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The stress response of red sea bream (*Pagrus major*) at harvest: Toward the development of a humane slaughter method

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ABSTRACT

This study uses a multifactorial experimental design to investigate the combined effects of rearing temperature, harvest, and slaughter methods on the stress response and *post-mortem* muscle quality in red seabream (*Pagrus major*) under both controlled laboratory and industrial conditions. Results showed significant interactions among these factors in primary stress parameters. These include plasma cortisol, glucose, lactate, chloride, and potassium. Environmental and procedural stressors interactively modulate physiological homeostasis. Stressful combinations, especially crowding during harvest, followed by asphyxia slaughter, caused the most severe physiological disruptions. This was evidenced by heightened HPI-axis activation (elevated plasma cortisol and glucose), exhausted anaerobic capacity (high plasma lactate and sodium concentrations), and osmoregulatory failure (high plasma chloride and potassium concentrations). Severe pre-slaughter exhaustion led to faster *post-mortem* deterioration. This was shown by a rapid decline in muscle pH and an accelerated onset and progression of *rigor mortis*, which directly compromised fillet quality. In contrast, low-stress protocols, such as rapid netting or hook-and-line harvest and humane slaughter methods like ikigun or application of electrical stunning before slaughter, minimized the stress response. These methods preserved muscle energy reserves, resulting in more stable *post-mortem* muscle quality profiles. Our results underscore the importance of optimizing harvest and slaughter methods to minimize stress and preserve product integrity in aquaculture. Rearing temperature also emerges as a key conditioning factor shaping physiological status at harvest. Together, these findings link humane handling practices, including the potential use of electrical stunning, to improved fillet quality in farmed fish.

1. Introduction

The red seabream (*Pagrus major*) is a high-value marine species that is extensively farmed throughout Asia and has recently been introduced into Mediterranean aquaculture (Saad et al., 2022). Native to the Northwest Pacific, this demersal species inhabits shallow coastal areas as juveniles before migrating to deeper offshore waters as adults (Law and Sadovy De Mitcheson, 2017). Japan remains the world's leading producer, with aquaculture output reaching 68,000 tons in 2023 after remaining stable at approximately 60,000 tons since 2014 (Kato, 2023; Ministry of Agriculture, Forestry, and Fisheries, 2024). Despite its economic importance, knowledge of the species' stress physiology under farming and harvest conditions remains limited, representing a critical gap in improving welfare standards and optimizing aquaculture production (Ciliberti et al., 2024; Samaras et al., 2022).

Stress sensitivity is a central challenge in aquaculture. Teleosts, including the red seabream, are physiologically vulnerable to acute environmental and handling stressors such as temperature fluctuations, harvest, and slaughter methods (Papaharisis et al., 2019; Samaras et al., 2018). These stressors can impair oxygen transport, disrupt endocrine balance, and weaken immune function (Alfonso et al., 2023; Islam et al., 2022; Roychowdhury et al., 2024). Thermal stress in particular has been studied in a range of fish species, including silver catfish (*Rhamdia quelen*) (Lermen et al., 2004), largemouth bass (*Micropterus salmoides*)

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(Ma et al., 2025), milkfish (*Chanos chanos*) (Chang et al., 2020), Mekong giant catfish (*Pangasianodon gigas*) (Phinrub et al., 2023), and cherry salmon (*Oncorhynchus masou*) (Lee and Balasubramanian, 2023). However, studies in red seabream reveal a distinct, temperature-dependent stress response, characterized by an inverse cortisol-temperature relationship within the species' optimal thermal range, contrasting with the typical elevation of cortisol observed at warmer temperature in most teleosts (Samaras et al., 2022). The response in red seabream showed a rapid cortisol response and recovery following acute stress, with plasma glucose increasing at higher temperatures while cortisol levels decreased in both plasma and scales (Biswas et al., 2006; Samaras et al., 2022; Van der Salm et al., 2006). This inverse relationship suggests temperature-dependent cortisol retention, consistent with prior findings where lower temperatures were associated with higher cortisol concentrations (Hwang et al., 2012; Papaharisis et al., 2019). Common harvesting methods constitute major additional stressors that can significantly elevate the physiological stress response and adversely affect muscle quality (Fazio et al., 2015; Goes et al., 2019; Maria Poli, 2009; Merkin et al., 2010). Prolonged crowding and netting significantly increase physiological stress, leading to a faster *post-mortem* decline in muscle pH that subsequently accelerates the onset and progression of *rigor mortis*. These effects have been documented in red seabream, European seabass (*Dicentrarchus labrax*), barramundi (*Lates calcarifer*), and rainbow trout (*Oncorhynchus mykiss*) (Brijs et al., 2018; Papaharisis et al., 2019; Wilkinson et al., 2008). In contrast, low-stress harvests, such as hook-and-line, result in lower stress markers, which demonstrate the advantages of humane harvesting methods (de la Rosa et al., 2021; Papaharisis et al., 2019). These findings indicate that harvest methods have a direct impact on both immediate physiological responses and the resulting muscle quality characteristics.

Slaughter methods represent the final and often the most critical stage influencing the physiological stress response and overall product quality (de la Rosa et al., 2021; Łosiewicz and Szudrowicz, 2024). Asphyxia in air remains common in commercial operations but induces intense stress and severe physiological disruption (Giuffrida et al., 2007; Robb, 2002; Schuck-Paim et al., 2025). The ikejime or "ikigun" method, although labour-intensive, minimizes neural and muscular activation, thereby reducing stress responses and improving *post-mortem* product quality (Cabrera-Álvarez et al., 2026; Mood et al., 2023). Live-chilling in ice slurry is widely applied in commercial fisheries and can preserve flesh texture compared to air asphyxiation. However, it is also associated with severe acute stress responses in teleosts, underscoring trade-offs between product quality and animal welfare. (Acerete et al., 2009; Hoyo-Alvarez et al., 2025; Ntzimani et al., 2022; Papaharisis et al., 2019). These contrasting techniques underscore the importance of assessing slaughter methods based on their physiological consequences and their capacity to modulate pre- and *post-mortem* stress dynamics.

Several physiological parameters respond directly to stress, providing insight into both acute responses and their implications for fish welfare and product quality. Cortisol, the principal glucocorticoid in teleosts, is consistently elevated following activation of the hypothalamic-pituitary-interrenal (HPI) axis (Carbajal et al., 2023; Kalamarz-Kubiak, 2018). This increase in cortisol mobilizes energy reserves and alters osmoregulatory and immune functions, making it the most widely recognized systemic indicator of stress in fish, particularly during harvest and slaughter stages of aquaculture production (Carbajal et al., 2023; Eissa and Wang, 2013; Mommsen et al., 1999; Pickering and Pottinger, 1989). Plasma glucose typically increases as a downstream effect of HPI and catecholamine activation, with elevated levels supplying rapid energy through adenosine triphosphate (ATP) production. While short-term increases fuel escape and survival responses, prolonged elevated glucose levels indicate severe or chronic stress (Alfonso et al., 2021; Samaras et al., 2016; Roychowdhury et al., 2024). Handling, acute temperature changes, and pre-slaughter stress elevate plasma glucose in fish via cortisol-mediated energy mobilization, making glucose a reliable indicator of physiological stress (Fantini et al., 2020; Silva et al., 2025). Lactate also rises during stress, particularly under anaerobic muscular activity or oxygen deprivation, such as during the harvest stages wherein crowding and brailing are commercially practiced, reflecting enhanced glycolysis (Daskalova, 2019; Fazio et al., 2015). Elevated lactate is also associated with rapid *post-mortem* pH decline and compromised flesh quality (Omlin and Weber, 2010; Raposo De Magalhães et al., 2020).

Stress from intense handling and confinement further disrupts osmoregulatory balance (Stewart et al., 2016; Young et al., 2019). Plasma sodium (Na^+), chloride (Cl^-), and overall osmolality rise when gill or renal function is compromised, epithelial permeability is altered, or metabolic demand increases (Lee et al., 1983; Gräns et al., 2016; Retter et al., 2018; Phinrub et al., 2023). Similarly, plasma potassium (K^+) increases extracellularly during stress. Muscle cell damage or acidosis leads to leakage from intracellular reserves. Such elevations are implications of tissue stress and cellular breakdown (Schwieterman et al., 2021; Owolabi et al., 2021). *Post-mortem* muscle pH decline is accelerated from pre-slaughter stress because of the elevated lactate production and ATP depletion. This directly affects fillet texture, colour, and water-holding capacity (Milligan, 1996; Samaras et al., 2016; Anders et al., 2020). The onset and progression of *rigor mortis* are also more rapid when stress reduces energy reserves before slaughter. This serves as a practical indicator of ATP depletion and handling-related energy loss (Daskalova, 2019; Gasco et al., 2014; Papaharisis et al., 2019; Zhang et al., 2023).

While previous studies have examined the effects of individual stressors, such as harvest, slaughter method, or rearing temperature, on fish physiology and *post-mortem* muscle quality (Angelakopoulos et al., 2024; Bagni et al., 2007; Papaharisis et al., 2019; Matos et al., 2010), the combined effects of these factors on the red seabream have not been extensively investigated. This lack of integrated research limits our understanding of the species' cumulative stress response. Furthermore, little is known about how these combined stressors affect key physiological parameters such as plasma cortisol, glucose, lactate, electrolyte balance, and *post-mortem* muscle quality, including muscle pH and *rigor mortis* (Papaharisis et al., 2019; Samaras et al., 2022).

To address this gap, the present study evaluates how three rearing temperatures (15, 20, and 25 °C), harvest (rapid netting/hook-and-line versus crowding/brailing), and slaughter methods (pithing/ikigun, ice-slurry immersion, and air asphyxia) affect physiological stress response and *post-mortem* muscle quality in red seabream. This study, conducted in both controlled laboratory and industrial settings, integrates hormonal, metabolic, behavioural, and *post-mortem* muscle quality indicators to evaluate the stress response in red seabream at harvest and to assist in the development and refinement of humane slaughter methods applicable in industrial-scale settings at open net-pen sea cages.

2. Materials and methods

2.1. Ethical statement

The Animal House Facility at the Department of Biology, University of Crete, is certified by the Veterinary Unit of the Region of Crete for the rearing (EC91-BIObr-09) and use of laboratory animals for scientific purposes (EL91-BIOexp-10). All experimental procedures in this study were approved by the Departmental Animal Care Committee with license No. 145418/09-05-2023, following the Three Rs principle, in accordance with Greek (PD 56/2013) and EU (Directive 63/2010) legislation on the care and use of experimental animals.

2.2. Laboratory-scale experiment

2.2.1. Experimental animals and conditions

Red seabream, aged approximately 1 year (mean weight ± S.E.M.: 332.6 ± 5.0 g), were provided by the Hellenic Centre for Marine Research (HCMR), Institute of Marine Biology, Biotechnology &

Aquaculture, from sea cage facilities at Souda Bay, Chania, and transferred to the Animal House of the Department of Biology, University of Crete, Heraklion. Fish were randomly assigned to 3 temperature groups (as described below) and stocked at a density of 20 kg m^{-3} in 2 × 400-l tanks per group (six tanks total), with 24 individuals per tank. They were acclimated for two weeks, shown to be sufficient for fish to recover from transport and handling stress and restore baseline physiological homeostasis under laboratory conditions (Aswani and Trabucco, 2019; Hwang et al., 2012), at an initial water temperature of approximately 19 °C, consistent with the temperature at Souda Bay. Fish were fed daily with commercial feed at a constant quantity of 2% average body weight (ABW) (New, 1987). Water parameters were monitored daily for dissolved oxygen (5.0–6.0 mg L^{-1}), and weekly for ammonia [NH_3/NH_4^+] (0–0.5 mg L^{-1}), nitrite [NO_2^-] (0–0.5 mg L^{-1}), nitrate [NO_3^-] (0–0.5 mg L^{-1}), and water pH (7.5 ± 0.8).

2.2.2. *Experimental design*

To investigate the effects of rearing temperature (15, 20, and 25 °C), harvest (control harvests: rapid netting – no stress group versus crowding with brailing - common harvesting practice), and slaughter method (pithing using ikigun, Adept Ltd., New Zealand; hypothermia through live-chilling with ice-slurry at a 1:1 seawater-to-ice ratio, 0 ± 0.5 °C, as per Ntzimani et al., 2022; and asphyxia in air), a total of 144 red seabream were used in the experiment. Fish were distributed across 6 tanks, with 24 fish sampled per tank and two tanks per temperature treatment. The temperature for each group was gradually adjusted at 1 °C every two days to reach the desired temperatures of 15, 20, and 25 °C. After reaching the target temperature, fish were given another week to acclimatise. From each temperature, fish were harvested and slaughtered accordingly ($n = 8$ fish per group): (Group 1) control harvest × ikigun, (2) control harvest × ice-slurry, (3) control harvest × asphyxia, (4) crowding × ikigun, (5) crowding × ice-slurry, and (6) crowding × asphyxia. Fish in the control harvest were rapidly netted (within 3–5 s) to ensure minimal handling and prompt capture to mimic low-stress harvesting conditions, and were immediately slaughtered accordingly. For the crowding groups, fish were subjected to crowding for 30 min using a net that raised the fish to the surface, exposing their dorsal fins to mimic common commercial practices.

Behavioural responses were assessed through continuous observation of the indicators, with the assessment method being described in Table 1. Three observers monitored each fish from the cessation of self-initiated movements until no further response was recorded (Kestin et al., 2002). The time to loss of behavioural responses (LBR) was defined as the moment when all indicators had ceased completely. Although behavioural indicators provide a practical method of assessment, they may not always align precisely with the cessation of neural activity (Gräns et al., 2025; Jung-Schroers et al., 2020; Wahltinez et al., 2024) and are therefore interpreted carefully as proxies of the overall loss of behavioural responsiveness, rather than as definitive confirmation of loss of consciousness and death.

Following the loss of behavioural responses (varying between slaughter methods), approximately 1.0 mL of blood was extracted via caudal venipuncture using a syringe and subsequently transferred into 1.3 mL heparinized microtubes (SARSTEDT AG & Co. KG, Germany). Plasma was separated by centrifugation (2000 ×*g* for 10 min) and stored at −20 °C until analysis. After blood extraction, fish were placed in a polystyrene box with ice flakes to mimic industrial-scale packaging conditions during the muscle pH and *rigor mortis* assessments.

2.3. *Industrial-scale trials*

To evaluate the practical relevance and applicability of laboratory findings under commercial production conditions, an industrial-scale trial was conducted to assess the effects of harvest and slaughter methods on the physiological stress response and *post-mortem* muscle quality of red seabream. The trials took place at a commercial fish farm located in Western Greece. Red seabreams were reared in rectangular sea cages (7 × 7 m, 8 m deep) with a stocking density of 10 kg m^{-3}, at a water temperature of 20–21 °C. The trial compared two harvest methods: hook and line (control) versus crowding with brailing, and five slaughter methods: (1) pithing with ikigun, (2) live-chilling with ice-slurry, (3) low-current electrical stunning/anaesthetic (low E/A, 1.8 V cm^{-1} at 36 A), (4) high-current electrical stunning (high E/A, 2.0 V cm^{-1} at 40 A), and (5) air asphyxia ($n = 10$ per method). Electrical stunning was applied using a commercial in-water flow-through stunner (HSU stunner; Ace Aquatec), in which fish are conveyed through the stun area while remaining immersed in seawater with a conductivity ranging from 50,000 to 55,000 µS cm^{-1}. Fish were crowded in the net and were pumped to the stunner with a fish pump (1080-P; Aqua Life) at a passage velocity of 1.6 m s^{-1}. The stunner delivered a sinusoidal alternating current (AC) at 50 Hz, with an average exposure duration of 20 s per fish. Full electrode design specifications, including configuration and spacing, are proprietary to Ace Aquatec and are therefore not described here (Ace Aquatec, 2025; Papaharisis et al., 2019). In contrast, fish in the hook-and-line group were individually captured using baited fishing hooks and immediately slaughtered by ikigun, ice-slurry immersion, and asphyxia.

Blood samples were collected immediately after loss of behavioural responses: for the ikigun group, blood was extracted as soon as the fish were slaughtered; for the electrical stunning and ice-slurry groups, immediately after the confirmed loss of all behavioural responses in the ice-slurry; and for the asphyxia group, following complete absence of vital signs. Blood collection and storage, as well as all *post-mortem* assessments, including handling, packaging, and measurements of muscle pH and *rigor mortis*, followed the protocols established in the laboratory-scale experiment.

Table 1

Behavioural indicators monitored to assess the time to total loss of behavioural responsiveness.

Indicator	Biological Significance	Assessment Method	Assessment Frequency
Equilibrium	Reflects intact postural control and vestibular function	Fish gently manually repositioned; loss of equilibrium recorded only when the fish consistently failed to right itself upon repositioning. This approach was adopted to exclude the possible effect of ice physically restricting movement in ice-slurry, which may immobilise fish without reflecting true loss of postural control (Lambooij et al., 2015; Wahltinez et al., 2024)	Every 5 min from the cessation of self-initiated movements until no further response was recorded
Visual response to stimulus	Reflects intact sensory awareness and environmental perception	A moving object presented within the fish's visual field; any orienting or avoidance reaction recorded as a positive response (Kestin et al., 2002)	Every 5 min from the cessation of self-initiated movements until no further response was recorded
Opercular movements	Reflects brainstem-mediated autonomic respiratory control; cessation indicates severe physiological impairment	Continuous visual observation without handling (Kestin et al., 2002; EFSA, 2009; Jung-Schroers et al., 2020)	Continuously from the cessation of self-initiated movements until no further response was recorded
Vestibulo-ocular reflex (VOR)	Reflects intact vestibular and central sensorimotor pathways	Fish gently lifted and rotated; presence of compensatory eye movement recorded as a positive response (Kestin et al., 2002; Lambooij et al., 2015)	Every 5 min from the cessation of self-initiated movements until no further response was recorded

To evaluate the effectivity of pre-slaughter stunning protocols currently applied in the industry, time to loss and potential recovery of behavioural responses were assessed specifically in these four groups: (1) hook-and-line × ice-slurry, (2) crowding × ice-slurry, (3) crowding × low E/A, and (4) crowding × high E/A, wherein 10 separate fish per group were monitored using the behavioural indicators and assessment protocol described in the laboratory experiment (Table 1). For electrically stunned groups, fish were transferred immediately after stunning to a recovery tank containing aerated seawater maintained at harvest-site temperature. For ice-slurry groups, transfer occurred once fish exhibited complete loss of equilibrium, absence of response to external stimuli, and loss of the vestibulo-ocular reflex. Opercular movement was continuously monitored as the most persistent indicator of residual vital function, with its absence used as a final proxy of cessation of vital cardiorespiratory activity, while acknowledging that behavioural indicators alone cannot definitively distinguish unconsciousness from death in the absence of neurological validation (Bowman et al., 2020; Gräns et al., 2025). Following transfer, fish were continuously observed, and the duration to the first observed motor activity, defined as any spontaneous, self-initiated movement distinct from passive physical displacement, including fin movement, body undulation, or attempted righting, was recorded as the indicator of potential behavioural recovery (Clemente et al., 2023; Robb et al., 2000).

2.4. *Analytical procedures*

Plasma cortisol levels were estimated using a commercial enzyme immunoassay kit (Neogen® Cortisol ELISA kit, NEOGEN Corporation, USA). Plasma glucose and lactate concentrations were measured by commercial enzymatic colorimetric kits (SPINREACT, S.A./S.A.U, Spain). Plasma electrolytes (Na^+, K^+, and Cl^-) were determined using an electrolyte analyser (i-Smart 30 VET Electrolyte Analyser, Woodley Equipment Company Ltd., UK). Plasma osmolality was determined using an osmometer (Osmomat 3000, Gonotec GmbH, Germany). Muscle pH was measured at 0, 1, 2, 3, and 4 h *post-mortem* using a WTW pH 3110 m (Xylem Analytics, Germany GmbH). *Rigor mortis* progression was evaluated at the same time points using the vertical tail drop method described by Bito (1983).

2.5. *Statistical analysis*

All statistical analyses were performed in R (v4.3.1; R Core Team, 2024) and RStudio integrated (v2025.09.1 + 401) using Quarto (v1.7.32) to ensure fully reproducible workflows.

2.5.1. *Physiological parameters*

To analyze plasma physiological variables (cortisol, glucose, lactate, osmolality, Na^+, K^+, Cl^-), a model-based framework was applied, comparing 12 candidate models per response. These included Gaussian LMs (identity and log-link) and Gamma GLMs (log and identity link) across three levels of complexity: additive, two-way, and full three-way interactions (Bates et al., 2015; Chen et al., 2018; Motulsky et al., 2025). TankID was included as a fixed effect to control for baseline differences, as the six-tank design provided insufficient levels for reliable random-effect variance estimation, as it requires a larger number of levels (commonly ≥5–6, and preferably >10) to reliably estimate among-group variance (Bolker et al., 2009; Gomes, 2022). Temperature and TankID were excluded from industrial-scale models because all fish were sampled from a single sea cage at uniform ambient temperature within one sampling period, eliminating both thermal and between-unit variation. Model selection was performed using the Akaike Information Criterion (AIC), with the model exhibiting the lowest AIC considered the best fit (Burnham and Anderson, 2002; Cavanaugh and Neath, 2019; Mazerolle, 2023).

Model assumptions were verified using Q-Q plots, Shapiro-Wilk, and Breusch-Pagan tests for linear models, while Gamma GLMs were assessed via DHARMa simulation-based diagnostics (Hartig, 2024). To ensure valid inference regardless of homoscedasticity, HC3 (Heteroscedasticity-Consistent standard errors, type 3) was applied to all linear models ($n \leq 250$) (Long and Ervin, 2000).

For the laboratory-scale experiment, which employed a complete factorial design, Type III ANOVA with sum-to-zero contrasts was used for hypothesis testing (Fox and Weisberg, 2019). For the industrial-scale trials, which had an incomplete factorial design with structurally missing cells, Type II ANOVA was applied (Langsrud, 2003).

Effect sizes were quantified as partial η^2 (Olejnik and Algina, 2003; Lakens, 2013). Significant effects ($\alpha = 0.05$) were further analysed using Tukey-adjusted pairwise comparisons of estimated marginal means (EMMs), with standard errors computed from the HC3 robust covariance matrix when available (Lenth et al., 2024). Following the principle of marginality, higher-order interactions were prioritized over main effects in interpretation (Nelder, 1977).

2.5.2. *Muscle pH*

Post-mortem muscle pH, measured repeatedly over 0 to 4 h *post-mortem*, was analysed using linear mixed-effects models (LMMs) to account for within-fish correlations (Pinheiro and Bates, 2000). FishID was included as a random intercept, with *post-mortem* time, temperature, harvest, and slaughter methods as fixed effects. Candidate models evaluated additive, two-way, and three-way interactions. Model selection was guided by AICc and likelihood-ratio tests, and final models were refitted using Restricted Maximum Likelihood (REML) for unbiased variance component estimation. Model diagnostics included residual normality checks, inspection of variance components, and calculation of intraclass correlation coefficients (ICC). Fixed effects significance was assessed by applying Type III ANOVA using Kenward-Roger degrees of freedom, and pairwise EMM comparisons were Tukey-adjusted. Conditional R^2 and ICC were reported to quantify variance explained by fixed and random effects (Nakagawa and Schielzeth, 2013).

2.5.3. *Rigor mortis*

Rigor mortis progression, expressed as a proportion of muscle stiffness (0–1), was analysed using Bayesian zero-one-inflated beta (ZOIB) regression (Bürkner, 2017; Liu and Kong, 2015; Ospina and Ferrari, 2012). Models simultaneously estimated three components: mean *rigor* intensity (μ), probability of no-*rigor* (zoi), and probability of complete *rigor* (coi). Fixed effects included *post-mortem* time, temperature, harvest, and slaughter method, while random intercepts and slopes (Time | FishID) captured individual-level variability. TankID was included as a fixed effect in the μ component. Weakly informative priors were applied, and posterior distributions were estimated using Markov Chain Monte Carlo (MCMC) with four chains, 4000 iterations per chain, 1000 warm-up iterations, adapt_delta = 0.99, and max_treedepth = 15. Convergence was assessed via Gelman-Rubin statistics ($\hat{R} < 1.01$) and effective sample sizes (ESS > 400). Model fit was evaluated using posterior predictive checks, and component-specific posterior estimates were used to compute EMMs and pairwise treatment comparisons (Armelloni et al., 2025).

2.5.4. *Loss of behavioural responses (LBR)*

Survival analysis was applied to quantify time to loss of behavioural responses (seconds) under different rearing temperatures, harvest, and slaughter methods (Denfeld et al., 2023). Kaplan-Meier estimators were used to visualize survival functions and estimate median times with 95% confidence intervals. Group differences were assessed using log-rank tests (Kaplan and Meier, 1958; Mantel, 1966).

Cox proportional hazards models (Cox, 1972) were fitted to evaluate covariate effects on the hazard of losing behavioural responses. Candidate models included main effects, two-way interactions, and tank-level frailty terms. Model selection was based on AIC, likelihood ratio tests, and concordance statistics. Interaction terms with $p < 0.05$ were retained in the final model. The proportional hazards assumption was tested using scaled Schoenfeld residuals (Grambsch and Therneau, 1994; Therneau and Grambsch, 2000; Vuong, 1989); variables violating this assumption were stratified.

Hazard ratios (HR) with 95% confidence intervals are reported, where $HR > 1$ indicates faster loss of behavioural responses. When significant interactions were present, survival curves were plotted for combined treatment groups to accurately reflect conditional effects (Royston and Sauerbrei, 2008).

3. Results

3.1. Laboratory-scale experiment

3.1.1. Plasma cortisol

Cortisol concentrations were significantly affected by temperature ($F_1 = 19.84$, $p < 0.001$, partial $\eta^2 = 0.14$), harvest ($F_1 = 15.61$, $p < 0.001$, partial $\eta^2 = 0.14$), slaughter method ($F_2 = 7.48$, $p < 0.001$, partial $\eta^2 = 0.08$), and tank ($F_1 = 4.35$, $p = 0.039$, partial $\eta^2 = 0.03$). All two-way interactions were significant, including temperature × harvest ($F_1 = 10.89$, $p = 0.0012$, partial $\eta^2 = 0.08$), temperature × slaughter ($F_2 = 6.82$, $p = 0.0015$, partial $\eta^2 = 0.12$), and harvest × slaughter ($F_2 = 10.93$, $p < 0.001$, partial $\eta^2 = 0.13$). The influence of rearing temperature on cortisol depends on the harvest method, wherein under rapid netting, levels decreased with increasing temperature (slope = −0.146, $p < 0.001$). While under crowding, the decline was minimal but still significant ($p = 0.001$), except for ice-slurry slaughter showing an opposite pattern, with concentrations increasing at higher temperatures (slope = 0.553, $p < 0.05$). At the lowest temperature, cortisol was lower in ikigun and ice-slurry than in asphyxia ($p < 0.05$), while at intermediate and higher temperatures, cortisol was lowest in ikigun and did not differ between ice-slurry and asphyxia (all $p < 0.001$ for ikigun vs other slaughter methods) (Fig. 1).

3.1.2. Plasma glucose

Glucose concentrations were significantly influenced by temperature ($F_1 = 11.22$, $p = 0.001$, partial $\eta^2 = 0.07$), harvest ($F_1 = 27.40$, $p < 0.001$, partial $\eta^2 = 0.17$), and slaughter method ($F_2 = 12.86$, $p < 0.001$, partial $\eta^2 = 0.20$). Glucose concentrations increased with temperature across all harvest and slaughter methods. Higher concentrations were also observed under crowding than rapid netting ($p < 0.0001$). In terms of the impact of slaughter method, asphyxia resulted in higher glucose than ikigun ($p < 0.0001$) and ice-slurry ($p < 0.001$), while ikigun and ice-slurry did not differ ($p = 0.15$) (Fig. 1).

3.1.3. Plasma lactate

Lactate concentrations were significantly affected by temperature ($F_1 = 14.23$, $p < 0.001$, partial $\eta^2 = 0.10$), harvest ($F_1 = 54.68$, $p < 0.001$, partial $\eta^2 = 0.26$), and slaughter method ($F_2 = 262.82$, $p < 0.001$, partial $\eta^2 = 0.71$). Temperature × harvest ($F_1 = 11.53$, $p < 0.001$, partial $\eta^2 = 0.09$) and harvest × slaughter ($F_2 = 9.14$, $p < 0.001$, partial $\eta^2 = 0.08$) were significant. A significant three-way interaction among temperature, harvest, and slaughter was also detected ($F_2 = 8.79$, $p < 0.001$, partial $\eta^2 = 0.07$). Under rapid netting, lactate concentrations were stable across temperatures for ikigun (slope = −0.0296) and ice-slurry (0.0057), whereas asphyxia resulted in a significant increase with temperature (slope = 0.0352, $p < 0.05$). In contrast, under crowding, lactate was consistently higher than rapid netting ($p < 0.05$), showing a significant increase with temperature across slaughter methods (average slope = 0.1275, $p < 0.05$) (Fig. 1).

3.1.4. Plasma sodium

Sodium (Na^+) concentrations were significantly affected by temperature ($F_1 = 31.59$, $p < 0.001$, partial $\eta^2 = 0.21$), harvest ($F_1 = 47.63$, $p < 0.001$, partial $\eta^2 = 0.20$), and slaughter method ($F_2 = 49.23$, $p < 0.001$, partial $\eta^2 = 0.30$). Significant interactions were detected for temperature × harvest ($F_1 = 10.60$, $p = 0.001$, partial $\eta^2 = 0.08$) and temperature × slaughter ($F_2 = 14.73$, $p < 0.001$, partial $\eta^2 = 0.13$). A

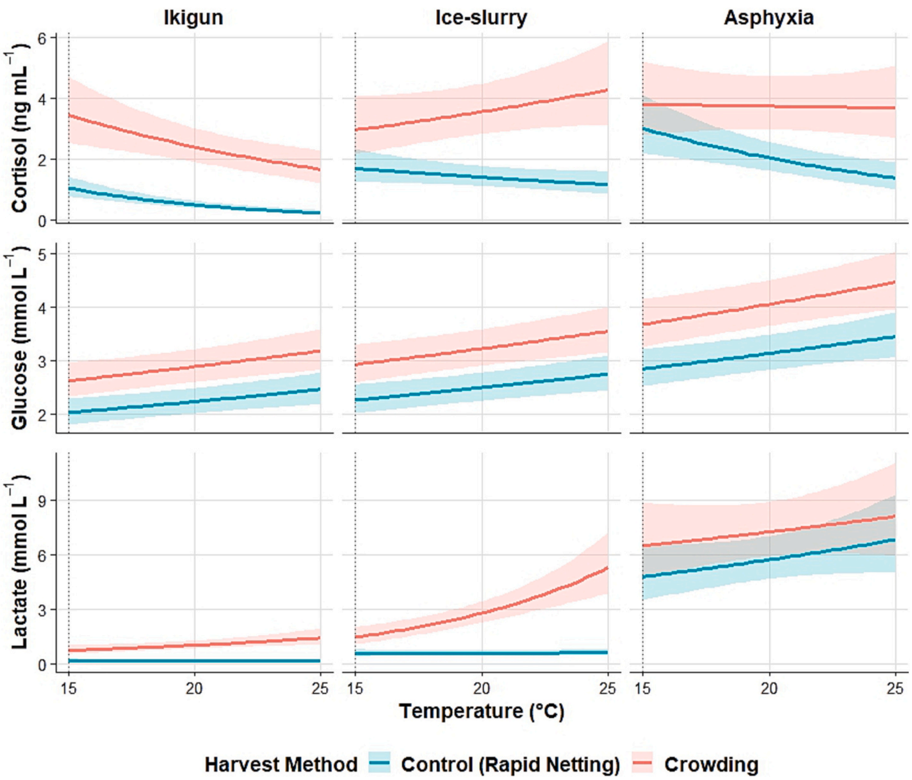

Fig. 1. Predicted plasma cortisol (ng mL^{-1}) and glucose concentrations (mmol L^{-1}) across rearing temperatures (15–25 °C) and harvest methods, faceted by slaughter method. Values represent estimated marginal means ±95% confidence intervals.

significant three-way interaction among temperature, harvest, and slaughter was observed ($F_2 = 4.12$, $p = 0.018$, partial $\eta^2 = 0.07$. Under rapid netting, Na^+ increased with temperature for both ice-slurry (slope $= 0.0045$, $p = 0.06$) and asphyxia (slope $= 0.0054$, $p = 0.02$), but showed a non-significant decline for ikigun (slope $= -0.0027$, $p = 0.26$). In contrast, under crowding, concentrations were significantly higher than rapid netting ($p < 0.05$), and increased with temperature across all slaughter methods (slopes: ikigun $= 0.0045$, ice-slurry $= 0.0185$, asphyxia $= 0.0043$), however the increase was only significant under ice-slurry ($p < 0.0001$), but not with ikigun and asphyxia (both $p > 0.05$) (Fig. 2).

3.1.5. *Plasma potassium*

Potassium (K^+) concentrations were significantly affected by temperature ($F_1 = 6.97$, $p = 0.009$, partial $\eta^2 = 0.04$), slaughter ($F_2 = 32.26$, $p < 0.001$, partial $\eta^2 = 0.26$), and tank ($F_1 = 8.08$, $p = 0.005$, partial $\eta^2 = 0.06$). Significant interactions were detected for temperature × harvest ($F_1 = 21.47$, $p < 0.001$, partial $\eta^2 = 0.12$) and temperature × slaughter ($F_2 = 12.03$, $p < 0.001$, partial $\eta^2 = 0.10$). A significant three-way interaction among temperature, harvest, and slaughter method was observed ($F_2 = 17.41$, $p < 0.001$, partial $\eta^2 = 0.20$). Under rapid netting, K^+ concentrations showed a significant decrease with increasing temperature under ice-slurry (slope $= -0.1235$, $p < 0.05$), while ikigun (slope $= -0.0738$) and asphyxia (slope $= -0.0319$) showed a non-significant decline (both $p > 0.05$). In contrast, under crowding, temperature effects differed among slaughter methods, wherein ice-slurry exhibited a significant increase with temperature (slope $= 0.1548$, $p < 0.0001$), asphyxia showed a stable trend (slope $= 0.0090$, $p = 0.75$), while ikigun resulted to a significant decrease with increasing temperature (slope $= -0.1009$, $p < 0.05$) (Fig. 2).

3.1.6. *Plasma chloride*

Chloride (Cl^-) concentrations were significantly affected by temperature ($F_1 = 34.85$, $p < 0.001$, partial $\eta^2 = 0.21$), harvest ($F_1 = 67.75$, $p < 0.001$, partial $\eta^2 = 0.33$), and slaughter method ($F_2 = 73.54$, $p < 0.001$, partial $\eta^2 = 0.45$). Significant interactions were detected for temperature × harvest ($F_1 = 15.25$, $p < 0.001$, partial $\eta^2 = 0.10$) and temperature × slaughter ($F_2 = 17.65$, $p < 0.001$, partial $\eta^2 = 0.21$). A significant three-way interaction among temperature, harvest, and slaughter method was observed ($F_2 = 3.53$, $p = 0.032$, partial $\eta^2 = 0.05$), wherein under rapid netting with ikigun, Cl^- concentrations remained stable across temperatures (slope $= -0.0011$, $p = 0.343$), while ice-slurry resulted to the steepest increase at warmer temperature (slope $= 0.0056$, $p = 0.0014$) (Fig. 2).

3.1.7. *Principal component analysis*

PC1 (67.9% variance) loaded strongly on Cl^-, Na^+, lactate, and K^+, representing a general axis of osmoregulation disturbance. PC2 (13.0% variance) was primarily associated with glucose and inversely with cortisol, indicating a distinct metabolic-endocrine dimension. Significant effects of temperature ($F_2 = 8.51$, $p < 0.001$), harvest ($F_1 = 37.79$, $p < 0.001$), slaughter ($F_2 = 68.49$, $p < 0.001$), and the harvest × slaughter interaction ($F_2 = 4.19$, $p = 0.017$) were detected in PC1. In contrast, PC2 scores were influenced only by rearing temperature ($F_2 = 23.53$, $p < 0.001$) (Fig. 3).

3.1.8. *Post-mortem muscle pH*

Muscle pH was significantly influenced by temperature ($F_{1,172.2} = 4.77$, $p = 0.030$), *post-mortem* time ($F_{1,564} = 4.70$, $p = 0.031$), harvest ($F_{1,172.2} = 10.52$, $p = 0.001$), and slaughter method ($F_{2,172.2} = 7.80$, $p < 0.001$). Several interaction terms were also significant, including temperature × time ($F_{1,564} = 16.75$, $p < 0.001$), temperature × harvest ($F_{1,172.2} = 16.35$, $p < 0.001$), time × harvest ($F_{1,564} = 7.35$, $p = 0.007$), temperature × slaughter ($F_{2,172.2} = 3.22$, $p = 0.042$), and time × slaughter ($F_{2,564} = 18.60$, $p < 0.001$). Significant higher-order interactions were also detected, including temperature × time × harvest ($F_{1,564} = 5.16$, $p = 0.024$), temperature × time × slaughter ($F_{2,564} = 14.16$, $p < 0.001$), time × harvest × slaughter ($F_{2,564} = 8.70$, $p < 0.001$).

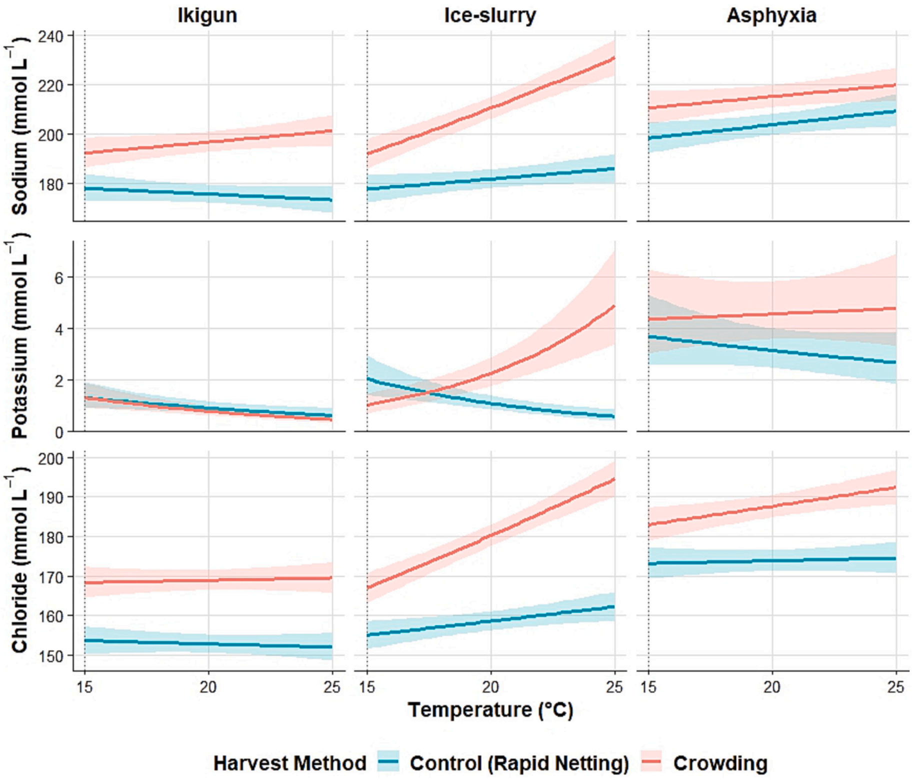

Fig. 2. Predicted plasma sodium, potassium, and chloride concentrations (mmol L-1) across rearing temperatures (15–25 °C) and harvest methods, faceted by slaughter method. Values represent estimated marginal means ±95% confidence intervals.

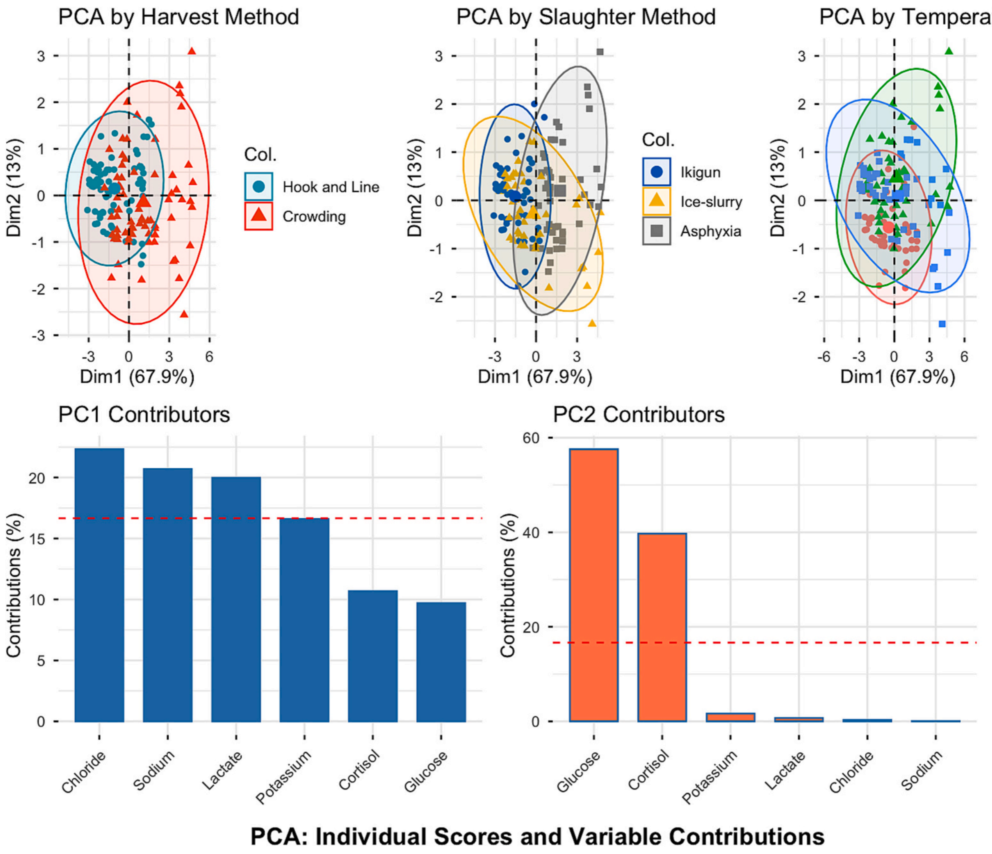

Fig. 3. Principal component analysis (PCA) of physiological stress parameters in fish. Individual points represent samples colored by harvest, slaughter, and temperature, with 95% confidence ellipses. Bar plots show the top six variables contributing to PC1 and PC2.

At 15 °C, rapid netting with ikigun exhibited a rapid pH decline (slope = −0.0179 h^{-1}), whereas crowded fish with ikigun declined more gradually (slope = −0.0080). Live-chilling with ice-slurry resulted in intermediate declines (slopes: rapid netting = −0.0095, crowded = −0.0080), while asphyxia had minimal decline (rapid netting = −0.0036, crowded = −0.0045). At 20 °C, the decline was moderate for ikigun (rapid netting = −0.0140, crowded = −0.0098), while ice-slurry showed a slower decline under rapid netting (−0.0103), but faster under crowding (−0.0107). In contrast, the rate of decline for asphyxia remained relatively slow in both harvest (rapid netting = −0.0088,

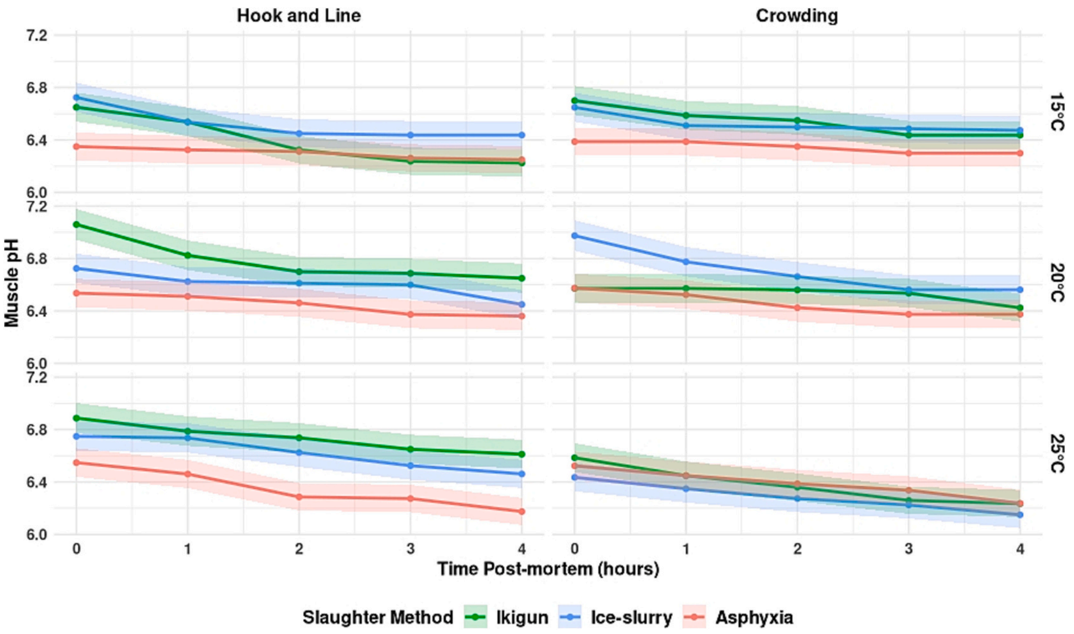

Fig. 4. Predicted muscle pH decline over post-mortem time (hours), faceted by temperature and harvest method with shaded ribbons indicating a 95% confidence interval (CI).

crowded = −0.0078). At 25 °C, crowding with ice-slurry showed the fastest decline (−0.0134), while rapid netting with ice-slurry (−0.0111) and ikigun (−0.0102) had intermediate rates, while asphyxia showed faster decline at higher temperature (rapid netting = −0.0141, crowded = −0.0111). These trends align with the temperature × time × slaughter interaction, wherein ikigun slopes gradually decreased with rising temperature (slopes: 15 °C = −0.0129, 20 °C = −0.0119, 25 °C = −0.0109), ice-slurry slopes increased moderately (15 °C = −0.0088, 20 °C = −0.0105, 25 °C = −0.0122), and asphyxia slopes accelerated with temperature (15 °C = −0.0040, 20 °C = −0.0083, 25 °C = −0.0126) (Fig. 4).

3.1.9. *Rigor mortis*

Rigor mortis progression increased over *post-mortem* time (Time_1: estimate = −1.26, 95% Credible Interval: −2.26, −0.25; Time_4: estimate = 1.09, 95% CrI: 0.07, 2.10). *Rigor* progression was higher under crowding (estimate = 1.12, 95% CrI: 0.76, 1.49) and asphyxia (estimate = 0.56, 95% CrI: 0.10, 1.01), but lower under ice-slurry (estimate = −0.40, 95% CrI: −0.75, −0.07) compared with ikigun. Temperature had no significant effect.

Rapid decline in the probability of no-*rigor* was observed over *post-mortem* time (Time_1: estimate = −3.39, 95% CrI: −4.05, −2.74) and under crowding (estimate = 1.96, 95% CrI: 1.54, 2.41), whereas higher temperature slightly increased this probability (estimate = 0.07, 95% CrI: 0.02, 0.12). The probability of full *rigor* increased significantly over *post-mortem* time (Time_4: estimate = 6.15, 95% CrI: 5.20, 7.20), and was higher under crowding (estimate = 1.19, 95% CrI: 0.50, 1.90) and asphyxia (estimate = 0.99, 95% CrI: 0.20, 1.80) compared with ikigun. Across all temperatures, asphyxia resulted in faster and more intense *rigor* indices than ikigun ($p < 0.05$), while ice-slurry consistently produced lower mean *rigor* at all *post-mortem* times (Fig. 5).

3.1.10. Loss of behavioural responses (LBR)

A significant interaction between harvest and slaughter methods was detected for the time to loss of behavioural responses (LBR; χ^2 (2) = 15.36, p = 0.0005). Significant main effects were observed for temperature (χ^2 (2) = 6.11, p = 0.047), harvest (χ^2 (1) = 5.46, p = 0.019), and slaughter method (χ^2 (2) = 214.93, $p < 0.001$),. When slaughter was performed using ikigun, the harvest method did not influence the rate of LBR, with both rapid-netting and crowding reaching LBR almost immediately (median: 9 to 15 s). In contrast, with ice-slurry slaughter, crowding increased the hazard of LBR by more than 5-fold compared with rapid netting (hazard ratio [HR] = 5.199, $p < 0.001$), reducing median LBR from 718 to 556 s. Similarly, under asphyxia, crowding resulted in a 4.1-fold higher hazard of LBR (HR = 4.130, p = 0.0009), with median times decreasing from 1409 to 828 s. In addition, LBR was faster at higher temperatures (HR: 20 °C = 0.738, at 25 °C = 0.601; all $p < 0.05$) (Fig. 6).

3.2. Industrial-scale trials

3.2.1. Plasma cortisol

Cortisol concentrations were significantly affected by both harvest ($F_{1,72}$ = 63.72, p < 0.001, partial η^2 = 0.47) and slaughter methods ($F_{4,72}$ = 37.19, p < 0.001, partial η^2 = 0.67). In addition, a significant harvest × slaughter interaction was detected ($F_{2,72}$ = 9.78, p = 0.00017, partial η^2 = 0.21). Crowded fish had higher cortisol than hook-and-line across all slaughter methods (p < 0.05), while application of electrical stunning (both low and high E/A) prior to live-chilling resulted in lower concentrations compared to asphyxia (both p < 0.05). Application of low E/A also resulted in significantly lower cortisol than direct ice-slurry slaughter (p < 0.05) (Fig. 7).

3.2.2. Plasma osmolality

Osmolality was significantly affected by both harvest ($F_{1,72}$ = 283.87, p < 0.001, partial η^2 = 0.80) and slaughter methods ($F_{4,72}$ = 327.51, p < 0.001, partial η^2 = 0.950, with a significant harvest × slaughter interaction ($F_{2,72}$ = 4.08, p = 0.021, partial η^2 = 0.10). Crowded fish showed higher osmolality than hook-and-line caught fish across all slaughter methods (p < 0.05). Low and high E/A resulted in significantly lower concentrations compared with asphyxia (p < 0.05). Application of low E/A was comparable to direct live-chilling (p = 0.87),

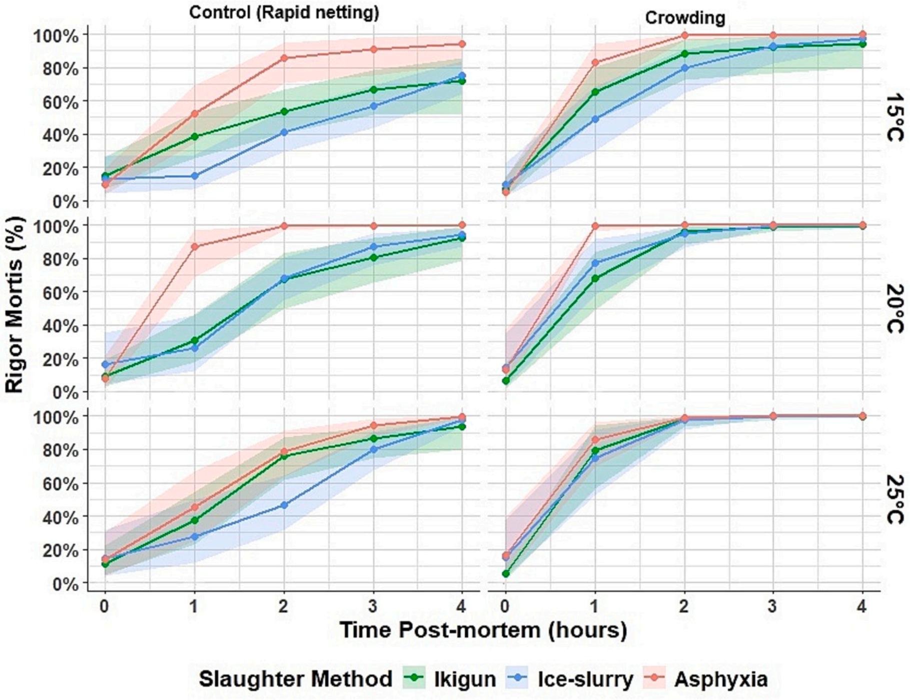

Fig. 5. Predicted rigor mortis progression over post-mortem time (hours), faceted by temperature and harvest method with shaded ribbons indicating 95% credible intervals [CrI].

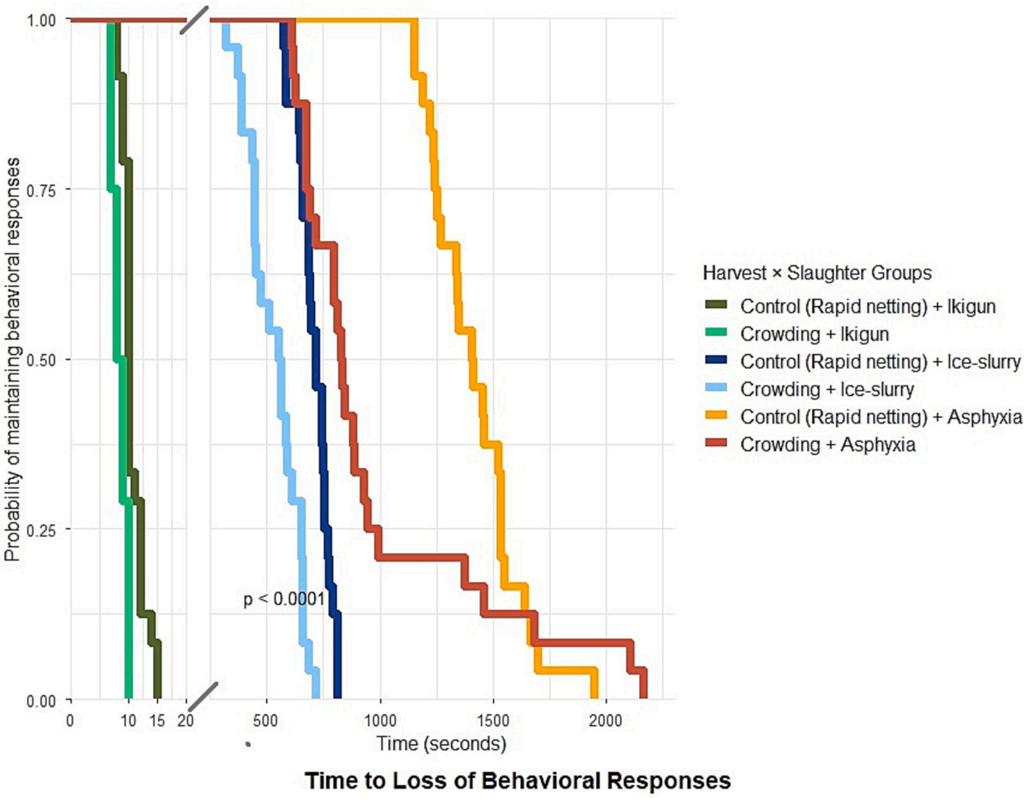

Fig. 6. Kaplan-Meier survival analysis for loss of behavioural responses (LBR) under harvest and slaughter. Survival curves represent the probability of loss of behavioural responses (seconds) with *p*-values indicate significant differences between groups. The x-axis is segmented, reflecting the significantly shorter time to loss of behavioural responses in ikigun (0 to 20 s), compared to ice-slurry and asphyxia groups (250 to 2300 s).

whereas high E/A resulted in significantly lower osmolality than ice-slurry alone ($p = 0.0058$) (Fig. 7).

3.2.3. *Plasma glucose*

Glucose concentrations were significantly influenced by slaughter method ($F_{4,75} = 49.06$, $p < 0.001$, partial $\eta^2 = 0.73$), whereas no significant effect of harvest was detected ($F_{1,75} = 1.33$, $p = 0.253$, partial $\eta^2 = 0.03$). Application of low E/A resulted in significantly lower glucose concentrations than direct ice-slurry and asphyxia (both $p < 0.05$) (Fig. 7).

3.2.4. *Plasma lactate*

Lactate concentrations were significantly influenced by both harvest ($F_{1,72} = 50.79$, $p < 0.001$, partial $\eta^2 = 0.41$) and slaughter methods ($F_{4,72} = 21.13$, $p < 0.001$, partial $\eta^2 = 0.54$). A significant harvest × slaughter interaction was also detected ($F_{2,72} = 11.44$, $p < 0.001$, partial $\eta^2 = 0.24$). Crowded fish had significantly higher lactate than hook-and-line for ikigun and ice-slurry (both $p < 0.05$), while application of electrical stunning (both low and high E/A) resulted in lactate concentrations comparable to both direct ice-slurry and asphyxia (all $p > 0.05$) (Fig. 7).

3.2.5. *Post-mortem muscle pH*

Muscle pH was significantly influenced by *post-mortem* time ($F_{1,195} = 288.11$, $p < 0.001$) and slaughter method ($F_{4,106} = 5.21$, $p = 0.0007$), with a significant time × slaughter interaction ($F_{4,195} = 4.41$, $p = 0.002$). Muscle pH declined over time in all harvest-slaughter combinations, wherein the fastest declines were observed in live-chilling with ice-slurry (slope $= -0.0228$) and asphyxia (slope $= -0.0212$), significantly faster than in the low E/A (slope $= -0.0119$, $p = 0.008$) and ikigun (slope $= -0.0131$, $p = 0.025$). High E/A showed an intermediate decline (slope $= -0.0177$), but did not differ significantly from the other slaughter groups (all $p > 0.05$) (Fig. 8).

3.2.6. *Rigor mortis*

Rigor mortis progression increased over *post-mortem* time (Time: estimate = 1.786, 95% Credible Intervals: 1.597, 1.964). Mean *rigor* intensity was lower under ice-slurry (estimate = −0.762, 95% CrI: −1.363, −0.180) relative to ikigun at Time 0, whereas low E/A produced higher mean *rigor* (estimate = 1.386, 95% CrI: 0.766, 1.981). High E/A did not differ from ikigun (estimate = 0.533, 95% CrI: −0.046, 1.129). A significant time × asphyxia interaction indicated that *rigor* progression accelerated over time under asphyxia relative to ikigun (estimate = 0.452, 95% CrI: 0.121, 0.793).

At Time 0, mean *rigor* was lowest under ikigun (estimate = 0.037, 95% CrI: 0.018, 0.072) and highest under asphyxia (estimate = 0.149, 95% CrI: 0.084, 0.235). By 4 h *post-mortem*, asphyxia reached the highest mean *rigor* (estimate = 0.980, 95% CrI: 0.955, 0.993), followed by low E/A (estimate = 0.966, 95% CrI: 0.930, 0.986), ice-slurry (estimate = 0.953, 95% CrI: 0.907, 0.981), ikigun (estimate = 0.887, 95% CrI: 0.804, 0.938), and high E/A the slowest (estimate = 0.870, 95% CrI: 0.770, 0.931). Asphyxia resulted in faster and more intense *rigor* development than ikigun across all *post-mortem* time points, while high E/A produced the most gradual progression (Fig. 8).

3.2.7. *Loss of behavioural responses (LBR)*

Results showed differences among stunning methods in the time required to lose behavioural responses. Fish subjected to electrical stunning (both low and high E/A) before ice-slurry immersion lost all the indicators immediately (≤ 5 s), whereas fish immersed in ice-slurry directly showed a longer time to LBR, occurring after 5 to 7 min in hook-and-line fish and 10 min in crowded fish.

Intermittent, non-directed motor activity was observed in all groups between 3 and 15 min after the initial LBR, noted earlier in electrically

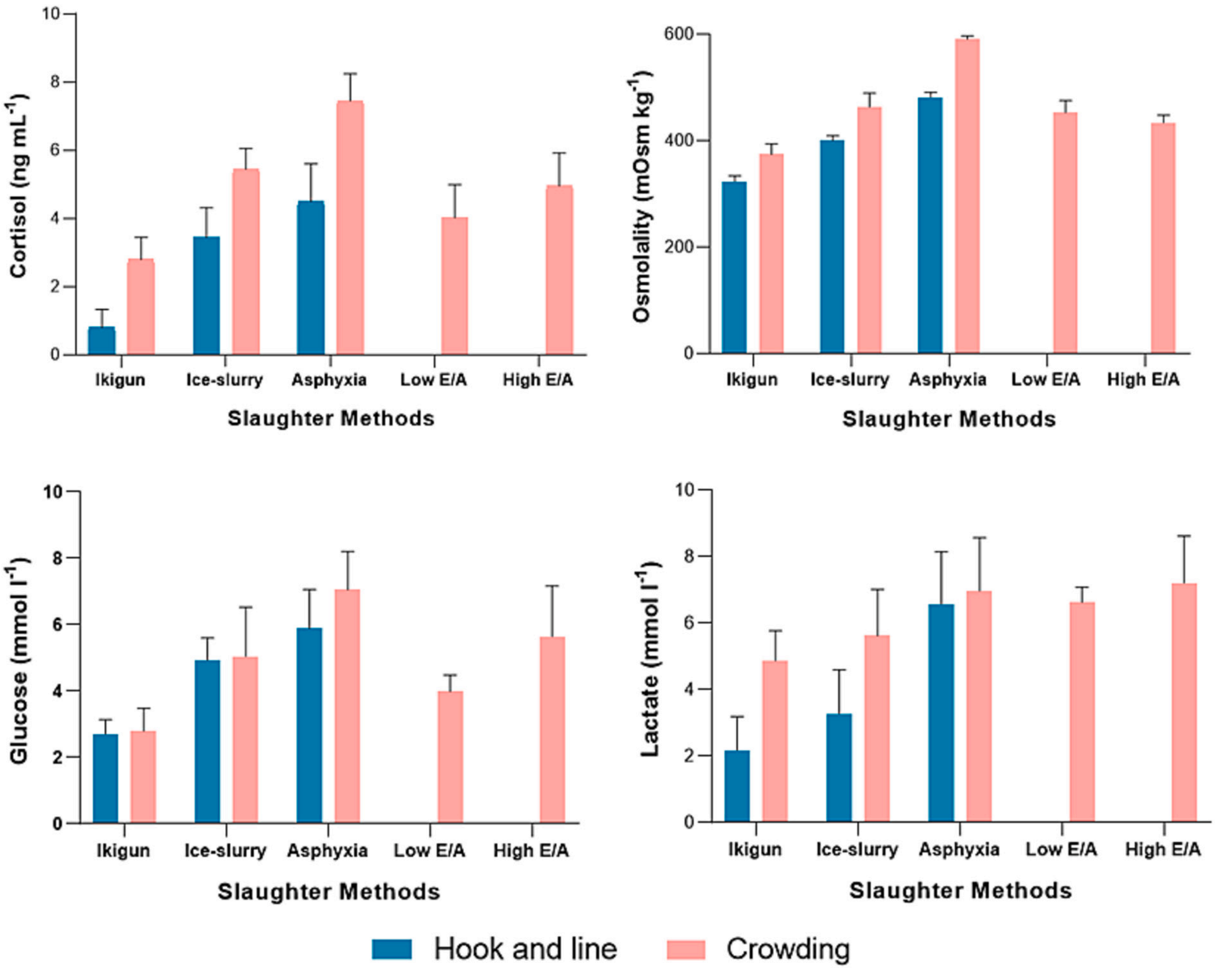

Fig. 7. Predicted (mean ± SEM) plasma cortisol (ng mL^{-1}) osmolality (mOsm kg^{-1}), glucose (mmol L^{-1}) and lactate (mmol L^{-1}) across harvest and slaughter methods at 20 °C.

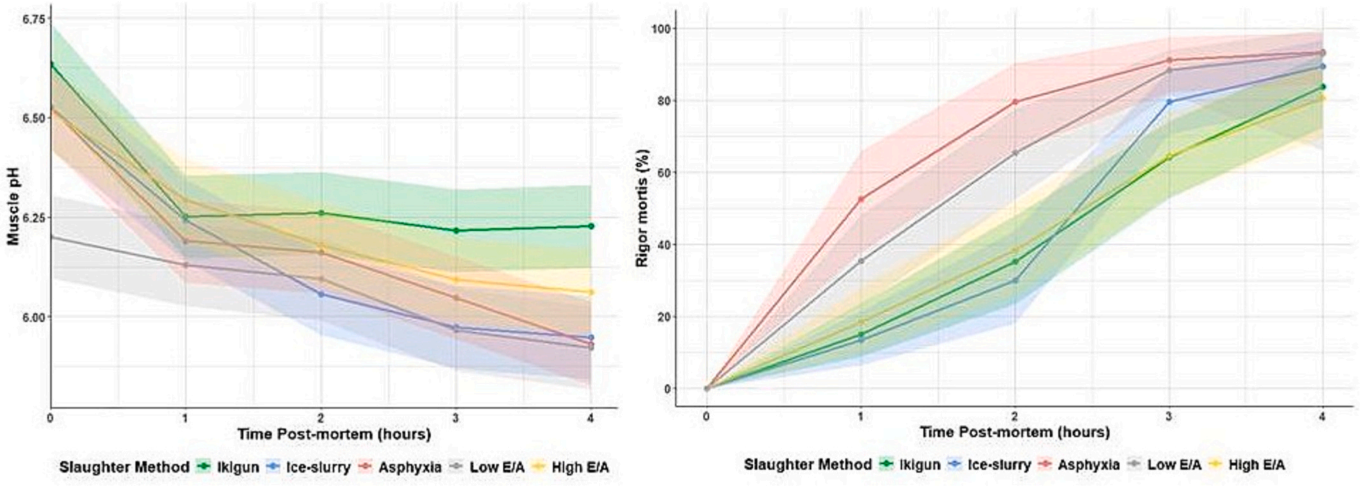

Fig. 8. Predicted post-mortem muscle pH and rigor mortis over time (hours) for different slaughter methods within the crowding harvest group. Regression lines represent model predictions, with shaded ribbons indicating 95% confidence intervals. Water temperature: 20 °C. Sample size: $n = 10$ per slaughter method.

stunned fish (3 to 7 min). These movements were not accompanied by full recovery of equilibrium, coordinated swimming, visual responsiveness, or VOR. No fish regained complete behavioural indicators during the 4-h observation period (Table 2).

4. Discussion

This study assessed the stress physiology response and *post-mortem* muscle quality in red seabream (*Pagrus major*) under varying pre-slaughter and slaughter conditions. In the laboratory-scale experiment,

Table 2

Loss of behavioural responses under different harvest and stunning methods.

Harvest method	Stunning methods	LBR	First motor activity
Hook and line	Ice-slurry	5–7 min	10–15 min
Crowding	Ice-slurry	10 mins	10 mins
	Low E/A	0 min	3–5 min
	High E/A	0 min	5–7 min

the combined effects of rearing temperature, harvest, and slaughter method were evaluated using a factorial experimental design, while in the industrial-scale trials, the assessment was focused on the effects of harvest and slaughter methods at a specific temperature. In the laboratory setting, significant interactions were observed in plasma cortisol, glucose, lactate, chloride (Cl^{-}), and potassium (K^{+}), which showed the interactive influence of environmental (temperature) and procedural (harvest and slaughter) stressors on physiological stress indicators. In the industrial-scale trials, significant interactions between harvest and slaughter methods were also evident, indicating their combined effects on stress response and muscle quality indicators in a commercial setting (Brijs et al., 2018; Daskalova, 2019).

Plasma cortisol concentrations, the primary indicator of HPI-axis activation (Carbajal et al., 2019; Mommsen et al., 1999), show that stress intensity was strongly influenced by the combined effects of harvest and slaughter methods. Across both laboratory and industrial settings, the harvest method emerges as a dominant driver of endocrine disturbance. Fish harvested using rapid netting (laboratory) or hook-and-line (industry) consistently maintain lower cortisol compared to those subjected to crowding and brailing (Papaharisis et al., 2019). These findings suggest that harvest triggers a stress response that influences all subsequent physiological reactions (Hultmann et al., 2012; Wilkinson et al., 2008).

The most pronounced neuroendocrine response occurs when crowding and brailing are combined with more severe slaughter methods, such as ice-slurry immersion or asphyxia. These findings indicate that stress induced during handling activates the HPI axis, and the subsequent application of an inhumane slaughter method further amplifies this response, highlighting the cumulative nature of stress and its implications for fish welfare (Mommsen et al., 1999; Samaras et al., 2022). Laboratory results show that rearing temperature modulates this response. In low-handling groups (rapid netting with ikigun), cortisol concentrations are notably lower at 20 °C and 25 °C compared with 15 °C, supporting the hypothesis that fish reared near their thermal optimum exhibit greater physiological resilience, which mitigates the endocrine response to stressors (Alfonso et al., 2023). In contrast, 15 °C likely represents a mild thermal challenge for red seabream, imposing a higher baseline endocrine burden even before harvest begins (Samaras et al., 2022). While concentrations decline with increasing temperature in rapidly netted fish, an opposite trend was observed in crowded fish subjected to ice-slurry immersion. This pattern suggests that at higher ambient temperatures, the "thermal shock" of moving fish from warm water to an ice-slurry is more physiologically stressful than at lower ambient temperatures (Foss et al., 2012; González-Garoz et al., 2025).

In the industrial trials, the effectiveness of pre-slaughter interventions is evident. Application of electrical stunning (both low and high E/A) prior to live-chilling results in cortisol concentrations that are significantly lower than those observed in asphyxia-slaughtered fish, while low E/A stunning also produces significantly lower cortisol than direct ice-slurry slaughter. These findings indicate that rapid induction of insensibility via electrical stunning limits further escalation of the HPI-axis response relative to the most extreme slaughter method. Although electrical stunning does not completely prevent endocrine activation, it clearly attenuates physiological stress during slaughter (González-Garoz et al., 2025; Mohammadi Dehcheshmeh et al., 2025).

Plasma glucose concentrations serve as a direct indicator of the secondary stress response, reflecting the mobilization of energy reserves to meet the metabolic demands of the "fight-or-flight" response (Biswal et al., 2021; Cheyadmi et al., 2023). Unlike cortisol, which shows variable trends with temperature, glucose exhibits a consistent positive correlation with temperature in the laboratory-scale experiment. This increase at higher temperatures likely reflects elevated basal metabolic rates in red seabream, where the energetic cost of responding to any procedural stressor is higher in warmer water (Abdel-Tawwab et al., 2025; Braz-Mota et al., 2024; Samaras et al., 2016).

The influence of harvest and slaughter methods on glucose is consistent across both laboratory and industrial settings. The significant elevation of glucose in crowded or brailed fish compared with those harvested by rapid netting or hook-and-line demonstrates that physical exertion and crowding trigger immediate glycogenolysis (Aketch et al., 2014; Basrur et al., 2010; Swain et al., 2022), which is further amplified by the inhumane slaughter method. In both settings, ikigun slaughter results in the lowest glucose concentrations. By instantly destroying the brain, the ikigun method prevents prolonged neural signaling that would otherwise stimulate additional catecholamine and glucocorticoid release, effectively "capping" the glucose surge (James et al., 2025; Robb et al., 2000; González-Garoz et al., 2025). In contrast, asphyxia and ice-slurry groups result in the highest glucose levels. The prolonged time to death of these methods, during which fish may remain conscious and struggling, imposes a sustained energy demand (Acerete et al., 2009; Compassion in World Farming, 2018; Davis, 2006).

Interestingly, the industrial trials reveal a significant difference between the two electrical stunning protocols. While low E/A results in significantly lower glucose than direct live-chilling, high E/A produces concentrations comparable to asphyxia. This likely reflects a galvanic effect associated with higher current exposure, in which excessive electrical stimulation triggers intense, involuntary tetanic muscular contractions throughout the body (Cabrera-Álvarez et al., 2026). These contractions accelerate glycogen breakdown and the release of glucose into the bloodstream (Bermejo-Poza et al., 2021; Cabrera-Álvarez et al., 2026; Yan et al., 2025). A similar metabolic effect occurs under stressful slaughter methods, such as asphyxia, suggesting that excessively high or improper electrical parameters may intensify the metabolic stress response rather than mitigate it, potentially undermining the welfare benefits of electrical stunning (Gräns et al., 2016).

Plasma lactate concentrations serve as a clear indicator of anaerobic muscular activity and physical exhaustion (Raposo De Magalhães et al., 2020). Across both laboratory and industrial trials, the harvest method emerges as a primary determinant of lactate accumulation, although laboratory results reveal a complex three-way interaction among rearing temperature, harvest, and slaughter method. The significant increase in lactate in crowded fish reflects intense burst swimming and thrashing during the crowding phase (Svalheim et al., 2020; Wells and Baldwin, 2006). Under high-density conditions, fish rely on anaerobic glycolysis to sustain rapid muscular contractions (Anders et al., 2020; Weber et al., 2016). In the laboratory, temperature further amplifies this response, with the steepest increases in lactate observed in crowded fish subjected to ice-slurry at higher temperatures. This pattern suggests that the combined stress of crowding and sudden immersion in near-freezing water imposes a metabolic demand that exceeds aerobic capacity (Anders et al., 2020; Skjervold et al., 2001).

The interaction between temperature and harvest in the laboratory-scale experiment highlights a clear distinction between low- and high-stress conditions. Under minimal handling, fish maintain efficient aerobic metabolism (Beuvard et al., 2021; Porter and Gamperl, 2023; Sfakianakis and Kentouri, 2010). In contrast, this pattern reverses under crowding, where higher temperatures are associated with increased lactate accumulation. This likely reflects the combined effects of reduced oxygen solubility and elevated metabolic demand in warmer water, which accelerate the onset of osmo-respiratory compromise (McBryan et al., 2013; Müller et al., 2023; Wood and Eom, 2021). In the industrial trials, asphyxia produces the highest lactate concentrations relative to ikigun, consistent with a prolonged time to death, characterized by sustained struggling and hypoxia, ultimately leading to metabolic exhaustion (Daskalova, 2019; Wang et al., 2023). Electrical stunning (both low and high E/A) results in intermediate lactate concentrations. Although these methods are more humane than asphyxia, the elevated lactate suggests that involuntary muscular tetany induced by the electric current still promotes anaerobic glycolysis (Bermejo-Poza et al., 2021; Castro et al., 2025; Yan et al., 2025).

Fluctuations in plasma Na^+, Cl^-, and K^+, together with total osmolality, provide clear evidence of homeostatic disruption under high-stress conditions (Breves et al., 2010; Stewart et al., 2016). Under rapid netting, osmoregulatory balance is largely maintained. Plasma K^+ declines moderately with temperature, Na^+ shows minor increases under asphyxia and ice-slurry, and Cl^-, although highest under ice-slurry, remains relatively stable across temperatures. This pattern aligns with the capacity of teleosts to upregulate branchial ion transport in response to thermally driven passive ion flux, indicating that under low-stress harvest conditions, fish retain sufficient physiological reserve to buffer environmental challenges (Vargas-Chacoff et al., 2020; Burton, 1986). In contrast, crowding significantly disrupts this balance, with Na^+ and Cl^- increasing with temperature across all slaughter methods. In the industrial trials, osmolality mirrors these electrolyte trends, with crowded fish exhibiting significantly higher osmolality than those harvested by hook-and-line, confirming substantial osmotic disturbance (Skjervold et al., 2001). The application of electrical stunning prior to live-chilling results in lower osmolality compared with asphyxia, suggesting that rapid immobilisation reduces the duration of intense ventilation and muscular activity, thereby limiting the severity of osmo-respiratory disturbance (Bermejo-Poza et al., 2021; Cabrera-Álvarez et al., 2026). This pattern is consistent with laboratory findings and supports the notion that the extent of osmotic disruption depends on how rapidly neural and physiological functions are suppressed at slaughter (Saraiva et al., 2024; Yan et al., 2025).

Plasma K^+ concentrations under ice-slurry slaughter exhibit a distinct temperature- and harvest-dependent pattern not observed for Na^+ or Cl^-, highlighting the need for further investigation. Whereas Na^+ and Cl^- show relatively consistent temperature-related responses under crowding across slaughter methods, K^+ displays opposing trends under ice-slurry. Plasma K^+ concentrations decline with increasing rearing temperature in rapidly netted fish but increase significantly in crowded fish. This pattern suggests that K^+ regulation during ice-slurry immersion is influenced by a more complex interaction among pre-slaughter physiological state, the magnitude of thermal shock at immersion, and the prolonged time to loss of consciousness associated with this method (González-Garoz et al., 2025; Hoyo-Alvarez et al., 2025). Because ice-slurry induces a comparatively slow dying process, fish remain physiologically active for an extended period during which harvest stress, acute cooling, and progressive hypoxia may act simultaneously (Wahltinez et al., 2024). The resulting K^+ dynamics therefore appear to reflect more than general osmotic disturbance, particularly given the absence of similar trends in Na^+ and Cl^-. Instead, results from this study suggest that plasma K^+ is especially sensitive to temperature-dependent shifts between intracellular and extracellular compartments, as well as to the energy reserves of the red seabream at slaughter (Balasubramanian et al., 2025; Madaro et al., 2024). Given that K^+ regulation in fish is mediated by specific ion transport mechanisms (Furukawa et al., 2012; Guh et al., 2015; Higgins et al., 2022), these results highlight a critical knowledge gap and underscore the need for targeted studies examining how harvest methods, ice-slurry immersion, and rearing temperature interact to influence plasma K^+ regulation.

The laboratory results reveal a complex interaction among *post-mortem* time, rearing temperature, harvest, and slaughter method, indicating that muscle pH trajectories are not solely determined by time but are conditioned by the fish's metabolic state at death. Fish subjected to low-stress handling often exhibit a steeper *post-mortem* pH decline from a higher baseline, reflecting greater intramuscular glycogen reserves at the time of death. These reserves provide the substrate for sustained anaerobic glycolysis, leading to prolonged lactic acid accumulation and a correspondingly larger decrease in muscle pH (Raposo De Magalhães et al., 2020). In contrast, crowded or asphyxiated fish generally display minimal *post-mortem* pH decline, as pre-slaughter thrashing has already depleted muscle glycogen, resulting in a lower baseline pH (Daskalova, 2019; Goes et al., 2019; Martínez Villalba et al., 2025). The difference between laboratory and industrial results, where muscle pH declines appear steeper in industrial trials, can be attributed to the scale and intensity of handling. In these trials, live-chilling with ice-slurry and asphyxia treatments induce much faster acidification than ikigun, a rapid decline that is particularly detrimental to product quality (Anders et al., 2020; Kim et al., 2014; Silva et al., 2025).

A particularly notable finding is that, although low E/A stunning resulted in the slowest pH decline and the lowest mean muscle pH at 4 h *post-mortem*, both the rate and overall pH levels were comparable to those observed under high E/A. This does not necessarily indicate greater metabolic disturbance under low E/A, but likely reflects the physiological effects of electrical exposure. Improper electrical current can induce electro-immobilisation rather than electronarcosis (Robb and Kestin, 2002). During electro-immobilisation, involuntary tetanic muscle contractions rapidly deplete high-energy phosphates, including ATP and creatine phosphate (Lindinger et al., 1987; Spriet et al., 1987). This pre-mortem energy depletion may limit the extent of subsequent *post-mortem* glycolysis, contributing to the slower pH decline observed (Daskalova, 2019; Martínez Villalba et al., 2025; Silva et al., 2025).

The onset of *rigor mortis* occurs when cellular ATP levels fall below a critical threshold, preventing the dissociation of actin and myosin filaments (Konno, 2017; Kristoffersen et al., 2006; Food and Agriculture Organization of the United Nations, 2019). In both laboratory and industrial trials, the rate of *rigor* progression was primarily determined by the severity of pre-slaughter stress. The most rapid transition from no *rigor* to full *rigor* occurred in fish subjected to crowding followed by asphyxia, reaching near-maximum *rigor* indices within three hours *post-mortem*. This accelerated stiffening reflects the high lactate concentrations and low muscle pH observed under these conditions (Daskalova, 2019; Kristoffersen et al., 2006). By contrast, fish harvested via rapid netting and slaughtered with ikigun exhibited a significantly slower and more gradual progression of *rigor*.

In the laboratory, ice-slurry immersion consistently resulted in lower mean *rigor* intensity compared to ikigun, whereas in the industrial trials, *rigor* progression followed an intermediate trajectory. This delay is likely due to the rapid reduction in core body temperature, which slows enzymatic reactions involved in ATP degradation and glycolysis, partially offsetting the metabolic stimulation caused by the stress of ice-slurry immersion (Mohammed et al., 2025; Nazir and Magar, 2006). In the industrial trials, the probability of remaining in a no-*rigor* state was highest in the asphyxia group and lowest in the ikigun group. Although initially counterintuitive, this likely reflects the distinction between active muscular contraction and *rigor mortis*, wherein fish killed by ikigun or immobilized from electrical stunning often undergo involuntary muscle contractions due to neural stimulation, whereas asphyxiated fish may reach a state of total muscular exhaustion or flaccidity just before death (Anders et al., 2020; Cabrera-Álvarez et al., 2026; Mohammadi Dehcheshmeh et al., 2025). Rapid *rigor* onset in stressed fish corresponds to reduced product flexibility and accelerated muscle degradation (Anders et al., 2020; Bordignon et al., 2024; Yan et al., 2025).

The time required to reach insensibility represents the most direct and welfare-relevant metric for assessing slaughter methods (Clemente et al., 2023; Mercogliano et al., 2024). Equally important is the duration of insensibility, as methods that rapidly render fish behaviourally unresponsive but fail to maintain unconsciousness throughout the killing process may pose severe welfare risks, with sensory awareness potentially returning before death (Hjelmstedt et al., 2022; Robb and Kestin, 2002; Wahltinez et al., 2024). In this study, the loss of behavioural responses (LBR) was used as a practical proxy for sensibility rather than as definitive evidence of death or complete absence of nociception. These indicators should be interpreted with caution, as the lack of observable responses does not necessarily confirm insensibility. Studies combining behavioural observations with electroencephalographic (EEG) recordings have demonstrated that the absence of behavioural responses alone does not reliably indicate unconsciousness (Retter et al., 2018; Wahltinez et al., 2024). This distinction is particularly relevant for electrical stunning, where electro-immobilisation suppresses voluntary movements without inducing the generalized epileptiform brain activity associated with true loss of consciousness (Hjelmstedt et al., 2022; Lambooij et al., 2010).

Electrical stunning (low and high E/A) applied prior to live-chilling or ikigun slaughter induced an almost instantaneous loss of responsiveness across all behavioural indicators (Anders et al., 2019; Cabrera-Álvarez et al., 2026; Compassion in World Farming, 2018). In contrast, ice-slurry and asphyxia resulted in substantially longer periods of sensibility. The faster onset of insensibility in crowded fish compared with those harvested by rapid netting or hook-and-line likely reflects the severe physiological stress and metabolic disturbance caused by intense crowding, which shifts energy metabolism toward anaerobic pathways, depletes energy reserves, and accelerates physiological collapse, rather than representing a welfare benefit (Anders et al., 2020). Similarly, higher temperatures accelerated the onset of insensibility during ice-slurry and asphyxia, consistent with elevated metabolic demand and faster oxygen depletion in warmer water, which promotes hypoxic neural failure rather than alleviating distress (Li et al., 2023; Madeira, 2025; Volkoff and Rønnestad, 2020).

A notable observation in the industrial trials was the occurrence of intermittent, non-directed motor activity 3 to 15 min after the initial loss of behavioural responses, appearing earlier in electrically stunned fish (3 to 5 min). The absence of coordinated swimming, restored equilibrium, and vestibulo-ocular reflex (VOR) indicates that these movements likely represent post-stun spinal reflexes or residual seizure-like motor discharges rather than recovered consciousness (Cabrera-Álvarez et al., 2026; James et al., 2025; Retter et al., 2018). However, this interpretation remains inferential, as behavioural indicators alone cannot confirm brain state. Similar to electro-immobilisation, voluntary movement may be suppressed independently of cortical activity, potentially leaving the fish conscious despite appearing unresponsive (Hjelmstedt et al., 2022; Lambooij et al., 2010). EEG-based studies have shown that improper electrical exposures can produce only short-lasting loss of brain function, with neurological recovery documented within seconds to minutes post-stun in some fish (Hjelmstedt et al., 2022; Robb and Roth, 2003). Therefore, the intermittent motor activity observed from the red seabream may coincide with partial neurological recovery, highlighting that cessation of behavioural responses does not reliably indicate sustained brain inactivation, which is essential for humane slaughter (Mocho et al., 2025; Saraiva et al., 2024).

Finally, the delayed loss of behavioural responses observed during ice-slurry immersion presents a distinct but equally important welfare concern. Unlike the risk of electro-immobilisation under electrical stunning, the welfare issue with ice-slurry comes from the gradual and prolonged nature of cold-induced neuromuscular suppression (Clemente et al., 2023; Wahltinez et al., 2024). This slow cessation of behavioural indicators should not be interpreted as a confirmed loss of consciousness, as cold shock can paralyse fish and suppress visible signs of distress without inducing rapid insensibility (Humane Slaughter Association, 2025; Lambooij et al., 2015). Physiological evidence supports this interpretation, as irregular heart rates have been recorded in fish immersed in ice-slurry even after observable movements cease (Lambooij et al., 2015; Wahltinez et al., 2024). Similar to electrically stunned fish, behavioural indicators are unreliable when physical immobilisation occurs, whether caused by electrical current or cold shock (Bowman et al., 2020; Humane Slaughter Association, 2025). The significant lag before the complete cessation of movement observed in the red seabream indicates that ice-slurry alone does not reliably induce insensibility. These findings reinforce the need for immediate pre-stunning protocols to ensure rapid and confirmed loss of brain function prior to slaughter (Humane Slaughter Association, 2025; Lambooij et al., 2015; Wahltinez et al., 2024).

5. Conclusion

This study demonstrates that the welfare and muscle quality of red seabream are tightly linked through a cascade of physiological responses initiated at harvest and completed at slaughter, with water temperature further modulating these effects. High-stress handling, such as crowding and brailing, acts as a metabolic trigger, causing an immediate neuroendocrine surge, disrupting osmoregulatory balance, and depleting cellular energy reserves before death. In contrast, electrical stunning, particularly when applied correctly, shows potential to mitigate these responses by inducing near-instantaneous behavioural insensibility, thereby limiting further HPI-axis activation, anaerobic metabolism, and osmoregulatory disturbance. Methods such as ikigun similarly produce rapid insensibility, whereas asphyxia and ice-slurry immersion prolong consciousness, exposing fish to sustained metabolic and physiological stress. These prolonged stressors directly impair muscle quality, evidenced by accelerated *post-mortem* pH decline and rapid *rigor mortis* progression. Improper stunning parameters may still induce involuntary muscular contractions without immediate brain inactivation, posing both welfare and quality risks. It should be noted that inferences regarding insensibility are primarily based on behavioural indicators and require validation via neurophysiological tools such as EEG. Overall, while rearing temperature modulates stress responses, the intensity of harvest and slaughter in industrial settings usually has a greater impact, emphasizing the need for standardized methods that render fish immediately insensible and maintain both welfare and product quality.

CRediT authorship contribution statement

Joey Arboleda: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation. **Athanasios Samaras:** Writing – review & editing, Validation, Methodology, Formal analysis, Data curation. **Nikos Papandroulakis:** Writing – review & editing, Visualization, Validation, Supervision, Methodology, Formal analysis. **Carlos Garcia de Leaniz:** Writing – review & editing, Visualization, Validation, Supervision, Methodology, Formal analysis, Data curation, Conceptualization. **Leonidas Papaharisis:** Writing – review & editing, Validation, Resources, Data curation. **Pavlos Pavlidis:** Writing – review & editing, Validation, Methodology, Formal analysis, Data curation. **Michail Pavlidis:** Writing – review & editing, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Funding acquisition, Formal analysis, Data curation, Conceptualization.

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Declaration of competing interest

The authors declare no conflict of interest.

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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.2026.744062.

Data availability

All data is provided in the article.

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