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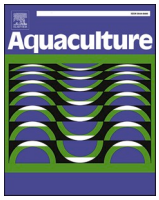

Assessing the effectiveness of percussive and electrical stunning in rainbow trout: Does an epileptic-like seizure imply brain failure?

P. Hjelmstedt [a,*], E. Sundell [a], J. Brijs [b], C. Berg [a], E. Sandblom [c], J. Lines [d], M. Axelsson [c], A. Gräns [a]

[a] Department of Animal Environment and Health, Swedish University of Agricultural Sciences, Skara SE-532 31, Sweden
[b] Hawai'i Institute of Marine Biology, University of Hawai'i at Mānoa, Honolulu, United States
[c] Department of Biological and Environmental Sciences, University of Gothenburg, Gothenburg SE-405-30, Sweden
[d] Ace Aquatec Ltd., Dundee, United Kingdom

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ABSTRACT

Both percussive and electrical stunning have been highlighted as methods that can be used to quickly render fish unconscious before being killed. However, accurately assessing unconsciousness in animals following stunning remains challenging, and thus methods for reliable interpretation and validation of different stunning methods are urgently needed. Here, we used a non-invasive technique to continuously record electroencephalograms (EEG) of rainbow trout (*Oncorhynchus mykiss*) enabling us to compare the effects of both percussive stunning, using a captive bolt gun, and various combinations of electrical stun parameters delivered in water. The EEG signals were assessed for the absence or presence of an epileptic-like seizure and for visually evoked responses (VERs). No epileptic-like seizures or VERs were observed after captive bolt stunning. We found that it is possible to reliably induce an epileptic-like seizure and an immediate, but short lasting, loss of VERs following a 1 s exposure to an electrical field strength of at least 2.8 V_{RMS} cm^{-1} and current density of 0.22 A_{RMS} dm^{-2} in water of conductivity of ~1000 μS cm^{-1} using a 50 Hz AC current. However, to avoid recovery of VERs shortly after the stun, it was necessary to increase the duration of the stun application (≥30 s), the electrical field strength (10.2 V_{RMS} cm^{-1}) and the current density (0.84 A_{RMS} dm^{-2} respectively). We found no clear relationship between presence and absence of ventilation and VERs following electrical stunning in rainbow trout, highlighting that loss of ventilation may not be a good indicator of brain failure in rainbow trout. Our results clearly show that the presence of an epileptic-like seizure following an electrical stun does not guarantee a prolonged period where the fish is unresponsive to visual stimulation (*i.e.* absence of VERs). It was further found that VERs can return before the end of the seizure. As both presence of a seizure and absence of VERs have been used independently as indicators of unconsciousness in fish, we emphasize the necessity to carefully consider and evaluate the reliability of neurophysiological indicators of unconsciousness when validating methods to stun fish.

1. Introduction

Due to a growing body of scientific evidence indicating that fish exhibit complex cognitive abilities including the capacity to suffer from fear, pain, distress, and anxiety (Braithwaite et al., 2013; Kohda et al., 2019), it has been argued that fish deserve the same level of animal protection as other livestock during slaughter (Huntingford et al., 2006; Huntingford and Kadri, 2014). Since the effectiveness and efficiency of many available stunning methods remains largely unknown, the slaughter process poses a major welfare hazard in aquaculture (Ashley, 2007; Gräns et al., 2016; Lines and Spence, 2012; Van De Vis et al., 2003). For humane slaughter, fish should be stunned before exsanguination in a way that induces immediate unconsciousness and insensibility that lasts until the fish has died, so that slaughter can be performed without avoidable fear, anxiety, pain, suffering and distress (EFSA, 2004; FAO, 2019). Here we will use the terms unconscious/unconsciousness defined 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 respond to normal stimuli, including pain*" (EFSA, 2004). Some methods commonly used for stunning and killing fish in

* Corresponding author at: Department of Animal Environment and Health, PO Box 234, 532 23, Skara, Sweden.
E-mail address: per.hjelmstedt@slu.se (P. Hjelmstedt).

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aquaculture (*e.g.* asphyxia, ice chilling and carbon dioxide narcosis) do not induce an immediate unconsciousness but instead elicit strong aversive behaviors that contrast with the concept of humane slaughter (Gräns et al., 2016; Kestin et al., 1995; Lambooij et al., 2002; Robb and Kestin, 2002). Therefore, there is an urgent need to comprehensively evaluate and validate alternative stunning methods to ensure the humane slaughter of fish in aquaculture.

Two such alternative methods are percussive and electrical stunning, both of which can, if applied correctly, induce an immediate loss of consciousness in a range of farm animals including some species of fish (EFSA, 2004). With percussive stunning, a severe blow is administered to the skull of the fish, either manually using a club or using an automated captive-bolt, causing a brain hemorrhage which is incompatible with brain function (Brijs et al., 2020; Lambooij et al., 2010; Lambooij et al., 2007; Robb et al., 2000).

Electrical stunning is done by sending an electric current through the brain and/or heart of the animal before slaughter. Based on mammalian and avian studies, an epileptic-like seizure (often referred to as an epileptiform insult, general epileptic insult, generalized tonic-clonic seizure or a *grand mal* seizure) is induced when sufficient current is passed through the brain of an animal (Devine et al., 1986; Devine et al., 1987; McKinstry and Anil, 2004). The epileptic-like seizure, at least in mammals, begins with a tonic (*i.e.* tension of muscles) and a clonic (*i.e.* rhythmic convulsions) phase that coincides with abnormally high electroencephalographic (EEG) fluctuations. This is followed by an isoelectric or silent phase with little to no neural activity (Lambooy and Spanjaard, 1982; Mason et al., 2018). Patterns similar to the epileptic-like seizures of mammals and birds have also been observed on the EEG in various species of fish following electrical stunning (Daskalova et al., 2015; Lambooij et al., 2013; Lambooij et al., 2012; Lambooij et al., 2002; Lambooij et al., 2010). During the seizure, all parts of the brain are assumed to lose normal function leaving the animal unconscious prior to killing. The animal should then remain in this state until death supervenes (Bager et al., 1992; Cook et al., 1995; Lambooy, 1982).

During the last decades, there has been a rapid development of large-scale electrical stunning equipment applied in the aquaculture industry. While the interest in the use of electrical stunning to improve the welfare of farmed fish during slaughter is widely welcomed, there are still knowledge gaps regarding how to reliably assess loss of consciousness in fish following electrical stunning. In this regard, it is important to consider the potential differences that exist between mammals and fish, such as the reported absence of an isoelectric phase following electrical stunning in some species of fish, including rainbow trout (*Oncorhynchus mykiss*) (Kestin et al., 1995; Robb et al., 2002). Therefore, the relationship between the presence of an epileptic-like seizure and the loss of brain function requires further investigation in fish species that do not show an isoelectric phase on the EEG following an epileptic-like seizure. To do so, a reliable method for assessing consciousness (or loss thereof) following electrical stunning in fish is needed. Today such assessments are normally done by monitoring for presence or absence of one or several visual indicators including: self-initiated behaviors (*e.g.* swimming and loss of equilibrium), responses to stimulation (*e.g.* response to handling and a needle prick), and clinical reflexes (*e.g.* ventilation and the vestibulo-ocular reflex/'eye-roll' reflex) (Anders et al., 2019; Grimsbø et al., 2014; Kestin et al., 2002). Such visual indicators have a practical use when evaluating stunning functionality in real slaughter situations, but only using these indicators when developing and validating the efficiency of new stunning methods can be problematic. This is because it is unclear whether these indicators can identify whether a fish merely becomes paralyzed by the stunning method while remaining sensible to pain or distress (Bowman et al., 2019; Daskalova et al., 2015; Kestin et al., 2002; Retter et al., 2018; Van De Vis et al., 2003).

An EEG can additionally be analyzed for consciousness by detecting the presence or absence of visually evoked responses (VERs), or by measuring changes in signal characteristics including EEG amplitude and frequency content. While various authors identify the significance of the amplitude in the high-frequency (8–32 Hz) and low-frequency bands (0.5–8 Hz) (Gibson et al., 2009; Lambooij et al., 2015; Verhoeven et al., 2015), the presence or loss of VERs was recently found to be the most robust and reliable indicator of brain failure when rainbow trout exposed to MS-222 transcended into insensibility (Bowman et al., 2019). VERs are normally detected in fish using repetitive visual stimulus (*e.g.* a flashing light) and is considered to be one of the last functions to be lost before the brain of a fish becomes completely unable to process input from its environment. Therefore, unless the eyes or the optic nerves of the animal is somehow non-functional, it is undoubtedly in a state of unconsciousness when VERs are lost (Bowman et al., 2019; Bowman et al., 2020; Daly et al., 1987; EFSA, 2004, 2009; Kestin et al., 1995; Robb et al., 2000; Van De Vis et al., 2003).

Here, we report a study designed to investigate the effect on rainbow trout brain activity, following percussive stunning, using a captive bolt gun, and various combinations of electrical stun parameters delivered in water. 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 induction, amplitude and duration of epileptic-like seizures, as well as the presence or absence of VERs. In addition, we investigated whether a loss of ventilation coincides with a loss of VERs, and thus can be used as a reliable indicator for determination of unconsciousness in rainbow trout following electrical stunning.

2. Materials and methods

2.1. Animals

80 rainbow trout of mixed sexes (weight 898 ± 27 g) were obtained from a commercial fish farm (Vänneåns fiskodling AB, Sweden) and housed in the animal facilities at the University of Gothenburg, Sweden. The animals were kept in a freshwater recirculation system at 10 °C with a 12:12 h light:dark cycle where they were fed to satiation three times a week and fasted 1–2 days prior to experimentation. After arrival, the fish were left undisturbed to acclimate to their new environment for at least one week prior to experimentation. At the end of the experiments, all fish were euthanized with a sharp blow delivered to the central skull bones, weighed and measured. The experimental protocols were designed in accordance with national regulations and approved by the regional ethical committee on animal research (permit number 1873–2018).

2.2. General description of the experimental design

Before the experiments, all fish ($n = 67$) were carefully hand netted and transferred into a bin with 10 °C water containing 100 mg l^{-1} MS222 (ethyl-3-aminobenzoate methane sulphonic acid, Sigma-Aldrich Inc., St. Louis, Missouri, USA) buffered with 200 mg l^{-1} $NaHCO_3$ (for electric stunning) or 0.07 ml l^{-1} isoeugenol (Aqui-S®, Lower Hutt, New Zealand) for captive bolt stunning. When the fish was lightly anaesthetized, the upper part of the head was lifted above the water and three electrodes mounted in a silicon suction cup were placed on the skull above the brain and optic lobe. The fish was then transferred by hand into an opaque glass flow-through experimental tank (dimensions 48 * 12 * 16 cm, volume ~ 9.2 l), where aerated water (10 °C) was gravity fed from a header tank at a rate of ~1 l min^{-1}. For electrical stunning, the water was prepared to a conductivity of ~1000 μS cm^{-1} by dissolving sea salt in tap water and measured by a conductivity meter (Hach HQ40d Portable Meter, Loveland, Colorado, United states). This was done because fish stunned in low conductivity tap water can receive as little as half of the electric field generated in the water (Lines and Kestin, 2004) (see Table 1 for details). When the fish had recovered from the anesthesia, determined by a visual inspection of the behavior and EEG recordings of the fish, the stun application was initiated.

The percussive stun was achieved using a handheld non-penetrative

Table 1
Stunning variables and biometrics. All electrical stun applications were done side-to-side using 50 Hz alternating current (AC). The percussive stun was done using a handheld non-penetrative pneumatic captive bolt gun driven by a pressured air of 125 psi. In Short application group S1 fish that did not show an epileptic-like insult or were deemed as failed. In Long application groups, L1–4 fish that shortly following the electrical exposure regained VERs were deemed as failed. Number in brackets include one individual when VERs returned 274 s after the stun, which is considerably later compared to other fish that regained VERs.

Stunning method	Stun application	Field strength	Water conductivity	Amperage	Voltage	Current density	Mass	n
Group	Seconds	V_{RMS} cm^{-1}	μS cm^{-1}	A_{RMS}	V_{RMS}	A dm^{-2}	g	Pass/total
S1	1	1.4 ± 0.01	974 ± 5	0.73 ± 0.01	15.4 ± 0.2	0.10 ± 0.002	1048 ± 110	2/4
S2	1	2.8 ± 0.01	983 ± 7	1.59 ± 0.02	31.0 ± 0.4	0.22 ± 0.003	647 ± 102	6/6
S3	1	5.1 ± 0.05	993 ± 11	2.87 ± 0.03	56.5 ± 0.5	0.43 ± 0.018	976 ± 89	4/4
S4	1	10.1 ± 0.04	985 ± 9	5.86 ± 0.06	110.9 ± 0.4	0.83 ± 0.008	823 ± 171	4/4
L2	30	2.9 ± 0.003	986 ± 1	1.56 ± 0.01	31.6 ± 0.4	0.22 ± 0.002	923 ± 74	1/3
L2	60	2.9 ± 0.01	996 ± 3	1.55 ± 0.01	32.0 ± 0.1	0.22 ± 0.002	938 ± 86	2/8 (3/8)
L3	30	5.0 ± 0.01	1000 ± 7	2.83 ± 0.01	55.5 ± 0.1	0.4 ± 0.001	1025 ± 93	0/3
L3	60	5.1	1004	2.74	55.7	0.39	1080	0/1
L4	15	10.2 ± 0.07	997 ± 1	5.83 ± 0.05	112.2 ± 0.7	0.83 ± 0.007	900 ± 165	2/3
L4	30	10.2 ± 0.03	1002 ± 2	6.02 ± 0.07	112.0 ± 0.4	0.85 ± 0.009	963 ± 51	10/10
L4	60	10.2 ± 0.03	995 ± 1	5.92 ± 0.03	112.7 ± 0.3	0.84 ± 0.005	935 ± 49	10/10
Captive bolt							788 ± 48	10/10

pneumatic captive bolt gun (Zephyr® F, Bock Industries, PA, US) driven by pressured air (125 psi) from a compressor (Herkules Walkair CE New, Siegen, Germany). Before percussive stun application, the silicone cup was removed and the fully awake fish (n = 10) was transferred to a plastic tray and firmly held in place by hand, where after a single shot from the captive bolt gun was used to cause brain hemorrhages and eliminate brain function. Immediately following the shot, the electrode cup was reattached to the head of the fish (within 30 s) and the fish was returned to the experimental tank and left in a continuous flow of aerated water for >15 min where its EEG was recorded and all movements including ventilation were noted by visual inspection.

For electrical stunning, we used a purpose-built electrical stunning device with two submerged stainless steel electrodes (dimensions 15*47 cm, area = 705 cm^2, separated by 11 cm of water) mounted in the experimental tank. All electric currents were applied in a side-to-side direction at 50 Hz with a water conductivity of ~1000 μS cm^{-1}. Although the current density and electric field strength is dependent on fish size/tissue conductivity and the effective conductivity between the plate electrodes will vary during each individual stun, a constant water conductivity ensure that environmental variables are comparable among individuals. Firstly, we investigated how short exposures to different electrical field strengths and current densities affects the brain activity of trout. This was done to determine the minimum field strength and current density needed to elicit an epileptic-like seizure on the EEG. To do so a subsample of fish (n = 18) were divided into four groups (S1-S4) and individually exposed to the electric field for 1 s, using four different stun settings (experiment group S1-S4, S = Short stun application, Table 1), starting with the lowest settings. Secondly, we investigated how the length of the stun application (15, 30 or 60 s) affected the duration of the period where the VERs are lost following electrical stunning. To do so, another subsample of fish was divided into three groups (L2-L4, L = Long stun application, n_{tot} = 38) and exposed to one out of the investigated exposures periods starting with experiment group L2 (see Table 1 for details). Immediately following the end of the stun application (<1 s), the fish was left in the flow of aerated water for >15 min while EEG was continually recorded and all movements including ventilation were noted. If a stun application failed to induce long lasting loss of VERs in an animal, *i.e.* if VERs reappeared shortly after the stun, the length of the stun application was increased for the following fish. This was done in order to minimize the number of animals used in the experiment.

2.3. Experimental set up and data collection

For all fish, presence and duration of an epileptic-like seizure and VERs was determined by measurements of EEG using a non-invasive technique recently developed for fish (for detailed description of the technique, see (Bowman et al., 2019; Bowman et al., 2020; Brijs et al., 2020)). Briefly, three 1 cm diameter silver chloride ECG electrodes (H98LG; Tyco/Kendall, Ratingen, Germany) were secured to the head of the trout with a custom-made silicone suction cup. The cup was firmly attached to the skull of the fish by negative pressure created by connecting a 2 mm diameter silicone tube, built into the cup, to a peristaltic pump. To improve electrode contact with the skin of the fish, the electrodes were covered with a thin layer of Ten20 conductive EEG paste (Weaver and Company, Aurora, Colorado, USA). The electrodes were connected to a bio amplifier (FE136; ADInstruments, Oxford, United Kingdom) connected to a power lab (ML 870, 8/30, ADInstruments) and a PC. The EEG signal was optimized in the bio-amplifier using ±2 mV sensitivity range, 50 Hz low-pass filter, 0.1 Hz high-pass filter and a 50 Hz notch filter. A custom made relay box allowed breaking the circuit between the electrodes and the recording hardware during stun application.

The plate electrodes used to create the electric field were placed on the long sides of the tank from top to surface, creating a full side-to-side exposure of the electric field. 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 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 ~1000 μS cm^{-1}. The voltages (V_{RMS}) and currents (A_{RMS}) going through the tank were determined using an AC/DC current probe (Fluke 80i-110 s, Fluke Corporation, Everett, Washington, USA) connected to a handheld oscilloscope (Fluke 123B). The electric field strengths and current densities were calculated using the following equations;

$$\text{Electric field strength } \left(V_{RMS}\ \text{cm}^{-1}\right) = \frac{\text{Electric potential diffrence } (V_{RMS})}{\text{Plate separation (cm)}}$$

$$\text{Current density } \left(A_{RMS}\ \text{dm}^{-2}\right) = \frac{\text{Current } (A_{RMS})}{\text{Electrode area } \left(dm^2\right)}$$

The experiments were carried out in a darkened room. A custom built battery-powered flashing LED-light (2 Hz light:dark cycle of 50:450 ms) placed approximately 0.5 m above the stunning tank was used to evoke responses on the EEG (*i.e.* VERs). In addition, a small solar panel (Velleman SOL1N, Gavere, Belgium), modified to function as a light detector and connected to the power lab was placed next to the tank and served as a trigger/reference point for subsequent recording and analysis of the EEG. To quantify periods of VERs, filtering and averaging of brain activity during stimuli was performed in LabChart as follows; the raw EEG

signal was continuously recorded throughout the protocol and simultaneously filtered using a bandpass filter of 13–32 Hz to obtain beta wave frequencies as this range has previously been observed to provide the best fit for attaining VER activity (Bowman et al., 2019). The Scope View function in LabChart (version 7.3.2, ADInstruments) was used to average 120 consecutive epochs (*i.e.* 450 ms time windows of the EEG, tie-locked to the light stimuli)which created an averaged image of the repetitive voltage fluctuations caused by the light stimulus (Kestin et al., 1991; Kumar et al., 2000; Trojaborg and Jørgensen, 1973). For each fish, 120 consecutive recordings (one minute of recording) were averaged to detect the repetitive responsesto the light stimuli. In total, each EEG recording resulted in 2-5 pre-stun averaged images with steady VERs, and a minimum of 15 post-stun images.

2.4. Assessment of epileptic-like seizures and VERs

The general structure of the epileptic-like seizures in the EEG was assessed and quantified relative to the pre-stun (p_0) amplitude. The EEG

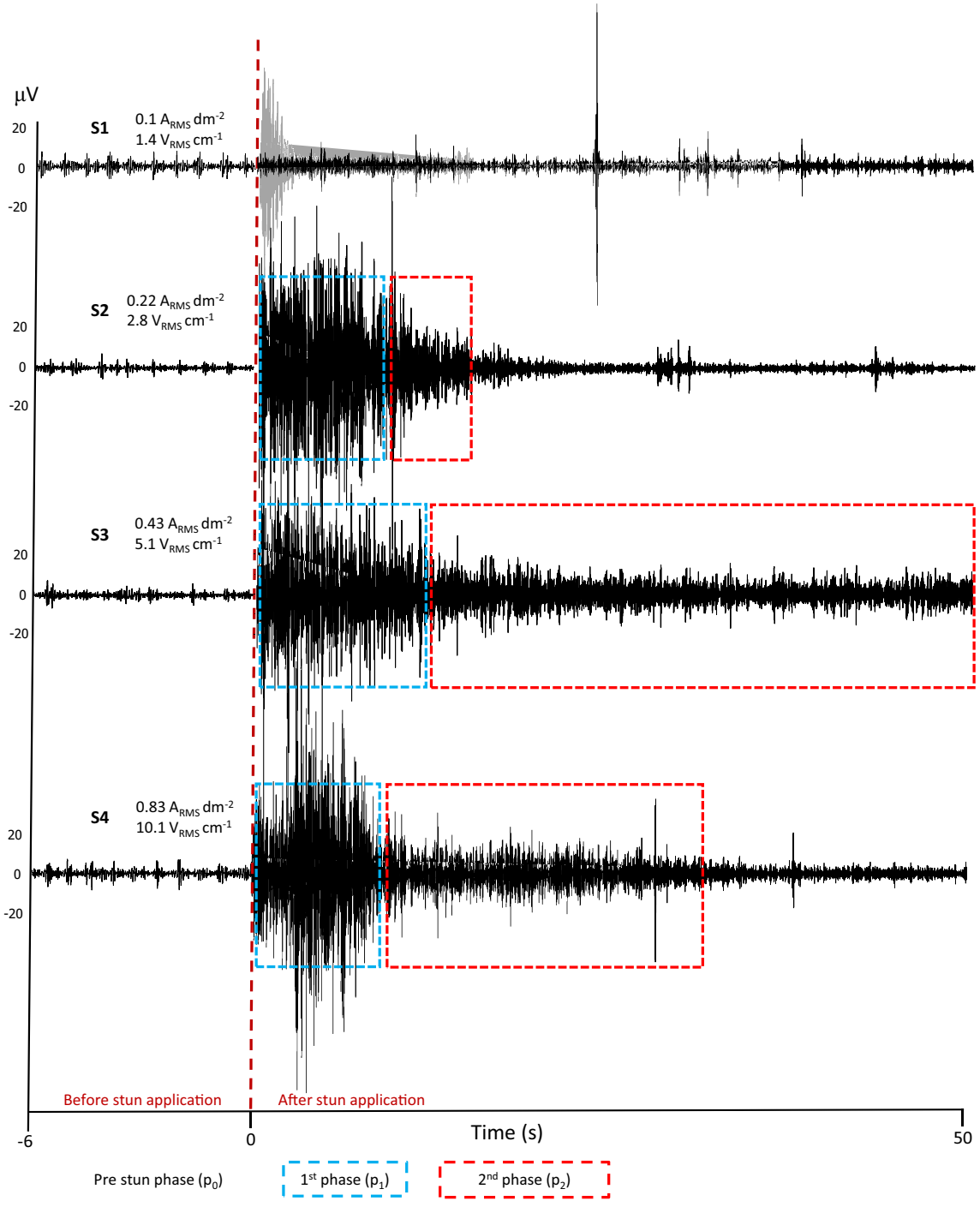

Fig. 1. Representative examples of individual EEG waves in the beta frequency before and after a 1 s long stun application with four different current and voltage settings. The setting used for experiment group S1 was not enough to induce an epileptic-like seizures in all animals. The grey trace in the background is an additional representative example of a short but clear epileptic-like seizure from this group. Settings used for experiment groups S2-S4 caused epileptic-like seizures and immediate loss of VERs in all animals. The blue hatched boxes show beginning and end of p_1 and red hatched boxes beginning and end of p_2 for each individual example. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

post stun-application was divided into a 1st (p_1) and a 2nd phase (p_2) by visually interpreting changes in amplitude, determined by decreases in amplitude between p_1 and p_2 and at the end of p_2 (Fig. 1). Mean amplitudes were calculated using a period of 10 s immediately prior to the stun (p_0), the whole duration of p_1 (~10 s) and the initial 10 s of p_2. The calculations were done using absolute values from the beta frequency EEG as this frequency provided more pronounced changes in amplitude with distinct phases during the seizure compared to lower (0.5–12 Hz) frequencies of the EEG. The durations of p_1 and p_2 were determined visually, as previously described, using information from raw EEG, beta frequency EEG and absolute values from beta frequency.

To ensure that the signal measured brain activity and did not deteriorate over time, a minimum of two averaged images displaying stable VERs were recorded before the stun application. The last image prior to the stun was used for the analysis. For the period immediately following the stun application, it was necessary to separate the image into epochs to find if and when the response returned, and to avoid missing transient periods of VERs. New images were created (using 30–120 consecutive epochs) for this period and analyzed. If no VER was present during this time, a full image of 120 consecutive epoch was used in the analysis. To define and quantify presence and absence of VERs, the image was assessed for amplitude during the first (0–200 ms, Amp_{Light}) and second (200–400 ms, Amp_{Dark}) period (Fig. 2). VERs were considered present when the amplitude quotient ($Amp_{Quot} = Amp_{Light} / Amp_{Dark}$) > 2 and a wave shaped reading was aligned with the light stimuli. Conversely, VERs were deemed absent if $Amp_{Quot} < 2$ following the stun application. The amplitudes were computed (max-min) and extracted from the scope view in LabChart.

2.5. Statistics

Statistical analyses were performed using SPSS Statistics version 24.0 (IBM Corp. Released 2016. IBM SPSS Statistics for Windows, Version 24.0. Armonk, NY: IBM Corp). All variables were checked for homogeneity of variances and normal distribution. Statistical significance was accepted at $P < 0.05$ and all data are reported as means ± SEM. Only groups that were deemed successful, *i.e.* where all animals within the group displayed clear signs of a seizure following a short stun (S1-S4) or did not recover VERs within a reasonable time following a long stun (L2-L4), were included in the analyses. To investigate whether the division of the epileptic-like seizure into distinct phases was statistically valid, paired *t*-tests (p_0 *vs* p_1, p_0 *vs* p_2 and p_1 *vs* p_2) was used to compare amplitudes between phases. To explore if increased current and voltage had an impact on amplitude during p_1 and p_2 of the epileptic-like seizure, a one-way ANOVA was performed. The three experiment groups (S2, S3 and S4) were set as factors and amplitudes during the phases (p_0, p_1 and p_2) as dependent variables. S2, S3 and S4 were also set as factors to analyze effects on duration of p_1 and p_2 from increased current and voltage using a one-way ANOVA. Significant variation among groups was further explored using an additional multiple comparison Bonferroni post-hoc test. To determine whether stun setting affected recovery times for VERs and ventilation after a 1 s stun application, one-way ANOVAs were equally performed. Success following captive bolt and a long stun application (15, 30 or 60 s stun application, experiment group L2, L3 and L4) was determined using binary scoring (fail/success) and settings were considered acceptable when 10/10 fish in the group did not recover within a time when subsequent bleeding could be accomplished.

3. Results

3.1. Induction of an epileptic-like seizure following 1 s electric field exposures

The settings used for the S1 group (electric field strength of 1.4 ± 0.01 V cm^{-1} with a current density of 0.10 ± 0.002 A_{RMS} dm^{-2}, $n = 4$) failed to induce epileptic-like seizures in 2 out of 4 fish and these animals remained fully awake with a short period of escape behavior following the stun application (Fig. 1). Ventilation was never lost except for one fish that lost ventilation for 17 s immediately following the exposure. This field strength and current density setting was therefore determined as insufficient to reliably induce epileptic-like seizures in this sized trout, and thus not further explored.

In contrast, all animals in experiment groups S2-S4 ($n = 14$), which were exposed to an electric field of ≥2.08 ± 0.01 V_{rms} cm^{-1} and a current density of ≥0.22 ± 0.003 A_{rms} dm^{-2} for 1 s (see Table 1 for details), were rendered immobilized and displayed epileptic-like seizures on the EEG immediately following the stun (see examples in Fig. 1). The amplitude during p_0 was 0.8 ± 0.05 μV to then increase to 15.8 ± 1.7 μV during p_1 and 4.0 ± 0.32 μV during p_2, *i.e* a relative 19.8 ± 2.0-fold and 5.1 ± 0.5-fold increase during p_1 and p_2 respectively (p_0 *vs* p_1; $t_{(13)} = -9.077$, $P < 0.001$, p_0 *vs* p_2; $t_{(13)} = -10.224$, $P < 0.001$, p_1 *vs* p_2; $t_{(13)} = 6.98$, see Fig. 3). There were no differences in mean amplitudes between stun groups (S2, S3 and S4) during any of the phases (p_0: $F_{2,11} = 1.972$, $P = 0.185$; p_1: $F_{2,11} = 0.283$, $P = 0.759$; p_2: $F_{2,11} = 2.369$, $P = 0.139$) and duration of p_1 was similar among groups ($F_{2,11} =$

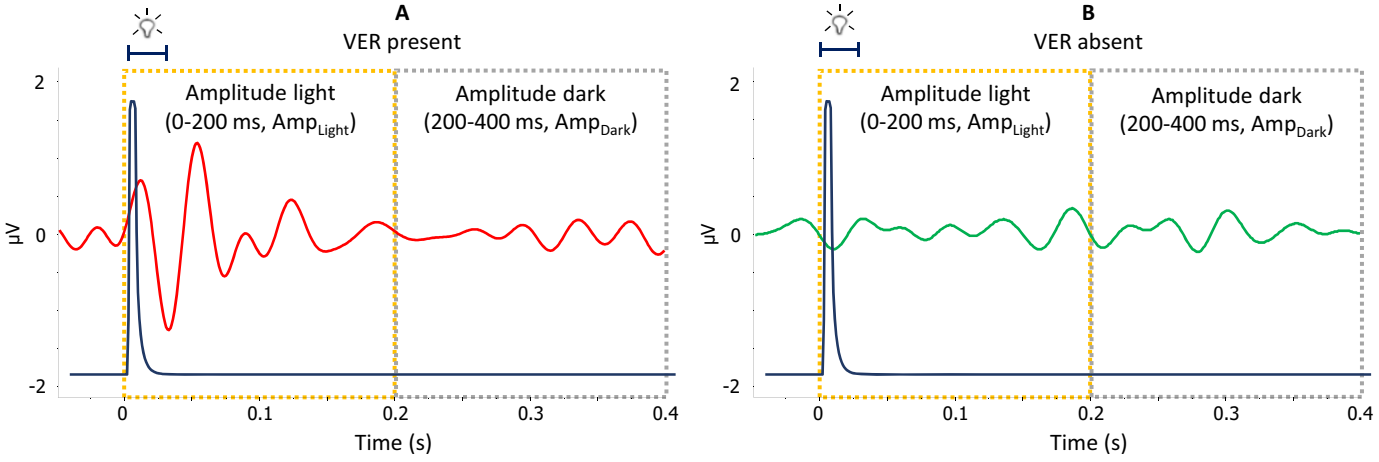

Fig. 2. Averaged images of 120 consecutive 500 ms recordings. Presence of VERs was determined both visually (sine shaped wave triggered by 50 ms light stimulus) and a relative amplitude (Amp_{Light}/Amp_{Dark}) > 2 (A). Loss of VERs was assumed when no sine shaped wave was present and relative amplitude was ≤2 (B). The blue line show onset and duration of light (amplitude scaled down to fit in the figure). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

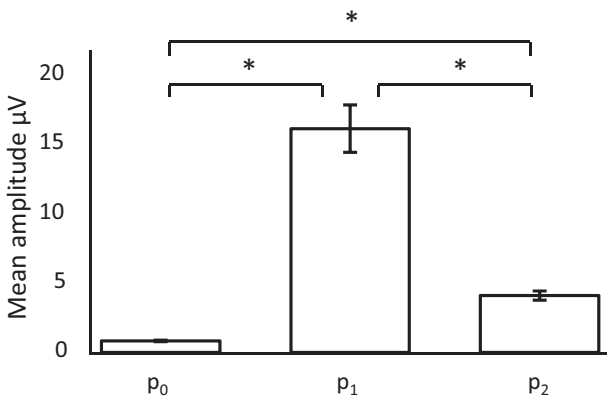

Fig. 3. Mean beta frequency amplitude from all individuals in groups S2-S4 (n = 14) for each phase. Amplitude of p_1 and p_2 was 19.8 ± 2.0 and 5.1 ± 0.5-fold higher compared to pre stun (p_0) mean. Asterisk * indicate significant difference in amplitude between groups (P < 0.001).

0.927, $P = 0.424$; 7.4 ± 1.8, 10 ± 1.3 and 9.9 ± 0.9 s for S2, S3 and S4 respectively). However, the duration of p_2 was significantly different among stun groups ($F_{2,11} = 11.626$, $P = 0.002$), and the post-hoc test revealed that settings used for experiment group S2 resulted in a significantly shorter p_2 duration (8.4 ± 2.6 s) compared to both S3 (35.3 ± 7.2 s; $P = 0.011$) and S4 (40.1 ± 7 s; $P = 0.004$).

VERs were absent immediately after the stun application for all fish and returned after 47 ± 12.8 s (range: 10–105 s), 56 ± 14.8 (16–87 s) and 170 ± 92.5 s (20–415 s) for group S2, S3 and S4, respectively ($F_{2,11} = 2.078$, $P = 0.175$). However, in 3 out of 14 fish the return of the VERs preceded the end of p_2 with 25, 35 and 49 s for the three individual fish respectively (a representative EEG of the latter can be seen in Fig. 4). In 4/14 fish, VERs disappeared >5 min post-stun while they remained present in 10/14 until the end of the 15 min post-stunning period. Ventilation in all animals exposed to a 1 s stun returned and remained present until euthanized. No difference among groups in time to return of ventilation was found ($F_{2,11} = 0.019$, $P = 0.978$).

3.2. *Effects of prolonged electric field exposures and captive bolt stunning*

It was found that 13/18 animals in experiment group L2 and L3 (30 and 60 s) and L4 (15 s) regained the VER shortly after stun application

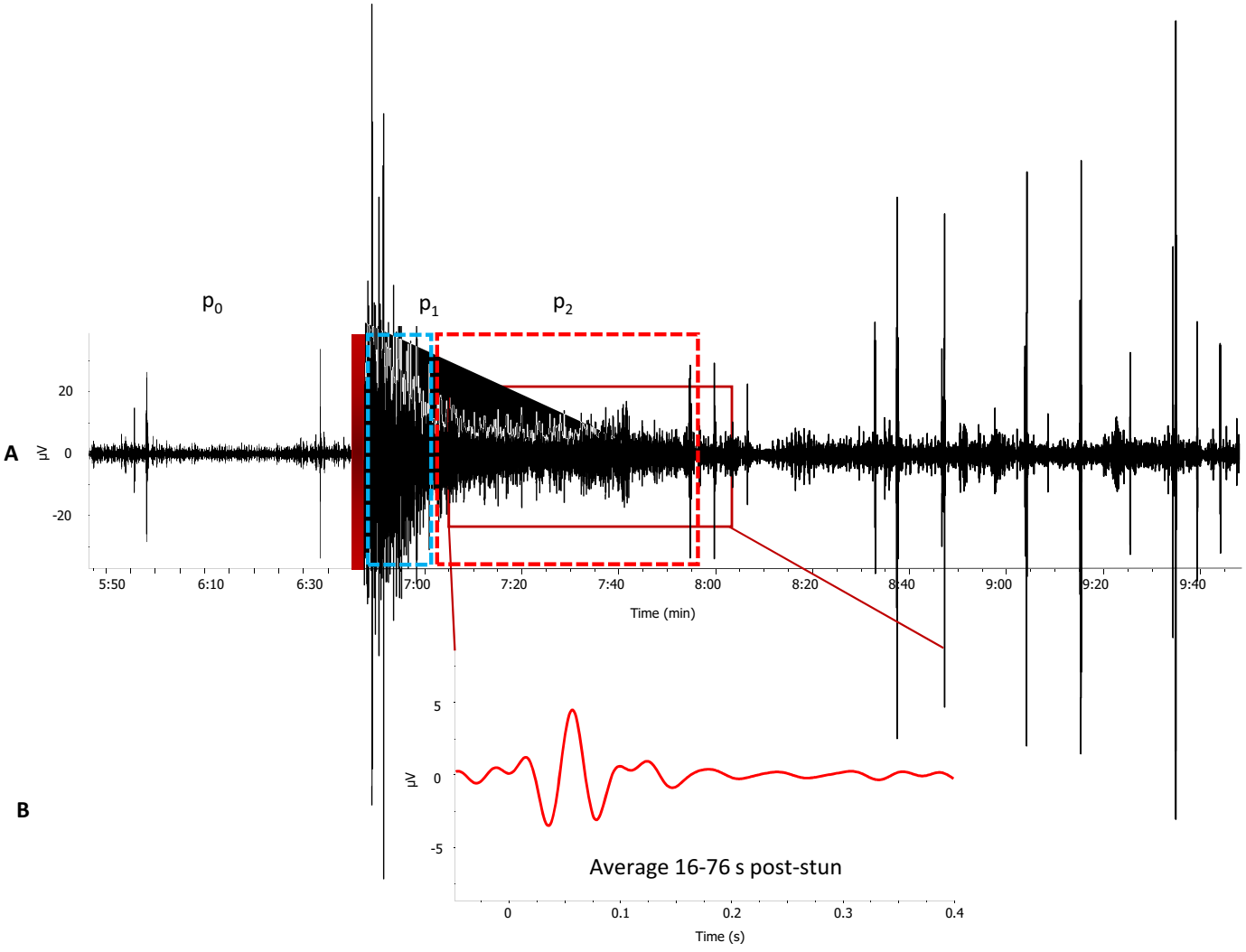

Fig. 4. Representative example of EEG in the beta frequency before and after a 1 s stun application (red bar) from one individual in experiment group S3 (A). In this individual, the epileptic-like seizure started with a 1st phase that lasted ~11 s followed by a 54 s long 2nd phase (blue hatched boxes). VERs appeared 16 s after the end of the stun application (red box indicate the trace 16–76 s after stun application) and remained present throughout the experimental protocol (B). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

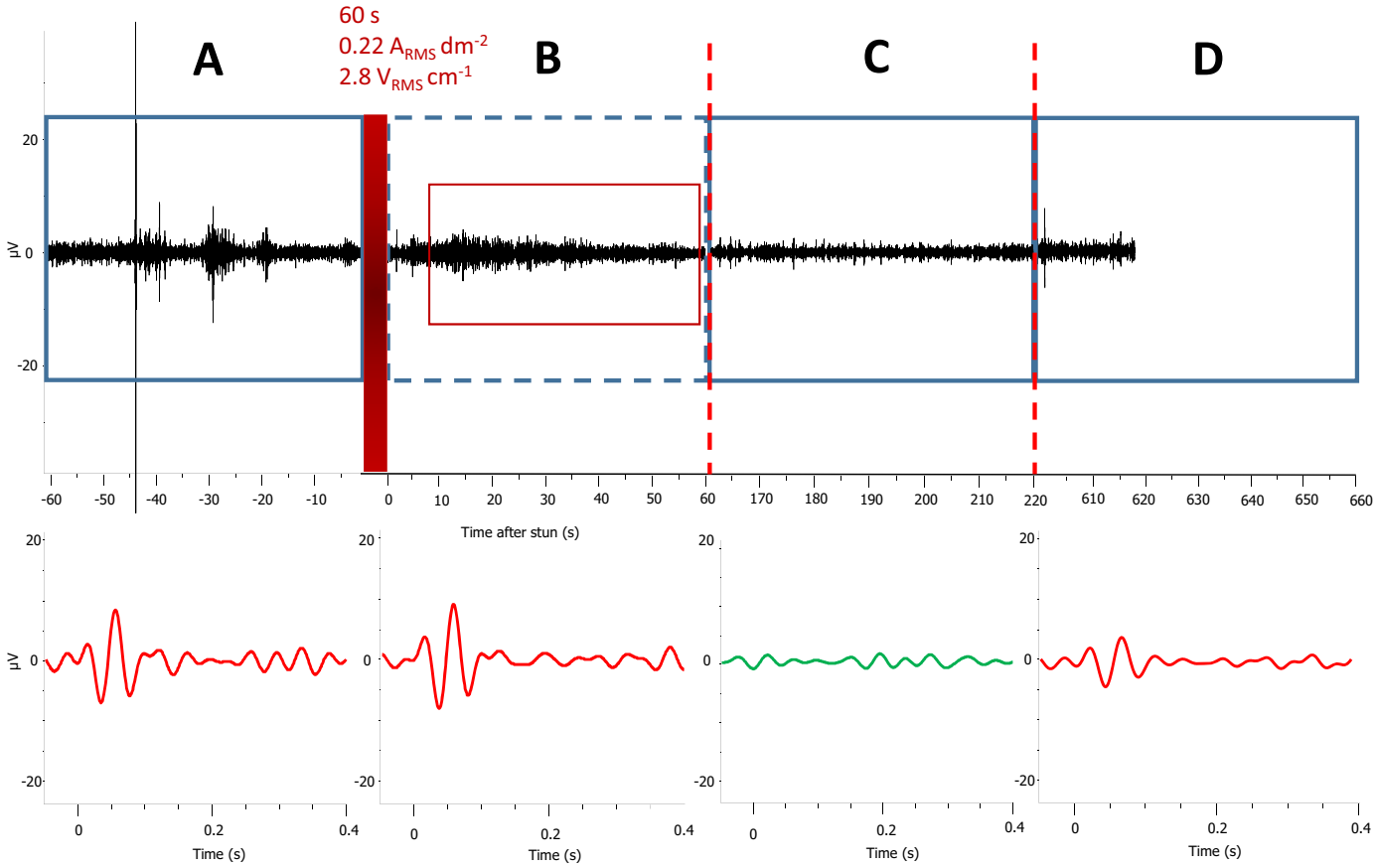

Fig. 5. A successful stun was determined by immediate loss of VERs compared to pre-stun (A) without recovery following stun. It was found that VERs returned shortly after the stun (within the first minute (B)) and often transient rather than a gradual and permanent recovery. This figure show a representative example from an individual in group L2, 60 s, that was deemed an unsuccessful stun where VERs returned 10 s after the stun application but ceased 47 s later (B, C). Steady ventilation returned 2 min post-stun and the fish had recovered completely after 14 min when VERs and swimming activity reappeared (D). No epileptic-like seizures were seen following stuns applications >15 s.

(Fig. 5, Fig. 6). Twelve of these animals regained VERs within the first minute following stun application, of which four responded to the light immediately after the electric field in the water was turned off. The period of VERs was transient and lasted for 56 ± 8 s (range: 20–124 s), except for one fish where the VER remained present throughout the whole trial once it returned. No pattern between recovery of VERs and recovery of ventilation was found, where recovery of the VER often, but not always, preceded the recovery of ventilation. It should be mentioned that in 5/12 animals, the transient period of VERs was already over when the ventilation returned 5).

Fish in group L4 exposed to a voltage of 10.2 ± 0.03 V cm^{-1} with a current of 0.85 ± 0.009 A_{rms} dm^{-2} for 30 s (n = 10) or 10.2 ± 0.03 V cm^{-1} with a current of 0.84 ± 0.005 A_{rms} dm^{-2} for 60 s (n = 10) all lost VERs permanently (Fig. 6). Ventilation was regained between 84 and 513 s post stun application for one 30 s fish, all other had lost ventilation throughout the 15 min post-stunning period. Similarly, all fish stunned with the bolt gun permanently lost VERs.

4. Discussion

This is the first study to non-invasively monitor EEG of rainbow trout during percussive and electrical stunning. Here we show that it is possible to immediately and permanently abolish VERs using a percussive captive bolt. Furthermore, it is possible to render rainbow trout immobilized and induce an epileptic-like seizure on the EEG with electric field strengths and current densities of 0.22 A_{RMS} dm^{-2} and 2.8 V_{RMS} cm^{-1}, respectively, in low salinity water using the described equipment. However, the level of consciousness in trout shortly (~10 s) after an epileptic-like seizure remains unclear as an isoelectric phase was only observed in a few individuals and most fish were responsive to visual stimulation (*i.e.* VERs were present) directly after the end of the seizure. Only when the electric field strengths and current densities were increased to 0.84 A_{RMS} dm^{-2} and 10.2 V_{RMS} cm^{-1}, respectively, and combined with an application duration of 30–60 s, fish became incapable of responding to their environment (*i.e.* the VERs were permanently lost). The details of our results and their potential implications to fish welfare are discussed below.

4.1. The effects on the EEG following percussive stunning and a 1 s electric field exposure

EEG was continuously recorded throughout the experiment (except

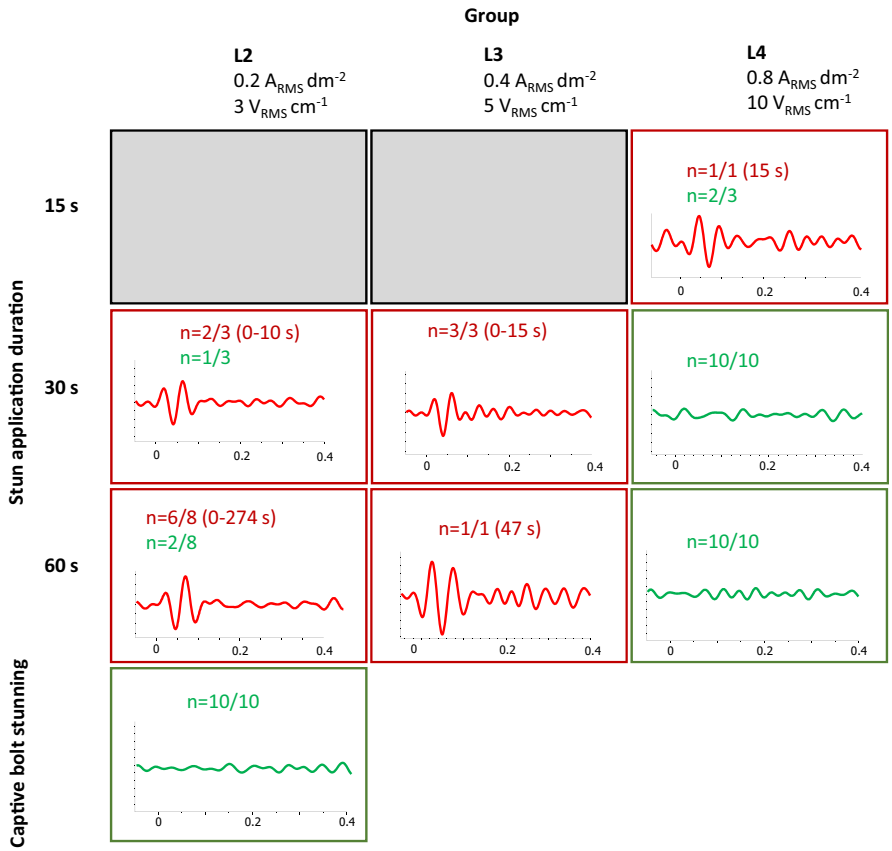

Fig. 6. Red boxes and lines indicate settings where the stun application did not manage to abolish VERs completely, with examples present from each group. Red font display the number of animals within each group that recovered VERs green font equals number of successfully stunned animals. Times in brackets mark range of time to start of VERs following stun application. Green boxes show the settings where 10/10 animals were successfully stunned, *i.e.* animals exposed to ~ 10.2 V_{RMS} cm^{-1} and 0.84 A_{RMS} dm^{-2} for 30 and 60 s and stunned with the 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.)

during percussive stun application) and the flashing light successfully induced responses in the form of VERs on the EEG that could be detected before the stun was applied. If the electrical exposure clearly failed to stun the fish (*i.e.* no epileptic-like seizures were observed and the fish showed clear aversive behaviors), VERs could be detected again immediately following the end of the exposure period.

When using a handheld non-penetrative pneumatic captive bolt gun, the blow administered to the head of the fish was always followed by a state with little to no neural activity on the EEG and the VERs were immediately and permanently lost. When using a 1 s electrical field exposure all fish exposed to ≥2.8 V_{RMS} cm^{-1} and ≥ 0.22 A_{RMS} dm^{-2} for 1 s displayed an epileptic-like seizure on the EEG. During an epileptic-like seizure two distinct phases (p_1 and p_2) were observed on the EEG. This is similar to previously reported descriptions of EEG in fish during an electrically induced seizure (Lambooij et al., 2010; Lambooij et al., 2006; Lambooij et al., 2007). The duration and amplitude of the first phase of the seizure (p_1) was unaffected by strength of the electric field and current density. The duration of p_1, (~9 ± 1 s) also corresponds relatively well with previously reported seizures in other fish species such as carp (*Cyprinus carpio*) (11 ± 4 s), sea bass (*Dicentrarchus labrax*) (8 ± 4 s), Nile tilapia (*Oreochromis niloticus*) (9 ± 3 s), Atlantic salmon (*Salmo salar*) (20 ± 12 s) and African sharptooth catfish (*Clarias gariepinus*) (7 ± 3 s) (Lambooij et al., 2008a, Lambooij et al., 2008b; Lambooij et al., 2010; Lambooij et al., 2006; Lambooij et al., 2007). In contrast, the duration of p_2 could be prolonged when the strength of the exposure was increased. This is probably one of the reasons why previously reported duration of a second phase is much more variable among species and studies (Lambooij et al., 2008a, Lambooij et al., 2008b; Lambooij et al., 2010; Lambooij et al., 2006; Lambooij et al., 2007). The time it took for VERs to return after a 1 s stun application varied significantly among individuals (10–415 s, $n = 14$), which is similar to the variability reported for other species such as Atlantic salmon (58–400, 44–478 and 56–310 s, for different currents and durations) and African sharptooth catfish (102–294 s) (Brijs et al., 2020; Robb and Roth, 2003).

We found that VERs can return directly at the end of an epileptic-like seizure and sometimes even within the second phase (p_2) of the seizure, (*i.e.* when the brain of the fish still show increased neural activity). This is the first time this phenomenon has been reported in fish, and our results resembles the findings of Gregory and Wotton (1989) who showed presence of somatosensory evoked potentials in chickens during seizures induced by electrical stunning. The author of that study discussed the possibility that what they observed in the chickens may not have been a *grand mal* seizure but rather a *petit mal* seizure (nowadays commonly called "absence seizure") that in humans does not necessarily indicate unconsciousness (Gregory and Wotton, 1987, 1989). In humans, absence seizure is described as a sudden but short (3–30 s.) poly-spike activity with varying symptoms, *i.e.* with or without impairment of consciousness and tonic and/or clonic components (Bancaud et al., 1981; Panayiotopoulos, 2008; Sadleir et al., 2009). Whether or not our results are indicative of "only" an absence seizure also for rainbow trout is unknown and merits further investigation, but this unexpected finding is beyond the scope of this study and comparisons among animal groups must be done cautiously. In addition, to fully understand how these results may affect the welfare of the animals, a deeper understanding of the underlying mechanisms that explain these dissimilarities in neural responses among animal groups is needed.

4.2. The effects on the EEG following longer electric field exposures

In order to achieve a permanent loss of VERs throughout the 15 min recovery protocol employed in the present study the electrical field strength and current density needed to be increased to ~10.2 V_{RMS} cm^{-1} and ~ 0.84 A_{RMS} dm^{-2}, respectively, with a stun application duration of

≥30 s. Shorter stun application (15 s) or lower electrical field strengths and current densities all failed to abolish VERs permanently. The recovery of VERs in rainbow trout using longer stun applications was quick but often transient, *i.e.* the majority (12/13) of the fish that recovered VERs did so within the first minute and their VERs remained present for approximately 1 min (although these settings was shown to induce a seizure following a 1 s stun application). The rapid but transient recovery of VERs are findings that require further investigation. It is unclear if the fish is "drifting" in and out of consciousness or if the fish during this period remain unconscious, and so, unaware of the events that takes place during this period. From an animal welfare perspective, this distinction is critical, as the few seconds following the stunning exposure is normally the time when the fish's gills/throat are cut before being bled to death (Gräns et al., 2016; Lines and Spence, 2012). Using conservative indicators such as VERs to determine efficacy of a stun may therefore be a reliable tool to safeguard fish welfare, but it must also be recognized that it is possible that the animal becomes unconscious before the VERs are lost. In such scenarios, there is a risk that stunning methods that actually do fulfill the criteria for humane slaughter of animals are rejected (Raj et al., 1991; Verhoeven et al., 2015).

The consequence of using the relatively high field strength (~10 V_{RMS} cm^{-1}) and current density (~0.85 A_{RMS} dm^{-2}), in combination with a low frequency AC current (50 Hz) and long stun application times (30 s) that were needed for long-lasting effects in the present study, increases the risk of carcass damage (Jung-Schroers et al., 2020; Robb et al., 2002; Roth et al., 2003; Roth et al., 2004). However, it must be recognized that efficiency of electrical stunning of fish is dependent on more variables than were tested here. For example, using different electrode positions and/or water conductivities could potentially affect the outcome of the stun efficiency (Lambooij et al., 2008a, 2008b; Lines and Kestin, 2004; Robb and Roth, 2003). Moreover, sensitivity to electrical stunning is species-specific, but individual size and tissue composition within a species is also discussed to affect impact of the stun (Brijs et al., 2020; Lines and Kestin, 2004), which could potentially be explained by differences in tissue impedance that is known to affect the efficiency of different AC frequencies (Grimsbø et al., 2016). Even though this is outside the scope of this study, this all needs to be taken into consideration and validated when evaluating a common, or designing a novel, species-specific protocol for electrical stunning. An alternative to using stun settings that could potentially downgrade the end-product is *via* the use of a combination of stunning methods. As even a short electrical stun is enough to render the fish immediately unconscious, there are possibilities to prolong this period by using *e.g.* a two-stage electrical stun (Lines and Kestin, 2005). Other alternatives include combining electrical stunning with subsequent ice chilling (Brijs et al., 2020; Daskalova et al., 2015; Grimsbø et al., 2014; Lambooij et al., 2006; Llonch et al., 2012; Sattari et al., 2010), or follow up electrical stunning with percussive stunning (Brijs et al., 2020; Lines and Spence, 2012; Van De Vis et al., 2003).

4.3. The relationship between VERs and ventilation

It is known that time to return of visual indicators, including ventilation, is dependent on stun application duration and current density in rainbow trout (Robb et al., 2002) and that an increase in stun application duration can delay the time to recovery of VERs in African sharptooth catfish (Brijs et al., 2020). Similarly, it is possible to prolong the time to recovery of some visual indicators (*i.e.* ventilation, eye-roll reflex and equilibrium) in carp with increased stun application time, but, contrariwise, not time to recovery of VERs (Retter et al., 2018). In the present study, we, likewise, found no indications of a linear relationship between stun application duration and time to recovery of VERs in rainbow trout, but rather a threshold level of both stun application duration and electric field strength and current density. The pattern observed here is similar to what has been reported in studies investigating electrical stun duration in lamb and sheep (Berg et al., 2012; Cook et al., 1995).

These results suggest that the times to recovery of VERs and to recovery of ventilation are not related following electric stunning. Similar findings were recently reported for African sharptooth catfish (Brijs et al., 2020). For example, one individual in the present study regained VERs after 10 s while it took 194 s for ventilation to recover. This means that the brain of the fish was able to respond to external stimuli for >3 min before ventilation was resumed. That individual was observably rendered immobilized by the stun, but whether the brain of the fish was functional enough to experience pain or fear remains unresolved. Even though the light conditions during experimentation in this study stipulate that visual observations of behavior must be interpreted cautiously, the general impression was that the animals did not display any signs of being awake following longer stun applications, even during periods when VERs were present.

4.4. Possible welfare implications of our results

From an animal welfare perspective, the onset of unconsciousness is absolutely critical (Gräns et al., 2016; Poli et al., 2005; Retter et al., 2018; Robb and Kestin, 2002). Additionally, it is equally important to ensure that the fish remains unconscious long enough to avoid recovery before subsequent death. For fish, being ectotherms, this is especially important as it takes much longer time to induce brain failure by common killing methods, *i.e.* exsanguination, compared to mammals and birds. For example, Robb et al. (2000) showed that it can take more than 7 min for an Atlantic salmon to lose VERs after being gill cut at 6 °C. It has also been reported that handling time between stunning and killing during batch slaughter events can be substantial (Jung-Schroers et al., 2020), which further increases the risk of fish recovering before death and must be considered when evaluating appropriate stunning methods.

The question is how to determine unconsciousness in an accurate way. Several previous studies have shown that the use of different indicators may result in different conclusions regarding onset and duration of insensibility in fish (Bowman et al., 2020; Brijs et al., 2020; Daskalova et al., 2015; Kestin et al., 2002; Llonch et al., 2012; Retter et al., 2018). From the perspective of electrical stunning this can be a problem if an insufficient electric field is used, as there is a risk that the electrical exposure only induces contractions that exhaust the muscles and render the fish in an immobilized but neurologically functional state (*i.e.* electro-immobilization) (Bohlin et al., 1989; Reid et al., 2019; Vibert, 1963). For fish stunned using the bolt gun in this study the results were much clearer, as all fish lost neural activity (including VERs) and ventilation directly from the administration of the percussive blow. However, the extensive handling of each individual makes this method relatively labor-intensive and potentially stressful for the fish and therefore percussive stunning is often considered impractical for farms rearing smaller fish and fish of varying or atypical morphology (Lines and Spence, 2012). Moreover, from previous studies on Atlantic salmon and African sharptooth catfish, we know that also percussive stunning may render the fish immobilized yet in a neurologically functional state and responsive to visual stimulation (Brijs et al., 2020; Lambooij et al., 2010).

In addition, large dissimilarities regarding duration of unconsciousness was observed depending on what indicators were used in the present study. For example, one individual that was electrically stunned would be judged as unconscious for a period of 12, 34, 69 or 188 s depending on using end of p_1, return of VERs, end of p_2 or recovery of ventilation as indicator of consciousness. These results confirm that absence of VERs is a conservative indicator that can be used to say that the animal is undoubtedly in an unconscious state.

5. Conclusions

We show that both percussive stunning using a captive bolt gun and electric stun can interrupt brain function in rainbow trout manifested as

an epileptic-like seizure and/or an isoelectric phase and absence of VERs on the EEG. However, we also show that it is necessary to continuously assess the presence or absence of VERs as we show that the brain can become responsive to its environment long before visual indicators (*e.g.* ventilation) are resumed. Our study identifies the need for a well-defined and standardized protocol for assessment of unconsciousness when evaluating the efficiency of stunning methods for humane slaughter of fish. Although loss of VERs most likely does not equal onset of unconsciousness, abolition of evoked potentials provide a reliable measure that do not risk overestimating the efficiency of different stunning methods. Therefore, until a reliable visual indicator of consciousness (or lack thereof) is confirmed, it is necessary to perform laboratory studies examining brain function to confirm that the stunning protocol meet the requirements for humane slaughter. In addition, a deeper understanding regarding how sensations of fear, pain, distress, and anxiety relates to the presence and absence of different indicators of consciousness, including VERs and ventilation, in fish is urgently needed.

CRediT authorship contribution statement

P. Hjelmstedt: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing, Visualization. **E. Sundell:** Investigation, Writing – review & editing. **J. Brijs:** Writing – review & editing. **C. Berg:** Resources, Writing – review & editing, Project administration, Funding acquisition. **E. Sandblom:** Resources, Writing – review & editing. **J. Lines:** Resources, Writing – review & editing. **M. Axelsson:** Resources, Writing – review & editing. **A. Gräns:** Conceptualization, Methodology, Validation, Investigation, Resources, Writing – review & editing, Visualization, Project administration, Funding acquisition.

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.2022.738012.

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