

ANIMAL WELFARE

Title: The use of different gases and gas combinations to humanely euthanize young suckling pigs - #NPB #09-199 **Revised**

Investigator: Mhairi Sutherland

Institution: AgResearch Ltd

Date Submitted: 2/14/2011

Industry Summary:

There are times on a swine farm when pigs become ill or injured, and the animal care person must decide if euthanasia is necessary, and if so what method of euthanasia is most humane for the pig, while alleviating worker stress. Blunt force trauma to the head is currently the most commonly employed method of 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 led to the need for alternative methods to be developed. In contrast, gaseous methods of euthanasia, although requiring the use of specialized equipment in the form of a chamber and gas delivery system, may be both more reliable and less disturbing for the personnel involved and may therefore be a preferable method for on-farm euthanasia if managed well. Gas euthanasia has the additional advantage of not requiring the animal to be restrained by an additional person. Several gases are currently used to euthanize animals, such as rodents and poultry, including argon (Ar), nitrogen (N₂) and carbon dioxide (CO₂). However, animals exposed to CO₂ may display behavioral and/or physiological signs of distress in response to CO₂ inhalation. Using other gases, such as Ar or N₂, either alone or in combination with CO₂ appears to be preferable to CO₂ alone, as gauged by measures of animal well-being. The other advantage to using Ar or N₂ is that they are inert, non-flammable and non-explosive gases, making them practical choices from a work safety stand point. Few studies have been conducted evaluating the effectiveness of these gases (Ar or N₂) as a means of on-farm euthanasia in young pigs, therefore the objective of this study was to evaluate different gases and gas mixtures (CO₂, Ar and N₂) as a humane method for euthanasia of young suckling pigs. In experiment 1, piglets received one of five gas treatments: 100% carbon dioxide (CO₂), 90% argon in air (Ar), 90% nitrogen in air (N₂), a mixture of 30% carbon dioxide/60% argon in air (Ar/CO₂) or a mixture of 40% carbon dioxide/50% nitrogen in air (N₂/CO₂). In experiment 2 and 3, piglets received one of three gas treatments: 100% carbon dioxide (CO₂), 100% argon (Ar), or a mixture of 40% carbon dioxide/60% argon (Ar/CO₂). On each occasion a chamber was filled with the test gas and a piglet was placed inside. Throughout the experimental period, behavioral (e.g. escape attempts, vocalizations, loss of coordination, respiratory effort, convulsions) and physiological (e.g. brain activity as measured by electroencephalogram (EEG), cardiac activity as measured by electrocardiogram (ECG) and respiratory rate) data were continuously recorded until death. In addition, plasma cortisol and adrenaline levels (common markers of stress in pigs) were determined before treatment and immediately following death. A welfare index was established to assess the relative welfare compromise induced by each gas treatment. The index included five behavioral measures observed in the period prior to apparent loss of consciousness, beyond which there was no further potential for welfare compromise. These

These research results were submitted in fulfillment of checkoff-funded research projects. This report is published directly as submitted by the project's principal investigator. This report has not been peer-reviewed.

For more information contact:

National Pork Board • PO Box 9114 • Des Moines, IA 50306 USA • 800-456-7675 • Fax: 515-223-2646 • pork.org

measures were: latency to onset of convulsions, duration of escape behavior, duration of increased respiratory effort, respiratory effort grade and duration of squealing. In experiment 1, four of the five gas treatments evaluated (Ar, CO₂/Ar, N₂ and CO₂/N₂) contained 10% air and therefore 2.1% residual oxygen. The duration of labored breathing, indicative of respiratory distress, was greater in those treatments containing residual oxygen. Additionally, animals in three of the residual oxygen treatments exhibited conscious behavior after the initial onset of convulsions, representing potentially serious welfare compromise due to distress or injury sustained during convulsions. Based upon the latency to respiratory arrest in each treatment, it appears that the inclusion of residual oxygen may prolong the survival time of piglets when exposed to lethal amounts of Ar or N₂, without any apparent welfare benefit. It was thus decided to exclude residual oxygen from the gas treatments evaluated in the subsequent experiments the following three gas treatments were selected for further evaluation: 100% CO₂, 100% Ar and 40% CO₂/60% Ar. In experiment 1 and 2, the sum of ranks for each animal across the 5 measures yielded a single score indicative of welfare compromise, with a lower score equating to less compromise. According to this index, CO₂ induced greater welfare compromise than either Ar or Ar/CO₂. Furthermore, epinephrine concentrations in response to euthanasia were lower in piglets exposed to Ar/CO₂ as compared to Ar or CO₂ alone. These results suggest that 100% CO₂ may cause piglets' distress prior to loss of consciousness. Although Ar and the Ar/CO₂ mixture did not significantly differ in terms of welfare impact, times to loss of consciousness, isoelectric EEG and respiratory arrest were shorter with Ar/CO₂ than Ar, making this potentially more useful from a practical standpoint. However, the degree of welfare compromise observed in all treatments suggests that other alternatives to manually applied blunt trauma should be investigated.

Keywords: Behavior, Euthanasia, Gas, Humane, Suckling

Scientific Abstract:

When euthanasia of sick or injured animals is required on animal welfare grounds, a method that induces minimal welfare compromise should be chosen. Blunt force trauma to the head is currently the most commonly employed means 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 led to the need for alternative methods to be developed. The objective of this study was to evaluate different gases and gas mixtures (CO₂, argon and nitrogen) as a humane method for euthanasia of young suckling pigs. In experiment 1, piglets received one of five gas treatments: 100% carbon dioxide (CO₂), 90% argon in air (Ar), 90% nitrogen in air (N₂), a mixture of 30% carbon dioxide/60% argon in air (Ar/CO₂) or a mixture of 40% carbon dioxide/50% nitrogen in air (N₂/CO₂). In experiment 2 and 3, piglets received one of three gas treatments: 100% carbon dioxide (CO₂), 100% argon (Ar), or a mixture of 40% carbon dioxide/60% argon (Ar/CO₂). On each occasion a chamber was filled with the test gas and a piglet was placed inside. Throughout the experimental period, behavioral (escape attempts, vocalization, loss of coordination, respiratory effort, convulsions) and physiological (electroencephalogram (EEG), electrocardiogram (ECG), respiratory rate) data were continuously recorded until death. In addition, plasma cortisol and adrenaline levels were determined before treatment and immediately following death. A welfare index was established to assess the relative welfare compromise induced by each gas treatment. The index included five behavioral measures observed in the period prior to apparent loss of consciousness, beyond which there was no further potential for welfare compromise. These measures were: latency to onset of convulsions, duration of escape behavior, duration of increased respiratory effort, respiratory effort grade and duration of squealing. In experiment 1, four of the five gas treatments evaluated (Ar, CO₂/Ar, N₂ and CO₂/N₂) contained 10% air and therefore 2.1% residual oxygen. The duration of labored breathing, indicative of respiratory distress, was greater in those treatments containing residual oxygen. Additionally, animals in three of the residual oxygen treatments exhibited conscious behavior after the initial onset of convulsions, representing potentially serious welfare compromise due to distress or injury sustained during convulsions. Based upon the latency to respiratory arrest in each treatment, it appears that the inclusion of residual oxygen may prolong the survival time of piglets when exposed to lethal hypoxia or hypercapnic hypoxia, without any apparent welfare benefit. It was thus decided to

exclude residual oxygen from the gas treatments evaluated in the subsequent experiments the following three gas treatments were selected for further evaluation: 100% carbon dioxide, 100% argon and 40% carbon dioxide/60% argon. In experiment 1 and 2, the sum of ranks for each animal across the 5 measures yielded a single score indicative of welfare compromise, with a lower score equating to less compromise. According to this index, CO₂ induced significantly greater welfare compromise (49.8 ± 2.77 , mean \pm SEM) than either Ar (35.2 ± 3.56), or Ar/ CO₂ (37.0 ± 0.89). These results suggest that 100% CO₂ may cause piglets' distress prior to loss of consciousness. Although argon and the mixture did not significantly differ in terms of welfare impact, times to loss of consciousness, isoelectric EEG and respiratory arrest were significantly shorter with Ar/ CO₂ than Ar ($P < 0.05$), making this potentially more useful from a practical standpoint. However, the degree of welfare compromise observed in all treatments suggests that other alternatives to manually applied blunt trauma should be investigated.

Introduction:

In the course of swine husbandry there are occasions when young pigs become ill or injured and farm workers must decide if euthanasia is necessary, and if so which method is most appropriate. The American Veterinary Medical Association (AVMA) guidelines on euthanasia (American Veterinary Medical Association, 2007) endorse several methods of euthanasia as being acceptable for piglets less than 3 weeks of age. These include overdose of injected barbiturate, anesthetic overdose, carbon dioxide (CO₂) inhalation, non-penetrating captive bolt and blunt force trauma to the head (AVMA, 2007). Of these, both barbiturates and anesthetics are regulated substances that require appropriately qualified personnel to obtain and administer. In practical terms, this means employing the services of a veterinarian, which may not be practical when haste is required and may be cost prohibitive when large numbers of animals are involved. Whilst captive bolt, blunt force trauma and CO₂ do not pose this problem, there are nonetheless issues with their use. The AVMA Guidelines on Euthanasia stipulate that euthanasia methods should induce rapid loss of consciousness preceding death, and should minimize any distress and anxiety experienced prior to loss of consciousness (AVMA, 2007). In addition, the psychological well-being of personnel performing the procedure should be considered.

Captive bolt or blunt force trauma (in the form of a blow to the head), if delivered accurately and with sufficient force, should induce instant loss of consciousness in the animal and when directly followed by exsanguination represents a humane method of euthanasia. However, incorrect placement or the use of insufficient force may fail to cause loss of consciousness, whilst causing pain and distress to the animal. In addition, even when performed accurately, this method may be emotionally disturbing to those carrying out or witnessing the procedure. In contrast, gaseous methods of euthanasia, although requiring the use of specialized equipment in the form of a chamber and gas delivery system, may be both more reliable and less disturbing for the personnel involved and may therefore be a preferable method for on-farm euthanasia if managed well. Gas euthanasia has the additional advantage of not requiring the animal to be restrained by an additional person. Several gases are currently used to euthanize animals including argon, nitrogen and CO₂. Gaseous methods of euthanasia are routinely used for laboratory rodents, with CO₂ being the most commonly used agent. The use of CO₂ is becoming increasingly popular for the stunning of pigs in slaughter plants in the US and Europe (Raj & Gregory, 1995). High concentrations of CO₂ cause central nervous system depression leading to loss of consciousness and subsequent death. However, prior to this there is the potential for animals to experience severe breathlessness due to hypercapnia (Lansing, Gracely, & Banzett, 2009; Liotti, et al., 2001), as well as pain due to the formation of carbonic acid in the nasal and respiratory mucosa (Anton, Euchner, & Handwerker, 1992; Danneman, Stein, & Walshaw, 1997). Behavioral and/or physiological signs of distress in response to CO₂ inhalation have been reported in rats (Niel & Weary, 2006), broiler chickens (Lambooj, et al., 1999; McKeegan, et al., 2007) and pigs (Raj & Gregory, 1995). Other gases and gas mixtures involving CO₂ have been tested to reduce these behavioral signs of distress, including argon and nitrogen. Aversion to 90% argon and 90% CO₂ was tested in pigs (Raj and Gregory, 1995). Pigs did not show any aversion to inhalation of 90% argon, but the majority of pigs found 90% CO₂ to be aversive (Raj and Gregory, 1995). Euthanasia studies involving broiler chickens have been conducted using argon, nitrogen, or mixtures of these gases with CO₂ (Lambooj et al., 1999; McKeegan et al., 2007). Using other gases either alone or in combination with CO₂ appears to be preferable to CO₂ alone, as gauged by measures of animal well-being. The other advantage to using argon or nitrogen is that they are inert, non-flammable and non-explosive gases, making them practical choices from a work safety stand

point. Few studies have been conducted evaluating the effectiveness of these gases (argon and nitrogen) as a means of on-farm euthanasia in young pigs, therefore the objective of this study was to evaluate different gases and gas mixtures (CO₂, argon, and nitrogen) as a humane method for euthanasia of young suckling pigs.

Objectives:

To evaluate different gases and gas mixtures (CO₂, argon and nitrogen) as a humane method for euthanasia of young suckling pigs.

Materials & Methods and Results:

Experiment 1

Materials and methods

Animals

Crossbred (Landrace X Large white) piglets aged 18 days were sourced from a commercial swine farm on the morning of treatment. Prior to treatment, the animals were held in a 28°C temperature controlled ventilated room, containing straw bedding over non-slip concrete flooring.

Experimental procedure

Animals were randomly assigned to receive one of five gas treatments: 100% carbon dioxide (CO₂), 90% argon in air (Ar), 90% nitrogen in air (N₂), a mixture of 30% carbon dioxide/60% argon in air (Ar/CO₂) or a mixture of 40% carbon dioxide/50% nitrogen in air (N₂/CO₂).

A 52-liter plastic storage container (558 x 390 x 327mm) with lid was modified to create the euthanasia chamber. Perspex windows were inserted in the lid and one side to allow unobstructed viewing and video recording of behavior throughout the procedure. Gas inlet and outlet hoses were fixed in one end. A short plastic tube (20mm diameter) with a bung was fixed into the lid to allow instrument cables to be connected to a chart recorder.

The chamber was pre-filled with the appropriate test gas, sourced from cylinders of compressed CO₂, N₂, Ar and air (Air Liquide New Zealand Ltd, Penrose, Auckland) connected to the chamber. Gas flow was maintained throughout the experimental procedure at a rate of 10 liters per minute.

On each occasion one animal was instrumented and placed on its feet in the chamber. The lid was replaced and physiological and behavioral measures were continuously recorded until death, as defined by cessation of respiratory activity along with electrical silence in both the electrocardiogram (ECG) and electroencephalogram (EEG).

Physiological Measures

Prior to gas treatment, and immediately following death, a blood sample was collected from the jugular vein of each animal, using a vacutainer containing EDTA. Plasma was separated by centrifugation then stored at -80°C for later analysis of cortisol and epinephrine by enzyme immunoassay (R&D Systems Parameter Cortisol Assay KGE008, R&D Systems Minneapolis, MN; AlpcO AP17-EPIHU-e01 Epinephrine assay, AlpcO Diagnostics Salem, NH).

Respiratory rate was recorded using a custom-made adjustable belt incorporating a saline-filled pressure cuff connected to a force transducer, which was linked to a chart recorder. The belt was fitted around the animal's abdomen prior to placement in the chamber.

Electrocardiogram (ECG) was recorded by fitting stainless steel sub dermal needle electrodes were across the animal's chest prior to placement in the chamber, to allow continual recording of heart rate and cardiac electrical activity. Data was recorded digitally for off-line analysis at the conclusion of the experiment.

Electroencephalogram (EEG) was recorded by placing four stainless steel sub dermal needle electrodes into the animal's head just prior to placement in the chamber allowed electrical activity from both the right and left cerebral hemispheres to be recorded throughout the treatment period. Data was recorded digitally for off-line analysis at the conclusion of the experiment.

Behavioral Measures

The behavior of the pigs during gas exposure was recorded with two video cameras located outside the chamber. The first camera was fixed above the chamber to record a dorsal view, whilst the second recorded a side view. At the completion of the experiment both video recordings were analyzed and each animal scored on the following parameters:

1) Vocalization: Squealing and grunting. The time of occurrence, type, frequency and duration of vocalization was recorded for each animal.

2) Escape attempts (backing away, butting head or shoulders against the chamber walls or lid, raising forelegs on side of chamber (Raj & Gregory, 1996)). The time of onset, number and duration of attempts was recorded for each animal.

3) Loss of coordination. The time of onset of indicators of loss of coordination (loss of balance, stumbling, falling, diminished muscle control) was noted for each animal.

4) Respiratory effort: changes in breathing patterns during treatment were scored as follows:

Labored breathing: an increase in rate and/or depth of respiration. The time of onset of labored breathing was noted. The duration of labored breathing was calculated from the time of onset until apparent loss of consciousness (onset of convulsions after which no further conscious behavior was observed).

Gasping: low frequency ($\leq 4/\text{min}$) very deep breathing through open mouth, accompanied by large abdominal movement and stretching of neck. Considered an indicator of the onset of respiratory arrest (Rodriguez, et al., 2008). Time of onset and frequency were noted.

Respiratory arrest: cessation of respiratory effort, with no recovery (recording continued for a minimum of sixty seconds). Time of occurrence was noted.

Respiratory effort grade: a four point scale, similar to that of Raj & Gregory (1996) was devised, where 0=closed mouth, no increase in rate or depth; 1=closed mouth, increase in rate and/or depth; 2=open mouth with increased rate and/or depth; 3=gasping (as per above). This was used to rate respiratory distress intensity during the period of labored breathing. The highest observed score during this period was recorded as the overall grade.

5) Convulsions: defined as involuntary contractions of the voluntary muscles (Blood, Studdert, & Gay, 2007). Convulsions were classified by type and intensity (see below). The time of onset and duration were recorded for each individual bout (no convulsive activity for >1 second indicated the end of a bout), and for all bouts combined for each animal.

Tonic: prolonged generalized contraction of the skeletal muscles.

Clonic: alternating contraction/relaxation in quick succession.

Paddling: involuntary walking/running/galloping motion of the limbs (considered clonic convulsive activity).

Convulsive activity rating: a three point scale similar to that used by (Nowak, Mueffling, & Hartung, 2006) was adopted, where 1=weak paddling motion of the limbs, involving 1-4 limbs, only partial extension/flexion, slow speed (1 or less motions per second) OR twitching or extension of 1 or more limbs, or entire body lasting for one second or less; 2=running motion involving one or both pairs of legs, limbs may fully extend, more than 1 motion per second, no pronounced curvature of the spine, motion does not cause animal to shift position or prolonged extension of one or more limbs (more than 1 second), repeated jerking motions of entire body, may involve body lifting off floor but animal remains in original orientation; 3=running or galloping motion involving all four limbs, very rapid (2 or more per second), pronounced curvature of spine as hooves move toward belly, violent action causes movement of animal around chamber OR rapid and violent tonic/clonic spasms, including spine flexion in all planes, may involve head shaking, causing body to lift off floor and move around.

6) Urination or defecation. Time and frequency of occurrence were noted.

7) Head shaking, sneezing or coughing: these have been considered indicative of nasal irritation (Raj, 1999; Raj, Johnson, Wotton, & McInistry, 1997; Rodriguez, et al., 2008). Time and frequency of occurrence were noted.

Results

The latency to respiratory arrest was considerably greater in those treatments where residual oxygen remained (Ar, CO₂/Ar, N₂, CO₂/N₂), compared with 100% CO₂ (Table 1). In addition, behavioral evidence of consciousness (sitting, standing and deliberate movements) was observed following the onset of convulsions in the Ar, N₂ and CO₂/Ar treatments, as reflected by the discrepancies between onset of convulsions and apparent loss of consciousness in these treatment groups (Table 1). The time to both apparent loss of consciousness and respiratory arrest were shortest in the CO₂ treatment (15s and 114s, respectively).

Cortisol concentrations did not differ ($P>0.05$) among treatments. Epinephrine concentrations did not differ ($P>0.05$) among treatments prior to euthanasia, but were greater ($P<0.005$) after euthanasia for all gas treatments (Figure 1).

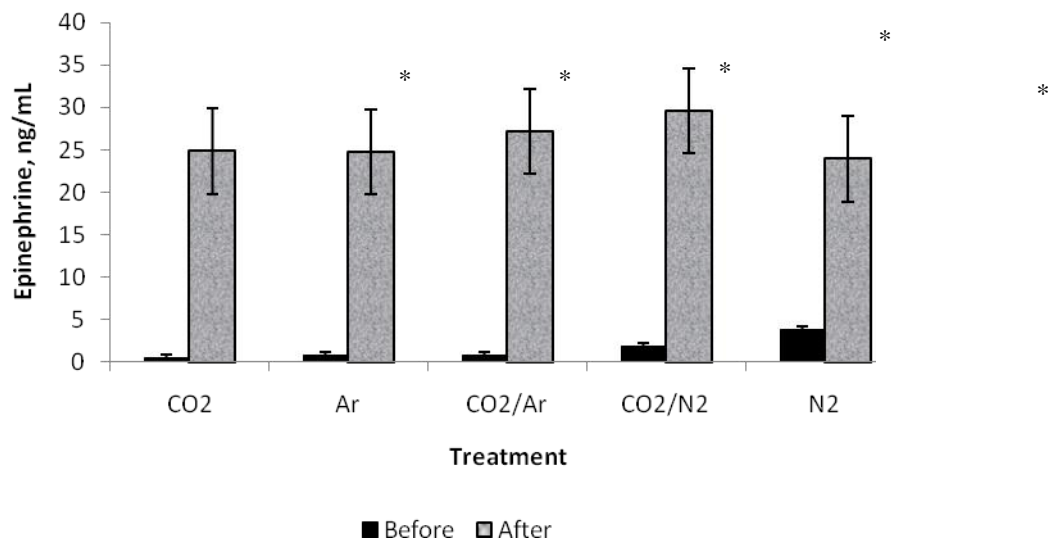
Based on the observations from this experiment, the following three gas treatments were selected for further evaluation: 100% carbon dioxide, 100% argon and 40% carbon dioxide/60% argon.

Table 1. Responses of piglets exposed to each of the 5 gas treatments

Treatment	CO ₂	Ar	CO ₂ /Ar	CO ₂ /N ₂	N ₂
escape attempts	2	1	8	1	2
total vocalizations	5	4	33	1	16
squeals	5	4	1	1	5
grunts	0	0	32	0	11
vocalization duration (s)	11.5	8	32	14	45.5
squeal duration (s)	11.5	8	16	14	33.5
escape onset (seconds from time of transfer)	11	20	178	10	12
escape duration (s)	3	4	15	3	38
convulsive bouts	9	20	11	15	13
convulsion onset (seconds from time of transfer)	15	25	199	60	365
convulsion duration (s)	42	161.5	63	63	104
labored breathing onset (seconds from time of transfer)	1	40	50	4	215
labored breathing duration (s)	14	45	318	56	218
gasping onset (seconds from time of transfer)	32	185	410	45	371
respiratory arrest (seconds from time of transfer)	114	470	630	527	780
apparent loss of consciousness (time to onset of convulsions after which no evidence of consciousness was observed)	15	85	368	60	433

Figure 1: Epinephrine concentrations before and after euthanasia in response to 5 gas treatments.

*Epinephrine concentrations within gas treatments differ at $P < 0.05$



Experiment 2

Materials and methods

Animals

Commercial crossbred (Landrace X Large white) male pigs aged 14-20 days were obtained from a commercial swine farm on the morning of treatment and housed together in a 28°C temperature controlled ventilated room, containing straw bedding over non-slip concrete flooring, until the time of treatment.

Experimental Procedure

Animals were randomly assigned to receive one of three gas treatments ($n = 5$ per treatment): 100% carbon dioxide (CO₂), 100% argon (Ar), or a mixture of 40% carbon dioxide/60% argon (Ar/CO₂). The order of treatment was randomly selected.

The euthanasia chamber was the same as that described in experiment 1. The chamber was pre-filled with the appropriate test gas, sourced from cylinders of compressed CO₂ or argon connected to the chamber. Gas flow was maintained throughout the experimental procedure at a rate of 10 liters per minute.

On each occasion one animal was instrumented and placed on its feet in the chamber. The lid was replaced and physiological and behavioral measures were continuously recorded until death, as defined by cessation of respiratory activity along with electrical silence in both the electrocardiogram (ECG) and electroencephalogram (EEG).

The physiological and behavioral measures recorded were identical to those described in experiment 1 above.

Data analysis

Heart rate was calculated from ECG data, using the rate meter function in Chart (ADInstruments Ltd). EEG recordings were visually inspected and classified into one of three categories: active, transitional or isoelectric (Gibson, et al., 2009). Active EEG represented normal cerebrocortical activity. Transitional EEG was classified as having amplitude of less than 50% of the baseline (pre-treatment) EEG. Isoelectric EEG was classified as a stable trace consisting of background noise with amplitude $< 1/8$ of baseline EEG.

A welfare index was established to assess the relative welfare compromise induced by each gas treatment. The index included five behavioral measures observed in the period prior to apparent loss of consciousness, beyond which there was no further potential for welfare compromise. The measures included in the index were: latency to onset of convulsions, duration of escape behavior, duration of

increased respiratory effort, respiratory effort grade and duration of squealing. The sum of ranks for each animal across the 5 measures yielded a single score indicative of welfare compromise, with a lower score equating to less compromise.

Analysis of variance was conducted using Prism 4 for Macintosh (GraphPad Software Inc) to determine the significance of any observed treatment differences (Kruskal-Wallis / Dunn's multiple comparison). The level of statistical significance was $P < 0.05$.

Results

As shown in Table 2, there were treatment differences ($P < 0.05$) in escape behavior, vocalization, convulsive activity, respiratory effort, gasping and time to respiratory arrest. Piglets in the CO₂ group made more escape attempts and spent more time engaging in escape behavior than piglets in the Ar group. In addition, the onset of escape behaviors was quicker in CO₂ than Ar piglets.

Piglets vocalized more frequently and for greater duration in the Ar than CO₂ treatment. More convulsive bouts occurred with Ar than with CO₂. Piglets breathing became labored quicker when exposed to CO₂ or Ar/CO₂ than Ar. Gasping occurred sooner in the CO₂ or mix treatments than Ar. Respiratory arrest occurred sooner in piglets exposed to CO₂ (113 ± 6.4 s) than in those exposed to Ar (331 ± 21 s).

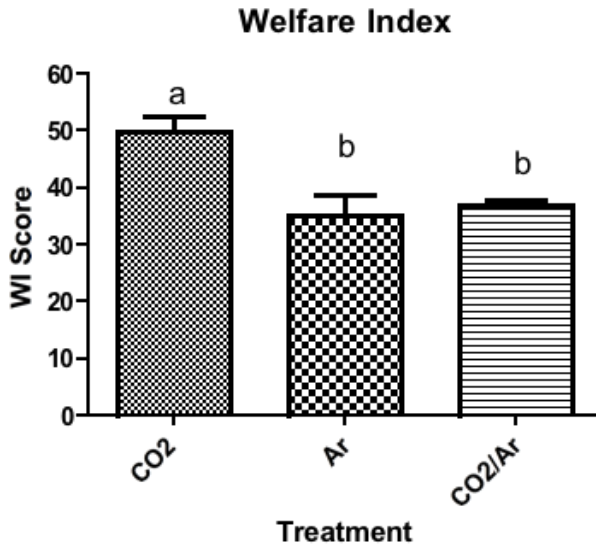
Table 2. Mean \pm SEM for each treatment across measures

Measured Variable	CO ₂	Ar	Ar/CO ₂
Number of escape attempts	2.4 ± 0.5^a	0.6 ± 0.2^b	1.2 ± 0.2^{ab}
Onset of escapes (s)	3.9 ± 1.5^a	19 ± 5.3^b	8.0 ± 1.3^{ab}
Duration of escape attempts (s)	7.2 ± 0.8^a	1.3 ± 0.7^b	2.8 ± 0.4^{ab}
Number of vocalizations	3.8 ± 0.8^a	6.6 ± 0.7^b	5.6 ± 0.5^{ab}
Number of grunts	0 ± 0.0^a	2.2 ± 0.4^b	1.0 ± 0.6^{ab}
Number of squeals	3.8 ± 0.8	4.4 ± 0.8	4.6 ± 0.6
Duration of vocalization (s)	6.3 ± 2.4^a	36 ± 6.9^b	12 ± 2.4^{ab}
Duration of grunts (s)	0 ± 0.0^a	11 ± 2.8^b	2.3 ± 1.5^{ab}
Duration of squeals (s)	6.3 ± 2.4^a	25 ± 5.0^b	9.9 ± 2.5^{ab}
Number of convulsions	4.6 ± 0.8^a	12 ± 1.2^b	5.4 ± 1.3^{ab}
Onset of convulsions (s)	14 ± 0.6^{ab}	21 ± 2.6^a	11 ± 0.8^b
Duration of convulsions (s)	20 ± 3.7^a	72 ± 7.4^b	37 ± 3.9^{ab}
Onset of loss of coordination (s)	9.5 ± 1.4	17 ± 3.5	8.5 ± 1.6
Onset of labored breathing (s)	5.8 ± 0.6^a	21 ± 1.8^b	4.6 ± 0.4^a
Onset of gasping (s)	33 ± 4.9^a	84 ± 2^b	30 ± 5.7^a
Respiratory arrest (s)	113 ± 6.4^a	331 ± 2^b	225 ± 1^{ab}
Welfare Index (ranked data)	49.8 ± 2.8^a	35.2 ± 3.6^b	37.0 ± 0.9^b

^{a,b}Means in the same row with different superscripts differ significantly ($P < 0.05$)

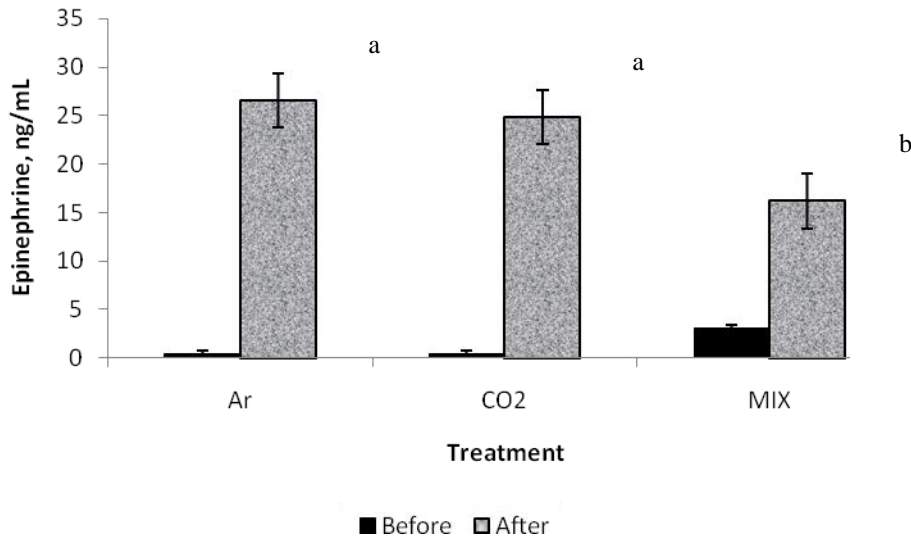
The mean welfare index scores were higher ($P < 0.05$) in piglets exposed to CO₂ (49.8 ± 2.8) compared with Ar or Ar/CO₂ (35.2 ± 3.6 and 37.0 ± 0.9) (Figure 2).

Figure 2: Mean Welfare Index scores for each gas treatment. Means with different superscripts differ significantly ($P < 0.05$)



Cortisol concentrations did not differ ($P > 0.05$) among treatments. Epinephrine concentrations did not differ ($P > 0.05$) among treatments prior to euthanasia, but were greater ($P < 0.005$) after euthanasia for all gas treatments (Figure 3). Epinephrine concentrations were lower ($P < 0.005$) in piglets exposed to Ar or CO2/Ar mixture compared with Ar or CO2 gas alone.

Figure 3: Epinephrine concentrations before and after euthanasia in response to 3 gas treatments. ^{a,b}Epinephrine concentrations after euthanasia differ at $P < 0.05$.



EEG data was unavailable from four of the fifteen piglets due to electrode displacement during the course of the experiment. In the remaining eleven data sets the times to appearance of transitional or isoelectric EEG could not be clearly defined. Movement of piglets in the chamber (passive, escape related, muscular convulsions or gasping) created noise artifacts in the EEG tracing which obscured cortical responses in the first 24-78 seconds after the start of treatment and intermittently beyond this. ECG tracings were also contaminated by movement artifact, however cessation of cardiac electrical activity (the experimental end point) could still be clearly determined.

The electrical activity generated by the brain can be recorded from inside the cranium using implanted electrodes or from outside the cranium using scalp electrodes. When recording from inside the cranium, the bones of the skull act as an attenuator for the muscular electrical activity that is generated when the animal moves and it is possible to make recordings from active animals, but this approach requires the

electrodes to be surgically implanted some time prior to the recording of data (usually a few weeks) and so is not suitable for studies requiring young animals. When recording from the scalp, the bones of the skull attenuate the EEG signal and not the muscular activity. This means that animals need to be anaesthetised to abolish muscular activity if a decent EEG signal is to be recorded. This approach allows immediate recording from replaced electrodes. This use of the EEG in anesthetized animals is a derivation of the minimal anesthesia technique that has to date been validated in 10 species of mammals including pigs (Murrell and Johnson, 2006; Gibson et al., 2007; McCracken et al., 2010). Due to the fact that EEG could not be accurately recorded in the experiment 2, it was decided to repeat this study using the minimal anesthesia technique so we could get accurate EEG and ECG recordings, in particular times to transitional or isoelectric EEG.

Experiment 3

Materials and methods

Animals

Fifteen commercial crossbred (Landrace X Large white) male pigs aged 14-20 days were obtained from a commercial swine farm on the morning of treatment and housed together in a 28°C temperature controlled ventilated room, containing straw bedding over non-slip concrete flooring, until the time of treatment.

Experimental Procedure

Animals were randomly assigned to receive one of three gas treatments (n = 5 per treatment): 100% carbon dioxide (CO₂), 100% argon (Ar), or a mixture of 40% carbon dioxide/60% argon (Ar/CO₂). The order of treatment was randomly selected.

Each piglet was placed into a perspex custom-built induction chamber and anesthesia induced with 8% Halothane vaporized in air (4l/min). Once adequate anesthesia was achieved (indicated by lateral recumbency and absence of withdrawal reflex to toe pinch), endotracheal intubation was carried out using a 3.5 - 5.0 mm cuffed endotracheal (ET) tube. After confirmation of successful intubation by capnometry, the ET tube was connected to a breathing circuit and anesthesia was maintained with halothane delivered in air. Piglets were mechanically ventilated (18-20 cm H₂O maximum inspiratory pressure) with end-tidal CO₂ maintained in the normocapnic range (40-50 mmHg). Halothane delivery was reduced until an end-tidal tension of 1.2% was obtained, and maintained at this level. Stainless steel needle electrodes were applied subcutaneously to monitor EEG and ECG activity (7 in total). A 22 gauge venous cannula was inserted in an auricular vein for the administration of atracurium (neuromuscular blocking agent). A digital rectal thermometer was used to continuously monitor body temperature. A Doppler plethysmograph was positioned over the radial artery in the foreleg to monitor movement of blood in the cardiovascular system. When end-tidal halothane was stable at 1.2% and end-tidal CO₂ was stable in the normocapnic range, baseline EEG/ECG was recorded for 10 minutes.

Atracurium (Tracrium; GlaxoSmithKline, Boronia, VIC) was injected (1 mg/kg) to prevent muscular movement during the treatment period. Following a ten-minute stabilization period, administration of test gas was begun. Treatment gas was administered from a separate anesthetic system, through a second vaporizer (calibrated to the first to ensure the same halothane concentration) to allow virtually instantaneous switch from room air to test gas (mimicking experiments 1 and 2 where piglets were placed in a pre-filled chamber). Continuous monitoring of heart rate, pupil reactivity and lacrimation was carried out to ensure maintenance of anesthesia throughout the procedure.

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 analyzed off-line at the conclusion of the experiment.

Data Analysis

Heart rate was calculated from ECG recordings, using the rate meter function in Chart (ADInstruments Ltd). EEG recordings were visually inspected and classified into one of three categories: active, transitional or isoelectric, as previously defined in experiment 2. Fast Fourier Transformation and calculation of median frequency (F50), 95% spectral edge frequency (F95) and total power (P_{tot}) indices

were performed using purpose-written software (Spectral Analyser; CB Johnson, Massey University, Palmerston North, 2002). Subsequent analysis was conducted using Microsoft Excel Mac 2008 (Microsoft Corporation, Redmond, WA, USA). Data from EEG spectral analysis are displayed as percentage change in specific indices, using mean values in the 5-minute period prior to gas administration as baseline values. Times to transitional EEG, isoelectric EEG and cessation of cardiac contractile activity were subjected to analysis of variance, using Prism 4 for Macintosh (GraphPad Software Inc), to determine the significance of any observed treatment differences (Kruskal-Wallis / Dunn's multiple comparison). The level of statistical significance was $P < 0.05$.

Results

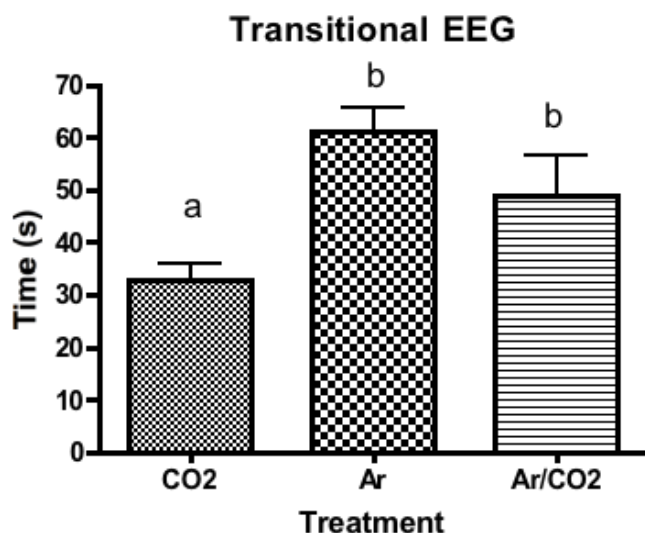
The time to cessation of cardiac contractile activity was recorded for 13 of the 15 animals tested, due the Doppler plethysmograph being unavailable during two treatment periods. The means across treatments were calculated, as shown in Table 3. Although the mean time to cessation of cardiac activity was shorter in the CO₂ than Ar or Ar/CO₂ treatments, these did not differ significantly, largely due to individual variation within treatments.

Table 3. Mean times to cessation of cardiac contractile activity in piglets euthanized with CO₂, Ar and Ar/CO₂

Treatment	Mean (s)	SEM
Ar	108.0	3.0
CO ₂	82.8	8.3
Ar/CO ₂	108.5	23.25

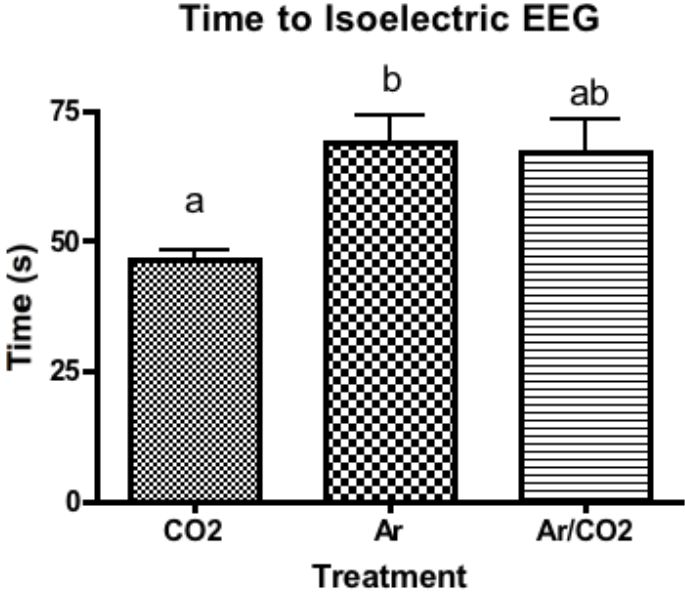
The appearance of transitional EEG occurred significantly sooner in the CO₂ treatment group (32.9 ± 2.9 s, mean \pm SEM) than either the Ar (61.1 ± 4.5 s) or Ar/CO₂ (48.9 ± 7.7 s) groups (Figure 4).

Figure 4: Mean times to transitional EEG in piglets euthanized with 100% CO₂, 100% argon and 40%CO/60% Ar. Means with different superscripts differ significantly ($P < 0.05$).



EEG traces became isoelectric following 46.2 ± 2.1 s exposure to CO₂, compared with 69.0 ± 5.0 s and 67.1 ± 6.3 s exposure to Ar and Ar/CO₂ respectively (Figure 5).

Figure 5: Mean times to isoelectric EEG in piglets euthanized with 100% CO₂, 100% argon and 40%CO/60% Ar. Means with different superscripts differ significantly ($P<0.05$).



Four of the five animals in the argon treatment exhibited slight stretching movements of the neck in the later stages of gas administration. These movements resembled those observed in non-anaesthetized pigs during terminal gasping, although much less pronounced. The movements were manually recorded on the EEG tracing.

Figure 6: Percentage change in the median frequency (F50), relative to pre-treatment values, of the EEG of piglets during euthanasia with 100% CO₂, 100% argon and 40%CO/60% Ar. Data are displayed as 5-point moving averages.

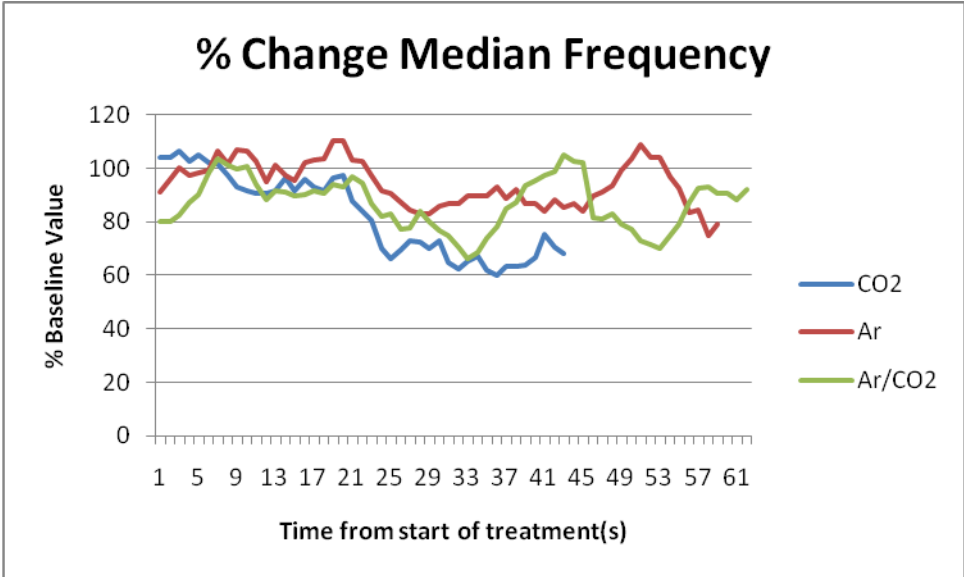


Figure 7: Percentage change in the 95% spectral edge frequency (F95), relative to pre-treatment values, of the EEG of piglets during euthanasia with 100% CO₂, 100% argon and 40%CO/60% Ar. Data are displayed as 5-point moving averages.

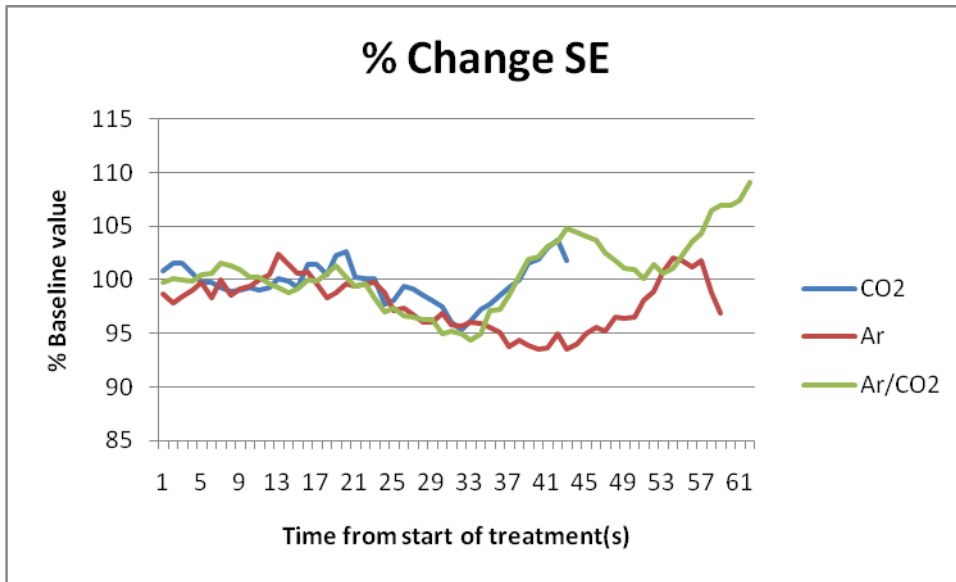
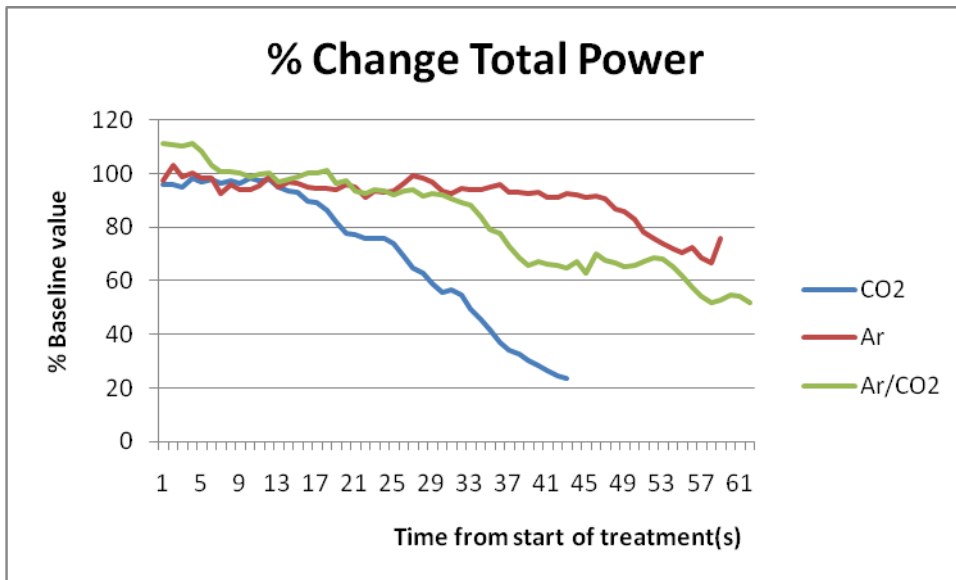


Figure 8: Percentage change in the total power (P_{tot}), relative to pre-treatment values, of the EEG of piglets during euthanasia with 100% CO₂, 100% argon and 40%CO/60% Ar. Data are displayed as 5-point moving averages.



As demonstrated by Figure 8, a reduction in the total power (P_{tot}) of the EEG occurred more rapidly in those piglets exposed to CO₂ than those exposed to either Ar or Ar/CO₂. The pattern of changes in median and spectral edge frequencies was similar between all three treatments (figures 6 and 7).

Discussion:

Experiment 1

No ECG or EEG data were obtained from this experiment, due the displacement of recording electrodes after transfer to the euthanasia chamber. As a result, time to isoelectric EEG and heart rate data could not be determined.

Four of the five gas treatments evaluated in this experiment (Ar, CO₂/Ar, N₂ and CO₂/N₂) contained 10% air and therefore 2.1% residual oxygen. The inclusion of oxygen in these treatments was based

upon protocols reported in previous studies, including those involving the stunning and euthanasia of adult pigs (Raj, 1999; Rodriguez, et al., 2008). In rodent studies, the inclusion of residual oxygen has been reported to reduce the respiratory distress observed in rats euthanized with CO₂ (Coenen, Drinkenburg, Hoenderken, & van Luijelaar, 1995). In the present study however, the duration of labored breathing, indicative of respiratory distress, was greater in those treatments containing residual oxygen. Additionally, animals in three of the residual oxygen treatments exhibited conscious behavior after the initial onset of convulsions, representing potentially serious welfare compromise due to distress or injury sustained during convulsions. Based upon the latency to respiratory arrest in each treatment, it appears that the inclusion of residual oxygen may prolong the survival time of piglets when exposed to lethal hypoxia or hypercapnic hypoxia, without any apparent welfare benefit. It was thus decided to exclude residual oxygen from the gas treatments evaluated in the subsequent experiments.

The necessity for evaluating both argon and nitrogen was reconsidered, in light of the prolonged duration of labored breathing and the reappearance of conscious activity post-convulsions observed with both of these gases. In the interest of minimizing the impact of future experiments, both in terms of welfare and the number of animals involved, a decision was made to exclude one of these gases from subsequent evaluations. The decision to retain argon and exclude nitrogen was made, based on both the shorter time to respiratory arrest observed with argon, along with the fact that argon is already being investigated for the commercial stunning of adult pigs at slaughter (Raj, 1999).

Based on the observations from experiment 1, the following three gas treatments were selected for further evaluation: 100% carbon dioxide, 100% argon and 40% carbon dioxide/60% argon.

Experiment 2

In the proposal we stated that we would use a total of 15 pigs (5 pigs per age group x 3 age groups = 15 pigs). However, in the present studies we only used 5 pigs per gas treatment. At the institute where this research was been conducted it was not possible to get ethics approval to euthanize more than 5 healthy pigs per gas treatment. It was not an option for this part of the study to use sick animals as this may have compromised and cofounded the results of the experiments. Recent research showed that age did not markedly affect the time to onset of heavy breathing or death or the cortisol response to euthanasia, suggesting that the effect of gas euthanasia is not significantly affected by age of the pig.

The results of experiment 2 indicate that euthanasia of young piglets with 100% carbon dioxide induces greater welfare compromise than does 100% argon or a mixture of 40% carbon dioxide/60% argon, based on observations of behavior prior to loss of consciousness. Although piglet's euthanized with argon squealed more and exhibited more convulsive activity than those euthanized with CO₂, these occurred after loss of consciousness and are therefore not welfare considerations. Although argon and the mixture did not significantly differ in terms of welfare impact, times to loss of consciousness, isoelectric EEG and respiratory arrest were significantly shorter with Ar/ CO₂ than Ar, making this potentially more useful from a practical standpoint.

Contamination of EEG traces due to muscular activity of the piglets during testing prevented the determination of times to transitional or isoelectric EEG and prohibited further analysis of EEG recordings. As explained in the results, when recording from the scalp, the bones of the skull attenuate the EEG signal and not the muscular activity. This means that animals need to be anesthetized to abolish muscular activity if a decent EEG signal is to be recorded. The appearance of transitional EEG has been associated with insensibility in calves following captive-bolt stunning (Blackmore & Delany, 1988) and specific changes in frequency parameters of the EEG are associated with the cortical processing of noxious stimuli (Murrell & Johnson, 2006). It would be of benefit in evaluating the relative welfare compromise of the three gas treatments in question to obtain uncorrupted EEG recordings during exposure of piglets to these gases for further analysis. Therefore, in a third experiment we repeated the above experiment (experiment 2) but put animals under light anesthesia so that we could get more accurate EEG results.

Experiment 3

The neuromuscular block administered in this study successfully eliminated movement artifacts in the EEG and ECG tracings resulting from muscle contraction that were observed in the previous study. However, small movements of the neck were observed in four of five pigs euthanized with argon alone.

These movements were independent of ventilation and did not interfere with analysis of electrophysiological data. Examination of the frequency and time of occurrence of these movements suggested an association with gasping movements observed in the non-anaesthetized animals euthanized with argon (experiment 2). The time from start of gas administration to final gasping movements in the four pigs was 5min 17s, 5min 19s, 5min 5s and 5min15s. This is comparable to the mean time of respiratory arrest (final respiratory movement) of $5m\ 31 \pm 21s$ (mean \pm SEM) for animals euthanized with argon in experiment 2, indicating equivalence in the data sets obtained in experiments 2 and 3. No gasping-type movements were observed in any of the anaesthetized pigs exposed to CO₂ or Ar/CO₂, possibly due to an interaction between halothane and CO₂.

The time to cessation of cardiac contractile activity did not differ significantly between the three gas treatments. However, individual variation within treatments was high, limiting interpretation of group differences. In terms of animal welfare, the time to cardiac arrest is not a welfare concern as this occurs after loss of consciousness. In terms of practical application of gas euthanasia, the time of exposure to the gas must be sufficient to ensure death in 100% of animals treated.

The time to loss of undoubted awareness following gas stunning or euthanasia in pigs has been assessed by a variety of measures, including loss of posture (Raj, 1999), loss of somatosensory evoked potentials and suppression of EEG signal amplitude (Raj & Gregory, 1996) and loss of auditory evoked potentials and corneal reflexes (Rodriguez, et al., 2008). To date, no single reliable measure of loss of awareness exists. In the present study, both behavioral and electrophysiological indicators of awareness were assessed. From these, the times to undisputed, probable and possible loss of awareness were determined as follows: The appearance of an isoelectric EEG indicates an absence of cortical activity and therefore represents undisputed loss of awareness. The appearance of transitional EEG trace is considered incompatible with consciousness (Blackmore & Delany, 1988) and represents probable loss of awareness. Using these parameters, probable loss of awareness occurred after 32.9, 48.9 and 61 sec exposure to CO₂, Ar/CO₂ and Ar respectively; with undisputed loss of awareness occurring after 46.2, 67.1 and 69 sec respectively. However, behavioral observations made during experiment 2 suggest that possible loss of awareness occurred even sooner than this. The onset of convulsions in response to all three gas treatments was coincident with loss of posture, which is considered an indicator of the onset of loss of consciousness (Raj, 1999). Convulsions began on average 14, 11 and 21 sec after exposure to CO₂, Ar/CO₂ and Ar respectively. Following the onset of convulsions, no behavioral signs of awareness were observed in any animal in any treatment group, lending weight to a possible earlier loss of awareness.

According to the welfare index, CO₂ induced greater welfare compromise than Ar or Ar/ CO₂. These results suggest that 100% CO₂ may cause piglets' distress prior to loss of consciousness. Although argon and the mixture did not significantly differ in terms of welfare impact, times to loss of consciousness, isoelectric EEG and respiratory arrest were significantly shorter with Ar/ CO₂ than Ar ($P < 0.05$), making this potentially more useful from a practical standpoint. However, the degree of welfare compromise observed in all treatments suggests that other alternatives to manually applied blunt trauma should be investigated.

In the proposal it was stated that the results from the first experiment would be assessed to determine if experiment 2 and 3 were necessary. From these studies we determined that Argon was preferable to using Nitrogen and that residual oxygen should not be included in the gas mixtures as this appears to prolong respiratory distress and time of death and therefore the suffering of the animals. The results from experiment 1 were positive enough to suggest that it was worth further investigating the potential of Argon and an Argon / CO₂ mixture as possible humane methods of euthanasia for young pigs. In experiment 2, extensive behavioral and physiological data were collected regarding the affects of the different gas mixtures on the welfare of the pigs. But, to achieve a more accurate understanding on the affects of the gas mixtures on the welfare of the pigs experiment 2 was repeated so that more detailed EEG data could be collected. From these results it appears as though Argon or an Argon/CO₂ mixture is preferable to CO₂ alone, however our results do not suggest that the benefits were significant enough to warrant further investigation into Argon or an Argon/CO₂ gas mixture as a method of humane euthanasia of young pigs. Furthermore, using Argon or an Argon/CO₂ mixture would markedly increase the cost of this procedure, so the benefits to the welfare of the pigs by using Argon would have to outweigh the extra cost to the producer to make it a recommendable method. Otherwise it is unlikely that producers would adopt this new method if they could not readily see the benefits. The results from this study do not suggest that using Argon or an Argon gas mixture substantially improves the welfare of the pigs during euthanasia. Therefore, from a welfare and an economic standpoint the researchers do not

see the necessity in further investigating the use of Argon or an Argon gas mixture as a practical method of humane euthanasia for young pigs on farm.

References

- American Veterinary Medical Association. (2007). AVMA guidelines on euthanasia: AVMA.
- Anton, F., Euchner, I., & Handwerker, H. O. (1992). Psychophysical examination of pain induced by defined CO₂ pulses applied to the nasal mucosa. *Pain*, 49, 53-60.
- Blackmore, D. K., & Delany, M. W. (1988). *Slaughter of stock. A practical review and guide*. Palmerston North, New Zealand: Veterinary Continuing Education Massey University.
- Blood, D., Studdert, V., & Gay, C. (2007). *Saunders comprehensive veterinary dictionary* (3 ed.). Edinburgh; New York: Elsevier Saunders.
- Carroll, J. A., Berg, E. L., Strauch, T. A., Roberts, M. P., & Kattesh, H. G. (2006). Hormonal profiles, behavioral responses, and short-term growth performance after castration of pigs at three, six, nine, or twelve days of age. *Journal of Animal Science*, 84, 1271-1278.
- Coenen, A. M. L., Drinkenburg, W. H. I. M., Hoenderken, R., & van Luijelaar, E. L. J. M. (1995). Carbon dioxide euthanasia in rats: oxygen supplementation minimises signs of agitation and asphyxia. *Laboratory Animals*, 29, 262-268.
- Danneman, P. J., Stein, S., & Walshaw, S. O. (1997). Humane and practical implications of using carbon dioxide mixed with oxygen for anesthesia or euthanasia of rats. *Laboratory Animal Science*, 47(4), 376-385.
- Gibson, T. J., Johnson, C. B., Murrell, J. C., Mitchinson, S. L., Stafford, K. J., & Mellor, D. J. (2009). Electroencephalographic responses to concussive non-penetrative captive-bolt stunning in halothane-anaesthetised calves. *New Zealand Veterinary Journal*, 57(2), 90-95.
- Hay, M., Vulin, A., Genin, S., Sales, P., & Prunier, A. (2003). Assessment of pain induced by castration in piglets: behavioral and physiological responses over the subsequent 5 days. *Applied Animal Behavior Science*, 82, 201-218.
- Lambooy, E., Gerritzen, M. A., Engal, B., Hillebrand, S. J. W., Lankhaar, J., & Pieterse, C. (1999). Behavioral responses during exposure of broiler chickens to different gas mixtures. *Applied Animal Behavior Science*, 62, 255-265.
- Lansing, R. W., Gracely, R. H., & Banzett, R. B. (2009). The multiple dimensions of dyspnea: Review and hypothesis. *Respiratory Physiology & Neurobiology*, 167, 53-60.
- Liotti, M., Brannan, S., Egan, G., Shade, R., Madden, L., Abplanalp, B., et al. (2001). Brain responses associated with consciousness of breathlessness (air hunger). *Proceedings of the National Academy of Sciences*, 98(4), 2035-2040.
- McKeegan, D. E. F., McIntyre, J. A., Demmers, T., Lowe, J. C., Wathes, C., van den Broek, P., et al. (2007). Physiological and behavioral responses of broilers to controlled atmosphere stunning: implications for welfare. *Animal Welfare*, 16, 409-426.
- Murrell, J. C., & Johnson, C. B. (2006). Neurophysiological techniques to assess pain in animals. *Journal of Veterinary Pharmacology and Therapy*, 29, 325-335.
- Niel, L., & Weary, D. M. (2006). Behavioral responses of rats to gradual-fill carbon dioxide euthanasia and reduced oxygen concentrations. *Applied Animal Behavior Science*, 100, 295-308.
- Nowak, B., Mueffling, T. V., & Hartung, J. (2006). Effect of different carbon dioxide concentrations and exposure times in stunning of slaughter pigs: Impact on animal welfare and meat quality. *Meat Science*, 75, 290-298.
- Raj, A. B. M. (1999). Behavior of pigs exposed to mixtures of gases and the time taken to stun and kill them: Welfare implications. *Veterinary Record*, 144, 165-168.
- Raj, A. B. M., & Gregory, N. G. (1995). Welfare implications of the gas stunning of pigs 1. Determination of aversion to the initial inhalation of carbon dioxide or argon. *Animal Welfare*, 4, 273-280.
- Raj, A. B. M., & Gregory, N. G. (1996). Welfare implications of the gas stunning of pigs 2. Stress of induction of anesthesia. *Animal Welfare*, 5, 71-78.
- Raj, A. B. M., Johnson, S. P., Wotton, S. B., & McIntistry, J. L. (1997). Welfare implications of gas stunning pigs: 3. The time to loss of somatosensory evoked potentials and spontaneous electrocorticogram of pigs during exposure to gases. *The Veterinary Journal*, 153, 329-340.
- Rodriguez, P., Dalmau, A., Ruiz de la Torre, J. L., Manteca, X., Jensen, E. W., Rodriguez, B. E., et al. (2008). Assessment of unconsciousness during carbon dioxide stunning in pigs. *Animal Welfare*, 17, 341-349.