INVESTIGATION OF WELFARE IMPACTS OF GASEOUS METHODS FOR ON-FARM EUTHANASIA OF SUCKLING PIGLETS

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Abstract

Blunt trauma (BT) to the head is the most common method used for on-farm euthanasia of pre-weaned piglets. When performed correctly, loss of consciousness is immediate, but the potential for delivery of sub-lethal blows, along with aesthetic unacceptability to many operators, has lead to the need for alternative methods to be developed.

One recommended alternative is exposure to 100% CO_2. Although gas euthanasia is potentially more reliable and less disturbing to perform than BT, there are concerns that CO_2 may induce breathlessness and pain before loss of consciousness, thus negatively affecting piglet welfare. This research aimed to evaluate the welfare impact of alternative gases, relative to CO_2, for piglet euthanasia.

A small pilot study was conducted to select appropriate gases for further evaluation. This identified 100% argon (Ar) and a mixture of 40% CO_2-60% argon (CO_2-Ar) as possible alternatives to 100% CO_2 (CO_2) for piglet euthanasia.

The relative welfare impacts of CO_2, Ar and CO_2-Ar were evaluated in two studies. These studies aimed to identify the interval following gas exposure in which the animal may be conscious, and to identify evidence of welfare compromise within this interval. Identifying the period of possible consciousness is important in evaluating welfare impact, as this defines the time period in which the animal is capable of perceiving potential negative experiences associated with euthanasia.

In the first study, conscious animals were exposed to the test-gases in a purpose-built chamber. Behavioural and physiological data including escape attempts, vocalisation, loss of coordination, loss of posture, respiratory effort, convulsions, gasping, and respiratory arrest were recorded until death. Loss of posture has previously been used to infer the onset of unconsciousness, whilst escape attempts, vocalisation and laboured breathing are associated with the experience of pain, aversion and distress in animals. Piglet behaviour was examined for evidence of negative experience prior to the onset of unconsciousness.

In the second study, EEG and ECG data were recorded from anaesthetised, immobilised pigs during exposure to the same test gases used in the first study. Changes in the amplitude of the raw EEG can provide information on the level of consciousness. Changes in the EEG power spectrum, derived from mathematical transformation of the raw EEG, can provide evidence of noxious stimulation in anaesthetised mammals. EEG recorded during exposure to test gases was analysed to determine the likely latency to loss of consciousness with each gas, and to determine

whether nociceptive processing occurred. Changes in heart rate, derived from the ECG, are frequently used as indicators of acute stress in mammals. ECG recorded during gas exposure was examined for indications of physiological stress responses.

Behavioural data suggested that the latency to onset of unconsciousness did not differ between gases. However, the changes in the amplitude of the EEG suggested that loss of consciousness may occur sooner with CO_2 than with Ar or CO_2-Ar. Behavioural data indicated that piglets found CO_2 exposure more aversive or unpleasant than exposure to either Ar or CO_2-Ar. However, CO_2-Ar induced greater respiratory stimulation than Ar alone, suggesting that Ar caused the least negative welfare impact of the 3 gases. ECG data showed that heart rate increased prior to likely loss of consciousness in piglets exposed to CO_2 and CO_2-Ar but not Ar alone, suggesting that Ar exposure does not induce a physiological stress response.

Contrary to expectations, there was no evidence of nociception in piglets exposed to either 40 or 100% CO_2, although this may have been influenced by the method used.

Together, these data suggest that whilst CO_2 induces more rapid loss of consciousness than Ar, it also results in significantly greater welfare impact prior to loss of consciousness. The addition of CO_2 to Ar may provide some welfare advantage over CO_2 alone, but not over Ar alone. From a welfare perspective, Ar is preferable to either CO_2 or CO_2-Ar for piglet euthanasia.

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Commonly used abbreviations

BT	Blunt trauma
CO_2	Carbon dioxide
ECG	Electrocardiogram / electrocardiographic
EEG	Electroencephalogram / electroencephalographic
N_2	Nitrogen
pCO_2	Partial pressure of carbon dioxide
pO_2	Partial pressure of oxygen
SEM	Standard error of the mean

Explanation of terms as applied in this document

Dyspnoea	The subjective experience of breathing discomfort, incorporating sensations such as the uncomfortable awareness of breathing, the sensation of breathlessness or the experience of air hunger
Hypercapnia	State in which arterial pCO_2 rises above the normocapnic range
Hyperventilation	Increase in ventilatory rate above normal resting values
Hypoxic	State in which arterial pO_2 falls below the normoxic range
Nociception	Neural process of encoding and processing noxious stimuli
Nociceptor	Sensory receptor that is activated by noxious stimuli, which then sends neural signals to the spinal cord and brain
Normocapnic	Within the normal arterial pCO_2 range of 36–44 mmHg
Normoxic	Within the normal arterial pO_2 range of 80–100 mmHg
Noxious stimulus	Stimulus that is damaging, or threatens damage, to normal tissues
Pain	Unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage
Respiration	Synonymous with ventilation; respiration rate thus refers to the number of breaths per minute
Ventilation	The process of filling and emptying the lungs with air, or breathing

1 General introduction

In the pig industry it is common for piglets born with poor survival odds, or those suffering severe injury or disease in the first weeks of life, to be euthanased via blunt trauma (BT) to the head. If performed accurately and with sufficient force this is a rapid, effective means of inducing concussion and subsequent death in piglets less than 3 weeks of age, with little or no negative impact on welfare. However, the potential for the delivery of sub-lethal blows, resulting in injury without loss of consciousness, along with the aesthetic unacceptability of this method to many operators, has lead the industry to investigate alternative euthanasia methods.

Although used less often, inhalation of high concentrations of carbon dioxide (CO_2) is also recommended for euthanasia of young piglets (AVMA 2007; National Pork Board 2008). Whilst overcoming some of the concerns associated with BT, there is evidence that exposure to high concentrations of CO_2 induces discomfort, pain and breathlessness in man (Dripps and Comroe 1947; Anton *et al*. 1992; Danneman *et al*. 1997; Liotti *et al*. 2001), and is distressing or aversive to rodents (Kirkden *et al*. 2005; Niel *et al*. 2008), poultry (Gerritzen *et al*. 2000) and adult pigs (Raj and Gregory 1995; Rodriguez *et al*. 2008).

It has been postulated that oxygen deprivation (hypoxia) may be a more humane form of gas euthanasia than CO_2 inhalation (Freed 1983). This can be achieved by replacing oxygen in air with inert gases such as argon or nitrogen. Rats and mice find argon aversive, but less so than CO_2 or mixtures of argon and CO_2 (Niel and Weary 2007; Makowska *et al*. 2008; Makowska *et al*. 2009). In contrast, few signs of aversion have been reported in either poultry or adult pigs exposed to 90% argon (Raj and Gregory 1995; Raj 1996; Gerritzen *et al*. 2000). To date, there are no data regarding the responses of pre-weaned piglets to exposure to hypoxic gases or gas mixtures.

Due to the global scale of commercial pig production, large numbers of piglets are likely to be identified as requiring emergency euthanasia. In 2010, 28 million piglet live births were recorded in the United States (National Agricultural Statistics Service 2010) and in excess of 40 million in the European Union (Marquer 2010). With an average pre-weaning mortality rate of 11–15% (Lay *et al*. 2002; O'Reilly *et al*. 2006; Shankar *et al*. 2009), this represents 7.5 –10 million pre-weaned piglet deaths per year in these regions alone. When it is considered that a percentage of these animals are identified as requiring euthanasia, improving piglet welfare during euthanasia has the potential to benefit a large number of animals worldwide on an ongoing annual basis.

This research investigated whether argon or nitrogen, alone or in combination with CO_2, provide any welfare advantages over 100% CO_2 for euthanasia of pre-weaned piglets.

2 Background and Literature Review

2.1 Piglet euthanasia: guidelines, industry practice and welfare concerns

2.1.1 Requirement for on-farm euthanasia

There are times on pig farms when the euthanasia of neonatal piglets may be required on animal welfare grounds. This may be due to the animal having suffered injuries during or after birth, or as a result of disease or infection contracted in the early postnatal period. When it is not possible or economically viable to rehabilitate these animals, farm workers must decide whether euthanasia is necessary and select the most appropriate means of doing so.

2.1.2 AVMA guidelines on euthanasia

The American Veterinary Medical Association (AVMA) Guidelines on Euthanasia are recognised internationally, both by government regulators and the animal care and use community, as the gold standard for acceptable procedures and agents for euthanasing a wide range of animal species (Nolen 2011).

The term euthanasia itself is derived from the Greek *eu* meaning good and *thanatos* meaning death. According to the AVMA, a 'good death' is one that occurs with minimal pain and distress. In the context of the Guidelines on Euthanasia, the term euthanasia is used to describe the act of inducing humane death in an animal (AVMA 2007). The main criteria for an acceptable, or humane, method of euthanasia are that it induces rapid loss of consciousness, followed by cardiac or respiratory arrest and ultimate loss of brain function; and

that it should cause minimal pain, distress or anxiety to the animal in the period prior to loss of consciousness (AVMA 2001). Based upon these criteria along with a number of others such as reliability, operator safety and the species, age and health status of the animal, available methods of euthanasia have been designated as acceptable, conditionally acceptable, or unacceptable (AVMA 2007).

According to the most recent Guidelines on Euthanasia, physical methods of euthanasia such as BT or captive bolt pistol are deemed conditionally acceptable, because they pose an inherent risk to both humans and animals (AVMA 2007). The use of CO_2 is deemed acceptable for euthanasing pigs, provided it is supplied from compressed cylinders using either pre-fill exposure to a concentration of 70% or more, or gradual induction at a rate of 20% of the chamber volume per minute, although it is acknowledged that high concentrations may be distressing to some animals (AVMA 2007). Due to evidence of distress prior to loss of consciousness in some species, the use of argon or nitrogen is deemed conditionally acceptable only, provided that a low oxygen concentration (< 2%) is achieved rapidly and the animals have previously been heavily sedated or anaesthetised (AVMA 2007).

2.1.3 Recommendations for euthanasia of suckling piglets

Suckling piglets are those that have not yet been weaned from the sow. In the New Zealand (NZ) swine industry weaning typically occurs around 3 weeks of age, therefore piglets less than 3 weeks of age are deemed suckling or pre-weaned.

In NZ, the Animal Welfare (Pigs) Code of Welfare, issued under the 1999 Animal Welfare Act, recommends the use of blunt trauma (BT) to the frontal region of the head, followed by severance of the arteries in the neck or under the foreleg, for the emergency humane destruction of suckling piglets (NAWAC,

2005). BT typically involves forcefully striking the frontal region of the pig's head with a blunt object such as a hammer, but may also be performed by lifting the pig by its hind legs and forcefully striking the head against a solid surface. The blow should be strong enough to cause traumatic injury, leading to rapid loss of consciousness and subsequent death without recovery of consciousness. BT is not recommended for pigs beyond 3 weeks of age, as the increased thickness of the cranium reduces the likelihood of an effective blow. The use of a secondary step (i.e. severance of the major arteries) ensures consciousness is not recovered before death if a sub-lethal blow has been delivered.

In the United States (US) on-farm euthanasia recommendations for producers (National Pork Board 2008) detail four acceptable methods for on-farm euthanasia of piglets less than 3 weeks of age. These are blunt trauma (without a secondary step), veterinarian-administered intravenous anaesthetic overdose, non-penetrating captive bolt to the head, and carbon dioxide (CO_2) inhalation.

2.1.4 Current industry practice

Because of the sensitive nature of this topic, literature regarding preferred on-farm methods of euthanasia is sparse. One survey of 47 swine farms in North Carolina revealed that BT was the most commonly used method for euthanasia of pre-weaned piglets, with 93.6% of employees reporting using BT compared with 2.1% using CO_2 and 1.0% using lethal injection (Matthis 2004). In New Zealand, BT is thought to be the sole method employed for euthanasia of pre-weaned piglets (Eric Neumann, personal communication[1]).

Although the use of a non-penetrating captive bolt (NCB) is deemed acceptable for pre-weaned piglets, its use appears to be restricted to nursery age pigs (approx 3 to 8 weeks of age) in the US (Matthis 2004). Whilst the reasons for

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this are not clear, it may be that BT is considered more practical and less time consuming than NCB, given that it does not require the use of specialised equipment. Alternatively, it may be that the smaller head size of pre-weaned pigs compared with nursery-age pigs makes NCB application more difficult, increasing the risk of injury to the operator. In New Zealand, the use of a gunpowder-driven captive bolt pistol requires that the operator hold a firearms license, which may be a deterrent to some operators. Regardless of the reasons for its infrequent use, NCB shares many of the limitations associated with BT: it requires the operator to restrain the animal, it is dependent on accurate placement for optimal success, and it induces traumatic injury, making it aesthetically unacceptable to some. Incorrect placement may render the animal only temporarily unconscious, thus NCB euthanasia should also be followed by a secondary step to ensure death without recovery of consciousness, although this is not currently recommended in the US.

The use of an anaesthetic injection is limited by restrictions on the acquisition and delivery of these substances. Anaesthetic agents may only be purchased, held and administered by veterinarians (National Pork Board 2008), which may result in unacceptable delays in the administration of euthanasia, as well as being cost prohibitive to many producers. In addition, consideration must be given to carcass disposal after anaesthetic use, to prevent incidental exposure of scavengers to drug residues in the carcass. For these reasons, the use of anaesthetics for euthanasia of piglets is unlikely to become commonplace in the swine industry.

2.1.5 Concerns regarding the use of BT

Despite the widespread implementation of BT euthanasia, concerns exist regarding its use both from an animal welfare perspective and in terms of acceptability to operators and the public. If delivered accurately and with sufficient force BT should induce rapid loss of consciousness and death, and

when directly followed by a secondary step such as bleeding out, death without recovery of consciousness is assured, which is desirable from a welfare perspective. However, incorrect placement or a lack of resolve on the part of the operator may result in the delivery of an insufficient blow, resulting in pain or distress to the animal as well as necessitating additional blows. Furthermore, when not followed by a secondary step there is the potential for recovery of consciousness prior to death if a sub-lethal blow has been delivered. Both of these scenarios represent potentially significant impacts on piglet welfare. In addition, the need to physically restrain the animal whist performing BT may be distressing to the animal, and may impede delivery of an accurate or sufficient blow should the animal resist restraint.

Aside from considering the welfare of the animal, the AVMA Guidelines on Euthanasia (AVMA 2007) recognise that the psychological well being of those personnel performing or witnessing the euthanasia procedure should also be taken into consideration. The routine performance of animal euthanasia can have a negative impact on the emotional well being, job satisfaction and job performance of animal care providers such as shelter employees (Arluke 1992; White and Shawhan 1996; Rogelberg *et al*. 2007) and it is possible that regularly performing euthanasia has a similar impact on pig farm workers. In support of this, Mathis (2004) reported that 63.5% of pig farm workers considered euthanasia to be a stressful part of the job.

The level of emotional impact may vary according to the euthanasia procedure employed, with more physical or traumatic methods being more stressful (Rollin 2009). Forty-four percent of pig-farm workers routinely using BT find the physicality of the act the most difficult, with 45% reporting they do not like having to hold the pig whilst the procedure is carried out (Matthis 2004). The implementation of alternative euthanasia methods that are more acceptable to workers could reduce the incidence of unsuccessful attempts resulting from the delivery of sub-lethal blows, thus improving piglet welfare, as well as having positive effects on morale and job satisfaction.

The pork industry relies on consumer support for sustainability. Consumers are becoming increasingly aware of, and concerned with, the welfare of agricultural animals, and these concerns are directly impacting on farm practices (Thompson *et al*. 2002). In the pig industry, this has been exemplified in recent years by the burgeoning public opposition to the use of gestational and farrowing stalls, fuelled by animal rights groups, which eventually lead to law changes abolishing or restricting their use in places such as the United Kingdom, NZ, Sweden, and a number of US states (Compassion in World Farming). In the US, public concern regarding the treatment of pigs by industry workers has increased over recent years, largely the result of publicity generated by animal rights groups; in particular the release to the public of undercover video footage depicting various forms of ill treatment, including the use of BT to euthanase adult pigs (a practise which contravenes industry guidelines (National Pork Board 2008)) (e.g. PETA 2006). It is in the interests of the swine industry to ensure public and consumer acceptance of all aspects of production, including emergency euthanasia of pigs. The development of alternatives to BT for euthanasia of pre-weaned pigs that meet animal welfare criteria and are acceptable to both the industry and the general public is therefore desirable to all parties.

2.1.6 Gaseous methods of euthanasia

Currently, CO_2 is the only gas recommended for piglet euthanasia (National Pork Board 2008). Commercial CO_2 euthanasia kits are available in the US, consisting of an opaque plastic chamber, gas bottle, hose and regulator. Euthanasia is performed by either pre-filling the chamber with 100% CO_2 and introducing the piglet (pre-fill), or placing the piglet into an air-filled chamber and gradually introducing CO_2 up to 100% at a controlled rate (gradual induction). In both instances gas flow is maintained for a period of time sufficient to ensure

death of the animal. CO_2 inhalation causes loss of consciousness and death through the direct depression of neurons in the central nervous system (CNS).

During either pre-fill or gradual induction CO_2 euthanasia, oxygen in air is displaced by the introduced CO_2. Whilst oxygen deprivation of the brain tissues can also induce loss of consciousness and death (Nunn 1969), the direct effects of CO_2 on the CNS (mediated by a reduction in cerebrospinal fluid pH) occur very rapidly so that loss of consciousness and death during CO_2 euthanasia results from the effects of CO_2 accumulation, rather than oxygen deprivation. Loss of consciousness in man occurs following inhalation of 15 to 20% inspired CO_2 (Nunn 1969; Levitzky 2003), with 30% CO_2 sufficient to produce general anaesthesia (Nunn 1969).

Gaseous methods of euthanasia, although requiring the use of specialised equipment in the form of a chamber and gas delivery system, may provide both welfare and practical advantages over traumatic methods. Unlike BT, gas euthanasia does not rely on operator precision or accuracy for success, and does not require a secondary step to ensure death. The reduced potential for human error means gas euthanasia methods are likely to be more reliable than traumatic methods. Wider use of gas euthanasia could result in a reduction in the incidence of pain or distress associated with poorly applied BT or NPB euthanasia. In support of this, Mathis (2004) reported that 65% of pig-farm employees perceive CO_2 euthanasia to be less painful than BT. Accurate performance of BT euthanasia should result in immediate loss of consciousness and therefore no further experience pain, therefore the perception of BT as more painful may relate to the delivery of inaccurate or insufficient blows. In addition, because gas euthanasia eliminates the need for operator restraint and physical participation, and does not result in traumatic injury, this method may also be more acceptable to personnel than BT.

2.1.7 Control of ventilation

Respiratory control centres in the brainstem are responsible for determining the rate and depth of pulmonary ventilation to ensure adequate supply of O_2 to, and removal of CO_2 from, the tissues. Central and peripheral chemoreceptor's in the body detect changes in the local chemical environment and signal the central controllers, which in turn effect changes in the rate and/or depth of ventilation through the action of the respiratory muscles (Levitzky 2003). The central and peripheral chemoreceptors are sensitive to alterations in the CO_2 or O_2 content of blood, or to changes in plasma pH. Changes in blood CO_2, O_2 or pH can occur in response to changes in the CO_2 or O_2 content of inspired air, or through changes in tissue metabolism (Levitzky 2003). Normal atmospheric air has an O_2 content of 21% and CO_2 content of 0.04%.

Under normal conditions, arterial pCO_2 (the partial pressure of CO_2 in the arterial blood) is the major factor controlling ventilation, through its effect on the pH of cerebrospinal fluid (CSF). As arterial pCO_2 increases above the normal range of 36 –44 mm Hg, CO_2 passes from the cerebral blood into the CSF, liberating H^+ ions, which stimulate central chemoreceptors located in the medulla, initiating an increase in ventilation. Elevated arterial pCO_2, or hypercapnia, is a very potent stimulus to ventilation (Levitzky 2003). The stimulatory effect of CO_2 on ventilation is most pronounced at inspired concentrations of 5 –10% (Levitzky 2003), with maximal ventilatory response achieved in response to 15 –20% CO_2 (Nunn 1969). Exposure to very high concentrations of CO_2 causes a marked increase in arterial pCO_2, resulting in respiratory depression, followed by respiratory and cardiac arrest when pCO_2 exceeds 70 –80 mm Hg (Nunn 1969). Hypoxia (a reduction in arterial pO_2 below normal) potentiates the ventilatory response to hypercapnia; for any given pCO_2, the ventilatory response is greater at a lower pO_2 (Levitzky 2003).

The peripheral chemoreceptors located in the carotid and aortic bodies are also involved in the regulation of ventilation. Although these respond to changes in pH or pCO_2, they are most sensitive to reductions in pO_2 below the normal

range of 80 –100 mm Hg. When pCO_2 is in the normal range, there is very little increase in ventilation in response to hypoxia until arterial pO_2 decreases to 50 –60 mm Hg, or around half the normal value (Levitzky 2003). However, concomitant hypercapnia increases the sensitivity of the peripheral chemoreceptors to hypoxia, resulting in a larger and earlier ventilatory increase when hypoxia occurs together with hypercapnia (Levitzky 2003).

Both hypoxia and hypercapnia induce the sensation of dyspnoea in humans (Buchanan and Richerson 2009). Dyspnoea is defined as the subjective experience of breathing discomfort, and incorporates sensations such as the uncomfortable awareness of breathing, the sensation of breathlessness or the experience of air hunger (American Thoracic Society 1999). Dyspnoea in response to hypoxia or hypercapnia is believed to result from the increased respiratory motor output generated by chemoreceptor activation, as well as through possible direct connections between the chemoreceptors and cortical and limbic areas of the brain (Buchanan and Richerson 2009). It is generally accepted that there is an emotional component to the perception of dyspnoea, meaning that dyspnoea may be perceived as unpleasant or even noxious in some instances (Lansing *et al*. 2009).

2.1.8 Welfare concerns regarding use of carbon dioxide

In both the US and Europe, CO_2 is being increasingly favoured over electrical methods for the pre-slaughter stunning of adult pigs in commercial slaughter plants (Raj and Gregory 1995), due to the reduced animal handling requirements and improved meat quality associated with its use (Verlarde *et al*. 2007). The incidence of pale, soft and exudative (PSE) meat and haemorrhage is lower in pigs stunned with CO_2 than those stunned electrically. CO_2 stunning typically involves a small number of pigs (2–8, depending on the model) being loaded into a crate which is subsequently lowered into a pit containing 70 –90%

CO_2 in air. Exposure to 70% CO_2 or more renders the animals unconscious for a period of time, during which sticking may be safely carried out.

However, research into the welfare implications of CO_2 stunning of pigs has identified a number of behaviours indicative of reduced welfare in the interval prior to loss of consciousness. These include escape attempts, retreats, vocalisation, hyperventilation and head shaking (Raj 1996; Verlarde *et al*. 2007; Rodriguez *et al*. 2008). Aversion studies involving slaughter weight pigs have demonstrated that these animals find a CO_2 concentration of 90% (that which is typically used for pre slaughter stunning) unpleasant, illustrated by the unwillingness of 88% of animals tested to enter a familiar feed box containing this concentration for a food reward, even when fasted for 24 hours prior (Raj & Gregory, 1995). The immediate withdrawal of the head observed in the majority of test animals lead the authors to conclude that aversion was due to the pungent odour of concentrated CO_2, rather than a result of CO_2 induced ventilatory changes. Of those pigs tested, 38% refused to enter the same box on the following day for a food reward when it contained air only, highlighting the strength of this aversion (Raj & Gregory, 1995).

Carbon dioxide is routinely used to euthanase laboratory rodents. CO_2 is preferred over many other agents due to its ready availability, relatively low cost, ease of use, safety for operators and suitability for euthanasing large numbers of animals simultaneously (Conlee *et al*. 2005). However, loss of consciousness upon exposure to high concentrations of CO_2 is not immediate and there is concern that animals may experience discomfort and/or pain prior to loss of consciousness. Humans report dyspnoea in response to as little as 8% inhaled CO_2 (Dripps and Comroe 1947; Liotti *et al*. 2001). Exposure to concentrations of 40 –55% CO_2 reportedly causes pain in the eyes, nose and throat through the formation of carbonic acid, which stimulates nociceptors located in the mucosa of the cornea, nose and upper respiratory tract (Anton *et al*. 1992; Danneman *et al*. 1997). Experimental studies on non-human mammals have shown that nociceptors in the nasal mucosa of rats are

activated by 37-50% CO_2, (Peppel and Anton 1993), and corneal nociceptors in the cat by 40% CO_2 (Chen *et al*. 1995), suggesting that CO_2 induced mucosal pain is a common feature of mammalian physiology. Other unpleasant symptoms associated with hypercapnia in man include headache, restlessness, dizziness and faintness (Dripps and Comroe 1947; Levitzky 2003). Physiological and anatomical similarities suggest that other mammals may experience similar unpleasant or noxious effects during CO_2 exposure, as do the results of experimental studies in various species (e.g. Conlee *et al*. 2005).

Behaviours indicative of distress or aversion in rodents have been reported during both pre-fill and gradual-induction CO_2 euthanasia, including agitation, gasping, vocalisations, rearing, escape attempts and laboured breathing(Coenen *et al*. 1995; Smith and Harrap 1997; Niel and Weary 2006). Moreover, rodents find CO_2 aversive at inhaled concentrations of 15-20%, well below that at which nociceptor stimulation occurs, suggesting that aversion is related to dyspnoea rather than pain (Leach *et al*. 2004; Niel and Weary 2007).

There is therefore an increasing body of evidence indicating that CO_2, even at moderate concentrations, is unpleasant or aversive to mammals. This has prompted research into the use of alternative gases or gas mixtures for stunning or euthanasia, to improve animal welfare.

2.1.9 Alternatives to carbon dioxide

It has been suggested that oxygen deprivation may be a more humane method of euthanasia than CO_2 inhalation (Freed 1983). This can be accomplished with the use of inert gases such as nitrogen or argon that displace oxygen in air, with the resultant progressive hypoxia leading to loss of consciousness and subsequent death as neurons become starved of oxygen (AVMA 2007). Because nitrogen and argon are odourless, non-irritant, inert gases, it is thought that loss of consciousness and death via nitrogen or argon induced anoxia may

occur without aversion or distress and thus be preferable to using CO_2. Whilst hypoxia does provoke an increase in ventilation, the response does not occur until pO_2 falls below 50 –60 mm Hg (Nunn 1969), meaning that exposure to inert gases such as nitrogen or argon may induce a later ventilatory response, and therefore a lesser sensation of breathlessness prior to loss of consciousness than CO_2.

There has been considerable research into the use of argon as an alternative to CO_2 for the pre-slaughter stunning of pigs and poultry, and for euthanasia of laboratory rodents. Approach-avoidance studies in farm animals have demonstrated that turkeys (Raj 1996), broiler chickens (Gerritzen *et al*. 2000) and adult pigs (Raj and Gregory 1995) will freely enter a chamber containing 90% argon or greater, typically losing consciousness without attempting to exit the chamber.

Rats and mice, unlike pigs and poultry, appear to find argon exposure aversive, although less so than CO_2. Niel and Weary (2007) reported that rats refused to enter, or immediately withdrew from a chamber containing 90% argon. Rats and mice that were free to enter and exit chambers containing various concentrations of CO_2, argon or air, were observed to spend more time in chambers containing argon than those containing CO_2, but significantly less time in an argon chamber than one with air (Leach *et al*. 2002; 2004). Other studies have demonstrated both aversion and physiological stress responses in rodents subjected to progressive argon-induced hypoxia (Makowska *et al*. 2008; Burkholder *et al*. 2010). Rats consistently left a chamber into which argon was being introduced when the oxygen concentration reached 7.7%, indicating hypoxia is aversive to rats at this level (Makowska *et al*. 2008; Burkholder *et al*. 2010). Because argon is odourless and physiologically inert, aversion is believed to result from the ensuing hypoxia.

Several studies have shown that moderate or high concentrations of argon alone are less effective than high concentrations of CO_2 for inducing loss of

consciousness (Raj 1996; Raj 1999) or death (Raj *et al*. 1997; Sharp *et al*. 2006). As a result, the use of argon in combination with CO_2 for euthanasia has been investigated, on the premise that inclusion of a moderate concentration of CO_2 may reduce the time to loss of consciousness, whilst preventing some of the negative effects associated with high concentrations of CO_2 (Leach *et al*. 2002). The inclusion of CO_2 with argon resulted in more rapid loss of consciousness and death in pigs and broilers (Raj 1996; Raj *et al*. 1997; Raj 1999; McKeegan *et al*. 2007), and less evidence of aversion was observed than with CO_2 alone (Raj 1996; 1999). However, there was also evidence of greater respiratory stimulation prior to loss of consciousness with a CO_2-argon mixture than with argon alone (Raj 1996; Raj and Gregory 1996; Lambooij *et al*. 1999; McKeegan *et al*. 2007). In rats and mice, a combination of argon and CO_2 was found to be more aversive than argon alone (Leach et al. 2002; 2004).

Nitrogen-induced hypoxia has been investigated for euthanasing rats (Hornett and Haynes 1984; Sharp *et al*. 2006), and for stunning poultry (McKeegan *et al*. 2006; Coenen *et al*. 2009). A comparison of 100% argon, 100% nitrogen or 100% CO_2 for euthanasia of rats concluded that nitrogen was an ineffective euthanasia agent, based on the extended period prior to loss of consciousness and death compared with CO_2 or argon (Sharp *et al*. 2006). Similarly, the time to collapse and death in rats during GI euthanasia was greater with nitrogen than CO_2 at lower flow rates, although these did not significantly differ at high flow rates (Hornett and Haynes 1984). The authors concluded that nitrogen was an unsuitable euthanasia agent, based upon the intense distress observed with nitrogen in comparison to CO_2. Argon and nitrogen, either alone or in combination with CO_2, were equally effective for stunning poultry, although struggling and wing-flapping were reported with all mixtures (McKeegan *et al*. 2007). Although there were no apparent differences in stunning efficacy between nitrogen and argon in poultry, the significant differences between these in terms of time to loss of consciousness and death in rats suggests the mechanism of action may differ between the two gases (Sharp *et al*. 2006).

There are no reports on the use of nitrogen alone for the euthanasia of pigs, possibly due to practical limitations. One study investigating the use of 90% argon, 70% nitrogen-30% CO_2 and 85% nitrogen-15% CO_2 to stun adult pigs concluded that both of the nitrogen-CO_2 mixtures were more aversive than argon alone, although 70% nitrogen-30% CO_2 induced more rapid loss of posture than argon alone (Dalmau *et al*. 2010b). Both of these observations are likely due to the effects of CO_2 in the nitrogen mixtures. Nitrogen alone was not included in this study, as previous research (Dalmau *et al*. 2010a) indicated that a sufficiently high concentration of nitrogen could not be maintained in a commercial dip-lift apparatus, making nitrogen alone impractical for commercial applications.

2.1.10 Gaps in the knowledge

Although data from adult pigs clearly identifies argon as being less aversive than CO_2, and as being an effective stunning agent (although not as rapid as CO_2 alone), it cannot be assumed that this will be the case across all age ranges. Neonatal tolerance to hypoxia has been well documented (e.g. Glass et al. 1944; Singer 1999). As a result of foetal adaptations to cope with the reduced oxygen environment in-utero, newborn mammals of various species have a far greater tolerance of hypoxia than their adult counterparts, meaning that hypoxic methods of euthanasia may be less effective in very young mammals. For example, when exposed to 99.9% nitrogen, newborn rabbits survived for 34 minutes, compared with 1.5 minutes for adult rabbits (Glass *et al*. 1944). Survival times were observed to decrease with increasing postnatal age, until the adult response occurred at 18 days of age. In pigs, the adult response to hypoxia does not develop until six weeks after birth (Novy *et al*. 1973). Such observations may mean that the times to loss of consciousness and death in young piglets exposed to hypoxic environments could be greater than those previously reported for adult pigs, which could have negative implications for welfare. To date there is little or no information available

regarding gas euthanasia of suckling piglets with CO_2 or alternative gases or gas combinations.

2.2 Assessment of animal welfare during euthanasia

2.2.1 What is animal welfare and how can it be measured?

Animal welfare may be defined as an experiential state within an individual, resulting from the integrated processing of sensory and other neural inputs arising from both the internal and external environments of the animal (Mellor *et al*. 2009). The welfare, or experiential state, of an animal is likely to include both positive and negative experiences and as such may vary on a continuum from extremely poor to very good (Mellor *et al*. 2009). Animal welfare status is thus dynamic, varying according to the summation of neural inputs at a given time. Because animal welfare relates to experience, it follows that an animal must be capable of experiencing. Therefore it must be sentient i.e. it must have a neural system of sufficient complexity to permit the transduction of neural impulses into experienced sensations (Mellor *et al*. 2009). In addition, the animal must be in a conscious state, as unconscious animals are incapable of subjective experience. It is generally accepted that all mammalian animals are sentient, or capable of subjective experience (Mellor *et al*. 2009). Pigs, being mammals, are therefore subject to variations in welfare status, so long as they are in a conscious state.

An animal's welfare state cannot be measured directly, however it can be inferred using a variety of indicators.

A number of approaches to welfare assessment have been proposed. Probably the most practical framework for assessing animal welfare is the needs-based model first proposed by Mellor and Reid, which uses the five domains of potential welfare compromise as a guideline (Mellor and Reid 1993). This approach considers the needs of an animal within the domains of nutrition,

environment, health, behaviour and mental experience, and assesses welfare status according to the extent to which these are (or are not) being fulfilled. Such a multidimensional approach to welfare assessment means that all factors that may affect welfare are considered. According to this model, sensory and neural inputs related to the functional domains of nutrition, environment, health and behaviour, along with other cognitive inputs, are processed and expressed in the mental domain as conscious subjective experience (Mellor *et al*. 2009). For example, prolonged food deprivation, representing welfare compromise in the nutritional domain, will give rise to the sensation of severe hunger, a negative experiential state. Similarly, tissue injury or damage, representing compromise in the health domain, may give rise to the sensations of pain, discomfort or nausea, which are negative experiential states. Overall welfare status is judged according to the nature of subjective experience in the mental domain and where it falls on the welfare continuum (Mellor *et al*. 2009).

In terms of welfare assessment during euthanasia, the absence of negative affective states such as fear, anxiety, pain or distress as a result of the procedure, rather than the presence of positive affective states, are the main criteria for an acceptable method (AVMA 2007). As such, this review will focus on the assessment of negative welfare states that may arise prior to loss of consciousness during the performance of animal euthanasia.

A wide range of behavioural and physiological indices have been utilised for the assessment of welfare status in domestic mammals. These include behavioural measures such as escape attempts and avoidance, and physiological measures such as changes in heart rate, respiration, adrenal activity and immune responses (Broom and Fraser 2007). Some measures are more relevant to short-term issues, such as brief exposure to adverse physical conditions, whereas others are more relevant to the assessment of long-term issues such as chronic pain as a result of disease or injury (Broom and Fraser 2007). In assessing welfare during euthanasia, only those measures that reflect acute

changes in welfare status induced by the performance of euthanasia itself are of interest.

2.2.2 Physiological indices of welfare

The initiation of physiological stress responses can result in measurable changes in some physiological variables, such as heart rate, ventilation rate and blood pressure. Stress itself has been defined as the biological response to a perceived threat to homeostasis, with the threat being defined as a stressor (Moberg 2000).

In terms of euthanasia, events such as handling, restraint, tissue damage, and hypercapnia or hypoxia may all invoke physiological stress responses. These events may also result in the experience of negative affective states, such as fear, anxiety, pain or breathlessness. Evidence of a physiological stress response during euthanasia is therefore suggestive of compromised welfare.

Acute stress causes autonomic nervous system (ANS) activation, resulting in alterations in physiological variables such as heart rate, blood pressure and ventilation rate through the actions of circulating catecholamines (Moberg 2000). Changes in heart rate, blood pressure, ventilation rate, or plasma catecholamine concentration can therefore provide evidence of physiological stress in response to a noxious or unpleasant stimulus (Moberg 2000).

Activation of the hypothalamic-pituitary-adrenal (HPA) axis can also occur in response to acute stress (Moberg 2000). Neurons in the hypothalamus are stimulated to release corticotrophin-releasing hormone (CRH), which initiates the release of adrenocorticotrophic-releasing hormone (ACTH) from the anterior pituitary, which in turn initiates the release of glucocorticoid hormones from the adrenal medulla (Moberg 2000). Whilst less rapid than the ANS response, glucocorticoid levels in plasma nonetheless begin to increase within 1.5 to 3 minutes of exposure to a stressor, making these a useful marker of stress in

studies examining the welfare impact of short-term animal management practices (Broom and Fraser 2007). For euthanasia methods that require minutes rather than seconds to perform, increases in circulating ACTH or glucocorticoid levels may provide additional evidence of a physiological stress response.

One limitation to the use of hormonal stress markers is the fact that sampling itself may be stressful, inducing a rise in both circulating catecholamine and glucocorticoid levels (Moberg 2000; Broom and Fraser 2007). This may be overcome by sampling with minimal disturbance to the animal, or by sampling from control animals that have not been exposed to the stressor in question, in order to isolate the effects of sampling itself.

2.2.3 Behavioural indices of welfare

In addition to hormonal changes, physiological stress frequently results in the initiation of behavioural responses, such as escape or avoidance actions in response to a stressor (Moberg 2000). Escape attempts and avoidance behaviours have been used to assess animal welfare in response to a wide range of short-term stressors (Broom and Fraser 2007). Avoidance of an object or event provides information on the animal's feelings toward the object/situation, with stronger avoidance indicating more negative feelings and therefore poorer welfare (Broom and Fraser 2007).

In pigs, escape behaviours, the duration and intensity of laboured breathing, and squealing have all been used as behavioural indicators of aversion or distress. For example, in adult pigs, escape attempts such as retreating or running at the walls of the chamber have been used as a measure of aversion in the evaluation of gas atmospheres for pre-slaughter stunning (Raj and Gregory 1995; Verlarde *et al*. 2007; Rodriguez *et al*. 2008; Dalmau *et al*. 2010b).

Regardless of whether behavioural or physiological measures are used to assess welfare, only those changes occurring prior to loss of consciousness are relevant, given that consciousness is a prerequisite for affective experience. Determining the point at which consciousness is lost during euthanasia is therefore vital to the assessment of welfare.

2.2.4 Assessing consciousness

An animal must be conscious in order to experience subjective phenomena that may affect its welfare. Euthanasia aims to induce rapid loss of consciousness, thus minimising the time frame for potential negative experiences such as pain, fear or anxiety. Therefore recognition of conscious states, and in particular of the point at which consciousness is no longer likely, is crucial to welfare assessment. Observations of behaviour, the presence or absence of certain physiological reflexes, and measurements of brain activity have all been used to assess consciousness in animals subjected to stunning or euthanasia (Raj and Gregory 1996; Raj *et al*. 1997; Raj 1999; Niel and Weary 2006; Sharp *et al*. 2006).

Loss of posture, involving loss of postural muscle tone and the subsequent emergence of a recumbent state, is considered to be the first behavioural sign of the onset of unconsciousness in pigs (Raj and Gregory 1996; Verlarde *et al*. 2007). Loss of posture is easily assessed by visual inspection, and is useful when physiological reflexes cannot be assessed, for example when animals are confined in a controlled atmosphere chamber.

The absence of certain physiological or behavioural reflexes is commonly used to determine adequate depth of anaesthesia, i.e. level of consciousness, in man and animals, as well as the adequacy of pre-slaughter stunning of farm animals. With regard to farm animals, the absence of corneal and/or palpebral reflexes, and the absence of a response to noxious stimulation, such as a nose prick or

toe pinch, have been used to determine loss of consciousness following stunning or slaughter of lambs (Newhook and Blackmore 1982), calves (Newhook and Blackmore 1982)and pigs (Raj 1999). Such assessments require that the operator has direct access to the animal and are frequently used to determine the duration of insensibility following stunning.

In mammals, the electroencephalogram (EEG) provides a record of the summated electrical activity of neurons located in the cerebral cortex, as recorded from electrodes located on the head or scalp (Murrell and Johnson 2006). In every mammalian species studied to date, there is a marked contrast in EEG activity between states of wakeful consciousness and deep sleep, a form of unconsciousness (Baars 2001). During wakeful states, the EEG typically exhibits a pattern of irregular, low amplitude fast activity, whereas during deep sleep the EEG typically consists of regular, high amplitude slow wave activity (Baars 2001). Importantly, such patterns of synchronised slow wave EEG activity are also evident during other states of global unconsciousness, such as general anaesthesia or coma (Baars 2001).

Alterations in the amplitude and frequency of the EEG waveform can provide information regarding the state of consciousness. For example, a reduction in the amplitude of the EEG has been associated with the onset of insensibility in sheep, calves and pigs following slaughter by ventral neck incision(Blackmore and Newhook 1981; Newhook and Blackmore 1982; Gibson *et al*. 2009). The onset of an isoelectric EEG waveform, indicating the absence of cortical electrical activity, is widely accepted as being incompatible with conscious states, therefore representing undisputed loss of consciousness.

2.2.5 Use of the EEG to assess nociception

The EEG provides a record of spontaneous electrical activity in the cerebral cortex. Fast Fourier Transformation (FFT) is a mathematical process that

transforms raw EEG from the time domain to the frequency domain, yielding a power spectrum. Variables derived from the EEG power spectrum following FFT include the total power, which is the total area under the frequency spectrum curve; the median frequency, which is the frequency below which 50% of the total power is located; and the 95% spectral edge frequency, which is the frequency below which 95% of the total power is located (Murrell and Johnson 2006). Changes in the power spectrum of the transformed EEG have been shown to reflect changes in cortical activity associated with the conscious perception of pain in man (Bromm 1984; Chen *et al*. 1989). In addition, changes in the EEG power spectrum in response to a known noxious stimulus correlate well with subjective accounts of pain intensity in conscious humans (Chen *et al*. 1989), and with behavioural responses to noxious stimuli in conscious sheep (Ong *et al*. 1997).

More recently, changes in the power spectrum of the EEG have been used to examine the acute noxiousness of painful events in anaesthetised horses (Murrell *et al*. 2003), lambs (Johnson *et al*. 2005a), calves (Gibson *et al*. 2007), rats (Murrell *et al*. 2007), deer (Johnson *et al*. 2005b), pigs (Haga and Ranheim 2005) and dogs (Kongara *et al*. 2010), using the Minimal Anaesthesia Model (MAM) developed by Johnson and co-workers (Murrell and Johnson 2006). Under minimal anaesthesia, noxious stimuli of sufficient intensity will produce nociceptive signals that can still reach cortical regions of the brain involved in the processing or perception of pain (Bromm 1984), although the animal is unable to consciously perceive this. The cortical processing of nociceptive signals gives rise to quantifiable changes in the EEG power spectrum (Murrell and Johnson 2006).

Changes in both the median frequency (F50) and total power (Ptot) of the EEG have been found to correlate strongly with nociception, with a number of studies reporting an increase in F50 and decrease in Ptot during noxious stimulation of lightly anaesthetised animals (e.g. Johnson *et al*. 2005b; Gibson *et al*. 2007; Kongara *et al*. 2010). Prior administration of local anaesthetic or analgesic

agents has been shown to abolish or attenuate the EEG response to noxious stimulation(Haga and Ranheim 2005; Gibson *et al*. 2007; Kongara *et al*. 2010), supporting the idea that changes in the EEG power spectrum result from the cortical processing of nociceptive signals.

2.3 Study objective and thesis structure

The overall objective of the current research was to evaluate the relative welfare impacts of CO_2 and a variety of alternative gas mixtures used for euthanasia on young suckling pigs, with a view to developing an improved method for on-farm euthanasia that prioritises animal welfare, whilst being acceptable to industry personnel, farm staff, and consumers of pork products.

A total of three studies were carried out. The first was a small pilot study to evaluate the experimental protocol and select gas mixtures for the main study (Chapter 3). The second (main study) involved collection of behavioural and physiological data from conscious, responsive animals during euthanasia with 3 selected gas treatments (Chapter 4), whilst the third involved collection of electrophysiological data from anaesthetised animals during euthanasia with the 3 selected gas treatments used in the main study (Chapter 5). A general discussion of the relative welfare impacts of the 3 gas treatments is presented in Chapter 6.

3 Experiment 1. Pilot study to evaluate the proposed protocol and potential gas mixtures for the main study

3.1 Introduction

A number of different gases and gas mixtures have been evaluated for the euthanasia of laboratory rodents, and for the pre-slaughter stunning of adult pigs and poultry, including carbon dioxide alone, and argon or nitrogen alone or in combination with CO_2 (e.g. Raj and Gregory 1995; Sharp *et al*. 2006; Makowska *et al*. 2009). The current research was undertaken to determine which of these gases or gas combinations is the most humane for use in on-farm euthanasia of pre-weaned piglets.

Due to the high ethical cost of the proposed study, a small pilot study was conducted whereby one animal was euthanased with each of the five proposed gas treatments, in order to evaluate the experimental protocol and determine whether any modifications were required before proceeding further.

Five gas treatments were selected for initial evaluation:

- 100% carbon dioxide
- 90% argon in air
- 90% nitrogen in air
- 30% carbon dioxide and 60% argon in air
- 40% carbon dioxide and 50% nitrogen in air

One hundred percent carbon dioxide represents current industry recommendations for gas euthanasia of piglets (National Pork Board 2008). In order to assess the relative welfare advantages of any alternative gases it was

necessary to include CO_2 in the evaluation. Argon and nitrogen were selected based on their inert properties, safety for operators and ready availability.

The combination of nitrogen or argon with carbon dioxide, and the concentrations used, were based on previous studies involving adult pigs and poultry, which indicated that the addition of CO_2 results in more rapid loss of consciousness and death than either nitrogen or argon alone (Raj and Gregory 1996; Raj *et al*. 1997; Lambooij *et al*. 1999; Raj 1999; Gerritzen *et al*. 2004; McKeegan *et al*. 2007). By reducing the carbon dioxide content to forty percent or less, we hoped to avoid activation of mucosal nociceptors in the cornea and respiratory tract and associated pain, thus producing unconsciousness with less welfare impacts than those associated with 100% carbon dioxide.

3.2 Materials and Methods

Approval for this study was granted by the Massey University Animal Ethics Committee (protocol # 10/22).

3.2.1 Animals and housing

Five male crossbred (Landrace X Large white) pre-weaned piglets aged 19 days, mean live weight 5.9 kg, were obtained from a commercial pig farm on the morning of the day of testing. Prior to testing, animals were group housed in a 28°C temperature controlled ventilated room, containing straw bedding over non-slip concrete flooring. Pigs had access to the sow until the time of collection from the farm, followed by ad libitum access to water until the time of testing. The pigs were transferred individually to the laboratory in a small transport cage immediately prior to testing.

3.2.2 Experimental procedure

Pigs were randomly assigned to receive one of the five gas mixtures:

- 100% carbon dioxide (CO_2)
- 90% argon in air (Ar)
- 90% nitrogen in air (N_2)
- 30% carbon dioxide/60% argon in air (CO_2-Ar)
- 40% carbon dioxide/50% nitrogen in air (N_2-CO_2).

A custom-built plastic euthanasia chamber was pre-filled with the appropriate test gas mixture, sourced from cylinders of compressed CO_2, N_2, Ar and air (Air Liquide New Zealand Ltd, Penrose, Auckland). Prior to being placed in the chamber, a blood sample was taken and each pig was instrumented for data collection. The animal was restrained in dorsal recumbency whilst 5ml of blood was collected from the jugular vein. Plasma was separated immediately by centrifugation (5000rpm, 10 minutes), and then stored at -80°C for later analysis of cortisol and epinephrine by enzyme immunoassay (Parameter Cortisol Assay KGE008, R&D Systems, Minneapolis, MN; AP17-EPIHU-e01 Epinephrine assay, Alpco Diagnostics, Salem, NH).

The animal was held gently whilst a custom-made respiratory belt incorporating a saline-filled pressure cuff was fitted around the abdomen for continual monitoring of ventilation rate. The belt was secured using a tape harness, as shown in Figure 1. Subcutaneous 27-gauge stainless steel needle electrodes (Viasys Healthcare, Surrey, England) were positioned to record electroencephalograph (EEG) and electrocardiograph (ECG) activity. A five-electrode montage was used to record EEG from both the left and right cerebral hemispheres, with inverting electrodes placed parallel to the midline over the zygomatic processes of the left and right frontal bones, non-inverting electrodes over the left and right mastoid processes and a ground electrode placed caudal to the occipital process. ECG was recorded using a base-apex configuration.

Both EEG and ECG signals were fed via breakout boxes to separate amplifiers (Iso-Dam isolated biological amplifier, World Precision Instruments Inc.). The signals were amplified with a gain of 1000 and a band-pass of 1.0 –500Hz and

digitised at a rate of 1kHz (Powerlab 4/20, ADInstruments Ltd, Colorado Springs, Co). The digitised signals were recorded on an Apple Macintosh personal computer for analysis off-line at the conclusion of the experiment.

Behaviour was video recorded from two different angles using separate digital camcorders (Sony Corporation, Tokyo, Japan), located above and to one side of the chamber, for analysis at the conclusion of the experiment.

After instrumentation, each animal was placed on its feet in the chamber (Figure 1). The lid was replaced and physiological and behavioural data continuously recorded until death was confirmed by cessation of respiratory activity along with electrical silence in both the electrocardiogram (ECG) and electroencephalogram (EEG). Gas flow was continued throughout at a rate of 10 L (20% of the chamber volume) per minute, to maintain stable gas concentrations. Immediately following confirmation of death, a second blood sample was collected from the jugular vein as previously described, for subsequent analysis of plasma cortisol and epinephrine concentrations.

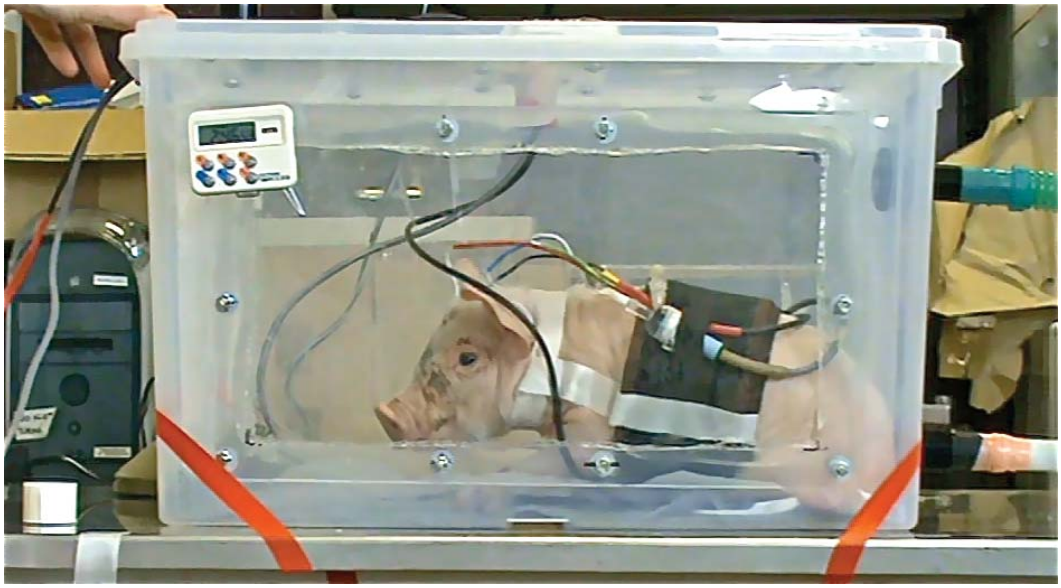

Figure 1 Video snapshot of an instrumented pig within the test chamber

3.2.3 Behaviour analysis

Selected behavioural indicators of pain, distress or aversion were based on those reported in previous studies of pigs or rodents exposed to hypoxic or hypercapnic gas atmospheres (Raj and Gregory 1995; Raj 1999; Conlee *et al*. 2005; Nowak *et al*. 2006; Verlarde *et al*. 2007; Rodriguez *et al*. 2008) and are described in Table 1. Following systematic review of data from both video cameras, the latency to onset, duration and/or frequency of those behaviours described in Table 1 were manually recorded.

The latency to onset of a specific behaviour was the time in seconds following transfer of the pig to the chamber that the behaviour was first observed. The duration of behaviour was the total time in seconds that the animal spent engaging in that behaviour following transfer to the chamber. The frequency of behaviour was the number of discrete occurrences.

In addition to determining the latency to onset and duration of laboured breathing, this was also scored according to the overall intensity. A four-point scale, similar to that employed by Raj and Gregory (1996), was used to grade the overall intensity of laboured breathing in the period prior to irreversible loss of posture, where:

0 = closed mouth, with no evidence of a change in rate or depth of ventilation relative to baseline (air)

1 = closed mouth, visible abdominal movement indicating increased breathing depth and rate >1 breath per second

2 = open mouth, increased rate and/or depth of ventilation

3 = gasping, as defined in Table 1

3.2.4 EEG and ECG analysis

Following placement in the chamber, all animals displayed some form of vigorous physical activity, which resulted in the displacement of EEG and/or ECG recording electrodes. As a result, electrophysiological data were incomplete and could not be analysed.

NB: Because there was only one animal per treatment group, no statistical analyses of data were carried out.

Table 1 Ethogram for the observation of piglet behaviour

Behaviour	Description	Measures
Squealing	The pig emits an audible squeal	Number of bouts[1] Total duration (s)
Grunting	The pigs emits an audible grunt	Number of bouts Total duration (s)
Escape attempts	Backing up, or Purposeful and vigorous butting of head, snout or shoulder into the chamber lid or walls, or Raising of forelegs against chamber walls	Latency to onset (s) Frequency of occurrence Total duration (s)
Laboured breathing	Increase in rate and/or depth of ventilation compared with baseline (prior to transfer to chamber)	Latency to onset (s) Total duration (s) Overall intensity
Loss of coordination	Loss of balance, stumbling, or diminished muscle control	Latency to onset (s)
Gasping	Low frequency (≤4/min), very deep breathing through a wide open mouth, accompanied by large abdominal movements and stretching of the neck	Latency to onset (s)
Respiratory arrest	Permanent cessation of respiratory movement (minimum of 60 seconds without a breath)	Latency to onset (s)
Irreversible loss of posture	The animal collapses into a recumbent position, with no evidence of postural control, and does not regain posture, or show further evidence of awareness	Latency to onset (s)
Convulsions	Involuntary contraction of the skeletal muscles, may be tonic (contraction only), clonic (repetitive contraction and relaxation) or both (Blood *et al*. 2007), includes paddling motions of the limbs	Latency to onset (s) Frequency of occurrence Total duration (s)
Urination	Evacuation of the bladder	Frequency of occurrence
Defecation	Evacuation of the bowels	Frequency of occurrence
Head shaking	Vigorous, rapid, purposeful movement of head from side to side (at least 2 consecutive movements)	Frequency of occurrence
Sneezing	The pig sneezes	Frequency of occurrence
Coughing	The pig coughs	Frequency of occurrence

[1]A bout was either a single discrete occurrence, or a period of uninterrupted activity. The absence of the behaviour for at least one second or indicated the end of a bout

3.3 Results

Table 2 shows the behavioural data collected from 5 piglets; 1 per treatment group. Because welfare cannot be affected following irreversible loss of consciousness, data for vocalisations, escape attempts and laboured breathing are reported for the period prior to the onset of irreversible loss of posture, which was the only available indicator of loss of consciousness.

Escape attempts, laboured breathing and convulsions were observed in all treatment groups. First loss of posture, considered to be the first sign of the onset of loss of consciousness (Raj and Gregory 1996), coincided with the onset of convulsions in all animals. However, piglets exposed to Ar, N_2 and CO_2-Ar demonstrated recovery of posture and evidence of awareness (e.g. opening of eyes, ocular movements, co-ordinated muscle movements) following earlier convulsive bouts. The time to irreversible loss of posture ranged from 15 seconds in CO_2 to 368 seconds in CO_2-Ar, with the time to respiratory arrest ranging from 114 seconds in CO_2 to 780 seconds in N_2. No animals in this study were observed to urinate, defecate, head shake, cough or sneeze.

The changes in plasma cortisol and epinephrine concentrations following euthanasia are shown in Table 3. The change in cortisol concentration was variable, with two animals showing an increase and three a decrease after euthanasia. Plasma epinephrine increased by 20 to 28 ng ml^{-1} in all animals following euthanasia.

Table 2 Latency to onset, frequency and/or duration of piglet behaviours during euthanasia with each of the five gas mixtures

Behaviour	CO_2	Ar	N_2	CO_2-Ar	CO_2-N_2
Number of squeals	0	3	4	1	1
Number of grunts	0	0	10	1	0
Duration of squeals (s)	0	7.5	29.5	16	6
Duration of grunts (s)	0	0	6.5	16	0
Number of escape attempts	2	1	2	8	1
Latency to first escape attempt (s)	11	20	12	178	10
Duration of escape attempts(s)	3	4	38	15	3
Number of convulsive bouts	9	20	13	11	15
Latency to first convulsion (s)	15	25	365	199	60
Duration of convulsions (s)	42	161.5	104	63	63
Latency to laboured breathing (s)	1	40	215	50	4
Duration of laboured breathing (s)	14	45	218	318	56
Intensity of laboured breathing	2	2	3	2	3
Latency to loss of coordination (s)	11	19	331	197	11
Latency to gasping (s)	32	185	371	410	45
Respiratory arrest (s)	114	470	780	630	527
Latency to irreversible loss of posture (s)	15	85	433	368	60

Table 3 Change in plasma cortisol and epinephrine from pre-treatment concentration (ng ml^{-1}) following euthanasia with each gas

Treatment	Cortisol before	Cortisol after	Cortisol change	Epinephrine before	Epinephrine after	Epinephrine change
CO_2	75.9	19.1	-56.9	0.25	23.15	22.9
Ar	117.2	8.2	-109.0	0.75	24.73	24.0
N_2	10.6	68.6	58.0	3.72	23.94	20.2
CO_2-Ar	13.3	149.7	136.4	0.70	27.23	26.5
CO_2-N_2	88.5	16.29	-72.3	1.75	29.66	27.9

3.4 Discussion

The purpose of the pilot study was to evaluate the experimental protocol and to determine whether any modifications were necessary before proceeding with the main study. Due to the very small sample size (n=1 per treatment), it is inappropriate to make any inferences regarding treatment effects from these results. However, in the light of some of the observed responses, we felt that several modifications were warranted before any further animals were tested.

Four of the five gas treatments evaluated in this study contained 10% air and therefore 2.1% residual oxygen. It appeared that the presence of residual oxygen prolonged piglet survival, given the marked difference in time to respiratory arrest observed between CO_2 and CO_2-Ar or CO_2-N2 (Table 2).

Residual oxygen also appeared to prolong the latency to onset of unconsciousness, potentially increasing the duration of respiratory distress experienced prior to loss of consciousness. In slaughter-weight pigs exposed to a CO_2-argon mixture with 5% residual oxygen, the time to collapse was significantly greater than exposure to the same mixture with 2% residual oxygen (Raj and Gregory 1996), supporting the role of oxygen concentration in prolonging the latency to loss of consciousness. In addition, behavioural evidence of consciousness (sitting, standing and deliberate movements) was observed following the onset of convulsions in three of the four residual oxygen treatments (Ar, N_2 and CO_2-Ar), suggesting that these animals may have been conscious during or between convulsions, representing potentially severe welfare compromise. The exclusion of residual oxygen may thus be desirable from both practical and welfare perspectives – reduced times to loss of consciousness and death mean less potential for negative experience in the animals, and greater efficiency for operators.

Much of the research conducted in pigs and poultry has focused on commercial stunning operations, where the maintenance of anoxic environments is not

viable. The use of a small sealed chamber such as that used in the present study however did allow for the provision of anoxic environments. Therefore it was decided to exclude residual oxygen from all gas treatments in the main study.

The intensity of laboured breathing prior to loss of consciousness was greater in the N_2 and CO_2-N_2 treatments than the other 3 treatments (Table 2). With N_2 alone, this probably reflects the prolonged time to irreversible loss of posture observed. However, laboured breathing intensity was rated higher for CO_2-N_2 than CO_2-Ar, despite the shorter time to irreversible loss of posture. This may have been due to the higher CO_2 content in the CO_2-N_2 mix (40%) versus the CO_2-Ar mix (30%). The observed differences may simply have been a feature of individual variation, given that only one animal was assigned to each treatment.

Loss of consciousness and respiratory arrest appeared to take longer with nitrogen than argon (433s versus 85s, and 780s versus 470s, respectively), suggesting nitrogen is a less rapidly acting euthanasia agent for piglets than argon. This is in agreement with previous studies that concluded nitrogen was ineffective for euthanasing rodents (Hornett and Haynes 1984; Sharp *et al*. 2006).

The apparent difference in efficacy of argon and nitrogen suggests these are not inducing CNS depression by the same mechanisms. At normal atmospheric pressures, both of these gases are inert and are expected to induce loss of consciousness and death via progressive hypoxia. Given the equivalent residual oxygen concentrations used in this study, the times to loss of consciousness and death were expected to be similar with both Ar and N_2. Although the lack of replication in the current study means that individual variation cannot be ruled out as the sole source of the observed differences, a more comprehensive study reported similarly inflated times to loss of consciousness and death in rats euthanased with nitrogen compared to those euthanased with argon (Sharp *et al*. 2006). In addition, the current study

demonstrated significant differences in cardiovascular responses of rats during euthanasia with N_2 and Ar, providing evidence for divergent modes of action. In light of the prolonged duration of consciousness observed with nitrogen in this and previous studies, and in the interests of reducing animal numbers, it was decided not to pursue the use of nitrogen as a euthanasing agent in subsequent experiments.

The usefulness of changes in plasma cortisol concentration as a welfare indicator during gas euthanasia is questionable, based upon the variation observed in this study (Table 3). The large variation in pre-treatment concentration (10.6 – 117.2 ng ml^{-1}) suggests that plasma cortisol was elevated prior to administration of euthanasia in at least some individuals. This may be due to individual differences in piglet responses to potential pre-treatment stressors such as separation from the sow, transport, handling and blood sampling. Because plasma cortisol can remain elevated for minutes or even hours following HPA activation (Moberg 2000), it may be less useful than epinephrine concentration for identifying acute stress during the procedure of gas euthanasia.

The displacement of EEG and ECG recording electrodes prevented the collection of data regarding heart rate and brain activity. In order to improve the recording of EEG and ECG data, a contact adhesive will be used to fix the electrodes in place for the main study.

4 Experiment 2. Behavioural assessment of the relative welfare impacts of 100% CO_2, 100% argon and a mixture of 40% CO_2-60% argon for euthanasia of pre-weaned piglets

4.1 Introduction

Based on the results of the initial pilot study, three gas mixtures were selected for further evaluation as potential agents for the on-farm euthanasia of young pigs. These were 100% CO_2 (CO_2), 100% argon (Ar) and a mixture of 40% CO_2 and 60% argon (CO_2-Ar). It was anticipated that the exclusion of residual oxygen from all treatments would reduce the times to both unconsciousness and death compared to those treatments with residual oxygen evaluated in the pilot study.

From a welfare perspective, the two key requirements for an acceptable form of euthanasia are that it induces rapid, irreversible loss of consciousness, and that it causes minimal pain, distress or anxiety to the animal in the period prior to loss of consciousness (AVMA 2001). In the present study, a range of behavioural and physiological measures were used to assess the relative welfare impacts of CO_2, Ar and CO_2-Ar, to determine whether argon or a CO_2-Ar mixture provide any welfare advantages over CO_2 alone for the euthanasia of pre-weaned piglets.

4.2 Materials and methods

Approval to undertake this study was granted by the Massey University Animal Ethics Committee (Protocol # 10/22).

4.2.1 Animals and housing

Fifteen commercial crossbred (Landrace X Large White) male pigs aged 14 –20 days, weighing between 2.75 and 6.0 kg (mean 4.2, SD 1.14 kg), were obtained from a commercial swine farm on the morning of the day of testing. Pigs were housed according to the protocol described in the pilot study.

4.2.2 Experimental procedure

Animals were randomly assigned to receive one of the following three gas treatments (n=5 per treatment): 100% carbon dioxide (CO_2), 100% argon (Ar), or a mixture of 40% carbon dioxide-60% argon (CO_2-Ar).

The euthanasia chamber was the same as that described in the pilot study (Figure 1). The chamber was pre-filled with the appropriate test gas, sourced from compressed gas cylinders connected to the chamber, and gas flow maintained throughout the experimental procedure at a rate of 10 litres (20% of the chamber volume) per minute.

Blood samples were collected prior to instrumentation and immediately following confirmation of death as described in Chapter 3, for subsequent analysis of plasma cortisol and epinephrine concentration. Plasma cortisol and epinephrine concentrations were determined by enzyme immunoassay, according to the manufacturers instructions (Chapter 3).

After blood sampling, each animal was instrumented. A respiratory belt was fitted around the abdomen to record respiration rate. ECG and EEG electrodes, for monitoring of cardiac and cerebral cortical electrical activity, were fitted as previously described and secured to the skin with Superglue to prevent displacement. After instrumentation, each animal was placed on its feet in the chamber. The lid was replaced and physiological and behavioural measures

were continuously recorded until death, as defined by the permanent cessation of respiratory activity along with electrical silence in both the ECG and EEG.

Behaviour was scored off-line at the completion of the experiment, according to the ethogram described previously (Table 1).

4.2.3 Data Analysis

Heart rate was calculated from the ECG recordings, using the rate meter function in Chart (Chart v5.5.6, ADInstruments Ltd, Colorado Springs, Co).

EEG recordings were visually inspected and classified into one of three categories: active, transitional or isoelectric (Gibson *et al*. 2009), to allow calculation of the mean latencies to the appearance of transitional and isoelectric EEG. Active EEG is indistinguishable from pre-treatment EEG (i.e. the same amplitude and frequency), and represents normal cerebrocortical activity. Transitional EEG is classified as having amplitude of less than 50% of the pre-treatment EEG. Isoelectric EEG is classified as a stable trace consisting of background noise only, with amplitude <12.5% of baseline EEG.

A welfare-impact index (WI) was calculated to compare the relative welfare impact of the 3 gas treatments (Appendix A (Table 9)). The index included five behavioural measures relevant to welfare observed in the period prior to apparent loss of consciousness, beyond which there was no further potential for welfare compromise. The measures were: latency to loss of consciousness, duration of escape behaviour, duration of laboured breathing, respiratory effort grade (indicative of the intensity of laboured breathing) and duration of squealing.

All fifteen animals were ranked for each of the 5 measures, with the lowest rank assigned to the animal with the lowest score for each measure. The sum of

ranks for each individual yielded a single score indicative of relative welfare impact, with a higher score indicating poorer welfare.

4.2.4 Statistical Analyses

Behaviour and plasma cortisol and epinephrine data were subjected to analysis of variance using Prism 4 for Macintosh (GraphPad Software Inc., La Jolla, CA, USA) to determine any significant treatment effects. Due to the relatively small sample size (n=5 per treatment) analysis of variance was conducted using the more conservative non-parametric Kruskal-Wallis test, with Dunn's test for multiple comparisons. In the case of the welfare-impact index data, one-way analysis of variance was used, with Bonferroni adjustment for multiple comparisons, as the data were already ranked. All values are reported as non-transformed mean ± standard error of the mean (SEM). Differences were considered significant at $p < 0.05$.

4.3 Results

4.3.1 Behaviour

Table 4 shows there were significant treatment effects for all behaviours except number and duration of squeals, latency to loss of coordination and duration of laboured breathing.

Only 3 of 15 piglets urinated or defecated during treatment, with this occurring after loss of posture on all occasions. Head shaking, coughing or sneezing were not observed in any animals. These behaviours are therefore not presented in Table 4.

Table 4 Treatment means (SEM) for piglet behaviour during euthanasia with 100% CO_2, 100% argon, or a mixture of 40% CO_2 and 60% argon. Means in the same row with different superscripts are significantly different ($p<0.05$)

Variable	CO_2	Ar	CO_2-Ar	H (2df)	p
*Number of escape attempts	2.4 ± 0.51^{a}	0.6 ± 0.24^{b}	1.2 ± 0.20^{ab}	8.2	0.017
*Latency to first escape attempt (s)	3.9 ± 1.5^{a}	19 ± 5.3^{b}	8.0 ± 1.3^{ab}	7.8	0.021
*Duration of escape attempts (s)	7.2 ± 0.8^{a}	1.3 ± 0.66^{b}	2.8 ± 0.37^{ab}	11	0.005
*Number of grunts	0 ± 0.0^{a}	1.0 ± 0.32^{b}	0 ± 0.0^{a}	10.0	0.007
*Number of squeals	0 ± 0.0	0.6 ± 2.4	0.6 ± 2.4	4.67	0.097
*Duration of grunts (s)	0 ± 0.0^{a}	11 ± 2.8^{b}	0 ± 0.0^{a}	10	0.006
*Duration of squeals (s)	0 ± 0.0	1.9 ± 0.98	0.4 ± 0.19	4.72	0.094
Number of convulsive bouts	4.6 ± 0.81^{a}	12 ± 1.2^{b}	5.4 ± 1.3^{ab}	8.5	0.015
Latency to first convulsion (s)	14 ± 0.6^{ab}	21 ± 2.6^{a}	11 ± 0.8^{b}	11	0.004
Duration of convulsions (s)	20 ± 3.7^{a}	72 ± 7.4^{b}	37 ± 3.9^{ab}	12	0.003
*Latency to loss of coordination (s)	9.5 ± 1.4	17 ± 3.5	8.5 ± 1.6	6.3	0.044
Latency to irreversible loss of posture (s)	14 ± 0.6^{ab}	21 ± 2.6^{a}	11 ± 0.8^{b}	11	0.004
*Latency to laboured breathing (s)	5.8 ± 0.58^{a}	21 ± 1.8^{b}	4.6 ± 0.4^{a}	11	0.005
*Duration of laboured breathing (s)	8.6 ± 0.81	3.0 ± 1.9	6.8 ± 0.9	4.8	0.09
*Intensity of laboured breathing	2.0 ± 0^{a}	0.4 ± 0.24^{b}	1.8 ± 0.20^{a}	11.4	0.003
Latency to gasping (s)	33 ± 4.9^{a}	84 ± 20^{b}	30 ± 5.7^{a}	8.7	0.013
Respiratory arrest (s)	113 ± 6.4^{a}	331 ± 21^{b}	225 ± 14^{ab}	13	0.002

* Measures recorded prior to irreversible loss of posture

CO_2 versus Ar

The time to irreversible loss of posture, indicative of the onset of loss of consciousness, did not significantly differ between CO_2 and Ar.

Piglets in the CO_2 group exhibited escape attempts sooner, with greater frequency and for a longer duration than piglets in the Ar group.

None of the piglets that were euthanased with CO_2 squealed, although the number and duration of squeals prior to loss of posture did not differ. The number and duration of grunts prior to loss of posture was less with CO_2 than Ar.

The latency to onset of convulsions did not differ, however there were fewer convulsive bouts, lasting for a shorter duration, in the CO_2 group. Convulsions occurred at or after loss of posture in both groups.

Laboured breathing occurred sooner with CO_2, and for a longer duration prior to loss of posture, than with Ar.

Both gasping and respiratory arrest occurred sooner with CO_2 than Ar, but occurred after loss of posture in both groups.

CO_2 versus CO_2-Ar

There was no significant difference between CO_2 and CO_2-Ar for any of the behavioural measures.

Ar versus CO_2-Ar

The latency to irreversible loss of posture, indicative of the onset of loss of consciousness, was longer with Ar than CO_2-Ar.

There were no differences in escape behaviour between the two groups.

The number and duration of grunts recorded was less with Ar than CO_2-Ar, although the number and duration of squeals did not differ.

The latency to onset of convulsions did not differ, however there were more convulsive bouts, lasting for a longer duration, in the Ar group. Convulsions in both groups occurred at or after loss of posture.

The latency to laboured breathing was longer with Ar, and the overall intensity less, although the duration prior to loss of posture did not differ between the two groups.

Both gasping and respiratory arrest occurred later with Ar than CO_2-Ar, but occurred after loss of posture in both groups.

4.3.2 Endocrine measures

On average, plasma cortisol concentration increased following euthanasia in all treatment groups. However the magnitude of change was extremely variable, as evidenced by the large standard errors of the means (Table 5). There was no significant effect of treatment on plasma cortisol concentration (H (2df)=0.42, p=0.81). Although not significant, there was a trend toward an effect of treatment on plasma epinephrine concentration (H (2df) =5.18, p=0.075), with a smaller mean increase observed following euthanasia with Ar/CO_2.

Table 5 Mean (±SEM) change in piglet plasma cortisol and epinephrine concentrations (ng ml-^{1}) following euthanasia with 100% CO_2, 100% argon and 40%CO2-60% argon mixture

Treatment Gas	Cortisol Before	Cortisol After	Cortisol Change	Epi*. Before	Epi. After	Epi. Change
CO_2	37.2 (10.4)	44.8 (16.6)	7.6 (25.2)	0.48 (0.22)	25.2 (0.44)	24.7 (0.37)
Ar	86.4 (20.1)	105.3 (19.3)	18.9 (23.7)	0.42 (0.12)	26.6 (0.88)	26.1 (0.87)
CO_2-Ar	65.0 (3.9)	89.0 (13.2)	24.0 (15.4)	3.08 (0.52)	20.0 (3.52)	16.9 (3.87)

*Epi. = epinephrine

4.3.3 EEG and ECG data

The displacement of recording electrodes occurred in five of fifteen animals, as a result of vigorous limb and body movements, meaning that no data were acquired from these. In the remaining ten traces movement artefacts caused by muscle contractions during escape attempts and convulsions periodically obscured EEG and ECG, preventing meaningful analysis of the data.

4.3.4 Welfare-impact Index

Piglets in the CO_2 treatment scored significantly higher on the welfare-impact index (mean 48.8 ± 2.6) than those in either the Ar or CO_2-Ar treatments (means 35.4 ± 3.6 and 35.6 ± 1.6 respectively) ($F(2,12)=7.79$, $p=0.007$) (Figure 2). The magnitude of the difference between mean WI scores is not indicative of the magnitude of differences in welfare impact, given that the index is based upon ranked data.

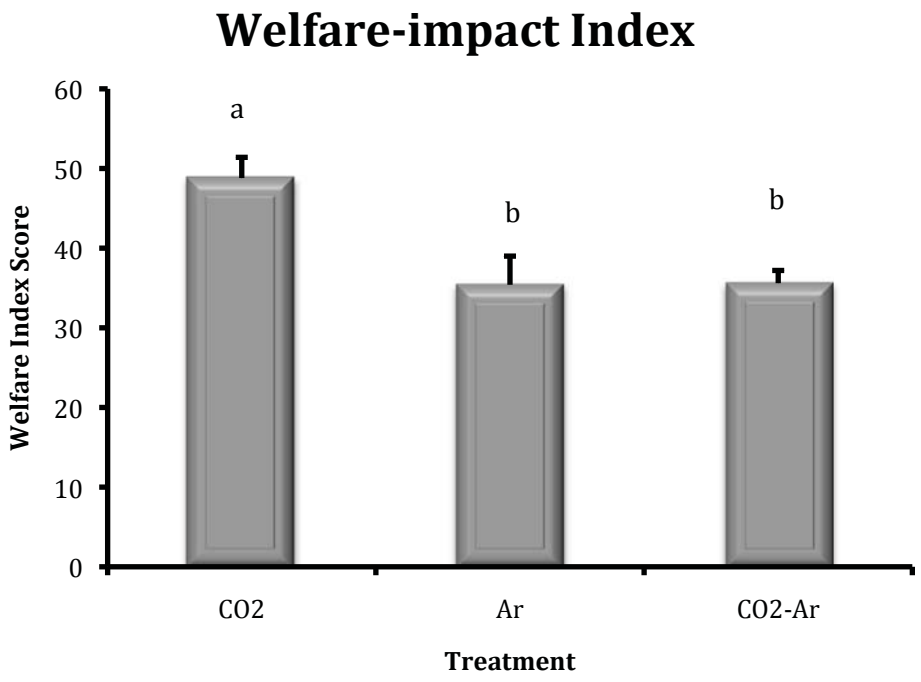

Figure 2 Mean (SEM) welfare-impact index scores for each group of piglets following euthanasia with 100% CO_2, 100% argon and 40%CO_2-60% argon. Means with different letters are significantly different ($p<0.05$)

4.4 Discussion

Based on behavioural measures alone, euthanasia with CO_2 resulted in greater impact on piglet welfare than euthanasia with Ar. Although latency to loss of posture (indicative of the onset of loss of consciousness) did not differ between

treatments, there was significantly greater evidence of distress prior to loss of posture with CO_2 than Ar. Piglets exposed to 100% CO_2 exhibited more escape behaviour and showed signs of laboured breathing sooner, and for a longer duration and greater intensity, than piglets exposed to argon. These data are reflected in the welfare-impact index scores; piglets in the CO_2 group scored significantly higher than those in either the argon or CO_2-argon groups, indicating welfare impacts were highest when 100% CO_2 was the euthanasing agent.

CO_2 versus Argon

Escape attempts occurred sooner and for a longer duration following exposure to CO_2 than Ar, indicating that piglets found CO_2 alone more aversive than argon alone. This is in agreement with the findings of Raj & Gregory (1996). Escape behaviours such as retreating, raising the forelegs against the sides of the chamber, and running or charging at the walls or lid of the chamber are considered evidence of aversion in pigs exposed to hypoxic or hypercapnic atmospheres(Raj and Gregory 1995; Verlarde *et al*. 2007; Dalmau *et al*. 2010b). In the present study, escape attempts were observed in all animals, suggesting that piglets find each gas mixture aversive to at least some degree.

Laboured breathing, indicative of respiratory distress, occurred sooner in piglets exposed to CO_2. In addition, the duration and intensity of laboured breathing was greater with CO_2 than Ar, suggesting that piglets euthanased with CO_2 experienced greater respiratory distress and therefore greater welfare compromise than those euthanased with argon alone. These results are consistent with a previous study on adult pigs, which reported that pigs exposed to various concentrations of CO_2 experienced respiratory distress sooner, and for a greater duration and intensity, than pigs exposed to argon (Raj and Gregory 1996).

Piglets that were euthanased with CO_2 exhibited fewer convulsions, for a shorter duration, than those euthanased with Ar. However, convulsions

occurred during or after loss of posture, suggesting that animals were not conscious at the time. The occurrence of convulsions is therefore unlikely to impact on piglet welfare.

Both gasping and respiratory arrest occurred sooner with CO_2 than Ar. Whilst not relevant to welfare (given that these occurred after loss of posture) both gasping and respiratory arrest indicate suppression of brain stem respiratory centres, suggesting that exposure to CO_2 will induce more rapid death than exposure to Ar.

The overall welfare impact prior to loss of consciousness, as measured by the welfare-impact index, was greater with CO_2 than Ar. The use of 100% argon is thus preferable to 100% CO_2 in terms of piglet welfare.

CO_2 versus CO_2-Argon

There were no significant differences in terms of the individual behaviours used to assess welfare. However, the overall welfare impact of CO_2 prior to loss of consciousness was deemed higher than CO_2-Ar, based upon the WI scores. From a welfare perspective, a CO_2-argon mixture may be preferable to CO_2 alone for euthanasia of pre-weaned piglets.

Argon versus CO_2-Argon

Irreversible loss of posture and the onset of convulsions occurred later with Ar than CO_2-Ar. Loss of posture is considered the first indicator of the onset of unconsciousness (Raj and Gregory 1996). Convulsions are thought to originate through the removal of descending inhibition of the caudal reticular formation (Ernsting 1965), reflecting the suppression of cortical regions associated with the onset of unconsciousness (Raj 1999). Based upon this, the latency to loss of consciousness was deemed to be longer with Ar than CO_2-Ar.

The latency to laboured breathing was also longer with Ar than CO_2-Ar, likely reflecting the more rapid ventilatory response to hypercapnia than hypoxia

(Levitzky 2003); however the duration of laboured breathing did not differ between the two. The only behavioural indicator of welfare impact prior to loss of consciousness that differed between Ar and CO_2-Ar was the intensity of laboured breathing, which was greater with CO_2-Ar than Ar.

The overall welfare impact prior to loss of consciousness, based on WI scores, did not differ between the two. However, given the higher score for laboured breathing intensity and the strong negative emotional impact associated with laboured breathing in humans (Davenport and Vovk 2009; Lansing *et al*. 2009), the use of argon may be preferable to a CO_2-argon mixture for piglet euthanasia, from a welfare perspective.

In the present study, there was no significant effect of treatment on squealing prior to apparent loss of consciousness, making this behaviour of limited use in discriminating between treatments. Squealing occurring after loss of consciousness is involuntary and therefore not relevant to animal welfare. In previous studies evaluating the suitability of hypoxic and hypercapnic gas atmospheres for stunning adult pigs, squealing prior to loss of consciousness has been interpreted as a sign of distress or aversion (Raj and Gregory 1995; Rodriguez *et al*. 2008; Llonch *et al*. 2012). Squealing in response to handling, teeth clipping, ear notching and tail-docking is also associated with distress in conscious pigs (Noonan *et al*. 1994). The absence of squealing in the CO_2 group and the low incidence in the CO_2-argon group may have been related to the marked hyperventilation observed in these animals, which may have affected vocal behaviour.

Grunting prior to loss of posture was only observed in the 100% argon group. This generally occurred soon after the animal was placed in the chamber, whilst it appeared to investigate the chamber environment. No grunting was recorded during escape behaviour or convulsions. Given that grunting appeared to be

associated with exploratory behaviour, it may indicate that argon is less aversive to piglets, at least initially, than CO_2 or a mixture of the two.

Convulsions occurred in all animals across all treatment groups. Given that these always occurred in conjunction with loss of posture, indicative of the onset of unconsciousness (Raj and Gregory 1996), it is unlikely that the piglets experienced any negative welfare effects as a result. However, our inability to record EEG data during convulsions means that consciousness during convulsions cannot be ruled out.

In the present study gasping, signifying suppression of brainstem respiratory centres (St. John 1996; Raj 1999), occurred sooner in the treatments containing CO_2, indicating the more rapid central effects of hypercapnic hypoxia than argon-induced hypoxia. The cessation of gasping, signifying respiratory arrest, occurred significantly sooner with 100% CO_2 than 100% argon, meaning that a shorter exposure time is required to ensure death with CO_2 than with Ar.

No animals in the present study exhibited head shaking, sneezing or coughing upon exposure to any of the test gases. In previous studies involving adult pigs head shaking, coughing or sneezing have been reported in conscious animals exposed to CO_2 (Raj *et al*. 1997; Raj 1999; Rodriguez *et al*. 2008). These were considered indicative of nasal irritation, due to the pungent odour and/or acidic properties of CO_2.

The reason for the absence of such behaviours in the current study is unclear. Piglets of similar age have been shown to exhibit behavioural signs of pain in response to tail docking and castration (Carroll *et al*. 2006; Sutherland *et al*. 2008), suggesting that pain pathways are functional at or soon after birth. As such, we would expect that the animals in the present study were capable of perceiving pain arising from acidification of the nasal mucosa. The absence of overt pain/irritation responses in the present study may have resulted from the rapid development of hypoxic hypercapnia, inducing rapid ventilatory

stimulation, such that the piglet's behavioural response to this may have overshadowed any response to the acidic effects of CO_2. Failure to obtain complete EEG recordings during this study meant that potential nociception could not be evaluated.

Endocrine measures

In the present study, the value of plasma cortisol and epinephrine measures as indicators of welfare impact prior to loss of consciousness was limited. Changes in the activity of the sympathetic nervous system (SNS) and hypothalamic-pituitary-adrenal (HPA) axis have previously been used to evaluate acute physiological stress responses of pigs to painful husbandry procedures such as castration and tail-docking (Prunier *et al*. 2005; Carroll *et al*. 2006; Sutherland *et al*. 2008).

In the present study plasma cortisol varied enormously between individuals, with some showing an increase following euthanasia and others a decrease, compared with pre-treatment values. The reason for these discrepancies is likely due to the effect of other stressors in the hours prior to treatment. Piglets in the current study had been exposed to the potential stressors of separation from the sow, transport from the farm to the laboratory, handling by unfamiliar personnel, introduction to a novel environment and restraint for blood collection in the hours or minutes preceding treatment. These events likely resulted in varying degrees of HPA activity, potentially obscuring any treatment effect on plasma cortisol levels.

In contrast, plasma epinephrine concentrations were similarly elevated in all animals following euthanasia, irrespective of treatment group. The tendency toward a smaller increase in the CO_2-argon group suggests this may have been less stressful to pigs than CO_2 or argon alone, although the influences of blood sampling and handling immediately prior to treatment cannot be excluded, limiting the usefulness of this data.

4.5 Limitations of the study

In the present study, our inability to record EEG activity in the period immediately following transfer to the chamber impaired our ability to determine the point after which consciousness was no longer a possibility. We relied upon the latency to loss of posture, which was coincident with the onset of convulsions, to define the earliest point at which consciousness may have been lost. If the animal did indeed lose consciousness at this point, then it could not experience any pain or distress as a result of convulsions, nor experience any further respiratory distress beyond this point. If, however it remained perceptive beyond this point, then the extent of welfare compromise may have been greater than that reported.

In addition, lack of EEG recordings prevented the evaluation of potential nociception in response to inhalation of CO_2.

In previous pig studies, contamination of EEG recordings due to muscle activity has been minimised either by surgically implanting electrodes in the skull, effectively increasing signal strength and reducing the extraneous influence of muscle electrical activity (Rodriguez *et al*. 2008). Alternatively, restraining the pigs in a hammock reduces the extent of body movements (Martoft *et al*. 2002). In the present study, surgical placement of electrodes was not possible, as we did not have appropriate facilities to house and feed the animals during the period required for recovery from surgery prior to testing. The use of a hammock to restrain pigs during the procedure was deemed inappropriate, as it was thought this would interfere with behavioural analysis, in particular the identification of escape attempts and respiratory distress, which were crucial to welfare evaluation. Therefore, a complementary study was undertaken in which piglets were anaesthetised and administered a neuromuscular block prior to gas exposure in order to prevent skeletal muscle activity and concomitant contamination of EEG recordings (Chapter 5).

4.6 Conclusion

Based on behavioural indicators of welfare impact, and using latency to loss of posture to define the likely period of consciousness, it was concluded that piglet welfare was poorest when CO_2 was the euthanasing agent. Although the use of a CO_2-argon mixture provided some welfare advantages over CO_2 alone, the impact on welfare was nonetheless greater than with argon alone.

From a welfare perspective, 100% argon is preferable to 100% CO_2 or a mixture of 40% CO_2-60% argon for on-farm euthanasia of pre-weaned piglets.

5 Experiment 3. Changes in EEG and ECG activity in halothane-anaesthetised piglets during euthanasia with 100% CO_2, 100% argon or a mixture of 40% CO_2-60% argon

5.1 Introduction

Changes in the EEG provide information on changes in the state of consciousness (Baars 2001), and assist in determining when consciousness is no longer likely or possible (Raj *et al*. 1997; Velarde *et al*. 2002). Determining the time to likely loss of consciousness during euthanasia is crucial to welfare evaluation, as this defines the point beyond which animal welfare can no longer be affected. In the previous study (Chapter 4), loss of posture was the only available indicator of loss of consciousness. The use of EEG data, in conjunction with behavioural data, allows a more comprehensive assessment of latency to loss of consciousness.

In addition, changes in the frequency spectrum of the EEG, obtained through mathematical transformation of the raw EEG signal, can provide information on the cortical processing of nociceptive inputs. Under minimal halothane anaesthesia, quantitative changes in the frequency spectrum of the EEG similar to those seen in unanaesthetised animals still occur, allowing us to make inferences about changes in consciousness and nociceptive processing in animals that are unaware (Murrell and Johnson 2006). This methodology, referred to as the minimal anaesthesia model, involves administering halothane to a stable plane of anaesthesia such that the animals are unconscious, but still able to generate EEG responses to noxious stimulation (Murrell and Johnson 2006). Under minimal anaesthesia, noxious stimuli of sufficient intensity will

produce nociceptive signals that can still reach cortical regions of the brain involved in the processing or perception of pain (Bromm 1984). The minimal anaesthesia model has been used to examine the acute noxiousness of painful stimuli in a range of mammals, including horses (Murrell *et al*. 2003), sheep (Johnson *et al*. 2005a), calves (Gibson *et al*. 2007) and pigs (Haga and Ranheim 2005).

In the previous study (Chapter 4), no behavioural evidence of nociception was observed following exposure of piglets to either 40 or 100% CO_2. Examination of the frequency spectrum of EEG data collected during euthanasia can provide an objective measure of nociception in response to CO_2, allowing a more comprehensive assessment of potential nociception.

Changes in heart rate, reflected in the ECG, can provide evidence of a physiological stress response to a noxious or unpleasant stimulus (Moberg 2000). ECG data collected prior to loss of consciousness may thus be useful in determining the relative welfare impacts of different gases for piglet euthanasia.

This study was therefore undertaken for the following reasons:

1) To accurately determine the relative times to the appearance of transitional and isoelectric EEG waveforms, indicative of the latencies to likely and undisputed loss of consciousness, in piglets being euthanased with 100% CO_2, 100% argon and 40% CO_2-60% argon.

2) To determine whether exposure to 40 or 100% CO_2 induces a nociceptive response in piglets, by conducting spectral analysis of transformed EEG data.

3) To examine changes in heart rates of piglets euthanased with 100% CO_2, 100% argon and 40% CO_2-60% argon in the period prior to likely loss of consciousness, to assess potential physiological stress responses.

5.2 Materials and methods

Approval to undertake this study was granted by the Massey University Animal Ethics Committee (protocol # 10/58).

5.2.1 Animals and housing

Fifteen commercial crossbred (Landrace X Large White) male pigs aged 14 –21 days, weighing between 3.3 and 6.1 kg, (mean 4.6, SD 0.89 kg) were obtained from a commercial swine farm on the morning of the day of testing. Pigs were housed according to the protocol previously described in Experiment 1.

5.2.2 Experimental procedure

Animals were randomly assigned to receive one of the three gas treatments (100% carbon dioxide, 100% argon, or a mixture of 40% carbon dioxide-60% argon, n=5 per treatment).

Each animal was placed into a custom-built perspex induction chamber and anaesthesia was induced with 8% halothane vaporized in air with a flow rate of 4 L min^{-1}. Once adequate anaesthesia depth was achieved (indicated by lateral recumbency and the absence of withdrawal reflex to a toe pinch) the animal was removed from the induction chamber and endotracheal intubation was carried out using a 3.5 – 5.0 mm cuffed endotracheal (ET) tube. Following confirmation of successful intubation by capnometry (MedAir RespSense Capnograph, Nonin Medical Inc., Plymouth, MN, USA), the ET tube was connected to a T-piece breathing circuit and anaesthesia was maintained with 1 –2% halothane delivered in air (4 L min^{-1}) with intermittent positive pressure ventilation (18 –20 cm H_2O maximum inspiratory pressure) (Ventimeter Controller II; Air Shields Vickers, Hatboro, PA, USA). End-tidal CO_2 was maintained in the normocapnic range (40 –50 mmHg) until euthanasia was

begun. Halothane delivery was reduced and maintained at an end-tidal tension of 1.2 ± 0.05%.

Stainless steel needle electrodes were positioned subcutaneously to monitor EEG and ECG activity, as previously described in Chapter 3. A 22-G cannula was positioned in an auricular vein for the administration of medication. Rectal temperature was monitored throughout anaesthesia using a digital thermometer (Q1437, Dick Smith Electronics, NZ) and body temperature was supported with the aid of a circulating warm-water heating blanket set at 38°C (Gaymar, New York, NY, USA). A Doppler plethysmograph was positioned over the foreleg radial artery to monitor arterial blood flow and to determine the time to cessation of cardiac contractile activity.

Continuous monitoring of heart rate, pupil reactivity and lacrimation was carried out to ensure maintenance of anaesthesia throughout the procedure.

When end-tidal halothane was stable at 1.2 ± 0.05%, baseline EEG and ECG were recorded for 10 minutes. Following baseline recording, 1 mg Kg^{-1} atracurium (Tracrium; GlaxoSmithKline, Boronia, VIC) was administered via the venous catheter to immobilise the animal and prevent the appearance of muscle spasms and convulsions that occurred in the unanaesthetised animals in the previous study. After a further ten-minute stabilization period, administration of treatment gas was begun. Administration was via a separate anaesthetic circuit, through a second vaporiser (calibrated to the first to ensure the same halothane concentration) to allow a virtually instantaneous switch from room air to test gas, mimicking experiments 1 and 2 where piglets were placed into a pre-filled chamber.

EEG and ECG data were recorded continuously until death (as determined by isoelectric EEG and the cessation of cardiac contractile activity), at which time

gas administration ceased. EEG and ECG data were analysed off-line at the conclusion of the experiment.

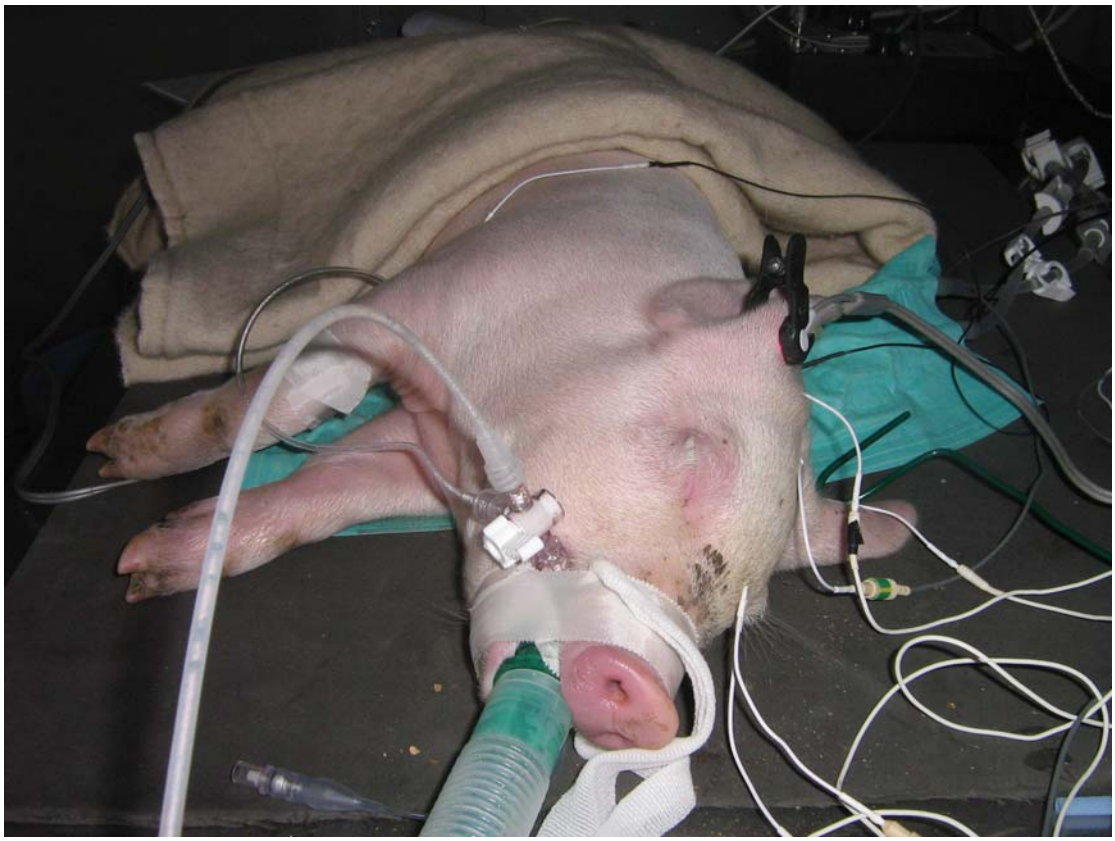

Figure 3 Example of an intubated, anaesthetised piglet. The cables from the EEG recording electrodes are visible in the foreground. The gas sampling tube on the left allowed continuous monitoring of end-tidal halothane and CO_2.

5.2.3 Data Analysis

EEG

Raw data from the EEG were inspected manually and any artefacts (under or over range) were excluded from further analysis. Based on visual inspection, EEG was classified into one of three categories: active, transitional or isoelectric (as defined in Chapter 3).

The total power (Ptot), median frequency (F50) and 95% spectral edge frequency (F95) were calculated for consecutive 1-second epochs for both EEG channels, after Fast Fourier Transformation (FFT) using purpose-written software (Spectral Analyser, CB Johnson, Massey University). For each FFT variable (F50, F95, Ptot), post treatment values were standardised into a percentage of baseline. A 5-point forward-moving average was applied to the standardised data, then a single mean value for F50, F95 and Ptot was calculated for the 5 minutes of baseline prior to gas administration, and for consecutive 5-second blocks following the start of gas exposure, using Microsoft Excel 2008 for Mac.

Cardiac activity

Heart rate was calculated from ECG recordings using the rate meter function in Chart. Heart rate over time was standardised for each individual and displayed as a percentage of baseline. Baseline was calculated as the mean heart rate for the five-minute period immediately preceding gas exposure.

The mean latency to cessation of forelimb blood flow, indicative of cessation of cardiac contractile activity, was calculated for each treatment.

5.2.4 Statistical analyses

Mean latency to the appearance transitional EEG, isoelectric EEG and cessation of cardiac contractile activity were calculated and subjected to non-parametric analysis of variance (Kruskal-Wallis ranks test), using Prism 4 for Macintosh (GraphPad Software Inc), to determine the significance of any observed treatment effects. Dunn's post-hoc test for multiple comparisons was conducted when a treatment effect was found.

Mean F50, F95 and Ptot for each 5-second block after gas exposure, up until 30 seconds, were compared to baseline (7 data points per pig) using repeated measures analysis of variance in SAS® 9.1 (SAS Institute Inc., Cary, NC, USA).

The linear mixed model for repeated measures included the fixed effects of treatment and time, and the random effect of animal. Where a significant effect was found, p values were manually adjusted for multiple comparisons by multiplying by the number of within-treatment comparisons with baseline (6 per treatment). Data from channel 1 and channel 2 (representing the right and left cerebral hemispheres respectively) were analysed separately.

Comparison of spectral variables beyond 30 seconds was not carried out, because EEG in the CO_2 group became transitional beyond this time. Frequency measures of the EEG power spectra depend upon the total power or amplitude of the EEG trace (Gibson *et al*. 2009). Therefore it is only appropriate to compare changes in frequency measures between periods of EEG with similar power or amplitude (Kongara *et al*. 2012). Frequency measures were thus compared between periods of active EEG only.

Heart rate at 5-second intervals from start of gas exposure until the time of appearance of transitional EEG, was compared to baseline for each treatment group, using repeated measures analysis of variance in SAS® 9.1 (SAS Institute Inc., Cary, NC, USA). The linear mixed model for repeated measures included the fixed effects of treatment and time, and the random effect of animal. Where a treatment effect was identified, p values were manually adjusted for multiple comparisons by multiplying by the number of within-treatment comparisons with baseline.

5.3 Results

EEG activity

During gas exposure, the amplitude of the piglet EEG typically remained stable in the period immediately following gas administration, and then exhibited a constant decline over time until it became isoelectric.

Both transitional and isoelectric EEG waveforms were observed significantly sooner in piglets exposed to CO_2 than those exposed to Ar (H (2df)=6.49, p=0.039, and H (2df)=7.98, p=0.019 respectively) (Figures 4 and 5). Latency to the appearance of either transitional or isoelectric EEG did not differ between CO_2-Ar and CO_2 or Ar alone.

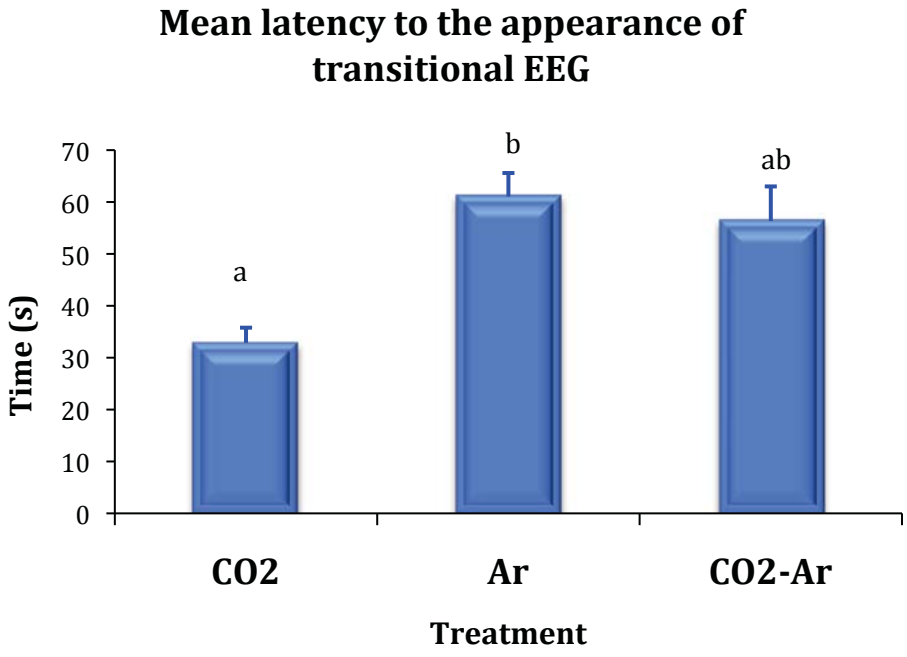

Figure 4 Mean (SEM) latency (seconds from start of exposure) to the appearance of a transitional EEG waveform in piglets exposed to 100% CO_2, 100% argon and 40% CO_2-60% argon (n=5 per treatment). Means with different letters differ significantly (p<0.05)

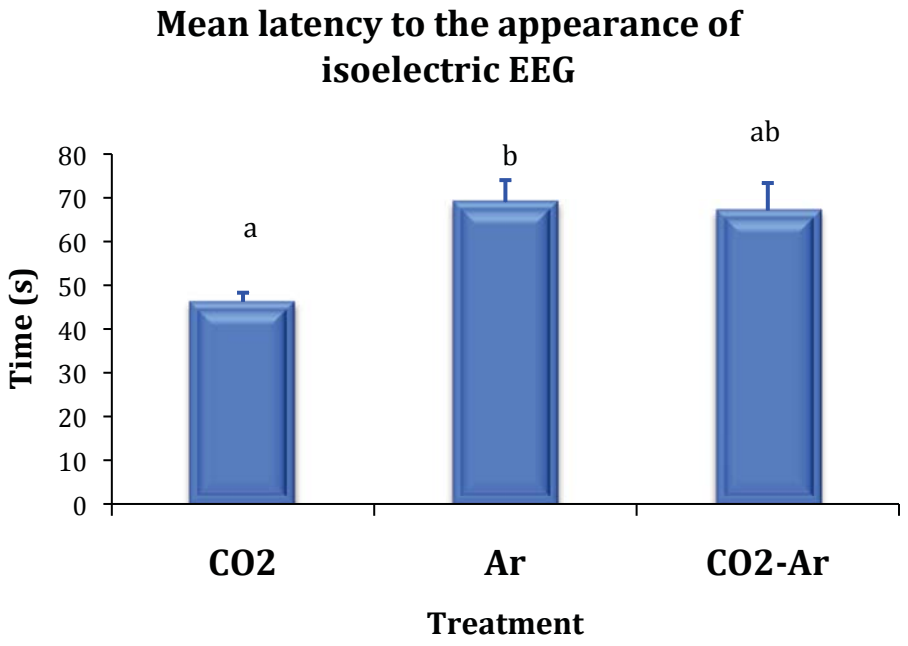

Figure 5 Mean (SEM) latency (seconds from start of exposure) to the appearance of an isoelectric EEG waveform in piglets exposed to 100% CO_2, 100% argon and 40% CO_2-60% argon (n=5 per treatment). Means with different letters differ significantly ($p<0.05$)

The results of the FFT analysis of piglet EEG from channel 2 (recorded from the left cerebral cortex) are presented below. Analysis of Channel 1 data (recorded from the right cerebral cortex) produced similar results. For convenience, these are presented in Appendix B.

There was no significant effect of gas treatment on the percentage change in mean F50 or F95 in the present study, although there was a tendency toward an effect of treatment on the change in Ptot (Table 6). There was a significant effect of time on the changes in F50 and Ptot, along with a significant treatment x time interaction on the change in Ptot (Table 6).

Table 6 Results of repeated measures analysis of variance, showing the influence of treatment and time on the change in mean F50, F95 and Ptot of the piglet EEG (channel 2 data)

	Treatment			Time			Treatment * Time		
	df	F	p	df	F	p	df	F	p
F50	2,12	0.08	0.9228	6,72	2.50	0.0298	12,72	1.48	0.1505
F95	2,12	2.45	0.1285	6,72	0.72	0.6336	12,72	0.88	0.5660
Ptot	2,12	3.42	0.0669	6,72	10.84	<0.0001	12,72	4.07	<0.0001

F50 decreased over time following gas exposure (Figure 6), with the mean across all treatments being lower than that of baseline at 25s (p=0.036) and 30s (p<0.0001) post-treatment.

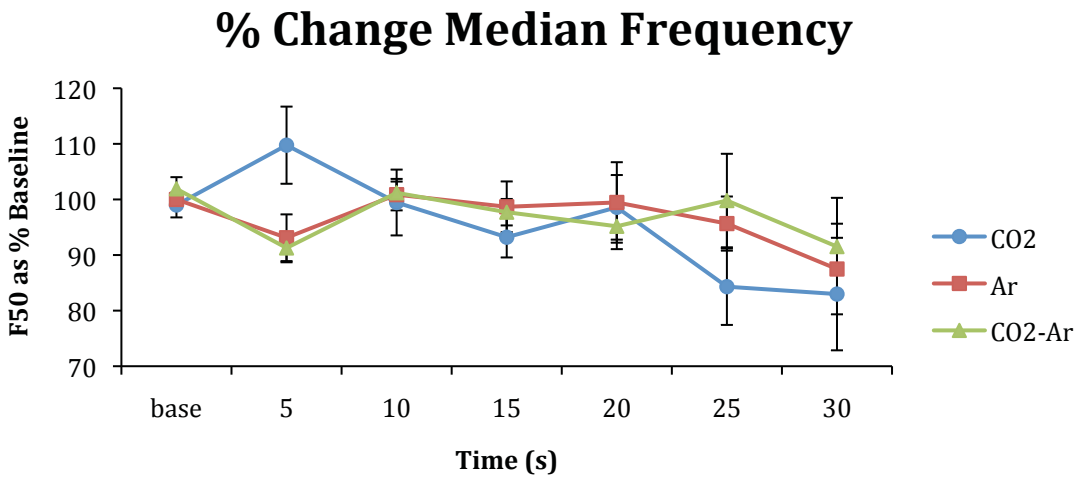

Figure 6 Percentage change in the mean median frequency (F50) of the piglet EEG, relative to baseline, for consecutive 5-second blocks following exposure to 100% CO_2, 100% argon and 40% CO_2-60% argon.

The total power of the piglet EEG also decreased over time, irrespective of treatment (Figure 7), with overall mean at 25 and 30 seconds post-treatment significantly below baseline (p<0.001).

Although mean Ptot in the Ar and CO_2-Ar groups did not differ to their respective baselines between 0 and 30 seconds, mean Ptot in the CO_2 group

was significantly less than baseline at 20 ($p=0.002$), 25 ($p<0.001$) and 30 ($p<0.001$) seconds post-treatment (Figure 7).

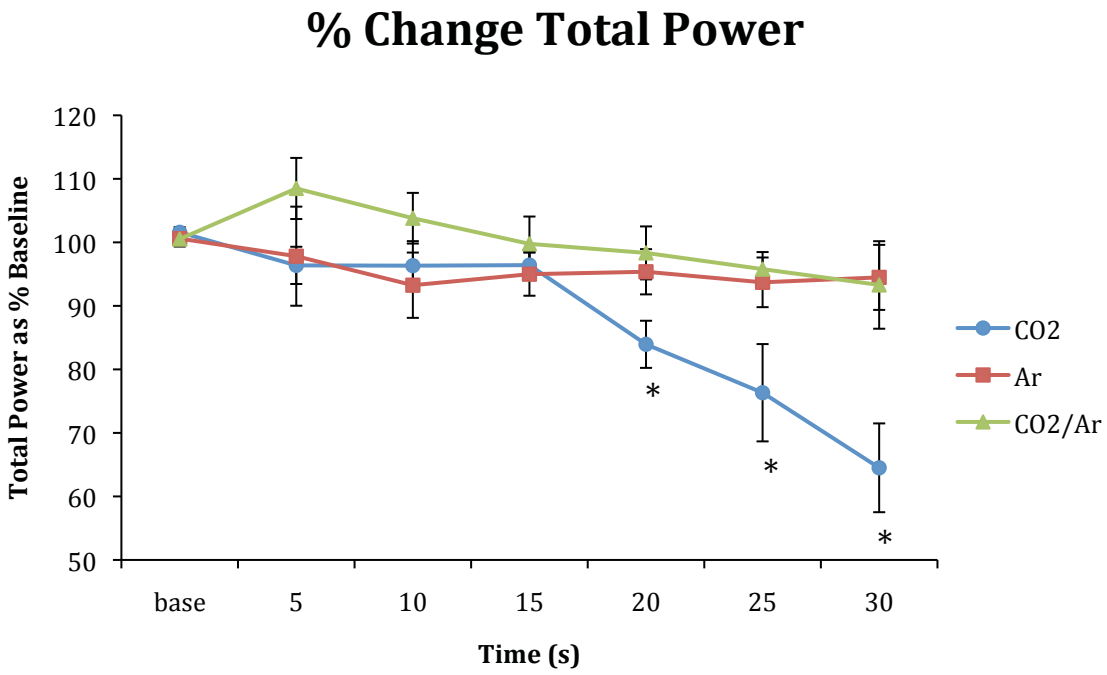

Figure 7 Percentage change in the mean total power (Ptot) of the piglet EEG, relative to baseline, for consecutive 5-second blocks following exposure to 100% CO_2, 100% argon and 40% CO_2-60% argon. An asterisk indicates the treatment mean differed significantly to baseline mean for the indicated gas at the indicated time.

There was no significant effect of treatment or time, or treatment x time interaction, on the change in mean F95 (Table 6).

Cardiac activity

Due to the unavailability of blood flow monitoring equipment, data was only collected from 4/5 animals in the Ar and CO_2-Ar groups. The mean latency to cessation of cardiac contractile activity, indicative of heart failure, did not differ significantly between treatments (H (2df)=3.01, p=0.22), although this often occurred sooner in those piglets euthanased with CO_2 (Table 7).

Table 7. Mean latency to the cessation of cardiac contractile activity in piglets' euthanased with 100% CO_2, 100% argon and 40% CO_2-60% argon

Treatment	Mean (SEM) (s)	Range (s)
CO_2 (n=5)	82.8 (8.8)	57 – 111
Ar (n=4)	181.8 (73.8)	102 – 403
CO_2-Ar (n=4)	108.5 (23.3)	65 – 161

Examination of the ECG was of limited value in determining cessation of cardiac activity, given that the tracings represent cardiac electrical activity rather than contractile activity. In the present study, rhythmic cardiac electrical activity persisted beyond the point where a radial pulse was no longer detectable in 10 of 13 animals, for an average of 105 s (range 4–336 s).

There was a significant effect of time on the mean change in heart rate following gas exposure (Table 8), with mean heart rate across treatments elevated between 35 and 60 seconds following start of gas exposure. There was also a trend toward a significant treatment x time interaction, suggesting that the effect of time was dependant on treatment gas. In the CO_2 and CO_2-Ar groups mean heart rate increased significantly above baseline in the period prior to the appearance of transitional EEG (Figure 8). Heart rate values beyond the time of appearance of transitional EEG in each group are not displayed.

Table 8 Results of repeated measures analysis of variance, showing the effects of treatment and time on the change in mean heart rate

	Treatment			Time			Treatment * Time		
	df	F	p	df	F	p	df	F	p
Heart Rate	2, 12	0.85	0.45	12, 144	10.81	<0.0001	24, 144	1.55	0.06

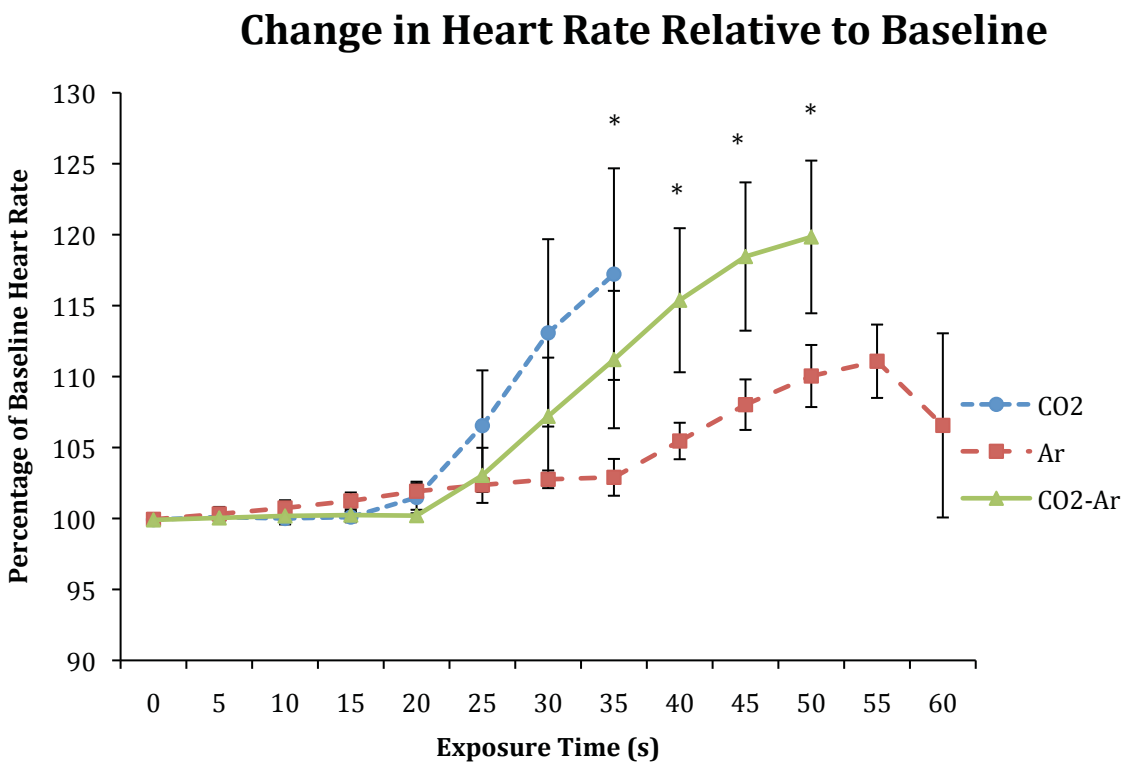

Figure 8. Mean (SEM) change in piglet heart rate, relative to baseline, in the period prior to the appearance of transitional EEG, following exposure to 100% CO_2, 100% argon and a mixture of 40% CO_2-60% argon. An asterisk indicates the treatment mean differed significantly to baseline mean for the indicated gas at the indicated time.

5.4 Discussion

Based on latency to the appearance of both transitional and isoelectric EEG, it was determined that loss of consciousness occurs more rapidly in piglets euthanased with 100% CO_2 than those euthanased with 100% argon.

Piglets in the present study were anaesthetised prior to gas exposure, meaning they were not conscious during the experiment. However, because the level of anaesthesia was minimal, changes in the EEG waveform related to altered states of consciousness in equivalent unanaesthetised animals were still apparent.

The appearance of both transitional and isoelectric EEG waveforms occurred significantly sooner when CO_2 was the euthanasing agent than when Ar was. The onset of a transitional EEG waveform illustrates a degree of cortical suppression likely representative of loss of awareness in an equivalent unanaesthetised animal (Gibson *et al*. 2009), whilst an isoelectric EEG waveform is incompatible with conscious awareness . This indicates that loss of consciousness would occur sooner following exposure to 100% CO_2 than 100% argon. From a welfare perspective, this means that the period during which the animal is capable of experiencing potential negative impact is shorter with 100% CO_2 than 100% argon. Argon did not differ to CO_2-Ar in latency to transitional or isoelectric EEG, suggesting no welfare advantage of CO_2-Ar over Ar alone in terms of speed of induction of unconsciousness.

Spectral analysis of EEG data collected from piglets during euthanasia revealed no clear evidence of nociception in the 30 seconds following gas exposure in any treatment, although this may have been due to methodological limitations.

The typical pattern of response to noxious stimulation is a transient increase in the F50 along with a transient decrease in the Ptot of the EEG in the period immediately following application of the noxious stimulus (Murrell and Johnson

2006). A number of mammalian EEG studies have identified an increase in F50 and decrease in Ptot of the EEG following application of a known noxious stimulus (e.g. Johnson *et al*. 2005b; Gibson *et al*. 2007; Kongara *et al*. 2010). Noxious stimulation in the form of a tail clamp applied to rats, or electrical stimulus applied to the hind limb of dogs, elicited changes in F50 and Ptot of the EEG within 10 seconds of stimulus application (Diesch *et al*. 2009; Kongara *et al*. 2010).

In the present study, F50 did not differ to baseline in the 30 seconds following exposure to any treatment gas. In the CO_2 group, F50 was elevated (but not significantly, $p>0.05$) relative to baseline at 5 seconds post-treatment (mean 109.9 (6.94)%; Figure 6).

There was however a significant treatment x time interaction on the change in Ptot, with Ptot in the CO_2 group being significantly lower than that of baseline at 20, 25 and 30 seconds post-treatment (Figure 7). In this group, piglet EEG remained active (of the same amplitude as baseline) until a mean of 32.9 (range 22–38) seconds post-treatment, meaning that the significant reduction in Ptot observed at 20 seconds was unlikely a result of cortical suppression induced by CO_2. However, the absence of a significant increase in F50 means that the change in total power alone cannot be taken as conclusive evidence of nociception.

Previous studies have shown that nociceptors in the nasal mucosa of man and rats respond to CO_2 concentrations of 30 –40% (Thurauf *et al*. 1991; Anton *et al*. 1992; Peppel and Anton 1993), and that inhalation of 40 –55% CO_2 reportedly causes pain in the eyes, nose and throat of humans, through stimulation of nociceptors in the mucosa of the corneal, nasal and upper respiratory regions (Anton *et al*. 1992; Danneman *et al*. 1997). Therefore the absence of a nociceptive response, particularly to 100% CO_2, in this study was unexpected.

Whilst the absence of a clear nociceptive response in the EEG of pigs exposed to either 40% or 100% CO_2 in this study may indicate that CO_2 does not induce a nociceptive response in young piglets, it is also possible that methodological limitations prevented the detection of any such response. The minimal anaesthesia model has previously been used to examine EEG responses to noxious physical and electrical stimuli (e.g. Johnson *et al*. 2005a; Kongara *et al*. 2010). It may be that the model is less sensitive to noxious chemical stimuli, thus failing to demonstrate a response to the CO_2 stimulus.

Alternatively, the use of a neuromuscular block and the necessity for endotracheal intubation in the present study meant that neither the corneal or nasal mucosae were exposed to CO_2, preventing potential activation of nociceptors in these regions, possibly accounting for the lack of a nociceptive response in the EEG of piglets in this study.

There was no difference in the latency to cessation of cardiac contractile activity between treatments, suggesting that time to death did not vary with treatment gas.

Analysis of ECG revealed an increase in mean heart rate prior to likely loss of consciousness (as determined by the appearance of a transitional EEG waveform), in piglets being euthanased with CO_2 and CO_2-Ar (Figure 9).

An increase in resting heart rate is commonly associated with an acute stress response in animals (Moberg 2000). The significant rise in heart rate following gas exposure in the CO_2 and CO_2-Ar groups indicates that stress responses were triggered as a result of gas exposure in these groups. The absence of a significant increase in heart rate in the Ar group indicates that physiological stress responses were not triggered in this group, suggesting welfare impact prior to loss of consciousness was less with Ar than either CO_2 or CO_2-Ar. This is in agreement with the welfare impact scores reported in Chapter 4.

5.5 Limitations

Spectral analysis of the EEG did not reveal any indication of nociception in response to either 40% or 100% CO_2, despite evidence indicating these concentrations stimulate nociceptors in the corneal and respiratory mucosa of man (Anton *et al*. 1992; Danneman *et al*. 1997), and the nasal mucosa of rats (Thurauf *et al*. 1991). However, the use of a neuromuscular block in the present study necessitated that the animals be intubated to allow mechanical ventilation; thus CO_2 was delivered directly to the trachea, precluding exposure of the corneal, nasal or upper respiratory tract mucosae as occurs during CO_2 euthanasia in a chamber. Therefore the possibility of a nociceptive response to CO_2 or CO_2-Ar euthanasia in piglets cannot be discounted based on the results of this study.

5.6 Conclusion

From a welfare standpoint, the use of CO_2 results in a shorter period of awareness following gas exposure than argon, thus reducing the duration of potential negative experience associated with gas exposure. The addition of CO_2 to Ar offered no benefit over Ar alone, in terms of induction of unconsciousness.

There was no conclusive evidence of a nociceptive response to the inhalation of either 40% or 100% CO_2, however this may have been due to the methodology employed. Further study is required to rule out a nociceptive response to these CO_2 concentrations in piglets being euthanased in an enclosed chamber.

Euthanasia of piglets with CO_2 or CO_2-Ar induces a stress response prior to the onset of unconsciousness, based on the increases in heart rate observed prior to the onset of a transitional EEG waveform. The lack of a significant increase in heart rate in the Ar group suggests that euthanasia with this gas is less stressful to pigs prior to loss of consciousness than either CO_2 or CO_2-Ar.

6 General discussion and conclusion

The purpose of this research was to determine the relative welfare impacts of 100% CO_2, 100% argon and a mixture of 40% CO_2-60% argon for euthanasia of pre-weaned piglets. Of the gases evaluated, it appears that 100% carbon dioxide, whilst inducing the most rapid loss of consciousness and death, also results in the greatest negative welfare impact prior to loss of consciousness. Argon, on the other hand, had the least impact on welfare of the gases evaluated.

Loss of consciousness may be inferred behaviourally, based on loss of posture (Raj and Gregory 1996) or by examining changes in the amplitude or power of the EEG(Blackmore and Newhook 1982; Gibson *et al*. 2009). In Chapter 4, the time to loss of posture did not differ between the 3 gases. However, analysis of EEG in Chapter 5 revealed that cortical suppression, leading to loss of conscious awareness, occurred significantly sooner with CO_2 than with Ar, based on the rate of development of transitional and isoelectric EEG waveforms. The CO_2-Ar mixture did not differ to either CO_2 or Ar in latency to the appearance of transitional or isoelectric EEG. It may be concluded that duration of potential negative experience is shortest when CO_2 is the euthanasing agent.

Despite the faster onset of unconsciousness with CO_2, there was significantly greater evidence of welfare compromise in the period prior to loss of consciousness than was observed with either Ar or CO_2-Ar.

Behavioural evidence of negative impact prior to loss of consciousness included escape attempts, laboured breathing and squealing (Chapter 4). Escape attempts, considered a sign of distress or aversion (Raj 1999; Dalmau *et al*. 2010b), were observed in response to all 3 gas treatments, but occurred sooner and for a longer duration in piglets exposed to CO_2 than those exposed to Ar. Laboured breathing, indicative of breathlessness, also occurred sooner and for

a longer duration in piglets exposed to CO_2 than those exposed to Ar, as well as being rated as more intense. Breathlessness in response to hypercapnia is described as being highly unpleasant in man (Liotti *et al*. 2001; Lansing *et al*. 2009), therefore laboured breathing was deemed indicative of reduced welfare in piglets. Squealing was only observed in the Ar and CO_2-Ar groups, however the number and duration of squeals did not differ significantly with treatment. Although CO_2 and CO_2-Ar did not differ in terms of individual behaviours assessed prior to loss of consciousness, the WI scores, based on ranked data, indicated that CO_2 resulted in greater negative welfare impact than CO_2-Ar (Chapter 4). Based upon the earlier onset and greater intensity of laboured breathing, CO_2-Ar was deemed to have greater negative welfare impact than Ar, despite these not differing in WI scores.

In addition to behavioural evidence of reduced welfare, increased heart rate prior to loss of consciousness was also recorded following exposure to both CO_2 and CO_2-Ar in anaesthetised piglets (Chapter 5). An increase in heart rate is commonly associated with acute stress in mammals (Moberg 2000). The absence of a significant increase in heart rate in the Ar group indicates that exposure to 100% argon does not initiate physiological stress responses prior to loss of consciousness, suggesting that welfare is less affected by exposure to Ar than to CO_2 or CO_2-Ar, which is consistent with the behavioural observations and WI scores in Chapter 4.

Plasma cortisol and epinephrine increased following euthanasia with all 3 gases, however there was no significant effect of treatment (Chapter 4). These measures were of limited value as indicators of welfare during euthanasia for 2 reasons. Firstly, there was considerable variation in pre-euthanasia values, likely due to the effects of other stressors such as separation from the sow, transport, handling and blood sampling. Secondly, post-treatment samples were taken post-mortem, meaning that changes occurring prior to loss of consciousness could not be distinguished from those occurring after loss of consciousness.

Although not directly relevant to welfare, determining the latency to death with each treatment gas is important in determining optimal exposure times for euthanasia. From a practical perspective, a shorter exposure time means that more animals can be euthanased within a defined time period, potentially improving efficiency when large numbers of animals require euthanasia. The criteria used to determine death in these studies included the appearance of isoelectric EEG, permanent cessation of respiration and permanent cessation of cardiac contractile activity. In the main study (Chapter 4), death was determined by respiratory arrest alone, due to the failure of EEG and ECG recordings in some animals. However, piglets were checked for absence of a detectable heartbeat after removal from the chamber. In the anaesthesia study piglets were mechanically ventilated, precluding evaluation of respiratory arrest; therefore death was determined by the cessation of arterial blood flow, in conjunction with isoelectric EEG.

Respiratory arrest occurred sooner in conscious piglets exposed to CO_2 than Ar, with CO_2-Ar not differing significantly to either. Although isoelectric EEG developed sooner in anaesthetised pigs following exposure to CO_2 than Ar, there was no apparent difference in latency to cessation of cardiac activity between groups, although the unavailability of monitoring equipment for some animals may have reduced our ability to detect any existing differences. The latency to respiratory arrest (Table 4) was greater than the latency to cessation of cardiac activity (Table 7) for all 3 gases tested. Exposure times must therefore exceed those of the latency to respiratory arrest in conscious pigs. Based upon this, a longer exposure time is required to ensure death with Ar than CO_2.

Spectral analysis of EEG from anaesthetised piglets revealed no changes consistent with nociception during euthanasia with any of the 3 gases (Chapter 5). Previous studies have shown that nociceptors in the nasal mucosa of man and rats respond to CO_2 concentrations of 30–40% (Thurauf *et al*. 1991; Peppel

and Anton 1993), with humans reporting the sensation of pain in the eyes, nose and throat following exposure to 50% CO_2 or more (Anton *et al*. 1992; Danneman *et al*. 1997). The absence of a nociceptive response in this study, particularly to 100% CO_2, was unexpected. However, the method employed required the animals to be intubated, preventing exposure of the corneal, nasal or upper respiratory tract mucosa to the test gas, which may have affected our results. Further study is required to rule out a nociceptive response to 40 or 100% CO_2 in pre-weaned pigs.

This study has identified that 100% argon is preferable in welfare terms to 100% CO_2 or a mixture of 40% CO_2-60% argon for the euthanasia of suckling piglets. However, it has also demonstrated that piglets euthanased with argon or a CO_2-argon mixture experience a degree of distress or aversion in the period prior to loss of consciousness, as evidenced by the elevation in heart rate, performance of escape attempts and squealing, and the incidence of laboured breathing. Bearing in mind that an 'ideal' euthanasia method should induce no (or at least minimal) pain, distress or anxiety prior to loss of consciousness, none of these agents can realistically be considered ideal.

However, as discussed previously, much of the concern regarding the use of BT stems from the degree of operator involvement, which may impact on the reliability of the method, and on the aesthetic acceptability, both to operators and to the public. Because gas euthanasia in an enclosed chamber effectively eliminates direct operator involvement in the act, as well as avoiding traumatic injury, it may be perceived as 'gentler' and more aesthetically acceptable, and therefore be preferable to both the industry and pork consumers.

Without accurate information on the reliability with which BT is performed, it is difficult to conclude whether argon euthanasia would provide any welfare advantage over BT. If it were demonstrated that there was a high incidence of insufficient blows administered during BT euthanasia of young piglets, with subsequent welfare impacts of greater intensity and duration than demonstrated

here, then the distress associated with argon euthanasia may be deemed an acceptable trade off for improved reliability.

7 Future Research

Having identified argon as the most welfare-friendly of the gases evaluated for on-farm euthanasia of pre-weaned piglets, it would be beneficial to evaluate the welfare benefits of argon across the entire age range of pre-weaned animals (newborn to approx 3 weeks). The present study tested piglets aged between two and three weeks, the upper end of the age range. Given the evidence for greater hypoxia tolerance in newborn mammals (Glass *et al*. 1944), along with the approximately four times greater blood oxygen affinity in newborn versus two-week old piglets (Novy *et al*. 1973), it may well be that the exposure time required to ensure death in newborn piglets is much greater than that required for 2 –3 week old piglets in the present study (331± 21s until respiratory arrest in conscious piglets (Chapter 4)). In order to recommend an exposure time that would ensure death over the entire birth to three-week age range, it is necessary to evaluate the responses of younger animals.

In order to comprehensively examine the welfare impact of CO_2 or CO_2-Ar, it would be useful to study piglet EEG responses to CO_2 exposure in a chamber, to determine whether nociception occurs. This would require an alternative method of immobilising the animal to prevent physical activity and concomitant artefacts in the EEG recordings. It may be possible to do so by pre-anaesthetising the animal with halothane prior to transfer to the pre-filled chamber. If the animal were to remain anaesthetised for a sufficient period after transfer to the chamber, much of the behavioural responses to gas exposure that were observed in the main study (Chapter 4) may be prevented. In addition, placing the animal in a hammock within the chamber may reduce the amount of physical activity were anaesthesia to wear off, as this reportedly reduces physical activity in conscious adult pigs during gas euthanasia (Martoft *et al*.

2002). Whist these measures may prevent or reduce artefacts due to voluntary muscle movements, artefacts due to convulsions, as observed in all gas treatments in the main study, would likely still occur. However, given that convulsions were associated with loss of posture, which is considered to indicate the onset of loss of consciousness (Raj and Gregory 1996), any nociceptive response to CO_2 exposure beyond this point will no longer impact on piglet welfare, as the animal is no longer capable of perceiving pain. Therefore prevention of convulsions may not be necessary for the evaluation of nociceptive responses to CO_2 prior to loss of consciousness.

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9 Appendix A

Table 9 Raw data used to calculate welfare-impact index scores

Pig #	Treatment	Latency to LoP (s)	Rank	Escape duration (s)	Rank	Lab. Breath. duration (s)	Rank	Lab. Breath intensity.	Rank	Duration of squealing (s)	Rank	Sum of ranks
6	CO_2	15	8.5	10	15	11	15	2	11	0	6	55.5
7	CO_2	13	5.5	6	12	6	6	2	11	0	6	40.5
8	CO_2	13	5.5	8	14	8	9	2	11	0	6	45.5
9	CO_2	15	8.5	6	12	9	12.5	2	11	0	6	50.0
10	CO_2	16	11	6	12	9	12.5	2	11	0	6	52.5
11	Ar	16	11	0	1.5	0	2	0	2	0	6	21.5
12	Ar	30	15	1	3	9	12.5	1	5	0	6	41.5
13	Ar	16	11	2	5	0	2	0	2	4.5	15	35.0
14	Ar	22	14	0	1.5	6	6	1	5	1	12.5	39.0
15	Ar	20	13	3.5	9	0	2	0	2	4	14	40.0
16	CO_2-Ar	10	1.5	3	7.5	6	6	2	11	0.5	6	32.0
17	CO_2-Ar	10.5	3	2	5	6.5	8	2	11	0.5	6	33.0
18	CO_2-Ar	14	7	2	5	9	12.5	1	5	0	6	35.5
19	CO_2-Ar	12.5	4	4	10	8.5	10	2	11	0	6	41.0
20	CO_2-Ar	10	1.5	3	7.5	4	4	2	11	1	12.5	36.5

NB: In the case of ties, the mean rank was assigned to both individuals

LoP = Loss of posture

Lab. Breath. = Laboured breathing

10 Appendix B

Results of spectral analysis of Channel 1 EEG data (Chapter 5)

There was a significant effect of time on the change in F50 of the piglet EEG (Table 10), with mean F50 across treatments decreasing over time. At 25 and 30 seconds post-treatment mean F50 was significantly below the baseline mean ($p=0.01$ and $p<0.0001$, respectively) (figure 9).

Table 10 Results of repeated measures analysis of variance, showing the influence of treatment and time on the change in F50, F95 and Ptot of the piglet EEG (Channel 1)

	Treatment			Time			Treatment * Time		
	df	F	p	df	F	p	df	F	p
F50	2,12	0.74	0.4998	6,72	8.24	<0.0001	12,72	1.10	0.3742
F95	2,12	0.17	0.8496	6,72	3.28	0.0065	12,72	0.50	0.9064
Ptot	2,12	4.43	0.0361	6,72	9.72	<0.0001	12,72	3.72	0.0002

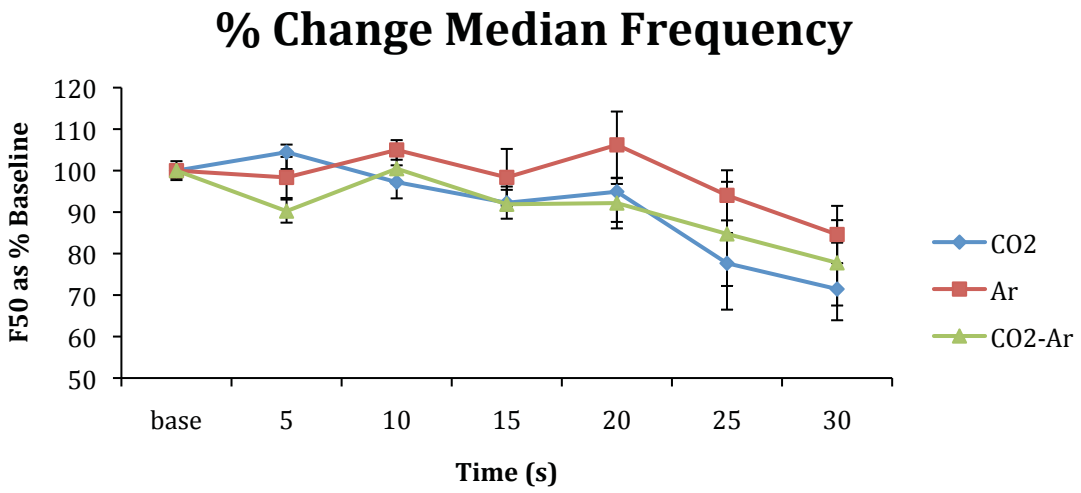

Figure 9 Percentage change in the mean median frequency (F50) of the piglet EEG (Channel 1), relative to baseline, for consecutive 5-second blocks following exposure to 100% CO_2, 100% argon and 40% CO_2-60% argon

There was no significant effect of treatment, time, or interaction on the change in mean F95 (Table 10).

The total power of the piglet EEG in channel 1 varied significantly with treatment and over time, as well as showing a significant treatment x time interaction (Table 10).

Total power in the CO_2 group differed significantly to that of the CO_2-Ar group ($p=0.048$), whilst the Ar group did not differ to either the CO_2 or CO_2-Ar groups ($p=0.13$ and $p=1.0$, respectively).

The mean total power across all treatments decreased over time (Figure 10), differing significantly to baseline mean at 20s ($p=0.062$), 25s ($p=0.0006$) and 30s ($p<0.0001$) post-treatment.

Although mean Ptot in the Ar and CO_2-Ar groups did not differ to their respective baselines between 0 and 30 seconds, mean Ptot in the CO_2 group was significantly less than baseline at 20 ($p=0.014$), 25 ($p<0.001$) and 30 ($p<0.001$) seconds post-treatment (Figure 10).

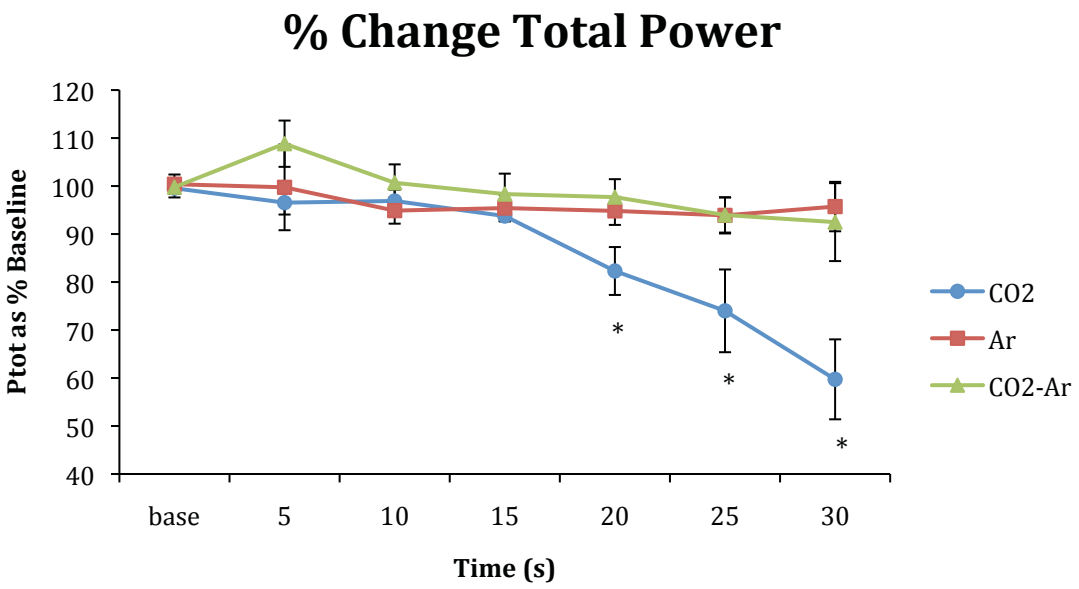

Figure 10 Percentage change in the mean total power (Ptot) of the piglet EEG (Channel 1), relative to baseline, for consecutive 5-second blocks following exposure to 100% CO_2, 100% argon and 40% CO_2-60% argon. An asterisk indicates the treatment mean differed significantly to baseline mean for the indicated gas at the indicated time