

From tradition to innovation: Effects of manual and automated *ikejime* on welfare and product quality of rainbow trout and hybrid striped bass

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ABSTRACT

Ensuring humane slaughter is essential for ethical and high-quality fish production. *Ikejime* is widely regarded as rapid and quality-preserving, yet its manual application is labour-intensive and anatomy-dependent, limiting scalability. An automated, AI-guided *ikejime* system could overcome these constraints, but its welfare efficacy is unvalidated. We provide the first neurological evaluation of manual and automated *ikejime* for rendering rainbow trout (*Oncorhynchus mykiss*, ~0.3–0.5 kg) and hybrid striped bass (*Morone chrysops* ♀ × *M. saxatilis* ♂, ~1.1–2.1 kg) insensible, using visually evoked responses (VERs) on the electroencephalogram (EEG). Results are compared with manual percussion and asphyxiation, together with exploratory product-quality analyses. Asphyxiation caused prolonged distress lasting up to 13–16 min in both species. In contrast, both *ikejime* methods rendered all trout irreversibly insensible within 2 min, although neither reliably achieved immediate insensibility. Efficacy declined in bass, with ~25–43 % of individuals remaining sensible after application. These findings demonstrate that *ikejime*, whether manual or automated, should be preceded by a stunning step ensuring immediate loss of sensibility. Reduced effectiveness in larger and more robust species underscores the need for species-specific refinements, including optimized spike dimensions and insertion techniques. While exploratory product-quality assessments indicated potential effects of slaughter method and effectiveness on *rigor mortis* and drip loss, these patterns should be validated in future studies using larger sample sizes and broader replication. Together, these data provide critical evidence to guide the refinement of slaughter methods aiming to enhance fish welfare while ensuring product quality.

1. Introduction

Fish play a crucial role in human nutrition, and their importance will only increase as the global population grows (UN, 2023). This dependence is reflected in the record-high global aquaculture and fisheries production of 223.2 million tonnes (FAO, 2024). This output is estimated to correspond to over 78–171 billion farmed and 1100–1200 billion wild-caught individuals harvested annually for food, feed, and bait (Mood et al., 2023; Mood and Brooke, 2024). In light of these vast numbers, international organisations for animal health and welfare have issued guidelines emphasizing the necessity of safeguarding fish welfare

during slaughter. Humane slaughter protocols recommend that fish be rendered immediately insensible (*i.e.*, unable to perceive, process, or respond to stimuli) and that they remain in that state until death (EFSA, 2004; WOAH, 2024). When insensibility is induced gradually, individuals must not experience pain, distress, or other negative states during the induction phase (EFSA, 2004; Lines and Spence, 2014; van de Vis et al., 2003). Despite these recommendations, methods deemed inhumane, such as asphyxiation, live chilling, CO₂ immersion, and exsanguination without prior stunning, remain widespread (Lines and Spence, 2014; Gräns et al., 2016; Wahltinez et al., 2024). The continued use of such practices highlights a major disconnect between established

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welfare standards and commercial realities, underscoring the urgent need to develop, validate, and implement more humane and scientifically grounded slaughter methods.

A central challenge in evaluating the humaneness of slaughter techniques lies in objectively determining whether, and when, a fish becomes insensible. The most reliable approach is neurological assessment using electroencephalography (EEG), which directly measures brain activity (EFSA, 2004; 2018; WOAH, 2024). Various neurological indicators have been used to describe transitions between sensibility and insensibility. These include marked reductions in signal amplitude ($\geq 50\%$ or $\geq 90\%$), shifts from high- to low-frequency brain waves (α and β [8–32 Hz] to δ and θ [0.5–8 Hz]), decreases in total power or median frequency, epileptiform seizures, and the disappearance of sensory-evoked potentials (Wahltnet et al., 2024). Among these, visually evoked responses (VERs), time-locked electrical responses of the visual cortex to flashes of light, are widely recognized as one of the most robust and objective measures of sensibility in fish (Kestin et al., 1991; EFSA, 2004; Wahltnet et al., 2024). VERs are typically the last brain responses to disappear before death and the first to reappear during recovery. They align closely with the disappearance or reappearance of operational indicators of sensibility such as self-initiated movements, reactions to handling or harmful stimuli, and clinical reflexes in a wide range of fish species (Kestin et al., 1991, 2002; Bowman et al., 2019, 2020; Brijs et al., 2021; Brijs et al., 2025; Gräns et al., 2025; Hjelmstedt et al., 2022, 2024; Hjelmstedt et al., 2025; Jung-Schroers et al., 2020; Retter et al., 2018; Sundell et al., 2024; van de Vis et al., 2003). Because VERs are time-locked and less affected by movement artefacts than spontaneous EEG activity, they provide an ideal neurological benchmark for validating both established and novel slaughter methods under controlled or commercial conditions.

One such technique that urgently requires neurological validation is *ikejime* (also known as *ikijime*, pithing, or brain spiking) (EFSA, 2004). Traditionally, *ikejime* involves inserting a rod, typically made of steel, plastic, or bamboo, into the cranial cavity by hand to physically destroy the central nervous tissues, resulting in rapid death when performed accurately (Davie and Kopf, 2006; Diggles, 2016). Mechanized versions of the technique also exist, such as pneumatically operated pistols (Robb et al., 2000; Kestin et al., 2002; Papaharisis et al., 2019; Tejada and Huidobro, 2002). Although *ikejime* is most commonly practiced in Japan and neighbouring regions where fish are consumed raw as *sashimi*, its use has expanded globally among small-scale aquaculture and fishery operations across a wide range of taxa, including salmonids (e.g., salmon, trout), sparids (e.g., seabreams), perciforms (e.g., tuna, yellowtail, seabass, jack mackerel), cichlids (e.g., tilapia), and flatfish (e.g., halibut, flounder) (Davie and Kopf, 2006; Diggles, 2016; Clouette, 2022; Ikejime, 2025; Ikejime Federation, 2025; James et al., 2025). This growing global interest stems from its reputation for improving welfare and product quality, two aspects that are closely intertwined (Lowe et al., 1993; Poli et al., 2005; Davie and Kopf, 2006; Diggles, 2016; Daskalova, 2019; Clouette, 2022). When performed correctly, *ikejime* can rapidly dispatch fish (Robb et al., 2000; Kestin et al., 2002; Sorensen et al., 2004; Duque et al., 2019; Cabrera-Álvarez et al., 2025; Hoyos-Álvarez et al., 2025) and, at least in some cases, induces lower stress levels compared to fish left to die in air, water, ice, or CO_2 , as indicated by stress indicators from the blood or plasma (Papaharisis et al., 2019; Atamanalp et al., 2020; Cabrera-Álvarez et al., 2025). Likewise, compared with these inhumane methods, *ikejime*-killed fish have been shown to exhibit slower *post-mortem* lactic acid accumulation, delayed *rigor mortis*, and fewer bruises, often resulting in superior texture, flavour, and shelf life (Boyd et al., 1984; Tejada and Huidobro, 2002; Sorensen et al., 2004; Poli et al., 2005; Davie and Kopf, 2006).

Despite its advantages, the small size and variable morphology of fish brains necessitate a high degree of anatomical precision and operator expertise to perform *ikejime* accurately, and thus humanely, on live fish (EFSA, 2004; Davie and Kopf, 2006; Diggles, 2016). Moreover, its manual application is labour-intensive and often incompatible with the

high-throughput demands of modern aquaculture and fishery operations. A promising innovation addressing these limitations is the recently developed automated *ikejime* system (2022 prototype, Shinkei Systems, CA, USA), which uses AI-based computer vision to recognize species, locate the brain, and execute the spiking procedure with precision. However, as with all slaughter methods, neurological validation is essential to confirm that the system can render fish insensible until death (EFSA, 2004; WOAH, 2024). According to EFSA guidance, such validation should first be performed under controlled laboratory conditions to establish proof-of-concept, followed by testing under realistic commercial scenarios (EFSA, 2018). This stepwise framework ensures that any novel system, including automated *ikejime*, is scientifically sound, ethically defensible, and suitable for widespread industry adoption.

Therefore, the primary objective of this study was to generate welfare-relevant neurological data to evaluate the effectiveness of *ikejime* applied either manually (i.e., manual use of an *ikejime* device by an expert) or automatically (i.e., a pilot prototype system leveraging AI-based computer-vision to perform *ikejime*) for humanely killing fish. The assessment centered on the presence or absence of VERs in the EEG of rainbow trout (*Oncorhynchus mykiss*) and hybrid striped bass (white bass, *Morone chrysops* ♀ \times striped bass, *Morone saxatilis* ♂) following the application of these methods. For context, we compared results to those from manual percussion and asphyxiation, methods regarded as effective and ineffective, respectively, for humanely rendering fish insensible (EFSA, 2004; 2009; Kestin et al., 1995; Kestin et al., 2002; van de Vis et al., 2003). As an additional exploratory aim, we examined whether specific slaughter methods and/or their effectiveness influenced key *post-mortem* product quality traits, including muscle pH, evolution of *rigor mortis* (i.e., *post-mortem* muscle stiffening caused by biochemical changes, particularly ATP depletion), drip loss (i.e., the release of water, ions, and proteins from ruptured myofibrils during the transition from muscle to meat), and fillet colour. Due to logistical constraints in the field and the resource-intensive nature of EEG-based experimentation, only a subset of fish could be assessed for product quality. These data are presented transparently and interpreted with appropriate caution to identify potential trends and inform future research directions.

2. Materials and methods

2.1. Fish and housing conditions

Rainbow trout ($n = 31$, body mass = 383 ± 13 g, fork length = 330 ± 4 mm, all data are presented as means $\pm 95\%$ confidence intervals unless otherwise stated) and hybrid striped bass ($n = 34$, body mass = 1645 ± 102 g, fork length = 466 ± 8 mm), hereafter referred to as trout and bass, respectively, of mixed sex, were selected for this study. These aquaculture species represent a spectrum of muscle types (i.e., pink vs. white muscle type, respectively) and inhabit vastly different environmental conditions (i.e., cold freshwater vs. warm euryhaline habitat, respectively). Trout and bass were obtained from fish hatcheries in Charlestown, New Hampshire (Blue Stream Aquaculture) and Aurora, North Carolina (North Carolina State University's Pamlico Aquaculture Field Lab). Fish were transported to the Ritzman Aquaculture Lab (University of New Hampshire, NH, USA) where they were held for at least two weeks prior to experimentation. Each species was stocked evenly into separate indoor recirculating aquaculture systems (RAS) comprised of six 1500 L tanks equipped with screen filters, bead filters, biological filters, protein skimmers, ultraviolet sterilizers and temperature control. Water quality was monitored daily for dissolved oxygen (>6 mg/L), pH (7.5–8), and salinity (0 ppt), while ammonia and nitrite (≤ 0.25 ppm) were monitored weekly for the entirety of the experiment. All systems were under a 12 h:12 h light:dark photoperiod. Trout were maintained at 10°C while bass were held at 18°C . Fish were hand fed twice a day for six days a week on a commercial finfish diet (6- or 9-mm fish pellets with a minimum protein and crude fat content of 50 and 18

%, respectively; Skretting, St. Andrew, NS, Canada). Animal care and all experimental procedures were performed in accordance with UNH Institutional Animal Care and Use Committee guidelines (Protocol #220701).

2.2. Overview of experimental protocol

This study evaluated four slaughter methods applied to trout or bass, which included (i) manual *ikejime*, (ii) automated *ikejime*, (iii) manual percussion, and (iv) asphyxiation. The *ikejime* treatments represented the primary focus, while percussion and asphyxiation served as humane and inhumane reference methods, respectively (EFSA, 2004; Kestin et al., 1995; Kestin et al., 2002; van de Vis et al., 2003). Details regarding sample sizes and body morphometrics for each species across the various treatments are summarized in Table 1.

For each species, fish were individually captured with a hand net and alternately assigned to one of the four treatments to maintain balanced replication while minimizing handling time and stress (see 2.3.1.–2.3.4 for detailed protocols). In the *ikejime* and percussion treatments, fish were first subjected to the assigned method and then immediately instrumented with electrodes to record EEG continuously for up to 5 min or until neurological insensibility was confirmed, whichever occurred first (see 2.4.1.–2.4.2. for electrode placement, signal recording, and analysis). In the asphyxiation treatment, fish were lightly sedated, instrumented, allowed to recover fully, and then exposed to asphyxiation until neurological insensibility was reached (see 2.3.4. for detailed protocol).

Following EEG assessments, all fish were euthanized with a percussive blow to the head, weighed, measured, and bled in an ice slurry for 15 min after severing the gill arches. To maximize information obtained from each individual, a subset of these fish was used for exploratory analyses of *post-mortem* product-quality parameters (see 2.5. for detailed protocols). For the asphyxiation treatment, these product-quality analyses were conducted on separate groups to avoid potential confounding effects of the light sedation required during EEG instrumentation. Details regarding sample sizes and body morphometrics for each species within the various treatments included in the product-quality analyses are summarized in Table 2.

2.3. Slaughter protocols

2.3.1. Manual *ikejime*

Following capture, individual fish were firmly held by hand on a

Table 1

Summary of rainbow trout and hybrid striped bass included in and excluded from the EEG analyses for each slaughter method.

Slaughter method	Fish included in EEG analyses			Fish excluded from EEG analyses	
	n	Body mass (g)	Fork length (mm)	n	Reason for exclusion
<i>Manual ikejime</i>					
Rainbow trout	8	395 ± 26	331 ± 8	0	
Hybrid striped bass	8	1818 ± 167	480 ± 12	0	
<i>Automatic ikejime</i>					
Rainbow trout	8	372 ± 24	332 ± 7	0	
Hybrid striped bass	7	1483 ± 284	456 ± 18	1	Poor quality EEG recordings
<i>Manual percussion</i>					
Rainbow trout	8	395 ± 30	334 ± 9	0	
Hybrid striped bass	8	1472 ± 157	447 ± 13	0	
<i>Asphyxiation</i>					
Rainbow trout	1	305	314	3	EEG electrodes were dislodged
Hybrid striped bass	5	1720 ± 233	477 ± 14	3	EEG electrodes were dislodged

Body mass and fork length are presented as means ± 95 % confidence intervals.

Table 2

Summary of rainbow trout and hybrid striped bass included in the exploratory product-quality analyses across slaughter methods and their respective effectiveness.

Product quality parameters across treatments	Rainbow trout			Hybrid striped bass		
	n	Body mass (g)	Fork length (mm)	n	Body mass (g)	Fork length (mm)
<i>Muscle pH & rigor mortis</i>						
Manual <i>ikejime</i> (MI)	3	368 ± 26	329 ± 11	3	1787 ± 329	467 ± 18
Automatic <i>ikejime</i> (AI)	3	358 ± 23	334 ± 8	3	1481 ± 454	459 ± 31
Manual percussion (MP)	3	393 ± 39	337 ± 18	3	1658 ± 194	459 ± 21
Asphyxiation (AS)	3	383 ± 39	323 ± 3	3	1738 ± 336	475 ± 25
Humanely killed	6	375 ± 27	336 ± 10	1	1838	480
Inhumanely killed	3	383 ± 39	323 ± 3	6	1688 ± 217	467 ± 19
Transient VERs	3	368 ± 16	329 ± 4	5	1605 ± 301	459 ± 13
<i>Drip loss & fillet colour</i>						
Manual <i>ikejime</i> (MI)	3	427 ± 41	335 ± 19	3	1850 ± 216	492 ± 13
Automatic <i>ikejime</i> (AI)	3	359 ± 39	325 ± 8	3	1544 ± 573	459 ± 33
Manual percussion (MP)	3	389 ± 28	330 ± 11	3	1455 ± 214	445 ± 22
Asphyxiation (AS)	3	378 ± 24	319 ± 3	3	1799 ± 308	477 ± 32
Humanely killed	8	397 ± 27	331 ± 8	2	1529 ± 272	456 ± 12
Inhumanely killed	3	378 ± 24	319 ± 3	6	1664 ± 304	470 ± 24
Transient VERs	1	344	319	4	1726 ± 324	472 ± 31

Body mass and fork length are presented as means ± 95 % confidence intervals. Humanely killed fish were those that remained insensible following the application of the slaughter method (*i.e.*, no VERs detected throughout the entire EEG monitoring period), whereas inhumanely killed fish remained sensible (*i.e.*, VERs were present throughout). Transient VERs refers to individuals that temporarily displayed VERs during monitoring but were deemed insensible at the end of the period. Fish showing transient VERs were excluded from product-quality analyses related to kill effectiveness due to the subjectivity of classification.

table top. A 0.50 cm thick stainless-steel spike (IkiPik, www.theikipik.com) was then inserted manually into the brain cavity of trout or bass and rotated for 1–2 s to destroy central nervous tissues. This procedure was consistently performed on the fish within 0.5 min of removal from the water by the same experienced member of the *Ikejime* Federation throughout the study (Ikejime Federation, 2025).

2.3.2. Automated *ikejime*

Following capture, individual trout or bass were inserted into a pilot prototype of the automated *ikejime* machine (2022 model, Shinkei Systems, CA, USA). Briefly, fish slid down a chute within the machine, were automatically restrained, and subsequently killed *via* the insertion of a 0.32 cm thick stainless-steel tool. The entire process (*i.e.*, from holding tank to application of automated *ikejime*) was conducted within 2 min by the same experienced member of the Shinkei Systems team throughout the study. The tool trajectory was determined *via* a computer-vision system that utilized a trained model to define brain location labels for each individual. Importantly, the system used was a prototype not originally designed for the two studied species, and thus the trained model was based on a limited data set. The system was run on trout utilizing a model originally trained on ~1.1 kg Coho salmon (*Oncorhynchus kisutch*), which was developed from 800 images of 300

individuals. In contrast, the system was run on striped bass using a newly trained model based on 150 images of 10 individual striped bass.

2.3.3. Manual percussion

Following capture, individual trout or bass were firmly held by hand on a table top and subjected to a sharp cranial blow by an aluminum fish bat (Offshore Angler™, Bass Pro Shop, Auburn, NY, USA). This procedure was consistently performed on the fish within 0.5 min of removal from the water by the same experienced researcher throughout the study to minimize variation in accuracy, impulse, or kinetic energy.

The maximum impact force (N), impulse (N·s) and kinetic energy (J) generated by a percussive blow from the aluminum fish bat were evaluated using a KAM force transducer (ProMess, Berlin, Germany) with a maximum load of 20 kN. The load cell was connected to a QuantumX MX440B amplifier (Hööttinger Brüel & Kjaer [HBK], Darmstadt, Germany) recording at 19200 Hz via Catman-AP (HBK), enabling the measurement of the force (N) produced by each percussive blow over time. To simulate the experimental conditions, the load cell was struck by the abovementioned experienced researcher aiming to replicate the force and accuracy of the blows previously delivered to trout and bass. A total of 10 percussive blows were analysed. The peak of the recorded force trace for each blow was determined to be the maximum impact force, while force traces were also integrated using Origin software (OriginLab Corporation, Northampton, USA) to calculate the impulse. The kinetic energy generated by each blow was subsequently calculated (*i.e.*, kinetic energy = $\frac{1}{2} m \times v^2$, where m was the mass of the aluminum fish bat [0.290 kg], and v was the velocity of the percussive blow, which was determined by dividing impulse with the mass of the bat) (Brijts et al., 2021). The maximum impact force generated by a percussive blow from the aluminum fish bat ranged between 3346 and 10,145 N (7735 \pm 1274 N), the impulse ranged between 2.67 and 4.89 N·s (4.08 \pm 0.37 N·s), and the kinetic energy ranged between 12.27 and 41.16 J (29.28 \pm 4.84 J).

2.3.4. Asphyxiation

Individual trout or bass were captured from their housing tanks and lightly sedated in water containing 5 mg/L metomidate (Aquacalm™, Syndel, Ferndale, Washington, USA). Upon sedation, as indicated by a loss of equilibrium, fish were instrumented with EEG electrodes and placed within a light exclusion container (see 2.4.1. for electrode placement). Fish were allowed to fully recover from sedation, as indicated by the recovery and maintenance of equilibrium, as well as the presence of VERs that were visually determined from the on-line analysis of the beta waves within the EEG (see 2.4.2. for EEG signal recording and analysis). Following recovery, the water was drained from the light exclusion container and the EEG of asphyxiating fish were continuously recorded in response to light flashes until the visual disappearance of VERs from the on-line analysis of the beta waves within the EEG was observed. Ambient air temperatures during these trials ranged between 1 and 4 °C. Once EEG recordings were obtained, fish were euthanized with a sharp blow to the head, weighed and measured.

Parameters related to product quality were evaluated in a separate group of trout and bass (see Table 2) that were captured from their housing tanks and immediately placed in a tote of ice to asphyxiate in air to avoid the potential confounding effects of the light sedation that was required to instrument fish with the EEG electrodes. Following at least 30 min of asphyxiation, fish were euthanized with a sharp blow to the head, weighed, measured, and bled out in an ice slurry for 15 min after severing their gill arches.

2.4. EEG methodology

2.4.1. EEG electrode instrumentation

EEG of fish were recorded via two stainless-steel needle electrodes tailored to the specific species (*i.e.*, 23-gauge needles for trout, 19-gauge needles for bass). The needle electrodes were carefully inserted

intracranially at positions \sim 0.5–1.0 cm caudal of the eyes, \sim 0.5–1.5 cm lateral of the sagittal suture, and \sim 0.5–1.0 cm deep depending on the size of the fish (Fig. 1A-D). A 23-gauge stainless-steel needle electrode was also inserted into the tissue near the tail of the fish to function as a common ground electrode. Fish were then immediately placed in a custom-made, light-exclusion container made out of PVC (length = 76.5 cm, width = 22.5 cm, height = 30.0 cm) that was filled with 15 L of water from the housing tank of the fish. The duration of EEG electrode instrumentation was 0.6 ± 0.1 min for trout and 1.1 ± 0.2 min for bass, with instrumentation taking longer in the latter due to the difficulty of inserting the electrodes through their thick scales and robust skull.

2.4.2. Recording, acquisition and analyses of EEG

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 bio-amplifier (FE136, ADInstruments), which was interfaced with a PowerLab (ML 870, 8/30, ADInstruments) and a PC equipped with LabChart Pro software (version 8.1.19, 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 60 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 analyzing 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; Bowman et al., 2020). The Scope View module within the LabChart Pro software averaged 30 to 120 non-overlapping consecutive epochs that displayed 50 ms before and 400 ms after the flash, representative of the beta wave for 15 to 60 s of recordings, respectively, to obtain specific determinations of when VERs were present or absent (Hjelmstedt et al., 2022; Brijts et al., 2025). 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. In this study, 'immediate' refers to the absence of VERs at the first post-application EEG measurement, while 'irreversible' indicates that VERs did not return at any point during the monitoring period.

2.5. Product quality measures

Following the procedures described above, six individuals per species per treatment were selected at random for a suite of product quality measures. Within each combination of species and treatment, three individuals were left whole and placed in plastic bags for muscle pH and *rigor mortis* analyses (see 2.5.1. and 2.5.2., respectively, and Table 2), while the other three individuals were immediately filleted and placed skin down in covered containers for analyses of drip loss and fillet colour (see 2.5.3. and 2.5.4., respectively, and Table 2). Filleting was conducted by the same experienced researcher to ensure consistency. Containers and bags were individually labeled and stored in a walk-in refrigerator at 4 °C for the entirety of the quality measure time courses.

2.5.1. Muscle pH

Muscle pH was measured in whole fish daily for 7 days using a portable pH meter (model HI98191, Hanna Instruments, Rhode Island, USA) equipped with an electrode designed for meat and semi frozen products (model FC230B, Hanna instruments). Briefly, a scalpel was used to make a small incision (\sim 1 cm) each day through the skin above

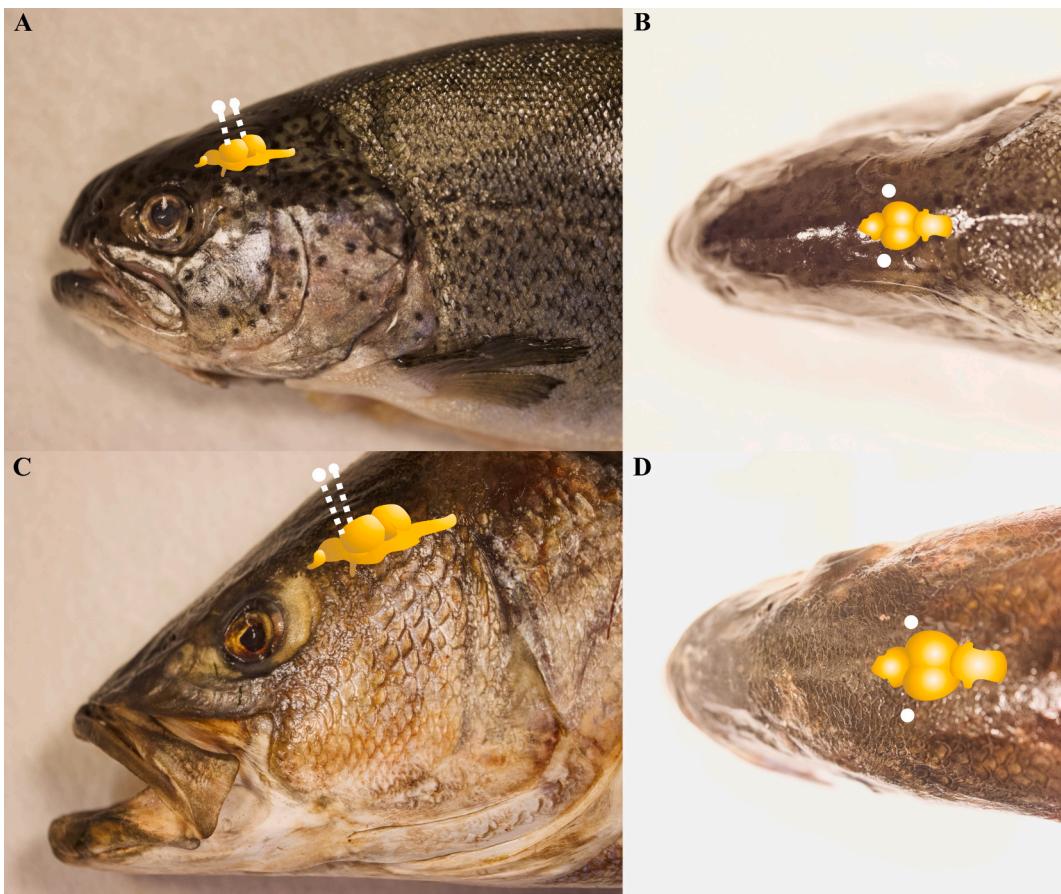


Fig. 1. Schematic illustration of brain location and EEG electrode placement in rainbow trout and hybrid striped bass. The brain (yellow schematic) and approximate placement of EEG electrodes (white pins) are shown from lateral and dorsal views in both rainbow trout (A–B) and hybrid striped bass (C–D). For both *ikejime* and manual percussion, the target is the centre of the brain, however, *ikejime* additionally requires destruction of all brain tissue to ensure effectiveness. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the lateral line on the anterior half of the fish so that the tip of the pH probe could be inserted into the muscle. The pH probe was cleaned and calibrated daily before each series of measurements.

2.5.2. Rigor mortis

Rigor mortis was assessed in whole fish using the Cuttinger's or tail drop method, from which a *rigor mortis* index was calculated as follows: $[(TD_0 - TD_T) / TD_0] \times 100$ (Diouf and Rioux, 1999). TD represents the vertical drop of the tail in cm when half of the fish's fork length is placed on the edge of a table, TD_0 is the initial tail drop at the start of the experiment, and TD_T represents subsequent measurements throughout the experiment.

Sampling was conducted continuously across the full *post-mortem* period, including overnight, to ensure uninterrupted tracking of the *rigor mortis* cycle for each fish. Measurements were taken at higher frequency during the early *post-mortem* phase, when the onset and peak of *rigor mortis* typically occur rapidly, and less frequently during the resolution phase, when changes progress more gradually. When multiple fish required attention simultaneously, individuals were visually monitored for signs of *rigor mortis* progression, and if no detectable change was observed, formal measurements were deferred to the next scheduled interval. Accordingly, the *rigor mortis* index was measured at $\sim 1.6 \pm 0.3$ h, 4.6 ± 0.6 h, 8.7 ± 0.9 h, 10.1 ± 0.8 h, 19.6 ± 1.4 h, 24.6 ± 1.2 h, 30.3 ± 1.2 h, 43.2 ± 2.0 h, 50.5 ± 1.2 h, 54.9 ± 1.2 h, 64.2 ± 3.3 h, and 72.0 ± 3.0 h *post-mortem*.

2.5.3. Drip loss

Drip loss was calculated using similar sized portions (~ 30 g) cut from

the anterior region of the loin using the opposite fillet required for colour analyses. Portions were placed individually in sterile, covered pyrex dishes at 4 °C and weighed every 48 h over a 10-day period. Drip loss was calculated as follows: $[(W_0 - W_T) / W_0] \times 100$, where W_0 represents the initial weight of a fillet portion and W_T represents subsequent measurements throughout the experiment (Kristoffersen et al., 2007).

2.5.4. Fillet colour

Colorimetric assessments were performed at two locations along the epaxial section of each fillet using a colorimeter (CR-10, Konica Minolta, Japan) every 2 days for 10 days. Measurements of the brightness (L), redness (a) and yellowness (b) of each fillet were collected anterior of the dorsal fin and above the anal fin. These measurements were subsequently averaged to account for potential colour differences between anterior and posterior regions of the fillet. The overall change in the colour of each fillet (ΔE) over time was calculated as the square root of $[(L_0 - L_T)^2 + (a_0 - a_T)^2 + (b_0 - b_T)^2]$, whereby L_0 , a_0 and b_0 were the initial averaged brightness and chromaticity value of the fish fillets, and L_T , a_T and b_T represents subsequent averaged measurements throughout the experiment (Schubring, 2009).

2.6. 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, as well as model output and inference, can be found in Supp. Info. 1–5, while the R scripts and figure codes can be found in Supp. Info. 6. Statistical significance was determined at a threshold of $p < 0.05$. Where applicable, post-hoc comparisons were adjusted for multiple testing using the Benjamini–Hochberg procedure (Benjamini and Hochberg, 1995).

The Kaplan–Meier method was conducted to evaluate the effects of the type of slaughter method (i.e., manual *ikejime*, automated *ikejime*, manual percussion, and asphyxiation) on the proportion of trout or bass that displayed VERs throughout the monitoring period (Supp. Info. 1). The outcome for each case was either when the ‘event’ occurred (i.e., when an individual lost VERs) or the individual was ‘censored’ (i.e., when an individual displayed VERs for the entire monitoring period). A Breslow test and log rank test were conducted to determine whether significant differences occurred between the Kaplan–Meier survival distributions of these groups, with the former emphasizing differences at earlier time points and the latter at later time points. The asphyxiation treatment group for trout was excluded from the statistical analysis, as only one reliable EEG recording was obtained. Effect size measures or hazard ratios between Kaplan–Meier survival distributions of individuals killed using the different methods were also calculated for each species using the Cox proportional hazards model.

In order to maximize the information obtained from each individual and minimize animal use, product-quality analyses (i.e., muscle pH, *rigor mortis*, drip loss, and fillet colour; Supp. Info. 2–5) were conducted as an exploratory component on a subset of fish. Given the logistical and ethical constraints associated with neurological assessments in a field setting, only a limited number of fish could be processed for product-quality assessments (Table 2). For method-type comparisons, all available data were included. For analyses based on kill effectiveness, individuals were classified as (i) humanely killed (i.e., when VERs were absent throughout the entire monitoring period; $n = 17$ of 48), (ii) inhumanely killed (i.e., when VERs were present throughout the entire monitoring period; $n = 18$ of 48), or (iii) transiently sensible (i.e., when VERs disappeared only after a delay; $n = 13$ of 48). Transiently sensible fish were excluded from the product-quality analyses because the level of stress or distress experienced during the period of sensibility could not be reliably determined without concurrent physiological stress measurements. Thus, by focusing on the two extremes (i.e., fish that either remained insensible or sensible throughout the entire monitoring period), enabled a clearer interpretation of how slaughter effectiveness may influence *post-mortem* product quality.

For slaughter method comparisons, linear mixed-effects models were used for parameters measured repeatedly over time (i.e., muscle pH, drip loss, and fillet colour, Supp. Info. 2, 4 & 5). These models included species, method, time, and their interactions, with individual fish included as a random intercept to account for repeated measures. The most suitable functional form of time (linear, quadratic, or cubic) was determined using model comparison criteria (AIC, BIC, R^2). For *rigor mortis*, which was analysed at distinct stages (onset, peak, resolution), linear models were applied with species, method, and their interaction as predictors (Supp. Info. 3). To ensure transparency, mean model estimates and 95 % confidence intervals are displayed alongside raw data points.

3. Results

3.1. Effectiveness of various slaughter methods

Rainbow trout and hybrid striped bass exhibited head shakes and tail flaps when removed from water and restrained (i.e., in an attempt to escape), followed by brief muscle tremors and/or fin flaring during manual *ikejime*, automated *ikejime* and manual percussion. These movements quickly subsided, with little to no activity observed for the remainder of the monitoring period, depending on the effectiveness of the method. In contrast, asphyxiation caused intense head shakes, tail

flapping, thrashing, and gasping in both species, lasting until VERs disappeared (see below). This behaviour was particularly pronounced in trout, restricting reliable EEG recordings to only one individual.

In trout, VERs were absent in 75 % of individuals at the time of instrumentation (i.e., within ~0.6 min) following the application of manual *ikejime*, automated *ikejime* and manual percussion (Fig. 2A). The proportion of irreversibly insensible trout increased to 100 % within 2 min of being subjected to these methods (Fig. 2A). In bass, VERs were absent upon instrumentation (i.e., within ~1.1 min) in a proportion of individuals subjected to automated *ikejime* and manual percussion (i.e., 14 and 50 %, respectively) but not following manual *ikejime* (Fig. 2B). However, by the end of the monitoring, VERs were absent in 75, 57 or 75 % of bass subjected to manual *ikejime*, automated *ikejime* or manual percussion, respectively (Fig. 2B). During asphyxiation, it took 12.75 min for the loss of VERs to occur in trout and 3.5 to 15.75 min in bass (Fig. 2A–B).

Survival distribution analyses of time to loss of VERs revealed no statistically significant differences among slaughter methods in trout (Breslow test: $\chi^2(2) = 0.10$, $p = 0.90$; Log-rank test: $\chi^2(2) = 1.60$, $p = 0.50$). In striped bass, the Breslow test suggested a trend toward differences among methods ($\chi^2(3) = 6.50$, $p = 0.09$), although this was not statistically significant, while the log-rank test similarly indicated no differences ($\chi^2(3) = 4.10$, $p = 0.30$). Pairwise Cox proportional hazards models corroborated these findings, with no significant differences after adjustment (all adjusted $p > 0.25$), but hazard ratios indicated that striped bass subjected to asphyxiation tended to lose VERs more slowly than those exposed to manual percussion (HR = 0.22, 95 % confidence intervals: 0.05–1.05).

3.2. Impact of various slaughter methods on product quality

The following results are based on exploratory product-quality analyses and should therefore be interpreted with caution. Given the small and/or uneven sample sizes, and baseline pigmentation differences between species, the findings are indicative rather than confirmatory and may have limited generalizability. To promote transparency, we emphasize effect sizes and corresponding 95 % confidence intervals (either in the text or figures) over p -values, focusing on the magnitude and direction of effects rather than strict statistical significance. This approach aims to highlight broad trends in *post-mortem* muscle pH, *rigor mortis*, drip loss, and fillet colour that can inform future hypothesis-driven studies, rather than drawing strong inferences about subtle species-, method-, or time-specific differences.

3.2.1. Post-mortem muscle pH

Muscle pH was analysed as a function of slaughter method, species, and time. When averaged across predictors, slaughter method ($\eta^2 = 0.07$, $\omega^2 = 0.03$; $F_{3,63.1} = 1.64$, $p = 0.19$) and species ($\eta^2 = 0.01$, $\omega^2 = 0.00$; $F_{1,63.1} = 0.86$, $p = 0.36$) showed only small to negligible effects. In contrast, time exerted a strong influence on *post-mortem* muscle pH ($\eta^2 = 0.39$, $\omega^2 = 0.37$; $F_{3,153} = 31.0$, $p < 0.001$), with values remaining relatively stable or slightly increasing for approximately 4–6 days *post-mortem* before declining toward the end of the monitoring period (Fig. 3A).

Interaction terms revealed moderate to large effect sizes consistent with the visual trends (Fig. 3A). Trout exhibited greater divergence among slaughter methods than striped bass (species \times method: $\eta^2 = 0.17$, $\omega^2 = 0.11$; $F_{3,16} = 3.37$, $p = 0.045$), and temporal trajectories differed among methods (method \times time: $\eta^2 = 0.10$, $\omega^2 = 0.06$; $F_{9,153} = 1.95$, $p = 0.049$). Muscle pH in fish killed via manual percussion tended to decline earlier (~3–4 days *post-mortem*) compared with other methods (~4–5 days).

Species-specific temporal effects were small (species \times time: $\eta^2 = 0.05$, $\omega^2 = 0.03$; $F_{3,153} = 2.61$, $p = 0.054$). Data were therefore pooled across species to evaluate potential differences in kill effectiveness irrespective of method. This exploratory comparison indicated no

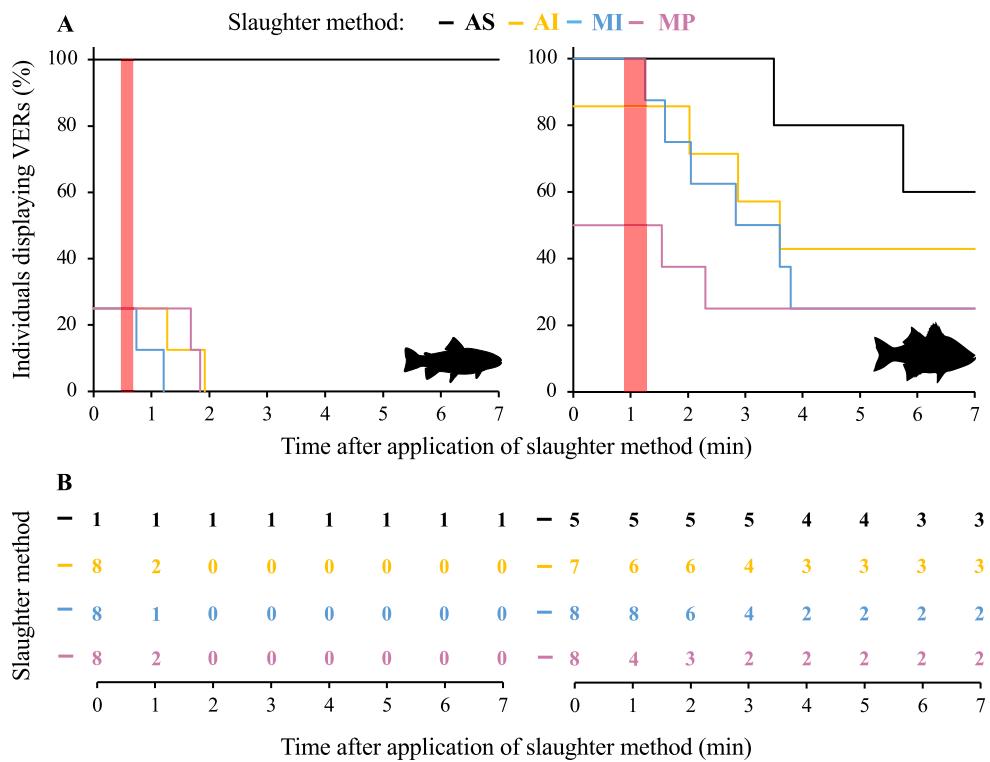


Fig. 2. Time taken for farmed fishes to be rendered irreversibly insensible following the application of various slaughter methods. (A) Kaplan–Meier survival distributions and (B) corresponding number-at-risk tables (i.e., individuals still displaying visually evoked responses [VERs] at each time point) for rainbow trout (left panels) and hybrid striped bass (right panels) during asphyxiation (AS) or following the application of automated ikejime (AI), manual ikejime (MI), and manual percussion (MP). The red shaded area in (A) indicates the 95 % confidence interval for when EEG monitoring commenced following electrode instrumentation. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

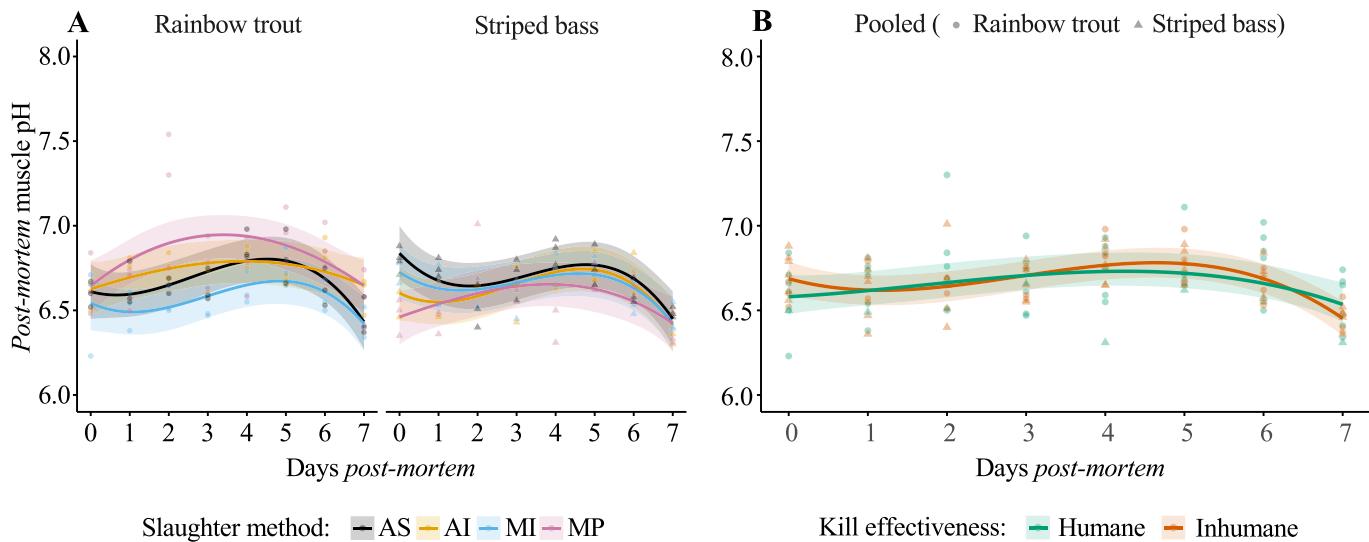


Fig. 3. Temporal changes in post-mortem muscle pH in rainbow trout and striped bass. (A) Post-mortem muscle pH of rainbow trout (left panel) or hybrid striped bass (right panel) subjected to various slaughter methods (asphyxiation [AS], automated ikejime [AI], manual ikejime [MI], manual percussion [MP]; $n = 3$ per method within each species) and (B) post-mortem muscle pH of humanely or inhumanely killed fish irrespective of method and pooled across species (rainbow trout: $n = 6$ humane, $n = 3$ inhumane; hybrid striped bass: $n = 1$ humane, $n = 6$ inhumane). Lines and shaded ribbons represent model-estimated means ± 95 % confidence intervals, while circles and triangles represent raw data points for individual rainbow trout and hybrid striped bass, respectively.

substantial differences between humanely and inhumanely killed fish, as both groups displayed overlapping trajectories that remained relatively stable or slightly increased during the first 4–5 days before declining toward the end of the monitoring period (Fig. 3B).

3.2.2. Temporal dynamics of rigor mortis

Onset, peak, and resolution of rigor mortis were analysed as a function of slaughter method and species. Onset of rigor mortis showed very strong effect of slaughter method ($\eta^2 = 0.91$, $\omega^2 = 0.86$, $F_{3,16} = 51.03$, $p < 0.001$), while species had a negligible effect when averaged across model predictors ($\eta^2 = 0.00$, $\omega^2 = 0.00$, $F_{1,16} = 0.06$, $p = 0.82$).

However, a very strong interaction effect indicated species-specific responses (species \times method: $\eta^2 = 0.68$, $\omega^2 = 0.57$, $F_{3,16} = 11.47$, $p < 0.001$). Asphyxiation and automated *ikejime* produced the fastest onset of *rigor mortis* (~ 1.4 – 2.2 h *post-mortem*), whereas manual *ikejime* and percussion resulted in slower development that varied between species (Fig. 4A).

The peak of *rigor mortis* was similarly dominated by slaughter method, showing an exceptionally strong effect ($\eta^2 = 0.99$, $\omega^2 = 0.99$, $F_{3,16} = 562.03$, $p < 0.001$), whereas the effect of species was moderate when averaged across model predictors ($\eta^2 = 0.16$, $\omega^2 = 0.08$, $F_{1,16} = 3.02$, $p = 0.10$). Furthermore, a strong interaction indicated species-specific responses (species \times method: $\eta^2 = 0.52$, $\omega^2 = 0.37$, $F_{3,16} = 5.76$, $p = 0.007$). Typically, the peak of *rigor mortis* occurred earliest following automated *ikejime* and asphyxiation (between ~ 4.9 – 6.2 h), intermediate after percussion (between ~ 9.5 – 11.7 h), and substantially later after manual *ikejime* (~ 21 – 23 h) in both species (Fig. 4B).

The resolution of *rigor mortis* showed a strong effect of species ($\eta^2 = 0.51$, $\omega^2 = 0.39$, $F_{1,16} = 16.40$, $p < 0.001$) and a moderate effect of method ($\eta^2 = 0.31$, $\omega^2 = 0.15$, $F_{3,16} = 2.41$, $p = 0.11$). The strong interaction effect (species \times method: $F_{3,16} = 3.19$, $p = 0.052$, $\eta^2 = 0.37$, $\omega^2 = 0.22$) indicated method-specific differences between species. Bass generally exhibited a later *rigor mortis* resolution (~ 53 – 71 h) than trout (~ 48 – 54 h), particularly after automated *ikejime* and percussion (Fig. 4C).

Given the lack of overall species differences in the timing of *rigor mortis* onset and peak (but not resolution), data were pooled across

species to explore potential effects of kill effectiveness irrespective of method. This exploratory analysis suggested that humanely killed individuals may show a delayed onset of *rigor mortis*, occurring at 4.3 h (95 % confidence intervals: 2.7–6.0) compared with 2.2 h (95 % confidence intervals: 0.7–3.6) in inhumanely treated fish (Fig. 4D). A similar tendency was observed for peak *rigor mortis*, which occurred at 11.8 h (95 % confidence intervals: 7.6–16.0) in humanely treated individuals and 5.9 h (95 % confidence intervals: 2.2–9.6) in inhumanely treated ones (Fig. 4E), although overlapping confidence intervals and limited sample sizes of humanely and inhumanely killed fish warrant cautious interpretation.

3.2.3. Post-mortem drip loss

Drip loss was analysed as a function of slaughter method, species, and time. When averaged across model predictors, the effects of slaughter method ($\eta^2 = 0.00$, $\omega^2 = 0.00$, $F_{3,63.9} = 0.10$, $p = 0.96$) and species ($\eta^2 = 0.02$, $\omega^2 = 0.00$, $F_{1,63.9} = 1.59$, $p = 0.21$) were negligible to small. In contrast, time exerted a strong effect on drip loss ($\eta^2 = 0.69$, $\omega^2 = 0.67$, $F_{2,86.0} = 94.32$, $p < 0.001$), with values increasing markedly throughout the *post-mortem* period (Fig. 5A).

Interaction terms revealed large to strong effects consistent with visual trends (Fig. 5A). Trout showed substantially higher drip loss following asphyxiation than after other methods, whereas striped bass displayed relatively uniform responses (species \times method: $\eta^2 = 0.38$, $\omega^2 = 0.23$, $F_{3,16.0} = 3.25$, $p = 0.05$). The rate of increase in drip loss also varied among methods (method \times time: $\eta^2 = 0.18$, $\omega^2 = 0.12$, $F_{6,86.0} =$

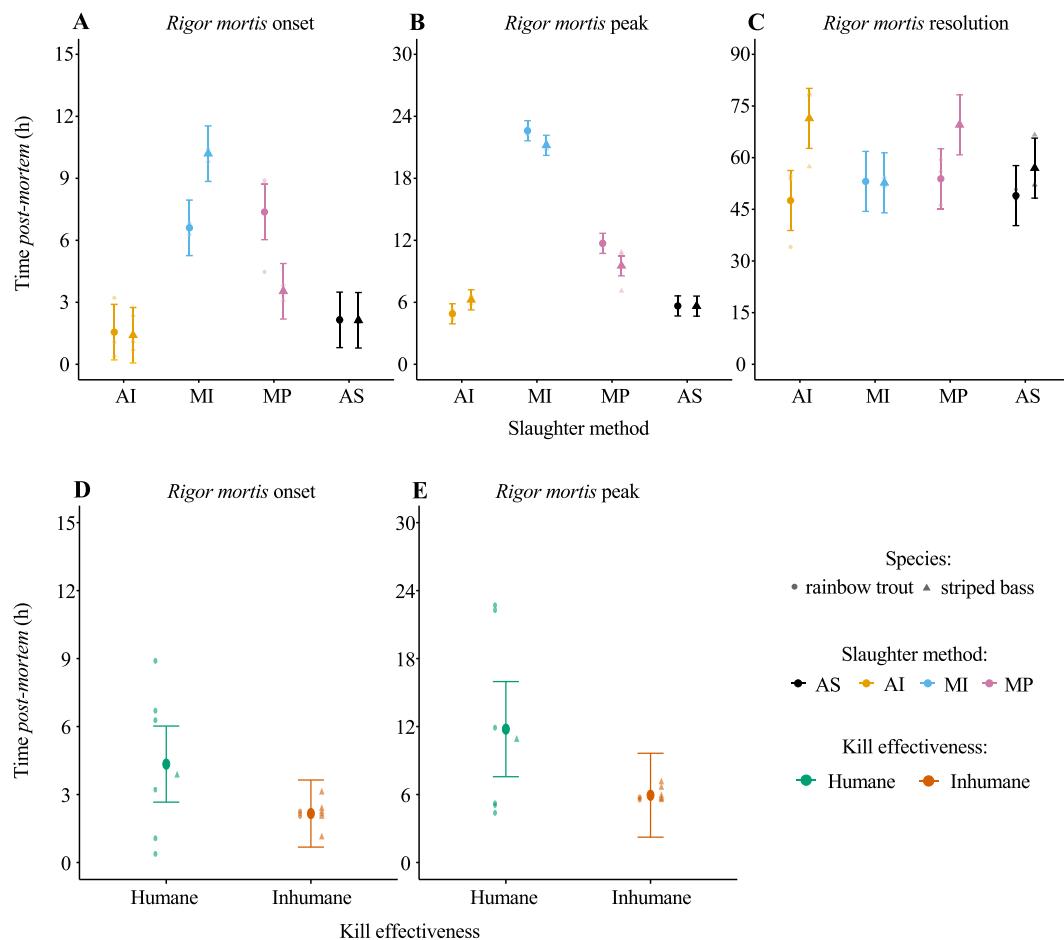


Fig. 4. Temporal dynamics of *rigor mortis* in rainbow trout and striped bass. Timing of the various stages of *rigor mortis* in rainbow trout and hybrid striped bass stratified by (A-C) slaughter method (asphyxiation [AS], automated *ikejime* [AI], manual *ikejime* [MI], manual percussion [MP]; $n = 3$ per method within each species) or (D-E) the effectiveness of the kill irrespective of method and pooled across species (rainbow trout: $n = 6$ humanely, $n = 3$ inhumanely; striped bass: $n = 1$ humanely, $n = 6$ inhumanely). Large symbols with error bars denote model-estimated means $\pm 95\%$ confidence intervals, while smaller transparent points show raw data for individual fish.

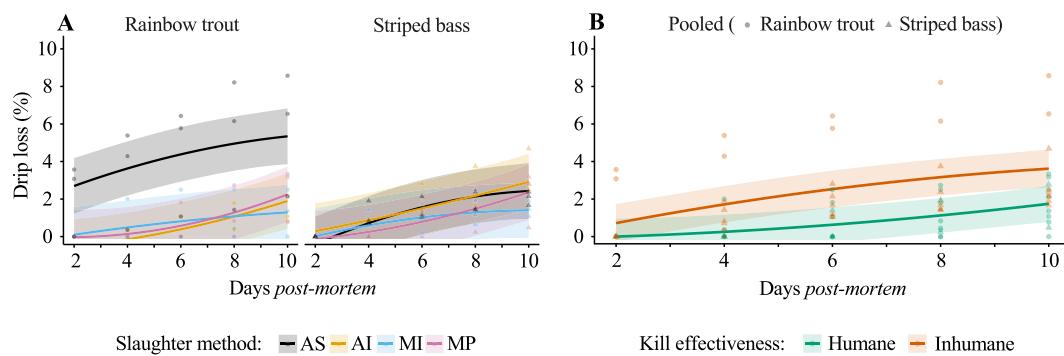


Fig. 5. Post-mortem drip loss from rainbow trout and hybrid striped bass fillets. (A) Post-mortem drip loss of rainbow trout (left panel) or hybrid striped bass (right panel) subjected to various slaughter methods (asphyxiation [AS], automated ikejime [AI], manual ikejime [MI], manual percussion [MP]; $n = 3$ per method within each species) and (B) post-mortem drip loss of humanely or inhumanely killed fish irrespective of method and pooled across species (rainbow trout: $n = 8$ humane, $n = 3$ inhumane; hybrid striped bass: $n = 2$ humane, $n = 6$ inhumane). Lines and shaded ribbons represent model-estimated means $\pm 95\%$ confidence intervals, while circles and triangles represent raw data points for individual rainbow trout and hybrid striped bass, respectively.

3.06, $p = 0.009$), most notably between asphyxiation and manual ikejime, which diverged strongly over time (Fig. 5A).

Since species-specific temporal effects were negligible (species \times time: $\eta^2 = 0.01$, $\omega^2 = 0.00$, $F_{2,86} = 0.43$, $p = 0.65$), data were pooled across species to evaluate potential impacts of kill effectiveness irrespective of method. This exploratory analysis indicated that while both humanely and inhumanely killed individuals showed steadily increasing drip loss over time, inhumanely killed fish consistently exhibited higher values, with only partial overlap of confidence intervals (Fig. 5B).

3.2.4. Post-mortem changes in fillet colour of trout and bass

Fillet colour was analysed as a function of slaughter method, species, and time. Fillet colour parameters showed clear differences between species and, to a lesser extent, among slaughter methods. Species effects on redness ($\eta^2 = 0.94$, $\omega^2 = 0.93$, $F_{1,20.43} = 318.30$, $p < 0.001$) and yellowness ($\eta^2 = 0.908$, $\omega^2 = 0.89$, $F_{1,24.70} = 224.96$, $p < 0.001$) were strong, while method effects on yellowness were large ($\eta^2 = 0.28$, $\omega^2 = 0.18$, $F_{3,24.70} = 3.15$, $p = 0.043$). The redness and yellowness of the fillets were approximately seven- and three-fold higher, respectively, in trout than in striped bass, while yellowness appeared lowest in individuals subjected to automated ikejime.

Beyond these main effects, all colour parameters were consistently shaped by time in a species-specific manner. Colour change ($\eta^2 = 0.15$, $\omega^2 = 0.13$; $F_{2,86} = 7.43$, $p = 0.001$), brightness ($\eta^2 = 0.14$, $\omega^2 = 0.13$; $F_{2,110} = 9.29$, $p < 0.001$), redness ($\eta^2 = 0.14$, $\omega^2 = 0.12$; $F_{2,110} = 8.81$, $p < 0.001$), and yellowness ($\eta^2 = 0.20$, $\omega^2 = 0.18$; $F_{2,110} = 13.62$, $p < 0.001$) all showed consistent species \times time interactions. Trout exhibited more dynamic trajectories, with early increases followed by stabilization or decline, whereas striped bass displayed more gradual, monotonic increases over time (Supp. Info. 5).

Given these clear species-specific differences in temporal trajectories, data could not be pooled across species to explore potential effects of kill effectiveness irrespective of method.

4. Discussion

This investigation represents the first neurological evaluation of the effectiveness of manual ikejime, shedding light on potential limitations of a method increasingly adopted by small-scale aquaculture and fishery operations. We also tested a pilot prototype of an automated system that uses AI-based computer-vision to identify fish species, locate the brain, and precisely perform the ikejime procedure. This automated approach showed promise and, with further refinement, could become a transformative tool for small-scale producers. However, in accordance with EFSA guidance, such novel technologies must undergo rigorous proof-of-concept trials under controlled laboratory conditions, as well as validation studies under realistic production settings, before any

commercial application can be considered (EFSA, 2018). By providing the first neurological data on the capacity of ikejime to induce insensibility in two morphologically distinct species (*i.e.*, rainbow trout and hybrid striped bass), our study represents an important step toward that validation process.

Inhumane slaughter methods such as asphyxiation remain widespread, despite causing fish to endure prolonged fear, anxiety, pain, and suffering in their final moments (EFSA, 2004; Lines and Spence, 2014; Wahltinez et al., 2024; WOAH, 2024). In this study, trout and bass subjected to asphyxiation displayed clear signs of severe distress, including vigorous head shaking, tail flapping, and gasping, before reaching a state of insensibility, which occurred after approximately 12.75 min and 3.5–15.75 min, respectively. Despite limited recordings, these durations align with previous findings that the time to loss of sensibility during asphyxiation is strongly temperature dependent. Kestin et al. (1991) reported that trout lost VERs after an average of 2.6 min at 20 °C, 3.0 min at 14 °C, and 9.6 min at 2 °C, demonstrating that colder conditions substantially prolong the period of sensibility. The protracted times till loss of sensibility observed in our study, conducted at ambient air temperatures of 1–4 °C, therefore align with this temperature-dependent effect. Similarly, gilthead seabream required an average of 5.5 min to lose VERs during asphyxiation (Kestin et al., 2002; van de Vis et al., 2003), although ambient temperature, likely considerably warmer than in our trials, was not reported. Collectively, these comparisons confirm that asphyxiation induces a slow and distressing death across species and environmental conditions, reinforcing the urgent need for the aquaculture and fishery sectors to phase out this method in favour of humane slaughter procedures that induce immediate and irreversible insensibility until death.

The manual application of ikejime is widely believed to offer a viable alternative, especially for small-scale aquaculture and fishery operations. Yet, neurological evidence supporting its effectiveness as a humane method has until now been lacking. In the present study, conclusions regarding the immediacy of insensibility are somewhat constrained by the time elapsed between the application of the method and EEG electrode placement. Nonetheless, given that it takes >1 s to complete the ikejime procedure when performed by hand (Duque et al., 2019; Cabrera-Álvarez et al., 2025; Hoyo-Alvarez et al., 2025), and VERs were still detectable upon instrumentation in at least a subset of individuals from both species, our findings indicate that the traditional administration of ikejime does not reliably induce an immediate loss of sensibility. Despite this limitation, our neurological assessments revealed that manual ikejime was relatively effective in inducing irreversible insensibility in trout (~ 0.3 –0.5 kg), with 75 % of individuals rendered irreversibly insensible upon instrumentation (within ~ 0.6 min) and the remaining 25 % within 2 min. In contrast, bass (~ 1.1 –2.1 kg) appeared more resilient, as none were immediately rendered

insensible upon instrumentation (within ~ 1.1 min), and 25 % retained VERs at the end of the monitoring period. Interestingly, the effectiveness of manual *ikejime* in both species was comparable to that of manual percussion in the present study, suggesting that the success of these manually applied methods may be governed by similar factors, particularly the accuracy of technique and/or the amount of force or kinetic energy imparted during application (Kestin et al., 1995; Kestin et al., 2002; Robb et al., 2000; van de Vis et al., 2003; Brijs et al., 2021). This in turn will likely be influenced by allometric and species-specific differences in brain size, position, and cranial morphology (Emerson and Bramble, 1993; van Staaden et al., 1994).

Previous neurological assessments have demonstrated that the success of manually applied *ikejime* is highly dependent on the accuracy of application. For example, Atlantic salmon ($\sim 3\text{--}5$ kg) could be rendered immediately and irreversibly insensible when accurately spiked using a modified pneumatic pistol, whereas inaccurate applications delayed the loss of sensibility by up to 4.5 min (Robb et al., 2000; Kestin et al., 2002; van de Vis et al., 2003). In the present study, inaccuracy may have contributed to the limited effectiveness of manual *ikejime* in at least some bass, as their robust skulls likely impeded spike penetration and compromised the accuracy of the spike angle during the initial struggle. However, accuracy alone may not fully explain the observed variability in outcomes. Despite the majority of both trout and bass being accurately targeted, some of these individuals still displayed residual VERs, mirroring patterns observed in the aforementioned salmon study where even precisely executed spikes occasionally resulted in delayed loss of VERs for up to 49 s (Robb et al., 2000; Kestin et al., 2002; van de Vis et al., 2003). Visual inspection further revealed that the hole created by the *ikejime* spike destroyed a larger proportion of the brain in trout than bass, suggesting that methods such as manual *ikejime* must not only accurately target the brain but also achieve extensive neural destruction to ensure immediate and irreversible insensibility. To comply with humane slaughter standards, it is therefore recommended that the manual administration of *ikejime* not only be preceded by a stunning method proven to induce immediate insensibility, but that operators also ensure sufficient destruction of central nervous tissue by using an appropriately sized tool and applying sufficient rotational motion within the cranium (EFSA, 2004; Davie and Kopf, 2006; Diggles, 2016).

Percussive stunning has previously been recommended as a method to induce immediate insensibility prior to the manual application of *ikejime* (EFSA, 2004; Davie and Kopf, 2006; Diggles, 2016). When applied accurately and with sufficient force, impulse, or kinetic energy, manually administered percussive stunning, whether by hand (using a fish priest or bat) or *via* pneumatic pistols, has been shown to induce an immediate and irreversible state of insensibility in species of carp (Lambooij et al., 2007), catfish (Brijs et al., 2021; Hjelmstedt et al., 2025), seabream (van de Vis et al., 2003), salmonids (Kestin et al., 1995, 2002; Robb et al., 2000; Kestin et al., 2002; van de Vis et al., 2003; Lambooij et al., 2010), and tilapia (Sundell et al., 2024). However, manual percussion may be less effective for certain species even when the blow is delivered accurately. In the present study, the robust skull of bass, and similarly that of catfish reported in other studies (Brijs et al., 2021; Hjelmstedt et al., 2025), appeared to offer enhanced protection against impact, potentially requiring greater maximum impact force, impulse, or kinetic energy than handheld devices such as fish priests or bats can reliably deliver. Species-specific differences in percussive stunning success, likely influenced by cranial morphology, have also been reported elsewhere. For instance, based on clinical reflex analyses (*i.e.*, presence or absence of opercular movements and the 'vestibulo-ocular' reflex), manual percussion effectively rendered 92 % of rainbow trout insensible compared to only 69 % of common carp across commercial facilities in Germany (Retter et al., 2018; Jung-Schroers et al., 2020). For larger or more robust fishes, these findings underscore the need for pneumatic systems, capable of delivering greater and more consistent impact energy to reliably induce an immediate and irreversible state of insensibility (Morzel et al., 2003; van de Vis et al., 2003;

Lambooij et al., 2010; Hjelmstedt et al., 2022; Sundell et al., 2024; Brijs et al., 2025).

However, as previously mentioned, the manual application of slaughter methods is labour-intensive and often incompatible with the high-throughput demands of modern aquaculture, necessitating the development of more automated systems. The automated *ikejime* prototype evaluated in this study represents an innovative step forward and, with further refinement, could potentially dispatch fish of varying sizes and species. Upon entry into the machine, trout and bass were automatically restrained, allowing the AI-based system to consistently and accurately target the brain. The system rendered 75 % of trout and 14 % of bass irreversibly insensible upon instrumentation. As with manual *ikejime* and percussion, all trout reached irreversible insensibility within 2 min, whereas this was not the case for bass, as 43 % of individuals still exhibited VERs at the end of the monitoring period. The observed differences in effectiveness between species suggests that the automated system likely faces similar challenges to its manual counterpart, particularly the need to induce immediate insensibility and ensure complete brain destruction. If allometric and species-specific differences in brain size and cranial morphology underlie the observed interspecific variation, future iterations of the system could incorporate a percussive element capable of delivering sufficient force to induce immediate insensibility, followed by an appropriately sized stainless-steel tool to ensure complete brain destruction. Alternatively, the injection of compressed air through the tool could assist in achieving full neural destruction, as this approach has shown considerable promise in captive needle pistols, where the injected air lacerates higher brain regions and produces immediate and irreversible insensibility in at least 93 % of European eels and African sharptooth catfish examined (Lambooij et al., 2002, 2003). While the present study provides proof-of-concept, expanding the training dataset (*e.g.*, >500 individuals and >5000 images per species) will be critical before commercial implementation to ensure maximal anatomical targeting accuracy, particularly given the individual variation in cranial morphology expected when slaughtering large numbers of fish. The adaptability of an AI-driven, computer-vision based automated *ikejime* system holds particular promise for more robust species, such as bass, where traditional slaughter methods often yield inconsistent results. Nonetheless, further testing of next-generation prototypes will be essential to validate these improvements and ensure compliance with humane slaughter standards.

Animal welfare and product quality are closely interlinked, as stressful conditions leading up to and during slaughter can compromise both fish welfare and the quality of the resulting product (Robb and Kestin, 2002; Poli et al., 2005; Daskalova, 2019). Acute stress immediately before or during slaughter elevates plasma cortisol and catecholamine concentrations (Merkin et al., 2010; Brijs et al., 2018; Papaharis et al., 2019; Hjelmstedt et al., 2021; Łosiewicz and Szudrowicz, 2024; Cabrera-Álvarez et al., 2025), accelerates glycogen depletion, and promotes lactic acid accumulation in muscle tissue, typically resulting in a reduced initial *post-mortem* pH across a wide range of fish species (Korhonen et al., 1990; Marx et al., 1997; Morzel et al., 2003; Kristoffersen et al., 2006; Ribas et al., 2007; Roth et al., 2007, 2009; Forgan et al., 2010; Daskalova and Pavlov, 2015; Lefevre et al., 2016; Cabrera-Álvarez et al., 2025). Although such stress likely occurred in the present study, particularly among fish killed using an inhumane method (*i.e.*, asphyxiation) or following an ineffective application of an alternative method (*i.e.*, inhumanely killed *via* *ikejime* or percussion), no clear or consistent reduction in initial muscle pH was detected *post-mortem*. Instead, muscle pH remained relatively stable throughout most of the monitoring period and declined only after $\sim 4\text{--}6$ days *post-mortem*. These findings should nevertheless be interpreted cautiously, as the limited sample sizes and substantial individual variability may have obscured subtle treatment effects or transient early-phase changes in muscle metabolism. In addition, the overall stability of *post-mortem* muscle pH observed in the present study may reflect effective cold-chain maintenance, since temperature strongly governs glycolytic enzyme activity

and lactic acid accumulation, resulting in a slower, more gradual decline in muscle pH under chilled conditions (Huss, 1995; Poli et al., 2005; Daskalova, 2019).

Although *post-mortem* pH trajectories did not differ markedly among treatments, clear differences were evident in the development of *rigor mortis*, suggesting that muscle energy metabolism was nonetheless influenced by slaughter method. Both manual *ikejime* and percussion delayed the onset and peak of *rigor mortis*, consistent with earlier findings that humane slaughter can preserve ATP stores, slow *post-mortem* energy depletion, and prolong the pre-*rigor* phase (Boyd et al., 1984; Korhonen et al., 1990; Lowe et al., 1993; Marx et al., 1997; Morzel et al., 2003; Davie and Kopf, 2006; Kristoffersen et al., 2006; Roth et al., 2007, 2009). This pre-*rigor* period is important because it improves fillet yield and reduces product damage during processing (Skjervold et al., 2001a, 2001b; Einen et al., 2002). Interestingly, this phase can be further extended by destroying the spinal cord immediately after brain spiking, as performed during the full *ikejime* procedure (Ando et al., 1996; Mishima et al., 2005). Conversely, the rapid onset of *rigor mortis* observed in asphyxiated fish aligns with prior research showing that exhaustive struggling accelerates ATP depletion and promotes early *post-mortem* stiffening (Korhonen et al., 1990; Lowe et al., 1993; Robb and Kestin, 2002; Poli et al., 2005; Kristoffersen et al., 2006; Roth et al., 2007, 2009). Notably, the automated *ikejime* system also produced a comparatively rapid *rigor mortis* response, likely due to extended handling and air exposure prior to and during machine processing, both known to elevate stress and metabolic demand (Robb and Kestin, 2002; Poli et al., 2005; Roth et al., 2009; Daskalova, 2019). These findings underscore the need to refine automated systems to minimize pre-slaughter handling and cycle time, thereby improving both welfare and product consistency.

The elevated drip loss observed in inhumanely killed fish further supports a link between stress physiology and *post-mortem* water-holding capacity. Prolonged struggling or distress prior to death depletes glycogen reserves and ATP, leading to impaired Ca^{2+} regulation within muscle fibers (Roth et al., 2006; Daskalova et al., 2016). The resulting increase in cytosolic Ca^{2+} activates Ca^{2+} -dependent proteases, including calpains and cathepsins B and L, which degrade cytoskeletal and myofibrillar proteins such as desmin, troponin T, and α -actinin (Ladrat et al., 2003; Roth et al., 2006; Hultmann et al., 2012). This proteolytic weakening of the muscle matrix, together with stress-induced changes in osmotic balance and membrane permeability, reduces the capacity of muscle to retain water and accelerates fluid loss during storage (Olsson et al., 2003; Bosworth et al., 2007). Comparable patterns have been reported in species of carp, catfish, cod, salmon, and seabream, where pre-slaughter stress increased drip loss, reduced fillet firmness, and/or shortened shelf life (Roth et al., 2006, 2009; Bosworth et al., 2007; Hultmann et al., 2012; Daskalova et al., 2016; Cabrera-Álvarez et al., 2025). Together, these results demonstrate that acute pre-slaughter stress accelerates *post-mortem* biochemical degradation, underscoring that welfare improvements confer not only ethical but also tangible product-quality and economic benefits.

No consistent differences were observed in *post-mortem* fillet colour among treatments, which is unsurprising given that colour expression is highly species-specific and influenced by factors such as myoglobin content, oxygenation state, and the rate of pH decline (Poli et al., 2005; Daskalova, 2019). Baseline pigmentation differences between trout and bass likely obscured subtle treatment effects, while the small sample sizes limited statistical power to detect minor but biologically meaningful trends. A brief prospective power evaluation ($\alpha = 0.05$, 80% power) based on medium effect sizes ($\eta^2 = 0.10$; Cohen's $f = 0.33$) indicated that, when analysed separately for each species, ~18 and 12 fish per group would be required in simple between-method and between-kill effectiveness comparisons, respectively, and ~12–15 and 10–12 fish for repeated-measures designs. These estimates provide an approximate benchmark for designing future, more statistically robust product-quality studies, although exact requirements will vary

depending on the specific experimental design.

Nevertheless, despite limited replication, our exploratory analyses revealed clear trends, as slaughter methods and their effectiveness influenced the progression of *rigor mortis* and drip loss, suggesting that the physiological and perceptual state of the fish at death can shape *post-mortem* muscle processes. By focusing on individuals that were either insensible or sensible throughout the entire monitoring period, we were able to contrast the two welfare extremes and demonstrate how differences in slaughter effectiveness may translate into measurable product-quality outcomes. Future studies should expand on these findings using larger, replicated designs and complementary biochemical, histological, or molecular assessments to strengthen mechanistic understanding. Incorporating intermediate cases, such as the fish that transiently displayed VERs prior to death, together with concurrent physiological stress measurements would further clarify how varying levels of stress or distress during slaughter influence muscle biochemistry and product stability. Expanding such integrative investigations under both experimental and commercial conditions will be essential for improving the reliability of welfare-quality assessments and advancing humane slaughter practices across species.

5. Conclusion

This study provides the first neurological assessment of *ikejime*, revealing both its potential and its limitations as a slaughter method. While *ikejime* improved welfare outcomes relative to asphyxiation and was relatively effective in rendering small rainbow trout (~0.3–0.5 kg) irreversibly insensible, it did not consistently induce immediate insensibility, a critical requirement for humane slaughter (EFSA, 2004; 2018; WOAH, 2024). Moreover, it was less effective in larger or more robust species such as hybrid striped bass (~1.1–2.1 kg), highlighting that brain size, cranial morphology, spike accuracy and insertion technique are key determinants of success. These findings reinforce that *ikejime* should be preceded by a stunning method proven to induce immediate insensibility to ensure compliance with welfare standards (EFSA, 2004; Davie and Kopf, 2006; Diggles, 2016; WOAH, 2024).

Species-specific validation and further mechanistic research are essential to clarify how variables such as anatomical variation, spike dimensions, percussive force, and application technique influence the likelihood of achieving immediate and irreversible insensibility. In line with EFSA (2018) guidance, both rigorous proof-of-concept trials under controlled laboratory conditions and validation studies under realistic production settings are recommended before automated systems such as that evaluated in the present study can be commercially implemented. With refinements including scaled tool dimensions, expanded AI training datasets, integration of an immediate stunning step, optimization of handling time, and improved cycle speed, automated *ikejime* holds considerable promise for improving welfare outcomes and processing speed in small-scale aquaculture and fishery operations.

By linking neurological verification of insensibility with *post-mortem* quality parameters, this study establishes an integrative framework for assessing both welfare and product outcomes. Future research should build on this foundation through species-specific validation and larger, statistically robust sample sizes to strengthen generalizability. Continued innovation and validation of humane slaughter methods will be critical to advancing ethical, sustainable, and economically viable aquaculture. Aligning welfare assurance with product quality and operational efficiency provides a clear pathway toward practices that benefit producers, consumers, and fish alike. Such integrations will ultimately support the development of evidence-based welfare standards across the global aquaculture industry.

Animal ethics statement

Animal care and all experimental procedures were performed in accordance with UNH Institutional Animal Care and Use Committee

guidelines (Protocol # 220701).

CRediT authorship contribution statement

Albin Gräns: Writing – review & editing, Writing – original draft, Visualization, Validation, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Linas Kenter:** Writing – review & editing, Writing – original draft, Visualization, Validation, Resources, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Waiman Meinhold:** Writing – review & editing, Writing – original draft, Visualization, Resources. **Andreas Säxer:** Writing – review & editing, Writing – original draft, Visualization, Validation, Resources, Methodology, Investigation, Formal analysis, Data curation. **Thorsten Schwerte:** Writing – review & editing, Writing – original draft, Visualization, Validation, Formal analysis, Data curation. **Jeroen Brijs:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

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 the manuscript. After using this tool/service, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: The authors declare that Waiman Meinhold is an employee of Shinkei Systems, the company developing the automated *ikejime* system, which may be considered a potential competing interest. However, Waiman Meinhold had no role in the study design, data collection, data analysis, or interpretation of the results. His contribution was limited to providing technical information on device operation and calibration and assisting in drafting and finalizing the manuscript.

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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.2025.743458>.

Data availability

The data that support the findings of this study are openly available in <https://doi.org/10.6084/m9.figshare.28540652>

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