Progress report validation of parameters to examine unconsciousness

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Validation of parameters used to assess consciousness in sheep at slaughter

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This research was conducted by Wageningen UR Livestock Research, commissioned and funded by the Ministry of Economic Affairs, within the framework of Policy Support Research theme 'Dierwelzijn'
project number BO-20-008-002.08

Wageningen UR Livestock Research
Lelystad, Mei 2014

CONFIDENTIAL

Livestock Research Report 380

Gerritzen, M.A., Verhoeven, M.T.W., Kluivers-Poodt, M., Reimert, H. and Anjema, D, 2014. *Progress report validation of parameters to examine unconsciousness: validation of parameters used to assess consciousness.* Lelystad, Wageningen UR (University & Research centre) Livestock Research, Confidential Livestock Research Report 380.

Confidential Livestock Research Report 380

Foreword

As part of the resolution of the Dutch parliament, that the welfare of slaughter animals has to improve, and following EU regulation 1099/2009, the Dutch minister of Economic Affairs has, in close compliance with concerned parties, listed areas which require further investigation to safeguard or improve animal welfare during slaughter, including animals that are slaughtered without stunning.

This work falls within the policy support programme (BO-20-008-002.08) of the Ministry of Economic Affairs. The progress report presents the results of a study on the validation of parameters used to assess unconsciousness in sheep at slaughter.

Summary

Introduction: New national legislation requires that unconsciousness at slaughter should be tested at 40 seconds in animals that have been neck cut without prior stunning. Animals that are not unconscious at 40 seconds following neck cut should be stunned immediately. The assessment of unconsciousness in animals at slaughter plants is in general performed on the basis of behavioural parameters (e.g. reflexes and voluntary movements). In experimental set-ups, the use of brain activity as presented in an electroencephalogram (EEG) can be used as well and is considered more objective. The validity and applicability of most behavioural parameters for assessing unconsciousness under different slaughter circumstances (with and without stunning) are under (inter)national debate and need further investigation.

Research question: The aim of this study (part a and b) was to validate the following parameters to assess unconsciousness in sheep: rhythmic breathing, eyelid reflex, ocular reflex to threat and pain withdrawal reflex (by pinching the ear). In part a of the study, the righting reflex was tested as well.

Material and methods: In part a of the study, mixed breed ewes (n=10, 36 ± 4 kg) were anaesthetised twice, six days apart, for ± 30 minutes after which they regained consciousness. Propofol (anaesthetic agent) was used to achieve different levels of (un)consciousness. After recording a baseline of five minutes, sheep received a bolus injection of propofol at T=0 min (8 mg kg^{-1}, intravenously) followed by continuous infusion of 8 mg kg^{-1} hr^{-1} for 28 minutes using an infusion pump. Administration of propofol stopped at T=31 min, where after sheep regained consciousness. Reflexes were tested every two minutes. Respiratory rate (RR) and EEG were recorded continuously. Loss and regain of consciousness was based on visual assessment of the EEG, as well as on analysis of derivatives of the EEG. In visual assessment of the EEG, five different stages were observed: baseline, transitional, unconscious, $transitional_{rec}$ and recovery (similar to baseline).

In part b, mixed breed ewes (n=21, 38 ± 4 kg) were exsanguinated by neck cut, severing both jugular veins and carotid arteries. Reflexes were tested every two seconds. RR and EEG were recorded continuously. Loss of consciousness was based on visual assessment of the EEG. In visual assessment of the EEG, three different stages were observed: baseline, unconscious and iso-electric (flat).

Results: In part a, sheep lost and regained consciousness after propofol administration (T=0 min) at 00:43 ± 00:06 min and 28:27 ± 06:20 min respectively, based on visual assessment of the EEG. Breathing was rhythmic (based on visual assessment of the RR), from 01:26 ± 0:21 min until 21:13 ± 05:46 min, whilst animals were unconscious. Ocular reflex to threat- and pain withdrawal reflexes were absent after 01:57 ± 0:31 and 02:48 ± 01:14 min and returned at 28:51 ± 06:14 and 13:36 ± 05:02 min, respectively. The eyelid reflex was absent at 04:40 ± 02:11 and returned at 10:45 ± 05:31 min, but did not disappear in 8 out of 20 observations. Based on the EEG, however, all sheep were considered unconscious during that time. Pearson correlations, showed strong and highly significant correlations between visual assessment of the EEG and derivatives of the EEG with correlations ranging from r=0.53 to r=0.97 (P<0.01), supporting the classification of different stages of unconsciousness as found in visual assessment of the EEG. Significant and strong correlations that were found between EEG- and behavioural parameters consisted of: $transitional_{rec}$ and recovery EEG with 1. ocular reflex to threat (r=0.58, P <0.01 and r=0.63, P <0.01); 2. righting reflex (r=0.78, P <0.001 and r=0.81, P <0.001) and 3. Change back to non-rhythmic breathing (r=0.81, P <0.001 and r=0.82, P <0.001). This indicates that only the presence of an ocular reflex to threat, presence of righting reflex and change in breathing pattern were parameters that indicated animals regained consciousness after propofol anaesthesia. Variation in the pain withdrawal- and eyelid reflex were difficult to interpret in the assessment of consciousness.

In part b, sheep lost consciousness 00:15 ± 00:04 min following neck cut (T=0 min), based on visual assessment of the EEG. The EEG was evaluated as iso-electric, indicating minimal brain activity, at 00:27 ± 00:08 min. Irregular breathing was observed in all animals at 00:43 ± 00:12 min. In seven

sheep, an ocular reflex to threat was present (until 00:07 ± 00:01 min) prior to loss of consciousness. No pain withdrawal reflex was seen following neck cut, during exsanguination. An eyelid reflex, however, was observed in all sheep until 01:14 ± 00:17 min.

Conclusions: The absence of the ocular reflex to threat and pain withdrawal reflex following neck cut, indicated that absence of these parameters does not necessarily indicate unconsciousness. When absence of regular breathing or the eyelid reflex was observed, animals were always unconscious. Absence of these parameters, however, occurred after 40 s following neck cut and it is therefore proposed to look at other, more valid, parameters that can indicate unconsciousness 40 seconds following neck cut.

Samenvatting

Inleiding: Nieuwe Nederlandse wetgeving vereist dat bewusteloosheid wordt getest op 40 seconden na aansnijden in dieren die voorafgaand niet verdoofd zijn. Dieren die op 40 seconden na aansnijden niet bewusteloos zijn, moeten dan alsnog onmiddellijk worden verdoofd. De beoordeling van bewusteloosheid tijdens het slachtproces wordt in het algemeen gebaseerd op gedrag parameters (zoals reflexen en willekeurige bewegingen). In een experimentele opzet kan het gebruik van hersenactiviteit, zoals weergegeven in een electroencephalogram (EEG), eveneens worden toegepast. Het EEG wordt beschouwd als een meer objectieve benadering om bewusteloosheid te beoordelen. De validiteit en toepasbaarheid van de meeste gedrag parameters zoals die gebruikt worden in de beoordeling van bewusteloosheid onder verschillende omstandigheden (met en zonder verdoving) worden (inter) nationaal bediscussieerd en dienen nader onderzocht te worden.

Onderzoeksvraag: Het doel van deze studie (deel a en b) was om de volgende parameters te valideren waarmee bewusteloosheid wordt vastgesteld: ritmische ademhaling, ooglidreflex, dreigreflex en pijnreflex. In deel a van de studie is de oprichtreflex ook getest.

Materiaal en methoden: In experiment 1a werden ooien (n = 10 , 36 ± 4 kg) tweemaal verdoofd, met zes dagen ertussen, gedurende ± 30 minuten waarna de dieren weer bijkwamen. Propofol (anestheticum) werd gebruikt om verschillende stadia van (on)bewustzijn te creëren. Na het opnemen van een basislijn van vijf minuten, kregen schapen intraveneus een propofol bolus (op T=0 min) van 8 mg kg^{-1}. Dit werd gevolgd door 28 minuten lang 8 mg kg^{-1} h^{-1} propofol toe te dienen. Op T=31 min werd de toediening gestopt waarna de schapen weer bij bewustzijn kwamen. Reflexen werden elke twee minuten getest. De ademhaling frequentie (RR) en het EEG werden continu geregistreerd. Het verlies en bijkomen van bewustzijn werden gebaseerd op visuele beoordeling van het EEG, en de analyse van de afgeleiden van het EEG. In de visuele beoordeling van het EEG werden de volgende vijf stadia waargenomen: basis, overgang, bewusteloos, overgang naar herstel en hersteld (vergelijkbaar met baseline) EEG.

In experiment 1b werden ooien (n = 21 , 38 ± 4 kg) verbloed door de hals, zowel de halsaders en halsslagaders, door te snijden. Reflexen werden na het aansnijden elke twee seconden getest. RR en EEG werden continu geregistreerd. Bewustzijnsverlies was gebaseerd op visuele beoordeling van het EEG. In de visuele beoordeling van het EEG werden de volgende drie stadia waargenomen: basis, bewusteloos en iso -elektrisch (vlak) EEG.

Resultaten: In experiment 1a verloren en herwonnen schapen het bewustzijn op 00:43 ± 00:06 min en 28:27 ± 06:20 min na propofol toediening (T=0 min), gebaseerd op de visuele beoordeling van het EEG. Ademhaling was ritmisch (op basis van visuele beoordeling van de RR) tussen 01:26 ± 00:21 en 21:13 ± 05:46 min, terwijl de dieren bewusteloos waren. De dreig- en pijn reflex waren afwezig vanaf 01:57 ± 00:31 en 02:48 ± 01:14 min en keerden terug na 28:51 ± 06:14 en 13:36 ± 05:02 min, respectievelijk. De ooglid reflex was afwezig tussen 04:40 ± 02:11 en 10:45 ± 05:31 min, maar bleef aanwezig tijdens 8 van de 20 waarnemingen. Op basis van het EEG, waren alle schapen echter bewusteloos in die tijd. Pearson correlaties toonden sterke en zeer significante correlaties tussen de visuele beoordeling van het EEG en afgeleiden van het EEG met correlaties variërend van r = 0.53 tot r = 0.97 (P<0.01). Dit onderbouwd de indeling van de verschillende stadia zoals gevonden bij visuele beoordeling van het EEG. Sterke correlaties die gevonden werden tussen EEG - en gedrag parameters waren: overgang naar herstel en hersteld EEG met 1. dreigreflex (r=0.58, P<0.01 en r=0.63, P<0.01); 2. Oprichtreflex (r=0.78, P<0.001 en r=0.81, P<0.001) en 3.Terugkeer naar niet - ritmische ademhaling (r = 0.81, P < 0,001 en r=0.82, P<0.001). Dit betekent dat alleen de aanwezigheid van een dreigreflex, oprichtreflex en een verandering van het ademhaling patroon parameters waren, die aangaven dat dieren weer bij bewustzijn kwamen na propofol anesthesie. Variatie in de pijn- en ooglid reflex waren moeilijk te interpreteren bij de beoordeling van bewustzijn.

In experiment 1b verloren schapen het bewustzijn 0:15 ± 0:04 min na de halssnede (T=0 min) op basis van visuele beoordeling van het EEG. Het EEG werd beoordeeld als iso–elektrisch (hersendood) 00:27 ± 00:08 min na de halssnede. Een onregelmatige ademhaling werd waargenomen na 00:43 ± 00:12 min. In 7 van de 21 schapen werd een dreigreflex waargenomen (tot 00:07 ± 00:01 min) voorafgaand aan het intreden van bewusteloosheid. In geen enkel schaap werd een pijnreflex waargenomen na aansnijden. De ooglid reflex, daarentegen, werd waargenomen tot 01:14 ± 00:17 min na aansnijden.

Conclusies: De afwezigheid van de pijn- en dreigreflex na aansnijden betekent niet noodzakelijk dat dieren bewusteloos zijn. Bij afwezigheid van een regelmatige ademhaling of ooglid reflex waren dieren altijd bewusteloos. Tijdens experiment 1b, waren deze laatstgenoemde parameters echter vaak later afwezig dan op de voorgeschreven 40 seconden na aansnijden. Het advies is dan ook, om te kijken naar andere, meer valide, parameters die met grote zekerheid bewusteloosheid aantonen op 40 seconden na aansnijden.

Table of contents

1 Introduction

European legislation (e.g. EU Council Regulation, 1099/2009) provides laws and regulations for the slaughter of farm animals, to safeguard animal welfare. To facilitate the implementation of the EU Council Regulation 1099/2009, the Dutch minister of Economic Affairs has, in close compliance with concerned parties, listed areas which require further investigation to safeguard or improve animal welfare during slaughter. These areas include:

1. Identification of critical control points
2. **Validation of parameters to examine unconsciousness**
3. Reducing time between neck cutting and unconsciousness
4. Number of neck cuts required
5. Method and duration of restraint

One of the areas of concern involves the assessment of unconsciousness with a focus on animals that have not been stunned prior to neck cutting as is mandatory following EU Council Regulation 1099/2009. This assessment of unconsciousness is important, since new national legislation requires that unconsciousness should be tested at 40 seconds after performing the neck cut, with an obligation to stun the animal if not unconscious at that point. To assess unconsciousness after neck cutting without prior stunning, a set of five (behavioural) parameters was agreed on in the ’Dutch agreement on non-stunning slaughter in accordance with religious rites’ of 2012. These 5 parameters are: 1. spontaneous eyelid reflex, 2. stimulation of the nasal septum (pain response), 3. spontaneous rhythmic breathing, 4. righting reflex and 5. the ocular reflex to threat. The agreement states that at least three of these five parameters need to be absent to consider an animal unconscious. The validity and applicability of these five parameters for assessing unconsciousness under different slaughter circumstances (with and without stunning) are, however, still under (inter)national debate and warrant further investigation.

Monitoring brain activity as presented in an electroencephalogram (EEG) is considered an objective method when assessing unconsciousness and is generally accepted as a ‘golden standard’ in the assessment of unconsciousness (Gerritzen and Hindle 2009; EFSA 2012); (Erasmus et al. 2010). The EEG reflects the sum of underlying electrical activity of populations of neurones supported by glia cells (Murrell and Johnson 2006). There are four different types of wave patterns (frequency bands) in the EEG that can be distinguished based on their respective frequencies and that are related to the state of consciousness: delta (0-4 Hz), theta (4-8 Hz), alpha (8-12 Hz) and beta (>12 Hz) waves. Both delta and theta (slow wave) activity is related to sleep or reduced consciousness. Alpha activity is prominent in subjects that are conscious, but mentally inactive (closing eyes and relaxation) and beta waves are associated with active movements and increased alertness (Kooi et al. 1978; Schomer and Da Silva 2012).

The onset of unconsciousness can be determined by visual assessment of changes in patterns, amplitude and frequency of the EEG, but also by calculating standardised EEG parameters or derivatives (Davidson 2006). Derivatives that can be derived from the EEG are: total power (PTOT), spectral edge frequency 95% (SEF) and power in the different frequency bands (Mota-Rojas et al.). PTOT is calculated as the total area under the power spectrum curve (Murrell and Johnson 2006). The SEF is the frequency below which 95% of the total power of the EEG is located (figure 1). These derivatives are known to be related to anaesthesia and clinical signs of loss of consciousness (Schwender et al. 1996; Schwilden 1989; Martin-Cancho et al. 2003; Martín-Cancho et al. 2006).

In order to study behavioural parameters that (supposedly) reflect loss of consciousness, induction of anaesthesia can be used to create different stages of (un)consciousness for longer periods of time, compared to stunning methods or exsanguination. The use of anaesthetic states can provide a model to validate different behavioural parameters when assessing unconsciousness and can provide detailed information on correlations between the presence / absence of behavioural parameters and the EEG.

Propofol is an anaesthetic agent that inhibits neuronal firing through GABA receptor mechanisms (Schomer and Da Silva 2012). It is used both for inducting and maintaining unconsciousness and the on- and offset are both quick (San-juan et al. 2010). By using propofol, the time frame during which animals lose consciousness can be controlled and extended, where this cannot be accomplished with the various stunning methods and exsanguination.

The objective of the present study was to evaluate the EEG- and behavioural parameters as described in the 'Dutch agreement on non-stunning slaughter in accordance with religious rites'(2012). Sheep were chosen as study animals, since they are one of the target species.

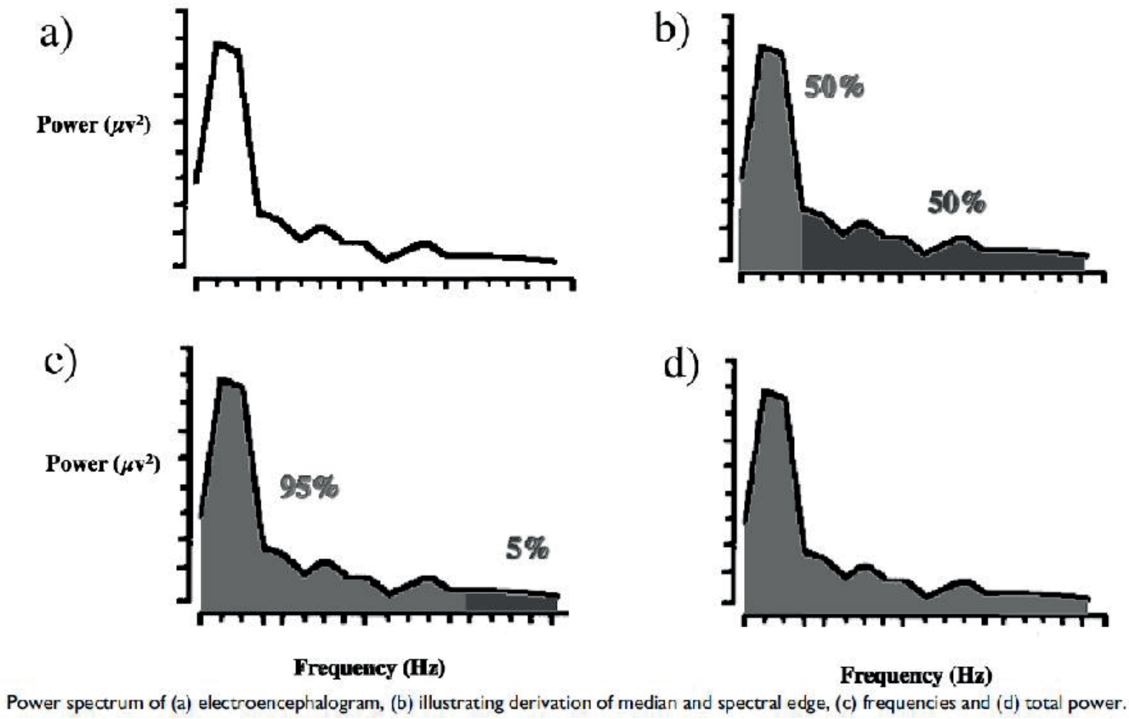

Figure 1. *Visualisation of EEG derivatives (parameters). Adopted from Johnson et al., 2012.*

An experiment, consisting of two parts, was set up to provide insight into 1. the validity and 2. the applicability of the five parameters proposed to assess unconsciousness at slaughter without stunning:

1a. An experiment under controlled circumstances to assess the 5 parameters in sheep that were anaesthetised with propofol during different levels of unconsciousness.

1b. An experiment under controlled circumstances to assess the 5 parameters in sheep that were exsanguinated without prior stunning.

The 5 parameters to be assessed included: 1. spontaneous eyelid reflex, 2. stimulation of the nasal septum (pain response), 3. spontaneous rhythmic breathing, 4. righting reflex and 5. the ocular reflex to threat.

The results of the two experiments are described in this progress report.

An experiment were the gained knowledge from experiment 1a and 1b will be applied under practical conditions at slaughter (in veal calves) is planned to take place end of 2014. During this experiment, there will also be more focus on the applicability of the different parameters, since the experiment will take place under practical conditions.

2 Material and methods

Four out of five of the parameters selected by the ’Dutch agreement on non-stunning slaughter in accordance with religious rites (2012) were included in the current experiment (part a and b) to study the validity and the feasibility of the parameters. The parameters included in the experiment were 1. (spontaneous) eyelid reflex, 2. stimulation of ear (pain response), 3. (spontaneous) rhythmic breathing, and 4. the ocular reflex to threat. The righting reflex was due to the set-up of the experiment only assessed during experiment 1a. The two experiments were performed with young sheep (ewes). The experiments were carried out under controlled circumstances at the research facilities of Wageningen UR Livestock Research, Lelystad. The experiment was performed under experimental conditions at the research facilities instead of at a commercial slaughter location to minimize the influence of the surroundings, acquire good quality EEG data and standardize the measurements as much as possible.

2.1 Approval

The experiment was approved (DEC 2013103.b) beforehand by the Ethical Committee of the Animal Sciences Group of Wageningen UR, The Netherlands.

2.2 Statistical set-up

Based on simulated response curves in GenStat, the recommended number of sheep for experiment 1a was ten, with a repetition of two times per animal. Twelve sheep were purchased: one sheep was used as pilot animal and one served as a spare animal, in case observations were interfered or propofol administration did not go according to schedule. The spare animal was used in the experiments.

The total number of sheep in experiment 1b was set at 22. The 12 sheep from experiment 1a were used plus ten new animals that were purchased solely for experiment 1b. One sheep was used as pilot animal for experiment 1b.

2.3 Animals and housing

In total, 22 ewes (36.0 ± 3.4 kg), mixed breeds, from a commercial trader, were used. Sheep arrived in two groups at the experimental site. The first group (group 1a, n=12) arrived four days prior to the start of experiment 1a. The pen at the experimental site measured 5.6 x 4.6 m (l x w) and was furnished with a layer of wood shavings of ± 5 cm (figure 2.3a,b). Ambient temperature was set at 18° C and lights were on between 06.00 h and 18.00 h. Sheep had free access to commercial feed, hay and water, except during the 16-h prior to anaesthesia of the first animal, when sheep only had access to water. The second group (group 1b, n=10) arrived five days prior to experiment 1b and was housed together with group 1a. Between the last day of experiment 1a and experiment 1b were 15 days. All sheep were shaved prior to their arrival at the experimental facilities.

__Figure 2.3a,b__. Housing of the animals at the experimental facilities

2.4 Experimental set-up

2.4.1 Experiment 1a (propofol)

In experiment 1a, sheep were anaesthetised with propofol twice, six days apart. Three to four sheep were anaesthetised per day and experiment 1a therefore comprised two times three days. Sheep were deprived of feed, but water was available until 07.00 h in the morning. Experimental days started at 08.00 h. When a sheep was taken out of the pen, it was transported in a cart to the operating room across the hall. After weighing, head and neck and legs were shaved with an electric clipper to enable careful installation of electrodes (both EEG and electrocardiogram, ECG) and catheter. Subsequently, the neck of the animal was anaesthetized locally: at the level of the jugular vein ~1 gram of the 2.5 % lidocaine and 2.5 % prilocaine topical anaesthetic EMLA cream (EMLA®, AstraZeneca, Zoetermeer) was applied. After ten minutes the catheter was placed. Respiratory rate (RR) was measured by placing a respiratory belt around the abdomen behind the sheep's last rib (figure 2.4.1.b). The sheep was then placed in a custom-made hammock (resting on the abdomen) with its legs hanging freely through four holes (figure 2.4.1a,b). After verifying the efficacy of the EMLA® cream by pinching the sheep's neck, an 18-gauge catheter (Braun, Germany) was placed into the jugular vein and flushed with heparinised saline solution (0.9% NaCl) and fixed to the skin (figure 2.4.1c). Subsequently, electrodes to record EEG and the electrocardiogram (ECG) were placed. Results of the ECG are not discussed in this report.

The recording started with a baseline of five minutes. Sheep thereafter (at T=0 min) received a bolus injection of propofol (8 mg kg^{-1}, intravenously) followed by continuous infusion of propofol of 8 mg kg^{-1} hr^{-1} for 28 minutes using an infusion pump. Administration of propofol stopped at T=31 min and sheep could regain full consciousness. Data was recorded until the EEG was similar to baseline EEG as seen live on screen and the sheep was taken back to a separate part of the pen for further recovery. At the end of the day all sheep were placed back with the flock. Six days later, every sheep followed the complete routine again.

Figure 2.4.1a,b. *A sheep placed in the hammock as used during experiment 1a and 1b.*

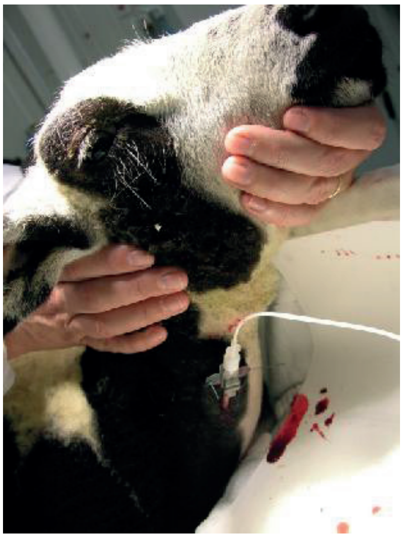

Figure 2.4.1c. *Placement of the catheter into the jugular vein to administer propofol*

2.4.2 Measurements

With the set-up of the experiments, it was only possible to assess the righting reflex during experiment 1a, where it was defined as the first lifting of the head after propofol anaesthesia. The spontaneous eyelid reflex has been replaced by the eyelid reflex. Animals that are slaughtered without prior stunning are often restrained and rotated before applying the neck cut. A spontaneous eyelid reflex may than be induced by blood that runs along the eye. In animals that are not rotated, it is expected that a spontaneous eyelid reflex may often not occur. If the reflex is induced when touching the eyelid, as can be done by a person, this parameter is thought to be more reliable. Responsiveness to the pain stimulus and the presence of eyelid- and ocular reflex to threat were assessed twice when sheep were conscious (during baseline recordings). Thereafter all three stimuli were applied every two minutes from start of administration of propofol (T=0 min) until the experiment was stopped. The experiment was stopped when the sheep showed a positive response to all three stimuli three times in a row after stop of propofol administration (T=31 min). The response to the pain stimulus was studied by pinching the sheep’s tip of the ear using two fingertips and determine if it responded by withdrawal of the ear or head. The ocular reflex to threat was assessed by checking the presence of a blinking reaction to an abrupt movement of the index finger to the eye ball without touching the eye. The eyelid reflex was assessed by a gentle touch of the eyelid, considered as present when a blinking reaction was observed and as absent when no response was observed. Stimuli to trigger responses were executed in a random order for each sheep.

An overview of experiment 1a (supported by pictures) can be found in appendix I.

2.4.3 Experiment 1b (exsanguination)

Five days prior to experiment 1b, ten healthy ewes, mixed breeds, from a commercial trader, reared under standard conditions arrived at the experimental facilities. They were placed in the pen, together with the 12 ewes from experiment 1a. Sixteen hours prior to experiment 1b, sheep were deprived of food. Housing and feeding regime was similar as for sheep in experiment 1a. At the day of experiment 1b, all sheep were deprived of water from 07.00 h onwards. The experiment started at 08.00 h. Weighing the sheep and recording the different parameters was similar as was done in experiment 1a. After recording a baseline of five minutes, sheep were exsanguinated (T=0 min) without stunning by severing both jugular veins and carotid arteries. The neck cut was performed by a certified and experienced person from a slaughter plant. Response to a pain stimulus, ocular reflex to threat and eyelid reflex were assessed in a randomized order. Responses to stimuli were assessed every two seconds until a negative response was observed five times in a row. EEG, ECG and RR were recorded up to five minutes after an iso-electric (flat) EEG was observed live on screen.

Blood was collected in a plastic gutter leading to a plastic bin, placed on a weighing scale. Cumulative blood loss was recorded every five seconds up to three minutes following neck cut. Video recordings were made to monitor the occurrence of convulsions at a later stage. These findings are not taken into account in this report.

An overview of experiment 1b (supported by pictures) can be found in appendix II.

2.5 Electrode placement

Parameters to be recorded continuously included brain activity (as presented by an electroencephalography, EEG), heart rate (as presented by an electrocardiogram, ECG) and respiratory rate (RR). A respiratory belt was placed around the abdomen to record the RR. Visual assessment of the RR after the experiments was used to observe presence of rhythmic breathing. Three newly developed EEG water surface electrodes (TMSi, Oldenzaal, The Netherlands) were placed left, right and on top of the midpoint of the head, between the caudal margin of the head and the line joining the caudal corners of the eye and ear (figure 2.5a). To secure these electrodes, tape was wrapped twice around the animals head, leaving the ears free to move. ECG electrodes (Ag/Cl from TMSi, Oldenzaal, The Netherlands) were snapped on belts that were placed around the legs ten cm above the knee (figure 2.5b). Once all ECG and EEG electrodes were placed properly and a good live signal was observed, a baseline was recorded for five minutes after which the propofol administration was started or the neck cut was performed.

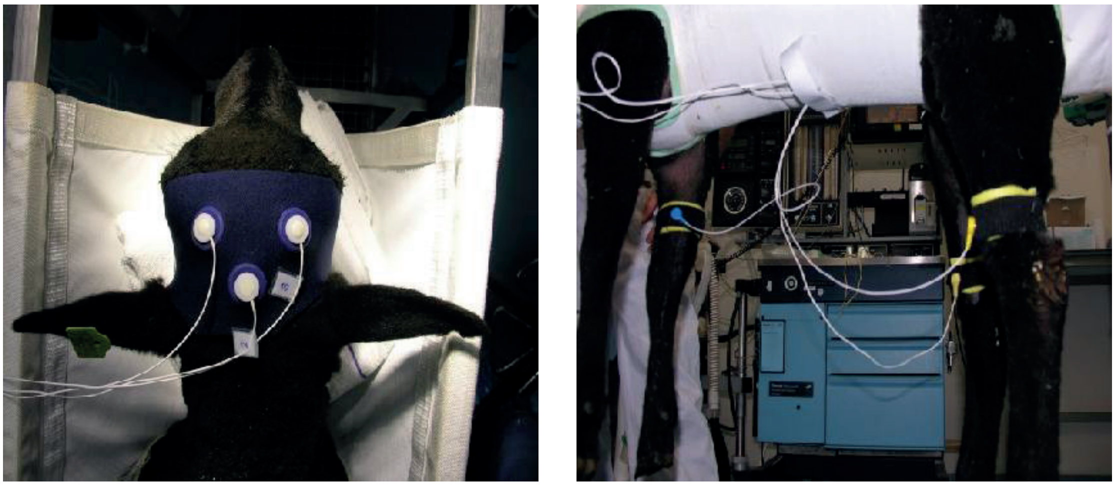

Figure 2.5a,b. *Placement of the EEG- (left) and ECG (right) electrodes in a sheep*

2.6 Data recording

EEG and ECG data were recorded using the Twente Medical Systems International (TMSi), Oldenzaal, The Netherlands, Porti system with 32 channels in a battery powered mode (figure 2.6). Electrode impedance was < 1kΩ due to the usage of shielded cables. The EEG was displayed at a high and low frequency cut off of 0.5 and 30 Hz, but saved to a computer unfiltered. Sample rate was set at 2 kHz.

Figure 2.6. *Porti system (32 channels) to record physiological parameters such as EEG, ECG and RR in sheep (figure adopted from www.tmsi.com).*

2.7 Data analysis

All physiological data were displayed, stored and analysed using PolyBench software (TMSi, Oldenzaal, The Netherlands). Changes in time in the EEG traces to a recovery (comparable to baseline), transitional$_{ind}$ (transitional during induction with propofol), transitional$_{rec}$ (transitional during recovery with propofol) suppressed and strongly suppressed or near iso-electric signal were located visually. Quantitative analysis of each recording using a Fast Fourier Transformation (FFT) was used to show the frequency composition of the time signal and calculate Total Power, SEF, and power in each frequency band per epoch of 2 seconds. RR was calculated automatically in rpm and visual scoring of these traces was used to detect apneas and the occurrence of (non) rhythmic or irregular breathing. Presence or absence of behavioural parameters were noted. Statistical analysis was performed using SAS. A Pearson correlation was run to analyze correlations between behavioural- and EEG parameters. All the tests were conducted at the 5% (P = 0.05) significance level.

3 Results

The result section can be subdivided into two main parts:

1. Responses during propofol anaesthesia
2. Responses during exsanguination

3.1 Results experiment 1a (propofol anaesthesia)

3.1.1 Animals

A total of 20 observations from 11 sheep were used in the analyses. Sheep weighed 35.2 ± 3.6 kg (n=9) on experimental day 1 and 36.3 ± 3.5 kg (n=11) on experimental day 2.

3.1.2 EEG parameters: visual assessment

A clear EEG signal was obtained from all sheep during propofol anaesthesia. During recovery though, the EEG was sometimes uninterpretable due to muscle activity. Figure 3.2.1a,e shows an example of a representative series of four seconds of EEG registration during the different stages that could be identified from visual assessment of the EEG during propofol anaesthesia. Baseline EEG consisted of a low amplitude, high frequency signal, indicating alert animals (a). After start of administrating propofol (T=0 min), changes in the EEG were seen at 00:33 ± 0:05 min, when slow wave activity (firing of neurons in a synchronized fashion) became more apparent, associated with reduced consciousness ($transitional_{ind}$ stage) (b). Slow wave activity was apparent from 00:43 ± 0:06 min onwards, indicating unconsciousness in sheep from that point onwards (c). Administration of propofol was stopped (at T=31 min), but changes back to a transitional EEG ($transitional_{rec}$ stage) were already seen at 23:54 ± 05:10 min (d) due to the low maintenance doses of 8 mg kg^{-1} hr^{-1}. An EEG comparable to baseline (recovery) was seen after 28:27 ± 06:20 min (e) (table 3.1.2.).

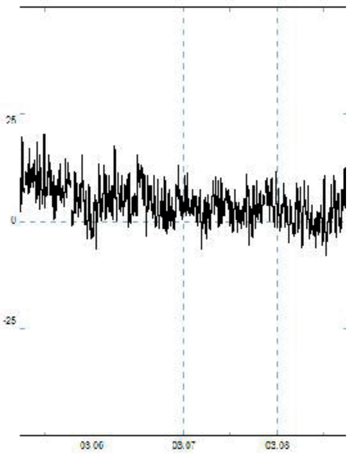

Figure a

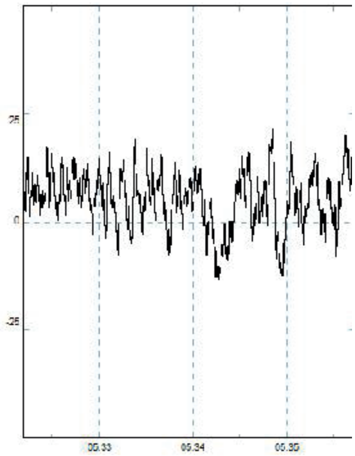

Figure b

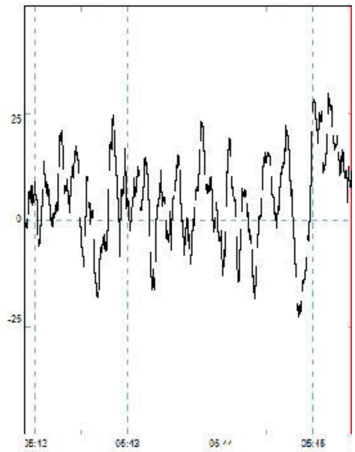

Figure c

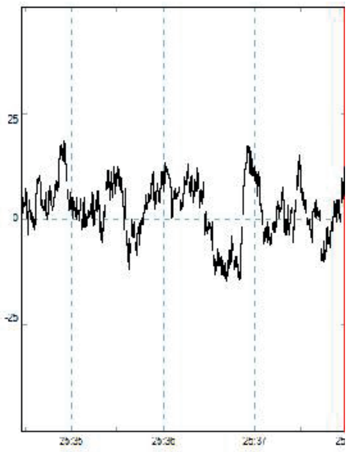

Figure d

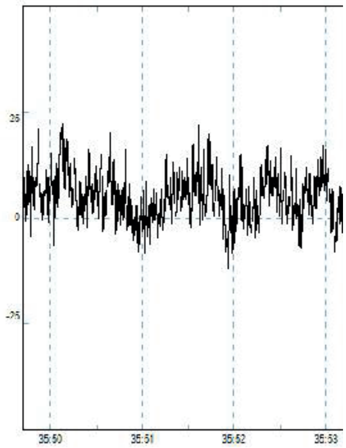

Figure e

Figure 3.1.2. *Typical example of the different stages identified with visual assessment of the EEG prior to, during and after propofol administration in sheep. The five stages from left to right and top to bottom: baseline (a),* transitional$_{ind}$ *(b), unconscious (c), transitional$_{rec}$ (d) and, recovery (similar to baseline) (e). X-axis represents ~4 seconds, Y-axis represents amplitude of the EEG-trace (µV).*

Table 3.1.2.

Start of the different stages (min) based on visual assessment of the EEG in 20 observations obtained from 11 sheep after propofol administration.

	Stages observed in the EEG			
	Transitional$_{ind}$ (min)	Unconscious (min)	Transitional$_{rec}$ (min)	Recovery (min)
Average	00:33	00:43	23:54	28:27
St.dv.	00:05	00:06	05:10	06:20

3.1.3 EEG parameters: EEG derivatives

Examining the EEG data in more detail, compared to visual assessment, was performed by computing a Fast Fourier Transformation (FFT) in order to look at spectral specifics. The output of a FFT represents the frequency composition of the signal, or formulated alternatively, how much power is presented in the different frequency bands. Figure 3.1.3a shows the mean total power (PTOT) of the EEG signal at the different stages: baseline, transitional$_{ind}$ (red line), unconscious (green line), transitional$_{rec}$ (black line) and recovery (purple line) in 20 observations from 11 sheep.

During baseline, the PTOT did not change (~50 µV^2), as the sheep were conscious during this time, but during induction of anaesthesia the mean PTOT progressively increased from 00:35 min onwards ($P < 0.001$). The PTOT values increased as sheep shifted from a conscious (low-amplitude, high-frequency EEG wave activity) to an unconscious state, in which EEG wave activity was synchronized and PTOT was at its highest (characterized by high-amplitude, low-frequency wave forms). The PTOT was higher than baseline until 31:00 min after start of propofol administration (all $P < 0.01$).

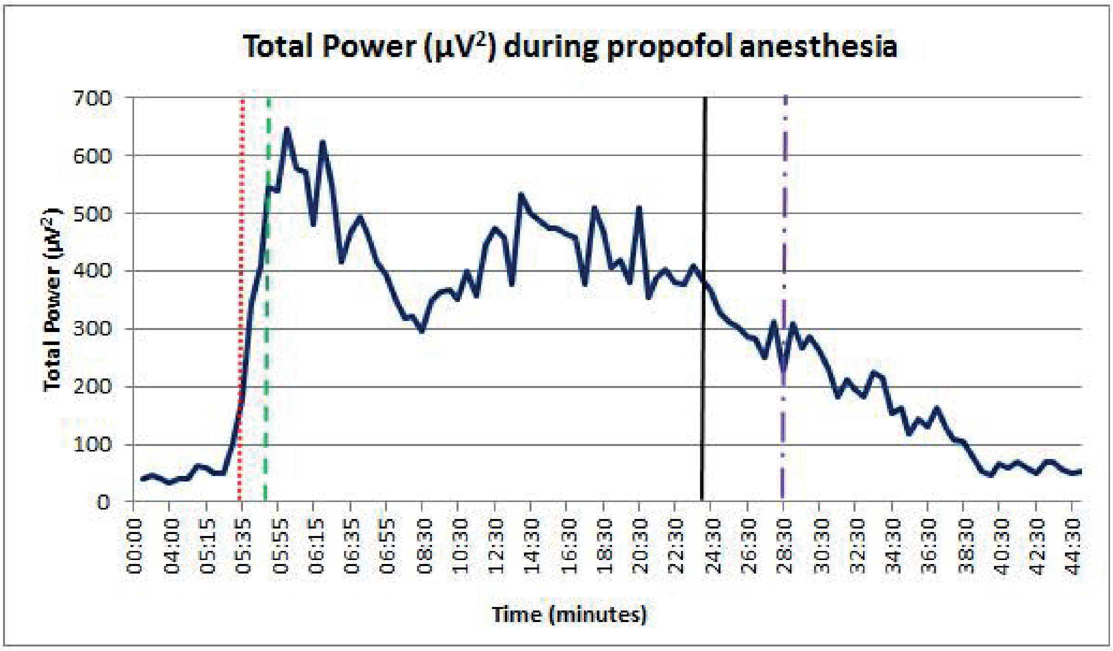

Figure 3.1.3a. *Mean Total Power (PTOT) of the EEG averaged over 20 observations from 11 sheep during propofol anaesthesia. Start of propofol anaesthesia in the graph is at T=5 min.*

Figure 3.1.3b shows the contribution of the different frequency bands within the EEG signal through time at the different stages: baseline, $\text{transitional}_{ind}$ (red line), unconscious (green line), $\text{transitional}_{rec}$ (black line) and recovery (purple line) in 20 observations from 11 sheep. During baseline, the four different frequency bands had stable, almost similar contributions in the EEG signal. During induction of anaesthesia contribution of slow wave frequency bands (delta: 0.5-4 Hz and theta: 4-8Hz) increased, indicating synchronizing of firing of neurons representative for loss of consciousness. From 25:00 minutes after propofol administration onwards, contributions of the alpha, and beta bands were similar compared to baseline. From 25:00 minutes after propofol administration onwards, contributions of the alpha, and beta bands were similar compared to baseline. From 29:30 minutes after propofol administration onwards, contributions of the delta and theta bands were similar compared to baseline.

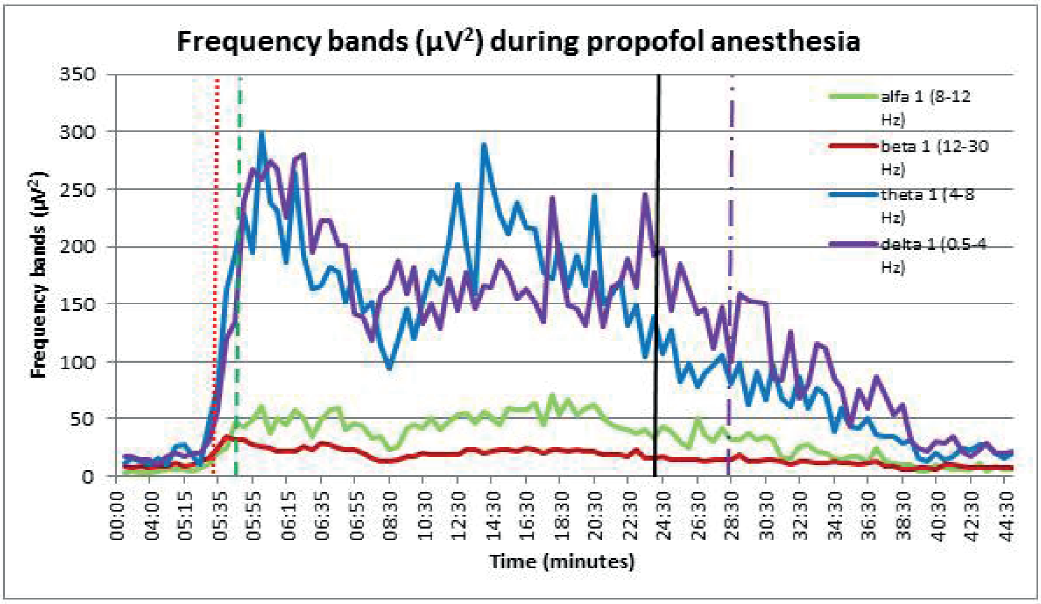

Figure 3.1.3b. *Average contribution of the different frequency bands of the EEG averaged over 20 observations from 11 sheep during propofol anaesthesia. Start of propofol anaesthesia in the graph is at T=5 min.*

Figure 3.1.3c shows the spectral edge frequency (SEF) through the different stages: baseline, transitional$_{ind}$ (red line), unconscious (green line), transitional$_{rec}$ (black line) and recovery (purple line) in 20 observations from 11 sheep. During baseline the SEF was on average 23.3 Hz. After induction this shifted quickly to 11-12 Hz, when delta and theta (low frequency waves) became dominant, as can be observed in figure 3.1.3b. From 19:30 min after propofol administration onwards SEF slowly increased to 18 Hz at the end of the recording.

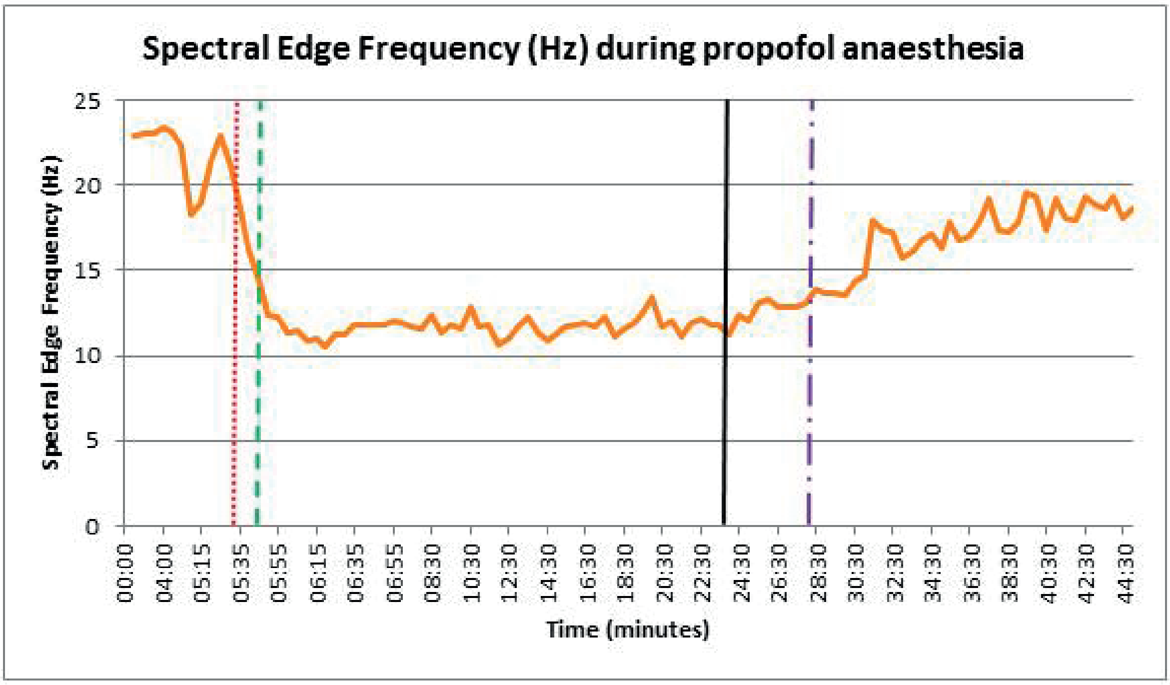

Figure 3.1.3c. *Spectral Edge Frequency (SEF) of the EEG of the EEG averaged over 20 observations from 11 sheep during propofol anaesthesia. Start of propofol anaesthesia in the graph is at T=5 min.*

3.1.4 EEG parameters: correlations

Pearson correlations were conducted between timing of the different stages based on visual assessment of the EEG and timing of the different stages based on (more standardised) EEG derivatives such as SEF, contribution of different frequency bands and PTot (all based on FFT).

Strong correlations were found between the two ways of analysing the EEG with correlations ranging from r=0.53 to r=0.97 (*P* <0.01).

3.1.5 Behavioural parameters: rhythmic breathing

Rhythmic breathing was assessed by visual assessment of the RR that was recorded for each sheep. During propofol anaesthesia, breathing was scored as rhythmic when, as a consequence of the propofol, breathing became even more rhythmic than during baseline. During rhythmic breathing, each breath was similar to the one before in both volume and frequency, as can be observed in the transition from figure 3.1.5a to figure 3.1.5b.

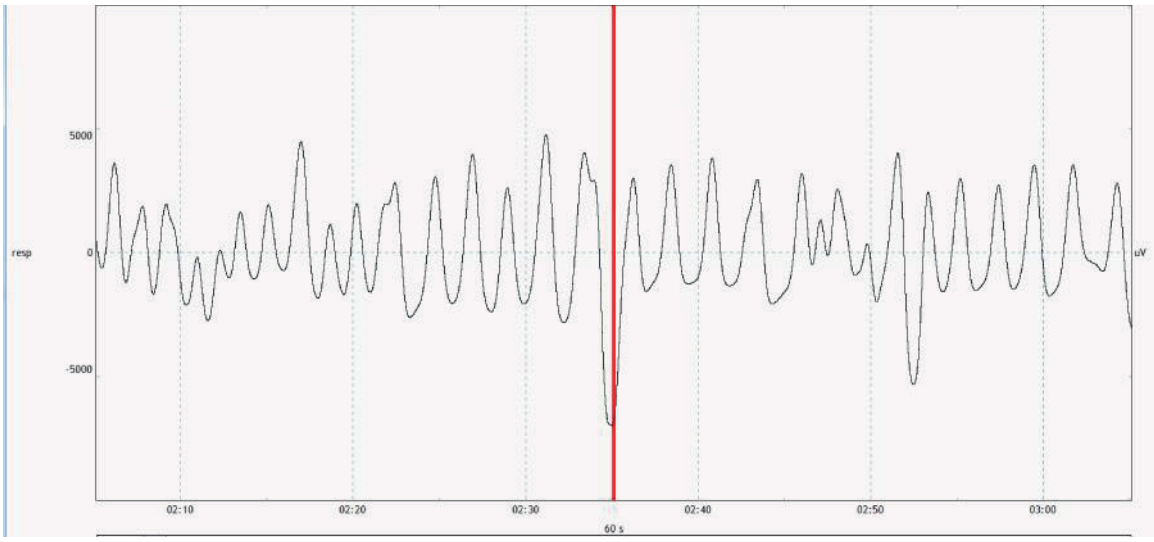

Figure 3.1.5a. *Typical example of 'non-rhythmic breathing' in sheep 3-1 during baseline recordings (start of figure, T=02:40 min. Complete time frame is 60 s).*

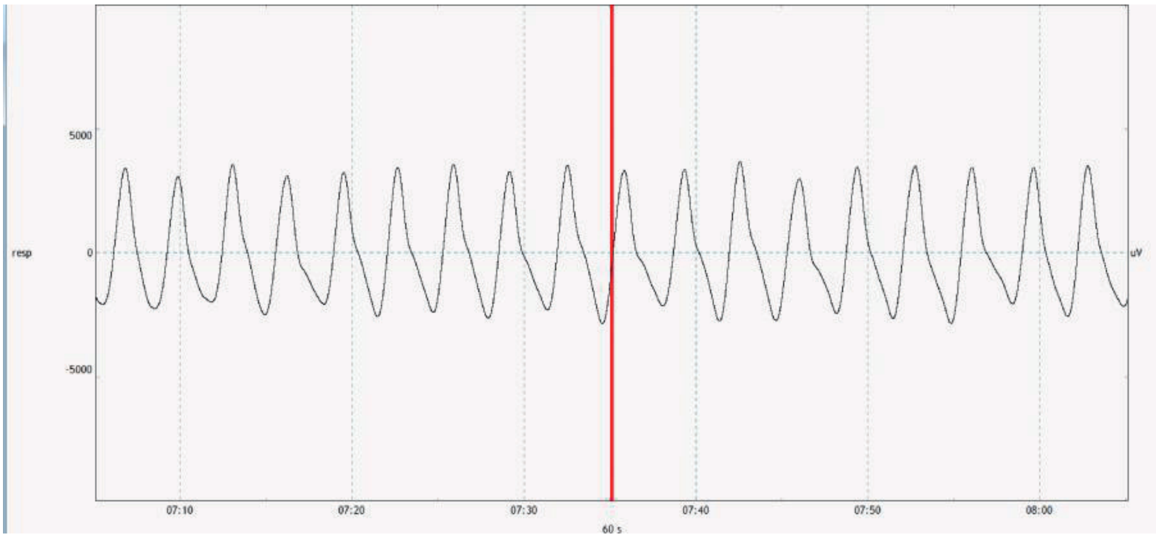

Figure 3.1.5b. *Typical example of 'rhythmic breathing' in sheep 3-1 during propofol anaesthesia (start of figure, T=07:40 min. Complete time frame is 60 s).*

Rhythmic breathing was observed from 01:26 ± 0:21 to 26:13 ± 05:46 min after start of propofol administration.

3.1.6 Behavioural parameters: presence of reflexes

All reflexes were tested and present in all sheep during baseline recordings. The first absence and presence of the different reflexes are summarised in figure 3.1.6 and table 3.1.6. First absence of the eyelid reflex occurred on average at 04:40 ± 02:11 min and with return of presence at 10:45 ± 05:31 min after start of propofol administration (T=0 min). In 8 out of 20 observations, however, the eyelid reflex was never absent. The average times to loss of and return of the pain withdrawal were 02:48 ± 1:14 and 13:36 ± 05:02 min. The average times to loss of and return of the ocular reflex to threat were 01:57 ± 00:31 and 28:51 ± 06:14 min. Sheep opened their eyes at 25:21 ± 05:09 min and showed a first righting reflex at 28:45 ± 05:57 min.

Table 3.1.6*.*
First absence and presence of three reflexes from 20 observation in 11 sheep anesthetised with propofol at T=0 min.

	First absence of reflex			First presence of reflex		
	Threat	*Pain*	*Eyelid*[1]	*Threat*	*Pain*	*Eyelid*
Average time (mm:ss)	01:57	02:48	04:40	28:51	13:36	10:45
Std. dev. (mm:ss)	00:31	01:14	02:11	06:14	05:02	05:31

[1]In 8 out of 20 observations, the eyelid reflex never disappeared.

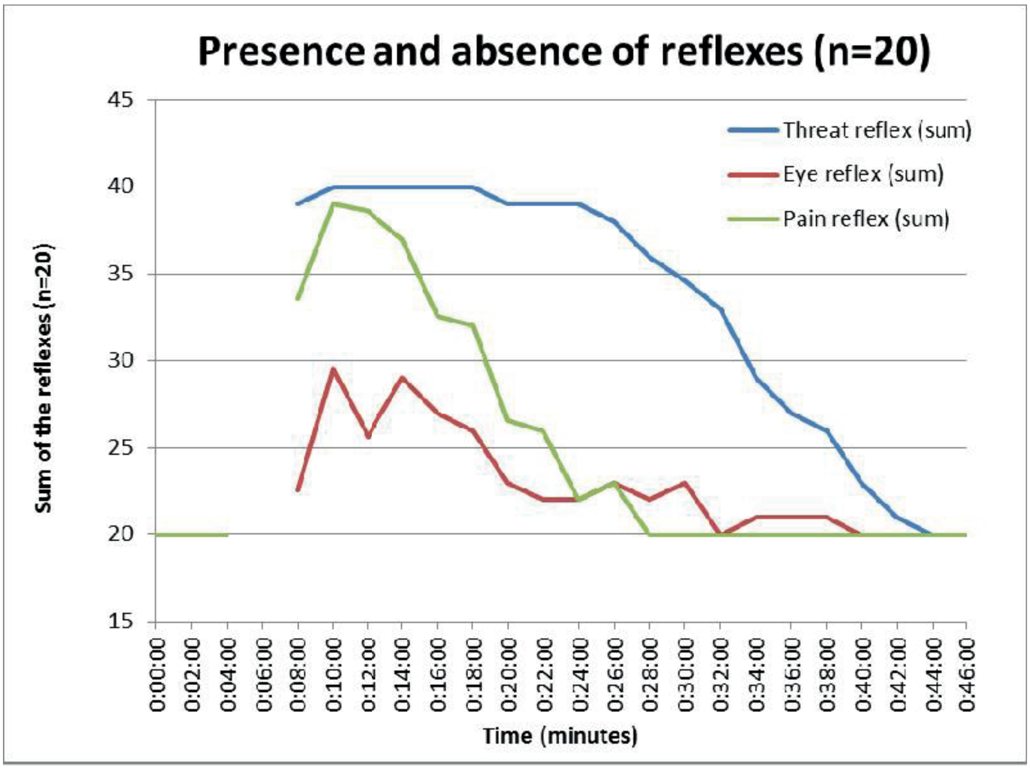

Figure 3.1.6*. Sum of absence and presence of three reflexes based on 20 observations in 11 sheep anesthetised with propofol at T=5 min. Presence of a reflex was scored '1', absence of a reflex was scored '2'. When a reflex was present in all 20 observations, a total score of 20 was observed (during baseline), when a reflex was absent in all 20 observations, a total score of 40 was observed. Reflexes were scored every 2 minutes.*

3.1.7 Correlations EEG parameters and behavioural parameters

Pearson correlations were conducted between, (non) rhythmic breathing, presence of different reflexes, visual assessment of the EEG and EEG derivatives (based on FFT).

Significant and strong correlations between EEG- and behavioural parameters were: transitional$_{rec}$ or recovery EEG with 1. an ocular reflex to threat (r=0.58, *P<0.01* and r=0.63, *P<0.01*); 2. The righting reflex (r=0.78, *P* <0.001 and r=0.81, *P* <0.001) and 3. the presence of 'non-rhythmic' breathing (r=0.81, *P* <0.001 and r=0.82, *P* <0.001).

3.2 Results experiment 1b (exsanguination)

3.2.1 Animals

The total number of sheep in experiment 1b was 22. A total of 21 observations from 21 sheep were used in the analyses. Sheep weighed 37.6 ± 3.5 kg.

3.2.2 EEG parameters: visual assessment

A clear EEG signal was obtained from all sheep during 5 min of baseline recordings, prior to applying the neck cut. Following neck cut, the EEG was often uninterpretable due to muscle activity. Figure 3.2.2 shows an example of a representative series of four seconds EEG recordings, during the different stages that could be identified from visual assessment of the EEG during exsanguination (data from sheep 10). Baseline EEG consisted of a low amplitude, high frequency signal, indicating alert animals (a). After applying the neck cut, changes in the EEG were seen at 00:15 ± 00:04 min, with slow wave activity apparent, indicating unconsciousness in all animals from that point onwards (b). No transitional stage was observed. The EEG was evaluated as iso-electric, indicating at least minimal brain activity, at 00:27 ± 00:08 s (c).

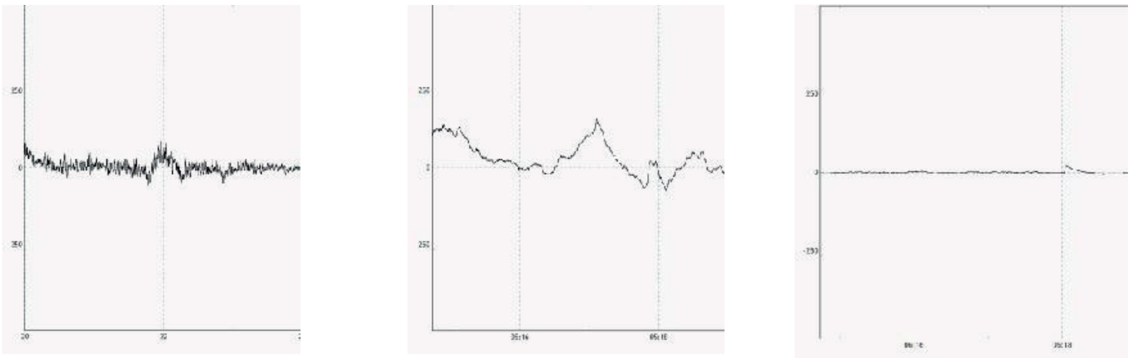

Figure a Figure b Figure c

Figure 3.2.2. *Typical example of the different stages identified with visual assessment of the EEG prior to, during and after applying the neck cut in sheep. The three stages from left to right: baseline (a), unconscious(b) and iso-electric or flat (c). X-axis represents 4 seconds, Y-axis represents amplitude of the EEG-trace (µV).*

3.2.3 EEG parameters: EEG derivatives

EEG's could only be partially analysed, due to movement artefacts that made FFT analyses difficult (figure 3.2.3). An expert consultation with Utrecht University, University of Amsterdam and TMSi, is currently conducted to assess the best way to analyse the EEG data.

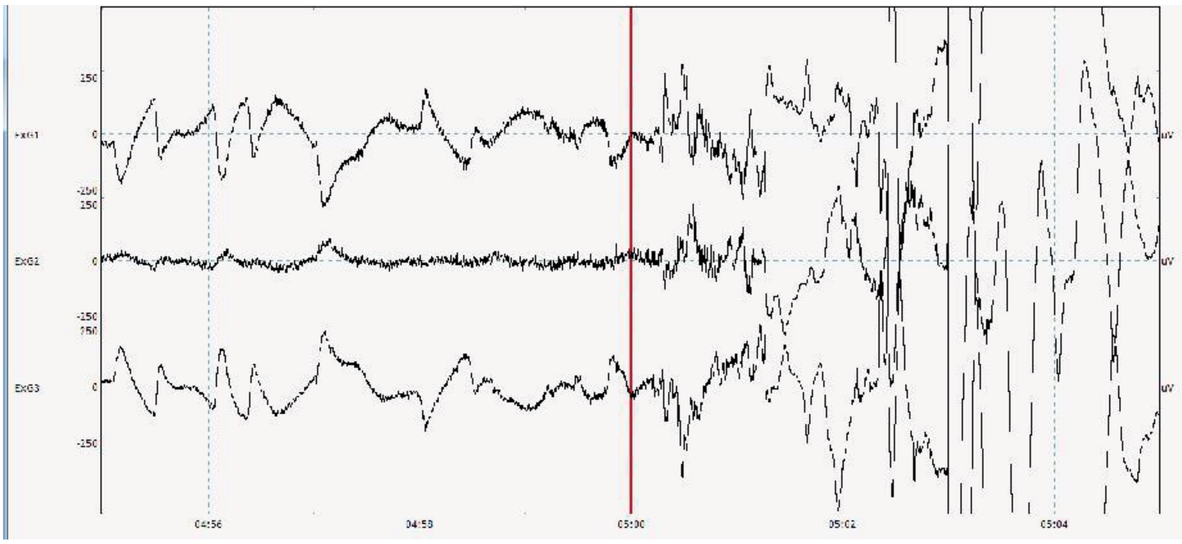

Figure 3.2.3. *Typical example of movement artefacts observed in EEG activity (three channels) of sheep 12 around the neck cut (red line, T=5 min).*

3.2.4 EEG parameters: correlations

Correlations between derivatives of the EEG and visual assessment of the EEG have not been performed, since we were not able to perform all FFT analyses yet (see 3.2.3).

3.2.5 Behavioural parameters: regular breathing

In experiment 1a we assessed (non) rhytmic breathing, were rhytmic breathing was observed during unconsciousness as an effect of the propofol anesthesia. In experiment 1b, rhytmic breathing was observed during baseline and following neck cut, non-rhytmic breathing could be observed. Since this is opposite to what was observed during experiment 1a, it was decided to refer to regular and irregular breathing in experiment 1b. Regular breathing was assessed by visual assessment of the RR, recorded in each sheep. Following neck cut, irregular breathing was defined as breathing changing from regular, normal breathing (figure 3.2.5a) to a breathing pattern where shallow breathing, taking big gulps of air, breathing rapidly and apnoea's alternated (figure 3.2.5b).

Start of irregular breathing was observed at 00:43 ± 00:12 min following neck cut.

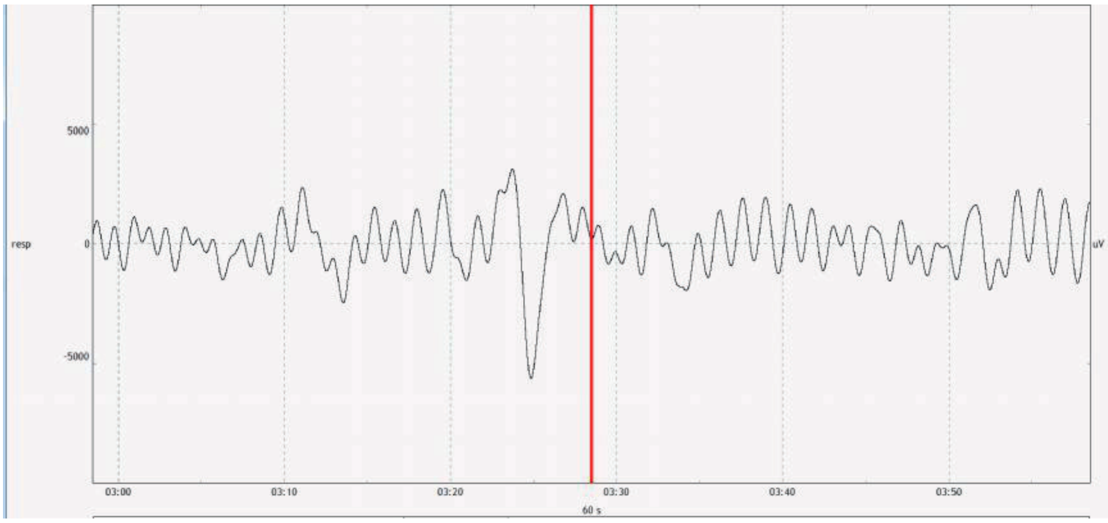

***Figure 3.2.5a**. Typical example of regular breathing in sheep 22 during baseline recordings (start of figure, T=3 min. Complete time frame is 60 s).*

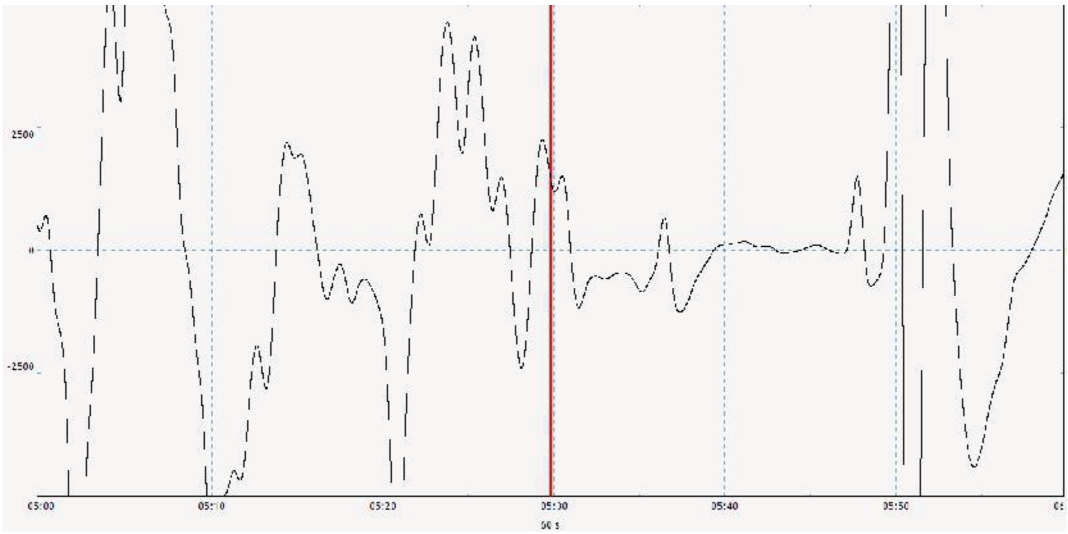

***Figure 3.2.5b**. Typical example of start of irregular breathing (red line) in sheep 22 after applying the neck cut (start of figure, T=5 min. Complete time frame is 60 s).*

3.2.6 Behavioural parameters: reflexes

All reflexes were tested and present in all sheep during baseline recordings. Sheep had rotating eye-balls after 00:10 ± 00:02 min and closed their eyes at 00:11 ± 00:02 min following neck cut. Seven sheep showed an ocular reflex to threat (until 00:07 ± 00:01 min) prior to loss of consciousness. No pain withdrawal reflexes were seen following neck cut, during exsanguination. An eyelid reflex, however, was observed until 00:74 ± 00:17 min.

3.2.7 Correlations EEG parameters and behavioural parameters

No correlations were found between visual assessment of the EEG and behavioural parameters.

4 Discussion

Four out of five of the parameters selected by the ’Dutch agreement on non-stunning slaughter in accordance with religious rites (2012) were included in the current experiments (1a and 1b) to study the validity and the feasibility of the parameters. The parameters included in the experiment were 1. (spontaneous) eyelid reflex, 2. stimulation of ear (pain response), 3. (spontaneous) rhythmic breathing, and 4. the ocular reflex to threat.

Eyelid reflex

Brain stem reflexes, such as the eyelid reflex, are regulated by 12 pairs of cranial nerves that enter or exit the brain and are not under cortical control. They therefor do not require consciousness (Carlson 1986; Rubin and Safdieh 2007). In both experiments, positive eyelid reflexes were observed when sheep were, according to the EEG and other behavioural parameters, unconscious or even brain-dead. During experiment 1b, the eyelid reflex disappeared in all animals at on average 74 s following neck cut. Expected is that, when testing this reflex at 40 s following neck cut in sheep, as requested in the agreement (“Agreement on non-stunned slaughtering in accordance with religious rites”, June 2012), it will be present in all sheep. The current findings support earlier statements that cranial nerve reflexes can be good indicators for impaired midbrain or brain stem activity, but have a limitation: when absent, it is very likely that the animal is unconscious, but when present, the animal does not necessarily need to be conscious (von Holleben et al. 2010).

Pain withdrawal reflex (pain response)

The pain withdrawal reflex is elicited by applying a painful stimulus to the animal, such as a nose- or ear prick. Experts claim that this is an important reflex to be assessed when establishing the effect of all types of stunning (EFSA 2004). In these experiment, the pain withdrawal reflex was often present in animals that were unconscious, based on the EEG, during propofol anaesthesia. No correlation was found between EEG parameters and the pain withdrawal reflex, indicating that during propofol anaesthesia it is not a reliable parameter. During exsanguination, no positive responses were observed, though animals were conscious until 15 s following neck cut based on the EEG. Possibly, the ventral-neck incision elicits a cerebrocortical pain response that during the duration of consciousness leave animals unresponsive to another source of pain (Johnson et al. 2012).

Rhythmic breathing

Presence of rhythmic breathing after stunning indicates that the corticospinal, ventral and lateral columns of the spinal cord are still intact and may thus indicate consciousness (Mitchell and Berger 1975). Absence of rhythmic breathing is generally accepted as a reliable parameter to assess consciousness, though may be difficult to observe in an animal (Gerritzen and Hindle 2009). During propofol anaesthesia, sheep that were unconscious showed rhythmic breathing, as a direct result of the anaesthetic agent on the respiratory control centre, housed in the medulla oblongata. There was a strong correlation between this parameter and the EEG derivatives. During exsanguination, regular breathing disappeared 00:43 ± 00:12 min following neck cut. These findings are similar to a study of Rodriguez et al. 2012 where regular breathing was lost at (on average) 44 s following neck cut. There was no correlation between loss of consciousness and loss of regular breathing. In 7 of 21 sheep, irregular breathing occurred later than 40 seconds following neck cut, indicating that when absent, it is likely that a sheep is unconscious or brain dead. It’s presence, however does not indicate consciousness.

Righting reflex

The righting reflex refers to any reflex that tends to bring the body into its normal upright position, it is also referred to as the head righting reflex. It is often assessed when animals are removed from the stunning box or are hung to the bleeding rail, but is difficult to assess when animals exhibit convulsions or involuntary body movements (Anil 1991; Blackmore and Newhook 1982). In our study design (sheep positioned in a hammock), it was not possible to reliably assess this parameter during exsanguination. However, during propofol anaesthesia, a righting reflex (lifting of head only) was observed as soon as sheep regained consciousness. Extrapolation of the validity of this parameter to slaughter without stunning is difficult and this parameter will need further evaluation in a next experiment.

Ocular reflex to threat

The limitation of this reflex is, that it can only be tested when the eye of an animal is open. During propofol anaesthesia, this reflex proved to be a valid parameter for sheep that had regained consciousness. During exsanguination, however, only few sheep had a positive response, which usually occurred only once (until 00:07 ± 00:01 s following neck cut). Based on the EEG, sheep did not lose consciousness immediately after applying the neck cut, but after 00:15 ± 00:04 min. These findings are similar to studies performed in sheep by Devine et al. 1986 (00:14 ± 00:07 min, n=10) and Gregory and Wotton 1984 (00:14 ± 00:05 min, n=20). The findings of the ocular reflex to threat indicate that this parameter is reliable at slaughter (without stunning) in one way only: when present, the animal is conscious, but when absent, the animal is not necessarily unconscious.

Correlations EEG- and behavioural parameters

During experiment 1a, high correlations were found between visual assessment of the EEG, derivatives of the EEG and certain behavioural parameters. These high correlations support the division of different stages as observed when visually assessing of the EEG. During exsanguination, however, no correlations were found. This can partially be explained by the absence of occurrence of some of the parameters, as well as the small variation in loss of consciousness between sheep. There are only few studies which have looked more closely at the relationship between EEG and absence / presence of reflexes. Rodriguez et al., 2012 also did not find correlations between the time to decreased brain activity and loss of reflexes. In this study, variation in loss of consciousness between sheep was also very little.

Applicability of the parameters

Applicability of parameters in an experimental set up is not necessarily a reflection of applicability in a slaughter plant. An experiment at a slaughter plant should point out, if these parameters can be tested under practical circumstances also. Standardization of the manner is important, indicating the importance of training people in charge of the assessment of unconsciousness.

Rhythmic (regular) breathing was recorded using a respiratory belt, changes from rhythmic to non-rhythmic breathing could be well established with this method. Previous experiments under practical circumstances have shown that visual assessment at a slaughter plant of this parameter is more difficult. Volume and frequency changes in the abdomen of the animal and changes in frequency of respiration are hard to establish due to movements of the animal or limited visibility when using a restrainer. The respiratory belt, however, is not applicable during slaughter of larger numbers of animals during a limited time frame.

Establishing the applicability of the righting reflex under practical circumstances requires further research. Previous experiments under practical circumstances have shown that visual assessment at a slaughter plant of this parameter is more difficult, since it may be masked by convulsions. When someone is certain of observing this parameter, there is no doubt that the animals is conscious.

5 Conclusion

Based on the results as obtained in the two experiments described in this report, it can be concluded that when a parameter is absent, it is very likely that the animal is unconscious, but when present, the animal does not necessarily need to be conscious. When looking specifically at absence of the different parameters to indicate unconsciousness, the following conclusions can be drawn from the two experiments:

1. Parameters that are reliable to establish unconsciousness in sheep at slaughter:

   - Absence of rhythmic breathing
   - Absence of eyelid reflex

It should be noted, however, that during the exsanguination experiment, these parameters often disappeared later than 40 seconds following neck cut. Therefore, to establish unconsciousness under practical circumstances at 40 seconds following neck cut, the absence of these parameters is not practical.

2. Parameters that are not reliable to establish unconsciousness in sheep at slaughter:

   - Absence of ocular reflex to threat
   - Absence of pain withdrawal reflex

Based on the results from these experiments, it should be considered to assess other parameters that could be used as (more) reliable parameters in the assessment of unconsciousness during slaughter.

6 Acknowledgements

The authors would like to thank Dr. H. Hopster (Wageningen UR Livestock Research, The Netherlands), Dr. K. van Elk (Wageningen UR Livestock Research, The Netherlands), Dr. A. Dalmau (IRTA, Spain) and Dr. T. Gibson (Royal Veterinary College, United Kingdom) for critically reviewing the draft version of this report.

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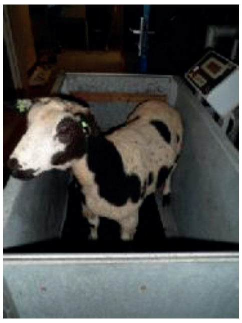
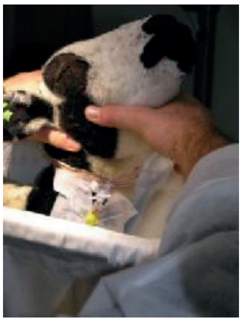
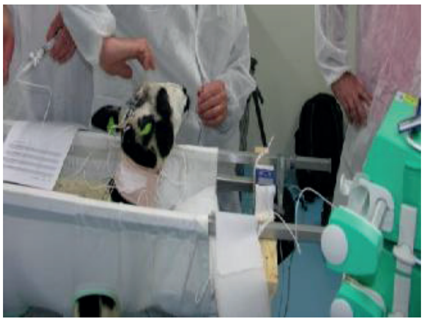
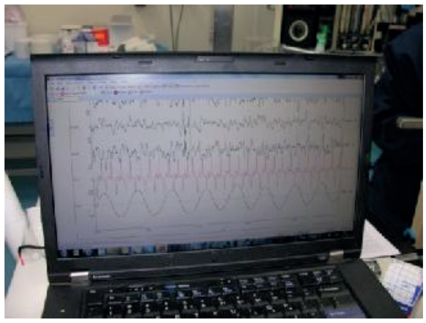
Appendix I 'Overview of experiment 1a'





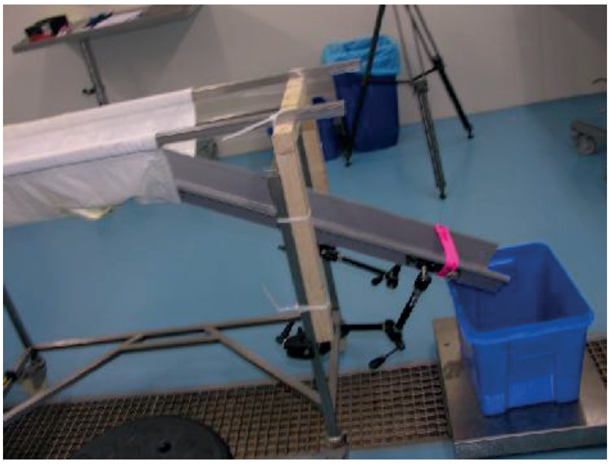




Description of pictures from left to right (and top to bottom):

1. Weighing of one of the ewes
2. Shaving the neck to place the catheter
3. Overview of the sheep in the hammock (side)
4. Overview of the sheep in the hammock (front)
5. Respiratory belt to measure rhythmic breathing
6. Placement of the EEG electrodes
7. Placement of the catheter to administer propofol (anaesthetic)
8. Testing of the ocular reflex to threat
9. Typical example of all signals that could be live viewed

Appendix II 'Overview of experiment 1b'



Description of pictures from left to right (and top to bottom):

1. Set up in the operation room with a slide to collect the blood
2. Example of a sheep equipped with electrodes and respiratory belt prior to exsanguination
3. Example of a sheep equipped with electrodes and respiratory belt prior to exsanguination
4. Example of a sheep equipped with electrodes and respiratory belt prior to exsanguination
5. Example of a sheep equipped with electrodes and respiratory belt prior to exsanguination
6. Example of a sheep equipped with EEG electrodes prior to exsanguination

To explore the potential of nature to improve the quality of life

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Livestock Research Report 380

Together with our clients, we integrate scientific know-how and practical experience to develop livestock concepts for the 21st century. With our expertise on innovative livestock systems, nutrition, welfare, genetics and environmental impact of livestock farming and our state-of-the art research facilities, such as Dairy Campus and Swine Innovation Centre Sterksel, we support our customers to find solutions for current and future challenges.

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