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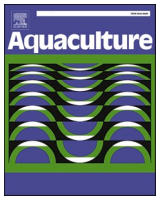

The quest for a humane protocol for stunning and killing Nile tilapia (*Oreochromis niloticus*)

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ABSTRACT

Considering the large number of individual Nile tilapia (*Oreochromis niloticus*) slaughtered yearly without adequate stunning, there is an urgent need for development and validation of stunning and killing methods that adhere to humane slaughter standards. In this study, we evaluated measurements of brain activity using electroencephalograms (EEG) to assess the effectiveness of percussive stunning, live chilling, electrical stunning, and a combination of electrical stunning followed by bleeding and chilling. Recordings were inspected for presence or absence of epileptic-like seizures, and for visually evoked responses (VERs). Percussive stunning using a pneumatic bolt gun resulted in both immediate and permanent loss of consciousness. This contrasts with live chilling, where it took up to 11 min for VERs to disappear, and where VERs recovered when the fish was returned to ambient water. Electrical stunning for 1 s at an intensity of ~8 V_{RMS} cm^{-1} and ~0.7 A_{RMS} dm^{-2} induced epileptic-like seizures, rendering the fish unresponsive to the light flashes for 16.3 ± 2.4 s, demonstrating immediate albeit transient stun effects. Increasing the electrical intensity, either by prolonging the stun duration or by increasing the electrical energy, prolonged the time that VERs were lost. However, to achieve a permanent loss of VERs, ~14 V cm^{-1} and ~1.1 A dm^{-2} for 30 s, followed by immediate throat cutting and chilling in an ice slurry, was required. Our results clearly show that percussive bolt gun stunning is the most effective method to render Nile tilapia immediately and permanently unconscious. However, this method can be stressful for the fish, and if the fish struggle when the blow is executed, the effectiveness of the stun is at risk. Combining electrical stunning with subsequent exsanguination and chilling was also successful. Adopting a sequential approach where different procedures are combined could pave a path for successful stunning and killing without inducing unnecessary fear, stress or discomfort. For this purpose, an even more fruitful combination could be to integrate electrical and percussive stunning techniques.

1. Introduction

In 2020, Nile tilapia (*Oreochromis niloticus*) ranked as the third most produced fish species in aquaculture, yielding over 4.4 million tonnes (FAO, 2022). The exact number of deaths resulting from this production remains unknown. However, considering typical harvest weights ranging from 250 to 800 g, this volume translates to approximately 5.5 to 17.6 billion Nile tilapia being slaughtered in 2020 (Eurogroup for Animals, 2018; Mood et al., 2023). The majority of these fish are killed in an inhumane fashion that cause immense and prolonged suffering such as asphyxiation (*i.e.*, fish is suffocated in air) and/or evisceration (*i.e.,* internal organs are removed; Lines and Spence, 2012; Robb and Kestin, 2002). Consequently, there are urgent needs to develop and implement humane methods for stunning and killing Nile tilapia (EFSA, 2009; OIE, 2023).

Humane slaughter entails the killing of animals in an unconscious state, as defined by EFSA (EFSA, 2004) as "a state of unawareness (loss of consciousness) in which there is temporary or permanent damage to brain function and the individual is unable to perceive external stimuli (which is referred to as insensibility) and control its voluntary mobility and, therefore, respond to normal stimuli, including pain'. Ideally, an effective humane stunning method should render the animal immediately unconscious, and persist for long enough for death to ensue before consciousness returns. Moreover, procedures preceding the stunning

Abbreviations: EEG, electroencephalography; VERs, Visually evoked responses.

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should not induce unnecessary fear, pain, suffering or distress (EFSA, 2004, 2009; FAWC, 1979; OIE, 2023; Robb and Kestin, 2002).

Assessing unconsciousness in fish presents a challenge due to limitations in communication, making us rely vastly on visual cues for determining loss of consciousness. However, some stunning methods can immobilize fish without actually rendering them unconscious (Brijs et al., 2021; Lambooij et al., 2010; Robb and Kestin, 2002). This can lead observers to mistakenly believe the fish are unconscious, resulting in the risk of performing slaughter procedures on conscious fish. Therefore, relying solely on inspecting the animal for absence of visual indicators of consciousness (*e.g.,* ventilation, equilibrium and the eye roll reflex) can be misleading, as these can all be lost without the fish entering a state of unconsciousness (Bowman et al., 2019, 2020; Brijs et al., 2021; van de Vis et al., 2003). Consequently, evaluating the fish brain functionality is crucial to determine the effectiveness of stunning methods. This can be achieved through measurements of brain activity using electroencephalograms (EEG), which have been conducted on numerous fish species, including Nile Tilapia (Bowman et al., 2019; Brijs et al., 2021; Hjelmstedt et al., 2022; Kestin et al., 1991; Lambooij et al., 2002a, 2008, 2010; Robb et al., 2000; Rucinque, 2021).

One approach to assess levels of brain activity and unconsciousness in farmed animals, including fish, is to assess evoked responses in the EEG. This approach relies on the absence of such potentials to indicate brain dysfunction and the inability to respond to internal or external stimuli (Gregory and Wotton, 1986; Regan, 2009). A specific type of evoked response is the visually evoked response (VER), elicited by a light stimulus. During a VER, neural signals from the fish retina transmit the light stimulus to the visual cortex for processing (Gregory and Shaw, 2000; Verhoeven et al., 2015). In this process, the primary wave indicates the arrival of the signal at the higher brain centres, while the secondary wave indicates further processing. Disruption of VERs suggests dysfunction in the cerebral cortex, which is incompatible with consciousness (Erasmus et al., 2010; Kumar et al., 2022). While it may be argued that a complete loss of VERs is too stringent a criterion for determining loss of consciousness, from an animal welfare perspective and in the absence of a direct measure of consciousness, the presence of VERs must represent the possibility that the animals are not unconscious (Kestin et al., 1991). Consequently, the abolition of VERs has been used as an objective and unequivocal indicator of brain dysfunction and, consequently, unconsciousness, across a diverse range of farmed fish subjected to various stunning and/or killing methods (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., 2002a; Retter et al., 2018; Robb and Roth, 2003; Robb et al., 2000). However, EEG measurements in fish are technically challenging and rarely performed during the development of stunning and killing methods. Consequently, significant uncertainties of how fish in general, and species slaughtered in great numbers in particular, can be slaughtered in a humane matter remains (; van de Vis et al., 2003).

Suggested stun or stun-and-kill methods include live chilling, electrical stunning, percussive stunning or a combination of methods. Live chilling involves rapid cooling of the fish and represents a procedure widely employed during Nile tilapia slaughter (Kumar et al., 2022; Rucinque et al., 2023). While this method is generally not considered a humane stunning method, much of this consensus stems from studies on salmonids, which are cold-water species (OIE (World Organisation of Animal Health), 2023; Robb and Kestin, 2002; Skjervold et al., 2001; van de Vis et al., 2003). Considering Nile tilapia's tropical nature and preference for warmer waters, the substantial temperature contrast between the rearing environment and the ice slurry used for live chilling may lead to rapid loss of consciousness in these warm-water fish. Although speculative, the efficiency of live chilling for warm-water species like tilapia warrants further investigation, especially given the widespread use of live-chilling in slaughter plants (Blessing et al., 2010).

Electrical stunning stands as a method capable of inducing immediate loss of consciousness in fish. Sufficient electrical current through the brain can trigger epileptic-like seizures, similar to the generalized tonic-clonic seizures, or *grand mals*, observed in humans and other mammals (Shaw, 1997; Terlouw et al., 2016). During these seizures, a surge of neuronal activity is detected as high-frequency activity on the EEG, followed by a period of very low brain activity (*i.e.,* the isoelectric phase in the EEG; Blumenfeld and Taylor, 2003; Cavanna and Ali, 2011; Zivotofsky and Strous, 2012). Both hyperpolarisation and subsequent isoelectric phases disrupt brain function and impair sensibility, either transiently or persistently (Blumenfeld and Taylor, 2003). EEG verification of seizures and immediate loss of sensibility has been successfully demonstrated across various species (Brijs et al., 2021; Cook et al., 1995; Kestin et al., 1995; Lambooij et al., 2013; Terlouw et al., 2016; van de Vis et al., 2014). However, recent research suggests that the fish EEG may not consistently exhibit a clear isoelectric phase after an epileptic-like seizure (Hjelmstedt et al., 2022), raising uncertainty about post-seizure responsiveness. This uncertainty, combined with the transient effects of electrical stunning, poses a risk that the fish may regain consciousness before subsequent killing methods are applied (Robb et al., 2002; van de Vis et al., 2003). Furthermore, electrical stunning is a complex process, involving various electrical parameters that interact and influence the outcomes, and where the effect of electricity can substantially vary among fish species due to differing sensitivities to electricity (Lines and Kestin, 2004; Marx et al., 1997; Robb and Roth, 2003; Roth et al., 2003).

Another method capable of inducing immediate loss of consciousness is physical destruction of the brain, typically achieved through spiking or percussive stunning in fish. Spiking, the practice of physically disrupting the brain with a knife or an awl, can quickly kill the fish if executed correctly (Rucinque, 2021). However, due to the small brains of many fish and the welfare risks associated with failed attempts, spiking is rarely used in aquaculture settings (Lambooij et al., 2003; Robb and Kestin, 2002; Robb et al., 2000; van de Vis et al., 2003). Percussive stunning, the delivery of a blow to the fish's head, on the other hand, is more widely used. This method aims to render the fish immediately and permanently unconscious through a severe concussion resulting from the head trauma. Such a trauma can cause alterations in blood flow, brain tissue lesions, depolarization of neurons and brain haemorrhaging, resulting in the rapid loss of consciousness (Brijs et al., 2021; Hjelmstedt et al., 2022; Marx et al., 1997). While this seems promising from an animal welfare perspective, percussive stunning presents a challenge both to workplace safety and to the welfare of the animal as both the operator and the fish may sustain injuries during the process, especially if performed manually without prior stunning. Additionally, percussive stunning typically involves air exposure, handling, and restraint of the fish, all of which can induce stress for both the fish and the operator (Lines and Spence, 2012). Consequently, this method is often considered labour-intensive and impractical for fish farms that must handle and slaughter large quantities of fish within a short timeframe (Robb and Kestin, 2002).

Both electrical and percussive stunning hold the potential to meet the high standards for humane slaughtering. However, given the vast diversity among fish species, it is crucial to validate slaughter protocols specific to each species. In this study, we aimed to assess the effectiveness (*i.e.,* the success in rendering Nile tilapia unconscious swiftly and permanently) of electrical and percussive stunning. Additionally, we aimed to evaluate live chilling as a potential stunning method for this warm-water species, given its broad application in Nile tilapia farming globally. We hypothesized that electrical stunning would induce immediate loss of consciousness but require a secondary stun or kill method due to its transient effects. We further hypothesized that percussive stunning would render fish immediately and permanently unconscious. As for live chilling, we hypothesized that there would be an induction time to render the fish unconscious. Furthermore, we also aimed to evaluate the effectiveness of a combination of steps already partly implemented in many slaughter facilities, *i.e.,* electrical stunning, throat cutting and chilling. Our hypothesis was that the electrical shock

in such a combination would render the fish immediately unconscious for long enough for the fish to be throat cut and immersed in ice slurry, wherein the cold temperature would prevent the fish from recovering consciousness before death by exsanguination. A similar slaughter protocol was recently proven successful for African sharptooth catfish (*Clarias gariepinus;* Brijs et al., 2021).

2. Material and methods

Male Nile tilapia (n: 42, body mass: 747 ± 33 g, fork length: 324 ± 4 mm) were transported for 3.5 h from a land-based fish farm (Gårdsfisk, Scandinavian aquaculture systems AB, Tollarp, Sweden) to the fish holding facility at the University of Gothenburg. Tilapia were housed in a freshwater re-circulation system with a water temperature of 21 ± 1 °C (unless otherwise stated, all data is presented as mean ± SEM) and a 12:12 h light:dark cycle. The re-circulating system consisted of a 600 L tank and a cleaning filter, and approximately 80% of the water was exchanged every second day. Fish were fed two to three times a week with commercial tilapia pellets (Til 300, Skretting, Stavanger, Norway). All animal handling and experimental procedures were in accordance with ethical permit ID# 001873 and Dr.#5.8.18–12,466/2018 issued by the regional animal ethics committee in Gothenburg Sweden.

2.1. Measurements of brain activity using EEG

EEG recordings were obtained prior to, during (whenever applicable), and following the stunning attempts. To record the EEG, the fish were sedated in 25 mg L^{-1} of metomidate (Aquacalm, Syndel, Canada) until movements and ventilation ceased. Two 21 gauge, stainless steel needle electrodes were inserted 1 cm caudal of the eyes and 0.5 cm lateral on each side of the sagittal suture, by gently lifting the scales and pressing the needle down through the skin and into the skull (Lambooij et al., 2002b; Fig. 1). The needle electrodes were soldered onto 1.5 mm shielded silver wires (MLAWBT9 EEG Flat Electrodes, ADInstruments, Oxford, United Kingdom), and the connection was waterproofed using a thick layer of silicone. In addition, a 29 gauge stainless steel needle electrode (ADInstruments, Oxford, United Kingdom) inserted into the tissue near the tail of the fish served as the reference electrode. EEG signals were amplified using a bio-amplifier (model FE136, ADInstruments) that relayed the signals to a 16SP PowerLab 8/30 system

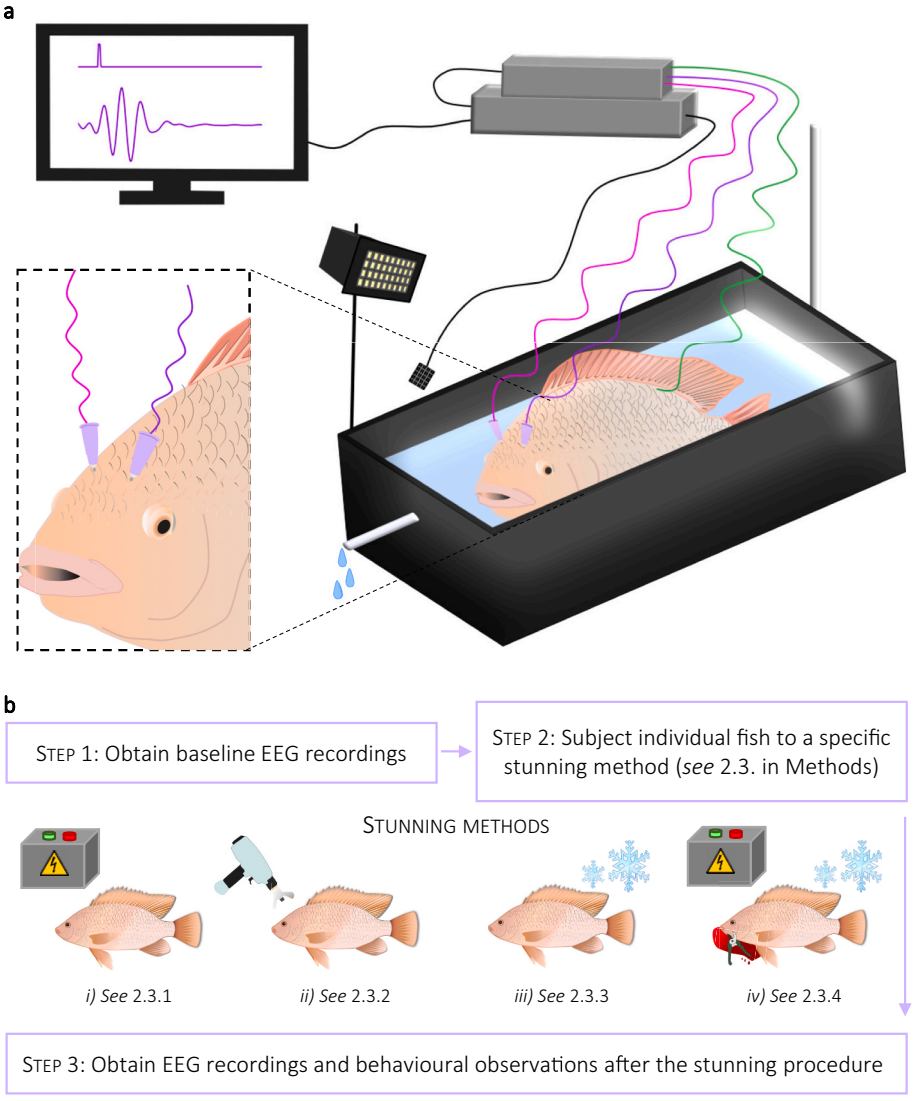

Fig. 1. A schematic of the experimental set up and design. a) A Nile tilapia instrumented with two active electrodes (pink and purple; *see* hatched rectangle for positioning) and one reference electrode (green) for measuring brain activity. b) When clear baseline recordings of visually evoked responses in the EEG (VERs) were obtained, the fish were subjected to a stunning protocol, followed by continuous evaluations of neurological and behavioral indicators of consciousness until the experiments where terminated.

(ADInstruments; Fig. 1). The EEG of the fish was monitored in real time using the data acquisition software Labchart Pro (7.3.2; ADInstruments, Castle Hill, Australia). In this software, a low-pass filter (50 Hz), high-pass filter (0.1 Hz) and 50 Hz notch filter was applied and the sensitivity range was set to ±2 mV (10). The EEG signal was then further filtered using a band-pass filter to obtain the beta wave frequencies (*i.e.,* 13–32 Hz), as this frequency range has been shown to be optimal for the detection and quantification of VERs in other fish species (Bowman et al., 2019; Brijs et al., 2021).

2.2. Assessing presence and absence of VERs in the EEG

Once equipped with the EEG electrodes, the fish were gently moved into a flow through tank of 9.2 L receiving aerated freshwater at 21 ± 1.0 °C gravity fed from a 200 L header tank in a dark room. A custom made strobe light was placed above the fish tank and flashed 5 ms of light at 2 Hz (*i.e.,* 5:495 ms light:dark cycle). Light flashes were detected using a custom made light detector made from a solar panel (Velleman SOL1N, Gavere, Belgium), which in turn relayed the signal to the Powerlab (Fig. 1). VERs were detected using the Scope View module in the software, which displayed a time window of 450 ms (starting 50 ms before, and ending 400 ms following each strobe-light flash). The last 50 ms of each cycle was excluded from the recordings to obtain a distinct separation between measurements. To reduce the effects of noise caused by strong muscular movements, all time windows where the amplitude of the beta wave exceeded 15 μV were automatically excluded from the analyses. The Scope View module was programmed to average 120 consecutive, non-overlapping time windows into a single 450 ms time window representative 60 s of recording (Fig. 2).To identify and define VERs in the EEG, the 450 ms recordings was divided into two phases: the light phase and the dark phase (Fig. 2). Quantitatively, VERs were defined as when the beta wave amplitude during the light phase was more or equal to twice the amplitude during the dark phase (Hjelmstedt et al., 2022). Importantly, VERs are formed in the shape of distinct waves, and so quantitative assessments using the amplitude quote was always accompanied by visual inspection to assure that a repetitive distinct wave form, indistinguishable from the rest of the beta wave, was present in close proximity to the light flash (Fig. 2).

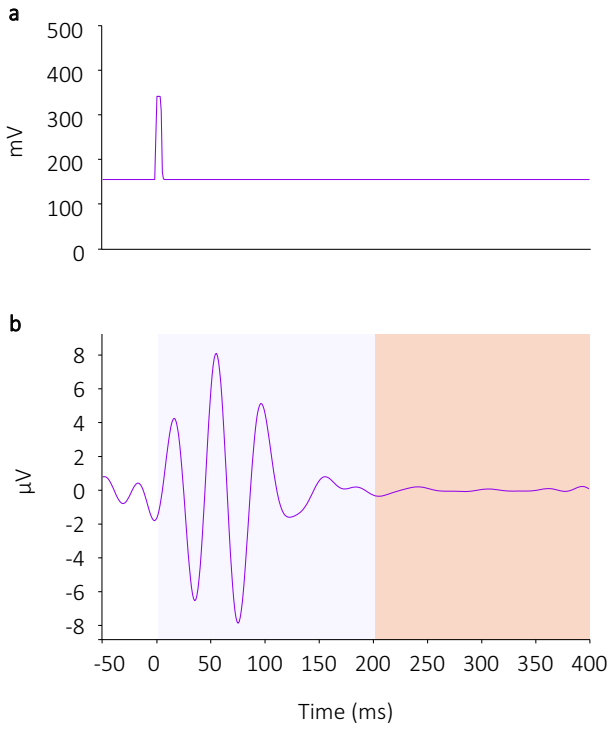

Fig. 2. Visually evoked responses (VERs) in the EEG of a Nile Tilapia. A light flash detected by a solar panel (a) and VERs in the beta wave frequencies of the EEG (b) triggered by the light are shown. Both (a) and (b) depict the average of 120 consecutive 450 ms recordings, with VERs appearing as a distinct waveform in the EEG trace immediately after the light stimulus. The shaded areas in (b) denote the light (purple) and dark (apricot) phases. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.3. Evaluation of different stunning methods

The effectiveness of four different stunning methods were evaluated by assessing the presence or absence of VERs during and/or after the stunning attempt. In this evaluation, the time to loss of VERs and the return of VERs if lost and recovered were noted. The stunning methods evaluated were i) percussive stunning, ii) electrical stunning, iii) live chilling, and iv) the sequential application of electrical stunning, exsanguination and live chilling. For all trials, a baseline period of clear VERs were recorded prior to each stunning protocol. Loss or onset of rhythmic ventilation and coordinated body movements, including fin movements, were also noted during and/or after a stunning attempt. For short electrical exposure, the generation of an epileptic like insult in the brain immediately post electrical exposure was assessed. For a schematic depicting the experimental design, *see* Fig. 1.

2.3.1. Electrical stunning

Electrical stunning was conducted using a custom-made in-water electrical stunning system (Ace Aquatec, Ltd., Dundee, United Kingdom) capable of supplying 50 Hz sinusoidal alternating current (AC). The electrical stunner was connected to two stainless steel plate electrodes (47 × 15 cm, separated by 11 cm) running in parallel along each side of the fish tank. An oscilloscope (123, 20 MHz, Fluke Corporation, Everett, USA) and current probe (801-110S, Fluke Corporation, Everett, USA) measured the voltage and current applied during the electrical stun. Water within the stunning tank was completely exchanged between trials, and water temperature and conductivity were recorded. Prior to stunning, the water inflow to the fish tank was turned off and immediately post stunning the flow of water was turned on, and the fish was left in ambient water for EEG measurements until VERs returned or for 30 min.

When electricity is applied in water, maintaining similar conductivities of both the water and the fish is important to optimize current conduction through the fish while minimizing energy wastage (Lines and Kestin, 2004). Therefore, a pilot study aiming to determine the conductivity of Nile tilapia was performed with the hypothesis that an applied current will be consistent with and without fish when the conductivities of the water and the fish is similar, whereas differing conductivities will lead to variations in current. Electricity was applied to a stunning bath with known water conductivity with and without a freshly killed fish, and the current delivered for each trial was noted. The water conductivities evaluated were 250, 500, 750, 1000, 1250 and 2000 μS cm^{-1} and the water level in the stunning tank was adjusted to be kept constant with and without fish. Fig. 3 reveal that when a water conductivity of 750 cm^{-1} was used, the current applied was similar with and without fish. Moreover, polynomial functions fitted to the data with and without fish revealed an intersection at 755 μS cm^{-1} (Eq. (1)), which was the water conductivity used in the stunning tank for all further test involving electricity.

$$-0.000002x^2 + 0.0097x + 0.3623 = 0.000002x^2 + 0.0087x + 1.1174 \quad (1)$$

To test whether electrical stunning can induce an immediate loss of consciousness, a 1 s stun was applied using two different electrical field strengths (*i.e.* 8.20 ± 0.03 and 12.05 ± 0.06 V_{RMS} cm^{-1}, achieved using the voltages and currents outlined in Table 1 at temperatures and

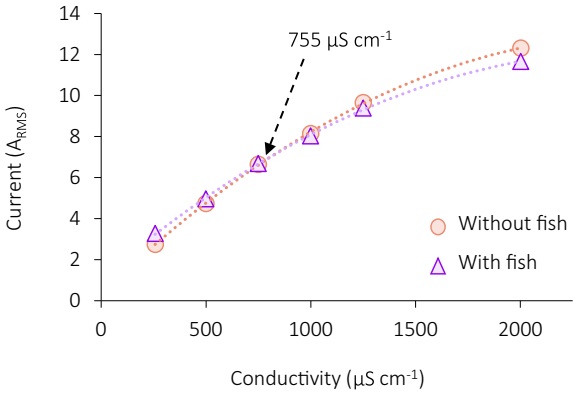

Fig. 3. Determining the optimal water conductivity for electrical stunning of Nile tilapia. The intersection point between the electrical currents (indicated by the hatched arrow) delivered through the stunning bath at different water conductivities with (triangles) or without (circles) Nile Tilapia represents the optimal water conductivity to electrically stun Nile tilapia.

Table 1
Electrical parameters for the four experimental groups used in the electrical stunning trial. The temperature and conductivity was kept constant at 21.1 ± 0.2 °C and 753.5 ± 1 µS cm^{-1}.

Group	*n*	*Current* (A_{RMS})	*Voltage* (V_{RMS})	*Current density* (A_{RMS} dm^{-2})	*Electric field* (V_{RMS} cm^{-1})
1					
1 s	8	4.80 ± 0.05	90.2 ± 0.3	0.68 ± 0.01	8.20 ± 0.03
30 s	6	4.77 ± 0.03	90.1 ± 0.3	0.68 ± 0.01	8.19 ± 0.03
2					
1 s	2	7.13 ± 0.08	132.6 ± 0.7	1.01 ± 0.01	12.05 ± 0.06
30 *s*	3	7.11 ± 0.06	131.3 ± 1.0	1.01 ± 0.01	11.94 ± 0.09

conductivities of 21.1 ± 0.2 °C and 753.5 ± 1 µS cm^{-1}, respectively, n = 10). The presence or absence of epileptic like seizures was determined visually from the EEG, and the amplitude of the raw EEG signal was determined for 10 s prior to electrical stunning, for the duration of the seizure, and for 10 s after the seizure was visually deemed complete. The filtered beta frequency in the EEG was evaluated for VERs before and after stunning, and the time for VERs to reappear after stunning was noted. To test whether the state of unconsciousness could be extended, a 30 s stun was applied using similar electrical field strengths as for the 1 s stuns (n = 9). Specific electrical parameters used for the different experimental groups are summarized in Table 1.

2.3.2. Percussive stunning

A non-penetrative captive bolt gun (Zephyr® F, Bock Industries, PA, US) driven by pressurized air (125 psi) from a compressor (Herkules Walkair CE New, Siegen, Germany) was used to perform the percussive stunning (n = 6). Prior to the stun, the EEG-electrodes were removed and the fish was taken out of the water. The tilapia was then firmly held and the percussive stun was applied directly over the brain. The fish was subsequently re-equipped with the electrodes and placed back in ambient water (21.0 ± 0.2 °C) in the stunning tank for at least 10 min.

2.3.3. Live chilling

To initiate live chilling, the inflow of warm water (21.0 ± 0.1 °C) was shut off and 15 L of ice slurry at <1 °C was added to the stunning tank causing the ambient water to overflow emptying out the majority of the warm water while being replaced by the ice slurry. During the live chilling, EEG was continuously recorded and the behavior of the fish in the tank was monitored (n = 4). Once VERs disappeared, the inflow of ambient water was turned on again in an attempt to recover the individuals. Time taken until loss or recovery of VERs during and after live chilling, respectively, was recorded.

2.3.4. Sequential approach: electrical stunning, exsanguination, and live-chilling

Fish were initially subjected to an electrical stun for 30 s using a range of electrical field strengths (*see* Table 2), followed by exsanguination *via* a throat cut and subsequent immersion in ice slurry for 30 min (n = 8). EEG was recorded prior to electrical stunning and throughout the protocol immediately upon the 30 s electrical exposure. When applicable, the time taken for the recovery of VERs following this sequential approach was recorded.

2.4. Statistical analyses

For statistical analyses, SPSS statistics (v. 27; IBM Corp., Armonk, NY, USA) was used and significance was accepted at $P \leq 0.05$. To determine the effectiveness of each stunning procedure, we statistically analysed changes in the averaged beta wave amplitude during the light phase before and after stunning for all stunning methods, as well as in the raw EEG beta wave trace before and after 1 s electrical stuns with paired *t*-tests. We also compared the time that VERs were lost caused by electrical stunning alone and by the sequential procedure of following electrical stunning with a throat cut and ice slurry immersion with an independent sample t-test. To abide by the assumptions of each test, it was necessary to transform the dataset for the experimental group that received the 1 s long electrical stun with ~8 V_{RMS} cm^{-1} using square root transformation, as unequal variances was found, and to logarithmically transform the dataset for the percussive stunning group as this data was positively skewed. For the comparison of beta wave amplitude before and after a 1 s long electrical stun with ~8 V_{RMS} cm^{-1}, one fish was excluded from the analysis due to high levels of background noise. That is, although a seizure could be visually determined in the trace, quantitative assessments of the amplitude was not possible.

3. Results

3.1. Electrical stunning

3.1.1. Effects of 1 s electrical stun on epileptic-like seizures and VERs

Electrical stunning for 1 s using an electrical field strength and current density of ~8 V_{RMS} cm^{-1} and ~0.7 A_{RMS} dm^{-2} respectively, induced epileptic like seizures in all individuals (n = 8, Fig. 4). In response to the stun, the amplitude of the averaged beta wave increased from 9 ± 2 µV to 660 ± 280 µV (t_6 = 4.660, P = 0.003). The epileptic like seizure lasted for 15.0 ± 2.4 s, after which the amplitude of the averaged beta wave decreased to a similar level observed prior to stunning (7 ± 1 µV, t_6 = 1.554, P = 0.171). Following the 1 s stun, all tilapia in this group lost VERs for 16.3 ± 2.4 s. Individuals that received a stronger electrical shock with an electrical field strength and current density of ~12 V_{RMS} cm^{-1} and ~1.0 A_{RMS} dm^{-2}, respectively, also displayed epileptic like seizures post stunning (n = 2). In these fish, VERs were lost for 22 and 28 s, which again matched the duration of their seizures.

Table 2
Electrical parameters applied during the sequential approach of electrical stunning, exsanguination, and ice slurry immersion. The temperature and conductivity was kept constant at 21.50 ± 0.04 °C and 754.5 ± 1.3 µS cm^{-1}.

Group	*n*	*Current* (A_{RMS})	*Voltage* (V_{RMS})	*Current density* (A_{RMS} dm^{-2})	*Electric field* (V_{RMS} cm^{-1})
1	2	4.77 ± 0.03	89.7 ± 0.9	0.62 + 0.01	8.15 ± 0.08
2	1	7.26	131.2	0.90	11.93
3	4	8.60 ± 0.05	146.5 ± 0.8	1.07 + 0.01	14.23 ± 0.07
4	1	9.82	178.5	1.23	16.23

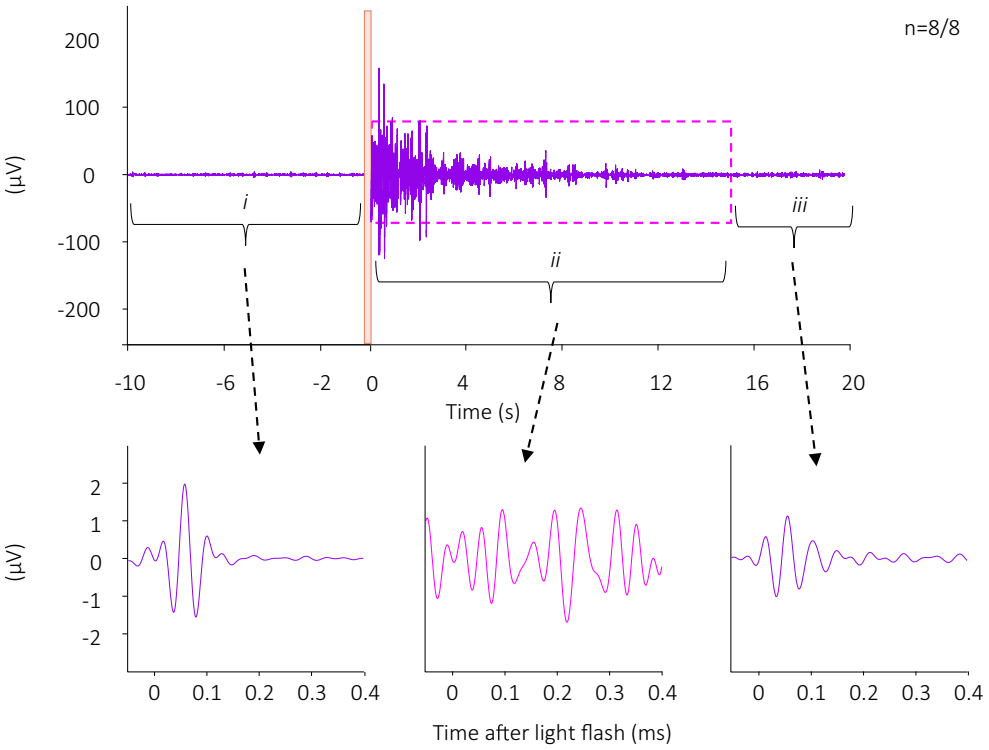

Fig. 4. Representative recording of an epileptic like seizure and visually evoked responses (VERs) in the EEG of a Nile Tilapia. *i*) Prior to a 1 s electrical stun (indicated by the apricot bar in the upper panel), clear VERs (lower left panel) were observed within the EEG. ii) Post stunning, a 15 s epileptic like seizure (indicated by the pink hatched rectangle) was observed during which no VERs were observed (lower middle panel). iii) Upon the completion of the seizure, the amplitude of the EEG returned to levels observed prior to stunning and VERs were observed (lower right panel). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.1.2. *Effects of 30 s electrical stun on VERs*

When increasing the stun duration to 30 s, all individuals stunned with ~8 V_{RMS} cm^{-1} and ~0.7 A_{RMS} dm^{-2} lost VERs for 30–39 s (34 ± 1 s, $n = 6$; Fig. 5a), during which signs of ventilation or movements were not observed. The amplitude of the averaged beta wave following the 30 s stun (0.4 ± 0.1 μV) was significantly lower than pre stunning levels (4.1 ± 1.0 μV, $t_5 = 3.667$, $P = 0.014$). With the exception of one individual, all fish recovered VERs between 30 and 39 s (34 ± 1 s), and ventilation between 120 and 390 s (216 ± 48 s) post stunning. Fish stunned with ~12 V_{RMS} cm^{-1} and ~1.0 A_{RMS} dm^{-2} lost VERs for 66, 96 and 151 s, respectively (104 ± 31 s, $n = 3$; Fig. 5b). The amplitude of the beta wave in the EEG during the light phase significantly decreased from 2.7 ± 0.3 μV prior to stunning to 0.3 ± 0.1 μV post stunning ($t_2 = 6.530$, $P = 0.023$). One fish started ventilating within 20 min post stunning.

3.2. *Percussive stunning*

No VERs, ventilation or body movements were observed in any fish stunned with the pneumatic captive bolt gun ($n = 6$; Fig. 6). The amplitude of the averaged beta wave was 45.0 ± 2.5 μV prior to stunning and decreased significantly to 0.4 ± 0.1 μV post stunning ($t_5 = 7.186$, $P < 0.001$).

3.3. *Live chilling*

Live chilling caused a loss of VERs between 60 and 650 s (295 ± 135 s, $n = 4$; Fig. 7), during which all fish remained calm and displayed no obvious aversive behavior. The amplitude of the averaged beta wave was 8.1 ± 5.5 μV prior to live chilling, and 0.4 ± 0.1 μV when VERs were lost during live chilling. When VERs were lost, ventilation appeared unchanged for one fish while opercular movements decreased significantly for the other three fish. When the water was warmed up again, fish recovered VERs between 240 and 1320 s (705 ± 226 s; Fig. 7). Ventilation increased again in the three fish with depressed ventilation between 150 and 450 s of warming. The fish that did not stop ventilating during chilling moved its pectoral fin throughout the protocol, whereas the other three fish became completely still during chilling but started to move their fins between 120 and 975 s of warming.

3.4. *Sequential approach: electrical stunning, exsanguination, and live chilling*

VERs recovered in fish that were subjected to the sequential approach when stunning intensity was below ~14 V_{RMS} cm^{-1} and ~1.1 A_{RMS} dm^{-2} (n = 3; Fig. 8a). Fish that were subjected to the sequential approach with a stunning intensity of ~8 V_{RMS} cm^{-1} and ~0.7 A_{RMS} dm^{-2} ($n = 2$), recovered VERs slower (*i.e.,* VERs recovered after 80 and 96 s, respectively) compared to fish that were only electrically stunned with those settings (*i.e.,* recovery between 30 and 39 s, n = 6; see *Effects of 30 s electrical stun on VERs*). The fish that was subjected to the sequential approach with a stunning intensity of ~12 V_{RMS} cm^{-1} and ~1.0 A_{RMS} dm^{-2} recovered VERs after 35 s ($n = 1$), which was faster than the fish that were only electrically stunned at those settings (*i.e.,* recovery between 66 and 151 s, n = 3; see *Effects of 30 s electrical stun on VERs*).

Fish subjected to the sequential approach at a stunning intensity of ~14 V_{RMS} cm^{-1} and ~1.1 A_{RMS} dm^{-2}, as well as ~16 V_{RMS} cm^{-1} and ~1.2 A_{RMS} dm^{-2}, did not recover VERs, ventilation or any movements throughout the 30 min protocol post electrical stunning ($n = 5$; Fig. 8b). The light phase amplitude of the beta wave in the EEG was significantly higher prior to electrical stunning (4.1 ± 1.2 μV) compared to the amplitude during both throat cutting (*i.e.,* immediately after the electrical stun was over; 1.1 ± 0.3 μV; $t_4 = 2.823$, $P = 0.048$), and following ice slurry immersion (0.3 ± 0.1 μV; $t_4 = 3.295$, $P = 0.030$).

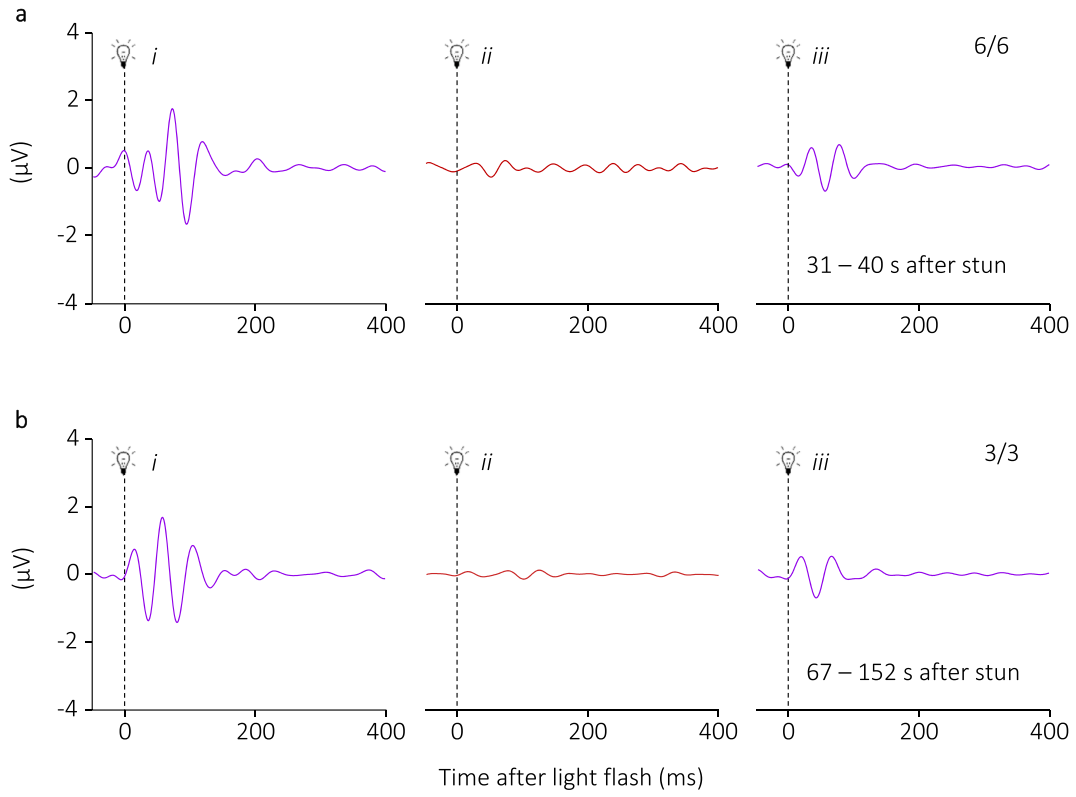

Fig. 5. State of consciousness of Nile tilapia before and after a 30 s electrical stun. The figure demonstrates the presence (purple) and absence (red) of VERs in the beta wave of the EEG prior to (i), directly after (ii), and during recovery from (iii) a 30 s electrical stun at either ~8 V_{RMS} cm^{-1} and ~0.7 A_{RMS} dm^{-2} (upper panel) or ~12 V_{RMS} cm^{-1} and ~1.0 A_{RMS} dm^{-2} (lower panel). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

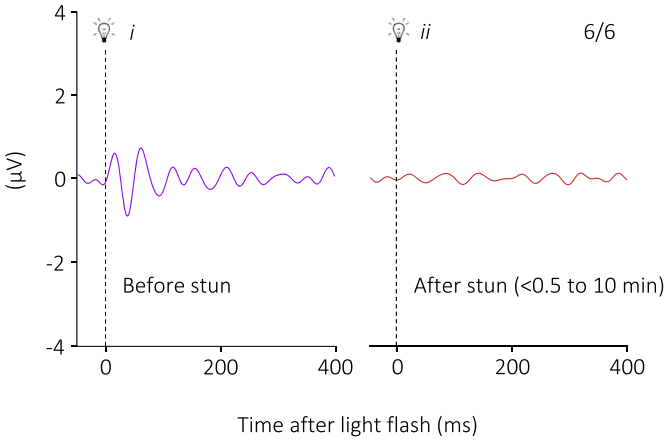

Fig. 6. State of consciousness of Nile tilapia before and after percussive stunning. The figure demonstrates the presence (purple) and absence (red) of VERs in the beta wave of the EEG before (i) and after (ii) percussive stunning with a pneumatic captive bolt gun. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4. Discussion

4.1. Electrical stunning

In this novel study, we evaluated a range of stunning methods on unrestrained Nile tilapia using measurements of EEG and assessment of VERs. Our findings further contribute towards the development of humane slaughter methods for Nile tilapia, which is essential considering that an estimated ~11.6 billion individuals are killed annually in aquaculture. Our results show that a 50 Hz sinusoidal side-to-side electrical stun for 1 s in water (electrical field strength of ~8 V_{RMS} cm^{-1}, current density of ~0.7 A_{RMS} dm^{-2}, water temperature of ~21.1 °C, water conductivity of ~754 μS cm^{-1}) can induce an epileptic-like seizure in Nile Tilapia. Inspections of the EEG revealed that brain activity of Nile tilapia was significantly elevated during the seizures. This finding mirrors previous reports across various fish species, including tilapia (Anders et al., 2019; Brijs et al., 2021; Daskalova et al., 2016; Hjelmstedt et al., 2022; Lambooij et al., 2008, 2010, 2012; Llonch et al., 2012), indicating that during the seizure period, the tilapia's brain experienced heightened stimulation from neuronal discharge rendering it unresponsive to environmental cues (van de Vis et al., 2014).

Increasing the stun intensity to ~12 V_{RMS} cm^{-1} and ~1 A_{RMS} dm^{-2}, and the duration to 30 s increased the minimum time that VERs were lost (*i.e.*, to 30 and 66 s, respectively). However, these periods of insensibility remain relatively brief, especially considering that stunning must be followed by a killing method, such as exsanguination, and endure sufficiently for death to occur before the fish regains consciousness. Increasing the stun intensity and duration was not deemed feasible considering the potential negative impacts on meat quality, as previously demonstrated by the increased proportion of broken bones and haemorrhages in Atlantic salmon (*Salmo salar*; Roth et al., 2003). Moreover, the electrical intensity used to stun tilapia in the present study was exceptionally high, and thus it becomes economically unfeasible to increase it further. For comparison, the electrical field used here was around 4 times higher than what was recently suggested to cause irreversible unconsciousness in juvenile Atlantic salmon (Bouwsema et al., 2022). Taken together our findings indicate a high resilience to electrical stunning in Nile tilapia, suggesting that electrical stunning might not be a suitable stand-alone method for humane slaughter of Nile tilapia.

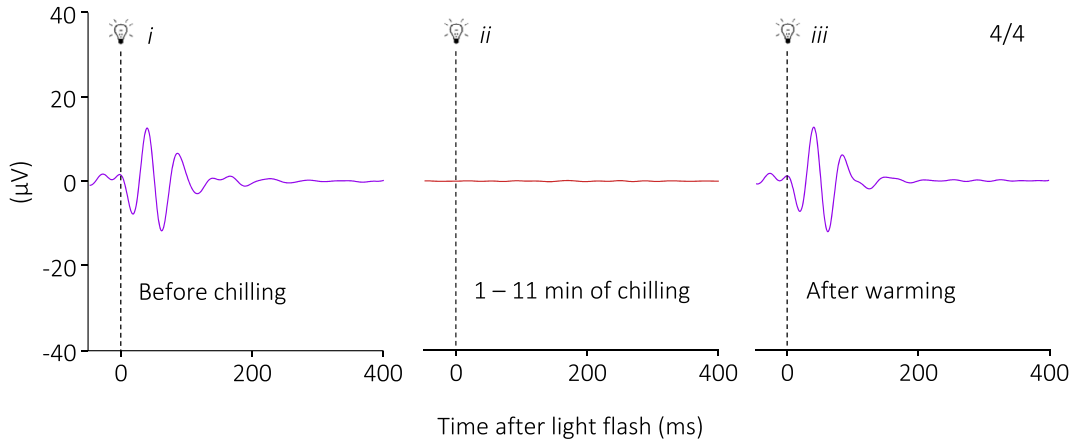

Fig. 7. State of consciousness of Nile tilapia before and after live chilling. The figure shows the presence (purple) and absence (red) of VERs in the beta wave of the EEG prior to (i) and during immersion in an ice slurry (ii), as well as during recovery in ambient water (iii). The time range for when fish lost VERs during chilling or recovered VERs during warming is displayed at the bottom of the respective panels. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4.2. Percussive stunning

Percussive stunning, using a pneumatic bolt gun, appears to be an efficient method to immediately and irreversibly stun Nile tilapia. As soon as the electrodes were reattached after stunning, the brain activity was low and no signs of VERs or other visual indicators of consciousness were observed. The advantage of using a pneumatic bolt gun is that the bolt can be positioned with high precision and deliver a consistent force (Hjelmstedt et al., 2022). In contrast, employing a handheld fish priest (a tool commonly used in small-scale aquaculture facilities) introduces higher potential for human errors during the stunning process and increases the risk of diminishing success rates, particularly as the operator experience fatigue towards the end of a long work shift. For example, Brijs et al. (2021) showed that 36% of African sharptooth catfish recovered VERs after percussive stunning using a handheld fish priest, likely due to variation in the impulse generated by each blow from the fish priest.

Regardless of percussive method used, a potential problem with percussive stunning is the requirement to handle and restrain the fish for the blow to be carried out effectively (Hjelmstedt et al., 2022; Roth et al., 2007). From a fish welfare perspective, this handling can be very stressful, not least as it usually involves taking the fish out of the water (EFSA, 2009). It can also infer a risk for the operators who may have to handle slaughter-sized fish that struggle. Such struggling can result in poor performance with applying the percussive blow, resulting in poor stunning efficiency. One way to bypass these practical challenges is to add a pre-stunning mechanism before the percussive stunning. Such a pre-stunning could alleviate struggles that might otherwise compromise work place safety as well as the effectiveness of the percussive blow, while at the same time safeguarding fish welfare by minimizing stress associated with exposure to air, handling and entrainment.

4.3. Live chilling

All tilapia subjected to live chilling lost VERs, with the induction time ranging between 60 and 660 s. Similar induction times during live chilling have been reported for other warm-water species including gilthead sea bream (*Sparus aurata*) and African sharptooth catfish (Brijs et al., 2021; van de Vis et al., 2003). For cold-water species, the time it takes for the cold water to have an anaesthetic effect (if at all) is usually much longer. For example, farmed turbot (*Scophthalmus maximus*) was reported to show vital signs after 90 min of in sub-zero water temperatures (Roth et al., 2009). While live chilling does not live up to the standards of an immediate or rapid loss of consciousness (EFSA, 2009), its impact on the welfare of Nile tilapia is difficult to interpret, as no obvious signs of aversive behavior was observed. Submersion in ice slurry may not be as stressful for Nile tilapia, as plasma cortisol levels of 22 ng mL^{-1} have previously been reported in this species following 20 min in ice water (Oliveira et al., 2015), which is similar to values reported in unstressed individuals (*e.g.*, > 17 ng mL^{-1}; Auperin et al., 1997). These findings are contrasting to that of many other warm-water species such as African sharptooth catfish (Brijs et al., 2021), grass carp (*Ctenopharyngodon idella*; Scherer et al., 2005), silver carp (*Hypophthalmichthys molitrix*; Zhang et al., 2017), common carp (*Cyprinus carpio*; Rahmanifarah et al., 2011) and gilt-head seabream (*Sparus aurata*; Bagni et al., 2007), which all exhibit aversive behavior and/or increases in plasma cortisol levels when submerged in ice slurry.

When working with ectothermic animals in cold waters the absence of observed behaviours can also be a direct effect of temperature that slows down the animal. In fact, in turbot, clear signs of physiological stress responses through altered blood ion balance and acidosis were seen after 90 min of live chilling. At this time the fish was still alive, yet it appeared dead, as it seemed to have entered a state of rigor (possibly caused by forced muscle contractions in the cold) and was stiff and cold (Roth et al., 2009). Live chilling also risks causing immobilisation, as previously seen in the African sharptooth catfish (Lambooij et al., 2006), which would mask any aversive behaviours otherwise displayed by the animal. Furthermore, it is imperative to consider that the stunning effect induced by live chilling is reversible, as evidenced by the recovery of VERs in all tilapia when being warmed up again. However, it is noteworthy that in this study, warm water circulation commenced within minutes following the loss of VERs, and thus it remains to be seen if extended immersion in ice slurry has fatal consequences.

While there may not be any clear physiological or behavioral signs of stress in tilapia subjected to live chilling, we do not know how this procedure affects their welfare, particularly since live chilling does not cause an immediate loss of consciousness. Therefore, further investigations are warranted to understand the potential to use live chilling as a humane stunning procedure for Nile tilapia.

4.4. Sequential approach: electrical stunning, exsanguination, live chilling

To achieve both immediate and permanent loss of VERs, a sequential approach of a 30 s electrical stun with an stunning intensity of at least ~14 V_{RMS} cm^{-1} and ~1.1 A_{RMS} dm^{-2} followed by an immediate throat cut and immersion in an ice slurry is required. While this is promising, two potential challenges arise. The first one is that such an electrical exposure entails a lot of electrical power, leading to high energy

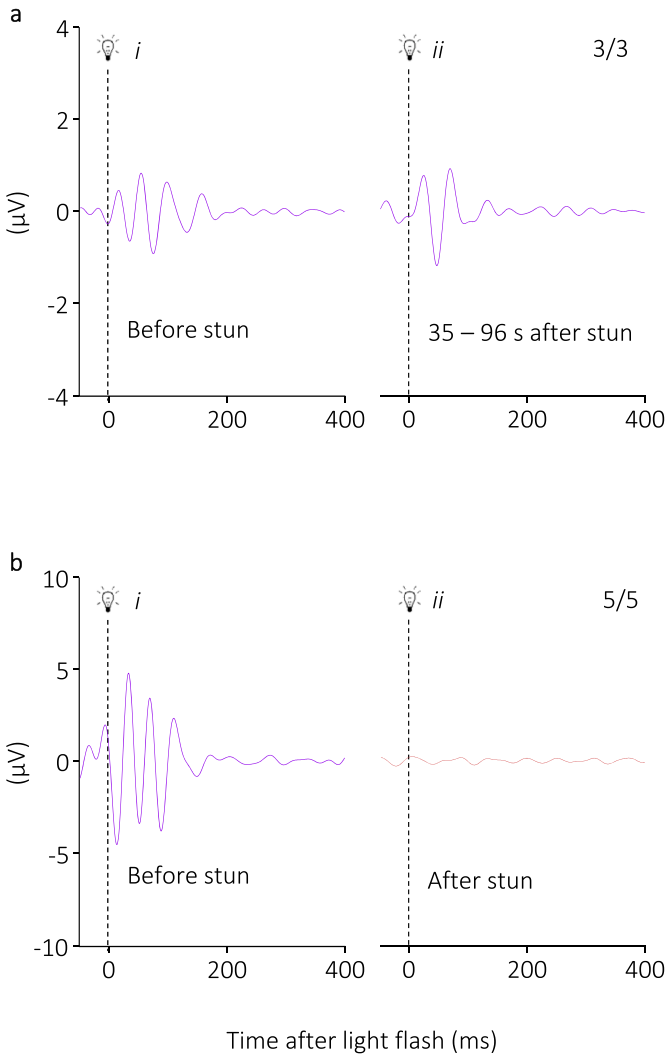

Fig. 8. State of consciousness of Nile tilapia before and after the sequential approach of electrical stunning, exsanguination and live chilling. The figure shows the presence (purple) and absence (red) of VERs in the beta wave of the EEG prior to (i) and after (ii) being subjected to a 30 s electrical stun followed by a throat cut and immersion in an ice slurry. The electrical settings were equal to and <12 V_{RMS} cm^{-1} and ~0.9 A_{RMS} dm^{-2} (upper panels) or or equal to and greater than ~14 V_{RMS} cm^{-1} and ~1.1 A_{RMS} dm^{-2} (lower panels) The time range for when fish recovered VERs during the sequential approach is displayed at the bottom of the panel when applicable. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

wastage, making solutions for batch stunning (*i.e.,* where many fish are stunned together in a large tank) difficult while posing a risk for poor meat quality. The second challenge is that throat cutting and ice slurry immersion have to be done immediately following the electrical stun in order to prevent a transient period of recovery. Our results indicate that to avoid risks for a transient recovery of consciousness during bleeding, the fish needs to be throat cut and placed in ice slurry within 90 s following the electrical stun. Both these challenges may be difficult to overcome in commercial tilapia farms, where many fish are to be killed in a very short period and processed manually. However, if these shortcomings can be resolved, that is, by manufacturing a system that manages the high stun intensities needed, and by ensuring that all individual fish are cut and immersed in ice slurry within the appropriate time, sequential methods like this one has the potential to live up to standards of humane slaughter of Nile tilapia.

4.5. Concluding remarks

Percussive stunning appears to be the most efficient way to immediately and permanently stun Nile tilapia. Alternatively, a side-to-side electrical shock utilizing an electric field of ~8 V_{RMS} cm^{-1} and a current density of ~0.7 A_{RMS} dm^{-2}, delivered as a 50 Hz sinusoidal (AC) pulse for 1 s can achieve immediate unconsciousness. However, this stun is only temporary, requiring higher intensity and prolonged exposure for permanent unconsciousness, followed by a subsequent killing method. Here, unconsciousness until death was achieved with an electric field of at least ~14 V_{RMS} cm^{-1} and a current density ~1.1 A_{RMS} dm^{-2} for 30 s followed by immediate exsanguination and immersion in ice slurry. Alternatively, a combination of percussive and electrical stunning could be employed, allowing the percussive force to be administered to an already unconscious fish, enhancing the efficiency of the procedure without compromising fish welfare or operator safety. With this novel information at hand, future studies should also look into how functional stunning procedures can be verified and validated using visual indicators of consciousness. That is, using neurological indicators for loss of consciousness as a first step in the lab are of utmost importance for evaluating stunning effectiveness. However, it is also important to acknowledge that the practicality of using neurological indicators is low and that future studies should look into how neurological indicators for loss of consciousness, like loss of VERs, relate to visual indicators for loss of consciousness such as loss of VORs and ventilation.

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Declaration of generative AI in scientific writing

During the preparation of this work, Erika Sundell used ChatGPT 3.5 by openai.com in order to improve the grammar and increase readability of parts of the introduction. Upon using this tool/service, the author reviewed and edited the content as needed and takes full responsibility for the content of the publication.

CRediT authorship contribution statement

Erika Sundell: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Jeroen Brijs:** Writing – review & editing, Visualization, Methodology. **Albin Gräns:** Writing – review & editing, Supervision, Resources, Methodology, Funding acquisition, Conceptualization.

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

The data supporting this article have been deposited in the Swedish national database (https://doi.org/10.5878/59q2-7h64)

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