Final report

DTS: Diathermic Syncope®: preparation of dossier for regulatory approval

Project code: V.SRP.0002

Prepared by: Alison Small
Commonwealth Scientific and Industrial Research Organisation

Date published: 26 April 2022

PUBLISHED BY
Meat & Livestock Australia Limited
PO Box 1961
NORTH SYDNEY NSW 2059

Meat & Livestock Australia acknowledges the matching funds provided by the Australian Government to support the research and development detailed in this publication.

Abstract

This project aimed to prepare a dossier of information pertaining to the development of DTS: Diathermic Syncope®, that would be suited to submission to multiple Regulatory Authorities for approval of the technology. Six peer-reviewed articles, 13 confidential unpublished project reports and 10 published supplementary material items, including factsheets and conference proceedings and/or presentations were included in the dossier. Overall integration of the findings of all studies indicated that 100 % insensibility was achieved when the desired energy package was delivered. The animal stunned using DTS can also return to consciousness, so this method of stunning is likely to be acceptable to the Halal and Kosher markets. Furthermore, blood flow was observed to be strong, and the rhythmic pumping of the heart was evident in the first few seconds of blood flow. Both these characteristics are desired by the Halal and Kosher markets. The updated applicator configuration allowed consistent repeatable induction of insensibility, which was sustained during Halal bleeding, without the need to apply an additional ventral cut. Approval to use the DTS: Diathermic Syncope® technology in an Approved Arrangement was granted by DAWE for use in abattoirs in Australia, providing that they comply with the relevant DAWE, State and Territory regulations and processes, and submission of the dossier to the European Food Safety Authority is planned.

Executive summary

Background

Development of the DTS: Diathermic Syncope® technology (DTS) for stunning of cattle has been proceeding for the past 15-20 years and consistent induction of insensibility has been reliably demonstrated. As at 2021, the information pertaining to the DTS system was in the form of multiple project reports and journal articles. For DTS to be commercialised as a stunning method available to the Australian beef industry, it must first be approved by the Department of Agriculture, Water and Environment (DAWE). For commercialisation in overseas markets, approval by the European Food Safety Authority (EFSA) is a vital step to facilitate approval in other jurisdictions. It was understood that the Australian approval process would be facilitated if the information pertaining to DTS were presented to DAWE as a coherent dossier of supporting evidence, while the EFSA approval process has a defined structured approach leading to preparation of such a dossier (EFSA 2018).

This project aimed to prepare such a dossier, following the structure required by EFSA, such that the dossier prepared would be suited to submission to multiple regulatory authorities for approval. As part of the project, the dossier would be submitted to DAWE, and the lodgement process with EFSA would be initiated.

Objectives

The original objectives of this project were:

- To collate the research outputs pertaining to DTS in a format suitable for submission to DAWE for approval of DTS as a stunning method (achieved);
- To prepare the remaining journal manuscript (meat quality comparison) (achieved);
- To submit the dossier to DAWE, and as part of their review process identify any remaining information gaps where further supporting evidence is required by DAWE (achieved);
- To lodge an 'intention to submit' application with EFSA, and
- To either:
    - Submit the dossier aligned to the EFSA guidelines for evaluation of stunning interventions (EFSA 2018); or
    - Draw up a forward plan to address identified knowledge gaps.

Methodology

Development of the dossier was a collaborative exercise, first collating all published and unpublished material pertaining to development of the DTS: Diathermic Syncope® technology. Written permission to utilise copyright and unpublished material in the process was gained from Wagstaff Food Services and AMPC.

The collation exercise resulted in six peer-reviewed articles (including the manuscript prepared during the course of this project), 13 confidential unpublished project reports and 10 published supplementary material items, including factsheets and conference proceedings and/or presentations.

Results/key findings

Overall integration of the findings of all studies indicated that 100 % insensibility was achieved when the desired energy package was delivered. Existing stunning techniques in Australia, where conditions are carefully controlled, are expected to deliver a 95-98 % first-stun efficacy (AMIC, 2009) but, particularly where adequate neck and head restraint is not used, can have up to 29 % first stun failure rates (Blackmore, 1979; Lambooy et al., 1981; Hoffmann, 2003; Endres, 2005; Octavio-Oliveira et al., 2018; Gibson et al., 2019). The animal stunned using DTS can also return to consciousness, so this method of stunning is likely to be acceptable to the Halal and Kosher markets. Furthermore, blood flow was observed to be strong, and the rhythmic pumping of the heart was evident in the first few seconds of blood flow. Both these characteristics are desired by the Halal and Kosher markets.

Use of the rotary box optimised handling of the stunned body, allowing a short stun-to-stick interval without the use of an immobiliser. The updated applicator configuration allowed consistent repeatable induction of insensibility, which was sustained during Halal bleeding, without the need to apply an additional ventral cut.

Benefits to industry

- DTS is an effective means of inducing insensibility in cattle, with a duration of insensibility suited to exsanguination using the Halal cut. The project was planned and executed under end-user-centred design principles, with the Halal and Kosher markets in mind. Key parameters that are likely to be viewed favourably when compared against the current stunning method are:
    - There was no requirement to perform a back-up ventral cut (thoracic stick), as the blood flow was strong and exsanguination was rapid;
    - Blood flow was visibly strong, and the pulsations associated with heart function were visible in the early stages of bleed-out;
    - Exsanguination could be performed safely, without the need for an immobiliser;
    - Visual inspection of the brains of carcases indicated no visible damage when energy deliveries of less than 220 kJ were applied, and the stun did not cause cracks in the skull, so eliminates a source of rejection against Halal requirements, and eliminates a potential source of breaches noted on audit when the auditors' interpretation of 'cracked' differs from the regular inspector;
- The stun is effective in heavy animals and bulls, which currently pose challenges to processors using percussive stunning;
- To date, no evidence of blood splash or ecchymosis has been encountered in any of the carcases processed.
- The animal stunned using DTS can also return to consciousness, so this method of stunning is likely to be acceptable to the Halal and Kosher markets.

Approval to use the DTS: Diathermic Syncope® technology in an Approved Arrangement was granted by DAWE for use in abattoirs in Australia, providing that they comply with the relevant DAWE, State and Territory regulations and processes. Submission of the technical dossier to EFSA is planned.

Future research and recommendations

In order to secure global acceptance of the technology and maintain market access as the technology is commercialised within Australia, communication with overseas regulatory authorities is vital.
In particular, engagement with the Halal and Kosher markets is important, as this technology may be suited.
Development of the technology for use in other species is warranted.

Table of contents

1. Background

Development of the DTS: Diathermic Syncope® technology (DTS) for stunning of cattle has been proceeding for the past 15-20 years and consistent induction of insensibility has been reliably demonstrated. As at 2021, the information pertaining to the DTS system was in the form of multiple project reports and journal articles. For DTS to be commercialised as a stunning method available to the Australian beef industry, it must first be approved by the Department of Agriculture, Water and Environment (DAWE). For commercialisation in overseas markets, approval by the European Food Safety Authority (EFSA) is a vital step to facilitate approval in other jurisdictions. It was understood that the Australian approval process would be facilitated if the information pertaining to DTS were presented to DAWE as a coherent dossier of supporting evidence, while the EFSA approval process has a defined structured approach leading to preparation of such a dossier (EFSA 2018).

This project aimed to prepare such a dossier, following the structure required by EFSA, such that the dossier prepared would be suited to submission to multiple Regulatory Authorities for approval. As part of the project, the dossier would be submitted to DAWE, and the lodgement process with EFSA would be initiated.

2. Objectives

The original objectives of this project were:

- To collate the research outputs pertaining to DTS in a format suitable for submission to DAWE for approval of DTS as a stunning method (achieved);
- To prepare the remaining journal manuscript (meat quality comparison) (achieved);
- To submit the dossier to DAWE, and as part of their review process identify any remaining information gaps where further supporting evidence is required by DAWE (achieved);
- To lodge an 'intention to submit' application with EFSA, and
- To either:
    - Submit the dossier aligned to the EFSA guidelines for evaluation of stunning interventions (EFSA 2018); or
    - Draw up a forward plan to address identified knowledge gaps.

3. Methodology

Development of the dossier was a collaborative exercise, first collating all published and unpublished material pertaining to development of the DTS: Diathermic Syncope® technology. Written permission to utilise copyright and unpublished material in the process was gained from Wagstaff Food Services and AMPC.

The collation exercise resulted in 6 peer-reviewed articles (including the manuscript prepared during the course of this project), 13 confidential unpublished project reports and 10 published supplementary material items, including factsheets and conference proceedings and/or presentations. The following section provides a redacted version of the dossier prepared for submission to EFSA, excluding confidential propriety information.

4. DTS: Diathermic Syncope® Technical dossier

Article 4 (2) of Council Regulation (EC) No 1099/20094 on the protection of animals at the time of killing allows the Commission to amend stunning parameters laid down in Annex I to this Regulation to take into account scientific and technical progress on the basis of an EFSA opinion. Any such amendments shall ensure a level of animal welfare at least equivalent to that ensured by the existing methods. This technical dossier summary presents a comprehensive overview of the use of electro-magnetic energy applied directly to the brain to induce volumetric heating (DTS: Diathermic Syncope®, hereafter referred to as 'DTS'), and presents the supporting scientific studies to address the criteria outlined in the EFSA Guidance on the assessment criteria for applications for new or modified stunning methods regarding animal protection at the time of killing (EFSA Journal 2018;16(7):534, hereafter referred to as 'EFSA guidance').

Council Regulation (EC) No 1099/2009 on the protection of animals at the time of killing defines "stunning" in Article 2 (f) as "any intentionally induced process which causes the loss of consciousness and sensibility without pain including any process resulting in instantaneous death". Article 4 on stunning interventions states that "animals shall only be killed after stunning in accordance with the methods and specific requirements related to the application of those methods set out in Annex I of the Regulation" and "that loss of consciousness and sensibility shall be maintained until the death of the animal".

As the use of DTS is a novel stunning system, no parameters for its use are currently defined by Council Regulation (EC) No 1099/2009.

4.1 Description of the stunning method

4.1.1 Name of the method

DTS: Diathermic Syncope® hereafter referred to as 'DTS'.

4.1.2 Description of the method including potential sources of pain, distress and suffering

4.1.2.1 Technical description of apparatus

The stunning system apparatus includes six critical components which are shown in Figure 1 and are listed below:

1. DTS Generator
2. User interface panel
3. Waveguide system
4. Applicator
5. Animal Restraint (not shown in Figure 1)
6. Faraday cage

1. **DTS generator**. This water-cooled generator contains a magnetron (an evacuated tube for generating microwaves, with the flow of electrons controlled by an external magnetic field), high power components, automation hardware and the electronics required for generating electromagnetic energy at a frequency of 922 MHz (or within the region of 890-925 MHz). Generator output is a minimum of 10 kW and maximum of 40 kW.
2. **User interface panel**. A software-based user control panel which allows pre-selection of the total amount of energy to be delivered to the animal. Energy calculations are based on variations of power (kW) and length of applied energy (seconds). Each dose of energy delivered to the animal is carefully monitored and recorded against a specific animal. The interface unit includes

emergency stop capability; indicators of magnetron status (standby, warming, on); fault alerts and reset. Fault diagnostics and permanent storage of energy transmission data are included in the software.

3. **Waveguide system**. These are used to transport the energy from the DTS generator to the Applicator. Electromagnetic energy leaves the DTS generator via rectangular aluminium tubing (waveguide). The cross-sectional dimensions of which are sized according to the frequency of the electromagnetic energy being produced. This rectangular waveguide is rigid and as such requires the energy to be transitioned to a more flexible apparatus, e.g., waveguide rotary and telescopic joints and/or coaxial cable. This flexibility allows for accurate positioning of the Applicator on the animal's head (Section 3.7.2). There is an auto tuner located on the generator side of the coaxial cable in the waveguide section, this ensures we optimise the energy transfer to the animal's head for the wide variety of head sizes and shapes.

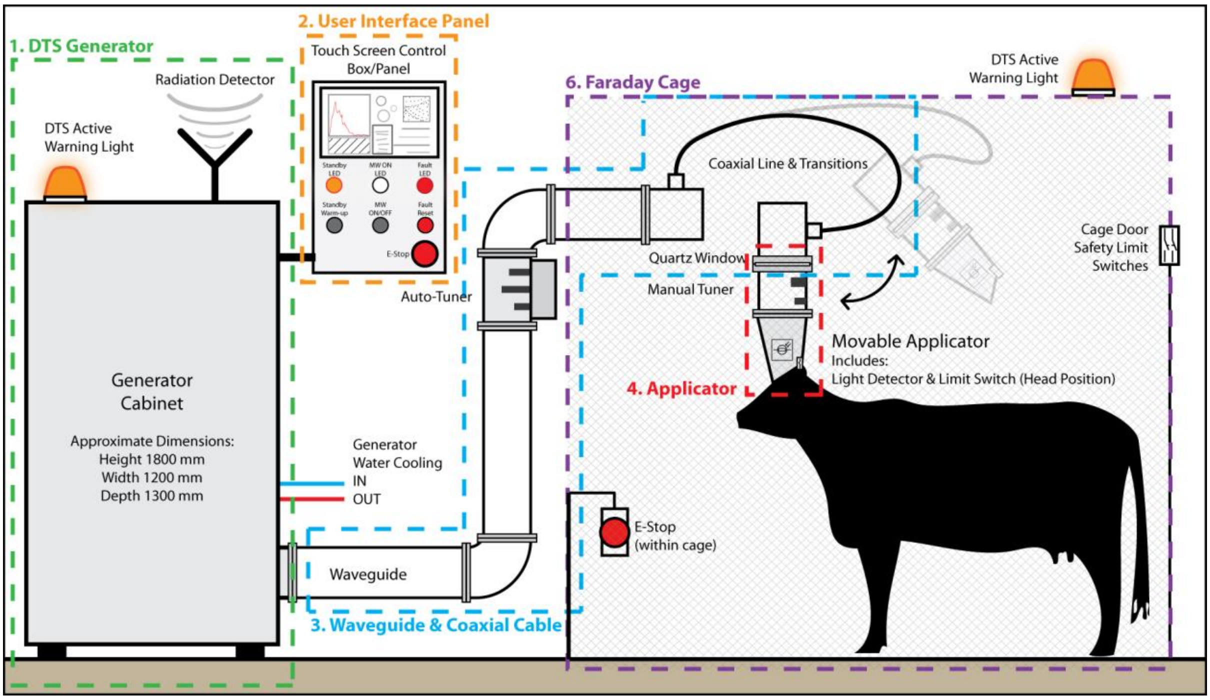
4. **Applicator**. This provides the direct interface between the DTS system and the animal's head. It is a unique design specific to the electromagnetic energy frequency and the type of animal being processed (e.g., cattle, calves, sheep). The waveguide energy is focused to a region considered the target area so that the penetrative energy is directed to the desired region of the brain in an efficient manner. Fixed tuning stubs are located just prior to the applicator ensuring that is well tuned into the animal's head for an acceptable range of animals. The autotuner corrects any small mismatch in tuning that may arise due to animal head variations. A quartz window protects the coaxial to waveguide transition piece from water, hair and foreign material.

NB: Poor tuning results in in-efficient energy transfer, which could lead to failure to induce unconsciousness, or inappropriate surface heating.

5. **Animal restraint**. (Section 3.6) The animal is restrained in an upright position in a mechanism that will allow animal rotation, e.g., in a tipping or rotating box (to allow rotation and ejection after unconsciousness has been confirmed). A rear pusher may be used to ensure that the animal is positioned with its head protruding through the neck yoke aperture. A neck capture with chin lift (based on the ASPCA Pen design) is applied, the chin lift is used to position the head such that the frontal bones are between 0 and 30 degrees from horizontal. The head capture unit is engineered with hydraulic rams, which are more rigid than the pneumatic rams, reducing the chance of compression during application.

6. **Faraday cage**. The entire rotary box is enclosed in a Faraday cage (an enclosure created by conducting materials that blocks static and non-static electric fields) to protect operators in the case of energy leakage. The Faraday cage consists of a steel mesh with holes less than 12mm in diameter. Personnel doors for access to the restraint unit are fitted at the level of the animal's head, from one side when in the upright position, and from the front when in the rotated position. These doors seal using electromagnets when the energy application is underway. The rear door that allows animal access is also sealed using electromagnets. Safety switches are installed at the doors such that energy application cannot proceed if the doors are not properly sealed (these safety interlocks cannot be manually over-ridden). An emergency stop button is fitted inside the Faraday cage in case of the unlikely event of personnel being trapped within. DTS Active warning lights are fitted in prominent positions on the DTS generator and the Faraday Cage.

Figure 1: Apparatus Overview (Animal Restraint Not Shown)



4.1.2.2 Stunning system operation sequence

Start-up sequence:

1. Turn on Cooling Water.
2. Switch on Mains Power.
3. Use the key to turn 'Key-Switch' ON.
4. Wait for the computer to start up and the DTS control program to initialise.
5. Press 'Continue' on the 'Open Com Port' box.
6. Press 'STANDBY' to begin the 3 min Warm Up Sequence.

Warm up is complete when STANDBY LED stays illuminated and Generator Status Reads "Generator Ready (Standby)".

Once the Start-Up Sequence is completed, DTS Energy can be applied and reapplied continuously without the need to go through the Start-Up Sequence again, until the system is shut down, either manually, as a result of an electrical fault or in the event of a power outage.

Applying Energy:

1. Before each energy application, select the correct DTS Profile (Power & Time Combination) on the Main Operations Page.
2. Applicator is manually put in place (fitted against animal's head) by the operator.
3. When the animal is ready, one of the 'APPLY ENERGY' buttons is pushed to initiate energy application. To cancel the energy application, push the 'CANCEL' button.

Note: Energy application is only possible when there are no generator faults, the animal is in place and all safety interlocks (E-Stop, gates, and gen doors) read OK. Some faults may result in the need to restart the warm-up sequence. If the animal is not in place and the applicator positioned on its forehead, energy escaping from the applicator will trigger the automatic safety cut-out and energy delivery will immediately cease.

Shut-down Procedure:

1. Exit Standby mode by pressing the STANDBY push button.
2. Press the touch screen Shutdown button to close the program.
3. Remove the key from the key switch.
4. Shut down the computer via the Windows start menu.
5. Allow 10 minutes to elapse for cooling
6. Turn off mains power.
7. Turn off cooling water.

4.1.2.3 Stunning apparatus cleaning and maintenance

Generator Care

Daily:

- Remove any build-up of rubbish from within and around the generator.
- Ensure that all air intakes and outlets are clear.

Weekly:

- Check water flow and arc detection devices are functional.
- Check for water leaks inside the generator.
- Check and replace (if required) air filters on generator side walls.
- Check waveguide for loose fittings and bolts.

Monthly**:**

- Check generator doors, limit switches and adjust to suit door stroke.
- Replace/wash air filters on generator side walls.

Applicator Care

Daily:

- Wipe out any moisture, dust or hair.
- Visually inspect for cracks or other damage.

Weekly:

- Check waveguide and applicator for loose fittings and bolts.

Restraint Unit Care

Daily:

- Wash out any faecal matter, blood, hair or excreta.
- Visually inspect for cracks or other damage.

- Grease moving parts.
- Visually inspect unit and faraday cage for damage.

Microwave safety

Primary microwave safety lies in the design of the Faraday cage which prevents any microwaves from escaping into the surrounding environment. All doors on the Faraday cage are fitted with specially designed microwave chokes which reflect energy back into the cage. The legal requirements pertaining to this screening differ in different countries, as do the requirements around the specific frequencies generated by the magnetron. Alternative frequencies should be validated prior to commercial use. In the case of a cage or choke failure, a radiation interlock system is placed on the outside of the cage and will instantly shut the DTS system down and prevent further operation, if an unsafe level of radiation is detected. All Faraday cage doors are to be fitted with double interlocks to prevent operation if a door is not correctly closed/sealed. An optional interlock may be fitted on the applicator which checks head placement is correct before energy can be applied.

Calibration of the apparatus

The stunning apparatus does not need regular calibration. However, care should be taken to inspect the waveguide, applicator and Faraday cage each day for damage and ensure everything is in good condition. Assessment of tuning should be carried out by a specialist technician annually, or in the event of a change in size of the applicator (e.g., for use on another species).

4.1.2.4 Technical description of restraining system construction and operation

The restraining system does not deviate substantially from that associated with conventional stunning and slaughter processes for cattle, e.g., using captive bolt or electric stun. The restraint unit is fed via a crowd pen and race, as per existing commercial restraint boxes used for mechanical or electrical stunning. The animal is restrained in an upright position in a mechanism that will allow animal rotation. A rear pusher may be used to ensure that the animal is positioned with its head protruding through the neck yoke aperture. A neck capture with chin lift is applied, the chin lift is used to position the head such that the frontal bones are between 0 and 30 degrees from horizontal. The head capture unit is engineered with hydraulic rams, which are more rigid than the pneumatic rams, reducing the chance of compression during application. The design of the restraining system allows for prompt ejection and immediate slaughter. Schematic diagrams of a rotating box restraining system are shown in Figures 2 - 3. Additional photographic images of a rotating box restraining system under construction are presented in Figures 4 – 7, and 3D representations of the rotating restraint box within the Faraday cage are shown in Figures 8 and 9.

Figure 2: Schematic diagram of restraint and ejection system onto bleed conveyor, front elevation. One animal is upright prior to DTS application (right), one is in the catching cradle post exsanguination (middle), and a third is on the bleed conveyor (left).

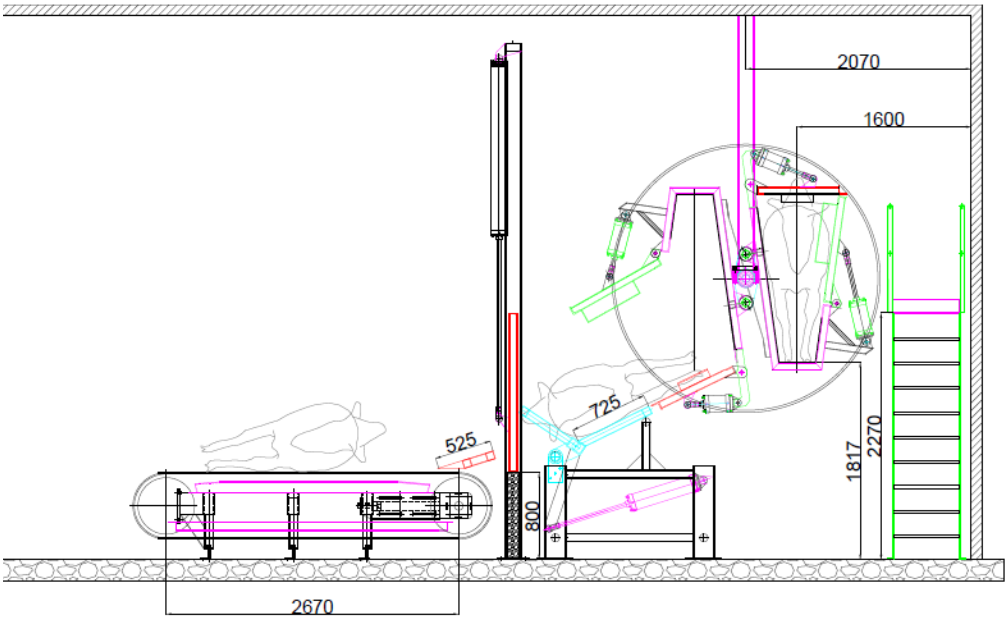


Figure 3: Schematic diagram of restraint and ejection system onto bleed conveyor, front elevation. One animal is upright prior to DTS application (right), one is in the tipped catching cradle post exsanguination (middle), in the process of being ejected onto the bleed conveyor (left).

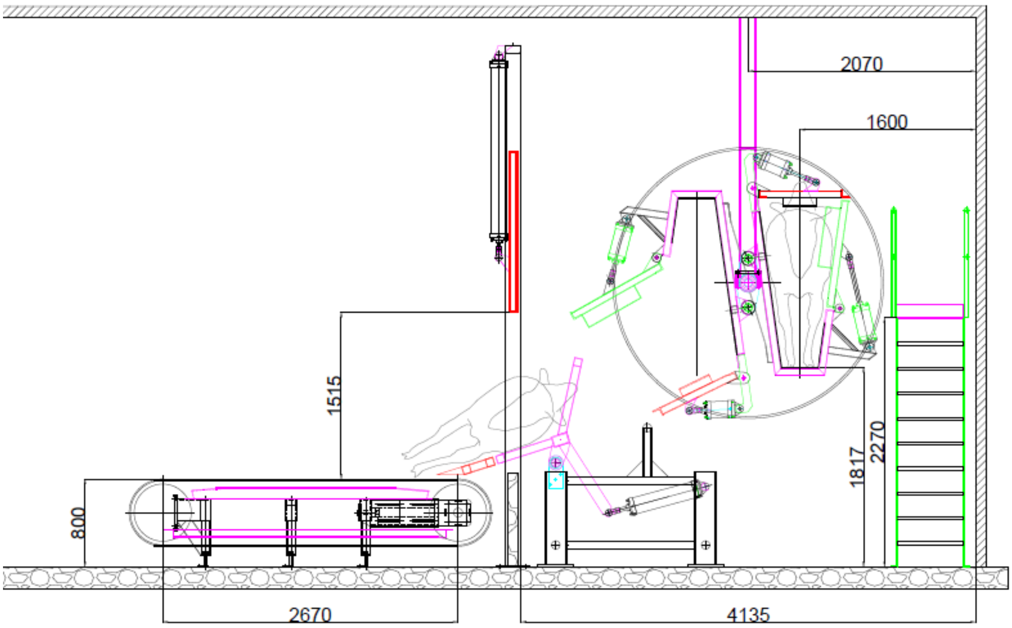


Figure 4: View of animal access to rotating restraint box, from rear. Image taken during workshop construction. In this image, the upright side is to the left, and the inverted side to the right.

Figure 5: View of the neck capture, side and 'top' doors opened to allow ejection of the animals when inverted. Image taken during workshop construction. In this image, the upright side is to the right, and the inverted side to the left.

Figure 6: Side view of the upright section of the rotating restraint box. Image taken during workshop construction. In this image, the animal would be facing to the left, with the head protruding through the 'U'-shaped aperture left of middle, upper portion.

Figure 7: View of the inverted part of the rotating restraint box, showing the side and 'top' doors opened to allow ejection of the animals when inverted. Image taken during workshop construction.

Figure 8: 3D representation of the rotating restraint box within the Faraday cage. Personnel access steps are on the right side of the image, with the bleed conveyor to the left.

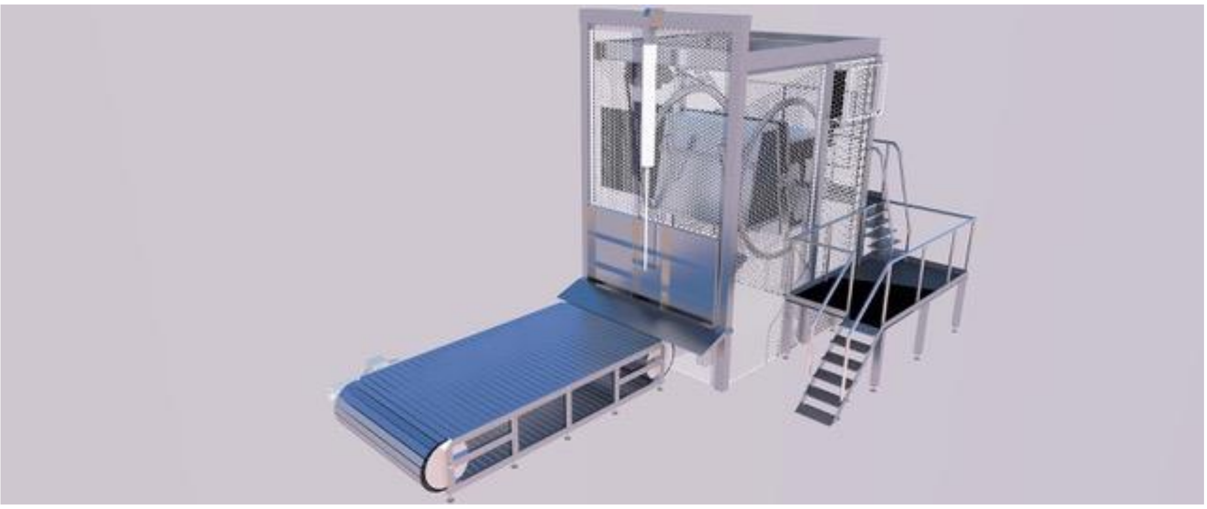
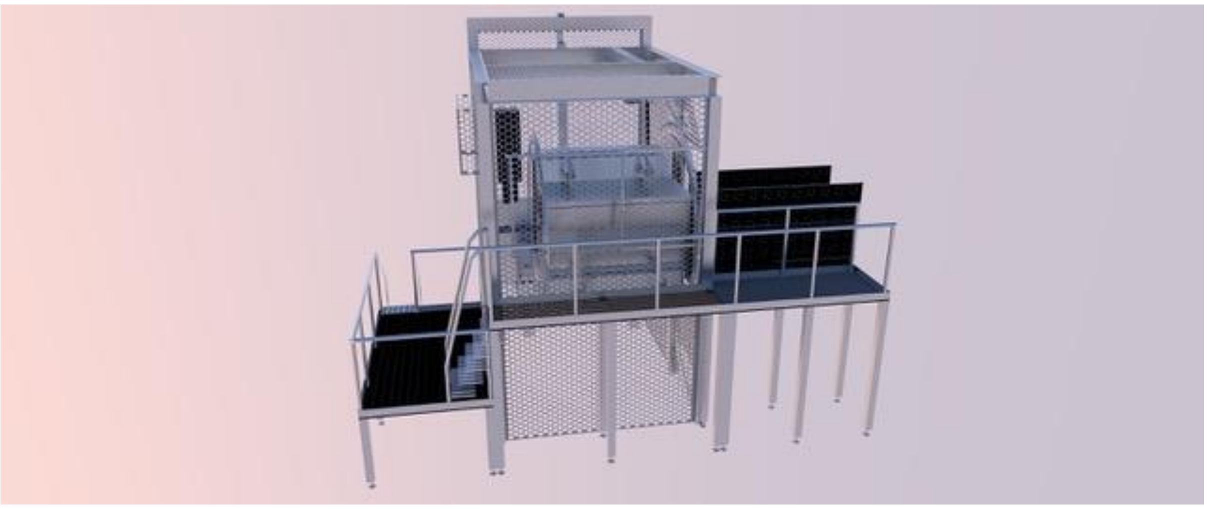


Figure 9: 3D representation of the rotating restraint box within the Faraday cage. Rear view showing personnel access.



Prolonged restraint in any system is stressful to animals (Ewbank, 1992). The restraint unit used with the DTS: Diathermic Syncope® system is a standard commercial restraint, with a neck capture and chin-lift system based on the ASPCA Pen design described by Grandin (1992) and Marshall (1963). Quiet handling and smooth application of the neck capture and chin lift apparatus is well tolerated by cattle (Dunn 1990, Grandin 1992) and the head position achieved allows accurate placement of the applicator onto the forehead of the animal. The animal is held in the chin lift for a maximum of 30 s while the applicator is positioned and DTS energy delivery initiated.

4.1.2.5 Technical description of waveguide position and application of energy

The applicator is manually positioned directly over the frontal lobes of the brain by the operator. Energy application is prevented if the animal's head is not pressed firmly against the applicator and if any of the access door limit switches are open or faults are triggered.

Contact between the Applicator and the animal's head

The Applicator is attached to a flexible waveguide system, allowing it to be applied at different heights and angles to match the various head shapes. Contouring within the Applicator head interface end also enables a uniform and continuous contact.

Position of the Applicator on the animal's head

The applicator is placed high on the forehead of the animal, on the frontal bones in front of the poll, such that the energy is directed into the brain mass (Figures 10 and 11).

Figure 10: Diagram of placement of applicator on the bovine head. Left: frontal view, hatched area shows applicator position; Right: sagittal view, hatched area represents the applicator, showing positioning relative to the brain and frontal sinuses (black).

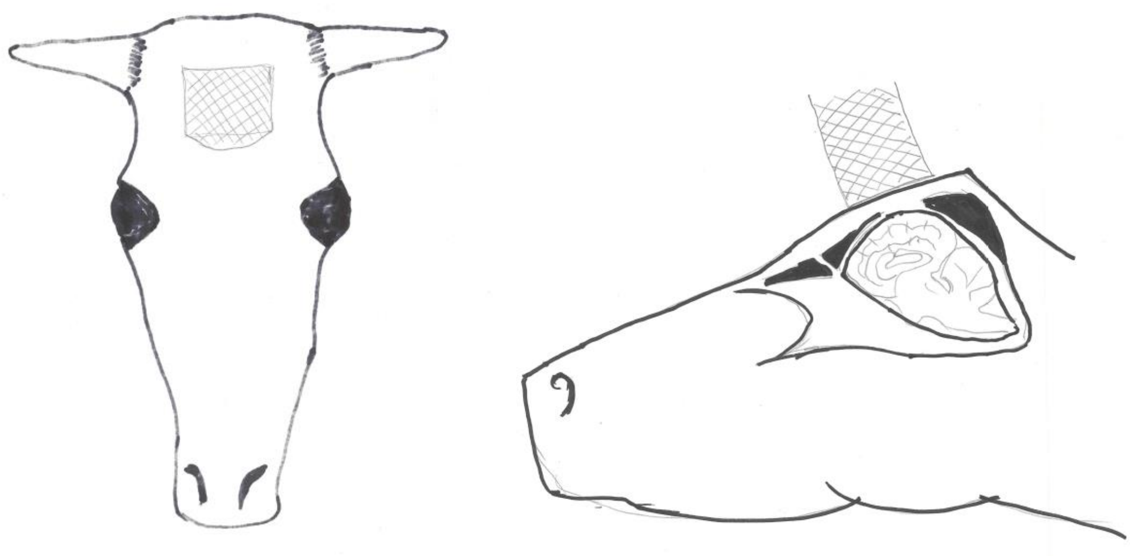


Figure 11: Animal in restraint with chin lift activated and applicator in position, immediately prior to DTS energy application.

Minimum energy (KJ) application

Although energy levels of 85 kJ have been shown to result in unconsciousness in some animals, the duration of unconsciousness is short. For commercial processing, energy levels of 160 kJ or above are recommended. Energy levels above 220 kJ can lead to damage to brain tissue, while energy levels of 300 kJ and above will result in death.

Duration of energy application

The duration of energy application is related to the incident power and the desired total energy 'package' to be delivered. The units of power (kW) is shorthand for 'kJ per second', so a higher power setting will reduce the time required to reach an energy delivery of 200 kJ than a lower power setting. However, the higher power setting may predispose to focal overheating, while a very low incident power may be insufficient to raise the temperature of the brain sufficiently rapidly to overcome the natural cooling provided by the blood circulation.

Our current data set indicates that a power setting of 18-20 kW is suitable. 18 kW will deliver 180 kJ in a period of 10 s, while 20 kW will deliver 180 kJ in a period of 9 s.

DTS, delivering 160-220 kJ at an incident power of 18-20 kW induces loss of posture within 8 seconds of onset of energy application, and the duration of unconsciousness recorded during validation was between 100 and 240 seconds, based on absence of corneal reflex, and duration of EEG changes.

For humane slaughter purposes, the insensibility should be of a duration that allows death through exsanguination prior to recovery. In cattle, death through exsanguination occurs within 4 min or less (Newhook & Blackmore 1982; Gregory et al 2010).

4.1.2.6 Additional commercial considerations

Animal characteristics

The DTS: Diathermic Syncope® system has been validated on over 300 cattle ranging from 160 to 413 kg liveweight **(PR12).** The cattle were predominantly *Bos taurus*, beef, dairy and cross-bred types. Heifers, steers, cows and bulls were represented; some were horned, some naturally polled, and some dehorned. Five Brahman (*Bos indicus*) animals were included in the validation. Animal type does not influence efficacy of induction of unconsciousness, providing that the applicator could be placed in contact with the forehead with no air gaps. The applicator design has been refined during the course of development to maximise the range of forehead shapes that can be presented to the applicator.

Animals with very wide horns may not fit onto the restraint unit: this is also the case with existing restraint units for captive bolt or electrical stun application.

While the DTS can be used for any medium to large animal, applicators are designed to suit specific animal head types. As of December 2021, there are applicators that have been developed for various types of bovine. Custom applicators will need to be designed and validated for use with other animals, such as sheep or calves.

Penetration depth can be predicted based on the dielectric properties of the tissues involved (**SS04 – McLean et al., 2017**). For the theoretical scenario where a bovine's head consists of 5 mm of skin and 12 mm of bone, brain penetration depth is approximately 38 mm. Penetration depth can vary depending on the size and shape of the bovine head. Penetration depth also changes with frequency of the microwave energy applied. Frequency is fixed by the magnetron used. Heat is distributed through the brain mass via direct electromagnetic heating, conduction and via circulating cerebrospinal fluid and blood.

All animals processed to date have been lairaged, so the skin of the forehead has been dry. Some animals have had a layer of dew in the hair coat. This moisture rapidly evaporates when energy application begins and has no influence on efficacy of induction of unconsciousness. There has been no evidence of differences related to thickness of hair coat.

Commercial throughput

Using a rotating box for animal restraint allows for a second animal to be loaded while the first (unconscious) animal is being exsanguinated. Under these conditions, animals have been processed at a rate of one each minute (60 per hr). The engineering team are working on an alternative design of box to reduce the footprint of the restraint unit and increase the speed of ejection of the unconscious animal from the restraint, with a view to achieving a processing rate of 2 per minute (120 per hr). This is in line with current processing rates in commercial beef abattoirs in Australia (60-120 per hr per restraint box).

4.1.3 Key parameters of the effective use of the method

DTS: Diathermic Syncope® uses electromagnetic energy to induce volumetric heating of the brain to a level that results in loss of consciousness (hyperthermic syncope, or 'fainting'). Hyperthermic loss of consciousness, or 'fainting' occurs in people when core body temperatures are in the range of 40-45 °C (Ohshima et al., 1992), while rats have been rendered unconscious (based on behavioural observations) after the brain temperature was elevated by 8 °C (Guy and Chou, 1982). Ikarashi et al.

(1984) demonstrated that neurotransmission (based on Acetylcholinesterase activity) abruptly diminished in rodent brain tissue when the brain was heated to temperatures above 45 °C. Bench-top cadaver work conducted as part of the development of the DTS: Diathermic Syncope® system indicated that the temperature of cattle (**SS04 - McLean et al., 2017**) and sheep (**SS02 - Small et al., 2013**) brains could be raised by the required amount (5-12 °C) to achieve induction of unconsciousness. This was then confirmed using electroencephalography (EEG) in anaesthetised sheep (**SS02 - Small et al., 2013**) and anaesthetised cattle (**SS03 - Rault et al., 2014**); and subsequently in conscious cattle (**SS05 - Small et al., 2019**).

According to (EC) Regulation 1099/2009, key parameters are defined as the critical factors for ensuring proper stunning of all animals subjected to the stunning process. Table 1 provides a summary of the key parameters associated with the use of DTS and associated values of the key parameters where appropriate. The key parameters related to various existing methods are provided in Annex A of the EFSA guidance. As DTS is a completely novel stunning method, key parameters have been established using a similar approach, using research to-date.

Table 1: Parameters considered relevant for DTS stunning method

Parameter	Component	Description
Position and application of the Applicator	Restraining system	Commercial rotating or tipping knocking box; Head restraint with chin lift is used which fixes the head and holds the neck in extension; Rear pusher may be used to help push smaller animals into the neck yoke;
	Applicator	Purpose built device that interfaces directly onto the animal's head and directs DTS energy to the correct region.
	Applicator positioning	Applicator positioned directly over the frontal lobes of the brain. Operation prevented if the animal’s head is not pressed firmly against the applicator.
	Energy penetration	Energy penetration through the skin and bone into frontal lobes.
Appropriate energy according to animal size and species	Microwave generator energy output	Subject to operator set-up of power level and time duration.
	Safety mechanism	Safety interlocks throughout include: microwave leakage monitors in appropriate areas, screened animal stunning room (faraday cage), interlocks on all doors and openings. Warning lights fitted on the cage and generator. Auto cut-off mechanism which operates if the applicator is not in contact with the animal’s head.
	Animal Type (e.g., beef or dairy cattle), size of animal, horned or polled	Validated on cattle ranging from 160 to 413 kg liveweight. Bos taurus, beef, dairy and cross-bred types. Heifers, steers, cows and bulls were represented; some were horned, some naturally polled, and some dehorned. Five Brahman (Bos indicus) animals

Parameter	Component	Description
	Minimum energy (KJ)	160 kJ for simple stun 300 kJ for kill
	Animal skin condition	Dry or damp
	Depth of penetration	A function of the animal's skin, bone and brain thickness and ability to absorb the applied energy. Energy frequency will impact the penetration depth. Energy frequency is fixed by the magnetron used.
Duration of intervention	Minimum time of exposure	Related to incident power. Recommend 18-20 kW, so approximately 9 seconds to achieve 160 kJ energy applied.
Equipment maintenance, cleaning and storage conditions		**Generator Care** *Daily:* Remove any build-up of rubbish from within and around the generator. Ensure that all air intakes and outlets are clear. *Weekly:* Check water flow and arc detection devices are functional. Check for water leaks inside the generator. Check and replace (if required) air filters on generator side walls. *Monthly:* Check generator doors, limit switches and adjust to suit door stroke. Replace/wash air filters on generator side walls. **Applicator Care** *Daily:* Wipe out any moisture, dust or hair Visually inspect for cracks or other damage *Weekly:* Check for loose bolts and connections **Restraint Unit Care** *Daily:* Wash out any faecal matter, blood, hair or excreta Visually inspect for cracks or other damage Grease moving parts Visually inspect for damage Visually inspect Faraday Cage for damage
Frequency of calibration of the equipment		No calibration required. Assessment of tuning should be carried out by a specialist technician annually, or in the event of a change in size of the applicator (e.g., for use on another species)
Maximum stun to stick/kill interval(s)		Stun to stick interval. Type of sticking method.

4.1.4 Scientific basis of induction and maintenance of unconsciousness

The purpose of the EU Slaughter Regulation 1099/2009 is to avoid pain and suffering during stunning and slaughter. Regulation 1099/2009 requires that stunning method should induce immediate loss of consciousness. If the loss of consciousness is not immediate, then the onset of unconsciousness needs to occur without causing avoidable pain and suffering. Council Regulation (EC) No 1099/2009 also states that unconsciousness and insensibility induced by stunning should last until the animal is dead. The submitted studies characterise the animals' responses (unconsciousness, absence of pain) using the most sensitive and specific methods available (e.g., electroencephalography (EEG), blood samples) and establishes the correlations between these measurements and non-invasive parameters (e.g., Behavioural observations) that can be applied in abattoirs (EFSA, 2018).

For DTS, the aim is to induce insensibility through selective heating of the brain tissue to a temperature above which neurotransmission cannot occur. Thermal unconsciousness, such as that induced by exercise heat stress or fever, is reported to occur when core body temperatures reach between 40 and 45°C (McDaniel et al., 1991; Ohshima et al., 1992; Mohanty et al., 1997; Roccatto et al., 2010; Lerman et al., 2014; Yoshizawa et al., 2016; Hjeresen et al., 1983). Under hyperthermic conditions, the extensive circulatory network of the brain assists in maintaining brain temperatures 1-2°C below core body temperature (Hjeresen et al., 1983), suggesting that thermal unconsciousness is achieved when the brain temperature reaches between 39-44 °C. Where destruction of neurological tissue is intended (e.g., for ablation of neoplastic material), temperatures above 43 °C for a duration of one hour are utilized (Ryan et al., 1994), and it appears that the maximum thermal tolerance of nervous tissue lies in the range of 40–60 min at 42°C, or 10–30 min at 43°C (Sminia et al., 1994; Fike et al., 1991; Lyons et al., 1984). Brain metabolism and neurotransmitter activity is affected by temperature. In rats, at a brain temperature of 41 °C (5.4 °C above control brain temperature of 35.6 °C), adenosine triphosphate (ATP) concentration was reduced by 29.2% and creatine phosphate (CP) concentration was reduced by 44% (Sanders and Joines, 1984). In the same study, nicotinamide adenine dinucleotide (NADH) fluorescence increased during microwave application and returned to baseline levels within one minute after cessation of energy application. Another study (Ikarashi et al., 1984) demonstrated that there was a rapid reduction in acetylcholinesterase activity in rat brains once a temperature of 50°C was reached.

Experimental work has shown that, using 2 to 10 kW of 915 MHz microwave radiation, rats could be stunned (Guy & Chou, 1982). It was demonstrated that the animals would be unconscious after a brain temperature rise of 8°C and would remain unconscious for 4 to 5 min post exposure. Consciousness was regained when brain temperature returned to within 1°C of normal values. Also, Lambooy et al. (1989) successfully induced unconsciousness in rats using a low-power microwave (2 kW): application for 1.5–2.0 s resulted in unconsciousness in 100% of the rats tested. In cattle, the core body temperature tends to be 1-2 degrees higher than in humans (38.6°C in resting cattle, 37°C in humans), so we expect that in cattle thermal unconsciousness would occur in the range 42-47°C. Thus, the required rise in temperature to achieve insensibility is in the range of 3.4 to 8.4 degrees. During development and testing of the DTS equipment utilised in this study, the temperature profiles of cadaver heads was tested, and a predictive equation describing the heating rate within the brain was generated (0.124 °C/kW.sec). From this equation, we can infer that for a 30 kW application, the time taken to achieve an 8 degree rise in temperature would be 2.15 sec (8/30/0.124); and for a 20 kW application, the time taken would be 3.23 sec (8/20/0.124). Both these time intervals are less than the time taken (4-8 sec) to observe physical changes through the camera installed within the faraday cage. In a commercial situation, the real-time monitoring system incorporated within the control panel will allow an objective record of energy delivered and rate of energy delivery to be maintained, and an alarm feature could be incorporated to use in conjunction with animal-based indicators (e.g., response to reflexes) when confirming insensibility prior to exsanguination.

Microwave irradiation is an accepted method of euthanasia of laboratory rodents (AVMA, 2013), and microwave heating is commonly used to inactivate enzymes and fix brain tissue for histological purposes (Moroji et al., 1977). Suggested microwave energy inputs for this application in mice are 3.2 kW for 1.1 s (Cosi and Marien, 1998) and 2.5 kW for 0.68 s (Nordgren et al., 1985), resulting in a brain temperature of 75–90°C. In rats, Delaney and Geiger (1996) used 10 kW for 1.25 s to achieve a brain temperature of 85°C, while Ikarashi et al. (1984) used 10 kW irradiation at 2,450 MHz to achieve a brain temperature of 90°C in less than 900 ms in rats, and Zeller et al. (1989) also euthanized chickens in less than 1 s using microwave irradiation at 2,450 MHz. During Development of the DTS: Diathermic Syncope® system, sheep and cattle have been successfully rendered unconscious though application of microwave energy at 922 MHz (**SS03-**Rault et al., 2014; **SS02-**Small et al., 2013; **SS05-**Small et al., 2019).

In the early stages of development, the risk of overheating of the skin of the forehead prior to induction of insensibility was identified. The current apparatus, when properly tuned and incorporating the auto-tuner system, directs the majority of the energy deep into the brain tissue, so that insensibility occurs rapidly (within 1-8 s of energy application), and heating of the skin is minimised. In rats, a transient skin and muscle necrosis was observed when the tissues were maintained at 44-45 °C for one hour; while maintaining the spinal cord at a temperature of 42.6 °C for one hour led to no neurological effects, and some ataxia was noted after the spinal cord was held for one hour at 43.0 °C (Franken et al., 1992).

4.1.5 Potential causes of system failure and chances of occurrence

Potential causes of system failure and the chance of occurrence are summarised in Table 2.

Table 2: Potential causes of system failure, likelihood of occurrence and correction

Problem	Cause	Likelihood	Action
Power outage	Infrastructure failure	Rare	Wait till power returns, then run the Start-Up-Sequence.
Door interlocks not activated	Operator error	Possible	Check that doors are fully closed, then re-start application.
Incorrect waveguide positioning	Operator error	Possible	Reposition waveguide, then re-start application
Electrical fault in generator cabinet	Component failure	Rare	Detailed diagnostics through user control panel. Work with tech support and electrician to fix.
Component failure	Plant failure	Rare	Detailed diagnostics through user control panel. Work with tech support to fix.

4.2 Description of the individual scientific studies submitted

The scientific studies submitted are outlined in Tables 3 and 4. A list of supplementary information is also included. Figure 12 indicates which Steps contributed to each of the submitted peer-reviewed publications.

Table 3: Peer reviewed publications

ID	Author	Title	Journal	Status
SS01	Small, A., McLean, D., Owen., J. S., Ralph, J.	Electromagnetic induction of insensibility in animals: a review	Animal Welfare 22: 287-290	Published 2013
SS02	Small, A., McLean, D., Keates, H., Owen., J. S., Ralph, J.	Preliminary investigations into the use of microwave energy for reversible stunning of sheep	Animal Welfare 22: 291-296	Published 2013
SS03	Rault, J.-L., Hemsworth, P., Cakebread, P., Mellor, D., Johnson, C.	Evaluation of microwave application as a humane stunning technique based on electroencephalography (EEG) of anaesthetised cattle	Animal Welfare 23: 391-400	Published 2014
SS04	McLean, D., Meers, L., Ralph, J., Owen, J. S., Small, A.	Development of a microwave energy delivery system for reversible stunning of cattle	Research in Veterinary Science 112: 13-17	Published 2017
SS05	Small, A., Lea, J., Niemeyer, D., Hughes, J., McLean, D., Ralph, J.	Development of a microwave stunning system for cattle 2: Preliminary observations on behavioural responses and EEG	Research in Veterinary Science 122: 72-80	Published 2019
SS06	Hughes, J., Small, A.	A comparison of beef carcases stunned using DTS: Diathermic Syncope® or captive bolt in terms of selected meat quality attributes and plasma biomarker concentrations	Meat Science	In preparation

Table 4: Project reports - not peer reviewed - CONFIDENTIAL

ID	Title	Date	Status
PR01	SRP.003 Investigate the Use of Microwave Technology for Stunning Beef.	2005	CONFIDENTIAL
PR02	Report on Microwave Technology Development Stage 1.	2009	CONFIDENTIAL
PR03	Microwave induced insensibility for animals Stage 2.	2010	CONFIDENTIAL
PR04	Development of applicator and autotuner	2011 - 2012	CONFIDENTIAL
PR05	Evaluation of microwave application as a humane stunning technique based on electroencephalography (EEG) of anaesthetised cattle.	2013	CONFIDENTIAL
PR06	Microwave results from the live testing conducted at Wagstaff Cranbourne in April 2012. Interim project report	2012	CONFIDENTIAL
PR07	Refinement of delivery apparatus and development of user interface software.	2014	CONFIDENTIAL
PR08	Dielectric induction of temporary insensibility in cattle - animal trials.	2015	CONFIDENTIAL
PR09	Meat quality from microwave stunned cattle.	2015	CONFIDENTIAL
PR10	Refinements to delivery system: applicators and coaxial cabling. Interim project report.	2016 - 2019	CONFIDENTIAL
PR11	DTS: Diathermic Syncope - commercial validation trials.	2018	CONFIDENTIAL
PR12	DTS: Diathermic Syncope® controlled trials.	2021	CONFIDENTIAL
PR13	Refinements to delivery system: applicators, coaxial cabling and tuning.	2018 - 2021	CONFIDENTIAL

Supplementary material

SUP1: Conference material. Evaluation of microwave application as a humane stunning technique based on electroencephalography. International Society for Applied Ethology 2013. Peer-reviewed. PUBLIC.

SUP2: Conference material. DTS®: a novel method for stunning cattle. Impacts on cortisol response and post slaughter meat quality attributes. Pan-Commonwealth Veterinary Conference 2015. Peer-reviewed. PUBLIC.

SUP3: Conference material. Meat quality attributes of beef carcases slaughtered using DTS: Diathermic Syncope®. International Congress of meat Science and Technology 2015. Peer-reviewed. PUBLIC.

SUP4: Conference material. DTS®: Diathermic Syncope for cattle stunning. Humane Slaughter Association 2015. Peer-reviewed. PUBLIC.

SUP5: Conference material. DTS: Diathermic Syncope® - induction of insensibility in cattle. Presentation Slides used for a number of stakeholder meetings. Not Peer-reviewed. PUBLIC.

SUP6: Conference material. DTS: Diathermic Syncope® - a new technology for pre-slaughter induction of insensibility. Royal Society for the Prevention of Cruelty to Animals Australia Conference 2018. Peer-reviewed. PUBLIC.

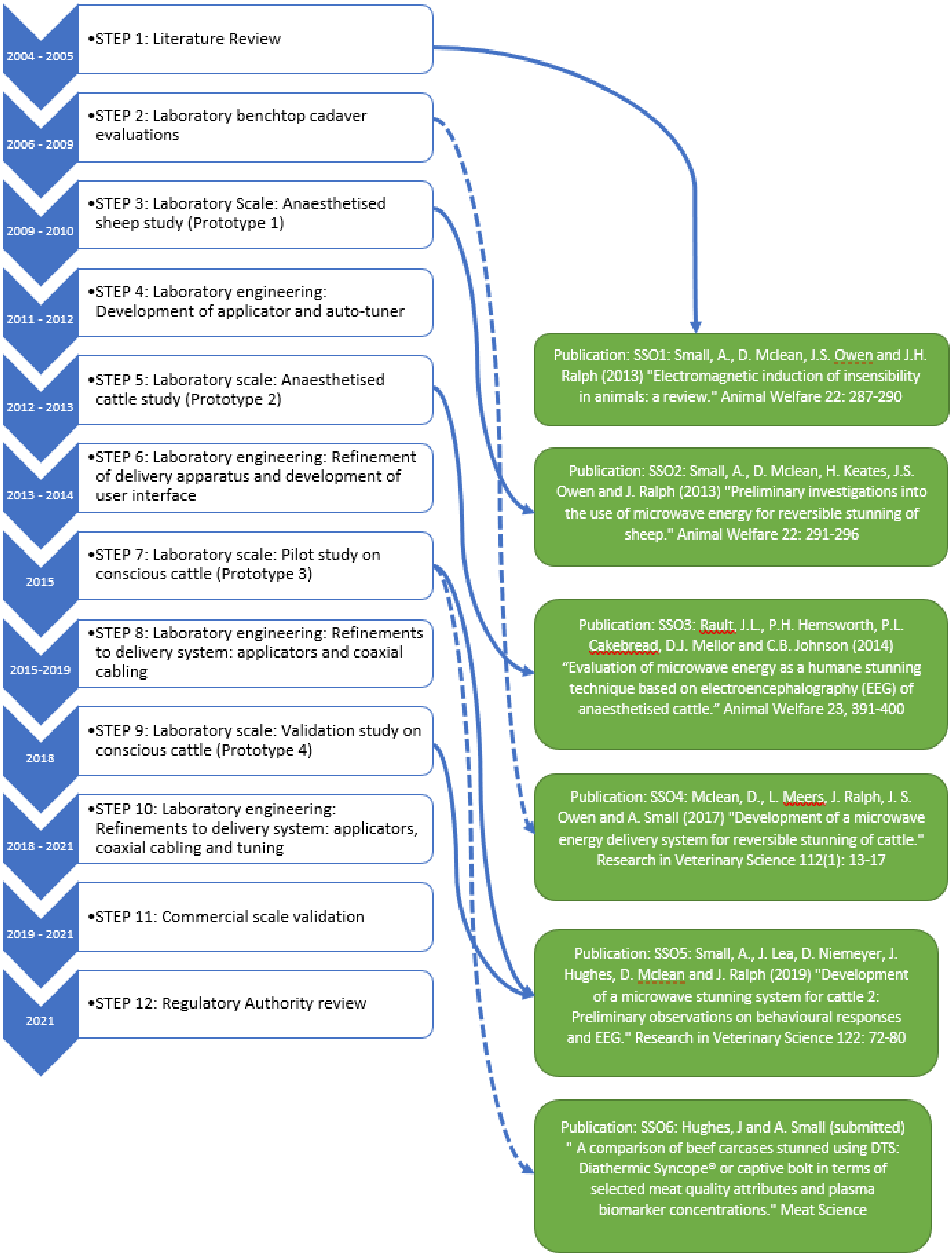
SUP7: Conference material. Applicator development for animal stunning. AMPERE Conference 2017. Peer-reviewed. PUBLIC.

SUP8: Factsheet. DTS: Diathermic Syncope®. Not peer-reviewed. PUBLIC.

SUP9: Conference material. DTS: Diathermic Syncope® - A new technology for pre-slaughter induction of reversible unconsciousness. North American Meat Industry Animal Care Conference 2021. Peer-reviewed. PUBLIC.

SUP10: Conference presentation recording. DTS: Diathermic Syncope® - A new technology for pre-slaughter induction of reversible unconsciousness. North American Meat Industry Animal Care Conference 2021. Peer-reviewed. PUBLIC.

Figure 12 DTS: Diathermic Syncope®: Flowchart representation of development timeline



STEP 1: Literature review

Submitted study: **SS01** Electromagnetic induction of insensibility in animals: a review.

Other reference documents: **PR01**: SRP.003 Investigate the Use of Microwave Technology for Stunning Beef. Project Report. Not peer-reviewed. CONFIDENTIAL. The Literature review component was revised and updated in 2013, for publication in a peer-reviewed journal (**SS01**).

Introduction

Some communities require that animals processed for human consumption are healthy, uninjured and normal at the moment of carrying out the slaughter cut. As a result, many methods of stunning used in modern commercial slaughter are not acceptable, because the animals could be considered injured, or because a proportion of animals would not recover from the stun. Head-only electrical stunning is used in sheep but is thought to be unsuitable for use in cattle because of concerns over the duration of insensibility being insufficient to allow death through total blood loss to occur prior to recovery from the stun (Blackmore & Newhook, 1982; Lambooy & Spanjaard, 1982; Anil et al., 1995a; Anil et al., 1995b), and also because of problems with blood splash in the meat (Gregory, 2005). The possibility of using microwave energy to heat the brain and thereby induce unconsciousness was suggested, leading to a scoping review being conducted.

Objective

To undertake a scoping review of scientific literature and patents and to consult with microwave engineers on the potential for using microwave energy for inducing unconsciousness in cattle.

Materials and methods

Literature searching was carried out using the CSIRO library service databases, e.g., Web of Science® Science Collection and Derwent Innovations Index. Consultation with Microwave engineers at the University of Melbourne was carried out.

Discussion and conclusions

The findings of this study phase were as follows:

PR01: From extensive studies with laboratory rodents there appears no doubt that microwave irradiation can provide a humane stun with unconsciousness (caused by a brain temperature increase of 8 – 10° C) being induced within milliseconds rather than seconds. Microwave irradiation can produce a reversible stun in rodents with the heart remaining beating and subsequent return of normal consciousness. This form of stun would appear to satisfy the principles of Halal slaughter. As with all reversible stunning methods, the stunning parameters and the subsequent sticking (exsanguination) procedures would need to be finely tuned to ensure welfare issues were addressed.

The incidence of convulsions and seizures following microwave irradiation of rodents is variable. It does seem possible for a successful stun to occur without convulsions. Similarly, the incidence of haemorrhages is variable. Microwave irradiation does not appear to invariably cause the intense generalized muscle body contractions which occur during electrical stunning and which are believed to be involved in the production of blood splash. While microwave radiation can cause thermal damage to the brain, it seems highly unlikely that there would be mechanical damage sufficient to cause release and subsequent transport of any BSE material which might be present in the brain.

Based on extrapolations of very limited data for microwave stunning of sheep and pigs available at the time of the review, it would appear that a ballpark figure for the energy requirements of a microwave generator for stunning cattle might be 100 kW. This is not a totally unrealistic proposition as presently 100 kW and 300 kW units are being trialed for use in the Australian timber industry.

SS01: Early attempts to induce insensibility and death in laboratory species were successful, but the technology to apply the technique to larger animals was not available at that time. More recently, however, technological advances have led to new work in the areas of transcranial magnetic stimulation and microwave irradiation, both of which are potential methods of inducing a recoverable stun in larger species.

STEP 2: Laboratory benchtop cadaver evaluations:

Submitted studies:

SS02 Preliminary investigations into the use of microwave energy for reversible stunning of sheep.

SS04 Development of a microwave energy delivery system for reversible stunning of cattle.

Other reference documents: **PR02**: Report on Microwave Technology Development Stage 1. Project report. Not peer-reviewed. CONFIDENTIAL.

Introduction

The scoping study described in **SS01** indicated that induction of unconsciousness in large animals may be feasible. Previous literature indicated that rats could be stunned successfully using 2–10kW of 915MHz microwave radiation (Guy and Chou, 1982). It was demonstrated that the animals would be stunned after brain temperature rise of 8° C and would remain unconscious for 4-5 mins post exposure. The regaining of consciousness occurred when brain temp dropped to within 1° C of normal values. In other studies, the figures of required brain temperature rise vary. Temperature increases required range from 4° C (Schwartz, 1974) to 10.3° C (Lambooy et al., 1989).

The next stage in development of the DTS: Diathermic Syncope® system was to understand in greater detail the dielectric properties of the tissues to be irradiated (brain, bone and skin), and then to conduct benchtop evaluations of the ability of microwave irradiation to selectively raise brain temperature. Two key parameters for evaluation were the temperature rise in the brain required to induce unconsciousness in the animal, and the length of time that unconsciousness could maintain effect.

There were three stages to this study: A literature review; an initial comparison of two microwave frequencies in terms of depth of penetration and heating in different parts of the head; and an evaluation of the heating profiles in different parts of the head.

Objective

Literature review objective: to outline any major findings or achievements in the field of biological microwave heating and from the literature highlight likely challenges for the development of a stunning system for cattle and sheep in an abattoir.

Stage 1 experimental work objective: to determine the optimum microwave frequency for future processing and determine the extent of heating at a range of locations around the head.

Stage 2 experimental work objective: to gain initial estimates into the depth of microwave penetration into the brain and magnitude of subsequent heating occurring through the brain and at skin level.

Materials and methods

Literature searching was carried out using databases such as Web of Science®, Scopus® and Google Scholar®.

Stage 1 experimental work

A test apparatus was built, which delivered energy from the generator to an applicator point, via hollow rectangular tubing called waveguide, through a series of components designed to optimize energy transmission. The generator contained the power supply and magnetron and could provide microwave power output levels of between 0 – 30kW at a frequency of 922MHz and a separate generator could supply 0 – 5kW at 2.45GHz. From the generator, energy was passed through a circulator, which eliminates problems associated with reflected energy. If microwave energy is allowed back into the launcher the reflected energy can be reabsorbed by the magnetron causing premature ageing and hence device failure. The circulator acts as a one-way valve allowing energy to pass from the magnetron to the rest of the circuit but prevents any reflected energy from being returned to the magnetron. The reflected energy is directed into the dummy load and is dissipated into a constant flow of water. The circulator was followed by a directional coupler, which enables the measurement of the forward and reflected powers at that point. The energy then passed through a tuner, which allows a wide range of varying load/applicator impedances to be matched to the microwave network. Reflected power is due to impedance mismatches and the tuner acts as a matching element allowing an impedance transformation to take place, thus reducing the reflected power and improving the net power transfer to the applicator/load. From the tuner, the energy is directed through the applicator into the test load. For this study, the test loads were cadaver cattle and sheep heads, sourced from a local abattoir, stored frozen until required for testing and defrosted overnight prior to testing.

Fiberoptic temperature probes (Neoptix, Canada) were inserted into the frontal skin immediately below the waveguide; nose; neck skin behind the jaw, ear (under the skin), eye (subconjunctival); and five locations within the brain to allow measurement of temperature immediately before and immediately after energy application. Two sheep heads were irradiated at 2.45 GHz, two sheep heads at 922 MHz, and eight cattle heads at 922 MHz.

Stage 2 experimental work

The same test apparatus as in stage 1 was used, applying energy at 922 MHz. Temperatures were continuously monitored using fiberoptic probes placed on and under the skin of the frontal area and in the brain at superficial, middle and deep locations. Four sheep heads and 12 cattle heads were irradiated, varying power applied and exposure time. For sheep, power levels varied from 3 to 6 kW and exposure times from approximately 10 to 20 seconds. For cattle heads, power levels were varied from 2.5 to 9kW and exposure times from approximately 15 to 60 seconds.

Reporting the results

Literature review

Two key parameters for development of a system for stunning animals will be the temperature rise in the brain required to stun the animal, and the length of time that stunning maintains effect. Experimental work has shown that using 2–10kW of 915MHz microwave radiation, that rats could be stunned successfully (Guy and Chou, 1982). It was demonstrated that the animals would be stunned after brain temperature rise of 8° C and would remain unconscious for 4-5 mins post exposure. The regaining of consciousness occurred when brain temp dropped to within 1° C of normal values. Figures of required brain temperature rise vary within literature. Temperatures required range from 4° C (Schwartz, 1974) to 10.3° C (Lambooy et al., 1989).

A wide range of microwave delivery apparatus were used in the literature studied. Applicator types include single antenna arrangements for poultry (Takamura and Ishida, 1997), multiple antennae for pigs (Lambooy et al., 1989), singular waveguide (Maruyama et al., 1986), and semi-circular waveguide arrays (Schwartz, 1992). Merritt et al. (1977) found that ensuring the electric field element of the radiation is parallel to the centre line of the head created much higher coupling rates. The microwave delivery system developed for stunning animals will be critical to the success of the technology, as it will affect energy coupling efficiency and focussing of the energy into the brain, without excessive external skin heating.

Basic theory regarding dielectric properties as relevant to stunning of animals was also collated and summarized. The dielectric properties of any material are the main parameters that influence how microwave energy is coupled into the material and how efficiently this energy is then converted into thermal energy. These properties are described by the following complex equation:

$$\varepsilon^* = \varepsilon' + j\varepsilon'' \quad \text{(Equation 1)}$$

The real part of the equation (ε'), the dielectric constant, represents the ability of the material to store energy when a microwave field is applied. The imaginary part of the equation (ε''), the loss factor, determines how readily the microwave energy is converted into heat within the material. The penetration depth refers to a wave passing into a dielectric material, whose amplitude is diminishing owing to power absorption as heat into the material. The wave's field intensity and power flux density fall exponentially with distance from the surface. The rate of decay is a function of the complex dielectric constant ε, which comprises the real component ε' and the loss factor ε'' as shown in Equation 1.

The penetration depth is defined as the depth into the material at which the power flux has fallen to 37% (1/e) of its value on the surface. penetration depth is proportional to the wavelength of the incident microwave energy, thus higher frequencies will achieve lower penetration into the workload given the dielectric properties are similar at both frequencies. In commercial applications in Australia the two frequencies available are 2.45GHz and 922MHz.

Stage 1 experimental work

Sheep heads irradiated at 2.45 GHz: little heating occurred in the five brain probe locations compared to the skin, and that the nose, eye, ear and neck temperature rose by similar amounts to the brain. The minimal microwave penetration into the brain caused only slight heating; confirming the dielectric theory was accurate. These results indicated that the 2.45 GHz frequency would not be suitable for this application.

Sheep heads irradiated at 922 MHz: there was far greater temperature rise within the brain compared with irradiation at 2.45 GHz. The brain temperatures rose by similar amounts to that of the skin. This increased heating of the brain is an effect of the higher penetration achieved at this lower frequency as was predicted by the dielectric theory. No significant heating of the neck, eyes or nose regions occurred.

Cattle heads irradiated at 922 MHz: similar results were obtained to those achieved with the sheep heads. Almost all the heating occurred in the brain and on the head directly above it. The cow head displayed slightly less penetration (compared with sheep) into the lower parts of the brain due to larger brain and skull size.

<u>Stage 2 experimental work</u>

Sheep heads: Heating rates of each area were linear for all test runs. The skin experienced greater heating than the brain and heating rate decreased with depth into brain. This correlates to what the dielectric data predicted. Upper brain temperature change was 40% of skin temperature change and that small reduction in heating was observed in the lower parts of the brain. These results show significant potential for 922MHz microwave energy to penetrate and heat the brain effectively and consistently.

Cattle heads: The cattle temperature profiles largely showed linear temperature changes. The brain heated slower than the skin on top of the head and the upper part of the brain heated by more than the lower parts as was predicted from dielectric data. Upper brain temperature change was 20% of skin temperature change and that significant reduction in heating was observed in the lower parts of the brain; 12% and 4% of skin temperature change for the middle and lower brain respectively.

The results for both sheep and cattle irradiation provided data from which approximate power-time curves could be formulated. These curves plot predicted power required versus desired process time for a given brain temperature rise.

The curves illustrate that cattle treatment requires much more power than sheep for equivalent brain temperature rise. This was largely caused by the higher degree of absorbed power into the thicker skin and bone layers of the cattle heads coupled with larger brain size. The power curves are asymptotic towards a process time of 0 seconds. The curves are estimates only and are based on a relatively small sample of trials, but do, however, give good indication of likely powers required. The curves also illustrate the significant reduction in required power if lower brain temperature change is needed or longer process times are acceptable.

During trials the weight of the whole cow corresponding to each test head was noted. This allowed further analysis of the temperature versus power relationship. The relationship between cow weight and temperature change gradient was established and used to plot new curves taking into account cow weight. This increases the accuracy of power level prediction and allows for estimation based on cattle weight. The relationship between weight and power required was found to be non-linear.

Discussion and conclusions

The findings of this study phase were as follows:

One of the main concerns in any microwave heating system is the penetration depth of the power flux into the workload. It gives a direct understanding to the heat distribution within the material and hence to heating uniformity. It is important to remember that the penetration depth only refers to the distance into the material where the power flux density has decreased to 37% of its surface value and that the corresponding temperature will be directly related to the exponential decay in power the deeper into the animal head.

The following are some general conclusions that may be derived from the published dielectric values of brain, bone and skin:

- These data represent a generalised set of loss factors ε'' that may be encountered. For a given electric field E the absorption of energy depends on ε''. The effectiveness of heating will depend as much on the design of the applicator as on the loss factor of the material.
- Penetration depths calculated from both dielectric data sources match quite closely at 922MHz but differ considerably at 2.45GHz. Generally, penetration depth at this frequency is approximately 30% of that at 922MHz due mainly to frequency effect.

The choice of frequency is basically between using multiple magnetrons operating at either 2.45 GHz maximum rated power 30kW or larger power magnetrons operating at 922MHz rated maximum power 100kW. Some facts about each approach are listed below:

- Magnetron efficiencies are between 50-70% for 2.45GHz compared to 80-87% for high power 922MHz,
- Applicator dimensions and complexity increases as the number of individual magnetron sources increases,
- In multiple magnetron installations, magnetron life is reduced due to cross coupling of power,
- Multiple magnetron systems provide a high degree of redundancy,
- Penetration depths are significantly larger at 922MHz than at 2.45GHz.

One key to success in the development of a system for animal stunning is to localise the heating to the brain as much as is possible. This preliminary experimental work suggests that this may be possible. The tests performed, particularly at 922 MHz, demonstrated that little heating took place in the regions of the eyes, ears, neck and nose when compared to the area around the brain. With further applicator design and development, it should be possible to reduce external heating even further. The trials also clearly demonstrated that 922MHz is the preferred frequency. It offers higher penetration into the brain and is commercially available with single generators up to 100kW.

While a large degree of attenuation was shown in the trial results, it should be stated that the applicator used was not designed to reduce this. Specific designs in later stages of work would endeavour to further focus the heating into the brain. These trials were undertaken to gain understanding into the nature of the temperature rise within the head. The trials founds that largely the temperature rise was highly linear within the brain and the skin. Linear temperature rise leads to easier control and repeatability in later development.

The experimental results clearly show that both cattle and sheep brains can be heated very quickly using microwave energy and that no excess heating of eyes, ears, nose and neck areas occur. The realisation of a commercial system appears to be very promising given the results from this initial study.

Literature suggests that there are many advantages to reversibly stunning livestock with microwave radiation; however previous attempts have been discontinued due to assumed risk of radiation exposure to operators or equipment cost. These issues may no longer be relevant due to information now available within this report.

Dielectric data predicted higher penetration into the brain at 922MHz. This was confirmed with early trials. The first phase of trials demonstrated that 922MHz energy penetrates further into the brain and also reduces heating to the other areas of the subject head. Based on this; 922MHz is the recommended frequency for future work.

The second phase of trials found that heating rates within the head were largely linear and that skin temperatures rose at higher rates than brain temperatures. This effect was less pronounced in the sheep head trials. This skin heating effect may require the design of an applicator which promotes high penetration into the head and focuses microwave coupling into the brain.

Likely power levels were predicted based on the phase 2 trial data. This revealed likely power levels of 4kW to 40kW to process sheep in 10 and 1 second respectively at 10° C temperature rise and 12kW to 100kW to process 300kg cattle in 10 to 1.5 seconds respectively at 10° C temperature rise.

A conceptual design was included in the phase 2 study report.

STEP 3: Laboratory scale: Anaesthetised sheep study (Prototype 1)

Submitted study: **SS02** Preliminary investigations into the use of microwave energy for reversible stunning of sheep

Other reference documents: **PR03**: Microwave induced insensibility for animals Stage 2. Project Report. Not peer-reviewed. CONFIDENTIAL.

Introduction

In recent years, microwave technology has developed to the point that high power equipment is available that can focus the energy into the animal's brain to produce a rapid rise in temperature. It is expected that controlling the brain temperature will result in insensibility and allow the animal to regain consciousness without any adverse effects when the temperature of the brain has returned to normal. This will give a recoverable insensibility that should be acceptable for religious slaughter. Benchtop cadaver work (Step 2) has demonstrated that both cattle and sheep brains can be heated very quickly using microwave energy and that no overheating of eyes, ears, nose and neck areas occur.

This project was intended to provide a proof-of-concept outcome, aiming to demonstrate whether or not induction of reversible insensibility, of sufficient duration to allow exsanguination during insensibility (or death before recovery) is indeed attainable, and thus to advise on the merit of pursuing development of this technology.

Objective

- To determine whether there is a significant effect on the heating effects of microwave energy application as a result of an active blood circulation in the brain. It is possible that the physiological thermoregulatory response of the animal will interfere with the heating effect;
- To adjust the power calculations to take account of this effect, if present;
- To ascertain that rapid heating of the brain can induce insensibility in sheep;
- To ascertain that this insensibility is maintained for a sufficient period to allow the animal to die, as a result of bleeding, prior to the onset of recovery.

Materials and methods

Ethical considerations: The study protocol was reviewed and approved by the CSIRO Animal Ethics Committee, reference 2-09, according to the Australian code for the care and use of animals for scientific purposes (NHMRC, 2013).

Study population: Four cross-breed ewes aged 3–4 years were used for the study. The study was carried out on a single day, with one sheep being anaesthetised, treated and euthanased prior to commencement of procedures on of a subsequent animal. **Randomisation** was in the form of a pre-assigned treatment schedule (table 1 in SS02) and sheep being brought to the restraint unit in the random order in which they were captured from the holding pen. Each sheep underwent general anaesthesia (premedicated with diazepam, induced with thiopentone and maintained on isoflurane in oxygen), and was placed in a custom-built v-restraint crate with chin support, presenting the forehead to the waveguide applicator. Respiratory rate and rhythm were observed and monitored by the anaesthesiologist, while pulse and haemoglobin oxygen saturation were monitored using a pulse oximeter (Nellcor N-595, Covidien, USA).

Sampling strategy: A single channel, 2-needle montage was used to collect electroencephalogram (EEG) traces on paper (Neocardiotrace, Australia). In two sheep, fiberoptic thermoprobes (Neoptix, Canada) were inserted into the brain at superficial, middle and deep levels, via a single trephine hole in the frontal bone.

Microwave energy at 922 MHz, 4 kW power was applied to each sheep for between 5 and 20 s. The two sheep from which brain temperature was measured were immediately euthanased (pentobarbitone overdose) after irradiation; while the two others were disconnected from inhalational anaesthesia, removed from the restraint unit and monitored until the swallowing, chewing and corneal reflexed had returned, at which point they were euthanased.

Heads were inspected and skinned to assess the physical appearance of the energy application site. Brains were removed after 3 days storage and underwent histological examination.

The study was a controlled observational pilot study, so there was no **blinding** and no statistical **analysis.**

Measurement of the outcome: The aim of the study was to confirm that unconsciousness could be induced based on EEG data. No attempt was made to estimate latency to onset or duration of unconsciousness. The sheep were anaesthetized, so no estimation of pain, distress or suffering could be made.

Reporting the results

Temperature profiles (sheep 1 and 2): In sheep 1, the energy was applied for 10 seconds, resulting in a temperature rise of 4.4°C at the top of the brain, and 2.15°C at the bottom of the brain. In sheep 2, the energy was applied for 20 seconds, resulting in a rise of 9.65°C (peak temperature 48.5°C) at the top and middle of the brain and 2.5°C to 40.95°C at the bottom of the brain. Again, by 10 seconds duration of application, the required temperature of 43°C (expected point of induction of hyperthermic unconsciousness, based in literature review, Step 1) was achieved. During and after this period, breathing remained rhythmic, the pulse regular, and haemoglobin oxygen saturation level at around 98%.

Electroencephalogram (sheep 1, 2 and 3): On the electroencephalogram (EEG) traces, it could be seen that brain activity in all three sheep changed from low amplitude before application of energy, to high amplitude after application of energy. This high amplitude activity is similar to the epileptiform activity induced by the current practice of electrical stunning, and suggests that the sheep, if they had not been anaesthetised, would have noticeably lapsed into unconsciousness. EEG was not recorded from sheep 4 due to failure of the battery pack in the unit.

The progress of recovery from deep to light anaesthesia in sheep 3 and 4 was uneventful; return of jaw tone and chewing movements were observed in both animals prior to euthanasia.

On dissection, there was no visible effect on the skin or skull where the energy had been applied, apart from one sheep (sheep 4) where excess heating of the skin had been observed, as a result of the prolonged application of energy (20 s compared with 10 s in sheep 3). In this sheep, the surface of the skull showed a light, tan-coloured scorch mark, and the overlying skin was noticeably detached and crisp. The brains were also grossly normal, apart from sheep 4, which showed an area of hyperaemia on the surface of the brain at the point of energy application. Histologically, tissues from sheep 1, 2 and 3 were generally normal, although signs of autolysis were evident. The exception was sheep 4, which had received overheating; two sections of brain were normal, but one section (taken from the hyperaemic area) showed malacia with loss of nuclear detail and fragmentation of the neuropil. The histopathologist concluded that “the malacia is similar to what would be expected with complete and sudden ischaemia and possibly caused by damage to and within associated blood vessels, as well as thermal effects on the parenchymal cells”.

As an observational pilot study, there was no statistical analysis performed. Sources of bias or confounding could include: small numbers of sheep involved in the study (n = 4) and the fact that they were all from a similar age and source cohort.

Discussion and conclusions

The findings of this study phase were as follows:

Hyperthermic insensibility, is predicted to occur when brain temperatures reach around 43-44 °C (Ohshima et al., 1992; Guy and Chou, 1982). A sheep’s core body temperature is around 38°C, so the temperature change desired is in the order of 6°C or more. The cadaver work described in Step 2 demonstrated that microwave energy at a frequency of 922 MHz could induce this temperature rise. However, the living brain does have an inherent cooling mechanism via the blood circulation (Niemark et al., 2007), and to induce unconsciousness without distress, it is imperative that this cooling mechanism is rapidly overcome by the energy applied.

The trials on anaesthetised sheep were highly successful. Application of microwave energy caused rapid increases in brain temperature to a point above which insensibility would be expected to occur (43°C), and below that which protein denaturation and damage would be expected to occur (50°C). There were no visible signs of damage to the surrounding tissues, or to the brain itself, except in one sheep in which the surface temperature was known to have been raised too high. In one sheep, there was a single convulsion on application of the energy, and she stopped breathing momentarily. Rhythmic breathing returned after about 10 seconds, and there was little effect on pulse rate and haemoglobin oxygen saturation.

STEP 4: Laboratory engineering: Development of applicator and auto-tuner

Submitted study: **PR04**: Development of applicator and auto-tuner. Interim project reports. Not peer-reviewed. CONFIDENTIAL.

Introduction

Previous work (Sections 4.2 and 4.3) indicated that the induction of a suitable rise in brain temperature to lead to insensibility was feasible. The next stage in development of the DTS: Diathermic Syncope® system was do develop suitable applicators and tuning systems to generate a 5-10 °C increase in brain temperature with minimal heating of skin and bone.

Objective

- To design three applicators that may be suited to induction of insensibility in animals (**PR04** part 1);
- To test these applicators on cadaver cattle heads (**PR04** part 3);
- To investigate the suitability of an auto-tuner system to be incorporated into the proposed system (**PR04** part 2).

The details of this step are confidential proprietary information.

STEP 5: Laboratory scale: Anaesthetised cattle study (Prototype 2)

Submitted study: **SS03**: Evaluation of microwave application as a humane stunning technique based on electroencephalography (EEG) of anaesthetised cattle.

Other reference documents: **PR05**: Evaluation of microwave application as a humane stunning technique based on electroencephalography (EEG) of anaesthetised cattle. Project Report. Not peer-reviewed. CONFIDENTIAL.

PR06: Microwave results from the live testing conducted at Wagstaff Cranbourne in April 2012. Interim project report. Not peer-reviewed. CONFIDENTIAL.

Introduction

Microwave application has been reported to induce loss of consciousness when applied to conscious rats, causing petit or grand mal seizures for 1 min after exposure and an unconscious state for the following 4 to 5 min with the animal ultimately recovering (Guy and Chou, 1982; Lambooy et al., 1989). However, Lambooy et al. (1989) deemed this technique unsuitable for pigs at that time, partly because of the capacity of the microwave generator being too low to deliver sufficient power. In recent years, microwave technology has developed to the point that high power equipment is available that can focus the energy to produce a rapid rise in temperature in cattle brains (Ralph et al., 2011. Patent number: PCT/AU2011/000527). It is expected that raising the brain temperature will stop brain function and result in insensibility, in other words eliminate the ability for the cows to feel pain, whilst still allowing the animal to regain consciousness after a period of time (reviewed by **SS01**-Small et al., 2013a). This is supported by preliminary evidence in sheep (**SS02**-Small et al., 2013b). Consequently, this may allow for a recoverable sensibility acceptable for religious slaughter since it would not have 'physically injured' the animal.

Objective

The aim of this project was to investigate the effectiveness of different settings of the microwave technique, power and duration of application, on anesthetized cows to induce insensibility, in other words eliminate the ability for the cows to feel pain, based on electroencephalography (EEG).

Materials and methods

Ethical considerations: The project was approved by the University of Melbourne Ethics Committee (approval number 1212620.1) in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (NHMRC, 2013).

Study population: Ten crossbred female cows, estimated liveweight 180 kg, were used over a total of 6 days. The animals were sourced from the daily intake of the abattoir. Each animal was processed individually. The animal was moved from the lairage area through a single chute race and into a restraining box. The head of the animal was restrained and anaesthesia was induced using intravenous ketamine and propofol. Once the animal was anaesthetised the animal was rolled out of the restraint box and intubated. The animal was then placed in dorsal recumbency onto a specifically designed V-restrainer rolling crate, and the endotracheal tube connected to the anaesthetic machine delivering halothane in oxygen via a circle breathing system using standard clinical flow rates and vapouriser settings. End-tidal halothane tension was maintained at 0.9%. Patient stability and depth of anaesthesia was monitored throughout the procedure using an anaesthetic agent monitor (Cardell® Veterinary Monitor Max-12HDim multiparameter monitor), recording the end-tidal carbon dioxide tension, end-tidal halothane tension, respiratory rate and heart rate every 5 minutes throughout the anaesthetic procedure.

Subdermal 27-G stainless steel needle electrodes were placed in a four-electrode montage to record two channels of EEG. For each channel, the common non-inverting electrode was placed midline between the medial canthi of the eyes, the inverting electrodes placed bilaterally over the mastoid processes, and the ground electrode placed caudal to the poll. The EEG was amplified using isolated differential signal amplifiers (Iso-Dam isolated physiological signal amplifiers; World Precision Instruments, Sarasota FL, USA), with a gain of 1,000 and pass-band of 0.1 to 500 Hz and digitised at a rate of 1 kHz (Powerlab/4sp; ADInstruments Ltd, Sydney, Australia). Data were analysed off-line after completion of the experiment. Insensibility was assessed by the appearance of seizure-like complexes in the EEG. Four variables were derived from combinations of the raw data and/or the variables derived from the frequency spectra: time to onset of EEG suppression (raw data), time to nadir of EEG suppression (95% spectral edge), duration of EEG suppression (combination of raw data, 95% spectral edge and total EEG power), and maximum effect (95% spectral edge) (see Figure 2). Time to onset of EEG suppression was measured from the start of the microwave application until the first appearance of seizure-like complexes in the EEG, hence including the duration of microwave application. Time to nadir of EEG suppression was measured from the start of the microwave application until the maximum depression of 95% spectral edge. Duration of EEG suppression was measured from the time of onset until the re-emergence of a normal EEG pattern similar to that seen prior to the application of the microwaves. The maximum effect was determined by the maximum reduction of the 95% spectral edge frequency.

Electrocardiogram (ECG) data were recorded by placing three ECG electrodes on the animal's body, for the last 4 animals. The electrodes were placed in a three-electrode montage. Adhesive electrode pads were adhered to the skin; the positive electrode was placed on the chest wall 5 cm behind the left point of the olecranon; the negative electrode was situated and 10cm out from midline of the thoracic back on the right-hand side; the ground electrode was placed in the same position as the negative electrode but on the left han2d side. Electrocardiogram recordings were acquired using Powerlab 4/25T (ADInstruments, Castle Hill, Australia) and Chart 5® software (version 5.5.5) (ADInstruments, Castle Hill, Australia). The ECG tracings were analysed using Chart 5 software to produce continuous heart rate recordings.

The microwave applicator was applied in contact with the top of the front head, on a mid-line half-way between the medial canthi of the eyes and the poll. The exact location varied slightly depending on the head shape. The microwave application was imposed at different powers and for different durations. It was repeated 35 min later at an identical dose to give a total of 2 applications per animal. This second application was used in an attempt to deliver multiple applications to a single animal maintained under anaesthesia, hence allowing the collection of additional data while reducing the number of animals needed for the experiment. However, the second application resulted in more efficient microwave delivery and more profound EEG effects than the first. This does not accurately reflect field conditions (each animal would be subjected to a single application). Therefore, the data from the second applications for each animal were not included in the analysis. The animal was maintained under anaesthesia throughout and was euthanized 10 min after the last microwave application by administrating a lethal dose of barbital into the jugular vein. One animal did not respond to the anaesthetic agents and was euthanized before any treatment could be applied. Thus, data were collected from nine animals. Energy deliveries were 20 kW for 15 s (n = 2); 20 kW, 10 s (n = 2), 30 kW, 10 s (n = 3), 30 kW, 5 s (n = 1) and 12 kW, 25 s (n = 1).

The external head temperature was recorded after microwave delivery by using a digital electric probe placed on the front head of the animal approximately 5 cm below the application point on the surface of the skin, for the first 3 animals. The temperature was monitored continuously from 3 min after the first microwave delivery until the second microwave delivery.

Post-mortem autopsies were performed by a veterinary pathologist on the heads of the last four animals to determine histological changes in the skin and brain tissues. Of these animals, two animals were given two microwave applications whereas two other animals were only given one microwave application in order to reliably observe the effects of a single microwave application as its intended use in the field. The skin was examined after a haematoxylin and eosin staining. The parts of the brains that were examined consisted of the frontal and parietal lobes (meninges, cortex, white matter), the basal nuclei, the thalamus and hypothalamus, and the caudal colliculi. These tissues were assessed for different parameters: tissue necrosis, vascular necrosis, cavitation or rarefaction, vascular haemorrhage, vascular congestion or oedema, and thrombosis using a grading scale from none to mild, moderate or severe.

Study design: this was an observational study, with no statistical analysis performed.

Randomisation and blinding: Treatments were pre-determined, so randomization was related to the order in which animals presented themselves to the restraint unit. Observers were not blinded to treatment.

Measurement of the outcome: Onset and duration of unconsciousness were measured using EEG

EEG data were inspected for pain-related complexes. Magnitude of distress and suffering were not assessed as the animals were anaesthetized.

Reporting the results

EEG

Interference from the electromagnetic field prevented collection of EEG data during application and for approximately 3 s after. Data presented are based on time to visible EEG changes, which may have occurred before the onset of recording. All of the applications resulted in changes in the EEG pattern indicative of seizure-like activity. Animal 5 was an outlier and was the animal that had received only 12 kW incident power. When data for animal 5 are excluded, time to onset of EEG changes ranged from 12 – 50 s from the start of energy application (mean 21 s), and time to nadir effect was 20 – 65 s (mean 35 s). Animal 9 only received 5 s application and was also an outlier in terms of duration of EEG changes and maximum reduction in Spectral Edge (SE). When animals 5 and 9 are excluded, duration of EEG change was 81 – 215 s (mean 118.9 s) and maximum reduction in SE was 11 – 59 % (mean 194 %). When data were pooled by treatment (excluding animal 9, and one other in which the EEG was heavily contaminated with artefact), the data are summarized in Table 5.

Pain-related complexes were not evident in the EEG.

Table 5: EEG results pooled by treatment: time to onset of EEG suppression, time to nadir of EEG suppression, duration of EEG suppression, and maximum effect (average and (value1, value2)). Time is counted from the start of the microwave application

Treatment	**1** (n = 2)	**2** (n = 2)	**3** (n = 2)	**4** (n = 1)
Power (kW)	20	20	30	12
Duration (s)	15	10	10	25
Energy delivered (kW x s = kJ)	300	200	300	300
Time to onset of EEG changes (s)	37 *(24, 50)*	13 *(12, 14)*	15 *(14, 16)*	138
Time to nadir effect (s)	59 *(52, 65)*	25 *(22, 28)*	31 *(24, 37)*	142
Duration of EEG changes (s)	105 *(81, 129)*	109 *(78, 140)*	162 *(109, 215)*	37
Maximum effect (% reduction in SE)	22 *(18, 25)*	25 *(21, 29)*	45 *(31, 59)*	6

ECG

Baseline heart rate prior to microwave application was 92.2 (± 8.4) bpm. After the start of microwave application, heart rate dropped within 5.0 (± 2.4) sec to 65.8 (± 24.0) bpm, and then rebounded after 23.75 (± 1.9) sec from the start to 82.3 (± 10.3) bpm. It stabilised within 160 (± 37.4) sec to 73.2 (±12.1) bpm.

Frontal skin temperature

Temperature of the skin surface 5 cm below the point of microwave application was recorded from the first three animals, using an electric probe placed on the surface of the skin. Skin temperature increased quickly after microwave application and returned to baseline within 35 min (Table 6). Skin temperature increased less in Animal 3 (20 kW, 10 s), in comparison to Animals 1 and 2 (20 kW, 15 s).

Table 6: Skin temperature (°C) after microwave application, on the frontal head skin surface approximately 5 cm below the application point.

Time post microwave application (min)	5	10	15	20	25	30	35
Animal 1 (20 kW, 15 s)	44.3	39.7	37.0	35.6	34.8		
Animal 2 (20 kW, 15 s)	44.8	43.2	40.0	37.6	35.9	34.6	33.4
Animal 3 (20 kW 10 s)	33.1						

Post-mortem examinations

At the point of application there was an area of complete skin loss, surrounded by a larger region displaying grey-tan discolouration of the subcutaneous tissue. This skin within this area displayed full-thickness coagulative necrosis extending down to the skull. Beyond this area, there was rapid progressive decrease in the severity of necrosis, with normal unaffected skin present within 0.5cm of the margin. In the brain, Animal 8 (single application, 30 kW, 10 s) had medium to severe lesions in the frontal and parietal lobes and Animal 9 (single application, 30 kW, 5 s) had relatively minor to no lesions in the same regions. For both animals, the basal nuclei, thalamus, hypothalamus, and the caudal colliculus were relatively unaltered except for mild to moderate vascular congestion. Animal that had had two microwave applications showed severe lesions at various levels (meninges, cortex and white matter) in the frontal and parietal lobes, with severe tissue and vascular necrosis, cavitation, haemorrhage and vascular congestion. Minor or no changes were apparent in the basal nuclei, thalamus, hypothalamus, and the caudal colliculus.

A potential source of bias is the small group size (n=10), animals being sourced from a single mob presented at the abattoir.

Discussion and conclusions

The findings of this study phase were as follows:

Microwave application induced a pattern of seizure-like activity in the EEG, a pattern that is not considered to be compatible with continued awareness or the ability to feel pain (Devine et al., 1986; Coenen, 1998; Velarde et al., 2002). Hence, this pattern of seizure-like activity in the EEG is interpreted as an assessment of insensibility in this study. The interval between the time when effects started appearing ('Time to onset') and the maximum effects were seen ('Time to nadir') was within 4 to 22 sec depending on the treatments. These results confirm findings on other species (rodents: AVMA, 2013; sheep: Small et al., 2013b) that microwave irradiation is a relatively quick process in comparison to other reversible stunning procedures such as cattle electrical stunning, for which applications of up to 15 sec can be performed in order to depolarize the spine and reduce kicking (NAMI, 2017), or carbon dioxide stunning in pigs, with latency of about 25 sec to the loss of posture (Velarde et al., 2007). In comparison, non-penetrative captive-bolt stunning induced EEG changes in about 8 sec in calves, in a study which used a similar anaesthesia model (Gibson et al., 2009). Furthermore, the current results indicated that a shorter duration of application induced more rapidly developing EEG changes, in the range of duration tested (10-25 sec), as evidenced by shorter 'time to onset' and 'time to nadir'.

Another consideration for reversible stunning techniques is the duration of insensibility, or the time before the animal regains consciousness. Insensibility should last until death ensues through exsanguination. Following microwave application, EEG suppression lasted for at least 37 sec and up to more than 2 min. Our results also indicated that applying higher power extended the duration of insensibility. The search for a long period of insensibility is useful for cattle because consciousness can last from 1 to 2 min after exsanguination (Newhook and Blackmore, 1982; Gregory et al., 2010).

Unfortunately, the microwave application caused artefacts that rendered the EEG unreadable until after the end of treatment. This is an inherent limitation of using electroencephalographic technique to assess the microwave technique since both techniques interact with electric activity. This leaves a window of uncertainty regarding the aversiveness of the microwave technique during its application and the experience of the animal during that short period of time. Pain-related complexes were not evident in the post-DTS EEG. The animal's perception of the procedure up to the induction of insensibility (in the order of 10-15 sec) should be investigated with alternative scientific methods that allow for data collection during microwave application.

The abrupt bradycardia observed following microwave application is in agreement with the literature on the heart rate response to noxious stimuli (Woodbury et al., 2005; Johnson et al., 2005; Gibson et al., 2007). The magnitude of that drop differed between animals, but the heart rate rebounded within 24 sec, irrespective of the treatment applied. Interestingly, the heart rate stabilised to a different, lower level, following microwave application. The most plausible explanation is that this may be the result of temporary and longer-persisting effects on the brain-stem or thalamus (Benarroch, 2001).

Based on the post-mortem autopsies, most histological changes appeared in the upper regions of the brain, with the frontal lobe, adjacent to the zone of application of the microwave, being the most affected, closely followed by the parietal lobes which are located on the sides of the animal's brain. However, the regions of highest interest in regards to consciousness, the deeper regions of the brain, namely the basal nuclei, thalamus, hypothalamus, and the caudal colliculus, appeared to be relatively unaffected by microwave application, even following two microwave applications.

Further research is warranted regarding the dissipation of energy throughout the brain and whether this is a homogeneous process or not. The lesions observed would suggest that animals may be able to regain consciousness following microwave application, although the frontal regions of the brain would unlikely be intact.

This experiment provided novel and crucial knowledge regarding the effects of different power and duration of microwave applications on anesthetized cattle. However, only a small number of animals, hence a small number of settings, could be tested. A possibility for future research is to perform further anaesthesia trials to refine the duration of application (< 10 sec), the potential for higher power to lengthen the duration of insensibility, and the variation between animals.

STEP 6: Laboratory engineering: Refinement of delivery apparatus and development of user interface software

Submitted study: **PR07**: Refinement of delivery apparatus and development of user interface software. Interim project reports. Not peer-reviewed. CONFIDENTIAL.

Introduction

Subsequent to the anaesthetized cattle study, refinements to the delivery apparatus, auto-tuner and user interface software were required to bring the next prototype closer to a configuration that may be used in a commercial setting, on conscious animals.

Objective

- To design and fabricate a waveguide reticulation system suitable to application on standing animals in a commercial restraint unit.
- To integrate a high-speed auto-tuner into the system.
- To commission the system into the Wagstaff Garfield abattoir and test the system on cadaver heads and dummy loads (water bodies).

The details of this step are confidential proprietary information.

STEP 7: Laboratory scale: Pilot study on conscious cattle (Prototype 3)

Submitted studies:

SS05: Development of a microwave energy delivery system for reversible stunning of cattle 2: Preliminary observations on behavioural responses and EEG.

SS06: A comparison of beef carcases stunned using DTS: Diathermic Syncope® or captive bolt in terms of selected meat quality attributes and plasma biomarker concentrations.

Other reference documents: **PR08**: Dielectric induction of temporary insensibility in cattle - animal trials. Project Report. Not peer-reviewed. CONFIDENTIAL. **PR09**: Meat quality from microwave stunned cattle Project Report. Not peer-reviewed. CONFIDENTIAL.

Introduction

Some communities require that animals processed for human consumption are healthy, uninjured and normal at the moment of carrying out the slaughter cut. As a result, many methods of stunning used in modern commercial slaughter are not acceptable, because the animals could be considered injured, or because a proportion of animals would not recover from the stun. Head-only electrical stunning is used in sheep but is not suitable for use in cattle because of concerns over the duration of insensibility being insufficient to allow death through total blood loss to occur prior to recovery from the stun, and also because of problems with blood splash in the meat. Preliminary research has shown that electromagnetic energy technology is likely to induce recoverable insensibility in animals and could result in an effective reversible stunning method that is suitable for religious slaughter.

Background research has shown that electromagnetic energy can lead to unconsciousness in animals. In chickens, Lambooij et al. (2011) applied an electromagnetic field, and produced electroencephalogram (EEG) traces that indicated insensibility and the duration of unconsciousness was calculated to be in the range of 15 to 20 s. Guy and Chou (1982) similarly induced unconsciousness in rats that lasted for 4-5 minutes. The mechanism of action is essentially selectively increasing the temperature slightly in the brain, by only 7° C, to the point that hyperthermic syncope (fainting) occurs, but below the point at which irreversible brain damage and death occurs. Thermal unconsciousness such as that induced by exercise heat stress or fever is reported to occur when core body temperatures reach between 40 and 45°C.

Wagstaff Food Services Pty Ltd and Advanced Microwave technologies have designed a system for delivery of electromagnetic energy to sheep, goats, cattle, buffalo and camel (Ralph et al., 2011: Patent PCT/AU2011/000527), DTS: Diathermic Syncope®. To date, trails have been carried out on anaesthetised sheep and anaesthetised cattle.

The trials on four anaesthetised sheep were highly successful. Application of microwave energy caused rapid increases in brain temperature to a point above which insensibility would be expected to occur (43°C), and below that which damage would be expected to occur (50°C). The sheep breathed regularly and normally throughout the application of energy, and afterward, in contrast to electrically stunned sheep, in which breathing stops until the animal begins to recover from the stun. Two animals were allowed to partially recover from anaesthesia, to the point at which neck tone, jaw movements and swallowing reflexes had returned, indicating that the animals were likely to recover. Full recovery was not permitted under this trial, as a condition of the Australian Animal Ethics Approval.

In the anaesthetised cattle trial, nine cattle were lightly anaesthetised, and electroencephalogram (EEG) traces collected before and after microwave energy application. All applications resulted in EEG traces that indicated unconsciousness (seizure-like activity, similar to that seen when electrical stunning is used). There was a slight slowing of the heart rate during and after application, but the rhythm remained regular. In comparison, when electrical stunning is used, the heart rate drops while the stun is applied, but rises to above normal rates after application, during the unconscious period. Five combinations of microwave power and durations were tested. Higher power led to a longer period of EEG disruption; while shorter application led to more rapid onset of EEG disruption. The EEG traces were analysed for evidence of pain according to the method of Gibson et al. (2007), and no evidence of pain was found. These results indicated that microwave application to conscious animals would lead to rapid onset of insensibility.

Objective

- To provide a proof of concept demonstration of induction of unconsciousness in cattle;
- To determine what signs, comparative to other validated stunning processes, might eventually be examined experimentally for future assessment on the effectiveness of the device in rendering the animal unconscious; and
- To compare DTS against the industry reference standard (penetrative captive bolt stunning), in terms of:
    - physiological variables (Cortisol, ACTH, β-endorphin and catecholamines) and
    - meat quality (carcase characteristics, pH, Colour, shear force, drip loss and lipid oxidation) at slaughter, one week and ten weeks post slaughter (vacuum packaged primals).

Materials and methods

Ethical considerations: The studies were carried out under the authority of the Victorian State Government Wildlife and Small Institutions Animal Ethics Committee, Authority 29.13.

Study population: Eighteen Aberdeen Angus cross bred heifers (weight range 350-400 kg) with a quiet temperament were selected from the normal commercial intake at Wagstaff Food Services abattoir. They were fed and rested in lairage for 4 days prior to the trial, cared for by an experienced stockperson. On the day of the trial, each animal was individually brought to the restraint box, by the same familiar stockperson, using low-stress animal handling techniques. A baseline blood sample was taken from the tail vein, and the animal was restrained using a head restraint unit, lifting the forehead to be in contact with the DTS energy applicator. After head capture, baseline EEG measurements were recorded. The assigned treatment was then applied.

Experimental design: The treatments were:

- Control (captive bolt): animals 1, 2, 8, 9, 12, 13, 15
- High energy DTS, receiving greater than 290 kJ: animals 3, 4, 11
- Low energy DTS, receiving less than 200 kJ: animals 7, 10, 14, 16
- Intermediate and double-application DTS: animals 5, 6 and 18.

Animal 17 was excluded from analysis: it received a very low dose of energy (35.55 kJ), which did not render the animal insensible, and was euthanased by captive bolt stun and exsanguination.

Randomization and blinding: the order of treatment application was pre-determined, randomization was based on the order in which the animals presented themselves to the lead-in race to the restraint unit.

Personnel carrying out offline video behaviour recording, EEG, blood and meat quality sample analysis were blinded to treatment applied.

Sampling strategy: Following treatment, insensibility was assessed by corneal reflex, assessment of visual function and response to a painful stimulus of the nose, EEG measurements were repeated, the animal removed from the restraint box, terminal EEG measurements recorded and the animal exsanguinated. A back-up captive bolt stun was delivered in cases where a risk of recovery during exsanguination was perceived, the time elapsed between application of the treatment and exsanguination being prolonged by the need to capture post-treatment EEG recordings and behavioural measures. A second blood sample was collected from the free-flowing exsanguinate.

Blood samples were centrifuged and plasma extracted. The entire process was video recorded using six cameras, capturing animal movements and behaviours from above, at the head, and on the roll-out table, and these videos were subsequently annotated against an ethogram designed for the trial.

The carcase was then dressed as normal practice, chilled overnight, and de-boned the following day. pH measurements were taken from the carcases every hour from slaughter till below pH6, and again at 24 hours post slaughter, prior to de-boning. Heads were sections for inspection, and brain samples collected for histological analysis. Colour was measured on the cut surface of the m. longissimus lumborum 30 minutes after quartering. At de-boning, two samples each of loin (m. longissimus lumborum) and round (m. semitendinosus) was removed, vacuum packed and refrigerated. These samples were transported to the laboratory by refrigerated vehicle, within the first week post slaughter. At each of 1 and 10 weeks post slaughter, muscle samples were unpacked, and sectioned into subsamples for colour, pH, shear force, lipid oxidation and drip loss evaluation.

Meat Colour was measured using a MINOLTA CR300® colorimeter under light source D65; pH was measured using a WP-80 digital pH meter (TPS instruments, Springwood, QLD), with a combination electrode for temperature compensation; Warner-Bratzler (WB) shear force was measured according to the protocols outlined by Bouton et al. (1971) and Bouton and Harris (1972). Drip loss was measured using the method outlined by Honikel et al. (1986); Lipid oxidation was determined by the thiobarbituric acid-reactive substances (TBARS) method of Witte et al. (1970). Plasma samples were tested for cortisol (RIA), ACTH (EIA), β-endorphin (EIA) and catecholamines (ELISA) concentrations.

Method of analysis: Blood sample and meat quality data were analysed using R Studio (R Core Team, 2014). The EEG data were analysed offline using LabChart 8 (ADInstruments, Sydney, Australia). The Spectral Analysis Package within LabChart 8 was used to apply Fast Fourier Transformation (FFT), with multiplication using a Hann window in 1-second epochs with a 25% overlap. Total power (Ptot), median frequency (F50) and 95% Spectral Edge frequency (F95) were extracted. Epochs containing artefacts were identified and rejected manually, with reference to video footage to identify event-related artefact (e.g., animal movements, eye/ ear movements, personnel movement or movement of leads), and the first and last 2 s of each recording were removed to eliminate edge artefacts. Heavily contaminated recordings, and recordings in which poor electrode contact was present were discarded in entirety. For each animal the median value of Ptot during T1 was calculated and this was used as the baseline value. Baseline normalization was then carried out by transforming data for each 1-s epoch into decibel change from baseline according to the formula: dB=10*log10(value/ baseline), to bring all data sets into a comparable format. These data, and data for F95 and F50 were charted and inspected for EEG suppression and epileptiform activity, and where possible time to resolution of EEG suppression was recorded.

Measurement of the outcome: Live and video behavioural monitoring was utilized to assess the magnitude of pain, suffering and distress; latency to return of corneal reflex and duration of EEG suppression were recorded as indicators of onset and duration of unconsciousness and time to death.

Reporting the results

Under live observation of the animals, the clearest indicators of effective induction of insensibility were:

- Loss of corneal responses

- Loss of withdrawal response (pinprick)
- Eye staring, not following movement

Unreliable indicators are:

- Loss of posture: there is an initial loss of posture, but by the time the DTS cycle is complete, the legs have re-extended, and paddling or walking movements of the hind limbs in particular, are seen. This pushes the shoulders of the animal hard into the neck bail, jamming it into an uptight position. When the animal is removed from this jammed position, it is in lateral recumbency, entering a convulsive or clonic phase, after which reflexes do appear to begin to return.
- Breathing characteristics: breathing and heart function is maintained – the brain stem appears to be unaffected by the DTS application. Breathing tends to be fast and shallow.
- Vocalisation: it appears that some animals do vocalize, even when there are no responses from the eye (following movement, corneal response, palpebral response) nor withdrawal reflexes. This vocalization, when it occurs, is continuous, occurring on every exhalation, and is not associated with any external stimulus.
- Blinking: as in electric stun, random movements of the facial muscles and eyelids can occur, particularly as the animal enters the clonic (convulsive) phase. Therefore, it is difficult to interpret a blink as to whether it is a response, or a random movement.

From the video footage it was confirmed that on application of a captive bolt, all four limbs immediately tucked into the body, and the animal fell to the floor of the restraint box. As it was rolled out of the box, the forelegs first became rigidly extended, followed by the hind limbs. No rhythmic breathing was observed, and the animal's eye was fixed and staring. There was no corneal response, no pupillary response to light, and no response to nose prick. No vocalization and no righting reflex was observed.

For DTS animals, application of energy resulted in rapid blinking and flickering of the third eyelid, with nystagmus. In some animals the back arched and the muscles of the neck contracted, pulling the chin down into the chin lift. In the low energy applications, this was followed by a period of convulsive movements, lasting 10-20 seconds, then ataxia and loss of posture, particularly in the hindquarters. Following this ataxic phase, the body became tense and tetanic, with the four limbs slightly splayed. There was no response to efforts to topple the animal: the tail hung limp and flaccid, and the limbs remained motionless. The animal then progressed into slow walking and paddling movements, pushing forward into the head restraint unit. There was no vocalisation, and rhythmic breathing continued throughout. The eyes became fixed and staring, with no ocular following of movement, and no pupillary response to light. There was no withdrawal response to nose prick.

Head inspection of captive bolt animals revealed one or two (in the instances where the animal was re-stunned) circular penetrations of the frontal bone. Haemorrhage was evident within the skull cavity, with clotted blood pooling around the lower parts and brainstem. This contusive damage was also noticeable in histological sections from deeper regions of the brain. Heads from high-energy DTS showed varying degrees of heating damage. On the forehead, an extensive area of scorched, dried skin was easily sloughed, showing brown discolouration of the bone below.

On splitting the skull, the mucous lining of the sinus cavity and nostrils was noted to be dried and discoloured. The brain was discoloured and firm immediately below the application site, with the appearance of cooked brain tissue. On histological examination, marked evidence of vascular congestion and thrombosis, with loss of neuropil structure and malacia was found in upper sections, and less markedly in middle sections. Low energy DTS heads similarly showed some degree of heating damage to the forehead, but this was much reduced compared to those of High energy DTS. The skin at centre point of waveguide application was dry and leathery, with loss of hair. Brain tissues were essentially normal, although there was some malacia and hyperaemia of the upper 2mm of the surface of the cerebral cortex.

The EEG responses of both Captive bolt and DTS animals were similar. Qualitative assessment of the EEG traces indicated that immediate post treatment EEG traces showed a reduction in amplitude with intermittent activity (trace not compatible with sensibility), and post-rollout traces tended towards the isoelectric state (flat-line, trace not compatible with sensibility). There were no significant treatment differences between baseline, post-treatment and terminal values of Mean Power; Root Mean Square power (RMS); Amplitude; Median Power Frequency (F50); or 95% Spectral Edge Frequency (F95).

There were no significant differences between DTS and captive bolt animals in terms of cortisol, ACTH, β-endorphin and catecholamine responses. Both treatments resulted in an increase in cortisol from baseline (DTS 33.19 ±16.89 nmol/L; Captive bolt 61.43 ±12.59 nmol/L) to post-stun levels (DTS 150.38 ±17.79 nmol/L; Captive bolt 160.64 ±13.26 nmol/L), indicating physiological stress. However, it is unclear if this stress is due to the stunning methods; or to the head capture and restraint, which was longer than in a commercial situation due to the need to take pre-stun EEG recordings, or to a combination of both restraint and stun.

There were no significant differences between DTS and captive bolt carcases in terms of pHu (24 h post slaughter); pH, Warner Bratzler Shear Force and Drip loss at 1or 10 weeks post slaughter. DTS carcases were slightly yellower at quartering (MINOLTA b* 2.71 ± 0.59 DTS; 1.06 ± 0.44 control); DTS loins were slightly redder (MINOLTA a* 23.22 ± 0.92 DTS; 20.89 ± 0.69 control) and slightly yellower (MINOLTA b* 2.79 ± 0.93 DTS; 0.77 ± 0.70 control); and DTS rounds were slightly lighter (MINOLTA L* 43.32 ± 1.05 DTS; 40.94 ± 0.78 control) at week 1, than control samples ($P<0.05$). There were no differences between DTS and captive bolt meat colour measurements at 10 weeks post slaughter.

A potential source of bias is the small group size (n=18), animals being sourced from a single mob presented at the abattoir. All animals were female Australian Angus cattle.

Discussion and conclusions

The findings of this study phase were as follows:

Based on live observations at the time of stun application, the research team was satisfied that DTS induced a state of insensibility, of a sufficient duration to allow humane slaughter through exsanguination. Live observations indicated that the process was painless, and evidence of distress was not observed.

EEG data indicated that DTS induced insensibility. DTS animals in the current study remained unresponsive to stimuli and showed evidence of EEG suppression for 3-4 minutes post energy application. In a commercial situation, it would be expected that exsanguination would be carried out within 30-60 seconds of energy application.

Video footage demonstrated a convulsive phase during low energy DTS application, but it is unclear as to whether this is involuntary convulsion, or an attempt to escape. Facial expression seems to indicate that it is involuntary. A further convulsive or clonic phase occurred between 60 and 90 seconds post energy application.

Endocrine data indicated no differences between DTS and captive bolt. Meat quality parameters in DTS carcases did not differ from captive bolt carcases. pH declines suggested that there may be a potential for increased metabolic rate, which would be predicted to result in a "PSE-like" or "heat toughening" condition, but this was not corroborated by the meat quality analyses.

Brain lesions suggest that 300 kJ would result in a non-recoverable state, while behavioural observations suggest that less than 100 kJ gives a short-duration insensibility.

DTS animals maintained rhythmic breathing and a strong heart beat throughout the period of insensibility, which lasted for at least 3-4 minutes post application of energy. During this time, there was no corneal reflex, no response to a painful stimulus of the nose, and no evidence of the eye beginning to focus and follow movement.

Two animals showed evidence of return to consciousness:

> Animal 16 received 184.68 kJ. Return of blink and corneal reflexes were noted prior to captive bolt application, 228 seconds after DTS application.
>
> Animal 18 received 217.62 kJ. It was unresponsive to stimuli for the first 90 seconds post treatment, and then appeared to go into a clonic or convulsive phase. Following this, it lay quietly for a further 90 seconds, showing no response to stimuli. Towards the end of this period the eye was beginning to regain focus, followed closely by corneal reflex, and within 15 seconds, return of righting reflex.

It is evident that the duration of insensibility achieved in DTS animals is sufficient to allow exsanguination prior to recovery, but in a commercial situation it would be strongly recommended to maintain a back-up captive bolt instrument on the bleed rail, as the return from focusing of the eye, through corneal reflex to return of righting reflex is rapid.

STEP 8: Laboratory engineering: Refinements to delivery system: applicators and coaxial cabling

Submitted study: **PR10**: Refinements to delivery system: applicators and coaxial cabling. Interim project report. Not peer-reviewed. CONFIDENTIAL.

Introduction

The pilot study described in Step 7 drew some recommendations for further development:

Aspect 1. Modifications to the technology

If recoverability is desired, and the animal to return to herd life, as opposed to being immediately slaughtered, the desiccation and skin changes at the application point should be minimised. Modifications to the technology should be accompanied by measurement of rates of heating within the brain and in superficial tissue.

Aspect 2. Engineering aspects:

Development to improve:

- Ease of capturing and positioning head;
- Maintenance of contact between the animal's head and the applicator;
    - Consideration of potential for contouring or flexibility within the waveguide tip;
- Ease of extraction of body from box;
    - Consideration of side clamp and tipping technologies.

Note: designs must ensure compliance with OH&S regulatory standards and guidelines.

Aspect 3. Research aspects

Subsequent to development of Prototype 4, addressing particularly the surface heating aspect, commercial pilot trials are required to understand and define the critical limits of energy application that allow induction of insensibility, but minimise brain tissue damage, such that the animal may recover and function in a normal manner.

This phase of work aimed to address aspect 1 and item 2 of aspect 2 of these recommendations. Items 1 and 3 of aspect 2 were addressed by an engineering company specializing in livestock handling systems in abattoirs, and a description of that design is provided in PR11 (Step 9) and Section 3.6.

Objective

- To investigate alternative applicator designs to reduce the footprint on the bovine head, and to minimize surface skin and hair heating.
- To investigate potential system designs that improve manoeuvrability of the applicator.

The details of this step are confidential proprietary information.

STEP 9: Laboratory scale: Validation study on conscious cattle (Prototype 4)

Submitted study: **SS05**: Development of a microwave energy delivery system for reversible stunning of cattle 2: Preliminary observations on behavioural responses and EEG.

Other reference documents: **PR11**: DTS: Diathermic Syncope - commercial validation trials. Interim project report. Not peer-reviewed. CONFIDENTIAL. **PR12**: DTS: Diathermic Syncope® controlled trials. Not peer-reviewed. CONFIDENTIAL.

Introduction

Wagstaff Food Services Pty Ltd and Advanced Microwave technologies have designed a system for rendering an animal unconscious prior to slaughter, using radiofrequency electromagnetic energy (Ralph et al., 2011: PCT/AU2011/000527). The mechanism of action is essentially selectively increasing the temperature slightly in the brain to the point that hyperthermic syncope (fainting) occurs. Thermal unconsciousness such as that induced by exercise heat stress or fever is reported to occur when core body temperatures reach between 40 and 45°C (Ohshima et al., 1992). A pilot study carried out in 2014 indicated that DTS provides a humane method of inducing insensibility prior to exsanguination, of sufficient duration to allow exsanguination prior to recovery, and produces comparable post slaughter meat quality and physiological responses in treated cattle to those stunned using penetrative captive bolt (**PR08**, **PR09**). However, energy leakage occurred when the animal convulsed on entering the insensible state, leading to activation of the generator safety cut-out, and incomplete delivery of energy to the brain. Subsequent to the pilot study, the restraint, head capture and waveguide set-up have been re-engineered to improve animal handling and restraint, and to limit energy leakage and automatic cut-out of the generator. Prior to the conduct of more extensive commercial scale studies and statistically robust assessments, the latest version of the system (Prototype 4) should be validated in terms of ability to induce unconsciousness in cattle.

Objective

To validate the efficacy of the re-engineered system, termed 'Prototype 4', in terms of its ability to induce unconsciousness in cattle.

Materials and methods

Ethical considerations: The protocol and conduct of the experiment was approved by the DEDJTR Wildlife and Small Institutions Animal Ethics Committee under the VIC Prevention of Cruelty to Animals Act, 1986 (Animal Research Authority 30.16).

A total of 20 cattle were processed during the period 4th – 6th October 2017.

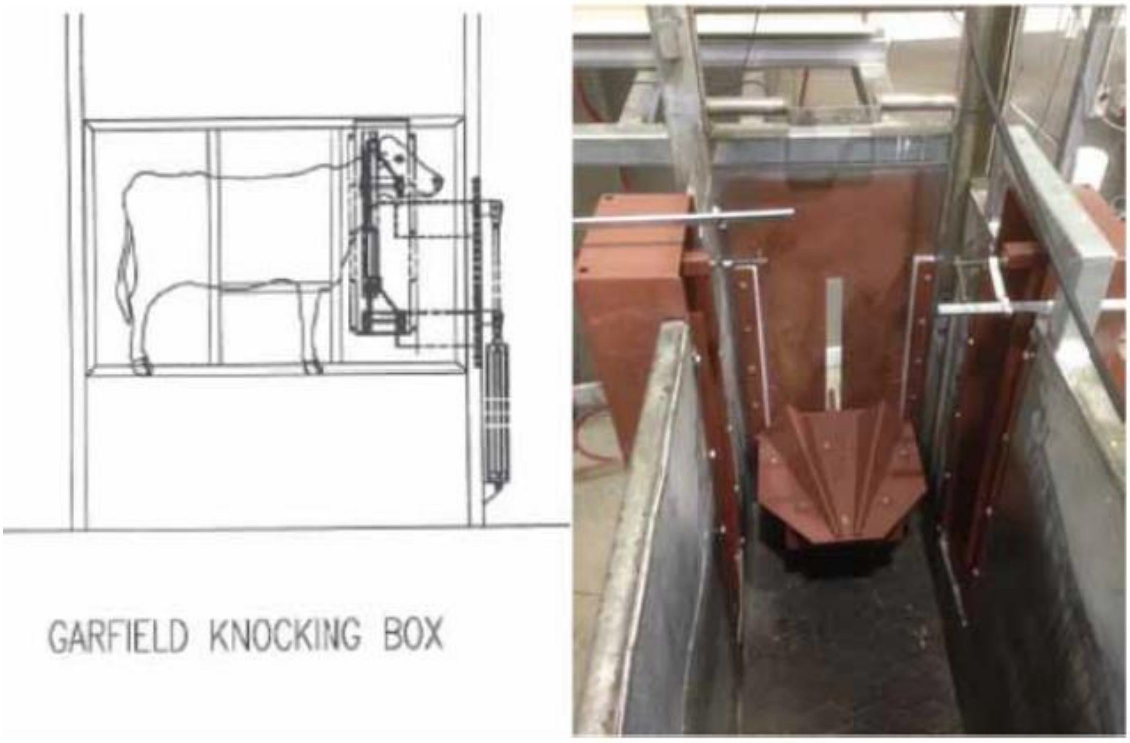
Study population: Animals were mixed breed, predominantly dairy crosses, some were aged cull cows, and others were poor-quality dairy cross steers, randomly drafted from the normal intake at the abattoir. Age and gender of individuals was not recorded.

Sampling strategy: Cattle were brought individually to the restraint unit. For the first three cattle, Electroencephalogram (EEG) recording pads were applied in the restraint crush in the lairage, and a baseline EEG recording taken. It was subsequently decided that it would be more efficient, and less stressful to the animal if the pads were applied and the baseline EEG recorded when the animal was restrained in the DTS restraint unit, obviating the need for a second handling event. This then was the procedure for animals 4-20. For application of DTS, the cattle were individually restrained in a stun box with neck yoke, head capture and chin lift (Figure 15), all of which was fully enclosed in a faraday cage.

EEG data were captured using a single channel, bi-hemispheric, four-electrode EEG montage (ground electrode on a bony prominence on one side of the poll; common reference electrode midline on the nose, midway between the nostrils and the eyes; and the inverting electrodes on each frontal bone, between the eye and the poll). RedDotMini electrode pads (3M Australia, North Ryde, NSW) were applied to the head, with good contact achieved by soaking the hair below the pad in superglue (Loctite 454, Loctite Australia, Caringbah, NSW). The EEG was recorded, using PowerLab and LabChart (ADInstruments, Sydney, Australia) for a period of up to 2 minutes, or until a stable, clear signal lasting for 30 seconds was observed on the screen, whichever occurred first, prior to application of the energy, and again for up to 90 seconds or until a stable, clear signal lasting for 30 seconds was observed on the screen prior to sticking, unless the animal was deemed to be regaining consciousness, in which case a back-up stun was delivered. EEG traces were qualitatively assessed during the course of the trial.

Following collection of baseline EEG data, the EEG leads were removed, because there is a risk of damage to the equipment if excess energy leakage occurs, and a baseline image taken using an infrared thermography (IRT) camera (ThermaCam T640, FLIR Systems AB, Danderyd, Sweden). The waveguide was then positioned on the forehead at the application point, checked for fit, the faraday cage shielding fastened, and personnel evacuated the immediate area in case of shielding failure.

Figure 13: Left: Engineering concept diagram of restraint unit; Right: photograph of chin lift unit, taken during installation, prior to enclosure with faraday cage shielding.



Experimental design: DTS was then applied to a pre-determined total energy delivery, at a defined power setting, which varied for each individual. Energy application began at 360 kJ, delivered using a power setting of 30 kW, and when each energy setting was confirmed to induce insensibility, the energy setting was sequentially reduced by increments of 25 kJ throughout the 20 animals, with a return to higher energy levels at the beginning of each processing day such that the settings applied were: one animal at 360 kJ, five at 300 kJ, five at 275 kJ, two at 250 kJ, five at 225 kJ, and two at 200 kJ. Towards the end of the study, power settings of 20 kW were attempted, with the total energy delivery set at levels (275 kJ and 200 kJ) that had produced insensibility when delivered using a power setting of 30 kW and finally one animal was processed using an energy delivery setting of 200 kJ and power setting of 15 kW.

Sampling strategy: Immediately the generator deactivated following energy application, the faraday cage was opened, the waveguide was removed, and the animal visually assessed for signs of distress (and in the event that distress was evident, which did not occur, the instructions were to immediately apply captive bolt). Corneal reflex was tested, a post-application thermal image taken, and the EEG leads reattached. Post-application EEG was recorded, with corneal or somatic withdrawal reflex tested at 30-second intervals. When 90 seconds (or 30 seconds of stable, clear signal was observed on the screen) of EEG had been recorded, the leads were again removed, and the body released from the restraint unit onto the bleed conveyor, where the oesophagus was sealed, and exsanguination was performed using the 'thoracic stick method' which severs the common carotid artery close to the thoracic inlet. If signs of returning consciousness were detected, namely the return of corneal reflex or eye focus and following movements, the animal was stunned using captive bolt, regardless of where in the process it was – i.e., if it was still in the restraint unit, it was stunned, then rolled out before exsanguination; if it was on the bleed conveyor, it was stunned there and exsanguinated immediately if this had not previously begun. Once the body was exsanguinated, it was processed and inspected for human consumption according to the normal practices at this abattoir.

During application of DTS, the animals were monitored using real-time video capture through a security camera system (Dahua HCVR4108HS-S3/8, Zhejiang Dahua Technology Co. Ltd, China) with one camera positioned over the animal's head within the head capture unit, one over the body within the restraint box unit, one over the control panel, and two over the bleed conveyor. Observations on animal reaction were recorded from the video footage in real time, then subsequently footage was played back at reduced speed (up to 16 times reduction) in order to prepare a detailed event log for each individual.

Randomization and blinding: the order of treatment application was pre-determined, randomization was based on the order in which the animals presented themselves to the lead-in race to the restraint unit.

Personnel carrying out offline video behaviour recordind, EEG and thermal imaging analysis were blinded to treatment applied.

Data analysis: EEG data were analysed offline using LabChart 8 (ADInstruments, Sydney, Australia). Artefacts were identified and rejected manually, with reference to video footage to identify event-related artefact (e.g., animal movements, eye/ear movements, personnel movement or movement of leads), and the first and last two seconds of each recording were removed to eliminate edge artefacts. Heavily contaminated recordings, and recordings in which poor electrode contact was present were discarded in entirety. A total of 11 usable recordings were generated: the post-DTS recording was not collected from animal 2 due to accidental shut-down of the laptop running LabChart, and in animal 13, corneal reflexes were present and the animal was stunned using the back-up captive bolt shortly after opening of the faraday cage. For animals 14 to 20, baseline EEG was not recorded, and post-DTS EEG was recorded on the bleed-conveyor during exsanguination. This change to protocol allowed an assessment of the time required to remove the unconscious animal from the restraint and exsanguinate, an important aspect for fit to the commercial environment. However, it meant that a baseline EEG for normalisation was not present, and the post-DTS EEG could only be used qualitatively.

For illustrative purposes, Total EEG power was converted to decibel change from lowest recorded value to bring it into a form that could be visually compared with traces from the other animals processed. In the event, the post-DTS EEG was heavily contaminated by artefact from the conveyor mechanism and from running water in a nearby drain, and once movement artefact was removed, there were very little EEG data available for animals 15 - 20 prior to the return of the corneal reflex and captive bolt application.

A band-pass filter of 0.1 to 30 Hz was applied to the raw data, and the Spectral Analysis Package within LabChart 8 was used to apply Fast Fourier Transformation (FFT), with multiplication using a Hann window in 1-second epochs with a 25% overlap. Total power (Ptot), median frequency (F50) and 95% Spectral Edge frequency (F95) were extracted. The median value of Ptot in the pre-DTS recording was calculated and this was used as the baseline value. Baseline normalization was then carried out by transforming data for each 1-s epoch into decibel change from baseline according to the formula: dB= 10*log10(value/baseline), to bring all data sets into a comparable format. These data, and data for F95 and F50 were charted and inspected for EEG suppression and epileptiform activity, and where possible time to resolution of EEG suppression was recorded.

Thermal image data were imported into ResearchIR (FLIR Systems AB, Danderyd, Sweden) for analysis. On each image, the forehead area was delineated to exclude the ears, eyes and muzzle area, the minimum, maximum and average temperatures within this area and the number of pixels represented by the delineated area was returned from the software. For post-DTS images, the image was then manipulated to show only areas where the surface temperature was greater than 45 °C (Figure 5); greater than 50 °C (Figure 6) or greater than 60 °C (Figure 7) and the number of pixels represented by each of these subsets returned from the software, and these were expressed as a proportion of the number of pixels in the delineated forehead area. Pre- and post-DTS minimum, maximum and mean temperatures were used along with the total energy delivered as recorded by the DTS control software to generate a ‘temperature change by energy delivered’ chart for each individual. Comparing ‘total energy delivered by time’ from the DTS control software with video annotation of behaviours allowed an estimation of the energy delivered at the point of loss of posture, and the associated surface temperature at that point could then be estimated from the ‘temperature change by energy delivered’ chart.

Measurement of the outcome: Live and video behavioural monitoring were utilized to assess the magnitude of pain, suffering and distress; latency to return of corneal reflex and duration of EEG suppression were recorded as indicators of onset and duration of unconsciousness and time to death.

Reporting the results

Of the 20 cattle processed, 17 were assessed as insensible following DTS application, based on loss of posture and loss of corneal and somatic withdrawal reflexes. These animals demonstrated behavioural signs consistent with an electrical stun – rapid blinking or flicking of the eyelids including the membrane nictitans (third eyelid), loss of posture, tonic (stiff) and clonic (convulsive) phases. Loss of posture occurred between 1 and 8 seconds after onset of energy delivery. In 4 animals, which received 300-360 kJ, this insensibility progressed to death. In the remaining 13 animals, absence of corneal reflex persisted for between 100 and 170 seconds, at which point captive bolt was administered in case of returning consciousness. For each of the three animals that were not deemed insensible, there had been problems with maintaining contact between the waveguide and the forehead, resulting in leakage of energy into the environment, rather than penetration into the brain. All three appeared partially conscious, with loss of eye focus and no visual following of movement, and a slow response to a touch on the cornea. All three were stunned using captive bolt immediately following assessment of consciousness. From video footage, loss of posture, which is considered to be a definitive indicator of unconsciousness, occurred between less than 1 second from DTS application to 19 seconds after the onset of DTS application (Table 9). It is unclear from the current data set whether leakage of energy contributed to the more prolonged intervals from onset of DTS application to loss of posture, but it could be expected that leakage of energy resulted in slower heating of the brain, and thus a delay in loss of posture. A number of animals (12 in total, all within the first 13 animals) returned to standing after initial loss of posture. This occurred between 5 and 144 seconds after loss of posture (mean 50.75; median 25.5 seconds), often prior to roll-out as the animals remained in the restraint for post-DTS EEG capture. The animals were considered unconscious due to lack of reflex responses, and the EEG data confirms this.

A summary of the surface temperatures recorded on the delineated forehead are of cattle pre- and post-DTS, and the percentage area post-DTS in which surface temperature was greater than 45, 50 and 60 °C is presented in Table 10. However, it must be borne in mind that the post DTS images were taken after the entire energy load had been delivered. From live and video observations, loss of posture usually occurred before the entire energy load had been delivered, so the remaining energy can be considered to be excess energy, not required for inducing insensibility. The continued application of energy would continue to heat tissues, resulting in the thermal maps produced. By comparing the data prior to energy application against the post-application data, it was possible to estimate a heating rate by energy delivered, and utilising this heating rate, estimate the surface temperature at the point of loss of posture. These data for each animal are shown in Table 11 but to summarise, the average surface temperature on the forehead at the point of loss of posture was estimated to be between 26 and 49 °C, although some animals (animals 6 and 7) that showed a delayed loss of posture also demonstrated hot-spots of up to 109 °C.

Of the 20 animals processed, 11 EEG recordings were generated: the post-DTS recording was not collected from animal 2 due to accidental shut-down of the laptop running LabChart, and in animal 13, corneal reflexes were present and the animal was stunned using the back-up captive bolt shortly after opening of the faraday cage, prior to EEG recording. For animals 14 to 20, baseline EEG was not recorded, and post-DTS EEG was recorded on the bleed-conveyor during exsanguination. Thus, baseline EEG for normalisation was not present, and the post-DTS EEG could only be used qualitatively. In the event, once movement artefact was removed, there were very little EEG data available for animals 15-20 prior to the return of the corneal reflex and captive bolt application.

Figure 16 shows an example of the EEG data collected, compared against a similar chart reproduced from Rault et al. (2014: **SS03**), both showing a period of 2.5 – 3 minutes post DTS application, scaled to similar length. Although the software and detail of smoothing methods used to generate these charts differ, clear similarities are evident: total EEG power (Ptot, green line) is dramatically increased and shows high amplitude spiking in the two-minute period post DTS application; 95% spectral edge frequency (SEF95, blue line) and 50% spectral edge frequency (SEF50, red line) drop following DTS application, and then begin to return towards baseline. In the DST pilot study, movement artefact posed a significant challenge to EEG recording, as the animals began to twitch and convulse, similar to the movements seen during the phases of an electric stun. This movement artefact was not present in the Rault study (**SS03**), as the animals were anaesthetised. Furthermore, in the DTS pilot study, background electrical noise from the abattoir infrastructure tended to interfere with the EEG signal, so interpretation of the data, particularly the spectral edge frequencies, is challenging

All the animals for which usable EEG recordings were collected showed changes from baseline, particularly in terms of Ptot, while for animal 14, for which EEG data was collected post-DTS only and captive bolt was not applied, high amplitude spiking of Ptot was observed. These patterns on EEG are considered to be incompatible with sensibility.

A potential source of bias is the small group size (n=20), animals being sourced from a single mob presented at the abattoir. A variety of breed, sex and age were presented.

Table 7: Time intervals from onset of DTS application to behavioural changes and interval from loss of posture to return of reflexes.

Animal number	*Time from onset of energy application to onset of physical response (and response character)*	*Time from onset of energy application to loss of posture*	*Time from onset of onset of physical response to loss of posture*	*Time from loss of posture to return of reflexes (last reflex tested before (or) captive bolt applied)*
1	< 1 sec (rapid blinking)	7 sec	<7 sec	Indefinite, animal died
2	< 1 sec (eye fixed open) 5 sec (rapid blinking)	5 sec	< 5 sec	2 min 5 sec
3	1 sec (loss of posture) 4 sec (rapid blinking)	1 sec	<1 sec	Indefinite, animal died
4	<1 sec (rapid blinking, loss of posture)	1 sec, regained feet then fell again at 6 sec	<1 sec	3 min 4 sec
5	2 sec (rapid blinking)	6 sec	4 sec	Indefinite, animal died
6	2 sec (hind limb movement) 19 sec (rapid blinking, loss of posture)	19 sec	17 sec	Indefinite, animal died
7	1 sec (eyes open wide) 2 sec (body movements)	15 sec	14 sec	3 min 36 sec
8	1 sec (eyes open wide)	9 sec	8 sec	3 min 37 sec
9	4 sec (eyes close tight then rapid blinking)	5 sec	1 sec	2 min 47 sec
10	2 sec (eyes closed) 5 sec (loss of posture)	5 sec	3 sec	2 min 52 sec

Animal number	Time from onset of energy application to onset of physical response (and response character)	Time from onset of energy application to loss of posture	Time from onset of onset of physical response to loss of posture	Time from loss of posture to return of reflexes (last reflex tested before (or) captive bolt applied)
11	1 sec (eyes close tight then open wide) 6 sec (body movements and blinking)	9 sec	8 sec	2 min 52 sec
12	1 sec (rapid blinking, loss of posture)	<1 sec	1 sec	2 min 12 sec
13	<1 sec (blinks hard)	4 sec	3 sec	2 min 25 sec
14	2 sec (rapid blinking) 4 sec (front legs paddling)	8 sec	6 sec	>3 min 45 sec (exsanguinated without application of bolt)
15	1 sec (blinks)	5 sec	4 sec	3 min 35 sec (back-up stun applied during exsanguination)
16	5 sec (blinks, loss of posture)	5 sec	<1 sec	2 min 14 sec
17	1 sec (blinks) 2 sec (loss of posture)	2 sec	1 sec	>2 min 34 sec (captive bolt applied early for personnel safety reasons as body was trapped in crate)
18	2 sec (blinks, loss of posture)	2 sec	<1 sec	>1 min 22sec (captive bolt applied early for personnel safety reasons as body was trapped in crate)
19	6 sec (eyes closed)	8 sec	2 sec	Estimated 3 min (visibility obscured by person carrying out reflex testing)
20	4 sec (pull back)	8 sec	4 sec	1 min 42 sec

Table 8: Surface temperatures recorded on the forehead of cattle pre- and post-DTS, and the percentage area post-DTS in which surface temperature was greater than 45, 50 and 60 °C.

Animal number	*Minimum temperature (°C)*	*Maximum temperature (°C)*	*Average temperature (°C)*	*Area > 45 °C (%)*	*Area > 50 °C (%)*	*Area > 60 °C (%)*
1 pre-DTS	20.4	35.7	27.8			
1 post-DTS	23.9	83.1	47.8	54	45	18
2 pre-DTS	21.8	38.1	29.6			
2 post-DTS	24.0	106.8	49.9	58	49	27
3 pre-DTS	19.8	35.5	25.0			
3 post-DTS	22.1	95.5	43.6	45	38	18
4 pre-DTS	17.1	34.4	24.4			
4 post-DTS	20.9	82.8	39.4	25	18	7
5 pre-DTS	18.2	34.6	24.1			
5 post-DTS	20.5	91.8	45.5	49	38	10
6 pre-DTS	20.8	36.4	27.9			
6 post-DTS	24.0	99.6	49.7	57	44	17
7 pre-DTS	20.0	37.5	26.9			
7 post-DTS	23.1	109.2	46.6	46	41	27
8 pre-DTS	23.2	34.4	29.4			
8 post-DTS	24.0	95.5	51.4	60	50	32
9 pre-DTS	21.9	35.5	28.5			
9 post-DTS	24.0	92.3	49.3	52	44	26
10 pre-DTS	21.9	34.9	28.1			
10 post-DTS	21.8	98.5	46.4	51	43	21
11 pre-DTS	20.4	39.4	27.9			
11 post-DTS	23.8	89.2	51.9	62	53	37
12 pre-DTS	18.4	34.3	24.7			

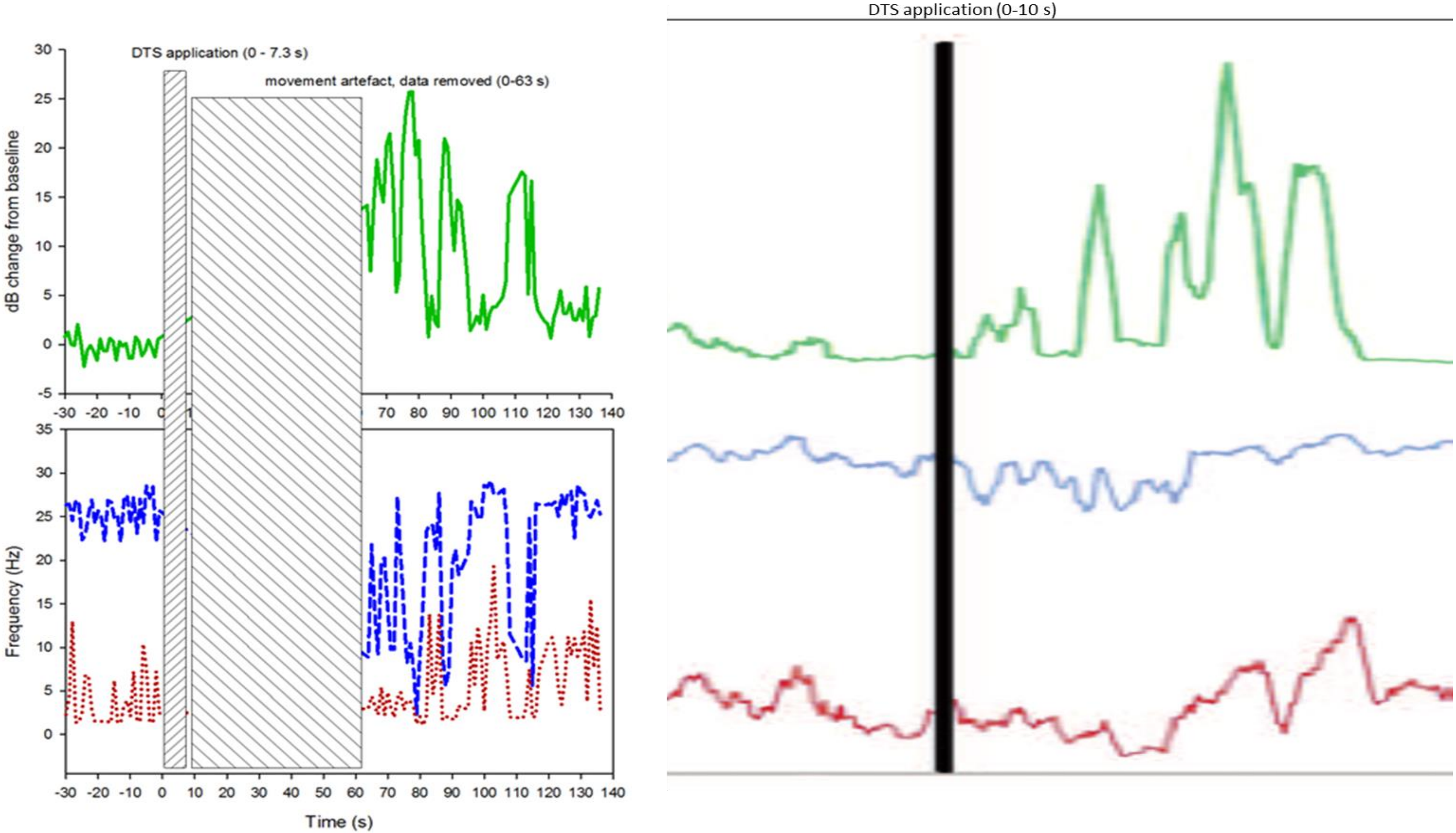
Animal number	*Minimum temperature (°C)*	*Maximum temperature (°C)*	*Average temperature (°C)*	*Area > 45 °C (%)*	*Area > 50 °C (%)*	*Area > 60 °C (%)*
12 post-DTS	21.4	77.6	31.6	10	7	3
14 pre-DTS	12.6	34.4	20.2			
14 post-DTS	15.2	91.1	41.4	32	23	13
15 pre-DTS	16.1	33.4	25.3			
15 post-DTS	17.4	77.3	44.2	46	27	9
16 pre-DTS	14.8	31.6	21.8			
16 post-DTS	17.0	90.1	43.1	41	33	11
18 pre-DTS	17.1	35.1	25.2			
18 post-DTS	15.5	62.9	39.1	23	08	0
19 pre-DTS	17.2	36.2	26.1			
19 post-DTS	16.6	80.3	43.3	45	32	7
20 pre-DTS	15.9	36.2	24.7			
20 post-DTS	17.7	79.9	42.9	36	25	13

Table 9: Summary of data collected from each animal.

Animal	*setting (kJ, kW)*	*energy delivered (kJ)*	*latency to react (s)*	*latency to loss of posture (s)*	*loss of posture to stand (s)*	*loss of posture to reflex return (s)*	*energy at loss of posture (kJ)*	*mean surface temp at loss of posture (°C)*	*min (°C)*	*max (°C)*	*unconscious*	*leakage*	*EEG DB change from baseline*	*Change in SEF95*	*Change in SEF50*	*time EEG trending towards baseline (s)*
1	360, 30	421	1	7	never	never	170	35.88	21.81	54.83	yes, then death	not observed	-10	+12	+5	> 90
2	300, 30	350	1	5	88	125	104	35.63	22.46	58.5	yes	evident on live observation	not recorded	not recorded	not recorded	not recorded
3	300, 30	351	1	1	69	never	135	32.16	20.69	58.57	yes, then death	evident on live observation	+10	-10	-8	> 140
4	300, 30	171	1	6	80	184	143	36.88	20.26	74.7	yes	evident on live observation	+4	0	-5	100
5	300, 30	338	2	6	17	never	138	32.82	19.13	57.9	yes, then death	visible on thermal image	-5	0	-5	> 110
6	275, 30	317	2	19	5	never	317	49.71	24	99.64	yes, then death	visible on thermal image	+30	+3	+2	160
7	250, 30	292	1	15	144	216	292	46.55	23.1	109.07	yes	not observed	-7	-20	-12	> 190
8	250, 30	284	1	9	17	217	233	47.39	23.85	84.38	yes	visible on thermal image	+8	-10	-5	> 140
9	225, 30	267	4	5	5	167	114	37.37	22.8	59.7	yes	evident on live observation	+3	-10	-8	> 130
10	225, 30	258	2	5	14	172	112	36.04	21.94	62.51	yes	evident on live observation	+15	-12	-2	130

Animal	*setting (kJ, kW)*	*energy delivered (kJ)*	*latency to react (s)*	*latency to loss of posture (s)*	*loss of posture to stand (s)*	*loss of posture to reflex return (s)*	*energy at loss of posture (kJ)*	*mean surface temp at loss of posture (°C)*	*min (°C)*	*max (°C)*	*unconscious*	*leakage*	*EEG DB change from baseline*	*Change in SEF95*	*Change in SEF50*	*time EEG trending towards baseline (s)*
11	225, 30	261	1	9	10	172	215	47.59	23.2	80.27	yes	evident on live observation	+3	+2	0	110
12	225, 30	125	1	1	34	132	6	25.02	18.54	36.29	partial	evident on live observation	+12	-18	-5	> 80
13	225, 20	247	1	4	126	145	58	not recorded			partial	evident on live observation	baseline EEG not recorded			
14	300, 30	351	2	8	never	> 225	196	32.04	14.05	66.03	yes	not observed	baseline EEG not recorded			
15	275, 30	310	1	5	never	215	110	32	16.23	48.95	yes	visible on thermal image	baseline EEG not recorded			
16	275, 20	310	5	5	never	134	80	27.28	15.37	46.66	yes	evident on live observation	baseline EEG not recorded			
17	275, 20	312	1	2	never	> 154	23	not recorded			yes	evident on live observation	baseline EEG not recorded			
18	275, 20	297	2	2	never	> 82	23	26.27	16.98	37.24	partial	evident on live observation	baseline EEG not recorded			
19	200, 20	226	6	8	never	180	135	36.36	17.56	62.51	yes	evident on live observation	baseline EEG not recorded			
20	200, 15	223	4	8	43	102	101	32.92	16.71	55.94	yes	visible on thermal image	baseline EEG not recorded			

Figure 14: Examples of EEG data. Left: Animal 10 in the current study, Right: Animal 6 from Rault et al. 2013 and 2014 (reproduction of an approximate 3-minute block). Green indicates total EEG power (Ptot) as change from baseline, blue indicates 95% spectral edge frequency (SEF95) and red indicates 50% spectral edge frequency (SEF50).



Discussion and conclusions

The findings of this study phase were as follows:

Seventeen of 20 cattle were assessed as insensible following DTS application. These animals demonstrated behavioural and EEG signs consistent with an electrical stun, loss of posture occurring between 1 and 8 seconds after onset of energy delivery. In 4 animals, which received 300-360 kJ, this insensibility progressed to death. In the remaining 13 animals, absence of corneal reflex persisted for between 100 and 170, at which point captive bolt was administered in case of returning consciousness. When these timeframes are compared with those reported for head-only electrical stunning in cattle (application of 4 to >20 sec, duration 31 to 90 seconds), it is evident that DTS provides a better welfare outcome.

For each of the three animals that were not deemed insensible, there had been problems with maintaining contact between the waveguide and the forehead, resulting in leakage of energy into the environment, rather than penetration into the brain. All three appeared partially conscious, with loss of eye focus and no visual following of movement, and a slow response to a touch on the cornea. All three were stunned using captive bolt immediately following assessment of consciousness.

Of the 20 animals processed, 11 EEG recordings were generated: the post-DTS recording was not collected from animal 2 due to accidental shut-down of the laptop running LabChart, and in animal 13, corneal reflexes were present and the animal was stunned using the back-up captive bolt shortly after opening of the faraday cage, prior to EEG recording. For animals 14 to 20, baseline EEG was not recorded, and post-DTS EEG was recorded on the bleed-conveyor during exsanguination. Thus, baseline EEG for normalisation was not present, and the post-DTS EEG could only be used qualitatively. In the event, once movement artefact was removed, there were very little EEG data available for animals 15-20 prior to the return of the corneal reflex and captive bolt application. All the animals for which usable EEG recordings were collected showed changes from baseline, particularly in terms of Ptot, while for animal 14, for which EEG data was collected post-DTS only and captive bolt was not applied, high amplitude spiking of Ptot was observed. These patterns on EEG are considered to be incompatible with sensibility.

Thermal imaging indicated that the average surface temperature on the forehead at the point of loss of posture would be between 26 and 49 °C, although some animals (animals 6 and 7) that showed a delayed loss of posture also demonstrated hot-spots of up to 109 °C. In context, Australian cattle may experience ambient air temperatures of 45 °C in summer and could experience higher surface temperatures when standing in in direct sunlight. Surface temperatures above 50 °C may be uncomfortable to cattle, although no literature on thermal contact sensitivity in cattle could be located to confirm or refute this supposition, while temperatures above 60 °C will result in skin tissue damage, as seen in hot branding which is commonly used for identification of cattle.

The results gathered were confounded by problems with waveguide to forehead contact, resulting in loss of energy to the environment instead of being transmitted into the brain. This variability in energy delivery may account for the variability in latency to loss of consciousness, using loss of posture as the proxy indicator, and problems with waveguide to forehead contact may have contributed to the variability in surface temperatures and the presence of hot spots on the forehead.

In conclusion:

- DTS Prototype 4 successfully rendered cattle insensible based on physical (reflex) and EEG data;
- Prolonged duration of insensibility suggests that DTS may be better than the existing commercially available head-only electrical stunning method for cattle;
- Some engineering modifications are required to ensure consistent delivery of energy to the brain and minimise leakage.

STEP 10: Laboratory engineering: Refinements to delivery system: applicators, coaxial cabling and tuning

Submitted study: **PR13**: Refinements to delivery system: applicators, coaxial cabling and tuning. Interim project reports. Not peer-reviewed. CONFIDENTIAL.

Introduction

The validation study (Step 9) identified some issues with energy leakage from the applicator, maintenance of contact between head and applicator, surface heating, and robustness of the coaxial cabling. Further engineering modifications were required to minimize or correct these issues. Subsequent to these initial modifications (**PR13** part1 and part 2), a series of further modifications were developed and tested during the course of the commercial-scale validation (Step 11).

Objective

Phase 1 (April-May 2018). To confirm functionality of coaxial cable components, bench-test a variety of alternative applicator designs and to optimize robust energy coupling to the animal's head and maximise the heating spread (**PR13** part 1 and part 2).

Phase 2 (October 2018). To design and bench-test a modified applicator to incorporate greater heating higher io the head, towards the poll (**PR13** part 3 and part 4).

Phase 3 (December 2020 – March 2021). To investigate reasons for failure of coaxial cable components (**PR13** part 5) To improve shielding around the restraint unit (**PR13** part 6); to investigate the potential for using metallic ducting as a flexible waveguide (**PR13** part 6); and to bench-test the latest applicator design (**PR13** part 7).

The details of this step are confidential proprietary information.

STEP 11: Commercial scale validation

Submitted study: **PR12**: DTS: Diathermic Syncope® controlled trials. Project report. Not peer-reviewed.

Introduction

A dielectric (electromagnetic) stunning system has been developed by Wagstaff Food Service Pty Ltd and Advanced Microwave Technologies. This system, trademarked DTS: Diathermic Syncope®, has the potential to address the requirements of the Halal and Kosher markets, without current disadvantages of existing stunning methods (e.g., the potential for cracked skulls associated with percussive stunning, the need to use an immobiliser to improve operator safety in electrical head-only stunning, the need for a second exsanguination cut following the neck cut to remove the risk of the animal regaining consciousness during bleed-out). Pilot trials on live animals have been successful in inducing electroencephalogram traces consistent with unconsciousness, of sufficient duration to allow exsanguination prior to recovery. DTS produced comparable post-slaughter meat quality and physiological responses in treated cattle to those stunned using penetrative captive bolt. This phase of development comprised trials on live animals in a controlled working environment, validating the previous outcomes in a larger number of animals, demonstrating repeatability. Ultimately the outcomes of this work will be used to gain industry stakeholder agreement on full commercialisation of the technology. The progress of research and development of this technology is evidence of the Australian Industry's commitment to continual improvement in Animal Welfare at processing, which in turn supports the continued social license to operate.

Objective

- Confirmation of previous science works in a commercial environment.
- Collection of data to support approval of the technology as a commercial means of inducing insensibility on cattle for the production of meat for human consumption.

Materials and methods

There were two parts to this phase of work – the first was a pre-commercial-scale evaluation in which cattle were processed using the restraint unit described in Step 9. Variations in input power and energy parameters and a variety of applicator head designs were tested. Finally, an evaluation of a proposed commercial set-up including a rotary box for handling of the stunned body was carried out.

Ethical considerations: The study was approved by the Commonwealth Scientific and Industrial Research Organisation (CSIRO) Wildlife and Large Animal Animal Ethics Committee (CWLA AEC), reference 2019-17.

For the first part of the study, a total of 235 cattle was processed in a series of small batches of between 8 and 50 cattle between October 2017 and October 2019. Between batches, modifications were made to the waveguide apparatus (Step 10) and/or restraint unit in order to optimise stun quality and ease of processing. These modifications led to reductions in the required input power (kW) and elimination of the surface overheating that had been observed during the previous study (Step 9). Data were collected on animal type, energy delivery parameters, presence/absence of indicators of unconsciousness, time of back-up stunning and behavioural observations.

Study population: The animals included steers, heifers, cows and bulls, of varying ages from milk teeth only to full mouth cull animals. The animals were predominantly *Bos taurus*, with 5 *Bos indicus* animals, and 13 had horns. Carcase weights ranged from 160 to 413 kg. Power settings for DTS application ranged from 15 to 40 kW, and energy settings from 100 to 371 kJ.

For Part 2, A rotary box was installed at the Wagstaff Garfield abattoir, VIC, and fully enclosed in a Faraday cage (Section 3.6). Over a three-day period from 1st to 3rd December 2020, 35 *Bos taurus* slaughter generation cattle of mixed breeding were processed using DTS: Diathermic Syncope® to induce insensibility, inverted using the rotary box and bled in the inverted position using the Halal cut delivered by a registered Halal slaughterman. The animals were processed to give carcase weights ranging from 242 to 413 kg (10 steers and 25 heifers). Breeds were crosses of Angus, Hereford, and Holstein/Friesian and eight had horns. Energy applications between 140 and 230 kJ were used in 33 animals, all of which were rendered insensible using DTS. Two animals received energy levels below 100 kJ as a result of equipment failure, these were immediately stunned using a non-penetrating device. Electroencephalogram (EEG) data were collected from 19 DTS animals using the protocol outlined in Step 7.

Study design and methods of analysis: this was an observational study, with no statistical analysis performed.

Randomisation and blinding: Treatments were pre-determined, so randomization was related to the order in which animals presented themselves to the restraint unit. Observers were not blinded to treatment.

Measurement of the outcome: Live and video behavioural monitoring were utilized to assess the magnitude of pain, suffering and distress. Absence of corneal reflex was used as the key indicator of unconsciousness.

Reporting the results

Part 1

Of 235 animals processed, 6 were assigned to penetrative captive bolt only as a result of their being collapsed in the restraint or overly agitated to securely restrain; and a further 6 were stunned and slaughtered at the end of the final batch using penetrative captive bolt because the current phase of DTS trials was complete and they were 6 remaining animals in the lairage when the plant was being decommissioned for refurbishment in preparation for the rotary box evaluation. These 12 animals were excluded from the evaluation of DTS, leaving 223 animals in the current evaluation.

Of the 223 animals included in the evaluation, a further 16 were excluded from analysis as a result of equipment failure resulting in generator shutdown and the required energy not being applied. These animals were also stunned using a penetrative captive bolt prior to exsanguination.

Of the remaining 207 animals, 201 were rendered fully insensible on first application of DTS (97.1%). This exceeds the expected minimum standard of 96% described in appendix 7 of the National Animal Welfare Standards for Livestock Processing Establishments (AMIC, 2009). The 6 other animals appeared sedated. Analysis of the data indicated that these cases were related to low Power (kW) settings, which were used during the study to determine the lower critical limits of expected operational parameters. In the 201 fully insensible animals, live observations suggested that there was rapid induction of insensibility, which lasted for between 2 and 4 minutes in the majority of cases.

Live observations indicated that low energy levels resulted in shorter duration of insensibility, while energy levels above 220 kJ appeared to result in early onset of intense convulsions. Surface overheating and visible blistering of the skin was evident when power settings were 25 kW and above.

Three entire Angus young bulls, three mature bulls and five Brahman animals (three of which were horned) were included in the data set. Energy applied to bulls ranged from 200 to 250 kJ, and all were rendered insensible using DTS. Forehead skin thickness in the bulls ranged from 5 mm (Brahman) to 24 mm (Angus).

In the absence of technical problems leading to interrupted delivery of energy, all 201 animals were rendered unconscious using DTS. On exsanguination, blood flow was noted to be strong, and the rhythmic pumping of the heart was evident in the first few seconds of blood flow. Both these characteristics are desired by the Halal and Kosher markets.

Visual inspection of a sample of brains, removed from the skull cavity immediately following head inspection, indicated that when energy delivery was 220 kJ or less, there was no visible damage to the brain.

The main challenge during this phase was extracting the stunned bodies from the restraint unit, which was a standard abattoir knocking box designed for use with captive bolt. Cattle that are stunned using DTS (or for that matter, electrical stunning) can quickly enter a stiff, tonic 'rocking-horse' position, with all four legs rigidly extended. If this happens before the body is rolled out from the box, the body is obstructed by the side door, which cannot open sufficiently to allow ejection from the box. After this 'rocking horse' phase, the stunned animal develops a convulsive or kicking phase (similar to that seen in an epileptic episode), followed by a recovery phase. In commercial processing it is important to exsanguinate the animal before the kicking phase begins – both to ensure that the animal does not recover during bleed-out, and because the size of the animal makes handling during that kicking phase very dangerous for the operator. The difficulties in extracting the body from the upright restraint box prevented prompt exsanguination during the tonic phase.

As such, installation of a rotary box was undertaken, so that the stunned animal could be tipped to allow rapid exsanguination.

Part 2

Energy levels of 140-160 kJ appeared to result in shorter durations of insensibility, while energy levels above 200 kJ appeared to result in early onset of intense convulsions.

The study reconfirmed consistent induction of insensibility, based on live observations of the animal and assessment of corneal and palpebral reflexes. The insensibility was sustained during bleeding using the Halal neck cut. The EEG data reconfirmed the high-amplitude spiking in Ptot following DTS application. This spiking persisted throughout bleeding. After sticking, Ptot rapidly dropped towards baseline, over a period of 20 to 60 seconds.

Use of the rotary box improved handling of the stunned body, and the updated applicator configuration allowed consistent repeatable induction of insensibility, which was sustained during Halal bleeding. When a delay to collect EEG data was not required, sticking was carried out within 30 seconds of DTS application.

Although attempts were made to include a wide range of animal type, sex, weight and age, numbers are still limited in a commercial sense (n=270), and the full range of cattle weights and feedbase origins that are processed commercially are not represented.

Discussion and conclusions

Combining data from Step 9 and Step 11, 258 cattle were presented, of which 242 cattle were processed using DTS: Diathermic Syncope® as the stunning method. The remaining 16 animals did not receive a DTS application due to technical faults resulting in generator shutdown prior to energy being delivered, and they were stunned using a captive bolt. Of the 242 animals, 8 received low energy deliveries due to technical faults and were deemed inadequately stunned, so were re-stunned using a penetrative captive bolt. Thus 234 of 242 (96.7%) cattle were stunned on first application of DTS, based on loss of posture and loss of corneal and somatic withdrawal reflexes (Von Holleben et al., 2010). Loss of posture, which is considered to be a definitive indicator of unconsciousness (EFSA, 2004; Muir, 2007; Von Holleben et al., 2010; EFSA, 2013), occurred between 1 and 8 seconds after onset of energy delivery.

The latency to onset of physical signs of insensibility was related to power setting. A higher power (kW), providing a more rapid delivery of the energy 'package', led to a shorter time to onset of physical signs of insensibility. The work on anesthetised cattle, carried out by Rault et al. (2014: **SS03**), indicated that shorter durations of applications resulted in more rapid onset of EEG suppression, while greater power settings resulted in a longer duration of insensibility. This is difficult to interpret fully, based on the small number of animals processed in their study and the fact that the animals in their study each received two applications of energy; however, there is a relationship between power setting and rate of heating. The units of power (kW) is shorthand for 'kJ per second', so a higher power setting will reduce the time required to reach an energy delivery of 200 kJ than a lower power setting. However, the higher power setting may predispose to focal overheating, as seen in the first phase of the current study.

Based on the outcomes of this project, we recommend that operational guidelines define the desired range of energy application to be between 160 and 200 kJ.

The stunned animals demonstrated behavioural signs consistent with an electrical stun – rapid blinking or flicking of the eyelids including the membrana nictitans (third eyelid), loss of posture, tonic (stiff) and clonic (convulsive) phases, so it is of value to compare the outcome of DTS against those reported for electrical stunning. In cattle, electrical stunning can be applied as head-only, which affects the brain, resulting in an epileptiform seizure, from which the animal can regain consciousness; or as head-to-chest, which incorporates a current passed through the heart, resulting in cardiac arrest, and the death of the animal. As DTS does not directly affect cardiac function, the comparison should be against head-only electrical stunning.

The biggest problem with head-only stunning of cattle is a very short duration of epilepsy, followed by strong convulsions (EFSA, 2004). The electrical current must be applied across the brain for sufficient duration to overcome the inherent impedance (resistance) across the head, and application of electrical stun ranges from 4 to greater than 20 seconds (Devine et al., 1986; Vonmickwitz et al., 1989; Schatzmann and Jaggin-Schmucker, 2000). The duration of unconsciousness in those studies was reported as 31 - 90 seconds. In contrast, DTS induces loss of posture within 8 seconds of onset of energy application, and the duration of unconsciousness recorded during the current study was between 100 and 240 seconds, based on absence of corneal reflex, and duration of EEG changes. All the animals for which usable EEG recordings were collected showed changes from baseline, particularly in terms of Ptot, showing high amplitude spiking. These patterns on EEG are considered to be incompatible with sensibility (EFSA, 2004) and during this period the animal cannot feel pain.

Return of reflexes are considered to be the earliest indication that consciousness is returning and was the point at which penetrative captive bolt was applied during the current study for animal welfare reasons. In the current study this occurred between 2 and 4 minutes, 30 seconds from loss of posture, which would allow ample time to exsanguinate the animal prior to recovery. Using the rotary box, when a delay to capture EEG data was not required, sticking was performed within 30 seconds of DTS application.

In the early stages of the project, overheating of the skin surface was identified. This was associated with incident power levels of > 25 kW. This was eliminated in the later stages of the project, through optimisation of the auto-tuning device and applicator apparatus such that incident power levels ranged from 18-20 kW. Australian cattle may experience ambient air temperatures of 45 °C in summer and could experience higher surface temperatures when standing in direct sunlight. Surface temperatures above 50 °C may be uncomfortable to cattle, although no literature on thermal contact sensitivity in cattle could be located to confirm or refute this supposition.

100 % insensibility was achieved when the desired energy package was delivered. Existing stunning techniques in Australia, where conditions are carefully controlled, are expected to deliver a 95-98 % first-stun efficacy (AMIC, 2009) but, particularly where adequate neck and head restraint is not used, can have up to 29 % first stun failure rates (Blackmore, 1979; Lambooy et al., 1981; Hoffmann, 2003; Endres, 2005; Octavio-Oliveira et al., 2018; Gibson et al., 2019). The animal stunned using DTS can also return to consciousness, so this method of stunning is likely to be acceptable to the Halal and Kosher markets. Furthermore, blood flow was observed to be strong, and the rhythmic pumping of the heart was evident in the first few seconds of blood flow. Both these characteristics are desired by the Halal and Kosher markets.

Use of the rotary box optimised handling of the stunned body, allowing a short stun-to-stick interval without the use of an immobiliser. The updated applicator configuration allowed consistent repeatable induction of insensibility, which was sustained during Halal bleeding, without the need to apply an additional ventral cut.

Key messages:

In the absence of technical problems leading to interrupted delivery of energy, all animals were rendered unconscious using DTS. 234 animals were successfully stunned on first application. At no time was Animal Welfare compromised by technical faults.

Power settings of 20 kW provided a rapid onset of insensibility, while a power setting of 18 kW led to a slower onset of insensibility. Power settings of 25 kW and above led to overheating at the skin surface.

Energy deliveries in the range of 160 to 200 kJ achieved insensibility of sufficient duration to allow exsanguination using a neck cut alone, with operator safety during exsanguination optimised. Energy deliveries of 160 kJ and less resulted in shorter duration of insensibility with a risk of early return to consciousness, while energy deliveries of 220 kJ and above resulted in early onset of intense convulsive activity, reducing operator safety.

Live observations indicated that DTS consistently induces insensibility, with no vocalisation, no evidence of pain or distress, and EEG data confirmed the development of a high-amplitude-low-frequency (HALF) epileptiform state.

There was no requirement to use an immobiliser during application of the exsanguination cuts, and the animal bled rapidly to brain death using the Halal cut alone. The blood flow on exsanguination was strong and rapid.

Visual inspection of the brains of carcases indicated no visible damage when energy deliveries of less than 220 kJ were applied.

STEP 12: Regulatory Authority review

Submitted study: **PR14**: Independent review of the new technology DTS: Diathermic Syncope. Report - With Australian Regulatory Authority Approval (letter). Not peer-reviewed. CONFIDENTIAL.

Introduction

Established researchers with expertise in animal welfare, cattle behaviour, and abattoir stunning and slaughter methods, were asked to provide the Department of Agriculture, Water and the Environment (the Department) with an independent review of the new technology, DTS: Diathermic Syncope.

The details of this step are confidential proprietary information.

Approval to use the DTS: Diathermic Syncope® technology in an Approved Arrangement was granted by DAWE for use in abattoirs in Australia, providing that they comply with the relevant DAWE, State and Territory regulations and processes.

4.3 Overall integration of findings from all studies

4.3.1 Demonstration of equivalence with existing methods

4.3.1.1 Welfare impact

Biomarkers of stress

The endocrine stress responses measured indicate activation of two physiological response systems. One is the 'fight – or – flight' response initiated by all animals when a threatening or 'emergency' situation is perceived: activation of this response leads to increases in catecholamines (adrenaline and noradrenaline). The catecholamines speed blood circulation and divert blood, and therefore oxygen, from internal organs to the brain and muscle, readying the animal for action. The second response is the HPA axis (Hypothalamic-Pituitary-Adrenal axis): activation of this response leads to the release of Adrenocortocitrophic hormone (ACTH) which leads to increased cortisol levels. This in turn increases the rate of production of glucose from glycogen reserves, so that the energy required for action is available. This speeding up of glycogen breakdown, circulation, and metabolism generally can have detrimental effects on meat quality: very fast metabolism at the point of slaughter can lead to pale meat colour or heat toughening; prolonged raised metabolism leads to reductions in glycogen stores, which in turn lead to dark cutting.

In a commercial slaughterhouse situation, the stresses associated with movement of the animal along the race into the knocking box and positioning the head into the head capture unit are common to all cattle, whether subjected to DTS, electrical stunning or captive bolt stun. Comparison against published reference ranges for various biomarkers, based on resting animals, is inappropriate, as there will always be some underlying effect of the pre-slaughter transport and handling phase. Even differences between abattoirs can strongly influence the stress response (Hemsworth and Barnett, 2001). For example, Zulkifli et al. (2014), working in a large commercial slaughterhouse, report higher, and a wider range in, cortisol concentrations than do Tume and Shaw (1992), from a small experimental facility (Table 12). This is why baseline samples are taken prior to treatment, against which the post-treatment samples are compared. Examples of published data are presented in Table 18 alongside the ranges found in the detailed evaluation of 18 cattle subjected to either DTS or penetrative captive bolt (Step 7), which align with the published values. The large range in values makes it difficult to draw firm statistical conclusions from small numbers of animals.

For the DTS pilot study, group mean and range (converted to SI units) for each sample point are summarised in Table 13. Baseline cortisol in the DTS group is significantly lower than post-treatment cortisol in that group, however, it is not significantly different from baseline captive bolt, and post-treatment cortisol in either group is not significantly different from the aggregate of all baseline cortisol results. Therefore, the fact that post-treatment cortisol is significantly different from baseline cortisol in the DTS group, and not in the control (captive bolt) group, is likely to be an artefact of small sample size.

Table 10: Published data on endocrine parameters measured in the DTS pilot study (Step 7)

	Rulofson et al. (1988)	*Mitchell et al. (1988)*	*Dunn (1990)*	*Shaw & Tume (1990)*	*Tume & Shaw (1992)*	*Zulkifli et al. (2014)*	*DTS pilot study*
Cortisol (nmol/L)		*25.0 ± 13.7 to 176.7 ± 65.5*	*124.8 ± 10.7 to 259.6 ± 104.0*	*44.9 ± 6.9 to 88.3 ± 10.8*	*41.0 ± 3.7 to 123.2 ± 5.3*	*175.11 to 274.08*	*14.36 to 212.46*
ACTH (pmol/L)		*20.6 ± 7.9 to 301.8 ± 142.9*				*922.1 to 4092.9*	*3.7 to 125.4*
Adrenaline (nmol/L)	*1.2 ± 0.1 to 81.3 ± 20.7*	*9.28 ± 0.44 to 49.12 ± 89.51*				*102.28 to 519.66*	*22.33 to 420.12*
Noradrenaline (nmol/L)	*1.9 ± 0.3 to 135.8 ± 31.6*	*0 to 37.82 ± 54.37*				*271.21 to 754.35*	*<0.12 to 503.64*
B-Endorphin (pmol/L)				*14.6 ± 1.8 to 30.7 ± 4.3*	*19.2 ± 1.5 to 20.9 ± 1.2*	*259.9 to 441.8*	*4.5 to 1559.8*

Table 11: Summary endocrine data

		DTS		**Captive Bolt**	
		Baseline	**Post-treatment**	**Baseline**	**Post-treatment**
Cortisol (nmol/L)	*Mean*	33.20	150.38	61.43	160.64
	Range	14.36 – 65.20	94.09 – 198.42	21.45 – 169.01	85.82 – 212.46
ACTH (pmol/L)	*Mean*	25.69	11.25	42.21	26.6
	Range	14.4 – 47.2	3.7 – 33.1	11.9 – 125.4	6.6 – 55.7
Adrenaline (nmol/L)	*Mean*	67.76	78.23	50.37	151.35
	Range	22.33 – 70.40	45.67 – 420.12	25.48 – 161.33	25.08 – 248.16
Noradrenaline (nmol/L)	*Mean*	17.84*	138.10	24.14*	80.32**
	Range	<0.12 – 56.39	19.18 – 503.64	<0.12 – 110.79	<0.12 – 262.88
b-endorphin (pmol/L)	*Mean*	794.8	646.1	686.8	614.1
	Range	11.8 – 1334.0	313.5 – 1059.9	177.8 – 1100.2	4.5 – 1559.8

* five of seven samples were below detection limit (0.12 nmol/L)

** one of seven samples was below detection limit (0.12 nmol/L)

Both stunning methods resulted in an increase in cortisol from baseline (DTS 33.19 ±16.89 nmol/L; Captive bolt 61.43 ±12.59 nmol/L) to post-treatment levels (DTS 150.38 ±17.79 nmol/L; Captive bolt 160.64 ±13.26 nmol/L), indicating a physiological response. However, it is unclear if this response is due to the methods; or to the head capture and restraint, which was longer than in a commercial situation due to the need to take baseline EEG recordings; or to a combination of both restraint and treatment. Shaw and Tume (1990) and Zulkifli et al. (2014) both report that cortisol levels in cattle are not affected by stunning (captive bolt), however, the former carried out their study in a highly controlled research abattoir environment, in which external stimuli are likely to have been minimised, while the latter carried out their study in a commercial slaughterhouse, and baseline cortisol levels prior to slaughter were already high, as a result of the preslaughter handling and environment. Both Dunn (1990) and Zulkifli et al. (2014) report post stun cortisol levels greater than those measured in the DTS pilot study, but it is important to note that these both relate to the case of unstunned slaughter. Indeed, the latter authors demonstrated an increase in cortisol levels associated with unstunned slaughter, but not in the case of captive bolt stunned slaughter. Mitchell et al. (1988) did find that slaughter (captive bolt) resulted in an increase in cortisol levels (+61.2 nmol/L), but this increase was less than in cattle that were handled through a race (+151.7 nmol/L). By inference, it is likely that the cortisol response seen in the DTS pilot study is predominantly due to the head capture and restraint. A tightly clamped head is required ensure delivery of the DTS energy; however, prolonged tight head capture is likely to be very stressful to the animal and struggling while restrained is considered to be an indication of excessive pressure (Grandin and Regenstein, 1994). The European Food Safety Authority recommends that "all restraining devices should use the concept of optimal pressure" (EFSA, 2004), however, the parameters that constitute 'optimal pressure' have not been determined.

In the DTS pilot study, there was neither a difference in ACTH levels between baseline and post-treatment samples, nor was there a treatment effect. The values generated in the DTS pilot study were lower than those reported by Zulkifli et al. (2014), but similar to those reported by Mitchell et al. (1988), who found that ACTH was, like cortisol, affected more by transportation and handling than by stunning alone.

Catecholamines (adrenaline and/or noradrenaline) have been reported to increase as a result of stunning (Mitchell et al., 1988; Rulofson et al., 1988; Zulkifli et al., 2014). Zulkifli et al. (2014) report no significant changes in adrenaline levels as a result of penetrative captive bolt stunning, or high powered percussive stunning, but an increase as a result of low powered percussive stunning and unstunned slaughter, while noradrenaline levels increased in all treatment groups; findings that concur with the DTS pilot study. However, Mitchell et al. (1988) and Rulofson et al. (1988) both report an increase in both adrenaline and noradrenaline associated with captive bolt slaughter. In the DTS pilot study, adrenaline levels tended to decrease between the pre-slaughter and post-slaughter samples, while noradrenaline levels tended to increase. There were no significant differences between treatments.

β-endorphins have a role in modulating the physiological stress response, and increases are associated with painful stimuli, fear and excitement. In the DTS pilot study, there was no significant effect of treatment on β-endorphin concentrations in either group, although the mean value appeared to decrease slightly. This finding concurs with that of Zulkifli et al. (2014), who demonstrated no significant change in concentrations as a result of penetrative captive bolt, percussive captive bolt, and unstunned slaughter at a commercial abattoir. The slight decline in β-endorphin concentrations also reflects the findings of Shaw and Tume (1990), who demonstrated a significantly lower concentration in captive bolt slaughtered cattle than in the live animals. Those authors however, carried out their study in a small research abattoir, and the live animal blood samples were collected on a separate occasion within a month prior to slaughter. Thus, the live animal values they report may not relate well to the baseline samples collected in the DTS pilot study and that of Zulkifli et al. (2014), which were collected immediately prior to stunning, in the restraint box.

Behavioural indicators of insensibility

In research, the gold standard of assessing consciousness is electroencephalography (EEG, in which electrodes are place on or into the skin of the scalp), or electrocorticography (ECoG, in which the electrodes are implanted into the superficial parts of the brain. However, in a commercial situation, it is impossible to utilise techniques such as EEG or ECoG to monitor the effectiveness of stunning, and behavioural indicators and responses to certain stimuli are used as surrogates. For example, captive bolt stunning is considered effective if the observer notes:

- Immediate collapse, hind legs tucked in then slowly extend, forelegs rigidly extended,
- Immediate and sustained absence of rhythmic breathing
- Fixed, staring eye with no corneal of palpebral reflex
- No righting reflex, no response to ear or nose pinch, no vocalisation

An effective electrical stun results in:

- Immediate collapse, hind legs tucked in, forelimbs rigidly extended
- Immediate onset of tonic (stiff) seizure that lasts for several seconds, followed by clonic (convulsing) seizure
- No rhythmic breathing
- Eyes rotated upwards, dilated pupils
- No response to nose prick

A summary of the indicators suggested by various international standards and guidelines, and indication of whether these are evident in DTS animals, is presented in Table 14 (mechanical stun) and Table 15 (electrical stun).

Table 12: Indicators of an effective mechanical stun

Behavioural sign	*DTS*	*OIE Terrestrial Animal Health Code*	*EFSA Scientific Opinion 2004*	*AMIC animal welfare standard*	*USDA Humane Handling Guidebook***
Immediate collapse		☑	☑	☑	
No attempt to stand up*		☑			☑
Body and muscles immediately rigid (tonic)		☑	☑	☑	
Lack of normal rhythmic breathing		☑	☑	☑	
Eye open and staring straight ahead / glazed expression	☑	☑		☑	
No attempt to raise head				☑	
Ears relaxed and drooping				☑	
Tongue loose and flapping / hanging out				☑	☑
Corneal reflex absent	☑		☑	☑	
No spontaneous eye blinking	☑			☑	
Straight back and floppy head					☑
No vocalisation of any kind					☑
No vocalisation in response to stimulus	☑				
No response to painful stimulus	☑		☑		
Gradual pupillary dilation			☑		

*The USDA Humane Handling Guidebook describes: “Absence of righting reflex, including an arched back”.

** The USDA Humane Handling Guidebook also lists indications that an animal is NOT properly stunned: Vocalisation, eye blinks, eye reflexes in response to touch, rhythmic breathing, curled tongue.

Table 13: Indicators of an effective electrical stun

Behavioural sign	*DTS*	*EFSA scientific opinion 2004*	*AMIC animal welfare standard*
Immediate collapse	☑*	☑*	☑*
Epileptiform seizure (described in detail)	☑†	☑	☑
Lack of normal rhythmic breathing	☑#	☑	☑
No spontaneous eye blinking	☑		☑
Gasping (breathing in without breathing out) sometimes occurs	☑		☑
Upward rotation of eyes		☑	
Dilated pupils		☑	
No response to nose prick (painful stimulus)	☑	☑	

*: Row 1: With regards to induction of insensibility prior to slaughter, there is much discussion over the 'immediacy' of the lapse into unconsciousness. in electrical stunning, the current must flow for a period of at least 2 (sheep) or 5 (cattle) seconds in order to ensure a sustained loss of consciousness (Warrington, 1974, Von Holleben et al., 2010).

†: Row 2, Column 1: Due to the fixed head restraint, full body collapse was not evident. However, the haunches initially dropped, followed by a tonic phase, and then convulsive movements or paddling of the limbs.

#: Row 3, Column1: In the DTS validation study, a short suspension of respiration was evident in some animals, but not all. However, when respiration was present it was much slower and deeper than in the conscious animal.

Unfortunately, there is no overall consensus as to which of these signs are the most reliable indicators of unconsciousness. For mechanical stun, Gracey and Collins (1995) cite 'no rhythmic breathing' as the cardinal sign, while Grandin (2002) advises observers to focus on "limp head, extended tongue and blank stare". Vocalisation during application of the stun is considered to be indicative of pain or distress (EFSA, 2004), but lack of vocalisation does not guarantee absence of pain or distress, some animals are naturally stoic, and disinclined to vocalise. Indeed, in the DTS pilot study, two animals vocalised during application of the head restraint and chin lift, but once in position, and not being handled by people, the animals fell silent, and remained so throughout the stun process. Similarly, the corneal reflex does not distinguish accurately between consciousness and unconsciousness, but if there is no corneal reflex, it is likely that the animal is unconscious (Anil and McKinstry, 1991; Gregory and Grandin, 2007). In the DTS pilot study, corneal reflex was present in animals prior to treatment, and absent following treatment, in both captive bolt and DTS groups.

The response to a painful event, such as a nose prick, is, however, is a good indicator of consciousness. Anil and McKinstry (1991) demonstrated that an animal that perceives pain will draw back from the nose prick, and during recovery from electrical head-only stunning, sheep that demonstrated this withdrawal reflex soon regained the righting reflex. In the DTS pilot study, prior to treatment, animals visibly flinched in response to the nose prick, but this response was absent post treatment in both captive bolt and DTS animals.

Other authors have reversed the approach and considered utilising indicators of ineffective stunning. For example, Gouveia et al. (2009), studying 2800 cattle stunned using captive bolt in a commercial Portuguese slaughterhouse, found that the most common signs of ineffective stunning were muscle tone of the ears (17.8%), absence of muscle spasms in the back and legs (11.5%), presence of rhythmic breathing (9.4%), and vocalisation (7.9%). They also observed animals that showed signs of recovery (e.g., corneal response and righting reflex returning), and suggested that this might be predicted by lack of immediate collapse (100%), eyes rotated rather than fixed (91.3%), rhythmic breathing (91%) and response to nose or ear pinch (84.6%).

For DTS animals, rhythmic breathing continued throughout, so this cannot be considered an indicator of effective induction of insensibility for this treatment. The eyes were fixed and staring, with no ocular following of movement, and there was no withdrawal response to nose prick. Initially, rapid twitching of ears and the third eyelid (nictitating membrane) was evident, with ataxia and loss of posture, particularly in the hindquarters. This was noted as the hind limbs slipping out from behind the animal and attempts to replace the foot showed incomplete stride length, such that the foot remained behind the centre of balance. The limbs did not suddenly tuck in tightly to the body as seen in mechanical and electrical stun (the main reason for 'immediate collapse', as there is no longer any support for the body): the effect was more similar to a heavily sedated animal attempting to maintain posture, similar to that seen in pigs slaughtered using gas inhalation. It is probable that, had the animal not been supported by the head restraint unit and the close confines of the restraint box, the animals would indeed have collapsed.

In the low energy applications, the animals at first arched its back and tensed the muscles in the neck, pushing the chin down into the chin lift, and then demonstrated a period of convulsive movements. This convulsive activity progressed to ataxia as described above. It is not clear whether the convulsive movements are deliberate escape attempts or involuntary convulsions, but the facial expression at the time is of rapid flickering of the third eyelid, with the eyeball partially drooping (a half-moon of white sclera visible in the medial corner of the eye), as opposed to eyes open, fearful and vigilant; and the back is arched rather than flat; and the hind limbs seem to kick forward and higher as compared with a pre-stun animal resisting the head restraint. Animal 4 resisted head capture strongly, and its struggles were characterised by a flat back, and limb movements pushing the body backwards from the neck bails.

Following this ataxic phase, the body became tense and tetanic, with the four limbs slightly splayed. During this phase, it was impossible to push or pull the animal onto its side, as the limbs were locked into place. There was no response to efforts to topple the animal: the tail hung limp and flaccid, and the limbs remained motionless. The animal then progressed into slow walking and paddling movements, pushing forward into the head restraint unit. At this point, the animal could be overbalanced and rolled out, but again there was no response in terms of tail flicking or vocalisation. Muscle tone in the neck persisted, with the head arching up and back, similar to an electrically stunned sheep or chicken. When rolled onto its side, the animal's limbs remained extended, performing slow walking or paddling movements, progressing into more rapid paddling and then convulsive motions.

Animals 14, 16 and 18 progressed from convulsions to a quiescent phase, lying still on the roll-out table, while the EEG was recorded, towards the end of which, return of corneal reflex was observed. This progression is very similar to that seen in electrical stunning – first there is the tonic (stiff) phase; followed by the clonic (convulsive or kicking phase). The convulsions lapse, and the animal enters a quiescent or quiet phase, in which the muscles relax, rhythmic breathing recovers, and then the reflexes return – first corneal, palpebral (blink) and withdrawal; and then righting.

Latency to insensibility

With regards to induction of insensibility prior to slaughter, there is much discussion over the 'immediacy' of the lapse into unconsciousness. Indeed, for both mechanical and electrical stun, normal neurological function is disrupted almost instantaneously following application of the equipment (Daly et al., 1987; Daly and Whittington, 1989). Estimates of 'immediacy' in these situations suggest loss of sensibility within less than one second following application, although in electrical stunning, the current must flow for a period of at least 2 (sheep) or 5 (cattle) seconds in order to ensure a sustained loss of consciousness (Von Holleben et al., 2010; Warrington, 1974). Even using EEG assessment, in those situations in which EEG can be measured, we can identify traces that are consistent with awareness and traces that are inconsistent with awareness, but these are separated by a transition trace (McKeegan et al., 2007), during which the ability of the animal to perceive external stimuli is unknown. At present the best option available is to calculate or infer the time taken to reach a point at which EEG changes indicative of insensibility are present. For example, in electrical stunning, Wotton et al. (2000) demonstrated that a minimum current of 1.15 Amps was required to induce EEG changes indicative of insensibility in cattle. Subsequently, Weaver and Wotton (2009) applied a current of 3.28 Amps or more to 287 cattle in a commercial situation, measuring the current flow between the electrodes, across the animal's brain. In all animals, a current flow of 1.15 Amps was achieved within 100 msec, and this figure has been accepted as the 'time to unconsciousness' for electrical stunning of cattle. In practice, the current flows for longer than 100 msec, to ensure that the insensibility is maintained.

In contrast, it is well known that gaseous inhalation (CO_2 and CO_2/N_2 mixtures) is not as dramatically 'immediate': the induction of unconsciousness is a gradual process as the inhaled CO_2 is absorbed into the circulation, progressively lowering the pH of the cerebrospinal fluid (CSF) to a threshold level at which neurotransmission fails (Woodbury and Karler, 1960), and the animal loses consciousness. At this threshold, the lapse from consciousness to unconsciousness is considered to be 'immediate'. In comparison, DTS utilises an increase in brain temperature to achieve the point at which neurotransmission fails (Small et al. 2013b: **SS02**). In cattle, the core body temperature tends to be 1-2 degrees higher than in humans (38.6°C in resting cattle, 37°C in humans), so we expect that in cattle thermal unconsciousness would occur in the range 42-47°C. Thus, the required rise in temperature to achieve insensibility is in the range of 3.4 to 8.4 degrees. During development and testing of the DTS equipment utilised in this study, the temperature profiles of cadaver heads was tested, and a predictive equation describing the heating rate within the brain was generated (0.124 °C/kW.sec). From this equation, we can infer that for a 30 kW application, the time taken to achieve an 8 degree rise in temperature would be 2.15 sec (8/30/0.124); and for a 20 kW application, the time taken would be 3.23 sec (8/20/0.124). In a commercial situation, the real-time monitoring system incorporated within the control panel will allow an objective record of energy delivered and rate of energy delivery to be maintained, and an alarm feature could be incorporated to use in conjunction with animal-based indicators (e.g., response to reflexes) when confirming insensibility prior to exsanguination.

There is substantial evidence that gaseous inhalation of CO_2 at concentrations above 30% (which are required to induce insensibility), is highly unpleasant (Dalmau et al., 2010; Llonch et al., 2012; Nowak et al., 2007; Raj, 1996; Raj and Gregory, 1995; Sandilands et al., 2011; Velarde et al., 2007). Furthermore, the gradual induction of insensibility by gaseous inhalation has been demonstrated to expose animals to a period of discomfort of 20 seconds or more prior to loss of consciousness. Llonch et al. (2012) described 'retreat attempts' in pigs 10 seconds after entry into a commercial Dip-Lift gas chamber mixtures; followed by 'escape attempts' and 'gasping' at 25 seconds exposure, with loss of balance at 30-32 seconds, muscular excitation at 33 seconds, and vocalisations at 35 seconds. Raj and Gregory (1996) described loss of posture occurring at 15-18 seconds of exposure to gas mixtures, vocalisations at 26-30 seconds and convulsions at 21-22 seconds (persisting for 17-33 s); while Raj et al. (1997) measured ECoG suppression beginning between 15 and 25 seconds of exposure, and an isoelectric trace (maximum effect) between 23 and 45 seconds. In comparison, for DTS energy exposures of 140-210 kJ (based on prototype 2 delivering only 70% energy as compared with prototype 3), Rault et al. (2014: **SS03**) estimated EEG changes to occur within 3 seconds of the end of each application, and the nadir (maximum effects) occurring within 4-22 s post application. In the DTS pilot study, by the time the Faraday cage was removed (approximately 20-30 s post energy application), DTS animals were unresponsive to stimuli, and the EEG recorded was suppressed. Furthermore, predictions of the time to threshold temperature in the DTS pilot study are 2.15 sec for a 30 kW application, and 3.23 sec for a 20 kW application. Thus, although DTS can also be considered a 'gradual' induction of insensibility, as the brain temperature is raised to the threshold level at which the 'immediate' lapse into unconsciousness occurs, the duration of this induction is substantially less than that experienced by animals undergoing gas inhalation.

Impacts on meat quality attributes

Carcase and quality assessments were carried out on 18 animals (Step 7). Visual inspection of the carcases showed no abnormality. Bruises were present on the bony tip of the pelvis on some animals, from both DTS and captive bolt treatment groups, and were deemed to be caused pre-slaughter during handling and restraint. No abnormalities of colour were noted. In general, there were no differences in pH declines between treatments, although five carcases showed a very rapid initial drop in pH post slaughter, reaching values below pH 6, while the carcase temperature was still above 35°C: Animals 1 (captive bolt), 5 (double application – total energy delivered 239.68 kJ), 7 (low energy DTS), 14 (very low energy DTS) and 16 (low energy DTS). This may be indicative of a raised metabolic rate and may suggest a tendency towards development of heat toughening, although this was not borne out by the meat quality analyses undertaken. There were also four animals for which the slope of the pH decline differed from others. These were animals: 1 (captive bolt); 14 (45.87 kJ); 16 (184.68 kJ) and 18 (217.62 kJ). Animal 14 received a very low amount of energy – there was an initial loss of posture, and convulsive movements began. There were no responses to stimuli, and the EEG trace was visibly suppressed, with intermittent bursts of activity. A risk of recovery was considered to be present after 5 minutes post energy application, and captive bolt was applied. Animal 16 spent a prolonged period in the head restraint prior to energy application (box entry to stun period 14 min 5 s) and return of blink and corneal reflexes were also noted prior to captive bolt application at 5 min 23 s post DTS application. Animal 18 similarly demonstrated return of reflexes, including the righting reflex, over 3 minutes post DTS application. It is possible that the return to consciousness process indicated by returning reflexes induces an increase in metabolic rate in these animals, when may contribute to alterations in the rate of pH decline.

After death, energy stored (as glycogen) in the animal's muscles is converted into lactic acid, making those muscles slightly acidic, i.e., lowering the muscle pH. Well-fed and well-rested animals normally have sufficient muscle energy reserves at processing to yield enough lactic acid to reduce the pH of muscles to near to 5.5. If an animal has lowered glycogen (usually as a result of tiredness, that could be due to 'exercise events' in the last few days before slaughter (e.g., long-distance transport, fighting, mustering) and has had insufficient time to re-establish the muscle energy reserves, the pH will not fall to below 5.8, and will yield what is known as 'dark-cutting' meat. When meat has an ultimate pH above 5.8:

- meat toughness increases;
- the colour of the meat is darker, and therefore less attractive to the consumer;
- the raw meat holds water, and feels dry or tacky to touch
- the meat becomes dry and chewy in texture when cooked;
- the meat oxidises (the muscle goes brown; and the fat becomes rancid) more rapidly

The converse quality problem encountered in meat is PSE – Pale, Soft, Exudative meat. It is more commonly associated with pigs and poultry, but has been reported in beef, particularly in young bulls, associated with slow chilling of the large muscles of the hindquarters while the metabolic rate is high. PSE occurs when the animal is well rested and has plenty of stored muscle energy at the time of slaughter – but something occurs that raises the metabolic rate. This results in a very rapid fall in pH, and sometimes to levels closer to pH 5.3. Events that could trigger this include acute stress immediately prior to slaughter (it has been demonstrated in pigs that have been pushed into 'fight or flight' mode through dreadful handling practices) and also activation of the nervous system by the stun process (it has been associated with electrical stun where the current has flowed for too long). PSE meat is characterised by:

- decreased meat toughness;
- pale colour;
- the meat does not hold water – it feels wet to touch, and a lot of fluid is seen in the packs, and when it is cooked, lots of water is released and the meat becomes dry and tough;
- the meat oxidises (the muscle goes brown; and the fat becomes rancid) more rapidly.

With regard to DTS, the concern is that either the electromagnetic field induced by the microwave energy, or the heating effect on the brain, or both, may induce an increase in metabolic rate, resulting in PSE-like changes in the meat. Therefore, the analyses carried out aimed to provide some information on toughness (shear force); colour; water holding capacity (drip loss); and oxidation (fat rancidity; TBARs) of DTS animals as compared with captive-bolt stunned animals.

In general, there were no significant differences between meat from DTS animals and meat from control (captive bolt stun) animals. Ultimate pH (pHu) in both groups was below pH 5.8, indicating that DFD was not induced, and pH values of loin and round at weeks 1 and 10 of storage lay within normal ranges (pH4 – pH 6).

There were some slight differences in meat colour at quartering, and in the first week after slaughter. DTS quarters were slightly yellower (greater MINOLTA b*) at quartering; and at 1 week post slaughter DTS loins were slightly redder (greater MINOLTA a*) and slightly yellower (greater MINOLTA b*); and DTS rounds were slightly lighter (greater MINOLTA L*) than control samples. However, these differences were marginal, and the values align with published data on MINOLTA colour attributes of loin (m. longissimus lumborum) and round (m. semitendinosus) (Onenc and Kaya, 2004; Sazili et al., 2013; Warner et al., 2007). In light of the small sample size (n=9 in each treatment group) these results should be interpreted with caution.

There was also a trend that DTS samples were more tender than control samples (Warner Bratzler Shear force measurement), but similarly, this trend should be interpreted with caution in light of the small sample size. Shear force values for loins at one week post slaughter in the DTS pilot study were 4.62 ± 0.34 kg and 4.01 ± 0.45 kg in control and DTS respectively, while at 10 weeks post slaughter these values were 3.45 ± 0.29 kg and 3.28 ± 0.39 respectively. These results lie within normal ranges: Warner et al. (2007) report values of 7.0 kg at 6 days post slaughter, and 4.8 kg at 21 days post slaughter; Gruber et al. (2010) report a range of 3.5 to 5.11 kg measured over a range of ageing periods from 3 to 28 days; while Sazili et al. (2013) report 9.19 ± 0.97 to 9.96 ± 0.72 kg at one week post slaughter. For rounds, the DTS pilot study measured 5.54 ± 0.25 kg for control and 4.84 ± 0.33 kg for DTS at one week post slaughter, and 5.46 ± 0.26 and 4.71 ± 0.36 kg respectively at week 10. These values again align with previously published ranges, for example 4 – 18 kg (Odusanya and Okubanjo, 1983), 4.12 ± 0.16– 6.63 ± 0.2 kg (Otremba et al., 1999) and 4.6 – 9.5 kg (Hwang et al., 2004).

The TBARs results are higher than would be expected from fresh meat. This is not reflective of the product or treatment but is due to a loss of temperature control during sample storage, after sample collection. The samples in week 1 underwent an unforeseen delay between collection and storage at -80°C, which will have resulted in excess lipid oxidation. For consistency, at week 10, we replicated the storage conditions encountered at week 1. Nevertheless, there were no significant differences in TBARs levels between the DTS and the captive bolt muscles.

Aversiveness of the system

The overall aversiveness of any stunning system can be considered to be made up of three main components:

- Pre-restraint handling;
- Restraint;
- The stunning method itself.

To minimize the adverse impacts of pre-restraint handling, low-stress stock handling by trained and competent personnel are recommended, according to normal commercial practice. Similarly, to minimize the adverse impacts of restraint, the units utilized of DTS: Diathermic Syncope are designed based on current best practice designs, specifically the ASPCA Pen design described by Grandin (1992) and Marshall (1963). Quiet handling and smooth application of the neck capture and chin lift apparatus is well tolerated by cattle (Dunn 1990, Grandin 1992) and the head position achieved allows accurate placement of the applicator onto the forehead of the animal. For DTS application, the is held in the chin lift for a maximum of 30 s while the applicator is positioned and DTS energy delivery initiated, as it is known that prolonged restraint in any system is stressful to animals (Ewbank, 1992).

Assessment of aversiveness of the final component, the stunning method itself, separate from the impacts of restraint and handling, is challenging, However, the fact that pain-related complexes were not evident in the EEG traces of minimally-anaesthetised animals (STEP 5; Rault, *et al.* 2014); and that endocrine and meat quality parameters from DTS cattle and carcases were not significantly different from captive bolt cattle and carcases when handled using the same pre-restraint handling and restraint unit (STEP 7), serve to indicate that the DTS:Diathermic Syncope system is no different in terms of overall aversiveness than captive-bolt stunning.

4.3.1.2 External validity

The purpose of the validation phase study (Step 11) was to assess on a pre-commercial scale, the potential for upscaling the technology to commercial beef processing.

Outcomes

During the entirety of the validation phase, 258 cattle were presented, of which 242 cattle were processed using DTS: Diathermic Syncope® as the stunning method (Table 16). The remaining 16 animals did not receive a DTS application due to technical faults resulting in generator shutdown prior to energy being delivered, and they were stunned using a captive bolt. Of the 242 animals, 8 received low energy deliveries due to technical faults and were deemed inadequately stunned, so were re-stunned using a penetrative captive bolt. Thus 234 of 242 (96.7%) cattle were stunned on first application of DTS, based on loss of posture and loss of corneal and somatic withdrawal reflexes (Von Holleben et al., 2010). Loss of posture, which is considered to be a definitive indicator of unconsciousness (EFSA, 2004; Muir, 2007; Von Holleben et al., 2010; EFSA, 2013), occurred between 1 and 8 seconds after onset of energy delivery.

Table 14: Summary of cattle stuns using DTS in all trials to date

Trial	Number presented for DTS	Number DTS delivered	Number stunned on first application	Percentage stunned on first application	Reasons for 'failures'
Rault et al. 2014 (anaesthetised cattle)	9	9	9	100	
P.PIP.0395	11	10	10	100	Technical fault 1 animal
P.PIP.0528 Upright knocking box	223	207	201	97.1	Technical fault 16 animals; Low energy delivery 6 animals
P.PIP.0528 Rotary box	35	35	33	94.3	Technical fault 2 animals - partial energy delivery
Totals	278	261	253	96.9	

The latency to onset of physical signs of insensibility was related to power setting. A higher power (kW), providing a more rapid delivery of the energy 'package', led to a shorter time to onset of physical signs of insensibility. The work on anesthetised cattle, carried out by Rault et al. (2014), indicated that shorter durations of applications resulted in more rapid onset of EEG suppression, while greater power settings resulted in a longer duration of insensibility. This is difficult to interpret fully, based on the small number of animals processed in their study and the fact that the animals in their study each received two applications of energy; however, there is a relationship between power setting and rate of heating. The units of power (kW) is shorthand for 'kJ per second', so a higher power setting will reduce the time required to reach an energy delivery of 200 kJ than a lower power setting. However, the higher power setting may predispose to focal overheating, as seen in the first phase of the DTS pilot study.

Based on the outcomes of this project, we recommend that operational guidelines define the desired range of energy application to be between 160 and 200 kJ.

The stunned animals demonstrated behavioural signs consistent with an electrical stun – rapid blinking or flicking of the eyelids including the membrana nictitans (third eyelid), loss of posture, tonic (stiff) and clonic (convulsive) phases (Table 17), so it is of value to compare the outcome of DTS against those reported for electrical stunning. In cattle, electrical stunning can be applied as head-only, which affects the brain, resulting in an epileptiform seizure, from which the animal can regain consciousness; or as head-to-chest, which incorporates a current passed through the heart, resulting in cardiac arrest, and the death of the animal. As DTS does not directly affect cardiac function, the comparison should be against head-only electrical stunning.

Table 15: Behavioural indicators of unconsciousness following DTS application, as compared with electrical stun.

Behavioural sign	*DTS*	*EFSA scientific opinion 2004*	*AMIC animal welfare standard*
Immediate collapse	☑*	☑*	☑*
Epileptiform seizure (described in detail)	☑†	☑	☑
Lack of normal rhythmic breathing	☑#	☑	☑
No spontaneous eye blinking	☑		☑
Gasping (breathing in without breathing out) sometimes occurs	☑		☑
Upward rotation of eyes		☑	
Dilated pupils		☑	
No response to nose prick (painful stimulus)	☑	☑	

*: Row 1: With regards to induction of insensibility prior to slaughter, there is much discussion over the ‘immediacy’ of the lapse into unconsciousness. in electrical stunning, the current must flow for a period of at least 2 (sheep) or 5 (cattle) seconds in order to ensure a sustained loss of consciousness (Warrington, 1974, Von Holleben et al., 2010).

†: Row 2, Column 1: Due to the fixed head restraint, full body collapse was not evident. However, the haunches initially dropped, followed by a tonic phase, and then convulsive movements or paddling of the limbs.

#: Row 3, Column1: In the DTS validation study, a short suspension of respiration was evident in some animals, but not all. However, when respiration was present it was much slower and deeper than in the conscious animal.

The biggest problem with head-only stunning of cattle is a very short duration of epilepsy, followed by strong convulsions (EFSA, 2004). The electrical current must be applied across the brain for sufficient duration to overcome the inherent impedance (resistance) across the head, and application of electrical stun ranges from 4 to greater than 20 seconds (Devine et al., 1986; Vonmickwitz et al., 1989; Schatzmann and Jaggin-Schmucker, 2000). The duration of unconsciousness in those studies was reported as 31 - 90 seconds. In contrast, DTS induces loss of posture within 8 seconds of onset of energy application, and the duration of unconsciousness recorded during the DTS validation study was between 100 and 240 seconds, based on absence of corneal reflex, and duration of EEG changes.

Return of reflexes are considered to be the earliest indication that consciousness is returning and was the point at which penetrative captive bolt was applied during the DTS validation study for animal welfare reasons. In the DTS validation study this occurred between 2 and 4.5 minutes from loss of posture, which would allow ample time to exsanguinate the animal prior to recovery. Using the rotary box, when a delay to capture EEG data was not required, sticking was performed within 30 seconds of DTS application.

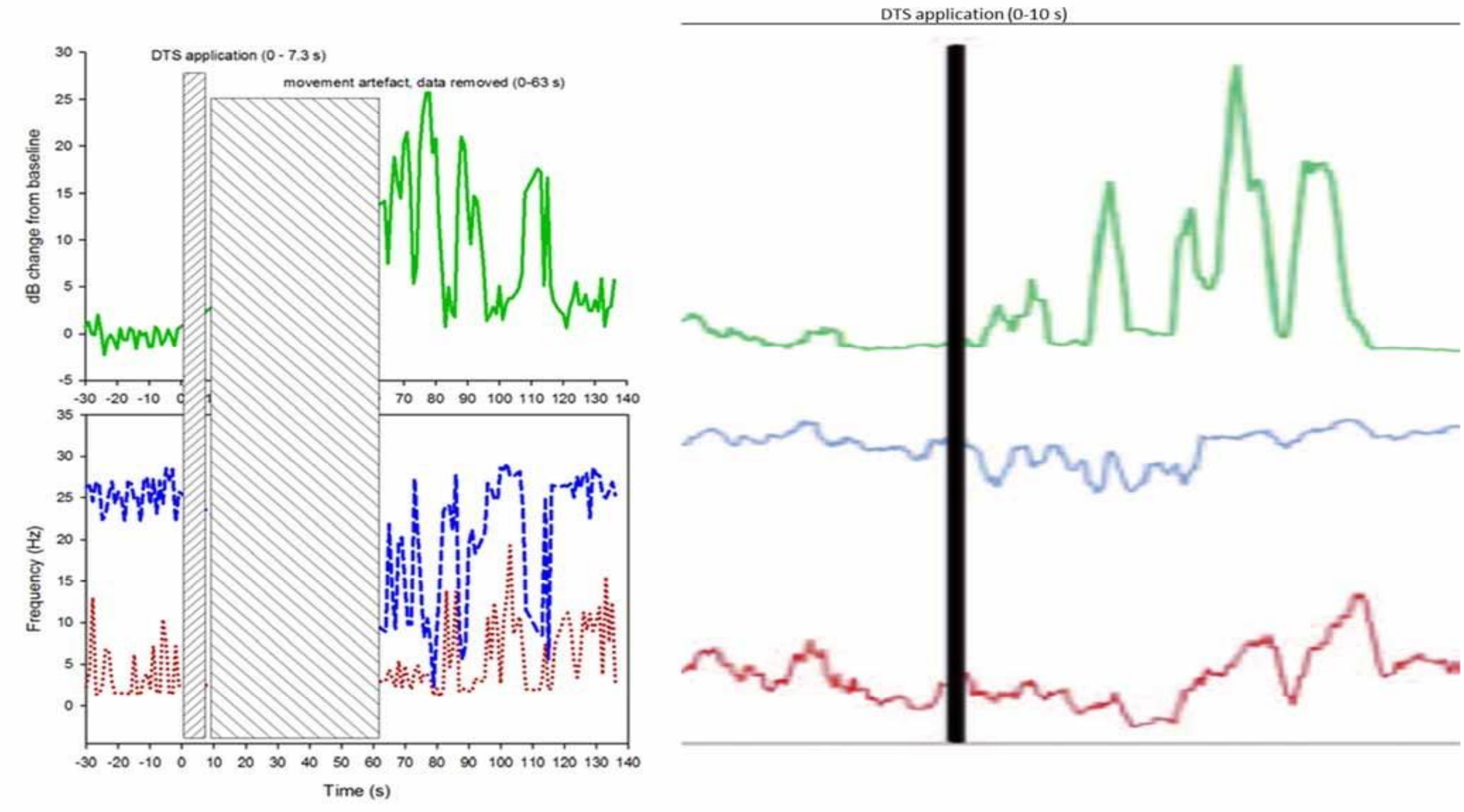
In the early stages of the project, overheating of the skin surface was identified. This was associated with incident power levels of > 25 kW. This was eliminated in the later stages of the project, through optimisation of the auto-tuning device and applicator apparatus such that incident power levels ranged from 18-20 kW. Australian cattle may experience ambient air temperatures of 45 °C in summer and could experience higher surface temperatures when standing in direct sunlight. Surface temperatures above 50 °C may be uncomfortable to cattle, although no literature on thermal contact sensitivity in cattle could be located to confirm or refute this supposition.

Figure 15 shows an example of the EEG data collected during the preliminary confirmation phase, compared against a similar chart reproduced from Rault et al. (2014: **SS03**), both showing a period of 2.5 – 3 minutes post-DTS application, scaled to similar length. Although the software and detail of smoothing methods used to generate these charts differ, clear similarities are evident: total EEG power (Ptot, green line) is dramatically increased and shows high amplitude spiking in the two-minute period post-DTS application; 95% spectral edge frequency (SEF95, blue line) and 50% spectral edge frequency (SEF50, red line) drop following DTS application, and then begin to return towards baseline. These EEG patterns were re-confirmed during the rotary box phase (Step 11).

In the DTS validation study, movement artefact posed a significant challenge to EEG recording, as the animals began to twitch and convulse, similar to the movements seen during the phases of an electric stun. This movement artefact was not present in the Rault et al. study (2013: **SS03**), as the animals were anaesthetised. Furthermore, in the DTS validation study, background electrical noise from the abattoir infrastructure tended to interfere with the EEG signal, so interpretation of the data, particularly the spectral edge frequencies, was challenging.

All the animals for which usable EEG recordings were collected showed changes from baseline, particularly in terms of Ptot, showing high amplitude spiking. These patterns on EEG are considered to be incompatible with sensibility (EFSA, 2004) and during this period the animal cannot feel pain.

Figure 15: Examples of EEG data. Left: Animal 10 in the DTS validation study, Right: Animal 6 from Rault et al. 2014 (reproduction of an approximate 3-minute block). Green indicates total EEG power (Ptot) as change from baseline, blue indicates 95% spectral edge frequency (SEF95) and red indicates 50% spectral edge frequency (SEF50).



4.3.1.3 Equivalence summary

Table 16: Equivalence of DTS: Diathermic Syncope® with existing stunning methods for cattle

	DTS	**Mechanical stunning (penetrative)**	**Electrical head-only Stunning**	**Electrical head-to chest stunning**
Per-restraint handling	Forcing pen and single-file race feed to restraint	Forcing pen and single-file race feed to restraint	Forcing pen and single-file race feed to restraint	Forcing pen and single-file race feed to restraint
Restraint type	Upright individual restraint with neck capture and chin lift	Upright individual restraint. Neck capture and chin lift improve first-shot efficacy	Upright individual restraint with neck capture and chin lift	Upright individual restraint with neck capture and chin lift
Minimum restraint duration (prior to onset of application)	10 s	10 s	10 s	10 s
Duration of application	9 – 10 s (simple stun) ≥ 15 s (kill)	< 1 s	4 - >20 s	4 - >20 s
Latency to behavioural response	3 s (eye and ear twitching) 8 s (loss of posture)	< 1 s	4 - >20 s	4 - >20 s
Latency to changes in EEG	12 – 50 s from onset of energy application	0 – 14 s post application	Data not located	Data not located
Duration of insensibility	120 – >240 s (simple stun) Indefinite (kill)	indefinite	31 – 90 s	indefinite

5. Conclusion

Overall integration of the findings of all studies indicated that 100 % insensibility was achieved when the desired energy package was delivered. Existing stunning techniques in Australia, where conditions are carefully controlled, are expected to deliver a 95-98 % first-stun efficacy (AMIC, 2009) but, particularly where adequate neck and head restraint is not used, can have up to 29 % first stun failure rates (Blackmore, 1979; Lambooy et al., 1981; Hoffmann, 2003; Endres, 2005; Octavio-Oliveira et al., 2018; Gibson et al., 2019). The animal stunned using DTS can also return to consciousness, so this method of stunning is likely to be acceptable to the Halal and Kosher markets. Furthermore, blood flow was observed to be strong, and the rhythmic pumping of the heart was evident in the first few seconds of blood flow. Both these characteristics are desired by the Halal and Kosher markets.

Use of the rotary box optimised handling of the stunned body, allowing a short stun-to-stick interval without the use of an immobiliser. The updated applicator configuration allowed consistent repeatable induction of insensibility, which was sustained during Halal bleeding, without the need to apply an additional ventral cut.

5.1 Key findings

- In the absence of technical problems leading to interrupted delivery of energy, all animals were rendered unconscious using DTS.
- Power settings of 18 kW led to a slower onset of insensibility, while power settings of 20 kW provided a rapid onset of insensibility. Power settings of 25 kW and above led to overheating at the skin surface.
- Energy deliveries of 160 kJ and less resulted in shorter duration of insensibility with a risk of early return to consciousness, while energy deliveries of 220 kJ and above resulted in early onset of intense convulsive activity.
- Live observations indicated that DTS consistently induces insensibility, with no vocalisation, no evidence of pain or distress, and EEG data confirmed the development of a high-amplitude-low-frequency (HALF) epileptiform state.
- There was no requirement to use an immobiliser during application of the exsanguination cuts, and the animal bled rapidly to brain death using the Halal cut alone. The blood flow on exsanguination was strong and rapid.
- Visual inspection of the brains of carcases indicated no visible damage when energy deliveries of less than 220 kJ were applied.

5.2 Benefits to industry

- DTS is an effective means of inducing insensibility in cattle, with a duration of insensibility suited to exsanguination using the Halal cut. The project was planned and executed under end-user-centred design principles, with the Halal and Kosher markets in mind. Key parameters that are likely to be viewed favourably when compared against the current stunning method are:
- There was no requirement to perform a back-up ventral cut (thoracic stick), as the blood flow was strong and exsanguination was rapid;
- Blood flow was visibly strong, and the pulsations associated with heart function were visible in the early stages of bleed-out;
- Exsanguination could be performed safely, without the need for an immobiliser;

- Visual inspection of the brains of carcases indicated no visible damage when energy deliveries of less than 220 kJ were applied, and the stun did not cause cracks in the skull, so eliminates a source of rejection against Halal requirements, and eliminates a potential source of breaches noted on audit when the auditors' interpretation of 'cracked' differs from the regular inspector;
- The stun is effective in heavy animals and bulls, which currently pose challenges to processors using percussive stunning;
- To date, no evidence of blood splash or ecchymosis has been encountered in any of the carcases processed.
- The animal stunned using DTS can also return to consciousness, so this method of stunning is likely to be acceptable to the Halal and Kosher markets.

Approval to use the DTS: Diathermic Syncope® technology under an Approved Arrangement in the Australian Meat Industry was granted.

Submission of the technical dossier to EFSA is planned.

6. Future research and recommendations

In order to secure global acceptance of the technology and maintain market access as the technology is commercialised within Australia, it is vital that communication with overseas regulatory authorities is communicated.
In particular, engagement with the Halal and Kosher markets is important, as this technology may be suited.
Development of the technology for use in other species is warranted.

7. References

AMIC (2009). "Industry Animal Welfare Standards: Livestock Processing Establishments." Crows Nest NSW, Australian Meat Industry Council: 63 pages.

ANIL, M.H. AND J.L. MCKINSTRY (1991). "Reflexes and loss of sensibility following head-to-back electrical stunning in sheep." Veterinary Record **128**: 106-107.

ANIL, M.H., J.L. MCKINSTRY, S.B. WOTTON AND N.G. GREGORY (1995)a. "Welfare of calves 1 - Investigations into some aspects of calf slaughter." Meat Science **41**(2):101-112.

ANIL, M.H., J L. MCKINSTRY, N.G. GREGORY, S.B. WOTTON AND H. SYMONDS (1995)b. "Welfare of Calves 2 - Increase in Vertebral Artery Blood Flow Following Exsanguination by Neck Sticking and Evaluation of Chest Sticking as an Alternative Slaughter Method." Meat Science **41**(2): 113-123.

AVMA (2013). "The AVMA Guidelines for the Euthanasia of Animals: 2013 Edition." American Veterinary Medical Association. Schaumburg, IL: 102.

BENARROCH, E.E. (2001). "Pain-autonomic interactions: a selective review." Clinical Autonomic Research **11**: 343-349. http://dx.doi.org /10.1007/BF02292765.

BLACKMORE, D.K. (1979). "Non-penetrative percussion stunning of sheep and calves." The Veterinary Record **105**: 372-375.

BLACKMORE, D.K. AND J.C. NEWHOOK (1982). "Electroencephalographic studies of stunning and slaughter of sheep and calves - Part 3: The duration of insensibility induced by electrical stunning in sheep and calves." Meat Science **7**: 19-28.

BOUTON, P.E. AND P.V. HARRIS (1972). "A comparison of some objective methods used to assess meat tenderness." Journal of Food Science **37**:218-221.

BOUTON, P.E., P.V. HARRIS AND W.R. SHORTHOSE (1971). "Effect of ultimate pH upon the water-holding capacity and tenderness of mutton." Journal of Food Science **36**:435-439.

COENEN, A.M.L. (1998) "Neuronal phenomena associated with vigilance and consciousness: from cellular mechanisms to electroencephalographic patterns." Consciousness and Cognition **7**: 42-53. http://dx.doi.org/10.1006/ccog.1997.0324.

COSI, C. AND M. MARIEN (1998). "Decreases in mouse brain NAD(+) and ATP induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP): prevention by the poly(ADP-ribose) polymerase inhibitor, benzamide." Brain Research **809**(1): 58-67.

DALMAU, A., P. RODRIGUEZ, P. LLONCH AND A. VELARDE (2010). "Stunning pigs with different gas mixtures: aversion in pigs." Animal Welfare **19**: 325-33.

DALY, C.C., N.G. GREGORY AND S.B. WOTTON (1987). "Captive bolt stunning of cattle: effects on brain function and role of bolt velocity." British Veterinary Journal **143**: 574-580.

DALY, C.C. AND P.E. WHITTINGTON (1989). "Investigation into the principal determinants of effective captive bolt stunning of sheep." Research in Veterinary Science **46**: 406-408.

DELANEY, S.M. AND J.D. GEIGER (1996). "Brain regional levels of adenosine and adenosine nucleotides in rats killed by high-energy focused microwave irradiation." Journal of Neuroscience Methods **64**(2): 151-156.

DEVINE, C.E., K.V. GILBERT, A.E. GRAAFHUIS, A. TAVENER, H. REED AND P. LEIGH (1986). "The effect of electrical stunning and slaughter on the electroencephalogram of sheep." Meat Science **17**: 267-281. http://dx.doi.org/10.1016/0309-1740(86)90045-8.

DUNN, C.S. (1990). "Stress reactions of cattle undergoing ritual slaughter using two methods of restraint." The Veterinary Record **126**: 522-525.

EC (2009). "Council Regulation (EC) No 1099/2009 of 24 September 2009 on the protection of animals at the time of killing." EC1099/2009. EC: L303/301-L303/330.

EFSA (2004). "Welfare aspects of animal stunning and killing methods." Parma, Italy, European Food Safety Authority: 241 pages.

EFSA (2013). "Guidance on the assessment criteria for studies evaluating the effectiveness of stunning interventions regarding animal protection at the time of killing." EFSA Journal **11**(12): 3486-3527.

EFSA PANEL ON ANIMAL HEALTH AND ANIMAL WELFARE, S. MORE, D. BICOUT, A. BØTNER, A. BUTTERWORTH, P. CALISTRI, K. DEPNER, S. EDWARDS, B. GARIN-BASTUJI, M. GOOD, C. GORTÁZAR SCHMIDT, M.A. MIRANDA, S. SAXMOSE NIELSEN, A. VELARDE, H.-H. THULKE, L. SIHVONEN, H. SPOOLDER, J.A. STEGEMAN, M. RAJ, P. WILLEBERG, C. WINCKLER, R. MARANO, F. VERDONCK, D. CANDIANI AND V. MICHEL (2018). "Guidance on the assessment criteria for applications for new or modified stunning methods regarding animal protection at the time of killing." EFSA Journal **16**(7): e05343.

EFSA PANEL ON ANIMAL HEALTH AND ANIMAL WELFARE, NIELSEN, S.S., J. ALVAREZ, D.J. BICOUT, P. CALISTRI, K. DEPNER, J.A. DREWE, B. GARIN-BASTUJI, J.L. GONZALES ROJAS, C.G. SCHMIDT, V. MICHEL, M.A. MIRANDA CHUECA, H.C. ROBERTS, L.H. SIHVONEN, H. SPOOLDER, K. STAHL, A. VELARDE, A. VILTROP, D. CANDIANI, Y. VAN DER STEDE AND C. WINCKLER (2020). "Welfare of cattle at slaughter." EFSA Journal **18**(11): 6275.

ENDRES, J.M. (2005). “Effektivität der Schuss-Schlag-Betäubung im Vergleich zur Bolzenschussbetäubung von Rindern in der Routineschlachtung (Effectiveness of percussive versus penetrative stunning in cattle).” Doctorate, Ludwig-Maximilians-Universität.

EWBANK, R., M.J. PARKER AND C.W. MASON (1992). "Reactions of cattle to head-restraint at stunning: a practical dilemma." Animal Welfare **1**: 55-63.

FIKE, J.R., G.T. GOBBEL, T. SATOH AND P.R. STAUFFER (1991). "Normal brain response after interstitial microwave hyperthermia." International Journal of Hyperthermia **7**(5): 795-808.

FOOD REGULATION STANDING COMMITTEE (2007). “Australian Standard for the Hygienic Production of Meat and Meat Products for Human Consumption.” SCARM report 80. S. CSIRO Publishing. 4696:2007: 72 pages. http://www.publish.csiro.au/pid/5553.htm.

FRANKEN, N.A.P., H.H. DEVRIND, P. SMINIA, J. HAVEMAN, D. TROOST AND D.G. GONZALEZ (1992). "Neurological complications after 434 MHz microwave hyperthermia of the rat lumbar region including the spinal-cord." International Journal of Radiation Biology **62**(2): 229-238.

GIBSON, T.J., JOHNSON, C.B., MURRELL, J.C., MITCHINSON, S.L., STAFFORD, K.J. AND MELLOR, D.J. (2009) “Electroencephalographic responses to concussive non-penetrative captive-bolt stunning in halothane-anaesthetised calves.” New Zealand Veterinary Journal **57**: 90-95. http://dx.doi.org/10.1080/00480169.2009.36884.

GIBSON, T.J., JOHNSON, C.B., STAFFORD, K.J., MITCHINSON, S.L. AND MELLOR, D.J. (2007) “Validation of the acute electroencephalographic responses of calves to noxious stimulus with scoop dehorning.” New Zealand Veterinary Journal **55**: 152-157. http://dx.doi.org/ 10.1080/00480169.2007.36760.

GIBSON, T.J., S.E. OCTAVIO OLIVEIRA, F.A. DALLA COSTA AND N.G. GREGORY (2019). "Electroencephalographic assessment of pneumatically powered penetrating and non-penetrating captive-bolt stunning of bulls." Meat Science **151**: 54-59.

GOUVEIA, K.G., FERREIRA, P.G., DA COSTA, J.C.R., VAZ-PIRES, P., DA COSTA, P.M. (2009). “Assessment of the efficiency of captive-bolt stunning in cattle and feasibility of associated behavioural signs.” Animal Welfare **18**: 171-175.

GRACEY, J.F., COLLINS, D.S. (1992) “Meat Hygiene.” London, UK. Bailliere Tindall. ISBN 0702014958, 9780702014956.

GRANDIN, T. (1992). "Observations of cattle restraint devices for stunning and slaughtering." Animal Welfare **1**: 85-90.

GRANDIN, T. (2002). “Animal welfare during transport and slaughter.” Animal Welfare and Animal Health: proceedings of Workshop 5 on Sustainable Animal Production, organized by the Institute for Animal Science and Animal Behaviour, Federal Agricultural Research Centre (FAL), Mariensee, held September 4-5, 2000, Braunschweig, Germany, Bundesforschungsanstalt für Landwirtschaft (FAL): pages 47-56.

GRANDIN, T. AND J.M. REGENSTEIN (1994). “Slaughter: Religious slaughter and animal welfare, a discussion for meat scientists.” Meat Focus International **March**: 115-123.

GREGORY, N.G. (2005). "Recent concerns about stunning and slaughter." Meat Science **70**: 481-491.

GREGORY, N.G., H.R. FIELDING, M. VON WENZLAWOWICZ AND K. VON HOLLEBEN (2010). "Time to collapse following slaughter without stunning in cattle." Meat Science **85**(1): 66-69.

GREGORY, N.G. AND T. GRANDIN (2007). “Stunning and slaughter.” *In* Animal welfare and meat production. *Eds* N.G. GREGORY AND T. GRANDIN. Wallingford, Oxfordshire, UK, CABI publishing: 191-212.

GRUBER S.L., J.D. TATUM, T.E. ENGLE, P.L. CHAPMAN, K. E. BELK AND G.C. SMITH (2010). “Relationships of behavioral and physiological symptoms of preslaughter stress to beef longissimus muscle tenderness.” Journal of Animal Science **88**, 1148-1159.

GUY, A.W. AND C.K. CHOU (1982). “Effects of high-intensity microwave pulse exposure of rat-brain.” Radio Science **17**: S169-S178.

HEMSWORTH, P.H. AND J.L. BARNETT (2001). “Human–animal interactions and animal stress. The biology of animal stress – Basic principles and implications for animal welfare.” G. P. Moberg and J. A. Mench. Oxon, UK, CABI Publishing: 309-336.

HJERESEN, D.L., A.W. GUY, F.M. PETRACCA AND J. DIAZ (1983). "A microwave-hyperthermia model of febrile convulsions." Bioelectromagnetics **4**(4): 341-355.

HOFFMANN, A. (2003). “Implementierung der Schuss-Schlag-Betäubung im zugelassenen Schlachtbetrieb [Implementation of concussion stunning in EU approved abattoirs].” Doctorate, Ludwig-Maximilians-Universität.

HONIKEL, K.O., C.J. KIM, R. HAMM AND P. RONCALES (1986). "Sarcomere shortening of prerigor muscles and its influence on drip loss." Meat Science **16**(4): 267-282.

HWANG I.H., PARK B.Y., CHO S.H., LEE J.M. (2004). “Effects of muscle shortening and proteolysis on Warner-Bratzler shear force in beef longissimus and semitendinosus.” Meat Science **68**: 497-505.

IKARASHI, Y., MARUYAMA, Y. AND STAVINOHA, W. B. (1984). “Study of the use of the microwave magnetic-field for the rapid inactivation of brain-enzymes.” Japanese Journal of Pharmacology **35**: 371-387.

JOHNSON, C.B., K.J. STAFFORD, S.P. SYLVESTER, R.N. WARD, S. MITCHINSON AND D.J. MELLOR (2005). “Effects of age on the electroencephalographic response to castration in lambs anaesthetised using halothane in oxygen.” New Zealand Veterinary Journal **53**: 433-437. http://dx.doi.org/10.1080/00480169.2005.36589.

KILKENNY, C., W.J. BROWNE, I.C. CUTHILL, M. EMERSON and D.G. ALTMAN (2010). "Improving Bioscience Research Reporting: The ARRIVE Guidelines for Reporting Animal Research." Plos Biology **8**(6): e1000412.

LAMBOOIJ, E., M.H. ANIL, S.R. BUTLER, H. REIMERT, L. WORKEL AND V. HINDLE (2011). "Transcranial magnetic stunning of broilers: a preliminary trial to induce unconsciousness." Animal Welfare **20**(3): 407-412.

LAMBOOY, B., J. LAGENIJK AND G. VAN RHOON (1989). "Feasibility of stunning slaughter pigs with microwaves at 434 MHz." Fleischwirtschaft. **1989**(6): 1030-1032.

LAMBOOY, E. AND W. SPANJAARD (1982). "Electrical stunning of veal calves." Meat Science **6**(1): 15-25.

LAMBOOY, E., W. SPANJAARD AND G. EIKELENBOOM (1981). "Concussion stunning of veal calves." Fleischwirtschaft **61**(1): 128-130.

LERMAN, O., Y. BRUCHIM, E. KELMER AND I. LENCHNER (2014). "Concurrent Heatstroke and Insulinoma in a Dog: A Case Report." Israel Journal of Veterinary Medicine **69**(1): 45-49.

LLONCH, P., A. DALMAU, P. RODRIGUEZ, X. MANTECA AND A. VELARDE (2012). "Aversion to nitrogen and carbon dioxide mixtures for stunning pigs." Animal Welfare **21**: 33-39.

LYONS, B.E., R.H. BRITT AND J.W. STROHBEHN (1984). "Localized hyperthermia in the treatment of malignant brain-tumors using an interstitial microwave antenna-array." IEEE Transactions on Biomedical Engineering **31**(1): 53-62.

MARSHALL, M., E.E. MILLBURY AND E.W. SHULTZ (1963) "Apparatus for holding cattle in position for slaughtering." US Patent 3,087,195. Washington DC.

MARUYAMA, Y., H. KIZAKI AND K. OTAKE (1986). "Microwave heating appts. for irradiating animals in biochemistry has waveguide forming directional coupler with loops detecting reflected and incident microwave energy." New Japan Radio Co Ltd; New Japan Nitrogen Kk.

MERRITT, J.H., A F. CHAMNESS, R.H. HARTZELL AND S.J. ALLEN (1977). "Orientation effects on microwave-induced hyperthermia and neurochemical correlates." Journal of Microwave Power and Electromagnetic Energy **12**(2): 167-172.

MCDANIEL, H.B., R.L. JENKINS AND H.G. MCDANIEL (1991). "Experimental hyperthermia - protective effect of oxygen carrying fluorocarbon and crystalloids intraperitoneally." American Journal of the Medical Sciences **301**(1): 9-14.

MCKEEGAN, D.E.F., J.A. MCINTYRE, T.G.M. DEMMERS, J.C. LOWE, C.M. WATHES, P.L.C. VAN DEN BROEK, A.M.L. COENEN AND M.J. GENTLE (2007). "Physiological and behavioural responses of broilers to controlled atmosphere stunning: implications for welfare." Animal Welfare **16**: 409-426.

MCLEAN, D., L. MEERS, J. RALPH, J. S. OWEN AND A. SMALL (2017). "Development of a microwave energy delivery system for reversible stunning of cattle." Research in Veterinary Science 112(1): 13-17.

MITCHELL, G., J. HATTINGH, AND M. GANHAO (1988). "Stress in cattle assessed after handling, after transport and after slaughter." Veterinary Record **123:** 201-205.

MOHANTY, D., J. GOMEZ, K.Y. MUSTAFA, M. KHOGALI AND K.C. DAS (1997). "Pathophysiology of bleeding in heat stress: An experimental study in sheep." Experimental Hematology **25**(7): 615-619.

MOHER, D., A. LIBERATI, J. TETZLAFF, D.G. ALTMAN and P.G. THE (2009). "Preferred Reporting Items for Systematic Reviews and Meta-Analyses: The PRISMA Statement." PLOS Medicine **6**(7): e1000097.

MOROJI, T., K. TAKAHASHI, K. OGURA, T. TOISHI AND S. ARAI (1977). "Rapid microwave fixation of rat-brain." Journal of Microwave Power and Electromagnetic Energy **12**(4): 273-286.

MUIR, W.W. (2007). "Considerations of General Anaesthesia. Lumb and Jones' Veterinary Anesthesia and Analgesia." Eds: W.J. TRANQUILLI, J.C. THURMON AND K.A. GRIMM. Chichester, UK, Wiley-Blackwell.

NAMI (2017). "Recommended animal handling guidelines & audit guide: A systematic approach to animal welfare." North American Meat Institute. 134 pages. http://www.animalhandling.org/sites/default/files/forms/animal-handling-guidelines-Nov32017.pdf.

NEWHOOK, J.C. AND D.K. BLACKMORE (1982). "Electroencephalographic studies of stunning and slaughter of sheep and calves. II." Meat Science **6**:295-300.

NHMRC (2013). "Australian code for the care and use of animals for scientific purposes. 8th Edition. Canberra: National Health and Medical Research Council." 96 pages. ISBN Online: 1864965975. https://www.nhmrc.gov.au/about-us/publications/australian-code-care-and-use-animals-scientific-purposes.

NIEMARK, M.A., A.-A. KONSTAS, A.F. LAINE AND J. PILE-SPELLMAN (2007). "Integration of jugular venous return and circle of Willis in a theoretical human model of selective brain cooling." Journal of Applied Physiology **103**: 1837-1847. http://dx.doi.org/10.1152/japplphysiol.00542.2007.

NORDGREN, I., G. LUNDGREN, G. PUU, B. KARLEN AND B. HOLMSTEDT (1985). "Distribution and elimination of the stereoisomers of soman and their effect on brain acetylcholine." Fundamental and Applied Toxicology **5**(6): S252-S259.

NOWAK B., T.V. MUEFFLING AND J. HARTUNG (2007). "Effect of different carbon dioxide concentrations and exposure times in stunning of slaughter pigs: Impact on animal welfare and meat quality." Meat Science **75**, 290-298.

OCTAVIO OLIVEIRA, S.E., F.A. DALLA COSTA, T.J. GIBSON, O.A. DALLA COSTA, A. COLDEBELLA AND N.G. GREGORY (2018). "Evaluation of brain damage resulting from penetrating and non-penetrating stunning in Nelore Cattle using pneumatically powered captive bolt guns." Meat Science **145**: 347-351.

ODUSANYA S.O. AND A.O. OKUBANJO (1983). "Shear Force Values for Steaks from the Semitendinosus Muscle of Pre-Rigor Leg-Twisted Beef Carcasses." Journal of Food Science **48**, 1577-1578.

OHSHIMA, T., H. MAEDA, T. TAKAYASU, Y. FUJIOKA AND T. NAKAYA (1992). "An autopsy case of infant death due to heat-stroke." American Journal of Forensic Medicine and Pathology **13**: 217-221.

OIE (2015). "Terrestrial Animal Health Code." Paris, France, World Organisation for Animal Health: 739. http://www.oie.int/international-standard-setting/terrestrial-code/access-online/.

ONENÇ A., AND A. KAYA (2004). "The effects of electrical stunning and percussive captive bolt stunning on meat quality of cattle processed by Turkish slaughter procedures." Meat Science B: 809-815.

OTREMBA M.M., M.E. DIKEMAN, G.A. MILLIKEN, S.L. STRODA, J.A. UNRUH AND E. CHAMBERS (1999). "Interrelationships among evaluations of beef longissimus and semitendinosus muscle tenderness by Warner-Bratzler shear force, a descriptive-texture profile sensory panel, and a descriptive attribute sensory panel." Journal of Animal Science **77**: 865-873.

R CORE TEAM (2014). "R: A Language and Environment for Statistical Computing." Vienna, Austria, R Foundation for Statistical Computing. http://www.R-project.org.

RAJ, A.B.M. (1996). "Aversive reactions of turkeys to argon, carbon dioxide and a mixture of carbon dioxide and argon." Veterinary Record **138**: 592-593.

RAJ, A.B.M. AND N.G. GREGORY (1995). "Welfare implications of the gas stunning of pigs .1. Determination of aversion to the initial inhalation of carbon-dioxide or argon." Animal Welfare **4**: 273-280.

RAJ, A.B.M. AND N.G. GREGORY (1996). "Welfare implications of the gas stunning of pigs .2. Stress of induction of anaesthesia." Animal Welfare **5**(1): 71-78.

RAJ, A.B.M., S.P. JOHNSON, S.B. WOTTON AND J.L. MCINSTRY (1997). "Welfare implications of gas stunning pigs .3. The time to loss of somatosensory evoked potentials and spontaneous electrocorticogram of pigs during exposure to gases." Veterinary Journal **153**: 329-339.

RALPH, J.H., J.S. OWEN, A.H. SMALL, D.W. MCLEAN AND D.J. GAILER (2011). "Animal Stunning." Patent AU 2010901954 (20100507), 9th May 2011. Australian Patent Office: Canberra, Australia. www.patentscope.wipo.int.

RAULT, J.L., P.H. HEMSWORTH, P.L. CAKEBREAD, D.J. MELLOR AND C.B. JOHNSON (2014). "Evaluation of microwave energy as a humane stunning technique based on electroencephalography (EEG) of anaesthetised cattle." Animal Welfare 23, 391-400.

ROCCATTO, L., A. MODENESE, V. OCCHIONERO, A. BARBIERI, D. SERRA, E. MIANI AND F. GOBBA (2010). "Heat stroke in the workplace: description of a case with fatal outcome." Medicina Del Lavoro **101**(6): 446-452.

RULOFSON, F.C., D.E. BROWN AND R.A. BJUR (1988). "Effect of Blood Sampling and Shipment to Slaughter on Plasma Catecholamine Concentrations in Bulls 1." Journal of Animal Science **66**: 1223-1229.

RYAN, T.P., B.S. TREMBLY, D.W. ROBERTS, J.W. STROHBEHN, C.T. COUGHLIN AND P.J. HOOPES (1994). "Brain hyperthermia .1. Interstitial microwave antenna-array techniques - the Dartmouth experience." International Journal of Radiation Oncology Biology Physics **29**(5): 1065-1078.

SANDERS, A.P. AND W.T. JOINES (1984). "The effects of hyperthermia and hyperthermia plus microwaves on rat-brain energy-metabolism." Bioelectromagnetics **5**(1): 63-70.

SANDILANDS, V., A.B.M. RAJ, L. BAKER AND N.H.C. SPARKS (2011). "Aversion of chickens to various lethal gas mixtures." Animal Welfare **20**: 253-262.

SAZILI A.Q., B. NORBAIYAH, I. ZULKIFLI, Y.M. GOH, M. LOTFI AND A.H. SMALL (2013). "Quality Assessment of Longissimus and Semitendinosus Muscles from Beef Cattle Subjected to Non-penetrative and Penetrative Percussive Stunning Methods." Asian-Australasian Journal of Animal Sciences **26**: 723- 731.

SCARM (2002). "Model code of practice for the welfare of animals: livestock at slaughtering establishments." Collingwood, Primary Industries Standing Committee: Standing Committee on Agriculture and Resource Management: 34 pages.

SCHATZMANN, U. AND N. JAGGIN-SCHMUCKER (2000). "Electrical stunning in cattle before exsanguination." Schweizer Archiv Fur Tierheilkunde **142**(5): 304-308.

SCHWARTZ, W. (1974). "Pig Stunning System for Abattoirs Using Microwave Beam Directed at Temple." Patent DE 4116670A1. German Patent Office. www.depatisnet.dpma.de.

SCHWARTZ, W. (1992). "Microwave animal stunning equipment for slaughter has electromagnetic radiation units in semicircle around head of animal". Patent DE4116670-A. German Patent Office. www.depatisnet.dpma.de.

SHAW, F.D. AND R.K. TUME (1990). "Beta-endorphin and cortisol concentrations in plasma of cattle." Australian Veterinary Journal **67**: 423-424.

SMALL, A., J. LEA, D. NIEMEYER, J. HUGHES, D. MCLEAN AND J. RALPH (2019). "Development of a microwave stunning system for cattle 2: Preliminary observations on behavioural responses and EEG." Research in Veterinary Science 122: 72-80.

SMALL, A., D. MCLEAN, J.S. OWEN AND J.H. RALPH (2013)a. "Electromagnetic induction of insensibility in animals: a review." Animal Welfare 22: 287-290.

SMALL, A., D. MCLEAN, H. KEATES, J.S. OWEN AND J. RALPH (2013)b. "Preliminary investigations into the use of microwave energy for reversible stunning of sheep." Animal Welfare 22: 291-296.

SMINIA, P., J. VANDERZEE, J. WONDERGEM AND J. HAVEMAN (1994). "Effect of Hyperthermia on the Central-Nervous-System - a Review." International Journal of Hyperthermia **10**(1): 1-30.

TAKAMURA, F. AND K. ISHIDA (1997). "Killing Domesticated animals, e.g., Poultry Uses Magnetron Oscillator to Irradiate Brain Tissue with Microwave." Patent JP 09-140324 A. Japanese Patent Office. www.ipdl.inpit.go.jp.

TUME, R. K. AND F. D. SHAW (1992). "Beta-endorphin and cortisol concentrations in plasma of blood samples collected during exsanguination of cattle." Meat Science **31**(2): 211-217.

USDA-FSIS (2009). "Humane Handling Guidebook". Washington DC, USDA-FSIS.

VELARDE, A., J. CRUZ, M. GISPERT, D. CARRION, J.L. RUIZ DE LA TORRE, A. DIESTRE AND X. MANTECA (2007). "Aversion to carbon dioxide stunning in pigs: effect of carbon dioxide concentration and halothane genotype." Animal Welfare **16**: 513-522.

VELARDE, A., J.L. RUIZ-DE-LA-TORRE, C. ROSELLO, E. FABREGA, A. DIESTRE AND X. MANTECA (2002). "Assessment of return to consciousness after electrical stunning in lambs." Animal Welfare **11**: 333-341.

VON HOLLEBEN, K., M. VON WENZLAWOWICZ, N. GREGORY, H. ANIL, A. VELARDE, P. RODRIGUEZ, B. CENCI-GOGA, B. CATANESE AND B. LAMBOOIJ (2010). "Report on good and adverse practices - Animal welfare concerns in relation to slaughter practices from the viewpoint of veterinary sciences." **1**.3, 81. https://www.dialrel.net/dialrel/images/veterinary-concerns.pdf.

VONMICKWITZ, G., A. HEER, T. DEMMLER, H. REHDER AND M. SEIDLER (1989). "Slaughter of cattle, swine and sheep according to the regulations on animal-welfare and disease-control using an electric stunning facility (Schermer, Type_EC)." Deutsche Tierarztliche Wochenschrift **96**(3): 127-133.

WARNER, R.D., D.M. FERGUSON, J.J. COTTRELL AND B.W. KNEE (2007). “Acute stress induced by the preslaughter use of electric prodders causes tougher beef meat.” Australian Journal of Experimental Agriculture **47**: 782-788.

WARRINGTON, R. (1974). “Electrical stunning.” The Veterinary Bulletin (Oct 1974) **44**(10):617-635.

WEAVER, A.L. AND S.B. WOTTON (2009) “The Jarvis Beef Stunner: Effects of a prototype chest electrode.” Meat Science **81**: 51-56.

WITTE, V.C., G.F. KRAUSE AND M.E. BAILEY (1970). "A new extraction method for determining 2-thiobarbituric acid values of pork and beef during storage." Journal of Food Science **35**: 582-585.

WOODBURY, D.M. AND R. KARLER (1960). “The role of carbon dioxide in the nervous system.” Anesthesiology **21**: 686-703.

WOODBURY, M.R., N.A. CAULKETT, C.B. JOHNSON AND P.R. WILSON (2005). “Comparison of analgesic techniques for antler removal in halothane-anaesthetized red deer (Cervus elaphus): cardiovascular and somatic responses.” Veterinary Anaesthesia and Analgesia **32**: 72-82. http://dx.doi.org/10.1111/j.1467-2995.2005.00227.x.

WOTTON, S.B., N.G. GREGORY, P.E. WHITTINGTON AND I.D. PARKMAN (2000). “Electrical stunning of cattle.” Veterinary Record **147**: 681-684.

YOSHIZAWA, T., K. OMORI, I. TAKEUCHI, Y. MIYOSHI, H. KIDO, E. TAKAHASHI, K. JITSUIKI, K. ISHIKAWA, H. OHSAKA, M. SUGITA AND Y. YANAGAWA (2016). "Heat stroke with bimodal rhabdomyolysis: a case report and review of the literature." Journal of Intensive Care **4**: 71-76.

ZELLER, W., D. METTLER AND U. SCHATZMANN (1989). "Stunning poultry with microwaves (2450 MHz)." Deutsche Tierarztliche Wochenschrift **96**(6): 311-313.

ZULKIFLI, I., Y.M. GOH, B. NORBAIYAH, A.Q. SAZILI, M. LOTFI, A.F. SOLEIMANI AND A.H. SMALL (2014). "Changes in blood parameters and electroencephalogram of cattle as affected by different stunning and slaughter methods in cattle." Animal Production Science **54**(2): 187-193.

8. Appendix

8.1 Glossary of terms

Term	Definition
ACTH	Adrenocorticotrophic hormone
AMIC	Australian Meat Industry Council
AMT	Advanced Microwave Technologies Pty Ltd
AVMA	American Veterinary Medical Association
CSIRO	Commonwealth Scientific and Industrial Research Organisation
CCTV	Closed-Circuit Television
DTS	DTS: Diathermic Syncope®
DVR	Digital Video Recording
EC	European Commission
ECG	Electrocardiogram
EEG	Electroencephalogram
EFSA	European Food Safety Authority
HPA	Hypothalamic-Pituitary Axis
NAMI	North American Meat Institute
NHMRC	National Health and Medical Research Council (Australia)
PR	Project Report
SS	Scientific Study
SUP	Supplementary material

8.2 Determination of intervention parameters for DTS - Establishing equivalency with the parameters to be provided when applying a mechanical or electrical stunning intervention, based on Annex I of Council Regulation (EC) No 1099/2009

Intervention	Parameters	Component	Equivalent DTS parameters	Equivalent DTS Component
Mechanical stunning – Penetrative captive bolt	Position and direction of the shot	Restraining system	Position and application of the applicator	Restraining system
		Position of the captive bolt gun		Position and contact of the waveguide
		Bolt penetration site		Wave penetration site
	Appropriate velocity, bolt length and diameter of the bolt according to animal size and species	Captive bolt gun characteristics	Appropriate power (kW) and energy (kJ) according to animal size and species	Design of applicator
		Cartridge or compressed air specifications		Microwave generator output
		Type (e.g., beef or dairy cattle), size of animal		Type (e.g., beef or dairy cattle), size of animal
		Equipment maintenance, cleaning and storage conditions		Equipment maintenance, cleaning and storage conditions
	Maximum stun to stick/kill interval(s)[1]		Maximum stun to stick/kill interval(s)	
Mechanical stunning – Non-penetrative captive bolt	Position and direction of the shot	Restraining system	Position and application of the waveguide	Restraining system
		Position of the captive bolt gun		Position and contact of the waveguide
		Bolt impact site		Wave penetration site
	Appropriate velocity and shape of the bolt according to animal size and species	Captive bolt gun characteristics	Appropriate energy according to animal size and species	Design of waveguide
		Cartridge or compressed air specifications		Microwave generator output
		Bolt dimensions, mass and velocity		Design of waveguide
		Type (e.g., beef or dairy cattle), size of animal		Type (e.g., beef or dairy cattle), size of animal
		Equipment maintenance, cleaning and storage conditions		Equipment maintenance, cleaning and storage conditions

1

	Maximum stun to stick/kill interval(s)		Maximum stun to stick/kill interval(s)	
Head-only and head-to-body electrical stunning	Minimum current (A or mA)	Current type	Minimum energy (KJ)	
		Waveform		
		Minimum current		
		Latency		
	Minimum voltage (V)	Exposed minimum voltage (V)	Minimum power (kW)	
		Delivered minimum voltage (V)		
	Maximum frequency (Hz)	Maximum frequency (Hz)	Maximum frequency (Hz)	
		Minimum frequency (Hz)	Minimum frequency (Hz)	
	Minimum time exposure		Duration of intervention	
	Minimum stun to stick interval		Minimum stun to stick interval	
	Frequency of calibration		Frequency of calibration	
	Optimisation of the current flow	Electrode characteristics	Optimisation of the energy applied	Waveguide characteristics
		Electrode appearance		
		Animal restraining		Animal restraining
	Prevention of electrical shocks before stunning			
	Position and contact surface area of electrodes	Position of the electrodes	Position and application of the waveguide	Position of the waveguide
		Type of electrode		Design of waveguide
		Animal skin condition		Animal skin condition

8.3 Animal-based measures for assessment of 'State of consciousness' after DTS: Diathermic Syncope® application.

Animal-based measures	**Description**
Posture	Attempts to regain posture when rolled out of the box.
Breathing	Breathing is deep and slow. A transient cessation of breathing may be observed. Breathing does not alter in response to stimulation, e.g., through incision of the skin.
Tonic seizures	Lack of stiff, rigidly extended legs; lack of stiff extended neck. Clonic seizures and paddling develop, particularly when there is a delay before exsanguination.
Corneal reflex	Blinking response elicited by touching or tapping the cornea with a finger or paint brush.
Palpebral reflex	Blinking response elicited by touching or tapping a finger on the inner/outer eye canthus or eyelashes.
Ocular tracking	Eye movements that follow movement of a hand passed in front of the eye.
Muscle tone	Unconscious animals will show general loss of muscle tone after the termination of tonic–clonic seizures coinciding with the recovery of the corneal reflex if not previously exsanguinated. Loss of muscle tone can be recognised from the completely relaxed legs, floppy ears and tail and relaxed jaws with protruding tongue. Ineffectively stunned animals and those recovering consciousness will show a righting reflex and attempts to raise the head.
Vocalisations	Grunting, bellowing or mooing. Passive vocalization may occur when the unconscious animal is inverted, due to tension in the vocal cords.
Body movements	Intentional or purposeful kicking or body or head movements as a response to incision of the skin and/or insertion of the knife.
Spontaneous blinking	Animal opens/closes eyelid on its own without stimulation. Involuntary flicking of the eyelids and third eyelid may be seen in the unconscious animal.

8.4 Hazard identification over the phases in different stunning methods

		DTS	Mechanical stunning*	Electrical Stunning*
Pre-intervention phase	Description	Cattle are handled through a forcing pen and race system into an individual restraint unit which incorporates a neck capture and chin lift	Cattle are handled through a forcing pen and race system into an individual restraint unit. Neck capture and chin lift are recommended to optimize stun placement	Cattle are handled through a forcing pen and race system into an individual restraint unit which incorporates a neck capture and chin lift (automated systems); or neck capture and chin lift are recommended to optimize tong placement (manual systems)
	Hazard	Poor handling practices in forcing pen and race Improper design, construction and maintenance of premises Inappropriate restraint Unexpected loud noise	Poor handling practices in forcing pen and race Improper design, construction and maintenance of premises Inappropriate restraint Unexpected loud noise	Poor handling practices in forcing pen and race Improper design, construction and maintenance of premises Inappropriate restraint Unexpected loud noise
Application phase	Description	Microwave energy is applied via a waveguide applicator positioned on the forehead	A concussive blow is applied to the forehead	An electrical current is applied to the head either via manually applied tongs or via the head restraint system
	Hazard	Inappropriate restraint Incorrect placement of applicator Incorrect DTS parameters (prevented via the control panel software)	Inappropriate restraint Incorrect position and direction of the shot Incorrect captive bolt parameters	Inappropriate restraint Incorrect placement of electrodes Poor electrical contact Exposure time too short Inappropriate electrical parameters
Unconscious phase	Description	Unconscious animal is rolled out of the restraint unit and exsanguinated	Unconscious animal is rolled out of the restraint unit and exsanguinated	Unconscious animal is rolled out of the restraint unit and exsanguinated
	Hazard	Prolonged stun-stick interval	Prolonged stun-stick interval	Prolonged stun-stick interval

* adapted from EFSA 2020.