantial suppression of coping mechanisms in the brain when exposed to asphyxiation. The suppression of coping mechanisms is a consequence of the decreased ability of the brain to adapt to asphyxiation and the impaired expression of stress-related proteins. The ischemic conditions resulting from asphyxiation, characterized by a lack of oxygen and nutrient supply, have been demonstrated to promote neural necrosis in fish (Li et al., 2022). This necrosis arises from the deprivation of essential resources, contributing to the disruption of normal cellular processes. Consequently, there is an overall reduction in metabolic activity, which includes the synthesis and degradation of proteins. This overall reduction in protein activity and expression aligns with the absence of an integrated stress and immune response. Considering that this is one example where the interest of the industry and the welfare of the animals walk hand in hand (Saraiva and Arechavala-Lopez, 2019), the common use of asphyxia as a slaughter method is nothing short of surprising.

Finally, the FC method was intended to be tested as a promising method to induce unconsciousness by a strong temperature shock using equipment already tested for *Salmo carpio* (Pulcini et al., 2022). In theory, the thermal shock could create a temperature gradient so strong that it could penetrate into the deeper regions of the body and brain, evoking quick heart failure and cessation of brain function. In zebrafish older than 28 days this method resulted quick and apparently 100% effective within 30 s with water at 0–4 °C (Wallace et al., 2018), probably due to a combination of their small size and warm-water adaptation (Matthews and Varga, 2012). In our study, the apparatus was able to create a water temperature of −8 °C still in liquid state. This was possible by adding salt at ~300 ppt (tenfold higher than seawater) and the fish were immersed in this ultra-low temperature, ultra-high salinity solution for 2 min. However, our results demonstrate that FC was not effective at all; only 13% of tested fish lost VOR, and even these recovered consciousness quickly. On the other hand, the brain proteome evidenced a significant enrichment of sensory reactions after this stunning method. There were signs of intense suffering in all animals, with very high amounts of mucus release, haemorrhage, and injuries, both internal and external. All individuals had their vitreous humour frozen during the stunning process, which thawed during recovery. While there was a large variation in circulating levels of cortisol, FC showed the most significant activation of cellular stress pathways in the brain as compared with the remaining stunning methods (Fig. 9). This was the method that undoubtedly induced more and most significant changes at brain proteome level. It may be that the rapid reduction in temperature may have led to a more immediate and severe stress response in brain cells. The brain is a highly sensitive organ that plays a crucial role in coordinating various physiological functions; this sudden drop in temperature may have disrupted cellular homeostasis and triggered a cascade of molecular events associated with stress, including cellular metabolism, protein synthesis, and enzymatic activities levels, leading to those proteomic changes. In addition, both "Caspase mediated cleavage of cytoskeletal proteins" and "Apoptosis" were over-represented in FC; these pathways have a high energy demand that could explain the low ATP level of FC fish similar to the one observed in the ASP group. This led to an early *rigor mortis* onset (80% after 3 h *post-mortem*) and resolution, which could be commented on a fish welfare point of view but also from the retailer/consumers' perspective. Indeed, the presence of *rigor mortis* contributes to the overall quality score using the Quality Index Method for freshness by attributing 0 points on the demerit scale (Diler and Genç, 2018). In addition, it has been previously observed how increasing fish stress at slaughter may decrease fish freshness fast, thus reducing fish shelf-life (Zampacavallo et al., 2015). However, it is important to note that further research is needed to fully understand the underlying mechanisms and specific reasons for these differences. Interestingly, an increase in osmolality would be expected, given the high salinity of the water, yet this was not found. This may have to do with the freezing of gill rakers and other membranes that enable ionic exchanges. In fact, the haemorrhages found in these fish were mainly found in the gills. It is probable that the freezing ruptured the fine blood vessels in gill rakers, which then bled when thawed.

6. Conclusions

Our study provides unprecedented detail and scientific evidence supporting the following findings:

a) Electrical stunning emerges as a viable method for the humane stunning of rainbow trout, demonstrating comparable welfare outcomes and favourable fillet quality parameters when compared to anaesthesia. Furthermore, electrical stunning exerts a less pronounced impact on brain cellular processes compared to the alternative stunning methods tested in this trial;
b) Asphyxia in air continues to be associated with high levels of suffering and a concomitant reduction in fillet quality. This reiterates the importance of avoiding asphyxia-based stunning methods due to their negative impact on fish welfare and product quality;
c) The fast-chilling method is unsuitable for stunning rainbow trout, exhibiting similarities to asphyxia in terms of welfare outcomes. Moreover, fast-chilling demonstrates the highest and most

significant impact on brain cellular mechanisms, emphasizing its limitations as a suitable stunning method for rainbow trout.

We further confirm that welfare at slaughter and fillet quality parameters are very closely aligned. Fish that experienced lower levels of stress and exhibited more humane stunning responses displayed better fillet quality characteristics. This close alignment between welfare at slaughter and fillet quality highlights the need for industry practices to prioritize animal welfare during the stunning process. These findings serve as a reminder of the ethical and economic significance of welfare considerations in the aquaculture industry, fostering sustainable and responsible practices, and which ultimately would benefit both the industry and the consumers.

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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 deposited at ProteomeXchange Consortium via the PRIDE repository with the identifier PXD044301 and doi: 10.6019/PXD044301.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2023.740443.

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