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Humane slaughter of African sharptooth catfish (*Clarias gariepinus*): Effects of various stunning methods on brain function

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ABSTRACT

Common slaughter procedures for African sharptooth catfish (*Clarias gariepinus*) include asphyxiation, ice chilling and exsanguination, which may all cause substantial suffering over prolonged periods of time before death. Therefore, comprehensive evaluations of potentially more humane slaughter procedures for this species are urgently needed. Here, we use a non-invasive electroencephalographic (EEG) method to assess the state of sensibility in African sharptooth catfish in response to various stunning methods (*e.g.* ice chilling, electrical stunning, electrical stunning followed by exsanguination, percussive stunning, and immersion in isoeugenol). Based on the abolition of visually evoked responses (VERs) on the EEG, ice slurry immersion induced insensibility between 2.6 and 7.6 min, during which catfish exhibited aversive behaviours. Once VERs were lost, they remained absent so long as catfish remained immersed in the ice slurry. Electrical stunning (*i.e.* exposure to ~1.7 A dm^{-2} at a water conductivity of ~997 μS cm^{-1}) induced insensibility immediately but not irreversibly. Depending on the duration of the stun (*i.e.* from 1 to 10 s), catfish either regained VERs immediately or within 4.9 min after the completion of the electrical insult. However, when a 10 s electrical stun was immediately followed by exsanguination and immersion in an ice slurry, the duration of insensibility was sufficient to humanely kill catfish. When administered correctly, manual percussive stunning with a fish priest induced insensibility immediately and irreversibly. However, 36% of catfish regained VERs, which is likely explained by the difficulty associated with administering an accurate manual percussive stun of sufficient force on a live and struggling catfish. Catfish appeared to be sedated following immersion in isoeugenol (*i.e.* catfish were calm and easy to handle), yet VERs remained present at doses exceeding that recommended for euthanasia in salmonids, which indicates that this substance may not be suitable for stunning catfish. However, the potential for using isoeugenol as a pre-stunning sedative for improving handleability and reducing handling stress of this species warrants further investigation. In conclusion, this study clearly demonstrates that when singularly administered, none of the abovementioned stunning methods could reliably induce insensibility immediately and/or irreversibly without welfare implications. Yet, our findings indicate that these shortcomings can be resolved by using a combination of methods. This could include an electrical or percussive stun to immediately induce insensibility that should be immediately followed by exsanguination and immersion in an ice slurry to maintain insensibility until death.

1. Introduction

For humane slaughter, fish should be immediately rendered insensible prior to killing and remain so until death without avoidable fear, anxiety, pain, suffering and distress (EFSA, 2004; OIE, 2019). However, if insensibility is gradually induced, then it should be ensured that fish do not experience the abovementioned negative states during the induction phase (EFSA, 2004; Lines and Spence, 2012; OIE, 2019; Robb and Kestin, 2002; van de Vis et al., 2003). To establish whether different commercial stunning methods can be considered humane, a

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range of behavioural indicators have been implemented to evaluate the degree of sensibility in fish (EFSA, 2004; Kestin et al., 2002; Lines and Spence, 2012; van de Vis et al., 2003). These behavioural measures include coordinated swimming and escape behaviours, ability to maintain equilibrium, reactions to painful stimuli, the vestibulo-occular or 'eye roll' reflex, and ventilatory reflexes (EFSA, 2004; Kestin et al., 2002). However, it has become increasingly clear that behavioural measures alone are not sufficient to assess insensibility, as some commercially used methods may induce sedation and/or paralysis without analgesia or anaesthesia prior to insensibility (EFSA, 2004; van de Vis et al., 2003). Therefore, it is necessary to obtain neurophysiological or neurochemical evidence of insensibility to ascertain the impact of various commercial slaughter procedures (EFSA, 2004).

Electroencephalographic (EEG) methods have successfully been used to record the electrical activity of the brain to determine the state of sensibility in mammals, birds and fish during slaughter procedures (EFSA, 2004). In fish, EEG recordings were traditionally obtained *via* the surgical implantation of electrodes into the brain cavity (Enger, 1957; Kestin et al., 1991; Lambooij et al., 2002a, 2002b, 2002c, 2003, 2004, 2006, 2007, 2008, 2010, 2015; Retter et al., 2018; Robb and Roth, 2003; Robb et al., 2000a). However, a non-invasive method consisting of a custom designed suction cup fitted with electrodes that is attached externally to the head of the fish has recently been developed and validated by Bowman et al. (2019, 2020). The non-invasive nature of this method not only improves the welfare of the research animal during the experiment since there is no need for invasive surgery, but it may also reduce the potentially aversive effects of the surgically implanted electrodes on the EEG recordings itself (Readman, 2015). Furthermore, a unique feature of this method is that it allows researchers to obtain baseline recordings for extended periods of time in fish that are not physically restrained (Bowman et al., 2019, 2020). This information can be used to better understand what the EEG of a calm and conscious fish during the pre-stun phase looks like, as well as the subsequent changes that occur in the EEG in response to different stunning methods. For example, previous studies have demonstrated that the transition to insensibility in fish can be observed in EEG recordings *via* reductions in the amplitude of the raw EEG signal, a transition from high- (8–32 Hz) to low-frequency waves (0.5–8 Hz), or by the lack of responses to pain stimuli (Bowman et al., 2019, 2020; Lambooij et al., 2002a, 2002b, 2002c, 2003, 2004, 2006, 2007, 2008, 2010, 2015). However, these indicators can potentially be confounded or remain undetected due to the sensitivity of EEG recordings with regards to background noise or the noise generated by body and/or ventilatory movements (Bowman et al., 2019, 2020). A relatively robust approach for gauging the state of sensibility in fish from EEG recordings is by assessing the presence or absence of averaged visually evoked responses (VERs) in the brain (Kestin et al., 1991). VERs represent the measurable changes in the electrical potential of the brain in response to a visual stimulus (*e.g.* a flashing light), which in a conscious animal produces a distinct waveform in the EEG recordings milliseconds after a visual stimulus (Daly et al., 1986; Gregory, 1998). This makes it a powerful tool for evaluating the effectiveness of stunning methods, as the abolition of VERs has been previously confirmed as an objective and unequivocal indicator of brain dysfunction and hence, loss of sensibility, in fish species such as Atlantic salmon, *Salmo salar* (Robb and Roth, 2003; Robb et al., 2000a), rainbow trout, *Oncorhynchus mykiss* (Bowman et al., 2019, 2020; Kestin et al., 1991), common carp, *Cyprinus carpio* (Readman, 2015; Retter et al., 2018), goldfish, *Carassius auratus* (Quick and Laming, 1990) and eel, *Anguilla* (Lambooij et al., 2002c).

Due to the vast variety of ecological adaptations and evolutionary histories among fishes, different species of fish can react very differently to the potentially stressful situations that arise from common farming and/or slaughter practices in aquaculture (EFSA, 2004). Therefore, it is necessary to develop and optimize slaughter procedures with respect to species-specific welfare needs. The global production of African sharptooth catfish (*Clarias gariepinus*) has rapidly increased over the last 20 years to reach an estimated annual harvest of 231,090 t in 2016 (FAO, 2020). However, the predominant slaughter procedure for this species (*e.g.* asphyxiation, ice chilling and exsanguination) exposes individuals to substantial suffering over a prolonged period of time (EFSA, 2004; Lambooij et al., 2004, 2006; OIE, 2019). Thus, there is an urgent need to comprehensively evaluate and validate alternative and more humane methods for this species.

In the present study, we evaluated the effectiveness of various stunning methods (*i.e.* ice chilling, electrical stunning, electrical stunning followed by exsanguination, percussive stunning, and immersion in isoeugenol) by monitoring changes in the state of sensibility of African sharptooth catfish. This was achieved using the non-invasive method for continuously recording EEG prior to, and following, the application of the different stunning methods to assess the presence or absence of VERs (Bowman et al., 2019, 2020). In addition, we investigated the relationship between behavioural indicators such as coordinated body movement and rhythmic ventilation in relation to the presence or absence of VERs following stunning in this increasingly important aquaculture species.

2. Material and methods

2.1. Experimental animals and housing

African sharptooth catfish were obtained from a local aquaponic operation (n: 58, Stadsjord, Aquaponics Slakthuset, Gothenburg, Sweden) and transported to the aquarium facilities at the University of Gothenburg in Sweden. The catfish were held at 23–25 °C on a 12:12 h light:dark photoperiod in a 1000 L tank containing recirculating aerated freshwater and were allowed $>$ 1 week to recover prior to experimentation. Fish were fed *ad libitum* once a week each Friday afternoon to ensure that fish were fasted for $>$ 60 h prior to stunning, as no experiments were performed on the weekends. Animal care and all experimental procedures performed at the University of Gothenburg were in accordance with national regulations and covered by an ethical permit (5-8-18-12466/2018) approved by the regional ethical committee on animal research in Gothenburg, Sweden. All data in the materials and methods section are presented as means ± s.d.

2.2. Description and placement of the non-invasive EEG recording device

The EEG signals of fish were recorded using a device that consists of a custom made silicone suction cup fitted with a 2 mm silicone tube and three 1 cm diameter silver chloride electrodes (Electrode ARBO H98LG MOD, Tyco Healthcare, Ratingen, Germany) soldered to 1.5 mm diameter shielded wires (MLAWBT9 EEG Flat Electrodes, ADInstruments, Oxford, United Kingdom) (Fig. 1A, Bowman et al., 2019, 2020). The silicone tube was connected to a peristaltic pump to provide enough suction required to keep the device in place and the electrodes in firm contact with the skin during the experiment, while the shielded wires were connected to a bio-amplifier (model FE136, ADInstruments). Prior to placing the device on the catfish, 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 catfish and the electrodes. In addition, a small amount of cooking oil was applied to the rim of the suction cup, as this ensured a good seal and prevented water from entering the suction cup during the experiment.

To correctly place the device, catfish were individually captured from the holding tank and lightly anaesthetised in freshwater containing 75 mg L^{-1} of MS222 (ethyl-3-aminobenzoate methanesulphonic acid, Sigma-Aldrich Inc., St. Louis, Missouri, USA) buffered with 150 mg L^{-1} of $NaHCO_3$. Once the catfish was sedated, it was gently held with only the top of its head above the surface of the water (*i.e.* the gills remained underwater). The device was then positioned so that the

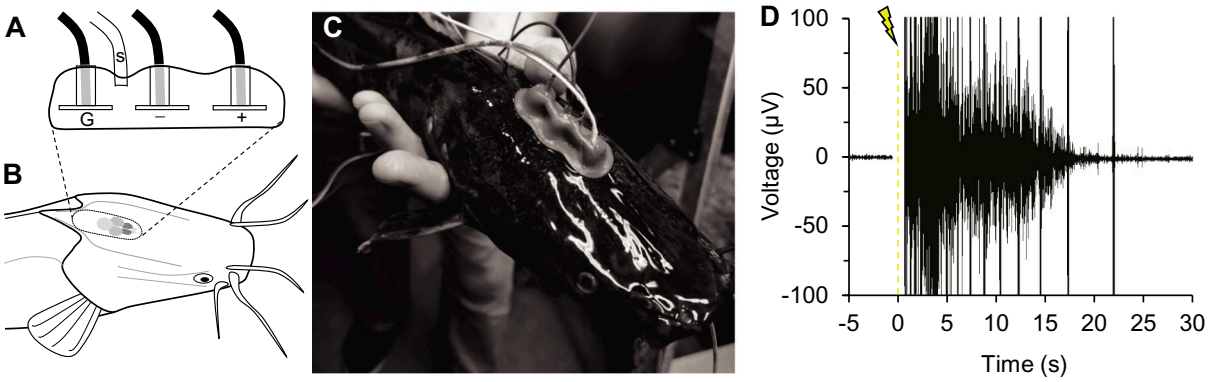

Fig. 1. Design and placement of the non-invasive EEG recording device on the African sharptooth catfish, as well as an example of the general epileptiform insult that is induced by electrical stunning. (A) A schematic of the non-invasive recording device, which consists of a suction cup containing ground (G), negative (−) and positive (+) electrodes, as well as a silicon tube (s) connected to a peristaltic pump to provide enough suction to keep the device in place during the experiment. (B) A schematic and (C) photo demonstrating the approximate positioning of the device on the head of an African sharptooth catfish. (D) An example of the general epileptiform insult that is induced by a 1 s electrical stun at ~1.7 A dm^{-2} (electrical field strength of ~16.0 V cm^{-1} r.m.s.).

positive and negative electrodes were centered above the approximate location where the optical nerves enter the neurocranium (the protective case around the brain) and secured in place using the suction generated by the peristaltic pump (Fig. 1A–C). The placement of the device on the head of the sedated catfish took less than 30 s. Each catfish was then carefully transferred to a flow-through experimental chamber (length: 47 cm, width: 11 cm, water depth: 15 cm) with a glass lid. The water flowing through the chamber was aerated and gravity fed from a 200 L header tank with a flow rate of ~1 L min^{-1}. An air stone supplying additional aeration was also present in the corner of the experimental chamber. Catfish were given at least 20 min to recover from the light anaesthesia prior to experimentation.

2.3. Recording, acquisition and analyses of EEG signals to determine VERs

To detect the presence or absence of VERs in catfish prior to, and following, the application of different stunning methods, EEG signals were continuously recorded *via* the bio-amplifier in response to light flashes from an LED strobe-light (150 ms light flashes at 2 Hz) in a dark room. The sensitivity range (± 2 mV), low-pass filter (50 Hz), high-pass filter (0.1 Hz) and 50 Hz notch filter were set in the bio-amplifier to optimize EEG signals. Signals from the bio-amplifier and a custom-made light detector (used to detect the light flashes from the strobe-light) were relayed to a PowerLab 8/30 system (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 (12–32 Hz), as VERs were found to be most distinct within this frequency range (Bowman et al., 2019, 2020). 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 strobe-light 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. The Scope View module was then used to average 120 consecutive, nonoverlapping time windows into a single 500 ms time window representative of the beta wave for 60 s of recording. If present, the VER could be visually determined from the representative time window and the amplitude of the VER calculated by subtracting the lowest signal value of the VER from the highest signal value (*i.e.* peak-to-peak amplitude). To determine when VERs disappeared or reappeared, 60 s moving averages of the representative time windows were visually analysed. VERs were considered lost when no distinguishable pattern in response to light flashes could be identified within the beta wave frequency (Bowman et al., 2019, 2020). The latency of the VER was calculated by recording the duration of time between the light flash and the first peak of the VER.

2.4. Evaluating the effectiveness of different stunning methods

2.4.1. General outline of stunning protocol

All stunning protocols were performed on weekdays between 9.00 am and 5.00 pm (CET or UTC + 1) from the 23rd of September to the 20th of December 2019. Prior to each stunning procedure (described in detail below), EEG signals of catfish were continuously recorded in response to light flashes for 10 min in water with a temperature of 19.6 ± 1.2 °C to determine the presence of VERs (*via* visual determination from the on-line analysis of the beta waves within the EEG signal). Following this period, catfish were subjected to a stunning procedure and EEG signals were continuously recorded in response to light flashes to evaluate if, and how long, it took for VERs to disappear and then reappear (when applicable). In addition, behavioural indicators such as the disappearance and reappearance of ventilatory and body movements were recorded throughout the protocols, as well as visual observations of the occurrence of aversive behaviour. At the end of each experiment, catfish were stunned by a cranial blow with a fish priest, subsequently killed by a second cranial blow, and then weighed to determine body mass. If it was suspected that an individual was not killed, then a successive cranial blow was administered. Sample sizes and body masses of catfish for the various treatment groups described below are reported in Table 1 and the EEG recordings for each individual are provided in the supplementary information (see Supp. Info. 1–5).

2.4.2. Ice chilling

Ice chilling was achieved by turning off the inflow of water into the experimental chamber, siphoning out the remaining water while simultaneously filling the chamber with an ice slurry (*i.e.* ~50% ice and 50% water, 0.9 ± 0.3 °C). This procedure took less than 30 s. EEG signals of the catfish immersed in the ice slurry were continuously recorded for 40 min. The inflow of water was then turned on in an attempt to recover the individual. However, since the first two individuals did not recover from this lengthy immersion in the ice slurry, the time of immersion for subsequent individuals was reduced to 30 min, which resulted in the successful recovery of all remaining individuals. The EEG signals and behavioural indicators of these catfish were then continuously recorded for 30 min.

Table 1
Sample size, body mass (mean ± s.d.) and the range of time taken for the different indicators of sensibility (*i.e.* VERs, ventilation and body movements) of African sharptooth catfish to be lost or to return following the various stunning protocols.

Stunning protocol	n	Mass	VERs lost	VERs return	Body movements lost	Body movements return	Ventilation lost	Ventilation return
		(g)	(min)	(min)	(min)	(min)	(min)	(min)
Ice chilling								
Immersed in ice slurry	6	472 ± 92	2.6–7.6	no	1.9–6.3	no	–	–
Recovering in water (19.6 °C)	4	454 ± 112	–	3.4–11.9	–	7.7–15.6	–	4.1–16.3
Electrical stunning								
2 s electrical stun	4	787 ± 48	0	< 0.5	0	0.4–0.8	0	0.3–0.6
5 s electrical stun	8	775 ± 80	0	< 0.5–4.3	0	0.6–4.5	0	1.5–4.3
10 s electrical stun	8	724 ± 262	0	1.7–4.9	0	1.8–6.4	0	2.4–3.7
Electrical stunning + throat cut								
In water after protocol (19.6 °C)	2	625 ± 29	0	2.2–4.7	0	3.2–6.7	0	2.1–6.3
In ice slurry after protocol (0.9 °C)	7	473 ± 93	0	no	0	no	0	no
Percussive stunning								
Successful percussion	9	664 ± 191	0	no	0	no	0	no
Failed percussion	5	769 ± 203	0	2.1–11.1	0	1.7–9.5	0	2.4–13.2
Immersion in isoeugenol								
10 mg L^{-1} isoeugenol	1	600	no	–	no	–	no	–
20 mg L^{-1} isoeugenol	1	377	no	–	no	–	no	–
30 mg L^{-1} isoeugenol	1	407	no	–	no	–	no	–
60 mg L^{-1} isoeugenol	1	556	no	–	7.3	no	no	–
100 mg L^{-1} isoeugenol	5	490 ± 218	no	–	1.3–3.3	no	5.5–23.1	no

In some cases, indicators of sensibility were immediately lost in response to a stunning method (represented by '0'), or did not return once lost (represented by 'no'), or were not evaluated/not applicable for the specific situation (represented by '–').

2.4.3. Electrical stunning

For electrical stunning, two 47 cm × 15 cm stainless steel plate electrodes were placed in the experimental chamber parallel to the fish (*i.e.* side to side stun) and spaced 11 cm apart. The steel plate electrodes covered the full tank width and water column to ensure a uniform electric field across the tank. The electrodes were subsequently connected to a purpose-built electrical stunning device assembled by Ace Aquatec Ltd. (Dundee, United Kingdom), which consisted of a variable AC transformer connected to an isolating transformer that was capable of delivering 50 Hz smooth sinusoidal AC from 0 to 350 V. A timing switch was also connected to the power supply of the variable AC transformer to control the duration of the output. The conductivity of the water within the experimental chamber was 997 ± 41 μS cm^{-1}. The stunning device was set to deliver an electrical current of 1.69 ± 0.09 A dm^{-2} (electrical field strength of 15.98 ± 0.64 V cm^{-1} r.m.s.), which was based on recommendations outlined by Lambooij et al. (2006) to induce an immediate loss of sensibility. Similar to Lambooij et al. (2006), a 1 s electrical stun using these settings was observed to induce the characteristics of a general epileptiform insult on the EEG of catfish (Fig. 1D). Voltage and current were measured using a digital oscilloscope (Model: 123, 20 MHz) and a current probe (Model: 801-110S) from Fluke Corporation (Everett, USA).

Catfish were subjected to one of three different electrical stunning protocols, which included 2 s, 5 s or 10 s electrical stuns. Prior to stunning, the inflow of water was turned off and the stun duration was set in the timing switch. Following stunning, the inflow of water was turned back on and EEG signals and behavioural indicators were continuously recorded for 30 min. During the experiments, it was clear that the 2 s stun duration could not render individuals insensible for a sufficient period of time to allow the humane application of a killing method, and thus this part of the experiment was aborted earlier to abide with 3R guidelines (see 3.2. Electrical stunning). This resulted in a lower sample size for this treatment group compared to the 5 s and 10 s electrical stun treatment groups (Table 1).

2.4.4. Electrical stunning and throat cut

Using the electrical stunning settings described above, catfish were subjected to a 10 s electrical stun, which was followed by a throat cut (*i.e.* the ventral aorta was severed). Following the throat cut, catfish were either placed back in water with a temperature of ~19.6 °C or placed in an ice slurry. EEG signals and behavioural indicators of the catfish were then continuously recorded for 30 min. When fish were placed in the water following the combination of electrical stunning and a throat cut, it was clear that fish recovered sensibility prior to death and thus this part of the experiment was aborted earlier to abide with 3R guidelines, which resulted in a low sample size for this treatment group (see Table 1 and 3.3. Electrical stunning and throat cut).

2.4.5. Percussive stunning

Prior to percussive stunning, the suction cup was removed by reversing the peristaltic pump. Catfish were subsequently removed from the experimental chamber and subjected to a sharp cranial blow by a fish priest. Due to the clear imprint that the suction cup leaves on the head of the catfish, it was possible to replace the suction cup on the exact same location before returning them to the experimental chamber. EEG signals and behavioural indicators were then continuously recorded for 30 min.

The impulse (N s) and kinetic energy (J) generated by a percussive blow from the fish priest was evaluated after the experiment by striking a U2 tension/compression load cell (Hottinger Baldwin Messtechnik GmbH, Darmstadt, Germany) with a fish priest. The load cell was connected to a Spider8 amplifier recording at 9600 Hz with HBM Catman 3.1 software (Hottinger Baldwin Messtechnik GmbH), which allowed the measurement of the force (N) produced by a percussive blow over time. The load cell was struck by a researcher attempting to apply blows of similar force and accuracy as that previously administered to the catfish. A total of 30 separate percussive blows were evaluated. Integration of the recorded trace from each blow was performed using Origin software (OriginLab Corporation, Northampton, USA) to determine the impulse (N s) generated. The kinetic energy generated by each blow was subsequently calculated (*i.e.* kinetic energy = ½·m·v^2, where m was the mass of the fish priest, 0.188 kg, and v was the velocity of the percussive blow, which was determined by dividing impulse with the mass of the fish priest).

2.4.6. Immersion in isoeugenol

Isoeugenol (540 g L^{-1}) is the active ingredient in a food grade fish anaesthetic (AQUI-S®, AQUI-S New Zealand Ltd., Lower Hutt, New Zealand) that has been approved for use to lightly sedate, anaesthetize and/or euthanize farmed fish in Australia, Chile, Costa Rica, Faroe Islands, Honduras, Iceland, South Korea, New Zealand, and Vietnam (Aqui-s.com, 2020). Here, the effects of 10, 20, 30, 60 or 100 mg L^{-1} isoeugenol were tested on catfish by turning off the inflow of water, adding the calculated amount of AQUI-S® to the experimental chamber (*i.e.* 20, 40, 60, 120 or 200 mg L^{-1} of AQUI-S®, respectively), and then continuously recording EEG signals and behavioural indicators for 30 min. Since the dosage of isoeugenol required to anaesthetize African sharptooth catfish was unknown, we initially tested the lowest concentration (*i.e.* 10 mg L^{-1} isoeugenol or 20 mg L^{-1} AQUI-S®, a dose recommended for anaesthetizing salmonids) on an individual catfish. If the VERs of the individual were not lost within the 30 min period, the catfish was euthanized and weighed, and then the concentration was increased for the next individual (*i.e.* 20 mg L^{-1} isoeugenol or 40 mg L^{-1} AQUI-S®). This process was continued up until the highest concentration was reached (*i.e.* 100 mg L^{-1} isoeugenol or 200 mg L^{-1} AQUI-S®, a dose exceeding that recommended for euthanizing salmonids).

2.5. Statistical analyses

Statistical analyses were performed using SPSS Statistics 26 (IBM Corp., Armonk, NY, USA). All data used were assessed to ensure that they did not violate the assumptions of the specific models outlined below. F-, t- and *P*-values obtained from the statistical analyses are reported throughout the text and all *P*-values < 0.05 were considered statistically significant.

For the statistical analyses regarding ice chilling, failed percussive stunning attempts and exposure to 100 mg L^{-1} of isoeugenol, paired-samples *t*-tests were used to determine whether VERs changed in amplitude and latency in response to the stunning method (*e.g.* following immersion in the ice slurry or water containing 100 mg L^{-1} of isoeugenol) or following recovery from the stunning method (*e.g.* when catfish recovered in water with a temperature of ~19.6 °C following ice chilling or when recovering from a failed percussive stunning attempt). A paired-samples t-test was also used to determine whether differences existed with regards to the duration of time it took for VERs and body movements to cease following ice chilling. One-way repeated measures ANOVAs with Bonferroni adjusted *post hoc* tests were used to determine whether differences existed in the time it took for VERs, ventilation and body movements to recover following ice chilling and the failed percussive stunning attempts.

For the statistical analyses regarding electrical stunning, an independent-samples t-test was used to determine whether differences existed in the time taken for VERs to recover following a 5 s or 10 s stun. One-way repeated measures ANOVA with a Bonferroni adjusted *post hoc* test was used to determine whether VERs changed in amplitude and latency in response to electrical stunning for each stun duration. To determine whether stun duration affected the recovery time for the behavioural indicators, a Welch ANOVA with a Games-Howell *post hoc* test was used for ventilation (due to the violation of homogeneity of variances) and a one-way ANOVA with a Tukey *post hoc* test was used for body movements. To determine whether differences existed with regards to the duration of time it took for VERs, ventilation and body movements to recover a 5 s or 10 s electrical stun, one-way repeated measures ANOVAs with Bonferroni adjusted *post hoc* tests were used.

3. Results

The range of time taken for the different indicators of sensibility (*i.e.* VERs, ventilation and body movements) of individual African sharptooth catfish to disappear or reappear (when applicable) following the various stunning protocols are summarized in Table 1. Unless otherwise specified, all data in the results section are presented as means ± s.e.m.

3.1. Ice chilling

The VERs of catfish gradually increased in latency (*i.e.* the time between the light flash and the first peak of the VER) and decreased in amplitude before becoming indistinguishable from the rest of the beta wave in response to ice chilling (Fig. 2A). One minute prior to disappearing, the latency of VERs had increased by 103 ± 37 ms ($t_5 = 2.813$, $P = 0.037$) while the amplitude of VERs had decreased by 66 ± 8% ($t_5 = 4.126$, $P = 0.009$) when compared to VERs prior to ice chilling. It took 5.8 ± 0.8 min for the VERs of catfish to disappear in response to ice chilling, but once lost, VERs remained absent throughout the rest of the immersion period in the ice slurry. When catfish were allowed to recover in water with a temperature of ~19.6 °C, VERs returned after 7.0 ± 1.8 min (Fig. 2B). VERs initially returned with a reduced amplitude (49 ± 5% smaller, $t_3 = 6.311$, $P = 0.008$) but no latency ($t_3 = 2.231$, $P = 0.112$) when compared to the fully recovered VERs observed between 12.6 and 23.8 min (average: 16.4 ± 2.9 min).

All catfish were relatively calm prior to ice chilling. Directly following ice slurry immersion, catfish displayed intermittent bouts of aversive behaviour (*e.g.* thrashing around and trying to escape vigorously) before eventually becoming motionless at the bottom of the experimental chamber after 4.2 ± 0.8 min. The loss of body movements in response to ice chilling occurred 1.5 ± 0.6 min prior to the loss of VERs ($t_5 = 2.587$, $P = 0.049$). The total duration of the bouts of aversive behaviour were 2.1 ± 0.3 min or 55 ± 10% of the time it took individuals to become motionless. Body movements remained absent while catfish were left immersed in the ice slurry. Unfortunately, the combination of the flashing strobe light and the presence of ice prevented accurate determination of when ventilatory movements ceased in the catfish following immersion in the ice slurry and thus this parameter was not evaluated. When catfish were allowed to recover in water with a temperature of ~19.6 °C, ventilation recovered after 9.2 ± 1.8 min, while body movements recovered after 11.4 ± 1.9 min. The mean time taken for the different indicators (*i.e.* VERs, ventilation and body movements) to recover from ice chilling did not significantly differ ($F_{2,6} = 1.874$, $P = 0.233$), yet considerable variation is apparent at the individual level with regards to the time taken for VERs to recover when compared to the behavioural indicators (see deviation of blue diamonds from 1:1 line in Fig. 3A–B).

3.2. Electrical stunning

Catfish displayed clear VERs with amplitudes of 0.56 ± 0.03 μV prior to electrical stunning (Fig. 4A–C). Following a 2 s electrical stun (Fig. 4A), VERs were observed in all catfish directly following the cessation of the general epileptiform insult. By increasing the duration of the stun to 5 s (Fig. 4B), VERs were observed to be present directly following the cessation of the epileptiform insult in one catfish, whereas VERs disappeared in the other seven catfish for 2.0 ± 0.4 min. By further increasing the duration of the stun to 10 s (Fig. 4C), VERs were observed to immediately disappear in all catfish and for a significantly longer period of time when compared to a 5 s stun (3.3 ± 0.4 min, $t_{14} = 2.405$, $P = 0.031$, Fig. 4F).

In contrast to ice chilling, no obvious differences were observed in the latency of VERs in response to electrical stunning (4A–C). With regards to amplitude, VERs observed directly following a 2 s stun were of similar amplitude as those observed prior to the stun, as well as after a period of recovery ($F_{2,6} = 0.022$, $P = 0.978$, Fig. 4A). In contrast, when the VERs initially returned following the 5 s and 10 s stuns, they were reduced in amplitude by ~26–29% when compared to VERs observed prior to the stun and after full recovery (5 s stun: $F_{2,14} = 15.511$, $P < 0.001$, 10 s stun: $F_{2,14} = 10.826$, $P = 0.001$, Fig. 4B–C).

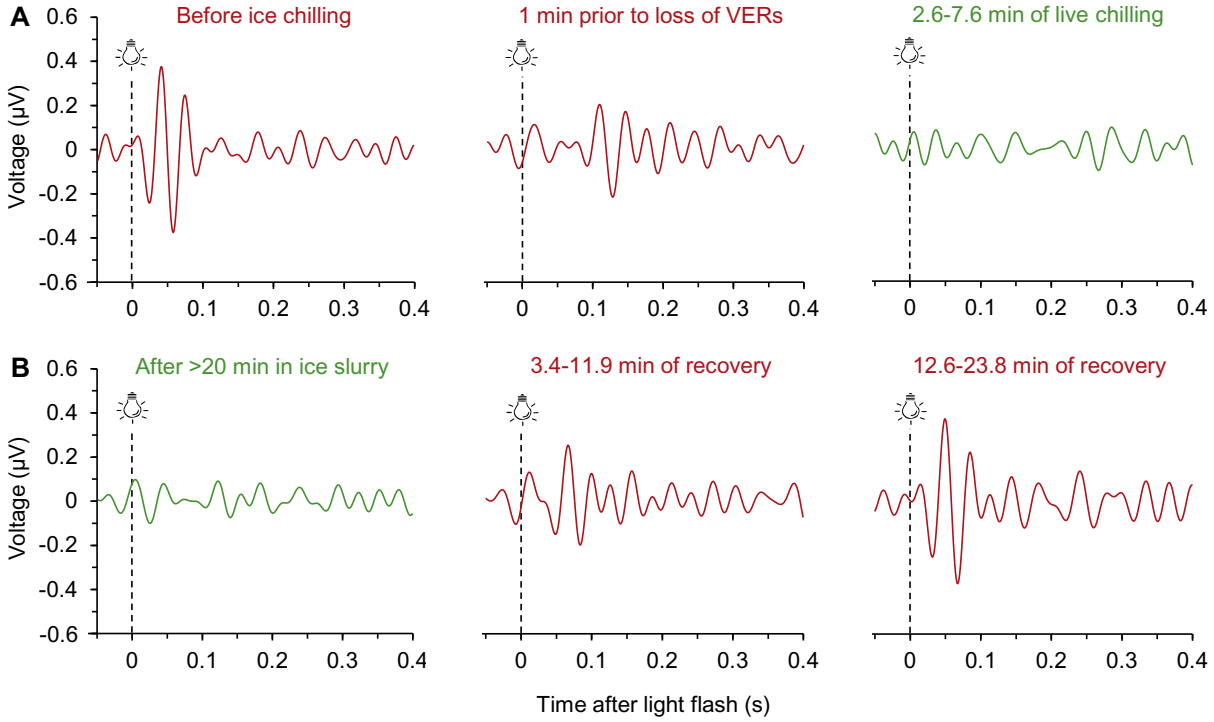

Fig. 2. Visually evoked responses (VERs) of individual African sharptooth catfish during (A) ice chilling and (B) subsequent recovery in water. (A) VERs of catfish gradually increased in latency and decreased in amplitude during ice chilling until they were indistinguishable from the rest of the beta wave (from left panel to right panel of A). (B) When catfish were allowed to recover in water with a temperature of ~19.6 °C, VERs gradually returned albeit with an initial reduction in amplitude compared to the VERs observed after an extended period of recovery (from left panel to right panel of B). The times displayed in each panel represent the range of times it took catfish to get to the specific stage highlighted in the respective panel following immersion in an ice slurry (A, $n = 6$) or following recovery in water (B, $n = 4$). Lines in (A) and (B) are green or red to illustrate when catfish are insensible or sensible, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

The duration of the electrical stun significantly affected the length of time taken for the recovery of ventilation (Welch's $F_{2,10.210} = 1.539$, $P < 0.001$) and body movements ($F_{2,17} = 6.019$, $P = 0.011$). The time taken for the recovery of ventilation was significantly shorter following a 2 s stun (0.5 ± 0.1 min) when compared to the statistically similar recovery times observed following a 5 s and 10 s stun (2.9 ± 0.2 min). The time taken for the recovery of body movements following a 2 s stun (0.6 ± 0.1 min) was significantly shorter than that observed after a 10 s stun (3.3 ± 0.6 min) but not after a 5 s stun (1.9 ± 0.5 min). The mean time taken for the different indicators (*i.e.* VERs, ventilation and body movements) to recover following the 5 s and 10 s stuns did not significantly differ (5 s: $F_{2,14} = 0.997$, $P = 0.394$, 10 s: $F_{2,14} = 0.068$, $P = 0.935$), yet considerable variation was apparent at the individual level with regards to the time taken for VERs to recover when compared to the behavioural indicators (see deviation of black circles and grey squares from 1:1 line in Fig. 3A–B).

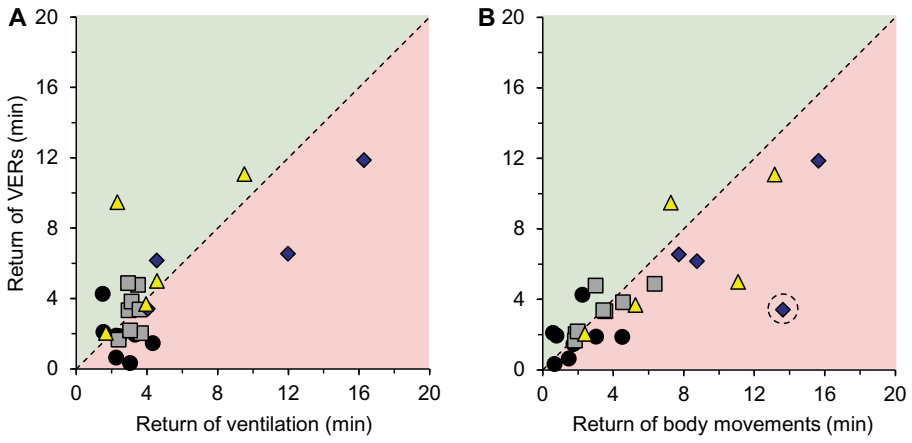

Fig. 3. Recovery of VERs, ventilation and body movements following ice chilling, electrical stunning and failed percussive stunning. The time taken for VERs and (A) ventilatory movements or (B) body movements to return following ice chilling (blue diamonds), 5 s electrical stuns (black circles), 10 s electrical stuns (grey squares), and failed percussive stuns (yellow triangles). The black dashed line is a 1:1 line and has been included to demonstrate the welfare implications of using ventilatory and body movements as behavioural indicators of sensibility. Considerable variation exists between the recovery of the visual indicators and the return of VERs, which from an individual welfare point of view during killing can be acceptable (markers in green shaded section, visual indicators return before VERs) or unacceptable (markers in red shaded section, VERs return before visual indicators). For example, the blue diamond within the black dashed circle in (B) represents a situation where the use of body movements as an indicator would suggest that the individual was insensible for ~13 min, while the use of VERs would indicate that the individual was only insensible for < 4 min. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

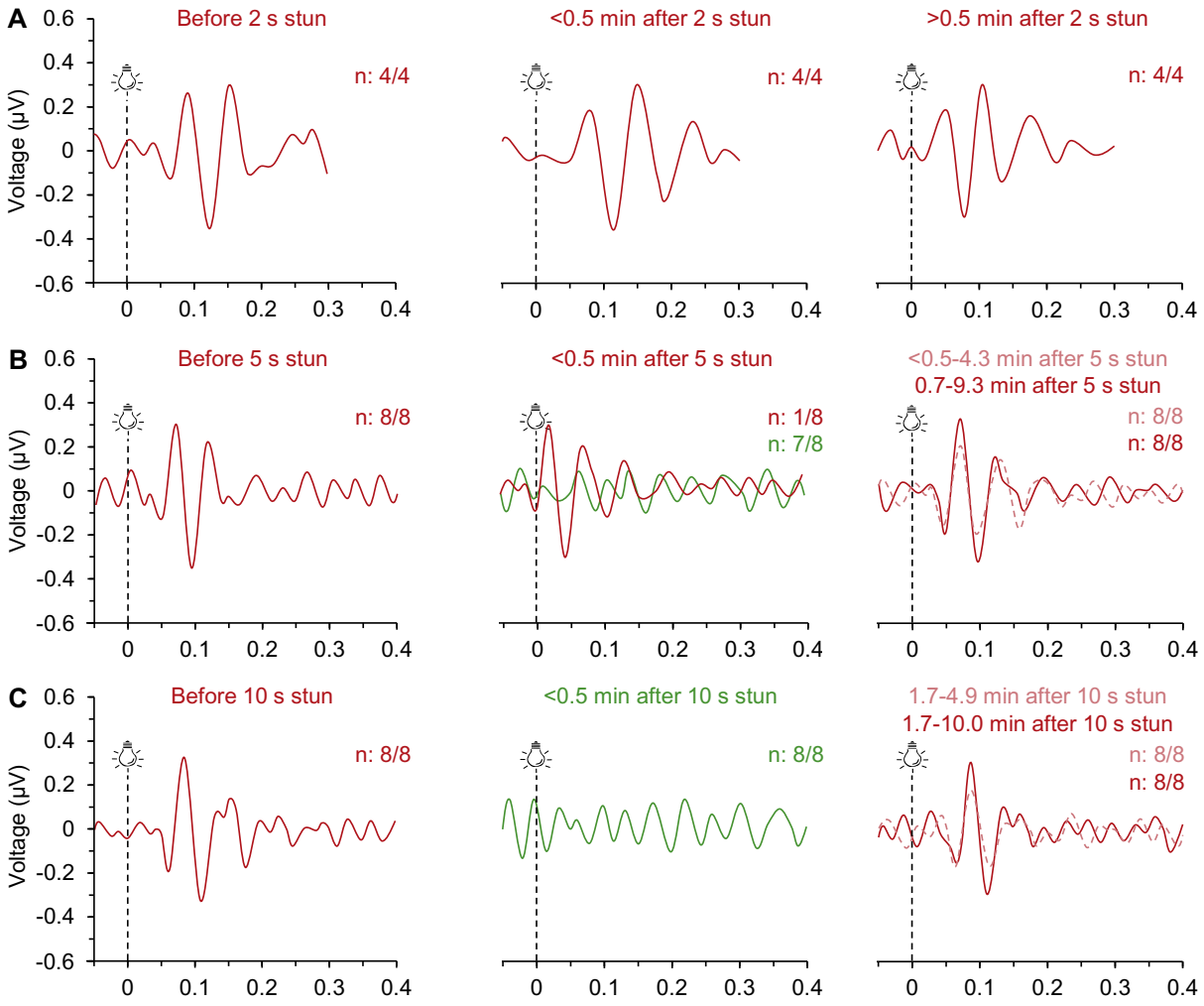

Fig. 4. Visually evoked responses (VERs) of individual African sharptooth catfish before and after electrical stunning. Clear VERs were observed in catfish directly prior to a 2 s, 5 s and 10 s electrical stun (left panels of A, B and C, respectively). Directly following the completion of the epileptiform insult on the EEG (< 0.5 min of the electrical stun), VERs were present in all individuals subjected to a 2 s stun (middle panel of A), in one of the individuals subjected to a 5 s stun (middle panel of B), and in none of the individuals subjected to a 10 s stun (middle panel of C). When the VERs initially returned following the 2 s stun they were of similar amplitude to VERs observed prior to the stun and after a period of recovery (middle panel *c.f.* left and right panel of A). In contrast, when the VERs initially returned following the 5 s and 10 s electrical stuns (see light red, dashed lines in right panels of B and C, respectively), they were reduced in amplitude when compared to VERs observed prior to the stun and after full recovery (see red lines in left and right panels of B and C, respectively). The range of time that it took individuals to initially recover VERs (light red text), as well as for VERs to fully recover (red text), following a 5 s and 10 s electrical stun are displayed in right panels of (B) and (C), respectively. Lines in (A), (B) and (C) are green or red to illustrate when catfish are insensible or sensible, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.3. Electrical stunning and throat cut

Following a 10 s electrical stun and subsequent throat cut (Fig. 5A), VERs immediately disappeared in all catfish. However, VERs were observed to recover after 3.4 ± 1.3 min for the two catfish that were returned to water with a temperature of ~19.6 °C following the throat cut, whereas VERs did not recover in catfish placed in the ice slurry following the throat cut.

All catfish were relatively calm prior to the 10 s electrical stun and throat cut. However, for the two catfish that were returned to water following the throat cut, strong ventilatory attempts (*i.e.* gasping) and highly aversive body movements (*i.e.* thrashing around) were observed after 4.2 ± 2.1 min and 5.0 ± 1.7 min, respectively. When this was observed, the experiments were immediately terminated and catfish were killed by multiple cranial blows with a fish priest. For the catfish that were instead placed in the ice slurry, only minor, sporadic and uncoordinated ventilatory and body movements were observed between 1.2 and 10.3 min.

3.4. Percussive stunning

In the present study, the impulse generated by a percussive blow from a fish priest ranged between 0.69 and 3.05 N s (average:2.3 ± 0.6 N s, mean ± s.d.), while the kinetic energy ranged between 1.3 and 24.7 J (average:15.2 ± 6.6 J, mean ± s.d.). All catfish displayed clear VERs with amplitudes of 0.60 ± 0.06 μV, which immediately disappeared following percussive stunning (Fig. 5B). However, VERs were observed to recover in 36% of the catfish after 6.3 ± 1.7 min albeit with a reduced amplitude (45 ± 9% smaller, t_4 = 4.422, P = 0.011), whereas VERs never recovered in the other catfish.

With regards to behaviour, all catfish were relatively calm prior to

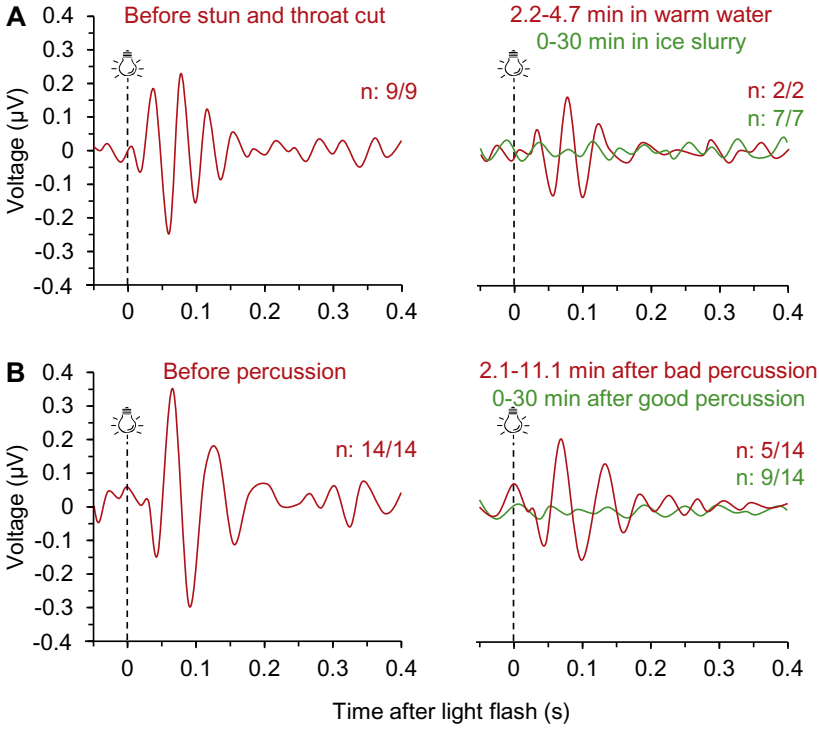

Fig. 5. Visually evoked responses (VERs) of individual African sharptooth catfish before and after (A) an electrical stun followed by a throat cut, and (B) percussive stunning. (A) Clear VERs were observed in all catfish directly prior to a 10 s electrical stun followed by a throat cut (left panel of A). VERs were observed to return in the catfish returned to water with a temperature of ~19.6 °C, whereas they did not return in the catfish placed in the ice slurry (right panel of A). (B) Clear VERs were observed in all catfish directly prior to percussive stunning (left panel of B). Although percussive stunning resulted in the initial loss of VERs in all catfish, VERs of reduced amplitude were observed to return in some individuals, while VERs did not return prior to death in the other catfish (right panel of B). Lines in (A) and (B) are green or red to illustrate when catfish are insensible or sensible, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

percussive stunning. For the catfish that recovered from percussive stunning, rhythmic ventilation recovered after 4.4 ± 1.4 min while coordinated body movements recovered after 7.8 ± 2.0 min. No ventilatory or body movements were observed in the catfish in which VERs did not return during the 30 min monitoring period. The mean time taken for the different indicators (*i.e.* VERs, ventilation and body movements) to recover in poorly percussed individuals did not significantly differ ($F_{2,8}$ = 3.613, P = 0.076), yet considerable variation was apparent at the individual level with regards to the time taken for VERs to recover when compared to the behavioural indicators (see deviation of yellow triangles from 1:1 line in Fig. 3A–B).

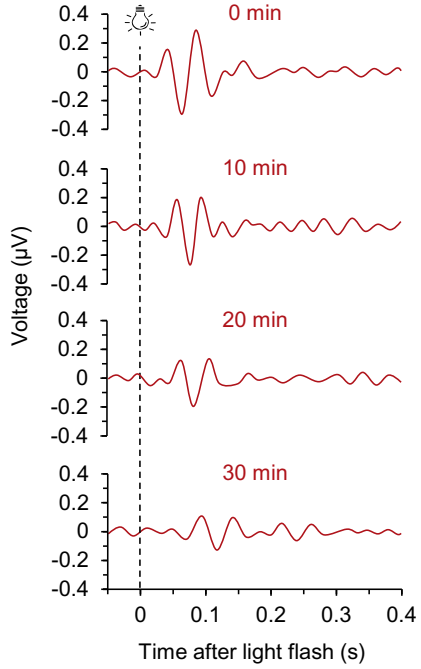

Fig. 6. Visually evoked responses (VERs) of individual African sharptooth catfish following immersion in 100 mg L^{-1} isoeugenol. Clear VERs were observed prior to immersion in 100 mg L^{-1} isoeugenol (top panel). During a 30 min period of immersion in isoeugenol, VERs gradually increased in latency and decreased in amplitude (from top to bottom panel). However, VERs were still distinguishable from the rest of the beta wave after 30 min (n = 5, bottom panel). Lines are red to illustrate that catfish are sensible throughout the immersion period. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.5. Immersion in isoeugenol

Catfish displayed clear VERs with amplitudes of 0.74 ± 0.09 μV prior to exposure to isoeugenol. Exposure to 10, 20 and 30 mg L^{-1} of isoeugenol did not have any obvious effects on the brain activity of catfish, as VERs of similar amplitude and latency were still present after 30 min of exposure. During the 30 min exposure to 60 and 100 mg L^{-1} of isoeugenol, the latency of VERs had increased (39 ± 13 ms more latent in 100 mg L^{-1} of isoeugenol, t_4 = 3.000, P = 0.040) while the amplitude of VERs had decreased (45 ± 7% smaller in 100 mg L^{-1} of isoeugenol, t_4 = 3.918, P = 0.017). However, even after 30 min of exposure to 60 and 100 mg L^{-1} of isoeugenol, VERs remained clearly distinguishable from the rest of the beta wave (Fig. 6).

Although catfish were calmer and easier to handle following exposure to 10, 20 and 30 mg L^{-1} of isoeugenol, rhythmic ventilation and coordinated body movements were observed throughout the 30 min recording period. Body movements of the individual catfish that was exposed to 60 mg L^{-1} of isoeugenol ceased after 7.3 min while rhythmic ventilation was observed for the entire 30 min. Following exposure to 100 mg L^{-1} of isoeugenol, body movements ceased after 2.5 ± 0.5 min, while ventilation ceased after 14.5 ± 3.0 min.

4. Discussion

This study is the first to non-invasively monitor VERs of unrestrained African sharptooth catfish in response to various stunning methods (*i.e.* ice chilling, electrical stunning, electrical stunning followed by exsanguination, percussive stunning, and immersion in isoeugenol). Based on the findings discussed below, species-specific evaluations of humane slaughter procedures are essential, as some fish (*e.g.*

African sharptooth catfish) appear to be relatively resistant to a wide range of stunning methods when compared to others (*e.g.* salmonids). This poses serious welfare hazards for this species, as methods either do not immediately induce insensibility in an ethical manner or for a sufficient period of time, which may result in prolonged periods of suffering during slaughter. However, our findings suggest that these shortcomings can be resolved by using a well-designed combination of methods.

4.1. Ice chilling

Ice chilling is widely used when stunning warm-water adapted fish species, as the rapid decrease in body temperature results in a relatively rapid cessation of struggling (EFSA, 2004; FAO, 2020; Lines and Spence, 2012). In the present study, body movements of catfish ceased within 1.9 to 6.3 min when acutely immersed in an ice slurry. However, for ~55% of the time taken to become motionless, catfish exhibited vigorous escape behaviours. This clearly demonstrates that this method does not induce insensibility without avoidable fear, anxiety, pain, suffering and distress (EFSA, 2004; OIE, 2019). Furthermore, ice chilling also immobilized catfish before rendering them insensible, as body movements ceased prior to the loss of VERs (Bagni et al., 2007; Kestin et al., 2002; Robb and Kestin, 2002; Roth et al., 2009; van de Vis et al., 2003). This finding represents a serious welfare risk in practice, as the use of behavioural indicators by slaughterhouse personnel would indicate that fish are insensible, when in reality they are not (EFSA, 2004).

The VERs of catfish gradually increased in latency and decreased in amplitude during ice chilling, which is most likely related to the decreases in ion conductance associated with cooling (Janssen, 1992). Based on the loss of VERs, it took between 2.6 and 7.6 min for catfish to lose sensibility in response to ice chilling. This appears to be quicker than a previous estimate of 5 to 20 min in the same species, which was based on the registration of pain responses on the EEG (Lambooij et al., 2006). However, it has been shown that catfish and eels lose sensibility in response to ice chilling when their body temperature drops by 9–10 °C (Lambooij et al., 2002b, 2006). As the catfish were > 3-fold larger in Lambooij et al. (2006) when compared to catfish in the present study, the difference in induction time may be explained by the negative relationship that exists between body mass and cooling coefficients of fish (Stevens and Fry, 1974). Therefore, it is likely that the larger catfish in the previous study took longer to cool to the physiological point of becoming insensible.

Once VERs of catfish were lost during ice chilling, they remained absent as long as catfish were immersed in the ice slurry. However, the loss of brain function from cooling is known to be reversible (Robb and Kestin, 2002). This is consistent with the response of catfish in the present study, as they recovered VERs between 3.4 and 11.9 min when transferred to water with a temperature of ~19.6 °C following an immersion in ice slurry for up to 30 min. Again, the use of behavioural indicators to evaluate the sensibility of catfish during recovery could potentially pose a serious welfare risk, as rhythmic ventilation and coordinated body movements were observed to recover up to 5.4 and 10.2 min, respectively, after the recovery of VERs. Although ice chilling itself is not a humane method of stunning catfish, it could potentially be used in combination with other more immediate and humane stunning methods, as insensibility appears to be maintained until death when this sized catfish remains immersed in the ice slurry for at least 40 min.

4.2. Electrical stunning

A 1 s exposure to an electrical current of ~1.7 A dm^{-2} through water with a conductivity of ~997 μS cm^{-1} instantaneously induced a general epileptiform insult in African sharptooth catfish. This indicates that all parts of the brain were stimulated during which the individual is assumed to be insensible (Lambooij et al., 2006; Lopes da Silva, 1983). However, a previous study demonstrated that catfish rapidly regained sensibility following a 1 s exposure to a similar electrical current (Lambooij et al., 2006). Therefore, the duration of subsequent stuns in the present study was extended, as this is a common approach to extend the duration of insensibility in salmonids (Robb et al., 2002; Robb and Roth, 2003). Based on both the neurophysiological and behavioural indicators of sensibility used in the present study, the duration of insensibility of catfish significantly increased with stun duration. Importantly, while all catfish recovered VERs within 30 s following a 2 s stun (*i.e.* directly after the completion of the general epileptiform insult), VERs disappeared for 1.7 to 4.9 min following a 10 s stun. Yet, the duration of insensibility provided by the 10 s stun was not sufficiently long enough for catfish to die from the subsequent exsanguination employed in the present study before they regained sensibility. This contrasts with the findings of Lambooij et al. (2006), where all fish that were bled to death *via* decapitation after the electrical stun displayed a depressed brain activity and no responses to pain stimuli (*e.g.* needle scratches). However, it is not entirely clear from that study how the brain of the catfish could have responded to needle scratches applied to the skin of the tail following decapitation. Nevertheless, in the present study, the problem associated with catfish regaining sensibility prior to death following electrical stunning and exsanguination was rectified by subsequently placing the catfish in an ice slurry, as all catfish remained insensible until death using this combination of methods. It has been recommended for farmed fish such as salmon, trout, char, bass and bream that they be placed on ice rather than in an ice slurry following electrical stunning to avoid any flow of oxygenated water across the gills, which would prolong the time until death (Lines and Spence, 2012). However, with regards to catfish, chilling on ice following electrical stunning only resulted in a short period of insensibility (Sattari et al., 2010), whereas in the present study, we demonstrate that the state of insensibility is adequately maintained as long as the stunned catfish remain immersed in the ice slurry. The reason for the relatively brief period of insensibility when placed on ice was suggested to result from insufficient contact between the ice flakes and the fish body surface, which results in a reduced thermal conductance and thus a slower rate of decrease in body temperature compared with ice slurry immersion (Sattari et al., 2010).

In the present study, a side to side electrical stunning technique was used, which required an electrical current or electrical field strength of ~1.7 A dm^{-2} or ~ 16.0 V cm^{-1} r.m.s., respectively, to render catfish insensible. The current or voltage required for this type of stunning would have significant safety and financial consequences, and so it is most likely not feasible for the stunning of catfish on a commercial scale. However, alternative electrical stunning techniques such as head to tail stunning (Lambooij et al., 2008) or dry stunning (Sattari et al., 2010) require lower electrical outputs to obtain a similar effect, and may therefore be more feasible for commercial use. Finally, from an animal welfare perspective, the findings of the present study suggest that evaluations and validations of electrical stunning methods should be based primarily on neurophysiological indicators, as visual assessment of behavioural indicators can in some cases greatly overestimate the true duration of insensibility.

4.3. Percussive stunning

When correctly applied, percussive stunning represents an efficient and humane stunning method for many species of fish, as sensibility is lost immediately and, in most cases, irreversibly (EFSA, 2004; Lines and Spence, 2012; van de Vis et al., 2003). However, it has been suggested that due to the skull morphology of fish such as sea bream, eels and catfish, there is a high risk that insufficient energy from the percussive stun reaches the brain to render the animal insensible (EFSA, 2004; van de Vis et al., 2001). In the present study, 64% of catfish were rendered insensible immediately and irreversibly following manual percussive stunning with a fish priest, as judged from the complete abolition of

VERs. Unfortunately, it is difficult to determine how much force is required to render a specific individual insensible, as well as to accurately and repeatedly apply manual percussive stuns of sufficient force (EFSA, 2004; van de Vis et al., 2001). This is highlighted in the present study by the variability in the impulse and kinetic energy produced by separate percussive blows with a fish priest, despite the researcher attempting to apply percussive blows of similar force and accuracy under a reasonably controlled and non-urgent situation. Thus, the large variation in the impulse and kinetic energy produced from manual percussion likely explains why 36% of the individuals in the present study recovered VERs between 2.1 and 11.1 min, and why 13–31% of fish recovered behavioural or neurophysiological indicators of sensibility following manual percussion in previous studies (Kestin et al., 1995; Retter et al., 2018). The reliability of percussive stunning can be improved by utilizing pneumatic devices that are set to deliver a specific amount of force (Lambooij et al., 2002a, 2003, 2007, 2010; Robb et al., 2000a; van de Vis et al., 2003). However, the accurate application of a percussive blow on a live and struggling catfish still remains a challenge in many cases. Thus, from an animal welfare perspective, it may be necessary to sedate or stun catfish using another method prior to percussion (EFSA, 2004).

4.4. Exposure to isoeugenol

The present study suggests that mature African sharptooth catfish are substantially more resistant to isoeugenol when compared to salmonids (Robb and Kestin, 2002; Robb et al., 2000b), as concentrations of 10–30 mg L^{-1} had no appreciable effects on neurophysiological or behavioural indicators of sensibility. Even following immersion in 100 mg L^{-1} isoeugenol for 30 min (a dose exceeding that recommended for euthanasia of salmonids), VERs remained present in catfish albeit with a reduced amplitude and increased latency. Interestingly, previous studies on zebra fish (*Danio rerio*) and gilthead sea bream (*Sparus aurata*) have demonstrated circadian rhythms in the effectiveness of anaesthetics such as eugenol or tricaine methane sulfonate (*i.e.* MS-222), with an increased effectiveness (*i.e.* reduced induction time for anaesthesia) observed during the time of day when fish were active (Sánchez-Vázquez et al., 2011; Vera et al., 2010). Since African sharptooth catfish are a nocturnal species (Manuel et al., 2016) and the experiments in the present study were performed during the day, further investigations into the effectiveness of isoeugenol during the night are warranted to determine whether or not similar circadian rhythms in the effectiveness of anaesthetics also exists in this species. However, our findings clearly demonstrate that, at least during the day, catfish may not become insensible when immersed in isoeugenol at concentrations of up to 100 mg L^{-1}, and that other stunning methods should therefore be applied before killing catfish during this time.

Nonetheless, the lack of aversive behaviour and ease of handling of catfish following immersion in isoeugenol suggests that it could represent a suitable sedative in preparation for stunning. In fact, previous studies have demonstrated that channel catfish (*Ictalurus punctatus*) sedated with isoeugenol appear to suffer far less distress than unsedated fish during handling procedures (Bosworth et al., 2007; Small and Chatakondi, 2005; Small, 2003). The decrease in muscle activity prior to slaughter in fish sedated with isoeugenol has been suggested to result in a slower *post mortem* pH decrease, which has been associated with a range of subsequent benefits with regards to fillet quality (Bosworth et al., 2007; Robb and Kestin, 2002; Robb et al., 2000b). However, further investigations on the effects of sedation on stress responses and consequences for fillet quality in African sharptooth catfish during common harvesting practices are warranted.

5. Conclusions

Our study suggests that African sharptooth catfish is an extremely robust fish species that is comparatively resistant to all the investigated stunning methods when administered singularly. Based on our findings, no stunning method alone could sufficiently induce insensibility immediately and until death without avoidable fear, anxiety, pain, suffering and distress. However, when applied correctly, electrical or percussive stunning induced insensibility immediately, which when combined with exsanguination and subsequent immersion in ice slurry substantially reduced the risk of fish temporarily regaining sensibility prior to death. Depending on its legal status with regards to the use on food grade fish, the use of isoeugenol may be beneficial to facilitate the handling of live fish, and thus improving the success rate of manual percussive stunning, as well as reducing stress and improving fillet quality. Finally, validation of stunning methods should be based on neurophysiological indicators, as the use of behavioural indicators alone can have serious welfare implications especially when evaluating methods that can potentially result in paralysis or immobilization (*e.g.* ice chilling, failed percussive stunning or immersion in isoeugenol). In that respect, further development of the non-invasive method to assess brain activity for field-use is warranted to allow accurate on-site validations of the varying stunning methods used in aquaculture.

Declaration of Competing Interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2020.735887.

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