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Effects of stocking rate on measures of efficacy and welfare during carbon dioxide gas euthanasia of young pigs

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Abstract

*The objective of this study was to evaluate the effects of chamber stocking rate on facets of animal welfare and efficacy during gas euthanasia of young pigs (*Sus scrofa domesticus*). Crossbred pigs (390 neonatal and 270 weaned) designated for euthanasia at production farms were randomly assigned to group sizes of one, two, four, or six pigs. Gas euthanasia of each piglet group was performed in a Euthanex® AgPro chamber. The chamber air was gradually displaced with CO_2 gas over 5 min to establish an in-chamber concentration of approximately 80% CO_2. Pigs remained in that atmosphere for an additional dwell period of at least 5 min. Higher stocking rates were associated with higher CO_2 concentrations after gradual fill for both age groups. While there was no evidence of an effect of stocking rate on latencies to loss of posture or last movement in neonatal pigs, there was evidence of an effect on all measured efficacy variables in weaned pigs, with grouped pigs faster to succumb than solitary pigs. This finding is consistent with expected consequences of higher CO_2 concentration at increased stocking densities. Aversive states and behaviours of focal pigs in the chamber were scored from video. Weaned solitary pigs displayed a high incidence of pacing and may have experienced isolation distress. Escape attempts were absent in neonates and not linearly affected by stocking rate in weaned pigs. Although the risk of hazardous interactions was correlated with group size, this study provided no evidence that isolation during gas euthanasia would benefit animal welfare.*

Keywords: *animal welfare, behaviour, carbon dioxide, euthanasia, pigs, stocking rate*

Introduction

Exposure to carbon dioxide gas (CO_2) can be used as a method of euthanasia in which acute respiratory acidosis and hypoxia lead to rapid nervous system depression and eventual respiratory and cardiac arrest (Forslid 1987). Induced hypercapnia is commonly used as a single-step method of euthanasia for young pigs and as a method of pre-slaughter stunning for adult swine (Atkinson *et al* 2012). Prolonged exposure to a concentration of 80–90% CO_2 in air is regarded by the American Veterinary Medical Association (AVMA) as an acceptable form of euthanasia for pigs (2013), although animal reactions to gas euthanasia appear to be closely tied to procedural details that vary widely within the industry and in published guidelines. Experimental comparison of physiological and behavioural indices of efficacy and welfare during procedural variations is needed to establish evidence-based recommendations for swine euthanasia.

Gas euthanasia methods are commonly chosen when large groups of animals need to be humanely and efficiently put to death, such as during disease outbreaks. Nevertheless, carbon dioxide gas does not provide instantaneous loss of consciousness and is believed to cause aversion and discomfort in animals, including pigs (Nowak *et al* 2007; Dalmau *et al* 2010; Mota-Rojas *et al* 2012). While the capacity to euthanise animals in groups is a major advantage of gas methods (Atkinson *et al* 2012), group euthanasia demands consideration of both direct and bystander effects.

CO_2 exposure elevates stress hormones in swine (Gregory *et al* 1987; Forslid & Augustinsson 1988; Kohler *et al* 1998) and increases vocalisation, agitation, and chamber escape attempts (Raj 1999; Velarde *et al* 2007; Rodríguez *et al* 2008). Numerous studies of pigs and other mammals show that visual, auditory, and olfactory alarms can transmit fear and stress to conspecifics in proximity to an animal undergoing a distressing procedure (Vieuille-Thomas & Signoret 1992; Talling *et al* 1996; Amory & Pearce 2000; Düpjan *et al* 2011). Carbon dioxide exposure also causes periods of neuromuscular excitation with myoclonus, which can appear violent and sometimes results in bone fractures in poultry (Raj 2006; McKeegan *et al* 2007). The onset of insensibility produced by CO_2 varies between pigs in unpredictable patterns that can only be ascribed to individual variation

Table 1 Summary of pigs euthanised by treatment (one-pig, two-pig, four-pig, or six-pig group sizes) and experiment (neonate or weaned). Data were collected from focal pigs with additional fill pigs creating variable chamber stocking rates.

	TRT 1	TRT 2	TRT 4	TRT 6
Experiment 1 (390 neonatal pigs)	30 focal, 0 fill	30 focal, 30 fill	30 focal, 90 fill	30 focal, 150 fill
Experiment 2 (270 weaned pigs)	30 focal, 0 fill	30 focal, 30 fill	–	30 focal, 150 fill

(Holst 2001). If loss of consciousness is asynchronous within a shared euthanasia chamber, there is potential for conscious pigs to be exposed to violent convulsions, distress vocalisations, and alarm pheromones of conspecifics, to the possible detriment of welfare (Raj 2006).

The density and positioning of animals also influence air flow within an enclosed space (Smith *et al* 1999), and crowding or piling of pigs in the chamber could create variable microenvironments that impede consistent gas exposure. Piling of pigs in the chamber, as with piling of poultry during mass euthanasia (Kingston *et al* 2005), may even result in some animals dying of asphyxiation without the anaesthetic benefits of carbon dioxide narcosis (Gerritzen *et al* 2008).

Alternatively, euthanasia in small groups could provide a calming effect for social animals like pigs during the periods of loading and retained consciousness (Sharp *et al* 2003; Atkinson *et al* 2012; Mota-Rojas *et al* 2012), and prevent the isolation distress to which young pigs are susceptible (Fraser 1975; Kanitz *et al* 2009). In a review of studies related to swine aversion toward CO_2 gas, the European Food Safety Authority offered that the isolation of market-weight pigs may have triggered escape attempts observed during solitary gas stunning, and recommended that pigs be maintained in a stable social group throughout the slaughter process (EFSA 2004).

Current AVMA Guidelines warn against performing individual euthanasia in the presence of sensitive conspecifics, but advise administering inhaled agents "under conditions where animals are most comfortable (eg, […] for pigs, in small groups)" (2013; p 19). The AVMA recommendations for inhalant euthanasia agents include cautions that "[i]f animals need to be combined, they should be of the same species and compatible cohorts, and, if needed, restrained or separated so that they will not hurt themselves or others. Chambers should not be overloaded" (2013; p 19). Canadian Council on Animal Care (CCAC) Guidelines also recommend sheltering conspecific bystanders from the euthanasia process, but advise euthanising groups of familiar animals together to avoid isolation of social species and buffer against stressors (2010). Given reports of asynchronous loss of consciousness, it is unclear whether administrators of CO_2 euthanasia should follow the prescriptions for individual euthanasia to avoid intensifying distress, or should strive to maintain a comforting social group during the procedure. Toward the latter goal, very little information is available regarding target stocking density or ideal group size during chamber euthanasia.

This study was designed to begin addressing those questions by assigning pigs to variable group sizes during gas euthanasia. Objectives of this study were to determine whether chamber stocking rate affects efficacy and animal welfare during CO_2 euthanasia of young pigs and to test the hypothesis that ongoing stimulation from cohorts may delay the onset of unconsciousness and increase the potential for distress when pigs are euthanised in groups.

Materials and methods

Study protocol

Animal procedures were performed with the approval of the Iowa State University Institutional Animal Care and Use Committee.

Study design

This study involved collection of physiological and behavioural data from two age groups of non-randomly selected cull pigs (*Sus scrofa domesticus*) during euthanasia. Experiment 1 used neonatal pigs (under 72 h of age) and Experiment 2 used weaned pigs (3–10 weeks of age). A power analysis in SAS® (PROC POWER, SAS Inst Inc, Cary, USA) using standard deviations from a previous euthanasia study in this laboratory indicated that at least 28 replicates would be required to provide 80% statistical power for the detection of a 30-s difference in latencies to loss of posture and 15-s difference in latencies to onset of ataxia. Based on that result, 30 replicates were used for each experiment.

To avoid delaying euthanasia for welfare-compromised animals, pigs were enrolled in the study each day in order of health instability and euthanised in the same sequence. The treatment order applied to consecutively enrolled pigs was randomly pre-determined. Treatments consisted of group sizes during euthanasia, specifically one pig (TRT 1), two pigs (TRT 2), four pigs (TRT 4), or six pigs (TRT 6). Based on a lack of significant findings from Experiment 1 and the relative difficulty of sourcing large numbers of nursery-aged pigs in need of euthanasia, TRT 4 was not used during Experiment 2. Each experimental unit ('piglet group' or PG) was thus comprised of one, two, four, or six pigs of the same treatment and replicate that were euthanised together, creating variable chamber stocking rates. One randomly selected pig in each piglet group was designated as the observational unit ('focal pig') for collection of behavioural observations. See Table 1 for a summary of all animals euthanised.

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Experiment 1 — Animals, housing and procedures

Crossbred ([Duroc] × [Large White × Landrace]) pigs of mixed sexes were utilised for this study only after being designated for euthanasia at commercial swine production farms due to animal welfare concerns or low commercial viability. A total of 390 pigs under 72 h of age (mean bodyweight [± SD]: 0.79 [± 0.28] kg; range: 0.1 to 2.2 kg) were euthanised in Experiment 1. Prior to selection, pigs were birthed and housed in indoor farrowing crates following standard husbandry practices of the producers. Upon designation for euthanasia at one of six proximal farrowing facilities, neonatal pigs were transported to a neighbouring building and group-housed with wood-shavings and heat lamps during study enrolment.

Pigs were enrolled in the study shortly before euthanasia by identification and physical examination. Those animals with unstable vital signs or acute injuries (bone fractures, open wounds, or contusions) were granted antecedence for enrolment and pigs that appeared unable to withstand the enrolment process were rejected from the study and euthanised immediately in accordance with farm procedures. The enrolment examination included assessing and recording weight, sex, heart and respiratory rate, rectal body temperature, apparent reasons for selection, body condition, activity level, hydration status, and oral, ocular, and nasal discharges. Each enrolled pig was marked with a PaintStik® (ALL-WEATHER®, Elk Grove, USA), to correspond to its consecutive identification number. Body condition scoring was based on guidelines provided in Straw *et al* (1999).

When assigning apparent reasons for selection during enrolment, researchers mimicked record-keeping systems used in some US swine production facilities (eg, PigCHAMP) and made subjective determinations of any obvious abnormalities for each piglet, such as injury (visible lacerations, fractures, or contusions), runt (noticeably below average weight for age), or low viability (visible signs of illness or poor vitality that would impair the pig's ability to compete for resources or survive). These determinations were not intended to be diagnostic and although there was regular communication between researchers and farm managers regarding major herd health concerns, the reasons stated may not have matched the reasoning of the caretaker making the designation for euthanasia.

Gas euthanasia was performed in a 43 × 60 × 30 cm (length × width × height) Euthanex® AgPro chamber with Smartbox™ control system (Value-Added Science & Technology, Mason City, USA) modified with transparent acrylic on the top and front panels for observation. The Smartbox™ control system includes timers and regulators that allow for customisable and precise administration of flow rates and delivery times (Sadler 2013). Industrial grade CO_2 was supplied from compressed gas cylinders via a volumetric high-output heated two-stage regulator (Euthanex, Allentown, USA). A clean rubber mat and wood-shavings were provided on the floor of the chamber prior to each trial to improve traction and comfort. The chamber was fitted with a carbon dioxide sensor (CO2Meter, Ormond Beach, USA) that was connected to a personal computer for recording and live observation of CO_2 levels in the AgPro chamber using CO_2 Meter DAS100 software (v 1.0.4867.20353 CO2Meter, Ormond Beach, USA). Video recording was achieved with a portable observation lab (Noldus Information Technology, Wageningen, The Netherlands). Two colour digital video cameras (model WV-CP484, Panasonic, Kadoma, Japan) were positioned perpendicularly to view activity in the chamber from above and beside it. The cameras were connected to a multiplexer that enabled capture of a dual recording at 30 frames s^{-1} on a personal computer using HandiAvi software (v 4.3, Anderson's AZcendant Software, Tempe, USA). Between each PG, the chamber was cleaned of bodily excretions and vacuumed to restore an ambient air composition.

During each trial, all of the pigs in a PG were enclosed in the chamber and the automated control box was used to time the administration of gas for 5 min. On the basis of the National Pork Board and American Association of Swine Veterinarians guidelines (National Pork Board 2008), a gradual-fill gas euthanasia protocol was implemented. A medium chamber volume exchange rate of 35% was selected based on the results of prior research evaluating efficacy and animal welfare implications of different gas flow rates (Sadler *et al* 2014a). A flow duration of 5 min was established in pre-trial testing as the necessary time to reach an in-chamber concentration of 80% CO_2 at this flow rate (1.59 m^3 per h or 35% chamber volume exchange per min) when the chamber was empty.

Based on National Pork Board recommendations to producers (2008), a 5-min in-chamber dwell period after reaching 80% CO_2 was considered adequate to achieve death. The first two neonatal piglet groups in Experiment 1 were left in the closed chamber for an additional 5 min in-chamber dwell; however, three of these six pigs (50%) had a detectable heartbeat upon removal. In an effort to achieve a lower failure rate, the dwell procedure was modified over the course of Experiment 1. The dwell period was first extended to 7 min for 10 PG and seven of these 25 pigs (28%) had a detectable heartbeat upon removal. The dwell period was further extended to 9 min for the next 23 PG, and 18 of these 80 pigs (23%) had a detectable heartbeat upon removal.

Since all piglets euthanised to that point had been insensible upon removal and all but one piglet had reached permanent respiratory arrest and presumably brain death by removal, it was decided that cardiac activity detectable by stethoscope was not indicative of unsuccessful gas euthanasia. All remaining piglets in Experiment 1 (85 PG, n = 279) were subject to 5 min of medium rate gas flow followed by a 5-min in-chamber dwell period during which a minimal gas flow rate (0.42 m^3 per h, or 10% chamber volume exchange per min) was used to prevent a decline of the in-box CO_2 concentration below 80%.

Upon removal from the chamber, all pigs were immediately tested for signs of sensibility, including corneal reflex, withdrawal reflex to nose prick and leg pinch, and pupillary light reflex (National Pork Board 2008). Pigs were also observed

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Table 2 Ethogram used when scoring focal pig behaviour and status from video recordings (adapted from Sadler 2013).

Behaviour	Definition
Ataxic movement	An apparent loss of co-ordination during voluntary movement, such as stumbling, dropped hocks, or crossed-leg stance
Crowded	Pig's head is more than 50% obstructed from free air flow by the presence of another pig(s); or attempts at normal posture, ambulation, or rising from recumbency are restricted by the presence of another pig(s)
Neuromuscular excitation	Period of seemingly involuntary excitement with unproductive, repetitive muscular contractions, including trembling, hyperresponsivity, thrashing, head shaking, paddling, or kicking
Onset of gasping	First point at which piglet begins reflexive gasping, characterised by rapid inspiration and gradual expiration at long and possibly irregular intervals, with exaggerated thoracic movements and corresponding sudden opening and slow relaxation of the jaw
Onset of open-mouth breathing	First point at which piglet begins breathing rapidly through continuously open mouth (panting)
Other	Animal behaviour or status does not fit into any other category
Out of view	Animal cannot be seen clearly enough to identify behaviour or status
Pacing	Repetitive, patterned locomotion along an interior wall of the chamber
Potential escape attempt	Apparently voluntary effort to escape the chamber, such as rearing with raised forelegs while pawing at chamber or in air, pushing with head or nose on the chamber lid, or forceful co-ordinated movement against interior of chamber
Righting attempt	Apparent attempt to restore standing, sitting, or sternal posture from sitting or recumbent position that was unsuccessful in maintaining the posture
Struck	Receives potentially harmful or offensive contact as a result of action by another pig, including being stepped on, bitten, kicked, shoved, or crushed

for cessation of breathing and auscultated with a stethoscope for at least 30 s to confirm cardiac arrest. Pigs that had not permanently ceased respiration before removal from the chamber were immediately euthanised using methods approved in the AVMA Guidelines, namely additional gas exposure or blunt force trauma for neonates (2007). Pigs with a heartbeat but no detectable respiration upon removal were observed for a 10-min, out-of-chamber dwell period in ambient air and then re-auscultated to confirm cardiac arrest. Pigs for which cardiac activity had not ceased after 10 min out-of-chamber were also secondarily euthanised with alternate, approved methods. No pigs recovered reflexes or resumed respiration during the out-of-chamber dwell period.

Experiment 2 — Animals, housing and procedures

Methods used in Experiment 2 were the same as those used in Experiment 1 except as specified here. Experiment 2 used 270 weaned pigs between 3–10 weeks of age (mean bodyweight [± SD]: 4.0 [± 3.0] kg; range: 1.6 to 26 kg). Weaned pigs were enrolled and euthanised on a single farm while housed in indoor group nursery pens following standard husbandry practices of their producer. After triaging for welfare, ten atypically large pigs (> 10 kg) with stable health statuses were enrolled on a rotational basis with pens of younger pigs in order to avoid exceeding the capacity of the chamber, although treatment group size for these animals was still subject to the pre-trial randomisation.

Experiment 2 utilised a 61 × 91 × 46 cm chamber. In order to closely parallel Experiment 1, the flow rate needed to reach an in-chamber CO_2 concentration of 80% in 5 min. This was determined in pre-trial testing to be 4.53 m^3 per h, or 30% chamber volume exchange per min, when the chamber was empty. Because weaned pigs have been observed to lose posture and achieve last movement later than neonates (Sadler *et al* 2014a), all weaned pigs in Experiment 2 were left in the closed chamber for the 5 min gradual fill time plus an 8-min in-chamber dwell period prior to removal for sensibility testing.

Pigs that had not permanently ceased respiration by removal from the chamber were immediately euthanised using a penetrating captive bolt device per AVMA guidelines (2007). Pigs with a heartbeat but no detectable respiration upon removal were observed for a 10-min out-of-chamber dwell period in ambient air and then re-auscultated to confirm cardiac arrest. No weaned pigs were found to have cardiac activity lasting greater than 10 min after removal from the chamber and no pigs recovered reflexes or resumed respiration during the out-of-chamber dwell period.

Behavioural observation

During exposure to CO_2, a single unblinded observer recorded focal pig latency to loss of posture and latency to last movement. Latency to loss of posture was defined as the elapsed time from start of gas flow until the recumbent focal pig stopped attempting to lift its head. This behaviour was regarded as a non-invasive approximation of the time required to reach unconsciousness. Because pigs often fall and rise repeatedly during induction with CO_2 and because some cull pigs never stood in the chamber, the posture of the head was judged to be the most consistent time-point to use

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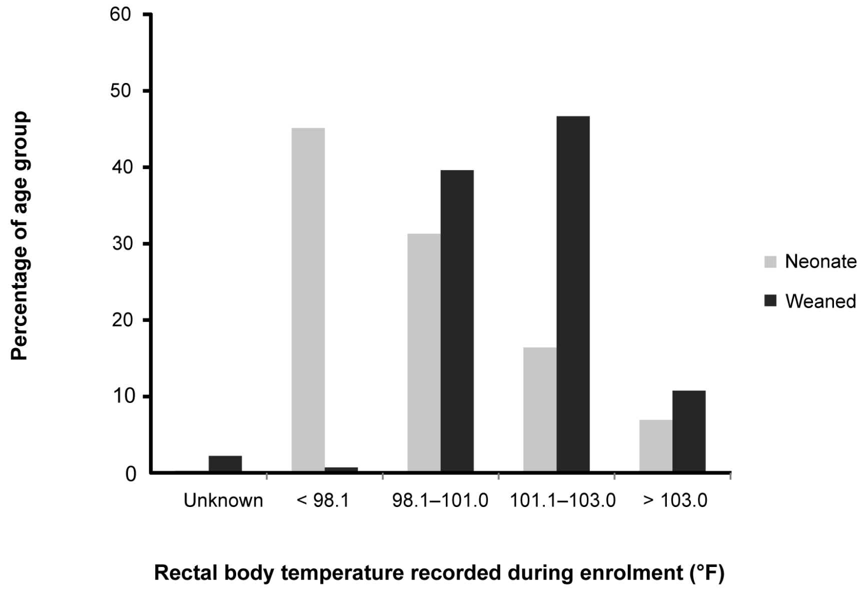
Figure 1



Rectal body temperatures of neonatal and weaned pigs recorded during enrolment.

for this estimate. Latency to last movement was defined as the elapsed time from start of gas flow to complete and permanent cessation of focal pig movement including respiratory arrest. This behaviour was regarded as a non-invasive approximation of the time required to achieve brain death.

Video recordings were scored by the same individual using Observer XT (Noldus, Wageningen, The Netherlands) for the frequency or duration of focal pig behaviours and statuses tied to specific animal welfare considerations. See Table 2 for video ethogram. An intra-observer reliability test performed in Observer generated a Cohen's kappa of 1.00 with a tolerance window of 3 s.

Statistical analysis

All statistical analyses were performed in SAS® using a significance level of 0.05. The CO_2 concentrations recorded at conclusion of gradual fill (5 min from gas start) for each piglet group were analysed for differences in treatment means using a general linear model (PROC GLM) with stocking rate as a categorical variable and the sole fixed effect.

Survival analysis with Weibull distribution (PROC LIFEREG) was used to test whether there was a multiplicative effect of CO_2 concentration at conclusion of gradual fill on latencies to loss of posture, onset of gasping (scored from video), and last movement (right censored at time of removal). The LIFEREG procedure was also used to determine whether there was a multiplicative effect of stocking rate on latencies to loss of posture, onset of gasping, or last movement. All clinical variables assessed during enrolment were examined as potential covariates. The final models were selected based on best fit and included combinations of day of trial, weight, body condition score, heart rate, respiratory rate, and body temperature as specified in *Results*. All three latency variables were log-transformed to improve model fit. Pair-wise comparisons were made between the behavioural latencies of solitary pigs and latencies observed at other stocking rates.

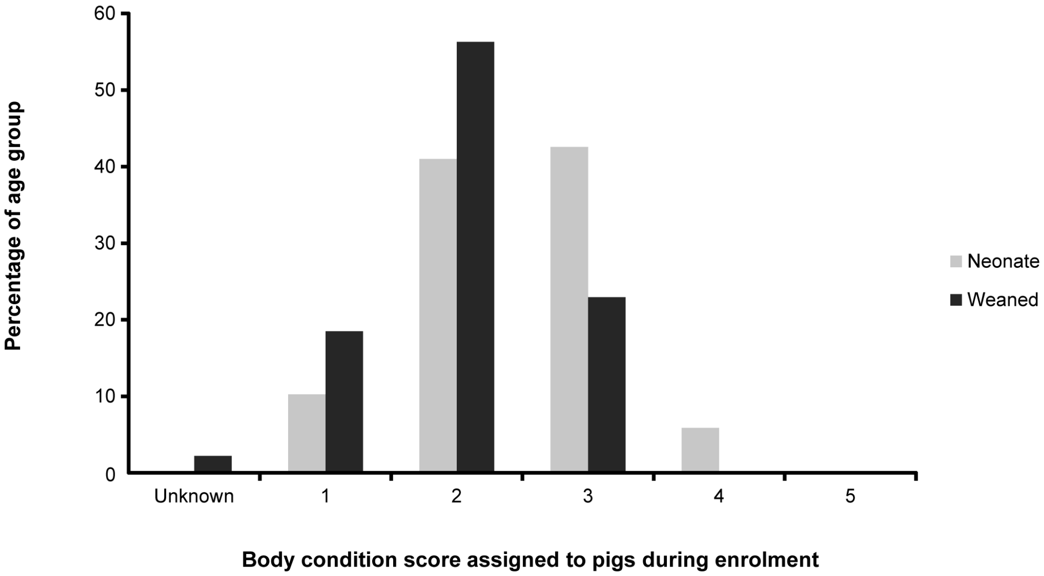
Levene's test for homogeneity of variance was used to test whether grouped focal pigs were more variable in latencies to loss of posture than solitary pigs (PROC GLM). Differences in failure rates (binary for heartbeat upon removal) by treatment were also analysed using survival analysis (PROC LIFEREG). Data analysis for failure rate during Experiment 1 used only the subset of 85 neonatal focal pigs that experienced a 5-min in-chamber dwell period with additional slow-flow CO_2.

Poisson regression (PROC GENMOD) was used to test for an effect of stocking rate on righting attempt and escape attempt counts, as scored from video. General linear modelling (PROC GLM) was used to test for an effect of stocking rate on the durations of open-mouth breathing, neuromuscular excitation, and pacing.

Results

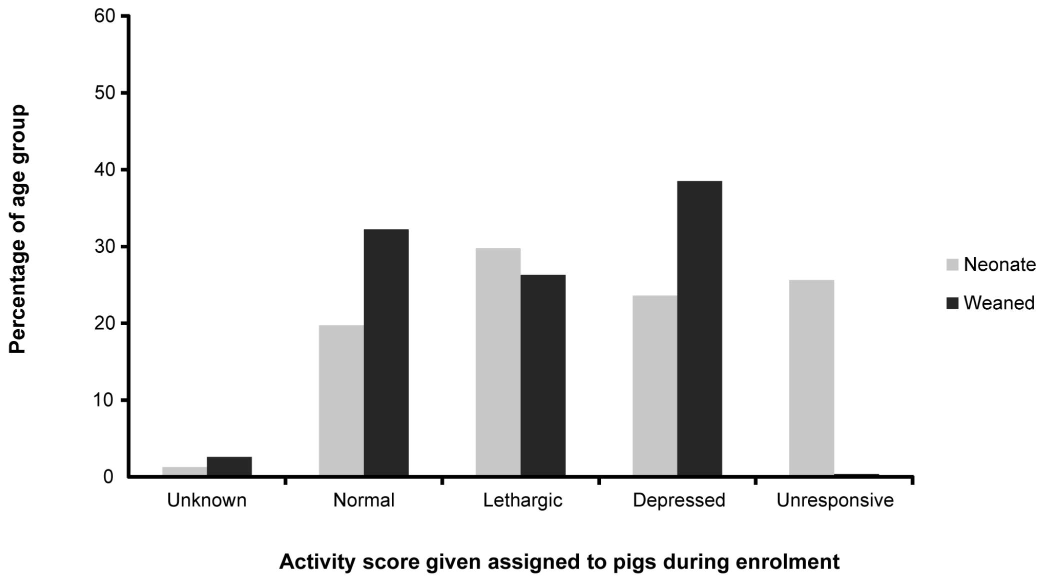
Differences in results between age groups should be considered in light of the characteristics of the pig populations sampled for each experiment. For example, a large proportion of neonatal pigs were hypothermic at the time of euthanasia and 26% were moribund and unresponsive. Over 70% of weaned pigs showed signs of chronic illness (respiratory, dermatological, or gastrointestinal) and many were undersize for their age, but the majority of pigs in that age group had a normal or only mildly reduced level of activity. Rectal body temperatures, body condition scores, activity scores, and potential reasons for euthanasia recorded for both experiments are summarised in Figures 1 to 4.

Figure 2



Body condition scores of neonatal and weaned pigs recorded during enrolment.

Figure 3



Activity scores of neonatal and weaned pigs recorded during enrolment.

Experiment 1

Higher stocking rates in neonate trials were associated with higher CO_2 concentrations at conclusion of gradual fill (P < 0.01). The differences in gas concentration observed most likely reflect the change in stocking density (and thus reduction of air volume) created by increasing stocking rate; see Table 3 and Figure 5[a] for depictions of mean total bodyweight and CO_2 concentration by treatment. There was no significant evidence of an effect of CO_2 concentration at conclusion of gradual fill on the log of any behaviour latencies in neonatal pigs.

There also was no significant evidence of an effect of stocking rate on any behaviour latencies in neonates after controlling for day of trial. See Table 4 for mean latencies by treatment. There was no significant evidence of an effect of stocking rate on the proportion of neonatal pigs with heartbeat upon removal.

The latencies to loss of posture for the first and last pig to succumb in each neonatal piglet group were recorded during live observation, and the difference between these values was calculated as an estimate of response asynchrony. Overall, the mean (± SEM) for the difference in

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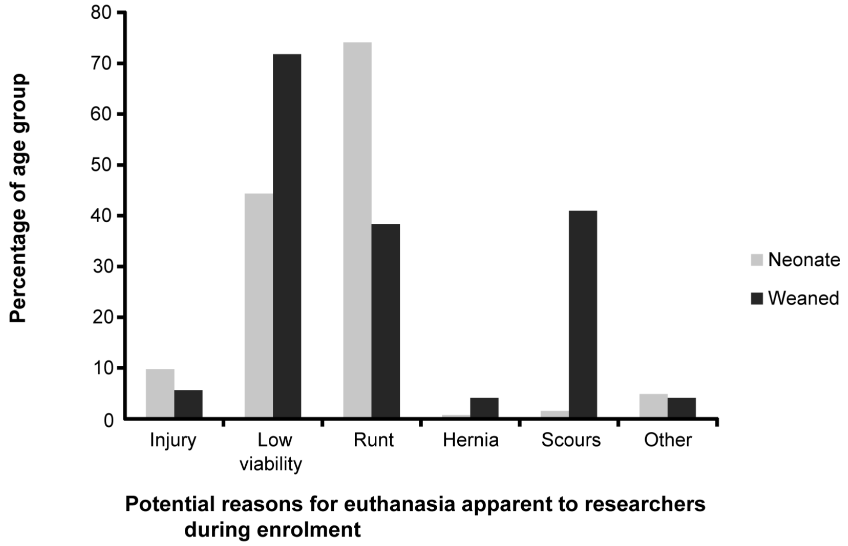
Figure 4



Potential reasons for euthanasia as recorded by researchers during enrolment of neonatal and weaned pigs (multiple reasons allowed per animal).

Table 3 Summary of piglet group mean (± SEM) total bodyweight (kg) and mean (± SEM) CO_2 concentration (%) at conclusion of gradual fill*.

	TRT 1	TRT 2	TRT 4	TRT 6
Experiment 1: neonatal pigs in 0.8 m³ chamber				
Total weight (n = 120)	0.8 (± 0.1)	1.6 (± 0.1)	3.0 (± 0.1)	4.9 (± 0.1)
CO_2 at 5 min (n = 111)	78.2 (± 0.5)	78.2 (± 0.5)	79.2 (± 0.5)	80.5 (± 0.5)
Experiment 2: weaned pigs in 0.25 m³ chamber				
Total weight (n = 90)	5.0 (± 1.4)	7.3 (± 1.4)	–	24.4 (± 1.4)
CO_2 at 5 min (n = 86)	78.5 (± 0.4)	79.6 (± 0.4)	–	82.6 (± 0.4)

* Reduced sample sizes reflect software malfunctions during data collection.

responses within non-solitary neonatal piglet groups was 28 (± 2) s (range 2 to 66 s), with one-third of PG in TRT 6 having a difference between first and last latency to loss of posture that was greater than 45 s. There was no significant evidence of an effect of stocking rate on variability in latencies to loss of posture for neonatal pigs.

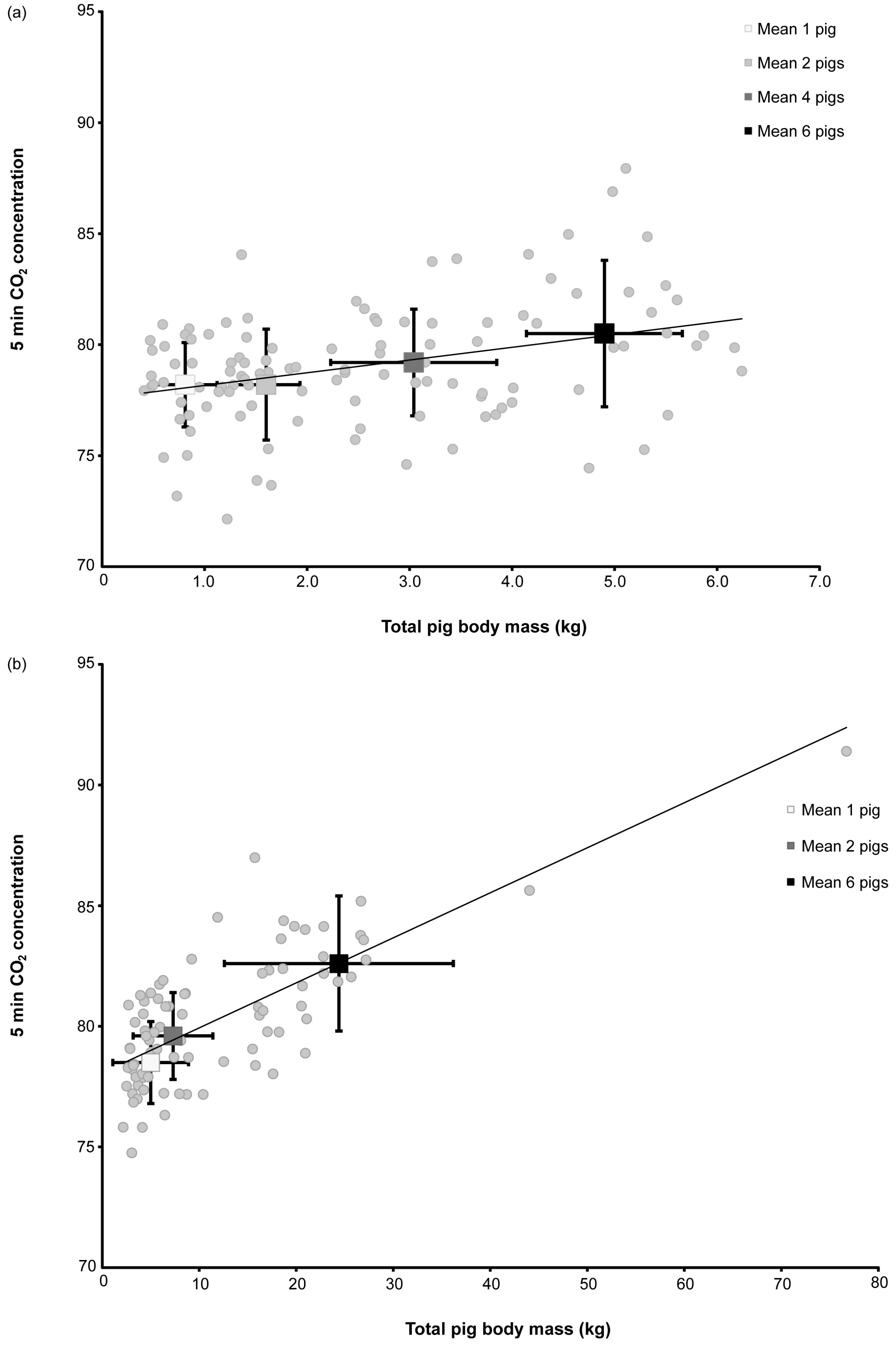
Neonatal pigs were generally quiescent in the chamber and displayed few behaviours from which welfare could be assessed. Mean righting attempt counts were higher in solitary focal pigs than grouped focal pigs ($P = 0.02$). There was no significant evidence of an effect of stocking rate on durations of open-mouth breathing in neonates. Escape attempts and pacing were not observed in this age group. Visible neuromuscular excitation was observed in only two of 120 focal pigs. Ataxia was not scored in this age group due to the prevalence of paresis and other causes of abnormal starting motor co-ordination in cull neonates. See Table 5 for a summary of behavioural data from neonatal focal pigs.

It was noted that the proportion of neonatal pigs receiving some type of potentially offensive contact from another pig while in the chamber ('strikes', eg, kicked, stepped on, or shoved) increased from 3% of focal pigs in TRT 2 to 30% of focal pigs in TRT 6. The average number of strikes received by each neonatal focal pig remained fairly low, but increased from 0.14 in TRT 2 to 0.60 in TRT 6.

Experiment 2

As in neonate trials, higher stocking rates in weaned pig trials were associated with higher CO_2 concentrations at conclusion of gradual fill ($P < 0.001$); see Table 3 and Figure 5(b) for CO_2 concentration by total bodyweight of piglet groups. There was no significant evidence of an effect of CO_2 concentration at conclusion of gradual fill on log latency to loss of posture in weaned pigs after controlling for body condition score, weight, and starting heart rate, but there was evidence of an association between the CO_2

Figure 5



CO_2 concentration (%) at conclusion of gradual fill varied with the total bodyweight (kg) of (a) neonatal and (b) weaned pigs in the chamber.

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Table 4 Least square means (± SEM) of latencies to loss of posture, onset of gasping, and last movement in focal pigs.

	TRT 1	TRT 2	TRT 4	TRT 6
Experiment 1: neonatal pigs				
Loss of posture (n = 120; *P* = 0.06)	83 (± 3.4)	84 (± 3.3)	85 (± 2.9)	76 (± 3.0)
Onset of gasping (n = 114; *P* = 0.13)	89 (± 3.5)	84 (± 2.6)	89 (± 2.7)	86 (± 6.2)
Last movement (n = 114; *P* = 0.61)	368 (± 15)	355 (± 11)	390 (± 17)	367 (± 13)
Experiment 2: weaned pigs				
Loss of posture (n = 90; *P* < 0.01)	107 (± 1.8)	109 (± 3.5)	–	100 (± 2.1)
Onset of gasping (n = 88; *P* < 0.001)	122 (± 3.1)	116 (± 3.1)	–	111 (± 2.4)
Last movement (n = 82; *P* < 0.001)	488 (± 28)	510 (± 29)	–	412 (± 15)

Reduced sample sizes for onset of gasping reflect focal pigs that did not display gasping or could not be clearly viewed on video. Reduced sample sizes for last movement reflect 14 focal pigs for which values were censored at time of removal from the chamber. *P*-values are included for linear effect of stocking rate.

Table 5 Behaviours of neonatal focal pigs scored from video in Experiment 1.

	Righting attempts (P)	Righting attempts (C)	Open-mouth breathing (D)
TRT 1	0.43	0.87 (± 0.24) ↑	46 (± 3)
TRT 2	0.24	0.41 (± 0.17) ↑	44 (± 3)
TRT 4	0.31	0.52 (± 0.18) ↑	51 (± 3)
TRT 6	0.17	0.33 (± 0.21) ↑	48 (± 6)

Arrows indicate direction of statistically significant positive effect of stocking rate, if any.
P = proportion of focal pigs exhibiting behaviour, C = mean count with standard error, D = mean duration (s) with standard error.

Table 6 Proportion of focal pigs for which a heartbeat was detectable by stethoscope upon removal from the chamber.

	TRT 1	TRT 2	TRT 4	TRT 6
Experiment 1 (neonatal, *P* = 0.48)	0.47	0.45	0.59	0.38
Experiment 2 (weaned, *P* = 0.04)	0.20	0.30	–	0.07

P-values are included for linear effect of stocking rate.

concentration and log latencies to onset of gasping ($P < 0.001$) and last movement ($P < 0.001$) after controlling for body condition score, weight, and starting heart rate.

There was evidence of an effect of stocking rate on the log of latency to loss of posture in weaned pigs after controlling for day of trial and activity level ($P < 0.001$), but neither group size had an estimated effect that was significantly different from that of solitary pigs. There was evidence of an effect of stocking rate on the log of latency to onset of gasping in weaned pigs after controlling for day of trial, weight, starting heart rate, and body temperature ($P < 0.001$), where grouping pigs accelerated the onset of gasping relative to solitary pigs ($P = 0.01$). There was also evidence of an effect of stocking rate on the log of latency to last movement in weaned pigs after controlling for day of trial, body condition score, starting respiratory rate, and body temperature ($P < 0.001$), where grouping pigs accelerated the cessation of respiration relative to solitary pigs ($P = 0.02$). See Table 4 for mean latencies by treatment. There was also evidence of an effect of stocking rate on the proportion of weaned pigs with heartbeat upon removal using survival analysis ($P = 0.04$), with grouped focal pigs more likely to achieve cardiac arrest before removal. See Table 6 for proportions of pigs removed with heartbeat.

As in Experiment 1, differences between first and last latencies to loss of posture within PG were calculated to estimate response asynchrony. Weaned pigs had lower within-group variability than neonates. Across all non-solitary treatments, the mean (± SEM) for the difference in responses within weaned piglet groups was 17 (± 2) s (range 1 to 53 s), with only one PG in any treatment having a difference between first and last latency to loss of posture that was greater than 45 s. There was no significant evidence of an effect of stocking rate on variability in latencies to loss of posture for weaned pigs.

Table 7 Behaviours of weaned focal pigs scored from video in Experiment 2.

	Righting attempts (P)	Righting attempts (C)	Open-mouth breathing (D)
TRT 1	0.76	1.6 (± 0.3) ↓	59 (± 4)
TRT 2	0.83	2.4 (± 0.4) ↓	48 (± 3)
TRT 6	0.93	2.6 (± 0.3) ↓	52 (± 3)
	Pacing (P)	**Pacing (D)**	**Neuromuscular excitation (D)**
TRT 1	0.90	23 (± 2.9) ↑	64 (± 5) ↑
TRT 2	0.21	3.3 (± 1.4) ↑	54 (± 6) ↑
TRT 6	0.04	0.2 (± 0.2) ↑	39 (± 6) ↑
	Escape attempts (P)	**Escape attempts (C)**	**Ataxia (D)**
TRT 1	0.47	0.93 (± 0.23)	31 (± 3)
TRT 2	0.31	0.55 (± 0.18)	28 (± 3)
TRT 6	0.37	0.89 (± 0.26)	25 (± 3)

Arrows indicate direction of statistically significant positive effect of treatment, if any.
P = proportion of focal pigs exhibiting behaviour, C = mean count with standard error, D = mean duration (s) with standard error.

In Experiment 2, mean righting attempt counts were higher in grouped focal pigs than solitary focal pigs ($P = 0.04$). There was no significant evidence of an effect of stocking rate on escape attempt counts. Mean duration of pacing was higher at lower stocking rates ($P < 0.001$), as was mean duration of neuromuscular excitation ($P = 0.02$). There was no significant evidence of an effect of stocking rate on durations of open-mouth breathing or ataxia. See Table 7 for a summary of behavioural data from weaned focal pigs.

Weaned pigs were both more active and more crowded in the chamber, and potentially negative interaction was much more common in weaned pigs than in neonatal pigs. The proportion of weaned pigs receiving some type of potentially offensive contact from another pig while in the chamber ('strikes', eg, kicked, stepped on, or shoved) increased from 34% of focal pigs in TRT 2 to 81% of focal pigs in TRT 6. The average number of potentially offensive contacts increased from 1.1 per pig in TRT 2 to 4.0 per pig in TRT 6. Many kicks were the result of neuromuscular excitation, which was observed before, during, and after loss of posture during CO_2 euthanasia, so it is not known how many of these strikes were consciously perceived.

Discussion

Humane euthanasia should rapidly induce unconsciousness and death (Raj 2006). The measures of efficacy used in this study were latencies to loss of posture, onset of gasping, and last movement, which represent critical transitional points during the induction of unconsciousness and death by hypercapnic hypoxia (Forslid 1987; Raj 1999; Llonch *et al* 2012; Sadler *et al* 2014a). In both age groups, higher stocking rates were associated with higher concentrations of CO_2 at the conclusion of gradual fill, and tests for effect of gas concentration on efficacy variables suggest that this difference was likely a major factor in other significant results observed. Those results included a trend toward negative effect of increased stocking rate on latency to loss of posture in neonates and evidence of negative effects of increased stocking rate on latencies to loss of posture, onset of gasping, and last movement in weaned pigs. Since a similar correlation between increased chamber stocking rate and more rapid air composition change has been observed by this laboratory in studies using argon gas (unpublished data), the reduction of air volume is assumed to be the primary mechanism behind this association.

Corroborating the variable loss and recovery of reflexes described for market weight pigs (Holst 2001), young pigs did not lose posture simultaneously when euthanised together in this study. The speed of response varied considerably in some cases, especially within grouped neonates, and was reflective of the wide range of individual characteristics that exist within a single cull selection in the production environment. Increasing the size of a euthanasia group will increase the probability of combining pigs with unusually low and high response rates, risking one or more conscious witness to conspecific distress. However, there was no evidence that stocking rate affected individual variability in latency to loss of posture.

Humane euthanasia should also strive to minimise aversion and distress, so several physiological responses associated with poor welfare were quantified in this study. Ataxia was commonly observed during CO_2 induction of weaned pigs from approximately the onset of hyperpnea until loss of posture. Ataxia during hypercapnia could be caused by impairment of the motor cortex, loss of sensory and proprioceptive signals, or both (Oliver *et al* 1997). Because ataxia occurs prior to complete loss of consciousness, the impaired mobility may be distressing to pigs and thus duration of ataxia was used in this study as a measure of welfare. Hyperpnea, or open-mouth breathing as it appears in pigs, is a form of respiratory compensation in response to decreased pH of blood and cerebrospinal fluid (Gerritzen *et al* 2008; Mota-Rojas *et al* 2012). Humans exposed to carbon dioxide often report an uncomfortable sense of breathlessness and an insatiable drive to breathe more (Llonch *et al* 2012). The prolonged period of hyperpnea observed in both neonatal and weaned pigs may constitute a form of respiratory distress and so was included as a measure of welfare. We did not find evidence of an effect of stocking rate on durations of ataxia or open-mouth breathing, although there was a trend toward decreased duration of open-mouth breathing with increased stocking rate in weaned pigs.

Neuromuscular excitation (including hyper-responsivity, trembling, myoclonus, and convulsions) may be caused by loss of inhibition from the cerebral cortex and rostral reticular formation on the caudal reticular formation (Raj 2006; Llonch *et al* 2012). Neuromuscular excitation was observed consistently in weaned pigs but not in neonates. At first onset, these periods of excitation often coincided with escape attempts and righting attempts, suggesting that pigs retained some degree of consciousness. This conclusion agrees with Raj *et al* (1997) and Rodríguez *et al* (2008), both of whom noted muscular excitation in pigs beginning prior to significant changes in brain function when exposed to CO_2. The experience of involuntary excitation while conscious may be distressing to pigs (Dalmau *et al* 2010), so total duration of excitation was included in this study as a measure of welfare. This study found that increased stocking rate was associated with decreased duration of neuromuscular excitation in weaned pigs.

We counted behavioural signs of aversion and distress including pacing, escape attempts, and righting attempts. Urgent pacing along the transparent front panel of the chamber was strongly suggestive of a desire to leave the space. Pacing by pigs often included multiple testing contacts between snout or front hoof and the panel and sometimes rearing and digging behaviours that were counted as potential escape attempts. Pacing occurred in the majority of solitary weaned pigs immediately upon enclosure in the chamber. After a period of pacing, solitary pigs would usually freeze suddenly and back into a corner. Retreat from a noxious stimulus is a characteristic behaviour of pigs (Dalmau *et al* 2010; Llonch *et al* 2012), so this interruption appeared to correspond to first detection of carbon dioxide gas and suggests that pacing was separate from gas aversion. It could be argued that grouped pigs, being more crowded, had neither a clear view of the transparent panel nor an unobstructed path to pace along it; however, paired pigs generally had ample room in the chamber and the incidence of pacing in that treatment was still significantly lower than in solitary pigs.

Escape attempts from a hypercapnic environment may be a reaction to the respiratory discomfort of hyperpnea described above or to direct irritation of mucous membranes by carbon dioxide (Llonch *et al* 2012). Potential escape attempts were observed only in weaned pigs and were least common in the paired focal pigs. The highest potential escape attempt counts were observed in solitary pigs, some of which accrued early during the procedure and may have been related to isolation distress rather than gas aversion. The total incidence of potential escape attempts in grouped focal pigs was similar to that of solitary pigs, although in the case of grouped pigs, the increase over paired pigs may have been caused by the relative averseness of higher CO_2 concentrations (Nowak *et al* 2007; Velarde *et al* 2007; Gregory 2008), since pacing and other signs of early agitation were very rare in the grouped treatment.

Righting attempts were often associated with a sudden change in posture, especially falling or being knocked over, although sleeping neonates would also wake and attempt to stand up at elevated CO_2 levels. Righting attempts, as with ataxia, suggest conscious awareness of a physically impaired state, which may be distressing (Webster & Fletcher 2004). Specifically, righting attempts indicate perception of lost posture and a desire to change posture, presumably due to either discomfort or a desire to be more readily mobile. For these reasons, righting attempt counts were included as a measure of welfare in this study. The effect of increased stocking rate was negative on this behaviour in neonates, but positive on this behaviour in weaned pigs. The reasons for this difference are unclear. One hypothesis, given the high rate at which weaned pigs were shoved and stepped on within the chamber, is that some righting attempts in the older age group were prompted by stimulation from another animal, rather than by an elevated stress level in the chamber.

We did not observe deliberate piling behaviour by any pigs in the chamber, and obstruction of one pig's head by another collapsed pig was rare and transient, so asphyxiation seemed unlikely at these stocking densities. We also did not observe any fighting between pigs in the chamber, despite grouping unfamiliar animals. Swine aggression in lairage usually becomes apparent after about 10 min (Weeks 2008), so if unfamiliar animals must be combined for euthanasia, it may be preferable to do so immediately upon placement in the chamber. Kicks and other potentially offensive strikes received by focal pigs from group-mates were quantified for a descriptive picture of the physical risk associated with confinement and neuromuscular excitation in this species. These events predictably increased with stocking rate and were essentially assured within six-pig groups. When euthanasia cohorts were similarly sized, kicks and shoves did not appear to be injurious. Minor bleeding from the nostrils was the only unexpected clinical change occasionally noted in pigs upon removal, but it is unknown whether this was the result of facial trauma or a side-effect of changes in air composition and pressure.

The low-birthweight, feeble neonates used in this study were described by farm managers as typical of the population of cull pigs at the farrowing stage, so their behaviour provides high relevance to the swine industry, but may differ from the behaviour of more robust littermates or older pigs. Previous research by our group showed that very small piglets (0.8 kg) displayed decreased latencies to respiratory arrest and cardiac arrest than their larger littermates (Sadler *et al* (2014b). Differences in health status likely contributed to variations in the results between the two experiments, although faster response to the gas by neonates was expected based on the results of Sadler *et al* (2014a). Such hastening by immaturity, along with very small animal size relative to the chamber used, may have made effects of stocking rate more difficult to detect in the neonate age group. Although available clinical variables were offered as covariates during statistical analysis of procedural efficacy, it cannot be ruled out that subpopulations of swine that differ in health status from those used in this study could have different degrees of positive and negative interaction when grouped in the chamber.

In conclusion, CO_2 gas concentrated fastest in densely stocked chambers, and for the weaned cull pigs used in this study, this difference was capable of accelerating loss of posture and respiratory arrest and reducing the duration of some aversive states.

Animal welfare implications

This study provided no convincing evidence that isolation during CO_2 gas euthanasia would benefit the welfare of neonatal or weaned cull pigs. Stocking rate did not appear to be a significant factor in efficacy or welfare of gas euthanasia in neonatal pigs, within the range of one to six animals. Although solitary weaned pigs avoided the frequent kicks and shoves experienced by most grouped pigs at various points during induction, almost all of them exhibited pacing and early escape attempts before gas potency had been established, which lends support to the conclusion that isolation distress may outweigh the benefits of seclusion when euthanasia of multiple pigs is necessary (CCAC 2010; AVMA 2013). This study also demonstrated improved efficacy and minor welfare benefits from even slightly accelerated concentration of carbon dioxide within the chamber. High frequency of contact in grouped pigs, when combined with faster gas concentration, did not increase the duration of neuromuscular excitation or delay loss of posture. As an investigation of on-farm procedures, our study design did not attempt to separate the effects of density and social interaction, so questions remain regarding the direct impact of group size as a source of comfort or stress during euthanasia of young pigs.

The provision of ten atypically large, weaned pigs presented a logistical and ethical dilemma during Experiment 2. These animals challenged the capabilities of our portable CO_2 chamber regardless of the group size to which they were assigned, while the unmodified Euthanex® Ag Pro™ 255 is marketed with a capacity of 50 kg. Large pigs appeared to pose a crushing risk to smaller pigs in the chamber, although kicks were often minimised by the mobility restriction faced by these animals. Common sense dictates grouping animals of similar size and health status in the chamber whenever possible, and the AVMA guidelines for CO_2 euthanasia recommend grouping animals in compatible cohorts with restraint or separation when necessary to avoid injury (AVMA 2013; p 19).

Our chamber allowed adequate floor space for lateral recumbency of six average-weight study pigs in our 'high stocking rate' treatment. The welfare implications of exceeding this stocking density, such as by stacking animals in a tall chamber with limited floor space, have never been examined to the authors' knowledge. Ekkel *et al* (2003) looked at space requirements for recumbent pigs when allowing for a voluntary amount of space-sharing and supported using 0.033 × (bodyweight in kg)$^{0.66}$ as a starting estimate for floor area in m^2, which would equate to about 0.03 m^2 per 1 kg neonatal pig or 0.08 m^2 per 4 kg weaned pig. This formula correlated well with our subjective assessment of a reasonably 'full' chamber during the planning process of this study and may be useful for on-farm estimates of chamber capacity. When euthanising fewer or unusually small animals, a proportionately reduced chamber volume (via smaller chamber or space-filling) would be expected to provide comparable efficacy without the need to alter timing or flow rate of gradual-fill procedures.

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