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Behavioural and neurological profiles after electrical stunning and cold shock in Pacific whiteleg shrimp (*Penaeus vannamei*) to inform humane slaughter

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ABSTRACT

Worldwide shrimp aquaculture is dominated by Pacific whiteleg shrimp (*Penaeus vannamei*). While legislative protection is limited, there is increasing interest in developing humane slaughter practices. Current commercial methods include cold shock (CS) using ice slurry, followed by placing animals in ice. To assess whether a prior electrical stun (ES) would be more humane, we compared in-water ES followed by CS vs CS at different temperatures (−2.5 to +5 °C). Effectiveness was assessed by examining behavioural responses and electrophysiological recordings from the brain (supraoesophageal ganglion). Behaviourally, CS animals displayed a series of tail flips after ice slurry immersion, whereas ES animals displayed one vigorous tail flip upon first contact. However, after subsequent CS immersion, a subset of ES animals responded with further tail flips, which was most prevalent in animals stunned at lower voltages for a shorter duration (2–2.5 V cm^{1} for 5 s). By contrast, animals exposed to higher voltages for a longer duration (2.5–3 V cm^{-1} for 20 s) were less likely to tail flip after CS immersion. Electrophysiological recordings between these two groups were substantially different and insensibility could only be confirmed (total power, Ptot <10%) in the animals that did not respond to subsequent CS. Additionally, reducing the ice slurry temperature to below 0 °C for CS animals reduced the time to insensibility (Ptot <10%). Our results show that the absence of tail flipping after cold exposure is aligned with neurological insensibility in *P. vannamei* after in-water ES and highlights the importance of CS after ES.

1. Introduction

Legal protection on the treatment of decapod species currently exists in countries such as Australia, Austria, New Zealand and Norway (Powell et al., 2024). In the UK, while decapod species are not legally protected, they are recognised as 'sentient' and have been included in the updated Animal Welfare (Sentient) Act 2022 (DEFRA, 2022). So far, this has not translated into increased welfare protection, but will most likely drive further guidance to the Welfare at the Time of Killing (WATOK) Regulations in England, Wales and Northern Ireland, as well as potentially driving changes to the existing Animal Welfare Act 2006 (which only protects vertebrates) and the Animals in Scientific Procedures Act 1986 (which only protects vertebrates and cephalopods). Current activities funded by the European Union, such as the establishment of the European Reference Centre for the Welfare of Aquatic Animals (EURCAW-Aqua), which is focused on welfare improvements of fish, cephalopods and decapods (please see https://www.eurcaw-aqua.eu/) may also improve decapod welfare in European States.

Typically, one of the aspects that is prioritised in animal welfare is the development of humane slaughter practices. As reflected by Browning and Veit (2020), death itself is harmful to welfare and therefore slaughter cannot be humane. However, improvements to the slaughter processes are important to protect animal welfare to the best of our ability in both research and commercial settings.

From a pragmatic perspective, humane slaughter is defined as a process in which the animal is killed instantly or rendered insensible until death ensues, without pain, suffering or distress (RSPCA, 2019). Therefore, stunning animals before slaughter aims to ensure

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insensibility until death and either (1) induce immediate loss of sensibility, or (2), if the loss of sensibility is gradual, ensure that the process does not induce distress, suffering and/or pain (EFSA, 2005).

Insensibility can be defined as the inability to perceive and respond to stimuli leading to incapability of processing sensory information (McCreary, 2008). For decapod species, electrical stunning has been suggested as a humane method for achieving insensibility (Conte et al., 2021). However, as summarised in Neil et al. (2024) most of the studies where the effectiveness of electrical stunning has been tested are in cold water species. As such, there is lack of information on the effect of electrical stunning in warm water decapod species. A preliminary study conducted by Weineck et al. (2018) compared physiological responses (electrocardiograms and octopamine and serotonin levels in the hemolymph) to cold shock (temperatures between 0 and 4 °C) and in-water electrical stunning (AC 60 Hz, 120 Volts, 20 amps) in Pacific whiteleg shrimp (*Penaeus vannamei*). In this species, cold shock by ice slurry induced sedation (behavioural immobility) and loss of cardiac function within one minute, while electrical stunning for 10 s induced immediate cardiac arrhythmia. However, the use of heart rate as an indicator might not be sufficient to ascertain with confidence that decapods are insensible (Neil et al., 2024). In decapod crustaceans, the heart is neurogenic, which means that contraction is directed from cardiac pacemaker cells situated in the cardiac ganglion rather than from the cardiac muscle itself (Wiersma and Novitski, 1942). Therefore, theoretically the heart rate could be a good proxy for neural functioning. However, studies in brown crab (*Carcinus maenas*) and European lobster (*Homarus gammarus*) have shown that cardiac activity can resume to some extent shortly after stunning, even when there is no central and peripheral neural activity (Neil et al., 2024). Therefore, signs of insensibility for decapod species, including *P. vannamei*, are still in development.

Approaches to ascertain insensibility indicators in other species have typically included measuring spontaneous behaviours, responses to stimulation such as no reaction to mechanical stimulation (prodding) in different parts of the body, and absence of reflexes (as recommended by RSPCA, 2024). However, EFSA states that to demonstrate effectiveness, stunning methods should be confirmed by assessing neuronal function, which can be measured through recording electrical activity in the brain (electroencephalography, EEG) (EFSA, 2018; 2004). Raw EEG waveforms are typically fast Fourier transformed (FFT) to generate a power spectrum. This power spectrum can be described using derivatives such as total power (Ptot), which can be used to assess the level of insensibility. From this, a 90% reduction of the normalised total power post-stun compared to pre-stun (Ptot <10%) is an approach used to confirm insensibility (Bowman et al., 2019; EFSA, 2018; Ramirez-Rodriguez et al., 2026; Rucinque et al., 2023). Alternatively, following stunning, it is possible to assess insensibility by measuring the absence of evoked responses, such as visual evoked responses, VERs (see Bowman et al., 2019; Hjelmstedt et al., 2022 for examples of this approach applied in fish species) or by recording the presence of an epileptic-like insult (seizure) followed by a period of minimal brain activity (Terlouw et al., 2016).

For decapods, global shrimp aquaculture production is dominated by *Penaeus vannamei*, with production estimated at around 6.8 M tonnes in 2022 alone (FAO, 2024), translating to around 300–340 billion animals produced yearly (Waldhorn and Autric, 2022).

Advice on humane slaughter protocols for shrimp from certification bodies such as the Aquaculture Stewardship Council (ASC) for commercial farms include immersion in an ice slurry or electrical stunning followed by ice (ASC, 2025). However, as already mentioned, only one study (Weineck et al., 2018) has attempted to compare the effectiveness of these methods in *P. vannamei*.

The current lack of information highlights an urgent need to evaluate stunning and/or stun-kill methods using both neurological and behavioural approaches to assess insensibility in decapods, and particularly in warm-water species such as *P. vannamei*. Therefore, in this study, we compared the effect of cold shock (CS) on its own vs the combination of electrical stunning (ES) followed by cold shock (ES + CS) in *P. vannamei*. Insensibility was assessed by performing behavioural observations (recovery from stunning) and by recording spontaneous electrophysiological activity from the brain (supraoesophageal ganglion), to assess if post-stun Ptot fell below 10% of the baseline (90% reduction in brain activity). We also tested neurological and behavioural responses to ES alone, to assess whether ES would be sufficient to induce insensibility. Overall, results from this study aim to contribute to the development of humane methods of slaughter for this species.

2. Material and methods

2.1. Animal supply

P. vannamei were sourced from a local commercial supplier (Rastech Ltd., St Andrews, Scotland, UK) and transported by car to the Niall Bromage Freshwater Research Unit, University of Stirling, in 200 L water containers supplied with oxygen (transport time 1.5 h approx., water temperature 28 °C, salinity 28 ppt). Animals were kept in a recirculation system (TMC 9000 RAS module) consisting of 6 white rounded tanks (volume 700 L/tank), where salinity was gradually reduced over a period of two weeks to 21.5 ± 0.2 PSU, water temperature of 29.1 ± 0.1 °C and photoperiod regime of 12:12 h. Water quality was monitored daily (Ammonia, TAN 0.15 ± 0.04 mg/L; nitrite 0.16 ± 0.01 mg/L). Animals were fed 3 times per day, with equal rations per feed (1–2% body weight) with a Biomar Larvia crustacean diet. The average weight of the animals when experiments took place was 22.6 ± 1.0 g. Experiments were approved by the Animal Welfare and Ethical and Review Body (AWERB) at the University of Stirling (AWERB 2023 14,150 9818).

2.2. Electrical stunning

Electrical stunning was conducted using a custom-made in-water electrical stunning system (Ace Aquatec, Ltd., Dundee, United Kingdom). The electrical stunner could supply a 50 Hz sinusoidal alternating voltage (AC) and included built-in electronics to control the timing of the stun duration. The electrical stunner was connected to two stainless steel plate electrodes mounted on opposite ends of an experimental tank (length = 40 cm, width = 15 cm and height = 25 cm). The separation distance between electrode plates was 40 cm. This arrangement was specifically designed to ensure that the electric field was applied along the length of the animal, from head to tail.

Behavioural observations reported in section '2.4 ES behavioural recovery' were done using a similar system. The only differences were that in this custom-made in-water electrical stunning system (Ace Aquatec, Ltd., Dundee, United Kingdom) the stun unit was connected to two stainless steel plate electrodes mounted on a plastic frame in a folding tank and the electrodes (31 cm × 17 cm) were spaced 30 cm apart.

An oscilloscope (123B, 20 MHz, Fluke Corporation, USA) and current probe (801-110S, Fluke Corporation, USA) measured the voltage and current applied during the electrical stun.

The experimental tank was filled with water sourced from the holding tanks to a depth of 5 cm and the salinity adjusted to 21 PSU, corresponding to a conductivity of ~31 mS/cm at 25 °C. Water temperature during experimentation was 24.6 ± 0.3 °C. Stunning was conducted on individual animals to maintain controlled conditions. Prior to the application of the electric stun, animals were aligned head to tail, and a mesh cover (non-conductive) was gently placed on the surface of the water. This precautionary measure was employed to prevent the animal from jumping out of the tank during the procedure. Following the stunning, the mesh cover was removed, to facilitate video recording.

2.3. Electrophysiological recordings

2.3.1. Insertion of electrodes

Individual animals were randomly selected from the holding tanks. To minimise stress and ensure adequate handling, animals were anaesthetised prior to inserting the needle electrodes. Animals were anaesthetised in (1.8 mL/L; stock solution prepared by mixing 1:9 with ethanol>99.8%) Eugenol (Reagent Plus 99%, Sigma Aldrich, UK) for <3 min (average anaesthesia time 2 min 38 s) in aerated water from the holding tanks. Animals were considered adequately anaesthetised when the righting reflex was lost and there was no response to handling. Animals were then dipped for 1 s in a cool bath (10–15 °C). This step aimed to reduce recovery times by flushing off the anaesthetic. To prevent desiccation during the procedure of inserting he needle electrodes, animals were partially covered with wet tissue paper that had been moistened using cooled seawater.

Needle electrodes were prepared from stainless steel subdermal needles diameter 0.4 mm, length 13 mm (Neurodart, Spes Medica, Italy), which come complete with 150 cm of wire connected to a DIN42802 connector for direct connection to the data acquisition system. The needle electrodes were cut to length depending on the size of the animal, to ensure appropriate depth of insertion. Needles were cut to a length of 3.5 mm for animals with weights <20 g and 5 mm for animals with weights >20 g.

Two needles were inserted either side of the supraoesophageal ganglion, posteriorly to the base of the eyestalks. Before insertion, attachment sites were dried with a cotton swap. The needles were pushed through the carapace into position. A third needle (length = 13 mm) used as the reference electrode was inserted under the second abdominal carapace segment. All needles were fixed externally using a cyanoacrylate adhesive (superglue) to prevent removal during tail flipping or other vigorous actions.

Animals were placed into a clear glass experimental tank (length = 40 cm, width = 15 cm and height = 25 cm) filled with ~5 cm of aerated water sourced from the holding tank and allowed to recover for at least 10 min and they had righted themselves prior to any experimental manipulations and data reported. Baseline recordings were taken from this point onwards for ~10 min, before ES or CS took place.

2.3.2. Data recording

To record the electrophysiological data the signal from the needle electrodes were amplified using a bioamplifier (FE231, ADInstruments, Oxford, England). The needle electrodes were connected to the bioamplifier via a custom-built device which protected the bioamplifier during electrical stunning. The bioamplifier interfaced with a Powerlab data acquisition system (PL 4/35, ADInstruments) controlled using a PC running LabChart Pro 8 software (version 8.1.22, ADInstruments). The sensitivity of the bioamplifier was set to ±2 mV with a 50 Hz low pass filter and 0.1 high pass filter. Data was collected at a sampling rate of 1 Hz. Please see Table S1 for electrical parameters and experimental group information.

2.3.3. Experimental groups

2.3.3.1. ES + CS. For ES + CS animals, electrophysiological recordings were first taken to gather data from baseline spontaneous activity, ~10 min pre-stun. After applying ES, animals were left undisturbed for approximately 40 s (range 12–64 s, mean = 41.9 s) to record neurological activity post-stun and then placed in a CS ice slurry tank (0 °C) and left undisturbed for 30 min.

After ES animals were immersed in ice slurry (CS), 100 and 86% of animals stunned for 5 s at 2 and 2.5 V cm^{-1} respectively responded to CS by tail flipping. In contrast, 50% of animals stunned at 2.5 V cm^{-1} for 20 s and 29% at 3 V cm^{-1} for 20 s responded to CS by tail flipping. From these observations, we categorised electrophysiological recordings into two different groups: 1) ES-CS-R at 2–2.5 V cm^{-1} for 5 s (containing only CS responders after ES; $n = 6$) and 2) ES-CS-NR at 2.5–3 V cm^{-1} for 20 s (containing only CS non-responders after ES; n = 4) (Table S1 and Fig. 1).

2.3.3.2. CS. For animals that were directly placed in CS ice slurry electrophysiological recordings were first taken to gather data from baseline spontaneous activity, ~10 min pre-CS exposure. Thereafter, individuals were moved to a CS ice slurry tank and were left undisturbed for the duration of the recording period (30 min). During this time temperature was monitored using a hand-held thermometer. Data was recorded in animals exposed to CS ice slurry at temperatures of −2.5 °C ($n = 5$), 0 °C (n = 5), +2.5°- 5 °C (n = 5) (Table S1 and Fig. 1).

2.3.3.3. ES alone. Animals ES (2 V cm^{-1} for 5 s) showed immobility after ES. However, these animals would typically respond to CS exposure. To further evaluate this group, animals were left in the stun tank ($n = 6$) undisturbed instead of being moved to the CS tank. In this group, animals were treated as described in ES + CS but were left in the stun tank undisturbed and electrophysiological recordings took place during the period that animals were behaviourally immobile (3 min 41 s; see section ES behavioural recovery for movement descriptions) (n = 6). All animals in this category were stunned at 2 Vcm^{-1} for 5 s (Table S1 and Fig. 1).

2.3.4. Signal processing and data visualisation

Electrophysiological data was analysed with the LabChart Pro software (version 8.1.22, ADInstruments). Total electrophysiological power (Ptot) was calculated from 0.5 to 32 Hz. Additionally, Ptot was calculated for the delta (0.5–4 Hz), theta (4–8 HZ), alpha (8–12 Hz), beta (12–32 Hz) frequency ranges. Data extracted from LabChart was sorted using the statistical programme R (R Core Team, 2026). To filter out noise and artefacts, we applied a Median Absolute Deviation filter (MAD) to the power traces, as this has been found to be an accurate smoothing method (Nguyen Cong et al., 2025). In the baseline period, values were removed if outside the median 0.5+/-MAD, while during CS, median ±1 MAD values were removed. The MAD filter applied was stricter in the baseline data, to account for higher amplitudes due to the animals being upright (artefacts of minor appendage movement more present) compared to during CS when animals were on their backs and fully immobile. The MAD filter was applied from the start of the electrophysiological recordings up to 100 s before ES or CS started (baseline section), and then 120 s after being placed in CS. A median MAD filtered Ptot value was calculated from the baseline section per animal (Table S2), which was then used to convert the rest of the filtered Ptot data into a Ptot percentage (MAD filtered Ptot %) from the baseline. MAD filtered Ptot % data was then converted to epochs, which are averaged sections of percent data. To calculate the baseline epoch, the time window was narrowed to between 100 s after electrophysiological recordings started and 100 s before ES or CS. These values were then averaged (see Table S2). For post-CS epochs, data was averaged every minute, using a maximum of 20 s of data. In cases where data was missing due to the MAD filter, the start time of each epoch was the value closest to the 1 min bin, and no minimum data points were used for the 20 s average (Table S3). Finally, the post-CS epochs were divided by the refined baseline epoch and multiplied by 100 for the final normalised Ptot % average epoch values.

The time interval before and after moving the animals into the CS (0–2 min) was not included in the MAD filtering as this data contained confounding factors such as the transfer of the animals from the stun tank to the ice slurry tank, tail flipping and muscle twitching observed in some of the experimental groups. However, after CS immersion, visual identification of tail flipping and muscle twitching was noted, enabling the labelling of the behavioural time stamps on raw electrophysiological recordings. As these sections were highly affected by muscular

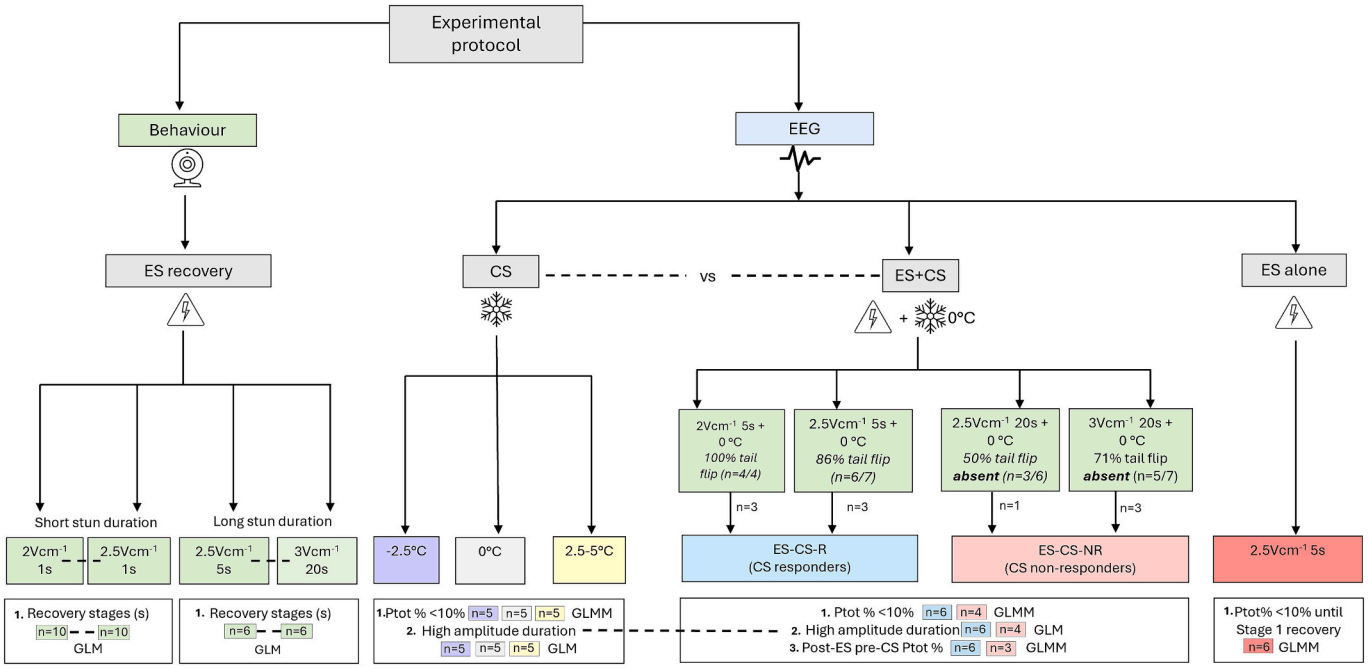

Fig. 1. Schematic of experimental design and treatments. Experimental groups are shown in the last row of coloured boxes, with the descriptions of statistical analyses below. Statistical comparisons across different experimental groups are shown with a dashed line. ES-CS-R refers to animals that responded to CS by tail flipping post- ES while ES-CS-NR refers to animals that did not respond to CS post-ES. For the ES alone group, stage 1 refers to uncoordinated movement (Table S3).

movement, they were defined as the "high amplitude" and were compared across protocols. These sections were characterised by an increasing amplitude of around 1 mV from a starting amplitude range of 0.01–0.1 mV. The end of the "high amplitude period" was visually noted (stop of tail flipping and muscle twitching) and corresponded with amplitudes values falling to the starting amplitude range. As ES-CS-NR animals did not tail flip, we labelled these animals as having a 1 s high amplitude period ($n = 4$). For ES + CS animals, after ES, animals remained visibly immobile before placement into the CS tank. Therefore, the corresponding Ptot trace was assessed as an additional epoch in both ES-CS-R ($n = 6$) and ES-CS-NRs (n = 4) groups. Due to this shortened section of data, raw traces were subsampled at 0.5 Hz per animal, where one animal with only 12 s of data was subsequently removed from the ES + CS-NR group ($n = 3$) due to a small number of data points, where animals with data in the range of 21–64 s were then analysed (Table S4). The data was processed as described above, where the MAD filter (±1 MAD) was instead applied after an 8 s buffer to account for the recovery of the signal (range 0–8 s, mean 5 s) once ES stopped and 5 s before CS immersion. Two epochs were subsequently calculated, which contained the average Ptot (%) data for the subsampled baseline and post-stun section respectively.

Finally, for ES only animals, as there was no immediate confounding effect of movement due to animals remaining in the stun tank and being immobile, the MAD filter was instead applied 8 s after stun. For these animals (n = 6), we analysed Ptot data until minor uncoordinated movement was first seen, (stage 1 recovery, see *Es behavioural recovery* for recovery stages), which was 3 min 41 s. Epochs were analysed using 20 s bins with a maximum average of 10 s of data per epoch, from 0 (+8 s) up to 220 s.

2.4. ES behavioural recovery

In a separate study, the effect of two electrical field strengths (2 and 2.5 V cm^{-1}) for 1 s and behavioural observations post-stunning were determined ($n = 10$/experimental group) (Table S1).

Based on a pilot study, common post-stunning behaviours, namely 'coordinated pleopod movement, stage 2' and 'regaining righting position, stage 3' were identified and selected for scoring (note that stage 0 would be total immobility and stage 1 would entail uncoordinated movement of appendages). This data was collected using the same electrical stunning procedure as above, using similar equipment (details in Section 2.2). This trial was performed at the Oceanloop Kiel GmbH, Kiel, Germany.

A further subset of behavioural data was also recorded, which was based on ES parameters used for the electrophysiological data within the main study, where time to recovery for stages 2 and 3 were also assessed. Animals from Rastech Ltd., St Andrews, Scotland, UK, were used for these experiments. The conditions tested were: 2.5 V cm^{-1} for 5 s and 3 V cm^{-1} for 20 s ($n = 6$/experimental group) (Table S1). For the former group, all animals were able to recover within the 3 min observation period, whereas only 29% of the animals exposed to 3 V cm-1 for 20 s recovered to stage 2 within the observation period (3 min) with no animals reaching stage 3 within that time. In this case, the observation period was extended, and all animals, including the animals that showed initial signs of recovery died, within 2 h.

2.5. Statistical analysis

All analyses were conducted using the statistical programme R (R Core Team, 2026). See link for R scripts and data at https://github.com/j-somerville/Whiteleg-shrimp-EEG-and-behavioural-analysis.

2.6. Electrophysiological data

As each epoch was average percentage data, we square root transformed values per individual. Individuals were treated as a random effect in a general linear mixed effects model (GLMM), to account for individual variation and repeated measures of individuals. Epochs were a fixed effect from 2 to 30 min for CS and ES + CS protocols and were compared to the baseline epoch to assess change over time after exposure to a slaughter protocol. For ES alone, epochs were from +8 s to 3 min 40 s in 20 s intervals (up to 220 s).

When assessing electrophysiology activity immediately after ES before CS (ES + CS groups) (21–64 s section; ES + CS groups), epochs

were analysed using a GLM, where fixed effects were stunning category (ES + CS-R 2–2.5 V cm^{-1} 5 s and ES + CS-NR 2.5–3 V cm^{-1} 20 s). Epoch values were square root transformed from percentage before analysis.

For differences in the high amplitude period between ES + CS and CS animals, we used CS data of animals exposed to 0 °C temperature only, as these were the CS temperatures that ES + CS animals were exposed to. For these comparisons, we used a general linear model (GLM), where the fixed effect was the stunning protocol (0 °C, ES + CS-R: 2–2.5 V cm^{-1} for 5 s and ES + CS-NR: 2.5–3 V cm^{-1} for 20 s). CS temperature categories were analysed separately using a GLM, as these animals experienced different ice slurry temperatures.

2.7. Behavioural data

For animals that were stunned for shorter (1 s) = or longer (5 or 20 s) durations, we used a GLM to assess whether the time (in secs) taken to reach stage 2 or 3 recovery differed between stun parameters, where stun parameter and recovery stage were fixed effects. However, animals stunned at 3 V cm^{-1} 20 s did not recover to stage 3 and therefore null and residual deviance was very high, which should be noted when interpreting results.

3. Results

3.1. Electrophysiological data

3.1.1. ES + CS: effect of ES before CS

When assessing electrophysiology activity immediately (21–64 s, see Table S4) after ES, before animals were placed in ice slurry there was a significant increase in the average Ptot compared to the baseline for both ES-CS-R (2–2.5 V cm^{-1} 5 s; $n = 6$) and ES-CS-NR (2.5–3 V cm^{-1} 20 s; $n = 3$) animals (b = 85.53, SE = 29.01, $t = 2.948$, $p = 0.009$). However, there was no significant difference between stunning parameters, although ES-CS-NR (2.5–3 V cm^{-1} 20 s) animals (b = −40.03, SE = 30.77, t = −-1.301, $p = 0.21291$) had a negative estimate compared to ES-CS-R (2–2.5 V cm^{-1} 5 s) animals (Intercept; b = 23.34, SE = 22.94, $t = 1.018$, $p = 0.32492$ Fig. 2).

3.1.2. ES + CS: effect of ES after CS

Ptot % in the ES + CS groups after placing the animals in the CS was significantly lower than the baseline for ES-CS-R (2–2.5 V cm^{-1} 5 s, $n = 6$) ($p < 0.001$ per epoch; Table S5; Fig. 3; 179 epochs) and ES-CS-NR animals (2.5–3 V cm^{-1} 20s, $n = 4$) ($p < 0.001$ per epoch; Table S6; Fig. 3; 112 epochs). However, individual variation was much higher for ES-CS-R (+ − 4.99 SD) compared to ES-CS-NR (+ − 0.399 SD). Additionally, only in 3 out of 6 ES-CS-R animals (2–2.5 V cm^{-1} 5 s), did the Ptot reach values consistently below 10%. In contrast, all ES-CS-NR animals (2.5–3 V cm^{-1} 20 s) reached values below 10% after 3 min (Fig. 3).

3.1.3. CS

For CS protocols, compared to the baseline, each subsequent epoch was significantly lower for animals in −2.5 °C ($p < 0.001$ per epoch; Table S7; Fig. 4; 109 epochs, $n = 5$)) and 0 °C ($p < 0.001$ per epoch; Table S8; Fig. 4; 146 epochs, n = 5). For animals placed in 2.5–5 °C, not all epochs significantly differed from the baseline. At this temperature range, only after 6 min ($p = 0.02$; Table S9) did epochs start to significantly decrease, with more a consistent decrease in power across epochs after 14 min (Table S9; Fig. 4; 139 epochs, n = 5). Individual variation was highest in this group (+ − 10 SD), compared to −2.5 °C (+ − 0.536 SD) and 0 °C CS (+ − 0.613 SD). This is likely due to the one individual that has a consistently high Ptot % epoch values. All animals directly placed in CS at temperatures of −2.5 and 0 °C reached Ptot <10% in 4 and 9 min respectively. In contrast it took 28 min for 4 out of the 5 animals exposed to 2.5–5 °C to reach Ptot <10% (Fig. 4).

Epoch data was also plotted for separate frequencies alpha, beta, delta and theta (see Fig. S1-S5).

3.1.4. ES alone

There was a significant increase between the baseline and the first epoch (5–20 s post-stun; $p < 0.001$) and no significant difference between the baseline and subsequent epochs for the rest of the immobility duration ($p > 0.05$, Table S10; Fig. 5; 77 epochs, $n = 6$).

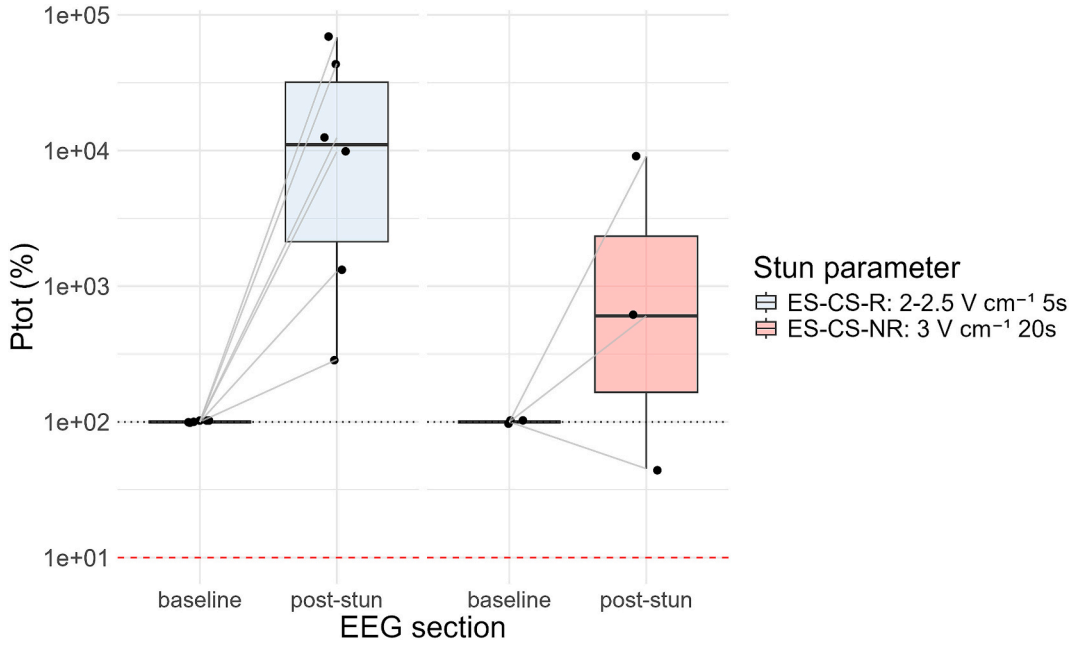

Fig. 2. Total power (Ptot) % of pre-stun levels (baseline) and after ES before exposure to CS. Data is presented for ES-CS-R (2–2.5 V cm^{-1} for 5 s) and ES-CS-NR (3 V cm^{-1} for 20 s) animals. Signal filtered at: 0.5–32 Hz. The y axis is a log scale; The horizontal black dashed line represents the 100% 'Ptot' baseline, and the red dashed line represents a reduction in 'Ptot' below 10% of baseline. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

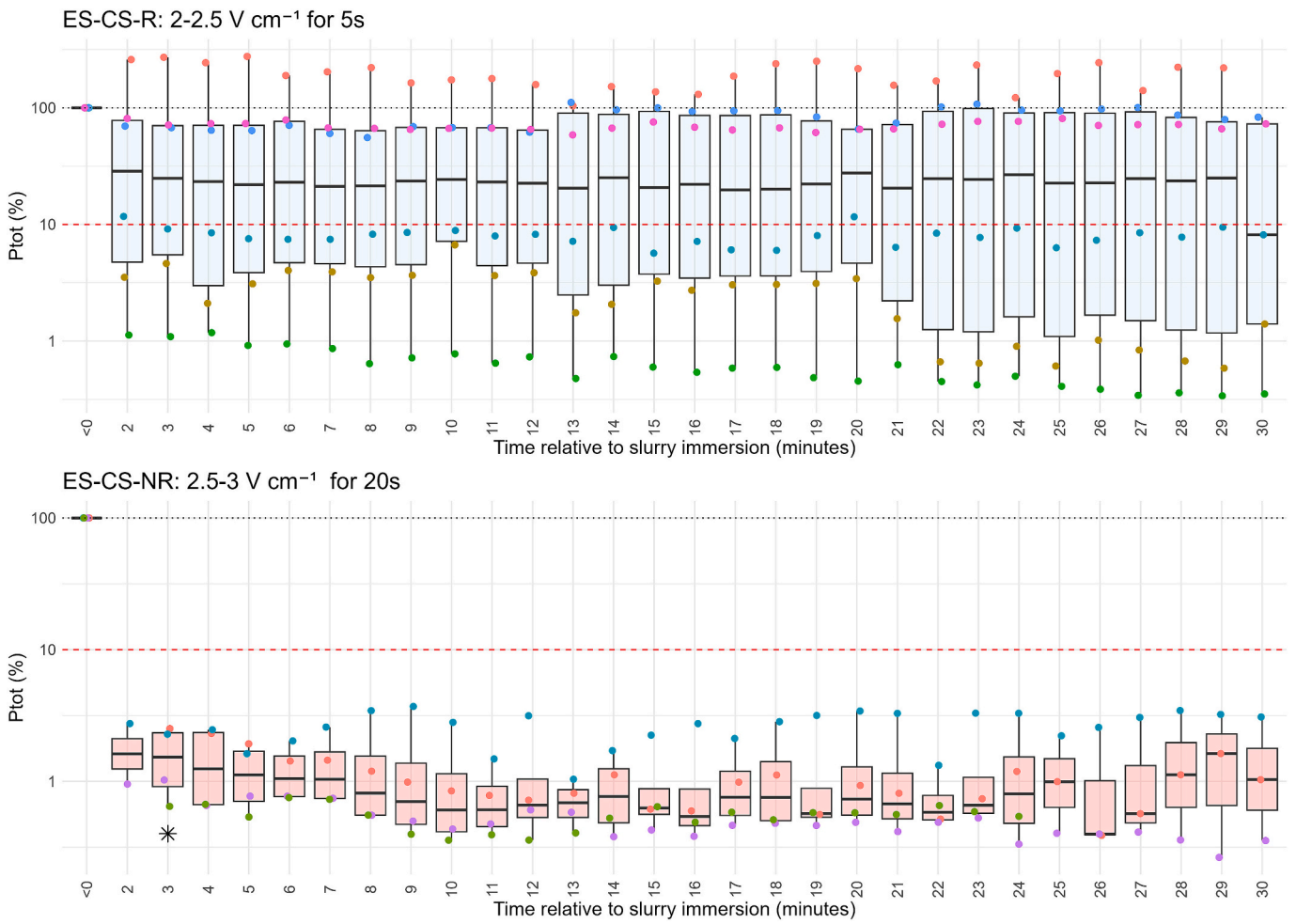

Fig. 3. Total power (Ptot) % of pre-stun levels (baseline). In top panel: ES-CS-R animals (2–2.5 V cm^{-1} for 5 s, n = 6) and bottom panel ES-CS-NR animals (2.5–3 V cm^{-1} for 20 s, n = 4). Signal filtered at: 0.5–32 Hz. The horizontal black dashed line represents the 100% 'Ptot' baseline, and the red dashed line represents a reduction in 'Ptot' below 10% of baseline. Note: log10 scale applied to y-axis. The x-axis starts at <0, where 0 represents immersion in CS, and baseline values were obtained prior to this. Time taken for all animals to reach below 10% is marked by a black asterisk *. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.1.5. *Post-CS high amplitude period*

Animals in the CS and ES-CS-R groups initially responded by a series of tail flips and muscle twitching when exposed to CS. This response was delayed in time as shown for example in Fig. 6A (delayed tail flip response of around 12 s). When comparing the duration of the high amplitude period, activity was significantly shorter for both ES-CS-R (2–2/5 V cm^{-1} 5 s, n = 6) ($b = -64.10$, SE = 10.29, $t = -6.23$, $p < 0.001$) and ES-CS-NR (2.5–3 V cm^{-1} 20 s, $n = 4$) animals ($b = -84.60$, SE = 11.40, $t = -7.42$, $p < 0.001$), compared to CS animals (0 °C, $n = 5$) (intercept; $b = 85.60$, SE = 7.60, $t = 11.26$, $p < 0.001$) (Fig. 6B).

When only comparing CS temperatures, the high amplitude period was significantly reduced when animals were immersed at the lowest temperature tested; −2.5 °C (intercept; $b = 47.40$, SE = 16.23, $t = 2.921$, $p = 0.0128$, n = 5), compared to the highest temperature tested; 2.5–5 °C ($b = 100.8$, SE = 22.95 $t = 4.393$, $p < 0.001$, n = 5). There were no significant differences in high amplitude period between the −2.5 and 0 °C slurry temperatures ($b = 38.20$, SE = 22.95, $t = 1.665$, $p = 0.122$, n = 5).

3.2. *ES behavioural recovery*

All *P. vannamei* exposed to ES responded to the actual stun with one very strong tail flip (Suppl. Video 1).

Animals that were electrically stunned at 2 V cm^{-1} for 1 s recovered significantly faster ($b = -24.45$, SE = 11.57, $t = -2.112$, $p = 0.04$) compared to animals electrically stunned at 2.5 V cm^{-1} for 1 s (intercept; $b = 20.02$, SE = 10.02, $t = 1.998$, $p = 0.05$). Recovery to stage 3 was also significantly longer in both protocols compared to stage 2 (intercept; $b = 35.45$, SE = 11.57, $t = 3.063$, $p < 0.01$). All animals tested ($n = 10$/stunning condition) recovered their righting reflex between 5 s to less than 3 min (Table 1).

When comparing time to each recovery stage, animals that were exposed to 2.5 V cm^{-1} for 5 s ($n = 6$) took significantly longer to reach stage 3 ($b = 1256.0$, SE = 202.6, $t = 6.198$, $p \leq 0.001$) compared to stage 2 (intercept; $b = 179.7$, SE = 143.3, $t = 1.254$, $p = 0.2359$), whereas we were unable to make this comparison for 3 V cm^{-1} 20 s animals (n = 6). When comparing stage 2 times between groups, 3 V cm^{-1} 20 s animals took significantly longer to reach stage 2 compared to animals stunned at 2.5 V cm^{-1} for 5 s ($b = 1303.8$, SE = 286.6, $t = 4.55$, $p < 0.001$; Fig. 7).

4. Discussion

In this study, for the first time in *P. vannamei*, we have evaluated stunning methods relevant for the aquaculture industry, which include CS alone (different temperatures), in-water ES followed by CS (0 °C), and ES alone.

For ES alone, behaviourally, we found that in-water ES at voltages of 2.5 V cm^{-1} for 1–5 s delivered a reversible stun in *P. vannamei,* with

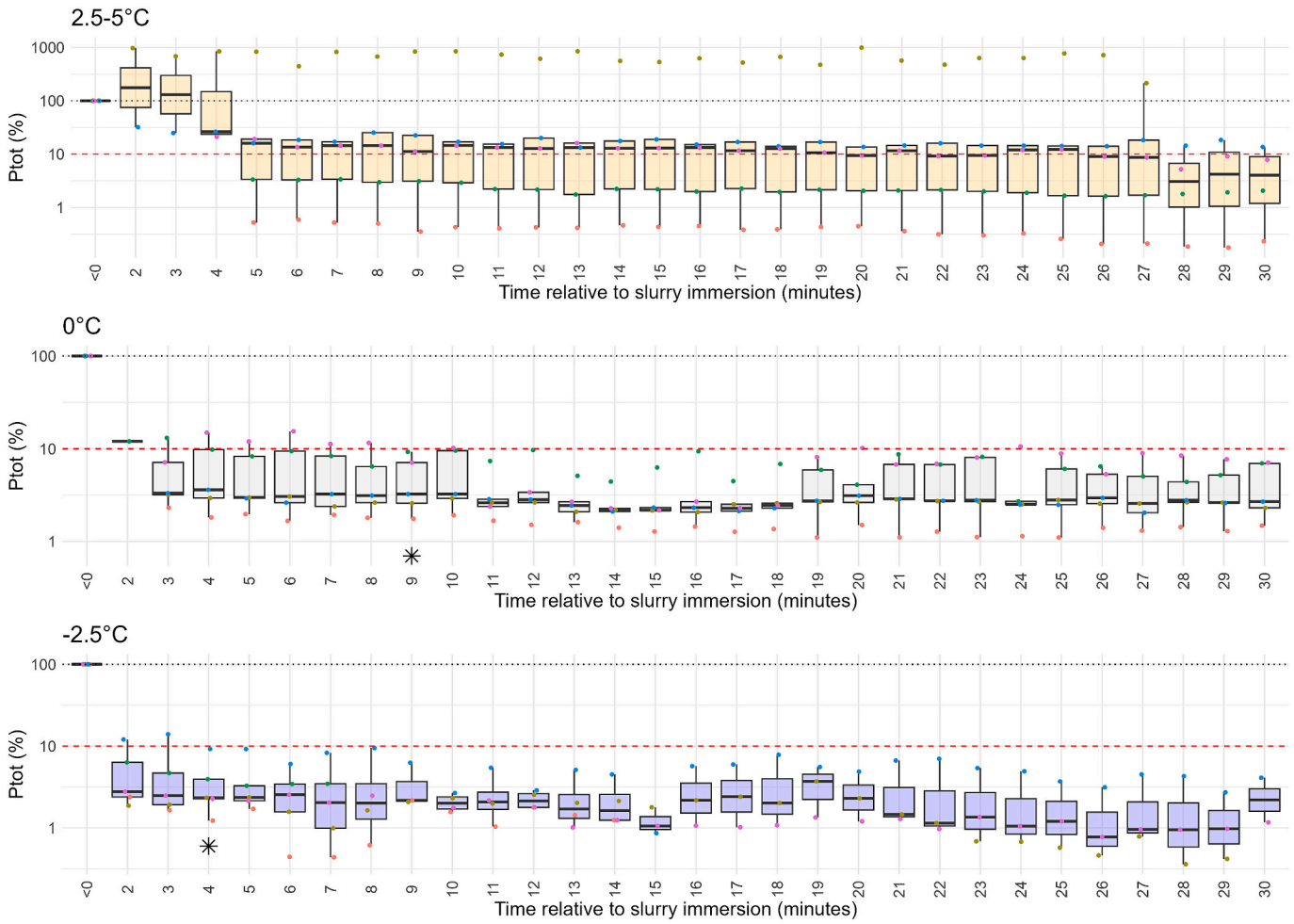

Fig. 4. Total power (Ptot) % of pre-stun levels (baseline) in *P. vannamei* exposed to CS at different temperatures, top panel) 2.5–5 °C (n = 5), middle panel) 0 °C (n = 5) and bottom panel) -2.5 °C (n = 5). Signal filtered at: 0.5–32 Hz. The horizontal black dashed line represents the 100% 'Ptot' baseline, and the red dashed line represents a reduction in 'Ptot' below 10% of baseline. n = 5 (2.5–5 °C), n = 5 (0 °C) and n = 5 (−2.5 °C). The x-axis starts at <0, where 0 represents immersion in CS, and baseline values were obtained prior to this. Time taken for all animals to reach below 10% is marked by a black asterisk *. All animals tail flipped for every slurry temperature. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

animals recovering their righting reflex within seconds to 3 min, while a lower voltage of 2 V cm^{-1} for 1 s was not sufficient to deliver an immediate stun. Previous studies on ES devices (Crustastun™ and dry ES) found that stunning in large decapods delivers behaviourally immobility from 5 min to several hours, but thereafter animals typically showed signs of recovery (reversible stunning) (Atanasoff et al., 2022; Fregin and Bickmeyer, 2016; Roth and Øines, 2010). Our results showed it is possible to generate behavioural immobility in *P. vannamei* using in-water electrical stunning even using 1 s stun duration and that time to recovery could be extended by applying a higher voltage and duration time. This effect has been reported for some species of fish (Lines et al., 2003; Robb et al., 2002) but not others (Hjelmstedt et al., 2025).

The single vigorous tail flip that ensued after *P vannamei* were electrically stunned has not always been reported, most likely because devices such as the Crustastun™ are enclosed and therefore do not allow for such observations. However, a tail flip in *P. vannamei* was previously reported by Weineck et al. (2018) and also agrees with data from Albalat et al. (2022) for electrostunned Norway Lobster (*Nephrops norvegicus*), where animals had faster muscle ATP interconversions (most likely due to muscle contraction). Previous studies in other decapod species have also shown how exposure to repeated low electrical fields can trigger a series of tail-flips (rapid backward movements) (Fossat et al., 2015). Tail flipping is a stereotypical escape response that is observed in many decapod species and consists of a series of precisely co-ordinated rapid flexions and extensions of the abdomen to escape predators or threating stimuli. In 1947, Wiersma first described that this response can be triggered by a single action potential in any of the four giant fibres, which consist of two pairs of giant interneurons, lateral (LG) and medial (MG). These interneurons course from the brain to the last abdominal ganglion in the ventral nerve cord (Wiersma, 1947). Regardless of whether the tail flip response is mediated by MG or LG, in both cases, a typical characteristic of giant-fibre mediated tail flips is their short latency (3–10 ms to flexor muscle activation) (Reichert and Wine, 1982; Wine and Krasne, 1972). It is therefore likely that the powerful tail flip seen in *P. vannamei* occurs from direct stimulation of at least one of the giant interneurons by the high electrical current from ES.

From a neurological perspective, post-ES behavioural immobility was coupled with a significant increase in the Ptot signal across all ES protocols. In vertebrate species, successful insensibility post electrical stunning has been linked with an epileptiform insult, which is characterised by the presence of high amplitude activity in the electroencephalogram (EEG) (Cook, 1992), a trait observed in different vertebrate groups (Lambooij et al., 2022; Lambooij et al., 2014; Small et al., 2023). However, from our data, it is difficult to ascertain whether the high amplitude activity observed post-electrical stunning in the higher voltage conditions tested would qualify as an epileptic insult, and to confirm immediate neurological insensibility, further work should be carried out on 1 s as a stun duration. Nevertheless, in some fish species, it

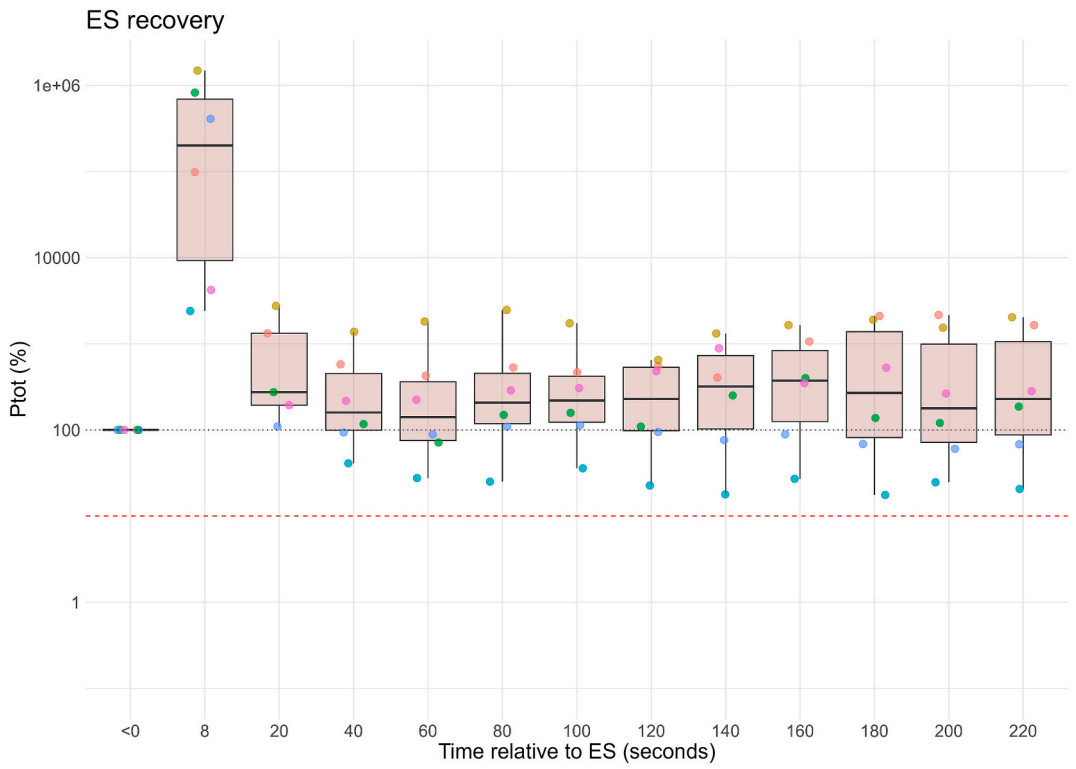

Fig. 5. Total power (Ptot) % of pre-stun levels (baseline) in *P. vannamei* exposed to ES (at 2 V cm $^{-1}$ for 5 s) and left undisturbed in the stun tank. Signal filtered at: 0.5–32 Hz. The horizontal black dashed line represents the 100% 'Ptot' baseline, and the red dashed line represents a reduction in 'Ptot' below 10% of baseline. The x-axis starts at <0, where 0 represents ES, and baseline values were obtained prior to this. Once ES stopped, epochs were calculated only after an 8 s buffer to account for residual signal, and for every 20 s until 220 s epoch (n = 6). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

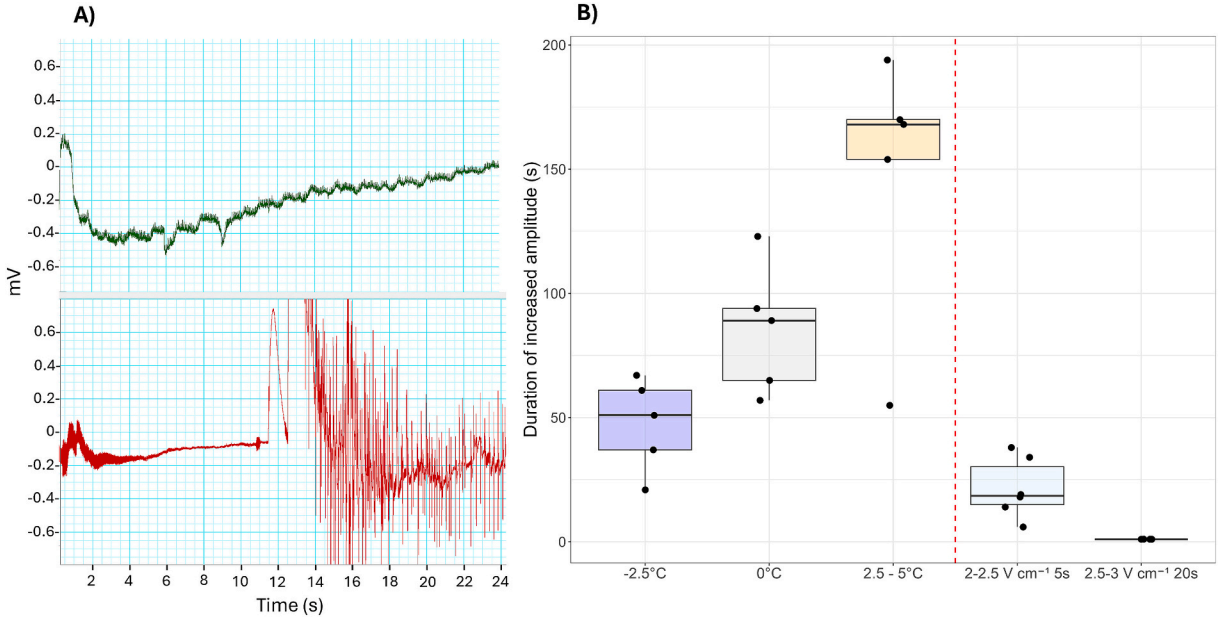

Fig. 6. A) Representative trace of high amplitude (mV) period in seconds (0.5–32 Hz) in *P. vannamei; top trace*; ES-CS-NR (3 V cm $^{-1}$ 20 s), an animal not responding to CS after placement in ice slurry at 0 s (0 °C); bottom trace; an animal directly placed in ice slurry at 0 s and responding to CS (0 °C) at 12 s with tail flipping. The recordings are from the moment the animals are placed in slurry ice (at 0 °C); 6B). Post-CS high amplitude activity duration from raw traces in *P. vannamei* placed directly in ice slurry at different temperatures (−2.5, 0 and 2.5–5 °C) or electrically stunned prior to placing the animals in ice slurry (0 °C). This high amplitude period is dominated by spikes and corresponds to the time when animals are tail flipping and muscle is twitching irregularly. Median duration and range are as follows; −2.5 °C; 51 s, 21–67 s, 0 °C; 89 s, 57–123 s, 2.5–5 °C;168 s, 55–194 s, 2–2.5 V cm^{-1} 5 s; 18.5 s, 6–38 s, 3 V cm^{-1} 20s; 1, 0 s.

has been demonstrated that the presence of an epileptic-like seizure following an electrical stun does not guarantee a prolonged period of

Table 1
Recovery time calculated as mean and range (min to max) in *P. vannamei* to reach stage 2 (coordinated pleopod movement) and stage 3 (righting reflex) of recovery after being ES using two different voltages (2 V and 2.5 V cm^{-1}) for 1 s. n = 10 animals per protocol, with all animals reaching stage 3.

Stun conditions	Recovery stage	Mean time to stage (s) ± SD	Range (s)
2 V cm^{-1}, 1 s	Stage 2	5.9 ± 7.17	0–24
2 V cm^{-1}, 1 s	Stage 3	20.7 ± 26.1	5–92
2.5 V cm^{-1}, 1 s	Stage 2	9.7 ± 4.6	4–18
2.5 V cm^{-1}, 1 s	Stage 3	65.8 ± 65.4	12–167

unresponsiveness to sensory stimulation such as light (i.e. absence of Visual Evoked Responses, VERs). In fact, VERs can return before the end of the seizure (Hjelmstedt et al., 2022).

In our study, a subset of ES animals showing behavioural immobility were still able to process external stimuli (extreme cold temperatures) and responded accordingly by a series of tail flips. Moreover, when applying these ES conditions, animals did not only respond to CS but also showed a return to Ptot baseline levels within a relative short period of time (20 s). Therefore, data from our study show that visual indicators such as immobility after electrical stunning is not sufficient to confirm full insensibility.

While the tail flip response is referred to as a reflex, studies have shown that decapods can modulate this behaviour. For instance, habituation, animal size, and the physical and behavioural state of the individuals can all influence the degree of the tail flip response (Kellie et al., 2001). Further studies have shown that there is direct central nervous (CNS) and possible endocrine regulation of the tail flip response, with factors affecting these mechanisms impinging the tail flip response (Page et al., 2007).

In line with this, our results show that the time taken for the Ptot to reach values below 10% was much faster in non-responder animals (ES-CS-NR) and values stayed below this threshold most consistently over time, suggesting neural insensibility in the whole nervous system. This contrasts with responder animals (ES-CS-R), which showed more electrophysiological activity. In some of the responder animals, the neurological activity remained substantially elevated and sustained over CS, which was unexpected. It is unclear why that effect was observed in some of the responder animals. Therefore, further work in this area is required. In this context, evaluating evoked responses would be add another layer to the analysis performed here. We note that tail flipping can occur in abdomens from decapitated *P. vannamei* that are placed in a cold shock solution whilst being mechanically stimulated, as shown by Weineck et al. (2018). However, in the present study, tail flipping was solely triggered by the change in temperature, and the total power (Ptot) obtained from the electrophysiological recordings in the supra-esophageal ganglion was substantially different between animals that responded to CS post-electrical stunning and animals that did not respond to CS post-electrical stunning. However, in both cases, the duration of the tail flipping and muscle twitching was affected by the electrical stun as the high amplitude period compared to CS alone was shorter and observationally less vigorous.

The mechanisms by which decapod crustaceans detect changes in temperature are still to be fully understood. Initial studies reported the presence of cool-sensitive neurons in the ventral nerve cord of several decapod species (Tani and Kuramoto, 1998). More recently, other studies have shown the presence of transient receptor potential (TRP) channels that respond to changes in temperature with tissue distribution especially apparent in neural tissues (Qian et al., 2024). This would explain the responses observed to CS. The fact that ES-CS-R animals also partially responded to cold exposure indicates that this neurological pathway is still functional to some extent. Cold temperatures especially around 0 °C and lower (−2.5 °C) were effective in reducing the duration of the tail flipping and reduction in Ptot in all animals to <10% achieved within a time frame between 9 and 4 min for 0 °C and − 2.5 °C respectively, most likely due to the compounding effect of a faster

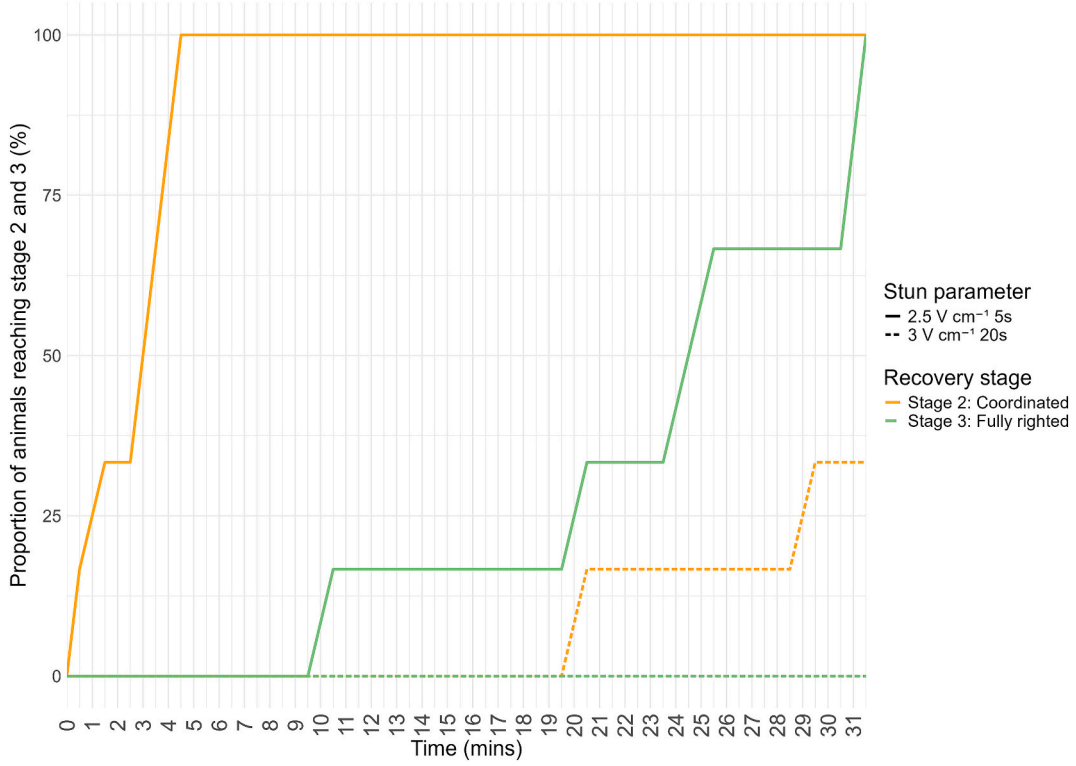

Fig. 7. Kaplan Meier curves of the recovery of *P. vannamei* to stages 2 (coordinated movement of pleopods) and 3 (righting reflex) for two different stun parameters (2.5 V cm^{-1} for 5 s or 3 V cm^{-1} for 20s, n = 6 animals for each stunning condition). Recovery time(s) in seconds were binned to the nearest 60 s interval to calculate the proportion of animals at stage 2 or 3 per minute. The first animal to reach stage 2 was at 42 s (at 2.5 V cm^{-1} for 5 s).

reduction in the metabolic rate directly triggered by the low temperatures (Zhu et al., 2024). Complementary to this work, the study done by Weineck et al. (2018) in *P. vannamei* showed a very fast decrease in heart rate, as well as a sharp decrease within a sensory-CNS-cardiac response when animals were exposed to cold temperatures (0–4 °C). However, data from our study highlights the improvements in CS effectiveness when animals are exposed to temperatures closer to 0 °C or lower (−2.5 °C) compared to temperatures closer to 5 °C.

For ES, the parameters we used showed that the combination of higher voltage and prolonged stunning duration increased insensibility likelihood. However, further research should evaluate whether neurological insensibility can be confirmed in 1 s electrical stuns. Moreover, in a commercial field setting, increasing voltage and stunning duration may be logistically challenging (Denicourt et al., 2023) and might lead to changes in quality (Dwi Anggo and Suharto, 2020; Robb et al., 2002). It is also important to note that our experiments tested one animal at a time. In commercial settings, tonnes of shrimp can be processed during a harvest, depending on the intensity classification of farms (Elwin et al., 2020). Hence, the percentage of animals that receive an effective electrical stun may be different to a controlled laboratory setting. Therefore, field trials should be conducted to ensure that voltage and stunning efficiency are optimised depending on the farm operations and conditions. In a commercial setting, as a validation method for neural insensibility after ES, we propose that the absence of a tail flip response to CS would be a reliable indicator.

Similarly, the increased number of animals that are subjected to CS in commercial settings may also affect the stability of CS temperatures in the field. In our study, all CS animals responded by vigorous tail flipping when immersed in ice slurry, so for this method of stunning, immediate insensibility is not achievable. With our data, it is also not possible to ascertain the level of distress, pain and discomfort experienced during the time animals are in the process of cooling down, a process that renders the animals insensitive. Therefore, further work is required to ascertain pain perception and responses associated with pain in decapod species especially within the context of stunning.

Overall, while further work is required, especially in confirming loss of immediate insensibility following ES (1 s stun duration), laboratory trials indicate that ES + CS is more effective than CS alone in inducing quicker insensibility (Ptot <10%). For humane slaughter of *P. vannamei*, effective ES should be always followed by CS as animals have the capacity to recover from ES alone. With current knowledge, optimisation of ES can be done by ensuring that no tail flip response is observed upon subsequent CS exposure. Finally, when ES is not used and hence the perception to extreme cold temperatures will occur, CS temperatures should be 0 °C or lower to reduce the time taken to reach neural insensibility, as shown by the faster decrease in Ptot activity duration from our electrophysiological data.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2026.744375.

CRediT authorship contribution statement

Jasmine Somerville: Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation. **Maureen Ellis:** Software, Methodology, Investigation, Data curation, Conceptualization. **Endre Putyora:** Writing – review & editing, Software, Methodology. **Nasser Ayaril:** Investigation, Data curation. **Sonia Rey Planellas:** Writing – review & editing, Methodology, Investigation, Funding acquisition. **Amaya Albalat:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Data curation.

Declaration of competing interest

Professor Amaya Albalat reports financial support was provided by Coefficient Giving LLC. If there are other authors, they 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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Data availability

We have shared a link in the manuscript to data and R script

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