The Old School, Brewhouse Hill, Wheathampstead,
Hertfordshire AL4 8AN, UK
www.ufaw.org.uk

Animal Welfare 2016, 25: 83-89
ISSN 0962-7286
doi: 10.7120/09627286.25.1.083

Effects of stocking rate on measures of efficacy and welfare during argon gas euthanasia of weaned pigs

KJ Fiedler[‡], RL Parsons[§], LJ Sadler[§] and ST Millman*[†‡§]

[†] 2201 Lloyd Veterinary Medical Center, Ames, Iowa 50011-1250, USA
[‡] Department of Biomedical Sciences, Iowa State University, Iowa, USA
[§] Veterinary Diagnostic and Production Animal Medicine, Iowa State University, Iowa, USA
* Contact for correspondence and requests for reprints: smillman@iastate.edu

Abstract

*The objective of this study was to evaluate the effects of chamber stocking rate on facets of animal welfare and efficacy during euthanasia of weaned pigs (*Sus scrofa domesticus*) with argon gas. Two hundred and thirty-three weaned pigs designated for euthanasia at a commercial production farm were randomly assigned to group sizes of one, two, or six pigs. Gas euthanasia of each piglet group was performed in a Euthanex® AgPro chamber. The chamber was filled with argon gas for 6 min in order to reduce the oxygen concentration to less than 2%. Pigs were then placed into the pre-filled chamber and gas flow was continued at a high rate to displace introduced air and re-establish a fatally low residual oxygen concentration. Pigs remained in the chamber for 10 min and were then removed to test for signs of sensibility and life. There was no significant evidence of an effect of stocking rate on focal pig latencies to onset of neuromuscular excitation or last movement, as scored from video recordings. Solitary pigs were more likely to pace and make righting attempts in the chamber than paired or grouped focal pigs, although pigs in higher stocking rate treatments tended to retain posture longer. The results of this study do not support seclusion during argon gas euthanasia as a method of improving animal welfare. The portable chamber did not facilitate a truly pre-filled atmosphere for euthanasia, although the procedure used in this study was consistently effective.*

Keywords: *animal welfare, argon, behaviour, euthanasia, pigs, stocking rate*

Introduction

Argon has been proposed as a less-aversive alternative to carbon dioxide gas for stunning and euthanasia of swine and poultry (Raj & Gregory 1995; Mota-Rojas *et al* 2012). Like carbon dioxide, argon euthanasia enables producers to euthanise animals in groups, although such practice must take into consideration non-instantaneous loss of consciousness (Dalmau *et al* 2010) and the potential distress of hypoxia (American Veterinary Medical Association [AVMA] 2013).

Argon exposure has been reported to increase vocalisation, righting attempts, and chamber escape attempts relative to either chamber confinement alone (Sadler 2013) or exposure to carbon dioxide (Sadler *et al* 2014b,c), and anoxia induces convulsions prior to brain death (Raj 2006; McKeegan *et al* 2007). Numerous studies of pigs (*Sus scrofa domesticus*) and other mammals have provided evidence 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). If loss of consciousness is asynchronous within a shared euthanasia chamber, there is potential for conscious pigs to be exposed to squealing and violent convulsions of conspecifics, to the possible detriment of welfare.

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). Current AVMA Guidelines generally warn against performing individual euthanasia in the presence of sensitive conspecifics, but also 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). The document advises separation or restraint of animals to prevent injuries during euthanasia with carbon monoxide gas, although this suggestion is not made for argon. Canadian Council on Animal Care (CCAC) Guidelines (2010) 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. Previous research by our group revealed that weaned solitary pigs displayed greater incidence of pacing and escape attempts during gradual-displacement carbon dioxide euthanasia than those exposed in groups (Fiedler *et al* 2014). It is unclear whether administrators of gas 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.

The objective of this study was to determine whether variation in stocking rate has an effect on measures of efficacy and welfare during gas euthanasia of weaned pigs using argon gas.

Materials and methods

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

Experimental procedure

Crossbred (Landrace × Yorkshire cross × Duroc) pigs of mixed sexes were utilised for this study after being designated for euthanasia at a commercial swine production farm due to animal welfare concerns or low commercial viability. Our request for locally available weaned pigs slated for euthanasia, but medically stable for transport, obtained 233 animals for the study. The pigs were between 3 and 10 weeks of age (mean [± SD] bodyweight: 7.7 [± 3.3] kg; range: 3.0 to 16.6 kg) and most showed symptoms of chronic respiratory, neurological, or dermatological disease. Cohorts of selected pigs were transported by livestock trailer and group-housed in an indoor biocontainment facility with *ad libitum* food and water during study enrolment.

Pigs were enrolled in the study each day in order of health instability (with potentially painful injuries and severe clinical signs of illness granted antecedence) 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), or six pigs (TRT 6). Each experimental unit ('piglet group' or PG) was thus comprised of one, two, 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. TRT 1 included 27 focal pigs and 0 companion pigs, TRT 2 included 25 focal pigs and 25 companion pigs, and TRT 6 contained 26 focal pigs and 130 companion pigs.

The enrolment examination included assessing and recording sex, heart and respiratory rates, rectal body temperature, apparent reasons for selection, body condition score, activity level, hydration status, respiratory score 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 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 the reasons stated may not have matched the reasoning of the caretaker making the designation for euthanasia.

Gas euthanasia was performed in a 91 × 61 × 46 cm (length × width × height) Euthanex® AgPro chamber (Value-Added Science & Technology, Mason City, USA) modified with transparent acrylic on the top and front panels for observation. Argon gas is approved by the AVMA (2013) for pigs only if pigs are placed directly into an atmosphere of less than 2% residual oxygen, which necessitates pre-filling the chamber. A layer of transparent 20 gauge vinyl was fitted across the top of the chamber and attached with Velcro® tape to provide a barrier against air flow when the acrylic lid was opened after pre-filling. An aperture of approximately 0.09 m^2 was cut through the vinyl layer in order to introduce animals. Industrial grade argon was supplied from compressed gas cylinders via a volumetric high-output heated two-stage regulator. 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 an oxygen sensor (model TR25OZ, CO_2Meter, Ormond Beach, USA) attached to a HOBO data logger (model U23-001, Onset, Cape Cod, USA) that was set to record oxygen concentrations at 10-s intervals continuously on the days of the experiment. Between each PG, the chamber was cleaned of bodily excretions and vacuumed to restore an ambient air composition. A light was directed toward the front of the chamber with the room otherwise darkened to minimise visual disturbance of pigs in the chamber.

During each trial, argon gas was used to pre-fill the empty chamber at a flow rate of 0.12 m^3 per min, or 49% chamber volume exchange per min, for 6 min. This established a residual O_2 concentration between 1 and 2%. After 6 min, the chamber lid was opened for approximately 20 s, during which time the piglet group was placed inside along with enough 7.6 L plastic barrels to bring the total group size to 6. Plastic barrels were used to simulate the placement of additional animals in lower stocking rate treatments with the aim of minimising differences in oxygen concentrations between treatments. When used, the barrels were always introduced first and were positioned along the back panel of the chamber. The barrels were weighted with sand to prevent movement by the pigs. The focal pig was placed last followed by immediate closure of the chamber lid. Gas flow was continued for approximately 2.5 min to displace introduced oxygen and re-establish an O_2 concentration between 1 and 2%. Pigs remained in the chamber for a total of 10 min in order to ensure exposure to $< 2\%$ oxygen for at least 7 min as recommended by Raj (1999).

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

https://doi.org/10.7120/09627286.25.1.083 Published online by Cambridge University Press

Table 1 Ethogram used when scoring focal pig behaviour and status from video recordings (adapted from Sadler 2013).

Behaviour	Definition
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, such as rapid vertical thrusting of the head or snout, foot stamping, paddling, or kicking
Open-mouth breathing	Prior to the onset of neuromuscular excitation, pig is breathing rapidly through continuously open mouth (panting)
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 pawing at chamber 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 (counted each independent effort between which posture regressed)
Struck	Prior to the onset of neuromuscular excitation, pig receives potentially harmful or offensive contact as a result of action by another pig, including being stepped on, bitten, kicked, shoved, or crushed
Swaying	Repetitive lateral or cranial-caudal rocking motion while stationary

breathing and auscultated with a stethoscope for at least 20 s to confirm cardiac arrest. Carcase weights were measured by hanging scale after confirming death.

Two PG trials were terminated due to interruptions of gas flow after animal placement. In one of these trials, the solitary pig was removed and euthanised immediately using a penetrating captive-bolt device as per AVMA Guidelines for Euthanasia (2013). In the other trial, gas flow was restored and the paired pigs allowed to remain in the chamber until death had occurred. Behavioural data from these two trials were not used in analyses. All other pigs (n = 230) were insensible, without respiration, upon removal. One pig had a detectable heartbeat upon removal and was observed in ambient air and then re-auscultated 3 min later, by which time cardiac arrest had occurred.

Behavioural observation

During exposure to argon, a single researcher directly observed the focal pig and recorded latency to loss of posture, which was defined as the elapsed time from lid closure until the focal pig stopped attempting to lift its head. All trials were also video-recorded using a Noldus portable 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 per second on a personal computer using HandiAvi software (v 4.3, Anderson's AZcendant Software, Tempe, USA).

Video recordings of pigs exposed to argon were continuously sampled by the same individual using The Observer software (The Observer, Version 10 Noldus Information Technology, Wageningen, The Netherlands). Focal pig latencies to onset of neuromuscular excitation and last movement were determined as additional measures of procedural efficacy. Latency to onset of neuromuscular excitation was defined as the elapsed time from closure of the chamber lid until the focal pig began exhibiting stereotypic movement characteristic of involuntary excitation, most often stepping in place or repeatedly thrusting the head upward. Latency to last movement was defined as the elapsed time from closure of the chamber lid to last visible gasp (mandibular abduction). The frequencies or durations of focal pig behaviours and statuses that have been previously associated with animal welfare considerations were also scored from video. See Table 1 for video ethogram. The observer could not be blind to treatments due to the visibility of group size in the chamber.

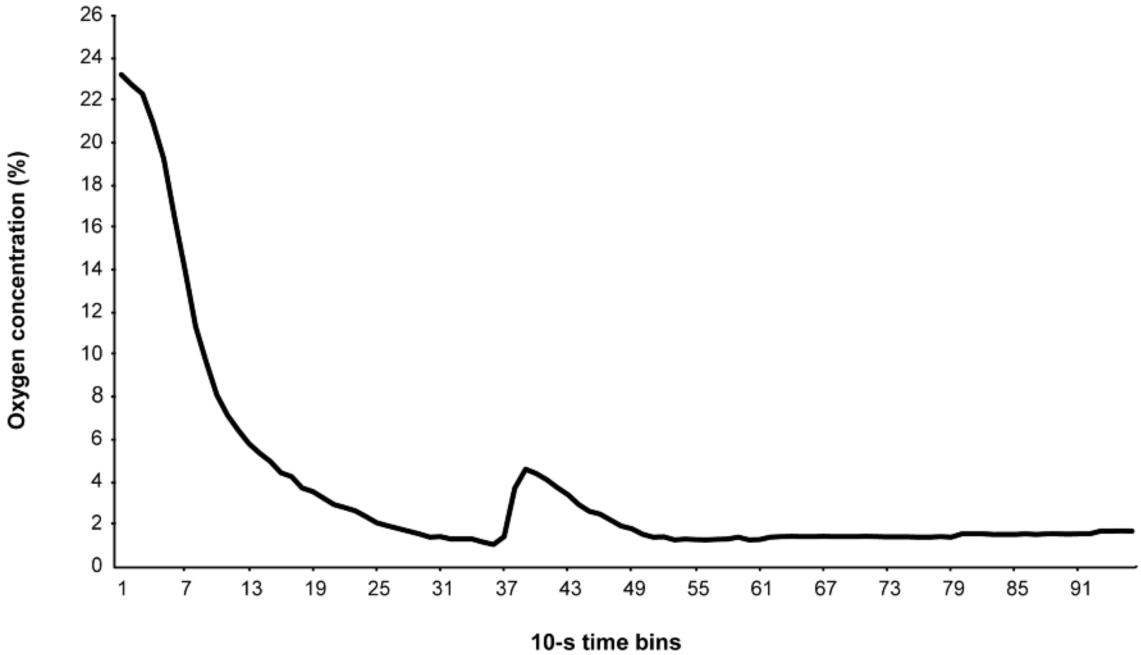
Statistical analysis

All statistical analyses were performed in SAS® (SAS Inst Inc, Cary, USA) using a significance level of 0.05. Oxygen concentrations recorded at 10-s intervals during the 10-min gas exposure were averaged to obtain one O_2 exposure value per PG trial. These O_2 exposure values were analysed for differences in treatment means using a general linear model (PROC GLM) with stocking rate and day of trial as categorical variables and fixed effects.

In behavioural data analyses, stocking rate was used as a categorical variable. Clinical variables assessed during enrolment were examined as potential covariates along with day of trial. The final models were selected based on best fit and included day of trial and starting body temperature. Survival analysis with Weibull distribution (PROC LIFEREG) was used to determine whether there was an effect of stocking rate on latencies to onset of neuromuscular excitation, loss of posture, or last movement. All three latency variables were log-transformed to improve model fit. Levene's test for homogeneity of variance was used to test whether grouped and paired focal pigs were more variable in latencies to onset of neuromuscular excitation than solitary pigs (PROC GLM). Poisson regression (PROC GENMOD) was used to test for an effect of stocking rate on

doi: 10.7120/09627286.25.1.083

Figure 1



Typical pattern of oxygen concentration in the chamber. Oxygen concentration (%) values collected during the euthanasia of PG 15 (TRT 1) show a typical pattern of gradual decline during pre-fill, small peak during pig placement and re-establishment of critical oxygen level over a total of 16 min (depicted as 96 10-s time bins).

righting attempt counts. General linear modelling (PROC GLM) was used to test for an effect of stocking rate on the duration of neuromuscular excitation. Fisher's exact test (PROC FREQ) was used to test for an association between treatment and proportion of focal pigs receiving strikes from conspecifics in the chamber.

Results

Oxygen data were successfully recorded during 44 of 76 PG trials. Within this subset, increased stocking rate was associated with higher average O_2 concentrations after controlling for day of trial ($P < 0.0001$). The difference between TRT 6 and TRT 1 was smaller in magnitude than the reported accuracy for the sensor (± 0.5% oxygen concentration); however, least square means for average O_2 concentration during exposure were 2.2, 2.3 and 2.4% for TRT 1, TRT 2, and TRT 6, respectively, with standard errors of 0.04%. The estimated difference between TRT 6 and TRT 1 was 0.2 (± 0.05)%. See Figure 1 for a representation of the pattern of O_2 concentration change during pre-fill, pig placement, and in-chamber dwell period.

Escape attempts were not observed from any focal pigs during argon exposure. Brief periods of open-mouth breathing were scored in seven of 74 focal pigs (9%). Typically, weaned pigs stood quietly until the onset of neuromuscular excitation. Twenty-one focal pigs (28%) displayed brief periods of swaying before beginning stereotypic movements. Initial excitatory movements often included head-thrusting upward and stepping motions that progressed into vigorous paddling of the front limbs with extended or kicking hind limbs. These patterned myoclonic movements often began while standing, but were not accompanied by efforts to maintain or restore posture, so that the pig might lurch forward for several steps before losing balance and sliding into lateral recumbency with extended limbs and uninterrupted paddling, or the pig might rear or tip onto its rump with extended hind limbs and appear to be digging frantically in the air until eventually falling over. Since loss of posture was defined by the cessation of head-lifting and dorsal head movement was common during neuromuscular excitation, loss of posture was usually scored after the onset of neuromuscular excitation and upon full lateral recumbency.

There was no significant evidence of an effect of stocking rate on latencies to onset of neuromuscular excitation ($P = 0.18$) or last movement ($P = 0.69$) after controlling for day of trial and starting body temperature. There was evidence of an effect of stocking rate on latency to loss of posture after controlling for day of trial and starting body temperature ($P = 0.03$). See Table 2 for mean latencies by treatment. There was no significant evidence of an effect of stocking rate on the variability of latency to onset of neuromuscular excitation ($P = 0.10$).

We quantified the number of potentially offensive contact 'strikes' received by focal pigs from conspecifics, such as being kicked, stepped on, or shoved. Nine focal pigs were scored as receiving strikes prior to onset of neuromuscular excitation (three of 23 scored pigs in TRT 2 and six of 26 scored pigs in TRT 6), with each of those pigs receiving an average of four strikes each. The difference in proportions was not statistically significant ($P = 0.60$).

https://doi.org/10.7120/09627286.25.1.083 Published online by Cambridge University Press

See Table 3 for behavioural data by treatment. Pigs had a generally subdued demeanour during the early induction period with minimal interaction despite crowding. No fighting or deliberate piling was observed.

Ten focal pigs, seven of which were in TRT 1, exhibited pacing or a weight-shifting movement while facing the transparent front panel that resembled an inclination to pace. Space restriction in the chamber from other animals or barrels made turning locomotion difficult for larger pigs in this study, so pacing behaviour was not analysed as a reliable indicator of distress in the chamber. There was evidence of an effect of stocking rate on righting attempt counts after controlling for day of trial and starting body temperature ($P < 0.001$), with fewer righting attempts by focal pigs at higher stocking rates. There was no significant evidence of an effect of stocking rate on duration of neuromuscular excitation in focal pigs ($P = 0.93$).

Table 2 Mean (± SEM) latencies (s) to focal pig onset of neuromuscular excitation, loss of posture and last movement.

	TRT 1	TRT 2	TRT 6
Onset of neuromuscular excitation (P = 0.18)	63 (± 4)	62 (± 6)	63 (± 4)
Loss of posture (P = 0.03)	75 (± 3)	77 (± 4)	81 (± 4)
Last movement (P = 0.69)	264 (± 12)	263 (± 15)	244 (± 14)

Table 3 Behaviours of focal pigs scored from video.

	Righting attempts (P)	Righting attempts (C) (P < 0.001)	Pacing (P)
TRT 1	0.44	1.32 (± 0.46)	0.28
TRT 2	0.46	0.96 (± 0.27)	0.08
TRT 6	0.12	0.27 (± 0.17)	0.04
	Strikes (P) (P = 0.60)	**Strikes (C)**	**Neuromuscular excitation (D)** (P = 0.93)
TRT 1	–	–	55 (± 4)
TRT 2	0.13	0.65 (± 0.38)	54 (± 3)
TRT 6	0.24	0.81 (± 0.37)	48 (± 5)

P: Proportion of focal pigs exhibiting behaviour; C: mean count with standard error; D: mean duration (s) with standard error.

Discussion

Rapid loss of consciousness is a key criterion of humane euthanasia (AVMA 2013), and variation in latency to loss of consciousness during procedural variations would have major animal welfare implications. The onset of neuromuscular excitation and convulsive activity during anoxic euthanasia signals a release of inhibition on the caudal reticular formation from higher brain centres and can, thus, be used as a non-invasive indicator of an altered level of consciousness (Raj 2006). This study did not find significant evidence that stocking rate had an effect on latency to onset of neuromuscular excitation, nor did it provide evidence that stocking rate has an effect on latency to last movement, an approximation of the time required to achieve brain death.

Some variation of loss of posture or loss of balance has been used previously in several gas euthanasia studies as a behavioural indicator of unconsciousness (Dalmau *et al* 2010; Llonch *et al* 2012; Sadler *et al* 2014a,b,c). In this study, grouped pigs tended to lose posture later than solitary pigs, despite little variation in onset of neuromuscular excitation. Loss of posture, as defined in this study, may not be a reliable index of efficacy for argon gas euthanasia using a pre-filled chamber, since the assumption of lateral recumbency was largely dependent on the positioning of the pig at the onset of neuromuscular excitation relative to other animals and objects in the chamber. To the extent that loss of posture can be considered a definitive marker of lost consciousness, then any effect of treatment would be confounded by higher average oxygen exposure values at higher stocking rates, which was most likely caused by the relative amount of chamber manipulation needed to place multiple animals. Excluding the differences in air composition between treatments, interactions between animals that could be responsible for a delay in loss of consciousness, such as strikes or displays of distress behaviour, were not observed in the majority of groups, although less-visible factors that were not examined in this study, such as distress pheromones, could be responsible.

The time-points discussed in the preceding two paragraphs were selected to represent the earliest likely change in consciousness (the onset of involuntary movement labelled neuromuscular excitation, which most commonly appeared as the first behaviour not normally seen in conscious swine), and a relatively undisputed sign of unconsciousness (loss of posture, defined here as a cessation of attempts to lift the head). The difference between these time-points creates a relatively short window (less than 15 s, on average) in which it is probably reasonable to assume consciousness was actually lost. This is consistent with prior research using physiological data to estimate when consciousness is lost during gas euthanasia, the reports from which differ in their exact conclusions but generally point to the window of time between initial recumbency and complete stillness (Herin *et al* 1978; Forslid 1987; Raj *et al* 1997; Martoft 2002; McKeegan 2007; Gerritzen 2008).

The event of 'going down' (with varied nomenclature including loss of posture and loss of righting reflex) has been more commonly reported as indicative of loss of consciousness; however, these terms have been vulnerable to differing interpretation (eg, 'loss of posture' was defined by Raj [1999] as 'recumbency' but it is unclear from this and subsequent uses of the term whether it refers to the first time a pig falls or to the last time a pig makes an attempt to rise) and the event can be difficult to measure consistently in unhealthy animals. Regardless of the exact definition, the point of a pig 'going down' in this study would also typically fall within the window described above. While choosing a single time-point to represent loss of consciousness would maximise clarity for reporting purposes, it risks under- or over-estimating the duration of potential animal suffering and may belie the lack of consensus among researchers as to what such terms mean and reliably indicate. Comparing a number of transition points is intended to acknowledge that our understanding of animal consciousness and our ability to measure it during complex neurophysiological changes are still imperfect, while achieving the purpose of testing for an effect of treatment at multiple consistently measurable transition points.

We measured the frequency of occurrence of voluntary righting attempts as an indicator of potential distress. The lowered frequency of righting attempts in grouped animals relative to solitary animals could, like the increased latency to loss of posture, have been the result of crowding in the chamber. Righting attempts most often occurred when pigs fell over from pronounced swaying behaviour immediately prior to the onset of neuromuscular excitation, and visible swaying was rarely observed in grouped pigs. If righting attempts reflected an elevated level of distress and stronger desire to be readily mobile during a gradual loss of control, then we might also expect a delay in latency to loss of posture; however, grouped pigs typically retained posture the longest.

The majority of weaned pigs squealed during euthanasia with argon gas. Squealing typically occurred during the first minute after onset of neuromuscular excitation and manifested at the same time as cessation of voluntary movements and initial signs of cortical disinhibition. Although the timing suggests that squealing during argon euthanasia may not be a genuine indicator of perceived distress, squealing was regarded by many observers as the most disconcerting behaviour associated with argon euthanasia, so the duration of squealing might be considered a relevant index of efficacy. Since squeals from focal pigs could not be isolated or identified reliably from audio and video recordings of groups in the chamber, squealing duration was not compared between treatments in this study.

Animal welfare implications

Stocking chambers with pig group sizes of one, two or six animals did not appear to have a biologically significant effect on the efficacy or welfare indices used in this study for argon gas euthanasia of weaned cull pigs. This study did not provide convincing evidence that isolation during argon gas euthanasia would benefit the welfare of weaned pigs. Achieving a true pre-filled environment for euthanasia in a portable chamber is probably not feasible, although the least oxygen would be introduced by rapid placement of a single animal.

Acknowledgements

This research was funded by grant 12-100 from the National Pork Board. The authors wish to thank Dr Rowles and employees of Elite Pork Partnership, LLC for their collaboration on this project. Technical assistance by L Maldonado, N White, S Ball, L Labeur, and B Woods during animal trials was greatly appreciated.

References

American Veterinary Medical Association (AVMA) 2013 *AVMA Guidelines for the Euthanasia of Animals: 2013 Edition*. American Veterinary Medical Association: Schaumburg, USA. https://www.avma.org/KB/Policies/Documents/euthanasia.pdf

Amory JR and Pearce GP 2000 Alarm pheromones in urine modify the behaviour of weaner pigs. *Animal Welfare 9(2)*: 167-175

Atkinson S, Velarde A, Llonch P and Algers B 2012 Assessing pig welfare at stunning in Swedish commercial abattoirs using CO_2 group-stun methods. *Animal Welfare 21(4)*: 487-495. http://doi.org/10.7120/09627286.21.4.487

Canadian Council on Animal Care (CCAC) 2010 CCAC guidelines on: euthanasia of animals used in science. In: Charbonneau R, Niel L, Olfert E, von Keyserlingk M and Griffin G (eds) *Canadian Council on Animal Care*. CCAC: Ottawa, CA. http://www.ccac.ca/Documents/Standards/Guidelines/Euthanasia.pdf

Dalmau A, Rodríguez P, Llonch P and Velarde A 2010 Stunning pigs with different gas mixtures: aversion in pigs. *Animal Welfare 19*: 325-333

Düpjan S, Tuchscherer A, Langbein J, Schön P-C, Manteuffel G and Puppe B 2011 Behavioural and cardiac responses towards conspecific distress calls in domestic pigs (*Sus scrofa*). *Physiology & Behavior 103*: 445-452. http://doi.org/10.1016/j.physbeh.2011.03.017

Fiedler KJ, Sadler LJ, Parsons RL and Millman ST 2014 Effects of stocking rate on measures of efficacy and welfare during carbon dioxide gas euthanasia of young pigs. *Animal Welfare 23(3)*: 309-321. http://dx.doi.org/10.7120/09627286.23.3.309

Forslid A 1987 *Pre-slaughter CO_2-anaesthesia in swine: Influence upon cerebral electrical activity, acid/base balance, blood oxygen tension and stress hormones*. Swedish University of Agricultural Sciences, Uppsala, Sweden

Fraser D 1975 Vocalization of isolated piglets II. Some environmental factors. *Applied Animal Ethology 2(1)*: 19-24. http://doi.org/10.1016/0304-3762(75)90062-0

Gerritzen MA, Kluivers-Poodt M, Reimert HGM, Hindle V and Lambooij E 2008 Castration of piglets under CO_2-gas anaesthesia. *Animal 2(11)*:1666. http://doi.org/10.1017/S1751731108002887

Herin RA, Hall P and Fitch JW 1978 Nitrogen inhalation as a method of euthanasia in dogs. *American Journal of Veterinary Research 39(6)*: 989-991

Kanitz E, Puppe B, Tuchscherer M, Heberer M, Viergutz T and Tuchscherer A 2009 A single exposure to social isolation in domestic piglets activates behavioural arousal, neuroendocrine stress hormones, and stress-related gene expression in the brain. *Physiology & Behavior 98*: 176-185. http://doi.org/10.1016/j.physbeh.2009.05.007

Llonch P, Rodríguez P, Gispert M, Dalmau A, Manteca X and Velarde A 2012 Stunning pigs with nitrogen and carbon dioxide mixtures: effects on animal welfare and meat quality. *Animal 6(4)*: 668-675. http://doi.org/10.1017/S1751731111001911

Martoft L, Lomholt, L, Kolthoff C, Rodríguez BE, Jensen EW, Jørgensen PF, Pedersen HD and Forslid A 2002 Effects of CO_2 anaesthesia on central nervous system activity in swine. *Laboratory Animals 36*: 115-126. http://dx.doi.org/10.1258/0023677021912398

McKeegan DEF, Abeyesinghe SM, McLeman MA, Lowe JC, Demmers TGM, White RP, Kranen RW, van Bemmel H, Lankhaar JAC and Wathes CM 2007 Controlled atmosphere stunning of broiler chickens II. Effects on behaviour, physiology and meat quality in a commercial processing plant. *British Poultry Science 48(4)*: 430-442. http://doi.org/10.1080/00071660701543097

Mota-Rojas D, Bolanos-Lopez D, Concepcion-Mendez M, Ramirez-Telles J, Roldan-Santiago P, Flores-Peinado S and Mora-Medina P 2012 Stunning swine with CO_2 gas: Controversies related to animal welfare. *International Journal of Pharmacology 8(3)*: 141-151. http://doi.org/10.3923/ijp.2012.141.151

National Pork Board 2008 *On-farm euthanasia of swine. Recommendations for the producer*. National Pork Board: Des Moines, USA. http://www.aasv.org/aasv/documents/SwineEuthanasia.pdf

Raj ABM 1999 Behaviour of pigs exposed to mixtures of gases and the time required to stun and kill them: welfare implications. *The Veterinary Record 144*: 165-168. http://doi.org/10.1136/vr.144.7.165

Raj ABM 2006 Recent developments in stunning and slaughter of poultry. *World's Poultry Science Journal 62*: 467-484. http://doi.org/10.1017/S0043933906001097

Raj ABM and Gregory NG 1995 Welfare implications of the gas stunning of pigs 1. Determination of aversion to the initial inhalation of carbon dioxide or argon. *Animal Welfare 4*: 273-280

Raj ABM, Johnson SP, Wotton SB and McInstry JL 1997 Welfare implications of gas stunning pigs 3. The time to loss of somatosensory evoked potentials and spontaneous electrocorticogram of pigs during exposure to gases. *Veterinary Journal 153*: 329-340. http://doi.org/10.1016/S1090-0233(97)80067-6

Sadler LJ 2013 *Effect of flow rate, gas type and disease status on the welfare of suckling and nursery pig during gas euthanasia*. PhD Thesis, Iowa State University, Ames, USA

Sadler LJ, Hagen CD, Wang C, Widowski TM, Johnson AK and Millman ST 2014a Effects of flow rate and gas mixture on the welfare of neonate and weaned pigs during gas euthanasia. *Journal of Animal Science 92*: 793-805. http://dx.doi.org/10.2527/jas.2013-6598

Sadler LJ, Karriker LA, Johnson AK, Schwartz KJ, Widowski TM, Wang C and Millman ST 2014b Swine respiratory disease minimally affects responses of nursery pigs to gas euthanasia. *Journal of Swine Health and Production 22(3)*: 125-133

Sadler LJ, Karriker LA, Schwartz KJ, Johnson AK, Widowski TM, Wang C, Sutherland MA and Millman ST 2014c Are severely depressed suckling pigs resistant to gas euthanasia? *Animal Welfare 23*: 145-155. http://dx.doi.org/10.7120/09627286.23.2.145

Sharp J, Zammit, T, Azar T and Lawson D 2003 Stress-like responses to common procedures in individually and group-housed female rats. *Continuing Topics in Laboratory Animal Science 42*: 9-18

Straw BE, Meuten DJ and Thacker BJ 1999 Physical examination. In: Straw BD, Allaire, S, Mengeling W and Taylor D (eds) *Diseases of Swine, 8th Edition* pp 3-5. Iowa State Press: Ames, USA

Talling JC, Waran NK, Wathes CM and Lines JA 1996 Behavioural and physiological responses of pigs to sound. *Applied Animal Behaviour Science 48*: 187-202. http://doi.org/10.1016/0168-1591(96)01029-5

Vieuille-Thomas C and Signoret JP 1992 Pheromonal transmission of an aversive experience in domestic pig. *Journal of Chemical Ecology 18(9)*: 1551-1557. http://doi.org/10.1007/BF00993228