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Effect of ice stunning versus electronarcosis on European sea bass (*Dicentrarchus labrax*) muscle structure, ultrastructure and quality traits

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In recent years, the aquaculture industry has seen an increasing concern regarding the welfare of fish, particularly the slaughter and stunning processes, one of the most significant wellbeing issues. Despite the critical role of stunning methods in fish processing, studies examining their impact on the structural integrity of muscle tissue and the overall quality of fish have been relatively scarce. In this context, the current study subjected adult European sea bass with an average weight of 693.8 ± 71.6 to two distinct stunning methods prior to slaughter: immersion in ice water and electronarcosis (with a voltage of 40 V and frequency of 50 Hz for 15 s) to examine their effect on muscle and quality parameters. Immersion in ice water resulted in better and more preserved muscle organization. Electronarcosis exhibited higher K values ($p < 0.05$) and a softer texture ($p < 0.05$) with less gumminess ($p < 0.05$), including changes at electrolyte levels (P: $p < 0.001$; K: $p < 0.036$). No significant differences were found in plasma stress biomarkers, including cortisol (814 vs. 966 ng/mL for ice-water and electronarcosis, respectively), glucose (7.34 vs. 7.28 mmol/L), and lactate (9.12 vs. 10.12 mmol/L). In conclusion, stunning in ice water seemed to induce minimal effects on the muscle structure and ultrastructure and maintain product quality, while electronarcosis appeared to diminish quality traits.

Ensuring fish welfare is a critical concern in aquaculture, both for ethical and practical reasons. Among the most significant stages in the production process is slaughter, particularly the method of stunning used prior to killing[1]. For a stunning method to be considered humane, it must render the animal unconscious and insensible before death[2]. This step is not only ethically important but also commercially relevant, as optimal stunning contributes to the high quality of fish as a food source[3].

Currently, two primary stunning methods are employed in aquaculture: immersion in ice water and electronarcosis. However, there is no European regulation recommending one method over the other[4]. EFSA (2009)[5] has advised phasing out ice-water immersion due to welfare concerns. Despite this, the absence of specific regulatory frameworks allows aquaculture facilities to continue using ice-water immersion as a practical method[6]. A critical limitation of this approach is the extended time required to induce complete unconsciousness. Previous research has shown that immersing fish in ice water lowers body temperature and reduces brain activity until unconsciousness is achieved[4]. Cold-tolerant species like rainbow trout (*Oncorhynchus mykiss*) exhibit prolonged post-mortem processes[4], compared to warm-water species such as gilthead seabream (*Sparus aurata*) and European sea bass (*Dicentrarchus labrax*)[6]. Seasonal variations also influence intra-specific responses, as ambient water temperature fluctuations modulate post-mortem biochemical and physiological kinetics[7].

Rapid temperature changes can induce stress, elevate plasma cortisol levels, and disrupt plasma osmolality[8]. Increased physical activity before unconsciousness raises heart rate and lowers muscle pH[4,9], promoting detachment of myofibres from adjacent structures and contributing to reduced muscle integrity and texture[10]. Post-mortem biochemical changes are critically influenced by ATP depletion, which is closely linked to pH decline. This acidification, driven by lactic acid accumulation from anaerobic glycolysis, inhibits glycolytic enzymes and impairs ATP synthesis[11]. Reduced ATP availability accelerates rigor mortis and structural degradation, ultimately affecting muscle texture and quality[12]. Despite its limitations, ice-water immersion

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remains widely used across species[8], and is the standard technique for assessing muscle alterations in European sea bass.

Electronarcosis, by contrast, is suitable for large-scale operations and has been shown to induce immediate and irreversible unconsciousness in fish, as confirmed by electroencephalogram recordings[13]. Electrical stunning, when applied immediately after harvesting, is considered efficient and humane[13]. It is fast, seemingly causes less suffering, and allows fish to remain in water until unconscious or dead. However, the stun duration is short and must be followed quickly by a killing method[6]. The technique works by passing sufficient current into the brain to induce loss of brain function[14], resulting in immediate unconsciousness that persists until death. This method stimulates muscles[15], causing strong contractions[16] and lowering pH[17]. It requires skill, and there is limited information about its effects on fish and flesh quality[18,19].

Although electrical stunning has been extensively investigated and is currently regarded as the benchmark method for ensuring humane slaughter in aquaculture species, it often fails to induce consistent unconsciousness across species[2,14], raising concerns about both welfare and product quality. Moreover, key structural aspects such as sarcomere integrity, perifibrillar detachment, and intrafibrillar damage remain underexplored, especially in relation to stress-induced biochemical shifts[20]. Addressing these gaps is essential to align industry practices with evolving ethical standards and consumer expectations. The implementation of effective, specific stunning regulations is essential to ensure the use of humane slaughter practices. Such measures would not only prevent prolonged suffering and pain in fish but also uphold ethical standards and enhance product quality and sustainability. This is particularly relevant considering growing consumer awareness and concern regarding the welfare of animals used for food production[1].

Stress during stunning and slaughter is known to affect flesh quality, leading to early onset of rigor mortis[21], texture softening[22], gaping[23], drip loss[24], and reduced shelf life[25]. However, practical knowledge about how current stunning methods influence muscle structure and quality traits remains limited. This is largely due to a historical focus on post-mortem product evaluation, which has hindered the development of welfare-conscious protocols.

The irreversible binding and degradation of actin-myosin complexes during rigor mortis critically affects muscle texture and quality. Stress induced by slaughter methods can accelerate these proteolytic processes[26,27]. Consequently, the breakdown of myofibrillar proteins has become a central focus in understanding muscle softness[28], with implications for developing new ingredients or meat products based on actin functionality[29]. HSP70 is a key biomarker of stress in fish, responding to environmental and handling stressors by aiding in protein repair and cellular protection. Its upregulation reflects physiological strain and can be used to assess both welfare and postmortem muscle quality[30,31].

In the current study, we evaluated how electronarcosis influences muscle preservation and quality traits throughout shelf life when used alongside the primary stunning method in aquaculture for European sea bass—immersion in ice water. A comprehensive histological analysis, including actin degradation, heat shock proteins, and ultrastructure, along with biochemical parameters, sensory assessment, K value, and texture, helps clarify the connections between pre-mortem stress, post-mortem metabolic progression, and potential alterations in fish muscle quality indicators.

Material and methods

Ethical statement

The animal experiments comply with the guidelines of the European Union Council (2010/63/EU) for the use of experimental animals. The procedures carried out have been positively informed by the Animal Experimentation Ethics Committee (CEEA) of the University of Las Palmas de Gran Canaria (ULPGC; Canary Islands, Spain) and authorized by the Department of Agriculture, Livestock and Fisheries of the Government of the Canary Islands (OEBA-ULPGC-07/2023). Given the severity of the project, upon its completion, a retrospective evaluation of the procedure was submitted to the competent authorities. In addition, the study was carried out in compliance with the ARRIVE guidelines (https://arriveguidelines.org).

Stunning protocol

The experimental fish were reared in the Aquaculture Facilities of the Scientific and Technological Park Foundation of the ULPGC. The experimental fish were obtained from the stock of this species maintained in the facilities. Fifty fish, with an average weight of 693.8 ± 71.6 g, were divided into two batches of 25 individuals. The sample size was calculated using the G*Power 3 statistical software, based on a Cohen's d of 0.8—indicative of a large effect size—a significance level (α) of 0.05, and a statistical power (1–β) of 0.80. This calculation indicated a minimum of 21 fish per group. To account for potential contingencies, the number was increased to 25 fish per group. One batch was stunned by thermal shock (TS), simulating the current methodologies employed by European sea bass producers in Spain, adjusting the conditions following the Guide to Correct Practices for Slaughter—Fish Farming UNE 173300[32]. Thus, the fish were introduced into a 300-l stunning tank with a seawater/ice ratio of 1:1 (v/v) which maintained the temperature of the slaughter medium below 2 °C after the fish were introduced. This ensured that death occurred by hypothermia rather than asphyxiation, as no individuals became trapped in the ice. The electrical stunning (ES) batch was made using a prototype supplied by the company Fish Management Systems (Belfast, Northern Ireland), designed and manufactured according to the specified characteristics referring to the size of the bass to be stunned. Before this study, various combinations of voltage (30, 40, 60, and 70 V) and pulse duration (5, 15, and 20 s) were tested at a constant frequency of 50 Hz to determine the optimal parameter configuration. The final selected parameters—50 Hz, 15 s, and 40 V—were chosen to ensure immediate stunning that lasted long enough to result in the animal's death, thereby preventing any recovery of consciousness during the killing process. Additionally, these conditions did not cause adverse effects on the external appearance of the fish, such as reddening of the head and abdomen or persistent opening

of the mouth and operculum. Fish were introduced individually into the stunning tank and subjected to 40 V at 50 Hz for 15 s, conditions that ensured immediate stunning and were maintained for a sufficient duration to culminate in death, thus guaranteeing no recovery of consciousness during the killing process. The confirmation of loss of consciousness during the stunning process, as well as its potential recovery, was assessed through behavioral monitoring, focusing on opercular movement, response to caudal puncture, and the presence of the vestibulo-ocular reflex[4]. After stunning, the fish were slaughtered in a tank with a seawater/ice ratio of 1:1 (v/v).

Sampling procedures

Twenty-five farmed European sea bass per treatment were analyzed for plasma biochemistry parameters. Afterwards, the samples were packed as whole ungutted fish with flaked ice into polystyrene boxes with holes for drainage and stored at 4ºC for 14 days postharvest (dph) at the High Specialization Aquaculture and Biotechnology Service (SABE) at the ULPGC (University of Las Palmas de Gran Canaria). At 0, 2, 6, 9 and 14 dph, five fish per stunning method, randomly chosen, were removed, and individually subjected to Quality Index Method (QIM), Texture Profile Analysis (TPA) and K-value.

Sampling intervals (0, 2, 6, 9, and 14 dph) were selected based on reported changes in quality parameters: a sharp decline in texture occurs between 0–2 dph[33], metabolite accumulation begins around 6 dph[34], and 14 dph marks the typical shelf-life limit for farmed specimens. Fish carcasses and fillets were visually inspected for assessment of spinal damage and incidence of hemorrhages. Each dph, muscle samples of 2 × 1.5 cm, including skin, from under the central lateral line were removed for morphological muscle studies. In the same location, samples of type II muscle were collected for ultrastructural analysis.

Morphological studies

For histochemical and immunohistochemical studies, after 48 h in 4% paraformaldehyde at 4 °C, muscle samples were dehydrated, embedded in paraffin, and sectioned at 3 µm. Samples were stained with hematoxylin and eosin (HE[35]) for general examination including artifacts, fixation, representativeness, muscle fibres arrangement or integrity, with Phosphotungstic Acid Hematoxylin (PTAH[36]) to evaluate muscle striations (banded fibre arrangement), with modified Gomori for collagen or connective tissue[37] labelling and with periodic acid-Schiff (PAS[38]) to evaluate glycogen storage.

For immunohistochemistry, actin (diluted 1:50, Invitrogen, Carlsbad, CA, USA) and Heat shock protein 70 (HSP 70, Biorbit 1/300) antibodies. The antibodies were selected for working in fish, and the suitability for European sea bass was assessed via BLASTp, comparing human epitopes with European sea bass orthologs. High sequence similarity suggested likely cross-reactivity. After antigen retrieval (High pH, Dako, Denmark), immunohistochemical staining was carried out using an anti-rabbit EnVision + System (Dako, Denmark). AEC (3, 3 amino-etil-carbazol) (Dako, Denmark) was used as chromogen, counterstaining with Mayer hematoxylin. Negative controls were processed simultaneously by replacing primary antibodies with primary antibody diluent, and a section of European sea bass liver for HSP70 sections was used as a positive control. The stained sections were scanned with a MoticEasyScan Pro digital scanner (Motic, Xiamen, China) run using the Motic DS Assistant software (Motic VM V1 Viewer 2.0). Six sections per stunning method including muscle type I and II, diet and experimental dph were compared. Changes in the muscle structure: glycogen, actin and HSP70 immunolabeling were quantified, utilizing the analySIS® software package for Windows (Image Pro Plus® V. 4.5.0.29) (Media Cybernetics, Silver Spring, MD, USA).

Three independent assessors, blinded to experimental treatments, conducted a semi-quantitative evaluation of the tissue sections. They assessed features such as fibrillar cracks, endomysium, and eosinophilic deposits using a predefined scoring system based on labeling intensity: 0 (not present), 1 (weak), 2 (moderate), and 3 (strong). Prior to the assessment, the assessors participated in a calibration session to harmonize scoring criteria and reduce subjectivity. Inter-rater reliability was confirmed through the intraclass correlation coefficient. From each treatment group, a representative section was chosen and included in the figure panels.

Samples for ultrastructural studies of type II muscle were cut into small pieces and immediately fixed for 24 h at 4 °C in 2.5% glutaraldehyde in 0.2 M phosphate buffer (pH = 7.2). Afterwards, post fixed for 1 h in 2% osmium tetroxide in 0.2 M potassium ferrocyanide, dehydrated in graded acetone series and embedded individually in an Epon/Araldite resin block. Ultrathin (50 nm) sections were qualitatively evaluated with a Field Emission Scanning Electron Microscope (FESEM; Carl Zeiss, Sigma 300 VP), using the transmission mode with STEM in BF detector at the Advanced Confocal and Electron Microscopy Research Service (SIMACE) of the ULPGC. Samples were evaluated for sarcomere integrity, collagen organization, and mitochondrial preservation. Sarcomere integrity was considered preserved when muscle fibres exhibited clear, uninterrupted striations with well-aligned Z-lines, without disruption, fragmentation, or swelling. Collagen organization was defined by the presence of densely packed, parallel collagen fibres with minimal waviness, disarray, or fragmentation. Mitochondrial preservation was assessed by the presence of intact membranes and cristae, with uniform shape and size, and without swelling, vacuolization, or fragmentation. FESEM micrographs of European sea bass type II muscle were descriptively analyzed across multiple fields per sample (25 fields). Representative fields were selected to illustrate typical morphological features as well as any observed alterations.

Plasma biochemistry

After stunning and before killing, blood was collected from the caudal vein, immediately chilled on ice, and centrifuged at 3000 g at 4 °C for 5 min to obtain plasma stored at − 80 °C until further analysis. Plasma glucose, lactate, pH, calcium, phosphorus, magnesium, potassium, sodium, and chlorine were determined by an automated analyser SMT-120VP (Seamaty, Chengdu, China). Serum cortisol was measured by using Vcheck V200 Veterinary Immunoassay Analyzer (Bionote, Minnesota, USA).

Sensory assessment

At 0, 2, 6, 9 and 14dph, five randomly chosen fish per stunning method were obtained and individually subjected to QIM. Six laboratory-trained panelists with prior experience in fish quality assessment were selected and trained according to ISO 8586–2:2009 guidelines. Sensory evaluations were conducted blindly, unaware of stunning treatments and using the species-specific QIM protocol[39], which includes 10 parameters scored on a 22-point demerit scale[40].

K value

The ATP-related breakdown compounds (adenosine-5′-diphosphate ADP, adenosine-5′-monophosphate, AMP, inosine-5′-monophosphate, IMP, inosine, HxR and hypoxanthine, Hx) were determined by high-performance liquid chromatography using a 1260 Infinity II LC System (Agilent Technologies, Santa Clara, USA) according to Özogul et al.[41]. 5 g of fillet were homogenized in 25 ml of 0.6 M perchloric acid for 2 min at 0°C. The homogenized sample was centrifuged at 2 °C at 3000 rpm for 10 min; the supernatant was neutralized to a pH of 6.7–6.9 and stored at -80°C until analysis. The K value is expressed as a percentage and is calculated using the following formula: K value % = 100 x (HXR + Hx) / (ATP + ADP + AMP + IMP + HXR + Hx). The K value allows for classifying and grading the freshness of fish: below 20% indicates excellent freshness, values up to 40% denote good freshness, and values above 60% suggest the fish is no longer recommended for consumption[41,42].

Texture profile analysis

The TPA was made using a TA.XT2 Texture Analyzer (Stable Micro Systems Ltd., Surrey, UK). The left fillet of each fish was unskinned and divided into three-square pieces (cranial, central, and caudal, 2.5 × 2.5 × 1.5 cm) above the lateral line, to account for variation in muscle structure and fat distribution in the different fillet regions. Due to potential variation in muscle fibre structure and fat distribution along the fillet, it is recommended to collect samples from the cranial, central, and caudal regions to obtain representative texture values. This approach helps ensure a more accurate and consistent assessment of overall fillet texture. The force–deformation curve was analysed to determine texture parameters. The compression plate and speed were 100 mm Ø and 0.8 mm/s, applying a deformation of 60% of the fillet thickness[43].

Statistical analysis

Data was analyzed with IBM SPSS Statistics for Windows, Version 26.0 (Armonk, NY: IBM Corp.). Plasma biochemical, QIM, K value and texture variables were checked for normality and homogeneity of variance using the Kolmogorov–Smirnoff and the Levene's test, respectively. To evaluate the effects of treatments, pairwise comparisons were conducted using *t*-tests. A significance level of $p < 0.05$ was used for all statistical tests. Morphological data assessed in a semi-quantitative scale were compared by the non-parametric Kruskal–Wallis's test, considering $P < 0.05$ as the level of significant differences.

Results

Morphological studies

No fractures or paravertebral and fillet hemorrhages were observed during the sampling process. With HE, on the 0th day post-hatch (0dph), histological examination revealed that cross sections of type II fibres exhibited a distinctive mosaic pattern with balanced distribution of fibre types with consistent size, shape, and spacing, without clustering or irregularities. This pattern was characterized by diffuse myofibrillar microtears revealing a cracked appearance with discontinuities or fissures in the muscle fibre structure, comparing treatments (Fig. 1a, e, i). In ES, the cracks were less evident (Fig. 1b, $p < 0.05$), and in contrast, some eosinophilic deposits appeared within dispersed fibres, but this was not significant. In type I fibres comparable findings were detected (Fig. 1c–d). This outcome was also noticeable with PTAH in type II fibres (Fig. 1e–f), as well as in type I fibres (Fig. 1g–h) where the cracked appearance was marked on TS ($p < 0.05$) (Fig. 1g). ES changes were less noticeable and only in isolated fibres. With Gomori staining (Fig. 1i–l), the endomysium seemed slightly more detached and spaced between fibres. The collagen of the interfibrillar septa, endo and perimysium appears stained green, especially visible with ES (Fig. 1j, l). Stunning with ES, in type I and II fibres, frequent images of isolated fibres ($p < 0,05$) with a greenish intrafibrillar infiltrate (Fig. 1j, l) were noticeable. PAS staining (Fig. 1m–p) revealed a greater amount of glycogen noticeable in the type II fibres of the fish stunned with TS treatment ($p < 0,01$) (Fig. 1m). The PAS positive depots were also visible in the transition zones between type II and type I muscle, termed pink muscle (IIb) with intermediate characteristics (Fig. 1m; white arrows). This result was also marked for type I muscle (Fig. 1o), where large groups of PAS positive fibres were found ($p < 0.05$).

Anti-actin antibody (Fig. 2a–d) displayed different immunostaining comparing experimental methods ($p < 0.05$). TS treatment showed a greater proportion of fibres with a fingerprint pattern resembling muscle bands ($p < 0.05$) (Fig. 2a, c). The stress protein HSP70 (Fig. 2e–h) with TS presented a diffuse distribution and a higher number of fibres showing expression in type II muscle ($p < 0.01$) (Fig. 2e). With ES, the expression was local, more focused on particular fibres following the configuration seen with the modified Gomori (Fig. 2f). In type I muscle the HSP70 distribution was reduced and not showing differences comparing treatments (Fig. 2g–h).

The ultrastructure of the type II myofibres showed that at 0dph, myofibres retained its myofibrillar arrangement (Fig. 3a–h). In the transversal sections (Fig. 3a–b), the interdigitated myofilaments of actin and myosin composing the sarcomere displayed an intact, regular structure. In myofibres subjected to ES treatment, an intermittent presence of band remnants, describing deteriorated, fragmented or partially visible sarcomeric bands, was observed (Fig. 3b). In the longitudinal Sects. (3c–d), the myofibrils exhibited intact, well-organized bands and parallel alignment. Sarcomere structures, including the M-line, Z-line, A-band, and I-band, were maintained clear and intact in TS samples (Fig. 3c). With ES treatment, the Z and the M-line appeared deteriorated, less marked and somewhat blurred (Fig. 3d). Slight gaps in the endomysium between myofibres were observable, but

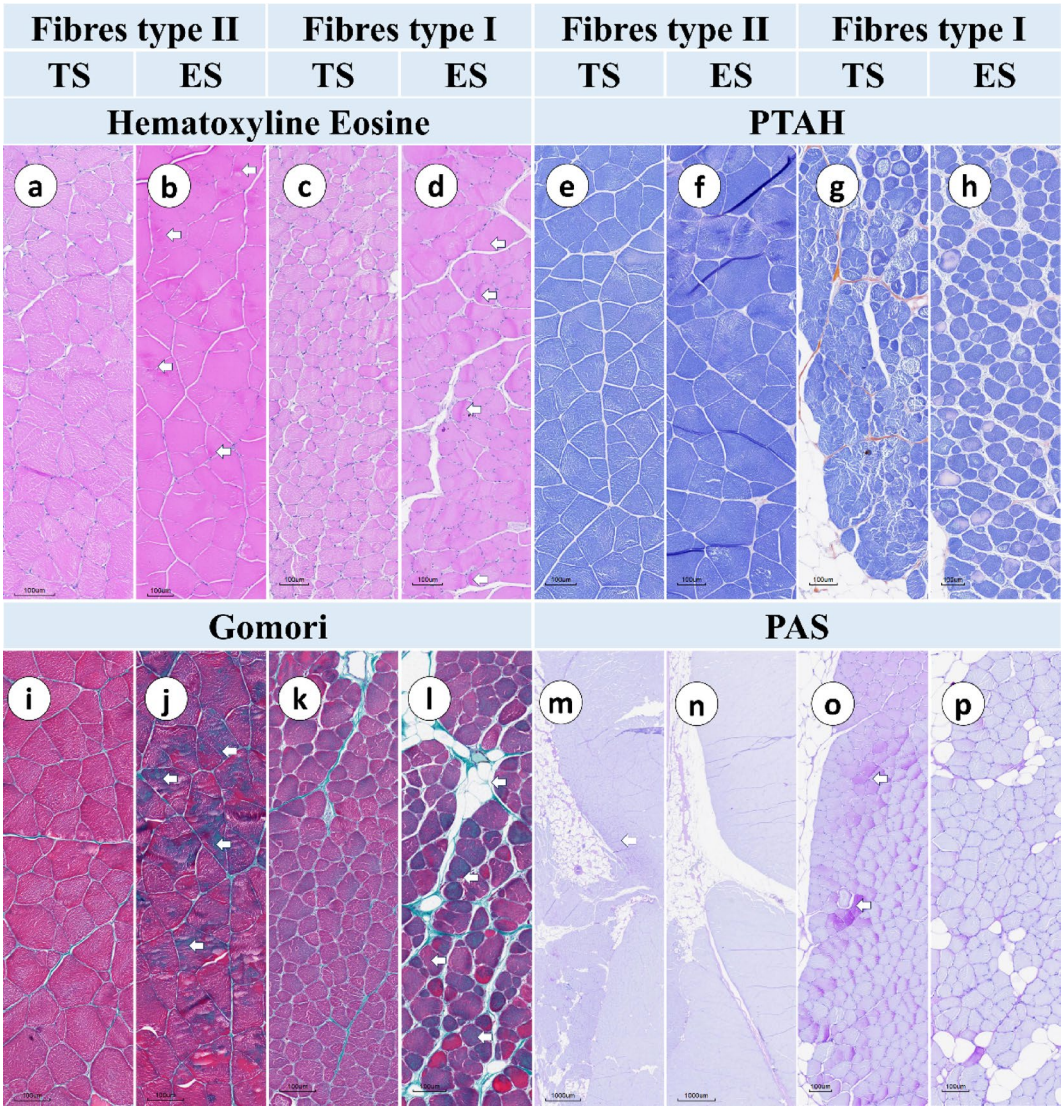

Fig. 1. 0 days postharvest. European sea bass muscle fibres histochemistry. HE in muscle type II and type I fibres. PTAH in muscle type II and in type I fibres. Modified Gomori in muscle type II and I. PAS in muscle type II and type I fibres. White arrows show intrafibrillar deposits. Scale bar 100um.

not frequently. Well-arranged collagen remained visible and continuous (Fig. 3e–f). The examination revealed the presence of abundant, compressed into an irregular shape, subsarcolemmal mitochondria ranging between 0.5 and 1um (Fig. 3g–h). Double-layered membranes were observable in most of the mitochondria, retaining an intact, undamaged and uniform shape. The cristae compartments displayed a compact and well-organized structure. In both treatments, scarce interfibrillar mitochondria and lipid droplets were present.

Throughout the shelf life, on the 2dph, the histochemical pattern demonstrated consistency, maintaining distinct fibrillary microtears patterns across both experimental treatments. The wider interfibrillar detachment was only found on type I fibres, particularly with HE (Fig. S1d) and PAS stain ($p < 0.05$) (Fig. S1p). With PAS the TS treatment preserved a larger glycogenic storage ($p < 0.05$) (Fig. S1m, o).

Actin showed an increase in immunostaining, which was less specific and displayed a less marked fingerprint pattern in TS type II fibres (Fig. S2a–d). HSP70 positivity was almost absent in type II fibres and residual in type I fibres, with no noticeable differences (Fig. S2g–h). With TEM, the myofibrils' actin-myosin pattern on transversal sections mirrored that of the 0dph (Fig. S3a–b). The remnants of the bands from the ES treatment persisted, although becoming increasingly diffuse (Fig. S3b). Similarly, the sarcomere longitudinal pattern in both treatments (Fig. S3c–d) showed signs of deterioration, with the ES treatment particularly displaying a broader Z line and a less evident M band (Fig. S3d). After two days of storage, the collagen packing appeared less compact (Fig. S3e–f). The mitochondrial morphology was slightly impacted by membrane loss and cristae alterations, with early indications of a few dense granules (Fig. S3g–h), but no differences attributable to the treatments were observed.

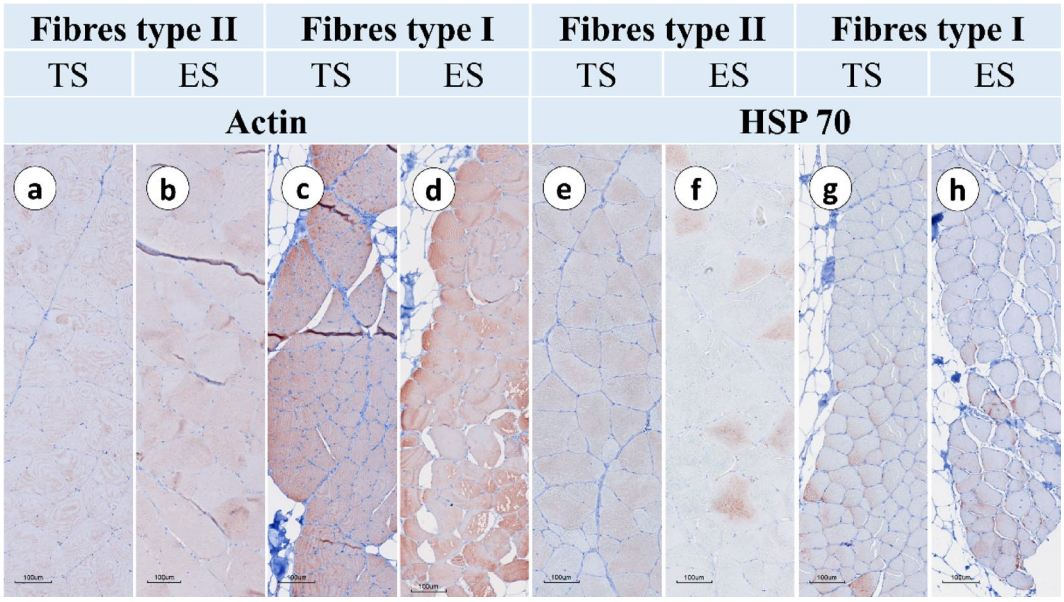

Fig. 2. 0 days postharvest. European sea bass muscle fibres immunohistochemistry on 0 dph. Anti-actin expression in muscle type II and type I fibres. Anti-HSP70 expression in muscle type II and type I fibres. Scale bar 100um.

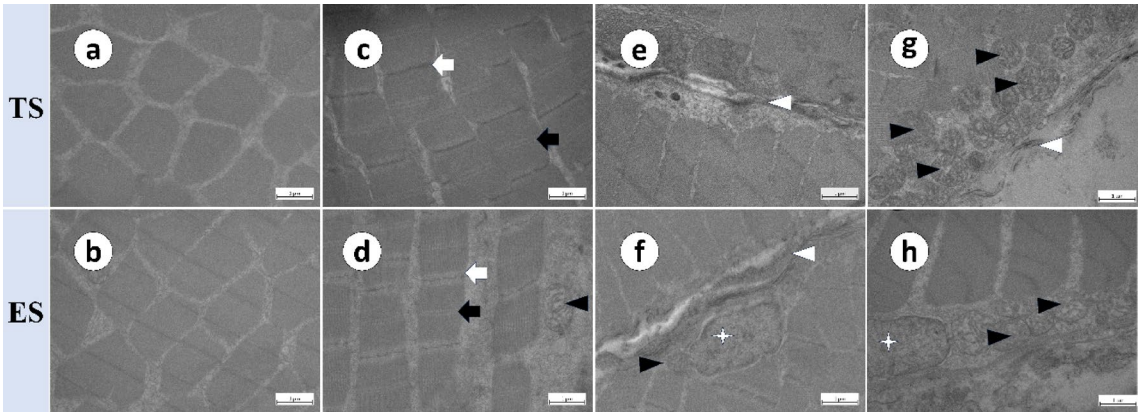

Fig. 3. 0 days postharvest. European sea bass muscle fibres electron microscopy. Type II muscle fibres transverse plane (**a**–**b**); type II muscle fibres longitudinal plane (**c**–**d**); endomysium collagen (**e**–**f**); subsarcolemmal mitochondria (**g**–**h**). Magnification is × 15,000 (Scale bar 1um). Z line white arrows, M line black arrows, collagen white arrowheads, mitochondria black arrowheads, nucleus white star.

On the 6dph, the patterns observed through histochemical analysis on day 0 and the 2dph remained consistent, demonstrating an ostensibly preserved fibrillar structure. The disparities observed with the histochemical techniques (Fig. 4a–p) were attenuated, resulting in a more unified appearance of fibres in both treatments. The intrafibrillar infiltrate identified with Gomori stain was less pronounced (Fig. 4i–l). The accumulation of glycogen, as detected using PAS staining, diminished in visibility but remained significantly prominent ($p < 0.05$) in TS type II muscle fibres (Fig. 4m).

Immunohistochemistry for actin in type II fibres persisted, displaying a conserved fingerprint outline in TS (Fig. 5a) than in ES ($p < 0.05$) where the pattern was absent (Fig. 5b). In type I fibres, the fingerprint arrangement was better preserved (Fig. 5c–d). HSP70 positivity was lacking in type II fibres and residual in a few scattered type I fibres (Fig. 5e–h).

At 6dph, the ultrastructure continued to exhibit the remnants of transversal bands, which were increasingly deteriorated, less defined in the ES treatment (Fig. 6b) than in TS (Fig. 6a). With the ES treatment, the M-band was nearly inexistent (Fig. 6d) compared to TS (Fig. 6c). In both transverse and longitudinal planes, expanding gaps between myofibres indicated the deterioration of connections between adjacent myofibres (Fig. 6b, d, e–h). Collagen packaging at this stage was dispersed over extensive spaces (Fig. 6e–h). Mitochondrial features appeared swollen with discontinuous membranes, damaged in some areas (Fig. 6g–h). The cristae were elongated, tubular,

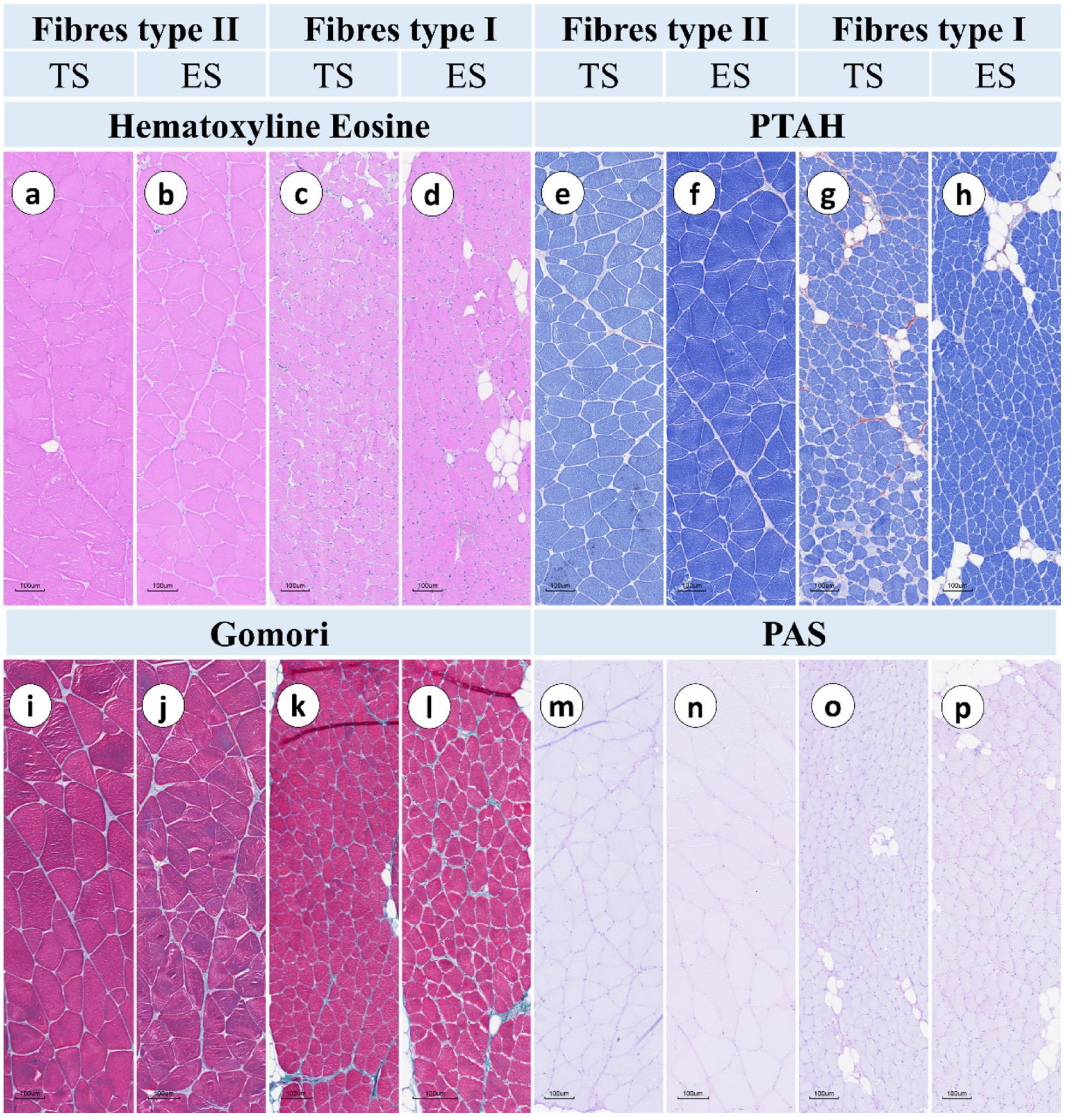

Fig. 4. 6 days postharvest. European sea bass muscle fibres histochemistry. HE in muscle type II and type I fibres. PTAH in muscle type II and in type I fibres. Modified Gomori in muscle type II and I. PAS in muscle type II and type I fibres. White arrows show intrafibrillar deposits. Scale bar 100um.

disorganised, and had nearly vanished in some mitochondria. The matrix exhibited a more electron-lucent appearance with an increased presence of dense granules, and images of fusion of nearby mitochondria were frequent. The treatments did not result in any noticeable variations.

On the 9dph (Supplementary Fig. S4), the patterns previously described were maintained, albeit with a progressively deteriorating structure. The disparities observed with all the histochemical techniques (Fig. S4a–p) were attenuated, resulting in a more consolidated and homogeneous appearance of fibres in both treatments. The primary changes observed were an increased detachment of fibres with separated myocommata in the ES treatment ($p < 0.05$) with HE and PTAH (Fig. S4b, d, f, h). These modifications were also detected with Gomori (Fig. S4i–l). Enhanced PAS positivity with TS ($p < 0.05$) (Fig. S4m–p) were also notable. Immunohistochemistry for actin was pronounced, like that observed on the 6dph (Fig. S5a–d), displaying a more preserved fingerprint-like appearance in TS than in ES, where the pattern was lost. In type II fibres, the pattern was better preserved, although no significant differences were found. HSP70 marking was absent, as seen from the 2dph onwards, with sporadic positivity, especially in type I fish muscle (Fig. S5g–h).

At 14dph, muscle fibre preservation was greater than expected. (Fig. 7a–p). At this juncture, the cracked appearance was not evident, with possible remnants in ES with HE, PTAH, and Gomori (Fig. 7a–l), accompanied by a presence of detachment. The intrafibrillar infiltrate with Gomori stain, previously noted in ES, was absent (Fig. 7i–l). The connective tissue remained marked with PTAH and Gomori but slightly faded. PAS glycogen storage was preferentially maintained in TS, albeit was only noticeable in type I fibres (Fig. 7o).

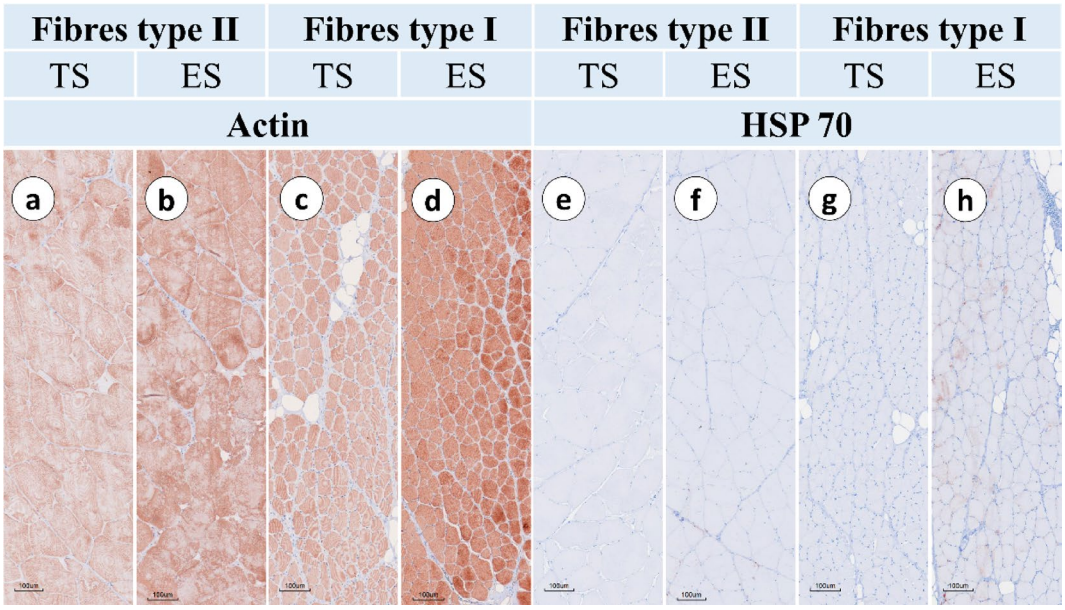

Fig. 5. 6 days postharvest. European sea bass muscle fibres immunohistochemistry. Anti-actin expression in muscle type II and type I fibres. Anti-HSP70 expression in muscle type II and type I fibres. Scale bar 100um.

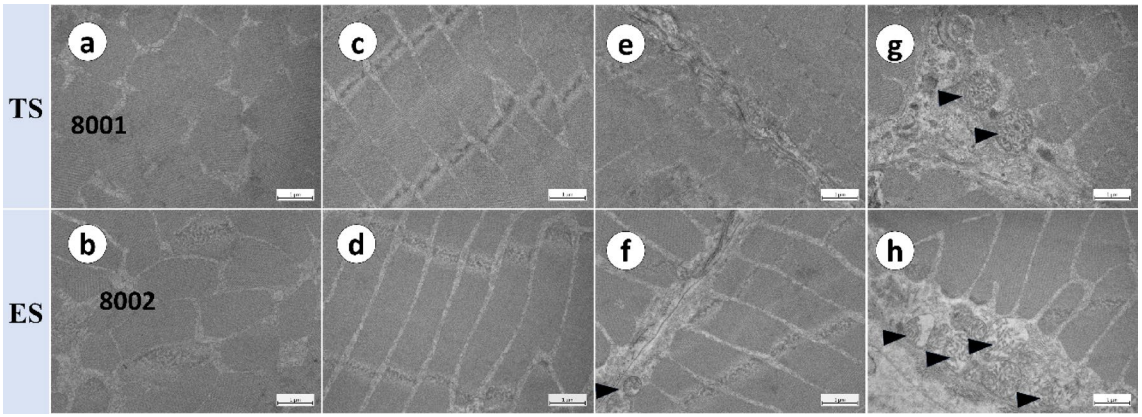

Fig. 6. 6 days postharvest. European sea bass muscle fibres electron microscopy. Type II muscle fibres transverse plane (**a**–**b**); type II muscle fibres medial plane (**c**–**d**); endomysium collagen (**e**–**f**); subsarcolemmal mitochondria (**g**–**h**). Magnification is × 15,000 (Scale bar 1um). Mitochondria black arrowheads.

Immunohistochemistry for actin exhibited persistent positivity (Fig. 8a–d), but similar to the 9dph, the fingerprint outline was almost negligible, more preserved in TS than in ES where the pattern was lost in both type II and I fibres (Fig. 8a, c). HSP70 positivity was absent in both type II and type I fibres (Fig. 8e–h).

Plasma biochemistry

The results of the plasma samples analysis are presented in Table 1. No significant differences were observed in stress markers, such as cortisol, glucose, or lactate, when comparing experimental methods. However, statistically significant variations were detected in the levels of phosphate and potassium, with the highest values observed in fish subjected to ES.

Sensory assessment

The Quality Index Method (QIM) scoring for European sea bass at 0dph, when subjected to the electrical stunning (ES) protocol, revealed the highest values ($p < 0.05$), indicating reduced quality (Fig. 9). Over the course of shelf life, these values gradually increased, and the differences between ES and TS methods persisted as the storage period advanced. The highest scores were consistently observed in the ES group, although they were not statistically significant.

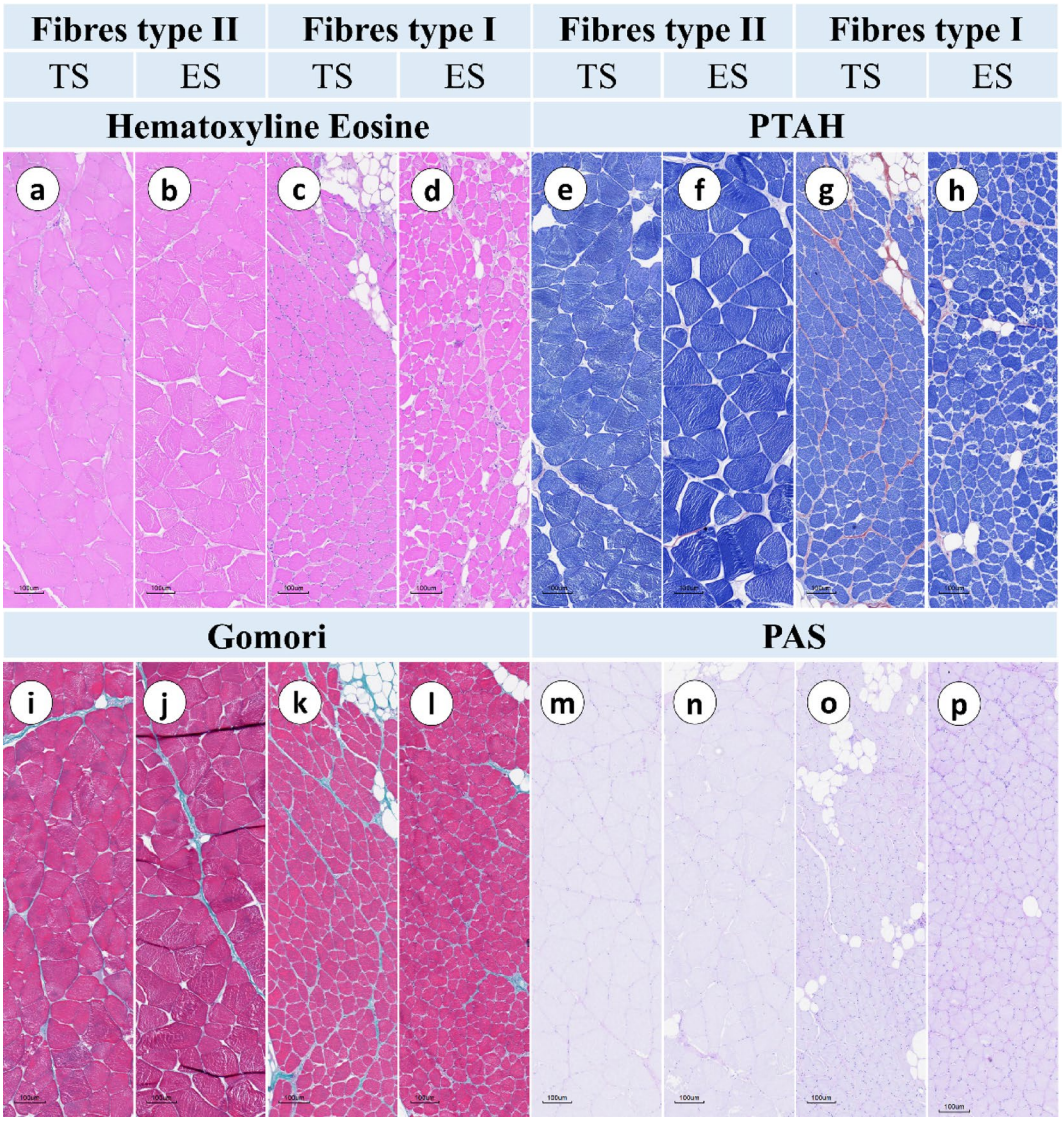

Fig. 7. 14 days postharvest. European sea bass muscle fibres histochemistry. HE in muscle type II and type I fibres. PTAH in muscle type II and in type I fibres. Modified Gomori in muscle type II and I. PAS in muscle type II and type I fibres. White arrows show intrafibrillar deposits. Scale bar 100um.

K value

The K value did not differ significantly between the stunning methods during the initial sampling at 0 and 2dph (Fig. 10). However, starting from 6dph, significant differences ($p < 0.05$) were observed both across days (as expected) and when comparing the stunning methods. The lowest K values, indicative of the highest freshness, were consistently found in fish stunned using the TS method.

Texture profile analysis

Only hardness and gumminess are reported, as other TPA parameters showed no significant differences between treatments. The maximum compression force required to deform the fillet to 60% of its thickness (hardness) was higher in fish stunned by the TS method ($p < 0.05$) (Fig. 11). Interestingly, this difference persisted not only immediately after slaughter but also throughout the shelf life. Notably, the loss of resistance to compression decreased significantly at 2dph in fish stunned with ES ($p < 0.05$), while those stunned with TS showed a progressive reduction in fillet hardness up to 9dph. Beyond that day of ice storage, no differences were observed between the stunning methods. Similarly, the gumminess values were higher in fish stunned using the TS method ($p < 0.05$) (Fig. 12).

Discussion

Morphological studies

Postmortem degradative changes in fish muscle have economic implications, as they lead to a decline in overall fish quality[44]. Some of the observed differences in muscle properties can be attributed to muscle stimulation

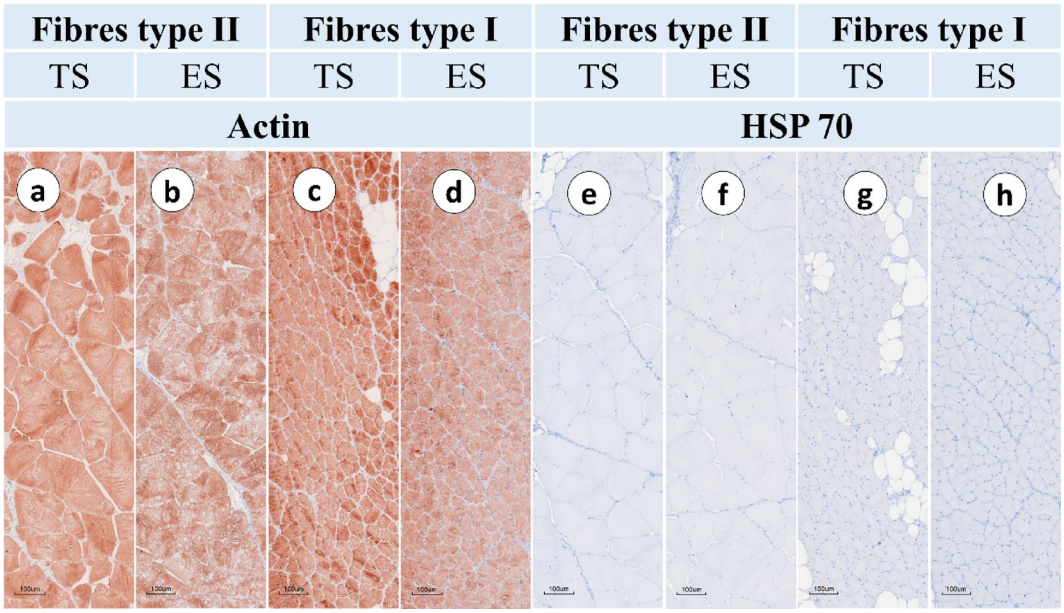

Fig. 8. 14 days postharvest. European sea bass muscle fibres immunohistochemistry. Anti-actin expression in muscle type II and in type I fibres. Anti-HSP70 expression in muscle type II and in type I. Scale bar 100um.

	TS	ES	*P-value*
Cortisol	814.34 ± 257.99	966.69 ± 221.94	0.169
Glucose	7.34 ± 1.77	7.28 ± 1.79	0.922
Lactate	9.12 ± 1.78	10.12 ± 1.19	0.098
pH	7.35 ± 0.1	7.36 ± 0.1	0.913
Calcium	3.47 ± 0.25	3.66 ± 0.28	0.064
Phosphorus	3.51 ± 0.44	4.08 ± 0.39	0.001
Magnesium	1.91 ± 0.60	1.59 ± 0.14	0.054
Potassium	5.83 ± 0.76	6.37 ± 0.52	0.036
Sodium	151.38 ± 2.45	150.55 ± 2.44	0.359
Chloride	170.25 ± 8.48	172.26 ± 4.45	0.438

Table 1. Plasma parameters in European sea bass (mmol/l except cortisol in ng/ml) according to the stunning protocol (mean ± SD).

by electrical current and muscle contractions, which trigger metabolic processes, accelerating rigor mortis. Electrical stimulation primarily depletes energy reserves in type II muscle fibres but also affects type I fibres, resembling the effects of escape swimming or forced exercise[45], as effectively labelled using the PAS technique. Notably, acute local electric stimulation has been associated with a significant decrease in glycogen content in muscle fibres[46]. Additionally, muscle post-mortem acidification occurs due to glycogen conversion to lactate[47], a phenomenon observed in sea bass studies by Zampacavallo et al.[6]. This process also results in a notable reduction in initial pH levels in type I muscle fibres[13,39]. Likewise, in farmed Atlantic cod (*Gadus morhua*), electrical stunning can induce an early onset of rigor mortis[48].

The presence of fibrillar cracks observed throughout the shelf life in fish muscle samples embedded in paraffin, suggests variations in fibrillar costameric or sarcomere protein cohesion. This observation, together with the pronounced perifibrillar space in ES samples, may reflect ultrastructural alterations in the Z line that compromise myofibrillar cohesion. Such changes can weaken the structural integrity of the muscle, reduce water-holding capacity and increase drip loss[49], critical factors for shelf-life, potentially affecting market value and consumer acceptance[50].

Additionally, the presence of oedema, a common occurrence after stunning[51,52], was noticeable through Gomori staining in the perimysium. Oedema may compromise muscle integrity by increasing tissue fluid content, which can weaken structural cohesion and assist enzymatic and microbial activity. This contributes to muscle degradation, negatively affecting texture[53] and reducing shelf life[54,55]. Notably, despite these changes, the overall structure of the muscle fibres remained relatively preserved in both treatments, with minimal detachment of muscle fibres from the myocommata. The degradation of transversal cytoskeletal actin filaments can lead to sarcolemma detachment from the basal lamina and the extracellular matrix network[56], compromising the

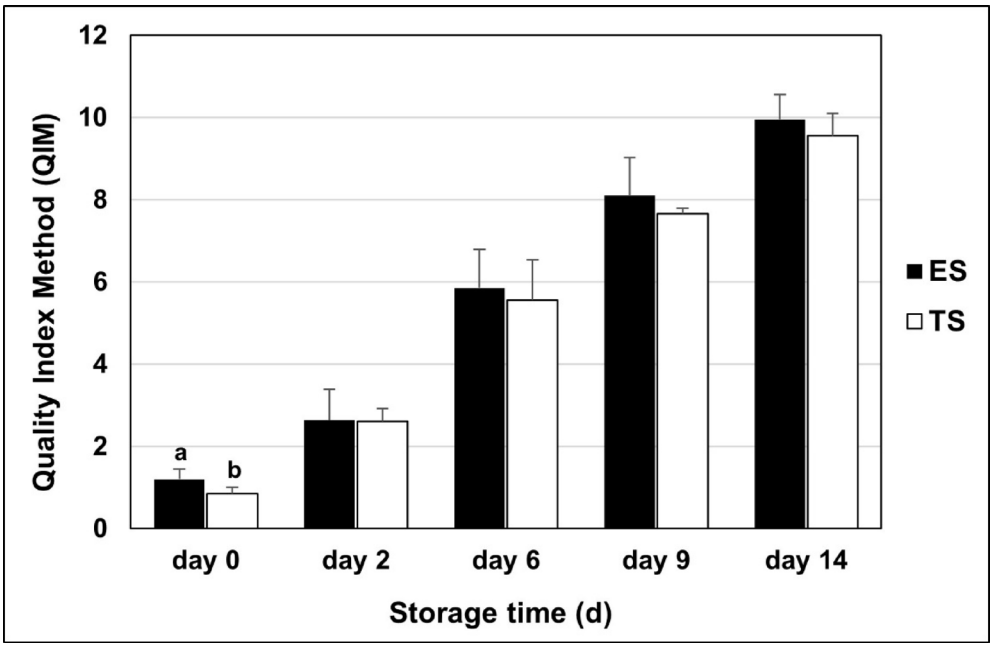

Fig. 9. QIM evolution during ice storage days in the electrical stunning (ES) and thermal shock stunning (TS). Different letters in the same day denote significant differences among stunning method ($p < 0.05$).

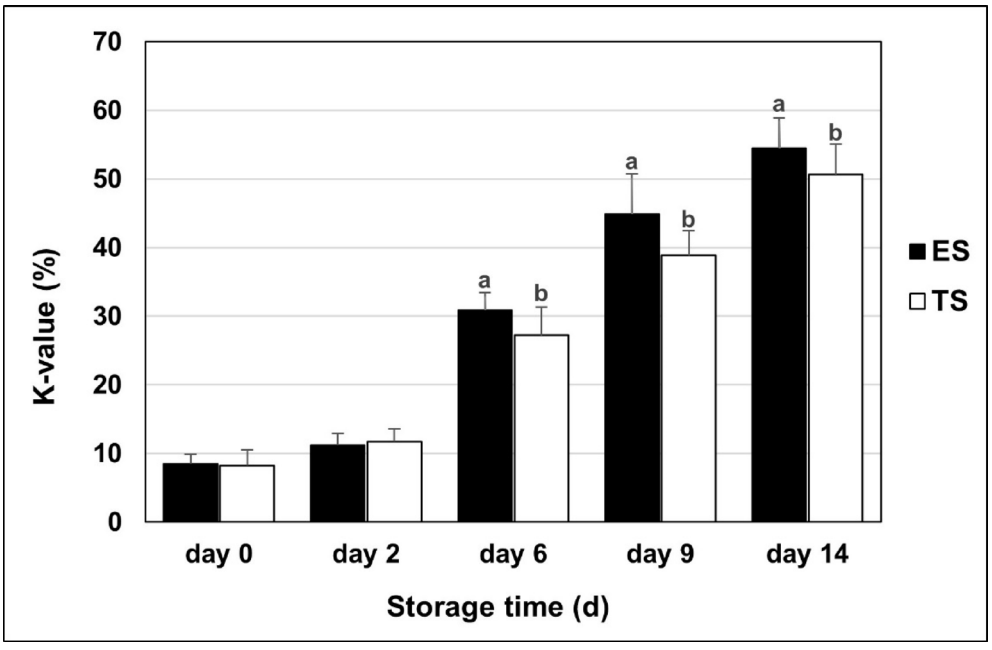

Fig. 10. K-value evolution during ice storage days in the electrical stunning (ES) and thermal shock stunning (TS). Different letters in the same day denote significant differences among stunning method ($p < 0.05$).

muscle's structural integrity[57]. Throughout storage, persistent actin staining in the cytoplasm of muscle fibres underscores the robust resistance to proteolysis exhibited by actin filaments. However, proteinaceous concretions observed with the Gomori stain may be associated with microscopic-scale leakage facilitated by electroporation, as observed in electrofishing[48]. Electrical stunning before slaughter induces muscular and vascular spasms that can lead to muscular and vascular damage in broilers[58]. In the context of this fibrillary damage, fish exhibit adaptive mechanisms, including HSP70, which help them cope with stressors and maintain homeostasis[59]. HSP70 activation depends on the stress situation, but its expression decreases after postmortem changes, as observed in the present study. HSP70 plays a crucial role in cellular protection and repair mechanisms under stress conditions, including postmortem muscle degradation. Its upregulation has been linked to enhanced

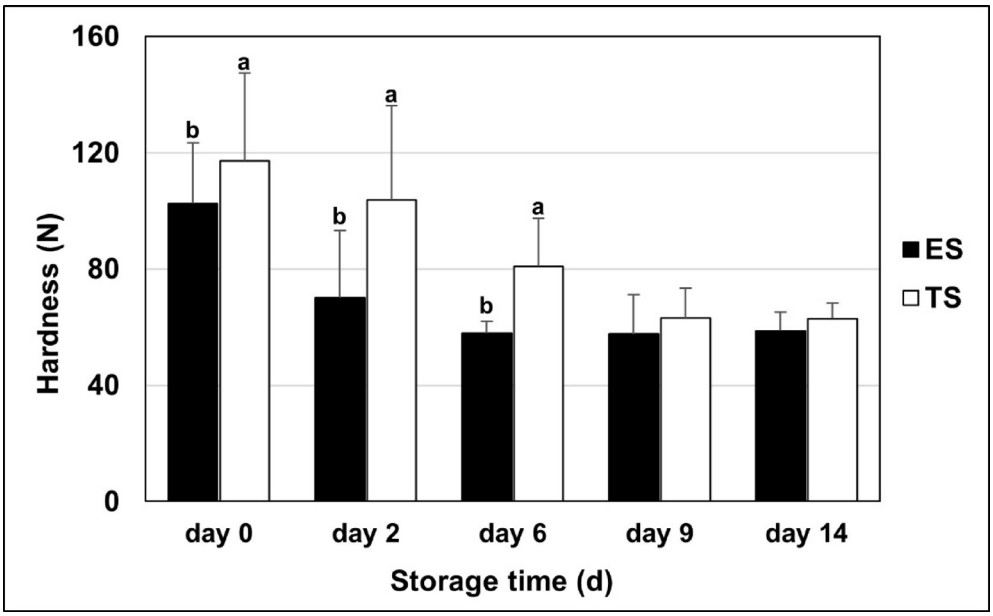

Fig. 11. Hardness evolution during ice storage days in the electrical stunning (ES) and thermal shock stunning (TS). Different letters in the same day denote significant differences among stunning method ($p < 0.05$).

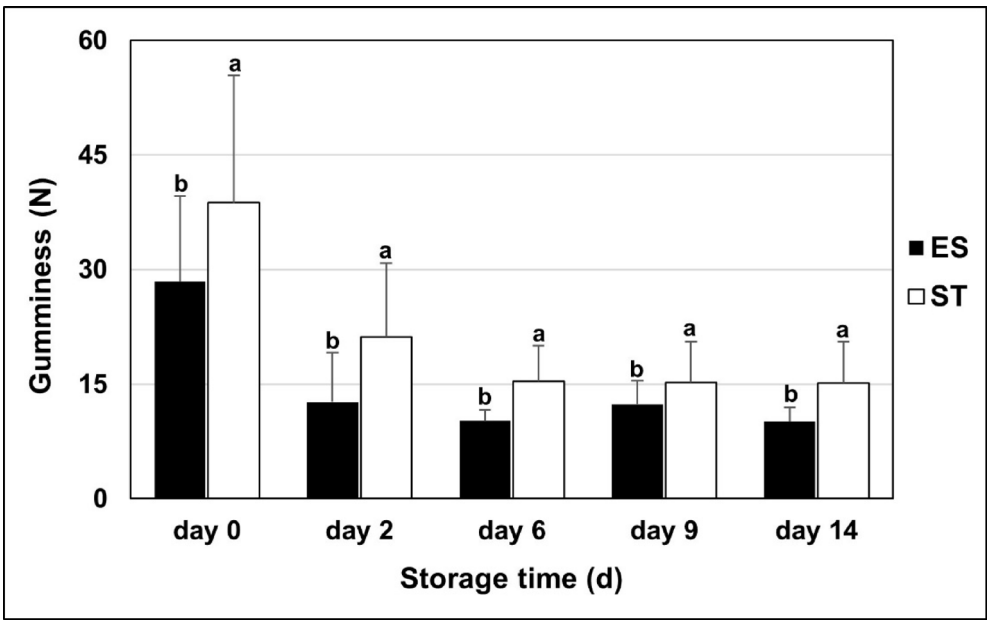

Fig. 12. Gumminess evolution during ice storage days in the electrical stunning (ES) and thermal shock stunning (TS). Different letters in the same day denote significant differences among stunning method ($p < 0.05$).

muscle integrity and recovery following stress or injury[60]. In fish, HSP70 expression is associated with adaptive responses to environmental stressors, including temperature and chemical exposure, which can influence muscle texture and quality. For instance, elevated HSP70 levels have been correlated with improved tolerance and cellular repair in fish exposed to stress, suggesting a protective role in maintaining muscle quality[61]. Thus, HSP70 has been identified as a key biomarker in fish muscle, reflecting physiological responses that may impact postmortem characteristics such as tenderness and structural integrity[60]. In seabream[62], immunohistochemistry with an inducible HSP70 antibody revealed moderate reactivity in skeletal musculature, but only in fry or adults of stressed fish during a three-hour transport.

Ultrastructure observations on day 0 were consistent with previous studies in sea bream, which described the good preservation of muscle myofilament fillets at 0dph[33–63]. No marked ultrastructural differences were found

in the sarcomere structure as fish sarcomeres are favored by major stability than mammalian. While fish I bands are seldom broken[64], the less densely packed Z line could influence the microscopic appearance of myofibres, which also correlates with texture results. In this context, collagen and the rupture of the myocommata have also been suggested as determining factors for the textural attributes of fish flesh, particularly regarding fracturability[33]. Papa et al.[65] studying sea bass muscle ultrastructure found that at 1dph most of the sarcolemma appeared detached, although myofibres appeared almost unchanged.

In fast-twitch type II muscle, the limited occurrence of interfibrillar mitochondria and intracellular lipid droplets suggests reduced aerobic activity, which is more frequent in type I red-slow muscle, reflecting differences in locomotor style. Researchers studying changes in myofilament structure in fish muscle have also described variations in mitochondrial morphology during shelf life. For instance, in European sea bass[66] and gilthead sea bream fillet[67], swelling of mitochondria and sarcoplasmic reticulum was observed 3 h *postmortem*. Similarly, Roy et al.[68] reported altered mitochondria with swollen cristae in Pacific bluefin tuna (*Thunnus orientalis*) muscle cells on day 0. As shelf life progressed, mitochondria continued to swell, exhibiting disrupted membranes and dense granules within the mitochondrial matrix by 6dph. Ayala et al.[67] also noted granular intramitochondrial structures from 5 days postmortem in gilthead sea bream. These dense granules result from the accumulation and precipitation of calcium in the mitochondrial matrix[69]. During cell death processes, disturbed calcium homeostasis leads to massive calcium entry into the mitochondria[3]. Initially, this influx causes organelle swelling, followed by the precipitation of calcium as insoluble phosphate and hydroxyapatite, contributing to mitochondrial damage and cell death[3,70]. Despite these changes, mitochondrial activity persists during ice storage, albeit with a decreasing trend under postmortem conditions, with the increase in storage duration correlated with an increase in mitochondrial swelling[70,71]. Alterations in mitochondria are sensitive to the length of storage, suggesting that the modifications in mitochondria occurred concurrently with the changes in myofibrillar structural proteins[72], indicating a close relationship between mitochondria and tenderness[28]. Exploring the interplay between mitochondrial function, sarcoplasmic reticulum integrity, and storage is crucial for enhancing seafood quality[28]. These ultrastructural changes reflect postmortem cellular responses that may also be influenced by pre-slaughter physiological conditions. To further explore this relationship, we examined plasma biochemical parameters as indicators of stress and metabolic activity associated with different stunning methods.

Plasma biochemistry

Previous studies have demonstrated that both stunning methods can alter stress parameters measured in blood. However, our results showed no significant differences in blood levels of cortisol, lactate, or glucose when comparing these experimental stunning methods. During slow stunning, insensibility is gradually induced[52], and the struggle movements may influence muscle activity, potentially leading to an accumulation of metabolites, including plasma lactate[9]. In European sea bass subjected to electrical stunning, no differences in plasma cortisol levels were found compared to those stunned on ice[73]. Contrarily, Poli et al.[74] reported that electrically stunned sea bass had higher cortisol levels when compared to other stunning methods. This response was attributed to the procedure being conducted in air rather than in a saline water medium. Under those conditions, the electrical current was transmitted through the residual film of saline water remaining on the fish post-capture, rather than via full immersion, potentially intensifying the physiological stress response. European sea bass has shown higher basal cortisol levels compared to other Mediterranean aquaculture species[75,76]. This physiological stress response, related to aquaculture practices, has led to this species being considered sensitive[77]. In our study, we found that the handling process before stunning, specifically fish crowding and capture prior to introduction into the stunning tank, had a more significant impact on the stress response than the stunning practices themselves. Therefore, our results support the notion that ice stunning, despite not being associated with an immediate loss of consciousness, does not elicit a greater stress response, as evidenced by the levels of stress metabolites measured in blood.

Plasma electrolyte concentrations can vary in response to exogenous factors, such as stress[78]. For instance, long-term low-lipid diets have been associated with elevated plasma phosphorus and potassium levels, which correlate with poor growth and physiological stress[79]. Likewise, acute stress has been observed to reduce plasma calcium and potassium concentrations following confinement[80]. In the present study, we found that electrical stunning increased plasma potassium and phosphorus concentrations. This is likely due to the altered cellular homeostasis caused by the electrical shock and heightened neuromuscular excitability. Most potassium is located intracellularly, with only a small proportion present extracellularly, necessary for maintaining membrane potential differences. High-frequency muscle cell stimulation has been found to reduce potassium concentration in T-tubule membranes, resulting in extracellular hyperkalemia[81].

Sensory assessment

The impact of stunning procedures on sensory attributes stems from the fact that rigor mortis is established and resolved earlier in fish subjected to electrical stunning[82,83]. This effect is likely due to the higher stress conditions experienced by muscle during electrical stunning. Other potential changes related to electrical stunning include variations in the eyes of electrically stunned sea bass[13], where the eyes appear less dark and less convex. Interestingly, throughout the subsequent storage period on ice, the spoilage processes exhibit similar performance regardless of the two stunning methods utilized. These findings align with other research exploring various electrical stunning protocols for this species[6,39]. The evaluation of spoilage, and consequently the acceptability of the fish during ice storage, exhibited a strong linear correlation between the mean QIM scores recorded for each storage day and the duration of storage in ice[84].

K value

The delayed onset of rigor mortis in fish stunned with thermal shock compared to electrical stunning helps preserve cellular energetic reserves at the time of death[85]. Consequently, fish stunned by electricity exhibit a faster initial rate of ATP degradation and enter rigor mortis earlier[48]. Considering that ATP decomposition plays a predominant role in fish muscle changes after death[86], electrical stunning may have a more negative impact on maintaining fish freshness during ice storage than thermal shock. Zampacavallo et al.[6] previously reported differences in K value in sea bass after electrical or thermal shock stunning, with a higher accumulation of HxR and Hx in the muscle of fish stunned with electricity during ice storage. This process evolves throughout the shelf life[34], with ATP degrading to inosine monophosphate (IMP) soon after death, while Hx is not detected until 6dph. From this point, European sea bass stunned by electricity consistently exhibit significantly higher K values than fish stunned by thermal shock. On day 0dph, ATP depletion and elevated levels of IMP do not significantly differ between sea bass stunned by electricity or ice slurry[39], following a similar reduction pattern in both stunning procedures during ice storage. However, as the accumulation of HxR and Hx progresses and the K value reaches around 30%, the effects of stunning become evident. Notably, in the results reported by Knowles et al.[39], no differences in K value between electrical and thermal shock were detected. Possible variations in stunning methodologies or handling operations before stunning could explain these effects, leading to stress-related accumulation of HxR and Hx compared to the total pool of ATP and related compounds, resulting in a higher K value[87].

Texture profile analysis

Texture is considered one of the most critical quality attributes of fish, valued by producers, processors, and consumers alike[88]. It is also a crucial factor in assessing the quality of raw fish products during ice storage, particularly freshness[89]. While flesh texture is primarily determined by muscle fibres, intramuscular connective tissue, and intramuscular fat[90], the degradation of these structures during shelf life is influenced by the procedures of stunning and slaughter. In this context, electrical stunning has shown a negative impact, requiring less force to compress the fillet compared to thermal shock stunning. This has been previously described in sea bass[73] and other species such as turbot[87]. The rapid onset and resolution of rigor mortis in electrically stunned fish result in an earlier loss of myofibrils' structural integrity, promoting the enzymatic degradation of proteins involved in intermyofibrillar linkages and linkages between myofibrils and the sarcolemma[64]. This effect is particularly noticeable when determining the energy needed to disintegrate the muscle structure, showing a dramatic fall between 0 and 2 days post-mortem[33]. Connective tissues, particularly collagen-rich structures such as the perimysium and endomysium, play a critical role in maintaining muscle integrity and texture. During postmortem storage, especially under iced conditions, these tissues undergo enzymatic breakdown due to the activity of endogenous and digestive proteases, including collagenases[91]. This degradation contributes to the softening of muscle and the development of mushiness, directly influencing sensory attributes such as gumminess and hardness. For instance, the breakdown of collagen reduces structural resistance, leading to decreased hardness and increased gumminess, as the muscle becomes less cohesive and more deformable[89]. Particularly in the case of gumminess, the differences observed between fillets according to the stunning method persisted throughout the entire ice storage period. This behavior, in which gumminess differs slightly from the other texture parameters, as previously observed when comparing sea bass fillets from feeding treatments that may alter the normal structure and strength of connective tissue40, is influenced by the loss of cohesion between muscle fibres caused by electrical stunning.

In conclusion, our findings indicate that electrical stunning had a greater impact on muscle quality than thermal shock. It altered muscle structure at the microscopic level, showing distinct patterns of intrafibrillar cracks, eosinophilic deposits, and perifibrillar detachment. These changes were associated with early onset of rigor mortis, likely due to energy substrate depletion and ultrastructural disruptions in sarcomere and connective tissue integrity. However, mitochondrial morphology suggested that cellular respiration and energy production were not significantly impaired. Plasma stress parameters—including cortisol, glucose, and lactate—showed elevated levels overall but did not differ significantly between stunning methods, suggesting that pre-stunning handling may have had a greater influence on physiological stress. Minor differences were observed in electrolytes related to neuromuscular excitability. Quality traits were negatively affected by electrical stunning, with higher QIM scores at 0 dph and increased K values from 6 dph onward. The decline in hardness and gumminess impacted sensory quality throughout shelf life. In contrast, ice-water stunning had minimal effects on the evaluated parameters. The reduced shelf life associated with electronarcosis may have economic implications for the aquaculture industry. Future studies should explore the long-term effects of electrical stunning on consumer acceptability and investigate alternative stunning techniques that better preserve muscle quality while maintaining welfare standards.

Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Conceptualization and methodology: P.L.C., I.M. and R.G; validation, P.L.C. and R.G; formal analysis, P.L.C., I.M. and RG.; data curation, P.L.C. and R.G.; writing—original draft preparation, P.L.C. and R.G.; writing—review and editing, P.L.C. and R.G.; visualization, P.L.C. and R.G; supervision, P.L.C. and R.G; project administration, R.G.; funding acquisition, R.G. All authors have read and agreed to the published version of the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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