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Stunning challenges: Operational indicators flag failures, but neurological validation is needed to confirm stunning effectiveness in seabass and seabream

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ABSTRACT

Humane slaughter in aquaculture depends on accurate assessments of insensibility, yet commonly used operational indicators remain poorly validated against neurological benchmarks. This study compared operational indicators such as the loss or recovery of equilibrium, 'eye-roll' reflex, and ventilation reflex with visually evoked responses (VERs) in European seabass (*Dicentrarchus labrax*) and gilthead seabream (*Sparus aurata*) subjected to anaesthetic immersion or in-water electrical stunning. Operational indicators consistently preceded or lagged behind VERs, risking misjudgment of fish sensibility. Notable species differences emerged, with seabass losing and recovering indicators faster than seabream, whose indicator sequences varied depending on stunning method. Among all indicators, the ventilation reflex aligned most closely with VERs in both species, suggesting it may be the most reliable operational proxy. Despite the increasing use of electrical stunning in commercial settings, rapid recovery times (within seconds to minutes) observed in most fish raise serious welfare concerns. These findings underscore the urgent need to improve and validate electrical stunning methods for seabass and seabream before they can be considered humane and are widely implemented. Until neurological tools are feasible for commercial use, thorough species- and method-specific validation of operational indicators remains essential for safeguarding fish welfare.

1. Introduction

Despite the centrality of humane slaughter in EU legislation, welfare assessments in seabass and seabream remain critically under-validated. Ethical and welfare concerns in aquaculture, as in other animal production sectors, are often greatest at the point of slaughter, with an estimated 78–171 billion fish affected annually (Mood et al., 2023). It is therefore imperative that humane slaughter practices are developed, validated and employed to minimize fear, anxiety, pain, and suffering of fish leading up to, and during, their final moments of life (Ashley, 2007; Franks et al., 2021). For stunning and/or killing methods to be considered humane, fish should be rendered insensible (*i.e.*, unable to perceive and respond to stimuli) immediately before killing and should remain so until death, avoiding any unnecessary pain, suffering, fear, anxiety, or distress (EFSA, 2004; WOAH, 2024). If insensibility is induced gradually, it is critical that fish do not experience the above-mentioned negative states during the process (EFSA, 2004; WOAH, 2024).

The gold-standard for evaluating the state of sensibility in farmed animals, including fish, during stunning and/or killing is the use of neurological indicators obtained through electroencephalography (EEG) (EFSA, 2004; More et al., 2018; WOAH, 2024). The transition from, or

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incompatibility with, a state of sensibility has been assessed in fish using a range of neurological indicators, including significant reductions in EEG signal amplitude (*i.e.*, >50 % or >90 %), a shift from high-frequency (*i.e.*, α and β [8–32 Hz]) to low-frequency brain waves (*i.e.*, δ and θ [0.5–8 Hz]), reductions in EEG derivatives (*i.e.*, total power, median frequency, and spectral edge frequency), the presence of an epileptic-like seizure (also referred to as an epileptiform insult, generalized tonic-clonic seizure or grand mal seizure), and/or the absence of evoked responses (*i.e.*, visual or somatosensory evoked responses, VERs or SERs, respectively) (Wahltinez et al., 2024). Among these, VERs are widely recognized as a robust and objective measure of sensibility in fish, as they reflect the integrity of primary sensory pathways and are among the last responses to disappear before brain death, while their return often coincides with the recovery of other indicators of sensibility (Bowman et al., 2019, 2020; Brijs et al., 2021, 2025; Hjelmstedt et al., 2022, 2024, 2025; Jung-Schroers et al., 2020; Kestin et al., 1991, 2002; Retter et al., 2018; Sundell et al., 2024; van de Vis et al., 2003). Moreover, unlike spontaneous EEG signals, VERs are time-locked to specific stimuli, enabling precise tracking of transitions between states of sensibility and reducing susceptibility to noise and artifacts. However, these types of neurological assessments require specialized equipment, as well as expertise in obtaining and interpreting EEG recordings, and so they are not currently feasible for personnel in commercial slaughter conditions.

Consequently, operational indicators, such as self-initiated movements (*e.g.*, coordinated swimming and maintenance of equilibrium), reactions to handling or harmful stimuli, and clinical reflexes (*e.g.*, ventilatory and 'eye-roll'/vestibulo-ocular reflexes), are more commonly used to evaluate the effectiveness of stunning and/or killing methods in commercial settings (Kestin et al., 2002). While these indicators offer a practical, rapid means of assessing stunning and/or killing outcomes under commercial conditions (hence the term operational), their reliability has been questioned. There are concerns about the temporal gap between the loss or recovery of operational indicators and the cessation or return of brain activity, as well as the possibility that these indicators may reflect immobilization rather than true insensibility (Bowman et al., 2020; Robb et al., 2000; van de Vis et al., 2003; Wahltinez et al., 2024). Such discrepancies pose a significant risk of misjudging a fish as insensible based on operational indicators, while remaining sensible according to neurological assessments. This misjudgment can severely compromise fish welfare, particularly during slaughter procedures such as exsanguination, evisceration, or decapitation (EFSA, 2004; WOAH, 2024). Thus, neurological validation of operational indicators is necessary, ideally at a species-specific level, to ensure their reliability and determine whether they accurately reflect insensibility in a commercial setting (More et al., 2018).

Despite growing recognition of this hazard, the relationship between operational and neurological indicators remains poorly understood for many fish species and under different slaughter conditions. This is especially true for the widely farmed European seabass (*Dicentrarchus labrax*) and gilthead seabream (*Sparus aurata*), which account for ~344,000 and 294,000 tonnes of annual production, respectively (FAO, 2024a,b), but for which neurological validation of stunning and/or killing methods remains limited. To address this knowledge gap, we evaluated and compared these indicators in both species, examining their responses during a gradual induction into insensibility (*i.e.*, during anaesthetic immersion), as well as during recovery from a rapid induction (*i.e.*, following in-water electrical stunning).

The decision to evaluate indicator loss during anaesthetic immersion and indicator recovery following electrical stunning was a deliberate and scientifically justified design choice. Anaesthesia allows a gradual, observable transition into insensibility, enabling precise tracking of the temporal sequence and relative timing of indicator loss. In contrast, electrical stunning induces an immediate loss of sensibility, which precludes assessment of indicator loss but provides an appropriate context for evaluating the duration of insensibility. These methods therefore address complementary research objectives and mirror real-world practice, as anaesthetics are typically applied until fish are fully insensible and remain so until removed from the solution, whereas electrical stunning must ensure that insensibility is maintained long enough to allow for slaughter. By integrating both approaches, we aimed to maximise the scientific robustness and practical relevance of our findings for welfare validation and implementation. By clarifying the reliability of operational indicators against neurological benchmarks, this study aims to determine whether, and to what extent, operational indicators can be used in practice to evaluate the effectiveness of commercial stunning and killing methods for seabass and seabream.

2. Materials and methods

2.1. Fish and housing conditions

Seabass (n = 37, body mass = 1137 ± 91 g, all data are presented as mean ± 95 % C.I.) and seabream (n = 41, body mass = 865 ± 63 g), were bred and housed in earthen ponds at the Estação Piloto de Piscicultura de Olhão (EPPO) of the Instituto Português do Mar e da Atmosfera (IPMA, Olhão, Portugal). Each species was collected from the earthen ponds and held for a minimum of one week before experimentation in a separate 3000 L tank within an outdoor recirculating aquaculture system (RAS) equipped with screen filters, bead filters, biological filters, protein skimmers, and ultraviolet sterilizers. Water quality parameters, including temperature, dissolved oxygen, pH, and salinity, were monitored daily, while ammonia and nitrite levels were checked weekly throughout the experiment. Fish were hand-fed twice daily, six days a week, with 10 mm commercial pellets (Standard Orange 8, AquaSoja, Portugal).

All animal care and experimental procedures were conducted in compliance with 2023DGV/000066293 issued by Direção Geral de Alimentação e Veterinária, Ministério da Agricultura, Florestas e Desenvolvimento Rural, Portugal in compliance with the European (Directive 2010/63/EU) and Portuguese (Decreto-Lei no. 113/2013 de 7 de Agosto) legislation for the use of laboratory animals.

2.2. Experimental protocols

Operational and neurological indicators were assessed in both species during either a gradual induction into insensibility (*i.e.*, anaesthesic immersion, see *2.2.1.*) or following an immediate induction into insensibility (*i.e.*, in-water electrical stunning, see *2.2.2.*, *2.2.3.*). An overview of the experimental protocols used, including the purpose of each, the indicators assessed, the timing of neurological recordings, and their relevance to real-world slaughter contexts, is provided in Table 1. The operational indicators assessed in this study included the ability of fish to maintain equilibrium, ventilatory reflexes, and the 'eye-roll' reflex (also referred to as the vestibulo-ocular reflex), as described by Kestin et al. (2002). A summary of these indicators and their descriptions for both the loss (during anaesthetic immersion) and recovery (following electrical stunning) phases is provided in Table 2. The neurological indicator selected was the presence or absence of VERs within EEG recordings. Morphometric data, water quality parameters, and stunning parameters for each protocol are provided in Table 3.

2.2.1. Loss of indicators during anaesthetic immersion

Seabass and seabream were captured from their holding tanks using pole nets and transferred to a rectangular plastic container (length = 600 mm, width = 400 mm, height = 320 mm) containing 48 L of saltwater from the holding tanks (salinity = 8–10 ppt, temperature = 22.8–26.8°C) with an anaesthetic solution (600 ppm 2-phenoxyethanol; Sigma–Aldrich, USA). The progressive loss of operational indicators was then visually monitored during anaesthetic immersion according to the ethogram outlined in Table 2. Upon loss of the final operational indicator, fish were removed from the anaesthetic and instrumented with

Table 1
Overview of experimental protocols used to assess insensibility in European seabass and gilthead seabream.

Protocol	Stunning/Induction method	Purpose	Assessed indicators	Neurological recording	Represents real-world context?
1. Loss of indicators during anaesthetic immersion	Anaesthetic (2-phenoxyethanol)	Determine the temporal sequence of indicator loss	Equilibrium, ventilation, 'eye-roll' reflex, VERs	After loss of final indicator	No (controlled gradual model)
2. Recovery after in-water electrical stunning (with anaesthesia)	Electrical (post-recovery from anaesthesia)	Assess recovery of neurological signals after stunning	VERs	Continuous (pre-instrumented)	Partially (idealised setup)
3. Recovery after in-water electrical stunning (no anaesthesia)	Electrical (direct from holding tank)	Assess recovery of operational and neurological indicators	Ventilation, 'eye-roll' reflex, VERs	Continuous (post-instrumented)	Yes (mimics commercial context)

Table 2
Overview of the operational indicators used to assess insensibility induction and recovery in European seabass and gilthead seabream. A description of the operational indicators employed to monitor the gradual induction into insensibility in seabass and seabream, as well as the recovery phase following an acute induction into insensibility.

Indicator	Description of indicator
Loss of indicators during gradual induction into insensibility	
Loss of equilibrium	cessation of vertical body position in water column
Loss of rhythmic ventilation	cessation of rhythmic opercular movement
Loss of 'eye-roll' reflex	cessation of vestibulo-ocular reflex
Loss of ventilation	cessation of opercular movement
Recovery of indicators following an acute induction into insensibility	
Recovery of ventilation	resumption of opercular movement
Recovery of rhythmic ventilation	resumption of rhythmic opercular movement
Recovery of 'eye-roll' reflex	resumption of vestibulo-ocular reflex

EEG electrodes (see *2.3.1.*). Fish were then placed in a custom-made, darkened, in-water electrical stunning chamber (length = 500 mm, width = 150 mm, height = 300 mm; Ace Aquatec Ltd, UK) containing 13.5 L of saltwater obtained from the holding tanks (salinity = 9–10 ppt, temperature = 21.3–25.3°C). An EEG was then continuously recorded and monitored in response to light flashes to evaluate the presence or absence of VERs (see *2.3.2*).

The abovementioned protocol differed slightly between species, as the loss of the 'eye-roll' reflex was initially presumed to be the operational indicator of sensibility most closely aligned with the neurological response. However, after completing trials on seabream, we observed that the complete loss of ventilation corresponded more closely with the loss of VERs, as ventilation consistently ceased during EEG electrode instrumentation. Consequently, for seabass, we adjusted our approach and waited until ventilation had ceased before instrumenting the fish with EEG electrodes to assess VERs.

2.2.2. Recovery of indicators following in-water electrical stunning (with prior anaesthesia)

To rapidly assess neurological recovery following in-water electrical stunning, a subset of fish from the abovementioned protocol were used. The fish that were selected had fully recovered from anaesthesia and exhibited reliable EEG signals—defined as recordings with minimal artefacts and clearly distinguishable VERs in response to light stimuli (confirmed via real-time EEG monitoring, see *2.3.2*). Since these fish had already been instrumented with EEG electrodes, it was possible to begin EEG recording immediately after electrical stunning, allowing precise detection of the recovery of VERs.

Following recovery from anaesthesia, seabass and seabream were exposed to an in-water electrical stun for 30 s using a 50 Hz AC source, delivering an electric field of ~1.7 V cm^{-1} and a current density of ~3.0 A dm^{-2}. The stun was conducted in the stunning chamber containing water obtained from the holding tanks (salinity = 9–10 ppt, temperature = 21.3–25.3°C). Directly following the stun, EEG responses to light flashes were continuously recorded within the stunning chamber for 10 min to assess the recovery of VERs (see *2.3.2*). At the end of the monitoring period, fish were euthanized by a sharp blow to the head, weighed, and measured.

Electrical stunning was conducted using stainless steel plate electrodes (width = 135 mm, height = 200 mm; Ace Aquatec Ltd., UK) positioned 495 mm apart on opposite ends of the stunning chamber to create a uniform head-to-tail electric field. The electrodes were connected to a custom-built stunning device by Ace Aquatec Ltd., delivering smooth sinusoidal AC at 50 Hz, with stun duration controlled by a timing switch. Detailed stun settings, based on manufacturer recommendations are provided in Table 3. Voltage and current were monitored with a digital oscilloscope (Model 123, 20 MHz) and a current probe (Model 801–110S) from Fluke Corporation, USA.,

2.2.3. Recovery of indicators following in-water electrical stunning (without prior anaesthesia)

To provide a more representative and unbiased assessment of recovery from in-water electrical stunning under typical slaughter

Table 3
Overview of protocol specifics. Morphometric data, water parameters, and electrical stunning parameters for the various experimental protocols conducted in the present study. All data are presented as mean ± 95 % C.I.

		Morphometrics		Water parameters		Electrical stunning parameters	
Species	n	Body mass	Fork length	Temperature	Salinity	Current density	Field strength
		(g)	(mm)	(°C)	(ppt)	(A dm^{-2})	(V cm^{-1})
Gradual induction into insensibility							
Seabream	14	1038 ± 115	359 ± 13	24.8 ± 0.8	9.1 ± 0.2	n/a	n/a
Seabass	16	1132 ± 154	422 ± 19	23.4 ± 0.3	9.3 ± 0.2	n/a	n/a
Acute induction into insensibility (with prior anaesthesia)							
Seabream	10	986 ± 97	353 ± 11	24.5 ± 0.9	9.1 ± 0.2	3.06 ± 0.09	1.67 ± 0.01
Seabass	16	1132 ± 154	422 ± 19	23.4 ± 0.3	9.3 ± 0.2	2.98 ± 0.04	1.68 ± 0.01
Acute induction into insensibility (without prior anaesthesia)							
Seabream	27	776 ± 49	328 ± 19	23.0 ± 0.5	9.3 ± 0.3	3.00 ± 0.04	1.69 ± 0.01
Seabass	21	1141 ± 112	430 ± 16	23.4 ± 0.3	9.3 ± 0.2	3.04 ± 0.04	1.68 ± 0.01

conditions, an additional group of seabass and seabream were tested without prior anaesthesia. These fish were directly subjected to an in-water electrical stun using the same parameters and equipment described above and in Table 3.

Immediately after stunning, fish were instrumented with EEG electrodes and returned to the stunning chamber to enable continuous monitoring for up to 10 min. During this time, both operational and neurophysiological indicators of recovery were assessed. Recovery of operational indicators were visually monitored according to the ethogram outlined in Table 2, however, due to the limited space within the stunning chamber, it was not possible to monitor the recovery of equilibrium following in-water electrical stunning. In parallel, EEG responses to light flashes were continuously recorded to assess the recovery of VERs (see *2.3.2*). At the end of the monitoring period, fish were euthanized by a sharp blow to the head, weighed, and measured.

2.3. EEG methodology

2.3.1. EEG electrode instrumentation

EEG of fish were recorded via two 20 or 23 gauge, stainless steel needle electrodes for seabass and seabream, respectively. The needle electrodes were carefully inserted ~0.5–1.0 cm caudal of the eyes and ~0.5–1.5 cm lateral of the sagittal suture depending on the size of the fish. Another electrode was clipped onto the posterior end of the dorsal fin using a 5.6 mm gator clip to function as a common ground electrode. Fish were then immediately placed in the darkened, in-water electrical stunning chamber. The duration of EEG electrode instrumentation was 0.57 ± 0.07 min for seabass and 0.54 ± 0.06 min for seabream.

2.3.2. Recording, acquisition and analyses of EEG signals

The abovementioned EEG electrodes were securely connected to 1.5 mm shielded EEG wires (MLAWBT9 EEG Flat Electrodes, ADInstruments, Oxford, United Kingdom) using WAGO connection terminals (WAGO 221–412, WAGO GmbH, Minden, Germany). The EEG wires were then connected to a custom made bio-amplifier, which was interfaced with a PowerLab (PL 8/35, ADInstruments) and a PC equipped with LabChart Pro software (version 8.1.28., ADInstruments).

EEG of fish were continuously recorded in response to 10 ms light flashes at 2 Hz from an LED strobe-light within the custom-made, light exclusion container. The sensitivity range of the bio-amplifier was ±2 mV with a 120 Hz low-pass filter, a 0.1 Hz high-pass filter, and a 50 Hz notch filter activated to optimize the EEG recording. Signals from the bio-amplifier and a custom-made light detector (*i.e.*, from a solar panel, Velleman SOL1N, Gavere, Belgium) were relayed to the PowerLab and collected on the PC at a sampling rate of 1 kHz. When analysing the EEG recordings in the LabChart Pro software, a bandpass filter was used to separate the beta waves (12–32 Hz), as VERs in fish are found to be most distinct within this frequency range (Bowman et al., 2019, 2020). The Scope View module within the LabChart Pro software averaged 10–120 non-overlapping consecutive epochs that displayed 50 ms before and 400 ms after the flash, representative of the beta wave for 5–60 s of recordings, respectively, to obtain specific determinations of when VERs were present or absent (Hjelmstedt et al., 2022). To reduce the effects of noise caused by strong muscular movements, all epochs exceeding 0.1 mV were automatically excluded from the analyses. VERs were determined to be present or absent when the peak-to-peak amplitude of the respective VER was greater or less than double the peak-to-peak amplitude of the rest of the beta wave.

2.4. Statistical analyses

All statistical analyses were performed using the statistical software R, version 4.2.3 (http://www.r-project.org). A comprehensive description of the statistical analyses, including details about the R packages employed and the procedures associated with data exploration, assumption testing, parameter transformation, as well as model output and inference, can be found in the supplementary information (Supp. Info. 1A-E). Statistical significance was determined at a threshold of $p < 0.05$.

Accelerated Failure Time (AFT) models were used to assess differences in the timing of indicator loss or recovery within and between seabass and seabream during anaesthetic immersion (Supp. Info. C) or following in-water electrical stunning (Supp. Info. D). The best fitting distribution (*i.e.*, Weibull, log-logistic, or log-normal) and fixed effects (*i.e.*, indicator, species, body mass, and/or their interactions) for these models were based on Akaike's Information Criterion (AIC). Model fit and assumptions were verified using deviance residuals, residual-versus-fitted plots (log-linearity), and log-log plots (proportional hazard acceleration). In cases where specific model assumptions (*e.g.*, homoscedasticity or normality of residuals) appeared violated (Supp. Info. D), parameter estimates were validated through bootstrapping (1000 replicates), providing robust and reliable inference. Robust clustered standard errors accounted for repeated measurements within individuals. Overall model significance was assessed using Wald tests. Following significant results, time ratios (TR), representing the relative median times to operational indicator loss or recovery compared to VERs, were computed by exponentiating model coefficients. Pairwise comparisons between indicators were conducted with *p*-values adjusted for multiple testing using the False Discovery Rate (FDR) (Benjamini and Hochberg, 1995).

To assess differences in the timing of VER recovery following in-water electrical stunning between instrumented seabass and seabream individuals (Supp. Info. E), a Mann–Whitney *U* test was performed due to the non-normal distribution of the data. The suitability of this non-parametric method was confirmed through visual inspection of histograms and density plots, which indicated similar distribution shapes between species.

3. Results

3.1. Loss of indicators during anaesthetic immersion

During anaesthetic immersion, seabass and seabream were gradually rendered insensible. In both species, all operational indicators disappeared before VERs, confirming that operational indicators of insensibility precede the loss of the neurological indicator (Fig. 1). However, as outlined below, the timing and sequence of indicator loss varied across indicators and between species.

In seabass, the gradual induction into insensibility began with the loss of equilibrium, followed by the loss of the 'eye-roll' reflex, rhythmic ventilation, ventilation, and finally VERs (upper panel of Fig. 1). VERs were lost between 4.0 and 8.6 min (median = 5.5 min). All operational indicators were lost significantly earlier than VERs in seabass: equilibrium was lost ~8.0 times faster (TR = 0.125, $p < 0.001$), the 'eye-roll' reflex ~3.1 times faster (TR = 0.320, $p < 0.001$), rhythmic ventilation ~2.0 times faster (TR = 0.509, $p < 0.001$), and ventilation ~1.4 times faster (TR = 0.702, $p < 0.001$).

Seabream displayed a slightly different sequence of indicator loss during the gradual induction into insensibility. In this species, equilibrium was also lost first, followed by rhythmic ventilation, then the eye-roll reflex, and finally VERs (lower panel of Fig. 1). VERs were lost between 5.8 and 9.5 min (median = 8.1 min). All operational indicators were also lost significantly earlier than VERs in seabream: equilibrium was lost ~8.1 times faster (TR = 0.124, $p < 0.001$), rhythmic ventilation ~2.3 times faster (TR = 0.429, $p < 0.001$), and the 'eye-roll' reflex ~1.3 times faster (TR = 0.774, $p < 0.001$).

Comparing the two species, seabass generally lost indicators faster than seabream during anaesthetic immersion. For example, loss of equilibrium was ~1.4 times faster in seabass (TR = 0.730, $p = 0.018$), loss of rhythmic ventilation tended to be ~1.2 times faster (TR = 0.855, $p = 0.053$), loss of 'eye-roll' reflex was ~3.4 times faster (TR = 0.297, $p < 0.001$), and loss of VERs was ~1.4 times faster (TR = 0.721,

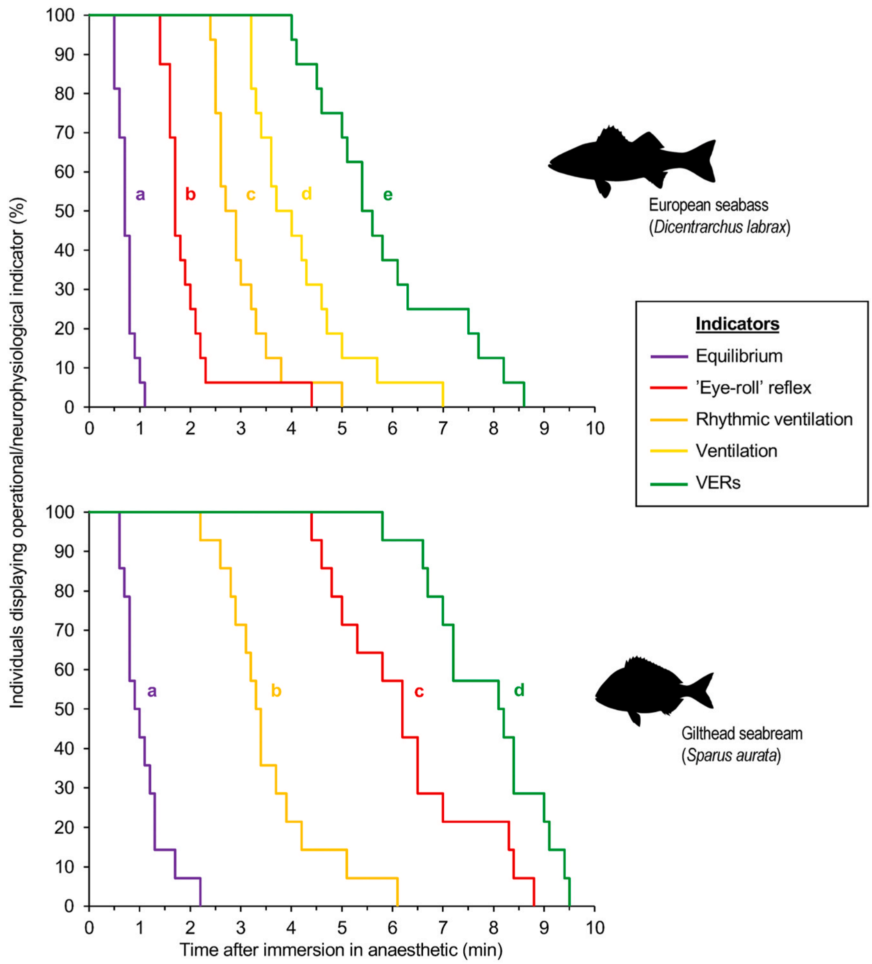

Fig. 1. Gradual loss of sensibility in European seabass and gilthead seabream during anaesthetic immersion. The time until the loss of equilibrium, 'eye-roll' reflex, rhythmic ventilation, ventilation, and/or visually evoked responses (VERs) in seabass (upper panel, n = 16, Wald $\chi^2 = 690.15$, $p < 0.001$) and seabream (lower panel, n = 14) during immersion in 600 ppm 2-phenoxyethanol. Based on the most parsimonious AFT model (AIC = 304.33; Wald $\chi^2 = 1688$, $p < 0.001$), time until insensibility during anaesthetic immersion was significantly influenced by the specific indicator used, the species tested, and their interaction, indicating that the temporal transition to a state of insensibility varied depending on the specific combination of indicator and species. Different lowercase letters indicate statistically significant differences in the median time until loss of sensibility between the various indicators (adjusted $p < 0.05$).

$p < 0.001$).

3.2. *Recovery of indicators following in-water electrical stunning (with prior anaesthesia)*

In the subset of individuals that were electrically stunned after recovering from anaesthesia (and already equipped with EEG electrodes), VERs were observed to recover rapidly in both species. In seabass, recovery occurred between 0.2 and 0.9 min (median = 0.5 min), while in seabream, VERs returned between 0.2 and 1.4 min (median = 0.4 min). There was no statistically significant difference in VER recovery time between species (Wilcoxon rank-sum test: $W = 98$, $p = 0.349$).

3.3. *Recovery of indicators following in-water electrical stunning (without prior anaesthesia)*

Following electrical stunning, both species recovered the neurological indicator before the operational indicators (Fig. 2). However, as outlined below, the timing of indicator recovery following electrical stunning varied across indicators and between species.

In seabass, VERs recovered between < 0.5–1.25 min (median = 0.7 min), while in seabream, they recovered between < 0.5–1.5 min (median = 0.9 min) (upper and lower panels of Fig. 2). However, these estimates are likely overestimated, as 71 % of seabass (15 out of 21 individuals) and 56 % of seabream (15 out of 27 individuals) already exhibited VERs at the start of EEG recording.

All operational indicators took longer to recover than VERs, and the recovery followed a consistent pattern in both species (Fig. 2). Ventilation recovered first, followed by rhythmic ventilation and then the 'eye-roll' reflex, with these indicators taking at least 1.4–2.7, 1.8–4.5, and 3.3–4.7 times longer than VERs, respectively (TR of 1.428–2.688, 1.814–4.516, and 3.233–4.713, $p < 0.001$ for all pairwise comparisons). In addition, recovery was significantly faster in seabass than seabream across all operational indicators (Fig. 2), as ventilation, rhythmic ventilation and the 'eye-roll' reflex recovered ~2.2, 3.0 and 1.7 times faster in the former (TR = 0.445, 0.336, 0.574: $p < 0.05$ for all pairwise comparisons).

4. Discussion

This study provides the first comprehensive comparative analysis of operational and neurological indicators of insensibility in seabass and seabream. By directly evaluating the alignment between commonly used operational indicators and a robust neurological benchmark, this research addresses a critical knowledge gap in the assessment of fish welfare. The findings have important implications for evaluating the effectiveness of stunning methods and refining welfare practices in

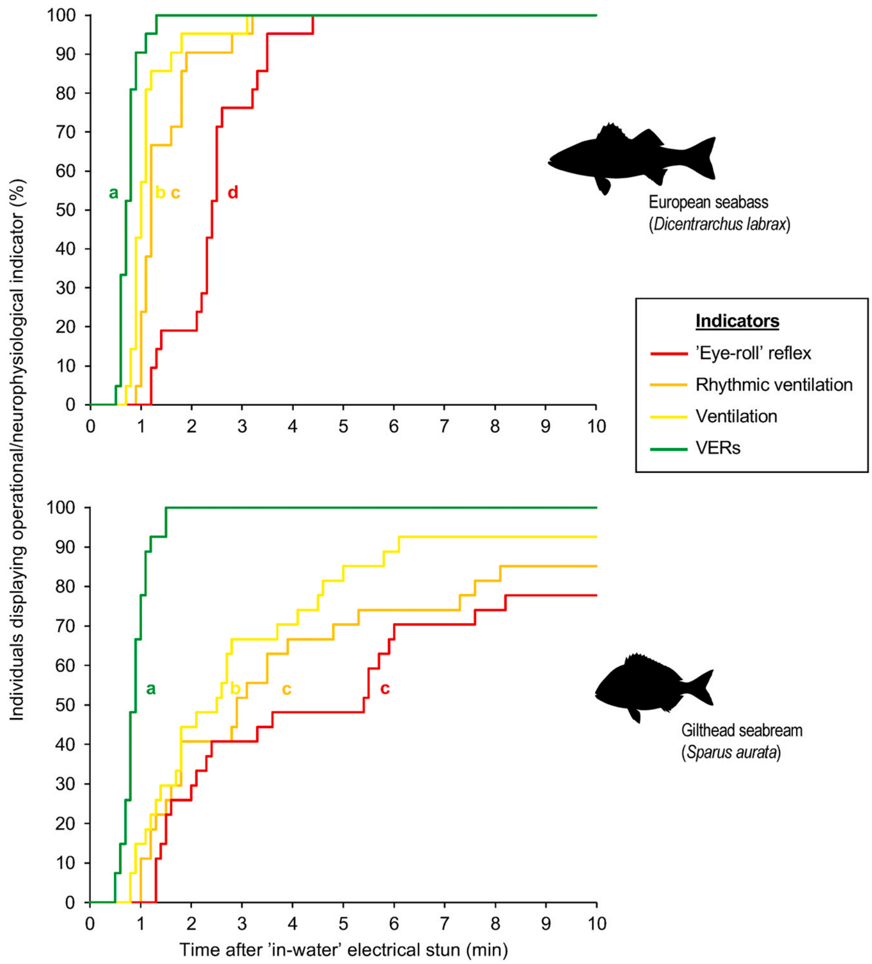

Fig. 2. Recovery of sensibility in European seabass and gilthead seabream following in-water electrical stunning. The time until the recovery of visually evoked responses (VERs), ventilation, rhythmic ventilation, and 'eye-roll' reflex in seabass (upper panel, n = 21) and seabream (lower panel, n = 27) following an in-water electrical stun for 30 s using a 50 Hz AC source, delivering an electric field of ~1.7 V cm^{-1} and a current density of ~3.0 A dm^{-2}, at a water temperature of ~24°C, salinity of ~9.2 pp, and conductivity ~14000 µS cm^{-1}. Based on the most parsimonious AFT model (AIC = 513.48, Wald $\chi^2 = 399.18$, $p < 0.001$), time until sensibility following in-water electrical stunning was significantly influenced by the specific indicator used, the species tested, and their interaction, indicating that the temporal transition to a state of sensibility varied depending on the specific combination of indicator and species. Different lowercase letters indicate statistically significant differences in the median time until recovery of sensibility between indicators (adjusted $p < 0.05$).

commercial aquaculture involving these economically significant species.

Operational indicators remain widely used in aquaculture, yet their accuracy in identifying transitions to, and recovery from, insensibility is uncertain without neurological validation (Bowman et al., 2020; Robb et al., 2000; van de Vis et al., 2003). Our results clearly demonstrate a consistent temporal mismatch between operational and neurological indicators in both seabass and seabream. Specifically, operational indicators were lost well before VERs during anaesthetic immersion and reappeared considerably later than VERs following in-water electrical stunning. This discrepancy highlights that while the presence of operational indicators reliably signals sensibility, flagging failed stunning attempts, their absence alone does not guarantee successful induction of insensibility. In some cases, such as after electrical stunning in seabream, operational indicators failed to recover despite the rapid return of VERs, further underscoring the risk of mistaking immobility for insensibility. These results reinforce the critical need for neurological validation to safeguard welfare during slaughter. (EFSA, 2004; More et al., 2018; WOAH, 2024). They also highlight the importance of developing robust and user-friendly methods for real-time neurological assessments to support both scientific evaluation and commercial application of humane stunning and/or killing practices.

Nonetheless, until neurological monitoring becomes practically feasible in commercial settings, operational indicators will remain essential for assessing stunning effectiveness (Kestin et al., 2002). Our findings suggest that these indicators may reflect a graded continuum of sensibility states rather than a binary shift from sensible to insensible. As in previous studies involving Atlantic salmon (*Salmo salar*, Kestin et al., 2002; van de Vis et al., 2003), common carp (*Cyprinus carpio*, Retter et al., 2018), European eel (*Anguilla anguilla*, van de Vis et al., 2003), and rainbow trout (*Oncorhynchus mykiss*, Bowman et al., 2020; Jung-Schroers et al., 2020), ventilation reflexes in our study showed the strongest temporal alignment with the loss or recovery of VERs. This suggests that this indicator may serve as a more reliable proxy for insensibility when compared to the loss or recovery of equilibrium and/or 'eye-roll' reflex. Recognizing this hierarchical relationship among operational indicators could help refine welfare assessments by prioritizing those that more closely reflect underlying neural states (More et al., 2018).

Importantly, it must be noted that the timing and sequence of operational indicators along this continuum can vary considerably depending on species, stunning method, and characteristics of the methodology used. For example, in the present study, seabass consistently lost and recovered all indicators more rapidly than seabream, and in seabream the sequence of loss and recovery varied depending on the stunning method. Inconsistencies have also been reported in other species and contexts, including African sharptooth catfish (*Clarias gariepinus*) subjected to live chilling, electrical stunning, and percussive stunning (Brijs et al., 2021), and rainbow trout subjected to 'in-air' electrical stunning or anaesthesia (Bowman et al., 2019; Brijs et al., 2025; Hjelmstedt et al., 2022). These findings underscore the importance of validating operational indicators not only by species but also by stunning method. If operational indicators indeed reflect distinct physiological stages towards or away from insensibility, then species- and stunning method-specific benchmarks are essential to ensure reliable interpretation. Tailoring welfare protocols in this way could substantially reduce the risk of misclassifying sensibility and ultimately improve welfare outcomes during commercial slaughter.

Despite the growing interest in electrical stunning as a humane stunning method for seabass and seabream, particularly among producers in Spain, Greece, and Türkiye (van Pelt et al., 2024), its widespread adoption currently rests on a limited scientific foundation. To date, only a few published studies, in addition to the present work, have neurologically evaluated the effectiveness of electrical stunning in these species (Kestin et al., 2002; Lambooij et al., 2008; van de Vis et al., 2003). Outcomes vary markedly with electrical parameters and species-specific responses. For instance, while a 1 s in-water stun at 3.0–3.3 A dm^{-2} (50 Hz AC or 133 Hz pulse wave) induced immediate insensibility in all seabass tested, 21–62 % of these fish regained brain activity within 0.5 min, while 80 % of unrestrained seabass recovered equilibrium and swimming within 1.3 min on average following a 10 s stun at ~5.0 A dm^{-2} (Lambooij et al., 2008). In contrast, only 10 % of seabream displayed immediate insensibility when stunned with 80 Vrms (50 Hz AC) for 1 s, and nearly half recovered within 0.3 min after a 10 s stun (Kestin et al., 2002; van de Vis et al., 2003). Our findings further highlight these limitations, with the majority of seabass and seabream regaining VERs within 1 min following a 30 s in-water stun at ~3.0 A dm^{-2} (50 Hz AC). These results demonstrate that the electrical parameters employed in both past and present studies—typically derived from manufacturer-default settings—are not sufficient to ensure prolonged insensibility in all individuals. This poses a serious welfare concern, as fish may regain sensibility during subsequent killing procedures, thereby undermining both ethical standards and consumer trust.

To mitigate this risk, the combination of electrical stunning and live chilling has been proposed as a strategy to extend the duration of insensibility. In seabass, a 10 s stun at 4.0–5.0 A dm^{-2} (50 Hz AC) followed by live chilling maintained an isoelectric EEG for the 15 min monitoring period (Lambooij et al., 2008). While there are no published neurological studies investigating the combined approach of electrical stunning and live chilling on seabream, a recent study using operational indicators demonstrated that the combined approach of electrical stunning (2.9 A dm^{-2}, 50 Hz AC, for 15 s) and live chilling was shown to be ineffective in seabream, as opercular movements resumed within 1 min and persisted for > 10 min, while 40 % of individuals regained the 'eye-roll' reflex within 0.5 min, lasting ~2.5 min (Cabrera-Álvarez et al., 2025). These findings raise serious concerns about the suitability of current electrical stunning protocols, particularly when applied without species-specific validation.

Compounding the issue, most electrical stunning systems currently deployed for seabass and seabream have not undergone rigorous scientific evaluation (van Pelt et al., 2024). As interest in electrical stunning continues to grow among producers in countries such as Spain, Greece, and Türkiye, there is an urgent need for evidence-based refinement. This includes systematic testing across species and sizes, precise documentation of electrical parameters and environmental conditions, and outcome reporting that includes both operational and neurological endpoints with appropriate statistical detail (*e.g.*, ranges, raw values, confidence intervals). Ideally, such evaluations should be performed under both experimental and commercial conditions to determine the minimum effective parameters required to induce immediate and irreversible insensibility in the majority, if not all, individuals across the range of environmental conditions encountered during slaughter. Without these targeted validations, reliance on default manufacturer settings may result in inconsistent outcomes and substantial welfare risks, as fish may regain sensibility during subsequent slaughter procedures. Moving forward, the development and adoption of standardized, transparent assessment protocols will be essential—not only to ensure compliance with national and international animal welfare legislation, but also to maintain public confidence in the ethical standards of aquaculture (Wahltinez et al., 2024). Ultimately, improving the reliability and validation of stunning methods will require coordinated efforts across research, industry, and policy domains, underpinned by practical tools for real-time neurological assessment and a commitment to continuous refinement based on species- and method-specific evidence.

5. Conclusion

This study reveals significant discrepancies between operational and neurological indicators of sensibility in seabass and seabream, underscoring the challenges of reliably assessing insensibility during slaughter. While operational indicators consistently disappeared before the loss of VERs during anaesthetic induction and reappeared after their return following electrical stunning, the timing and sequence of these changes varied between species. These interspecific differences highlight the necessity of species-specific validation when applying operational indicators in welfare protocols.

Relying solely on operational cues without neurological confirmation risks misclassifying sensibility status and may compromise animal welfare. Such confirmation should be achieved using robust and objective neurological methodology, such as assessing the presence or absence of VERs, which currently provides an accurate measure of stunning effectiveness under controlled conditions and, with further methodological refinement, could be applied at commercial scale. To safeguard welfare during slaughter, there is a clear need for these rigorous, context-specific evaluations of stunning methods—grounded in both neurological benchmarks and practical applicability—across the diverse species used in aquaculture.

CRediT authorship contribution statement

Albin Gräns: Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Cabrera-Álvarez María J:** Writing – review & editing, Writing – original draft, Validation, Resources, Project administration, Methodology, Investigation, Formal analysis, Data curation. **Oliveira Gonçalo D. C.:** Writing – review & editing, Writing – original draft, Visualization, Validation, Investigation, Formal analysis. **João L. Saraiva:** Writing – review & editing, Writing – original draft, Visualization, Validation, Resources, Investigation, Formal analysis. **Pablo Arechavala-Lopez:** Writing – review & editing, Writing – original draft, Visualization, Validation, Formal analysis. **Martina Bortoletti:** Writing – review & editing, Writing – original draft, Visualization, Validation, Investigation, Formal analysis. **Thorsten Schwerte:** Writing – review & editing, Writing – original draft, Visualization, Validation, Formal analysis. **Jeroen Brijs:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Animal ethics statement

All animal care and experimental procedures were conducted in compliance with 2023DGV/000066293 issued by Direção Geral de Alimentação e Veterinária, Ministério da Agricultura, Florestas e Desenvolvimento Rural, Portugal in compliance with the European

(Directive 2010/63/EU) and Portuguese (Decreto-Lei no. 113/2013 de 7 de Agosto) legislation for the use of laboratory animals.

Declaration of Generative AI and AI-assisted technologies in the writing process

During the preparation of this work the authors used ChatGPT 4.5 by openai.com in order to improve the grammar and readability of sections within the introduction and discussion of this manuscript. After using this tool/service, the author reviewed and edited the content as needed and takes full responsibility for the content of the publication.

Declaration of Competing Interest

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

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.aqrep.2025.103189.

Data availability

The data that support the findings of this study are openly available in 10.6084/m9.figshare.28816445

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