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Effects of electrical and percussive stunning on neural, ventilatory and cardiac responses of rainbow trout

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ABSTRACT

From an ethical standpoint, it is imperative that rainbow trout (*Oncorhynchus mykiss*) are humanely slaughtered, which entails that they are rendered unconscious immediately by a stunning method and remain so until death. The efficacy of electrical stunning following dewatering (*i.e.*, in-air electrical stunning at intensities of 50 to 920 mA and durations of 5 to 30 s) and percussive stunning, both advocated as humane stunning and/or killing methods, are evaluated here for this species *via* the presence or absence of visually evoked responses (VERs). In addition, ventilatory and cardiac responses were evaluated to elucidate the physiological basis for the lethality of both methods. While the present study was unable to determine the capability of in-air electrical stunning to induce immediate unconsciousness, our findings revealed that irreversible stuns were induced by both in-air electrical stunning (*i.e.*, ~25 to 70% of individuals did not recover VERs across the various combinations of stunning intensities and/or durations) and percussive stunning (*i.e.*, ~100% of individuals did not recover VERs). The efficacy of in-air electrical stunning for permanently abolishing VERs was marginally, but significantly, impacted by stun intensity (*i.e.*, explained 8% of the variation). Furthermore, due to substantial inter-specific variability and a limited sample size, significant impacts of stun intensity and/or duration on the recovery of VERs in reversibly stunned individuals were not detected in the present study (*i.e.*, VERs recovered between <0.5 to 28.8 min). Further investigation is therefore necessary before in-air electrical stunning can be endorsed as a standalone humane slaughter method for rainbow trout. This includes determining its capacity to induce immediate unconsciousness, as well as to identify additional factors that could be modified or enhanced to improve its efficacy. Furthermore, since death following in-air electrical stunning likely entails a prolonged process involving ventilatory failure, hypoxemia, and subsequent vital organ malfunction, rather than immediate cardiac arrest or central nervous system failure, the sequential use of methods such as percussive stunning is recommended to safeguard the welfare of rainbow trout during slaughter.

1. Introduction

Fish are a vital source of sustenance for humanity, which will only intensify as we strive to nourish the growing global population (GSDR, 2023). This is highlighted by the fact that global farmed finfish production has escalated from ~9 million tonnes in 1990 to ~56 million tonnes in 2019 (FAO, 2022). Consequently, this equates to ~78–171 billion individual farmed finfish that were harvested for consumption in 2019 (Mood et al., 2023). From an ethical standpoint, it is therefore imperative that humane stunning and killing practices are developed, validated and employed to mitigate concerns related to animal welfare during the slaughter process (OIE, 2023).

It is recommended that farmed fish undergo stunning before being killed to ensure that slaughter can be performed without avoidable fear, anxiety, pain, suffering and distress (OIE, 2023). The chosen stunning method should achieve both an immediate and irreversible loss of

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consciousness, or in cases where stunning is not irreversible, the humane killing of fish should occur prior to the recovery of consciousness (OIE, 2023). The abovementioned loss of consciousness is defined here as ‘a state of unawareness in which there is temporary or permanent impairment to brain function whereby the individual is unable to respond to normal stimuli, including pain’ (EFSA, 2004). Regrettably, a significant proportion of globally farmed fish are currently slaughtered using methods that demonstrate limited consideration for their welfare (Lines and Spence, 2014; Mood et al., 2023). This includes a range of prolonged stunning methods (*i.e.*, live chilling, CO_2 immersion, and exposure to salt/ammonia baths) and killing methods without prior stunning (*i.e.*, asphyxiation and exsanguination) that clearly deviate from the prescribed guidelines of the OIE (2023). While potentially more humane stunning and killing methods (*i.e.*, electrical and percussive stunning) have been recommended by the OIE, the essential species-specific parameters ensuring the efficacy of these alternative methods remain largely insufficient (Lines and Spence, 2012, 2014; Mood et al., 2023; OIE, 2023).

The lack of species-specific information and regulatory frameworks aimed at ensuring welfare during time of slaughter likely stems from the vast diversity of farmed fish species and the inherent difficulty in assessing whether stunning and/or killing methods result in an immediate and irreversible loss of consciousness. Numerous visual indicators have historically been used to assess the level of consciousness in farmed fish, encompassing various behavioural measures (*e.g.*, coordinated swimming, escape behaviours, equilibrium maintenance, reactions to painful stimuli, and the ‘eye-roll’ reflex) and physiological responses (*e.g.*, ventilatory responses) (Kestin et al., 2002; Lines and Spence, 2012; Van De Vis et al., 2003). However, it has become increasingly clear that these visual measures are inadequate for accurately assessing changes in the state of consciousness during stunning and/or killing of fish (Bowman et al., 2019, 2020; Brijs et al., 2021), and that reliance on these indicators may even pose significant welfare concerns (*e.g.*, during situations where a stunning method induces paralysis without achieving unconsciousness; Brijs et al., 2021; E. Lambooij et al., 2002). Consequently, it has become imperative to obtain neurophysiological evidence of unconsciousness to comprehensively assess the impact of various commercial slaughter procedures (EFSA, 2004; van de Vis et al., 2003).

Electroencephalographic (EEG) methods have successfully been used to record the electrical activity of the brain to determine unconsciousness in a wide range of fish species during slaughter procedures. Prior investigations across various fish species have demonstrated that an immediate transition to unconsciousness following electrical and/or percussive stunning can be discerned *via* the appearance of epileptiform insults, and/or the appearance of theta waves, delta waves and spikes in the EEG recordings (Daskalova et al., 2016; Hjelmstedt et al., 2022; B. Lambooij et al., 2006; Lambooij et al., 2008a; E. Lambooij et al., 2007; Lambooij et al., 2008b; Lambooij et al., 2010). Furthermore, the duration of unconsciousness induced by these stunning methods can be subsequently discerned using analyses of EEG waveforms, frequency and amplitude, as well as *via* the recovery of somatosensory evoked potentials or visually evoked responses (VERs) (Brijs et al., 2021; Daskalova et al., 2016; Hjelmstedt et al., 2022; Jung-Schroers et al., 2020; Kestin et al., 1991; B. Lambooij et al., 2006; Lambooij et al., 2008b; E. Lambooij et al., 2007; Lambooij et al., 2008b; Lambooij et al., 2010; Retter et al., 2018; Robb et al., 2000; Robb and Roth, 2003).

Rainbow trout (*Oncorhynchus mykiss*), a globally significant farmed salmonid species, reached a production of ~917,000 t or 1.4 billion individuals in 2019 (Mood et al., 2023). This species is currently stunned and/or killed using a range of inhumane methods (*i.e.*, live chilling, CO_2 immersion, asphyxiation, and exsanguination/evisceration), as well as potentially more humane methods (*i.e.*, electrical and percussive stunning) (EFSA, 2009; EU, 2017). With regards to the latter methods, recent neurophysiological investigations have demonstrated that rainbow trout subjected to electrical stunning while submerged in water (*i.e.*, in-water electrical stunning) were immediately rendered unconscious (*i.e.*, based on the presence of epileptiform insults following a 1 s stun; Hjelmstedt et al., 2022) and remained so until death at sufficient stunning intensities and durations (*i.e.*, based on the absence of VERs; Hjelmstedt et al., 2022; Jung-Schroers et al., 2020). Furthermore, it has also been demonstrated that rainbow trout were irreversibly rendered unconscious following percussive stunning with a handheld, non-penetrative, pneumatic captive bolt gun based on immediate and substantial reductions in EEG amplitude, and the failure to recover VERs (Hjelmstedt et al., 2022). However, knowledge gaps still exist for this species with regards to the efficacy of electrical stunning following dewatering (*i.e.*, in-air electrical stunning), as well as the underlying physiological basis for the lethality of both electrical and percussive stunning.

In the present study, we aim to address these knowledge gaps *via* assessments of neural, ventilatory and cardiac responses of rainbow trout before and after the application of *i)* in-air electrical stunning across various stun intensities (*i.e.*, from 50 to 920 mA) and/or durations (*i.e.*, from 5 to 30 s), and *ii)* percussive stunning using a handheld, non-penetrative, pneumatic captive bolt gun. Neural responses were determined using a non-invasive method that allows the continuous recording of EEG in unrestrained animals before and after stunning for extended periods of time (Bowman et al., 2019, 2020; Hjelmstedt et al., 2022), while ventilatory and cardiac responses were determined visually and/or *via* continuous recordings of electrocardiograms (ECG).

2. Materials and methods

2.1. Animals

Rainbow trout of mixed sex subjected to in-air electrical stunning in the present study (n: 52, body mass: 792 ± 45 g, fork length: 36 ± 1 cm, Fulton’s condition factor: 1.7 ± 0.1, all data are presented as means ±95% C.I. unless otherwise stated) and percussive stunning in our previous study (n: 10, body mass: 788 ± 94 g, fork length: 36 ± 2 cm, Fulton’s condition factor: 1.7 ± 0.1, Hjelmstedt et al., 2022) were all obtained from a local fish hatchery (Vänneåns Fiskodling AB, Knäred, Sweden). Fish were transported to the aquarium facilities at the University of Gothenburg, Gothenburg, Sweden. Trout were held at 10–11 °C on a 12 h:12 h light:dark photoperiod in a 2000 L tank containing recirculating aerated freshwater for a minimum period of 1 week prior to experimentation. During this period, trout were fed dry commercial trout pellets (9 mm Protec Trout pellets, Skretting, Stavanger, Norway) three times per week. Animal care and all experimental procedures were performed between June and November 2020 (for in-air electrical stunning) and January and March 2020 (for percussive stunning). The separate trials were in accordance with national regulations and covered by the same ethical permit approved by the ethical committee on animal research in Gothenburg, Sweden (1873–2018).

2.2. Experimental protocols

2.2.1. Pre-stunning protocol

All trout that were to be subjected to in-air electrical stunning in the present study, as well as those subjected to percussive stunning in Hjelmstedt et al. (2022), were individually captured from their holding tanks and lightly anaesthetized in freshwater containing 4 mg L^{-1} iso-eugenol (Aqui-S®, Lower Hutt, New Zealand). Upon the onset of a loss of equilibrium, fish were instrumented with the EEG recording equipment (see *2.3. Description and placement of EEG and ECG recording equipment*) and transferred to an opaque monitoring chamber (length: 48 cm, width: 12 cm, water depth: 16 cm) covered with a glass lid. The chamber received gravity fed, aerated, 10 °C freshwater at a flow rate of ~2 L min^{-1} from a 200 L header tank. Fish were allowed to fully recover from light anaesthesia prior to pre-stun monitoring, 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 signal (see *2.4. Recording, acquisition and analyses of EEG and ECG signals*). Following recovery, EEG signals of trout were continuously recorded in response to light flashes for an additional 5 min. The EEG recording equipment was subsequently removed and trout were subjected to one of the stunning techniques described below (see *2.2.2 In-air electrical stunning protocols* or *2.2.3 Percussive stunning protocol*).

2.2.2. *In-air electrical stunning protocols*

In-air electrical stunning involved administering a combination of AC and DC electrical currents to the fish when out of the water using a custom-made in-air electric stunner (Optimar, Norway) with four different stunning settings (see Table 1 for AC/DC voltages, current and frequency associated with each stun setting).

To investigate whether in-air electrical stunning induces immediate unconsciousness in rainbow trout, EEG recordings need to be obtained before and immediately after administering a 0.5–1 s electrical stun. These recordings allow for the assessment of the onset, intensity, and duration of epileptiform insults (*e.g.*, see the methodology employed in Hjelmstedt et al., 2022 for in-water electrical stunning). However, the in-air electrical stunner used in the present study had a startup delay of ~1–2 s before reaching the desired stunning frequency. Consequently, it was not feasible to position instrumented fish within the stunner to administer a 1 s stun, neither was it feasible to pass them through an operational live stunner since the EEG electrodes would detach when passing through the array of stunning electrodes.

In-air electrical stunning has induced epileptiform insults in all farmed fish species studied thus far (Daskalova et al., 2016; E. Lambooij et al., 2004, 2007, 2010, 2012; Erikson et al., 2012; Llonch et al., 2012; Sattari et al., 2010), across a range of electrical parameters similar to those employed in our study. Therefore, our investigation aimed to determine whether extending the intensity and/or duration of the stun could either induce an irreversible stun or sufficiently prolong the period of unconsciousness for fish to succumb to a sequential killing method. This assessment relied on the presence or absence of VERs within the EEG signal, as the abolition of VERs has previously been established as an objective and unequivocal marker of brain dysfunction and, consequently unconsciousness, across a diverse range of farmed fish species (Bowman et al., 2019, 2020; Brijs et al., 2021; Hjelmstedt et al., 2022; Jung-Schroers et al., 2020; Kestin et al., 1991; Lambooij et al., 2002; Retter et al., 2018; Robb and Roth, 2003; Robb et al., 2000). However, it could be argued that using the abolition of VERs as a criterion for determining loss of consciousness may be overly stringent. This is because the primary wave of the VER signifies the arrival of the signal at the higher centers of the brain and the subsequent waves indicate further processing, with the latter expected to occur to some extent in a conscious animal (Daly et al., 1987; Gregory and Wotton, 1983; Kestin et al., 1991). However, accurately determining the transition from the primary wave to the subsequent waves, or pinpointing when all subsequent waves disappear, was not possible from the EEG data obtained in the present study. Therefore, given the lack of a direct measure of consciousness, the presence of VERs must represent the possibility that an animal is conscious from an animal welfare perspective (Kestin et al., 1991).

To mimic a commercial slaughter situation, trout were passed through the electrical stunner head first, and subjected to an electrical stun *via* various parts of the head and/or body upon contact with the three rows of hanging electrodes while the other side of the body was lying on the conveyor belt which acted as the other electrode. Electrical voltages (AC V_{rms} and DC V) and current (A_{rms}) passing through the stunner when the fish touched the electrodes were determined using an AC/DC current probe (Fluke 80i-110 s, Fluke Corporation, Everett, Washington, USA) and a handheld oscilloscope (Fluke 123B).

The efficacy of in-air electrical stunning was investigated *i*) across a range of stun intensities (*i.e.*, trout were stunned for 5 s using stun settings 1, 2, 3 or 4), *ii*) across a range of stun durations (*i.e.*, trout were stunned for 5, 10, 20 and 30 s using stun setting 3, which is the recommended stun setting for salmonids according to the manufacturer), or *iii*) at a combination of maximum stun intensity and duration (*i.e.*, trout were stunned using stun setting 4 for 30 s). Information regarding sample size, body mass, electrical voltages and current for each investigation can be found in Table 1.

2.2.3. *Percussive stunning protocol*

Percussive stunning of rainbow trout was performed in a previous study (see Hjelmstedt et al., 2022) using a handheld, non-penetrative, pneumatic captive bolt gun (Zephyr® F, Bock Industries, PA, USA) driven by pressurized air (125 psi) from a compressor (Herkules Walkair CE New, Siegen, Germany). In that study, trout were transferred from the opaque monitoring chamber to a plastic tray, and firmly held while a

Table 1

Sample sizes and body masses of rainbow trout subjected to in-air electrical stunning at various combinations of stun intensities and durations. Electrical voltages (AC and DC) and current were determined during each stun using an AC/DC current probe connected to a handheld oscilloscope. All data are presented as means±95%C.I., with the exception of the recovery time of reversibly stunned fish presented as minimum - maximum. It must be noted that upon real-time analysis of VERs, it became evident that 5 s electrical stuns, regardless of intensity, were unsuitable for commercial use. Since this aligned with previous research (*e.g.*, in-water electrical stunning of rainbow trout, see Hjelmstedt et al., 2022), further evaluation of short duration stuns was discontinued, and the focus was shifted to thoroughly investigating the efficacy of prolonged stuns at settings recommended by the manufacturer or higher.

Group	n	Body mass	Stun setting	Stun duration	Voltage	Voltage	Frequency	Current	Irreversibly stunned fish	Recovery time of reversibly stunned fish
		(g)		(s)	(AC V_{rms})	(DC V)	(Hz)	(mA_{rms})	(%)	(min)
1	6	851 ± 68	1	5	4.23 ± 0.03	41.4 ± 0.4	100	117 ± 17	50	<0.5–12.5
2	4	641 ± 26	2	5	8.00 ± 0.14	70.0 ± 1.2	100	163 ± 15	50	1.2–12.5
3	4	685 ± 58	3	5	10.85 ± 0.20	106.7 ± 0.7	100	330 ± 35	25	<0.5–28.8
4	4	711 ± 41	4	5	14.20 ± 0.31	139.1 ± 1.0	100	560 ± 50	25	0.6–1.2
5	8	805 ± 50	3	10	11.06 ± 0.15	106.2 ± 0.4	100	376 ± 31	25	<0.5–2.5
6	8	821 ± 33	3	20	11.16 ± 0.09	106.4 ± 0.4	100	381 ± 25	25	0.6–27
7	8	792 ± 40	3	30	11.38 ± 0.09	104.8 ± 0.3	100	426 ± 24	38	<0.5–16.5
8	10	835 ± 33	4	30	15.55 ± 0.24	138.7 ± 0.6	100	701 ± 40	70	<0.5–7

single blow from the captive bolt gun was delivered directly over the brain.

2.2.4. Post-stunning protocol

Directly after stunning, EEG and ECG recording equipment were put into their respective positions on the trout within ~30 s. The trout was then placed back into the monitoring chamber for 30 min during which they were subjected to the flashing light while EEG and ECG were continuously recorded. Previously reported EEG data and unpublished ECG data obtained from percussively stunned rainbow trout were analysed in a similar manner as in-air electrically stunned trout in the present study (see *2.4. Recording, acquisition and analyses of EEG and ECG signals*). At the end of the monitoring period, trout were euthanized with a sharp blow to the head, weighed and measured.

2.3. Description and placement of EEG and ECG recording equipment

The EEG signals of trout were recorded using a custom-made, non-invasive device that consisted of a silicone suction cup fitted with a 2 mm silicone tube and three 1 cm diameter silver chloride plate electrodes (Electrode ARBO H98LG MOD, Tyco Healthcare, Ratingen, Germany) soldered to shielded wires (MLAWBT9 EEG Flat Electrodes, ADInstruments, Oxford, United Kingdom) (Bowman et al., 2019, 2020). The silicone tube was connected to a peristaltic pump to provide suction for keeping the silicone cup in place and the electrodes in firm contact with the skin during the experiment. The EEG wires were then connected to a bio-amplifier (FE136, ADInstruments). Prior to the placement of the device on individual trout, a thin layer of conductive paste (Ten20, Weaver and Company, Aurora, Colorado, USA) was applied to the surface of each electrode to ensure good contact between the skin of the trout and the electrodes. The device was then positioned on either a lightly anaesthetized or stunned trout (*i.e.*, before or after stunning, respectively, see *2.2. Experimental protocols* for more details) so that the plate electrodes were centered above the approximate location where the optical nerves enter the neurocranium and the reference-ground electrode was positioned slightly behind the brain of the fish (Bowman et al., 2019). The silicon cup and the plate electrodes were then secured in place using the suction generated by the peristaltic pump.

Electrical signals from the heart (electrocardiogram, ECG) were recorded using needle electrodes (MLA1213, ADInstruments, Oxford, United Kingdom) inserted between the pectoral fins in a medial position (*i.e.*, one electrode in a caudal direction and the other directed anteriorly orienting the tip close to the heart), as well as a reference-ground electrode that was inserted on the side of the fish between the lateral line and the anal fin. The needle electrodes were then connected to another bio-amplifier (model FE136, ADInstruments).

2.4. Recording, acquisition and analyses of EEG and ECG signals

EEG signals were continuously recorded *via* the bio-amplifier in response to light flashes from an LED strobe-light (10 ms light flashes at 2 Hz) in a dark room. The sensitivity range of the bio-amplifier was (±2 mV) with a low-pass filter (50 Hz), highpass filter (0.1 Hz) and 50 Hz notch filter activated to optimize EEG signals. Signals from the bio-amplifier and a light detector were relayed to a PowerLab (ML 870, 8/30, ADInstruments). Data were subsequently collected on a PC for analyses using LabChart Pro software (version 7.3.2, ADInstruments) 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 wave frequency (13–32 Hz). This is because activity in this frequency range relates to awareness and normal alert consciousness, and is consequently also where VERs are found to be most distinct (Bowman et al., 2019). VERs were detected using the Scope View module in the software, which was set to display time windows starting 50 ms before and ending 450 ms after the strobelight flash total time window of 500 ms). To reduce the effects of noise caused by strong muscular movements, 500 ms time windows where the amplitude of the beta wave exceeded 10 μV were automatically excluded from the analyses. To obtain specific determinations of when VERs were present or absent, the Scope View module was used to average 30 to 120 consecutive, nonoverlapping time windows into a single 500 ms time window representative of the beta wave for 15 to 60 s of recordings, respectively. 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 addition, the recovery of ventilation following electrical stunning could be visually determined, as well as *via* the emergence of rhythmic wave patterns within the raw EEG signal (0.1–50 Hz).

ECG signals were continuously recorded *via* the bio-amplifier and relayed to the PowerLab. The sensitivity range (±100 mV), low-pass filter (1 kHz), highpass filter (0.3 Hz) and 50 Hz notch-filter were set in the bio-amplifier to optimize ECG signals. Data were subsequently collected on a PC for analyses using LabChart Pro software at a sampling rate of 1 kHz. When analyzing the ECG recordings in the LabChart Pro software, a bandpass-filter was used (*i.e.*, high cut-off frequency range: 15–40 Hz; low-pass range: 1–4 Hz) to attain the best quality signal for identification of the QRS complex, from which the R-R wave intervals could be determined to calculate heart rate using the cyclic measurements module within the LabChart Pro software.

2.5. Statistical analyses

All statistical analyses were performed using R. The best-fitting models described below were selected based on Akaike's Information Criterion (Anderson and Burnham, 2002), and were carefully assessed to ensure that no assumptions were violated. A comprehensive description of the statistical analyses, including details about the R packages employed and the procedures associated with model fitting, selection, checking, parameter transformation and inference, can be found in the supplementary information (Supp. Info. 1 A-G). Throughout the text and figures, inferences and predictions derived from the various models, such as significant interactions and main effects not included within an interaction, are reported. Statistical significance was determined at a threshold of $p < 0.05$.

The Kaplan-Meier method was conducted to evaluate the effects of stun intensity (*i.e.*, trout were stunned for 5 s using stun settings 1, 2, 3 or 4), stun duration (*i.e.*, trout were stunned for 5, 10, 20 or 30 s using stun setting 3), and a combination of maximum stun setting and duration (*i.e.*, trout were stunned with stun setting 4 for 30 s) on the proportion of individuals that did not recover VERs throughout the monitoring period (Fig. 1A-D). The outcome for each case was either when the 'event' occurred (*i.e.*, when an individual recovered VERs) or the individual was 'censored' (*i.e.*, when an individual did not recover VERs throughout 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.

To determine the morphometric (*i.e.*, body mass, fork length or Fulton's condition factor) and/or stunning factors (*i.e.*, stun setting and/or duration) contributing to the substantial variation in electrical current passing through trout during in-air electrical stunning with stun settings 3 and 4 (see Table 1 and Fig. 2A), a linear regression model was employed. In addition, morphometric and stunning factors contributing to the likelihood of an irreversible stun or recovery time (*i.e.*, time taken for VERs to recover following stunning) was explored using best-fitting binomial logistic and linear regression models, respectively. Recovery time in the latter model was inversely transformed to coax it towards normality.

A simple linear regression model examined the relationship between the recovery of rhythmic ventilation and VERs within individuals that

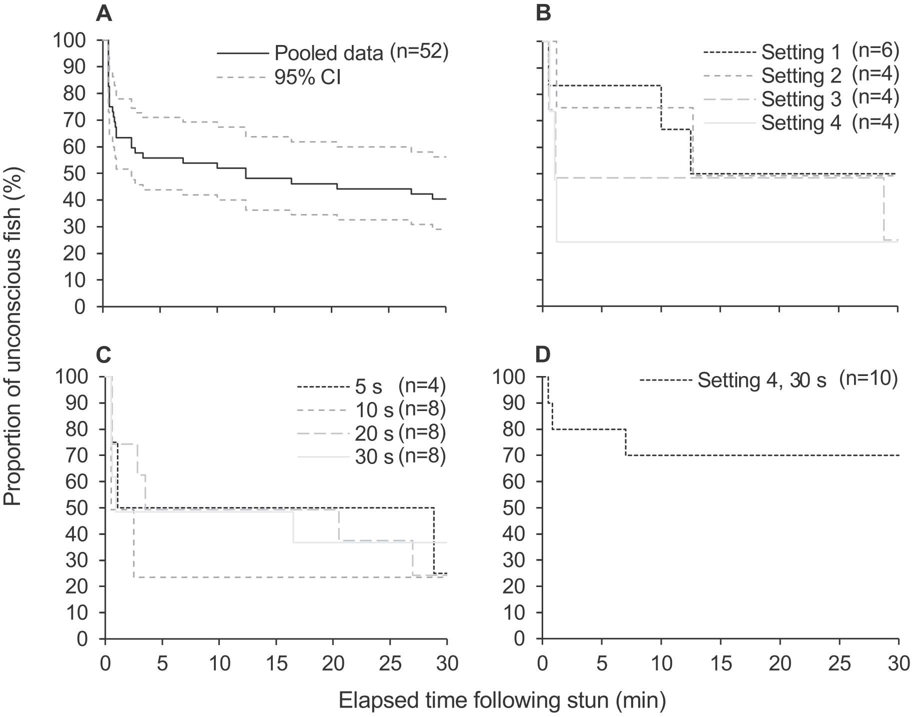

Fig. 1. Temporal recovery of VERs in rainbow trout following in-air electrical stunning at various intensities and/or durations. (A) The proportion of in-air electrically stunned rainbow trout (±95% C.I.) that exhibited an absence of VERs throughout the monitoring period. Trout that did not exhibit VERs for 30 min following stunning were deemed irreversibly stunned. No significant differences were observed in the Kaplan-Meier survival distributions across various (B) stun settings (*i.e.*, when stun duration was held at 5 s), (C) stun durations (*i.e.*, when stun intensity was held at the setting recommended by the manufacturer), or (D) the combination of maximum stun setting and duration (Log rank test: $\chi 2(7) = 6.6$, $p = 0.5$; Breslow test: $\chi 2(7) = 7.3$, $p = 0.4$).

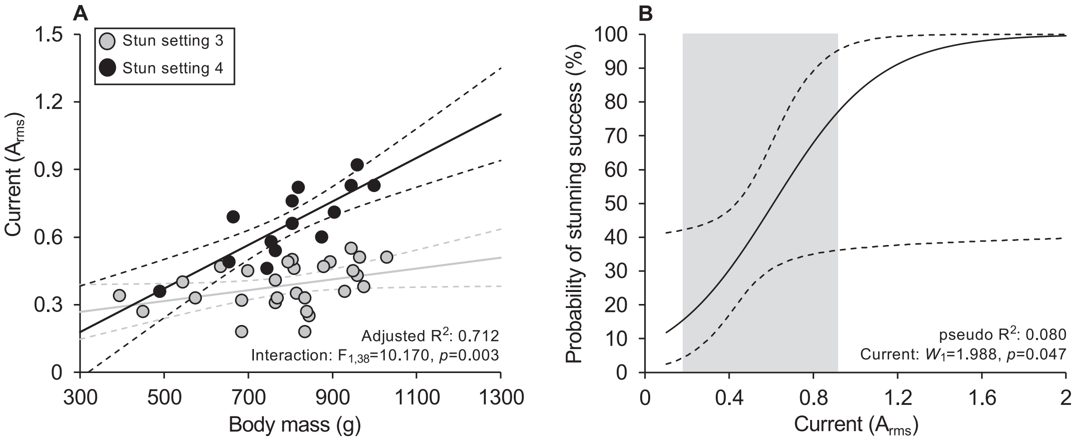

Fig. 2. Electrical current passing through trout during in-air electrical stunning and its effects on stunning success. (A) The electrical current passing through trout when using stun setting 3 (grey circles) and 4 (black circles) was largely explained by stun setting, body mass and the interaction between the two factors. (B) Stunning success (*i.e.*, the probability that rainbow trout did not recover VERs) was only partially explained by the amount of electrical current passing through the trout. Solid and dashed lines in (A) and (B) represent the predicted mean ± 95% C.I. from regression models 1 and 2, respectively (see Supp. Info. 1), while the grey shaded area in (B) represents the range of electrical current that passed through rainbow trout in the present study.

recovered both parameters following in-air electrical stunning. Cardiac responses of rainbow trout following electrical stunning were assessed using the best-fitting linear mixed-effects model, which incorporated the recovery status of trout (*i.e.*, permanent, transient, or no recovery), elapsed time following stunning, the interaction between recovery status and time, and individual as a random intercept. Post-hoc tests evaluating temporal changes in heart rate within each recovery group employed separate linear mixed-effects models with elapsed time as the main effect. Planned contrasts were conducted to discern differences between recovery groups at pre-specified time points (*i.e.*, 1, 5, 10, 20, and 30 min). Resulting *p*-values underwent Bonferroni correction to address multiple testing. Cardiac responses of rainbow trout following percussive stunning were assessed using the best-fitting linear mixed-effects model, which incorporated elapsed time following stunning and individual as a random intercept. Heart rate was log-transformed to best meet the assumptions of the abovementioned linear mixed-effects models.

3. Results

3.1. In-air electrical stunning success and the impact of stun setting and/or duration

The proportion of rainbow trout exhibiting an absence of VERs following in-air electrical stunning decreased throughout the 30 min post-stunning monitoring period (Fig. 1A). For example, ~83, 56, 52 and 40% of individuals displayed an absence of VERs at 0.5, 5, 10 and 30 min post-stunning, respectively, with the latter representing irreversibly stunned individuals (Fig. 1A). The proportion of individuals exhibiting an absence of VERs throughout the recovery period did not appear to be affected by stun setting and/or duration, as evidenced by the lack of significant differences between the corresponding Kaplan-Meier survival distributions (Log-rank test: $\chi 27 = 6.6$, $p = 0.5$; Breslow test: $\chi 27 = 7.3$, $p = 0.4$; Supp. Info. 1A; Fig. 1B-D). However, caution is warranted in interpreting this finding due to the small sample sizes in certain treatment groups (Table 1).

Rainbow trout that were not irreversibly stunned (*i.e.*, ~60% of individuals) recovered VERs between <0.5 to 28.8 min (median duration: 12.5 min; Fig. 1A). In ~19% of these trout, VERs only persisted for a limited period of time before they were subsequently lost for the remainder of the monitoring period (*i.e.*, transient recovery). The duration of time that VERs persisted in these individuals ranged from 0.33 to 1.66 min.

3.2. Influence of electrical current passing through trout on stunning success

Based on the most parsimonious linear regression model (Supp. Info. 1B), the amount of electrical current passing through a trout was affected by the interaction between stun setting and body mass (Interaction: $F_{1,38} = 10.170$, $p = 0.003$, adj. $R^2 = 0.712$; Fig. 2A). Model coefficients demonstrated that the electrical current passing through a trout increased more in response to body mass when using the maximum stun setting (Fig. 2A).

Based on the most parsimonious binomial logistic regression model (Supp. Info. 1C), the amount of electrical current passing through trout affected stunning success (Current: $W_1 = 1.988$, $p = 0.047$), yet it could only explain 8% of the variation (Fig. 2B). In addition, the time taken for VERs to return in individuals that recovered from electrical stunning was not significantly affected by any of the examined morphometric (*e.g.*, body mass, fork length or Fulton's condition factor) and/or stunning factors (*e.g.*, electrical current and/or stunning duration) (Supp. Info. 1D).

3.3. Ventilatory and cardiac responses of electrically stunned trout

Rhythmic ventilation was observed in ~81% of the trout that recovered VERs following in-air electrical stunning (*i.e.*, 25 out of 31 individuals, see red circles in Fig. 3A). Rhythmic ventilation recovered within 1.6 ± 0.4 min in these individuals, and a weak relationship was observed between the recovery of rhythmic ventilation and VERs ($F_{1,23} = 9.745$, $p = 0.005$, adj. $R^2 = 0.267$; Supp. Info. 1E). However, visual inspection of the data clearly shows that the recovery of rhythmic ventilation cannot be reliably used to predict the recovery of VERs (*c.f.*, position of red circles with 1:1 black dashed line in Fig. 3A), especially when one considers situations when individuals did not recover rhythmic ventilation while they transiently recovered VERs (*i.e.*, 6 out of 52 individuals, see orange crosses in Fig. 3A) or when rhythmic ventilation recovered in the absence of VERs (*i.e.*, 11 out of 52 individuals, see green crosses in Fig. 3A).

Based on the most parsimonious linear mixed-effects model (Supp. Info. 1F), heart rate of trout following in-air electrical stunning was significantly affected by the interaction between recovery status (*i.e.*, individual recovered VERs permanently, transiently or not at all) and the elapsed time following the application of the stun (Interaction: $F_{2,869.37} = 80.764$, $p < 0.001$; Fig. 3B). The fixed and random effects of the linear mixed-effects model explained 63% and 18% of the variation in heart rate, respectively, following in-air electrical stunning. Model coefficients and post-hoc tests revealed that the heart rate of permanently recovered rainbow trout (*i.e.*, heart rate increased from ~40 to 62 beats min^{-1}, Time: $F_{1,327.67}$:104.25, $p < 0.001$) was significantly higher than the heart rate of individuals that either transiently recovered (*i.e.*, heart rate decreased from ~23 to 12 beats min^{-1}, Time: $F_{1,327.67}$:104.25, $p < 0.001$) or not at all (*i.e.*, heart rate remained at ~20 beats min^{-1}, Time: $F_{1,391.81}$:3.889, $p = 0.150$) throughout the monitoring period (see planned contrasts in Fig. 3B).

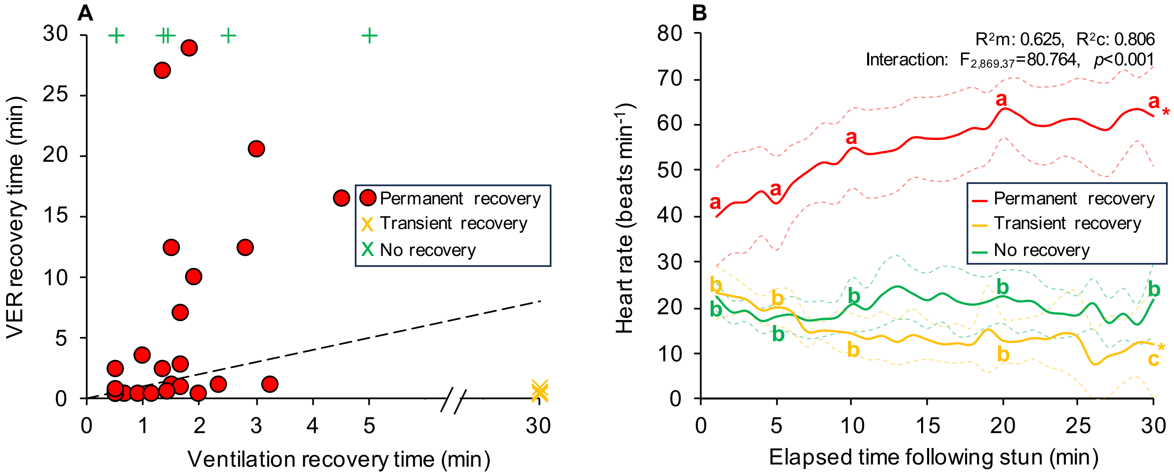

Fig. 3. Ventilatory and cardiac responses of rainbow trout following in-air electrical stunning. (A) The recovery of rhythmic ventilation following in-air electrical stunning is poorly related to the recovery of VERs in rainbow trout (*c.f.*, position of red circles in relation to 1:1 line of best fit [black dashed line]). This is further highlighted during situations when individuals transiently recovered VERs but not ventilation (orange crosses) and *vice versa* (green crosses). (B) Heart rate of rainbow trout was significantly higher in individuals that permanently recovered VERs (red lines) when compared to those that only transiently recovered VERs (orange lines) or not at all (green lines). Solid and dashed coloured lines in (B) represent the mean ± 95% C.I. calculated from the raw data, while statistical differences ($p < 0.05$) between recovery groups at specified time points (*i.e.*, 1, 5, 10, 20 and 30 min, represented by different lower case letters) or within recovery groups (*i.e.*, significant temporal changes in heart rate, represented by an *) are based on model 5 (see Supp. Info. 1). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.4. Brain, ventilatory and cardiac responses of percussively stunned trout

All rainbow trout exhibited an absence of VERs and rhythmic ventilation throughout the entire monitoring period following percussive stunning (*i.e.*, 10 out of 10 individuals; Fig. 4A-B). Based on the most parsimonious linear mixed-effects model (Supp. Info. 1G), heart rate of trout significantly decreased over time following percussive stunning (*i.e.*, heart rate decreased from ~64 to 26 beats min^{-1}, Time: $F_{1,197.23}$:287.83, $p < 0.001$; Fig. 4B). The fixed and random effects of the linear mixed-effects model explained 45% and 26% of the variation in heart rate following percussive stunning.

4. Discussion

This investigation marks the first electroencephalographic assessment of the efficacy of in-air electrical stunning for rainbow trout. Our findings demonstrate the potential of in-air electrical stunning as a humane stunning and/or killing method, as well as its limitations, while providing insights into the physiological mechanisms underpinning lethality following stunning.

4.1. Can in-air electrical stunning induce an immediate and irreversible stun?

When electrical parameters or settings exceed species-specific thresholds, in-air electrical stunning has been demonstrated to immediately induce epileptiform insults (*i.e.* a neurological indicator incompatible with consciousness) in a range of farmed fish species such as Atlantic cod (*Gadus morhua*; Erikson et al., 2012), African sharptooth catfish (*Clarias gariepinus*; E. Lambooij et al., 2004; Sattari et al., 2010); Atlantic salmon (*Salmo salar*; E. Lambooij et al., 2010), common carp (*Cyprinus carpio*; E. Lambooij et al., 2007), common sole (*Solea solea*; Daskalova et al., 2016; Llonch et al., 2012), haddock (*Melanogrammus aeglefinus*; E. Lambooij et al., 2012), pike perch (*Stizostedion lucioperca*; Llonch et al., 2012), turbot (*Scophthalmus maximus*; Daskalova et al., 2016), and yellowtail kingfish (*Seriola lalandi*; Llonch et al., 2012). However, the immediate induction of epileptiform insults by in-air electrical stunning in rainbow trout could not be determined in our study due to technical limitations. Nevertheless, ~17% of the trout subjected to in-air electrical stunning displayed VERs immediately upon instrumentation. This observation indicates that these fish were either not immediately rendered unconscious or regained consciousness within the 30 s instrumentation period. Although the latter scenario is plausible (*i.e.*, electrically stunned rainbow trout have been documented to recover VERs within 10 s; Hjelmstedt et al., 2022), it is imperative that future research investigates the efficacy of in-air electrical stunning in immediately inducing unconsciousness in this species.

While the efficacy of in-air electrical stunning in immediately inducing unconsciousness remains in question, our study demonstrates its ability to induce an irreversible stun in rainbow trout, a phenomenon also observed in the aforementioned farmed fish species (Daskalova et al., 2016; Erikson et al., 2012; E. Lambooij et al., 2004, 2007, 2010, 2012; Llonch et al., 2012; Sattari et al., 2010). However, despite its potential to induce an irreversible stun, our data reveals that ~44, 48 and 60% of rainbow trout recovered VERs at 5, 10 and 30 min post-stunning, respectively. Considering the welfare implications of this recovery rate, it becomes imperative to identify factors that could be modified or enhanced to improve the efficacy of this stunning and/or killing method in rainbow trout.

4.2. Increasing stunning intensity and duration of in-air electrical stunning has limited impact

Factors such as the intensity and duration of the electrical stun have been highlighted as crucial factors that underlie stunning success (EFSA, 2004). Comprehensive assessments in rainbow trout indicated that the duration of unconsciousness (based on the absence of VERs) following in-water electrical stunning could be extended by increasing stun intensity and/or duration (Robb et al., 2002). However, Robb et al. (2002) also demonstrated that beyond a specific threshold in stun intensity (*i.e.*, ≥100–150 mA), the period of time during which VERs were absent tended to plateau while a proportion of stunned individuals were irreversibly stunned (*i.e.*, up to 60% of trout never recovered VERs). In the present study, rainbow trout were subjected to a range of stunning intensities (*i.e.*, from 50 to 920 mA) and durations (*i.e.*, from 5 to 30 s), yet neither factor significantly affected the period of time during which VERs were absent in rainbow trout that recovered from electrical stunning. While the combination of maximum stunning intensity and

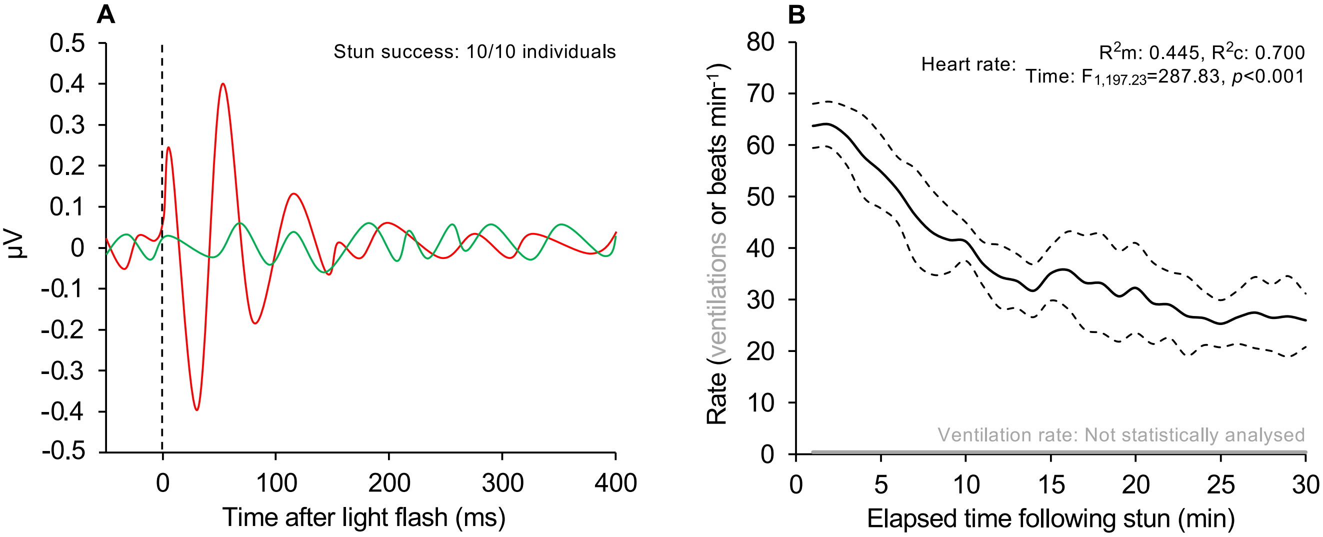

Fig. 4. Brain, ventilatory and cardiac responses of rainbow trout following percussive stunning. (A) All rainbow trout that were percussively stunned did not recover VERs throughout the monitoring period (red and green lines represent example VERs before and after stunning, respectively, while the black dashed line represents the light flash). (B) In addition, individuals did not recover rhythmic ventilation (grey line), while heart rate was significantly elevated directly following stunning and then decreased throughout the monitoring period (solid and dashed black lines represent mean ± 95% C.I. calculated from the raw data, while the model output for temporal changes in heart rate is based on model 6 in Supp. Info. 1). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

duration was observed to induce the highest proportion of irreversibly stunned trout (*i.e.*, 70% of individuals), regression analyses revealed that the likelihood of trout being irreversibly stunned was only marginally affected by stun intensity (*i.e.*, stun intensity explained 8% of the variation in stunning success) but not stun duration. This data, along with findings from Robb et al. (2002), suggests that enhancing the efficacy of both in-air and in-water electrical stunning cannot solely rely on increasing stunning intensity and/or duration. Instead, it underscores the need to identify other contributing factors for the continued improvement of these stunning techniques.

4.3. Individual variation in susceptibility to electrical stunning requires further investigation

Significant variability in the electrical current passing through trout was recorded in the present study despite the predefined settings of the stunner that generates specific AC and DC voltages (see Table 1). Although body mass played a role in explaining part of this variation, additional explanatory factors should be investigated to enhance the effectiveness of in-air electrical stunning. For instance, a previous study on Atlantic mackerel (*Scomber scombrus*) highlighted the importance of electrode positioning in relation to the brain of the fish, as the only unsuccessful stun occurred when the electrodes were not positioned correctly on the head of the individual (Anders et al., 2019). Electrode positioning may have contributed to the variation in stunning success in our study, as trout were not restrained within the stunner with the electrode placed directly over the brain, but were rather passed through the stunner in a manner that mimicked a commercial slaughter situation. Future research should therefore delve into understanding the relationship between the electrical current generated from the stunner and its passage through the brain of the fish, considering factors such as electrode positioning (Anders et al., 2019) and/or the tissue composition/impedance of individual fish (Grimsbø et al., 2016; Sattari et al., 2010). This is crucial to unravel the individual variation in the susceptibility of rainbow trout to electrical stunning observed in the present study and others (Hjelmstedt et al., 2022; Robb et al., 2002). Only through such comprehensive examinations can technological refinements be implemented to elevate the success rate of in-air electrical stunning in rainbow trout.

4.4. Sequential killing method required to ensure human slaughter

While it has been shown that in-air electrical stunning can be used to successfully stun numerous species of farmed fish, the likelihood that all individuals are irreversibly stunned is not guaranteed (Daskalova et al., 2016; Erikson et al., 2012; E. Lambooij et al., 2010, 2012; Llonch et al., 2012). Therefore, it has been recommended that a killing method be immediately employed following electrical stunning to ensure that individuals do not regain consciousness prior to death. Previous studies investigating the efficacy of in-air electrical stunning have proposed sequential killing methods such as immersion in ice water (*e.g.*, common sole, pike perch, turbot and yellowtail kingfish; Daskalova et al., 2016; Llonch et al., 2012), exsanguination (*e.g.* Atlantic cod and haddock; Erikson et al., 2012; E. Lambooij et al., 2012), decapitation (*e.g.*, African sharptooth catfish, *Clarias gariepinus*; Sattari et al., 2010), and percussion (*e.g.*, Atlantic salmon; E. Lambooij et al., 2010). Our findings strongly suggest that the sequential killing method should be instantaneous, as some rainbow trout showed signs of recovery within 0.5 min following in-air electrical stunning. The sequential application of a percussive stun fulfills this criterion, as percussively stunned fish have been observed to lose consciousness immediately and irreversibly (based on neurological evidence of immediate brain trauma and permanent abolition of VERs, Hjelmstedt et al., 2022; Lambooij et al., 2010). However, it is crucial that the application of a percussive stun is performed accurately and with sufficient force, as fish can otherwise recover VERs (Brijs et al., 2021; Hjelmstedt et al., 2022; Jung-Schroers et al., 2020; E. Lambooij et al., 2010; Retter et al., 2018; Robb et al., 2000). Thus, the sequential approach of in-air electrical stunning (assuming it can induce an epileptiform insult in rainbow trout) followed by percussive stunning may represent a potentially humane stunning and killing method. This is because in-air electrical stunning may render rainbow trout immediately unconscious and immobile, which would subsequently increase the accuracy of either manual percussive stunning (*i.e.*, fish become easier and safer to handle for aquaculture personnel) or automated percussive stunning (*i.e.*, fish do not struggle or thrash around and enter the percussive stunner in the correct orientation/position) to ensure a humane slaughter (Hjelmstedt et al., 2022; Jung-Schroers et al., 2020; Retter et al., 2018).

4.5. Insights into the physiological basis for the lethality of electrical stunning

While rhythmic ventilation appeared to be a poor indicator of recovery in the present study and others (Bowman et al., 2019, 2020; Brijs et al., 2021; Hjelmstedt et al., 2022), it nonetheless offers valuable insights into the underlying physiological mechanisms governing lethality following stunning. In the present study, all trout that permanently recovered VERs exhibited a return to rhythmic ventilation within 5 min post-stun, while rhythmic ventilation was absent in fish that transiently recovered VERs or in the majority of fish that failed to recover VERs altogether (*i.e.*, ~62 and 100% of individuals irreversibly stunned using in-air electrical and percussive stunning, respectively). Irrecoverable cardiac arrests were not observed in any rainbow trout following electrical or percussive stunning. Following in-air electrical stunning, there was an elevation in heart rate from ~40 to 62 beats min^{-1} in trout that recovered VERs permanently, while trout that did not permanently recover VERs displayed varied responses, with heart rates either remaining at ~20 beats min^{-1} or decreasing throughout the monitoring period (*i.e.*, from ~23 to 12 beats min^{-1}). Following percussive stunning, rainbow trout exhibited a similar pattern as previously reported in Atlantic salmon (E. Lambooij et al., 2010), with cardiac fibrillation followed by a gradual decrease in heart rate throughout the monitoring period (from ~64 to 26 beats min^{-1}).

The cardio-ventilatory responses of rainbow trout to in-air electrical stunning closely mirror those observed in electrically stunned Arctic char (*Salvelinus alpinus*; Sandblom et al., 2012), suggesting that death following electrical stunning primarily stems from ventilatory failure rather than an immediate and irreversible cardiac arrest (Daskalova et al., 2016; E. Lambooij et al., 2010; Sandblom et al., 2012). Ventilatory failure, attributed to disturbances in the ventilatory rhythm generation in the central nervous system (Kestin et al., 2002), likely results in hypoxemia and subsequent malfunction of vital organs, such as the heart and brain (Daskalova et al., 2016; Sandblom et al., 2012). In contrast, the powerful pressure waves or rapid oscillations in pressure induced within the cranial cavity by percussive stunning lead to a cerebral hemorrhage, which either directly or indirectly (*e.g.*, *via* ventilatory failure) results in death (E. Lambooij et al., 2010).

5. Conclusion

While our findings demonstrate that in-air electrical stunning can irreversibly stun rainbow trout, further research and/or technological refinements are required to determine whether this method can immediately induce unconsciousness in rainbow trout, as well as to enhance its success rate before it can be recommended as a standalone humane slaughter method. Moreover, a sequential approach commencing with in-air electrical stunning (provided it immediately renders fish unconscious) followed by percussive stunning, likely offers a more humane approach. This strategy would capitalize on in-air electrical stunning promptly rendering rainbow trout unconscious and immobilized, thereby improving the safety and precision of both manual and automated percussive stunning techniques to safeguard the welfare of rainbow trout during the slaughter process.

Animal ethics statement

Animal care and all experimental procedures were performed in accordance with national regulations and were covered by an ethical permit approved by the ethical committee on animal research in Gothenburg, Sweden (1873–2018).

CRediT authorship contribution statement

Jeroen Brijs: Writing – review & editing, Writing – original draft, Visualization, Validation, Formal analysis, Data curation. **Per Hjelmstedt:** Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Conceptualization. **Charlotte Berg:** Writing – review & editing, Validation, Supervision. **Erik Sandblom:** Writing – review & editing, Validation, Methodology, Investigation. **Albin Gräns:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization.

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

During the preparation of this work Jeroen Brijs used ChatGPT 3.5 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.

Data availability

Raw data supporting this study has been included in the supplementary information

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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.2024.741387.

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