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Effects of different stunning methods on the welfare and carcass quality of finisher pigs in South African pork abattoirs

by

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DECLARATION

I, Naomi Lupton, hereby declare that this dissertation, submitted for the degree: MSc (Agric) (Magister Scientiae Agriculturae) Animal Science: Production Physiology and Product Quality, at the University of Pretoria, is my own work and has not been submitted by me for a degree at any other university.

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Miss Naomi Lupton
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2024

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LIST OF ABBREVIATIONS

ADP	-	Adenosine Diphosphate
ARC	-	Agricultural Research Centre
ATP	-	Adenosine Triphosphate
Ar	-	Argon
Ar-CO_2	-	80% Argon 20% Carbon Dioxide admixture
Ca^{2+}	-	Calcium
°C	-	degrees Celsius
CO_2	-	Carbon Dioxide
CK	-	Creatine Kinase
cm	-	centimetre
CP	-	Creatine phosphate (phosphocreatine)
DFD	-	Dark, firm and dry meat
EEG	-	Electroencephalogram
G6P	-	Glucose-6-Phosphate
g	-	gram
GDARD	-	Gauteng Department of Agriculture and Rural Development
H^+	-	Hydrogen
H_2O	-	Water
$HC\ell O_4$	-	Perchloric acid
h	-	hour(s)
KOH	-	Potassium Hydroxide
l	-	litre
L.o.c.	-	LOC
M	-	Molarity
min	-	minute(s)
$MgC\ell$	-	Magnesium Chloride
mℓ	-	millilitre
mm	-	minutes
N	-	Normality (g/L)
NAD	-	Nicotinamide Adenine Dinucleotide
NADH	-	Nicotinamide Adenine Dinucleotide + Hydrogen

NADP	-	Nicotinamide Adenine Dinucleotide Phosphate
NaOH	-	Sodium Hydroxide
NSPCA	-	National Council of the Society for the Prevention of Cruelty to Animals
PSE	-	Pale, soft and exudative meat
RMAA	-	Red Meat Abattoir Association
R.o.c.	-	Return of consciousness
Rpm	-	rotations per minute
s/ss	-	s
sig.	-	significance
SOP	-	Standard Operating Procedure
µℓ	-	microlitre

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ABSTRACT

The purpose of this research was to study the effects of different stunning systems on the welfare of pigs in South African abattoirs. Four stunning systems were evaluated: 84% CO_2, emergency head-only electrical, standard head-to-heart electrical, and an experimental 80% Argon 20% CO_2 (Ar-CO_2) admixture. pH data, pig behaviour inside the stunner and muscle metabolites were studied in this research. Both the Ar-CO_2 and emergency head-only stunning methods were deemed problematic on the basis of animal welfare. The Ar-CO_2 method had the sharpest and fastest pH decline. The 84% CO_2 and standard electrical head-to-heart stunning methods had similar rates of pH decline, with CO_2 having a lower pH than electrical stunning at every point. The behaviour displayed during gas stunning treatments was recorded by way of cameras located inside the stunner. Those stunned by 84% CO_2 lost their consciousness significantly faster than those stunned by Ar-CO_2. During the Ar-CO_2 stunning, four out of five pigs squealed while under the admixture's influence, indicating that the animals were distressed during this process. Squeals were not heard while the pigs were exposed to the 84% CO_2. pH profiles did not differ significantly between head-to-heart electrical stunning and 84% CO_2 stunning, implying that the stunning treatment itself did not have a big effect on the *post mortem* pH and its decline. The author recommends that further research in the South African pork industry be focussed on improving and refining current CO_2- and electrical stunning systems. Pre-slaughter handling plays a big role in the animal's psychological- and physiological state during stunning and must be further improved upon.

CHAPTER 1: INTRODUCTION

Across the globe, rendering animals unconscious before slaughter is a legal requirement, and can be done in several ways according to guidelines and requirements set out by various governing bodies and animal cruelty prevention groups (Channon *et al.*, 2002; More *et al.*, 2018; Marcon *et al.*, 2019). In South Africa, this is mandatory under the Meat Safety Act (Act No. 40 of 2000, Government Gazette Notice No. 1106, Parliament of the Republic of Southern Africa) and the Animals Protection Act (Act No. 71 of 1962, Government Gazette Notice No. 379, Parliament of the Republic of South Africa) (Petty, 2015). This is to ensure that animals are slaughtered humanely, aiming to prevent as much unnecessary pain, and suffering as possible. Animal welfare is a crucial aspect of the meat industry, however, economics, commercial practicality, worker safety and meat quality must also be considered, as the stunning method used will be influenced by its effects on these factors (Velarde *et al.*, 2000; Warner *et al.*, 2010). Due to welfare implications, gas stunning (CAS; controlled atmosphere stunning) systems are controversial, with many not in favour of itsuse.

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In South Africa, CO_2 stunning is only permitted under special conditions whereas stunning by captive bolt or electronarcosis is approved for the commercial slaughter of pigs (Meat Safety Act, 2000; Petty, 2015). If performed correctly, both these methods produce an instant state of unconsciousness after stunning. Electrical stunning is done utilising either the head-only method or a head-to-body method. Both require that an electric current of sufficient strength be passed through the brain, with the recommended settings being 1.3 A sine wave AC at 50 Hz (Nielsen *et al.*, 2020). During head-only, the stunner is applied on both sides of the pig's skull, between the eye and base of the ear (Grandin, 2013). During head-to-body electrical stunning, the current is passed through both the head and the body simultaneously. Head-only electric stunning induces both a tonic and clonic phase, which are the indications that a seizure has taken place. During the tonic phase, the leg muscles are rigid, and during the clonic phase, clear leg-paddling can be observed (Grandin, 2013; Gerritzen *et al.*, 2021). These phases are generally masked once cardiac arrest has taken place.

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CO_2 stunning has come under fire on the grounds of animal welfare, as pigs often react aversively when exposed to high concentrations thereof (Raj & Gregory, 1995, 1996; Raj *et al.*, 1997a; Raj, 1999; Velarde *et al.*, 2001; Dalmau *et al.*, 2010c; Llonch *et al.*, 2012a; Atkinson *et al.*, 2012; Verhoeven *et al.*, 2016; More *et al.*, 2018), despite yielding superior carcasses

and meat quality compared to electrical stunning methods (Velarde et al., 2000b; Channon et al., 2002).

During the pre-slaughter phase, many stressors can negatively influence swine welfare and meat quality. Transport, lairage, pre-slaughter handling techniques and the stunning system used can cause stress, potentially resulting in injury, carcass damage and poor meat quality (Dreyer *et al.*, 1972; Channon *et al.*, 2000; Velarde *et al.*, 2000; Støier *et al.*, 2001; Grandin, 2003; Van de Perre *et al.*, 2010; Adzitey & Nurul, 2011; Brandt & Aaslyng, 2015). Often the focus is placed solely on the stunning method with little to no regard for the handling techniques that precede it (Velarde *et al.*, 2000; Grandin, 2013).

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Stress is primarily classified as either acute or chronic stress. Acute, or short-term stress, is experienced just before slaughter on the way to the stunner and is the leading cause of PSE (pale, soft, exudative) pork (D'Souza *et al.*, 1998; Bowker *et al.*, 2000; Sionek & Przybylski, 2016). Chronic, or long-term stress, is experienced during transport and lairage and is associated with the development of DFD (dark, firm, dry) pork (Guàrdia *et al.*, 2005; Sionek & Przybylski, 2016).

The objectives of this pilot study was to establish the state of different stunning methods used in the South African pork production sector, while also investigating the possible use of an alternative gas stunning method. Lastly, the feasibility of and need for future studies in this field was to be considered.

The aims of the pilot study were to compare CO_2- and Ar-CO_2 stunning with electrical stunning from an animal welfare perspective, based on parameters like carcass pH, *post mortem* muscle metabolite levels and behaviour. The aims of the follow-up study remained the same as for the pilot, but only CO_2- and electrical stunning methods were considered.

CHAPTER 2: LITERATURE REVIEW

2.1 Pork muscle physiology

2.1.1 Post mortem energy metabolism

After slaughter, many biological processes work in an interdependent fashion to produce meat from muscle (Ouali *et al.*, 2006). During this turnover, many changes take place: (1) available energy is depleted, (2) shift from aerobic to anaerobic metabolism, (3) pH decline in muscle tissues, (4) rising ionic strength due to malfunctioning ionic pumps, and (5) the cell is unable to maintain these changing conditions (Huff Lonergan *et al.*, 2010; Ertbjerg & Puolanne, 2017).

The events that take place during the perimortem period (just before slaughter) influence *post mortem* proteolysis and tenderization (Lonergan *et al.*, 2018). This typically encompasses the physiological effects due to stress brought on by transport to the slaughter plant, lairage and feed withdrawal before slaughter, as well as handling methods up to slaughtering (Grandin, 1997; Chulayo *et al.*, 2012; Chulayo & Muchenje, 2015). These processes influence the animal's muscle glycogen content, which determines the rate and extent of *post mortem* proteolysis (Lonergan *et al.*, 2018). Between the point of harvest and onset of rigor, the pH of the muscle tissue must decline from around 7.2 to about 5.6.

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After slaughter and exsanguination, blood circulation stops and circulation of oxygen ceases (Matarneh *et al.*, 2017; Chauhan & England, 2018), bringing about a shift from aerobic to anaerobic metabolism (Huff Lonergan *et al.*, 2010; Ertbjerg & Puolanne, 2017; Chauhan & England, 2018). Three main pathways exist by which ATP generation in the muscle takes place, namely the phosphagen system, glycolysis, and oxidative phosphorylation (Matarneh *et al.*, 2017; Lonergan *et al.*, 2018).

Under anaerobic conditions, the concentration of adenosine triphosphate (ATP) remains stable via the utilization of phosphocreatine (PCr), an immediate source of energy (Matarneh *et al.*, 2017). Catalysed by the enzyme creatine kinase (CK), an inorganic phosphate (Pi) is transferred to an adenosine diphosphate (ADP) molecule, forming ATP and creatine. Stores of ATP and PCr are limited in muscle tissue, meaning that PCr can maintain *post mortem* ATP concentrations for a short time (Scheffler *et al.*, 2011; Ferguson & Gerrard, 2014). Soon the majority of PCr is degraded, and rates of ATP hydrolysis have likely exceeded those of resynthesis. The result thereof is that ADP is produced in excessive quantities, leading to the activation of adenylate kinase (AK). The decline in ATP concentrations is buffered by the action

of AK after which ATP and adenosine monophosphate (AMP) are produced from two ADP molecules (Ferguson & Gerrard, 2014). In turn, AMP is deaminated by adenosine monophosphate deaminase (AMPD), producing inosine monophosphate (IMP). The reaction is irreversible, and IMP accumulates in the muscle (Scheffler *et al.*, 2011).

Metabolites such as AMP, ADP, and Pi function as activators for the rate-limiting enzymes during glycolysis. Glycolysis becomes the dominant pathway by which ATP is produced. It is during this pathway that glycogen is converted to pyruvate, also yielding ATP, reduced nicotinamide adenine dinucleotide (NADH), H^+ and water.

Glycogenolysis can be defined as the process by which glycogen is broken down into glucose monomers. This degradation takes place by way of two enzymes – glycogen phosphorylase (GP) and glycogen debranching enzyme (GDE). GP cleaves the α-1,4-glycosidic bonds found at the non-reducing ends of the chain. An P_i is attached to a newly cleaved glucose monomer, yielding glucose-1-phosphate (G1P). GDE extends glycogen chains by transferring three terminal glycosyl residues from the branches (characterised by α-1,6-linkages) to said chains. Another catalytic activity of GDE involves the hydrolysis of the last remaining glucose residue of the branch by α-1,6-glucosidase, releasing a free glucose molecule. G1P is readily converted to glucose-6-phosphate (G6P) by the enzyme phosphoglucomutase, and free glucose molecules are either converted to G6P by hexokinase as part of the first step of the glycolytic pathway or accumulated in the muscle. The reaction driven by hexokinase involves ATP hydrolysis, thus also yielding ADP and H^+ (Matarneh *et al.*, 2017).

Under these anaerobic conditions, pyruvate is reduced to lactate by lactate dehydrogenase (LDH). This reaction consumes two H+ ions (by oxidising NADH + H^+), yielding the NAD^+ necessary to allow the reaction by glyceraldehyde phosphate dehydrogenase to continue. Without this step, anaerobic metabolism would not be able to continue. Lactate accumulates in the muscle, as no blood circulation takes place. The production of H^+ during ATP hydrolysis, coupled with lactate production, causes the pH in muscle to fall (Huff Lonergan *et al.*, 2010; Ferguson & Gerrard, 2014; Matarneh *et al.*, 2017). The accumulation of lactate has long been regarded as the process driving the *post mortem* pH decline (Heffron, 1973; Koohmaraie, 1992), but it has been established that it is the associated accumulation of H+-ions during this process that causes the decline in pH (England *et al.*, 2013; Kim *et al.*, 2014).

It seems unlikely that oxidative phosphorylation would play a role in *post mortem* metabolism, due to the cessation of oxygen delivery. However, the aerobic activity that takes place for a short time after harvesting, while oxygen is depleted, is still able to improve maintenance of ATP concentrations (Matarneh *et al.*, 2017). Oxidative phosphorylation can produce 10 times more ATP than anaerobic glycolysis. The mitochondrial capacity varies between species,

breeds, individuals and even muscles, and is thus partially responsible for variation in pH decline rates.

Shortly after slaughter and exsanguination, the hide is removed from the carcass. Many abattoirs trim the excess fat, and bruised tissue is cut away or condemned. The internal organs are removed and soon the carcass is stored in a cooler to facilitate *post mortem* temperature decline. This decline in temperature enhances Ca^{2+} release from the sarcoplasmic reticulum (SR), causing Ca^{2+} concentrations to rise in the cytosol (Matarneh *et al.*, 2017; Ertbjerg & Puolanne, 2017). This rise in Ca^{2+} concentrations causes an increase in the rate of ATP hydrolysis and enables muscle contraction previously inhibited by the troponin-tropomyosin complex. This allows the myosin cross-bridges to interact with the active sites on the adjacent actin fibres. This is the principle by which electrical stimulation, not often used in pork production, is used to accelerate the development of rigor mortis. The electrical current liberates elevated concentrations of Ca^{2+}, which then accelerates ATP depletion (Matarneh *et al.*, 2017).

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2.1.2 Post mortem proteolysis and tenderization

Several endogenous proteolytic systems are responsible for the degradation of muscle fibres, of which the calpain system has been shown to influence muscle fibre degradation the most (Goll *et al.*, 1992; Dransfield, 1994; Bhat *et al.*, 2018). The drop in temperature *post mortem* stimulates the gradual release of Ca^{2+} ions into the cytosol (Matarneh *et al.*, 2017). This increase in Ca^{2+} concentrations, slow at first, is what activates the calpain enzymes (Goll *et al.*, 1992; Matarneh *et al.*, 2017). Two important calpain isoforms are found in meat, and these are named according to the level of Ca^{2+} to activate each. Calpain I, or μ-calpain, is activated by μM's of Ca^{2+} (Goll *et al.*, 1992; Koohmaraie, 1992). Calpain II, or m-calpain, is activated by mM's of Ca^{2+}. This implies that μ-calpain is activated first, as it requires less Ca^{2+} to be activated. In the study by (Dransfield, 1994), it was found that μ-calpain activation starts around pH 6.3, or at 6 h after slaughter in beef. It is estimated that the calpains are responsible for around 95% of *post mortem* proteolytic tenderisation during the first 7-14d (Goll *et al.*, 1998).

The enzyme inhibitor of the calpains, calpastatin, can bind to the calpains at Ca^{2+} concentrations similar to that required for activation of μ-calpain (Goll *et al.*, 1992; Dransfield, 1994). In beef, low calpain-to-calpastatin ratios *post mortem* result in increased meat toughness (Goll *et al.*, 1998) due to lower calpain proteolytic activity.

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It has been hypothesised that the development of rigor takes place in phases. This process starts with the delay phase and continues until completion in the onset phase (Ferguson & Gerrard, 2014; Lonergan *et al.*, 2018). *Post mortem*, due to the temperature and pH decline,

the sarcoplasmic reticulum loses its ability to retain Ca^{2+}, and this Ca^{2+} ends up in the sarcoplasm where it fuels the activity of the calpains and calpastatin (Huff Lonergan *et al.*, 2010).

After slaughter, the carcass is left to cool down to about 7˚C before being transported. During this time, the calpains degrade muscle proteins such as titin and desmin, and the actomyosin bonds become less heat soluble and more irreversible. The onset of rigor mortis takes place when a muscle fibre's actomyosin bonds are no longer able to dissociate. The decline in temperature during this time causes the pH decline rate to decrease, until the pH_u is reached (Steen *et al.*, 1997; Huff Lonergan *et al.*, 2010).

2.2 Pork carcass defects

2.2.1 Dark, firm and dry meat

The dark, firm and dry (DFD) meat defect occurs when animals are exposed to long-term (chronic) stress such as transportation over long distances and overcrowding during lairage (van der Wal *et al.*, 1989; Adzitey & Nurul, 2011; Manalo & Gabriel, 2020). This defect is characterised by dark muscle colour, a dry surface appearance and a firm texture (van der Wal *et al.*, 1989; Sionek & Przybylski, 2016). These carcasses are more susceptible to microbial spoilage due to the higher pH (Guàrdia *et al.*, 2005; Adzitey & Nurul, 2011; Rey-Salgueiro *et al.*, 2018). Chronic stress before slaughter depletes the animal's glycogen stores (Guàrdia *et al.*, 2005), resulting in low concentrations of glycolysis and H^+-ion production and a high pH (above 6.0) (Scheffler *et al.*, 2011; Manalo & Gabriel, 2020) 12 – 48 hours *post mortem* (Adzitey & Nurul, 2011). Due to this high pH, little protein denaturation takes place, resulting in almost no shrinkage of the myofilament lattice. DFD meat has exceptional WHC, with almost no fluid loss (Čobanović *et al.*, 2016b). This condition makes the meat appear darker, due to the reduced differences in refractive indices of the myofibrils and sarcoplasm (Adzitey & Nurul, 2011). This causes the muscle to absorb light, instead of reflecting it, thus causing the darker colour. It is common for DFD meat to appear more brown and less red, due to oxygen depletion (Čobanović et al., 2016b; Stajkovic et al., 2019)(Čobanović et al., 2016b; Stajkovic et al., 2019).

Electrical stimulation can be used to decrease the severity of DFD. An electrical current would provide energy required for the utilisation of any remaining glycogen reserves and promote faster pH decline rates (Offer, 1991; Hopkins *et al.*, 2014). However, due to stress-susceptible genotypes and a large presence of fast-glycolytic muscle fibres (Adzitey & Nurul, 2011), electrical stimulation is not recommended for pig carcasses.

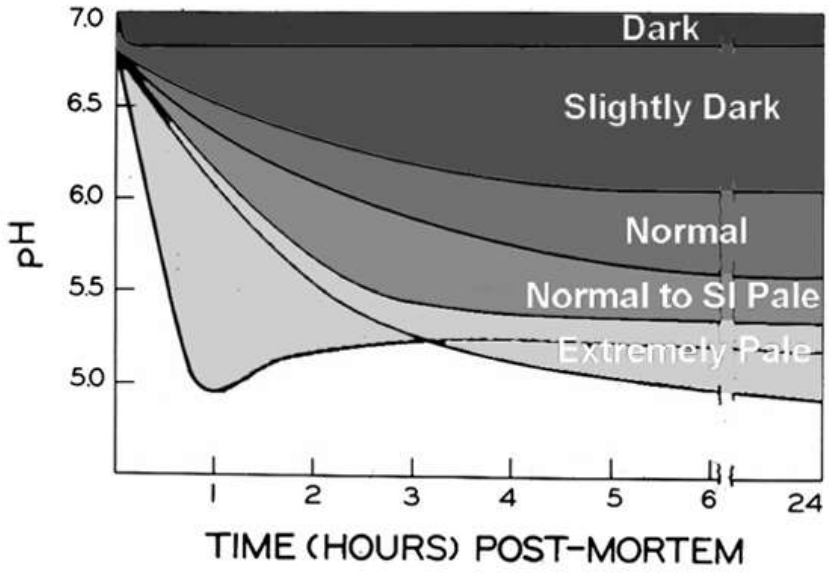

Figure 2.1 *Different post mortem pH decline rates and their expected effects on pork colour (Briskey, 1964; Scheffler & Gerrard, 2007).*

2.2.2 Pale, soft, and exudative meat

Pale, soft and exudative (PSE) meat is often caused by a rapid *post mortem* pH decline whilst the carcass temperature is still high (Klingbiel & Naudé, 1972; Offer, 1991; Adzitey & Nurul, 2011). This carcass defect develops when muscle pH is below 6.0 while still having an internal temperature of 35˚C or higher at 45 min. *post mortem* (McLoughlin, 1970; Scheffler *et al.*, 2011). Many factors influence the onset hereof, including stress susceptibility, short-term (acute) ante-mortem stress and *post mortem* carcass handling (Offer, 1991; Fisher & Mellett, 1997; Rosenvold & Andersen, 2003a; Manalo & Gabriel, 2020).

This condition is characterised by pale muscle colour, excessive amounts of free fluids (also known as juices and/or exudatives) due to a compromised WHC and a soft texture (Heffron, 1973; van der Wal *et al.*, 1989; Warner *et al.*, 1993; Adzitey & Nurul, 2011; Manalo & Gabriel, 2020) and is thus considered as a product of inferior quality by consumers (Warner *et al.*, 1993; Troy & Kerry, 2010; Sionek & Przybylski, 2016; Stufft *et al.*, 2017). The production of products of inferior quality result in economic losses (Adzitey & Nurul, 2011). Consumers rely on colour to discern between products of inferior and superior quality and would rather select normal and darker products as opposed to lighter (paler) products (Topel *et al.*, 1976; Brewer *et al.*, 1998). Free fluids are also viewed in a negative light, as many consumers believe it to be blood.

Muscle fiber types, pH decline rates, carcass- and rigor temperatures as well as the extent of glycolysis each play a key role in the development of PSE meat. According to (Offer, 1991), it is not a given that the rate of pH decline is accelerated in a PSE carcass. At extended durations of glycolysis, even normal rates of pH decline can lead to a low pH_u. An increase in metabolic heat is associated with glycolysis, further increasing the carcass temperature before it can decline. Accordingly, insufficient carcass cooling regimes also lead to the onset of PSE, as elevated temperatures over lengthy periods of time increase pH decline rates. Lactate and H^+ accumulation occurs more rapidly, thus driving the faster pH decline. Muscles with a higher concentration of fast-glycolytic muscle fibres are at a higher risk of developing PSE. In these muscles, glycogen concentrations are higher, and thus glycolysis takes place for a longer period, than the muscles with a higher concentration of oxidative muscle fibres. Oxidative muscles, having less glycogen, have shorter extents of glycolysis (Adzitey & Nurul, 2011).

Protein denaturation is more severe in PSE carcasses as the proteins are exposed to low pH values (Offer, 1991). This compromises the WHC of the muscle, increasing the degree of shrinkage of the myofilament lattice, thereby expelling fluids from the muscle fibres. It must be noted, however, that during *post mortem* proteolysis, some degree of myofilament lattice shrinkage takes place, bringing about basal ("normal") fluid losses. This fluid is lost both before (as drip loss) and during cooking (cooking loss) (van der Wal *et al.*, 1989; Offer, 1991).

The fast pH decline results in quicker activation of the calpain enzymes which break down the muscle proteins. However, these enzymes are quickly denatured as the pH continues to decline (Dransfield, 1994), often past the preferred pH_u of 5.5. This results in sub-optimal tenderness values. This can be ameliorated by decreasing the *post mortem* carcass temperature, which will further inactivate the enzymes responsible for the decrease in pH Various chilling methods are used to achieve this goal (Savell *et al*., 2005).

2.3 Factors leading to acute- and chronic stress responses

2.3.1 Porcine stress syndrome (PSS)/Malignant hyperthermia (MH) syndrome

PSS is a genetic disorder resulting in the sudden death of pigs when exposed to stressful preslaughter conditions as well as higher incidences of PSE meat (Webb *et al.*, 1982; Bašić *et al.*, 1997; Fisher & Mellett, 1997; Soma *et al.*, 2014a). Pigs predisposed to this defect used to be identified via the halothane challenge test, during which pigs between the ages of 6 and 15 weeks were exposed to a gas mixture containing about 5% halothane gas. Halothane positive pigs developed rigidity in the hindlegs, whereas halothane negative pigs showed no indications of stress. The test is then stopped to prevent the onset of MH, then considered to be a secondary phase reaction in stress-susceptible pigs (Webb *et al.*, 1982). Besides

muscular rigidity, other symptoms included fever and hypermetabolism (Heffron, 1973; Bašić *et al.*, 1997).

It has been established that this condition is inherited as a single recessive gene (n), where individuals with the homozygous recessive genotype (nn) exhibit the symptoms associated with PSS/MH (Bašić *et al.*, 1997). The homozygous dominant genotype (NN) are thus not carriers of this defect and not as susceptible to extreme reactions during stressful conditions. This syndrome affects the genes in the sarcoplasmic reticulum encoding the Ca^{2+} release channels. RYR1 (ryanodine receptor gene) encodes the release channels in skeletal muscle, whereas RYR2 encodes those found in the cardiac muscle and brain. RYR1 has been established as a candidate gene for MH in both pigs and humans. It has been proposed that, in order to reduce PSE meat incidences, the halothane mutation must be eliminated (Velarde *et al.*, 2001; Grandin, 2003). Genomic testing has aided the pork industry in nearly eradicating the mutation from breeding stock, thereby reducing the likelihood of producing PSE meat (Soma *et al.*, 2014a). MH does not appear to have significant effects on DFD incidence (Guàrdia *et al.*, 2005).

From a physiological perspective, the symptoms associated with this condition are the results of abnormal Ca^{2+} regulation in skeletal muscle (Bašić *et al.*, 1997; Soma *et al.*, 2014a). In skeletal muscle, Ca^{2+} concentrations regulate muscle contraction, relaxation, and energy metabolism. Depolarization of the sarcoplasmic reticulum tubules brings about an increase in Ca^{2+} concentrations within the sarcoplasm, initiating muscle contraction. Ca^{2+} ATPase pumps transport Ca^{2+} back into the sarcoplasmic reticulum to allow the muscle to relax. In MH pigs, these Ca^{2+} channels are more sensitive to lower concentrations of Ca^{2+}-releasing stimulants. Ca^{2+} is released at higher rates and these channels are not able to readily close. The Ca^{2+} pump is overwhelmed, and muscle contractions are sustained for longer periods of time (Heffron, 1973; Bašić *et al.*, 1997). This causes the skeletal muscles to permanently produce lactic acid, CO_2, and heat as products of glycolytic and aerobic metabolism. Oxygen uptake is also greatly increased. Cell membranes are damaged and ion transport is in a near-constant state of disequilibrium, amounting to death in severe MH episodes (Bašić *et al.*, 1997).

Some economic advantages have been associated with the MH gene. Production traits such as larger eye muscle area, increased leanness and metabolic efficiency have caused breeders to select for carriers of this gene in order to improve production (Webb *et al.*, 1982; Bašić *et al.*, 1997; Channon *et al.*, 2000). Two explanations have been proposed for the improved production traits associated with the MH gene (Bašić *et al.*, 1997). Firstly, it could be that the increased concentrations of Ca^{2+} in the sarcoplasm bring about spontaneous muscle contractions, thereby toning the muscles. This would also result in increased oxygen and

energy usage, preventing fat deposition. Secondly, it could be that the MH gene is closely located to other genes related to these improved production traits. By maintaining linkage disequilibrium (LD), this mutation would be indirectly selected for when selecting for production traits.

(Soma *et al.*, 2014a) found an extremely low prevalence of carrier- (Nn) and homozygous recessive (nn) individuals among slaughtered South African pigs (3.4% and 0.2%, respectively). Some breeds with excellent carcass characteristics do seem to have more carriers of the defect, such as the Pietrain (Rey-Salgueiro *et al.*, 2018). Whilst agreeing that the eradication of this defect would be beneficial, (Soma *et al.*, 2014a) have noted that attention must still be given to reduce the amount of stress pigs experience during handling, transport and lairage. These factors are often the reason for poor pork quality (Fisher & Mellett, 1997; Channon *et al.*, 2000; Soma *et al.*, 2014a), especially in South Africa.

2.3.2 Transportation of pigs

Transportation from the farm to the abattoir encompasses many factors that influence the welfare and meat quality of pigs (Brandt & Aaslyng, 2015). The mortality rate is of utmost concern, as it reflects the welfare of the animals in question and affects profitability. Both the duration and quality of transport influences the rate of mortality, with quality potentially having a larger influence than duration. Stressful conditions during loading and offloading must also be taken into consideration when discussing transportation-related stress. Stress during the transportation phase is considered to be chronic and can lead to an increase in DFD development (Guàrdia *et al.*, 2005; Čobanović *et al.*, 2016a). In a study utilising salivary concentrations of cortisol and corticosterone as stress biomarkers, it was found that transport caused the pigs to experience a medium level of stress, having an average of 3 µg/L salivary cortisol (concentrations above 4 µg/L indicated high stress concentrations) (Rey-Salgueiro *et al.*, 2018). A study comparing the effects of sex and time of transportation and lairage on salivary, serum and urine cortisol concentrations found that after having been transported for two hours, boars had significantly higher ($P < 0.001$) salivary cortisol concentrations than gilts (38.6 ± 0.07 ng/ml vs. 15.2 ± 0.07 ng/ml, respectively) (Jama *et al.*, 2016). If the threshold values given by (Rey-Salgueiro *et al.*, 2018) are taken into consideration, then both the gilts and boars in the study by (Jama *et al.*, 2016) were severely stressed after transportation. This could also indicate that boars experienced more stress during the transport period compared to the gilts. Considering the baseline concentrations measured before slaughter (gilts = 3.9 ± 0.06 ng/ml, boars = 4.5 ± 0.06 ng/ml), both sexes experienced severe stress during transport (Jama *et al.*, 2016).

It is well documented that mixing of unfamiliar pigs is a stressful process. Pigs will attempt to establish a new group hierarchy – often in an aggressive manner (Grandin, 2003; Barton Gade, 2008; Brandt & Aaslyng, 2015). This aggression can result in skin damage, bruises, and broken bones in severe cases (Faucitano, 2018). Although the establishment of a new group hierarchy is a natural process, it is still a stressful process and precautions should be made in order to avoid it as far as possible (Barton Gade, 2008; Brandt & Aaslyng, 2015). Grandin (2003) suggests that, instead of avoiding the mixing of separate groups, one should monitor the animals based on the amount of skin damage. Regrouping or mixing often takes place at the on-farm pick-up facility and at the abattoir between offloading and lairage (Brandt & Aaslyng, 2015).

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During transport, the absence of disease is an important criterion that must be adhered to. Transport-induced illness such as motion sickness violates this criterion, and care must be taken by the driver to drive in such a way as to prevent the onset thereof (Grandin, 2003; Brandt & Aaslyng, 2015). Sudden braking and changes in speed can result in the pigs losing their balance. This can lead to broken bones, bruising, skin damage and an increased mortality rate, depending on the severity of the fall, the stocking density and whether the pigs can get up.

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Smaller groups of pigs have been known to move easier than larger groups, with less aggressive reactions between the members of the group (Grandin, 2003; Dalla Costa *et al.*, 2019). (Barton Gade & Christensen, 1998) found that more than 0.35m^2/100kg pig increased the probability of skin damage due to trampling or fighting. This stocking density was also found to lower risks of PSE development over varying transport durations when compared to higher (0.2, 0.25 and 0.3m^2) and lower (0.4m^2) stocking densities (Gajana *et al.*, 2013). Higher stocking densities may have lower instances of skin damage and displays of aggression simply because pigs do not have enough room to carry out these actions (Moss, 1980). Increasing stocking densities during transportation from 0.5 to 0.37 m^2/100 kg pig was found to lower the risk of producing DFD pork by 11% (Guàrdia *et al.*, 2005). Both excessive and insufficient space during transportation negatively affect swine welfare and pork quality ($P < 0.05$), as 26.62% of pigs subjected to high stocking densities (<0.3m^2/100kg) developed PSE, and 18.18% developed DFD (Čobanović *et al.*, 2016a). In the same study, 47.66% of pigs in the low stocking density category (>0.5m^2/100kg) developed DFD. In the medium stocking density (0.3 – 0.5m^2/100kg) group, 94.07% had normal carcasses. Pigs subjected to low stocking densities had the highest skin lesion scores (Čobanović *et al.*, 2016a).

From a meat quality perspective, transport over shorter distances is generally recommended above longer distances in order to decrease incidences of DFD and PSE meat (Guàrdia *et al.*,

2005; Gajana *et al.*, 2013; Sommavilla *et al.*, 2017). Pigs subjected to transport durations between 8 h and 16 h become exhausted and may not be able to recover during the lairage period. In some cases, it is possible that exhaustion could manifest itself on journeys shorter than eight hours. In the study by (Gajana *et al.*, 2013), transportation periods shorter than one hour have been shown to yield a higher proportion of normal pork carcasses, whereas transportation periods longer than one and a half hours tend to yield a higher proportion of potentially PSE carcasses. Increases in transport duration is also associated with higher mortality rates (Dalla Costa *et al.*, 2019). Very short transport durations (<15min) may cause pigs to exhibit a more intense stress response, negatively influencing meat quality compared to trips of three hours (Pérez *et al.*, 2002).

2.3.3 Lairage conditions

A variety of recommendations exist with regards to lairage duration (De Smet *et al.*, 1996; Dall Aaslyng & Barton Gade, 2001; Pérez *et al.*, 2002; Dokmanovic *et al.*, 2017). Durations of two to four hours are recommended, for two reasons. Firstly, to allow the pigs enough time to recover from the stress experienced before arriving at the abattoir, and, secondly, to avoid long-term food deprivation and dehydration (Pérez *et al.*, 2002; Čobanović *et al.*, 2016a).

After being in lairage for 20h, the salivary cortisol concentrations of boars did not differ significantly from that of gilts (16.5 ± 0.06 ng/ml vs. 16.3 ± 0.06 ng/ml, respectively) in the study by (Jama *et al.*, 2016). Compared to the concentrations seen after transportation (mentioned earlier), the gilts were unable to recover from the stress experienced during transportation. This could be due to mixing that took place with the boars, as gilts experience stress during physical contact with boars as is evidenced by the associated increase in cortisol secretion during boar pheromone transfer (Pearce & Paterson, 1992; Jama *et al.*, 2016).

Some authors have found that lairage time did not significantly influence meat quality when low-stress handling was used in largely halothane-free castrates and gilts (Dall Aaslyng & Barton Gade, 2001). They did, however, note that under warmer climatic conditions more PSE spots where seen in the leg muscles when less than 30min were spent in lairage. Other authors found that, compared to lairage periods shorter than three hours, lairage periods up to 14h had negative effects on animal welfare as these pigs had overall higher blood lactate concentrations and skin blemish scores (Dokmanovic *et al.*, 2017). The incidence of PSE in this study was, however, lower in the longer lairage period than the brief period.

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Pigs kept in lairage overnight (>20h) had lower carcass quality than those in lairage for periods shorter than one hour, as was evident in their lower slaughter weights, dressing percentage and backfat thickness, and higher skin lesion scores, pH_{45} and DFD incidence (Čobanović *et*

al., 2016a). During the shorter lairage period, pH_{45} values where lower and PSE incidence increased ($P < 0.05$).

Lairage periods of less than one hour are not recommended as pigs do not have sufficient time to recover from the transportation period (Vermeulen *et al.*, 2015; Čobanović *et al.*, 2016a). Likewise, excessively long periods (more than four hours) of lairage are not preferable either as this is associated with chronic stress, thereby increasing the likelihood of DFD development (Čobanović *et al.*, 2016a; Jama *et al.*, 2016). After short (<15min) periods of transportation, pigs may need longer lairage time in order to recover from the stress associated with travel (Pérez *et al.*, 2002).

2.3.4 Ante-mortem (pre-slaughter) handling techniques

Low-stress handling techniques before slaughter has been proven to increase pork quality by delaying the early *post mortem* pH decline and decreasing the amount of drip loss (Støier *et al.*, 2001). Stress caused by rough handling techniques (fast driving pace to stunner, use of electric goads) just prior to slaughter has been shown to decrease the pH_{45} (45 min *post mortem*) (de Oliveira *et al.*, 2018), but seem insufficient to significantly alter pork quality. The authors have suggested that the period during which the pigs were subjected to rough handling may have been too short to affect meat quality characteristics. Pre-slaughter handling techniques become more critical as one gets closer to the stunning phase, as this is when the pH (of the *m. longissimus thoracis*) and PSE prevalence is most likely to be influenced (Vermeulen *et al.*, 2015). From an animal welfare point-of-view, rough handling techniques can lead to additional bruises and fractures due to increased displays of aggression, use of electric goads and contact with equipment and structures (Dalla Costa *et al.*, 2019).

Sounds that pigs are unaccustomed to, and the loudness thereof also appear to be a source of stress pre-slaughter. This, however, can also be present during transport and lairage, not just on the way to the stunner (Warriss *et al.*, 1994). Systems that were classed as more stressful (loud noises, design and construction, handling techniques) had higher incidences of both PSE and DFD development, indicating that pigs experienced both acute and chronic stress before being stunned and slaughtered.

2.4 Physical stunning methods

2.4.1 Mechanical stunning (captive bolt stunning)

These methods induce unconsciousness by causing physical damage to the brain. A penetrating captive bolt gun has a retractable rod which, when applied correctly, fractures the cranium and thereby brings about immediate unconsciousness after stunning (Grandin, 2013). The velocity of the bolt must be high enough to cause an extensive concussion (Gibson *et al.*, 2015).

Poorly restrained animals, insufficient maintenance (of the bolt pistol), insufficient air pressure in pneumatic captive bolt guns, wet cartridges in cartridge-fired captive bolt guns and human error are the leading causes of unreliable captive bolt stunning (Grandin, 2013). Captive bolt pistols must be cleaned and maintained as per the manufacturer's recommendation (Gibson *et al.*, 2015). Regular breaks must be issued for those manning the stunner, as fatigue negatively impacts their accuracy. Furthermore, prior to stunning, animals must be handled in a manner that prevents as much unnecessary stress to the animals as possible. Animals that aren't agitated or skittish will be calmer inside the restrainer, and thus allow for a more accurate and effective stun.

Captive bolt stunning is employed in most beef plants and is not a standard stunning method in pork abattoirs. In South Africa, it is recommended that large boars be stunned using the captive bolt method, even though PSE may result thereafter. This is the only exception to the recommendation that pigs be stunned using one of the electrical stunning methods (LWCC, 2018).

2.4.2 Electrical stunning

Electrical stunning is performed by passing an electric current of sufficient magnitude through the pig's brain, bringing about a state of epileptiform of the Grand Mal type that leads to what many would call an 'instant' LOC after stunning (Wotton & O'Callaghan, 2002; McKinstry & Anil, 2004; Gerritzen *et al.*, 2021). Both insensibility and unconsciousness are induced by disrupting the natural neuron functions in the thalamus and cerebral cortex, and death is brought about by bleeding, thereby removing the blood from the carcass and with it, the oxygen required to keep the tissues and organs alive.

Electrical stunning is characterised by three (3) phases: the tonic-, clonic- and recovery phases (Gerritzen *et al.*, 2021). The tonic phase is characterised by the collapsing of the hindquarters, outstretched forelegs, and rigid posture. During this stage, signs of regular breathing are absent. During the clonic phase, the body trunk remains rigid while kicking motions are visible in the hindlimbs (Grandin, 2013; Gerritzen *et al.*, 2021). The recovery phase is avoided by ensuring that the stun-to-stick interval and bleeding is sufficiently short to prevent the return of consciousness. This results in the death of the pig. Recovery would normally occur if sticking doesn't take place but can start as soon as 30s after stunning (Gerritzen *et al.*, 2021). If a pig doesn't bleed out fast enough, its consciousness can return. It is of extreme importance that sticking take place as soon as possible, to allow for sufficient bleeding of the carcass that the state of unconsciousness be maintained until the onset of death.

Two main methods of electrical stunning can be used: head-only-, and head-to-body electrical stunning.

1) Head-only

During head-only electrical stunning, the electrodes are placed on either side of the head, between the eyes and ears, as to span the brain. It is recommended that pigs be stunned using a minimum current of 1.3 Amps for two to three (2 – 3)s in order to deliver an effective stun. Stunning for longer times will not bring about a deeper state of unconsciousness, but it may make sticking easier by causing longer muscular immobilization (Lambooij, 2004; Gerritzen *et al.*, 2021). When stunning pigs weighing more than 150 kg, a current of 1.8 – 2 Amps is recommended (based on a frequency of 50 – 100 Hz). This is to ensure that pigs are stunned effectively while minimising unnecessary pain and suffering on the pig's part (Gerritzen *et al.*, 2021). As mentioned earlier, the electrodes are to span the brain in order to provide an effective stun. Placement of the electrodes more caudally results in an insufficient current intensity and would require a higher voltage. Since the current moves into the brain via the nerves and carotid arteries, the electrodes aren't to be placed more than 5cm behind the ears (Gerritzen *et al.*, 2021).

2) Head-to-body

Head-to-body stunning utilizes a third electrode in addition to the electrodes spanning the brain, causing fibrillation of the heart and reducing the risk of consciousness returning (Lambooij, 2004; Gerritzen *et al.*, 2021). Sometimes, cardiac arrest is induced. This third electrode can be placed either on the chest (or near the heart and is often referred to as head-to-heart stunning) or the back (and is then referred to as head-to-back stunning). The method was developed to overcome the short duration of unconsciousness experienced in the head-only electrical stunning method.

The method still requires the animal to be killed by bleeding within a short time after stunning. The addition of a third electrode requires the system to be more automated than many head-only systems, and it is for this reason that many utilise a conveyor system to bring the pigs to the stunning area, where the brain-spanning electrodes are either placed automatically, or by a human stunner. In the head-to-heart stunning method, the third electrode is placed on the chest, near the heart, with the aim of inducing ventricular fibrillation.

Head-to-back stunning was developed as an alternative to the traditional head-only stunning method, aiming to improve animal welfare and carcass quality (Wotton *et al.*, 1992). This is done by placing electrode tongs over the pig's head, encompassing the brain, and placing another electrode on the pig's back. This both stuns the pig and fibrillates the heart, preventing the regaining of consciousness associated with the head-only method and its variable stun-

to-stick intervals (Channon *et al.*, 2002; Lambooij, 2004). In South Africa, the majority of head-to-body stunning systems have an automatic electrode at the chest area, and a human handler places the electrode tongs over the pig's head (personal observation). Objections to the head-to-back stunning method are based on the broken vertebrae that result when the voltage and current used is too high (Wotton *et al.*, 1992; Channon *et al.*, 2002).

2.5 Controlled atmosphere stunning (CAS) methods

Stunning using gas mixtures, especially CO_2, are common in commercial systems in Europe, Australia, Brazil, and the USA. Gas stunning is hailed to have benefits with regards to animal and labourer welfare, as well as product quality. Some gases, however, are criticised on the grounds of animal welfare (Raj & Gregory, 1995; Dalmau *et al.*, 2010c).

Different experimental mixtures of gases have been proposed to stun pigs before slaughter, including gases such as CO_2, argon (Ar) and nitrogen (N_2). Each has its own array of advantages and disadvantages that must be considered to stun and slaughter pigs as animanely (Webb & Webb, 2022) as possible. An advantage for all gas stunning methods is that the need for animal restraint is largely bypassed (Velarde *et al.*, 2000; Becerril-Herrera *et al.*, 2009), as animals are stunned in groups and handling stress is thus reduced (Rodríguez *et al.*, 2008).

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The stability of a gas mixture within the stunning pit, is defined as the capability of the gas to be sustained within the pit without being displaced by O_2 (Dalmau *et al.*, 2010b). This is influenced by each gas' density (kg/m^3) relative to air (relative density = 1.00), with heavier gases (relative density >1.00; such as Ar & CO_2) being more stable than lighter gases (relative density <1.00; such as N_2). Less stable gas mixtures tend to be less effective at inducing unconsciousness than more stable mixtures.

2.5.1 Commercial carbon dioxide (CO_2) stunning

CO_2 stunning is the most widely used gas stunning method (Velarde *et al.*, 2000; Becerril-Herrera *et al.*, 2009), and is used across Europe (Raj & Gregory, 1995; Dalmau *et al.*, 2010b; More *et al.*, 2018), South America (Marcon *et al.*, 2019) and the United States (Grandin, 2013). Despite its wide usage, there exists much controversy regarding its effects on animal welfare (Raj & Gregory, 1995, 1996; Raj *et al.*, 1997a; Raj, 1999) despite its positive effects on pork quality – especially when compared to stunning methods such as electrical stunning. CO_2 has a high vapour density (1.53), causing it to displace oxygen at lower altitudes and thus enabling its containment at the bottom of a dip-lift or paternoster stunning system (Dalmau *et al.*, 2010b).

The inhalation of high concentrations of CO_2 causes the blood CO_2 concentrations to increase, while the O_2 concentrations decrease. Respectively, these phenomena are termed

'hypercapnia' and 'hypoxia' (Raj *et al.*, 1997b). Physiologically speaking, this state is referred to as 'hypercapnic hypoxia'. When CO_2 is inhaled, HCO_3^- forms, leading to lower blood pH and increased blood CO_2 (Hambrecht *et al.*, 2004; Rodríguez *et al.*, 2008). Not only does blood pH drop, but the pH of cerebrospinal fluid also decreases, thereby inducing unconsciousness (Rodríguez *et al.*, 2008).

Different concentrations of CO_2 require different exposure times and stun-to-stick intervals. It is generally accepted that the higher the [CO_2], the shorter the necessary exposure time (Nowak *et al.*, 2007). When using an atmosphere of 80% CO_2, exposure times of >100s are recommended, along with a short stun-to-stick interval (<40s). 30% CO_2 seems to induce a tolerable level of respiratory distress as no attempts at escape were made during a study by (Raj & Gregory, 1996). Therefore, this could theoretically be combined with other inert gases such as Ar for a more humane stunning option compared to 90% CO_2.

Advantages of this gas include reductions in PSE- and petechiae incidences when compared to electrical stunning (Velarde *et al.*, 2000, 2001; Channon *et al.*, 2002). However, CO_2 stunning in and of itself does not affect the incidence of DFD, indicating that other factors, such as handling techniques and genotypes, must be improved upon to improve pork quality (Channon *et al.*, 2000). Compared to electrical stunning, stunning by CO_2 can lead to less partial carcass condemnations but may increase condemnations by lung congestion. Overall, CO_2 stunning is a highly effective stunning system, as long as sufficient exposure time is allowed, a short stun-to-stick interval is maintained and bleeding is effective (Von Wenzlawowicz *et al.*, 2012).

Only one South African abattoir uses the CO_2 stunning method (Petty, 2015). The abattoir can stun groups of 4-5 pigs per gondola in a four-gondola paternoster system. A concentration of 84% CO_2 is maintained in a pit to where the gondolas descend, exposing the pigs to the gas for 160s. The time required to reach the maximum CO_2 concentration is approximately 47s, which is too long based on recommendations by other researchers (Von Wenzlawowicz *et al.*, 2012).

2.5.2 Experimental Argon (Ar) stunning

Ar is widely experienced as both a tasteless and odourless gas, and induction of anaesthesia by anoxia is not as unpleasant as CO_2 (Raj & Gregory, 1995). It may be more expensive than CO_2 as it occurs naturally in minute quantities (0.9%) and must be extracted from atmospheric air (Raj & Gregory, 1995; Dalmau *et al.*, 2010b). Ar has a relative density of 1.38, and like CO_2, can be contained in a pit (Dalmau *et al.*, 2010b).

Stunning of pigs using a 90% Ar atmosphere proved to be more animane than 90% CO_2 as the pigs showed no signs of aversion and continued to feed under the influence, almost to the

point of LOC. The pigs showed no signs of posture loss and showed no recollection of an unpleasant experience, eagerly entering the same box the next day. However, the same authors do not recommend 90% Ar with 5% residual O_2 for commercial stunning as it induced mild respiratory distress after 60s of exposure time (Raj & Gregory, 1996). Decreasing the residual O_2 concentration to 2%, on the other hand, induced very little respiratory distress. On animal welfare grounds, this may be more acceptable when compared to the 12s of severe hyperventilation caused by 90% CO_2. Argon-induced anoxia is recommended due to the lack of aversive properties and its rapid abolition of brain responsiveness (Raj *et al.*, 1997a).

A mixture of 30% CO_2, 60% Ar and 2% residual O_2 induced a less severe state of hyperventilation compared to 90% CO_2, and a more rapid onset of unconsciousness compared to anoxia induced by 90% Ar (Raj & Gregory, 1996; Raj *et al.*, 1997a).

Stunning by Ar and Ar mixtures proved to be ineffective at killing pigs when compared to high concentration CO_2, as pigs exposed to Ar and its mixtures for 3min rapidly regained consciousness when returned to atmospheric air (Raj, 1999). This can be overcome by sticking (bleeding) the pigs within 25s of leaving the gas atmosphere. Moreover, unsightly convulsions took place while the carcasses were bleeding. Such convulsions could be a hazard to the slaughterhouse staff and could indicate that animals are regaining consciousness (Raj, 1999). On the other hand, pigs exposed to Ar and its mixtures for 5min and bled within 45s did not regain consciousness and no convulsions took place during bleeding. Increasing the exposure time to 7min killed most of the pigs exposed to the Ar atmosphere, and the remainder were bled within 45s to prevent return of consciousness. Exposing pigs to the 30% CO_2 / 60% Ar (2% residual O_2) for 7min resulted in a 100% mortality rate.

2.5.3 Experimental Nitrogen (N_2) stunning

N_2 makes up 78% of our atmosphere, and has a relative density of 0.97, rendering it slightly less dense than air. It's large portion of the atmosphere may also make it a more economic gas of choice than Ar (Dalmau *et al.*, 2010b). However, it must be used in a mixture with heavier gases such as CO_2. As N_2 is lighter than air, it will accumulate in the top portion of the stunning pit, and air (which is comparatively heavier) will accumulate at the bottom, and induction of unconsciousness would not be possible.

From an animal welfare point of view, gas mixtures of N_2 and CO_2 are less aversive to pigs and induce lower degrees of breathlessness in pigs (Llonch *et al.*, 2012b, 2013). Based on the percentage of animals gasping, mixtures containing up to 20% CO_2 induced a lower sense of breathlessness compared to mixtures containing 30% or more CO_2 (Llonch *et al.*, 2012b). As mentioned previously, the stun-to-stick interval is of utmost importance. If N_2 mixtures are

to be used, a very short stun-to-stick interval is needed in order to kill the animal before the return of consciousness. Pigs stunned using N_2 mixtures showed signs of returning consciousness before sticking could take place (Llonch *et al.*, 2012b, 2013). A mixture containing 85% N_2 and 15% CO_2 caused the shortest duration of unconsciousness, with rhythmic breathing returning at 17.7 ± 4.10s after stunning. In the mixture containing 70% N_2 and 30% CO_2 rhythmic breathing returned at 29.7 ± 3.47s after stunning, whereas pigs exposed to the 90% CO_2 treatment remained unconscious until sticking (Llonch *et al.*, 2012b). Mixtures containing more than 85% N_2 are not recommended for the stunning of pigs, and when mixtures containing 70-80% N_2 are used, longer exposure times (up to 5min) are recommended to induce unconsciousness (Llonch *et al.*, 2013).

Regarding meat quality, (Llonch *et al.*, 2012a) found that pigs exposed to an atmosphere of 80% N_2 and 20% CO_2 exhibited less muscular excitation. This is contrasted by (Llonch *et al.*, 2012a) who found that mixtures with 80-85% N_2 showed longer periods of muscle excitation and had lower pH_{45} values compared to mixtures containing less N_2. While no PSE carcasses were found in that study, the incidence of RSE (red, soft, exudative; a milder version of PSE) pork increased as the concentration of N_2 in the mixture decreased. Mixtures containing N_2 also had higher incidences of ecchymosis in the ham muscles. A definite correlation between pork quality and percentage N_2 cannot be established based on these studies as some did not consider the effects of the gas mixtures on meat (Llonch *et al.*, 2012a, 2013).

2.6 Welfare concerns regarding CO_2 stunning

CO_2 gas is acidic and has a strong odour, causing an unmissable sense of breathlessness when inhaled in large concentrations (Raj & Gregory, 1995; Llonch *et al.*, 2013). When given the choice, most pigs (88%) avoided the 90% CO_2 atmosphere (even when a reward in the form of apples was offered) (Raj & Gregory, 1995). The authors noted that, even after 24h of fasting, the pigs still avoided the 90% CO_2 atmosphere. Atmospheres containing >80% CO_2 induce severe respiratory distress, albeit for a short duration due to the rapid onset of unconsciousness (Raj & Gregory, 1996). CO_2 is an irritant to the nasal mucosal membranes, induces hyperventilation and suffocation before the onset of unconsciousness (Rodríguez *et al.*, 2008).

As the concentration of CO_2 increases, the duration of respiratory distress decreases (30s for 40% CO_2 and 12s for 90% CO_2) while the intensity remained constant regardless of CO_2 concentration. Attempts at escape are an observatory parameter also used to judge aversiveness. It was found that, at >80% CO_2, no attempts at escape were made, but this could be due to the quick onset of unconsciousness (Raj & Gregory, 1996). All pigs studied displayed moderate to severe hyperventilation before loss of posture was achieved (Raj &

Gregory, 1995, 1996). Pigs exposed to 80-90% CO_2 in air exhibited physical activity for 15s and were conscious for at least 16s and at most 36s. The welfare implication hereof is that pigs would have to endure severe respiratory distress for this period of time before they are rendered unconscious (Raj *et al.*, 1997a). (Rodríguez *et al.*, 2008) recommends a maximum stun-to-stick interval of 104s after 76s exposure to 90% CO_2 to avoid unnecessary pain and suffering due to return of consciousness during slaughter. It is necessary to mention that because many CO_2 systems stun pigs in groups of up to 8 pigs at a time, implying that the first pig to undergo sticking will have the shortest stun-to-stick interval, whereas the last pig would have the longest (Von Wenzlawowicz *et al.*, 2012). The above observations describe the aversiveness of CO_2 when inhaled in high concentrations, and it is on these grounds that many oppose the use of high concentration CO_2 stunning.

At lower concentrations, CO_2 can be used in anoxic (containing less than 2% residual O_2) gas mixtures, which are less aversive than 90% CO_2, to lower the severity of respiratory distress (Dalmau *et al.*, 2010a; c; Llonch *et al.*, 2012a; b, 2013). However, this requires that the exposure time to the gas mixture be increased to ensure a sufficiently deep state of unconsciousness, and that the stun-to-stick interval is drastically shortened to prevent return of consciousness.

2.7 Muscle Metabolites

Glycolytic metabolites that can be extracted from the muscle include glycogen, G6P, lactate (Dalrymple & Hamm, 1973), ATP and CP (Lamprecht *et al.*, 1974). Alongside pH and glycolytic potential, these parameters can indicate exposure to chronic or acute stress. High pH and low glycolytic potential is associated with chronic stress experienced during transport and lairage (Hambrecht *et al.*, 2004; Simela *et al.*, 2004), which could result in higher incidences of DFD meat (van der Wal *et al.*, 1989; Gonzalez-Rivas *et al.*, 2020; Manalo & Gabriel, 2020). High lactate concentrations directly after slaughter are indicative of acute stress (Simela *et al.*, 2004; Brandt *et al.*, 2013), and could lead to increased incidence of PSE meat (D'Souza *et al.*, 1998; Gonzalez-Rivas *et al.*, 2020; Manalo & Gabriel, 2020). Muscle metabolites and glycolytic intermediates can be measured at pre-determined intervals throughout the *post mortem* conversion of muscle to meat, allowing one to track its progression (Henckel *et al.*, 2002). Increased lactate concentrations following slaughter are associated with lower glycogen concentrations (Choe *et al.*, 2008, 2015). Muscle metabolite concentrations *post mortem* can be influenced by the species, breed or genetic line, pre-slaughter handling, stunning and *post mortem* processing like the application of electrical stimulation and chilling regime (Huff-Lonergan & Johnson, 2001; Hambrecht *et al.*, 2005).

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CHAPTER 3: MATERIALS AND METHODS

3.1 Experimental Design

Ethical approval for this research was obtained from the Ethics Committee of the faculty of Natural and Agricultural Sciences and from the Animal Ethics Committee of the University of Pretoria with ethics approval number NAS307/2020. The experimental design of the Pilot Study and the Follow-Up studies are presented in Table 3.1 and the parameters studied are presented in Table 3.2.

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Table 3.1 *Stunning methods used at each abattoir during each phase of study.*

Phase	Abattoir A	Abattoir B
Pilot	Standard 84% CO_2	Not applicable
	Experimental 80% CO_2 20% Ar	
	Emergency head-only electrical stunning	
Follow-up	Standard 84% CO_2	Standard head-to-heart electrical stunning

Table 3.2 *Data collected at each abattoir during each phase of study.*

Phase	Abattoir A	Abattoir B
Pilot	Carcass pH	Not applicable
	Behaviour	
Follow-up	Carcass pH	Carcass pH
	Carcass temperature	Carcass temperature
	Behaviour	Behaviour
	Muscle metabolites	Muscle metabolites

3.2 Phase 1: Pilot study

3.2.1 Handling procedures during loading and transportation

The pigs in this study were sourced from Molare Meats' Ede farm, outside Middelburg, in the Mpumalanga province of South Africa, to use pigs from a similar genetic background.

The pigs were herded, pen-by-pen, in smaller groups by way of a system of plastic boards, black bags and whistles. The pigs exhibited curiosity yet were still willing to walk to the truck. A few individuals were startled on the way to the truck and attempted to return to their pen. The pigs were moved in smaller groups as this causes less stress compared to movement in

large groups. No prodders were used. Pigs that have tired on the way to the truck were kept aside and loaded after they have recovered. Some pigs showed signs of bruising and others had scratches.

The trucks used are double-deckers, capable of holding 88 pigs in both the top and bottom compartments. The dense stocking allows the pigs to stand up, without risk of falling about when the truck moves. Pigs from different pens are mixed on the truck, which inevitably results in the re-establishment of their social hierarchy. Before leaving the farm, the truck was weighed and sealed. This seal was only removed at the abattoir before offloading. The truck travelled 160km from Middelburg to Olifantsfontein. This remained the same for the pigs used in the second phase of the study.

3.2.2 Data collection at abattoir A

Three stunning treatments were administered, namely emergency head-only electrical stunning, 84% CO_2 and an admixture consisting of 80% Ar and 20% CO_2. Video footage of the pigs' behaviour during stunning was recorded for both the 84% CO_2 and 80% Ar 20% CO_2 treatments.

Pigs were selected randomly. These were loaded onto a double-decker truck and transported to abattoir A and left to rest in lairage overnight. For the 84% CO_2 treatment, five groups of five pigs were moved towards the stunner (Butina Backloader 4), according to the abattoir's standard operating procedures (SOP's). As this was a pilot study, the number of pigs used was chosen, initially to determine whether the experimental design was feasible, and whether a larger trial was feasible. Each group was exposed to 84% CO_2 for approximately 160s (excluding the time used for loading, moving and unloading). One pig from each group was randomly selected for EEG monitoring and future carcass pH monitoring. A surveillance camera (GoPro 9 Black) was attached to the top left corner of the stunning box, to provide a view of the whole box. This was used to record the pigs' reaction to the stunning atmosphere.

Commented [AO12]: How did you decide on this number?

A temporary stunning box was constructed to perform the electrical stunning procedure, according to abattoir A's SOP's. Seven pigs were stunned in this manner, at 1 Amp and 240V for 15s. Duration of stun was determined and checked by a veterinarian. Directly after stunning, EEGs were recorded, and the carcasses were processed. pH monitoring was done on all seven electrically stunned carcasses.

Due to labourer safety and animal welfare concerns, it was decided to stun five pigs, one at a time, during the 80% Ar 20% CO_2 treatment. Each pig was exposed to the gas mixture until an acceptable appearance of unconsciousness was reached. All five of these pigs had EEG

monitoring done and were used for carcass pH monitoring. The EEGs were used to determine state of unconsciousness after pigs have come out of the stunner. The research team was unable to record EEGs throughout the whole stunning process due to the design of the equipment.

Carcass pH was recorded from the *M. longissimus thoracis*, between the 11th and 12th ribs, at hourly intervals of 1-, 3-, 6-, 12- and 24-hours *post mortem*. Muscle samples of approximately 2cm x 2cm x 2cm were collected from the same area as the pH measurements during carcass evisceration and were placed in liquid nitrogen immediately. This was done for all treatments. The muscle samples were stored in the Agricultural Research Council (ARC) Irene's Meat laboratory freezer (-80℃) for subsequent metabolomic analyses.

Commented [AO13]: Depending on your analysis -80 should be suggested

3.2.3 Video review and scoring

Video footage recorded during the 84% CO_2- and 80% Ar 20% CO_2 treatments was reviewed and scored by a panel comprised of the research team and industry representatives from different regulatory bodies, including, but not limited to, the NSPCA (National Council of Societies for the Prevention against Cruelty of Animals), the RMAA (Red Meat Abattoir Association) and GDARD (Gauteng Department of Agriculture and Rural Development). Aversive behaviour was identified and scored according to a scoring sheet set up based on the studies by Llonch et al. (2013) and Atkinson et al. (2015). Attention was given to times at which certain behaviours were exhibited by the pigs.

Commented [AO14]: This is actually difficult. Were your panellists male or female and other contributing factors like age and experience that could influence their perception.

3.3 Phase 2: Comparison of CO_2- with electrical stunning

3.3.1 Data collection at abattoir B

The pigs used in the study had been standing in lairage for about two hours before being herded towards the stunning block. Filming of pigs' behaviour during stunning started promptly at 08:00. 25 pigs were filmed from the front, with their facial expressions and the placement of the electric nodes visible. Representatives from the NSPCA Animal Ethics Unit were present for consultation on the necessary angles of the videos. Filming ended at 08:12, when 25 pigs had been stunned and slaughtered.

The videos were recorded with a Nikon D500 camera and Nikon 17-35mm lens, as well as a RodeMic Pro+ microphone for accurate sound recording. The videos were immediately handed over to abattoir B's management team, who now own all existing copies of, and rights, to said videos. All videos and data were recorded under normal commercial conditions, i.e., under abattoir B's standard operating procedures (SOPs). No part of the process from the lairage area, stunning area, or processing inside the abattoir or chiller was altered during video recording or data collection.

At 09:00, from the 25 recorded pigs, 10 carcasses were selected at random for sample-, pH- and temperature data collection. One muscle sample was collected per carcass (10 in total) from the *longissimus thoracicis* on the left half of the carcass (facing the dorsal region), 3 cm from the vertebrae, 2 cm deep and between the 11^{th} and 12^{th} ribs. pH- and temperature readings were taken from the same area where the muscle sample was collected from. pH- and temperature data from the right side of the carcasses were also taken, but no muscle sampling was done on the right side of the carcasses.

pH- and temperature data was recorded at 1-, 3-, 6-, 9-, 12-, and 24-hours *post mortem*, to determine rate of pH decline and carcass cooling. Thus, the relevant pH- & temperature data was recorded at 09:00, 11:00, 14:00, 17:00, 20:00 and 08:00 (next day). Muscle samples were stored in numbered plastic bags and put in liquid nitrogen for immediate freezing, to stop the conversion of muscle to meat. These samples were transported to the ARC Meat Laboratory in Irene, Pretoria, where muscle metabolites were analysed from each of these collected samples.

3.3.2 Data collection at abattoir A

The recording of behavioural footage commenced at 09:00. Representatives of the NSPCA's Animal Ethics Unit were present for the recording of footage to be used in the study.

25 pigs were filmed inside the CO_2 stunner from two opposing angles, in five groups of five pigs each. Filming from two angles was done to capture most of the pigs' behaviour and facial expressions, providing a realistic view of the activity inside the stunner. The videos were recorded using two action cameras (GoPro 9 Black, GoXtreme Rebel) mounted on opposite ends inside one of the cradles inside the Butina.

As far as possible, all videos and data were recorded under normal commercial conditions, i.e. under abattoir A's standard operating procedures (SOPs). Except for the pausing of the stunner in order to adjust the cameras on two occasions, no other part of the process from the lairage area, stunning area, processing inside the abattoir or the chiller was altered during either video recording or data collection.

Due to technical difficulties, more than five groups of pigs were recorded. In total, 10 groups were recorded. Two of these groups were not recorded fully due to technicalities with one of the cameras used. Five groups were recorded fully, and an extra group was recorded in case there was an error with one of the aforementioned five recordings. The first five recordings that have recorded fully will be used as part of the study (thereby adhering to the ethical

clearance agreement) and, along with the other three videos, as part of an evaluation of the CO_2-stunning method.

Of the 25 pigs recorded, two were randomly selected from each group for pH-, temperature- and muscle sample recording. As was the case at abattoir B, one muscle sample was collected per pig, from the *longissimus thoracicis* on the left half of the carcass (facing the dorsal region), 3 cm from the vertebrae, 2 cm deep and between the 11th and 12th ribs. pH- and temperature readings were taken from the same area where the muscle sample was collected from. pH- and temperature data from the right side of the carcasses were also taken, but no muscle sampling was done on the right side of the carcasses.

Carcass pH- and temperature data were recorded at 1-, 3-, 6-, 9-, 12-, and 24 hours *post mortem*, to determine rate of pH decline and carcass cooling. As the pigs were stunned at various times between 09:00 and 10:00, the relevant pH- & temperature data was recorded at 10:00-11:00, 10:00-11:00, 12:00-13:00, 15:00-16:00, 18:00-19:00, 21:00-22:00 and 09:00-10:00 (next day). Muscle samples were stored in numbered plastic bags and put in liquid nitrogen for immediate freezing, so that the muscle to meat process can be stopped. These samples were transported to the ARC Meat Laboratory in Irene, Pretoria, where muscle metabolites were analysed from each of these collected samples.

3.3.3 Video review and scoring

A review panel was formed, with representatives from the NSPCA, RMAA and GDARD as well as the research team and their research consultants. The aversion scoring sheets (tables 4.1-4.5) used in the Pilot study were revised and improved upon (tables 4.27-4.30). A similar, yet adapted, approach was used to evaluate the welfare during stunning at abattoir B. The videos were reviewed at abattoir A. Attention was given to times at which certain behaviours were exhibited by the pigs.

3.3.4 Metabolomic analyses

20 samples, 10 samples from each treatment, were used for muscle metabolite analyses by way of spectrophotometer analysis. This was conducted in the ARC Meat Science laboratories. The Agilent 8453 UV-Visible Spectroscopy system was used along with the Agilent Chemstation Software.

Muscle metabolites that were analysed were L-lactate, glycogen, glucose, creatine phosphate (CP), adenosine triphosphate (ATP) and glucose-6-phosphate (G6P). Buffers that had to be prepared beforehand included perchloric acid, a lactate buffer, an acetate buffer, potassium

hydroxide, a triethyl (glycol) buffer, sodium hydroxide, a triethyl (CP, G6P, ATP) buffer, $MgCl_2$, glucose, and methyl orange.

The specifications and use for each buffer were as follows:

- Perchloric acid (0.6M, $HClO_4$), which was used for sample extraction and was to be stored at 4°C until use.
- Lactate buffer (Hydrazine/glycine, pH 9), used for L-Lactate analysis and stored at 25°C.
- Acetate buffer (0.2M, pH 4.8), used for glycogen analysis and stored at 25°C.
- 1N KOH, used for glycogen analysis and stored at 25°C.
- KOH (5.4M) used for extraction and neutralising of samples, and to be stored at 25°C.
- Triethyl (glycol) buffer (Triethanolamine HCl/Mg, 0.3M, pH 7.5) stored at 25°C.
- 1N NaOH, used for CP analysis and stored at 25°C.
- Triethyl (CP, G6P & ATP) buffer (Triethanolamine HCl/NaOH, 0.05M, pH 7.5) stored at 25°C.
- $MgCl_2$, used for analysis of G6P and stored at 4°C.
- Glucose, used for analysis of CP and stored at 4°C.
- Methyl orange (indicator) stored at 25°C.

Solutions that had to be made on the day of use included amyloglucosidase (AGS), b-nicotinamide adenine dinucleotide (NAD), glycogen buffer, nicotinamide adenine dinucleotide phosphate (NADP), adenosine diphosphate (ADP) and creatine kinase (CK). These all were made in distilled water (H_2O) and stored at 4°C until needed.

20 sample extractions were prepared by adding 10 ml cold 0.6M $HCℓO_4$ and 2g of meat sample to each test tube. These where then homogenised and centrifuged at 10 000 rpm for 15 minutes.

For the glycogen extraction, another set of test tubes was used, and to each of these 0.1 ml 0.6M $HCℓO_4$, 50ul KOH and 1ml AGS solution was added. 100ul of the extracted sample prepared earlier was added to this AGS solution and heated for two hours at 40°C. The resulting reaction was stopped after heating by adding 0.5ml 0.6M $HCℓO_4$ to the test tube. A few drops of Methyl Orange indicator were added to the extracted glycogen samples to indicate when the solution reaches neutrality. 5.4M KOH is added in drops until the solution turns yellow. Filter paper and glass filters are used to remove solids from the solution. The volume of the solution was measured, and the mass used for calculating extraction (ml) per muscle weight (g).

Metabolic analyses:

1. L-lactate:

L-lactate was analysed on a method based on that by Gutmann and Wahlefeld (1974). The spectrophotometer was calibrated against an empty quartz cuvette. To the 1st cuvette (the control), 2.5ml of the lactate buffer, 200ul of the NAD solution and 20ul of the 0.6N perchloric acid solution was added. To the rest of the cuvettes, 2.5ml of the lactate buffer, 200ul of the NAD solution and 20ul of the yellow samples was added. The solutions in the cuvettes were stirred and read at 340nm at 37°C. 20ul L-lactate dehydrogenase solution was added to each cuvette. The solutions in the cuvettes were stirred, and after 30min, the L-lactate values were read.

2. Glycogen:

Glycogen was analysed based on the methodology in Keppler and Decker (1974). The spectrophotometer was calibrated against an empty micro cuvette. Two sample sets were used for the determination of the glycogen content, a clear glycogen sample and a yellow glucose sample. 1ml of the glycogen buffer was added to 50ul of each sample in the micro cuvettes. The solutions in the cuvettes were mixed, left to stand for five minutes, and read at 340nm. 5ul Hexokinase is added to each of the sample solutions, and then mixed thoroughly. After resting for 10 minutes, the glucose and glycogen values were read. (Glycogen values were determined by subtracting the glucose content values in the clear samples from those in the yellow samples).

3. Glucose-6-Phosphate:

The spectrophotometer was calibrated against an empty micro cuvette. 2.5ml of the Triethyl (CP, G6P, ATP) buffer, 100ul NADP, 100ul $MgC\ell_2$, 20ul ADP was added to 50ul of each of the (yellow) samples in the micro cuvettes. The solutions were mixed and allowed to stand for five minutes. 5ul Glucose-6-phosphate dehydrogenase suspension solution was added to the samples. The solutions were mixed and allowed to stand for five minutes, after which the G6P values were read. These solutions are to be used to determine the adenosine tri-phosphate content, as detailed by Lamprecht *et al*. (1974).

4. Adenosine Triphosphate:

Still using the methodology in Lamprecht *et al.* (1974), 100ul glucose was added to the solutions (as prepared for the determination of glucose-6-phosphate content). After being thoroughly mixed together, 5ul hexokinase was added to the solutions. The solutions were mixed, allowed to stand for five minutes, after which the adenosine triphosphate values could be read. These solutions are to be used to determine the creatine phosphate content.

5. Creatine Phosphate:

The solutions (as prepared for the determination of adenosine triphosphate content) were left to stand for a further 20 minutes. Thereafter, 20ul creatine kinase was added to the solutions. These solutions were mixed thoroughly, and left to stand for 10 minutes, after which the creatine phosphate content could be determined. This was done following methodology by Lamprecht *et al.* (1974).

Commented [AO15]: Just remember to add references for these analyses

3.4 Statistical analysis

The data was analysed using IBM SPSS Statistics (IBM SPSS Statistics Version 29.0.0.0. [241]) and Microsoft Excel (Microsoft® Excel® for Microsoft 365 MSO (Version 2311 Build 16.0.17029.20028) 64-bit). Differences between treatment means were tested by means of the Linear Mixed methods procedure in SPSS and by using the Repeated Measures option. The Bonferroni Multiple Range test was employed to test between different treatment means at a level of significance of 95% ($P<0.05$).

CHAPTER 4: RESULTS

4.1 Introduction

The study was conducted under commercial conditions in two phases. During the pilot study, pigs were exposed to three different stunning treatments, namely standard 84% CO_2, experimental 80% Argon-20% CO_2 gas admixture and emergency head-only electrical stunning. Data was collected in the form of pH data, video recordings of animal behaviour during stunning, electroencephalograms (EEGs) directly after stunning and *m. longissimus thoracis* (LT) samples, which were analysed for *post mortem* muscle metabolites. The video footage was scored by a review panel consisting of the research team, their research consultants and industry representatives from the RMAA, NSPCA and GDARD. Regrettably, due to load-shedding and cable theft, the freezer at the ARC was not able to store the muscle samples at the optimum temperature for the analysis of the muscle metabolites. This caused the samples to thaw prematurely, and they were thus discarded. This motioned the proposal for a follow-up study to the originally approved pilot study.

The follow-up study differed from the pilot study in that the experimental Argon admixture and emergency electrical stunning treatments were discontinued on the grounds of poor animal welfare and unfair industry representation. In the follow-up study, pigs were stunned by means of the standard 84% CO_2 stunning and standard head-to-heart electrical stunning. Data collected included carcass pH profiles over time, carcass temperature data over time, LT muscle samples and video recordings of animal behaviour during stunning. The LT muscle samples were analysed for the following metabolites: L-lactate, glucose, glycogen, G6P, ATP and CP. The video footage was scored by a review panel consisting of the research team, their research consultants and industry representatives from the RMAA, NSPCA and GDARD. This data was analysed for effects of the individual researcher and the stunning procedure used.

Commented [AO16]: You don't really explain how this was analysed statistically

4.2 Pilot study

4.2.1 Panel review of behavioural footage

The behaviour visible on the video footage was evaluated by a team representing different aspects of the pork industry, i.e. welfare, slaughtering process, veterinarians, and researchers. Scoring was done in accordance with the displayed behaviours and their perceived intensity according to the following guidelines (Atkinson *et al*., 2015):

Table 4.1 *List of defined behaviours that may be displayed in video footage of pigs during stunning in the Butina with standard 84% CO_2 atmosphere.*

Commented [AO17]: You need a reference for these observations. How did you decide on these?

Behaviour parameters	Definition
Retreat/escape attempt	First pig in group backs up/turns around/moves forward in cage
Neck stretch	First pig in group lifts its head outwards/stretches the neck, either when standing/sitting/laying
Gasping	First pig in group opens and closes his mouth while standing upright
Swaying	First pig in group to lose balance without falling over
Sitting	First pig in group sits with rump on floor
First fall	First pig to fall over
First reaction	Any of the above behaviours that could be identified as the first reaction to the gas
Gagging	First pig in group periodically opens and closes its mouth while laying on ground
Last fall	Last pig to fall over
First significant muscle contractions	First pig starts shaking and convulsing – whole body or just parts
Last significant muscle contractions	Last pig finishes shaking and convulsing – whole body or just parts

Table 4.2 *Aversive behaviours & their severity (indicated by scores)*

Commented [AO18]: You need a reference

Expected behaviour (0) *	Slight/moderate discomfort	Severe/extreme discomfort
• Swaying • Sitting • Falling • Involuntary muscle contractions • LOC	• Neck stretching (1) • Gasping (1) • Gagging (2) • Minor vocalisations (2) • Other voluntary movements before LOC** (2)	• Convulsions (2) • Severe vocalisations (3) • Escape attempt (3) • Consciousness (voluntary movements after supposed LOC***) (4)

* Expected behaviours not indicative of stress, and so doesn't contribute towards aversion score.
** Includes thrashing, kicking, etc.
*** Voluntary movements after supposed LOC indicate that consciousness has either been regained or that it has not been lost in the first place (insufficient stun)

Table 4.3 *Aversion classes & their associated severity score ranges.*

Commented [AO19]: How were these analysed?

Welfare Class	5	4	3	2	1
Severity of discomfort/stress	No aversion	Slight	Moderate	Severe	Extreme
Point range	0	1-4	5-8	9-12	13-20

4.2.1.1 84% CO_2 controlled atmospheric stunning

The first group exhibited gasping-, swaying- and neck-stretching movements in reaction to the CO_2. All five pigs fell and lost their consciousness. After this, involuntary muscle contractions

were visible. The second group exhibited gasping-, swaying- and neck-stretching movements while exposed to the CO_2. An escape attempt was also recorded.

Table 4.4 *Stunning scores and their corresponding welfare classes for CO_2 stunning.*

Group	Sample size	Stunning treatment	Reviewer #	Score	Class
1-1	5	CO_2	1	2	4
			2	2	4
			3	4	4
			4	4	4
			5	3	4
			6	2	4
			7	2	4
			Average	**2.71**	**4.00**
1-2	5	CO_2	1	2	4
			2	4	4
			3	4	4
			4	3	4
			5	4	4
			6	1	4
			7	1	4
			Average	**2.71**	**4.00**
1-4	5	CO_2	1	4	4
			2	4	4
			3	3	4
			4	4	4
			5	10	2
			6	4	4
			7	3	4
			Average	**4.57**	**3.71**
Individual 1	1	CO_2	1	0	5
			2	0	5
			3	4	4
			4	4	4
			5	2	4
			6	0	5
			7	3	4
			Average	**1.86**	**4.43**
Individual 2	1	CO_2	1	6	3
			2	1	4
			3	4	4
			4	4	4
			5	4	4
			6	1	4
			7	3	4
			Average	**3.29**	**4.00**

All five pigs fell and were rendered unconscious. Involuntary muscle contractions were visible after this. The fourth group exhibited swaying-, gasping-, gagging- and neck-stretching movements during stunning. All five pigs fell and lost their consciousness. Involuntary muscle contractions were visible after the onset of unconsciousness.

Regrettably, there was a technical malfunction with the camera, and as such, the third group was not recorded fully, and the fifth group was not recorded at all.

Two pigs were stunned individually to determine the order of behaviours in reaction to the CO_2 gas. The first pig was not fully recorded due to a camera malfunction. However, the pig sat upon entry into the CO_2. It lost its balance before displaying any other behaviours. Most visible behaviours could be attributed to involuntary muscular contractions. The second pig exhibited neck-stretching-, gasping-, sitting-, gasping-, swaying- and gagging behaviour. It lost its balance, was rendered unconscious, and displayed involuntary muscle contractions after the onset of unconsciousness.

Table 4.4 represents the scores given by each reviewer (kept anonymous) based on the behaviours visible and the perceived intensity of each. Included is the average score and class for each group.

4.2.1.2 80% Ar 20% CO_2 controlled atmospheric stunning

The first pig exhibited swaying-, gasping- and gagging movements, of which the latter two were observed throughout the stunning period. Squeals were observed. Reviewers reported that some jumping movements looked like escape attempts. The pig fell and displayed thrashing and kicking movements. Gasping and neck movements were visible until ejectionfrom the gondola. The pig was not stunned irreversibly, i.e. signs of returning consciousness were evident at the time of sticking.

The second pig displayed gasping-, gagging- and swaying behaviours when exposed to the gas admixture. After falling, gasping, and gagging was observed until the crate was emptied. Squealing was observed. The pig lost its balance and consciousness. As was the case for the first pig, the second pig was not stunned irreversibly. Muscular contractions were visible until ejection from the gondola.

The third pig exhibited swaying-, backing- and neck-stretching when exposed to the admixture. After it lost its balance and fell, neck-stretching, kicking, and thrashing was visible. These behaviours took place before LOC was observed in the form of a loss of control over the ears and are thus assumed to have been voluntary movements. Muscular contractions ceased before ejection from the gondola.

Table 4.5 *Stunning scores and their corresponding welfare class for Ar-CO_2 stunning.*

Pig	Stunning Treatment	Reviewer #	Score	Class
1	Ar-CO_2	1	8	3
		2	7	3
		3	9	2
		4	7	3
		5	7	3
		6	6	3
		7	12	2
		Average	**8.00**	**2.71**
2	Ar-CO_2	1	6	3
		2	12	2
		3	7	2
		4	4	4
		5	12	2
		6	6	3
		7	8	3
		Average	**7.86**	**2.71**
3	Ar-CO_2	1	10	2
		2	7	3
		3	5	3
		4	3	4
		5	6	3
		6	5	3
		7	10	2
		Average	**6.57**	**2.86**
4	Ar-CO_2	1	9	2
		2	12	2
		3	9	2
		4	6	3
		5	7	3
		6	6	3
		7	6	3
		Average	**7.86**	**2.57**
5	Ar-CO_2	1	5	3
		2	6	3
		3	7	3
		4	6	3
		5	4	4
		6	11	2
		7	6	3
		Average	**6.43**	**3.00**

The fourth pig exhibited backing-, swaying- and gasping behaviours when exposed to the admixture. The pig fell, and lost its consciousness, again indicated by the loss of control over the ears. Before this, thrashing, kicking, and squealing was observed. Muscle tremors were observed until ejection form the gondola.

The fifth pig displayed swaying- and backing movements when exposed to the admixture. It lost its balance and, later, its consciousness. Between falling and losing consciousness, squealing, gasping, and kicking was observed. Muscle contractions ceased before ejection from the gondola.

As these five pigs were not stunned irreversibly (i.e. killed inside the gondola after losing consciousness), the time interval between stunning and sticking was closely monitored. Sticking took place as soon as possible after ejection from the stunner.

Table 4.5 describes the scores and associated welfare classification attributed by each reviewer (kept anonymous) for each pig.

4.2.1.3 Emergency head-only electrical stunning

The emergency head-only electrical stunning method was ruled as unsatisfactory by the review panel. Thus, this method could not be used as a reliable control for the pilot. It was not filmed like the gas stunning methods, as a temporary stunning box was used for this.

It was noted on two out of seven occasions, that the tongs were not placed at the correct position for head-only stunning. The depth of unconsciousness in these cases are called into question, as are the intervals between stunning and sticking. On one occasion, the animal was not properly restrained, which may have resulted in an improper stun.

4.2.2 Descriptive statistics for carcass pH profiles of pigs

Table 4.1 summarises the minimum and maximum pH values recorded at each time stamp (hours *post mortem*), as well as the means and standard deviations. In total, 19 pigs were used in the pilot study.

The maximum values for the Ar-CO_2 admixture at 1-, 3-, and 21-hours *post mortem* were 6.79, 5.84 and 5.62, respectively. The corresponding minimum values, in the same order, were 6.12, 5.58 and 5.41, respectively. The mean values and standard deviations at 1-, 3- and 21-hours *post mortem* were 6.50 ± 0.25, 5.72 ± 0.01 and 5.52 ± 0.09, respectively.

The maximum pH values for the CO_2 stunning treatment at 1-, 6-, 9-, and 24-hours *post mortem* were 6.90, 6.61, 6.09 and 5.86, respectively. The corresponding minimum pH values, in the same order, were 6.31, 6.26, 5.65 and 5.43, respectively. The mean pH values and

standard deviations at 1-, 6-, 9-, and 24-hours *post mortem* were 6.65 ± 0.18, 6.46 ± 0.15, 5.88 ± 0.19 and 5.58 ± 0.16, respectively.

Table 4.6 *Descriptive statistics for pH measurements at 1-, 3-, 6-, 9-, 21- and 24 h post mortem for different stunning methods.*

Time	Stunning Treatment	Minimum	Maximum	Mean	Std. Deviation
1	Ar-CO_2	6.12	6.79	6.50	0.25
	CO_2	6.31	6.90	6.65	0.18
	Electrical	6.10	6.61	6.36	0.23
3	Ar-CO_2	5.58	5.84	5.72	0.09
	Electrical	6.12	6.56	6.28	0.16
6	CO_2	6.26	6.61	6.46	0.15
	Electrical	5.32	6.13	5.84	0.27
9	CO_2	5.65	6.09	5.88	0.19
21	Ar-CO_2	5.41	5.62	5.52	0.09
24	CO_2	5.43	5.86	5.58	0.16
	Electrical	5.32	5.74	5.58	0.15

The maximum pH values for electrical stunning at 1-, 3-, 6- and 24-hours *post mortem* were 6.61, 6.56, 6.13 and 5.74, respectively. The corresponding minimum pH values, in the same order, were 6.10, 6.12, 5.32 and 5.32, respectively. The mean pH values and standard deviations were 6.36 ± 0.23, 6.27 ± 0.16, 5.84 ± 0.27 and 5.58 ± 0.15, respectively.

Commented [AO20]: I assume there were no significant differences?

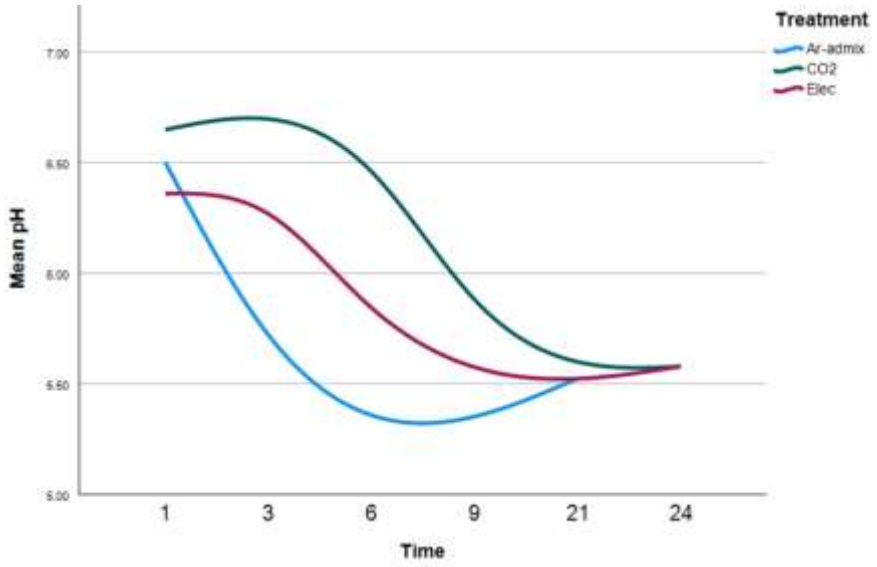

Figure 4.1 *Effect of stunning method on carcass pH from 0 to 24 h post mortem.*

Figure 4.1 depicts and compares the rate of pH-decline over a 24-hour period among the three stunning methods. The Ar-CO_2 admixture is represented by the blue line, 84% CO_2 is represented by the green line, and emergency electrical stunning is represented by the red line.

Both the 84% CO_2 and electrical stunning methods depicted similarly-shaped decline curves, whereas the Ar-CO_2 admixture, comparatively, depicted a much sharper and earlier rate of decline. Despite having different starting mean pH-values, both the 84% CO_2- and electrically-stunned carcasses had a similar pH_u at 24-hours *post mortem*.

4.2.3 Video Statistics

Timings and durations of important behavioural events were recorded by noting the time on the video when certain events took place or certain behaviours were exhibited and measuring the time difference between these points. This was done for the videos of both the 84% CO_2- and 80% Ar 20% CO_2 stunning treatments.

"Time to exposure" was measured as the time between the start of the descent into the pit and the first reactions to the gas. "Total time in stunner" is defined as the time between entry into and exit from the gondola. "Total time in gas" is defined as the time between the first exposure to gas and ascent out of the pit. "First reaction" is defined as the first possible reaction to the gas (by any or all the pigs). Time until LOC was measured as the time between exposure to the gas and the last head drop and/or loss of control over ears. Complete LOC was assumed to have taken place when a pig was no longer able to hold up its head voluntarily, or, if its head was not visible to the camera, the permanent drooping of its ears. "Time to fall" is defined as the time between the entry into the gas and the pigs losing their balance. "Muscle contractions" is defined as the time during which muscle contractions, which can be attributed to involuntary movements, take place. Descriptive statistics for this video data will be discussed with those of Follow-up study in the next section.

4.2.3.1 84% CO_2 Stunning

Table 4.7 *Timing of major events and behaviours observed in the footage recording of Group 1-1.*

Event/behaviour	**Time (hh: mm: ss)**
Entry into the stunner	10:33:57
Gondola starts descending, one pig jumps on another	10:34:25
Vocalisation by pig being jumped on	10:34:27
At least one pig gasping	10:34:38

All pigs start exhibiting swaying movements – theorised entry into CO_2	10:34:42
First pig to lose balance, falls	10:34:47
Potential jump/escape attempt, next two pigs fall	10:34:49
Attempts to regain posture unsuccessful	10:34:53
Gasping, all pigs on ground	10:34:54
Short vocalisation (bark), gasping	10:34:59
One pig lying on another, looks like an escape attempt (but not able to prove as such)	10:35:03
Kicking and thrashing movements, involuntary shivers visible	10:35:07
First pig to drop head – possible onset of LOC	10:35:15
Muscle contractions - Most cease - Some still present - Last muscle contraction	10:35:21 – 10:37:31 - 10:36:10 - 10:36:55 - 10:37:31
Ascent out of pit	10:37:26 – 10:38:14
Ejection from gondola	10:38:19

Table 4.8 *Durations of major events and behaviours during stunning of Group 1-1.*

Event	**Duration/time to (mm: ss) (s)**
Time to exposure	00:27 (27)
Total time in stunner	04:22 (262)
Total time in CO2	02:44 (164)
First reaction (gasping)	00:07 (7)
Time until assumed LOC	00:33 (33)
Time to fall First pig falls Second & third pigs fall Fourth & fifth pigs fall	(8.6) 00:05 (5) 00:07 (7) 00:12 (12)
Muscle contractions	02:10 (130)

The pigs reluctantly entered the gondola. At the start of the descent, one of the pigs jumped on another, which had vocalised in return. During the descent, at least one pig could be seen gasping. Exposure to CO_2 was assumed at the time when all five pigs suddenly displayed a reaction – in this case, swaying movements. After the first pig had lost its balance, the rest lost their balance and fell after an average time of 4.5s. At 10:34:49, a jump was witnessed. Whether or not this was an escape attempt could not accurately be determined as the thrashing and kicking movements, along with the inability to regain posture, made the pig land on another. All five pigs were gasping while they were lying down. At 10:34:59, a short vocalisation (between a grunt and a squeal) was heard while the gasping continued. When pigs lie on another, kicking and thrashing movements can look like escape. At 10:35:15, a definite head drop was witnessed. This was likely an involuntary action, and a sign that consciousness had been lost. Involuntary muscle contractions persisted for 130s from 10:35:21 until they ceased at 10:37:31.

Commented [AO21]: Well done to be able to describe these stressful events in so much detail

Table 4.9 *Timing of major events and behaviours observed in the footage recording of Group 1-2.*

Event/behaviour	Time (hh: mm: ss)
Entry into the stunner	10:39:55
Gondola starts descending	10:40:26
One pig starts swaying	10:40:36
Rest of pigs start swaying – theorised entry into CO_2	10:40:38
Gasping	10:40:38
One pig jumps and knocks the camera	10:40:39
At least one pig loses balance and falls, thrashing movements are visible	10:40:44
Another pig falls, thrashing movements visible	10:40:50
Growl-grunt-like vocalisation heard	10:40:55
One pig drops its head	10:41:06
Kicking	10:41:10
No voluntary movements visible – LOC assumed	10:41:14
Muscle contractions - Convulsions - Last muscle contraction	10:41:18 – 10:43:33 - 10:42:03 - 10:43:33
Signs of breathing	10:43:15
Ejection from the stunner	10:44:20

Table 4.10 *Durations of major events and behaviours during stunning of Group 1-2*

Event	Duration/time to (mm: ss) (s)
Time to exposure	00:12 (12)
Total time in stunner	04:25 (265)
Total time in CO_2	Not determined
First reaction (swaying)	00:10 (10)
Time until assumed LOC	00:30 (30)
Time to fall - First visible fall - Last visible fall	(9) - 00:06 (6) - 00:12 (12)
Muscle contractions	02:15 (135)

The pigs were somewhat reluctant to enter the gondola without the assistance of the automatic push arm. 10s after the start of the gondola's descent, one pig starts swaying. This is likely due to exposure to CO_2, as the rest of the pigs also displayed swaying movements two seconds later. This was assumed as the time of entry into the CO_2 gas. At 10:40:39, a pig jumped and, in doing so, knocked the camera. This was considered to be an escape attempt. The camera was knocked in such a way that the view of the pigs in the gondola was obscured and incomplete. Due to the obscured view, some data could not be collected. Thrashing and kicking was observed periodically until 10:41:10. Gasping was observed periodically until 10:40:38. Breathing movements (chest movements) were observed until 10:43:15. It is assumed that death took place shortly after this.

Table 4.11 *Timing of major events and behaviours observed in the footage recording of Group 1-4.*

Event/behaviour	**Time (hh: mm: ss)**
Entry into the stunner	11:03:14
Gondola starts descending	11:03:40
Some swaying movements observed	11:03:52
Pigs start losing balance – theorised entry into CO_2	11:03:54
Grunts heard	11:03:56
Neck stretching visible	11:04:00
One pig jumps	11:04:03
First pig falls	11:04:04
Next pig falls, thrashing movements	11:04:06
Rasping noises, last three pigs fall	11:04:11
Gasping	11:04:17
Head drop visible	11:04:25
Grunt	11:04:26
Assumed LOC	11:04:28
Muscle contractions - Gasping after LOC - Last visible gasp - Last muscle contractions	11:04:33 - 11:05:53 - 11:04:33 - 11:05:39 - 11:05:53
Ascent out of pit	11:06:50
Ejection from the stunner	11:07:26

Table 4.12 *Duration of major events and behaviours during stunning of group 1-4.*

Event	**Duration/time to (mm: ss) (s)**
Time to exposure	00:14 (14)
Total time in stunner	04:12 (252)
Total time in CO_2	02:56 (176)
First reaction (swaying)	00:12 (12)
Time until assumed LOC	00:34 (34)
Time to fall - First pig falls - Second pig falls - Third, fourth and fifth pig falls	(8.4) - 00:04 (4) - 00:06 (6) - 00:11 (11)
Muscle contractions	01:20 (80)

The pigs were reluctant to enter the gondola without the aid of the automatic push arm's aid. Their ears were up, indicating that they were alert. Despite this, they appeared calm once inside the gondola. Shortly after the descent started, they seemed somewhat uneasy, but this could be due to the movement of the gondola. Swaying movements started 12s after the gondola started descending into the pit. Some started losing their balance and tried to counter this by moving sideways. Vocalisations were heard at 11:03:56, 11:04:11 and 11:04:26. Two of these were grunts, the other was a rasping noise as the last pigs lost their balance and fell. One of these grunts took place after a pig dropped its head (11:04:25), and since the loss of

voluntary movement is indicative of the LOC, it is unlikely that the pig that dropped its head was responsible for the grunt. Gasping movements after the onset of unconsciousness (11:04:28) are assumed to be involuntary movements. Involuntary movements ceased after 11:05:53.

Table 4.13 *Timings of major events and behaviours observed in the footage recording of the first individual pig.*

Event/behaviour	**Time (hh: mm: ss)**
Entry into the stunner	11:32:36
Gondola starts descending	11:33:04
Loss of balance, pig sits – theorised entry into CO_2	11:33:20
Complete loss of posture, falls, trashing, and neck stretching visible	11:33:22
Face not visible on camera	11:33:27
Thrashing stops, back legs extended	11:33:30
Most voluntary movements ceasing – possible LOC	11:33:47
Gasping	11:34:00
Muscle contractions	11:34:11 – 11:35:00
Ascent out of pit	Not visible
Ejection from stunner	11:37:00

Table 4.14 *Duration of major events and behaviours during stunning of the first individual pig.*

Event	**Duration/time to (mm: ss) (s)**
Time to exposure	00:16 (16)
Total time in stunner	04:24 (264)
Total time in CO_2	Cannot accurately be determined
First reaction (sitting)	00:16 (16)
Time until assumed LOC	00:27 (27)
Time to fall	(2.0)
Muscle contractions	01:11 (71)

Upon entry, the pig was curious and inspected its new surroundings. During the descent, having a lot of space available, it moved around easily. The pig lost its balance quickly and sat down; this was assumed to be the point of entry into the CO_2 gas. Shortly after entry into the gas, loss of posture took place, and was accompanied by thrashing and neck stretching. At 11:33:27, the pig's body had moved in such a position that its head was obscured from the camera's view. The rest of the body was visible for further observation of behaviour in reaction to the CO_2. Thrashing and extension of the back legs was observed at 11:33:30. By 11:33:47, most voluntary movements had ceased, signalling the start of the onset of unconsciousness. Muscle contractions ceased at 11:35:00.

Table 4.15 *Timing of major events and behaviours observed in the footage recording of the second individual pig.*

Event/behaviour	Time (hh: mm: ss)
Entry into the stunner	11:41:18
Gondola starts descending	11:41:49
Gasping – theorised entry into CO_2	11:42:01
Slipping, onset of loss of posture	11:42:04
Neck stretch, foam visible at mouth	11:42:09
Sitting down	11:42:11
Lie down (on stomach)	11:42:14
Gasping, neck stretch	11:42:21
Head drop – assumed LOC	11:42:37
Gasping	11:42:48
Muscle contraction, pig moved onto its side	11:44:11
Ascent from pit	11:44:50
Ejection from stunner	11:45:40

Table 4.16 *Duration of major events and behaviours during stunning of the second individual pig.*

Event	Duration/time to (mm: ss) (s)
Time to exposure	00:12 (12)
Total time in stunner	04:22 (262)
Total time in CO_2	02:49 (169)
First reaction (gasping)	00:12 (12)
Time until assumed LOC	00:36 (36)
Time to fall	(13.0)
Muscle contractions	01:23 (83)

The last individually stunned pig entered the gondola reluctantly. 12s after the start of descent, the pig started gasping; this was assumed to be the point of entry into the CO_2 gas. Three seconds later, the pig started slipping, and losing its balance. At 11:42:09, foam was visible at the pig's mouth. A neck stretch was visible as well. Two seconds later, the pig sat down, and another two seconds later, lay down on its stomach. Further gasping and neck stretches were observed. At 11:42:37, the pig dropped its head. It was assumed that LOC occurred at this point. Muscle contractions were visible until 11:44:11, when a final muscle contraction caused the body to be moved onto its side.

4.2.3.2 80% Ar 20% CO_2 Stunning

Prior to this study, Argon gas mixtures have only been tested under laboratory (experimental) conditions. Due to concerns over the effectiveness of the mixture in a commercial gas stunner, it was decided to stun five pigs individually. If the gas proved effective within reason, group stunning would commence as initially planned. Problems experienced on the day included excessively long times to fill the chamber with the mixture, difficulty ensuring that the O_2

concentrations remained below 2% and difficulty keeping the gas inside the chamber. Those who were required to stand or work near the stunner during this time were all required to wear O_2 meters. This could potentially constitute a labourer safety violation if the admixture is not properly contained.

Table 4.17 *Timing of major events and behaviours observed in the footage recording of the first pig stunned by the Argon admixture.*

Event/behaviour	**Time (hh: mm: ss)**
Entry into the stunner	16:18:54
Gondola starts descending	16:19:23
Swaying starts – theorised entry into CO_2	16:19:39
Backing into corner	16:19:48
Loss of posture and fall	16:19:54
Stifled grunt	16:19:55
Squealing	16:19:57 – 16:20:12
Violent kicking, thrashing	16:19:57 – 16:20:12
Neck stretch	16:20:22
Gasp	16:21:18
Periodic gasping and neck movements	16:22:13 – 16:26:30
Ascent from pit starts	16:25:41
Ejection	16:26:30

Due to the difficulty associated with filling and maintaining the necessary gas concentrations within the stunner, the first pig was only stunned at 16:18:54. The last pig to undergo stunning with Argon exited the stunner at 17:22:16. It was concluded that the Butina-system used was not designed for use with any gas other than CO_2. The experimental mixture proved ineffective at delivering a sufficiently deep stun, and so no further pigs were stunned using the mixture. It was also determined to be unnecessarily stressful to the pigs, and so was considered to be a poor welfare practice. Therefore, gas stunning using Argon gas was not investigated any further in Follow-up study of this study.

Table 4.18 *Duration of major events and behaviours during stunning of the first pig stunned by the Argon admixture.*

Event	**Duration/time to (mm: ss) (s)**
Time to exposure	00:16 (16)
Total time in stunner	07:36 (456)
Total time in Ar-CO_2	06:02 (362)
First reaction (swaying)	00:16 (16)
Time until assumed LOC	Not accurately determinable
Time to fall	(15.0)
Muscle contractions	04:17 (257)

The pig appeared anxious when entering the gondola. Swaying started shortly after exposure and was followed by backing into a corner. It is likely that the atmosphere, not as aversive as 84% CO_2, caused the pig discomfort, the level of which it normally would avoid, but not necessarily flee from. At 16:19:54, the pig fell, and let out a grunt directly after. A squeal, accompanied by kicking and thrashing, lasted 15s. From 16:21:18 until ejection from the stunner at 16:26:30, periodic gasping and neck stretching was observed. Some muscle contractions were visible at ejection, indicating that the pig was still alive (and a short stun-to-stick interval was thus needed to ensure that the pig's consciousness did not return before the onset of death through bleeding). The latency to onset of unconsciousness was unclear, as behaviour such as head dropping or loss of posture in the ear, which could be used as an indication of the loss of voluntary action, was not observed.

Table 4.19 *Timing of major events and behaviours observed in the footage recording of the second pig stunned by the Argon admixture.*

Event/behaviour	**Time (hh: mm: ss)**
Entry into the stunner	16:33:20
Gondola starts descending	16:33:38
Swaying – theorised entry into CO_2	16:33:47
Sitting	16:33:50
Continues to lose balance	16:33:56
Loss of posture and fall	16:33:58
Squealing	16:33:59 – 16:34:02
Regains posture, falls immediately on side	16:34:04
Squealing	16:34:05 – 16:34:29
Thrashing & kicking	16:34:06 – 16:34:29
Position change (from side to stomach, onto other side)	16:34:34
Periodic gasping and neck movements	16:34:58 – 16:40:55
Muscle contractions	16:34:34 – 16:41:08
Ascent from pit starts	16:40:23
Ejection	16:41:08

Table 4.20 *Duration of major events and behaviours during stunning of the second individual pig stunned by the Argon admixture.*

Event	**Duration/time to (mm: ss) (s)**
Time to exposure	00:09 (9)
Total time in stunner	07:48 (468)
Total time in Ar-CO_2	06:36 (396)
Time until first reaction (swaying)	00:09 (9)
Time until assumed LOC	Not accurately determinable
Time to fall	00:09 (9)
Duration of muscle contractions	06:34 (394)

The pig appeared reluctant to enter the gondola and paced during its descent. The first reaction to the gas was swaying movements, which resulted in the pig sitting down. Further swaying movements were observed until loss of posture. Attempts to regain posture were observed directly after falling. A 3s squeal was heard from 16:33:59 – 16:34:02. The pig managed to regain some posture, but immediately fell on its side afterwards. Squealing continued and was accompanied by thrashing and kicking movements from 16:34:04 – 16:34:29. Based on this behaviour, the onset of unconsciousness could not be accurately determined. However, it would have taken place after the squeal and thrashing episode. At 16:34:34, the pig had moved from its side onto its stomach, and then onto its other side due to a kick-like muscular contraction. The pig was likely still conscious at this point. From 16:34:58 onwards, periodic gasping and neck stretching took place until ascension from the pit. Mild muscle contractions were still visible upon exit from the gondola at 16:41:08.

Table 4.21 *Timings of major events and behaviours observed in the footage recording of the third pig stunned by the Argon admixture.*

Event/behaviour	**Time (hh: mm: ss)**
Entry into the stunner	16:50:50
Gondola starts descending	16:51:26
Backing – theorised entry into CO_2	16:51:38
Neck stretching	16:51:42
Loss of balance and fall	16:51:47
Neck stretching, kicking, thrashing	16:51:49
Thrashing	16:51:52
Thrashing ceased	16:52:08
Periodic gasping	16:52:14
Periodic muscle contractions	16:53:05 – 16:56:18
Gasping more frequent	16:54:38
Ears dropped – theoretical LOC	16:54:41
Kicking	16:55:40
Neck stretching ceased	16:56:18
Ascent from pit starts	17:02:07
Ejection	17:02:30

Table 4.22 *Duration of major events and behaviours during stunning of the third pig stunned by the Argon admixture.*

Event	**Duration/time to (mm: ss) (s)**
Time to exposure	00:12 (12)
Total time in stunner	11:40 (700)
Total time in Ar-CO_2	10:29 (629)
Time until first reaction (backing)	00:12 (12)
Time until assumed LOC	03:03 (183)
Time to fall	00:09 (9)
Duration of muscle contractions	03:13 (193)

The pig appeared curious as it entered the gondola. During the descent, it started backing into a corner and neck stretching was visible shortly after. This is regarded as the point of entry into the Ar-CO_2. Loss of balance came about, and was followed by neck stretching, kicking, and thrashing movements. Thrashing movements ceased at 16:52:08. Periodic gasping and gagging movements were visible from 16:52:14. Periodic muscle contractions were visible from 16:53:05 until 16:56:18. At 16:54:41, a clear drop of the ears was visible which indicates that unconsciousness had set in. Kicking-like muscle contractions were visible at 16:55:40, and neck stretching movements ceased at 16:56:18. No movement was visible at ejection out of the stunner. This pig was exposed to the Ar-CO_2 gas mixture for 10min 29s. This was done to induce a deeper state of insensibility. Maintenance of consistent gas concentrations inside the Butina is crucial to ensure that a sufficient stun is administered.

Table 4.23 *Timing of major events and behaviours observed in the footage recording of the fourth pig stunned by the Argon admixture.*

Event/behaviour	**Time (hh: mm: ss)**
Entry into the stunner	17:04:11
Gondola starts descending	17:04:42
Backing – theorised entry into CO_2	17:05:01
Difficulty maintain posture	17:05:03
Fall, followed by kicking movements	17:05:06
Attempt to stand, fail	17:05:09, 17:05:12
Thrashing, kicking	17:05:15 – 17:05:26
Squealing	17:05:17 – 17:05:30
Pig moved out of view	17:05:31
Pig moved back into view	17:09:06
Periodic gasping, kicking, and thrashing	17:09:07 – 17:11:48
Ears picked up, slowly dropped – theorised onset of unconsciousness	17:09:08
Ascent from pit starts, movement ceases	17:11:48
Ejection	17:12:31

Table 4.24 *Duration of major events and behaviours during stunning of the fourth pig stunned by the Argon admixture.*

Event	**Duration/time to (mm: ss) (s)**
Time to exposure	00:19 (19)
Total time in stunner	08:20 (500)
Total time in Ar-CO_2	06:47 (407)
Time until first reaction (backing)	00:19 (19)
Time until assumed LOC	04:07 (247)
Time to fall	00:05 (5)
Duration of muscle contractions	Not accurately determined

While entering, the pig seemed curious and was not reluctant to enter the gondola. 19s after the gondola started descending, the pig started moving backward into a corner, and quickly

lost its balance and fell. This is assumed as the theorised entry into the Ar-CO_2 and was followed by two failed attempts to regain posture. A 13s squeal was heard, starting at 17:05:17, and was accompanied by thrashing and kicking movements. Not all the pig's behaviours could be documented as it rolled out of the camera view at 17:05:31, only returning to view at 17:09:06. Further thrashing and kicking movements were observed upon re-entry into camera view, followed by the picking up and slow drop of the pig's ears. This, theoretically, implies a loss of voluntary control of the ears, and thus, the LOC. Periodic kicking movements were observed until the gondola started its ascent out of the pit at 17:11:48. Hereafter, no more movements were observed.

Table 4.25 *Timing of major events and behaviours observed in the footage recording of the fifth pig stunned by the Argon admixture.*

Event/behaviour	**Time (hh: mm: ss)**
Entry into the stunner	17:14:12
Gondola starts descending	17:14:45
Backing – theorised entry into CO_2	17:14:56
Slipping	17:15:01
Loss of balance and fall	17:15:06
Attempt to regain posture, thrashing, kicking	17:15:08 – 17:15:31
Grunt	17:15:15
Squealing	17:15:18 – 17:15:30
Gasping	17:16:11
Kicking	17:16:30
Thrashing	17:16:46, 17:16:57, 17:17:35
Muscle contractions	17:17:55 – 17:19:01
Ascent from pit starts	17:21:26
Ejection	17:22:16

Table 4.26 *Duration of major events and behaviours during stunning of the fifth pig stunned by the Argon admixture.*

Event	**Duration/time to (mm: ss) (s)**
Time to exposure	00:11 (11)
Total time in stunner	08:04 (484)
Total time in Ar-CO_2	6:30 (390)
Time until first reaction (backing)	00:11 (11)
Time until assumed LOC	04:49 (299)
Time to fall	00:10 (10)
Duration of muscle contractions	01:06 (66)

The last pig was curious when entering the gondola. 11s later, the pig started backing into a corner, shortly after which it started slipping and lost its balance. This was followed by an unsuccessful attempt to stand up. Vocalisations, in the forms of grunting and squealing, were

heard, with the squealing accompanied by kicking and thrashing. It looked like these behaviours were exhibited in response to the difficulty of standing up while exposed to the Ar-CO_2. Thrashing and kicking movements were observed until 17:17:35. LOC set in after this, when the thrashing movements ceased. Hereafter, muscle contractions were visible for 66s. At the time of ascent, no further movements were visible. This pig was inside the stunner for 8 min and 4s and exposed to the Ar-CO_2 gas mixture for 6 min and 30s.

4.3 Follow-up study

4.3.1 Panel review of behavioural footage

A panel consisting of industry representatives from the RMAA, NSPCA, GDARD and practicing veterinarians scored the behaviour recorded while pigs were stunned. In total, the behaviour of 50 pigs were scored, 25 pigs per stunning method. 25 pigs were filmed and scored individually as part of the standard head-to-heart electrical stunning. A further 25 pigs were filmed and scored in five groups consisting of five pigs each as part of the 84% CO_2 stunning method.

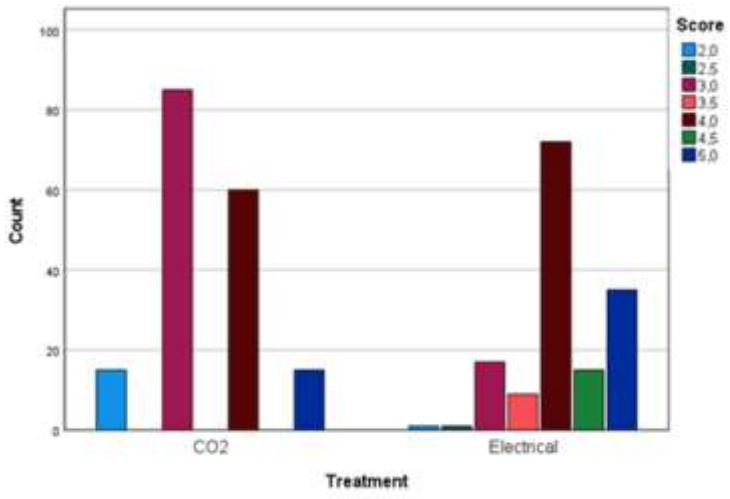

Figure 4.2 *Figure depicting the spread of scores assigned by the review panel for both carbon dioxide- and electrical stunning.*

Due to the subjective nature of human perception, a scoring system was developed with the hopes of yielding more objective data. It was, however, left up to the reviewers to determine the acceptability of the welfare of the pigs based on the behaviours they exhibited during stunning.

The most numerous scores assigned by the review panel during carbon dioxide stunning was 3.0, with the least assigned being 2.0 and 5.0. Fewer scores were assigned to CO_2 stunning than to electrical stunning, as the group is scored rather than each individual pig. The most numerous scores assigned by the review panel during electrical stunning was 4.0, with the least assigned being 2.0 and 2.5.

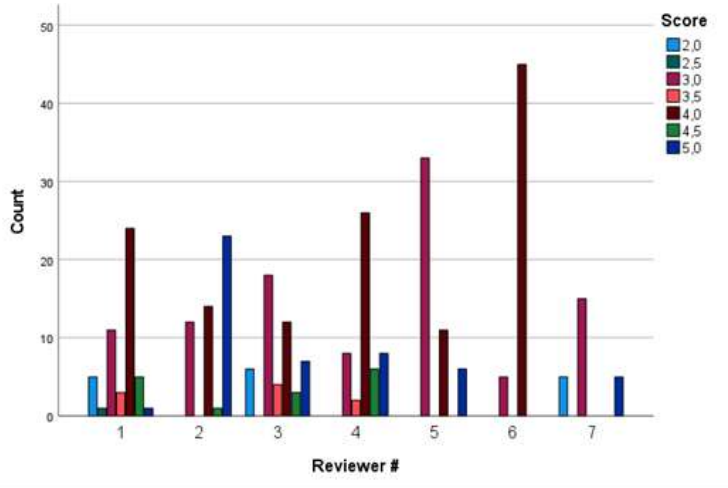

Figure 4.3 *Figure depicting the spread of scores between reviewers over both stunning treatments.*

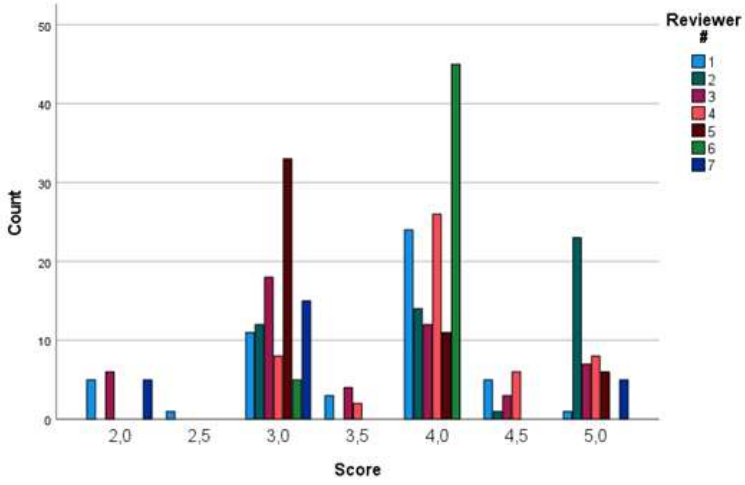

Figure 4.4 *Figure depicting reviewer's total assigned scores.*

4.3.4.1 84% CO_2 controlled atmospheric stunning

Table 4.27 *Stunning scores and corresponding welfare classes as assigned by the review panel for pigs stunned using CO_2.*

Commented [AO22]: These are averages and were not really statistically analysed. It is difficult to draw conclusions on the averages only.

Group	Group size	Reviewer #	Score (/18)	Class (/5)
2-1	5	1	4	4
		2	5	3
		3	3	4
		4	4	4
		5	3	4
		6	4	4
		7	3	4
		Average	**3.72**	**3.86**
2-2	5	1	3	4
		2	3	4
		3	2	4
		4	3	4
		5	3	4
		6	4	4
		7	2	4
		Average	**2.86**	**4.00**
2-3	5	1	3	4
		2	4	4
		3	3	4
		4	4	4
		5	3	4
		6	4	4
		7	3	4
		Average	**3.43**	**4.00**
2-4	5	1	2	4
		2	3	4
		3	3	4
		4	4	4
		5	3	4
		6	3	4
		7	3	4
		Average	**3.00**	**4.00**
2-5	5	1	4	4
		2	5	3
		3	4	4
		4	4	4
		5	3	4
		6	4	4
		7	5	3
		Average	**4.14**	**3.71**

The behaviour visible on the videos was scored according to the same five-category scoring system used in the pilot study, adapted from the behaviours used and documented in Atkinson *et al.* (2015). Each behaviour was scored according to its associated level of aversiveness. Behaviours expected during the LOC, such as swaying and falling, were assigned a value of '0', as these aren't signs of aversiveness to the change in atmosphere. Behaviours like gasping and neck stretching were assigned a value of '1', as these may indicate a conscious state of breathlessness, but within the literature is still considered to be a natural physiological reaction in the absence of oxygen (Raj & Gregory, 1996; Atkinson *et al., 2015)*. Behaviours such as squealing and attempting to escape were assigned a value of '3' as these are conscious exhibitions of discomfort in the changing atmosphere. After consciousness has been lost, and voluntary movements are seen returning, it is to be assumed that the state of unconsciousness is waning. The return of consciousness is assigned a value of '4' and is to be considered the most severe infringement on animal welfare during this stage of the slaughtering process. A higher score (out of 18) was indicative of a lower welfare class (out of 5). During the follow-up study, two camera angles were used for behavioural footage recording, from opposite sides of the gondola.

Commented [AO23]: You had no literature when you described the scores and classes in your materials and methods, and once again you do not cite literature. You do need references here.

Group 2-1 was awarded an average score of 3.71, and an average welfare class of 3.86. The highest score was 5/18, with a respective welfare class of 3/5. The lowest score and welfare class was 4/18, with a respective welfare class of 4/5.

Group 2-2 was awarded an average score of 2.86, and an average welfare class of 4.00. The highest score was 4/18, with a respective welfare class of 4/5. The lowest score was 2/18, with a respective welfare class of 4/5.

Group 2-3 was awarded an average score of 3.43, and an average welfare class of 4.00. The highest score was 4/18, with a respective welfare class of 4/5. The lowest score was 3/18, with a respective welfare class of 4/5.

Group 2-4 was awarded an average score of 3.00, and an average welfare class of 4.00. The highest score was 4/18, with a respective welfare class of 4/5. The lowest score was 2/18, with a respective welfare score of 4/5.

Group 2-5 was awarded an average score of 4.14, and an average welfare class of 3.71. The highest score 5/18, with a respective welfare class of 3/5. The lowest score was 3/18, with a corresponding welfare class of 4/5.

4.3.4.2 Head-to-heart electrical stunning

Table 4.28 *Possible behaviours observed shortly before-, during- and after electrical stunning.*

Commented [AO24]: You need references here

Behaviour/Term	Definition
Flight behaviour	Reluctance to move forward, backing/reversing
Escape attempts	Attempts to jump out of raceway and-/or stunner
Vocalisations	Noise made by the pigs, can range from simple grunts to excessive high-pitch squealing
Sitting	Relaxation of the rump on the floor (or on top of another pig) in response to exhaustion
Restlessness	Small excited movements in one place (due to confinement in raceway & line)
Loss of balance	Slipping, falling, difficulty to retain posture
° Convulsions	Involuntary jerking & contraction of muscles (not indicative of a seizure in and of itself)
° Seizure	Electrical disturbance in the brain, expected in electrical stunning and can theoretically take place without convulsions
° Epilepsy	Repeated seizures due to electrical disturbance in the brain
° LOC	Loss of ability to perceive and voluntarily react to sensory stimulation, often characterised by loss of posture
Return of consciousness	Sensibility & ability to react to sensory stimulation returns (characterised by display of voluntary movements and reactions like vocalisations after stunning)
° Apnoea	Temporary cessation of breathing (as opposed to regular breathing and actions that indicate possible breathing, like gasping)
° Tetanus	Rigidly stretched front- and flexed hind limbs, rigid body trunk
Spontaneous blinking	Repeated closing and opening of eyelids

° desirable behaviour during stunning

Due to the short duration of stunning, the behaviours exhibited shortly before stunning was also considered. The duration of the applied stun was also taken into consideration. Unlike the scoring system developed for the CO_2 stunning system, each reviewer was allowed to class the stun based on the duration of the stun, placement of the tongs and the behaviour visible on the video. No scoring system was used. These behavioural parameters were adapted from those used in Atkinson *et al*. (2015).

Table 4.29 *Electrical stunning score card.*

Score	1	2	3	4	5
Class	Unacceptable	Poor	Average	Good	Outstanding
Definition	Severe stress before, insufficient stunning	Moderate stress, insufficient stunning	Some stress, stunning is sufficient	Very little stress, sufficient stunning	No stress exhibited, sufficient stunning
Examples	Escaping, squealing, r.o.c.	Squealing, flight responses, r.o.c.	Restlessness, flight responses, sitting	Some restlessness, minor flight response, grunts	Grunts, pig calm

Table 4.30 *Method-related practices that influence quality of stun during electrical stunning.*

Practice	**Definition**
Use of automatic gate & push arm	Gate closing on pigs, position of pig when push arm activates
Stunner clamp	Lifting and restraining of pigs for effective stunning, stance of pig when activated (sitting vs standing)
Placement of electrodes	Either side of head, between eyes and base of ears (spanning the brain)
Duration of stun delivery	EU recommendation: Minimum current of 1.3 Amps, for 2-3s at a maximum frequency of 50Hz sine wave (AC) (Head-only stunning)
Stun-Stick Interval	Sticking ideally to take place directly after stunning, or at least within 15s of the end of stunning (*not able to accurately assess due to space on abattoir floor and camera angle*)

Table 4.31 *Stunning scores as assigned by the review panel for pigs stunned using electrical stunning.*

	Reviewer #						
Pig #	**1**	**2**	**3**	**4**	**5**	**6**	**Average**
1	4.0	4.0	4.0	4.0	4.0	4.0	4.00
2	5.0	5.0	5.0	5.0	4.0	4.0	4.67
3	4.5	5.0	5.0	4.5	4.0	4.0	4.50
4	4.0	5.0	5.0	5.0	5.0	4.0	4.67
5	3.5	4.0	4.5	4.0	4.0	4.0	4.00
6	4.0	5.0	5.0	5.0	4.0	4.0	4.50
7	3.0	4.0	3.5	3.0	3.0	4.0	3.42
8	4.0	4.0	4.0	5.0	5.0	4.0	4.33
9	4.0	4.0	3.5	3.5	3.0	4.0	3.67
10	3.5	3.0	3.5	3.0	3.0	4.0	3.33
11	4.0	5.0	5.0	5.0	5.0	4.0	4.67
12	4.0	5.0	4.0	4.0	4.0	4.0	4.17
13	4.5	5.0	4.5	4.0	4.0	4.0	4.33
14	4.0	4.0	3.0	4.0	4.0	4.0	3.83
15	4.0	4.0	4.0	4.0	4.0	4.0	4.00
16	2.5	3.0	2.0	3.0	3.0	4.0	2.92
17	4.0	5.0	4.5	4.5	5.0	4.0	4.50
18	4.5	5.0	3.0	4.5	3.0	4.0	4.00
19	4.0	5.0	5.0	4.5	5.0	4.0	4.58
20	4.0	4.0	5.0	5.0	4.0	4.0	4.33
21	4.5	5.0	4.0	4.5	5.0	4.0	4.50
22	4.0	5.0	4.0	4.5	3.0	4.0	4.08
23	4.0	4.5	3.0	4.0	3.0	4.0	3.75
24	4.5	5.0	4.0	5.0	4.0	4.0	4.42
25	3.5	4.0	3.5	3.5	3.0	4.0	3.58
Average	3.98	4.46	4.06	4.24	3.92	4.00	

All reviewers agreed that the welfare of pigs 1 and 15 was ‘Good’, as all 6 reviewers attributed a class of 4/5. Pigs 2, 4 and 11 had the highest average class score of 4.67 (good-outstanding). Pig 16 had the lowest average class score of 2.92 (poor-average). Of the 25 pigs, 18 had good or better welfare scores, whereas 6 had welfare scores between average

and good. One pig had a score between poor and average. Reviewer 2 had the highest average attributed score of 4.46, while reviewer 5 had the lowest average attributed score of 3.92. Reviewer 6 had attributed the same class score of 4/5 to each stunning. Reviewer 3 had the biggest range in class scores, ranging from 2/5 to 5/5.

4.3.2 Descriptive statistics for pH

Table 4.32 *Descriptive statistics for muscle pH measurements during the follow-up study.*

Stunning Treatment	Side	Time	Maximum	Minimum	Mean	Std. Deviation
CO_2	L	1	6.86	5.74	6.29	0.34
		3	6.66	5.58	5.99	0.31
		6	6.49	5.44	5.88	0.29
		9	6.24	5.44	5.69	0.23
		12	6.06	5.45	5.63	0.18
		24	6.06	5.41	5.69	0.20
	R	1	6.47	5.98	6.19	0.15
		3	6.18	5.94	6.03	0.22
		6	6.00	5.57	5.80	0.14
		9	5.92	5.52	5.73	0.12
		12	5.70	5.49	5.60	0.06
		24	5.77	5.45	5.59	0.12
	Total	1	-	-	6.24	0.25
		3	-	-	6.01	0.26
		6	-	-	5.84	0.23
		9	-	-	5.72	0.18
		12	-	-	5.62	0.13
		24	-	-	5.64	0.17
Electrical	L	1	6.86	6.08	6.41	0.26
		3	6.82	5.74	6.18	0.32
		6	6.86	5.61	6.04	0.38
		9	6.18	5.47	5.81	0.23
		12	5.91	5.50	5.69	0.15
		24	5.86	5.50	5.72	0.18
	R	1	6.63	6.02	6.23	0.21
		3	6.66	5.62	6.06	0.30
		6	6.47	5.52	5.89	0.30
		9	5.91	5.49	5.69	0.15
		12	6.16	5.43	5.64	0.22
		24	6.11	5.43	5.73	0.23
	Total	1	-	-	6.32	0.25
		3	-	-	6.12	0.31
		6	-	-	5.96	0.34
		9	-	-	5.75	0.20
		12	-	-	5.67	0.19
		24	-	-	5.73	0.20

Table 4.32 summarises the minimum and maximum pH values recorded at each time stamp (hours *post mortem*), their means and standard deviations. In total, 20 pigs were used in Follow-up study.

The maximum pH values for the CO_2 stunning treatment at 1-, 3-, 6-, 9-, 12- and 24-hours *post mortem* were 6.86, 6.66, 6.49, 6.24, 6.06 and 6.06, respectively. The corresponding minimum pH values, in the same order, were 5.74, 5.58, 5.44, 5.44, 5.45 and 5.41, respectively. The mean pH values and standard deviations at 1-, 3-, 6-, 9-, 12-, and 24-hours *post mortem* were 6.24 ± 0.26, 6.00 ± 0.26, 5.84 ± 0.23, 5.72 ± 0.18, 5.62 ± 0.14 and 5.64 ± 0.17, respectively.

The maximum pH values for the electrical stunning treatment at 1-, 3-, 6-, 9-, 12- and 24-hours *post mortem* were 6.86, 6.82, 6.86, 6.18, 6.16 and 6.11, respectively. The corresponding minimum pH values, in the same order, were 6.02, 5.62, 5.52, 5.47, 5.43 and 5.43, respectively. The mean pH values and standard deviations at 1-, 3-, 6-, 9-, 12- and 24-hours were 6.32 ± 0.25, 6.12 ± 0.31, 5.96 ± 0.34, 5.75 ± 0.20, 5.67 ± 0.19 and 5.73 ± 0.20, respectively.

Figure 4.5 depicts and compares the rate of pH-decline over a 24-hour period among the two stunning methods. The 84% CO_2 pH decline is represented by the blue line, and standard electrical stunning is represented by the red line.

Commented [AO25]: Were there any significant differences?

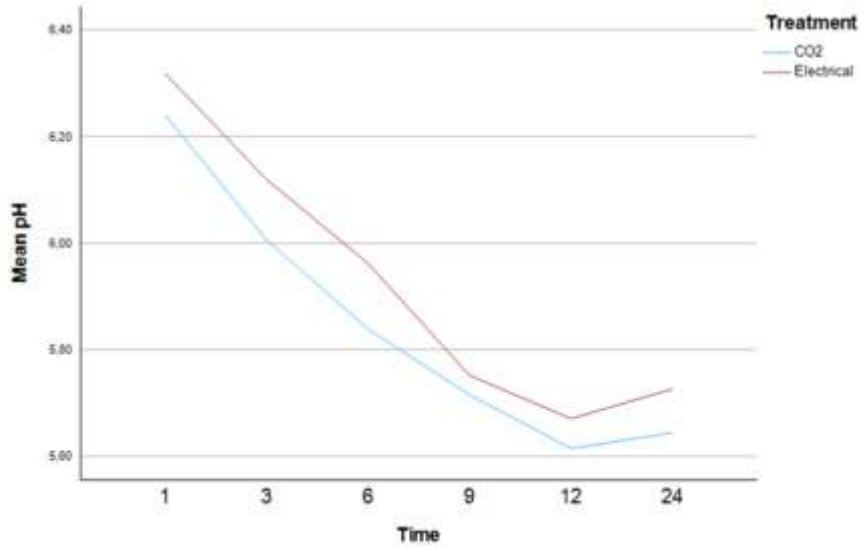

Figure 4.5 *Effect of stunning method on pH decline rate over 24 h period post mortem.*

Both the 84% CO_2 and electrical stunning methods depicted similarly-shaped decline curves. While both methods had different starting- and ultimate pH values, the curves depicted similar rates of pH decline. Carcasses from electrically-stunned pigs had higher pH values overall than the carcasses from CO_2-stunned pigs. However, there weren't any significant differences regarding the pH measurements between these stunning procedures.

Commented [AO26]: Was it significant?

4.3.3 Descriptive statistics for carcass temperature

The maximum carcass temperatures recorded during CO_2 stunning at 1-, 3-, 6-, 9-, 12- and 24-hours *post mortem* were 32.8, 23.4, 15.1, 9.8, 8.1 and 6.0, respectively. The corresponding minimum carcass temperatures, in the same order, were 24.0, 18.2, 12.0, 7.3, 5.3 and 2.7, respectively. The mean pH values and standard deviations at 1-, 3-, 6-, 9-, 12- and 24-hours *post mortem* were 30.31 ± 2.09, 21.15 ± 1.15, 13.59 ± 0.82, 8.24 ± 0.63, 6.12 ± 0.57 and 3.15 ± 0.74, respectively.

The maximum carcass temperatures recorded for the standard electrical stunning treatment at 1-, 3-, 6-, 9-, 12- and 24-hours *post mortem* were 29.7, 18.1, 12.8, 10.8, 6.7 and 4.5, respectively. The corresponding minimum carcass temperatures, in the same order, were 24.4, 13.9, 9.9, 8.0, 3.2 and 0.1, respectively. The mean pH values and standard deviations 1-, 3-, 6-, 9-, 12- and 24-hours *post mortem* were 27.63 ± 1.48, 16.82 ± 1.31, 11.51 ± 0.79, 9.29 ± 0.69, 5.29 ± 0.85 and 1.09 ± 0.93, respectively.

Table 4.33 *Descriptive statistics for muscle temperature measurements at 1-, 3-, 6-, 9-, 12-, and 24 h post mortem.*

Stunning Treatment	Side	Time	Maximum	Minimum	Mean	Std. Deviation
CO_2	L	1	32.7	24.0	29.71	2.62
		3	22.3	18.2	20.99	1.22
		6	14.6	12.6	13.56	0.70
		9	9.2	7.6	8.23	0.57
		12	8.1	5.5	6.21	0.72
		24	6.0	2.7	3.28	0.99
	R	1	32.8	28.5	30.91	1.25
		3	23.4	19.7	21.30	1.11
		6	15.1	12.0	13.61	0.96
		9	9.8	7.3	8.25	0.72
		12	6.6	5.3	6.02	0.39
		24	3.9	2.7	3.01	0.36
	Total	1	-	-	30.31	2.09
		3	-	-	21.15	1.15
		6	-	-	13.59	0.82
		9	-	-	8.24	0.63
		12	-	-	6.12	0.57

		24	-	-	3.15	0.74
Electrical	L	1	29.7	25.4	27.68	1.53
		3	18.0	14.4	16.83	1.34
		6	12.8	10.2	11.62	0.76
		9	10.8	8.1	9.35	0.76
		12	6.7	3.6	5.34	0.86
		24	4.5	0.2	1.26	1.20
	R	1	29.7	24.4	27.57	1.50
		3	18.1	13.9	16.80	1.35
		6	12.5	9.9	11.40	0.84
		9	9.9	8.0	9.24	0.65
		12	6.3	3.2	5.23	0.89
		24	2.2	0.1	0.91	0.57
	Total	1	-	-	27.63	1.46
		3	-	-	16.82	1.31
		6	-	-	11.51	0.79
		9	-	-	9.30	0.69
		12	-	-	5.29	0.85
		24	-	-	1.09	0.93

Figure 4.6 depicts and compares the rate of carcass chilling over a 24-hour period among the two stunning methods. The 84% CO_2 temperature decline is represented by the blue line, and standard electrical stunning is represented by the red line.

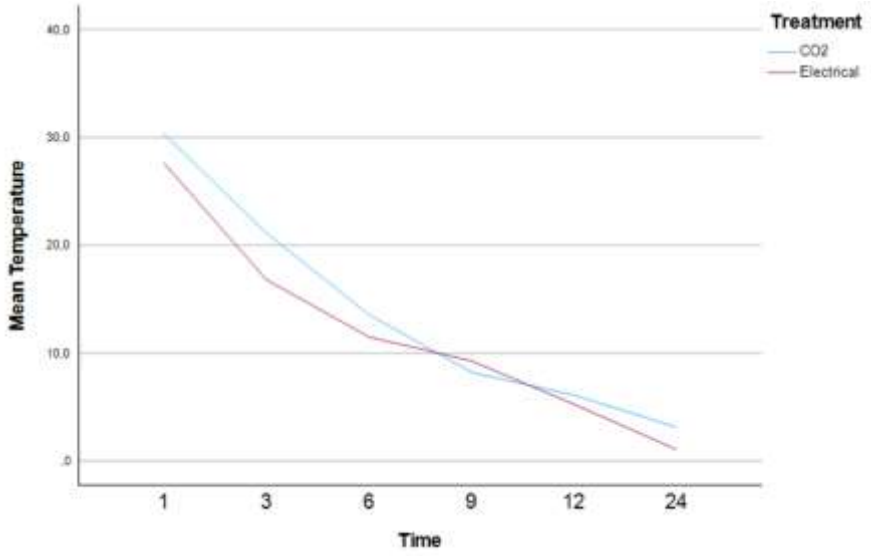

Figure 4.6 *Effect of stunning method on carcass temperature decline rates over a 24 h period post mortem.*

Both the 84% CO_2 and electrical stunning methods depicted similarly-shaped chilling curves. While both methods had different starting- and ultimate carcass temperatures, the curves depicted similar rates of chilling. Carcasses from electrically-stunned pigs had higher carcass temperatures overall than the carcasses from CO_2-stunned pigs.

Commented [AO27]: You could also draw a pH over temp graph to predict the risk for PSE or maybe DFD

4.3.4 Descriptive statistics for muscle metabolites

Table 4.34 *Descriptive statistics for muscle metabolites from muscle harvested 1 h post mortem.*

Commented [AO28]: You should define your abbreviations at the bottom of a table

	Stunning Treatment	Minimum	Maximum	Mean	Standard Deviation
Lactate	Electrical	23,87	52,83	33,35	8,14
	CO_2	20,49	36,29	27,62	5,02
Glucose	Electrical	0,59	2,02	1,56	0,42
	CO_2	0,75	1,41	1,21	0,23
Glycogen	Electrical	7,92	19,89	11,44	3,33
	CO_2	3,84	14,59	9,03	3,79
G-6-P	Electrical	1,08	3,27	1,94	0,66
	CO_2	0,56	1,98	1,20	0,45
ATP	Electrical	5,93	8,65	7,10	0,88
	CO_2	6,28	11,16	7,57	1,54
CP	Electrical	2,44	4,68	3,62	0,80
	CO_2	2,76	4,23	3,67	0,49

G-6-P: Glucose-6-phosphate; ATP: Adenosine Triphosphate; CP: Creatine Phosphate;

4.3.3.1 L-lactate

The maximum value of L-lactate in the CO_2-stunned samples was 36.29 µmol/g muscle; the minimum value was 20.49 µmol/g muscle. The mean was 27.62 µmol/g muscle, and the standard deviation was 5.02 µmol/g muscle.

The maximum value of L-lactate in the electrically stunned samples was 52.83 µmol/g muscle; the minimum value was 23.87 µmol/g muscle. The mean was 33.35 µmol/g muscle, and the standard deviation was ±8.14 µmol/g muscle.

4.3.3.2 Glucose

The maximum value of glucose in the CO_2-stunned samples was 1.41 µmol/g muscle; the minimum value was 0.75 µmol/g muscle. The mean was 1.21 µmol/g muscle, and the standard deviation was 0.23 µmol/g muscle.

The maximum value of glucose in the electrically stunned samples was 2.02 µmol/g muscle; the minimum value was 0.59 µmol/g muscle. The mean was 1.56 µmol/g muscle, and the standard deviation was ±0.42 µmol/g muscle.

4.3.3.3 Glycogen

The maximum value of glycogen in the CO_2-stunned samples was 14.59 µmol/g muscle; the minimum value was 3.84 µmol/g muscle. The mean was 9.03 µmol/g muscle, and the standard deviation was 3.79 µmol/g muscle.

The maximum value of glycogen in the electrically stunned samples was 19.89 µmol/g muscle; the minimum value was 7.92 µmol/g muscle. The mean was 11.44 µmol/g muscle, and the standard deviation was ±3.33 µmol/g muscle.

4.3.3.4 Glucose-6-phosphate (G6P)

The maximum value of G6P in the CO_2-stunned samples was 1.98 µmol/g muscle; the minimum value was 0.56 µmol/g muscle. The mean was 1.20 µmol/g muscle, and the standard deviation was 0.45 µmol/g muscle.

The maximum value of G6P in the electrically stunned samples was 3.27 µmol/g muscle; the minimum value was 1.08 µmol/g muscle. The mean was 1.94 µmol/g muscle, and the standard deviation was ±0.66 µmol/g muscle.

4.3.3.5 Adenosine triphosphate (ATP)

The maximum value of ATP in the CO_2-stunned samples was 11.16 µmol/g muscle; the minimum value was 6.28 µmol/g muscle. The mean was 7.57 µmol/g muscle, and the standard deviation was 1.54 µmol/g muscle.

The maximum value of ATP in the electrically stunned samples was 8.65 µmol/g muscle; the minimum value was 5.93 µmol/g muscle. The mean was 7.10 µmol/g muscle, and the standard deviation was ±0.88 µmol/g muscle.

4.3.3.6 Creatine phosphate (CP)

The maximum value of CP in the CO_2-stunned samples was 4.23 µmol/g muscle; the minimum value was 2.76 µmol/g muscle. The mean was 3.67 µmol/g muscle, and the standard deviation was 0.49 µmol/g muscle.

The maximum value of CP in the electrically stunned samples was 4.68 µmol/g muscle; the minimum value was 2.44 µmol/g muscle. The mean was 3.62 µmol/g muscle, and the standard deviation was ±0.80 µmol/g muscle.

4.3.5 Video Statistics

For the CO_2 stunning treatment during the follow-up study, the same criteria were used to assess the behaviour as was used during the pilot study. Thus the definitions and parameters remained the same.

Table 3.35 *Timing of major events and behaviours observed in the footage recording of group 2-1.*

Event/behaviour	**Time (hh: mm: ss)**
Entry into the stunner	09:02:50
Gondola starts descending	09:03:02
One pig starts thrashing – theorised entry into CO_2	09:03:17
First pig falls	09:03:21
Second pig falls, gasping, stretches neck	09:03:24
Third pig falls, gasping	09:03:26
Fourth pig falls, gasping, stretches neck	09:03:27
Fifth pig falls, gasping	09:03:30
Thrashing, kicking	09:03:32
Gasping	09:03:37
Most movements ceased	09:03:42
One pig kicks, the rest respond	09:03:47
Head drop – theorised onset of unconsciousness	09:03:52
Muscle contractions - Kick-like movement - Periodic gasp-like movements - Last contraction	09:03:47 – 09:05:55 - 09:03:47 - 09:04:07 – 09:05:50 - 09:05:55
Ascent out of pit	09:06:05
Ejection from gondola	09:06:30

Table 4.36 *Durations of major events and behaviours during stunning of group 2-1.*

Event	**Duration/time to (mm: ss) (s)**
Time to exposure	00:15 (15)
Total time in stunner	03:40 (220)
Total time in CO_2	02:48 (168)
First reaction (gasping)	00:15 (15)
Time until assumed LOC	00:35 (35)
Time to fall - First pig - Second pig - Third pig - Fourth pig - Fifth pig	(8.6) - 00:04 (4) - 00:07 (7) - 00:09 (9) - 00:10 (10) - 00:13 (13)
Muscle contractions	02:08 (128)

While entering the gondola, the pigs were curious and not reluctant to enter. During the descent, they were calm. 15s after descent, one pig started thrashing, falling over shortly after. Within 13s of exposure to the CO_2, all five pigs had lost their posture. Four out of the five pigs

started gasping after falling, and two of these also stretched their necks. Thrashing and kicking movements were observed at 09:03:32, further gasping at 09:03:37. Most voluntary movements ceased at 09:03:42. A head drop was visible at 09:03:52, which indicates that unconsciousness has set in. All movements after this can be contributed to involuntary muscle contractions, which ceased at 09:05:55. The pigs were inside the stunner for 3 minutes and 40s and were exposed to the CO_2 for 168s. On average, the pigs lost their posture and fell 8.6s after entering the CO_2, and LOC is estimated at 35s after entering the CO_2.

Table 4.37 *Timing of major events and behaviours observed in the footage recording of group 2-2.*

Event/behaviour	Time (hh: mm: ss)
Entry into the stunner	09:08:20
Gondola starts descending	09:08:51
Swaying – theorised entry into CO_2	09:09:06
Kicking, thrashing and an escape attempt	09:09:09
First pig fall	09:09:15
Second pig fall	09:09:17
Third and fourth pigs fall	09:09:20
Fifth pig falls	09:09:22
Gasping, neck stretch, shivering	09:09:28
Voluntary movements cease	09:09:44
Muscle contractions - Periodic gasp-like movements - Muscle contractions cease	09:09:53 – 09:11:49 - 09:09:55 - 09:11:49
Ascent out of pit	09:12:15
Ejection from gondola	09:12:45

Table 4.38 *Duration of major events and behaviours during stunning of group 2.*

Event	Duration/time to (mm: ss) (s)
Time to exposure	00:15 (15)
Total time in stunner	04:25 (265)
Total time in CO_2	03:09 (189)
First reaction (swaying)	00:15 (15)
Time until assumed LOC	00:38 (38)
Time to fall - First pig - Second pig - Third and fourth pig - Fifth pig	(12.8) - 00:09 (9) - 00:11 (11) - 00:14 (14) - 00:16 (16)
Muscle contractions	01:56 (116)

While entering the gondola, the pigs were curious and not reluctant to enter. 15s after descent, the pigs started swaying. This is the point of entry into the CO_2. The pigs started kicking and thrashing, and there was one escape attempt. Within 16s of exposure to the CO_2, all five pigs had lost their posture, the first pig after 9s, and the last pig after 16s. After the pigs fell, gasping

and neck stretching movements were observed. Shivering was observed as well. Voluntary movements ceased at 09:09:44, indicating that unconsciousness had set in. All movements after this can be contributed to involuntary muscle contractions, which ceased at 09:11:49. The pigs were inside the stunner for 4 minutes and 25s and were exposed to the CO_2 for 189s. On average, the pigs lost their posture and fell 12.8s after entering the CO_2, and LOC is estimated at 38s after entering the CO_2.

Table 4.39 *Timing of major events and behaviours observed in the footage recording of group 2-3.*

Event/behaviour	Time (hh: mm: ss)
Entry into the stunner	09:14:16
Gondola starts descending	09:14:45
Swaying – theorised entry into CO_2	09:14:56
Neck stretching, gasping	09:15:04
First pig falls, thrashing, kicking	09:15:06
Second, third and fourth pig falls	09:15:07
Neck stretching	09:15:13
Fifth pig falls, gasping	09:15:14
Thrashing movements stop	09:15:20
Muscle contractions - Periodic gasp-like movements - Muscle contractions cease	09:15:26 – 09:17:15 - 09:15:35 – 09:17:12 - 09:17:15
Ascent out of pit	09:18:16
Ejection from gondola	09:18:37

Table 4.40 *Durations of major events and behaviours during the stunning of group 2-3.*

Commented [AO29]: These durations could have been analysed statistically (for publication purposes perhaps?)

Event	Duration/time to (mm: ss) (s)
Time to exposure	00:10 (10)
Total time in stunner	04:21 (261)
Total time in CO_2	03:20 (200)
First reaction (swaying)	00:10 (10)
Time until assumed LOC	00:30 (30)
Time to fall - First pig - Second, third and fourth pig - Fifth pig	(12.8) - 00:10 (10) - 00:11 (11) - 00:18 (18)
Muscle contractions	01:49 (109)

While entering the gondola, the pigs were somewhat reluctant to enter. 10s after descent, the pigs started swaying. This is the point of entry into the CO_2. The pigs started gasping for air and stretching their necks. Within 18s of exposure to the CO_2, all five pigs had lost their posture, the first pig after 10s, and the last pig after 18s. After the pigs fell, gasping, thrashing, kicking, and neck stretching movements were observed. Voluntary movements ceased at 09:15:20, indicating that unconsciousness was starting to set in. All movements after this can

be contributed to involuntary muscle contractions, which ceased at 09:17:15. The pigs were inside the stunner for 4 minutes and 21s and were exposed to the CO_2 for 200s. On average, the pigs lost their posture and fell 12.2s after entering the CO_2, and LOC is estimated at 30s after entering the CO_2.

Table 4.41 *Timing of major events and behaviours observed in the footage recording of group 2-4.*

Event/behaviour	Time (hh: mm: ss)
Entry into the stunner	09:37:16
Gondola starts descending	09:37:57
One pig climbs onto another	09:38:07
Squeal, uneasiness from all pigs – theorised entry into CO_2	09:38:12
First & second pig falls	09:38:19
Third pig falls	09:38:23
Fourth and fifth pig falls	09:38:25
Gasping, kicking	09:38:27
Voluntary movements cease, unconsciousness starts setting in	09:38:35
Muscle contractions - Periodic gasp-like movements - Muscle contractions cease	09:38:36 – 09:40:36 - 09:38:36 - 09:40:36
Ascent out of pit	09:41:00
Ejection from gondola	09:41:50

Table 4.42 *Duration of major events and behaviours during stunning of group 2-4.*

Event	Duration/time to (mm: ss) (s)
Time to exposure	00:15 (15)
Total time in stunner	04:34 (274)
Total time in CO_2	02:48 (168)
First reaction (climbing)	00:10 (10)
Time until assumed LOC	00:23 (23)
Time to fall - First and second pig - Third pig - Fourth and fifth pig	(10.2) - 00:07 (07) - 00:11 (11) - 00:13 (13)
Muscle contractions	01:49 (109)

The pigs were curious and didn't hesitate to enter the gondola. 10s after descent, one of the pigs climbed on another. This is unlikely to have been caused by the CO_2 gas, as only one pig exhibited this behaviour. It could be due to limited space inside the gondola. The group displayed squealing and uneasiness during the descent. This is the point of entry into the CO_2. Shortly after, the first two pigs lost their balance after 7s of CO2 exposure. Within 13s of exposure to the CO_2, all pigs had lost their posture. After falling, gasping and kicking

movements were observed. Voluntary movements ceased at 09:38:35, indicating that unconsciousness was starting to set in. All movements after this can be contributed to involuntary muscle contractions, which ceased at 09:40:36. The pigs were inside the stunner for 4 min and 34s and were exposed to the CO_2 for 168s. On average, the pigs lost their posture 10.2s after entering the CO_2, and LOC is estimated at 23s after entering the CO_2.

Table 4.43 *Timing of major events and behaviours observed in the footage recording of group 2-5.*

Event/behaviour	Time (hh: mm: ss)
Entry into the stunner	09:50:22
One pig shoves another	09:50:47
Descent into pit	09:50:54
Uneasiness and swaying – theorised entry into CO_2	09:51:10
Neck stretching, kicking	09:51:11
First pig falls	09:51:16
Second pig falls	09:51:19
Third and fourth pig falls	09:51:20
Fifth pig falls	09:51:24
Thrashing largely stops	09:51:40
Muscle contractions - Muscles contract periodically - Contractions cease	09:51:42 – 09:53:50 - 09:51:42 - 09:53:50
Ascent out of pit	09:54:08
Ejection from gondola	09:54:45

Table 4.44 *Duration of major events and behaviours during stunning of group 2-5.*

Event	Duration/time to (mm: ss) (s)
Time to exposure	00:16 (16)
Total time in stunner	04:23 (263)
Total time in CO_2	02:58 (178)
First reaction (swaying)	00:16 (16)
Time until assumed LOC	00:32 (32)
Time to fall - First pig - Second pig - Third and fourth pig - Fifth pig	(9.8) - 00:06 (06) - 00:09 (09) - 00:10 (10) - 00:14 (14)
Muscle contractions	02:08 (128)

While entering the gondola, the pigs were curious and calm. Before descent, one pig pushed another. 16s after descent, the pigs started swaying and were uneasy. This is the point of entry into the CO_2. The pigs started displaying kicking- and neck stretching movements. Within 14s of exposure to the CO_2, all five pigs had lost their posture, the first pig after 6s, and the last pig after 14s. After the pigs fell, kicking and neck stretching movements were observed.

Voluntary movements ceased at 09:51:40, indicating that unconsciousness was starting to set in. All movements after this can be contributed to involuntary muscle contractions, which ceased at 09:53:50. The pigs were inside the stunner for 4 minutes and 24s and were exposed to the CO_2 for 178s. On average, the pigs lost their posture and fell 9.8s after entering the CO_2, and LOC is estimated at 32s after entering the CO_2.

Table 4.45 summarises the descriptive statistics for the video data collected during both the pilot study and Follow-up study.

The mean time spent in the stunner during the Ar-CO_2 stunning was 592s. The 95% confidence interval upper bound value was 683.47s, and the lower bound value was 500.53s. The standard error was 39.67s, and the standard deviation was 152.74s. The mean time spent in the stunner during CO_2 stunning was 257.38s. The 95% confidence interval upper bound value was 303.11s, and the lower bound value was 211.64s. The standard error was 19.834s, and the standard deviation was 16.248s.

The mean time to exposure [to the gas] during the Ar-CO_2 stunning was 11.5s. The 95% confidence interval upper bound value was 19.20s, and the lower bound value was 3.80s. The standard error was 3.34s, and the standard deviation was 0.71s. The mean time to exposure during CO_2 stunning was 15.50s. The 95% confidence interval upper bound value was 19.32s, and the lower bound value was 11.65s. The standard error was 1.67s, and the standard deviation was 5.04s.

Table 4.45 *Descriptive statistics for the duration of different major events during gas stunning.*

Commented [AO30]: Ignore previous comment

Dependent Variable	Stunning Treatment	Mean	Std. Error	Std. Deviation	95% Confidence Interval	
					Lower Bound	Upper Bound
Time in stunner	Ar-CO_2	592.00	39.67	152.74	500.53	683.47
	CO_2	257.38	19.83	16.25	211.64	303.11
Time to exposure	Ar-CO_2	11.50	3.34	0.71	3.80	19.20
	CO_2	15.50	1.67	5.04	11.65	19.35
Time exposed to gas	Ar-CO_2	509.50	43.03	168.99	410.27	608.73
	CO_2	176.50	21.52	12.35	126.88	226.12
LOC	Ar-CO_2	241.00	20.73	82.02	193.19	288.80
	CO_2	32.63	10.37	4.59	8.72	56.53
Time to fall average	Ar-CO_2	9.50	1.36	0.71	6.37	12.63
	CO_2	10.53	0.68	2.04	8.96	12.09
Muscle contractions	Ar-CO_2	129.50	25.96	89.80	69.65	189.35
	CO_2	110.38	12.98	19.69	80.45	140.30

LOC: Time to loss of consciousness

The mean time exposed [to the gas] during the Ar-CO_2 stunning was 509.50s. The 95% confidence interval upper bound value was 608.73s, and the lower bound value was 410.27s. The standard error was 43.03s, and the standard deviation was 168.99s. The mean time exposed during CO_2 stunning was 176.50s. The upper bound value was 226.12s, and the lower bound value was 126.88s. The standard error was 21.52s, and the standard deviation was 12.35s.

The mean time to fall during the Ar-CO_2 stunning was 9.50s. The 95% confidence interval upper bound value was 12.63s, and the lower bound value was 6.37s. The standard error was 1.36s, and the standard deviation was 0.71s. The mean time to fall during CO_2 stunning was 10.53s. The 95% confidence interval upper bound value was 12.09s, and the lower bound value was 8.96s. The standard error was 0.68s, and the standard deviation was 2.04s.

Commented [AO31]: It seems like there were significant differences, yet they are not indicated

The mean time to LOC during the Ar-CO_2 stunning was 241s. The 95% confidence interval upper bound value was 288.80s, and the lower bound value was 193.20s. The standard error was 20.73s, and the standard deviation was 82.02s. The mean time to LOC during CO_2 stunning was 32.63s. The 95% confidence interval upper bound value was 56.53s, and the lower bound value was 8.72s. The standard error was 10.37s, and the standard deviation was 4.60s.

Table 4.46 *Pairwise comparisons of the duration of different major events between different gas stunning systems.*

Commented [AO32]: Please ignore previous comment, remember to define abbreviations at the bottom of a table

Commented [AO33]: This is also not a typical way to represent data in table. But not incorrect.

Dependent Variable	(I) Stunning Treatment	(J) Stunning Treatment	Mean Difference (I-J)	Std. Error	Sig.[b]
Time in stunner	Ar-CO_2	CO_2	334.625*	44.349	<,001
	CO_2	Ar-CO_2	-334.625*	44.349	<,001
Time to exposure	Ar-CO_2	CO_2	-4.000	3.734	.315
	CO_2	Ar-CO_2	4.000	3.734	.315
Time exposed to gas	Ar-CO_2	CO_2	333.000*	48.112	<,001
	CO_2	Ar-CO_2	-333.000*	48.112	<,001
LOC	Ar-CO_2	CO_2	208.375*	23.177	<,001
	CO_2	Ar-CO_2	-208.375*	23.177	<,001
Time to fall average	Ar-CO_2	CO_2	-1.025	1.519	.519
	CO_2	Ar-CO_2	1.025	1.519	.519
Muscle contractions	Ar-CO_2	CO_2	19.125	29.018	.528
	CO_2	Ar-CO_2	-19.125	29.018	.528

LOC: Time to loss of consciousness
Based on estimated marginal means
*. The mean difference is significant at the ,05 level.
b. Adjustment for multiple comparisons: Bonferroni.

The mean duration of muscle contractions (involuntary movements typically after LOC) during the Ar-CO_2 stunning was 129.50s. The 95% confidence interval upper bound value was 189.35s, and the lower bound value was 69.65s. The standard error was 25.96s, and the standard deviation was 89.80s. The mean duration of muscle contractions during CO_2 stunning was 110.38s. The 95% confidence interval upper bound value was 140.30s, and the lower bound value was 80.45s. The standard error was 12.98s, and the standard deviation was 19.69s.

Based on Table 4.46, the differences between the two gas stunning treatments were significant ($P < 0.005$) for the time spent in the stunner, the duration of exposure to the gas, and the time to LOC.

CHAPTER 5: DISCUSSION

5.1 Behavioural footage

5.1.1 84% CO_2 stunning

84% CO_2 stunning was tested during both phases in the current study and will be discussed here together. During the pilot study, each pig stunned using CO_2 spent an average of 260.06s inside the stunner. Average time that it took the gondola to enter the gas was 17.24s. On average, the pigs were exposed to the CO_2 for a duration of 169.91s, with the longest time attributed to group 3 (176 s) and the shortest to group 1 (164 s). On average, the pigs lost their balance after having been exposed to the CO_2 for 8.59 ± 3.31s. The two individually stunned pigs had both the shortest (2 s) and the longest time to fall (13 s). Theoretical LOC, based on the pigs' observable behaviour inside the stunner, took place after 32.24 ± 2.24s.

During the follow-up study, the pigs spent 256.60 ± 18.83s inside the stunner. The average time to first exposure to the CO_2 gas was 14.20 ± 2.14s. The average total time exposed to the gas was 180.60 ± 12.42s. Mean time to fall was measured at 10.72 ± 3.16s, and time to LOC was estimated at 31.60 ± 5.08s.

Due to the movement of the animals inside the stunner and the camera angle, it was not possible to attribute individual measurements to each pig in the groups. Many of the behaviours were concealed from the camera, and this affected the accuracy of behaviour evaluations done using this footage. Verhoeven et al., (2016) found that pigs immersed in 80% CO_2 and 90% CO_2 lost their consciousness on average after 47 and 33s, respectively. Similarly, Llonch et al., (2013) reported that pigs exposed to 90% CO_2 lost their consciousness after an average of 37.6s after descent into the stunner started. The results obtained for pigs stunned using 84% CO_2 in the current study are comparable to the results of these previous studies, as average time to LOC was measured at 32.24 ± 2.24s and 31.60 ± 5.08s during the first and second phases, respectively. The studies differed in the following aspects: the current study tested an 84% CO_2 stunning system, under commercial conditions, whereas previous studies tested 80% and 90% CO_2 stunning under experimental conditions.

Our results disagreed with those obtained by Verhoeven et al., (2016) with regards to the time to fall, which they referred to as 'latency to first lying [down]'. In their study, they defined 'loss of posture' as being 'in a recumbent position with total loss of control of posture', as opposed to being 'in a recumbent position, still having partial control of posture' for lying [down]. In the

current study, the average time to fall was measured as 8.59 ± 3.31 and 10.72 ± 3.16s after first exposure to the 84% CO_2 during the pilot- and follow-up studies, respectively. In comparison, Verhoeven et al., (2016) reported a latency to first lying of 34 ± 5 and 17 ± 3s for 80% CO_2 and 90% CO2, respectively. Another major difference in the design of these two studies is that in the current study, pigs stunned using the 84% CO_2 stunning method were stunned in groups of five, as opposed to groups of two. The only exception for this was the two pigs that were stunned individually during the first phase.

Furthermore, the results of the present study are based on observable exhibited behaviour (Lechner *et al.*, 2021) and did not make use of EEGs. During the first phase, attempts were made to incorporate EEGs, but useful data could not be retrieved during stunning due to the design of the equipment, the design of the abattoir floor and the ambient interference from the mechanical machinery around the stunner (Verhoeven *et al.*, 2016; Steiner *et al.*, 2019). In future, this would be of use to further investigate the relationship between exhibited behaviour and LOC, if interference from the environment inside and around the stunner could be minimised.

Apart from one individually stunned pig, the pigs were reluctant to enter the gondola, and had to be pushed in by the automatic push-arm. This reluctance probably stemmed from the unfamiliarity of the gondola and the noises inside the abattoir (Terlouw *et al.*, 2008; Becerril-Herrera *et al.*, 2009; Lechner *et al.*, 2021). Lairage conditions, while not considered in this study, could also have impacted the pigs' willingness to enter the gondola. There was an abnormally large crowd of on-lookers around the stunner during the pilot study. This could also have contributed to the noise concentrations inside the abattoir. During the descent, a pig in group 1-1 vocalised after one of its groupmates jumped on it. It is unlikely that this jump, and the resulting vocalisation by extension, was due to the CO_2 as the gondola had just started its descent into the pit and was still above the gas. A few groups looked uneasy while descending, but this is likely not due to the gas but rather due to the movement of the gondola. The pig that was not reluctant to enter the gondola proceeded to sniff and explore the gondola upon entry.

Inside the gondola, entry into CO_2 was assumed to have taken place when the whole group had exhibited a reaction, either at the same time, or in very short succession of one another. The most common first reaction to the gas was swaying movements, indicating that the immersion into the CO_2 was quick. Shortly after this, gasping, neck-stretching, and jumping, in response to the inevitable breathlessness that followed, were observed in all groups. Vocalisations ranging from grunts to severe squealing were heard. Grunting is often associated with the normal day-to-day vocalisations that pigs make (Manteuffel *et al.*, 2004), and is not necessarily indicative of stress. Longer vocalisations like screaming and squealing

are associated with stressful conditions, whereas short vocalisations like grunts and barks are used in more positive communication (Tallet *et al.*, 2013; Friel *et al.*, 2019). During the first phase, vocalisations were heard during the CO_2 stunning of the groups. No vocalisations were recorded during the stunning of the two individual pigs. Low frequency sounds like grunts (groups 1-2 & 1-4), barks (group 1-1), growling (group 1-2) were heard during the pilot study. A short, high-pitched squeal was heard during the second phase, from a pig in group 2-4. It is likely that entry into the CO_2 took place when that happened, as the first pig lost its balance and fell 7s later, with the rest of the group following suit within 6s after the first pig. This was the only vocalisation recorded by the cameras during the follow-up study. With the noise concentrations inside the stunner, it is possible that more of the low-frequency vocalisations were drowned out during both phases. LOC would have taken place after vocalizations have ended, as vocalizing is a conscious response to the pigs' environment (EFSA (European Food Safety Authority), 2013; Dalmau *et al.*, 2016).

Commented [AO34]: Spelling

Behaviour inside the stunner can be either voluntary or involuntary, and this influences how aversiveness is evaluated. Gasping, for example, occurs due to residual medullary activity in the brainstem during exposure to high concentrations of CO_2. Thus, it is a natural physiological response to the breathlessness that occurs when high concentrations of CO_2 are inhaled (Verhoeven *et al.*, 2016). The author of the current study would argue that it's expected and, in the context of gas stunning, an appropriate reaction. That is not to say that the sensation does not cause the animals discomfort while they are still conscious, just that the animals' body will physiologically respond to its environment, sometimes involuntarily. These behaviours can be considered aversive depending on their duration and intensity and whether the animal is conscious (Verhoeven *et al.*, 2016). Stunning effectiveness inside the abattoir can be assessed using vocalisations, righting reflexes (any reflex that lets the animal regain its posture), corneal reflexes and rhythmic breathing (Verhoeven *et al.*, 2014; Dalmau *et al.*, 2016). Righting reflexes, along with vocalisations, are associated with an animal that is still fully conscious, whereas corneal reflexes and rhythmic breathing are indicators of returning consciousness after stunning (Rodríguez *et al.*, 2008).

Commented [AO35]: You need a reference here

When pigs are stunned in groups, they can land and lie on each other after falling. This unnatural position made the identification of exhibited behaviour and its evaluation difficult. For example, while pigs were losing their balance, it became difficult to distinguish between jumping (perhaps to reach for air), escape attempts and convulsions. Distinguishing between these behaviours is important, as jumping can merely be a behavioural response to breathlessness whereas escape attempts are indications of stress so severe that the animal's fight-or-flight response is triggered. Both are indications of stress, but potentially differ in their severity. Convulsions are involuntary muscular excitations that indicate the loss of higher

motor control (Lambooij, 2004). However, none of these physical behaviours, though they are associated with the LOC, should be used to determine the state of unconsciousness by themselves (Verhoeven *et al.*, 2014). Using an array of behaviours likely will increase the accuracy with which abattoir managers can determine unconsciousness in slaughter animals. Between the loss of balance and the theorised LOC, kicking and thrashing movements were seen during most CO_2 stunnings. It is unlikely that these movements were part of the convulsions described above, as they seemed coordinated. As the onset of unconsciousness in a gas stunning system is gradual, it can be theorised that higher motor control is also lost gradually. This could allow for convulsions to start before consciousness has been lost entirely and continue past this point.

The pigs appeared lifeless when the gondola started its ascent out of the CO_2, and all movement, both voluntary and involuntary, had ceased by the time the pigs were ejected from the gondola. This was expected, as the stunning system employed by this specific abattoir is used to irreversibly stun the pigs. No corneal reflexes or rhythmic breathing were observed. From an animal welfare point-of-view, this renders a strict stun-to-stick interval unnecessary, as the animals have already been killed and cannot experience pain during slaughtering.

Commented [AO36]: I do think it is still relatively important as you still want the heart to pump out the most amount of blood possible

The reasoning behind the use of higher concentrations of CO_2 in gas stunning systems is the associated shorter time to LOC as the concentration of CO_2 increases (Raj & Gregory, 1995; Dalmau *et al.*, 2010c), despite the animals reacting more aversively during this time (Raj *et al.*, 1997a). Shorter times to LOC means that the pig experiences a shorter period of stress during stunning. In abattoirs where irreversible gas stunning is used, an earlier onset of unconsciousness means the animal will be killed faster, which allows for higher production throughput in commercial pork abattoirs and shortens the time during which the animal will experience pain and fear. (Nowak *et al.*, 2007). The debate on whether lower or higher concentrations of CO_2 used during stunning is more advantageous continues due to the high variability in observations and results reported across the globe. Nowak et al., (2007) reported that 90% CO_2 was better for both animal welfare and meat quality. Due to the quality of the stun, a longer stun-to-stick interval could be used without the risk of returning sensibility. pH and impulse impendence values were more favourable when animals were stunned with 90% CO_2 for 100s and stuck between 40 and 50s thereafter. However, from a behavioural perspective, Verhoeven et al., (2016) reported that there were not enough significant differences between the impacts of 80- and 90% CO_2 stunning atmospheres on pigs.

Commented [AO37]: And from an animal welfare point of view

The current study did not classify the behaviour parameters as in depth as others have. In many cases, specific behaviours were not counted, but merely mentioned based on varying intensities. The use of behaviours to determine LOC has been criticized based on the

variability thereof (Steiner *et al.*, 2019). The behaviour animals exhibit prior to slaughter often varies between groups and individuals as it is influenced by many different factors, including but not limited to genetics and previous experiences (Terlouw, 2005), environment (Grandin, 1982) and handling (Van de Perre *et al.*, 2010; Vermeulen *et al.*, 2015). Ideally, as many of these factors as possible must be considered when evaluating behaviour.

5.1.2 80% Argon 20% CO_2 admixture

The mean time spent exposed to the Ar-CO_2 mixture was 436.80 ± 97.24s, with the maximum duration of 629s and the minimum duration of 362s. The average latency to exposure was 13.40 ± 3.61s, with the longest latency being 19s and the shortest latency being 9s. Pigs lost their balance on average 9.60 ± 3.20s after first exposure to the admixture, with the longest latency being 19s and the shortest, 9s. Time to LOC could only be determined in three pigs due to certain behavioural queues being absent in the other two. On average, it is estimated that consciousness was lost after being exposed to the admixture for 243 ± 47.44s.

Behaviours observed included swaying, avoidance behaviours like backing, kicking and thrashing, vocalisations, gasping, neck movements and muscle contractions after LOC. No escape attempts or jumps were observed. 80% of pigs stunned using this mixture vocalised while being stunned. High pitched, long squeals were heard during each of these, and were often accompanied by or followed by violent thrashing and kicking movements. In two of these, the squeals were preceded by grunts. One pig in this stunning treatment displayed muscular contractions until the point of ejection from the stunner, where these movements had ceased in the others.

In response to the aversive behaviours witnessed during the stunning of pigs using CO_2, researchers have investigated alternative gas stunning methods that could utilise the group-stunning welfare benefits and improve meat quality as seen in CO_2 stunning (Raj & Gregory, 1995, 1996; Dalmau *et al.*, 2010c; Llonch *et al.*, 2012a; b, 2013). It has been reported that gasses like argon and nitrogen could improve animal welfare during gas stunning (Raj & Gregory, 1995; Dalmau *et al.*, 2010c; Llonch *et al.*, 2012b). Others reported that high concentrations of argon caused some aversion to the animals during stunning (Dalmau *et al.*, 2010c).

These results differed from that reported by Raj (1999), who observed a loss of posture after 15 ± 9.2s when pigs were stunned with 90% Argon, and 18 ± 4.6s when stunned with a 60% Ar 30% CO_2 mixture in air. He also reported that pigs stunned using 90% Ar had to be exposed for 5 minutes (300 s) and bled within 45s, to prevent the return of consciousness. Otherwise, an exposure time of more than 7 minutes (420 s), which would kill the pigs and forego the need of a strict stun-stick interval. For the 60% Ar 30% CO_2 mixture, a stunning time of 7

minutes (420 s) was recommended to kill the animals. The first pig stunned using this mixture displayed gasping- and neck movements until it was ejected from the stunner, having been exposed to the gas for 362s. The second pig was exposed to the gas for 396s, which was too short as muscle contractions were visible at the time of ejection. Neither of these two pigs displayed behaviour that was clearly indicative of unconsciousness. The third pig stunned with Ar-CO_2 was exposed to the gas for 629s, 209s longer than needed for 90% Ar, according to Raj (1999), and lost its consciousness after being exposed to the gas for 183s. This pig was kept under the influence of the gas for longer than was theoretically necessary to ensure that the animal died before being ejected from the stunner. The fourth pig was exposed for a total of 407s and lost its consciousness after being exposed to the gas for 247s. Bodily movements ceased 43s before ejection from the stunner. It was deemed likely that this pig was still alive after ejection, and so a 15s stun-to-stick interval was adhered to. The fifth pig was exposed to the gas for 390s and lost its consciousness after being exposed to the mixture for 299s. All movements ceased 195s before ejection from the stunner, and sticking took place 15s afterwards.

As the concentration of CO_2 in the admixture was lower than that used by Raj, (1999) and increasing concentrations of CO_2 improved the time to unconsciousness, it was concluded that a longer time of exposure would be required to induce unconsciousness under experimental conditions. What was not known, however, was how long the animals needed to be exposed to induce unconsciousness in a commercial gas stunner. The results from this study proved that 80% Ar 20% CO_2 was capable of stunning one pig after approximately 390s (6 m 30 s). This is shorter than the second pig, which was exposed for 9s longer, but still displayed movements at ejection. The Butina Backloader used by this abattoir proved incapable of maintaining consistent gas concentrations, particularly those of O_2, which had to remain under 2%. This could have influenced the time at which the pigs lost their consciousness.

410s (6 m 50 s) passed between the ejection of the first pig and the loading of the second pig. 582s (9 m 42 s) passed between the ejection of the second pig and the loading of the third pig. This was due to difficulties in maintaining constant gas concentrations inside the stunner, and likely affected the efficiency of the stun delivered to the first two pigs as well as their demeanor inside the stunner. Thereafter, the gas concentrations were kept under control long enough to finish the rest of the Ar-CO_2 stunnings.

Commented [AO38]: I would say that this "waiting time" for the next pig may also have an influence on the animal itself

Table 5.1 *Times between the ejection and loading of pigs during Ar-CO_2 stunning*

Ejected pig – loaded pig	Time in minutes (m:s)	Time (s)
1st – 2nd	6:50	410
2nd – 3rd	9:42	582

3^{rd} – 4^{th}	1:41	101
4^{th} – 5^{th}	1:41	101

It was reported that stunning pigs with more than 30% CO_2 resulted in aversive behaviour, but higher concentrations of CO_2 resulted in a more efficient stun and deeper state of unconsciousness (Raj *et al.*, 1997a; Dalmau *et al.*, 2010c; Atkinson *et al.*, 2020). This is one example of a trade-off between gas concentrations. Less aversive behaviour and a shorter time to LOC is preferred from an animal welfare point of view. However, these two gas stunning methods have proved that a trade-off must be made. Even if gas admixture concentrations could be kept constant in this specific commercial stunner, one would still have longer times to loss of unconsciousness, even if less aversive behaviour is exhibited. The observation of increased frequencies of high pitched, longer squeals during Ar-CO_2 admixture stunning compared to CO_2 calls into question whether the pigs really experienced the mixture as less aversive or not. What an animal experiences while it is conscious is of importance for animal welfare (Verhoeven *et al.*, 2016). According to the abattoir's current standard operating procedures, 5 pigs are loaded per gondola, and each gondola spends about 4 m 30s inside the stunner. This is sufficient to kill the pigs before they are ejected from the stunner. Vocalisations during CO_2 stunning were rare. It was previously observed that pigs agitated prior to entering the stunner are more likely to react aversively and vocalise than calm pigs. While pre-stunning mental state was not evaluated as part of this study, it likely had an effect on the behaviour exhibited inside the stunner. Handling prior to stunning and slaughter remains one of the main reasons for poor meat quality (Van de Perre *et al.*, 2010; Soma *et al.*, 2014b).

5.2 pH and Temperature

5.2.1 Pilot study

Pigs stunned using either of the gas stunning methods (Ar-CO_2 or CO_2) yielded carcasses that exhibited pH-values associated with the DFD carcass defect, as the mean pH in the *m. longissimus thoracis* at one hour *post mortem* (pH_1) for Ar-CO_2 and CO_2 was 6.50 and 6.65, respectively.

Commented [AO39]: The rate of pH decline over carcass temperature is a better predictor of PSE and DFD

Of the seven groups stunned using 84% CO_2, only Group 2 had a mean pH_1 above 6.0 and below 6.4 (pH_1 = 6.13). The rest had pH_1 values higher than 6.4, which at 1h post mortem posed a risk for the development of DFD, since pH values above 6.0 at 45min are typically considered to be at risk (Adzitey & Nurul, 2011). Of the seven pigs stunned via emergency electrical head-only stunning, four carcasses exhibited carcass pH_1 values between 6.0 and 6.4, and the remaining three had pH_1 values above 6.4, posing a risk for DFD. The first pig stunned using the Ar-CO_2 admixture had a pH_1 of 6.12, whereas the remaining four carcasses

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all had pH_1 values higher than 6.4. However, after nine hours *post mortem*, none of the treatments displayed pH values above 6.0. The mean ultimate pH (pH_u) value for Ar-CO_2 stunning was 5.52 (at 21 h), and the mean pH_u for CO_2- and electrical stunning was 5.58 (at 24 h) for both treatments.

Table 5.2 *Distribution of potential carcass defects according to pH_1 during the pilot study.*

Stunning method	DFD *($pH_1 > 6.4$)*		Normal *($6.0 \leq pH_1 \leq 6.4$)*		PSE *($pH_1 < 6.0$)*		Total	
	N	%	n	%	n	%	n	%
Ar-CO_2	4	80.00	1	20.00	0	0.00	5	100.00
CO_2	6	85.71	1	14.29	0	0.00	7	100.00
Electrical	3	42.86	4	57.14	0	0.00	7	100.00
Total	**13**	**68.42**	**6**	**31.58**	**0**	**0.00**	**19**	**100.00**

Table 5.3 *Distribution of potential carcass defects according to pH_u during the pilot study.*

Stunning method	DFD *($6.0 < pH_u$)*		Normal *($5.4 \leq pH_u \leq 6.0$)*		PSE *($pH_u < 5.4$)*		Total	
	N	%	N	%	n	%	n	%
Ar-CO_2	0	00.00	5	100.00	0	0.00	5	100.00
CO_2	0	00.00	7	100.00	0	0.00	7	100.00
Electrical	0	00.00	6	85.71	1	14.29	7	100.00
Total	**0**	**00.00**	**18**	**94.74**	**1**	**5.26**	**19**	**100.00**

In normal carcasses, the *post mortem* muscle pH drops from 6.4 (at 45 minutes *post mortem*) to 5.5 (at 24 hours *post mortem*). Ante-mortem stress negatively affects the normal rate of pH decline and the conversion of muscle to meat. Dark, firm, and dry (DFD) meat is a result of chronic (long-term) stress (Viljoen *et al.*, 2002; Adzitey & Nurul, 2011), caused during transportation and long periods of lairage. After slaughter, DFD meat is characterised by pH_1 values above 6.4, and pH_u values above 6.0. By contrast, pale, soft and exudative (PSE) meat is a result of acute (short-term) stress shortly prior to slaughter (Adzitey & Nurul, 2011). pH_1 values below 6.0 and pH_u values below 5.3 are associated with PSE carcass defects (Cobanovic *et al.*, 2019). Based on these definitions, it is to be expected that extremely stressful conditions just prior and during stunning would result in the development of PSE-like characteristics. However, none of the treatments had mean pH_1 values below 6.0 or mean pH_u values below 5.4, and only one electrically stunned carcass had a pH_u of 5.32.

None of the carcasses that had pH_1 values indicative of DFD had high pH_u values at 24 hours *post mortem*. Furthermore, based on graph 4.1, the decline in pH for both the CO_2- and electrical stunning treatments followed the expected curve, and had similar mean pH_u values. By contrast, the pH decline in the Ar-CO_2 stunning treatment did not have a normal curve, but instead declined rapidly shortly after only one hour *post mortem*. It is normal for the muscle

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temperature to increase directly after slaughter as the muscle is actively converting glycogen to lactate (Lindahl *et al.*, 2006). This typically takes place in the first 30min *post mortem* and continues as long as energy can be derived from the hydrolysis of creatine phosphate and ATP. The temperature starts declining once sources of creatine phosphate have been depleted (Lindahl *et al.*, 2006) and the chilling system starts to decrease the carcass temperature. Additionally, the pigs stunned with the Ar-CO_2 admixture endured prolonged stressful conditions during stunning. It is well-known that stressful conditions shortly before slaughter negatively affect the rate of glycolysis during the first few hours *post mortem* (Lindahl *et al.*, 2006; Scheffler & Gerrard, 2007). This results in higher muscle glycogen content, elevated muscle temperatures and, thus, faster pH declines. Higher temperatures in the muscles catalyse the reactions during glycolysis, resulting in faster rates of pH decline for a longer period. Muscle temperature increases following stressful conditions inside the stunner on top of normal increases in *post mortem* muscle temperature (Hambrecht *et al.,* 2004) could explain the immediate drastic pH decline seen in carcasses in the Ar-CO_2 group.

The carcasses from pigs stunned with CO_2 maintained marginally but consistently higher pH than those from pigs that were stunned electrically, but with similar mean pH_u values at 24 hours *post mortem*. Previous DFD research (Guàrdia et al., 2005; Scheffler et al., 2011; Manalo & Gabriel, 2020) indicates that chronic stress prior to slaughter results in lower muscle glycogen content compared to normal- and acutely stressed muscles. Although the pH_u did not indicate DFD, the higher pH concentrations throughout *post mortem* muscle metabolism indicate a low level of chronic stress prior to slaughter. Lower muscle glycogen content is associated with long term stress during long distance travel, prolonged food deprivation and overcrowding during long lairage periods (Adzitey & Nurul, 2011). It is speculated that, while these carcasses likely had lower muscle glycogen content compared to those stunned electrically, there was still sufficient glycogen to be metabolised. This would prevent the muscle temperature from rapidly declining, allowing pH decline to take place for a longer period compared to classical DFD.

While the emergency electrical head-only stunning method was criticised because it was not conducive to good animal welfare practices, the carcasses had the best pH decline curve of the three treatments. However, this stunning method had the lowest pH_u value, indicative of PSE (5.31). Based on the reviewed literature, this group likely had more glycogen available for *post mortem* metabolism than pigs in the CO_2-stunned group, and comparatively cooler muscle temperatures, slowing the decline of muscle pH and limiting the extent of muscle metabolism.

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5.2.2 Follow-up study

Overall, the carcasses in the 84% CO_2 stunning group had a lower mean pH than those in the electrical stunning group at every measurement. Both treatments, based on the pH decline curves in graph 4.2, exhibited similar pH decline rates. This could indicate that the pigs stunned using the head-to-heart stunning method experienced less stress directly prior to slaughter than those stunned using CO_2. It is worth noting however that six out of 20 carcass-halves exhibited pH_1 values above 6.4, which could indicate the possibility of DFD in those carcasses, whereas three out of 20 carcass-halves from pigs stunned using CO_2 exhibited pH values above 6.4 at 1 hr *post mortem*.

Table 5.4 *Distribution of potential carcass defects according to pH_1 during the follow-up study.*

Stunning method	DFD ($pH_1 > 6.4$)		Normal ($6.0 \leq pH_1 \leq 6.4$)		PSE ($pH_1 < 6.0$)		Total	
	n	%	n	%	n	%	n	%
CO_2	3	15.00	13	65.00	4	20.00	20	100.00
Electrical	6	30.00	13	65.00	1	5.00	20	100.00
Total	**9**	**22.50**	**26**	**65.00**	**5**	**12.50**	**40**	**100.00**

Table 5.5 *Distribution of potential carcass defects according to pH_u during the follow-up study.*

Stunning method	DFD ($6.4 < pH_1$)		Normal ($6.0 \leq pH_1 \leq 6.4$)		PSE ($pH_1 < 6.0$)		Total	
	n	%	n	%	n	%	n	%
CO_2	1	5.00	19	95.00	0	0.00	20	100.00
Electrical	3	15.00	17	85.00	0	0.00	20	100.00
Total	**4**	**10.00**	**36**	**90.00**	**0**	**0.00**	**40**	**100.00**

However, DFD is more accurately determined based on the pH_u, measured at 24 h *post mortem* (Boler *et al.*, 2009; Manalo & Gabriel, 2020). pH_u values higher than 6.0 are thus considered indicative of the development of DFD. Only one CO_2-stunned carcass-half exhibited a pH_u over 6.0 (pH_u = 6.06), whereas three carcass-halves in the electrical stunning group exhibited a pH_u above 6.0 (pH_u = 6.01, 6.08 and 6.11).

As previously discussed, DFD is a carcass defect that develops due to muscle glycogen reserves being depleted prior to slaughter, brought on by high concentrations of chronic stress. The expected defect for acute stress (as could theoretically be experienced during stunning) is thus PSE, caused by elevated body temperatures and prolonged periods of faster rates of glycolysis. This would drive the pH_u down to below 5.4 at 24 h *post mortem*. However, no carcasses exhibited pH_u values below 5.4, indicating that neither stunning method resulted in the development of PSE. It is thus clear that long-term stress remains a problem, even though it was quite small in this study. According to Soma et al., (2014), far more progress will be made with regards to improving pork quality in South Africa by improving the environments

Commented [AO48]: It would be nice if you had carcass characteristics like warm, cold carcass mass and drip loss or even WHC to make this statement with assurance.

pigs are kept in and the techniques with which these pigs are handled, both on-farm and at the abattoir.

Both stunning methods had similar temperature decline rates. Except for the values measured at 9 h *post mortem*, CO_2-stunned carcasses had slightly higher muscle temperatures than those stunned using the electrical stunning method.

5.3 Muscle metabolites

Overall, the *m. longissimus thoracis* from carcasses from the standard head-to-heart electrical stunning method had higher lactate content, but lower glucose- and glycogen content. The differences in muscle lactate- and -glycogen content, however, were not significant (P = 0.075 and P = 0.149, respectively), whereas the difference in muscle glucose concentrations was more significant (P = 0.034). Furthermore, the *m. longissimus thoracis* from carcasses stunned using the electrical stunning method had lower G-6-P concentrations and higher ATP concentrations compared to the carcasses stunned using CO_2. The difference in G-6-P content was significant (P = 0.009), whereas the difference in ATP concentrations was not (P = 0.410). Creatine phosphate concentrations were similar between the two treatments, and the difference was not significant (P = 0.868).

Commented [AO49]: These are costly lab analyses but should be analysed for different periods post mortem

Research by Henckel et al., (2002) indicated that stunning by CO_2 causes a decrease in muscle glycogen, irrespective of glycogen concentrations before stunning, but still influenced by physical stress prior to stunning. They also reported decreases in creatine phosphate concentrations, caused by CO_2 and possibly influenced by pre-slaughter handling. ATP concentrations increased, independent of pre-slaughter treatment. Increases in lactate may have been dependent on pre-slaughter treatment. It is possible that pre-slaughter treatment influenced the animals' reaction during stunning.

In the present study, muscle glycogen content was higher (11.44 ± 3.33 µmol/g) after electrical stunning than CO_2 (9.03 ± 3.79 µmol/g). Compared to the results obtained by Henckel et al. (2002) at 1 h *post mortem*, the pigs in the current study had experienced severe glycogen depletion under both stunning regimes. It is to be expected that CO_2 stunning, having a longer period of activity before LOC than electrical stunning, would have a higher rate of pH decline *post mortem*, and thus more depleted glycogen reserves at 1 h *post mortem*. However, the glycogen content observed in this study was still much lower than the values reported by (Henckel *et al.*, 2002). The closest value reported by these researchers at 1 h *post mortem* was 13.91 µmol/g, which was recorded from 20 pigs subjected to a treatment designed to represent a severely stressed animal prior to slaughter. It is known that long-term chronic stress results in depleted glycogen reserves, putting the carcass at risk of developing DFD (van der Wal *et al.*, 1989; Guàrdia *et al.*, 2005). This may explain the high pH_1 values recorded,

Commented [AO50]: How long after the last meal were the animals slaughtered? This can also have an effect. How long were the animals kept in lairage?

of which six were from pigs stunned using electricity, and three were from the pigs stunned using CO_2. As the difference in glycogen concentrations was not significant between these two treatments, it is not likely that stunning method had greatly influenced muscle glycogen content *post mortem*. Muscle lactate content was generally lower in CO_2-stunned pigs compared to electrically stunned pigs, but not significantly so. Increased lactate concentrations are associated with higher concentrations of stress (Hambrecht *et al.*, 2004; Barton Gade, 2008; Brandt et al., 2013; Sommavilla et al., 2017) before slaughter, and coincides with lower concentrations of glycogen (Choe *et al.*, 2015). The pigs in the current study had higher creatine phosphate concentrations in the *m. longissimus thoracis* at one hour *post mortem* than those reported by (Henckel *et al.*, 2002). This would be used for the production of ATP most-mortem (Scheffler & Gerrard, 2007) as creatine phosphate typically is present at higher concentrations in the *m. longissimus thoracis* than ATP (Brendall, 1973). Significant differences were found when comparing glucose- and G-6-P concentrations between the two stunning methods. Glucose and G-6-P are both metabolic intermediates in the biochemical process of glycolysis, by which glycogen is converted to lactate (Scheffler & Gerrard, 2007; Spires *et al.*, 2023). Higher glycogen concentrations were found in the carcasses stunned by CO_2 compared to those stunned by electrical stunning. The opposite was true for lactate concentrations, with the carcasses stunned using electrical stunning having higher lactate concentrations. It is likely that the rate of glycolysis was slightly faster in the carcasses stunned by electrical stunning at this point in the *post mortem* process, as the glycogen stores were smaller, but the metabolic intermediate pools were higher than those in carcasses stunned by CO_2. This would further explain the difference in lactate concentrations between the stunning methods. Although the differences in glucose concentrations and G-6-P concentrations between the stunning methods are significant ($P < 0.05$), it is unlikely that this would imply significantly different rates of *post mortem* metabolism. The pH declines between the two stunning methods were similar, although electrically stunned carcasses generally had a slightly higher pH than CO_2-stunned carcasses. These where the only glycolytic intermediates measured in this study.

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Commented [AO53]: It is also in indication of how fast the metabolic rate it.

Commented [AO54]: G6P does indeed indicate the rate of metabolism post mortem. But you need the other time intervals post mortem to draw a conclusion

5.4 Panel review

Based on the criteria given, the panellists agreed that the behaviour observed during 80% CO_2 20% Ar stunning indicated that the pigs were severely distressed during exposure. It was noted that the pigs moved around and vocalised more before and after the LOC (when it was determinable) compared to pigs stunned by CO_2. Both the depth and quality of the stun were questioned at least twice due to pigs displaying behaviour that was considered indicative of returning sensibility. These movements took place near the end of the stun and involved movements of the head and ears. It is possible that some of these movements could be due

to the movement of the gondola as it stopped and continued along its course. However, the veterinarian responsible for checking for signs of returning consciousness after stunning confirmed that, based on the 'eye tap' test, the stun was not deep enough. A short stun-to-stick interval of 15s or less was adhered to, preventing the pigs from regaining consciousness too early. Unlike the CO_2 stunning, most of these pigs were still alive after ejection from the stunner. The CO_2 treatment consistently had lower average aversion scores (out of 18, based on exhibited behaviour inside the stunner) than the Ar-CO_2 treatment. Where the maximum average aversion score during both phases' CO_2 treatments was 4.57/18, with an associated average welfare class of 3.71 (moderate welfare), the lowest average aversion score during Ar-CO_2 stunning was 6.43/18, with an associated average welfare class of 3 (also moderate). The highest average aversion score was 8/18, which equates to an average welfare class of 3 (moderate).

The individual scores, however, showcase a range of perceptions and opinions regarding pig behaviour inside the stunner. The panellists were requested to record the behaviours that they saw, when they saw it (based on the time on the video), and to add additional comments that they thought were necessary. Some panellists only recorded the behaviours that they considered to be problematic. As all panellists were representatives of different aspects of the South African red meat industry, they each had some knowledge of farm animal behaviour. However, it is possible that they could not accurately distinguish between two or more types of behaviour because they have not been exposed to it at any other point in the industry. Gas stunning is not a standard stunning method in South Africa, and so the country lacks experts, academic or in industry, in this field. If gas stunning is to be adopted as an alternative stunning method in South Africa, technicians, veterinarians, animal welfare officers and animal scientists will have to be trained in the system.

Commented [AO55]: I would suggest to put this in a separate chapter for limitations and recommendations

Individual scores (out of 18) for CO_2 stunning during the pilot ranged from 0/18 (no aversion) to 10/18 (severe aversion), indicating a wide range of perceptions regarding aversiveness between the panellists. It has been established earlier in this study that it theoretically is not possible to have absolutely no aversion during gas stunning systems, not with the given criteria. All animals displayed gasping and other movements prior to LOC, which, according to the developed criteria, should have added three points to the score in each case.

Individual scores (out of 18) for CO_2 stunning during the follow-up study ranged from 2/18 (slight aversion) to 5/18 (moderate aversion), indicating a closer range of values. The panel largely stayed the same between the two phases, and the behaviour seen in the footage recordings were like those witnessed in the pilot study. This narrow range indicates a positive learning curve by the panellists, as the behaviours were better identified. However, two

panellists during this phase consistently differed from the rest. Reviewer #6 gave the same scores throughout the follow-up study (welfare class 4, slight aversion). Reviewer #2 overall gave better welfare scores when compared to the other reviewers. This could indicate some bias against certain stunning methods.

Two methods of electrical stunning were used as controls in the study. During the pilot study, head-only emergency electrical stunning was used at abattoir A alongside the gas stunning treatments. This stunning was not recorded like the gas stunnings, but veterinarians and animal welfare officers were present to ensure that the pigs were sufficiently stunned. The method was ruled as unsatisfactory, not only as a suitable control against the gas stunning methods, but also on animal welfare grounds. As mentioned earlier, two out of seven pigs had improper tong placement during stunning. One animal was improperly restrained, which could have influenced tong placement and stun quality. The pigs were reluctant to willingly enter the temporary stunning crate. When using the emergency stunning method, pigs are herded by human handlers and not an automatic push system (compared to the way they are prior to CO_2 stunning). As mentioned earlier, there was a rather large crowd of onlookers at the abattoir during this phase. This could have intimidated the animals and added to their reluctance to enter the stunning crate. In preparation for the second phase of the study, this emergency stunning method was replaced by a standard head-to-heart stunning method at a different abattoir.

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During the follow-up study, no scoring system was used to evaluate the head-to-heart electrical stunning at the second abattoir. Due to the stunner design and spatial allowances, the camera angle was unable to record the stunning over the brain and the chest stunning. The pigs were stunned for 4s, during which very little behaviour besides a rigid posture can be observed. The pigs' behaviour while walking up to the stunning area was visible on the recording, and this was taken into consideration when a welfare classification score was assigned. One pig lost its footing before it could be restrained and had to be helped up before stunning could commence. Another was very reluctant to approach the stunner. The rest displayed curiosity while approaching and entering the stunning area. At this abattoir, pigs are mainly herded by human handlers until they approach the stunning area. Tong placement over the brain was consistent and met the requirements for best animal welfare practices. No animals showed signs of premature return of consciousness post-stunning. Only one stunning was classed as 'Poor', with an average class score of 2.92. This was the only stunning to have received individual class scores below 3 (out of 5). The highest average welfare scores were 4.67 (3 pigs), 4.58 (1 pig) and 4.50 (3 pigs). The lowest average welfare scores were 2.92 (1 pig), 3.42 (1 pig) and 3.58 (1 pig). The panellists deemed the standard of welfare witnessed during electrical stunning to be satisfactory. Reviewer #6 scored all the pigs the same, giving

a class score of 4 for each pig. On average, reviewer #2 awarded the highest scores, and reviewer #5 awarded the lowest scores. The scores given during electrical stunning were closer to each other compared to the gas stunning methods, with an average score of 4.12 ± 0.64. By comparison, the average welfare score given to CO_2 during the follow-up study was 3.43 ± 0.77. According to the panellists, welfare during electrical stunning was perceived to be marginally better than that of CO_2.

Electrical- and gas stunning systems were developed to address certain shortcomings in the methods previously used in the abattoir industry. Each method, by design, works differently compared to the other methods. This makes accurate and objective comparison between the methods challenging. Electrical stunning was developed to address the animal welfare- and meat quality concerns associated with captive bolt stunning (Klingbiel & Naude, 1972). Likewise, CO_2 stunning was developed to address some of the animal welfare- and meat quality concerns associated with electrical stunning (Velarde *et al.*, 2000). However, both methods still deliver suboptimal animal welfare and meat quality. Head-only electrical stunning is known for producing carcasses with broken backs, haemorrhages, and PSE (Velarde *et al.*, 2001; Channon *et al.*, 2002; Rosenvold & Andersen, 2003b; Van de Perre *et al.*, 2010). CO_2 is known to cause some pigs to react aversively during stunning before LOC sets in (Raj, 1999) while yielding better meat quality (Velarde *et al.*, 2001; Channon *et al.*, 2002). Based on the literature- and video reviews, comparing the systems is challenging because the physiological basis upon which each operates, differs. CO_2 has long boasted a welfare advantage over traditional electrical stunning systems in that the pigs are moved and stunned in groups. This reduces the stress experienced directly prior to slaughter as the animals can stay in a form of herd. Most CO_2 stunners are automated, meaning that fewer people are needed to herd and stun animals and the human error factor is reduced. Recently, automated electrical stunners have been developed that aim to reduce the human-animal interaction prior to slaughter as well as the human error factor (Gerritzen *et al.*, 2021).

From these aversion scores, it is clear that, without proper training and well-defined criteria, animal welfare evaluations are quickly reduced to the opinions and views of the reviewer. On the one hand, this will negatively affect the animal's wellbeing as poor welfare could be overlooked. On the other hand, pedantic concerns over welfare that is not considered perfect will undermine the line speed in an abattoir. Either ditch is undesirable. The aim should be to improve animal welfare while also bearing in mind the production criteria of the abattoir (Velarde *et al.*, 2000). The criteria used for the panel review, in the author's opinion, were not sufficient to accurately score the aversion experienced by the pigs during stunning. A redesign wherein the intensity, duration and counts of different behaviours are considered could improve the accuracy of aversion evaluations. The association of an aversion score with a

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welfare class sounded like a good idea in theory. Overall, the scores given based on the panellists' observations were associated with moderate to good welfare classes. These scores and their associated welfare classes give an estimate of the state of welfare inside the stunner, but a more accurate assessment must be developed for continuous monitoring of this system in South Africa. This is an endeavour that will require the cooperation of animal welfare officers, veterinarians, abattoir personnel and animal scientists (Hernandez *et al.*, 2023).

Another important factor affecting animal welfare during gas stunning systems, is pre-slaughter animal handling. The animal's mental state also seems to influence their reaction to the conditions inside the stunner (personal observation). The environment prior to stunning is just as important, if not more, as the stunning system used. It is also clear at this stage in time, that trade-offs might need to be made when considering the different benefits and shortcomings of different stunning systems. For example, high concentration CO_2 stunning has the ability to stun animals in groups rather than on their own, render a strict stun-to-stick interval irrelevant and produce pork carcasses void of haemorrhages and PSE meat (Velarde *et al.*, 2000). However, many consider this system to be a concern for animal welfare as the LOC is not instantaneous, and therefore the animals probably experience some level of discomfort during this time (Raj *et al.*, 1995). Electrical stunning, when used in an abattoir with good animal welfare practices and regularly maintained stunning equipment, can produce pork that is fit for human consumption and of a good quality. However, in electrical stunning systems that are not automated and rely on human handlers, a definite risk of poor stunning efficiency exists, which can result in the pig regaining its consciousness before it has bled out sufficiently. Neither system is perfect, but both can be refined with improved and consistent animal handling practices before slaughter.

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CHAPTER 6: FURTHER RESEARCH AND RECOMMENDATIONS

In South Africa, only one abattoir utilises carbon dioxide stunning as part of the slaughter process. This research endeavour set out to establish concentrations of aversiveness to carbon dioxide during stunning and investigated the potential use of an alternative gas mixture. It was established that 84% carbon dioxide stunning was objectively better than an 80% Argon 20% carbon dioxide admixture from animal welfare-, carcass quality- and worker safety standpoints. However, the scope for the study was limited to the stunning method, and so there is very little accounting to be done for other equally, if not potentially more, important events in the production chain. It is a well-known fact that the pre-slaughter environment and -handling, starting on the farm and extending up to the point of stunning, are crucial aspects of the pork production industry.

In this study, biological parameters like pig size, -sex and -breed were not taken into consideration, though these likely influenced the results obtained. While no definite PSE- or DFD carcasses were reported, there were more pigs with very high pH_1 values than very low pH_1 values, which indicate that more pigs had depleted glycogen reserves at the point of stunning, which is indicative of chronic stress. It is likely that, while the stunning process did contribute to the stress concentrations experienced by the pigs, the environment and associated handling during transport and lairage contributed more. Behavioural differences inside the Butina stunner were observed between different groups of pigs from different farms.

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Based on the results and observations made in this pilot study, it is recommended that more research be conducted on this subject under commercial conditions in the South African pork industry. Comparative studies between male and female pigs, breeds and genetic lines, different sizes and stocking densities on the truck, in lairage and inside the stunner must be conducted. Weather extremes between summer and winter months in South Africa differ tremendously and should be considered when designing abattoir facilities and drafting management schemes. Attention must be given to handling techniques on the farm and at the abattoir as this affects the animals' countenance and, by implication, future meat quality. Improving conditions and the time spent during transport and lairage is an important starting point. The susceptibility to stress was not investigated in this study, and although other authors have stated that genes like the Halothane gene have largely been eradicated from the global commercial pork industry, a better understanding of the genetic predisposition to stress can

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help South African pig farmers and abattoir personnel to better manage the animals under their care.

The correlation between blood- and muscle metabolites could produce a more accurate picture of the animal's mental and physical state at the time of slaughter compared to relying on either one alone. It is also recommended that more muscle metabolite studies be undertaken throughout the post-slaughter conversion of muscle to meat, in order to further understand how different pre-slaughter factors influence pH decline and, ultimately, meat quality. Electroencephalograms (EEGs) have proven to be a useful tool under experimental circumstances, but at present, the technology is inefficient and ineffective under commercial conditions. Using EEGs during commercial slaughter can more accurately confirm the depth of consciousness during and after stunning if the design and durability of the equipment can be improved upon. Studies focussing on meat quality aspects like tenderness, drip loss and colour are needed to establish the effects of pre-slaughter conditions and post-slaughter processing. Carcass inspections, including inspections of the lungs, liver, and heart, must be incorporated to further understand the effects of carbon dioxide stunning in pigs on their physiology.

In this study, the *m. longissimus thoracis* was used for the measurement of pH values, carcass temperature and collection of muscle samples for the study of muscle metabolites due to its high ratio of glycolytic to oxidative muscle fibres. Studies on metabolism in other muscles may be useful to understand the relationship between stunning, stress and meat quality.

Finally, both stunning systems require improvements. Electrical stunning systems are driven by human labourers, which increases the likelihood of human error playing a role in the effectiveness of stunning. Carbon dioxide stunning systems are criticised for the long latency to the onset of unconsciousness. There is a need for methods to reduce this latency while minimising aversive behaviour. These methods must be practical in a commercial abattoir setting, not be a cause for danger towards abattoir staff, and improve animal welfare.

CHAPTER 7: CONCLUSION

The improvement of animal welfare in animal-based production systems remains a challenge for various reasons. Interspecies genetic variation, varying animal handling regimes, challenging climates and human perception all play a role in the exhibited behaviour and the interpretation thereof. It is likely that the relationship between carcass characteristics and expressed behaviour is not strong or linear, i.e. aversive behaviour during stunning does not necessarily imply that the carcass will exhibit inferior characteristics. Neither the electrical stunning methods nor the studied CO_2 stunning method is ideal, and these methods require improvement and refining in order to minimise the unnecessary stress experienced by the pigs and produce pork products of superior quality. It follows that the alternative gas admixture to high concentration CO_2 was not effective nor an efficient means of stunning at commercial pig abattoirs. The stunning method employed must ideally induce unconsciousness immediately (as is the case with electrical stunning) or within a short time (as should be the goal with gas stunning methods). The stun must also be deep enough to prevent the pig from regaining its consciousness before it has been sufficiently bled out. In systems employing reversible stunning methods, a strict stun-to-stick interval must be adhered to. With regards to CO_2 stunning methods, the current study found a shorter time to LOC using a lower concentration of CO_2 compared to others. This implies that further improvement of the system is possible at a commercial level by considering the total animal (pig) weight in the box, and CO_2-atmosphere, which should be researched further. Having said that, there remains a need for better animal handling techniques, from the farm to the abattoir. Severe stress during loading at the farm, transport to the abattoir and the lairage period will likely lead to higher incidences of DFD carcasses, whereas severe stress between lairage and slaughter is likely to lead to higher incidences of PSE carcasses. This is also influenced by the pigs' conditioning and genetics. The personnel responsible for monitoring and managing animal welfare must be sufficiently trained to accurately judge the animal's physical- and mental state. Objective methods to measure stress, which are practically applicable in the commercial sector, must be developed and adopted throughout the pork industry. More research on this topic is required in South Africa as the influence of different genetic lines, sex, age, weight, ambient temperature and weather on the animals' reaction to the stunning system is unknown.

REFERENCES

Adzitey, F., & Nurul, H. 2011. Pale soft exudative (PSE) and dark firm dry (DFD) meats: Causes and measures to reduce these incidences - A mini review. Int Food Res J 18, 11–20.

Atkinson, S., Algers, B., Larsen, A., Llonch, P., & Velarde, A. 2015. Group stunning of pigs during commercial slaughter in a Butina pasternoster system using 80% nitrogen and 20% carbon dioxide compared to 90% carbon dioxide. https://www.researchgate.net/publication/280026394

Atkinson, S., Algers, B., Pallisera, J., Velarde, A., & Llonch, P. 2020. Animal welfare and meat quality assessment in gas stunning during commercial slaughter of pigs using hypercapnic-hypoxia (20% co2 2% o2) compared to acute hypercapnia (90% co2 in air). Animals 10, 1–16 https://doi.org/10.3390/ani10122440.

Atkinson, S., Velarde, A., Llonch, P., & Algers, B. 2012. Assessing pig welfare at stunning in Swedish commercial abattoirs using CO2 group-stun methods. Animal Welfare 21, 487–495 https://doi.org/10.7120/09627286.21.4.487.

Barton Gade, P. 2008. Effect of rearing system and mixing at loading on transport and lairage behaviour and meat quality: Comparison of free range and conventionally raised pigs. Animal 2, 1238–1246 https://doi.org/10.1017/S1751731108002565.

Barton Gade, P., & Christensen, L. 1998. Effect of different stocking densities during transport on welfare and meat quality in Danish slaughter pigs. Meat Sci 48, 237–247 https://doi.org/10.1016/S0309-1740(97)00098-3.

Bašić, I., Tadić, Z., Lacković, V., & Gomerčić, A. 1997. Stress syndrome: Ryanodine receptor (RYR1) gene in malignant hyperthermia in humans and pigs. Period Biol 99, 313–317.

Becerril-Herrera, M., Alonso-Spilsbury, M., Lemus-Flores, C., Guerrero-Legarreta, I., Olmos-Hernández, A., Ramírez-Necoechea, R., & Mota-Rojas, D. 2009. CO2 stunning may compromise swine welfare compared with electrical stunning. Meat Sci 81, 233–237 https://doi.org/10.1016/j.meatsci.2008.07.025.

Bhat, Z. F., Morton, J. D., Mason, S. L., & Bekhit, A. E. A. 2018. Food Science and Human Wellness Role of calpain system in meat tenderness : A review. Food Science and Human Wellness 7, 196–204 https://doi.org/10.1016/j.fshw.2018.08.002.

Boler, D. D., Gabriel, S. R., Yang, H., Balsbaugh, R., Mahan, D. C., Brewer, M. S., McKeith, F. K., & Killefer, J. 2009. Effect of different dietary concentrations of natural-source vitamin E in grow-finish pigs on pork quality and shelf life. Meat Sci 83, 723–730 https://doi.org/10.1016/j.meatsci.2009.08.012.

Bowker, B. C., Grant, A. L., Forrest, J. C., & Gerrard, D. E. 2000. Muscle metabolism and PSE pork. J Anim Sci 79, 1 https://doi.org/10.2527/jas.00.079es1001c.

Brandt, P., & Aaslyng, M. D. 2015. Welfare measurements of finishing pigs on the day of slaughter: A review. Meat Sci 103, 13–23 https://doi.org/10.1016/j.meatsci.2014.12.004.

Brandt, P., Rousing, T., Herskin, M. S., & Aaslyng, M. D. 2013. Identification of post-mortem indicators of welfare of finishing pigs on the day of slaughter. Livest Sci 157, 535–544 https://doi.org/10.1016/j.livsci.2013.08.020.

Brendall, J. R. 1973. Postmortem changes in muscle. Pages 227–274 in The structure and function of muscle. Bournes, G.H., ed. Academic Press, New York.

Brewer, S., Boler, D. D., & Moeller, S. J. 1998. Consumer Attitudes Towards Color and Marbling of Fresh Pork Defining Color. Facts: Pork Quality. National Pork Board., 1–8.

Briskey, E. J. 1964. Etiological status and associated studies of pale, soft, exudative porcine musculature. Adv Food Res 13, 89-178.

Channon, H. A., Payne, A. M., & Warner, R. D. 2000. Halothane genotype, pre-slaughter handling and stunning method all influence pork quality. Meat Sci 56, 291–299 https://doi.org/10.1016/S0309-1740(00)00056-5.

Channon, H. A., Payne, A. M., & Warner, R. D. 2002. Comparison of CO 2 stunning with manual electrical stunning (50 Hz) of pigs on carcass and meat quality. Meat Sci 60, 63–68.

Chauhan, S. S., & England, E. M. 2018. Postmortem glycolysis and glycogenolysis: insights from species comparisons. Meat Sci 144, 118–126 https://doi.org/10.1016/j.meatsci.2018.06.021.

Choe, J. H., Choi, Y. M., Lee, S. H., Shin, H. G., Ryu, Y. C., Hong, K. C., & Kim, B. C. 2008. The relation between glycogen, lactate content and muscle fiber type composition, and their influence on postmortem glycolytic rate and pork quality. Meat Sci 80, 355–362 https://doi.org/10.1016/j.meatsci.2007.12.019.

Choe, J. H., Choi, M. H., Ryu, Y. C., Go, G. W., Choi, Y. M., Lee, S. H., Lim, K. S., Lee, E. A., Kang, J. H., Hong, K. C., & Kim, B. C. 2015. Estimation of pork quality traits using exsanguination blood and postmortem muscle metabolites. Asian-Australas J Anim Sci 28, 862–869 https://doi.org/10.5713/ajas.14.0768.

Chulayo, A. Y., & Muchenje, V. 2015. A balanced perspective on animal welfare for improved meat and meat products. South African Journal of Animal Sciences 45, 452–469 https://doi.org/10.4314/sajas.v45i5.2.

Chulayo, A. Y., Tada, O., & Muchenje, V. 2012. Research on pre-slaughter stress and meat quality: A review of challenges faced under practical conditions. Applied Animal Husbandry & Rural Development 5, 1–6.

Čobanović, N., Bošković, M., Vasilev, D., Dimitrijević, M., Parunović, N., Djordjević, J., & Karabasil, N. 2016a. Effects of various pre-slaughter conditions on pig carcasses and meat quality in a low-input slaughter facility. South African Journal of Animal Sciences 46, 380–390 https://doi.org/10.4314/sajas.v46i4.6.

Čobanović, N., Karabasil, N., Stajković, S., Ilić, N., Suvajdžić, B., Petrović, M., & Teodorović, V. 2016b. The influence of pre-mortem conditions on pale, Soft and Exudative (PSE) and Dark firm and dry (DFD) pork meat. Acta Vet Brno 66, 172–186 https://doi.org/10.1515/acve-2016-0015.

Čobanović, N., Stajković, S., Grkovic, N., Suvajdzic, B., Vasilev, D., & Karabasil, N. 2019. Effects of RYR1 gene mutation on the health, welfare, carcass and meat quality in slaughter pigs.in IOP Conference Series: Earth and Environmental Science. Institute of Physics Publishing.

Dall Aaslyng, M., & Barton Gade, P. 2001. Low stress pre-slaughter handling: Effect of lairage time on the meat quality of pork. Meat Sci 57, 87–92 https://doi.org/10.1016/S0309-1740(00)00081-4.

Dalla Costa, O. A., Dalla Costa, F. A., Feddern, V., Lopes, L. dos S., Coldebella, A., Gregory, N. G., & de Lima, G. J. M. M. 2019. Risk factors associated with pig pre-slaughtering losses. Meat Sci 155, 61–68 https://doi.org/10.1016/j.meatsci.2019.04.020.

Dalmau, A., Llonch, P., Manteca, X., Dalmau, A., Llonch, P., Rodríguez, P., Ruíz-De-La-Torre, J. L., Manteca, X., & Velarde, A. 2010a. ClearFarm: Co-designed Welfare Monitoring Platform for Pig and Dairy Cattle View project Red Bienestar: Scientific Network on Animal Welfare in Spain View project Stunning pigs with different gas mixtures: gas stability. Animal Welfare 19, 315–323.

Dalmau, A., Llonch, P., Rodríguez, P., Ruíz-de-la-Torre, J. L., Manteca, X., & Velarde, A. 2010b. Stunning pigs with different gas mixtures: Gas stability. Animal Welfare 19, 315–323.

Dalmau, A., Llonch, P., Velarde, A., Dalmau, A., Rodríguez, P., Llonch, P., & Velarde, A. 2010c. Stunning pigs with different gas mixtures: Aversion in pigs. Animal Welfare 19, 325–333.

Dalmau, A., Nande, A., Vieira-Pinto, M., Zamprogna, S., Di Martino, G., Ribas, J. C. R., da Costa, M. P., Halinen-Elemo, K., & Velarde, A. 2016. Application of the Welfare Quality® protocol in pig slaughterhouses of five countries. Livest Sci 193, 78–87 https://doi.org/10.1016/j.livsci.2016.10.001.

Dalrymple, R. H., & Hamm, R. 1973. A method for the extraction of glycogen and metabolites from a single muscle sample. Int J Food Sci Technol 8, 439–444 https://doi.org/10.1111/j.1365-2621.1973.tb01730.x.

de Oliveira, E. A., Dall'olio, S., Tassone, F., Arduini, A., & Nanni Costa, L. 2018. The effect of stress immediately prior to stunning on proglycogen, macroglycogen, lactate and meat quality traits in different pig breeds. Ital J Anim Sci 17, 879–883 https://doi.org/10.1080/1828051X.2018.1449672.

De Smet, S. M., Pauwels, H., De Bie, S., Demeyer, D. I., Callewier, J., & Eeckhout, W. 1996. Effect of Halothane Genotype, Breed, Feed Withdrawal, and Lairage on Pork Quality of Belgian Slaughter Pigs. J Anim Sci 74, 1854–1863 https://doi.org/10.2527/1996.7481854x.

Dokmanovic, M., Ivanovic, J., Janjic, J., Boskovic, M., Laudanovic, M., Pantic, S., & Baltic, M. Z. 2017. Effect of lairage time, behaviour and gender on stress and meat quality parameters in pigs. Animal Science Journal 88, 500–506 https://doi.org/10.1111/asj.12649.

Dransfield, E. 1994. Optimisation of tenderisation, ageing and tenderness. Meat Sci 36, 105–121 https://doi.org/10.1016/0309-1740(94)90037-X.

Dreyer, J. H., Gouws, P. J., & Naudé, R. T. 1972. The influence of slaughter technique and histological treatment on muscle fibre diameter of low and high pH1 pork muscle. S Afr J Anim Sci 2, 109–112 https://doi.org/10.1017/CBO9781107415324.004.

D'Souza, D. N., Dunshea, F. R., Warner, R. D., & Leury, B. J. 1998. The Effect Of Handling Pre-Slaughter And Carcass Processing Rate Post-Slaughter On Pork Quality. Meat Sci 50, 429–437 https://doi.org/10.1016/S0309-1740(98)00055-2.

EFSA (European Food Safety Authority). 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 https://doi.org/10.2903/j.efsa.2013.3486.

England, E. M., Scheffler, T. L., Kasten, S. C., Matarneh, S. K., & Gerrard, D. E. 2013. Exploring the unknowns involved in the transformation of muscle to meat. Meat Sci 95, 837–843 https://doi.org/10.1016/j.meatsci.2013.04.031.

Ertbjerg, P., & Puolanne, E. 2017. Muscle structure, sarcomere length and influences on meat quality: A review. Meat Sci 132, 139–152 https://doi.org/10.1016/j.meatsci.2017.04.261.

Faucitano, L. 2018. Preslaughter handling practices and their effects on animal welfare and pork quality. J Anim Sci 96, 728–738 https://doi.org/10.1093/jas/skx064.

Ferguson, D. M., & Gerrard, D. E. 2014. Regulation of post-mortem glycolysis in ruminant muscle. Anim Prod Sci 54, 464–481 https://doi.org/10.1071/AN13088.

Fisher, P., & Mellett, F. D. 1997. Halothane genotype and pork production. 1 Growth, carcass and meat quality characteristics. S Afr J Anim Sci 27, 22–26.

Friel, M., Kunc, H. P., Griffin, K., Asher, L., & Collins, L. M. 2019. Positive and negative contexts predict duration of pig vocalisations. Sci Rep 9 https://doi.org/10.1038/s41598-019-38514-w.

Gajana, C. S., Nkukwana, T. T., Marume, U., & Muchenje, V. 2013. Effects of transportation time, distance, stocking density, temperature and lairage time on incidences of pale soft exudative (PSE) and the physico-chemical characteristics of pork. Meat Sci 95, 520–525 https://doi.org/10.1016/j.meatsci.2013.05.028.

Gerritzen, M. A., Marahrens, M., Kongsted, H., & Bracke, M. B. M. 2021. Review of pig welfare in slaughterhouses at stunning and bleeding.

Gibson, T. J., Mason, C. W., Spence, J. Y., Barker, H., & Gregory, N. G. 2015. Factors Affecting Penetrating Captive Bolt Gun Performance. Journal of Applied Animal Welfare Science 18, 222–238 https://doi.org/10.1080/10888705.2014.980579.

Goll, D. E., Thompson, V. F., Taylor, R. G., & Christiansen, J. A. 1992. Role of the calpain system in muscle growth. Biochimie 74, 225–237 https://doi.org/10.1016/0300-9084(92)90121-T.

Goll, D. E., Thompson, V. F., Taylor, R. G., & Ouali, A. 1998. The calpain system and skeletal muscle growth. Can J Anim Sci 78, 503–512 https://doi.org/10.4141/A98-081.

Gonzalez-Rivas, P. A., Chauhan, S. S., Ha, M., Fegan, N., Dunshea, F. R., & Warner, R. D. 2020. Effects of heat stress on animal physiology, metabolism, and meat quality: A review. Meat Sci 162, 108025 https://doi.org/10.1016/j.meatsci.2019.108025.

Grandin, T. 1982. Pig behavior studies applied to slaughter-plant design. Applied Animal Ethology 9, 141–151.

Grandin, T. 1997. Assessment of Stress during Handling and Transport. J Anim Sci 75, 249–257 https://doi.org/10.2527/1997.751249x.

Grandin, T. 2003. The welfare of pigs during transport and slaughter. Res Vet Sci 24, 83–90.

Grandin, T. 2013. Making slaughterhouses more humane for cattle, pigs, and sheep. Annu Rev Anim Biosci 1, 491–512 https://doi.org/10.1146/annurev-animal-031412-103713.

Guàrdia, M. D., Estany, J., Balasch, S., Oliver, M. A., Gispert, M., & Diestre, A. 2005. Risk assessment of DFD meat due to pre-slaughter conditions in pigs. Meat Sci 70, 709–716 https://doi.org/10.1016/j.meatsci.2005.03.007.

Gutmann, I. and Wahlefeld, A. W. 1974. Determination of the Concentration and Molecular Weight of L-Lactic Acid. Methods of Enzymatic Analysis 3, 1464-1468.

Hambrecht, E., Eissen, J. J., Newman, D. J., Smits, C. H. M., Verstegen, M. W. A., & Den Hartog, L. A. 2005. Preslaughter handling effects on pork quality and glycolytic potential in two muscles differing in fiber type composition.

Hambrecht, E., Eissen, J. J., Nooijen, R. I. J., Ducro, B. J., Smits, C. H. M., Den Hartog, L. A., & Verstegen, M. W. A. 2004. Preslaughter stress and muscle energy largely determine pork quality at two commercial processing plants. J Anim Sci 82, 1401–1409 https://doi.org/10.2527/2004.8251401x.

Heffron, J. J. A. 1973. The porcine, pale, soft, exudative muscle condition - What is the basic biochemical lesion? S Afr J Anim Sci 3, 177–181.

Henckel, P., Karlsson, A., Jensen, M. T., Oksbjerg, N., & Petersen, S. 2002. Metabolic conditions in Porcine longissimus muscle immediately pre-slaughter and its influence on peri-and post mortem energy metabolism. Meat Sci 62, 145–155.

Hernandez, R. O., Romero, M. H., & Sanchez, J. A. 2023. Assessment of slaughterhouse-based measures as animal welfare indicators in fattening pigs. Frontiers in Animal Science 4 https://doi.org/10.3389/fanim.2023.1064933.

Hopkins, D. L., Ponnampalam, E. N., Ven, R. J. Van De, & Warner, R. D. 2014. The effect of pH decline rate on the meat and eating quality of beef carcasses. Anim Prod Sci 54, 407–413.

Huff Lonergan, E., Zhang, W., & Lonergan, S. M. 2010. Biochemistry of postmortem muscle - Lessons on mechanisms of meat tenderization. Meat Sci 86, 184–195 https://doi.org/10.1016/j.meatsci.2010.05.004.

Huff-Lonergan, E., & Johnson, R. 2001. The Role of Carcass Chilling in the Development of Pork Quality.

Jama, N., Maphosa, V., Hoffman, L. C., & Muchenje, V. 2016. Effect of sex and time to slaughter (transportation and lairage duration) on the concentrations of cortisol, creatine kinase and subsequent relationship with pork quality. Meat Sci 116, 43–49 https://doi.org/10.1016/j.meatsci.2016.02.001.

Keppler, D. and Decker, K. 1974. Glycogen Determination with Amyloglucosidase. *In Methods of Enzymatic Analysis,* Vol 3, 1127-1131.

Kim, Y. H. B., Warner, R. D., & Katja Rosenvold. 2014. Influence of high pre-rigor temperature and fast pH fall on muscle proteins and meat quality : a review. Anim Prod Sci, 375–395.

Klingbiel, J. F. G., & Naudé, R. T. 1972. The effect of two stunning techniques on the pH1 values of muscles in carcasses of bacon pigs. S Afr J Anim Sci 2, 105–107.

Klingbiel, J. F. G., & Naude, R. T. 1972. THE EFFECT OF TWO STUNNING TECHNIQUES ON THE pH 1 VALUES OF MUSCLES IN CARCASSES OF BACON PIGS. S Afr J Anim Sci, 105–107.

Koohmaraie, M. 1992. The role of Ca2+-dependent proteases (calpains) in post mortem proteolysis and meat tenderness. Biochimie 74, 239–245 https://doi.org/10.1016/0300-9084(92)90122-U.

Lambooij, E. 2004. Electrical Stunning.Pages 1342–1348 in Encyclopedia of Meat Science. Jensen, D., Devine, D., Dikeman, M., eds. Elsevier, Oxford.

Lamprecht, W., Stein, P., Heinz, F., & Weisser, H. 1974. Creatine Phosphate.Pages 1777–1785 in Methods of Enzymatic Analysis, Vol. 3. Bergmeyer, H.U., ed. 2nd ed. Verlag Chemie, Weinheim.

Lechner, I., Léger, A., Zimmermann, A., Atkinson, S., & Schuppers, M. 2021. Discomfort period of fattening pigs and sows stunned with CO2: Duration and potential influencing factors in a commercial setting. Meat Sci 179 https://doi.org/10.1016/j.meatsci.2021.108535.

Lindahl, G., Henckel, P., Karlsson, A. H., & Andersen, H. J. 2006. Significance of early postmortem temperature and pH decline on colour characteristics of pork loin from different crossbreeds. Meat Sci 72, 613–623 https://doi.org/10.1016/j.meatsci.2005.09.014.

Llonch, P., Dalmau, A., Rodríguez, P., Manteca, X., & Velarde, A. 2012a. Aversion to nitrogen and carbon dioxide mixtures for stunning pigs. Animal Welfare 21, 33–39 https://doi.org/10.7120/096272812799129475.

Llonch, P., Rodríguez, P., Gispert, M., Dalmau, A., Manteca, X., & Velarde, A. 2012b. Stunning pigs with nitrogen and carbon dioxide mixtures: Effects on animal welfare and meat quality. Animal 6, 668–675 https://doi.org/10.1017/S1751731111001911.

Llonch, P., Rodríguez, P., Jospin, M., Dalmau, A., Manteca, X., & Velarde, A. 2013. Assessment of unconsciousness in pigs during exposure to nitrogen and carbon dioxide mixtures. Animal 7, 492–498 https://doi.org/10.1017/S1751731112001966.

Lonergan, S. M., Topel, D. G., & Marple, D. N. 2018. The conversion of muscle to meat.Pages 163–174 in The science of animal growth and meat technology.

LWCC. 2018. A guideline for the use of prodders and stunning devices in abattoirs.

Manalo, M. R., & Gabriel, A. A. 2020. Influence of antemortem and slaughtering practices on the pH of pork and chicken meats. Philipp J Sci 149, 1–19 https://doi.org/10.56899/149.01.01.

Manteuffel, G., Puppe, B., & Schön, P. C. 2004. Vocalization of farm animals as a measure of welfare. Appl Anim Behav Sci 88, 163–182 https://doi.org/10.1016/j.applanim.2004.02.012.

Marcon, A. V., Caldara, F. R., de Oliveira, G. F., Gonçalves, L. M. P., Garcia, R. G., Paz, I. C. L. A., Crone, C., & Marcon, A. 2019. Pork quality after electrical or carbon dioxide stunning at slaughter. Meat Sci 156, 93–97 https://doi.org/10.1016/j.meatsci.2019.04.022.

Matarneh, S. K., England, E. M., Scheffler, T. L., & Gerrard, D. E. 2017. The Conversion of Muscle to Meat.Pages 128–156 in Lawrie's Meat Science. Toldra, F., Lawrie, R.A., eds. 8th ed. Woodhead Publishing.

McKinstry, J. L., & Anil, M. H. 2004. The effect of repeat application of electrical stunning on the welfare of pigs. Meat Sci 67, 121–128 https://doi.org/10.1016/j.meatsci.2003.10.002.

McLoughlin, J. V. 1970. Muscle contraction and postmortem pH changes in pig skeletal muscle. J Food Sci 35, 717–719 https://doi.org/10.1111/j.1365-2621.1970.tb01978.x.

Meat Safety Act. 2000. South Africa.

More, S., Bicout, D., Bøtner, A., Butterworth, A., Calistri, P., Depner, K., Edwards, S., Garin-Bastuji, B., Good, M., Gortázar Schmidt, C., Miranda, M. A., Saxmose Nielsen, S., Velarde, A., Thulke, H. H., Sihvonen, L., Spoolder, H., Stegeman, J. A., Raj, M., Willeberg, P., Winckler, C., Marano, R., Verdonck, F., Candiani, D., & Michel, V. 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 https://doi.org/10.2903/j.efsa.2018.5343.

Moss, B. W. 1980. The effects of mixing, transport and duration of lairage on carcass characteristics in commercial bacon weight pigs. J Sci Food Agric 31, 308–315 https://doi.org/10.1002/jsfa.2740310316.

Nielsen, S. S., Alvarez, J., Bicout, D. J., Calistri, P., Depner, K., Drewe, J. A., Garin-bastuji, B., Luis, J., Rojas, G., Michel, V., Angel, M., Chueca, M., Roberts, H. C., Sihvonen, L. H., Spoolder, H., Stahl, K., Viltrop, A., Winckler, C., Candiani, D., Fabris, C., Stede, Y. Van Der, & Velarde, A. 2020. Welfare of pigs at slaughter. EFSA Journal 18, 1–113 https://doi.org/10.2903/j.efsa.2020.6148.

Nowak, B., Mueffling, T. V., & Hartung, J. 2007. Effect of different carbon dioxide concentrations and exposure times in stunning of slaughter pigs: Impact on animal welfare and meat quality. Meat Sci 75, 290–298 https://doi.org/10.1016/j.meatsci.2006.07.014.

Offer, G. 1991. Modelling of the formation of pale, soft and exudative meat: Effects of chilling regime and rate and extent of glycolysis. Meat Sci 30, 157–184 https://doi.org/10.1016/0309-1740(91)90005-B.

Ouali, A., Herrera-mendez, C. H., Coulis, G., Becila, S., Boudjellal, A., Aubry, L., & Angel, M. 2006. Revisiting the conversion of muscle into meat and the underlying mechanisms. Meat Sci 74, 44–58 https://doi.org/10.1016/j.meatsci.2006.05.010.

Pearce, G. P., & Paterson, A. M. 1992. Physical contact with the boar is required for maximum stimulation of puberty in the gilt because it allows transfer of boar pheromones and not because it induces cortisol release. Anim Reprod Sci 27, 209–224 https://doi.org/10.1016/0378-4320(92)90059-M.

Pérez, M. P., Palacio, J., Santolaria, M. P., Aceña, M. C., Chacón, G., Gascón, M., Calvo, J. H., Zaragoza, P., Beltran, J. A., & García-Belenguer, S. 2002. Effect of transport time on welfare and meat quality in pigs. Meat Sci 61, 425–433 https://doi.org/10.1016/S0309-1740(01)00216-9.

Petty, D. 2015. Welfare concerns associated with CO2 stunning for pigs in the South African context.

Raj, A. B. M. 1999. Behaviour of pigs exposed to mixtures of gases and the time required to stun and kill them: Welfare implications. Veterinary Record 144, 165–168 https://doi.org/10.1136/vr.144.7.165.

Raj, A. B. M., & Gregory, N. G. 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., & Gregory, N. G. 1996. Welfare implications of the gas stunning of pigs 2. Stress of induction of anaesthesia. Animal Welfare 5, 71–78.

Raj, A. B. M., Johnson, S. P., Wotton, S. B., & McInstry, J. L. 1997a. Welfare Implications of Gas Sturming Pigs: 3. the Time to Loss of Somatosensory Evoked Potentials and Spontaneous Electrocorticogram of Pigs During Exposure to Gases. The Veterinary Journal 153, 329–340.

Raj, A. B. M., Johnson, S. P., Wotton, S. B., & McInstry, J. L. 1997b. 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 https://doi.org/10.1016/s1090-0233(97)80067-6.

Rey-Salgueiro, L., Martinez-Carballo, E., Fajardo, P., Chapela, M. J., Espiñeira, M., & Simal-Gandara, J. 2018. Meat quality in relation to swine well-being after transport and during lairage at the slaughterhouse. Meat Sci 142, 38–43 https://doi.org/10.1016/j.meatsci.2018.04.005.

Rodríguez, P., Dalmau, A., Ruiz-de-la-Torre, J. L., Manteca, X., Jensen, E. W., Rodríguez, B., Litvan, H., & Velarde, A. 2008. Assessment of unconsciousness during carbon dioxide stunning in pigs. Animal Welfare 17, 341–349.

Rosenvold, K., & Andersen, H. J. 2003a. Factors of significance for pork quality - A review. Meat Sci 64, 219–237 https://doi.org/10.1016/S0309-1740(02)00186-9.

Rosenvold, K., & Andersen, H. J. 2003b. Factors of significance for pork quality - A review. Meat Sci 64, 219–237 https://doi.org/10.1016/S0309-1740(02)00186-9.

Savell, J. W., Mueller, S. L., Baird, B. E. 2005. The chilling of carcasses. Meat Sci 70, 449-459 https://doi.org/10.1016/j.meatsci.2004.06.027

Scheffler, T. L., & Gerrard, D. E. 2007. Mechanisms controlling pork quality development: The biochemistry controlling postmortem energy metabolism. Meat Sci 77, 7–16 https://doi.org/10.1016/j.meatsci.2007.04.024.

Scheffler, T. L., Park, S., & Gerrard, D. E. 2011. Lessons to learn about postmortem metabolism using the AMPKγ3R200Q mutation in the pig. Meat Sci 89, 244–250 https://doi.org/10.1016/j.meatsci.2011.04.030.

Simela, L., Webb, E. C., & Frylinck, L. 2004. Post-mortem metabolic status, pH and temperature of chevon from indigenous South African goats slaughtered under commercial conditions. South African Journal of Animal Sciences 34, 204–207.

Sionek, B., & Przybylski, W. 2016. The Impact of Ante- and Post-Mortem Factors on the Incidence of Pork Defective Meat - A Review. Annals of Animal Science 16, 333–345 https://doi.org/10.1515/aoas-2015-0086.

Soma, P., van Marle-Köster, E., & Frylinck, L. 2014a. Frequency of the malignant hyperthermia gene in the south african pig industry. South African Journal of Animal Sciences 44, 384–387 https://doi.org/10.4314/sajas.v44i4.8.

Soma, P., van Marle-Köster, E., & Frylinck, L. 2014b. Frequency of the malignant hyperthermia gene in the south african pig industry. South African Journal of Animal Sciences 44, 384–387 https://doi.org/10.4314/sajas.v44i4.8.

Sommavilla, R., Faucitano, L., Gonyou, H., Seddon, Y., Bergeron, R., Widowski, T., Crowe, T., Connor, L., Scheeren, M. B., Goumon, S., & Brown, J. 2017. Season, transport duration and trailer compartment effects on blood stress indicators in pigs: Relationship to environmental, behavioral and other physiological factors, and pork quality traits. Animals 7, 1–12 https://doi.org/10.3390/ani7020008.

Spires, M. D., Bodmer, J. S., Beline, M., Wicks, J. C., Zumbaugh, M. D., Shi, T. H., Reichert, B. T., Schinckel, A. P., Grant, A. L., & Gerrard, D. E. 2023. Postmortem Metabolism and Pork Quality Development Are Affected by Electrical Stimulation across Three Genetic Lines. Animals 13 https://doi.org/10.3390/ani13162599.

Stajkovic, S., Vasilev, D., Teodorovic, V., & Karabasil, N. 2019. Postmortem glycolysis and pork quality. IOP Conf Ser Earth Environ Sci 333 https://doi.org/10.1088/1755-1315/333/1/012032.

Steen, D., Claeys, E., Uytterhaegen, L., De Smet, S., & Demeyer, D. 1997. Early post-mortem conditions and the calpain/calpastatin system in relation to tenderness of double-muscled beef. Meat Sci 45, 307–319 https://doi.org/10.1016/S0309-1740(96)00124-6.

Steiner, A. R., Flammer, S. A., Beausoleil, N. J., Berg, C., Bettschart-Wolfensberger, R., Pinillos, R. G., Golledge, H. D. W., Marahrens, M., Meyer, R., Schnitzer, T., Toscano, M. J., Turner, P. V., Weary, D. M., & Gent, T. C. 2019. Humanely ending the life of animals: Research priorities to identify alternatives to carbon dioxide. Animals 9 https://doi.org/10.3390/ani9110911.

Støier, S., Aaslyng, M. D., Olsen, E. V., & Henckel, P. 2001. The effect of stress during lairage and stunning on muscle metabolism and drip loss in Danish pork. Meat Sci 59, 127–131 https://doi.org/10.1016/S0309-1740(01)00040-7.

Stufft, K., Elgin, J., Patterson, B., Matarneh, S. K., Preisser, R., Shi, H., England, E. M., Scheffler, T. L., Mills, E. W., & Gerrard, D. E. 2017. Muscle characteristics only partially explain color variations in fresh hams. Meat Sci 128, 88–96 https://doi.org/10.1016/j.meatsci.2016.12.012.

Tallet, C., Linhart, P., Policht, R., Hammerschmidt, K., Šimeček, P., Kratinova, P., & Špinka, M. 2013. Encoding of Situations in the Vocal Repertoire of Piglets (Sus scrofa): A Comparison of Discrete and Graded Classifications. PLoS One 8 https://doi.org/10.1371/journal.pone.0071841.

Terlouw, C. 2005. Stress reactions at slaughter and meat quality in pigs: Genetic background and prior experience: A brief review of recent findings.Pages 125–135 in Livestock Production Science.

Terlouw, E. M. C., Arnould, C., Auperin, B., Berri, C., Le Bihan-Duval, E., Deiss, V., Lefèvre, F., Lensink, B. J., & Mounier, L. 2008. Pre-slaughter conditions, animal stress and welfare: Current status and possible future research. Animal 2, 1501–1517 https://doi.org/10.1017/S1751731108002723.

Topel, D. G., Miller, J. A., Berger, P. J., Rust, R. E., Parrish, F. C., & Ono, K. 1976. Palatibility and visual acceptance of dark, normal and pale coloured porcine M. longissimus. J Food Sci 41, 628–630.

Troy, D. J., & Kerry, J. P. 2010. Consumer perception and the role of science in the meat industry. Meat Sci 86, 214–226 https://doi.org/10.1016/j.meatsci.2010.05.009.

Van de Perre, V., Permentier, L., De Bie, S., Verbeke, G., & Geers, R. 2010. Effect of unloading, lairage, pig handling, stunning and season on pH of pork. Meat Sci 86, 931–937 https://doi.org/10.1016/j.meatsci.2010.07.019.

van der Wal, P. G., Bolink, A. H., & Merkus, G. S. M. 1989. Differences in Quality Characteristics of Normal, PSE and DFD Pork. Meat Sci 24, 79–84.

Velarde, A., Gispert, M., Faucitano, L., Alonso, P., Manteca, X., & Diestre, A. 2001. Effects of the stunning procedure and the halothane genotype on meat quality and incidence of haemorrhages in pigs. Meat Sci 58, 313–319.

Velarde, A., Gispert, M., Faucitano, L., Manteca, X., & Diestre, A. 2000. The effect of stunning method on the incidence of PSE meat and haemorrhages in pork carcasses. Meat Sci 55, 309–314.

Verhoeven, M. T. W., Gerritzen, M. A., Hellebrekers, L. J., & Kemp, B. 2014. Indicators used in livestock to assess unconsciousness after stunning: A review. Animal 9, 320–330 https://doi.org/10.1017/S1751731114002596.

Verhoeven, M., Gerritzen, M., Velarde, A., Hellebrekers, L., & Kemp, B. 2016. Time to loss of consciousness and its relation to behavior in slaughter pigs during stunning with 80 or 95% carbon dioxide. Front Vet Sci 3, 1–10 https://doi.org/10.3389/fvets.2016.00038.

Vermeulen, L., Van de Perre, V., Permentier, L., De Bie, S., Verbeke, G., & Geers, R. 2015. Pre-slaughter handling and pork quality. Meat Sci 100, 118–123 https://doi.org/10.1016/j.meatsci.2014.09.148.

Viljoen, H. F., De Kock, H. L., & Webb, E. C. 2002. Consumer acceptability of dark, firm and dry (DFD) and normal pH beef steaks. Meat Sci 61, 181–185.

Warner, R. D., Greenwood, P. L., Pethick, D. W., & Ferguson, D. M. 2010. Genetic and environmental effects on meat quality. Meat Sci 86, 171–183 https://doi.org/10.1016/j.meatsci.2010.04.042.

Warner, R. D., Kauffman, R. G., & Russel, R. L. 1993. Quality attributes of major porcine muscles: A comparison with the Longissimus Lumborum. Meat Sci 33, 359–372 https://doi.org/10.1016/0309-1740(93)90007-5.

Warriss, P. D., Brown, S. N., Adams, S. J. M., & Corlett, I. K. 1994. Relationships between subjective and objective assessments of stress at slaughter and meat quality in pigs. Meat Sci 38, 329–340 https://doi.org/10.1016/0309-1740(94)90121-X.

Webb, A. J., Carden, A. E., Smith, C., & Imlah, P. 1982. Porcine stress syndrome in pig breeding.Pages 588–608 in 2nd World Congress on Genetics Applied to Livestock Production V.

Webb, E. C., & Webb, E. M. 2022. Ethics of meat production and its relation to perceived meat quality. New Aspects of Meat Quality: From Genes to Ethics, 2nd Ed., 809-828.

Von Wenzlawowicz, M., Von Holleben, K., & Eser, E. 2012. Identifying reasons for stun failures in slaughterhouses for cattle and pigs: A field study. Animal Welfare 21, 51–60 https://doi.org/10.7120/096272812X13353700593527.

Wotton, S. B., Anil, M. H., Whittington, P. E., & Mckinstry, J. L. 1992. Pig slaughtering procedures: Head-to-back stunning. Meat Sci 32, 245–255.

Wotton, S. B., & O'Callaghan, M. 2002. Electrical stunning of pigs: the effect of applied voltage on impedance to current flow and the operation of a fail-safe device. Meat Sci 60, 203–208.