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Evaluation of the reliability of indicators of consciousness during CO_2 stunning of rainbow trout and the effects of temperature

Jennifer Bowman[1] | Nicole van Nuland[2] | Per Hjelmstedt[1] | Charlotte Berg[1] | Albin Gräns[1]

[1]Department of Animal Environment and Health, Swedish University of Agricultural Sciences, Skara, Sweden

[2]Institute for Life Sciences and Chemistry, University of Applied Sciences CH, Utrecht, Netherlands

Correspondence
Jennifer Bowman, Department of Animal Environment and Health, Swedish University of Agricultural Sciences, Box 234, 53223 Skara, Sweden.
Email: j.r.bowman.0.0@gmail.com

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Abstract

A two-part experiment was conducted to determine whether visual indicators of consciousness such as equilibrium, eye-roll reflex and ventilation are reliable for evaluating whether CO_2 stunning of rainbow trout (*Oncorhynchus mykiss*) is humane. In part 1, the time taken until the loss of visual indicators in rainbow trout during CO_2 stunning was monitored under field conditions at 14, 8 and 2°C. Here, we clearly demonstrate that it takes longer for visual indicators to disappear as temperature decreases, with significant differences in the time taken until the loss of equilibrium between 2 and 14°C, and significant differences between all temperatures in the time taken until the loss of eye-roll reflex and ventilation. In part 2, rainbow trout were equipped with external non-invasive electrodes for recording EEG prior to, and following, CO_2 stunning to assess the presence or absence of visually evoked responses (VERs), which are indicative of brain function and sensibility. The resulting EEG recordings during CO_2 stunning at 10°C demonstrated a poor relationship between visual indicators of consciousness and loss of sensibility, as VERs were present up to 3.5 min after ventilation was lost and up to 6.5 min after the fish lost equilibrium. Collectively, these results show that cold-water temperatures prolong the time taken until loss of consciousness and that visual indicators are insufficient for determining when sensibility is lost in rainbow trout during CO_2 stunning.

KEYWORDS

brain function, electroencephalography, sensibility, unconsciousness, welfare

1 | INTRODUCTION

Humane slaughter guidelines state that fish should be killed in a way that does not cause fear or pain (EFSA, 2004; OIE, 2018). This is generally accomplished by rendering the fish insensible with a stunning method followed immediately by killing. For a stunning method to be considered acceptable, loss of consciousness and/or insensibility must occur immediately and irreversibly so that slaughter personnel have enough time to kill the fish (EFSA, 2004; OIE, 2018). The only stunning methods proposed to meet the requirement for immediate

insensibility when used properly are percussive and electrical stunning (EFSA, 2004; OIE, 2018). However, there are many other methods of stunning still used in practice that do not meet the required standards (Gräns et al., 2016; Lines & Spence, 2012). One of these methods is carbon dioxide (CO_2) stunning, the use of which has declined in recent years but is still utilized in several European countries (Brijs et al., 2018; IBF, 2017). This method is easy and inexpensive to implement on large groups of fish, but does not meet OIE (2018) standards as it is known to cause severe aversive behaviours and requires long induction times before consciousness is lost in several fish species, including rainbow trout (*Oncorhynchus mykiss*; Kestin, Wotton, & Adams, 1995; Marx, Brunner, Weinzierl, Hoffmann, & Stolle, 1997; Robb & Kestin, 2002). CO_2 stunning is accomplished by bubbling CO_2 into water until the pH is reduced <5, which is a level associated with complete saturation (Anonymous, 1995). Fish submerged in this solution experience an increase in dissolved CO_2 in their bloodstream, which leads to narcosis, decreased ventilation and ultimately respiratory failure (Bernier & Randall, 1998; Kugino, Tamaru, Hisatomi, & Sakaguchi, 2016).

Verifying the functionality of a stunning method requires that consciousness, or lack thereof, is determined in a reliable way. As consciousness cannot be easily assessed, the presence or absence of visual indicators is often used by both researchers and farmers to determine whether a fish has lost consciousness (Table 1). The most commonly used visual indicators of consciousness such as loss of equilibrium, the eye-roll reflex and ventilatory changes can be difficult to observe and are easily misjudged, which results in the use of inadequate stunning methods that may cause unnecessary fear or pain (Lambooij et al., 2010; Robb & Kestin, 2002). Visual indicators are also unreliable when fish become paralysed or immobile without losing consciousness or sensibility. Loss of movements without loss of sensibility has been reported during CO_2 stunning of Atlantic salmon (*Salmo salar*; Robb et al., 2000). Currently, the only known method for accurately assessing a loss of sensibility in the case of paralysis is through electroencephalography (EEG) (Kestin, van de Vis, & Robb, 2002; Lambooij et al., 2010).

EEG is a method for recording and measuring the electrical activity of the brain through the use of electrodes that are either surgically implanted in the brain (Kestin et al., 2002; Kestin, Wotton, & Gregory, 1991; Quick & Laming, 1990), or attached externally to the head (Bowman, Hjelmstedt, & Gräns, 2019; Cho et al., 2017). EEG allows the investigator to detect changes in brain activity indicative of a loss of consciousness and/or sensibility. One such method involves assessing the presence or absence of averaged visually evoked responses (VERs), which in a conscious animal produces a distinct waveform in the EEG in response to visual stimulation from a flashing light (Kestin et al., 1991; Robb & Roth, 2003; Robb et al., 2000). The abolition of VERs is an objective and unequivocal indicator of brain dysfunction and hence, loss of sensibility, as the failure of this primary sensory pathway is one of the last responses to an external stimulus to be lost before brain death (Daly, Gregory, & Wotton, 1987; EFSA, 2004; Robb et al., 2000). While it is possible that the loss of consciousness occurs prior to the loss of VERs, the presence of VERs indicates that some level of rudimentary visual processing is still occurring in the brain, and thus from an ethical perspective, it must be assumed that there is a possibility the animal is still conscious (Kestin et al., 1991; Robb et al., 2000).

Other methods for assessing changes in consciousness include measurements of EEG signal amplitude and changes in brain frequencies. A ≥50% reduction in total signal amplitude compared to pre-treatment values has been cited as the stage at which calves transitioned from conscious to unconscious (Gibson et al., 2009). An even further reduction, such as when the amplitude of the EEG is less than 12% of pre-treatment values (sometimes defined as iso-electric EEG), has been used as an indicator of profound brain failure in calves and chickens (Gibson et al., 2009; Mcilhone, Beausoleil, Johnson, & Mellor, 2014).

A shift in consciousness can also be assessed by separating the EEG signal into beta, alpha, theta and delta frequency waves, and measuring their relative contribution to the overall signal. A transition from high-frequency beta and alpha brain waves to low-frequency theta and delta waves has been used as an indicator that a fish is no longer conscious (Lambooij et al., 2006, 2010, 2013). The shift from high- to low-frequency waves has also been assessed by using the median frequency of the total EEG signal in calves and chickens (Gibson et al., 2009; Martin & McKeegan, 2017).

While the effects of CO_2 stunning on fish are well documented, there has been little investigation of how different seasonal

TABLE 1 Description of visual indicators used to determine loss of consciousness in fish during stunning and slaughter (table modified from Readman, 2015; McFarland, 1959; Ross & Ross, 2008; Sneddon, 2012)

Visual indicator	Description	Sign of	Estimated comparative depth of anaesthesia
Loss of equilibrium	Unable to maintain upright swimming[1]	Depression of the midbrain[2]	Light anaesthesia[1,2]
Change in ventilation	A change from regular opercular movement to slowing and irregular movement[1]	Depression of the medulla[2], thought to be first sign of transition to an unconscious state	Light to deep anaesthesia[2]
Loss of eye-roll reflex	Unable to adjust eyes to compensate for tilt[1]	Loss of visual reflex and processing[1]	Deep to surgical anaesthesia
Loss of ventilation	No opercular movement[2]	Impending medullary collapse[2], thought to indicate unconsciousness	Surgical anaesthesia/ Medullary collapse[2]

Note: References: [1]Kestin et al. (2002); [2]McFarland (1959).

temperatures affects the efficacy of the method. Therefore, we investigated the effects of acclimation temperatures on the time to loss of visual indicators (i.e. loss of equilibrium, eye-roll reflex and ventilation) of consciousness in rainbow trout stunned in CO_2 saturated water. Part 1 was conducted at a commercial rainbow trout farm in southern Sweden in summer, autumn and winter with water temperatures of 14, 8 and 2°C respectively.

The aim of part 2 of the study was to test whether the loss of visual indicators used in part 1 of the study can be considered accurate indicators of a loss of consciousness. To do this, we fitted rainbow trout with three non-invasive electrodes to monitor brain function. EEG was recorded simultaneously with visual indicators when trout were submerged in CO_2 saturated water at 10°C using the same experimental setup as the field portion of the study. By investigating the relationship between the visual indicators and brain function, we aimed to determine if the external visual indicators used in commercial farm and slaughter situations during CO_2 stunning are accurate indicators of a loss of sensibility.

2 | MATERIALS AND METHODS

2.1 | Ethical statement

This study was performed in accordance with Swedish animal welfare legislation. Animal care and experimental procedures were approved by the ethical committee of Gothenburg, ethical permit number: 177-2013. No protected species were used during the experiment.

2.2 | Part 1: Field experiments

2.2.1 | Animals and husbandry

Rainbow trout of mixed sex with a mean weight ± SE of 1,087 ± 27.9 g were maintained in freshwater at Vänneåns fiskodling AB. Tests were conducted on three different occasions over a year to entail the effects of different seasonal temperatures on the stunning efficiency.

2.2.2 | CO_2 stunning

To measure the time to loss of visual indicators of consciousness during CO_2 stunning under field conditions at a commercial Swedish fish farm, rainbow trout (n = 30) were submerged in CO_2 saturated water at acclimation temperatures of 14, 8 and 2°C. Ten rainbow trout were used per acclimation temperature. CO_2 saturated water was circulated between a 50-L barrel and a 70 L aquarium using a hydraulic pump and kept at a consistent temperature by a refrigeration unit (CB 8-30E, Heto-Holten A/S). CO_2 was continuously bubbled into the barrel until the pH reduced to a steady state < 5, indicating the water was fully saturated. Temperature and pH were measured in the aquarium, before each fish was submersed, using a digital thermometer (Testo 108−-2, Testo North America) and a multifunctional pH meter (HANNA Instruments, HI981901 pH-ORP-OWE. Three point calibrated: 4.01, 7.01 and 10.01) to ensure that temperature and pH were consistent throughout testing. Fish were individually and quickly transferred from their holding tanks using a dip net to the 70-L tank containing CO_2 saturated water where they remained for 12 min while being monitored for loss of visual indicators. After treatment, fish were transferred by hand to a tank containing aerated water for 10 min and monitored for recovery, followed by euthanization via a percussive strike.

2.3 | Data analysis and statistics

Experiments were filmed for verification purposes. Time until loss of equilibrium, eye-roll reflex and ventilation was recorded during experiments. The eye-roll reflex was checked every 30 s until it was lost. A one-way ANOVA followed by Tukey's post hoc test was used to analyse difference between acclimation groups in SPSS (version 2017).

2.4 | Part 2: Laboratory experiments

2.4.1 | Animals and husbandry

Rainbow trout (n = 9) obtained from Vänneåns fiskodling AB, Sweden, of mixed sex and ranging in size between 560 and 905 g (mean ± SE: 673.9 ± 32.9 g) were housed in the animal facility at the Department of Biological and Environmental Sciences, University of Gothenburg between April and June 2018. Water was maintained at 10°C on a 12h:12h light:dark photoperiod.

2.4.2 | Placement of electrodes and recording of VER on the EEG

Fish were individually netted and submerged a tank containing 150 mg/L MS-222 (ethyl 3-aminobenzoate methanesulphonic acid, $C_{10}H_{15}NO_5S$) (Sigma-Aldrich Inc.) buffered with 300 mg/L sodium bicarbonate ($NaHCO_3$) dissolved in 12 L of water to induce light anaesthesia. A silicone cup with integrated electrodes was secured to the head of the fish using a peristaltic pump, and consistent low suction was maintained during the experiment to ensure the cup remained in place. For details of cup design and placement, see Bowman et al. (2019).

Fish were transferred to a 20 L tank containing 16 L of 10°C water and allowed to recover for 10 min. EEG was confirmed and recorded for 10 min without light stimulus to serve as a reference signal, ensuring that the fish was fully awake and the EEG signal was clear. EEG was then recorded for 10 min with light stimulus delivered

as two 250 ms light flashes per second using an LED strobe light and detected using an in-house designed light detector connected to a PowerLab system (ADInstruments Pty Ltd.) sampling at 1 kHz. The peristaltic pump was turned on high for 30 s before fish were gently manually transferred to a 70-L tank of 10°C water that was fully saturated with CO_2, as indicated by a pH meter (HANNA Instruments, HI981901 pH-ORP-OWE). EEG was recorded with light stimulation for 30 min in the treatment tank followed by 10 min without light. If the cup moved or became dislodged after transfer, it was reattached after the fish lost equilibrium. Visual indicators of loss of consciousness were determined during testing and times were recorded. Visual indicators included loss of equilibrium, change in ventilation and loss of ventilation. A transition from regular opercular movement to irregular and gradually slowing was deemed a change in ventilation. Fish were euthanized following treatment with a percussive blow to the head. Room lights were turned off for the entirety of the experiment.

2.5 | Data analysis and statistics

EEG recordings were assessed using LabChart 7 (version 7.3.8, AD Instruments), and a bandpass filter of 0.5–32 Hz was used to reduce signal noise. LabChart Scope View was set to record 500 ms time windows of EEG with the strobe light acting as the input trigger to begin recording. A 1-minute representative window of EEG signal was created for each minute or recording by averaging 120 consecutive, non-overlapping windows. To ensure that the signal accurately represented electrical activity in the brain and was not skewed by electrical artefacts caused by movement or gasping, LabChart was set to exclude windows where the EEG signal exceeded 10 μV from the averaging process. If representative minutes of EEG signal contained less than 60 averaged 500 ms windows (<50% of windows were included), they were removed from analysis.

For analysis of VERs, the EEG signal was filtered into beta waves (13–32 Hz) using a bandpass filter. The presence or absence of the VER waveform for each fish was determined through observations of measurable changes in the beta wave of the brain in response to a visual stimulus (i.e. a flashing light turning on and off) over time. When the VER wave amplitude became indistinguishable from the amplitude of the rest of the beta wave, the VER was considered absent (Figure 1).

Amplitude was measured as the maximum minus the minimum point of the total EEG signal (0.5–32 Hz) for each representative minute. The time to reduce the EEG signal amplitude to <50% of pre-treatment values was calculated using Microsoft Excel (version 2016). EEG signal median frequency and relative power were calculated using data that was transformed in LabChart using Fast Fourier transformation with a Hann (cosine-bell) window with 50% overlap. Relative power was analysed for changes between high-frequency (8–32 Hz) beta and alpha waves and low-frequency (0.5–8 Hz) theta and delta waves indicative of a loss of consciousness. SPSS (version 2017) was used to analyse EEG signal amplitude, median frequency and relative power using a linear mixed model with AR(1) as the covariance structure and time as the repeated variable. Amplitude and median frequency data were log-transformed to meet the assumptions of the model.

3 | RESULTS

All data are reported as mean (min-max value) unless otherwise stated.

3.1 | Part 1: Field experiments

There was a significant difference in weight between the temperature groups [$F_{(2,27)} = 10.96$, $p < .001$] where fish acclimated to 14°C

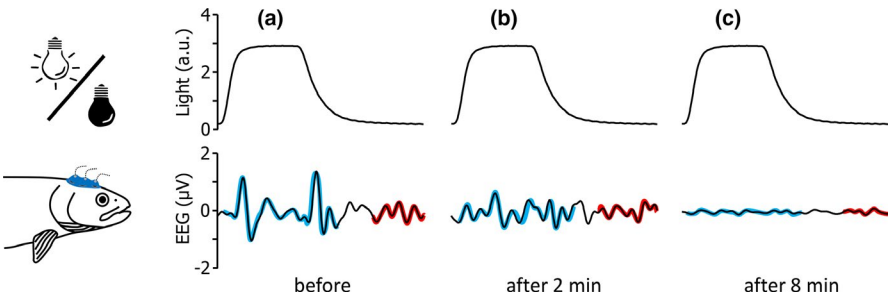

FIGURE 1 Changes in the visually evoked responses (VERs) in the EEG of rainbow trout during submersion in CO_2 saturated water. VERs represent the measurable changes in the electrical potential of the brain in response to a visual stimulus (i.e. a flashing light). In the present study, each VER represents the averaged response to 120 consecutive flashes delivered at one flash per 500 ms. (a) Directly prior to submersion in CO_2 saturated water, VERs can be observed in this specific individual as distinct waveforms in the EEG recordings milliseconds after the light flashes on and off (marked in blue). These waveforms have an amplitude ~3 times greater than the EEG recordings during the dark period (marked in red). (b) After 2 min of CO_2 exposure, VERs can still be observed as the waveform in the EEG repetitively appears following the light flashes. However, the amplitude of the waveform has decreased and is now only ~1.7 times greater than the EEG recordings during the dark period. (c) After 8 min of CO_2 exposure, VERs were abolished as no distinguishable waveform in response to light flashes could be identified within the EEG recordings (i.e. the amplitude of the EEG signal is the same before and after the light flash, ~1:1 ratio) [Colour figure can be viewed at wileyonlinelibrary.com]

were smaller, 945.5 g (805–1,090 g), compared to fish acclimated to 8°C, 1,168.5 g (985–1,315 g) and 2°C, 1,148 g (990–1,355 g). However, no interacting effects were found on any of the investigated variables; thus, mass was not included as a covariate in the final statistical analysis.

3.1.1 | Visual indicators

Overall, the time taken until the loss of visual indicators of consciousness increased as the temperature of CO_2 saturated water decreased (Figure 2). It took significantly longer for fish to lose equilibrium after submersion in CO_2 saturated water at 2°C compared to 14°C [$F_{(2, 27)} = 4.81$, $p = .016$]. The eye-roll reflex was lost shortly after equilibrium, taking 1.30 (1–2) min, 3.15 (2–4.5) min and 4.15 (3–5.5) min at 14, 8 and 2°C, respectively, and the time taken for the eye-roll reflex to be lost was significantly different between all temperature groups [$F_{(2, 27)} = 53.5$, $p < .001$]. Ventilation was the last indicator lost, taking nearly four times longer to cease than the time taken until the loss of equilibrium at all temperatures tested. Ventilation ceased 3.61 (2.90–4.55) min at 14°C, 5.74 (4.01–7.33) min at 8°C and 9.00 (7.33–11.33) min at 2°C, and the time taken for ventilation to cease was significantly different between all temperature groups [$F_{(2,27)} = 69.5$, $p < .001$].

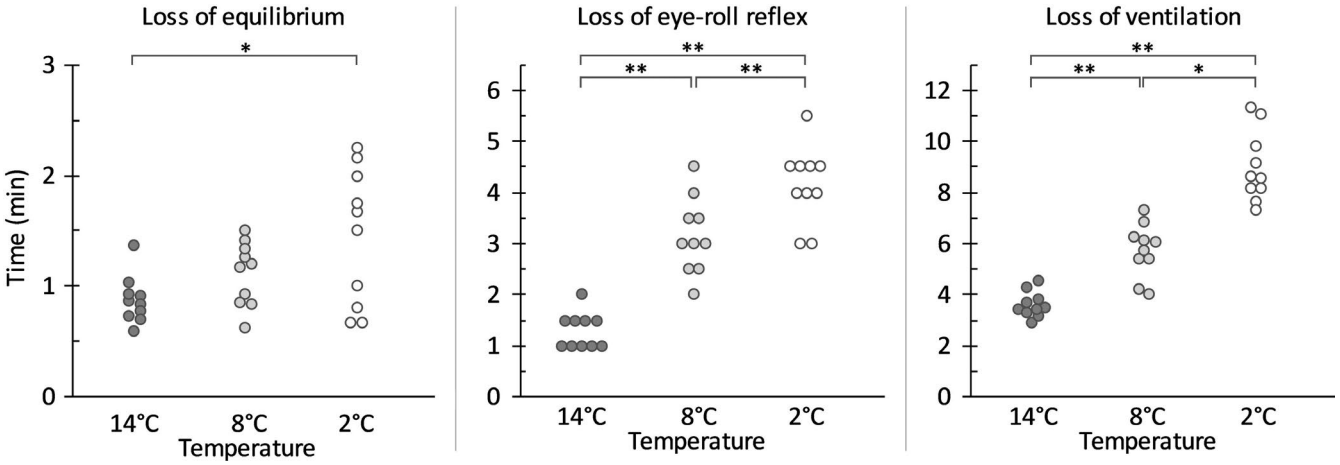

FIGURE 2 Differences in time (min) to loss of equilibrium, the eye-roll reflex and ventilation at 14, 8 and 2°C of individual rainbow trout during progressive loss of consciousness ($n = 30$). The eye-roll reflex was assessed every 30 s. Statistical significance indicated $^{*}p < .05$, $^{**}p < .001$

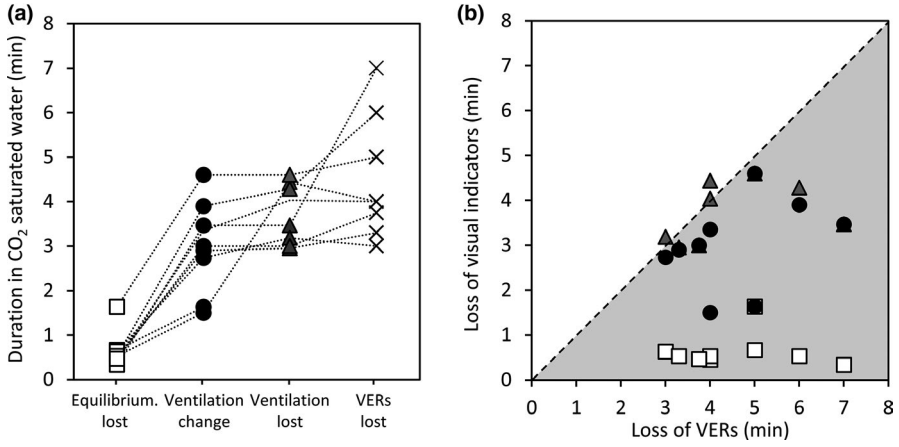

FIGURE 3 Relationship between the loss of VERs and loss of visual indicators of consciousness following submersion in CO_2 saturated water. (a) Time taken for trout ($n = 8$–9) to lose equilibrium (open squares), exhibit changes in ventilation (black circles), cease to ventilate (grey triangles) and lose VERs (X) following submersion in CO_2 saturated water. (b) The time taken for trout ($n = 8$–9) to lose VERs compared with the time taken to lose equilibrium (open squares), exhibit changes in ventilation (black circles) and cease to ventilate (grey triangles). The black dashed line is a 1:1 line and has been included to demonstrate the welfare implications of using visual indicators of consciousness to identify when an animal is insensible. Considerable variation exists between the loss of the visual indicators and the loss of VERs, which from an individual welfare point of view can be acceptable (markers white section, VERs are lost before the visual indicators) or unacceptable (markers in grey shaded section, visual indicators are lost before the VERs)

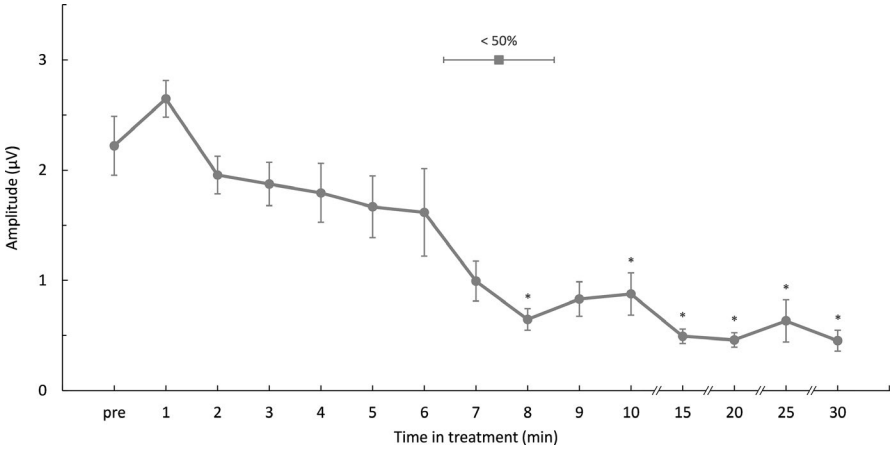

FIGURE 4 Amplitude ± SE of filtered EEG (0.5–32 Hz) during progressive loss of consciousness in rainbow trout ($n = 9$) for the averaged pre-treatment signal (pre) and each minute of treatment. '*' indicates that the amplitude of the EEG signal is significantly different ($p < .05$) from the amplitude of the pre-treatment mean. The marker and horizontal bars above the amplitude data indicate the average time ± SE it took for EEG signal amplitude to reach < 50% pre-treatment amplitude

3.2 | Part 2: Laboratory experiments

3.2.1 | Visual indicators and VERs

After submersion in the CO_2 saturated water, loss of equilibrium occurred after 0.64 (0.33–1.63) min (Figure 3a). Ventilation changed and ceased 3.01 (1.50–4.60) min and 3.74 (2.95–4.6) min, respectively, following submersion in CO_2 saturated water (Figure 3a). Ventilation loss failed to be recorded for one fish. VERs were lost 4.56 (3.30–7.00) min after submersion in the CO_2 saturated water (Figure 3a). Considerable variation was observed between the time to loss of different visual indicators of consciousness and the loss of VERs (Figure 3b). All trout lost equilibrium and altered their ventilation before the VERs were lost with a disparity of up to 6.7 and 3.5 min respectively (Figure 3b). Five out of eight trout ceased their ventilation before losing their VERs with a disparity of up to 3.5 min (Figure 3b).

3.2.2 | Signal amplitude

EEG signal amplitude increased immediately after transfer to the treatment tank compared to pre-treatment amplitude measurements. Time until amplitude declined to <50% pre-treatment amplitude was 7.44 (4–15) min after transfer. Time until amplitude reached <12% pre-treatment amplitude ranged widely, with one fish reaching <12% after 10 min and one fish not reaching < 12% at all during treatment.

Results of the linear mixed model (Figure 4) showed a significant decrease in amplitude during min 8 and min 10–30 min 7 and 9 were close to achieving significance ($p = .056$ and $p = .071$) but ultimately did not.

3.2.3 | Median frequency and relative power

No significant difference was found between pre-treatment mean and treatment values for EEG signal median frequency. Mean pre-treatment median frequency was 7.74 (2.53–22.3) Hz. After transfer and until min 10 mean median frequency stayed below pre-treatment mean, with the lowest frequencies occurring at min 4 and 7, measuring 3.87 (1.93–7.91) and 3.86 (1.27–11.1) Hz respectively. After this, median frequency fluctuated between 5.52 and 13.33 Hz for the remainder of the experiment.

Results of the linear mixed model showed no significant difference between the pre-treatment means and the relative power of beta and alpha (B/A) and theta and delta (T/D) frequencies over treatment. B/A frequencies decreased during the first minute after transfer from 46.8% to 32.9% of the total signal power, while T/D increased from 53.0% to 67.0%. B/A increased and T/D decreased slightly over the next min before reversing over the next 2 min. The lowest B/A and highest T/D powers occurred during min 4, comprising 18% and 81% of the total signal power respectively.

4 | DISCUSSION

This is the first study to use a newly developed, non-invasive technique to monitor brain function and sensibility via EEG recording in a fish to evaluate the effectiveness of a stunning method. The results of this study show great promise on how to validate stunning efficiency in fish using a non-invasive method. We have demonstrated that this method can be a useful tool for filling in the knowledge gaps needed for safeguarding the welfare of farmed fish at the time of slaughter. Furthermore, our results show that stunning rainbow trout with CO_2 clearly does not meet the OIE standards for humane slaughter and that cold-water temperature exacerbate the poor effectiveness of the method even further.

The results of part 1 clearly demonstrate that the time it takes until rainbow trout lose visual indicators of consciousness following CO_2 stunning is strongly dependent on temperature. Even at 14°C, the highest acclimation temperature tested, it took >30 s before the first visual indicator was observed (i.e. loss of equilibrium). Prior to the loss of equilibrium, all fish showed strong aversive behaviours in the form of repeated attempts to escape the tank and vigorous swimming. The

longest time it took for the last visual indicator of consciousness to be observed (i.e. loss of ventilation) increased from around 4.5 min at 14°C to more than 11 min at 2°C. CO_2 narcosis clearly does not induce immediate unconsciousness and triggers intense aversive behaviours before stunning is effective, with cold acclimation temperatures significantly prolonging the induction time of the narcosis, which ultimately leads to substantial animal welfare problems.

Similar negative effects of cold temperatures on stunning and killing efficiency have previously been demonstrated for other methods and can be explained by a decreased metabolic rate in fish at low water temperatures (Kestin et al., 1991; Robb & Kestin, 2002). For example, when asphyxiated in air it took around 11 min for rainbow trout to lose ventilation at 14°C, whereas at 2°C it took >3 h, more than 17 times longer, for ventilation to cease (Kestin et al., 1991). Another factor that plays into the strong effect temperature has on stunning efficiency is the decreased diffusion rate of gases at lower temperatures (Ott, Heisler, & Ultsch, 1980). This will potentially prolong the time it takes before enough CO_2 is absorbed into the bloodstream to reach the levels needed for the gas to induce unconsciousness.

The analyses of EEG signals showed that there was no defining minute in which consciousness was lost following CO_2 stunning. The analysis of changes in EEG signal amplitude indicates that the transition to unconsciousness occurred between 6.5 and 8.5 min after submersion, whereas the analyses of the lowest median frequencies and the period when high-amplitude, low-frequency waves comprised the largest portion of the signal indicate that the transition to unconsciousness occurred between 4 and 7 min. Thus, by collectively examining the time it takes for VERs to disappear and for changes to occur in the EEG signal (amplitude and median frequency), it seems that the transition to an unconscious state occurred 4.5–8.5 min after submersion in CO_2 saturated water. Unfortunately, these results confirm that visual indicators of consciousness may be unreliable from an ethical perspective (Lambooij et al., 2010; Robb & Kestin, 2002; Robb & Roth, 2003), as brain function was observed to continue in some individuals for up to 3.5 min after ventilation ceased and up to 6.5 min after the loss of equilibrium in rainbow trout.

Previous studies have shown that loss of VERs indicates a degree of brain failure indicative of insensibility in fish (Bowman et al., 2019; Bullock, Hofmann, New, & Nahm, 1991; Kestin et al., 1991, 1995; Readman, 2015; Retter et al., 2018; Robb & Roth, 2003; Robb et al., 2000; van de Vis et al., 2003), chickens and ducks (Gregory & Wotton, 1986) and livestock (Verhoeven, Gerritzen, Hellebrekers, & Kemp, 2014). While visual indicators of a loss of consciousness in fish are susceptible to misinterpretation from subjective visual observation, the use of EEG to assess VERs allows for a more definitive method of determining when sensibility is lost. The presence of a waveform as a response to flashing light indicates that there is some level of processing occurring in the brain, and so in the absence of a direct measure of consciousness, the presence of VERs must represent the possibility that the fish is not insensible (Kestin et al., 1991). When VERs are lost, it indicates that the insult to the brain was sufficient enough to inhibit a primary sensory pathway, which may represent a degree of brain failure that is inconsistent with sensibility (Daly et al., 1987; Kestin et al., 1991).

Taken together, these results highlight that visual indicators of a loss of consciousness in rainbow trout during CO_2 stunning are insufficient, and the prolonged time of induction for fish in cold-water temperatures increases the risk of misjudgements of the state of consciousness. To safeguard the welfare of farmed fish, it is critical to evaluate stunning methods using EEG to determine sensibility (or lack thereof) rather than relying on visual indicators alone and to do so at all possible water temperatures.

5 | CONCLUSION

A successful stun should induce insensibility in as short a time as possible, ideally immediately, and not cause any adverse reactions. In the present study, we found no evidence in support of this for CO_2 stunning as the long induction time and aversive behaviour indicate that fish are subjected to avoidable stress. Therefore, we recommend that CO_2 stunning continue to be considered an inhumane stunning practice. Additionally, we showed that there is a poor relationship between the loss of VERs and loss of visual indicators of consciousness, which suggests that when visual indicators alone are used fish risk being misjudged as insensible before sensibility is actually lost. Colder temperatures extend the time it takes for visual indicators of consciousness to be lost and aggravates the problem of determining when a fish loses brain function and sensibility. Our results highlight that stunning methods, like CO_2 stunning, that rely on human observation to determine when a fish loses sensibility are welfare hazards in aquaculture.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in figshare at http://doi.org/10.6084/m9.figshare.11356283

ORCID

Jennifer Bowman https://orcid.org/0000-0003-2227-2819
Per Hjelmstedt https://orcid.org/0000-0002-4797-748X
Charlotte Berg https://orcid.org/0000-0001-5671-5273
Albin Gräns https://orcid.org/0000-0003-0268-1199

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