Electroencephalogram (EEG) assessment of brain activity before and after electrical stunning in the Nile crocodile (*Crocodylus niloticus*)

by

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Submitted in fulfilment of the requirements for the degree

MSc (Veterinary Sciences)

in the Department of Paraclinical Sciences,
Faculty of Veterinary Sciences,
University of Pretoria

October 2019

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Declaration

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Date: October 2019

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ACKNOWLEDGEMENT

This project would not have been possible without the Exotic Leather Research Centre (ELRC). I would like to thank the ELRC for this opportunity and amazing experience.

I would like to thank my supervisor, Prof Gerry Swan, for the time put into this project.

Thank you to Prof Jan Myburgh and Prof Gareth Zeiler my co-supervisors for your time, expertise and all round positive attitude. Prof Zeiler provided extraordinary support in doing the EEG recordings and evaluation. Many thanks, to Mr Adam Kokkas at SSEM Mthembu Medical and his company for supplying the EEG equipment, knowledge and time.

I wish to thank Stefan van As and his experienced team at Le Croc, commercial crocodile farm, for allowing us to utilize your time, facilities and animals.

Tess Du Plooy, without you I wouldn’t be able to be here, thank you for always supporting me. Thank you to Albert de Wet for being my assist and for the endless support.

ABSTRACT

The purpose of the study was to assess and describe brain activity before (pre-stun) and after (post-stun) electrical stunning (e-stunning) in the Nile crocodile (*Crocodylus niloticus*) by using electroencephalogram (EEG) analysis to determine if stunned crocodiles demonstrate EEG signs of unconsciousness. Twenty-six cadaver *C. niloticus* heads were used to examine the topography of the brain in the cranium and skull thickness. 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. The type of EEG electrodes, portable EEG machine, electrical dose and e-stunner were established using 14 live captive-bred *C. niloticus* in two pilot trials. From the pilot trials, the EEG recording procedure was established which included determining the suitability of pre-selected EEG electrode placement sites and verifying EEG recording device settings. Fifteen live grower crocodiles were used to assess EEG brain activity before and after e-stunning, and behavioural observations during e-stunning were documented. An effective stun was identified by a loss of consciousness, observed in EEG as a decrease in alpha waves and increase in delta waves patterns, in crocodiles immediately after-stunning. The study confirmed that e-stunning at 171.4 ± 1.7 V and 50 Hz for 5 - 7 sec applied to the neck (behind the cranial plate) was able to immobilise all crocodiles and to achieve an effective stun in 6 out of 12 crocodiles with measurable EEG recordings. The delta power significantly increased ($p = 0.010$) and alpha power significantly decreased ($p = 0.015$) immediately after stunning in these crocodiles. In crocodiles that were not effectively stunned ($n = 6$) the delta power significantly decreased immediately after stunning ($p = 0.037$) whereas alpha power showed no significant change ($p = 0.336$) after stunning. A mean maximum electrical current of 1.1 ± 0.2 amps and a total electrical current of 5.5 ± 1.4 amps*sec were delivered to the crocodiles. Size and weight of crocodiles and the electrical current delivered to the crocodiles had no significant ($p > 0.05$) effect in achieving conscious and unconscious. The results of study indicate the importance of monitoring crocodiles e-stunned for clinical signs of consciousness. Identifying an unsuccessful stun is easier than identifying a successful stun (unconsciousness) using behavioural observations. Further studies are recommended to improve the efficiency of e-stunning to achieve immediate unconsciousness in *C. niloticus*, measured by the proportion of animals affected, and to examine the safety of repeated e-stunning of crocodiles for management purposes.

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ABBREVIATIONS

α	Alpha
β	Beta
^{0}C	Degrees Celsius
δ	Delta
μV	Microvolts, measurement of amplitude
μV^2	Microvolts squared, measurement of power
Ω	Ohms
θ	Theta
%	Percent, percentage
3D	Three dimensional
A. mississippiensis	*Alligator mississippiensis,* American alligator
Amp	Ampere
Amps*sec	Amperes per second of time
AUC	Area under the curve
C. sclerops	*Caiman sclerops,* spectacled caiman (*Caiman crocodilus*)
cm	Centimetre
C. niloticus	*Crocodylus niloticus,* Nile crocodile
C. porosus	*Crocodylus porosus,* Estuarine crocodile
Croc	Crocodile
EEG	Electroencephalogram
E-stunner	Electrical stunner
E-stunning	Electrical stunning
g	Gram
HL	Head length
Hz	Hertz
kg	Kilogram
min	Minute
m	Metre
mm	Millimetre
MRI	Magnetic Resonance Imaging
n	Sample size
PSD	Power spectral density (μV^2/Hz)
Q	Quarter
sec	Second
TL	Total length
V	Volts
Volts	Voltage

CHAPTER 1
INTRODUCTION

In South Africa, the farming of the Nile crocodile (*Crocodylus niloticus*) has developed because of growing local and international demand for the production of their skin and to a less extent their meat. Different husbandry systems are generally used on commercial crocodile farms ranging from closed (fully enclosed, climate controlled housing), semi-closed (from enclosed and climate controlled housing to outdoor enclosures after 24 months) and open (moved to outdoor enclosures after hatchling phase) communal housing systems. A growing number of farming operations also have unitised pen enclosures for housing of growers separately during the final phase of their growing period before harvesting. To operate commercial crocodile farming in all types of husbandry systems, consideration of animal welfare, health, environmental sustainability and effective management of crocodiles is essential. Grower *C. niloticus* on most commercial farms are electrically stunned to carry out routine management procedures.

Electrical stunning (e-stunning) is an efficient and less stressful method to capture and restrain crocodiles for the purpose of their handling and conduction of management procedures and for the humane killing of crocodiles (Davis *et al.*, 2000; Franklin *et al.,* 2003; Von Holleben *et al.*, 2010; Pfitzer *et al.*, 2014). E-stunning needs to render an animal unconscious (stunned) 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 (OIE, 2019a). Clinical evaluation of the level of consciousness and analgesia is difficult in any species and further complicated in reptiles because they have a high tolerance to noxious stimuli and hypoxia (Nevarez *et al.*, 2014). Behavioural indicators for immobilisation that are used in mammals may not be easily extrapolated to assess immobilisation in reptiles because of their physiological and behavioural peculiarities (Verhoeven *et al.,* 2014). Furthermore, a stunned (unconscious) state needs to be differentiated from the undesirable electro-immobilised only induced state where the animals remain conscious but immobile (OIE, 2019a). The differentiation between these states could be detected using electroencephalographs (EEG).

Currently there are few reports of EEGs used in large reptiles. The EEG has been used to diagnose and manage a seizing Chinese crocodilelizard (*Shinisaurus crocodilurus*) (Brady *et al.* 2016) and in American alligators (*Alligator mississippiensis*) during four methods of humane slaughter (Nevarez *et al.*, 2014). However, no EEG reports or studies could be found, by the investigators of this study, describing what happens during e-stunning of crocodiles; and to confirm if e-stunning renders them unconscious or in an electro-immobile state. This information is important in identifying behavioural indicators of immobilisation in crocodiles and confirming whether e-stunning practises on commercial crocodile farms are in accordance with ethical and welfare considerations, and meet compliance requirements governing slaughter practice and animal protection (EU, 2009; OIE, 2019a).

1.1. Hypothesis

Electroencephalogram (EEG) shows significant changes in brain wave patterns in *C. niloticus* after electrical stunning.

1.2. Justification

This study will investigate:

a. EEG effect of pre- and post e-stunning on consciousness in the *C. niloticus* because in accordance with EU regulation (No. 1099/2009) criteria, EEG confirmation of the efficacy of e-stunning in animals is required. Despite the wide use of e-stunning no such information has yet been published in crocodiles.
b. A baseline of electrical current, strength and frequency and the site and time of placement of the electrodes because validating e-stunners will improve the efficacy and safety of e-stunning and to validate e-stunning equipment used in crocodiles.
c. Behavioural indicators during e-stunning will improve validity of the EEG in assessing the loss of consciousness in *C. niloticus*, a combined parameter that needs further exploration (Verhoeven *et al.,* 2015).

1.3. Objectives of the Study

The objective was to assess and describe brain activity before (pre-stun) and after (post-stun) e-stunning in the *C. niloticus* by using electroencephalogram (EEG) analysis to determine if stunned crocodiles demonstrate EEG signs of unconsciousness. To achieve this objective, we divided our study into two parts.

<u>Part one</u> (Chapter 3) was the preparatory investigations to identify external sites for placement of the EEG electrodes on the head of the crocodiles by examining the topography of the brain and skull thickness by means of Magnetic Resonance Image (MRI) scans and anatomical measurements; and then to evaluate and establish the EEG recording procedure which included determining suitability of pre-selected EEG electrode placement sites and verifying EEG recording device settings.

<u>Part two</u> (Chapter 4) was the main EEG study aimed to examine the objective.

CHAPTER 2
LITERATURE REVIEW

2.1 Introduction

In South Africa, there are more than 85 commercial crocodile farms, with most farms being in Limpopo, North West Province and Kwa-Zulu Natal (Carpenter & Swan, 2017). The primary objective of crocodile farming is harvesting for skins, but meat and other by-products are also produced. Data collected from 40 farm surveys done between 2016 and 2018 showed an average of 72 753 crocodiles were harvested for their skin and meat (Carpenter & Swan, 2017). Most crocodile skins and meat are exported by South Africa with an average of 77 000 skins exported per annum during 2011 - 2015 and 57 786kg of crocodile meat exported per annum between 2013-2017(CITES Trade data base).

When handling crocodiles for the purpose of conducting different management procedures including capture restraining, stunning and culling there are general characteristics of reptiles that need to be considered (OIE, 2019a) such as: sensitivity and responsiveness stimuli (visual, sound, touch, vibration); ability to escape handling and restraint due to their strength and agility; danger and ability to inflict serious injuries to handlers; ectothermic nature; vocally absent but can hiss; and susceptibility to regurgitate or choke if noosed (if not stunned). Therefore, the proper capture, restraint and humane killing of crocodiles involves measures to minimise distress, fear, pain and any other form of suffering that the animal may experience and to protect the handler (EU, 2009).

The use of e-stunning has been shown to be an effective method in the capture and immobilisation of crocodiles and improving human safety during handling (Joanen & Perry, 1971; Davis *et al.,* 2000; Peuker *et al.,* 2004; Manolis & Webb*,* 2016). E-stunning was used for the first time to capture and restrain crocodilians by Joanen & Perry (1971). A pulsating unit (110 – 120V DC) was used and *A. mississippiensis* were immobilised for 15 – 25 minutes when partially exposed in water with low salinity. Peucker and co-workers (2005) subsequently modified and approved the method by applying 110 V via a metal fork wand e-stunner to the back of the neck of estuarine crocodiles (*Crocodylus porosus) (*Peucker *et al,* 2005). Crocodiles were immobilised for 5 – 10 minutes and the method resulted in less stressed animals, rapid return to normal eating and behaviour, and less skin damage.

The stress responses in captive crocodiles were shown to be lower in crocodiles immobilised by e-stunning compared to manual capture and restraint using physiological indicators (Franklin *et al.*, 2003; Pfitzer *et al.,* 2014). Estuarine crocodiles when captured manually (noosing), had significantly higher increases in haematocrit, haemoglobin, glucose, lactate and cortisone concentrations than when compared to crocodiles e-stunned at 110 V for approximately 6 seconds (sec) behind the neck, and their recovery time was faster (Franklin *et al.*, 2003). A significant decrease in serum lactate was shown in *C. niloticus* e-stunned at 135V, 50Hz for 5 – 11 sec applied behind the head of the crocodile compared to manual capture (noosing) (Pfitzer *et al.*, 2014). No significant difference between the two methods of capture was observed for serum corticosterone, glucose, alanineaminotransferase, alkaline phosphatase, aspartate aminotransferase and creatine kinase. Also, manual capture takes a significantly longer time versus e-stunning (Pfitzer *et al*., 2014). Manual capture not only causes stress in the noosed crocodile but causes agitation amongst the surrounding crocodiles (Davis *et al.*, 2000).

Several countries accept stunning as a routine pre-slaughter practice to facilitate the humane killing of animals. The methods of stunning in mammals includes penetrative captive bolt, non-penetrative captive bolt, head-only electrical stunning, head-to-body electrical stunning and electrical waterbath (poultry) (EU, 2009). Stunning as a method to facilitate slaughter of reptiles needs to: be species appropriate (age, size, and health);be reliable and reproducible; reduce stress, agitation and pain experienced by the animal; avoid injuries; cause immediate unconsciousness (OIE, 2019a). In South Africa, the 'Animal Protection Act No.71' stipulates that all animals must be stunned before slaughter, except animals which are to be slaughtered using religious methods. This Act also covers reptiles that are kept in captivity. 'The Humane Methods of Slaughter Act' of the United States of America is a Federal law that includes stunning for pre-slaughter of cattle, chickens, sheep and pigs (Welty, 2007). In the United Kingdom, pre-slaughter stunning for all species of animals killed in abattoirs is a legal requirement (Anil & Gregory, 2014). In Australia, only a small number of abattoirs have special permission to be exempt from pre-slaughter stunning for religious purposes, and for all other abattoirs' pre-slaughter stunning is mandatory (Pendergrast, 2015). E-stunning results in unconsciousness and requires an additional killing step (OIE, 2019a).

2.2 Electrical Stunner (Design and Function)

The purpose of an e-stunner is to cause immediate unconsciousness to decrease stress and facilitate handling of crocodiles by sending an electrical current through the brain without damaging the crocodile's skin (CSIRO, 2006). There are 2 types of e-stunner commonly used in crocodile farming, *viz.* mains powered and battery operated units (Davis *et al.*, 2000). There are disadvantages and advantages of both. The mains powered unit has long leads that may interfere with procedures, but has a consistent power source (Davis *et al.*, 2000). The battery operated units are portable but the power reduces as the battery gets depleted (Davis *et al.*, 2000). Both units have reduced conductivity when used on crocodiles not in water. Newer e-stunners designed for sheep, pigs and cattle include features such as: devices to set either volt or amperes; display and data recording devices for electrical current (Amp), voltage (V), frequency (Hz) and minimum stunning time; as well as computer software for data analysis (HAS, 2016). In aquaculture, e-stunning and semi-dry e-stunning are used as a stunning and killing methods for farmed fish (OIE, 2019b). E-stunning provides an electrical current of sufficient strength, frequency and duration to a large number of fish in a tank causing immediate unconsciousness (OIE, 2019b). Semi-dry e-stunning provide an electrical current to the head of an individual fish as it enters the device causing immediate unconsciousness (OIE, 2019b). New fish e-stunner equipment pumps fish through plastic tubes where an electrical current is applied to the water as either a method to slaughter or cause immediate unconsciousness for farmed fish (Smith-root, 2020).

The e-stunner used to stun crocodiles is made up of a pole/ wand with metal electrode ends (Manolis & Webb, 2016). The electrodes at the end of the wand can either have two stainless steel ballpoint pins, or noose and fork-like wand, or fork-like wand (Figure 2.1) (Davis *et al.*, 2000; Livestock Welfare, 2017). The fork-like metal prong (most commonly used) is designed to be pushed up against the neck or across the head to deliver the electric shock. There is no earth leakage therefore all current flows between the electrodes until intercepted by something (Livestock Welfare, 2017). Stunning is most effective when the contact area, neck behind the cranial plate or cranial plate, is wet (Manolis & Webb, 2016).

Figure 2.1: Electrodes of an e-stunner wand a) ballpoint pins, b) noose and fork-like wand and c) fork-like wand (Davis *et al.*, 2000; Livestock Welfare, 2017).

To induce the desired unconsciousness after e-stunning, sufficient amperage (current) must be applied to an animal's brain to cause an epileptic seizure (AVMA, 2013). The minimum ampere required to cause insensibility is 1.25 amps for pigs, 1 amp for sheep and 1.5 amps for cattle (Grandin, 2015). Amperage-regulated e-stunners where amperes are set and volts varies are better to use rather than e-stunners that are volt-regulated where the volt is set and amperes vary (Grandin, 2015). However, both designs should induce epileptic-like seizure.

2.3 Crocodiles and Electrical Stunning (Physiological Aspects)

Operator observed animal behavioural signs during e-stunning are primary indicators of a successful stun that render the animal immobile and unconscious (Verhoeven *et al*., 2015). Animals insufficiently stunned are conscious and display subtle behavioural indicators consistent with electro-immobilisation. The e-stunner during e-stunning applies a set volt over time to generate a current that is designed to incapacitate the brain, especially in regions of the brain where consciousness is thought to arise. The cerebral cortex and the thalamus form the thalamocortical complex is the region of the brain involved in consciousness and are regulated by the brain stem (Verhoeven *et al*., 2015). The centre of the brain stem consists of a large neural tissue network called the reticular formation which receives signals from the spinal cord (stimuli signals from the body are transferred to the brain via the spinal cord) and is responsible for keeping an animal in an awake state (Verhoeven *et al*., 2015).

E-stunning causes a temporary or permanent disruption of brain function where the animal experiences unawareness and is unable to respond to normal or painful stimuli. This causes electric shock waves moving through the brain, depolarising of neurons in a different location of the electrical application site, thus disrupting the normal ion channels functions and providing changes in the release of amino acid transmitter (Cook *et al*., 1995 & 1996; Somjen, 2001; Von Holleben et al., 2010; Verhoeven *et al*., 2015).

Consciousness is defined as the ability to perceive, interact and communicate with the environment; it is associated with an awake state (Verhoeven *et al*., 2015). Signs of consciousness can be monitored as reflexes and behavioural indicators. Reflexes originating from the brain stem include loss of eye reflexes or from the spinal cord resulting in loss of pedal reflexes. Brain stem reflexes are regulated through twelve pairs of cranial nerves that enter and exit the brain of which 2 cranial nerves (I and II) enter from the forebrain and the others (III - XII) enter and exit from the brain stem (Verhoeven et al., 2015). Loss of brain stem reflexes is shown when animals lose consciousness and when the EEG is suppressed or iso-electric for a period of time after sheep and calves are e- stunned (Verhoeven *et al*., 2015). Behavioural indicators include loss of posture, vocalisation and respiratory rhythm.

After e-stunning, animals should immediately collapse and exhibit no vocalisation. Vocalisation indicates consciousness, distress or pain (not to be confused with the involuntary passage of air along the vocal cords) (Verhoeven *et al*., 2015). Other signs of unconsciousness include a limp head, protruding tongue, and arrhythmic breathing, especially if the current traverses the brain stem (where the respiratory centre is found). Behavioural indicators of consciousness and unconsciousness could be used to help interpret EEG recordings to identify an effective stun versus an electro-immobilised state. Using behavioural indicators Verhoeven *et al*., (2015) reported that 3 – 14% of 37 00 animals (pigs and cattle) demonstrated indications of insufficient stunning in slaughter plants. Clinical signs used to assess consciousness were brain stem reflexes (cornea and palpebral reflexes), spinal cord reflexes (pedal withdrawal reflex) and behavioural indicators (loss of posture). Eight percent of e-stunned cattle showed signs of consciousness 20 and 90 sec after being stunned. It is recommended to kill pigs humanely within a 15 sec or less when e-stunning pigs to ensure unconsciousness throughout the killing process (Verhoeven *et al*., 2015).

In practice, crocodiles are incapacitated quickly, only showing signs of rigor and tail twitching for 5 – 10 sec after being stunned (NRMMC, 2009; CFAZ, 2012; Testronics, 2015). These signs are followed by total body musculature relaxation with the limbs splayed backwards parallel to the body (Peucker *et al*., 2005; NRMMC, 2009). There will be no movements for 2 – 3 minutes, the third eyelid opens and a deep breath between 2 and 3 minutes after stunning (NRMMC, 2009; Testronics, 2015). At 5 minutes, the crocodiles' feet will return to the normal position and regain normal jaw movements. Between 7 and 11 minutes the crocodiles will respond to movement and be mobile but will remain in a dazed state for a period of time (Testronics, 2015). Crocodiles can be immobile for5- 10 minutes, but most individuals regain movement after 3 minutes (Franklin *et al*., 2003; NRMMC, 2009; CFAZ, 2012; Manolis & Webb, 2016). However, it is unknown if the stunned crocodiles remain unconscious throughout the immobile state. Successful e-stunning of the animal avoids stress and pain during invasive routine management procedures (tooth extractions) and immediately prior to slaughter.

E-stunning of crocodiles at slaughter should render them unconscious before a required killing step is implemented (OIE, 2019a). Furthermore, signs of consciousness include pupillary response to light or movements (not a reliable indicator of unconsciousness as reptiles have striated pupillary muscles), blinking, blinking as a response to touch of the cornea, hissing as defence response and/ or tongue movements should be observed for (OIE, 2019a). The advantage of e-stunning crocodiles, if done effectively and sufficient current is used, is that they do not need to be restrained to induce the final killing step such as use of captive bolt (EFSA, 2004). The disadvantages of e-stunning are the duration of unconsciousness can be short (a few seconds) and the electric shock is painful and could be perceived if loss of consciousness is not induced immediately (EFSA, 2004).

2.4 Brain Anatomy and Topography

Magnetic resonance imaging (MRI) scans of the cranium provide images of sagittal, coronal and transverse planes to show the position of the brain and skull thickness (Joslyn & Hague, 2016). Cranial scans of crocodilians were taken from the rostral orbital border to the caudal tip of supraoccipital (medial posterior margin of cranial table) to ensure the brain is included in the image (Hall & Portier, 1994; Pearcy & Wijtten, 2011).

Due to the anatomical similarities between alligators and crocodiles (Pearcy & Wijtten, 2011), the topography and position of the brain within the skull of the *C. niloticus* is predicted to be similar to an alligator which is depicted in Figure 2.2.

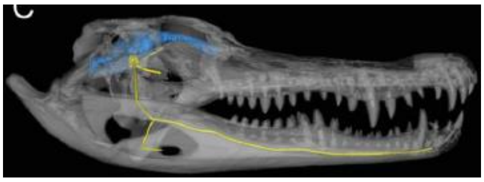

Figure 2.2: 3D rendering of an alligator skull. (George & Holliday, 2013).

The relationship between body growth (length) and brain growth (brain mass) of the *C. niloticus* was investigated (Ngwenya et al., 2013). Seventy crocodiles were selected from a commercial crocodile farm in South Africa. After the crocodiles were euthanized, the total length (TL) was measured from the rostral tip of the snout to the caudal tip of the tail. The brain, including the olfactory bulbs and tracts, was removed from the surrounding bone using various saws and rongeurs and then placed into a fixative solution overnight before being weighed. Brain growth slowed after reaching a body mass of 2 500g, where the brain tissue mass increased by 1g per 20 kg body mass increase. Crocodiles with a total body length of 126 to 158cm (body mass of 7720g to17100g) have an average brain mass of 4.91g (Figure 2.3). There were no significant differences between males and females (Ngwenya *et al*., 2013).

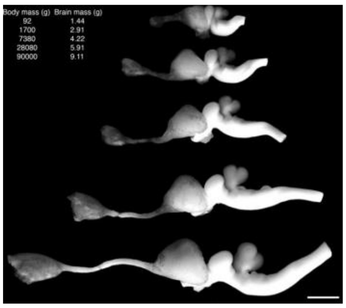

Figure 2.3: The brain of the *C. niloticus* at different body masses. (Ngwenya *et al.,* 2013).

2.5 Encephalogram (EEG) to Measure Brain Activity

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) (Verhoeven *et al.*, 2015).These stages can be used to evaluate the effectiveness of e-stunning and procedures used to kill animals at slaughter.

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. The wave patterns of raw EEG recordings are made up of four different wave types that oscillate at four different frequency bands: delta (0 to 4 Hz), theta (4 to 8 Hz), alpha (8 to 12 Hz) and beta (12 to 30 Hz) waves (Nevarez *et al.*, 2014; Sánchez-Barrera *et al.*, 2014; Verhoeven *et al.*, 2015).The frequency bands are associated with different states of consciousness that have characteristic pattern changes when an animal loses consciousness, depending on the stunning method (Verhoeven *et al.*, 2015).The slow waves, delta and theta activity have lower frequencies and higher amplitudes compared to the faster alpha and beta wave patterns and are associated with deeper stages of sleep, anaesthesia, and coma or reduced consciousness (Nevarez *et al.*, 2014; Verhoeven *et al.*, 2015). Alpha activity is associated with consciousness or an awake state of relaxation (closed eyes) and have a lower frequency and higher amplitude compared to beta waves which have the highest frequency and lowest amplitudes. Beta waves are associated with an awake and alert state (cognitive consciousness).

The transition from conscious alert awake state to an unconsciousness state to a state of deep sleep (from predominant beta frequency band to predominant delta and theta frequency bands) are shown as a change from high frequency and low amplitude (fast wave) to a low frequency and high amplitude (slow waves) EEG patterns (Nevarez *et al.*, 2014; Sánchez-Barrera *et al.*, 2014; Verhoeven *et al.*, 2015). An iso-electric (flatline pattern) EEG pattern can never be compatible with consciousness as it is associated with no brain activity and death in animals that have just been slaughtered. The precise point at which an animal loses consciousness is difficult to establish because of our incomplete understanding of consciousness (Verhoeven *et al.*, 2015).

To aid in our understanding of EEG patterns we had to review the current literature in species that are slaughtered for human consumption to identify potential EEG markers to identify an unconscious state. Defining this state will help differentiate a stunned state from an electro-immobilised state in the crocodiles.

2.6 Analysis of EEG Data

There are many ways to analyse raw EEG data by either examining the filtered waves over time (time domain analysis) or to examine wave characteristics (such as power) within each frequency band (frequency domain analysis) (Harighira, 2015). A section of filtered or raw EEG recording is often manually inspected to find a tracing in each channel that is clean of artefacts (movement, electrical hum etc.) and then cut from the entire recording (Levy & Warren, 1987). This cut snippet in time is called an epoch and can range from 1 to 40 sec of recording in time. The epochs are processed to allow analysis of their frequency domain (power spectrum analysis or power spectral density analysis). Regardless of the type of frequency domain analysis used, the filtered EEG tracing must undergo Fast Fourier Transformation (FFT) which calculates the absolute power within each of the frequency bands (Sreelekha &Sabi., 2016).

Power spectral density (PSD) analysis is more complex, but the fundamentals of frequency domain analysis remains the same whereby the EEG epoch tracing will undergo FFT (Sreelekha & Sabi, 2016), but also additional mathematics is applied that is outside the scope of this study. However, of relevance to the present study is the widely used Welch periodogram method of PSD analysis (Sreelekha & Sabi, 2016). The Welch method is where consecutive FFT signals per frequency over the time period of the epoch is averaged within a pre-selected frequency band of interest to determine the power within the frequency band. The PSD analysis has been used successfully to compare awake and asleep EEG epoch tracings to estimate the likelihood of consciousness or to assess the depth of anaesthesia (Verhoeven *et al.*, 2015).

2.7 EEG Studies in Crocodiles

Several EEG studies have been performed in crocodilians (Huggins *et al.*, 1968; Flanigan *et al.*, 1973, Meglasson & Huggins, 1978; Nevarez *et al*. 2014) to study brain activity. Most of these earlier studies examined spontaneous electrical activity of the brain and sleep in the spectacled caiman (*Crocodylus sclerops*); but EEG evaluations were qualitative rather than quantitative in nature. The study by Nevarez *et al*. (2014) examined the level of consciousness in alligators in evaluating four methods of inducing death during slaughter using quantitative analysis. No EEG studies in crocodilians to evaluate the effect of e-stunning could be sourced.

The EEG and behavioural continuum of the crocodilian during states that resembles 'sleep' was done by correlating behavioural observations and electrophysiological patterns (Flanigan *et al.*, 1973). The investigator implanted gold plated screw electrodes and electrode leads were connected to a connector cemented to their skull (Flanigan *et al.*, 1973). EEG recordings were taken over a 24 hour period before and after treatment (response to touch and visual stimuli, and temperature changes). There are four obvious postures observed in this species. Posture 1 show flexed limbs, body off the ground, eyes open, and head and neck elevated. This posture is accommodated by quick responses to stimulation and irregular throat palpitations. Posture 2 show flexed limbs, body flat on the ground, eyes open the majority of the time, and different degrees of head and neck elevation. There is less aggressive response to stimulation and increases of throat palpitations are observed. Posture 3 show less flexed limbs, body flat on the ground with 2 to 3 limbs parallel to the body, eyes are predominately closed, and head and neck positioned on the ground. The responses to the stimulations are delayed and throat palpitations are shallow and occur at irregular intervals. Posture 4 shows all limbs bent back against the body which is flat on the ground along with the head, closed eyes, and the total body is relaxed. Responses to stimulation are poorly coordinated and unenthusiastic as well as throat palpitations are shallow and often absent. Although they found an increase in EEG frequency and amplitude, throat palpitation, and eye openings (transient arousals), they were unable to record paradoxical sleep (relatively high frequency, low volt) in any of the specimens (Flanigan *et al.*, 1973). EEG patterns decreased slightly in frequency and amplitude for posture 3 and 4. The EEG showed large amplitude (up to 200μV) and arrhythmic spikes (fast waves) in all time periods (Flanigan *et al.*, 1973). These spikes increased in posture 3 and 4, and decreased or absent when treatment was applied. The findings from this study are important as it highlights the species' sensitivity to stimuli and the environment. The sensitivity of crocodilians is a factor that could influence EEG recordings and duration of immobilisation. The importance of this study is the use of behavioural observations in conjunction with EEG data.

Nevarez *et al*., (2014) used EEG power values (delta, theta, alpha and beta frequency bands, and expressed in microvolts) to evaluate whether four methods of inducing death of 24 *A. mississippiensis* during slaughter are a humane practice. The methods to induce death were: severance-of-the-spinal cord; severance of the spinal cord followed by pithing of the brain; application of a penetrating captive bolt; and application of a non-penetrating captive bolt. The investigators drilled holes (2mm diameter and 4 mm deep) through the skin and skull of the anesthetised alligators to implant self-tapping, stainless steel screw electrodes (2.8 mm diameter by 6.4 mm length). Four electrodes were placed overlying the brain (left rostral, right rostral, left caudal and right caudal) with one ground electrode between the orbit ridges. Electrodes were connected to an EEG machine and recordings were obtained with a Cadwell Easy II EEG system (recording sampling rate and electrode impedance were not reported). EEG recordings were taken at 3 different time periods during anaesthesia, before the killing method and after inducing death. From each time period for each alligator 10-second-long epochs were extracted and analysed. The EEG recordings were taken until an isoelectric line was observed from the EEG and spontaneous blinking and corneal reflex had stopped, indicative of death. The study concludes that EEG readings of predominantly beta wave patterns are associated with an alert-awake behavioural state, alpha wave patterns are associated with an awake-relaxed behavioural state and delta and theta wave patterns are associated with deeper sleep, anaesthesia or coma.

2.8 Conclusion

This study will investigate e-stunning of *C. niloticus* by studying the effectiveness of e-stunning to induce unconsciousness using EEGs which has not been done before.

Modern e-stunner equipment allows for electrical current to be delivered to the crocodile to be better controlled and to enable the logging of electrical parameters post-stunning. The placement of the electrodes needs to achieve an electrical current to flow through the brain to result in immediate unconsciousness after e-stunning.

The topography and position of the brain in crocodilians has previously been researched but further research needs to be done to identify the EEG electrode placement sites, EEG electrode type and quantity of EEG electrodes for the purpose of this study.

Identifying the point at which an animal loses consciousness and measuring unconsciousness by means of EEG is difficult and thus using behaviour assessments concurrently with EEG recordings is important to conclude a result. This will be one of the first studies to capture quantitative EEG data for crocodilians and document the effect that e-stunning has on brain activity of the *C. niloticus*.

CHAPTER 3

PREPARATORY INVESTIGATION FOR THE NILE CROCODILE EEG STUDY

3.1 Brain Topography

3.1.1 Introduction

The position, size of brain and skull thickness was studied using MRI and anatomical sagittal and coronal dissections. Four live crocodiles scheduled for humane euthanasia and twenty-two head specimens of captive-bred grower *C. niloticus* collected from commercial crocodile farms, were used. The head specimens were from mortalities or crocodiles slaughtered at harvesting for skins. The four crocodiles scheduled for euthanasia received an intravenous bolus of pentobarbitone (100 mg/ml; 4 ml/kg dose) immediately before undergoing MRI scans. The MRI scans were carried out at a private MRI hospital in Pretoria, Gauteng, South Africa using a 1.5 Tesla magnet machine operated by experienced human radiologists using echo time (TE) 93 repetition time (TR) 4730 for transversal and coronal plane images and echo time (TE) 15 and 107 repetition time (TL) 500 and 4500 for sagittal plane images. Images were taken, stored on a DVD and examined.

3.1.2 Magnetic Resonance Imaging and Anatomical Dissection

The position and size of the brain and skull thickness of the crocodiles were visualised on the MRI scans (Figure 3.1, Figure 3.2 and Figure 3.3).

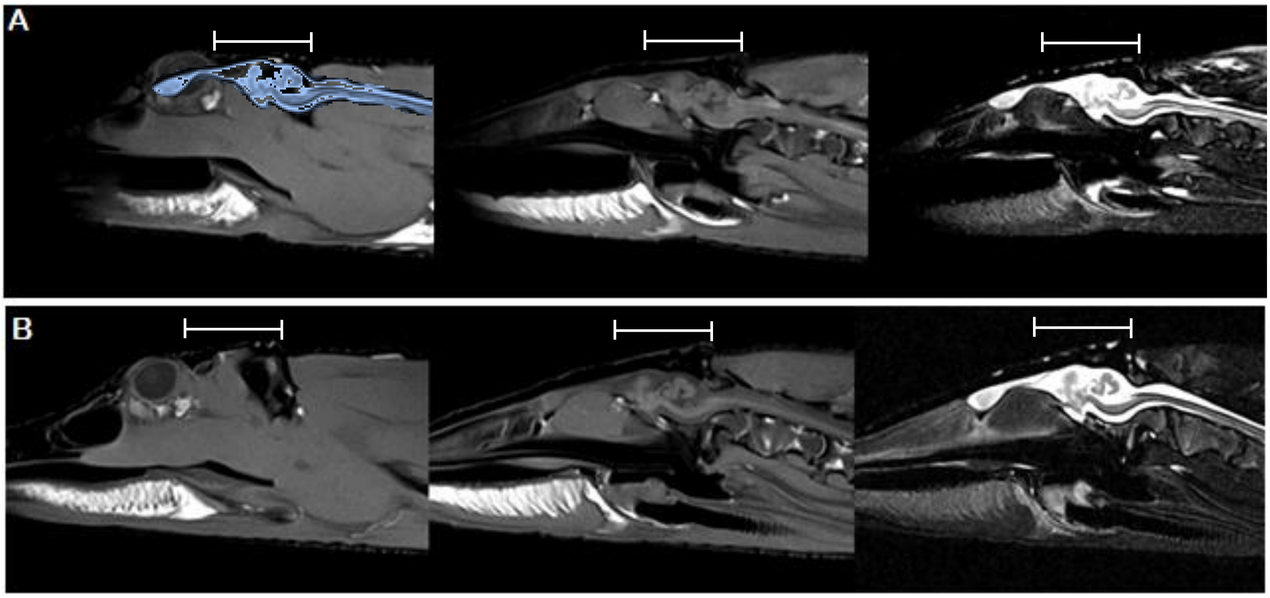

Figure 3.1: MRI lateral view of the sagittal sections of crocodile 1 (A) and crocodile 2 (B). Brain (blue) edited in the MRI scan to show the position of the brain in the cranium. I—I is the position of the cranial plate exterior to the cranium.

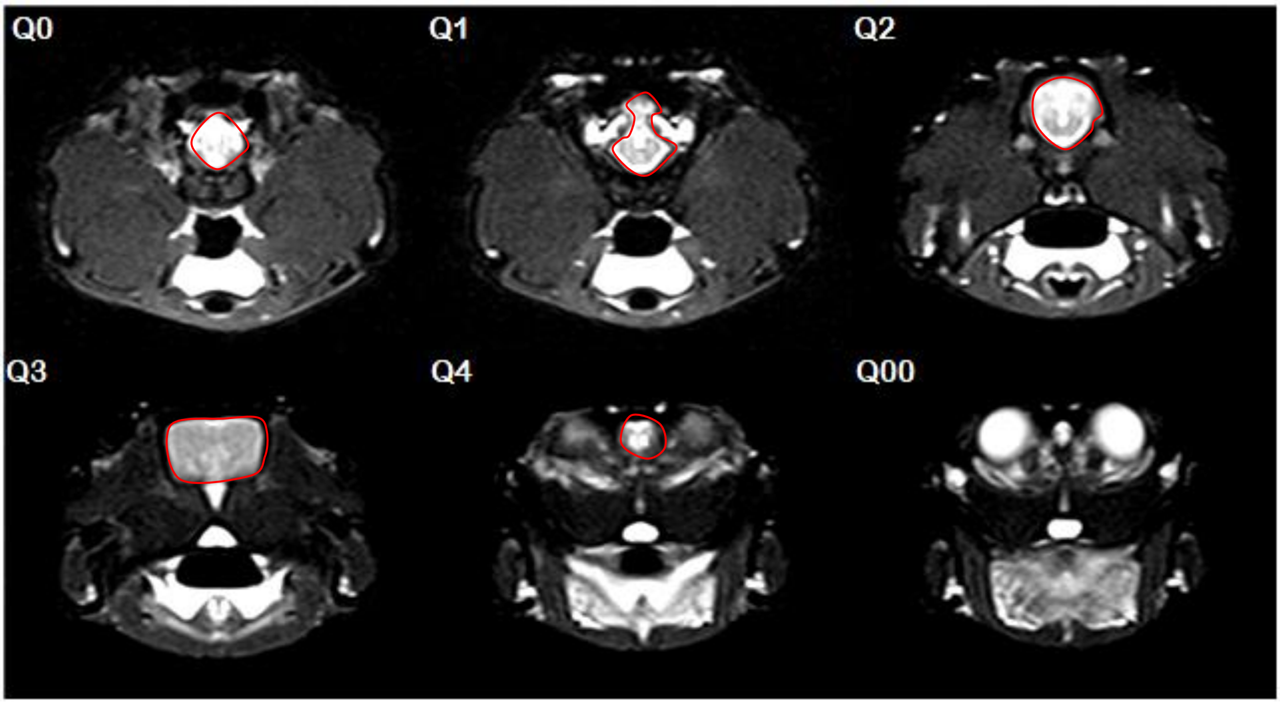

Figure 3.2: MRI occipital view of the coronal sections of crocodile 3. Q1-Q4 quarters of the cranial plate, Q0 caudal region of the cranium, Q00 rostral region of the cranium. Position of the brain the cranium (red).

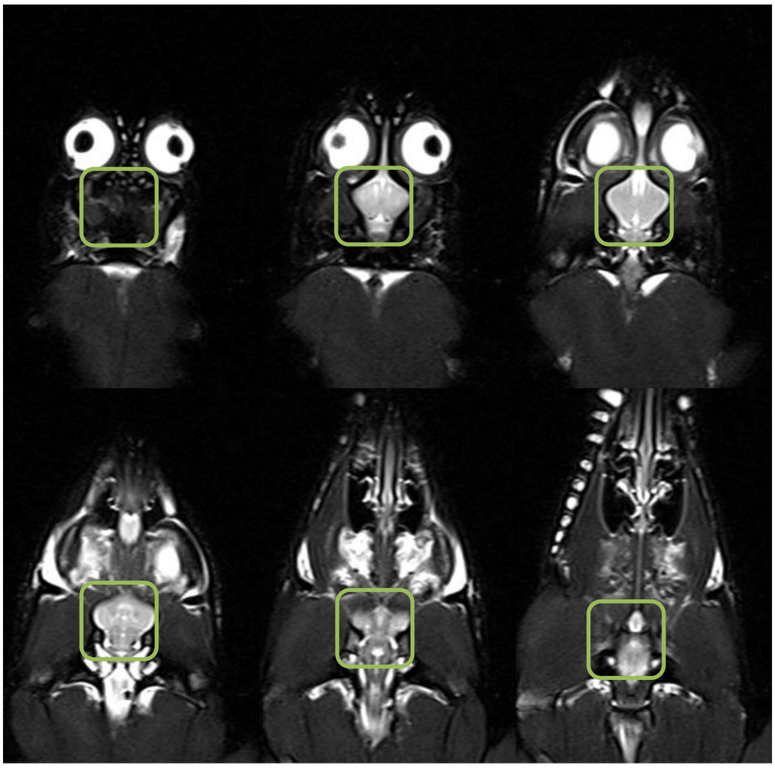

Figure 3.3: MRI dorsal view of the transverse sections of crocodile 4. Showing Position of the cranial plate that overlays the brain (green rectangle).

After completion of MRI scans, the heads were dissected by a single cut along the sagittal plane (n = 2) or coronal plane (n = 2) using a bandsaw for visual inspection and measurements. Similarly, frozen head specimens (n = 22) were also dissected along the sagittal (n = 11) and coronal (n = 11) planes for visual inspection and measurements. Coronal dissections of the heads were done by dividing then cutting the cranial plate into quarters. A self-calibrating electronic calliper was used to measure skull thickness and the brain cavity for each quarter.

Anatomical measurements on the sagittal and coronal dissections were performed as illustrated in Figure 3.4 and Figure 3.5, respectively. The sagittal dissection measurements taken from 13 cadaver crocodile heads and those of the coronal dissections from 13 cadaver crocodile heads represented in Table 3.1. Measurements with the value 0 represent specimens damaged during dissections or decapitation on the farm therefore, unable to measure.

The skull thickness dorsal to the brain, was shown to decrease from the caudal to rostral sides of the brain cavity. The mass of the brain is located on the rostral half of the brain cavity between Q 3 and Q 4 sections of the cranial plate (Figure 3.4). The observations made from the MRIs aided in the identification of the external landmarks and EEG electrode placement sites.

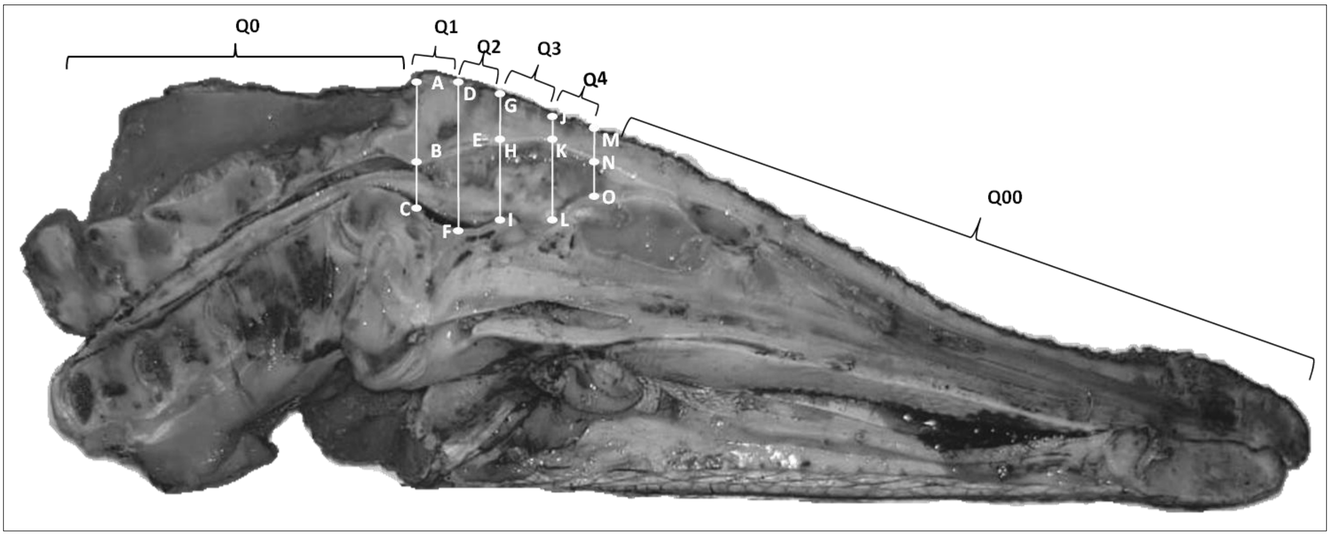

Figure 3.4: Sagittal dissection and anatomical measurements. Q0: Caudal region of the cranium/ neck. Q1-Q4: Cranial plate in quarters. Q00: Rostral region of the cranium. A-B, D-E, G-H, J-K, M-N: Skull thickness. B-C, E-F, H-I, K-L, N-O: Brain/ cranial cavity.

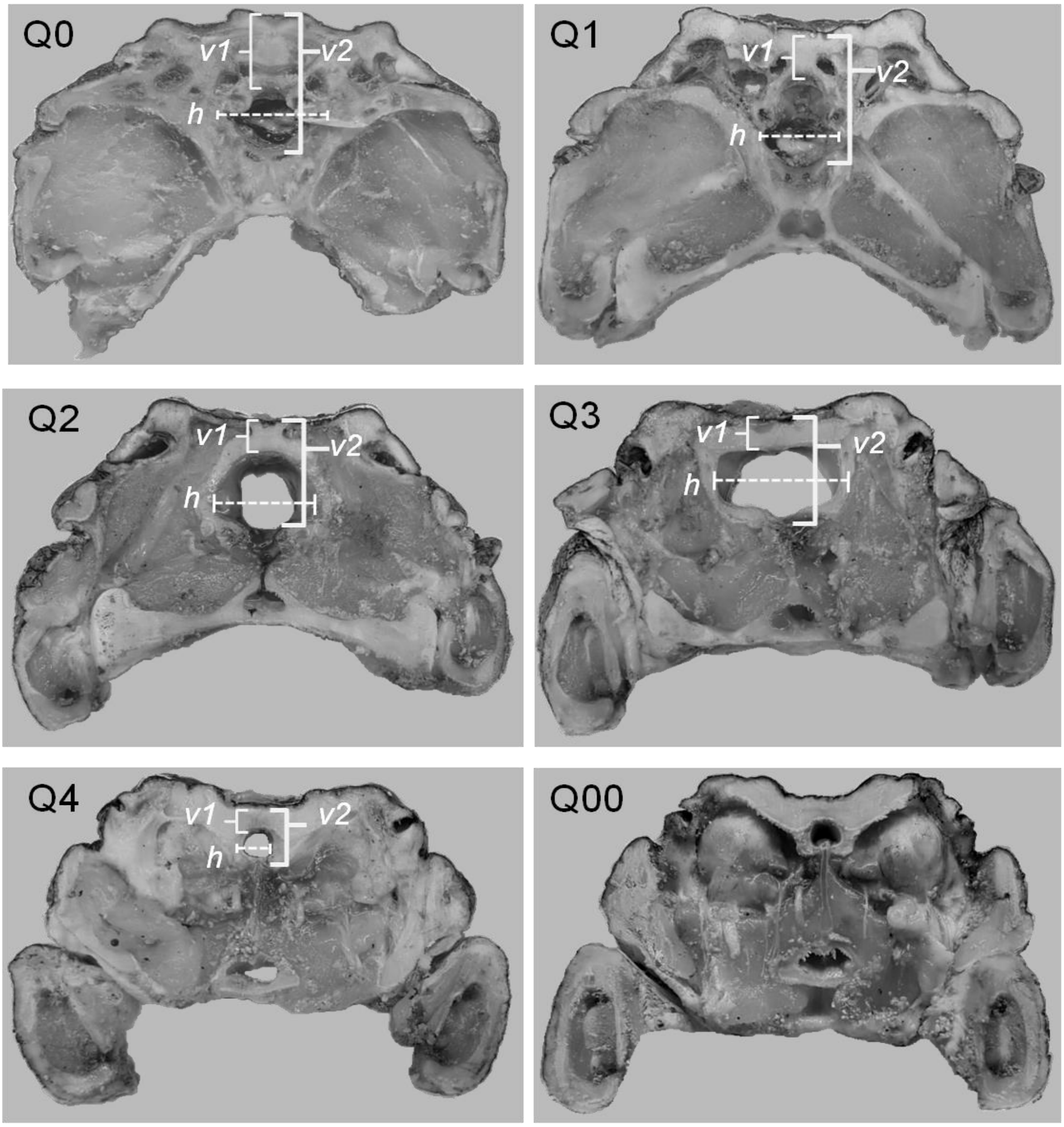

Figure 3.5: Coronal dissection sections with horizontal (h) and ventral (v1, v2) measurements of the cranial cavity. Q0, quarter 0 (caudal); Q1, quarter; Q2, quarter 2; Q3, quarter 3; Q4, quarter 4; Q00, quarter 00 with no measurements (rostral). v1= skull thickness, v2 – v1= height of the cranial cavity, h= width of cranial cavity.

Table 3.1: Sagittal and coronal anatomical cranial measurements.

Cranial Sites	Measurements per crocodile (mm)													Mean ± SD
Sagittal dissections (Figure 3.4)														
	S1*	**S2***	**S3**	**S4**	**S5**	**S6**	**S7**	**S8**	**S9**	**S10**	**S11**	**S12**	**S13**	
A-M	34.2	36.37	35.19	37.69	42.44	41.19	43.92	38.67	40.05	41.46	38.05	38.36	33.59	38.55±3.19
A-D	8.56	9.09	8.80	9.42	10.61	10.30	10.98	9.67	10.06	10.36	9.51	9.59	8.40	9.64±0.80
A-B	17.63	15.84	14.69	18.64	18.36	16.02	16.98	17.22	15.61	16.77	16.12	17.45	15.27	16.66±1.19
A-C	23.43	25.59	23.91	24.66	27.24	23.95	25.50	24.15	21.61	24.49	10.92	24.36	0	21.52±7.58
D-E	9.51	9.54	9.94	12.73	7.59	14.36	9.39	9.39	8.24	8.74	7.68	10.70	11.82	9.97±1.99
D-F	25.05	28.92	26.74	27.11	25.76	26.76	27.90	26.99	24.51	28.08	26.93	26.95	22.67	26.49±1.65
G-H	6.81	6.98	7.79	7.96	5.48	6.89	6.48	7.62	5.02	5.95	5.43	7.14	4.86	6.49±1.05
G-I	20.74	23.66	23.65	23.37	21.47	22.57	24.38	22.81	19.90	22.69	22.90	22.82	23.21	22.62±1.24
J-K	3.72	5.65	4.47	5.02	4.37	4.69	4.92	5.01	3.93	3.835	4.77	4.79	3.36	4.50±0.64
J-L	20.04	23.85	22.74	20.78	18.02	21.36	19.88	22.39	16.83	18.77	19.57	20.69	15.10	20±2.43
M-N	4.41	5.18	4.63	5.24	5.37	4.61	5.45	5.49	4.35	5.24	4.92	5.15	4.29	4.95±0.44
M-O	10.41	11.99	10.96	10.70	9.79	10.24	10.56	11.11	8.68	9.81	8.35	9.88	8.67	10.09±1.06
Coronal dissections (Figure 3.5)														
	C1	**C2**	**C3***	**C4***	**C5**	**C6**	**C7**	**C8**	**C9**	**C10**	**C11**	**C12**	**C13**	
Q0/V1	8.77	16.08	14.75	13.05	13.03	12.90	0	0	16.17	16.10	14.86	0	0	9.67 ±6.98
Q0/V2	16.7	27.92	19.45	23.14	20.69	18.90	0	0	23.99	22.26	0	0	0	13.31 ±11.27
Q0/h	12.29	15.55	7.47	10.34	11.21	10.73	0	0	12.54	11.88	0	0	0	7.08 ±6.08
Q1/V1	9.94	8.34	6.78	9.66	8.08	8.83	8.68	11.95	9.13	9.75	8.86	9.86	10.03	9.22 ±1.23
Q1/V2	26.22	26.04	20.81	23.28	23.48	24.28	24.9	27.28	26.42	26.03	27.05	27.18	0	23.31 ±7.25
Q1/h	12.58	14.13	11.52	12.02	12.72	12.26	12.32	12.70	13.05	11.74	13.30	12.26	0	11.58 ±3.55
Q2/V1	7.63	5.76	5.13	6.5	6.06	5.68	6.21	7.13	6.32	7.63	7.26	6.45	7.3	6.54 ±0.79
Q2/V2	21.8	22.19	20.72	23.24	19.45	18.25	19.59	21.31	21.25	23.16	22.02	25.47	23.22	21.67 ±1.92
Q2/h	13.06	19.58	17.24	17.06	13.39	13.82	14.1	14.55	13.75	12.33	13.55	16.5	15.79	14.98 ±2.10
Q3/V1	7.27	4.02	3.37	4.49	4.05	4.33	4.05	5.05	3.78	4.91	4.95	3.6	4.12	4.46 ±0.99
Q3/V2	20.81	16.08	13.75	15.76	20.54	15.55	18.9	18.75	19.65	22.93	21.93	21.18	21.24	19.01 ±2.86
Q3/h	13.81	23.66	19.97	21.61	21.84	22.41	23.3	22.39	22.56	20.88	22.8	21.72	21.26	21.40 ±2.49
Q4/V1	4.97	4.27	3.71	4.13	3.77	4.62	4.7	4.76	4.17	3.93	3.92	3.87	5.4	4.32 ±0.52
Q4/V2	9.56	9.68	7.25	8.96	10.34	8.81	9.04	9.95	10.33	10.98	11.16	12.71	10.58	9.95 ±1.34
Q4/h	7.37	6.62	4.66	5.64	7.88	6.09	7.25	6.86	8.47	11.33	9.23	11.28	7.82	7.73 ±1.98

*****Crocodiles used for MRI
n= 13 sagittal crocodile head dissections (S1-S13), n= 13 coronal crocodile head dissections (C1-C13), n= 26 total crocodile head dissections
A-M: length of the cranial plate. A-D: length of each quarter of the cranial plate. A-B, D-E, G-H, J-K, M-N: Skull thickness. A-C, D-F, G-I, J-L, M-O: length from the exterior dorsal side of the cranium to the ventral side of the brain cavity of each quarter of the cranial plate.
Q0: Caudal region of the cranium/ neck. Q1-Q4: Cranial plate in quarters (Q1= quarter 1, Q2= quarter 2, Q3= quarter 3, Q4= quarter 4). Q00: Rostral region of the cranium. V1: skull thickness. V2: exterior dorsal point of the cranium to the ventral point of the cranial cavity (V2 – V1 = height of cranial cavity). h: width of cranial cavity

3.1.3 Identification of External Skull Landmarks

External cranial landmarks (Figure 3.6) were measured and the individual and mean (SD) measurements recorded for all 26 cadaver heads prior to dissections (Table 3.2). The brain is positioned between cranial points IV-V and the olfactory bulb is positioned between points II-IV (Figure 3.6a). The cranial plate overlays the brain (Figure 3.6b), the posterior end of the frontal bone (i-ii), temporal bone (ii-iii and iv-i) and the parietal bone (iii-iv).

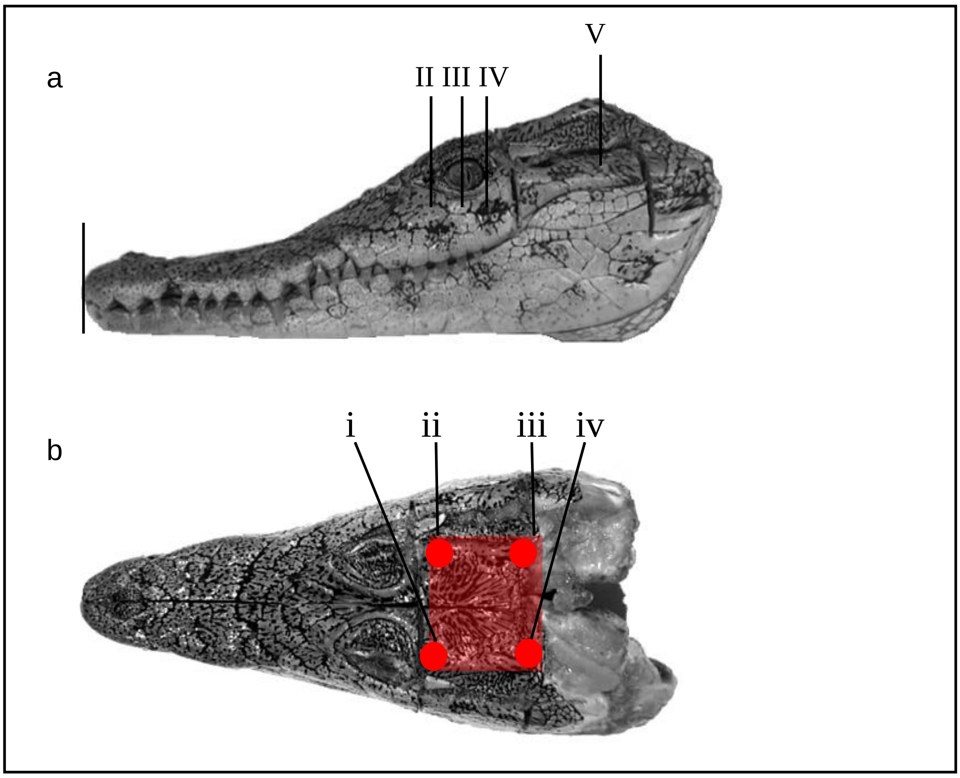

Figure 3.6: a) Lateral view of the head with external landmarks II to V and b) dorsal view of the head with external landmarks of the cranial plate i to iv of *C. niloticus*. a) **I-V: Total length of cranium. II: Rostral point of the eye. III: Mid-point of the eye. IV: Caudal point of the eye. IV-V: Length of the cranial plate. b) Area of the cranial plate. i-ii, iii-iv: Width of the cranial plate. ii-iii, iv-i: Length of the cranial plate**

Table 3.2: External landmark measurement (Figure 3.6).

Cranial Sites	Measurements per crocodile (mm)													Mean ± SD
Lateral view														
	S1*	**S2***	**S3**	**S4**	**S5**	**S6**	**S7**	**S8**	**S9**	**S10**	**S11**	**S12**	**S13**	
I-II	120	123	115	121	130	125	123	124	114	126	118	125	102	120.46±7.11
I-III	131.5	140	127.5	134	144.5	137.5	138.5	137	126	139	131.5	137	114	133.69±7.85
I-IV	143	158	140	147	159	150	154	150	138	152	145	149	126	147±8.91
I-V	185	202	185	190	197	196	199	192	171	194	180	187	158	187.38±12.20
	C1	**C2**	**C3***	**C4***	**C5**	**C6**	**C7**	**C8**	**C9**	**C10**	**C11**	**C12**	**C13**	
I-II	111	115	84	107	100.53	106.95	111.82	121.04	113	121	111	114	114	110.03±9.19
I-III	123	130.5	100	122.5	113.78	119.53	125.29	133.44	126	134.5	123.5	126.5	125.5	123.39±8.92
I-IV	135	146	116	138	127.03	132.11	138.76	145.84	139	148	136	139	137	136.75±8.47
I-V	180	184	148	164	160	170	173.32	183.32	178	193	175	183	180	174.74±11.90
Cranial plate														
	S1*	**S2***	**S3**	**S4**	**S5**	**S6**	**S7**	**S8**	**S9**	**S10**	**S11**	**S12**	**S13**	
i-ii	45.42	48.68	42.48	42.92	50.1	43.69	46.78	46.98	42.05	45.97	41.74	43.87	39.69	44.64±2.99
ii-iii	33.7	37.43	34.43	37.89	41.21	39.67	48.07	36.97	34.06	40.74	37.57	37.84	33.59	37.94±3.98
iii-iv	46.71	51.27	48.46	49.97	58.61	54.04	52.88	52.04	48.26	54.91	49.18	51.95	43.81	50.93±3.83
i-iv	34.7	35.31	35.95	32.49	43.66	42.7	39.77	40.36	46.04	42.17	38.53	38.87	33.59	38.78±4.18
i-iii	57.29	61.35	56.25	59.12	68.67	63.03	62.65	63.76	56.93	65.03	58.14	61.32	53.82	60.57±4.12
ii-iv	57.29	61.82	56.87	60.63	68.7	62.6	63.26	62.75	56.76	65.21	59.28	60.61	52.74	60.66±4.15
	C1	**C2**	**C3***	**C4***	**C5**	**C6**	**C7**	**C8**	**C9**	**C10**	**C11**	**C12**	**C13**	
i-ii	40.91	42.6	37.31	42.19	38.2	41.75	42.39	46.51	41.59	42.52	39.13	41.13	41.73	41.38±2.29
ii-iii	35.8	33.84	26.92	31.03	33.9	36.1	37.74	39.86	35.1	37.16	35.12	34.82	35.71	34.85±3.18
iii-iv	48.44	47.47	38.18	45.52	45	47.81	48.76	50.72	48.99	49.5	44.37	47.96	45.36	46.78±3.21
i-iv	35.87	34.25	26.37	29.41	34.7	35.99	38.43	39.34	36.49	37.06	35.05	37.25	38.43	35.28±3.66
i-iii	56.99	56.32	45.27	52.25	53.6	57.11	58.56	62.89	57	58.6	54.42	55.95	56.44	55.80±4.09
ii-iv	56.99	56.42	46.44	53	54.52	55.95	59.08	62.85	56.7	59.22	54.13	57.15	58.07	56.19±3.87

*Crocodiles used in MRI

n= 13 sagittal crocodile head dissections (S1-S13), n= 13 coronal crocodile head dissections (C1-C13), n= 26 total crocodile head dissections

Lateral view: I-V: Total length of cranium, II: Rostral point of the eye, III: Mid-point of the eye, IV: Caudal point of the orbital, IV-V: Length of the cranial plate

Cranial plate: i-ii: Width of the cranial plate, iii-iv: Width of the cranial plate, ii-iii: Length of the cranial plate, iv-i: Length of the cranial plate

3.1.4 Establishment of EEG Electrode Placement Sites

EEG electrode placement sites and appropriate quantity of electrodes were determined using sagittal and coronal measurements (Tables 3.1), and the MRI scans (Figures 3.1 through 3.3).The smallest mean of the horizontal length (h) (Table 3.1 and Figure 3.5) where found in Q3 and Q4, which represents where skull thickness is the thinnest (brain closed to the surface of the skull). Skull thickness was taken from points A- B, D- E, G- H, J-K and M-N from Figure 3.4 and V1 from Figure 3.5 (Table 3.1). The size of the brain can be taken from points B- C, E- F, H- I, K- L and N- O from Figure 3.4 and horizontal length of the brain cavity (h) from Figure 3.5.Based on skull measurements the smallest means (skull thickness at its smallest) were found from point G-H and J- K, and the largest mean for the brain cavity was found from point H- I and K- L (largest portion of the brain where brain activity would be picked up by the EEG). The skull thickness dorsal to the brain, was shown to decrease from the caudal to rostral sides of the brain cavity. The mass of the brain is located on the rostral half of the brain cavity between Q 3 and Q 4 sections of the cranial plate. The observations made from the MRIs aided in the identification of the external landmarks and EEG electrode placement sites. Seven EEG electrode placement sites were identified (Figure 3.7). The number of EEG electrodes was limited by the size of the cranial plate of the crocodiles (Figure 3.6 and Table 3.2).

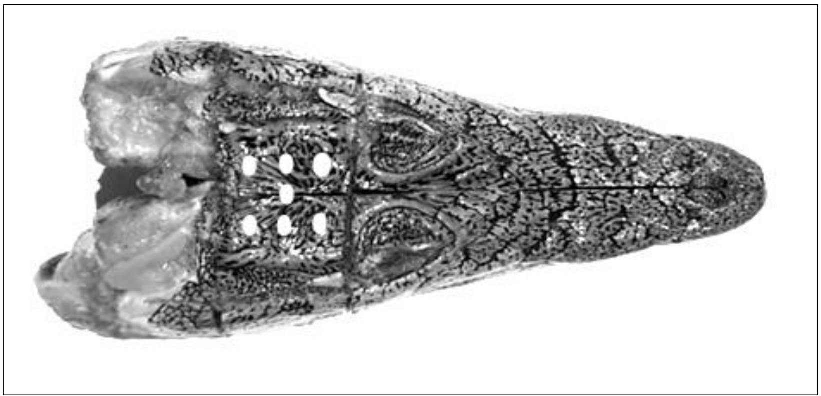

Figure 3.7: EEG electrode placement sites. The two EEG electrode placement sites (white dots) on the rostral side on the cranial plate are the neutral and reference electrodes. The remaining 5 EEG electrode placements sites (white dots) are the EEG channels 1-5.

3.2 EEG Pilot Investigations

3.2.1 Introduction

The EEG assessment procedure was established in crocodiles from communal pens using electrical stunning as per standard farm operating practice and equipment. Two pilot studies were conducted on a commercial crocodile captive breeding farm (Le Croc farm, Brits, North West, South Africa) over 3 different days. For both studies, crocodiles were captured manually from a communal enclosure and transferred to an experimental house that was adjacent to the room where all experimental procedures were performed.

3.2.2 First Pilot Study

The first pilot study occurred in two phases and was conducted using 10 crocodiles of 24-26 months of age and ranging from 148 cm to 172cm in length. The objective of the first phase was to observe the electrical stunning procedure employed by the farming operation to identify behaviour indicators for an effective stun and to place EEG electrodes. During the second phase the EEG equipment was tested.

For the first phase (n= 10) crocodiles were e-stunned in the experimental pens for 4 sec on the dorsal surface of the neck immediately behind the frontal bone, while in the water pond. The e-stunner settings were recorded at an average current reading of 0.8 amps, potential difference reading of 171 V and delivered at a frequency of 50 Hz. The behavioural observations (Table 3.3) post-stunning were recorded which supported that the crocodiles were effectively stunned, as confirmed by an experienced e-stunner operator of the farm where the pilot study was undertaken.

Table 3.3: Behavioural observations during the pilot studies during and post-stunning.

	Stun Site	During e-stunning	Post-stunning 0-1 min	1-2 min	> 2min
1st Pilot Study, Phase 1 (10 crocs)	Neck	Head up, muscle contraction, tail thrash	Body stiff, forearms extended, hind legs along body, floated to surface of the water. (1/10)*blinked,		
1st Pilot Study, Phase 2 (10 crocs)	5 Neck	Head up. (1/5) body stiff		(2/5) head lowered + started twitching	(1/5) pedal reflex >9min
	5Cranial plate	Head up	(1/5) head lowered to normal	(2/5) head lowered to a normal position+ started twitching.	(1/3) tail + back leg twitch
2nd Pilot Study (4 Crocs)	Neck	Head up. Tail thrashing.	(3/4) slight tail twitch +eyes open (1/4) blinked	(2/4) head lowered + eyes closing (1/4) eyes open (1/4) tail twitching	(1/4) 3min no movement, 5min head still elevated. (1/4) 3min tail curled then straightened, eyes open but nonresponsive, 9min almost jumped off table. (1/4) 7min no movements, 10min head still up. (1/4) 10 min, toes sprawled

x (y*)= number of crocodiles observed / total number of crocodiles*

After stunning, the crocodile snout was taped and the eyes covered and carried to the adjacent procedure room and placed on a low work bench. An analgesia was planned to be injected subcutaneously but due to the skin being attached to the skull, it was unsuccessful as there was no absorption site. Following capture, 7 stainless steel surgical sutures were placed under the skin (between the skin and skull) around the external cranial vault. Once the steel sutures were placed under the skin, the ends were joined together. This served as an attachment site for the 7 clip EEG electrodes. Four electrodes were placed at each corner of the cranial plate and one was positioned in the centre of the cranial plate. Two electrodes were then placed on the nose, approximately 2 cm cranial to the eye on each lateral aspect. The left nose electrode was the neutral electrode and the right nose electrode was the reference electrode. A referential montage was used which meant that all five electrodes of the cranial plate used one common reference electrode (electrode on the nose) to record the EEG per channel (measuring electrode to reference electrode). On completion of phase one, two crocodiles were placed in each pen (*n* = 5 pens) and allowed to recover for 7 days. The immobilisation of the crocodiles lasted for an average of 18.2 minutes (minimum 13 minutes and maximum 28 minutes).

In the second phase (n= 10) crocodiles were randomly selected from the individual experimental pens, manually captured, restrained, blindfolded and injected intramuscularly with 0.5 ml of a neuromuscular blocker, gallamine triethiodide (Flaxedil, 40mg/ml). The purpose of the neuromuscular blocker was an endeavour to obtain a normal resting EEG recording without muscular movement artefacts during the pre-stun recording period. After injection of the neuromuscular blocker at least 30 minutes was allowed for the drug to act and then the drug-immobilised crocodile was carried to the procedure room and placed on a low work bench and the steel suture clips examined. Where the surgical steel sutures had fallen out, 22 Gauge hypodermic needles (51mm length) were subcutaneously placed for the attachment of the clip electrodes. The mobile EEG recorder (Sleepwalker, Lifelines Ltd) was connected to the respective electrodes via a cable umbilicus custom designed to allow rapid disconnection and reconnection. Adam Kokkas at SSEM Mthembu Medical customed designed the cable to have a female and male connection between the end of the cable and the EEG device to allow the rapid disconnection and reconnection. A laptop (Inspiron, Dell) was coupled to the mobile EEG recorder to visualise the live EEG recordings using compatible software (TrackIt version 2.8.0.12; Lifelines Ltd). An experienced electroencephalographer operated the laptop and mobile EEG recorder for all recordings and an experienced veterinary anaesthesiologist assessed the raw recordings and did the post data collection evaluation of the EEGs.

First, an electrode impedance check was done to ensure it was less than 16 kΩ, and then a pre-programmed referential montage was opened to allow visual inspection and recording of the raw EEG data for all five channels at a sampling rate of 200Hz. If the tracing appeared acceptable then a seven-minute pre-stun recording was done. For stunning, the crocodiles were randomly allocated to be stunned by either applying the e-stunner (home-made 50Hz stunner) to the cranial plate (n = 5) or the dorsal surface of the neck (n = 5). Once the pre-stun EEG recording was obtained, the electrode cable umbilicus was disconnected and the crocodile stunned by an experienced stun operator applying the e-stunner wand electrodes to the allocated site; and discharging 160 V for 4 sec, which achieved an average of 0.33 amps as read from an attached digital reader. Immediately after the stun, the EEG cable umbilicus was reconnected to record the EEG for another 7 minutes. The behavioural indicators were also assessed and recorded but not included in the final discussion, used as a way to establish the methodology. After the procedures, the sutures and needles were removed and crocodiles returned to the individual experimental pens. Crocodiles were injected intramuscularly with 0.2 ml of neostigmine methylsulphate (2.5 mg/ml) as a neuromuscular blocker antidote and an 0.5 ml of meloxicam (Metacam 5mg/ml, 0.1 mg/kg; Boehringer Ingelheim) as an anti-inflammatory and analgesic to reduce immediate pain following the procedure. The crocodiles were returned to the commercial communal pen, after they were suitably responsive and adequately recovered as judged by the resident veterinarian.

The results of the first pilot study (phase 2) indicated that crocodiles stunned on the cranial plate were not successfully stunned (insufficient electrical current and no behavioural signs of immobilisation). Therefore, it was opted to discontinue any further investigation of this site of application in the main study. The results of the EEG data analysis were inconclusive and contrary to what were expected. Most recordings (n = 6) had many artefacts that could not be corrected through applying filters (pass band and notch filters) or through normalising the baselines. Furthermore, the average recorded amplitudes were often less than 10 μV in all five channels which made visual inspection of raw and filtered recordings a challenge.

Furthermore, when PSD was applied to various length epochs in the pre-stun and post-stun EEG recordings it became evident that the crocodiles had predominantly delta waves during the pre-stun period and predominantly alpha and beta waves during the post-stun period. The EEG analysis suggested that the crocodiles were in a relaxed and sleepy state during the pre-stun period and that the stunning process woke them up, rather than inducing a state of unconsciousness. It was speculated that the predominant delta wave pattern during the pre-stun period may have been due to the neuromuscular blocker and the blindfolding of the crocodiles; and that the alpha and beta wave pattern were predominating because of inconsistent e-stunning. The low amplitude of the EEG tracing being consistently less than 10 μV required adjustments and it was decided to incorporate all electrodes on the cranial plate. Therefore, a second pilot study was required to confirm that the proposed adjustments to the electrode positions without the use of the neuromuscular blocker and by removing the blindfold before e-stunning would resolve these issues.

3.2.3 Second Pilot Study

The second pilot trial was performed over one day. Four crocodiles that were transferred to the experimental pens the day before the study were manually captured, restrained by taping the snouts and eyes were covered and brought to the procedure room and placed on the low work bench. While restrained, seven 22G hypodermic needles of 51mm length were inserted subcutaneously in a similar pattern to the first pilot study, with the exception of the two nose electrodes which were also placed on the rostral margin of the cranial plate. The EEG cables were connected to the respective electrodes (subcutaneous needles) and coupled to the mobile EEG recorded and laptop (Inspiron, Dell). The electrode impedance was checked and if a stable EEG tracing was obtained from the five channels the blindfold was removed and a 5-minute pre-stun EEG recording was obtained. Thereafter, the EEG device was disconnected, and crocodiles were stunned on the neck behind the cranial plate for 4s at160V. After stunning the EEG device was immediately reconnected, and EEG recordings were captured for seven minutes. After completion all needles were removed, and the crocodiles were returned to the experimental pens to recover fully before being returned to the communal pen. An anti-inflammatory was not administered as this method was not as invasive as the method in 3.2.1. The behavioural observations (Table 3.3) post-stunning were recorded. The outcome of the second pilot study was encouraging, whereby very stable EEG recordings were obtained with larger wave amplitude and most of the recordings were artefact free. An expected wave pattern shift in power from the pre-stun to the post-stun period in two of the crocodiles was also detected. However, the e-stunner current was inconsistent, and the average amp reading was too low (0.38 A). Therefore, the researchers opted to obtain a research purpose-built e-stunner where the wand was also specifically modified to improve conductivity during stunning.

3.3 Conclusions

The MRI and anatomical dissection studies and the two pilot studies were used as preparatory investigation for the main EEG study. These studies confirmed:

a. The external sites and technique for the placement of the EEG electrodes to obtain a good quality raw EEG tracing of adequate amplitude without excessive artefacts and interference.
b. Behavioural indicators that can be used to evaluate the affectivity of e-stunning clinically for crocodiles in the final procedure.
c. That the EEG PSD analysis should demonstrate an increase in delta band power and a decrease in alpha band power after an effective stun to indicate a state of unconsciousness.

The effective performance of the purpose built stunned (fully describe under 4.3.2) was tested and confirmed using a custom built stun tester with a resistance of 220 Ωand on 40 crocodiles that were part of a routine slaughter carried out on the farm.

CHAPTER 4
MATERIAL AND METHODS

4.1. Model System

An open study design was performed in captive-bred grower *C. niloticus*.

4.2. Study Site and Facilities

The study was performed on an intensive commercial crocodile captive breeding farm (Le Crocodile farm, Brits, North West Province) that uses e-stunning routinely for animal capture and restraint and during slaughter. The study was performed at a time in which the environmental temperature was between 24^0C and 31^0C.

4.3. Experimental Design and Procedures

4.3.1 Animals

Fifteen healthy *C. niloticus* growers, 30 - 32 months of age, 143.4 – 159.9 cm total body length (TL) were randomly selected by farm management from a group of growers that were housed in a closed, thermal controlled communal pen at 27-30°C. 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 EEG recordings. The crocodiles were placed in 3 indoor pens (5 x 5 m in size) of 5 crocodiles each that contained a central water pond (0.5 m deep and half the size of the pen) in each.

4.3.2 Equipment

The EEG device was selected because it is portable, light and easy to work with. It has software that makes it possible to view live EEG recordings to ensure clean raw data was being collected.

Figure 4.1: Portable sleepwalker EEG machine.

A plastic 3D printed mould (Cavity Creation) was designed for the purpose of this study by a private company, Cavity Creation, based on findings from Chapter 3. It was made to fit on the cranial plate and to securely hold hypodermic needles for the attachment of EEG clip electrodes during the EEG assessment phase (Figure 4.2). The hypodermic needles were placed in position and then the tip (6 mm) was bent to form a 90-degree angle which improved the contact on the scale surface (not inserted subcutaneously). The positions of the electrodes were: four electrodes were placed at the caudal and rostral margins of the cranial plate; one was placed in the centre of the cranial plate; the left rostral electrode was the neutral electrode and the right rostral electrode was the permanent reference electrode. A five-channel referential montage was pre-programmed into the software and was used to visualise the live recording during the procedures.

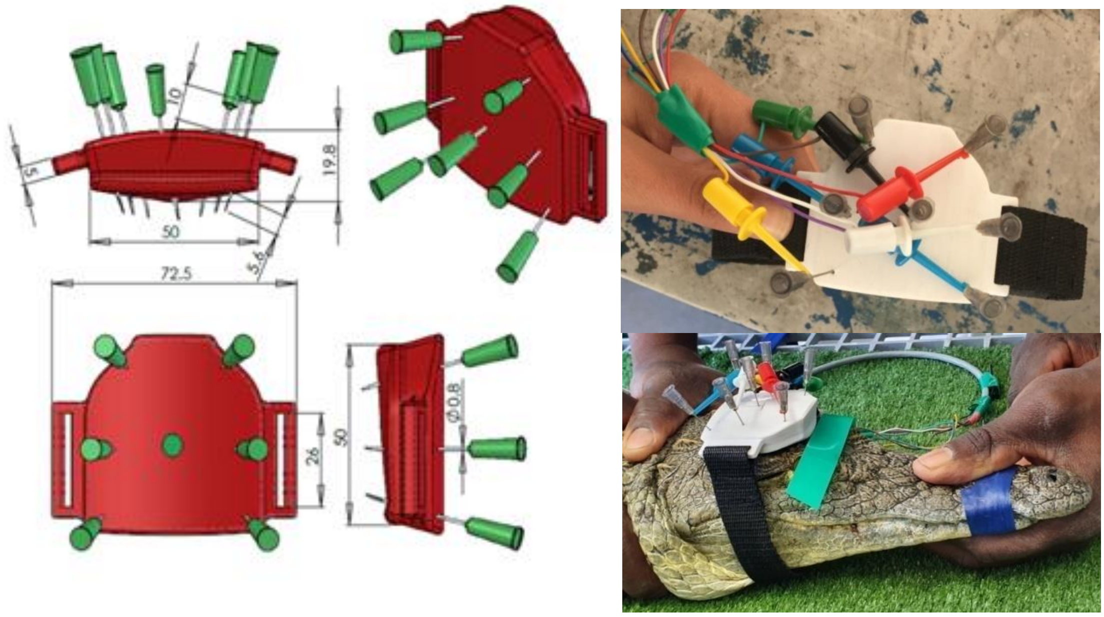

Figure 4.2: Plastic injection mould with hypodermic needles design, attached EEG clip electrode and placement on crocodile.

A research purpose-built e-stunner (I'Vimbi technologies) was custom built for this part of the study. The e-stunner included a stunning controller referred to as the stunner and stunning apparatus referred to as stunning wand (Figure 4.3).

The stunner was mains power operated and included the following features:

- A main power socket and electrical cable that was plugged into a 220V, 50Hz wall plug;
- A volt variable transformer (variator) from 0 – 260 volts;
- A main isolator switch that isolated the power from the socket outlet;
- A power-on light that illuminated when the main isolator is on;

- A stun-on light that illuminated when stunning is in progress;
- A stun timer that selected time required for stunning, adjustable in seconds with a digital count-down and adjusted using various selector switches on the instrument;
- A timer – off bypass which can switch off the timer instrument;
- A variable volt-out controller that was used to set the e-stunning volt selected by turning the hand-wheel;
- A data logger that indicated the value of the volts and amperes and has a menu which was used to set the preferences;
- A data USB port used to connect with a computer to download data in memory or when doing a live recording; and
- A digital volt/amp meter that indicated the actual volts and amperes during e-stunning.

Figure 4.3: E- stunner used in the EEG assessment phase.

The stunning wand was used to apply the electrical current to the crocodile. It was a hand-held plastic pole of approximately 1.4 m long with a push-button at the top, v-shaped stunning electrodes at the tip of the wand and a digital volt/amp meter fitted on the side. The push button activated the stunning sequence. It continued for the duration that the timer had been set. The stunning electrodes were the contact points that complete the electrical circuit. When the electrodes are applied on the crocodile there is a conductive resistance. Metal buttons (three on each electrode) were included to improve contact and electrical conductivity when applied to the crocodile. The digital volt/amp meter indicated to the operator that the stunning sequence had been activated and that the system is live.

4.3.3 Data Collection

On the day of the experiment, crocodiles were manually captured, one at a time, the snouts immediately taped and blindfolded. The captured crocodile was carried to a procedure room immediately adjacent to the experimental enclosure, placed on a low work bench and physically restrained by two animal caretakers, one holding the neck and the other the tail. Before the start of the EEG procedure the total body length and head length of each crocodile was measured. The custom-built EEG electrode mould was Velcro strapped to the head of the crocodile and the bent needle tips were pushed against the skin by fitting a foam filled lid on top of the mould and secured in place using additional Velcro straps. Then the electrode cables were connected to the mobile EEG recorder and laptop configuration, as described above. The raw tracing was visually inspected 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 five EEG channels (Ferree *et al.,* 2001). Once a stable tracing was obtained, the blindfold was removed from the crocodiles and a pre-stun EEG recording commenced for five minutes at a 200Hz sampling rate. Following the recording, a patient event (time tag used to identify events on the EEG recording software) was used to mark the time on the EEG recording when the electrode cable umbilicus was disconnected, and the crocodile was prepared for e-stunning.

The volt-out controller of the Stunner was set at 170V and the timer set at 5 sec stunning period for the first three crocodiles and 7 sec 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,

The neck and head region of the crocodile was then doused with water and the e-stunner wand electrodes were positioned at a 45 – 50 degree angle, on the dorsal surface of the neck immediately behind the frontal 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 electrode cable umbilicus was reconnected and a patient event was used to mark the time when the EEG recording continued for seven minutes during the post-stun period. After completion of the post-stun the EEG electrodes and electrode mould were removed, the crocodile weighed and returned to their experimental pen. The next crocodile was captured and brought to the procedure room. This same procedure was followed until all 15 crocodiles were examined. Crocodiles were returned to their communal pen following a recovery period of at least 1 hour.

Throughout the procedure, for each crocodile the principle investigator monitored and recorded behavioural indicators post-stunning and a second investigator made digital video recordings of each stunning to evaluate after the data collection period.

4.4. Data Analysis

4.4.1 Animal Data

A tape measure was used to measure the length (cm) of the head and total body length of each crocodile following manual capture and after they were blindfolded. Following the completion of post-stun EEG recordings, the crocodiles were weighed (kg) using a hang mat and a portable hanging scale.

4.4.2 E-Stunning Data

The data logger data recorded during e-stunning was downloaded to a laptop. The volts, amps and hertz were captured per second for the duration of the stun for each crocodile. The area under the curve (AUC) for amperes versus time was calculated using the Trapezoidal method ($AUC_{1\text{-}n}=\sum\{(\frac{Cp1+Cp2}{2})(t2-t1)\}+\{(\frac{Cp2+Cp3}{2})(t3-t2)\}+\cdots$) where Cp is the amperes and t is time (seconds). The purpose was to calculate to the amperes load delivered to each crocodile.

4.4.3 Crocodile Behaviour Data

The behavioural indicators of each crocodile after e-stunning was documented and assessed against previously reported behaviour signs following e-stunning. Furthermore, the behavioural indicators were assessed in conjunction with the post-stun EEG recordings to determine if there are behavioural cues that indicate unconsciousness.

4.4.4 EEG Recording Data

Raw EEG recordings, consisting of five channels per recording, were uploaded to an open source software (Brainstorm software package, freely available for download online under the GNU general public license at http://neuroimage.usc.edu/brainstorm) (Tadel*et al.*, 2011) for processing and analysis. The raw EEG recordings were reviewed manually for signal artefacts and excessive movement away from the isoelectric line. A band-pass filter (1 Hz and 35 Hz) was applied to the raw EEG tracing and was reassessed visually to detect the three best performing channels out of the five. If at least three channels could not be identified because of poor signal quality, low amplitude (<8 μV), excessive movement artefacts or excessive shifting away from the isoelectric line then the entire crocodile EEG recording was discarded and not analysed further nor included in the statistical analysis (Davis *et al*., 2018). Crocodile 2, 3 and 5 were removed from the study because the EEG recordings could not be analysed. The patient event markers were used to identify the pre-stun and post-stun time periods on the EEG recording.

A series of seven 4-second-long epochs were identified on stable, artefact free EEG recordings and marked, as follows: 1 pre-stun epoch (period 0); 1 post-stun epoch (period 1) identified within 1 – 7 sec after reconnecting the electrode cable umbilicus and 5 additional post-stun epochs (periods 2 – 6) exactly every 60 sec after the start of the first post-stun epoch. The seven epochs were imported into the software database for further analysis. First, each epoch was visually inspected for stability and to ensure they were artefact free. Then the three-channel epoch was standardised by applying an average reference montage. The three-channel epochs were then analysed using the Welch method of power spectral density analysis (time window: 4 sec; Hamming window length: 0.5 sec; window overlap ratio: 50%; decomposition into delta [2 – 4 Hz], theta [5 – 7 Hz], alpha [8 – 14 Hz] and beta [15 – 30 Hz] frequency bands). The three spectrum graphs were normalised and then averaged using root mean square averaging. The final averaged PSD analysis was used to obtain power values ($\mu V^2/Hz$) for each frequency band of interest for each epoch (Levy & Warren, 1987). The total power was calculated by adding all frequency band powers. The percentage of power within each frequency band was calculated by dividing the power within the band by the total calculated power and converted to a percentage. Additionally, a 40 second epochs were extracted from the filtered EEG recording during the pre-stun (40 sec before disconnecting electrode cable umbilicus) and post-stun (40 sec after reconnecting the electrode cable umbilicus) periods for visual comparisons and to identify tonic-clonic seizure activity.

The PSD data were assessed for normality through plotting of histograms, inspecting descriptive analysis and the Anderson-Darling test for normality. Data were non-parametric and therefore reported as median (interquartile range). The pre-stun power within each frequency band was compared to post-stun power using Kruskal-Wallis test. Crocodiles with EEG data that demonstrated a post-stun increase in delta power and a decrease in alpha power and showed good behavioural indicators of being stunned effectively were considered unconscious. The PSD analyses were compared and matched descriptively to their behavioural indicators that were recorded and interpreted with the aid of re-viewing the digital video recordings. Data was analysed using commercially available software (MiniTab 18.1; MiniTab Inc) and significances interpreted at $p < 0.05$.

4.4.5 Statistical Analysis of Demographic and Electrical Data of Conscious and Unconscious Crocodiles

The demographic data (TL, HL and weight) and electrical data of crocodiles that were evaluated on EEG analysis as conscious and unconscious were compared statistically.Normality and homogeneity of variance was assessed using the respective Shapiro-Wilks and Levene's tests. Pairwise differences were subsequently assessed using Student's t-tests. Statistical analyses were performed using IBM SPSS v26 (IBM Corp, USA).

CHAPTER 5

RESULTS

5.1. Crocodile Size Measurements

The total length (TL) and head length (HL) and weight (kg) of all crocodiles are summarised in Table 5.1. Crocodiles used in the study were uniform in TL, HL and weight with a covariance of 3.4 %, 5.2 % and 7.2 %, respectively.

Table 5.1: Individual and mean ± SD total length (TL), head length (HL) and weight of the crocodiles.

Crocodile	TL (cm)	HL (cm)	Weight (kg)
1	159.8	21.8	12.2
4	144.3	18.5	10.7
6	149.1	18.8	11.0
7	154.0	20.0	12.0
8	155.0	18.5	12.5
9	147.6	19.0	10.3
10	157.0	19.5	11.0
11	147.2	18.6	10.7
12	145.0	18.5	9.8
13	147.5	18.0	11.5
14	154.5	19.5	11.0
15	147.2	19.0	10.8
Mean ± SD	150.7 ± 5.1	19.1 ± 1	11.1 ± 0.8

5.2 Electrical Current Applied to Crocodiles

The electrical potential (V), electrical current (amps) and electrical frequency (Hz) each crocodile received as logged per sec over the duration of stunning are summarised in Table 5.2.The results of two stun tests of the stunner confirmed the expected electrical current using a stun tester with a resistance of 220Ω and the stunner set at 170V. Crocodile1 and 3 were stunned for 5sec. The stun time was increased to 7 sec in crocodiles 4 through 15 as electrical current recorded in these crocodiles were less than the desired current of at least 0.5 amps. Crocodile 2 was stunned for only 3sec due to the handler removing the stunner wand from the crocodile's neck prematurely and was excluded from the study (removed from study as well as crocodile 3 and 4 due to inability to analyse EEG recordings).

The volt delivered to the crocodiles (171.3 ±1.75 V) was consistent with the volts set (170 V) for stunning and varied on average <1 %. The highest volt average, 174V, was recorded for Crocodile 14 and the lowest average volt, 168.89V, was recorded for Crocodile 1 (Table 5.2). An electrical frequency of 49.8 – 50.1 Hz was recorded.

Table 5.2: Volts, amp range, hertz and duration of stun logged for stunner test and for each crocodile.

Stun test & Crocodile No.	Volt Ave ± SD	Amp Range	Amp Range Ave ± SD	Hz	Stun duration (seconds)
Test 1	167.84 ± 0.13	-	-	50.00	5
Test 2	168.36 ± 0.02	-	-	50.10	5
1	172.48± 0.53	0.23-0,60	0.47 ± 0.16	50.10	5
4	168.89 ± 0.52	0.51-1.04	0.84 ± 0.18	49.90	7
6	169.81± 0.55	0.30-0.95	0.76 ± 0.22	50.00	7
7	169.99 ± 0.42	0.75-1.21	1.03 ± 0.16	50.00	7
8	170.23 ± 0.17	0.87-1.17	1.06 ± 0.11	49.80	7
9	172.09 ± 0.51	0.34-0.91	0.78 ± 0.20	50.20	7
10	169.42 ± 0.59	0.72-1.35	1.12 ± 0.23	50.10	7
11	171.08 ± 0.43	0.94-1.29	1.18 ± 0.14	49.90	7
12	171.90 ± 0.50	0.64-1.15	0.92 ± 0.18	49.90	7
13	173.00 ± 0.58	0.22-0.94	0.72 ± 0.25	50.00	7
14	174.00 ± 0.69	0.48-1.18	0.95 ± 0.24	50.00	7
15	173.88 ± 0.50	0.83-1.32	1.13 ± 0.18	50.00	7
Mean ± SD	**171.4± 1.70**		**0.93 ± 0.21**		

Electrical current (amps) delivered increased over the period stunned and reached the maximum current delivered at the end of the stunning period in all crocodiles (Table 5.2). The total electrical current, as measured by AUC (amp*sec), and maximum electrical current (amp-max), delivered to each crocodile are provided in Table 5.3. Crocodiles that were stunned over 7 sec all had a larger AUC and were delivered a higher amp-max than crocodiles that were stunned for the shorter period (5 sec). The largest AUC (7.13 amp*sec) occurred in Crocodile 11 and the highest amp-max was delivered to Crocodile 15.

Table 5.3: Crocodiles mean ± SD of ampere verse time (AUC), maximum amperes (amp-max) achieved and time to maximum amperes.

Crocodile No.	AUC (Amp *Sec)	Amp-max (amps)	Time to Amp-max (sec)
1	1.92	0.60	5
4	5.13	1.04	7
6	4.69	0.95	7
7	6.22	1.21	7
8	6.39	1.17	7
9	4.82	0.91	7
10	6.82	1.35	7
11	7.13	1.29	7
12	5.57	1.15	7
13	4.43	0.94	7
14	5.79	1.18	7
15	6.85	1.32	7
Mean ± SD	**5.48 ± 1.44**	**1.09 ± 0.20**	**-**

5.3 Behavioural Observations

Identifying behavioural indicators of stunned crocodiles was difficult. Crocodiles were docile and showed little to no signs of distress after being captured manually and during pre-stun EEG recordings. This made identifying signs of immobilisation challenging. Behavioural observations in Table 5.4 were documented for the 15 crocodiles. There were no apparent adverse effects observed in crocodiles resulting from e-stunning.

Table 5.4: Behavioural observations observed during and post-stunning of all crocodiles. (x/12) = number of crocodiles observed /total number of crocodiles (12). Behavioural observations: effectively stunned crocodiles (dark grey), ineffectively stunned crocodiles (light grey) and observations seen in both (white).

During e-stunning	Post-stunning		
	0-1 min	1-2 min	> 2min
(12/12)Arched back. (12/12) Eyes closed. (12/12)Forearms extended initially (12/12) Tail twitches (5/12)limbs along body (3/12) forearms stiff alongside body (11/12) Head up. (2/12) forearms along body, hind legs extended (2/12) body twitches (2/12) toe twitches (2/12) hind leg muscle tremors (1/12) rapid tail twitch (1/12) tail thrash (1/12) head never lifted (1/12) eyes half open (1/12)hind legs stiff along body then extended	(12/12)Hind legs stiff. (5/12) muscle contractions legs "walking motion" (2/12) eyes open (2/12) increase tail twitch (2/12) tail 'wag' (2/12) head slightly lowered (1/12) toes twitch (1/12) subtle hind leg movements (1/12) forearms relax (1/12) reaction to touch (1/12)pupils contract (1/12) tail thrashing	(7/12) legs relax (5/12) head lowered (1/12) eyes open. (2/12) hind legs contracted then relaxed (2/12) tail twitch stop (1/12) blink (1/12) tail twitches (1/12) back relax flat (1/12) toes splayed (1/12) few deep breathes (1/12) body twitch (1/12) neck twitch (1/12)forearms along body (1/12) random body contractions	(1/12) 3min blinked and flicked ears (1/12)3 min subtle tail movements (1/12) 3min head down, toes relaxed (1/12) 3 min very still, eye movements (1/12) 3min toe twitch (1/12) 3min head slightly elevated (2/12) 3-4min no pedal reflex (1/12) 3-4min reaction to touch and movements but 5min no pedal reflex (2/12) 4min eyes relax (1/12) 4min shallow breathes (1/12) 5min shallow breathing (1/12) 6min aware of surroundings (8/12) 5-7min few deep breathes then norm breathing (2/12) 7min hissed when picked up (1/12)7min pedal reflex (1/12) >7min head slightly elevated (1/12) 8min blink (1/12) 8min no pedal reflex (1/12) 8min shallow breathes (1/12) 8min no pedal reflex but thrashed when picked up (1/12) 10min vigorous movements when picked up

5.4 EEG Recordings

Crocodile 2, 3 and 5 were removed from the study because the EEG recordings could not be analysed. Crocodile 2 was stunned for less time than required and had too many artefacts on the EEG recording to allow analysis. Crocodile 3 and 5 had poor quality and low amplitude EEG recordings thought to be because of poor electrode contact on the skin.

Table 5.5 shows the immediate effect of e-stunning on brain activity in the crocodiles. Power spectral density (PSD) in Figure 5.1, shows the overall effect in the crocodiles after e-stunning. An effective stun was identified by an increase in delta activity and a decrease in alpha activity (loss of consciousness). Six out of 12 crocodiles showed signs of an effective stun.

Table 5.5: PSD (µV²/Hz) and relative bandpower (%) for EEG frequency bands (δ,θ,α,β) before (period 0) and immediately after (period 1) e-stunning in the *C. niloticus.*

Croc	Effective Stun	Period	δ (µV²/Hz)	θ (µV²/Hz)	α (µV²/Hz)	β (µV²/Hz)	Total (µV²/Hz)	δ %	θ %	α %	β %
1	Yes	0	0.85	0.75	0.15	0.02	1.77	48.0	42.4	8.5	1.1
		1	1.49	0.18	0.11	0.04	1.82	81.9	9.9	6.0	2.2
4	No	0	0.55	0.29	0.42	0.50	1.76	31.3	16.5	23.9	28.4
		1	0.52	0.95	0.24	0.015	1.73	30.1	55.1	13.9	0.9
6	No	0	1.46	0.15	0.07	0.035	1.75	85.4	8.6	4.0	2.0
		1	1.15	0.46	0.1	0.08	1.79	64.2	25.7	5.6	4.5
7	Yes	0	1.15	0.36	0.18	0.05	1.74	66.1	20.7	10.3	2.9
		1	1.4	0.13	0.12	0.19	1.84	76.1	7.1	6.5	10.3
8	Yes	0	0.87	0.58	0.24	0.038	1.73	50.3	33.6	13.9	2.2
		1	1.1	0.33	0.18	0.16	1.77	62.1	18.6	10.2	9.0
9	Yes	0	0.92	0.36	0.3	0.14	1.72	53.5	20.9	17.4	8.1
		1	1.4	0.14	0.11	0.21	1.86	75.3	7.5	5.9	11.3
10	No	0	1.6	0.44	0.27	0.04	2.35	68.1	18.7	11.5	1.7
		1	0.25	1.3	0.13	0.1	1.78	14.0	73.0	7.3	5.6
11	Yes	0	0.64	0.36	0.68	0.12	1.80	35.6	20.0	37.8	6.7
		1	0.95	0.24	0.18	0.4	1.77	53.7	13.6	10.2	22.6
12	No	0	1.31	0.28	0.22	0.02	1.83	71.6	15.3	12.0	1.1
		1	1.14	0.31	0.11	0.19	1.75	65.1	17.7	6.3	10.9
13	No	0	1.17	0.32	0.22	0.05	1.76	66.5	18.2	12.5	2.8
		1	0.4	0.83	0.33	0.3	1.86	21.5	44.6	17.7	16.1
14	No	0	0.74	0.68	0.28	0.05	1.75	42.3	38.9	16.0	2.9
		1	0.65	0.41	0.21	0.55	1.82	35.7	22.5	11.5	30.2
15	Yes	0	0.68	0.78	0.23	0.06	1.75	38.9	44.6	13.1	3.4
		1	1.41	0.18	0.06	0.12	1.77	79.7	10.2	3.4	6.8

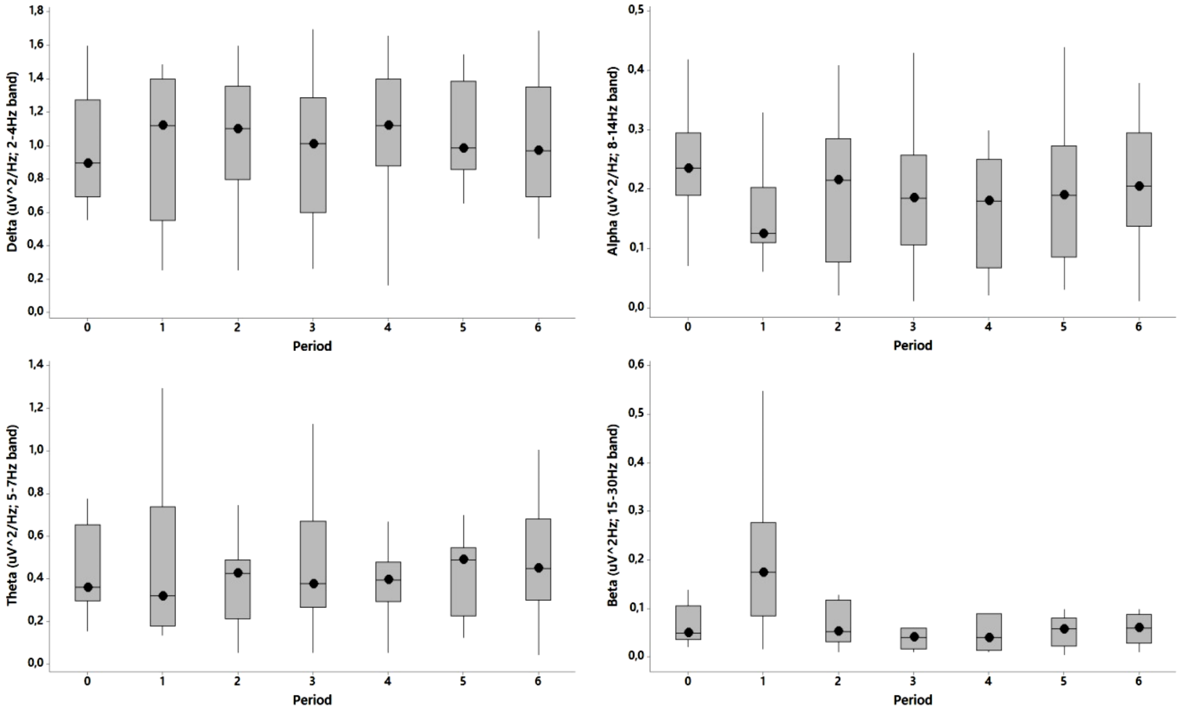

Figure 5.1: Power Spectral Density (PSD) for delta, theta, alpha and beta frequency bands before (period 0) and after (period 1-6) e-stunning in 12 *C. niloticus.*

The percent of each power band frequency in the EEG recordings for each crocodile in every period is shown in Table 5.6. The percent of each power band frequency in each period is shown in Figure 5.2. Table 5.6 and Figure 5.2 shows the power of the frequency bands present in the EEG recordings before (period 0) and after stunning (period 1 -6).

Table 5.6: EEG relative bandpower (%) per frequency band (δ,θ,α,β) per period per crocodile.

	Period	Crocodile 1	4	6	7	8	9	10	11	12	13	14	15
δ	0	48.0	31.3	85.4	66.1	50.3	53.5	68.1	35.6	71.6	66.5	42.3	38.9
	1	81.9	30.1	64.2	76.1	62.1	75.3	14.0	53.7	65.1	21.5	35.7	79.7
	2	95.2	14.0	58.1	61.3	84.2	68.9	40.2	60.1	62.9	35.4	64.2	78.1
	3	73.8	13.9	68.6	74.7	73.0	30.9	56.9	34.8	95.0	58.6	56.5	33.9
	4	80.1	9.1	71.6	51.1	52.3	66.3	63.6	39.7	95.4	65.1	48.9	88.6
	5	87.1	36.7	89.6	51.2	60.6	42.1	55.4	55.2	83.6	53.7	50.6	62.3
	6	66.3	36.0	75.3	61.1	41.6	45.4	39.0	63.8	96.6	43.7	24.7	92.1
θ	0	42.4	16.5	8.6	20.7	33.6	20.9	18.7	20.0	15.3	18.2	38.9	44.6
	1	9.9	55.1	25.7	7.1	18.6	7.5	73.0	13.6	17.7	44.6	22.5	10.2
	2	3.0	21.2	25.6	22.3	11.9	24.8	27.9	25.1	12.6	42.1	29.5	11.8
	3	19.1	13.9	12.0	17.2	16.3	42.1	29.9	39.8	2.8	24.7	25.4	64.9
	4	16.0	17.0	21.8	27.3	27.9	22.7	26.0	37.9	2.9	19.4	37.6	9.1
	5	11.0	15.3	6.9	28.9	27.5	39.3	32.0	30.9	11.9	29.1	29.3	21.1
	6	24.9	25.3	14.5	23.3	39.0	35.1	35.5	22.4	2.3	39.7	56.7	5.6
α	0	8.5	28.4	4.0	10.3	13.9	17.4	11.5	37.8	12.0	12.5	16.0	13.1
	1	6.0	0.9	5.6	6.5	10.2	5.9	7.3	10.2	6.3	17.7	11.5	3.4
	2	1.2	41.9	12.0	13.4	2.5	4.1	13.4	12.0	17.1	18.0	4.6	7.3
	3	6.5	58.8	10.3	6.0	8.4	24.2	10.9	22.1	1.7	14.4	14.7	0.6
	4	3.3	59.7	5.2	16.5	14.5	9.4	8.7	17.2	1.1	12.0	10.7	1.7
	5	1.7	23.2	2.6	15.1	8.8	15.7	10.9	9.4	3.4	13.7	16.7	10.9
	6	7.2	21.3	8.6	10.0	15.6	15.1	22.1	10.3	0.6	13.2	13.5	1.7
β	0	1.1	28.4	2.0	2.0	2.2	8.1	1.7	6.7	1.1	2.8	2.9	3.4
	1	2.2	0.9	4.5	4.5	9.0	11.3	5.6	22.6	10.9	16.1	30.2	6.8
	2	0.6	41.9	4.3	4.3	1.4	2.1	18.4	2.7	7.4	4.5	1.7	2.8
	3	0.6	58.8	9.1	9.1	2.3	2.8	2.3	3.3	0.6	2.3	3.4	0.6
	4	0.6	59.7	1.4	1.4	5.2	1.7	1.7	5.2	0.6	3.4	2.8	0.6
	5	0.2	23.2	0.9	0.9	3.0	2.8	1.7	4.4	1.1	3.4	3.4	5.7
	6	1.7	21.3	1.5	1.5	3.9	4.3	3.5	3.4	0.6	3.4	5.1	0.6

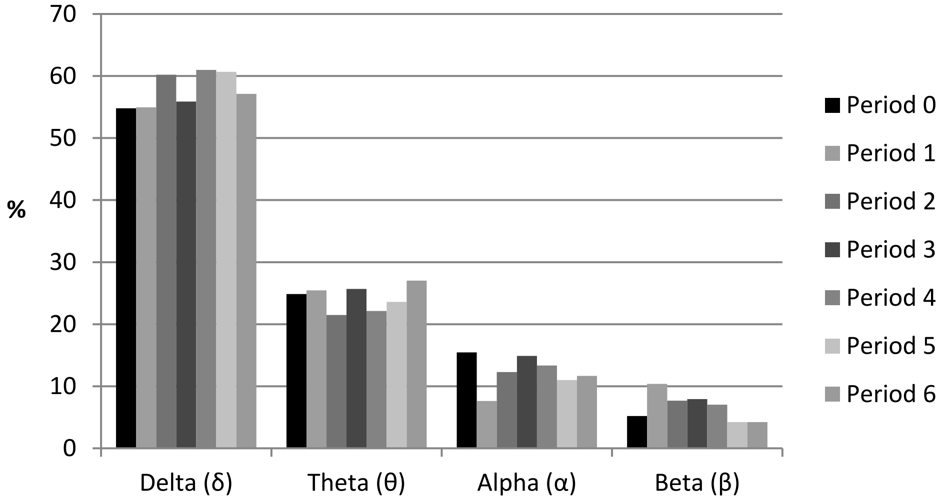

Figure 5.2: Graph showing the relationship between relative EEG frequency band power (%) before (period 0) and after (period 1-6) e-stunning in the *C. niloticus.*

The change in power of each frequency band can be seen before (period 0), immediately after (period 1) and after (period 1-6) e-stunned of crocodiles that were effectively and ineffectively stunned in Table 5.7.

Table 5.7: PSD (μV²/Hz) and relative bandpower (%) changes in EEG frequency bands (δ,θ,β,α) before (period 0) and after (period 1-6) e-stunning in the *C. niloticus.*

Croc	Effective stun	Period	δ (μV²/Hz)	θ (μV²/Hz)	α (μV²/Hz)	β (μV²/Hz)	δ %	θ %	α %	β %
		0	0	0	0	0	0	0	0	0
1	Yes	1	0.64	-0.57	-0.04	0.02	43	-317	-36	50
		1-6	0.57	-0.50	-0.073	-0.003	39	-395	-215	-131
		0	0	0	0	0	0	0	0	0
4	No	1	-0.03	0.66	-0.18	-0.485	-6	69	-75	-3233
		1-6	-0.14	0.15	-0.10	0.12	-75	19	-41	-524
		0	0	0	0	0	0	0	0	0
6	No	1	-0.34	0.31	0.03	0.045	-30	67	30	56
		1-6	-0.23	0.17	0.061	0.029	-20	40	30	-2
		0	0	0	0	0	0	0	0	0
7	Yes	1	0.25	-0.23	-0.06	0.14	18	-177	-50	74
		1-6	-0.042	0.008	0.018	0.042	-7	-20	-5	29
		0	0	0	0	0	0	0	0	0
8	Yes	1	0.23	-0.25	-0.06	0.122	21	-76	-33	76
		1-6	0.25	-0.15	-0.059	0.036	19	-61	-92	28
		0	0	0	0	0	0	0	0	0
9	Yes	1	0.48	-0.22	-0.19	0.07	34	-157	-173	33
		1-6	0.078	0.16	-0.076	-0.064	-3	3	-89	-173
		0	0	0	0	0	0	0	0	0
10	No	1	-1.35	0.86	-0.14	0.06	-540	66	-108	60
		1-6	-0.82	0.22	-0.057	0.058	-162	24	-43	19
		0	0	0	0	0	0	0	0	0
11	Yes	1	0.31	-0.12	-0.5	0.28	33	-50	-278	70
		1-6	0.27	0.15	-0.44	0.003	26	18	-210	-59
		0	0	0	0	0	0	0	0	0
12	No	1	-0.17	0.03	-0.11	0.17	-15	10	-100	89
		1-6	0.15	-0.13	-0.13	0.042	7	-262	-679	-21
		0	0	0	0	0	0	0	0	0
13	No	1	-0.77	0.51	0.11	0.25	-193	61	33	83
		1-6	-0.36	0.27	0.043	0.05	-62	40	14	24
		0	0	0	0	0	0	0	0	0
14	No	1	-0.09	-0.27	-0.07	0.5	-14	-66	-33	91
		1-6	0.085	-0.087	-0.069	0.09	1	-25	-59	17
		0	0	0	0	0	0	0	0	0
15	Yes	1	0.73	-0.6	-0.17	0.06	52	-333	-283	50
		1-6	0.60	-0.42	-0.16	-0.01	40	-292	-652	-238

The effective stun can be seen in a filtered EEG channel recording and in the PSD change over time. In the pre-stun EEG of ineffective stunned crocodiles there is excessive movement seen as the erratic and irregular increase in wave amplitude (Figure 5.3). In crocodiles effectively stunned, there is an increase in the wave amplitude and signs of tonic-clonic activity post-stun (Figure 5.4). The power for each frequency band from Figure 5.3 and Figure 5.4 are represented in Figure 5.5 and Figure 5.6.

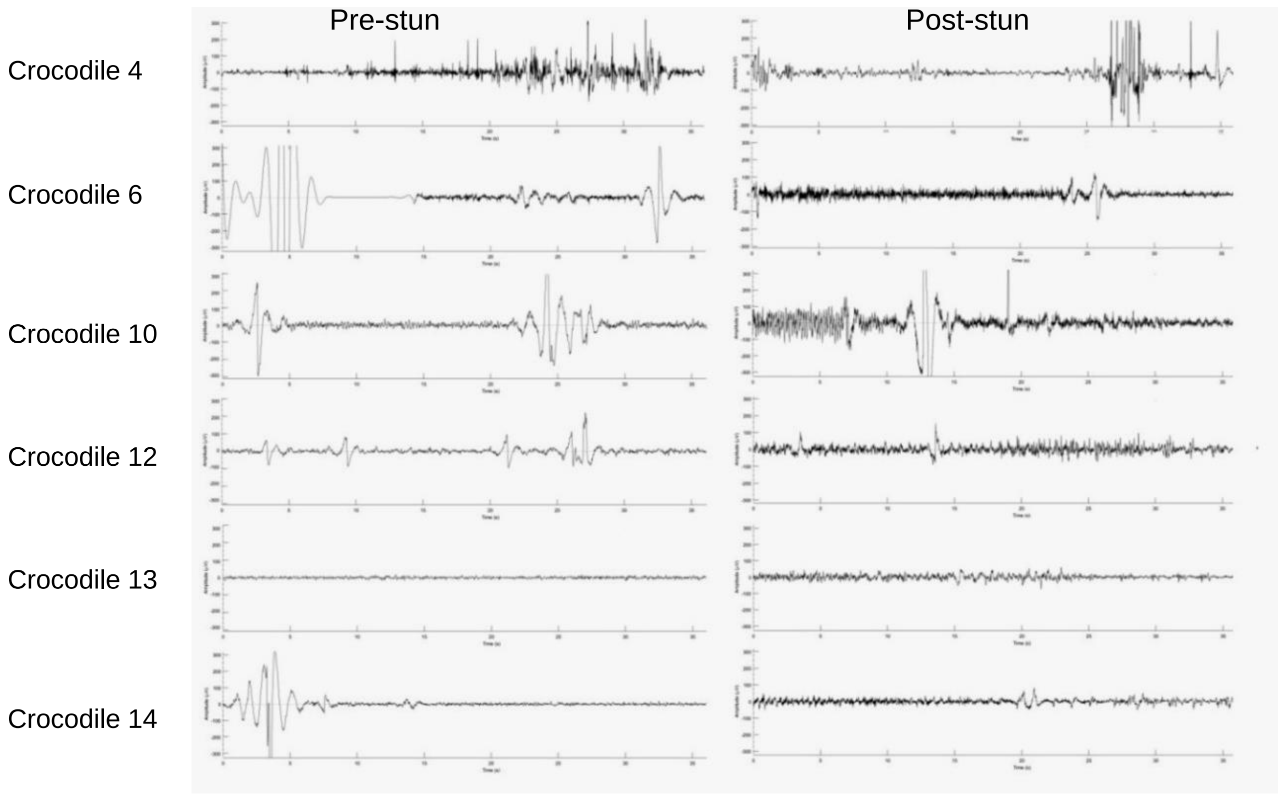

Figure 5.3: Raw EEG before and after e-stunning of the *C. niloticus* that were not effectively stunned.

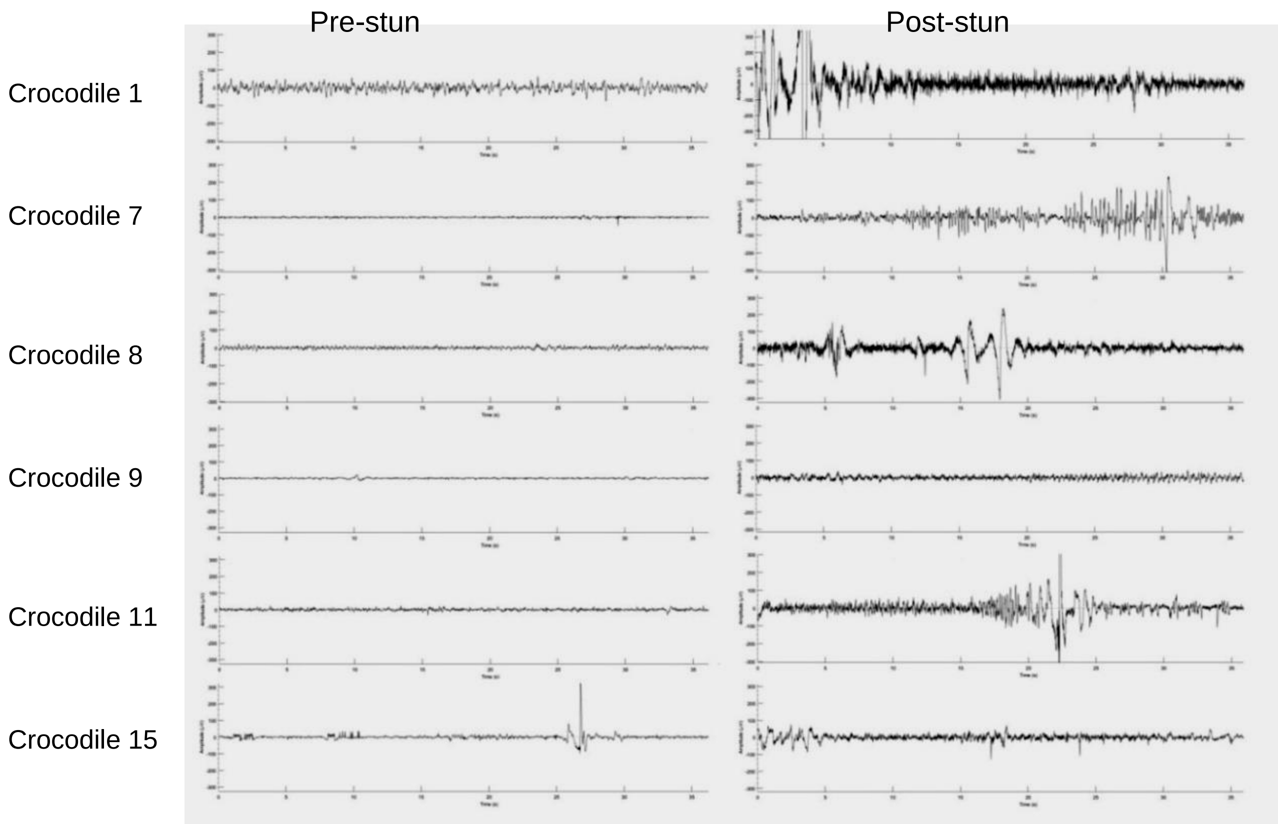

Figure 5.4: Raw EEG before and after e-stunning of the *C. niloticus* that were effectively stunned.

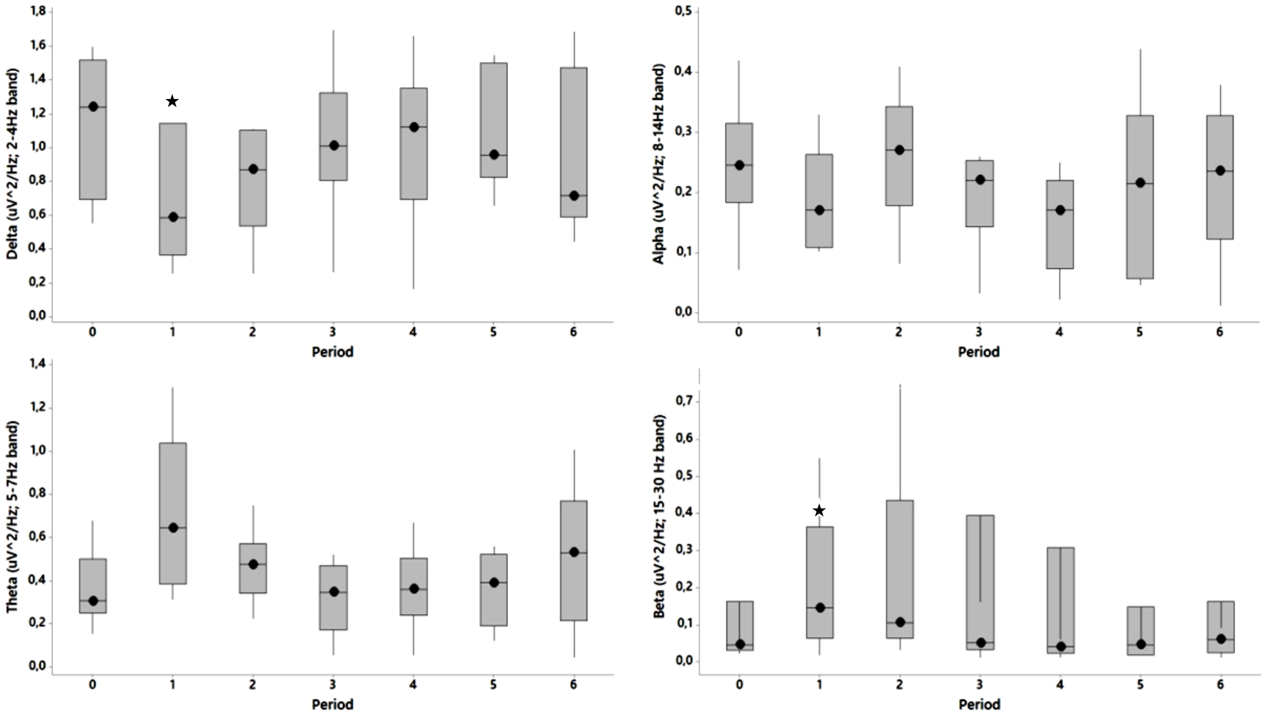

Figure 5.5: PSD (μV²/Hz) before and after e-stunning of the *C. niloticus* that were not effectively stunned. * = Significant. Period 0= Pre-stun. Period 1-6= Post-stun

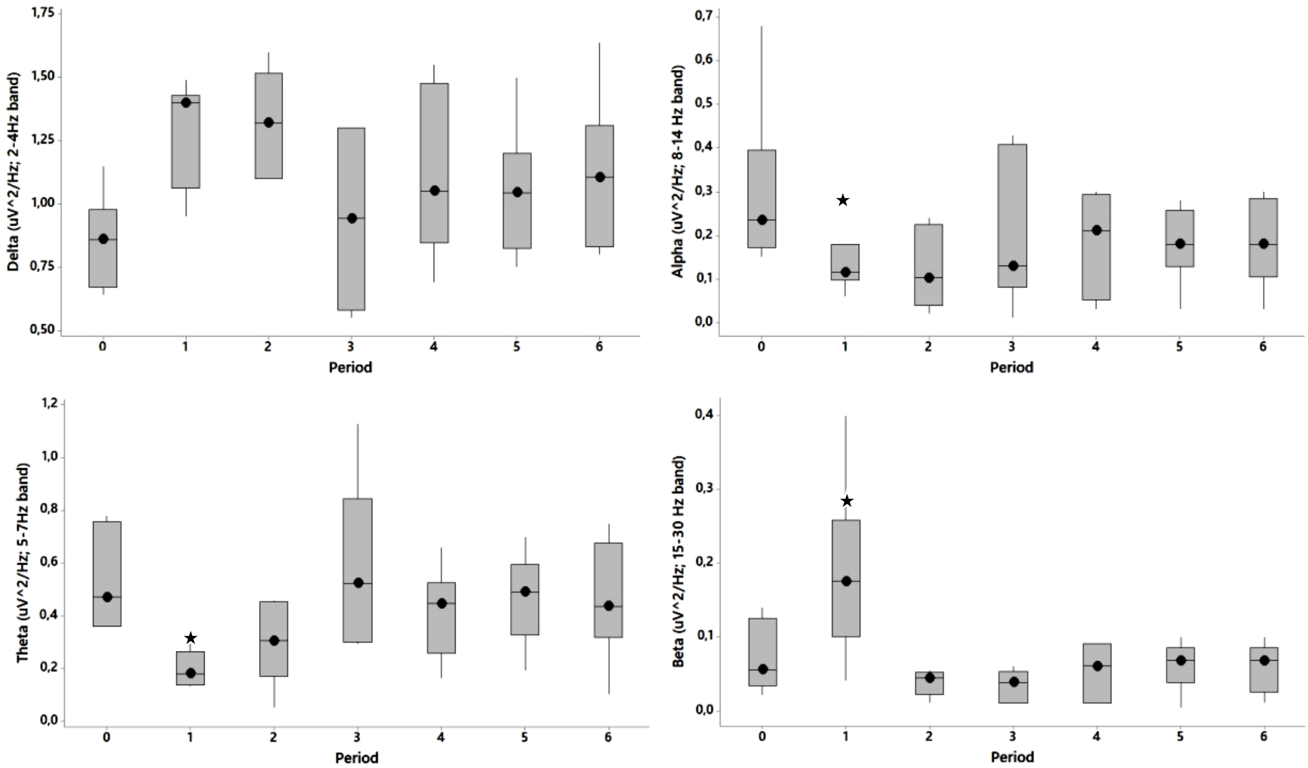

Figure 5.6: PSD (μV²/Hz) before and after e-stunning of the *C. niloticus* that were effectively stunned. * = Significant. Period 0= Pre-stun. Period 1-6= Post-stun

5.5 EEG Data Interpretation

There were no significant changes in delta or theta power after e-stunning amongst all crocodiles (n = 12) (p = 0.992, p = 0.969). Power for the alpha frequency band showed a significant decrease immediately after stunning (period 0-1, p = 0.016), but no significant change over the entire post-stunning period (period 1-6, p = 0.508). Beta power significantly increased immediately after stunning (period 0-1, p =0.028) and was the only frequency band to show a significant difference in the amount of power change (period 0-6, p =0.017) without having a significant overall change post-stunning (period 0-6, p = 0.098).

In crocodiles that were not effectively stunned (n = 6) the delta power significantly decreased immediately after stunning (period 0-1) (p = 0.037) whereas theta, alpha and beta power showed no significant change (period 0-1) (p = 0.055, p = 0.336 and p = 0.20) immediately after stunning.

For crocodiles that were effectively stunned (n = 6) the delta and beta power significantly increased (p = 0.010, p = 0.045) and theta and alpha power significantly decreased (p = 0.004, p = 0.015) immediately after stunning (period 0 -1). For the overall period before and after stunning (period 0-6); delta, theta and alpha power had no significant changes (p = 0.099, p = 0.051 and p = 0.381), whereas beta power significantly changed by initially increasing then decreasing (p = 0.046). Furthermore, the delta power returned to pre-stun values by time period 3, which is 120 sec after completion of the stun. Whereas, the alpha power returned to pre-stun values at time period 4, which is 180 sec after completion of the stun.

5.6 Demographic and Electrical Data Analysis of Conscious and Unconscious Crocodiles

The demographic data and electrical current delivered to conscious and unconscious crocodiles are summarised in Table 5.8.

Table 5.8: Mean ± standard deviation of demographic data and electrical current delivered to conscious and unconscious crocodiles.

Measurement	Mean ± SD	
	Conscious	Unconscious
Demographic data		
TL (cm)	149.6 ± 5.2	151.8 ± 5.3
HL (cm)	18.8 ± 0.6	19.5 ± 1.25
Weight (kg)	10.8 ± 0.6	11,4 ± 0.9
Electrical data		
Volts (v)	171.1 ± 0.07	172.7 ± 0.14
Amp-max (amp)	1.10 ± 0.16	1.08 ± 0.28
AUC (amp*sec)	5.41 ± 0.86	5.56 ± 1.95

There were no significant differences in AUC, amp-max, volt, TL, HL and weight among the crocodiles rendered conscious and those unconscious following stunning ($p > 0.05$, Student's t-test). Similarly, no significant differences in the aforementioned variables were observed when one of the crocodiles in the unconscious group, which was only stunned for 5 sec compared to all other crocodiles that were stunned for 7 sec, was removed from the dataset.

CHAPTER 6
DISCUSSION

Because of the size and strength of crocodiles the use of e-stunning as a method of immobilisation is important to ensure the safety of both handler and animal. Immobilisation by e-stunning should render an animal unconsciousness when capturing and restraining and for humane killing of crocodiles to reduce distress, fear, and pain (EU 2009; OIE, 2019a).

Countries such as South Africa, USA, UK and Australia have laws concerning the requirement of pre-slaughter stunning of animals. Also, welfare concerns have been raised about e-stunning such as: is the procedure painful; is it ineffective if the electrodes of the e-stunner wand are incorrectly placed; will it result in insensitivity if volts are too low; will the animal regain consciousness before the final killing step (Welty, 2007). For management procedures e-stunning has been shown to be a less stressful method to capture crocodiles compared to noosing (Franklin *et al.,* 2003; Pfitzer *et al.,* 2014).

The EEG procedure was established over 3 days and was adapted based on the preparatory study findings (Chapter 3). The considerations made for the adaptation of the procedure were thoroughly discussed and concluded within the team. E-stunning of *C. niloticus* on the dorsal surface of the neck immediately behind the frontal bone set at 170V for 5 – 7 sec achieved variable results. The wave amplitudes in the pre-stun raw EEG recording were high, irregular and erratic in crocodiles that were ineffectively stunned compared to effectively stunned crocodiles (Figure 5.3 and Figure 5.4). The stimulation of the animals may have contributed to the e-stunning being ineffective as crocodiles are highly sensitive to touch, sight, vibrations and sound (OIE, 2019a). Based on the raw EEG data care should be taken during the manual capture of the crocodiles and during the pre-stunning period by controlling the crocodile’s environment in order to reduce stress and stimulation of the animals. The design of the injection mould allowed the EEG electrodes to be positioned uniformly and firmly on the crocodile cranial plate that permitted collection of stable EEG data. The use of the data-logger to record the electrical output delivered to each crocodile provided a unique opportunity to compare the electrical current in crocodiles that were effectively and ineffectively stunned. The ability to log and record electrical current delivered to individual crocodiles allows the opportunity to investigate the impact electrical output on the effect of e-stunning on crocodiles when set at different volts, amperes and duration of stun and evaluating the site of placement stunner electrodes in future studies.

The mean volts, AUC (amp*sec) and amp-max delivered to all crocodiles in the current study were 171.4 ± 1.7 V, 5.5 ± 1.4 amp*sec, 1.1 ± 0.2 amp-max, respectively. Volts delivered to the crocodiles was higher than the volts used in published reports (NRMMC, 2009; CFAZ, 2012; Pfitzer *et al*., 2014) but were in accordance with that used on the commercial farm where the study was performed and those by Nile crocodile farms surveyed in South Africa (Carpenter & Swan, 2017).The minimum electrical current for head only e-stunning required for most species at slaughter is prescribed and varies from 0.25 amps in chickens to 1.28 amps in bovine 6 month and older (EU, 2009). No similar information have been published for crocodiles, although some farmers in South Africa aim to achieve a minimum electrical current of 0.4 amps for management procedures and above 0.8 amps for slaughter (Tahnee & Swan, 2017).The duration of stunning used in the study (5 – 7 sec) is similar to the duration of stunning (4 – 6 sec) reported (NRMMC, 2009; CFAZ, 2012; Pfitzer *et al*., 2014)

In order to capture EEG readings metal screw EEG electrodes were implanted in *C. sclerops* (8 electrodes) and *A. mississippiensis* (4 electrodes) (Flanigan *et al.,* 1973; Nevarez *et al.,* 2014). This method is more invasive than the method used in this study. In the Flanigan *et al.* (1973) study, 1 out of 5 study animals was removed due to poor quality EEG recordings. The quality of EEG recordings was also limited by available technology at the time the study where the power of each frequency band could not be quantified. In the Nevarez *et al.* (2014) study, 2 of the 4 electrodes were used to analyse the power of each frequency band (unless there was excessive artefacts then the other 2 electrodes were used), residuals plots were logarithmically transformed as they were not normally distributed and a single data point was removed as an outlier for 1 alligator. A less invasive procedure was used by investigators when obtaining EEG readings from sheep by using 2 needle electrodes which were placed subcutaneously (Sánchez-Barrera *et al.*, 2014). In the Sánchez-Barrera *et al*. (2014) study, no study animal were reported to be removed, the relative power of each frequency band was recorded and reported an epileptic crisis in the raw EEG readings after e-stunning that resemble the raw EEG readings after e-stunning in this study. The method of placing the electrodes subcutaneously was attempted in this study but was unsuccessful as the skin is tightly attached to the skull of the crocodile. In this study, 3 study animals were removed (2 due to the EEG recordings being unable to be analysed and 1 due to poor e-stunning practise). This method resulted in strong, clear EEG recordings whereby the different frequency bands could be derived and analysed, as opposed to EEG recordings that only show a change in brain waves. The use of the 3D electrode mould was less invasive and ensured that needles are placed similarly in all animals.

Power spectral density (PSD) analysis is a universal accepted method of analysing EEGs, especially with more powerful computers and large number of electrodes used (Levy & Warren, 1987). In this study, seven EEG electrodes were used which included neutral and reference electrodes and five channels. These were sufficient and included more channels than previously described in animals being stunned or slaughtered. It is difficult to compare the results of studies using exact power values because the range between studies varies due to different acquisition and analysis methodologies. Therefore, it is important to standardise the signals by means of spectrum normalisation using a relative power technique especially if more than one channel is being analysed. This method of analysis allows the opportunity to compare shifts in power within a frequency band to elucidating the state of unconsciousness.

There is no universal consensus statement describing the exact point of loss of consciousness. In this study unconsciousness was defined as an increase in delta and decrease in alpha frequency band power which resemble certain stages of sleep and general anaesthesia at surgical depths (Nevarez *et al.*, 2014; Sánchez-Barrera *et al.*, 2014; Verhoeven *et al.*, 2015).Verhoeven *et al.* (2014) described EEG brain activity of conscious livestock to have predominately fast low amplitudes and unconscious livestock to have predominately slow high amplitudes which can be seen in the raw EEG recordings of crocodiles (Figure 5.5 and Figure 5.6). A tonic-clonic wave pattern seen in EEG recordings indicates unconsciousness in mammals. During the tonic phase the hind legs are extend out, forelegs become stiff, neck muscles contract and respiration stop. During the clonic phase gradual relaxation occurs and legs have a walking-like movement 10 sec after e-stunning (Grandin, 1980). The tonic-clonic patterns were identified in the raw EEG recordings in the current study and behaviour described was also documented in the crocodiles after e-stunning. Alpha power decreases and delta and theta power increases at deeper levels of anaesthesia (Hagihira, 2015). Furthermore, EEG wave amplitude will increase after stunning and then within a few minutes decreases to return to pre-stun amplitudes (Hagihira, 2015; Verhoeven, 2015).

Disadvantages of using EEG to assess unconsciousness: unclear undefined division of stage of unconsciousness; electrode placement sites; skull thickness; equipment variations make it difficult to compare EEG between species and individuals, and artefacts in the EEG recordings caused by animal movements or interference of the equipment (Verhoeven *et al.,* 2015).

Half the crocodiles appear to have an effective stun and showed signs of unconsciousness. The EEGs of these crocodiles exhibited an increase in delta activity and a decrease in alpha activity immediately after e-stunning that lasted for approximately 120 sec, followed by frequency bands slowly stabilising (Figure 6.1). These EEG patterns are indicative of unconsciousness (Hagihira, 2015; Verhoeven, 2015). The other 6 crocodiles had an initial decrease in delta activity followed by unpredictable changes of frequency band power between periods.

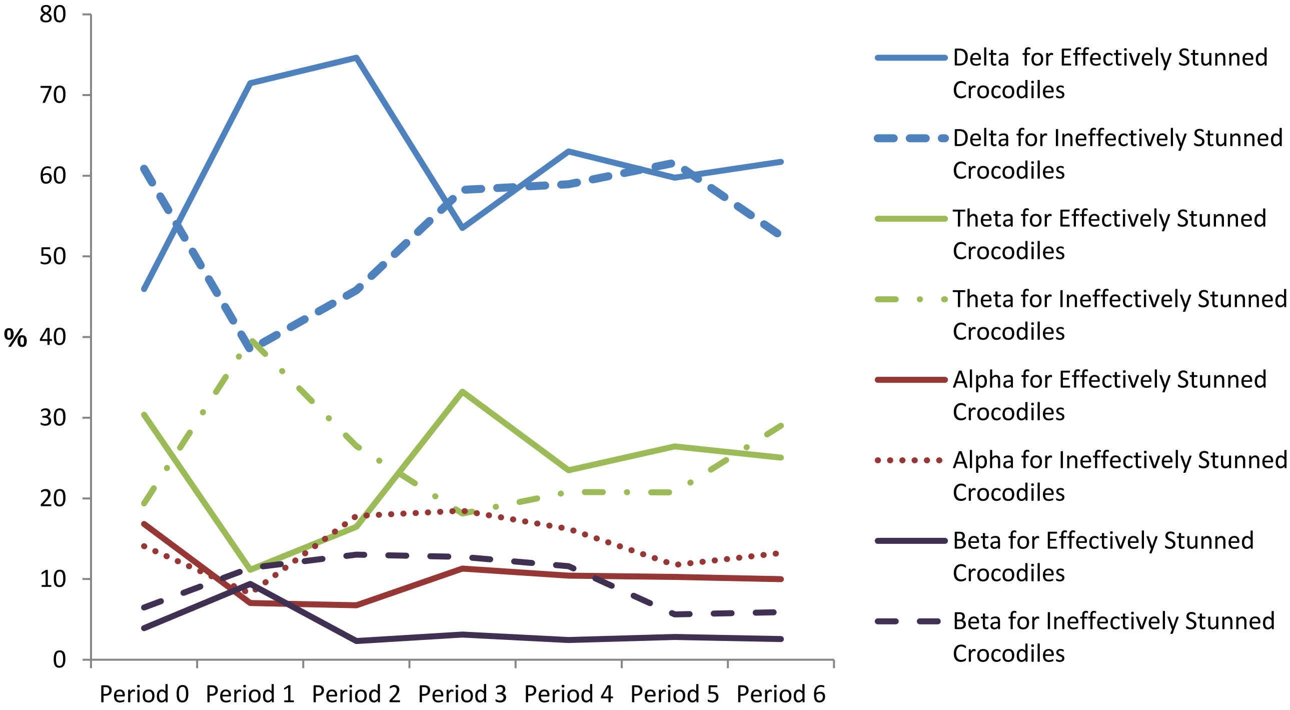

Figure 6.1: Relative power (%) for frequency bands (delta, theta, alpha and beta) in effectively and ineffectively stunned *C. niloticus.* Period 0: pre-stunning and period 1-6: post-stunning phases

The failure to cause loss of consciousness in half of the crocodiles was not related to their demographic profile (TL, HL and weight) or the applied electrical current (AUC, amp-max, volt). Therefore, other factors need to be considered and warrant further investigations to elucidate the factors that contribute to an ineffective stun. The increased activity highlighted in the pre-stun EEG tracings of the ineffectively stunned crocodiles suggests that increased movement could indicate pre-stunning anxiety, stress and that perhaps this demeanor prior to stunning could result in an ineffective stun.

The behaviour of crocodiles immediately after e-stunned have been described for *C. porosus* and *C. niloticus* (NRMMC, 2009; CFAZ, 2012; Manolis & Webb, 2016). Initiation of the stunned state is rapid and comprises an initial tonic phase, with rigor and tail twitching (NRMMC, 2009; CFAZ, 2012; Testronics, 2015). After this period, the stunned crocodile becomes relaxed with legs splayed backwards, parallel to the body (Peucker *et al.*, 2005; NRMMC, 2009). Stunned animals remain immobilized (though not necessarily unconscious) for 5-10 minutes, but most individuals regain movement after about 3 minutes (Franklin *et al.*, 2003; NRMMC, 2009; CFAZ, 2012; Testronics, 2015).

Identifying indicators of consciousness in *C, niloticus,* by analysing behavioural observations post-stunning and EEG patterns was inconclusive as all crocodiles were immobilised but only half loss consciousness. After stunning, in general vocalisation is absent (Verhoeven *et al*., 2015). These behavioural indicators were not observed in crocodiles. Crocodiles that showed EEG signs of unconsciousness showed similar, consistent behavioural observations post stunning (Table 6.1). Behavioural observations in crocodiles ineffectively stunned were erratic and inconsistent which are shown in the EEG pattern changes (Figure 6.1). Limb relaxation is a physical response to electrical stunning rather than a behavioural indicator of consciousness as it is seen in both groups of crocodiles. Signs of consciousness that could not be evaluated in the study due to the EEG procedure include: pupillary response to light or eye movements in response to objects and movement; blinking or nictitating membrane as a response to touch of the cornea, hissing as defence response and/ or tongue movements (OIE, 2019a).

Table 6.1: Behavioural observations of *C. niloticus* effectively and ineffectively stunned.

Behavioural observations	Crocodiles effectively stunned	Crocodiles ineffectively stunned
Leg position immediately post-stunning	4 crocs: forelimbs stiffened and extended away from the body 2 crocs: forelegs stiffened alongside the body	5 croc: all limbs stiffened alongside the body
Leg relaxation	All crocodiles: limbs relaxed 2-2.5min post-stunning	4 croc: limbs relaxed 2min post-stun 1 croc: limbs relaxed 1min post-stun 1 croc: limbs relaxed 3 min post-stun
Hind limb contraction followed by relaxation	5 croc: experienced hind leg contraction followed by relaxation less than 1 min post-stun	1 croc: experienced hind leg contraction followed by relaxation 2 min post-stun (other crocs did not exhibit this)
Lowering of head	5 crocs: lowered head1.5-2min post-stun	1 croc: head down 3min 1 croc: head down 1 min 1 croc: lowered 2min 1 croc: lowered 1min post-stun
Tail twitching	All crocodiles: experienced constant tail twitching for at least 2min post-stun	1 croc: increased tail movements after 1min post-stun 1 croc: tail began to sway 30s post stun 1 croc: tail thrash post-stun, stopped 3min post-stun 1 croc: tail swaying for 1min post-stun 1 croc: tail sway for 3min post-stun
Reactions	2 crocs hissed 6-7min post-stun, and 1 of those crocs thrashed its body when picked up 7 min post stun	1 crocodile thrashed its body when picked up 8min post-stun. 1 croc hissed when touched 3.5 min post-stun, both croc's limbs were non responsive
Return to normal breathing	3 crocs: normal breathing 6min post-stun 2 crocs: normal breathing 7min post-stun	3 crocs: normal breathing 5min post-stun 1 croc: normal breathing 8min post-stun

CHAPTER 7
CONCLUSION AND RECOMMENDATIONS

The study confirmed that e-stunning at 171.4 ± 1.7 V and 50 Hz for 5 – 7 sec was able to immobilise all crocodiles and to effectively stun 6 out of 12 crocodiles by rendering them unconscious, measured by EEG as an increase in delta wave brain activity and a decrease in alpha activity, immediately after being e-stunned and lasted for approximately 120 sec. A mean maximum electrical current of 1.1 ± 0.2 amps and a total electrical current of 5.5 ± 1.4 amps*sec were achieved in the crocodiles and are within the minimum amp ranges for other species (EU, 2009; Grandin, 2015).

The size and weight of crocodiles and the electrical current delivered to the crocodiles had no significant effect in achieving conscious and unconscious in the study. In practice, crocodiles are e-stunned in water, which increases conductivity and may result in more crocodiles to achieve loss of consciousness. The wand electrode placement may have been a contributing factor as head-only e-stunning is applied for other species (EFSA, 2004; Verhoeven *et al.*, 2015). However, in the pilot study poor and erratic e-stunning effect were found in crocodiles where the electrodes were placed on the cranial plate. The sensitivity of crocodiles to their surroundings could have contributed to ineffective e-stunning, especially as the crocodiles were manually captured before pre-stun EEG recordings and due to noise and interaction with crocodiles. In future studies, crocodiles should be partially submerged in water when e-stunned, either on the cranial plate or directly behind the cranial plate, in a strictly controlled environment, to restrict human contact to the minimum, and avoid noise in experimental environment

There were no specific behavioural indicators identified in the study that could be used to verify unconsciousness of *C. niloticus*.

The variability in achieving unconsciousness following e-stunning in *C. niloticus* as determined by EEG in this study confirmed the need to concurrently monitor crocodiles that are e-stunned for clinical signs of consciousness, including intentional defence responses, spontaneous eye opening or closing, eye movement, blink or nictitating responses and tongue movement (OIE, 2019a). No painful procedures should be performed on any e-stunned crocodile showing any signs of consciousness and the crocodile should be released as soon as possible. In the case of slaughter, crocodiles showing signs of consciousness following e-stunning must either be re-stunned or an alternative accepted killing method employed as back-up. Behavioural indicators of consciousness and unconsciousness can be further studied using a large sample size of crocodiles on commercial crocodile farms during routine e-stunning practices.

Based on the results of this study it is recommended that further EEG studies be performed to understand the factors why e-stunning was unable to cause unconsciousness in some animals, including the required electrical current and duration of stunning, modelling of the electrical current through the brain as determined by placement site of the stunner electrodes, the importance of stunning in water, the effect of successive and interval of e-stunning in crocodiles and the impact of stress of crocodiles during manual capture and pre-stunning EEG recordings.

Inducing unconsciousness is required before the killing of crocodiles whereas immobilisation without rendering crocodiles unconscious suffices during management practises such as moving, measuring and grading skins of crocodiles. ‘Unconsciousness’ can be further researched in commercially farmed crocodiles by investigating a large sample sizes’ response to stimuli and pain perception (analgesia) after routine e-stunning.

REFERENCES

AVMA, American Veterinary Medical Association. (2013)AVMA Guidelines for the euthanasia of animals: 2013 Edition.

Anil, M. and Gregory, N.(2014) Slaughter, ethics, and the law. *Encyclopedia of Meat Sciences***,** 280-283.

Brady, S., Harrison, T., Williams, C., Evola, M. and Wack, R. F.(2016) Diagnostic evaluation and treatment of a Chinese crocodile lizard (*Shinisaurus crocodilurus*) with seizures *Veterinary Record Case Reports* 4: e000368. doi: 10.1136/vetreccr-2016-000368.

Cook, C. J., Devine, C.E., Gilbert, K. V., Smith, D. D. and Maasland, S. A. (1995) The Effect of Electrical Head-only Stun Duration on Electroencephalographic-measured Seizure and Brain Amino Acid Neurotransmitter Release. *Meat Science,* 40. 137-147.

Cook, C. J., Devine, C.E., Gilbert, K. V., Maasland, S. A. and Blackmore, D. K. (1996) Changes in the release of amino acid neurotransmitters in the brains of calves and sheep after head-only electrical stunning and throat cutting. *Research in Veterinary Science,* 60**,** 255-26.

Carpenter, T. and Swan, G.E. (2017) Farm surveys and production output. South African Crocodile Industry Congress, August 10 & 11, Pretoria

CITES Trade Database (2018) UNEP World Conservation Monitoring Centre, Cambridge, UK). https://trade.cites.org

CFAZ (Crocodile Farmers Association of Zimbabwe) (2012) Codes of Practice. CFAZ: Harare, Zimbabwe.

CSIRO, (2006) Meat technology update: Electrical stunning of small stock. *Food Science Australia*, 98 (5).

Davis, B., Peucker, S. and Mayer, R.(2000) Crocodiles Restraining & Meat quality. RIRDC Publication No. 00/105.

Davis, K.A., Devries, S.P., Krieger, A., Mihaylova, T., Minecan, D., Litt, B., Wagenaar, J.B. and Stacey, W.C. (2018). The effect of increased intracranial EEG sampling rates in clinical practice. *Clinical Neurophysiology, 129(2),* pp. 360-367.

EFSA (2004) Opinion of the Scientific Panel on Animal Health and Welfare on a request from the Commission related to welfare aspects of the main systems of stunning and killing the main commercial species of animals. *The EFSA Journal,* 45**,** 1-29.

EU. (2009)EU Regulations No 1099/2009 of 24 September 2009. Official Journal of the European Union**,** L303/1 1-30.

Flanigan,W., Wilcox, R. and Rechtschaffen, A.(1973)The EEG and behavioral continuum of the crocodilian, *Caiman sclerops*. *Electroencephalography and clinical neurophysiology,* 34**,** 521-538.

Franklin, C.E., Davis, B.M., Peucker, S., Stephenson, H., Mayer, R., Whittier, J., Lever, J. and Grigg, G.(2003) Comparison of stress induced by manual restraint and immobilisation in the estuarine crocodile, *Crocodylus porosus*. *Journal of Experimental Zoology Part A: Comparative Experimental Biology,* 298**,** 86-92.

Ferree, T.C., Luu, P., Russell, G.S. and Tucker, D.M. (2001) Scalp electrode impedance, infection risk, and EEG data quality. *Clinical Neurophysiology*, *112*(3), 536-544.

George, I.D. and Holliday, C.M. (2013) Trigeminal nerve morphology in Alligator mississippiensis and its significance for crocodyliform facial sensation and evolution. *The Anatomical Record,* 296**,** 670-680.

Grandin, T. (1980) Mechanical, electrical and anesthetic stunning methods for livestock. *International Journal for the Study of Animal Problems*, 1(4), 242-263.

Grandin, T. (2015) Electric Stunning of Pigs and Sheep[Online]https://www.grandin.com/humane/elec.stun.html: Colorado State University. [Accessed 2019].

Hall, P.M. and Portier, K.M. (1994) Cranial morphometry of New Guinea crocodiles (Crocodylus novaeguineae): ontogenetic variation in relative growth of the skull and an assessment of its utility as a predictor of the sex and size of individuals. *Herpetological Monographs*, 203-225.

Harighira, S. (2015) Changes in the electroencephalogram during anaethsia and their physiological basis. *British Journal of Anaesthesia,* i27-i31.

HSA (2016) Electrical stunning of red meat animals. www.hsa.org.uk/publications/online-guide

Huggins, S. E., Parsons, L. C. And Pena, R. V. (1968) Further study of the spontaneous electrical activity of the brain of *Caiman sclerops. Physiol. Zool.,* 41, 371 – 383.

Joanen, T. and Perry, W. G. (1971) A new method for capturing alligators using electricity. In *Proc. 25th Ann. Conf. Southeast. Assoc. Game and fish Commissioners,* 124-130.

Joslyn, S. and Hague, D. (2016) Magnetic resonance imaging of the small animal brain. *In Practice,* 38, 373-385.

Levy, M.D. and Warren, J. (1987) Effect of epoch length on power spectrum analysis of the EEG. *Anesthesiology,* 66, 489-495.

Livestock Welfare, U.O.B.and S.L.S.L.(2017) Standard Operating Procedure Battery powered Electrical Stunning Equipment for Crocodiles.

Manolis, S.C. and Webb, G.J. (2016) Best Management Practices for Crocodilian Farming. IUCN-SSC Crocodile Specialist Group: Darwin, Australia.

Meglasson, M. D. And Huggins, S. E. (1979) Sleep in a crocodilians, Caiman sclerops. *Comparative Biochemistry and Physiology Part A: Physiology,* 63(4), 561 – 567.

Nevarez, J.G., Strain, G.M., Da Cunha, A.F. and Beaufrere, H. (2014) Evaluation of four methods for inducing death during slaughter of American alligators (Alligator mississippiensis). *American Journal of Veterinary Research* 75, 536-43.

Ngwenya, A.,Patzke, N.,Spocter, M.A., Kruger, J.L., Dell, L.A.,Chawana, R.,Mazengenya, P., Billings, B.K.,Olaleye, O. and Herculano-Houzel, S. (2013) The continuously growing central nervous system of the Nile crocodile (*Crocodylus niloticus*). *The Anatomical Record,* 296**,** 1489-1500.

NRMMC (Natural Resource Management Ministerial Council) (2009). Code of Practice for the Humane Treatment of Wild and Farmed Australian Crocodiles. NRMMC: Canberra, Australia.

OIE (2019a) Killing of reptiles for their skins, meat and other products..OIE Terrestrial Animal Health Code, 28th Edition, Volume 1, Chapter 7.14.www.o.i.e.int/standard-setting/terrestrial-code.

OIE (2019b) Welfare aspects of stunning and killing of farmed fish for human consumption., OIE Aquatic Animal Health Code, 22nd Edition, Volume 1, Chapter 7.3.www.o.i.e.int/standard-setting-aquatic code.

Pearcy, A. and Wijtten, Z. (2011) A morphometric analysis of crocodilian skull shapes. The Herpetological Journal*,* 21**,** 213-218.

Pendergrast, N. (2015) Live animal export, humane slaughter and media hegemony. Animal Studies Journal*,* 4**,** 99-125.

Peucker, S., Davis, B. and Van Barneveld, R. (2005) Crocodile farming research: hatching to harvest. Kingston, ACT: Australia: Rural Industries Research and Development Corporation.

Pfitzer, S.,Ganswindt, A.,Fosgate, G.T., Botha, P.J. and Myburgh, J.G. (2014) Capture of farmed Nile crocodiles (Crocodylus niloticus): comparison of physiological parameters after manual capture and after capture with electrical stunning. *Veterinary Record* doi: 10.1136/vr.102438.

Sánchez-Barrera, I.C., Albarracin, W. and Rojas, M.J. (2014) Electroencephalographic spectrum power of sheep's brain after stunning. *Journal of Applied Animal Research,* 42**,** 73-76.

Smith-root (2020) *Humane Fish Harvester - Smith-Root.* [online] Smith-Root.com. Available at: https://www.smith-root.com/aquaculture/humane-fish-harvester.

Somjen, G. (2001) Mechanisms of spreading depression and hypoxic spreading depression-like depolarization. *Physiological Reviews*, 81 (3): 1065-1096.

Sreelekha, S. and Sabi, S.(2016) Modified welch power spectral density compution with fast fourier transformation. *International Journal of Scientific Engineering and Research* 4**,** 92-96.

Tadel, F., Baillet, S., Mosher, J. C.,Pantazis, D.and Leahy, R.M.(2011)Brainstorm: A User-Friendly Application for MEG/EEG Analysis. *Computational Intelligence and Neuroscience*, vol. 2011, Article ID 879716, 13 pages, 2011. doi:10.1155/2011/879716.

Testronics (2015) Testronics electronic services, Crocodile Stunner2015 Operator Manual, Manual2, Rev 01.

Verhoeven, M.T.W.,Gerritzen, M.A.,Hellebrekers, L.J.and Kemp, B. (2015) Indicators used in livestock to assess unconsciousness after stunning: a review. Animal*,* 9**,** 320-330.

Von Holleben, K., Von Wenzlawowicz, M., Gregory, N., Anil, H., Velarde, A., Rodriguez, P., Cenci Goga, B., Catanese, B. andLambooij, B., (2010) Report on good and adverse practices: animal welfare concerns in relation to slaughter practices from the viewpoint of veterinary sciences. Dialrel Deliverable 02/2011. www.diarel.eu.

Welty, J. (2007) Humane Slaughter Laws. Law and contemporary problems*,* 70**,** 175-206.

ADDENDUM

Publication Name

Du Plooy, K.J., Swan, G.E., Myburgh, and J.G., Zeiler, G.E., (2019). Electroencephalogram (EEG) assessment of brain activity before and after electrical stunning in the Nile crocodile (*Crocodylus niloticus*)

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Ethical Approval V084-18 Certificate

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Animal Ethics Committee

PROJECT TITLE	**Electroencephalogram (EEG) assessment of brain activity after electrical stunning in the Nile crocodile (*Crocodylis Niloticus*)**
PROJECT NUMBER	**V084-18**
RESEARCHER/PRINCIPAL INVESTIGATOR	**KJ du Plooy**

STUDENT NUMBER (where applicable)	**U_12057356**
DISSERTATION/THESIS SUBMITTED FOR	**MSc**

ANIMAL SPECIES/SAMPLES	**Nile Crocodile (*Crocodylis Niloticus*)**	
NUMBER OF ANIMALS	**>17**	
Approval period to use animals for research/testing purposes		**September 2018 –September 2019**
SUPERVISOR	**Prof. GE Swan**	

KINDLY NOTE:

Should there be a change in the species or number of animal/s required, or the experimental procedure/s - please submit an amendment form to the UP Animal Ethics Committee for approval before commencing with the experiment

APPROVED	Date 8 October 2018
CHAIRMAN: UP Animal Ethics Committee	Signature [signature]

Pain category changed to "C"

S4285-15