Title: Evaluation of rate of administration of various gas mixtures using the Smart Box euthanasia device as a humane and effective method of piglet euthanasia **– NPB #09-197** Revised

Investigator: Chad D. Hagen

Institution: Value-Added Science and Technologies, LLC

Date Submitted: 2-3-2012

Industry Summary:

The objectives of this project were to evaluate the effectiveness and quality of gas euthanasia applied to suckling and nursery piglets at different flow rates, and with the use of different gas mixtures. Pigs were euthanized with the Smartbox™ system, which allowed for precise control of gas types, mixtures, flow rates and delivery times. Four flow rates were examined: slow, medium, fast and prefill (20%, 35%, 50%, and prefilled + 20%; chamber exchange rates per minute, respectively). The gas mixtures examined included 100% CO_2 and a 50:50 CO_2:Argon gas blend. Piglets were categorized and examined as two age groups: neonates (less than 72 hours old) and weaned (16 to 24 days of age). A control group was also included in each age group. The control piglets were allowed to remain in the euthanizing chamber for 10 minutes with ambient air passing through, after which blunt force trauma was applied. Effectiveness and quality of gas euthanasia were examined and analyzed using a variety of techniques, including behavior observations collected directly and from video, as well as physiologic (pulse, respiration and body temperature) and endocrine (cortisol, epinephrine and norepinephrine) responses. Live observation data was collected primarily to assess the effectiveness of the process including parameters such as latency to open mouth breathing, loss of posture and last movement (cessation of respiration). Live observation also included the occurrence of behavioral measures assumed to be associated with sensation and distress, including licking and chewing, nasal discharge, defecation, and urination. The video was utilized to collect behaviors in detail, specifically duration of behaviors, and added the ability to capture accurately, quickly occurring behaviors of interest. Video was scored primarily for behavioral traits which are difficult to collect live and mostly related to the quality of the euthanizing process, and sensation by the pig. These traits included duration of ataxia, duration of open mouth breathing, escape attempts and others.

As expected, piglets succumb to the effects of the gas quicker with the flow rates that create a rapid buildup of CO_2 concentrations within the chamber. To achieve loss of posture in the weaned piglet, 100% CO_2 at the slow flow rate took 3.33 minutes, whereas 100% CO_2 at a fast or prefilled

configuration took approximately 1.7 minutes. For the weaned piglet, addition of Argon increased the time to loss of posture by about 0.8 minute at the fast flow rate. Within the slow rate, this effect is even more dramatic, taking an additional 1.25 minutes. This same trend is seen for last movement with the addition of Argon increasing the latency time by 2 minutes for the fast flow rate and over 4 minutes for the slow flow rate. Between the age groups, it was observed that neonate piglets succumb to the effects of the gas as quickly as, or quicker than, weaned piglets, as judged by latency to open mouth breathing, loss of posture, and last movement. For example in the slow CO2 treatment, weaned piglets took approximately 3.33 minutes, while neonates averaged around 2 minutes for loss of posture. Differences were not observed between treatments for percentage of piglets displaying indicators of sensation or distress, but fast and prefill, 100% CO2 treatments resulted in decreased durations of these behaviors. Thus based on the data collected in this experiment, inclusion of 50% Argon with CO2 was not associated with benefits in terms of reduced distress, and is not recommended for gas euthanasia of pigs. Furthermore, slow fill rate (25% chamber volume exchange per minute) should be avoided due to prolonged distress. Additionally, differences were found between age groups, with neonate piglets succumbing to the effects of the gas as quickly for more quickly than weaned piglets.

For additional information please contact Chad D. Hagen. Value-Added Science and Technologies. 3782 9th Street SW. Mason City, IA 50401.

Keywords: carbon dioxide, euthanasia, argon, flow rates, piglets, animal welfare

Scientific Abstract:

The objectives of this study were to compare the effectiveness and response of 100% CO_2 gas, relative to a 50:50 CO_2:Argon (MIXED) gas mixture and different flow rates: slow (SL), medium (MD), fast(FT) and prefill (PF; 20, 35, 50, and prefill with 20%; chamber volume exchange per minute) as an effective tool for euthanizing when applied to piglets in two age categories: neonates (less than 3 days, n=160, BW 2.61 ± 0.81 kg) and weaned (16 to 24 days, n=180, BW 4.62 ± 0.76 kg). The slow flow rate 50:50 CO2:Argon gas mixture was not tested in neonates due to ethical concerns. A control treatment (CT) administered ambient air through the chamber followed by blunt force trauma. Male-female piglet pairs were placed in a chamber with lid and one side panel made of clear plastic to facilitate behavior observations. A Smartbox device (Euthanex Corp, Palmer, PA) was used to supply gas at controlled rates. Piglets were scored using direct observation and video for latency and duration of a variety of behavioral indicators of sensation, distress, insensibility, physiologic changes and efficacy. Behaviors were also scored as zero/one data to create percentages of piglets displaying for a variety of behaviors including loss of posture (LP), last movement (LM), gasping (GSP), open mouth breathing (OMB), defecation (DEF), urination (UR), vomiting (VM), licking and chewing (LC) and nasal discharge (ND). Piglet pair was the experimental unit. Analyses of zero/one and latency data were performed in R (v2.12.0, The R Foundation for Statistical Computing) as the univariate product-limit estimation of the survival curves, to determine significant differences. Significance was determined at $P < 0.05$. Analyses of durations were log transformed to equalize variance, when non-normal, and analyzed as a mixed model with fixed effects of sex and trt in SAS (v 9.2, SAS Institute Incorporated, Cary, NC). Significance was determined at $P < 0.05$ using a Sidak correction. Raw means were calculated using Proc Means of SAS. Differences were observed for behavioral indicators of insensibility. Latency (seconds) to LM was shortest for PF & FT, followed by MD then SL (269, 274, 313, and 529 respectively). LP and GSP followed a similar pattern (means ranged over all 4 flow rates LP: 97- 200, GSP: 46-159). All other behaviors were observed with the exception of VM. A difference ($P < 0.05$) was observed for OMB. No piglets displayed this behavior in the CT, while the gas treatments ranged from 80 – 100%; differences were not observed between the

flow rates or gas type for OMB. No differences ($P > 0.1$) were observed for any other measures: LC (5 – 70, %), ND (0 – 30, %), DEF (25 – 60, %), UR (5 – 35, %). Righting reflex occurred more frequently and for a longer duration ($P < 0.05$) with the ST MIXED treatment relative to other flow rates. Differences were observed for the duration of behavioral indicators of sensation and distress. OMB was shortest ($P < 0.001$) in duration for the PF treatment, followed by FT, MD and SL (19.6, 26.3, 33.7 and 44.8, seconds, respectively). OMB was also prolonged ($P<0.05$) with the addition of Argon gas for all flow rates. Ataxia was shortest ($P < 0.05$) in the PF, FT, and MD flow rates relative to SL (13.5, 18.7, 20.7, and 38.6. seconds, respectively). The addition of Argon increased the duration of ataxia within all flow rates ($P < 0.05$). Neonate piglets were euthanized as quickly as or faster than weaned piglets for all gases and flow rates (latency (sec): LP 99 vs. 142 ($P=0.001$); LM 360 vs. 392 ($P=0.045$); GSP 97 vs. 139 ($P<0.001$); for neonate and weaned piglets, respectively). Main effect of age was observed for the proportion of piglets displaying distress or discomfort for two of the four measured behaviors (% displaying: DEF 23 vs. 46 ($P<0.001$); ND 4 vs. 14% ($P=0.017$); OMB: 97 vs. 94 (0.116); for neonate and weaned piglets, respectively). Gas by age interactions were observed. Differences were observed between the two age groups, with neonates succumbing to the gas effects faster than weaned piglets In conclusion, MIXED and SL prolonged the duration to insensibility, as measured by LM, LP and GSP. When examined as percentage of animals displaying behaviors, OMB was the only behavioral measure to discern differences between treatments when examining percent of animals displaying, and this was for CT versus all other treatments. In general, more distress behaviors were observed for slower flow rates and with the use of MIXED gas. Thus, based on the parameters measured, 100% CO2 and a faster flow rate decreases the duration of sensation and distress to the gas.

Introduction:

Millions of piglets are euthanized annually in the U.S. swine industry. Euthanasia of these young pigs is the humane thing to do when their chances of survival are low and they are suffering due to injury or illness. The U.S. swine industry is in need of tools to accomplish this euthanasia quickly, economically, safely and humanely.

As the swine industry develops tools for euthanasia, awareness of how these techniques will be viewed by the general public and the customers of pork is an important consideration. Euthanasia is an important component of the credibility of "We Care" and it is critical that the swine industry continue to search for more acceptable, humane methods of euthanasia.

Little research has been published on gas euthanasia of piglets. Gas stunning has been implemented at processing facilities, for market hogs, over the past few decades (Gregory et al., 1987). This has lead to the majority of research in this field being conducted on market weight pigs, with CO_2 being studied for its effectiveness as a gas-stunning agent and its effects on meat quality (Dodman, 1977; Nowak et al., 2006). Raj (1999) found that the majority of these pigs died after exposure to CO_2 or a CO_2-argon gas mixture for duration of 7 minutes.The objectives of this trial were to investigate the effects of gas flow rate and a carbon dioxide/argon gas blend on effectiveness and animal welfare associated with euthanasia of piglets. At the conclusion of this study, the swine industry will have a better understanding of how to humanely euthanize piglets using gas euthanasia techniques. Ultimately, this information can be used to finalize the development of a safe, cost effective tool for the humane euthanasia of neonatal and weaned piglets.

Recently, researchers have begun to question the humanness of CO2 (Wright et al., 2009; Raj and Gregory, 1996). CO_2 is an acidic gas and therefore has been hypothesized that it could irritate the mucus membranes (Danneman et al., 1997). Prior to loss of consciousness, the piglet may experience severe breathlessness due to hypercapnia (Liotti et al., 2001). When using CO_2 at all levels and flow rates, some level of sensation or distress is observed for market weight pigs (Raj and Gregory, 1996), thus it is critical the procedures be researched, so those which induce minimal distress can be identified.

In the rat model, euthanasia with CO_2 causes irritation of the lungs. Danneman et al. (1997), found an inverse relationship of the severity of edema and hemorrhaging with increase concentrations of CO_2 (50 to 100, %). In their study, they also found several animals displaying nasal and oral hemorrhaging, with its presence related to a high incidence of edema and hemorrhaging observed in the lungs.

In this project, two gas treatments and four rates of administration were evaluated to determine the formula that provides the best combination of safety, economy, effectiveness and animal well-being. The trials evaluated both behavioral and physiological responses indicative of animal stress as well of the effectiveness of each technique in quickly bringing about unconsciousness and death.

Objectives:

1. To evaluate the effectiveness of CO_2 and a CO_2/argon blend euthanasia of suckling and peri-weaned pigs, as measured by stress responses and by latency to induce unconsciousness and death.
2. To evaluate the effects of rate of administration of CO_2, and a CO_2/argon blend on quality of euthanasia, as measured by stress responses and by latency to induce unconsciousness and death.
3. To determine potential interactions between gas blends and rate of gas administration.

Materials & Methods:

The protocol for this experiment was approved by the Iowa State University Institutional Animal Care and Use Committee. The experiment was conducted from May through September, 2010. A total of 340 mixed sex piglets (50% gilts, 50% boars/barrows) were used. A 2 x 9 experimental design, examining 2 age groups (**neonate** and **weaned**), and 9 treatments was utilized. The 9 treatments included a 2 x 4 factorial design crossed between 2 gas types (**100% CO2** and **MIXED**, 50:50 CO2:Argon) and 4 flow rates. The four gas flow rates examined were: **slow**, 20% of chamber volume, **medium**, 35% of chamber volume, **fast**, 50% of chamber volume per minute, and **prefilled**, where the chamber was allowed to fill with the gas before placement of the piglets, followed with a 20% chamber volume per minute flow rate. Additionally, a **control** treatment was examined in which ambient (AMB) air was passed through the chamber. Within an age group, each treatment was performed on 10 piglet pairs. Piglets were placed as matched litter (neonate) or pen-mate (weaned) pairs into the box. Piglet pairs were utilized to reduce the background noise that would occur due to isolation or the behavioral responses that may occur when meeting an unknown piglet. During a treatment day, all treatments were conducted once for a given piglet age. The order of treatments was randomly assigned prior to the day of treatment.

Animals & Housing

The experiment was performed on **two age groups of piglets**, neonates and weaned. **Neonates** were classified as suckling piglets less than 3 days of age. These piglets were housed and sourced from one of two locations, the Iowa State University Teaching Farm and a large integrated producer located in western Iowa, USA (Elite Pork Products, Carroll, IA). Genetics were a composite of purebred genetics and crosses of those genetics including Duroc, Landrace, Yorkshire, and Hampshire or a custom maternal x performance line (Landrace x Yorkshire cross x Duroc sire performance line) at the Teaching Farm and integrated producer respectively. Piglets in this age category were 1.40 ± 0.06 days of age and weighed 2.61 ± 0.06 kg. Piglet pairs were obtained from a litter. Enrolled piglets were penned with mother and other littermates in standard farrowing crates with metal flooring. The pen was also equipped with a rubber mat and heat lamp in the piglet area.

Exact ages of the individual **weaned** piglets were unknown, ranging from 16 to 24 days, with piglets weighing 4.64 ± 0.06 kg. These piglets were housed and maintained at the Iowa State University Swine Nutrition Farm. Prior to arriving to the farm, piglets had been processed, including castration and tail docking. Genetics were of a PIC commercial line. Weaned piglets were housed in a large group pen, with all other pigs enrolled in the trial for that day. The pen provided multiple feeders and two water nipples. Flooring was tender foot.

Smartbox

Gas was administered to the piglets via a Smartbox™ system. This system was modified by Euthanex Corporation (Palmer, PA), which is a leader in gas delivery systems for rodents and small animals. The system was modified for use with piglets and to allow behavioral observation. The Smartbox system allows for variable and precise delivery of gas types, mixtures, flow rates and delivery time. The box was constructed of clear plastic on the top and front, which permitted live observation and video capture of the piglets' behavior. The remaining 4 sides were constructed of opaque plastic. The floor was fitted with a rubber mat (Rubber floor mats, Kraco, Enterprises, LLC, Compton, CA) to help prevent slipping. The inside dimensions of the box were 43 cm wide, x 60 cm long, x 30 cm high. Gas was sourced through the Iowa State University Chemistry Store. The CO2 gas used was industrial grade (99% pure), while the Argon had a guaranteed analysis of 99.996% pure. A constant and precise gas flow was provided by compressed gas cylinders equipped with a compressed gas regulator and meter (Western Enterprises, Westlake, OH). Between each treatment the box was blown out using pressurized air from an air compressor.

Environmental Conditions

A HOBO data logger (U23-001, Onset Computer Corporation, Cape Cod, MS) was placed within the Smartbox to record temperature and relative humidity. The data logger was set to record every 10 seconds. Data was collected continuously throughout the treatment day.

Behavioral Observations

When enrolled into the study, piglet pairs were identified and marked with an animal safe marker (LA-CO Ind; Elk Grove, IL). This mark was then used to identify the piglets throughout the trial. The first piglet pair selected was assigned to the first treatment to be run, proceeding in this fashion sequentially until all treatments were filled. Piglets were then pulled from their home pen and carried to the testing room. This room was isolated from other piglets and efforts were made to minimize noise and distractions by physical movement within the testing room. Prior to treatment being applied, the piglet pair was placed in the box for 10 minutes to begin habituation to the novel environment. They were allowed a minimum of 1 hour before they were placed in the box again. For treatment they were placed into the box and gas was immediately started. Piglets were scored both live and via video. For live observation, an observer sat approximately 1.5 m away from the box. During this observation, piglets were scored for latency to behavioral indicators of distress and loss of brain function. Video was captured simultaneously to the live observation, utilizing a Noldus portable lab (Noldus Information Technology, Wageninger, NL). Two color Panasonic cameras (WV-CP484, Kadoma, Japan) were fed into a multiplexer, which then allowed the image to be recorded onto a PC using HandiAvi (v4.3, Anderson's AZcendant Software, Tempe, AZ) at 30 frames per second. Video was scored by two trained observers using Observer® (v10.1.548, Noldus Information Technology, Wageninger, NL). The trained observers were blind to treatments during scoring. Piglets were scored individually for latency, duration and event behavioral indicators of effectiveness, distress and irritation of the mucus membranes.

Signs of sensibility

The gas treatment was applied for 10 minutes. When in the box, last movement of the piglets was identified. Piglets were then allowed to remain in the chamber for 10 minutes after the last movement of both piglets. When pulled from the box, the piglets were immediately checked for sensibility. Three tests were conducted for sensibility: the eye was touched, and observed for a corneal reflex response, a flashlight was shown onto the eye, observing for a pupillary reflex, finally, the nose was pricked with a needle and the animal observed for a response. If no response was observed, the animal was checked for a heartbeat using a stethoscope. If the animal displayed a response to any of the sensibility tests or a heartbeat was detected, blunt force trauma would have been immediately performed. However, for all piglets, no responses were observed 10 minutes after last movement in this trial. If piglets did not have a last movement during the 10 minutes the gas was being applied, they were pulled from the chamber and checked for sensibility. If they were not sensible, they were placed back into the chamber for up to an additional 10 minutes to obtain last movement. All insensible piglets placed back into the chamber obtained a last movement within this 20-minute total gas exposure time. Piglets that were removed from the chamber after the 10-minute gas treatment and showed signs of sensibility were euthanized via blunt force trauma.

Modification of study design. In this study, 75% of the weaned piglets in the slow flow rate within the MIXED gas treatment had not achieved last movement during the initial 10 minutes of gas application. Of these, 53% were negative for all signs of sensibility, but maintained a heartbeat. These piglets were placed back in the chamber for up to an additional 10 minutes, to allow for last movement. All of these piglets achieved last movement within these additional 10 minutes. The remaining 47% of piglets from this group were still sensible, with blunt force trauma being immediately applied. Due to the high number of piglets in which additional measures were needed, creating ethical concerns, this treatment was not examined in the neonates, creating an unbalanced study design for the neonate age group.

Physiological measurements

Piglets that showed no signs of sensibility were checked for a heartbeat to confirm death. If dead, a blood sample was collected via a heart stick, collecting 6 to 12 mL of blood. Blood was collected from both the gas treatments and the control ten minutes after last movement to standardize collection time. The drawn sample was immediately placed in an EDTA collection tube, inverted 5 times and placed on ice. After all samples for the day had been drawn, samples were spun down and the plasma pulled off using a plastic pipette. The obtained plasma were placed in a freezer and stored at -20 °C until analyzed. The blood was analyzed for the stress hormones of cortisol, epinephrine and norepinephrine. Cortisol was run using the Coat-a-Count® radioimmunoassay kit (Diagnostic Products Corporation, Los Angeles, CA). Procedures of the kit were followed with no modifications to the protocol. Samples were run in duplicate. The ranges of the standards were from zero to 48. The epinephrine and norepinephrine were run with Alpco Immunoassays Bi-CAT RIA kit (Salem, NH). Again, procedures for the kit were followed with no modifications.

Statistical Analysis

The study design was a 2 x 9 experimental design, examining 2 age groups (neonate and weaned), and 9 treatments. The 9 treatments included 2 x 4 factorial design crossed between 2 gas types (100% CO_2 and MIXED) and 4 flow rates (slow, medium, fast, prefill) plus one control. Piglet pair was the experimental unit.

Live Observation. For live observations, latency to the behavior was scored. Latency for the gas treatments was considered from the point placed in the box. An exception of this was for the AMB treatment regarding last movement, which was from the point blunt force trauma was applied until last movement occurred. Sample means and percentages were calculated in SAS. These values are what will be presented. Utilizing a latency to behavior scoring technique creates censored data. For example if a behavior did not occur during the observation period it would receive the maximum observed time. These data were analyzed in R (version 2.13.1, The R Foundation for Statistical Computing) using a Cox model for survival analysis. Analysis from this model was used to provide statistical support for observed differences between treatments. For the behavior of last movement, which is not censored, the data was log transformed to stabilize variances. The transformed data was analyzed in SAS with a mixed model which included the fixed effects of treatment and age.
Video Observation Scored behaviors were analyzed as latency, duration or number of occurrences as appropriate for the measure. Piglet pair was the experimental unit. As was the case for live observation, some of the data were censored. Non-censored data were analyzed in SAS using a generalized linear mixed model, with the fixed effects of age and treatment. The P-values were adjusted post hoc, utilizing a Tukey adjustment. The censored data were analyzed as a Cox proportional hazard model, with fixed effects of age and treatment and random effect of piglet pair controlled.
Blood parameters. The analysis included all piglets, those who achieved last movement within the initial gas treatment, those who received additional time (not sensible, but not last movement) as well as those who blunt force trauma was applied (sensible after initial 10 minute gas treatment). The values of plasma cortisol levels were averaged for the two samples and log transformed to stabilize the variance. The plasma levels for Epinephrine and Norepinephrine values were also log transformed to stabilize the variance. The transformed data values were analyzed in SAS using a mixed model with fixed effect of age and treatments. Simple statistics, ran on the non-transformed data will be used to present the numeric values.

Results:

Weaned piglets

Efficacy. Examining flow rates within the gas type CO2, piglets displayed open mouth breathing (OMB) promptly upon being placed in the box (11 s). Latency to display this behavior then increased to approximately 60 s ($P < 0.05$) for Fast and Medium flow rates, this was then followed ($P < 0.05$) by the Slow flow rate with a mean of 87 s (Figure 1). Within the MIXED gas type, a similar pattern was observed, with latency to OMB greater ($P < 0.05$) for the slower flowing rates. Examining the differences between gas types, statistical differences were not seen at the prefill and fast flow rate, though in general most pigs in the MIXED treatment had high latencies to OMB relative to their matched flow rate of the CO2 gas. For the medium and slow flow rates, the differences between the two gas types was statistically different, with the difference in means of 26 s ($P < 0.05$) occurring at the slow flow rate. Latency to loss of posture (LP), between flow rates and gas type, varied from 97 s for the CO2 prefilled to 275 s in the MIXED slow treatment, showing a pattern similar to that observed with OMB (Figure 2). Ataxia, which occurred prior to LP, was shortest in duration for the Prefill CO2 (13 s) and within the CO2 treatment, increased with the slower flow rates, almost 3 times the rate. Ataxia, in the MIXED treatment relative to the CO2 treatment, was almost double within each flow rate, except in the slow rate where it increased from 39 s for the CO2 slow to 52 s for the MIXED slow. Gasping (GASP), within a treatment, often occurred within 10 s of LP, with the exception of CO2 Slow, in which GASP occurred 41 seconds prior to loss of posture. Latency to last movement (LM) was shortest in the Prefill and Fast flow rates (CO2 269 and 274 s, respectively), latency increased ($P < 0.05$) for the medium flow rate, and there was a rather large increase in the slow flow rates, which on average took 529 s (Figure 3). When argon was present, the latency to LM almost doubled within a flow rate. Blunt force trauma had the greatest latency to LM, 821 s, a full 552 s greater than CO_2 Fast.

Detection of gas and distress. Percent of piglets displaying OMB, did not differ between flow rates or gas type, with the majority of piglets displaying this behavior ranging between 80 and 100 %, though differences were observed for OMB between the AMB treatment and all gas treatments ($P < 0.001$), with no piglets in the AMB treatment displaying OMB. Significant differences between all treatments, including AMB, were not observed for percentage of piglets displaying a variety of indicators of sensory stimulation and distress, including Defecation, Urination, Salivation, Nasal discharge, and Licking and Chewing. After loss of posture, in some instances a righting reflex was observed. When this did occur within the CO_2 Prefill and Fast flow rate, it was less than 2 seconds. This increased dramatically for the slow flow rates and MIXED treatments, reaching over 13 seconds for the slow MIXED treatment. The number of occurrences of a righting movement was less than 1 for CO_2 treatments, while it was greater than 1 for MIXED treatment. The number of righting attempts by a single piglet ranged from zero, which occurred in all treatments, up to 12 for the Prefilled MIXED treatment. This was in contrast to the fast CO_2 treatment, in which the maximum observed was 1. Escape attempts were low in this trial, with attempts only being made in the MIXED treatments, with a 10% occurrence for the Fast treatment and 15% for all other flow rates. For the duration of Licking and Chewing (LC) within the gas types, a general pattern could not be discerned. However, the Prefill treatments were shorter ($P < 0.001$) than the other flow rates and AMB treatments (< 5 s vs. > 10).

Aesthetics and other measures. Muscle excitation occurred most frequently with the Prefill and faster flow rates occurring 60% within the CO_2 gas treatment and dropping to 40% for the slower flow rates. Rates were lower for the MIXED treatment, around 40% for the medium and fast flow rates and 30% and 25% for prefill and slow flow rates respectively. Standing and Locomotion (SL), one of the behaviors scored in which the piglets were not showing any signs of the effects of gas, did not show differences between gas types or flow rates. For the duration of Oral and Nasal (ON) behaviors displayed, statistical differences were observed ($P < 0.05$); however, the mean for the longest treatment (Slow MIXED) was only 6 seconds (data not reported).

Physiological parameters. Cortisol was similar ($P > 0.05$) for prefill, fast and medium flow rates (3.02 – 4.05 µg/dL). An increase was seen for the slow flow rate relative to fast and prefill ($P < 0.05$). Interestingly, the highest value was observed for blunt force trauma, 23.63 µg/dL, which was significantly different than gas treatments ($P < 0.05$). Differences were not observed between flow rates or gas type for Epinephrine or Norepinephrine values.

Neonate piglets

Efficiency. Open mouth breathing (OMB**)** occurred shortly upon the piglets being placed in the box during the CO_2 Prefill treatment. Latency increased significantly for the fast and medium flow rates, increasing 38 or more seconds. For the MIXED treatment, again the prefill resulted in a very short latency to OMB, with a significant increase of more than 40 seconds for the fast and medium flow rates. Between the gas mixtures, while within each flow rate, latency to OMB was longer for the MIXED treatment, no significant differences ($P > 0.05$) were found. Loss of posture (LP), within the CO_2 gas treatment, occurred quickest with the prefill treatment. This increased a minute for the fast and medium flow rates, while the slow flow rate took an additional 40 seconds. Within the MIXED treatments, a similar pattern was found with the prefill treatment resulting in the shortest latency to LP, followed by fast and medium flow rates. Between gas treatments, MIXED treatments always took longer than the CO_2 treatments, with the greatest difference occurring at the medium flow rate (94 s difference). The duration of Ataxia was shortest for the Prefill CO_2 treatment (14 s). Within the gas treatment CO_2 this increases for the Fast and Medium flow rates to around 26 s and 43 s for the slow flow rate. Within the MIXED treatment, again the shortest duration is seen in the Prefill (24 s) with increases observed at the fast (47 s) and medium flow rates (65s). Latency to gasping was shortest in the prefill treatments 47 s and 61 s for CO_2 and MIXED respectively. Within the CO_2

treatment, this then increased ($P < 0.05$) to 84 and 88 s for fast and prefill with another increase ($P < 0.05$) observed in the slow (121 s). The similar pattern was seen within the MIXED treatment, with increases observed for the fast (115 s) and again for the mixed (147 s) Last movement (LM) occurred at 226 seconds for the CO2 prefill treatment. This time increased ($P < 0.05$) for other fast and medium flow rates (293 and 296 respectively), with a sharp increase ($P < 0.05$) for the slow (427). For the MIXED treatment, last movement occurred several minutes after that of the CO2 treatment (significant within each flow rate $P < 0.05$), taking 397, 380, 452 seconds for the prefill, fast and medium flow rates respectively.

Detection of gas and distress. Percent of piglets displaying OMB, did not differ between flow rates or gas type, with the majority of piglets displaying this behavior ranging between 90 and 100 %, though differences were observed for OMB between the AMB treatment and all gas treatments ($P < 0.001$), with only 5% in the AMB treatment displaying OMB. Significant differences between all treatments, including AMB, were not observed for percentage of piglets displaying a variety of indicators of sensory stimulation and distress, including Defecation, Urination, Salivation, Nasal discharge, and Licking and Chewing (Table 1). When the righting response was observed, it was shortest in duration for the slow CO2 treatment. Again, MIXED increased the duration, with the longest duration at 8 s for the MIXED medium flow rate. The means for number of righting attempts was under 1 for all treatments, with the exception of Medium MIXED. The number of attempts ranged from zero, which was seen in all treatments, up to a maximum of 6 for the Medium MIXED treatment. Escape attempts were minimal, with no attempts made for most treatments. For both gas types, a 5% escape attempt rate was observed within the prefill treatment. Pigs exposed to ambient air showed a 10% escape attempt rate. Duration of Licking and Chewing (LC) did not follow a discernable pattern, as seen for many of the other variables. LC was not observed in the Prefill CO2 treatment, and was observed for around 5 seconds for the other flow rates. Duration was less than 3 s for all MIXED treatments. AMB treatments displayed this behavior for a longer duration, 7 ± 5 s.

Other measures. Muscle excitation, occurred on a limited basis, appearing only in the CO2 Fast, CO2 Prefill, and MIXED Prefill treatments at a rate of 5, 10 and 15% respectively. Standing and Locomotion (SL**)**, one of the behaviors scored in which the piglets were not showing any signs of the effects of gas, did not show differences between gas types or flow rates. For the duration of Oral and Nasal (ON) behaviors displayed, statistical differences were observed ($P < 0.05$); however, the mean for the longest treatment (Slow MIXED) was only 6 seconds (data not reported).

Physiological parameters. Differences ($P > 0.1$) were not observed between flow rates, including control, or gas types for the measures of cortisol, epinephrine**,** or norepinephrine.

Comparison between age groups

Efficacy. When comparing the weaned piglet to the neonate numerically, OMB occurred sooner within each flow rate and gas type. Statistical differences ($P < 0.05$) were observed between the two age groups for the slow flow rate for both gas types and the prefill within the MIXED gas. The duration of Ataxia, was the one parameter measured which did not fit the trend seen with all other measurements taken. Neonates performed ataxic movements longer than the weaned piglets, showing statistical differences ($P < 0.05$) for the fast CO2 and medium MIXED gases. Numerically, shorter latencies occurred for neonates relative to weaned piglets for the behavior of gasping. Statistical differences ($P < 0.05$) were seen in several of the flow rates within gas type, including slow, medium and fast within the CO2 and slow and fast flow rates within the MIXED gas type. Last movement also followed this pattern, with the neonates succumbing to the effects of the gas as fast, or faster than, the weaned piglets. Interestingly, when using blunt force trauma the neonates took longer than the weaned piglets (913 vs. 821 seconds) to die. Percent of piglets displaying OMB was similar between both age groups, ranging between 80 to 100 % within flow rates and gas types. Percent of piglets defecating was higher ($P < 0.05$) for the weaned piglets, with the weaned piglets ranging from 0 to 20 percent within flow rate and gas type, while the neonates highest observed rate was 5%. Differences were not observed for the number of piglets urinating, with this ranging between 5 and 35 %. Amount of nasal discharge was less ($P < 0.05$) for the neonates for the slow and medium flow rates within the CO_2 gas. Licking and Chewing was displayed longer in the weaned relative to the neonate piglets for the slow and fast CO_2 and fast MIXED gas (5, 5, 2 vs. 18, 21, 20 s neonates vs. weaned, respectively).

Discussion:

Overall, it was found that piglets succumb to the effects of the gas sooner when treatments are such that a faster build up of CO_2 is created in the chamber. The slow treatment had the longest latency to all observations of sensations, distress, and efficacy relative to the medium, fast, and prefill treatments. In line with this, the addition of Argon to the gas mixture also caused the process to take longer. When examining the percent of animals displaying indicators of sensation and distress, differences are not observed between flow rates. However, duration of these behaviors were generally prolonged for the slow treatment relative to the faster building flow rates. This same trend was seen for the addition of Argon, extending the duration of sensation and distress behaviors. Hence, a slow fill rate of 20% chamber volume exchange per minute is not recommended. Fast and prefilled produced very similar results, and due to the practicality and difficulty in maintaining a prefilled environment, it would be recommended a fast or medium flow rate be utilized. These results are critical in establishing best practices for euthanizing piglets when CO_2 is utilized.

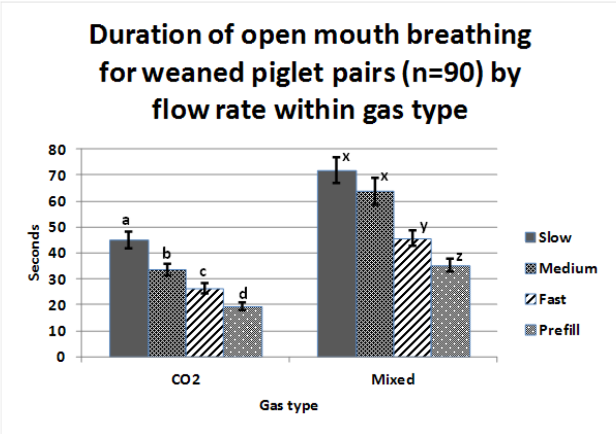

Figure 1; superscripts indicate differences ($P < 0.05$) between flow rates within a gas type. CO2 was provided at 100% within the flow rates. Mixed constituted a 50% CO2 and 50% Argon within the flow rates. Blunt force trauma (BFT), was performed on piglets after 10 minutes exposure to ambient air. Flow rates: slow = 20%, medium = 35%, fast = 50%, and prefilled = filled + 20%, chamber volume per minute.

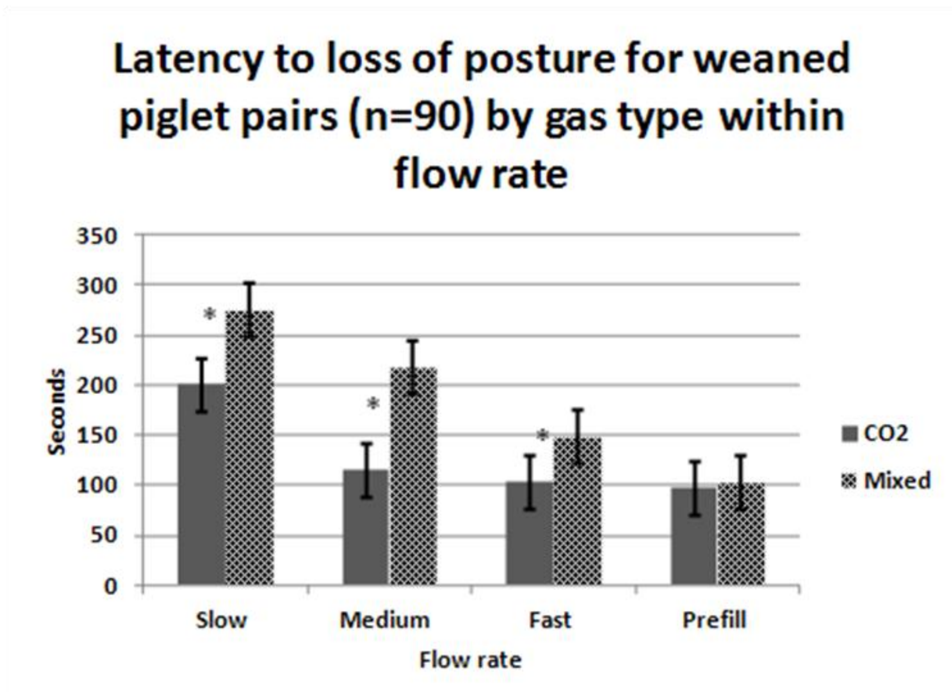

**Figure 1; ***= $P < 0.05$ between gas types. CO2 was provided at 100% within the flow rates. Mixed constituted a 50% CO2 and 50% Argon within the flow rates. Blunt force trauma (BFT), was performed on piglets after 10 minutes exposure to ambient air. Flow rates: slow = 20%, medium = 35%, fast = 50%, and prefilled = filled + 20%, chamber volume per minute.

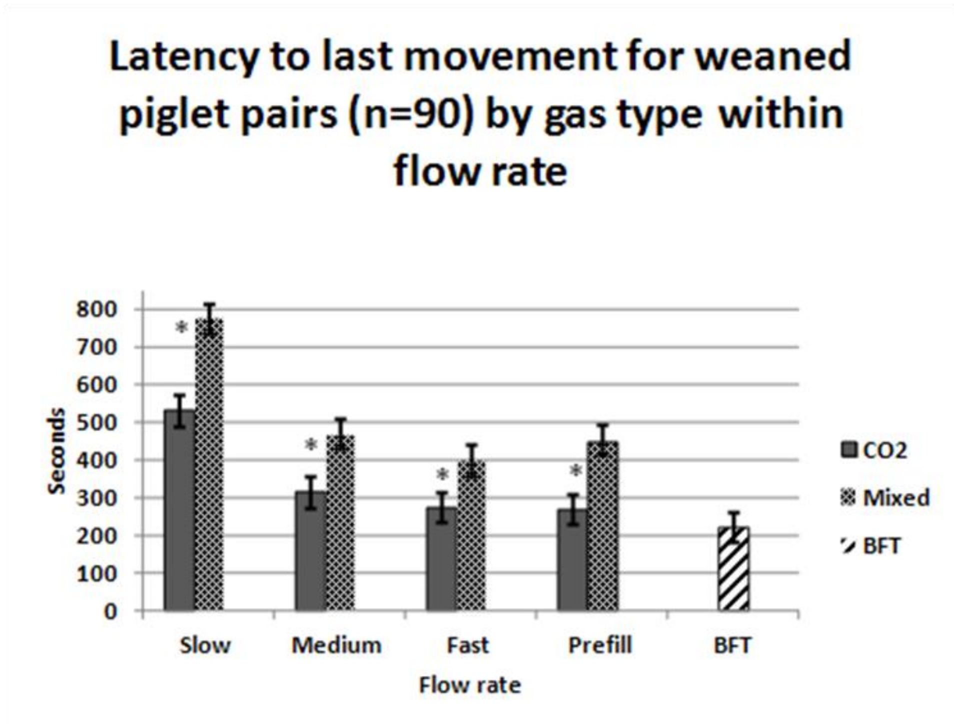

Figure 3; *= $P < 0.05$ between gas types. CO_2. CO2 was provided at 100% within the flow rates. Mixed constituted a 50% CO2 and 50% Argon within the flow rates. Blunt force trauma (BFT), was performed on piglets after 10 minutes exposure to ambient air. Flow rates: slow = 20%, medium = 35%, fast = 50%, and prefilled = filled + 20%, chamber volume per minute.

Table 1. Raw percentage of piglets displaying behavioral indicators of sensation and distress for gasping, open mouth breathing, nasal discharge, defecation, urination salivation, and licking and chewing by gas type and gas flow rate for weaned pigs

	Flow rate of the gas			
Efficacy measures	**Slow**	**Medium**	**Fast**	**Prefill**
Defecation (Def)- no sig				
CO_2	50	45	45	25
MIXED	45	50	60	50
AMB				35
Urination- (Urn)- no sig				
CO_2	10	10	20	15
MIXED	5	35	35	30
AMB				10
Salivation (Sal)- no sig				
CO_2	15	0	5	5
MIXED	50	30	10	10
AMB				15
Licking and Chewing (LCK)- no sig				
CO_2	45	60	70	5
MIXED	55	40	50	10
AMB				40
Nasal Discharge (ND)- so sig diff				
CO_2	25	20	10	0
MIXED	30	20	5	15
				10

Superscripts indicate differences ($P > 0.05$) within a behavior measure (all flow rates and gas types). CO2 was provided at 100% within the flow rates. Mixed constituted a 50% CO2 and 50% Argon within the flow rates. Blunt force trauma (BFT), was performed on piglets after 10 minutes exposure to ambient air. Flow rates: slow = 20%, medium = 35%, fast = 50%, and prefilled = filled + 20%, chamber volume per minute.