SRAS Final Report
Electroencephalogram (EEG) Assessment of Brain Activity Before and After Electrical Stunning in the Nile Crocodile (*Crocodylus niloticus*)

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ABSTRACT

The purpose of this study was to assess brain activity before and after electrical stunning in the Nile crocodile *(Crocodylus niloticus*) using electroencephalogram (EEG) analysis. EEG wave patterns of 15 captive-bred *C. niloticus* were captured and analysed. Identification of external sites for placement of the EEG electrodes on the head of the crocodiles was established by magnetic resonance imaging (MRI) and anatomical measurements taken from sagittal and coronal dissections. Seven EEG electrode placement sites were identified from studying the brain topography and skull thickness which was limited by the size of the cranial plate. A good technical stun (effective stun) was identified by a loss of consciousness, seen in EEG as a decrease in alpha waves and increase in delta waves patterns, of crocodiles immediately after-stunning. E-stunning at 171.40 ± 1.75 V and 50 Hz for 5 - 7 seconds applied to the neck (behind the cranial plate) with a mean maximum electrical current of 1.09 ± 0.37 A and a total electrical current of 5.48 ± 1.44 Amps x seconds was able to immobilize all crocodiles and to achieve an effective stun in half of the crocodiles with measurable EEG recordings.

Introduction

In South Africa, the farming of the Nile crocodile (*Crocodylus niloticus*) has developed because of local and international demand for the production of their skin and to a lesser extent their meat. Electrical-stunning (E-stunning) is an efficient method to capture and restrain crocodiles for the purpose of handling to conduct management procedures including as part of the humane killing procedure [3, 8, 16, 20]. E-stunning needs to render an animal unconscious to enable performing any painful management procedure, or for the killing of a crocodile by means of decapitation, or spinal cord severance followed by pithing of the brain during slaughter [15]. Physiological parameters in *C. niloticus* have been studied to compare manual (noosing) and electrical stunning capture methods [16]. The EU regulation criteria require electroencephalogram (EEG) confirmation of the efficacy of e-stunning in animals [6]. E-stunning should cause loss of consciousness. Despite the wide use of e-stunning on commercial crocodile farms, no such information has yet been published in *C. niloticus*.

Measuring brain activity through EEG wave patterns is used to illustrate the state of consciousness. There are four stages of the EEG that can be associated with levels of consciousness which are: active, transitional, unconscious and iso-electric (flat line pattern) [19]. These stages can be used to evaluate the effectiveness of e-stunning. Frequency (Hz), amplitude (μV) and power (μV^2) are all measurable constituents of an EEG recording and are used together to represent the amount of brain activity and allow meaningful interpretations of the recording when using frequency domain methods of analysis. The raw EEG tracing must undergo Fast Fourier Transformation before a frequency domain method of analysis can be applied. Fourier Transformation separates the raw EEG tracing into four different wave types that oscillate at four different frequency bands: delta (δ; 0 to 4 Hz), theta (θ; 4 to 8 Hz), alpha (α; 8 to 12 Hz) and beta (β; 12 to 30 Hz) waves [13, 17, 19]. In humans, deeper stages of sleep, anaesthesia, and reduced consciousness are associated with more activity (power) of the slower waves (delta and theta) compared to the faster waves (alpha and beta) when analysed using a power spectral analysis (a frequency domain method) [13, 19]. Production animal studies where e-stunning has been used to render the animal unconscious before killing has been investigated using a bipolar montage for raw EEG recording and observing behavioural indicators of loss of consciousness. The EEG recording and behaviour indicators assist in differentiating between effective e-stunning (achieving an unconscious state) and electro-immobilization (animal remains conscious but immobile).

These studies confirm that there is more activity in the slower waves compared to the faster waves based on power spectral analysis that indicated a state of unconsciousness [19]. We speculate that this outcome will be similar in crocodiles, however, their behavioural indicators may not be as easy to detect as in mammals because of their physiological and behaviour differences. A 5-channel referential montage was used in order to obtain high quality EEG recordings. This method of EEG acquisition has not been previously described for crocodiles therefore an anatomical component to this study was required to determine the position and size of brain and skull thickness to identify the best surface electrode position sites.

The aim of this study was to identify surface EEG electrode sites to be able to record a 5-channel referential EEG tracing and to record EEG and behaviour indicators pre- and post- e-stunning to identify a state of unconsciousness using a standardised e-stunner setting in *C. niloticus*.

Methods

Brain topography, skull thickness and electrode placement sites

Twenty-six head specimens were collected from captive-bred commercial crocodile farms after harvest and were dissected along various coronal (n = 13) and sagittal (n = 13) plane cuts made by a bandsaw. Four of the heads underwent magnetic resonance imaging scans before dissection (Fig. 1). The scans were carried out using a 1.5 T machine (Ingenia MRI system; Phillips, South Africa) operated by experienced human radiologists. The skull progressively decreased in thickness from caudal (16.7 ± 1.2 mm) to rostral (4.5 ± 0.8 mm) over the brain cavity. The cerebral hemispheres of the brain were located ventral to the centre point of the cranial plate and this part of the brain was the largest portion that was closest to the surface.

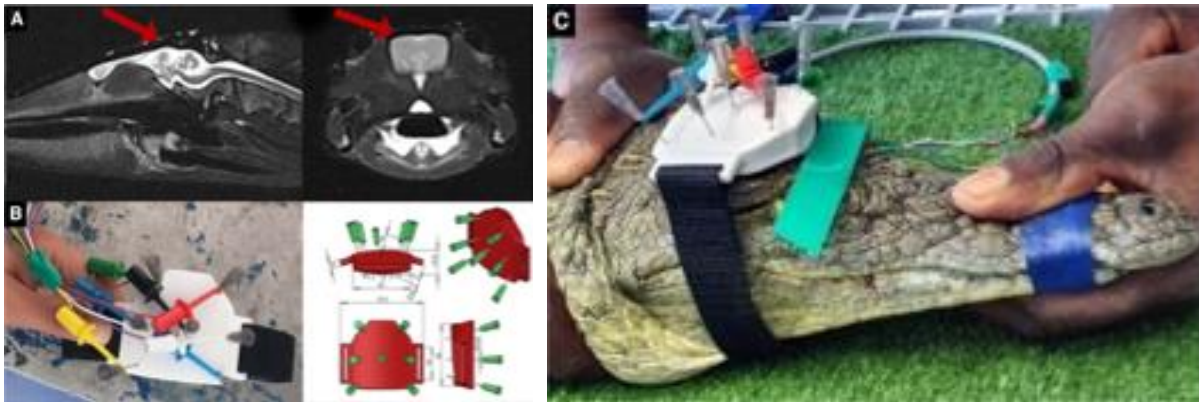


Figure 1. A series of figures depicting the stages of development of a custom 3-dimensional printed electrode placement skull-cap for continuous electroencephalography recordings. Midline sagittal and coronal magnetic resonance imaging (MRI) views of the brain parenchyma (white and light grey structures) within the cranial vault (dark grey) of a head specimen of a Nile crocodile (*Crocodylus niloticus*) illustrating that the best site to place the skull-cap was on the cranial plate (red arrows) (**A**). The skull-cap had 7 electroencephalogram electrode placement sites where the neutral and reference electrodes were the two rostral needles (**B**). The remaining 5 electroencephalogram electrode placements sites were for the electroencephalogram channels 1–5. A live crocodile with the electrode placement skull-cap placed on the cranial plate (**C**).

E-stunning procedure

Animals
Fifteen healthy crocodiles between 30–32 months of age and ranging from 144 to 160 cm in total body length were randomly selected by farm management from a group of growers that were housed in a closed communal pen. The crocodiles were captured manually and moved from the communal pen into an experimental house adjacent to the procedure room two days prior to the electroencephalogram recordings. A total of five crocodiles were placed in each of the three indoor concrete-floored pens that contained a water pond. The use of the live crocodiles in this study was approved by University of Pretoria Animal Ethics Committee (V084-18) and all study methods were performed in accordance with the relevant guidelines and regulations. The study is reported in accordance with ARRIVE guidelines. Once the data collection was complete, all crocodiles were returned to the owners who reintroduced them into the grower communal pens of the farm.

Procedure
A 7-hole plastic skull-cap that was custom made by 3D printing to fit similar sized crocodiles and strapped to the head of the crocodile was used to hold the electrodes in a fixed position during the EEG recording procedure. On the day of the EEG recordings, the skull-cap was assembled by placing injection needles (35 mm long; 21 gauge) through the 7 holes and 6 mm of the needle tip was bent to form a 90-degree angle which improved contact with the scale surface. On the crocodile end, the seven EEG cables were attached to the hub end of the needles using metal clips. On the EEG recorder end, the seven cables were attached to the mobile EEG recorder (Sleepwalker, Lifelines Ltd) and laptop (Inspiron, DELL). The seven cables were wrapped together to form an umbilicus and a quick-release connector was positioned midway along the 1.2-m-long cables to allow for rapid disconnection and reconnection.

Once the EEG recorder was setup, the crocodiles were manually captured, one at a time, their snouts immediately taped and blindfolded, and transferred to the procedure room. They were placed on a low work bench and physically restrained by two experienced animal caretakers, one holding the neck and the other the tail. The assembled skull-cap was placed onto the cranial plate of the crocodile and a 30 mm thick sponge was placed over the top to push the bent needles firmly against the scales. The entire assembly was secured using two self-adhesive material (Velcro) straps wrapped around the head. Then the raw tracing was visually inspected using compatible software (TrackIt version 2.8.0.12; Lifelines Ltd) for stability (not wandering off the isoelectric line or exhibiting excessive course oscillations around the isoelectric line) and an impedance check was done to ensure a value of less than 16 kΩ for each of the 5 EEG recoding channels. Once a stable tracing was obtained, the cloth blindfold was removed from the crocodile and a pre-stun EEG recording commenced for 5 min at a 200 Hz sampling rate. Following the pre-stun EEG recording, the cable umbilicus was disconnected, and the crocodile was prepared for e-stunning.

A research custom-built e-stunner (I'Vimbi technologies) that was powered by single phase mains electricity (220 V, 50 Hz) was used for this study. The e-stunner included a stunning controller referred to as the stunner and stunning apparatus referred to as stunning wand. The stunning wand was a hand-held plastic pole (1.4 m long) with a push-button at the operator-end and a V-shaped studded (three studs on each electrode) stunning electrodes at the crocodile-end of the wand. The volt-out controller of the Stunner was set at 170 V, and the timer set at 5 s stunning period for the first three crocodiles and 7 s for the remaining 12 crocodiles examined.

The data logger was set to record volts, amps and hertz applied for each crocodile and duration of stunning. For the stunning procedure, the neck and head region of the crocodile was doused with water and the stunning wand electrodes were pressed firmly at approximately 50° angle to the horizontal plane, on the dorsal surface of the neck immediately behind the occipital bone. The push-button was activated and the electrodes kept in the same position on the crocodile until the timer had run out. Immediately thereafter, the EEG cable umbilicus was reconnected, and the EEG recording continued for 7 min during

the post-stun period. Throughout the procedure, for each crocodile, the principal investigator digitally video recorded the pre- and post-stunning periods and recorded behavioural indicators.

After completion of the post-stun, meloxicam (0.5 mL; Metacam 20 mg/mL; Boehringer, South Africa) was administered subcutaneously and the skull-cap assembly were removed, the crocodile was weighed and then returned to their experimental pen. The next crocodile was captured and brought to the procedure room. This same procedure was followed until all crocodiles were examined on the same day. Crocodiles were returned to their communal pen the following day

Results

Identification of surface electrode sites

Seven EEG electrode placement sites were identified on the cranial plate based on magnetic resonance imaging study and dissections (Fig. 1). The number of electrodes used was limited by the size of the cranial plate. A 7-hole plastic skull-cap was custom made by 3D printing to fit similar sized crocodiles that was used to hold the electrodes in a fixed position during the EEG recording procedure.

EEG Recordings and behavioural indicators

A state of unconsciousness (effective stun) was identified by a simultaneous increase in delta activity and a decrease in alpha activity and was demonstrated in 6 out of 12 crocodiles. Three of the EEG recordings could not be analysed at the pre-planned epochs likely because of poor electrode contact. This occurred in crocodiles 2, 3 and 5. Crocodiles 2 and 3 were stunned for 5 s and crocodile 5 for 7 s. In the effectively stunned crocodiles, the delta ($p = 0.010$) and beta ($p = 0.045$) power significantly increased, and theta ($p = 0.004$) and alpha ($p = 0.015$) power significantly decreased immediately after stunning (period 0–1) (Fig. 2A). Furthermore, the delta power returned to pre-stun values by time period 3, which is 120 s after completion of the stun. Whereas the alpha power returned to pre-stun values at time period 4, which is 180 s after completion of the stun. The remaining 6 crocodiles were not effectively stunned and considered to be in a state of electro- immobilization. In crocodiles that were not effectively stunned, the delta power significantly decreased ($p = 0.037$) immediately after stunning (period 0–1) whereas theta ($p = 0.055$), alpha ($p = 0.336$) and beta ($p = 0.20$) power showed no significant change (period 0–1), immediately after stunning (Fig. 2B). The 40-s pre- and post-stun EEG waveforms were visually different between the crocodiles that were effectively (Fig. 3) stunned compared to those that were not effectively stunned (Fig. 4). The crocodiles that were not effectively stunned had more movement artefacts during the 40-s pre-stun recording because of escape attempts which was confirmed on the video recording. The post-stun recordings also demonstrated an increase in amplitude compared to the pre-stun recordings in all crocodiles.

Identifying behavioural indicators of stunned crocodiles was inconclusive because there were no behavioural observations specific to crocodiles effectively or not effectively stunned. The crocodiles were mostly docile and showed little to no signs of distress after being captured manually and during pre-stun EEG recordings, except of short bouts of head and limb movements in 5 of the crocodiles that were not effectively stunned. Thus, overall, the crocodile demeanour made identifying behavioural indicators of unconsciousness unreliable. There were no apparent adverse effects observed in crocodiles resulting from e-stunning.

E-stunning data between effectively and not effectively stunned crocodile

The mean volts, AUC (amp × seconds) and amp-max delivered to all crocodiles in the current study were 171.40 ± 1.75 V, 5.48 ± 1.44 AUC, 1.09 ± 0.34 amp-max, respectively, and not different between effectively and not effectively stunned crocodiles. There were no significant differences in the total length, head length and body mass between the effectively stunned and not effectively stunned crocodile.

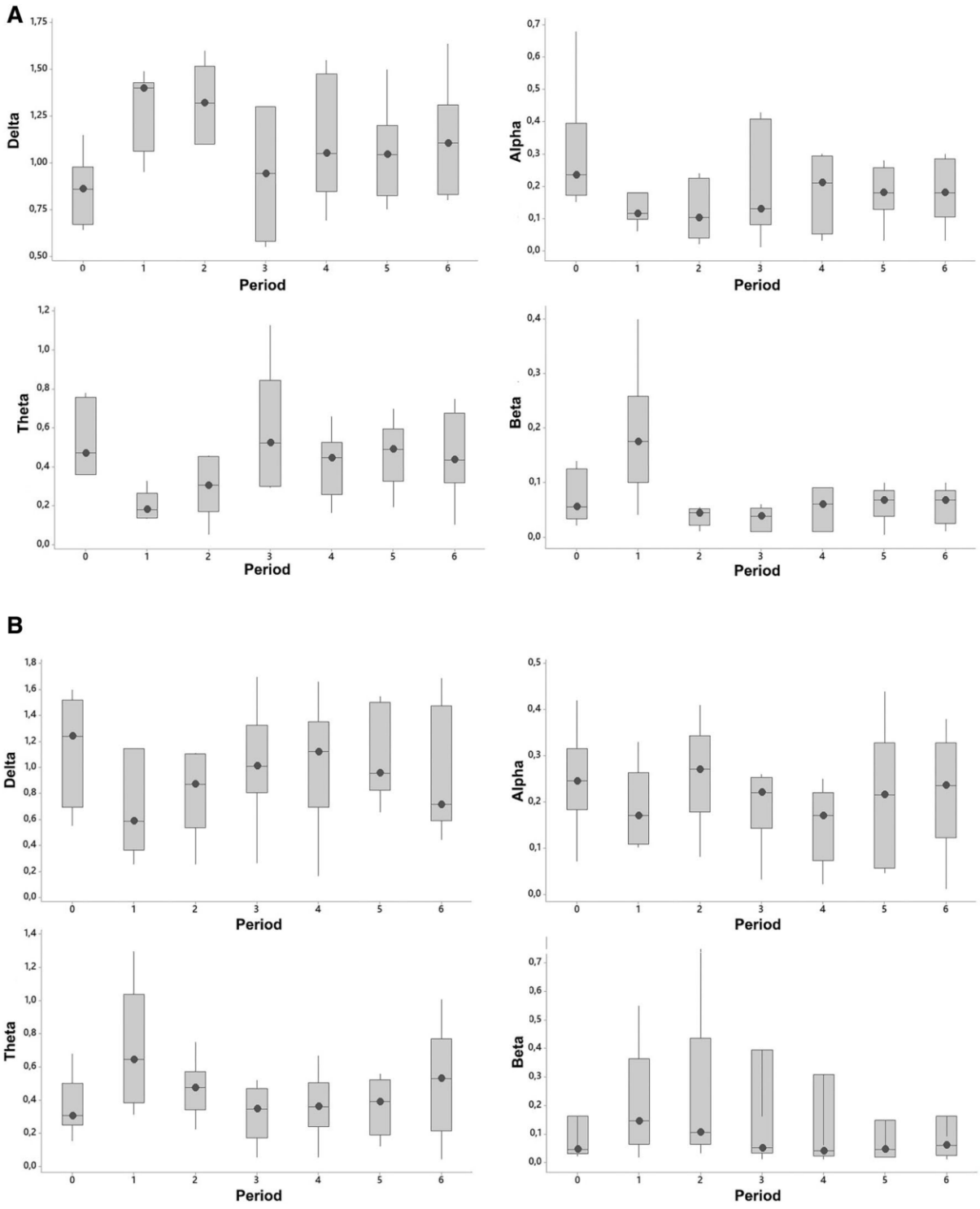


Figure 2. Power Spectral Density (PSD: µV2/Hz) for each frequency band (delta, theta, alpha and beta) before (period 0) and after (period 1–6) e-stunning of Nile crocodile (*Crocodylus niloticus*) that were effectively stunned (A) and not effectively stunned (B).

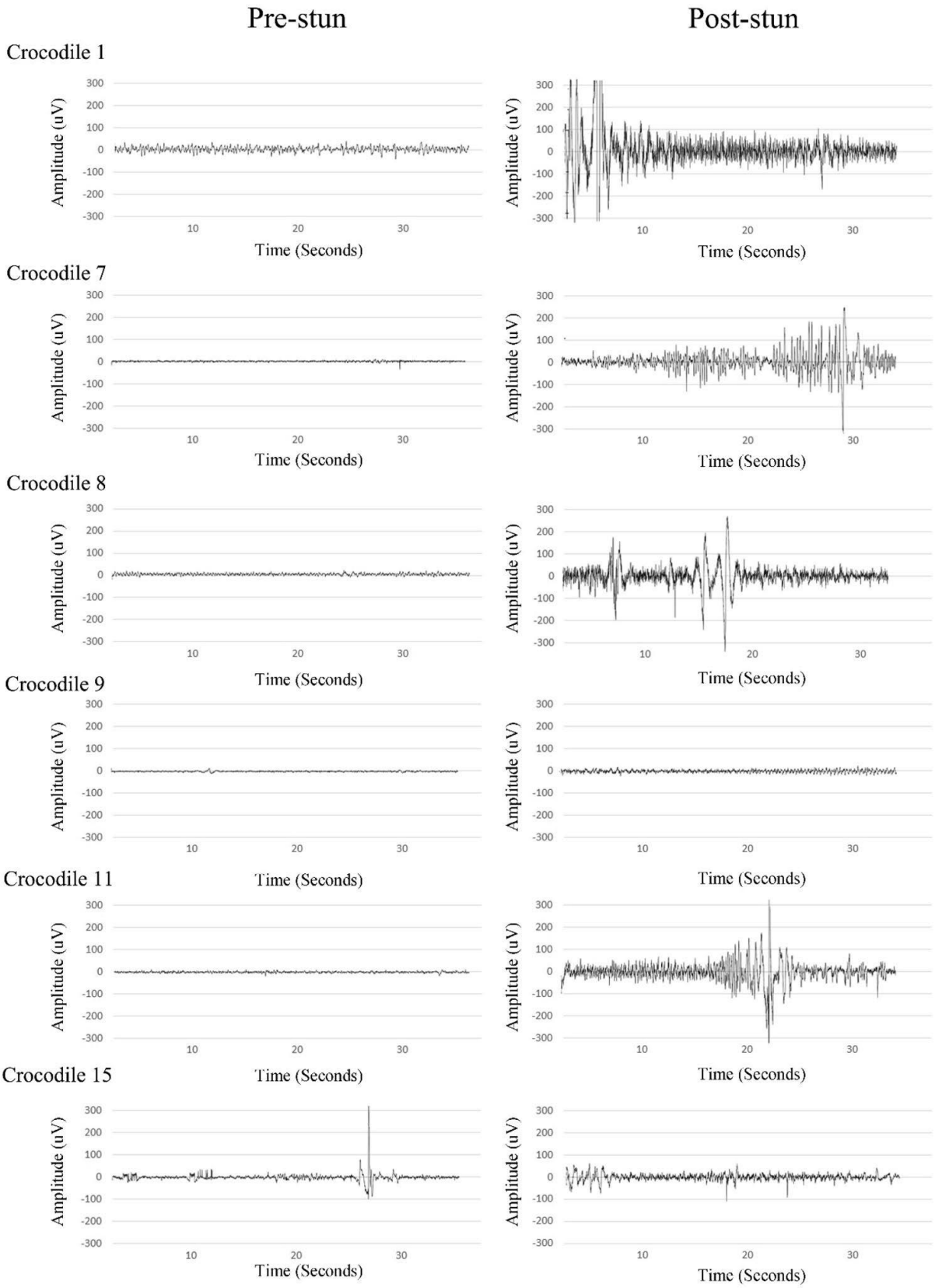


Figure 3. Raw electroencephalography tracings before and after e-stunning of Nile crocodiles (Crocodylus niloticus) that were effectively stunned.

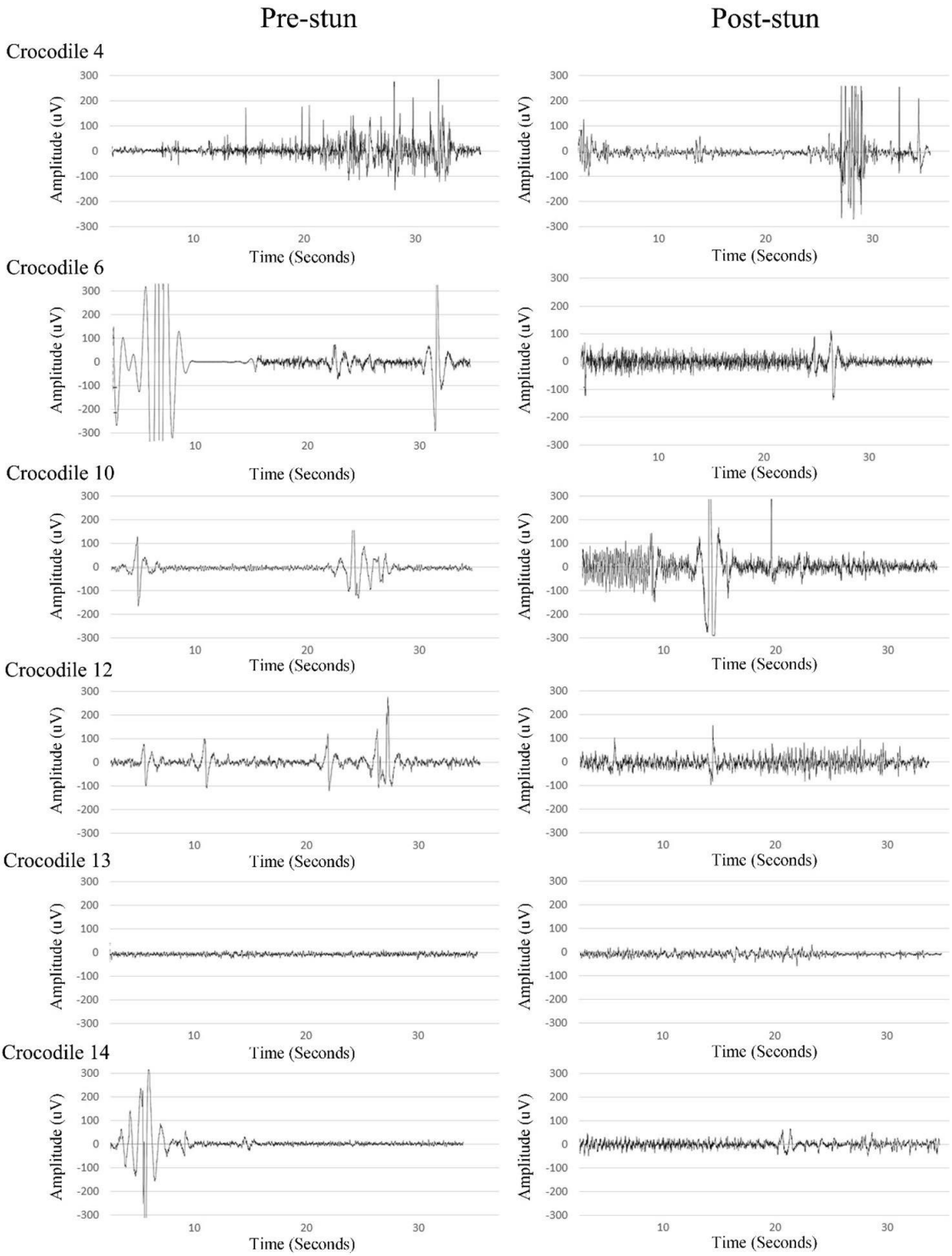


Figure 4. Raw electroencephalography tracings before and after e-stunning of Nile crocodiles (*Crocodylus niloticus*) that were not effectively stunned.

Discussion

Reliable EEG tracings were obtained using a referential EEG recording. A state of unconsciousness was identified through EEG analysis. E-stunning of Nile crocodiles on the dorsal surface of the neck immediately behind the occipital bone, set at 170 V for 5–7 s, achieved variable results, with only half of the study animals showing EEG indications of unconsciousness despite all of the study animals receiving a stable and standardised electrical stun.

Power spectral density analysis is a universally accepted method of analysing EEG recordings, especially with more powerful computers and large number of electrodes used [11]. In this study, seven EEG electrodes were used which included neutral and reference electrodes and five channels using a referential montage. Referential montages include more channels than bipolar montages which have been previously used in e-stunned animal studies [6]. Referential montages are advantageous, as the best raw EEG data can be selected for analysis. Bipolar montages use only two electrodes per channel making it impossible to select raw data for analysis.

EEG studies have been conducted to monitor the brain activity changes after e-stunning and during anaesthesia (unconsciousness) in different species. American alligators (*Alligator mississippiensis*) under anaesthesia, showed a decrease amongst all frequency bands (alpha, beta, delta, and theta waves) [3]. Whilst humans under surgical anaesthesia, the alpha waves decrease, and the delta and theta waves become predominate at deeper levels of anaesthesia [9]. Effects of e-stunning on the brain activity in cattle and sheep have been researched more often. The researchers observed that the amplitude of the post-stun raw EEG recordings increases and that there are often signs of tonic–clonic seizures activity [1,8,14]. Pre-stun EEG recordings (i.e., conscious state) results from these studies showed amplitude readings of 10–50 μV [1,8,14]. Whereas post-stun EEG indicators for unconsciousness were defined as an increase in amplitude (600 μV or at least an increase by fivefold), and/or brain wave patterns resembling a tonic-conic seizure immediately after e-stunning [1,8,14].

The European Union regulations stipulate that a head-only e-stun for all species and an electrified warm water bath for poultry require a tonic–clonic wave pattern on the EEG recordings as a description of an effective stun [15].

This study defined ‘unconsciousness’ by the change in power within the different frequency bands (an increase in delta waves and a decrease in alpha waves), as well as signs of tonic–clonic wave patterns in the raw EEG recordings. All crocodiles demonstrated an increase in waveform amplitude, but only 50% were considered to be effectively stunned and most had tonic–clonic seizure activity. However, these wave patterns were demonstrated after the delta and alpha waves had returned to pre-stun power. Therefore, we cannot confidently recommend an increase in post-stun wave amplitude or tonic–clonic seizure activity as a reliable indicator of unconsciousness in e-stunned Nile crocodile.

Behavioural indicators could give a better indication of immediate effect as the raw EEG tracing cannot be analysed immediately while the animal is being e-stunned. Behavioural signs of loss of consciousness following e-stunning have been identified in livestock and poultry. The behaviour of crocodiles immediately after e-stunned have been described for saltwater crocodiles (*Crocodylus porosus*) and Nile crocodiles [4,16]. Initiation of the stunned state is rapid and comprises an initial tonic phase, with rigor and tail twitching [4,16]. After this period, the stunned crocodile becomes relaxed with legs splayed backwards, parallel to the body [16.] Stunned animals remain immobilized for 5–10 min, but most individuals regain movement after about 3 min [4,16,17]. These behavioural indicators were similar to those observed in the present study. However, the period of unconsciousness, according to the study definition, only lasted for a maximum of 2 min despite the crocodiles remaining immobilized for at least 7 min after stunning. Therefore, the transition from an effective stun to a state of electro-immobilization was not obvious. The outcome of the present study indicates that painful procedures or killing of crocodiles after e-stunning should be done immediately after the stunning procedure to ensure that the crocodile is unconscious. Behavioural indicators of consciousness and unconsciousness warrant further investigation to identify reliable indicators that would confirm an unconscious state. Evaluating reflexes

(such as toe-pinch, tail- pinch, cloaca-pinch) could help determine conscious responses to noxious stimulation and should be included in future e-stunning research on crocodiles of a similar size to those used in the present study.

There were notable limitations to this study. The sample size was small and could not be accurately calculated because of the scarcity of similar studies in reptiles. However, the information in this study will allow for more suitable sample size calculations to be made in future. EEGs of crocodiles 2 and 3 stunned for 5 s and crocodile 5 stunned for 7 s could not be interpreted, therefore comment on the duration of stunning and level of consciousness cannot be made. However, crocodile 1 was effectively stunned using 5 s and no concerns on the remaining crocodiles' EEGs suggest the research team refined the data collection procedures as the day progressed. The study proposal set out the standardised setting for the e-stunner, however, at the start of data collection the stunning time variable was incorrectly set to 5 s which was noticed and corrected to 7 s before stunning the remaining 12 crocodiles. Because crocodile 1 was effectively stunned, we concluded that this change in stunning time did not impact the outcome of this study. We did not test other e-stunner electrical settings to determine if an increased current would cause an increase in e-stunning efficiency. Furthermore, although the crocodiles were doused with water before e-stunning, they were not submerged in water. However, in many farms, removing the crocodiles from water bodies and stunning after dousing with water is common practice to ensure the correct crocodile is stunned and to prevent any safety concerns such as drowning. Conversely, some farms adopt stunning while the crocodile is submerged in water, often in an isolation capture tank and our study did not emulate this e-stunning procedure. With the results of our study, future studies should compare these different stunning practices.

Conclusion

The use of electroencephalography can be used to measure brain activity in Nile crocodiles, and it is a tool that can be used to determine changes in frequency band power. In this study unconsciousness was defined as an increase in delta and decrease in alpha frequency band power. An unconscious state was identified in 50% of the e-stunned crocodile that lasted for a maximum of 120 s. An increase in post-stun wave amplitude and presence of tonic–clonic seizure activity were not reliable indicators of unconsciousness. Furthermore, Nile crocodile demonstrate typical behavioural indicators of being stunned. However, these behaviour indicators were not reliable indicators of unconsciousness in the current study. E-stunning is an effective tool that can be used to capture crocodile safely and effective stunning results in a state of unconsciousness, further research should be focused on improving the efficiency and reliability of e-stunning.

Acknowledgements
K.J, G.E, and J.G conceived the experiment. K.J conducted brain topography and skull thickness experiment, analysed results. K.J, G.E, J.G and G.E conducted the EEG experiment. J.E was responsible for the EEG capturing and analysis. K.J wrote up experiment, results and interpretation. G.E, J.G and G.E advised based on their expertise. All authors reviewed the manuscript. Financial assistance provided by the IUCN SSC Crocodile Specialist Group Student Research Assistance Scheme.

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