Gas alternatives to carbon dioxide for euthanasia: A piglet perspective1,2

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ABSTRACT: The search for alternative methods to euthanize piglets is critical to address public concern that current methods are not optimal. Scientific evidence supports that blunt force trauma is humane when performed correctly, but most people find it visually difficult to accept. The use of CO2 is often recommended; at the same time, it is criticized as being aversive to pigs. This research sought to 1) identify a method of scientifically determining if piglets find a gas aversive, using an approach-avoidance test, which relies on the perspective of the piglet, and 2) test different gas mixtures to determine if they are effective and humane for neonatal piglet euthanasia. Pigs were allowed to walk freely between 1 chamber filled with air and another chamber either gradually filled with gas mixtures (Exp. 1) or prefilled with gas mixtures (Exp. 2). Experiment 1 tested CO2 (90%) and air (10%), N2O (60%) and CO2 (30%), Ar (60%) and CO2 (30%), and N2 (60%) and CO2 (30%). Because piglets had to be removed when they started to flail, the test was shortest (P < 0.01) for the pigs in the CO2 treatment compared with pigs in the N2O/CO2, Ar/CO2, and N2/CO2 treatments, 3.1 ± 0.2, 8.5 ± 0.6, 9.6 ± 0.4, and 9.9 ± 0.1 min, respectively. Nonetheless, all gas mixtures adversely affected the pigs, causing the pigs to leave the test chamber. In Exp. 2, piglets were allowed to enter a chamber prefilled with N2/CO2 or N2O/CO2 (both 60/30%). Pigs exposed to the prefill chambers started to flail in fewer than 20 s, much faster in comparison with the gradual fill method, which supports that this method was more aversive. In Exp. 3, piglets were euthanized using a 2-step procedure. Pigs were first placed in a gradual fill chamber with 1 of 4 gas mixtures: 90% CO2, N2/CO2, N2O/CO2, or N2O/O2 (the last 3 mixtures at 60/30%) followed by placement into a 90% CO2 prefill chamber when the pigs started to flail or were anesthetized. All 3 gas treatments that contained CO2 killed pigs more quickly than N2O/O2 (P < 0.05). However, N2O/O2 was the only treatment that anesthetized the pigs instead of causing squeals or flailing although requiring about 12 min longer. Although longer, a 2-step procedure in which pigs are anesthetized with a mixture of N2O and O2 before being euthanized by immersion in CO2 may prove to be more humane than CO2 alone.

Key words: anesthesia, carbon dioxide, euthanasia, gas, nitrous oxide, piglet


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INTRODUCTION

The neonatal stage is a critical time in the life of pigs, when they are prone to become sick or weak. This is the stage at which most euthanasia procedures are required if the pig is judged unable to recover. Any euthanasia method should be humane, practical, economical, and socially acceptable. Blunt force trauma is widely used to euthanize neonatal piglets by administering a blow to the head, which results in severe brain damage given the thin skull bones and results in immediate loss of consciousness. Data support that blunt force trauma is humane when performed correctly (AVMA, 2007), but most people find it visually difficult to accept and it can be emotionally disturbing for the stockperson. Any alternative must allow for human safety, cause minimal pain and distress, and cause a rapid loss of consciousness and death. Gas euthanasia could fulfill these criteria. The use of CO₂ is often recommended (AVMA, 2007). At the same time, CO₂ is criticized as being aversive to swine (Raj and Gregory, 1995; Velarde et al., 2007) and causes a profound sense of breathlessness in humans (Gregory et al., 1990). Furthermore, little is known about the reaction of neonatal piglets to CO₂ euthanasia. Chemically inert gases such as N₂, Ar, and N₂O possess anesthetic properties without imparting any sense of breathlessness (Raj and Gregory, 1995).

This research sought to 1) identify a method of scientifically determining if piglets find a gas aversive, using an approach-avoidance test, which relies on the perspective of the piglet (Exp. 1 and 2), and 2) test different gas mixtures to determine if they are effective and humane for neonatal piglet euthanasia, using a 2-step anesthesia-euthanasia procedure (Exp. 3). We hypothesized that N₂O, and alternatively Ar and N₂O, would be less aversive than CO₂ and would induce anesthesia before using CO₂ to complete the euthanasia procedure.

MATERIALS AND METHODS

The project was approved by the Purdue University Animal Care and Use Committee, and animals were housed in accordance with Federation of Animal Science Societies (FASS, 2010) guidelines at the Purdue University Animal Science Research and Education Center.

Animals

All experimental piglets were the progeny of Yorkshire × Landrace dams bred to Duroc × Hampshire sires. Sows and their piglets were housed in conventional farrowing crates. For Exp. 1 and 2, healthy piglets were used and returned to their litter after testing. In Exp. 3, only compromised piglets destined to be euthanized by the Purdue University farm staff were used, with the justification that this type of neonatal piglet represented the population of interest, as that is when morbidity and the need for euthanasia are greater in production settings. Piglets were not screened for their halothane genotype, which may influence their sensibility to gas changes (Velarde et al., 2007), although the proportion of halothane gene in this herd is expected to be low.

Test Chambers

The pigs were tested in a 2-chamber custom built box (152 by 61 by 60 cm), which was designed such that medical air (80% N₂/20% O₂; “control chamber”) or treatment gas (“test chamber”) could be delivered separately into each chamber (Fig. 1). A third chamber (76 by 30 by 30 cm) outside of these 2 chambers was used to hold “companion” pigs, which could be viewed by the test pig through a clear plastic wall. A heat lamp was used to keep these companions comfortable. The entire top of the box was clear plastic to allow for observation and the 2 chambers were connected via a passage door (28 by 18 cm) with a see-through plastic door. Gases were delivered from compressed gas cylinders (Peoples Welding Supply, Lafayette, IN) through a 1 cm (i.d.) entry port located 9 cm from the top of the chamber. Each chamber had a 2 cm (i.d.) exit port 11 cm below the lid in which gas could freely escape by positive pressure to prevent the chamber from building pressure. A gas trap (23 by 8 cm) covered by
wire was placed in the doorway between the 2 chambers to serve as a sink in which heavier gases could escape. Carbon dioxide and O₂ sensors were placed 15 cm from the floor, approximately at the height of the head of the pig, on both the control side and the test side.

**Pilot Study**

A pilot study was conducted to determine if pigs could be preferentially attracted to 1 side of the box. This was required to set up the approach-avoidance test. Eleven 7-d-old pigs were placed into 1 chamber of the box devoid of attractants and the second chamber, which could be accessed through a clear plastic door, contained stimuli to attract the pig (Fig. 1). It was not the goal of this project to determine which attractant worked best but rather that all attractants presented together successfully attracted the piglets into the chamber. Several items were placed into the chamber to make the test chamber attractive, including a heat lamp to warm the chamber (23.3°C), a towel that had the odor of the dam of the test piglet, a speaker (AW811, Acoustic Research; Audiovox, Cambridge, MA), which played the nursing grunts of an unrelated sow, dry powdered milk spread on the rubber floor, and iron replacement meal (Sweet Iron; International Nutrition, Omaha, NE). In addition, the pig in the test chamber could see 3 littermates through a plexiglass wall. The odor of the sow was transferred to the towel before the test by rubbing the towel on the udder of the sow for at least 5 min. The control side of the chamber, which was designed to not attract the pig, was maintained at 19.4°C and contained a speaker as in the test side but turned off. Each chamber was designed with a glove (Neoprene sleeves; Coy Lab Products, Grass Lake, MI) in the wall (20 cm i.d.) such that an observer could put his or her hands into the chamber in case of emergency or to move the pig, without letting any gas escape. A predefined ethogram (Table 1) was used to record piglet behavior, which was captured on video recording.

**Experiment 1**

Four different gas mixtures were evaluated and targeted to create a gaseous environment that was 90% treatment gas and 10% residual air. The treatments were CO₂ (90%), N₂O (60%) and CO₂ (30%), Ar (60%) and CO₂ (30%), and N₂ (60%) and CO₂ (30%). The treatments were chosen for these reasons: a standard recommendation for gas euthanasia is 80 to 90% CO₂ (AVMA, 2007) although it has been shown to be aversive based on previous research in mature swine (Raj and Gregory, 1995; Velarde et al., 2007). Therefore, 90% CO₂ was chosen as a basis of comparison for the aversiveness of the alternative gas mixtures. Nitrogen, Ar, and N₂O are all inert gases and have anesthetic properties (Raj and Gregory, 1995; Dalmau et al., 2010; Rault and Lay, 2011); therefore, they were used as an alternative to CO₂. Alternatively, they could be used to induce anesthesia before the use of CO₂. Inclusion of 30% CO₂ does not appear specifically aversive to pigs, therefore allowing for a mixture of such concentrations (Raj and Gregory, 1995, 1996).

The aversion of the neonatal pig to inhaling 1 of the gas treatments was assessed using an approach-avoidance testing procedure, inspired by Makowska et al. (2009). The test was designed as a conflict situation between the willingness of the piglet to enter and stay in the test chamber (Fig. 1) containing the attractive stimuli, as described earlier, and the gas supplied in progressively increasing concentrations. The piglet could also choose to remain in the control chamber, which contained only air and no attractive stimuli.

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**Table 1. Ethogram used for behavioral observations**

<table>
<thead>
<tr>
<th>Category</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Location</strong></td>
<td></td>
</tr>
<tr>
<td>Treatment Side</td>
<td>When both piglet ears are in the plane of the test chamber side</td>
</tr>
<tr>
<td>Control Side</td>
<td>When both piglet ears are in the plane of the control, medical air, side</td>
</tr>
<tr>
<td><strong>Behavior</strong></td>
<td></td>
</tr>
<tr>
<td>Normal Behavior</td>
<td>Initial “baseline” behavior and condition of piglet when placed in box</td>
</tr>
<tr>
<td>Stressed</td>
<td>Heavy breathing, lethargy, can be standing, laying, or kneeling</td>
</tr>
<tr>
<td>Disoriented</td>
<td>Loss of stability, swaying of head or limbs with lethargy and heavy breathing; can transition between standing, laying or lateral recumbency</td>
</tr>
<tr>
<td>Flail</td>
<td>Erratic, uncontrolled movements, including sudden reversals of behavior, uncoordinated jumps and thrashing</td>
</tr>
<tr>
<td>Pronounced dead²</td>
<td>The heart beat can no longer be heard with the stethoscope. No palpebral reflex, pin prick reflex of the nose, or toe pinch reflex</td>
</tr>
<tr>
<td>Squeal²</td>
<td>High pitch vocalization</td>
</tr>
<tr>
<td><strong>Consciousness</strong></td>
<td></td>
</tr>
<tr>
<td>Anesthetized²</td>
<td>A pig is in lateral recumbency and lacks: a palpebral reflex, response to pin prick to the nose and response to toe pinch test</td>
</tr>
<tr>
<td>Not anesthetized</td>
<td>A pig has voluntary movement, including all postures, and is reflexive to touch and pin prick</td>
</tr>
</tbody>
</table>

¹Each piglet was recorded continuously. Each of the 4 categories was exhaustive and mutually exclusive.
²Measures only recorded in Exp. 3.
Therefore, in this experiment, 28 female pigs from 7 litters were randomly assigned within litter to 1 of 4 treatments: CO₂, N₂O/CO₂, Ar/CO₂, or N₂/CO₂. Due to different technical problems, the final sample size for each treatment was CO₂ (n = 6), N₂O/CO₂ (n = 7), Ar/CO₂ (n = 6), and N₂/CO₂ (n = 3). The mean pooled BW of the pigs for all treatments was 5.1 ± 0.3 kg and did not differ between treatments (P > 0.1).

Starting when the pigs were 10 d of age, the training procedure consisted of 2 training d, 1 rest d, 1 control-test d, and then 1 test d on which the treatment gas was used. At 10 d of age, 4 pigs from the same litter were acclimated to the chambers. The 4 pigs were taken from their dam to a different room and placed in a holding area (61 by 61 cm) with a heat lamp. This holding area had a clear plastic wall, which directly connected to the test chamber. After a 5 min acclimation period, 1 pig was placed into the control chamber for 5 min and allowed to learn to use the passage door through trial and error to obtain entry into the test chamber, which contained the attractive stimuli. If after 2 min the pig never entered the test chamber, a technician would gently push the pig through the chamber door and it was allowed to go between the chambers for the remaining 3 min. This procedure was repeated for the remaining 3 litters. The entire procedure was repeated on the next day by reintroducing the pigs in a randomized order. On the fourth day (d 13 of age), the pig was placed into the control chamber but only medical air was delivered in both the control and the test chamber. Data were collected on the use of each chamber over 10 min to validate that the pig was indeed spending more time in the test chamber containing the attractants.

On test day (14 d of age), after the 5 min acclimation period, each pig was picked up following a preset random order, fitted with a belt and heart rate recorder to collect heart rate interbeat interval data during the duration of the test (Polar RS800CX; Polar Electro Oy, Kempele, Finland), and placed into the center of the control chamber facing the door into the test chamber. The box was closed and the delivery of medical air was initiated into the control chamber as the treatment gas was delivered into the test chamber.

All gases were delivered at constant flow rate (Meter Model VFB-67; Dwyer Instruments, Inc., Michigan City, IN) of 40 L/min with the exception of N₂, which was delivered at 32 L/min to account for differences in gas densities. This rate was calculated as a 20% replacement rate per minute for the chambers used. Air samples were continuously analyzed for O₂ and CO₂ from both the control and test chambers using 2 dual combination infrared and electrochemical sensors (CM-0053 Wall Monitor NEMA; Gas Measurement Products, Ormond Beach, FL) to ensure the treatment gas was not escaping into the control chamber. For Exp. 1, the gas sensors could not measure either CO₂ or O₂ in the presence of N₂O; however, a new sensor was engineered for the next 2 experiments, which could measure CO₂ but not O₂ in the presence of N₂O. Therefore, data relative to gas concentrations are being reported relative to concentrations of CO₂, as most treatments contained CO₂.

The test was conducted for 10 min during which the pig was free to move between chambers. Behavior was recorded using a camera with a 2.8 to 12 mm lens (Color CCTV; Panasonic, Secaucus, NJ), which was mounted directly above the test box. Behaviors were characterized based on location and activity using a predefined ethogram (Table 1). Data were collected directly during the tests as well as from video. Vocalizations were not recorded as it was difficult to discern the vocalizations of the tested pig from its littermates and difficult to count vocalizations from outside the box. Observers were blind to the treatments and interobserver reliability between individuals was verified (>90% agreement) before the onset of experimental observations. Videos were analyzed using The Observer XT (Noldus, Wageningen, The Netherlands). Pigs were removed from the test either after 10 min or when they succumbed to the test gas and started to flail or collapsed in the test chamber.

Heart rate data were manually corrected for artifacts (Marchant-Forde et al., 2004) and 1 min sections containing more than 4% artifacts were excluded from the analysis. Behavioral and heart rate data were later paired with gas concentration data obtained from the CO₂ and O₂ monitors to assess the aversiveness of the pig to different concentrations of the gases.

**Experiment 2**

Experiment 2 followed the same protocol as in Exp. 1, except that the test chamber was prefilled with either N₂ (60%) and CO₂ (30%) or N₂O (60%) and CO₂ (30%). The CO₂ treatment was not included in Exp. 2 because preliminary data in which pigs were placed into 90% CO₂ showed it to be extremely aversive. In that test, pigs placed into 90% CO₂ began to flail within 5 s and had to be removed; in contrast, pigs placed in either N₂/CO₂ or N₂O/CO₂ took at least twice as long to start to flail (see results). We did not think that 5 s was enough time for a pig to make a choice to walk out of the test chamber; therefore, this treatment was not repeated for humane reasons. The same 5 d training/test protocol was followed starting when pigs were 10 d of age. Thirteen female pigs were randomly assigned to 1 of the 2 treatments; however, due to different complications, data could only be collected from 10 pigs: 4 N₂ pigs and 6 N₂O pigs. A typical complication would be when a pig entered (either head first or rear first) only partially
through the door, which caused the gas to leak into the control side. The mean pooled BW of the pigs in both treatments was 4.9 ± 0.4 (P > 0.1). As in Exp. 1, pigs were placed into the control side with medical air and allowed to enter and leave the test chamber at will. However in this experiment, the test chamber was filled with either N₂ (60%) and CO₂ (30%) or N₂O (60%) and CO₂ (30%). Behavioral data were recorded using real time video and analyzed using a predefined ethogram (Table 1). Latencies for the pig to become stressed once in the test chamber were recorded, as were the comparable time intervals between subsequent disoriented and flail behaviors. Concentrations of CO₂ and O₂ were continuously recorded in both chambers to validate that treatment gas was as prescribed in the test chamber and that no gas was leaking into the control chamber. When a pig entered the test chamber and could not exit due to it starting to flail or collapsing, it was immediately removed from the test chamber and placed back into the control chamber containing medical air.

**Experiment 3**

This experiment was designed to determine the effectiveness of a 2-step anesthesia-euthanasia procedure to euthanize pigs. Pigs were first exposed to 1 of 4 gas mixtures and then to 90% CO₂ to complete the euthanasia procedure. This approach was used to bypass the aversive effects of CO₂ (Raj and Gregory, 1995; Velarde et al., 2007) by presedating the pig with O₂. When pigs were identified by the farm staff as requiring euthanasia, they were euthanized using 1 of the 4 treatments within 5 h. Effort was made to balance the treatments by cause for euthanasia, gender, age, and BW as much as possible. The mean pooled age and weight of the pigs were 4.7 ± 1.6 d and 1.1 ± 0.4 kg and did not differ between treatments (P > 0.1). The average gender ratio of males to females for all 4 treatments was approximately 50:50. Pigs identified to be euthanized were brought to the laboratory and their rectal temperature and cause for euthanasia were recorded. They were classified as either sick/injured, starving, or splay leg and allocated across treatments. Body temperature was recorded as normal (39.4 to 38.6°C), hypothermic (38.5 to 36.4°C), or nearing death (below 36.4°C). Examining cause of death, it is clear that the majority of pigs were euthanized due to starvation (17 of the 28), with another 7 sick/injured and 4 sway leg. Comparing the categories strictly for body temperature indicates that the majority of the pigs were also hypothermic (19 of the 28). Before euthanasia, the pig was fitted with a belt to collect heart rate and placed into the test chamber that gradually filled with gas. The pig was removed from this chamber and immediately moved into the chamber with 90% CO₂ when 1 of 3 criteria was met: the pig squealed, flailed, or became anesthetized. To determine if pigs were anesthetized, their palpebral reflex, response to a pin prick on the nose, and response to a toe pinch test were determined every 30 s once they assumed a posture of lateral recumbency and appeared unresponsive. Behavior was recorded via direct observations and video recordings. Once the pig was placed into the CO₂ chamber, its reflexes were recorded every 30 s until the pig stopped responding. In addition, the heart rate of the pig was manually checked starting 2 min after the last reflex using a stethoscope that was mounted such that the diaphragm could be placed directly on the chest of the pig and the ear pieces could be worn by the observer on the outside of the chamber. The pig was determined to be dead when the observer could no longer hear the heartbeat. The stethoscope was required because the heart rate monitors are not reliable once the heart rate falls below 15 beats per minute (bpm) and becomes arrhythmic.

**Statistical Analysis**

All data were checked for normality and homogeneity of variance. Nonnormal data that could not be transformed were analyzed using the Kruskal–Wallis test statistic; these data were the latencies to perform behaviors in Exp. 1 and 2 and heart rate in Exp. 1. The model for this set of heart rate data included litter, time, and treatment. The remaining data were analyzed using mixed models with a heterogeneous first-order autoregressive covariate structure as appropriate (SAS Inst. Inc., Cary, NC) with a longitudinal analysis for the heart rate data, which were repeated measures collected over time. The model included the fixed effects of BW and treatment, with pig and litter (for Exp. 1) included as the random effect. The body temperature of the pig served as a covariate in Exp. 3. When significant differences (P < 0.05) were detected, Tukey-Kramer adjustments were used for pairwise comparisons between treatments.
Data were considered to exhibit a trend when $P > 0.05$ and $P < 0.10$. Data are presented as means ± SE.

RESULTS

Pilot Study

Pigs quickly learned to enter the test chamber, which was designed to attract them. During the first exposure of the pigs to the chamber, they easily were able to gain access through the door and spent 21.8 ± 3.1% of their time in the test chamber, and on their second trial they made marked improvements, spending 41.0 ± 13.1% in the test chamber. The attractants worked, with piglets choosing to spend 72.0 ± 0.8% of their time near the attractants on the test side of the chamber. Data collected on the day before testing piglets (d 14 of age) when medical air was delivered into both chambers indicate that the piglets chose to spend 59.3 ± 8.2% of their time near the attractants.

Experiment 1: Gradual Fill Method

Measurements of $O_2$ and $CO_2$ indicate that nonsignificant amounts of the treatment gas escaped into the control chamber (Fig. 2) and that concentrations of treatment gas mixtures in the test chamber were at 50% approximately 5 min into the test but never reached 100% before the end of the 10-min test (Fig. 2). The duration of testing was shortest for the pigs in the $CO_2$ treatment when compared with pigs in the $N_2O/CO_2$ ($P < 0.005$), $Ar/CO_2$ ($P < 0.003$), and $N_2/CO_2$ ($P < 0.01$) treatments, 3.13 ± 0.20, 8.46 ± 0.64, 9.64 ± 0.36, and 9.99 ± 0.01 min respectively, because pigs had to be manually removed when they started to fail. There were only 3 pigs in the $N_2/CO_2$ treatment and all 3 of these pigs finished this test in the control chamber after passing several times into the test chamber. All 6 of the pigs in the $CO_2$ treatment and 5 of the 7 pigs in the $N_2O/CO_2$ treatments had to be removed before the end of the 10 min test because they succumbed to the gas and started to fail or collapsed. Three of the 6 $Ar/CO_2$ pigs and none of the 3 $N_2/CO_2$ pigs had to be removed before the end of the test for the same reason. The 3 $N_2/CO_2$ pigs left the test chamber and finished the test in the control chamber. All pigs survived the treatments.

Examining the cumulative durations for time in the test chamber at different concentrations of $CO_2$ (used as a common measure across treatments), only pigs in the $N_2O/CO_2$, $N_2/CO_2$, and $Ar/CO_2$ treatments spent more than 1 min in the gas mixture when the $CO_2$ concentration ranged between 16 and 20% (the greatest range in which data from all 4 treatments could be collected; Fig. 3). Furthermore, the maximum duration in the test chamber was obtained for the $N_2O/CO_2$ treatment when pigs spent more than 3.5 ± 0.3 min in the test chamber at a similar (high) range of 16 to 20% of $CO_2$ (Fig. 3). Due to the variation in response between pigs in the free-choice test, we could not statistically compare specific behaviors between specific gas concentrations because pigs chose to enter and leave the test chamber at differing times corresponding to different gas concentrations, creating disparate data points, which are difficult to compare.

Heart rate means were compared based on activities defined in the ethogram. Because pigs could enter and leave each chamber at will, heart rate means could not be compared based simply on time in treatment. Initial heart rate mean was approximately 175 bpm and did not change statistically ($P > 0.1$; Fig. 4) throughout the test or in response to treatment ($P > 0.1$).

Experiment 2: Prefill Method

On the test day, once in the test chamber, pigs in the $N_2/CO_2$ treatment tended to have a shorter latency for the onset of stressed behavior compared with $N_2O/CO_2$ pigs

Figure 2. Concentrations of $CO_2$ and $O_2$ in the control (bottom) and test chamber (top) during the 10 min test in Exp. 1.
Rault et al. 1880

The latencies between the onset of stressed behavior and becoming disoriented and between becoming disoriented and failing were not different between treatments ($P > 0.1$). Overall, the duration of the total procedure showed that pigs submitted to the N$_2$/CO$_2$ treatment could endure that gas for 8 s longer than pigs in the N$_2$/CO$_2$ treatment before failing ($P < 0.09$). Heart rate data could not be obtained for this experiment because of defective equipment.

**Table 2.** Latency (number of seconds ± SEM) to specific behaviors in Exp. 2 based on when the pig first entered the test chamber

<table>
<thead>
<tr>
<th>Gas treatment</th>
<th>N$_2$/CO$_2$</th>
<th>N$_2$O/CO$_2$</th>
<th>$P$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enter test chamber to stressed</td>
<td>4.0 ± 0.7$^x$</td>
<td>7.3 ± 1.1$^y$</td>
<td>0.07</td>
</tr>
<tr>
<td>Stressed to disoriented</td>
<td>3.5 ± 1.0</td>
<td>4.8 ± 1.0</td>
<td>0.38</td>
</tr>
<tr>
<td>Disoriented to fail</td>
<td>3.3 ± 0.5</td>
<td>6.5 ± 1.8</td>
<td>0.28</td>
</tr>
<tr>
<td>Total duration of the test</td>
<td>10.8 ± 1.7$^x$</td>
<td>18.7 ± 2.7$^y$</td>
<td>0.09</td>
</tr>
</tbody>
</table>

$x,y$Means within a row with different superscript show a tendency to differ ($P < 0.1$).

The total duration of the test correspond to the time at which the pig entered the test chamber until it was removed when it started to fail.

**Experiment 3: Gas Effectiveness**

Measurements of O$_2$ and CO$_2$ indicate that by approximately 4 min, CO$_2$ concentrations had reached 15% in the N$_2$/O$_2$ and N$_2$/CO$_2$ treatments (representing 50% of the gas mixture) with the CO$_2$ treatment at 30% at the same time (Fig. 5). The time at which pigs were removed from the treatment chamber and placed into the CO$_2$ chamber differed ($P < 0.001$; Table 3), with the pigs in the CO$_2$ treatment being moved into the chamber prefilled with CO$_2$ sooner than for the N$_2$/CO$_2$ and N$_2$/O$_2$/CO$_2$ treatments ($P < 0.001$) and N$_2$/O$_2$ taking the longest of all treatments ($P < 0.004$). Nonetheless, all pigs in the N$_2$/O$_2$ treatment were moved into the CO$_2$ chamber because they became anesthetized. In contrast, none of the pigs in the other treatments became anesthetized and were moved into the CO$_2$ chamber because they started to either squeal or fail.

Once the pigs were transferred from the treatment chamber to the CO$_2$ chamber, the time to death was similar for all treatments ($P > 0.20$; Table 3). Overall, combining the 2 steps corresponding to each chamber, the total duration of the euthanasia procedure differed between treatments ($P < 0.005$; Table 3), with pigs in the N$_2$/O$_2$ treatment dying after the longest duration of treatment when compared with pigs in the 3 other treatments.

Heart rate did not differ by treatment when pigs were being exposed to the treatment gas ($P > 0.1$): 167.2 ± 2.3, 158.0 ± 2.2, 147.0 ± 3.1, and 134.6 ± 5.7 bpm for the N$_2$/CO$_2$, N$_2$/O$_2$/CO$_2$, N$_2$/O$_2$/CO$_2$, and CO$_2$ pigs, respectively (Fig. 6). However, once the pigs were placed into CO$_2$, heart rate did exhibit a treatment effect ($P < 0.002$) with pigs in the CO$_2$ and N$_2$/O$_2$ treatments having lower heart rates (98.3 ± 3.3 and 100.5 ± 4.2, respectively) than pigs in the N$_2$/CO$_2$ and N$_2$/O$_2$/CO$_2$ (149.6 ± 3.8 and 120.3 ± 2.2) treatments.

**DISCUSSION**

Data from these studies support the idea that exposure to a mixture of N$_2$O and O$_2$ before using CO$_2$ may be a more humane method of euthanasia than...
exposure to CO2 alone. This assertion is based on the observation that pigs exposed to N2O and O2 entered a state of anesthesia, which was not observed in any other treatments tested. In addition, when these pigs were subsequently placed into CO2, few started to flail. This observation was in sharp contrast to the squeals and flailing behavior exhibited by pigs that were exposed to the other treatment gases and then placed into CO2.

Experiment 1 was designed to measure the aversion that neonatal pigs had to the different treatment gases. Compared with aversion learning tests (Raj and Gregory, 1995; Jongman et al., 2000), we expected that our approach-avoidance paradigm would test a more spontaneous response from the piglet. This approach has proven successful to assess gas aversion from the point of view of the animal, a crucial aspect of animal welfare (rodents: Makowska et al., 2009). Piglets learned quickly to move between chambers and were successfully attracted to the test chamber. Experiment 1 did show that all gases overall were sufficiently aversive as to overcome the attractant stimuli, from the perspective of the piglet, as evidenced by the short time spent in the gas mixtures and the flailing behavior exhibited when the pig entered when concentrations were high. Experiment 1 also provided additional evidence to previous reports (Raj and Gregory, 1995; Velarde et al., 2007; Sutherland, 2011) that CO2 is more aversive than N2O, N2, or Ar because pigs in the latter treatments spent a longer duration in the test chamber when concentrations were elevated. It is important to note that the majority of the literature investigated the aversiveness of gas mixtures for mature, market-weight pigs whereas our experiments focused on gas aversiveness for piglets. It is unknown if differences between those ages affect the reaction to gases.

A previous study (Sutherland, 2010) suggested that the use of a prefill or “precharged” chamber may be more humane than gradual fill methods due the shorter time required to euthanize piglets. Therefore, Exp. 2 was designed to determine the relative aversiveness of 2 prefill gas treatments, N2/CO2 and N2O/CO2. Because in Exp. 1 the maximum duration in the test chamber was obtained for the N2O/CO2 treatment and because all pigs in the N2/CO2 treatment finished the 10 min test without flailing, we decided to proceed in Exp. 2 with these 2 gases and remove the Ar treatment. The prefill CO2 treatment was purposely omitted from this experiment because previous testing in our laboratory found that pigs reacted so quickly (less than 5 s) and violently to the prefill CO2 that they would flail and not have time to make the choice to return to the safe air chamber. In Exp. 2, this was also found to be the case for both N2/CO2 and N2O/CO2. None of the pigs that entered the chamber with either treatment successfully exited the test chamber on its own. All had to be “rescued” when they exhibited flailing behavior. Therefore, the prefill method was more aversive to the pigs than gradual fill. However, we found that pigs exposed to the N2O/CO2 tended to take a longer time to start to flail, indicating that the N2O was less aversive than N2 although the small difference of 8 s may carry only little biological relevance and suggests that both gases delivered under that prefill method were indeed highly aversive. Note that in Exp. 1 and 2, no pigs stayed long enough in the test chamber to lose consciousness. All expressed distress behaviors at some point that resulted in manual removal of the pig from the test chamber for ethical reasons.

Unfortunately, interpretation of the behavioral data collected in Exp. 1 and 2 is difficult as reasons for beings in either the test chamber or the control chamber

| Table 3. Duration (minutes ± SEM) to reach the different stages in Exp. 31 |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| Gas treatment   | CO2             | N2/CO2          | N2O/CO2         | N2O/O2          |
| Transfer from treatment to CO2 chamber | 2.9 ± 0.3 a     | 6.4 ± 0.6 b    | 6.7 ± 1.0 b    | 14.7 ± 2.1 c   | 0.001 |
| Time to death after transfer          | 7.8 ± 1.3 a     | 7.6 ± 1.0 a    | 5.6 ± 0.3 a    | 9.3 ± 1.2 a    | 0.20  |
| Total procedure duration              | 10.8 ± 1.3 a    | 13.9 ± 1.2 a   | 11.3 ± 1.4 a   | 24.0 ± 3.0 b   | 0.005 |

a,bMean within a row with different superscripts differ P < 0.05.

1The total procedure duration is from the time that treatment gas was initiated until death. Transfer from treatment to CO2 chamber indicates the amount of time the pigs were exposed to the treatment gas. Time to death after transfer is the time it took pigs to die after being placed into 90% CO2.
could vary. For instance, in some cases a pig would be in the test chamber for a longer duration than expected (when gas concentrations were high) because it could not make its way out of the chamber due to it starting to enter a state of flailing and not being able to make the choice to leave. This limitation is not unique to our test; indeed, exposure to stimuli that possibly impair cognitive processes (e.g., Jongman et al., 2000) question the validity of preference and motivation tests that rely on cognitive skills, particularly to assess procedures that are expected to lead to loss of consciousness.

In contrast to typical stressors that increase the heart rate of an animal, the heart rates of the pigs did not increase in response to any of the gases but stayed the same or fell when the pigs entered the CO2. Treatment effects could be seen after placement into the final CO2 chamber, with CO2 and N2O/O2 treatments resulting in decreased heart rates than N2/CO2 and N2O/CO2 treatments. Interestingly, the heart rate of rats exposed to Ar for 2 min stayed constant and equal to baseline values even though these animals exhibited seizure-like activity whereas the heart rate of rats exposed to CO2 decreased over a similar 2 min exposure (Burkholder et al., 2010).

Experiment 3 sought to determine the effectiveness of the gases previously evaluated for their aversiveness. However, because none of the gas mixtures tested in Exp. 1 and 2 induced anesthesia, we added a treatment consisting of N2O with O2 because previous research in our laboratory showed that this combination did induce anesthesia (Rault and Lay, 2011). The CO2 treatment was included as this is regularly used in the livestock industry and recommended (AVMA, 2007) as an approved method of euthanasia; therefore, it served as a control treatment. The mix of N2O with O2 was the only gas mixture able to induce anesthesia although that required 14 min. None of the other gas mixtures caused anesthesia, but in contrast, pigs showed stressed behavior or distress vocalizations, which required moving them to the CO2 prefill chamber.

In terms of expediency, treatments that contained CO2 led to the quickest death overall whereas the N2O with O2 treatment took the longest. However, the ability of N2O in combination with O2 to induce a state of anesthesia before euthanizing the pigs with CO2 indicates that it is the best agent tested in this study to humanely euthanize pigs. The fact that 90% CO2 leads to a quicker death than lower concentrations of CO2 or combinations with other gases is well known (Raj and Gregory, 1996; Velarde et al., 2007). Yet, whether expediency can compensate for aversiveness in determining what is a humane method of euthanasia remains ethically debatable. Raj and Gregory (1996) previously suggested a 2 step-process to euthanize mature pigs using Ar with 2% O2 before 90% CO2, rather than straight 90% CO2, to minimize distress. Our experiments show that N2O could be a better option than Ar for piglet euthanasia. Data on brain activity, such as the use of electroencephalogram, would help to confirm this finding.

Previous studies on gas euthanasia in pigs usually compare gases to each other and reported that 90% Ar caused less aversion than N2 with CO2 (with greater CO2 being associated with increased breathlessness), itself favored over 90% CO2 (Raj and Gregory, 1995; Raj, 1999; Dalmau et al., 2010). Our results, by using a control air chamber, showed that all gases mixtures are aversive compared with air, to various degrees. Yet N2O in O2 appeared to be less aversive than N2O or Ar all combined with low (30%) concentrations of CO2 or 90% CO2 by itself. Nitrous oxide has been proposed to reduce CO2 aversiveness in mature pigs (Channon et al., 2005) but this study used a very low concentration of N2O (10%) combined with a high concentration of

![Figure 6](image-url). Heart rate means [beats per minute (bpm) ± SEM] of pigs during gradual fill of gas treatment and euthanasia with 90% CO2 in Exp. 3. The treatments are indicated by their acronym located in the upper right corner of each plot. Pigs entered the test chamber at Time 0. Heart rate was analyzed per 30 s intervals. The vertical black line in each plot indicates the time at which the pig was moved from the test chamber into the CO2 chamber. This time was based on the behavior of the pig and decided when the pig either squealed, flailed, or became anesthetized. Missing data points are due to having only 1 or no heart rate data at that time. The gray arrow indicates the average time at which the pigs were declared dead.
CO₂ (90%) delivered in a prefill method. Our study is the first to investigate the use of N₂O at sufficiently high concentrations to cause anesthesia. These results and our previous experiment (Rault and Lay, 2011) refute the idea that hyperbaric conditions are required to induce anesthesia in piglets using N₂O (Weiskopf and Bogetz, 1984). Nitrous oxide, commonly referred to as laughing gas, has been widely used in human surgery and dental offices for its pain-relieving, sedative, and anxiolytic effects. It is cheap, nonflammable, nonexplosive, legally accessible, and not classified as a drug in the United States and is already commonly used in the food industry as a propellant for food products. Development of its use into an automated procedure will allow producers to implement it with little effort. Therefore, its use as an anesthetic/euthanasia agent may prove to be affordable, feasible, and more humane than other alternatives.

**LITERATURE CITED**


