Investigation of aversion associated with gas inhalants in pigs using cognitive paradigms

by

Luna KC

A thesis submitted to the graduate faculty

in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Major: Biomedical Sciences (Physiology)

Program of Study Committee:
Suzanne T. Millman, Major Professor
Vlastislav Bracha
Jesse P. Goff
Locke A. Karriker
Anna K. Butters-Johnson

Iowa State University

Ames, Iowa

2018

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Carbon dioxide (CO_2) is commonly used for stunning and euthanasia of pigs, but the practice is restricted in some countries due to concerns about pain and distress that appear to occur prior to loss of consciousness when pigs are immersed in CO_2. Argon-induced hypoxia is suggested as an alternative, but is associated with prolonged induction of unconsciousness during which pigs display behaviors suggestive of distress. The aim of this thesis was to assess pigs' aversion to inhalant euthanasia gases using approach-avoidance (AA) and conditioned place avoidance (CPA) paradigms. A preference-testing device was designed with two identical chambers: a control chamber (CC) with ambient air and a treatment chamber (TC) with different concentrations of CO_2 and O_2 gases, separated by a sliding door and an exhaust sink. Weaned crossbred commercial pigs were individually trained once daily for five days to enter the TC through the sliding door to obtain food rewards, with chambers maintained at ambient air conditions. Following entry to TC, the pig could move freely between chambers. Testing involved the same methods, with TC maintained at 10%, 20%, and 30% CO_2 (Chapter 2) or 6% and 2% O_2 (Chapter 3). Tests concluded when loss of posture (LOP) occurred, or after the test concluded. Pigs were systematically assigned to each gas treatment over three rounds (Chapter 2) or two rounds (Chapter 3). Each test round consisted of ambient air on baseline (B) day, assigned gas treatment on gas (G) day, followed by ambient air on washout (W) day. It was hypothesized pigs would avoid gas concentrations that were aversive on G and CPA would be observed on W. Behavioral outcomes were collected using live and video recordings. In Chapter 2, 10 of 12 pigs entered the TC on all B, G and W days, followed by six minutes during which pigs could move freely between chambers. Pigs displayed longer latency to enter TC, shorter latency to leave TC and longer latency to re-enter TC on G than B days. Five pigs at 20% and 4 pigs at 30% CO_2 remained in the TC until LOP. CPA was not observed on any of W. Hence, all CO_2 levels tested induced mild aversion compared to ambient conditions, but this aversion did not provoke marked avoidance or CPA. In Chapter 3, all 12 pigs entered TC on all B., G. and W days, followed by 10 minutes during which pigs could move freely between chambers. Pigs displayed shorter latency to leave TC and longer latency to re-enter the TC on G than B days. At 2% O_2, seven pigs remained in TC until LOP. CPA was not observed on any of W. Hence, hypoxia appears to be mildly aversive to pigs, however the degree of aversion was not pronounced to provoke marked avoidance or CPA. In conclusion, 30% CO_2 and 2% O_2 were not sufficiently aversive to provoke avoidance or CPP in weaned pigs, and are suitable for gradual-fill or two-step euthanasia protocols for on-farm euthanasia of pigs.

CHAPTER 1 GENERAL INTRODUCTION

1.1 Introduction

Animal welfare and ethical use of animals for various purposes have been a major global issue over the past half- century (Rollin, 2004). The raising concerns for animal suffering have brought positive attitudes among public and animal industry stakeholders towards humane killing of animals. Euthanasia is “usually used to describe ending of life of an individual animal in a way that minimizes or eliminates pain and distress” (Leary et al., 2013, pp6). It is considered one of the critical animal welfare issues of food producing animals (OIE, 2017 Article 7.1.4). My focus for this thesis is on the current on-farm euthanasia practice of using gas inhalants for young pigs (including suckling and nursery pigs), and how these gases affect pigs’ welfare.

Gas inhalants are widely used euthanasia agents for young pigs (Leary et al., 2013; Sadler et al., 2014). The American Veterinary Medicine Association (AVMA) Panel on Euthanasia (POE) lists gas inhalants as conditionally accepted euthanasia methods. Carbon dioxide (CO_2) is the commonly used on-farm gas inhalant. Physiologically, CO_2 causes hypercapnic hypoxia which depresses brain function resulting in loss of consciousness (LOC), and usually high (80% - 90%) CO_2 concentrations are used. However, pain and distress associated aversion are reported with such high concentrations in both human and animals (Conlee et al., 2005) including pigs (Raj and Gregory, 1995; Velarde et al., 2007; Dalmau et al., 2010) which raises animal welfare concerns. To minimize high CO_2 aversion, the AVMA recommends a 2-step method as an alternative where animals are first rendered unconscious followed by immersion into 100% CO_2 for death (Leary et al., 2013). There is a potential for using low CO_2 for anesthetizing pigs in a 2-step euthanasia method, since pigs’ behaviors vary with concentrations and low CO_2 are relatively less aversive (Raj and Gregory, 1996; Velarde et al., 2007). Inert gas, like argon is another suggested alternative to aversive high CO_2 (Raj and Gregory, 1995; EFSA, 2004; OIE, 2017). Physiologically, argon displaces oxygen to produce hypoxia. As a result of reduced O_2 in blood gases and brain tissues, LOC ensues.

Previous experiments have utilized forced gas exposure method to assess aversion associated with gas inhalants in pigs (Velarde et al., 2007; Fiedler et al., 2014; Sadler et al., 2014). In forced exposure, animals do not have choice but to get exposed to aversive gas concentrations. This may cause unnecessary suffering and may not give a clear evaluation of animals' affective states. Cognitive tests can be better alternatives to assess animals' affective states by 'asking' them their experience with gas exposure (Millman, 2013).

There are inadequate scientific works addressing the affective states of young pigs when exposed to gas inhalants. In addition, suitable methodologies capable to quantify the affective states are also lacking. In the following literature review, I will provide a synopsis of published gas euthanasia studies in pigs, discuss current gaps in our knowledge and introduce the ways to fulfill those gaps with a detailed background of my research question for the assessment of gas aversion in young pigs.

1.2 On-farm euthanasia needs in the US swine farms – an outlook

The US swine industry has a current pig inventory of 71.7 million head, 45% of which represents young pigs (USDA, 2017). A recent report published by the National Pork Board (NPB) estimates an average of 13.5 pigs are born per sow, of which 12.1 are born alive and 10 of the live pigs are weaned in the US swine farms constituting a 17.4% pre-weaning mortality (Stalder, 2016). Weaned pigs kept in farms to attain a body weight of 32 kg are called nursery pigs whose average mortality rate is 5.2%. Reasons for pig mortality are mainly respiratory problems (47%), failure to thrive (22%), CNS/meningitis (13%) and scours (9%) with grave prognosis (Stalder, 2016).

These mortality data suggest how millions of young pigs do not enter the production cycle, and need to be euthanized on-farm annually. However, there is a lack of sufficient information regarding reliable and consistent euthanasia methods for young pigs. The AVMA and the US swine industry have therefore, encouraged for a detailed assessment on this sensitive topic.

1.3 Current euthanasia guidelines

The AVMA POE (2013) is a guiding document for veterinarians performing euthanasia and it has expanded its publication for broad range of species, including pigs. In addition, the American Association of Swine Veterinarians (AASV) and National Pork Board (NPB) has also jointly published a set of on-farm euthanasia guidelines (2016) for pork producers. Both these documents provide guidelines for pigs' euthanasia, based on age/weight category on different settings with a focus on animal welfare.

A good euthanasia method should be humane to animals, practical to apply, economical, aesthetically pleasant and less stressful to workers (Leary et al., 2013; AASV and NPB, 2016). Whether a method is humane or not is determined by its ability to induce rapid LOC reliably and consistently with minimal pain and distress (Leary et al., 2013). Furthermore, the AVMA categorizes euthanasia methods as acceptable, acceptable with conditions, and unacceptable. Acceptable methods consistently provide humane death when used as a sole euthanasia technique. They include barbiturates and their derivatives and are considered the 'gold standard'. But they are not practical options for on-farm euthanasia due to veterinarian supervision need, costs and drug residue in animal tissues posing a threat to public health. Conditionally accepted methods require certain conditions to be met to be considered humane. Conditions may have a greater risk for human error or workers' safety, have very few published scientific literatures, or require a secondary method for complete death. They are considered to be as humane as acceptable methods when all required criteria are met. Examples mainly include manual blunt force trauma and gas inhalants (CO_2, inert gases like nitrogen, argon). Blunt force trauma is the most common method for suckling pigs, and if done properly can cause rapid LOC (Leary et al., 2013), but it is aesthetically unpleasant and cannot be applied consistently. Unacceptable methods are techniques that are known or have significant potential to ensue human or animal pain and suffering under any given conditions (Leary et al., 2013).

Although conditionally acceptable, gas inhalants are considered a practical option for on-farm euthanasia of pigs due to relatively inexpensive, direct vet supervision not needed for stockperson, multiple pigs euthanized at a time, aesthetically less distressing to workers, reduce handling stress in animals and readily available. However, concerns are raised with gases as they produce marked aversion and distress. At present, there is a lack of enough information on consistent and reliable euthanasia achieved with gas inhalants. Understanding how gases affect physiological and emotional well-being of animals while progressing to LOC is essential to revise, and refine the current euthanasia guidelines.

1.4 Gas inhalants- mechanism of action

1.4.1 Carbon dioxide (CO_2) -respiratory neurophysiology

CO_2 is directly involved in respiration, in excess it produces hypercapnic hypoxia. The mechanism by which CO_2 produces LOC involves alteration in normal respiratory neurophysiology. Gaseous exchange occurs at the alveoli which is the functional unit of lungs. The partial pressure of O_2 (PO_2) in the alveoli is 104 mm Hg whereas the PO_2 at the arterial end is around 40 mm Hg, due to this pressure difference O_2 diffuses through the capillary from alveoli. The O_2 rich blood combines with hemoglobin (Hb) which helps in transportation. After O_2 metabolism, CO_2 is produced as a byproduct which rises the PCO_2 to 45 mm Hg while PCO_2 at the alveoli is 40 mm Hg. The narrow PCO_2 difference diffuses CO_2 from the blood capillaries into the alveolus and out into the exterior. As oxygenated blood passes through tissues, CO_2 diffuses out into the blood forming carbonic acid and further converts into bicarbonate (HCO_3^-) and H+ ions, which in turn raises the H+ and lowers pH. The low pH allows O_2 to dissociate from the Hb, the process is called the Bohr Effect. Once O_2 is released, CO_2 readily attaches to the Hb as CO_2 affinity is 20 times higher than O_2 and is transported out from the pulmonary capillary to the exterior through exhalation (Hall and Guyton, 2011, p495-500). Respiration is regulated by neural and humoral component. Neural component has a respiratory center composed of different neuron groups, located in the medulla oblongata and pons of the brain stem. Neurons regulate the rhythmic pattern of breathing via phrenic nerve in the diaphragm, vagal and glossopharyngeal nerve. Mechanoreceptors monitor lung stretch and changes in airway and vasculature. Proprioceptors in respiratory muscles regulate breathing effort. Humoral component are blood chemicals such as CO_2, O_2 and H+ ions that can modify basic rhythm and alveolar ventilation. The central and the peripheral chemoreceptors are local chemical control sites. The peripheral chemoreceptors (carotid and aortic bodies) are primarily specialized in detecting arterial O_2 changes (below 80-100 mm Hg), and also respond to PCO_2 and H+ changes, thus stimulating breathing (Nattie, 1999). The central chemoreceptors are located on the ventral surface of medulla and are highly sensitive to changes in H+ ions in the interstitial fluid of the brain, cerebral blood flow and arterial PCO_2 (Reece et al., 2015, pp232-237). Any changes in blood gas, alter the interstitial fluid pH and ventilation (Nattie, 2006). When in excess, body's compensatory mechanism cannot remove the high CO_2 deposit. As a result, CO_2 crosses blood brain barrier, which drops pH in the cerebrospinal fluid, that is thought to depress neuronal excitability producing reversible anesthetic state (Hsu et al., 2000; Dulla et al., 2005; Erickson et al., 2015).

1.4.2. Inert gas – respiratory neurophysiology

Inert gases like argon or nitrogen are suggested as alternatives for pigs' euthanasia (EFSA, 2004; Leary et al., 2013). Argon produces hypoxia or anoxia based on O_2 displacement. As discussed in the physiology section, low O_2 in the arterial blood is detected by peripheral chemoreceptors in the carotid and aortic bodies. The sensory innervation to the chemoreceptor is part of glossopharyngeal nerve whose activity increases with even a small drop in arterial PO_2 within 0.2 to 0.4 sec after the onset (Prabhakar and Semenza, 2015). The peripheral chemoreceptors hence, affect the cardiovascular and respiratory system by transferring impulses from afferent nerve fibers of the glossopharyngeal nerves (from carotid body) and the vagus nerves (from aortic body) (Erickson et al., 2015). Brain O_2 depletion causes neuronal inhibition and death, resulting in brain failure (Aitken and Schiff, 1986; Neubauer et al., 1990).

1.5 Animal welfare status during gas euthanasia

Animal welfare is multifaceted concept and one of the facets is 'feelings' or affective states of animals. Feelings or affective states are interchangeably used in this thesis. Feelings which are subjective in nature (Duncan, 2005), are complex mental experiences of different body circumstances (Damasio and Carvalho, 2013) elicited as a result of any rewarding or challenging situation (Millot et al., 2014). "To be concerned about animal welfare is to be concerned with the subjective feelings of animals particularly the unpleasant subjective feelings of pain and suffering" (Dawkins, 1988). This statement suggest "how an animal feels" is an important welfare question (Fraser et al., 1997; Boissy et al., 2007). The Organization for Economic Cooperation and Development puts forward a view that if something is known to cause pain, distress or suffering in humans, it should be assumed to cause similar feelings in animals (OECD, 2000). Moreover, basic neuroanatomy, brain chemistry and other central nervous system attributes are remarkably similar between human and animals, hence it is likely animals feel the same way as humans do during pain and distress (Kirkden and Pajor, 2006; Boissy et al., 2007). During welfare assessment emphasis is put on negative feelings, as we strive to understand, identify and minimize those feelings (Mellor, 2016). Since animals cannot self-report their experience, feelings are recognized by behavioral responses. In addition to behavior, physiological responses are also used as proxy (Murphy et al., 2014) and are likely correspond to the strength of associated feelings (Desire et al., 2002; Paul et al., 2005; Kirkden and Pajor, 2006). These experiences occur as collective sensory outcomes and other neural inputs from within animal's body as well as its environment (Hemsworth et al., 2015).

Previous euthanasia studies have reported behavioral and physiological responses associated with pain, distress and aversion with gas inhalants. Animals are likely within a 'conscious' window and are possibly aware of the events that occurs before LOC. Throughout this thesis, LOC is indicated when an animal displays loss of righting reflex. Rapid LOC is an important euthanasia evaluation criteria (Leary et al., 2013), however, LOC is not immediate with gases which makes animals susceptible to prolong suffering. For better understanding of poor welfare associated with gas euthanasia, it seems logical to define and discuss the negative feelings of pain and distress.

1.5.1 Pain

Pain is defined as an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage (International Association for the Study of Pain, 1994). High CO_2 (80%-90%) commonly used for pigs' euthanasia in itself is not painful, when it combines with body fluids (water) forms a weak carbonic acid causing irritation of ocular and nasal mucosa which may be painful. Unlike CO_2, argon is chemically inert and therefore do not produce pain and can be a better alternative (Raj and Gregory, 1995). Humans report pain at ocular and nasal mucosa over 40% CO_2 (Anton et al., 1992; Chen et al., 1995; Feng and Simpson, 2003). In rats, nociceptors (pain sensory receptors) activate at 37%-50% CO_2 (Anton et al., 1992; Peppel and Anton, 1993).The primary afferent nociceptors are present in the skin and mucous membrane which are activated during chemical (carbonic acid) insult to tissues. The nociceptors transduce any pain stimuli into action potentials with the help of specialized Aδ and C fibers, which are then transmitted to dorsal horn of spinal cord. A second order neuron at the dorsal horn then modulates the encoded information and transmit it to the thalamus. Finally a third order neuron transmits the modified stimulus to higher brain centers, initiating appropriate behavioral and physiological responses (Dubin and Patapoutian, 2010; Hall and Guyton, 2011). Moreover, perception of pain has two components – a) sensory-discriminative and b) motivational-affective. The processing of sensory-discriminative mechanism occurs in the cortical and subcortical structures of brain. These enable to point out the location, intensity, duration and stimulus of pain. The processing of motivational-affective involves the ascending reticular formation for arousal, limbic system for perception of aversion, fear, anxiety and depression and hypothalamus-pituitary-adrenal (HPA) axis for autonomic nervous system modulation (Bromm, 2001; Leary et al., 2013). This explains how a pig may perceive pain associated with CO_2 exposure. However, the duration and intensity of pain perception and the associated negative feelings may vary depending on LOC. Somatosensory evoked potentials (SEP), indicative of brain responsiveness, were lost in 22 sec when pigs were exposed to 90% CO_2 (Raj et al., 1997), which elicit at least until that duration pigs perceived pain consciously.

1.5.2 Distress

Distress is negative feeling in response to a stressor, the result of which reduces well-being and comfort of any animal (Leary et al., 2013). During on-farm euthanasia, when pigs are euthanized with gas inhalants in an enclosed chamber, following elements as shown in Figure 1.1 may lead to distress:

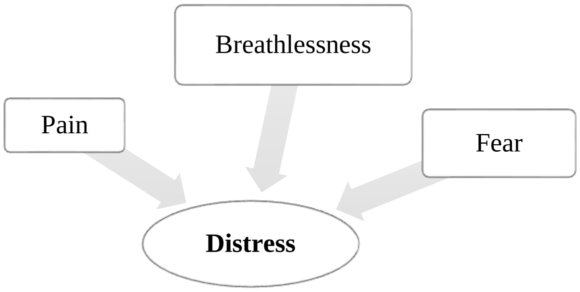

Figure 1.1 Factors causing distress during gas euthanasia

Depending on time lag between when an animal is introduced to gas until LOC is achieved, feeling of breathlessness occurs with both CO_2 and argon as hypercapnia and hypoxia respectively affects respiratory mechanism, which further stimulates negative feelings (Beausoleil and Mellor, 2015). Altered blood pH and ventilation initiate afferent feedback to respiratory center. It further triggers receptors in airways, lungs, inspiratory and expiratory muscles and chest wall to relay information to cortex and limbic structures which then modulate and generate feeling of breathlessness with cognitive component (Widdicombe, 1982; Gigliotti, 2010). Breathlessness is a subjective feeling of breathing discomfort associated with respiratory effort, air hunger and chest tightness (Lansing et al., 2000; Beausoleil and Mellor, 2015), however, in animals it is unlikely to detect every components of breathlessness reliably and consistently. Humans report breathlessness at 8% CO_2 which gets severe at 15% CO_2 (Dripps and Comroe, 1947; Liotti et al., 2001), with hypoxic levels breathlessness can occur as low as 7% O_2 during constrained respiration (Moosavi et al., 2003).

Raj and Gregory (1996) reported a subjective respiration score indicating mild to moderate hyperventilation along with audibly heavy breathing at 20% CO_2, the behaviors further elevated with higher CO_2. Velarde et al. (2007) defined gasping as first sign of breathlessness and found onset of gasping occurring earlier when pigs were stunned at 90% compared to 70%. Raj and Gregory (1997) suggested duration of respiratory distress should be evaluated until the onset of LOC to assess the gas merits for euthanasia. The authors reported pigs exposed to 80-90% CO_2 experienced moderate to severe respiratory distress until the induction of LOC occurred in 21 sec as measured by SEPs. When neonatal and weaned pigs were introduced to 100% pre-filled CO_2 in a chamber, Sadler et al. (2014) noted over 80% pigs from both groups displayed open-mouth breathing for 12 and 20 sec respectively. Pigs showed normal to mild hyperventilation when exposed to hypoxia (<2% O_2) (Raj and Gregory, 1996). Around 13% pigs exposed to 90% argon in a stunning unit showed onset of gasps in 13 sec (Dalmau et al., 2010). Sutherland (2011) noted labored breathing for 45 sec when suckling pigs exposed to 90% argon in a chamber. When weaned pigs were exposed to hypoxia in a chamber either singly or in groups of 2 or 6, brief periods of open mouth breathing was observed in 9% weaned pigs (Fiedler et al., 2016).

Fear is an emotional state that is induced by the perception of any actual (fear state) or potential (anxiety state) danger and threatens the well-being of an animal (Boissy, 1995). Physiologically, CO_2 induces fear responses due to activation of ‘fear’ centers in brain amygdala (Davis, 1992; Ziemann et al., 2009). Amygdala expresses acid sensing ion channel -1a (ASIC1a) and it is responsible for fear responses (Coryell et al., 2007). Ziemann and colleagues (2009) hypothesized amygdala would detect low pH and found mice when exposed to CO_2 induced brain acidosis and evoked fear responses. In humans, the sensation of breathlessness caused by 7% - 35% CO_2 induced fear responses (Bailey et al., 2005; Pappens et al., 2012). Around 60% pigs ‘backed away’ during initial exposure to high CO_2 (Dodman, 1977). Velarde et al. (2007) reported number of ‘retreat attempts’ increased on subsequent testing days when pigs were exposed to 90% CO_2 compared to when exposed to ambient air. After 90% CO_2 exposure on a previous test day, pigs hesitated to enter a chamber containing ambient air (Raj and Gregory, 1995). The unpleasantness associated with CO_2 exposure may have instilled fear in pigs.

1.6 Assessment of affective states in euthanasia studies

Objective assessment of affective states during gas euthanasia is difficult (Kirkden and Pajor, 2006). There is not a validated method to assess affective states during euthanasia, however some proxy measures such as behavior, physiology and hormonal profiles are commonly used to investigate and evaluate different affective states (Boissy et al., 2007; Mormede et al., 2007). Physiological measures such as heart rate, blood pressure, blood gas (White et al., 1995; Martoft et al., 2002; Rault et al., 2013) and neuroendocrinology measures such as cortisol, adrenocorticotropic hormone (ACTH), adrenaline, noradrenaline are often reported as candidates of stress. Activation of sympathetic-adrenal-medullary (SAM) and hypothalamus-pituitary-adrenal (HPA) axis occur during stress (Mormede et al., 2007; Martínez-Miró et al., 2016). SAM activation during stressful condition is responsible for changes in heart rate and blood pressure. Activation of HPA axis leads to the release of ACTH, which then activates the release of cortisol of adrenal cortex (Hall and Guyton, 2011). Plasma cortisol did not differ when suckling pigs were exposed to different gas treatments including 100% CO_2 and 90% argon in air and blood samples were collected prior and after gas euthanasia (Sutherland, 2011). Differences between pre and post euthanasia cortisol levels were lower when pigs were exposed to 100% CO_2 at slow flow rate compared to fast flow rate (10% vs 20% chamber volume/min respectively) (Meyer et al., 2013). Cortisol took about 30 minutes to reach its peak level and generally pigs attain LOC within 14 to 30 sec when exposed to 60%-90% CO_2 (Sutherland et al., 2012) which means cortisol level may not reflect the distress experienced by pigs while still conscious. Rault et al (2013) looked into heart rate to assess stress associated with different gas treatments in neonatal pigs but failed to record reliable data due to faulty heart rate monitors. However, in the last part of the experiment, the authors were able to evaluate heart rate using a stethoscope but did not find any difference within gas treatments. Behaviors are regarded more sensitive measure (Marchant-Forde et al., 2009) to evaluate affective states associated with pain and distress during gas exposure such as vocalization, open mouth breathing, gasping, flailing, escape attempts, withdrawal or aversion learning techniques (Raj and Gregory, 1995; Jongman et al., 2000; Rault et al., 2013; Sadler et al., 2014; Fiedler et al., 2016). Several variations exist across studies regarding which behaviors to measure and methods used to quantify them. When exposed to 90% CO_2 in a deep stunning unit for a cycle of 270 sec, more than 50% market age pigs showed escape attempts (Llonch et al., 2012), whereas Sadler et al. (2014) did not report any escape attempts when weaned pigs (piglet pair) were exposed to 100% CO_2 in a chamber. The reason pigs did not display escape attempts in the latter example even with high CO_2 could be pigs' age, euthanasia method and presence of companion. Respiratory neurophysiology is affected during gas euthanasia so outcome associated with respiratory distress is very important, however variability in terminology and behavior observation ethogram differ across studies which make it difficult for interpretation (O'Connor et al., 2014). For instance, the use of gasping by Llonch et al. (2012) and Sadler et al. (2014). Sadler et al (2014) used 'gasping' to describe later stages of respiratory distress near or after pig lost posture whereas Llonch et al. (2012) used gasping as an indicator of onset of breathlessness that occurred before loss of posture. Vocalizations are reported in pigs during gas euthanasia. Rodríguez et al. (2008) reported pigs vocalized when exposed to 90% CO_2 in a dip lift stunning unit within 26 sec after exposure. Raj and Gregory (1999) found majority of pigs stunned with

90% CO_2, while only few pigs exposed to 90% argon squealed before electrocorticogram suppression. Fiedler et al. (2016) reported majority of weaned pigs squealed during argon exposure in a chamber. With vocalizations, it is a challenge to interpret the associated affective state during euthanasia as we may not know what affective states are involved (Fraser, 2009). For instance, pigs may vocalize out of fear as CO_2 activates 'fear' centers in amygdala (Davis, 1992; Ziemann et al., 2009) or due to novelty, pain, isolation stress, ascending or descending of the stunning unit.

In research settings, when pigs are forced exposed to gases in a gas chamber or in a dip lift stunning unit (Velarde et al., 2007; Sadler et al., 2014; Fiedler et al., 2016), it becomes difficult to differentiate whether certain behaviors or biomarkers arise due to the gas or due to the forced exposure. In addition, the forced exposure paradigm are not designed to examine the gas aversion from pigs' perspectives. Aversion learning techniques are tools that can be used to assess animals' motivation to avoid anything that is unpleasant or aversive. In such tests, animals are given some control over their environment and observations are made based on animals' sensory-discriminative processing, but not based on affective component (Rushen, 1996; Kirkden and Pajor, 2006; Millman, 2013). Aversion is a tendency to extinguish a behavior or to avoid a stimulus or situation, especially a previously pleasurable one, because it is or has been associated with a noxious stimulus (Merriam-Webster, 2017). Pigs exposed to 90% CO_2, avoid the gaseous environment because it is aversive, hence avoidance is indicative of aversion to gas (Raj and Gregory, 1995). In such cases, animals respond to the aversive stimuli but it is unknown whether the stimuli is significant or trivial, in other words the strength of the aversion is unknown (Kirkden et al., 2008). Cognitive tests can be alternatives to measure negative affective states of animals by 'asking' them how they feel as well as to assess strength of that feeling (Dawkins, 1990; Paul et al., 2005).

1.7.Cognitive tests – assessment of negative feelings

Feelings or emotions have cognitive component (Mendl and Paul, 2004). Any response to a stimuli, in parts depends on the cognitive appraisal of animals, such appraisal is needed for decision making for instance, to approach or to avoid (Paul et al., 2005; Boissy et al., 2007). Appraisal may be influenced by the innate spontaneous responses that have evolved over many generations or by learning or memory of previous experiences (LeDoux, 1995). There is still a gap in our knowledge about young pigs' initial perception to low CO_2 and argon gas and the potential of experiencing aversion during induction of loss of consciousness. When assessing aversion to gas inhalants, cognitive tests such as approach-avoidance and conditioned place avoidance may provide insight from animal's perspectives. Approach-avoidance test determines the strength of animals' motivation to stay in contact with a positive incentive which is paired with an unpleasant stimulus, thus producing a motivational conflict (Kirkden et al., 2008; Ito and Lee, 2016) and has been used to determine neonatal pigs aversion to different gas combinations (Rault et al., 2013). Conditioned place avoidance (CPA) test is based on animals' past experience or memory. An aversive stimulus is paired with a neutral environment or place, animal associates the place with aversive experience upon re-exposure (Mathur et al., 2011; Millman, 2013). Conditioned place preference (CPP) has been used to determine the relative preference/aversion for different handling and blood collection experiences in piglets (Wahi et al., 2011).

1.8.Thesis objectives

The overall objective of this thesis was to address young pigs' aversion during gas euthanasia using cognitive tests, including approach avoidance and conditioned place avoidance tests. I planned to address the broad objectives with two specific objectives. The first objective was to assess aversion associated with exposure to and induction of LOC with CO_2 gas using approach-avoidance and conditioned place avoidance paradigms. The second objective was to assess aversion associated with exposure to and induction of LOC with argon induced hypoxia using approach-avoidance and conditioned place paradigms. Through these studies, I aimed to fulfill current gaps in knowledge associated with young pigs' perceived aversion to different gas inhalants by use of cognitive tests.

CHAPTER 2

AVERSION TO CO_2 GAS IN PIGS USING APPROACH-AVOIDANCE AND CONDITIONED PLACE AVOIDANCE PARADIGMS[1]

(This chapter is prepared as a manuscript for submission to **Journal of Animal Science**)

[1] The author would like to thank the USDA Agriculture and Food Research Initiative (Grant No. 2012-67021-19363) for the financial support to this project. The authors would also like to acknowledge the invaluable technical support from the following people: Rebecca Parsons, Isabelle Withrock, Kassi Miller, Kesley Grossman, Katie Tapper, and Laboratory Animal Resources personnel.

2.1 Abstract

The main objective of this study was to investigate pigs' aversion to CO_2 using approach avoidance (AA) and conditioned place avoidance (CPA) paradigms. A preference testing device was designed with two identical chambers separated by a sliding door and an exhaust sink. Twelve crossbred pigs were individually trained for 5 consecutive days to enter the treatment chamber (TC) to access food rewards when the sliding door was opened, followed by 6 min during which they could move freely between the chambers. The same methods were used during the testing phase, during which CO_2 in the TC was maintained at one of 3 concentrations: 10%, 20%, or 30%. Tests concluded when loss of consciousness (LOC) occurred or after 6 min. During each of the 3 rounds of testing, pigs experienced the assigned CO_2 treatment on gas day (G), and ambient air conditions on one baseline day (B) and on one washout day (W) prior to and following G respectively. We hypothesized pigs would display avoidance at 30% CO_2. When avoidance or loss of posture (LOP) occurred, we hypothesized conditioned place avoidance would be observed on W. Behavior data was collected using live observations and video recordings. Ten of 12 pigs entered TC on all B, G and W; 1 pig never entered TC on any day and 1 pig did not receive assigned 20% in round 1 and were removed from the analyses. On G, pigs were slower to enter TC ($P < 0.05$) and faster to leave TC ($P < 0.05$) than on B and slower to re-enter TC ($P < 0.05$) on W. On G, 5 pigs at 20% and 4 pigs at 30% CO_2 remained in the TC until LOP occurred. In conclusion, some degree of aversion was observed at all CO_2 concentrations compared to ambient conditions. However, aversion was not sufficient to provoke marked avoidance or conditioned placed avoidance.

Key words: pigs, aversion, carbon dioxide, approach-avoidance, conditioned place avoidance

2.2 Introduction

Euthanasia refers good death and the techniques should induce loss of consciousness and death with minimal pain and distress (Leary et al., 2013, pp 6). Use of carbon dioxide (CO_2) is conditionally accepted for young pigs (*Sus scrofa*) below 32 kg (Leary et al., 2013; AASV and NPB, 2016). However, the pain, distress and associated aversion with high CO_2 are welfare concerns (Conlee et al., 2005). The AVMA suggests an alternate 2-step euthanasia method where animals are first rendered unconscious and then immersed in 100% CO_2. The Canadian Code of Practice requires pigs below 32 kg must be heavily sedated before gases are introduced (NFACC, 2014). Since, the effectiveness of low CO_2 as a pre-anesthetic in pigs is not well documented, there is a critical need to determine CO_2 concentrations acceptable for young pigs' euthanasia.

Research exploring inhalants typically involves forced exposure paradigms that poses challenges for determining when pigs are responding with awareness (Fiedler et al., 2014; Sadler et al., 2014). Conversely, cognitive paradigms can be designed to 'ask' conscious animals how they perceive a stimulus or a previous experience. Approach-avoidance (AA) test measures strength of aversion when a positive stimulus is paired with a negative experience resulting a motivational conflict (Kirkden et al., 2008). Conditioned place avoidance (CPA) test measures responses when animals are re-exposed to an environment previously associated with a negative experience (Mathur et al., 2011).

The first aim was to examine CO_2 associated proximate avoidance using an AA test. We hypothesized pigs would not avoid 10% CO_2 but would avoid 30% CO_2. The second aim was to examine learned aversion to CO_2 using a CPA test. We hypothesized pigs would avoid a chamber previously associated with aversive CO_2 concentrations and/or where LOP was experienced.

2.3 Materials and methods

The protocol for this experiment was approved by the Institutional Animal Care and Use Committee at Iowa State University.

2.3.1 *Animals, housing and management*

Twelve healthy crossbred, newly weaned pigs were enrolled in the study, consisting of 11 barrows and 1 gilt. Pigs were group housed in two identical temperature controlled rooms in the Laboratory Animal Research facility at Iowa State University. The average room temperature was set between 26 – 29 ºC and relative humidity was between 60 – 65%. Six pigs were assigned to each room, balanced by weight. Average BW at the start of the experiment was 5.4 ± 0.3 kg and 6.9 ± 0.1 kg in room 1 and room 2, respectively. Each room contained a single pen (3.04 m x 3.65 m) housing all 6 pigs (1.84 m^2/pig) with concrete flooring and a single rubber mat (61 cm x 61 cm). A heat lamp was provided as a supplemental heat source for the first 5 days. Environmental enrichment included a rubber boot and a metal chain attached to either a toy or a ball. Pigs were provided with *ad libitum* access to water through a nipple drinker. A commercial starter diet (Heartland Co-op, Prairie City, IA) was scattered on the rubber mat. To facilitate behavioral observations, pigs were dorsally identified using a livestock marker (All Weather Twist Stick Livestock marker, LA-CO, Elk Grove, IL) that was reapplied each afternoon during testing.

2.3.2 *Experimental room and gas preference box*

All testing was conducted in an experimental room located in the same building where the pigs were housed. Testing was conducted in a custom-built preference box that consisted of two identical chambers (61cm x 61cm x 76 cm) separated by a sliding door (Fig. 1a; Fig. 1b). Each chamber was equipped a hinged door through which pigs could be placed into or removed from the chamber. The floor in each chamber was fitted with a commercially available non-slip rubber mat. Box doors and top panels were made of Plexiglas to facilitate behavior observations. Opaque side walls were fitted with gloves for guiding pigs through the sliding door, and walls contained perforations to evenly distribute gases.

Chamber ventilation systems were independently controlled to achieve desired CO_2 concentrations within the treatment chamber (TC) while maintaining CO_2 concentrations at near ambient levels (<1%) within the control chamber (CC). The TC ventilation was operated as a recirculating positive pressure system. This included two small square axial fans (Model 4WD47, Dayton Electric Mfg. Co. IL) drew air into the right sidewall of the chamber where CO_2 gas was injected and mixed into the air stream. The mixed gas was returned to the chamber through the left sidewall. The CC was operated as a positive pressure system. This included two small fans (Model 4WD47, Dayton Electric Mfg. Co. IL) that introduced fresh air into the left and right sidewalls. Air escaped the chamber through designed outlets on the top panel and door. A negative pressure exhaust sink was located between the two chambers to evacuate excess CO_2 and maintain separate TC and CC environments.

Compressed CO_2 gas cylinders (Industrial grade 100% Carbon dioxide CGA-320, Radnor, PA) were fitted with a volumetric high output heated two-stage CO_2 regulator (PRS-3008 Special, Euthanex, Allentown, PA) controlling injection rates into the recirculated TC air stream. The TC environment was monitored in real-time with a wide span CO_2 sensor (CO2ZIR, CO2Meter Inc, FL) and a real-time O_2 sensor (TR25OZ Oxygen sensor, CO2Meter Inc, FL) connected to an automatic data recording system (GASLAB, CO2Meter Inc, FL). The CC environment was monitored with a wide span CO_2 sensor and an ambient range CO_2 sensor (Vaisala CARBOCAP CO_2 Sensor 0 – 10,000ppm, CO). The CO_2 injection rates were adjusted to maintain TC target concentrations and CC near ambient conditions using real-time gas concentration displays.

2.3.3 Experimental design

A repeated measure design was utilized, with pigs assigned to receive each of three concentrations of CO_2 (10%, 20% or 30%) in 3 rounds. Sample size was based on a similar experiment conducted by Wahi et al (2011) for conditioned place preference with suckling pigs, with pig as the experimental unit and serving as its own control. Pig responses to CO_2 concentrations on gas (G) day (d0) were compared to responses during ambient air concentrations (<1% CO_2) on baseline (B) day (d-1) and washout (W) day (d+1). Testing order was assigned using a random number generator, and pigs were tested at the same time of day on all test d. Pigs were randomly assigned to receive either 10% or 20% CO_2 during round 1, systematically assigned to receive the alternate treatment in round 2, and all pigs received 30% CO_2 in round 3. This confounding of CO_2 concentration and round was not originally planned in our experimental design. The design was revised when technical concerns were identified regarding the ability to maintain near ambient conditions (<1% CO_2) in the CC when TC was held at 30% CO_2 with subsequent carryover effects.

2.3.4 Training procedures

Pigs were acclimatized to the research environment for 3 days in their home pens, followed by training within the home pen to desensitize them to social isolation and to familiarize them with food rewards. A 2.2 m x 1.3 m spindle bar panels training pen was assembled in each room, which allowed pigs to see and touch pen-mates. The first home pen training stage included all pigs simultaneously. All pigs were encouraged to enter the pen, which included a slow feeding bowl (Brake-fast, LLC, VA) containing starter diet, few mini marshmallows and raisins, and a Kong-rubber dog toy (The Kong Company, Golden, CO) smeared with strawberry fruit spread (Great Value, Batesville, IL) and attached to a spindle panel with a chain. Pigs were allowed a 6-min food interaction reward period. The second stage followed the previous training, but was done on an individual pig basis. The third stage introduced visual social isolation, which was achieved by affixing all training pen walls with solid plastic panels. Starting with the second stage, each pig had to meet a performance standard (snout contact with at least one food reward within 2 min after pen entry) to graduate to the next stage of home pen training. Pigs that failed to meet this standard continued with the same training on subsequent days until the performance criteria was met. Preference box training in the experimental room began once home pen training was completed.

All pigs were trained individually in the preference box for 4 days. To encourage pigs to cross through the sliding door, food rewards previously experienced in the home pen training were provided in the TC. In addition, strawberry jam was smeared on the mat. The sliding door separating the chambers was closed at the start of the training session. The pigs were individually carried to the experimental room and placed into the CC. After 2 min, the sliding door was opened which provided the pig with access to both chambers. The pig was gently guided through the sliding door into TC using the gloves if it did not enter the TC within 5 min after the sliding door was opened. Once the pig entered the TC, a 6-min time period was provided during which it could freely move about the chambers. The pig was removed and returned to the home pen after the test. The preference box was cleaned and disinfected with Accel (ACCDISC1G-US, Virox Technologies Inc, ON, Canada) between tests and food rewards were replenished. On day 4 of training, pigs were deprived of food for 5-6 h prior to the training session to increase foraging motivation and was included in all subsequent training and test sessions. On the same training day, a plastic curtain with a slit in the middle was added on the TC side of the sliding door to facilitate stable CO_2 concentrations in the TC.

2.3.5 Testing procedures

First day of testing was baseline day that followed day 4 of training day. During the testing phase, each pig was tested on baseline (B d), Gas (G d) and Washout (W d) days over 3 rounds for 9 days. The procedures on B and W d were identical to those described for preference box training. Prior to placing each pig in the CC on G d, the TC was prefilled and stabilized at the designated CO_2 treatment concentration. If LOC occurred, the pig was removed from the TC and placed on a rubber mat in a recovery pen located in the testing room. For ethical reasons and injury concerns, removal criteria were later modified to include situations where loss of posture (LOP) and neuromuscular excitation (NME) occurred prior to LOC. During recovery, affected pigs were observed for a minimum of 5 min after return to consciousness and standing posture. Pigs were then provided with a tablespoon of starter diet and returned to the home pen after normal foraging (rooting or eating) behavior occurred.

2.3.6 Behavior data collection

Behavior during testing was recorded using direct observations and video recordings. Direct observations were collected by two observers. One observer sat beside the preference box and out of the test pig’s view. The second observer sat approximately 0.5 m from the TC door from which the pig was visible. A black fabric curtain (2.1 m x 0.9 m) and lighting placement were used to obscure this observer from the test pig.

Continuous video was recorded using four color digital video cameras (Panasonic, Model WV-CP-484, Matsushita Co. Ltd., Kadoma, Japan) which were positioned to provide overhead and lateral views of CC and TC. The cameras were fed into a multiplexer using Noldus Portable Lab (Noldus Information Technology, Wageningen, The Netherlands) that enabled capture of a dual recording at 30 frames/s onto a computer using HandyAVI software (version 4.3 D, Anderson’s AZcendant Software, Tempe, AZ)

Data collected as direct observation included latency to enter TC, latency to leave TC, latency to re-enter TC, vocalization and elimination. Latency to enter, leave and re-enter TC were recorded using a digital timer (National Presto IND. Inc., Eau Claire, WI) and were collected as measures of avoidance. Vocalization was collected as counts using a commercially available manual counter (Great Star Tally counter). Vocalizations were separated into 2 categories-those that occurred before the sliding door was opened (VB) when the pig could see but not access feed rewards, and those that occurred after the sliding door was opened (VA) when the pig could freely move between chambers. Elimination (defecation and urination) was collected as binary data (yes/no). Both vocalization and elimination were collected as measures of distress.

Data collected by video recordings included behaviors on G: open mouth breathing (OMB), ataxia, escape attempts, loss of posture (LOP), righting response (RR) and neuromuscular excitation (NME) were collected as measures of induction of LOC and considered as distress behaviors. Data were collected from videos by a trained observer, blinded to the animal ID, date and test day, using Observer (version 10.1.548; Noldus Information Technology). A neutral individual performed the blinding procedures for the video recordings from all tests. The blinding procedures involved cutting the video recordings to remove identification presented at the beginning of each video, assigning a random number to each video segment and sorting for the purpose of providing a random sequence in which videos were to be scored. Seven videos were selected at random and duplicated within this sequence for the purpose of determining intra-observer reliability. Prior to data collection, the observer was trained to use the Observer XT program by repeatedly scoring 2 videos and an ethogram from an unrelated CO_2 study until reaching an inter and intra-reliability score of $k \geq 0.90$ as calculated by the Observer program. After reaching the desired level of competence, data collection began using blinded videos and ethogram (Table 2.1).

2.3.7 Statistical analysis

Of 12 pigs enrolled in the testing phase, 2 pigs did not enter TC on any of B, G or W in round 1 or round 2, and 1 pig did not enter TC on any of B, G or W d in Round 3. These pigs were excluded in the analyses for these respective rounds. On G, 1 of 10 pigs in round 1 did not receive the targeted 20% CO_2 in TC due to a technical problem and was excluded from this round. Therefore, there were 10 pigs on B and W in round 1 and 2; 9 pigs on G in round 1 and 10 pigs in round 2; and 11 pigs on B, G and W in round 3.

For analysis, behavioral data were assessed as latency, duration or frequency wherever appropriate. Latency to enter, latency to leave and latency to re-enter were calculated relative to the time the sliding door was opened. Because pigs moved in and out of TC more than once, measures of avoidance were analyzed for the first event only. Likewise, latencies for measures of induction of LOC were calculated relative to the time of last entry to TC for those pigs that displayed LOP and/or LOC. Duration was calculated as total time recorded over all bouts during testing.

Data were evaluated for normality using PROC UNIVARIATE (SAS Inst. Inc., Cary, NC). The model for all continuous variables of avoidance behavior included fixed effects of day (B, G and W). The model for analyzing continuous variables on behaviors during G included the fixed effect of CO_2 (10%, 20% and 30%) concentration. Pig was included as a random effect in both models. Latency and duration were analyzed using PROC GLIMMIX (SAS Inst. Inc., Cary, NC) with an autoregressive covariance structure and gamma distribution.

Mean differences were considered significant at $P < 0.05$, whereas $0.1 > P \geq 0.05$ was considered to represent a tendency for a difference. When significant differences were detected, Tukey-Kramer adjustments were used for multiple comparisons. Least square means estimates and standard errors (SE) are reported in the results and corresponding tables.

2.4 Results

All pigs successfully completed home pen training within 4 days. One pig never entered TC on any of the 4 training days in the preference box. On testing days, 2 pigs did not enter TC on any of B, G or W in round 1 or round 2, and 1 pig did not enter TC on any of B, G or W in round 3. LOP was displayed by 5 pigs and 4 pigs at 20% and 30% CO_2 while none displayed LOP at 10% CO_2. Three pigs that lost posture at 20% CO_2 also lost consciousness but none of the pigs lost consciousness at 30% CO_2. Two pigs that lost posture at 20%, also lost posture when exposed to 30% CO_2.

2.4.1 *Behaviors indicating avoidance*

Latency to enter TC differed significantly by day (*P* < 0.01), with pigs entering TC 6 times slower on G relative to B (Table 2.2). Within G, latency to enter TC tended (*P* = 0.06) to be faster at 20% CO_2 compared to 10% or 30% CO_2 (Table 2).

During testing some pigs remained in TC for the entire 6 min after entry. Pigs leaving TC included 15/31 on B, 13/30 on G and 11/31 on W. Latency to leave TC differed significantly by day (*P* < 0.01), with pigs leaving TC 2 times faster on G than on B or W (Table 2.2). On G, 5/10, 3/9 and 5/11 pigs left TC at 10%, 20% and 30% CO_2. Latency to leave TC significantly differed with CO_2 concentration (*P* = 0.05), with pigs leaving TC faster at 30% CO_2 (Table 2.2).

Pigs that left TC, re-entered on all testing days (Table 2.2). Pigs re-entering TC included 12/15 on B, 13/13 on G and 10/11 on W. Latency to re-enter TC differed by Day (*P* <0.02), with pigs re-entering TC 1.5 times faster on G than W. Re-entry was not affected by CO_2 concentration (*P* = 0.6).

Five pigs at 20% and 4 pigs at 30% CO_2 remained in TC until LOP. Remaining pigs, however left and re-entered TC more than once during testing.

2.4.2 Behaviors indicating distress and LOC

None of the pigs at 10% CO_2 presented behaviors associated with induction of loss of consciousness (Table 2.3). Open mouth breathing (OMB) was observed in 7 of 9 pigs at 20% CO_2 and 6 of 11 pigs at 30% CO_2. However, OMB was difficult to observe reliably due to camera placement, and for this reason data was not analyzed further.

Some pigs displayed ataxia and left TC prior to LOP. Seven of 9 pigs at 20% CO_2 and 9 of 11 pigs at 30% CO_2 showed ataxia. Of the total 9 pigs that displayed LOP and did not leave TC, latency to ataxia did not differ with CO_2 concentrations (*P* = 0.99) (Table 2.3). Latency to LOP was significantly longer at 20% CO_2 compared to 30% CO_2 ($P < 0.05$). Duration of ataxia was significantly longer at 20% CO_2 compared to 30% CO_2 (129.0 ± 33.2 s and 34.0 ± 9.7 s respectively, $P = 0.01$).

Escape attempts were observed in 2 of 5 pigs at 20% CO_2 and 1 of 4 pigs at 30% CO_2 that displayed LOP. Of 2 pigs, latency to escape at 20% was 108.1s and 82.4 s whereas for 1 pig at 30% the latency was 53.6 s. Frequency of escape attempts displayed by 2 pigs at 20% was 3 and 4 whereas at 30% CO_2, frequency of escape attempts was 4.

Righting response (RR) was observed in 2 of 5 pigs that displayed LOP at 20% CO_2. For 2 pigs, latency to RR was 174 s and 63 s. Frequency of RR displayed by 2 pigs was 1 and 4.

Neuromuscular excitation (NME) was observed in 4 of 5 pigs that displayed LOP at 20% CO_2 and 4 of 4 pigs that lost posture at 30% CO_2 (Table 2.3). Latency to NME was greater at 20% CO_2 compared to 30% CO_2 ($P < 0.05$).

Not all pigs defecated all days. Defecation differed by day ($P = 0.002$), with more pigs defecating on G than on B and W (22.8%, 14.1% and 8.7% respectively). Defecation did not differ by CO_2 concentration ($P = 0.17$). Of 9 pigs that displayed LOP, 2 pigs did not defecate.

Not all pigs urinated on all days. Urination tended to differ by day ($P = 0.08$) with more pigs urinating on G than on B and W d (8.7%, 4.3% and 2.1% respectively). Urination did not differ with CO_2 concentration ($P = 0.65$). Of 9 pigs that displayed LOP, 1 pig urinated.

Two pigs did not vocalize on any testing days before (VB) or after (VA) the sliding door was opened. VB was observed in 67% pigs on B, 50% pigs on G and 64% pigs on W. On G, VB was displayed by 47%, 33% and 20% pigs at 10%, 20% and 30% CO_2. Furthermore, VA was displayed by 29% pigs on B, 43% pigs on G and 19% pigs on W. On G, 70%, 50% and 27% pigs displayed VA at 10%, 20% and 30% CO_2.

2.5 Discussion

Results from this study suggest low CO_2 is mildly aversive to pigs. Degree of aversion associated with low CO_2 was not strong enough to provoke complete proximate avoidance or conditioned place avoidance.

The first objective was to determine proximate avoidance associated with CO_2. We hypothesized 10% CO_2 would not be aversive to pigs whereas avoidance would occur at 30% CO_2. Contrary to our hypothesis, all pigs entered TC and did not avoid the chamber at any tested CO_2 concentrations on G but displayed longer latencies to enter TC compared to ambient conditions on B. Pigs appeared to enter TC faster at 20% CO_2 which may be due to the current study design where pigs randomly experienced either 10% or 20% CO_2 in round 1 and systematically assigned to receive the alternate concentrations in round 2. Three pigs that received 20% in round 2 entered TC in 29s, 76s and 179s (results not shown) respectively at 10% CO_2 in round 1 and skewed the data. The tested concentrations especially 20% and 30% were mildly aversive (Raj and Gregory, 1996) but the feed restricted pigs entered TC to retrieve feed rewards (Jones et al., 1998; Rault et al., 2013), indicating feeding motivation was higher compared to motivation to avoid gas. The results supported the findings by (Velarde et al., 2007) where CO_2 (70% or 90%) aversion was assessed in adult pigs using dip lift stunning unit, pigs were first trained to enter the CO_2 stunning crate of the stunning system after which the crate was lowered into a pit containing CO_2 or air. Pigs showed longer latency to cross raceway and enter the stunning crate on treatment days compared to control day. However, in that study pigs received CO_2 for 3 consecutive days and may have learned to predict the negative CO_2 stimuli. Contrary to our results, when rats were exposed to a static concentration of 10%, 15%, 20% CO_2 or 90% argon in air for 300s, 1 rat at 20%

CO_2 and 3 rats at 90% argon refused to enter the test cage, suggesting aversion to those gases (Niel and Weary, 2007)

The design of the box allowed pigs to move in and out at their will. Due to pigs' inherent curious nature, some pigs left TC on all testing days including B (48% pigs) and W (35% pigs) possibly to explore their surroundings. Opposite to what we expected, pigs that did not display LOP left TC within a minute on G compared to >2 min on B suggesting proximate CO_2 exposure was aversive relative to ambient conditions. At relatively higher CO_2 (30%), pigs may have detected pungent CO_2 (Raj and Gregory, 1995) or may have experienced a feeling of 'breathlessness' (Liotti et al., 2001) causing a repelling or an aversive experience (Leach et al., 2004), prompting them to leave TC. In a different free choice study, adult pigs' latency to withdraw head from a Perspex box after being exposed to 30% CO_2, 90% CO_2, 90% argon or air (control) were examined. Pigs' withdrawal of head from the box was an indicator of CO_2 aversion. Of 16 pigs from trial 1 and trial 2, 87% withdrew their heads from the box in 18s and 47s respectively when exposed to 30% CO_2 and concluded the concentration produced mild aversion. In the present study, 45% pigs exposed to 30% CO_2 left TC in 24 s while the remaining pigs either displayed LOP or were rescued due to violent NME (Rault et al., 2013).

Pigs that left TC, re-entered (Raj and Gregory, 1995) on all days. Altogether 80% pigs that left TC on B re-entered while 100% re-entered on G. Latency to re-enter TC did not differ between G and B. In accordance, (Jongman et al., 2000) reported boars latency to re-enter the stunning crate did not differ between CO_2 treatments (60% or 90%) and control (air). However, in this study, boars were allowed to re-enter the stunning crate 30 minutes after CO_2 treatment. Within G, latency to re-enter did not change with CO_2 concentrations. The motivation to re-enter TC on B or W may be different than G. In addition to food motivation, the mild aversion caused by CO_2 may be a novel environment for pigs, forcing them to approach TC to retrieve feed rewards and at the same time forcing them to avoid TC temporarily when there is aversion (Leach et al., 2002) or feeling of breathlessness.

The second objective was to determine pigs' learned avoidance to CO_2 using CPA paradigm. Conditioned place preference task has been well established in pigs (de Jonge et al., 2008). Our hypothesis was pigs would avoid a chamber previously associated with aversive CO_2 concentrations and/or when LOP occurred. The current result did not support this hypothesis as all pigs that experienced CO_2 on G entered TC on subsequent W, including those that lost posture. Pigs avoidance behavior on W was similar to B. Aversion to CO_2 is dose dependent (Velarde et al., 2007) so low concentrations used in the present study could be mildly aversive to pigs (Raj and Gregory, 1995) affecting them transiently during proximate exposure. As a result, conditioned place avoidance was not observed on any W.

Open mouth breathing, ataxia, escape attempts, loss of posture and righting response are caused due to CO_2 induced hypercapnic hypoxia. Physiologically, these behaviors occur in a conscious animal and are likely associated with distress in previous studies (Raj and Gregory, 1995; Niel and Weary, 2006; Velarde et al., 2007; Sadler et al., 2014; Beausoleil and Mellor, 2015). Conditioned place avoidance was also not observed in pigs that lost posture, it is likely pigs were unconscious and due to apparent amnesia (Jongman et al., 2000), pigs may not have recalled the presumable CO_2 aversion (Velarde et al., 2007) and so conditioned placed avoidance was not displayed on any subsequent W.

Defecation and urination are physiological processes. In the present study, more pigs exhibited elimination behaviors on G which indicates the possible role of CO_2 inducing fear and distress. Previous studies have shown animals defecate and urinate more during fear or stress related arousal (Hall, 1934; Boissy, 1995). CO_2 induces fear responses in pigs due to activation of 'fear' centers in amygdala (Davis, 1992; Ziemann et al., 2009), which may have been another reason for more pigs to defecate or urinate on G. Amygdala expresses acid sensing ion channel -1a (ASIC1a) and it is responsible for fear responses (Coryell et al., 2007). Ziemann and colleagues (2009) hypothesized amygdala would detect low pH and they found mice when exposed to CO_2 induces brain acidosis in brain and evoked fear responses in mice.

LOP was evident in some pigs at 20% and 30% CO_2 and significantly faster LOP occurred at 30%. Previous studies have reported 30% CO_2 can render LOP with mild aversion and has been used with inert gases for faster induction of LOP (Raj and Gregory, 1995; Raj and Gregory, 1996; Rault et al., 2013). Though 30% CO_2 produced mild aversion in pigs, it may not be ideal for two-stage euthanasia as pigs displayed violent NME and needed to be rescued for welfare concerns.

In conclusion, the CO_2 concentrations used in this study are mildly aversive but may not be suitable for two-stage euthanasia due to violent neuromuscular excitation occurring before loss of consciousness therefore, posing a challenge to animal welfare. Further research is required to find a suitable CO_2 concentration that would reliably render pigs unconsciousness with minimal aversion and distress.

2.6 References

AASV, and NPB. 2016. On-Farm Euthanasia of Swine Recommendations for the Producer.

Aitken, P. G., and S. J. Schiff. 1986. Selective neuronal vulnerability to hypoxia in vitro. Neurosci. Lett. 67:92–96.

Anton, F., I. Euchner, and H. O. Handwerker. 1992. Psychophysical examination of pain induced by defined CO2 pulses applied to the nasal mucosa. Pain 49:53–60.

Beausoleil, N., and D. Mellor. 2015. Introducing breathlessness as a significant animal welfare issue. N. Z. Vet. J. 63:44–51.

Benson, E. R., R. L. Alphin, M. K. Rankin, M. P. Caputo, C. A. Kinney, and A. L. Johnson. 2012. Evaluation of EEG based determination of unconsciousness vs. loss of posture in broilers. Res. Vet. Sci. 93:960–964.

Boissy, A. 1995. Fear and Fearfulness in Animals. Q. Rev. Biol. 70:165–191.

Boissy, A., G. Manteuffel, M. B. Jensen, R. O. Moe, B. Spruijt, L. J. Keeling, C. Winckler, B. Forkman, I. Dimitrov, J. Langbein, M. Bakken, I. Veissier, and A. Aubert. 2007. Assessment of positive emotions in animals to improve their welfare. Physiol. Behav. 92:375–397.

Bromm, B. 2001. Brain images of pain. News Physiol. Sci. 16:244–9. Available from: http://www.ncbi.nlm.nih.gov/pubmed/11572930

Casey-Trott, T. M., S. T. Millman, P. V. Turner, S. G. Nykamp, P. C. Lawlis, and T. M. Widowski. 2014. Effectiveness of a nonpenetrating captive bolt for euthanasia of 3 kg to 9 kg pigs. J. Anim. Sci. 92:5166–5174.

Chen, X., J. Gallar, M. A. Pozo, M. Baeza, and C. Belmonte. 1995. CO2 stimulation of the Cornea: A Comparison Between Human Sensation and Nerve Activity in Polymodal Nociceptive Afferents of the Cat. Eur. J. Neurosci. 7:1154–1163.

Conlee, K. M., M. L. Stephens, A. N. Rowan, and L. A. King. 2005. Carbon dioxide for euthanasia: concerns regarding pain and distress, with special reference to mice and rats. Lab. Anim. 39:137–61.

Coryell, M. W., A. E. Ziemann, P. J. Westmoreland, J. M. Haenfler, Z. Kurjakovic, X. Zha, M. Price, M. K. Schnizler, and J. A. Wemmie. 2007. Targeting ASIC1a Reduces Innate Fear and Alters Neuronal Activity in the Fear Circuit. Biol. Pshychiatry 62:1140–1148.

Dalmau, A., P. Rodríguez, P. Llonch, and A. Velarde. 2010. Stunning pigs with different gas mixtures: aversion in pigs. Anim. Welf. 19:325–333.

Damasio, A., and G. B. Carvalho. 2013. The nature of feelings: evolutionary and neurobiological origins. Nat. Rev. Neurosci. 14:143–152.

Danneman, P. J., S. Stein, and S. O. Walshaw. 1997. Humane and practical implications of using carbon dioxide mixed with oxygen for anesthesia or euthanasia of rats. Lab. Anim. Sci. 47:376–385.

Davis, M. 1992. The Role of the Amygdala in Fear and Anxiety. Annu. Rev. Neurosci. 15:353–375.

Dawkins, M. S. 1988. Behavioural deprivation: A central problem in animal welfare. Appl. Anim. Behav. Sci. 20:209–225.

Dawkins, M. S. 1990. From an animal’s point of view: Motivation, fitness, and animal welfare. Behav. Brain Sci. 13:1–9.

Desire, L., A. Boissy, and I. Veissier. 2002. Emotions in farm animals:: a new approach to animal welfare in applied ethology. Behav. Processes 60:165–180.

Dixon, L. M., V. Sandilands, M. Bateson, S. Brocklehurst, B. J. Tolkamp, and R. B. D’Eath. 2013. Conditioned place preference or aversion as animal welfare assessment tools: Limitations in their application. Appl. Anim. Behav. Sci. 148:164–176.

Dodman, N. H. 1977. Observation on the use of the Wernberg dip-lift carbon dioxide apparatus for pre-slaughter anesthesia of pigs. Br. Vet. J. 133:71–80.

Dripps, R. D., and J. H. Comroe. 1947. The respiratory and circulatory response of normal man to inhalation of 7.6 and 10.4 percent CO2 with a compariosn of the maximal ventilation produced by severe muscular exercise, inhalation of CO2 and maximal voluntary hyperventilation. Am. J. Physiol. 149:43–51.

Dubin, A. E., and A. Patapoutian. 2010. Nociceptors: the sensors of the pain pathway. J. Clin. Invest. 120:3760–72. Available from: http://www.ncbi.nlm.nih.gov/pubmed/21041958

Dulla, C. G., P. Dobelis, T. Pearson, B. G. Frenguelli, K. J. Staley, and S. A. Masino. 2005. Adenosine and ATP link PCO2 to cortical excitability via pH. Neuron 48:1011–23.

Duncan, I. J. H. 2005. Science-based assessment of animal welfare: farm animals. Rev. sci. tech. Off. int. Epiz 24:483–492.

EFSA. 2004. Opinion of the Scientific Panel on Animal Health and Welfare (AHAW) on a request from the Commission related to welfare aspects of the main systems of stunning and killing the main commercial species of animals. EFSA J. 45:1–29.

Erickson, H. H., J. P. Goff, E. E. Uemura, and W. O. Reece, eds. 2015. Dukes' Physiology of Domestic Animals. 13th editi. John Wiley & Sons, New York.

Feng, Y., and T. L. Simpson. 2003. Nociceptive Sensation and Sensitivity Evoked from Human Cornea and Conjunctiva Stimulated by CO2. Invest. Ophthalmol. Vis. Sci. 44:529–532.

Fiedler, K. J., R. L. Parsons, L. J. Sadler, and S. T. Millman. 2014. Effects of stocking rate on measures of efficacy and welfare during carbon dioxide gas euthanasia of young pigs. Anim. Welf. 23:309–321.

Fiedler, K., R. Parsons, L. Sadler, and S. Millman. 2016. Effects of stocking rate on measures of efficacy and welfare during argon gas euthanasia of weaned pigs. Anim. Welf. 25:83–89.

Fraser, D. 2009. Can We Measure Distress in Animals? Ethol. Nonhum. Anim.:1–10. Available from: http://animalstudiesrepository.org/acwp_ena/7

Fraser, D., D. M. Weary, E. A. Pajor, and B. N. Milligan. 1997. A Scientific Conception of Animal Welfare that Reflects Ethical Concerns. Anim. Welf. 6:187–205.

Gerritzen, M. a, B. Lambooij, H. Reimert, a Stegeman, and B. Spruijt. 2004. On-farm euthanasia of broiler chickens: effects of different gas mixtures on behavior and brain activity. Poult. Sci. 83:1294–1301.

Gigliotti, F. 2010. Mechanisms of dyspnea in healthy subjects. Multidiscip. Respir. Med. 5:195.

Hall, C. S. 1934. Emotional behavior in the rat I. Defecation and urination as measures of individual differences in emtionality. J. Comp. Psychol. 18:385–403.

Hall, J. E., and A. C. Guyton. 2011. Guyton and Hall textbook of medical physiology. 12th ed. Saunders/Elsevier, Philadelphia, PA.

Hemsworth, P. H., D. J. Mellor, G. M. Cronin, and A. J. Tilbrook. 2015. Scientific assessment of animal welfare. N. Z. Vet. J. 63:24–30.

Hsu, K.-S., Y.-C. Liang, and C.-C. Huang. 2000. Influence of an extracellular acidosis on excitatory synaptic transmission and long-term potentiation in the CA1 region of rat hippocampal slices. J. Neurosci. Res. 62:403–415.

Ito, R., and A. C. H. Lee. 2016. The role of the hippocampus in approach-avoidance conflict decision-making: Evidence from rodent and human studies. Behav. Brain Res. 313:345–357.

Jones, J. B., C. M. Wathes, and A. J. F. Webster. 1998. Operant responses of pigs to atmospheric ammonia. Appl. Anim. Behav. Sci. 58:35–47.

de Jonge, F. H., S.-L. Tilly, A. M. Baars, and B. M. Spruijt. 2008. On the rewarding nature of appetitive feeding behaviour in pigs (Sus scrofa): Do domesticated pigs contrafreeload? Appl. Anim. Behav. Sci. 114:359–372.

Jongman, E. C., J. L. Barnett, and P. H. Hemsworth. 2000. The aversiveness of carbon dioxide stunning in pigs and a comparison of the CO2 stunner crate vs. the V-restrainer. Appl. Anim. Behav. Sci. 67:67–76.

Kirkden, R. D., L. Niel, G. Lee, I. J. Makowska, M. J. Pfaffinger, and D. M. Weary. 2008. The validity of using an approach-avoidance test to measure the strength of aversion to carbon dioxide in rats. Appl. Anim. Behav. Sci. 114:216–234.

Kirkden, R. D., and E. A. Pajor. 2006. Using preference, motivation and aversion tests to ask scientific questions about animals’ feelings. Appl. Anim. Behav. Sci. 100:29–47.

Lambooij, E., M. . Gerritzen, B. Engel, S. J. . Hillebrand, J. Lankhaar, and C. Pieterse. 1999. Behavioural responses during exposure of broiler chickens to different gas mixtures. Appl. Anim. Behav. Sci. 62:255–265.

Lansing, R. W., B. S. H. Im, J. I. Thwing, A. T. R. Legeda, and R. B. Banzett. 2000. The Perception of Respiratory Work and Effort Can Be Independent of the Perception of Air Hunger. Am. J. Respir. Crit. Care Med. 162:1690–1696.

Lawrence, A. B., M. C. Appleby, and H. A. Macleod. 1988. Measuring hunger in the pig using operant conditioning: The effect of food restriction. Anim. Prod. 47:131–137.

Leach, M. C., V. A. Bowell, T. F. Allan, and D. B. Morton. 2002. Aversion to gaseous euthanasia agents in rats and mice. Comp. Med. 52:249–257.

Leach, M. C., V. A. Bowell, T. F. Allan, and D. B. Morton. 2004. Measurement of aversion to determine humane methods of anaesthesia and euthanasia. Anim. Welf. 13:77–86.

Leary, S., W. Underwood, R. Anthony, S. Cartner, D. Corey, T. Grandin, C. Greenacre, S. Gwaltney-Brant, M. A. McCrackin, R. Meyer, D. Miller, J. Shearer, R. Yanong, G. C. Golab, and E. Patterson-kane. 2013. AVMA Guidelines for the Euthanasia of Animals: 2013 Edition. American Veterinary Medical Association, Schaumburg, IL.

LeDoux, J. E. 1995. Emotion: Clues from the Brain. Annu. Rev. Psychol. 46:209–235.

Liotti, M., S. Brannan, G. Egan, R. Shade, L. Madden, B. Abplanalp, R. Robillard, J. Lancaster, F. E. Zamarripa, P. T. Fox, and D. Denton. 2001. Brain responses associated with consciousness of breathlessness (air hunger). Proc. Natl. Acad. Sci. U. S. A. 98:2035–2040.

Llonch, P., P. Rodríguez, M. Gispert, A. Dalmau, X. Manteca, and A. Velarde. 2012. Stunning pigs with nitrogen and carbon dioxide mixtures: effects on animal welfare and meat quality. Animal 6:668–75.

Makowska, I. J., L. Niel, R. D. Kirkden, and D. M. Weary. 2008. Rats show aversion to argon-induced hypoxia. Appl. Anim. Behav. Sci. 114:572–581.

Makowska, I. J., L. Vickers, J. Mancell, and D. M. Weary. 2009. Evaluating methods of gas euthanasia for laboratory mice. Appl. Anim. Behav. Sci. 121:230–235.

Makowska, I. J., and D. M. Weary. 2009. Rat aversion to induction with inhalant anaesthetics. Appl. Anim. Behav. Sci. 119:229–235.

Marchant-Forde, J. N., D. C. Lay, K. A. McMunn, H. W. Cheng, E. A. Pajor, and R. M. Marchant-Forde. 2009. Postnatal piglet husbandry practices and well-being: the effects of alternative techniques delivered separately. J. Anim. Sci. 87:1479–92.

Martínez-Miró, S., F. Tecles, M. Ramón, D. Escribano, F. Hernández, J. Madrid, J. Orengo, S. Martínez-Subiela, X. Manteca, and J. J. Cerón. 2016. Causes, consequences and biomarkers of stress in swine: an update. BMC Vet. Res. 12:171. Available from: http://www.ncbi.nlm.nih.gov/pubmed/27543093

Martoft, L., L. Lomholt, C. Kolthoff, B. E. Rodriguez, E. W. Jensen, P. F. Jorgensen, H. D. Pedersen, and A. Forslid. 2002. Effects of CO2 anaesthesia on central nervous system activity in swine. Lab. Anim. 36:115–126.

Mathur, P., B. Lau, and S. Guo. 2011. Conditioned place preference behavior in zebrafish. Nat. Protoc. 6:338–345.

McKeegan, D. E. F., J. McIntyre, T. G. M. Demmers, C. M. Wathes, and R. B. Jones. 2006. Behavioural responses of broiler chickens during acute exposure to gaseous stimulation. Appl. Anim. Behav. Sci. 99:271–286.

Mellor, D. 2016. Updating Animal Welfare Thinking: Moving beyond the “Five Freedoms” towards “A Life Worth Living.” Animals 6:21.

Mendl, M., and E. Paul. 2004. Consciousness, emotion and animal welfare: insights from cognitive science. Anim. Welf. 13:17–25.

Merriam-Webster. 2017. Merriam-Webster Online Dict. Accessed 2017-04-25. Available from: https://www.merriam-webster.com/dictionary/aversion

Meyer, R. E. 2015. Physiologic Measures of Animal Stress during Transitional States of Consciousness. Animals 5:702–716.

Meyer, R. E., D. Acvaa, J. T. Whitley, W. E. M. Morrow, L. F. Stikeleather, C. L. Baird, J. M. Rice, B. V Halbert, D. K. Styles, and C. S. Whisnant. 2013. Effect of physical and inhaled euthanasia methods on hormonal measures of stress in pigs. J. Swine Heal. Prod. 21:261–269.

Millman, S. T. 2013. Behavioral Responses of Cattle to Pain and Implications for Diagnosis, Management, and Animal Welfare. Vet. Clin. North Am. Food Anim. Pract. 29:47–58.

Millot, S., M. Cerqueira, M. F. Castanheira, Ø. Øverli, C. I. M. Martins, and R. F. Oliveira. 2014. Use of conditioned place preference/avoidance tests to assess affective states in fish. Appl. Anim. Behav. Sci. 154:104–111.

Moosavi, S. H., E. Golestanian, A. P. Binks, R. W. Lansing, R. Brown, and R. B. Banzett. 2003. Hypoxic and hypercapnic drives to breathe generate equivalent levels of air hunger in humans. J. Appl. Physiol. 94:141–54.

Mormede, P., S. Andanson, B. Auperin, B. Beerda, D. Guenmene, J. Malmkvist, X. Manteca, G. Manteuffel, P. Prunet, C. G. Van Rennen, S. Richard, and I. Veissier. 2007. Exploration of the hypothalamic-pituitary-adrenal function as a tool to evaluate animal welfare. Physiol. Behav. 92:317–339.

Mota-Rojas, D., D. Bolanos-Lopez, M. Concepcion-Mendez, J. Ramirez-Telles, P. Roldan-Santiago, S. Flores-Peinado, and P. Mora-Medina. 2012. Stunning Swine with CO2 Gas: Controversies Related to Animal Welfare. Int. J. Pharmacol. 8:141–151.

Mount, L. E., and J. G. Rowell. 1960. Body size, body temperature and age in relation to the metabolic rate of the pig in the first five weeks after birth. J. Physiol. 154:408–416.

Murphy, E., R. E. Nordquist, and F. J. van der Staay. 2014. A review of behavioural methods to study emotion and mood in pigs, Sus scrofa. Appl. Anim. Behav. Sci. 159:9–28.

Nattie, E. 1999. CO2, brainstem chemoreceptors and breathing. Prog. Neurobiol. 59:299–331.

Nattie, E. 2006. Why do we have both peripheral and central chemoreceptors? J. Appl. Physiol. 100:9–10.

Neubauer, J. A., J. E. Melton, and N. H. Edelman. 1990. Modulation of respiration during brain hypoxia. J. Appl. Physiol. 68:441–51.

Niel, L., and D. M. Weary. 2006. Behavioural responses of rats to gradual-fill carbon dioxide euthanasia and reduced oxygen concentrations. Appl. Anim. Behav. Sci. 100:295–308.

Niel, L., and D. M. Weary. 2007. Rats avoid exposure to carbon dioxide and argon. Appl. Anim. Behav. Sci. 107:100–109.

O'Connor, A., R. Anthony, L. Bergamasco, J. Coetzee, S. Gould, A. K. Johnson, L. A. Karriker, J. N. Marchant-Forde, G. S. Martineau, J. McKean, S. T. Millman, S. Niekamp, E. A. Pajor, K. Rutherford, M. Sprague, M. Sutherland, E. von Borell, and R. S. Dzikamunhenga. 2014. Pain management in the neonatal piglet during routine management procedures. Part 2: grading the quality of evidence and the strength of recommendations. Anim. Health Res. Rev. 15:39–62.

OECD. 2000. Guidance Document on the Recognition, Assessment, and Use of Clinical Signs as Humane Endpoints for Experimental Animals used in Safety Evaluation. Organisation for Economic Co-operation and Development Office, Paris, France.

OIE. 2017. Terrestrial Animal Health Code Chapter 7.5 Slaughter of Animals Article 7.5.7. :1–18. Available from: http://www.oie.int/index.php?id=169&L=0&htmfile=chapitre_aw_slaughter.htm

Paul, E. S., E. J. Harding, and M. Mendl. 2005. Measuring emotional processes in animals: the utility of a cognitive approach. Neurosci. Biobehav. Rev. 29:469–491.

Peppel, P., and F. Anton. 1993. Responses of rat medullary dorsal horn neurons following intranasal noxious chemical stimulation: effects of stimulus intensity, duration, and interstimulus interval. J. Neurophysiol. 70:2260–75.

Pig Code of Practice Scientific Committee. 2014. The Code of Practice For the Care and Handlings of Pigs: Review of scientific research on priority issues. Ottawa, Ontario. Available from: http://www.nfacc.ca/pdfs/codes/pig_code_of_practice.pdf

Prabhakar, N. R., and G. L. Semenza. 2015. Oxygen Sensing and Homeostasis. Physiology 30:340–8.

Raj, A. B. M. 1999. Behaviour of pigs exposed to mixtures of gases and the time required to stun and kill them: welfare implications. Vet. Rec. 144:165–168.

Raj, A. B. M., and N. G. Gregory. 1991. Preferential feeding behaviour of hens in different gaseous atmospheres. Br. Poult. Sci. 32:57–65.

Raj, A. B. M., and N. G. Gregory. 1996. Welfare implications of the gas stunning of pigs 2. Stress of induction of anaesthesia. Anim. Welf. 5:71–78.

Raj, A. B. M., S. P. Johnson, S. B. Wotton, and J. L. M. Instry. 1997. Welfare Implications of Gas Sturming Pigs: 3. the Time to Loss of Somatosensory Evoked Potentials and Spontaneous Electrocorticogram of Pigs During Exposure to Gases. Vet. J. 153:329–339.

Raj, A., and N. Gregory. 1995. Welfare implications of the gas stunning of pigs 1. Determination of aversion to the initial inhalation of carbon dioxide or argon. Anim. Welf. 4:273–280.

Rault, J. L., K. A. Mcmunn, J. N. Marchant-Forde, and D. C. J. Lay. 2013. Gas alternatives to carbon dioxide for euthanasia: A piglet perspective. J. Anim. Sci. 91:1874–1883.

Rushen, J. 1996. Using aversion learning techniques to assess the mental state, suffering, and welfare of farm animals. J. Anim. Sci. 74:1990–5.

Sadler, L. J. 2013. Effects of flow rate, gas type and disease status on the welfare of sucking and weaned pigs during gas euthanasia. PhD Diss. Iowa State University, Ames, IA.

Sadler, L. J., C. D. Hagen, C. Wang, T. M. Widowski, A. K. Johnson, and S. T. Millman. 2014. Effects of flow rate and gas mixture on the welfare of weaned and neonate pigs during gas euthanasia. J. Anim. Sci. 92:793–805.

Stalder, K. 2016. 2010-2015 Summary Analysis of U.S. Pork Industry Productivity. Des Moines, IA. Available from: http://www.pork.org/wp-content/uploads/2016/08/2016-pork-industry-productivity-summary.pdf

Sutherland, M. A. 2011. The use of different gases and gas combinations to humanely euthanize young suckling pigs. National Pork Board, Des Moines , IA.

Sutherland, M. A., B. L. Davis, T. A. Brooks, and J. F. Coetzee. 2012. The physiological and behavioral response of pigs castrated with and without anesthesia or analgesia. J. Anim. Sci. 90:2211–21.

Sutherland, M., P. Bryer, and B. Backus. 2017. The effect of age and method of gas delivery on carbon dioxide euthanasia of pigs. Anim. Welf. 26:293–299.

USDA, N. A. S. S. (NASS). 2017. Quarterly Hogs and Pigs (March 2017). Available from: http://usda.mannlib.cornell.edu/usda/current/HogsPigs/HogsPigs-03-30-2017.pdf

Velarde, A., J. Cruz, M. Gispert, D. Carrión, J. L. Ruiz de la Torre, A. Diestre, and X. Manteca. 2007. Aversion to carbon dioxide stunning in pigs: Effect of carbon dioxide concentration and halothane genotype. Anim. Welf. 16:513–522.

Wahi, P., T. Widowski, and S. Yue Cottee. 2011. I'm not going there! using conditioned place preference to assess the aversiveness of restraint and blood sampling in piglets. In: J. N. Marchant-Forde and E. A. Pajor, editors. Proceedings of the 45th Congress of the International Society for Applied Ethology (ISAE). Wageningen Academic Publishers The Netherlands, Indianapolis, Indiana USA. p. 9.

White, R. G., J. A. DeShazer, C. J. Tressler, G. M. Borcher, S. Davey, A. Waninge, A. M. Parkhurst, M. J. Milanuk, and E. T. Clemens. 1995. Vocalization and physiological response of pigs during castration with or without a local anesthetic. J. Anim. Sci. 73:381–386.

Widdicombe, J. G. 1982. Pulmonary and respiratory tract receptors. J. Exp. Biol. 100:41–57.

Ziemann, A. E., J. E. Allen, N. S. Dahdaleh, I. I. Drebot, M. W. Coryell, A. M. Wunsch, C. M. Lynch, F. M. Faraci, M. A. Howard, M. J. Welsh, J. A. Wemmie, and J. A. Wemmie. 2009. The amygdala is a chemosensor that detects carbon dioxide and acidosis to elicit fear behavior. Cell 139:1012–21.

Table 2.1 Ethogram used for behavioral observations

Behavior	Variable type	Definition
Enter ^{D}	Latency, frequency	Pig’s ears and forelegs cross plastic curtain and pig enters treatment chamber
Leave ^{D}	Latency	Pig’s ears and forelegs cross plastic curtain and pig enters control chamber
Elimination ^{D}	Yes/No	Urination or defecation in the control or treatment chamber of the preference box
Vocalizations ^{D}	Frequency	High pitched squeals that occur in control chamber before and after the sliding door was opened
Open mouth breathing^{V,1}	Latency, duration	First point at which pig begins breathing rapidly through continuous open mouth (panting)
Ataxia ^{V,1}	Latency, duration	An apparent loss of co-ordination during voluntary movement such as stumbling, dropped hocks or crossed leg stance
Escape attempt^{V,1}	Latency, frequency	Apparent voluntary effort to escape from the treatment chamber after ataxia has occurred, such as pawing at chamber walls or curtain separating treatment and control chambers
Loss of posture^{V,2}	Latency	Pig slumps down after ataxia into lateral or sternal recumbency, may follow attempts to right itself, but is unsuccessful
Righting response^{V,1}	Latency, frequency	Apparent attempt to restore standing, sitting, or sternal posture from sitting or recumbent position that was unsuccessful in maintaining the posture
Neuromuscular excitation^{V,1}	Latency	Period of seemingly involuntary and unproductive muscular activity including tonic rigidity or clonic movements such as thrashing, paddling, kicking or lordosis
Loss of consciousness ^{V}	Latency	Pig has lost posture; shows no righting reflex and neck tension

^{D} Direct observations. Elimination were scored as binary (yes/no). Vocalizations were recorded separately before and after first entry to the treatment chamber

^{V} Video recordings

[1] Fiedler et al, 2014

[2] Sadler et al, 2014

Table 2.2 Pig avoidance behaviors [LSMeans ± SE (s)] in a preference box by Day and CO_2 concentration[1]

	Latency to enter TC (n = # pigs)	Latency to leave TC (n = # pigs)	Latency to re-enter TC (n = # pigs)
Day^{2,D}			
B	3.7 ± 1.3^{a} (n = 31)	217.8 ± 32.2^{a} (n = 15)	231.5 ± 28.0^{ab} (n = 12)
G	20.8 ± 8.4^{b} (n = 30)	73.3 ± 11.6^{b} (n = 13)	179.9 ± 20.9^{b} (n = 13)
W	11.7 ± 4.6^{ab} (n = 31)	293.9 ± 50.8^{a} (n = 11)	304.1 ± 42.5^{a} (n = 9)
CO_2 concentration^{3,G} (%)			
10	30.3 ± 20.0^{a} (n = 10)	124.8 ± 25.1^{a} (n = 5)	170.2 ± 38.2^{a} (n = 5)
20	3.5 ± 2.4^{b} (n = 9)	70.6 ± 18.3^{a} (n = 3)	147.6 ± 42.8^{a} (n = 3)
30	26.4 ± 16.6^{a} (n = 11)	23.6 ± 4.7^{b} (n = 5)	209.0 ± 46.9^{a} (n = 5)

[1] Different superscripts within a column indicate significant differences (*$P < 0.05$*).

[2] Days included baseline (B) on which outcomes were collected under ambient conditions during the day prior to testing, gas (G) on which outcomes were collected under CO_2 conditions during testing day, washout (W) on which outcomes were collected under ambient conditions during the day after testing.

[D] Effect of Day – Pigs' average latency to enter was analyzed for all pigs (n = # pigs) that entered TC over three rounds on each B, G and W. Average latency to leave TC was analyzed for all pigs that left TC over three rounds on each B, G and W. Average latency to re-enter TC was analyzed for all pigs that re-entered TC over three rounds on each B, G and W.

[3] CO_2 concentration included 10%, 20% and 30% CO_2. CO_2 concentration was randomly assigned to pigs on gas day (G). Pigs experienced assigned CO_2 concentration in the treatment chamber.

[G] Effect of Gas – Pigs' average latency to enter was analyzed for all pigs (n = number of pigs) that entered TC at three CO_2 concentration. Average latency to leave TC was analyzed for all pigs that left TC at three CO_2 concentration. Average latency to re-enter TC was analyzed for all pigs that re-entered TC at three CO_2 concentration

Table 2.3 Behaviors associated with loss of consciousness [LSMeans ± SE (s)] for pigs relative to last entry of treatment chamber that displayed loss of posture[1]

Behavior[2]	CO_2 concentration		
	10% CO_2 (n = #pigs)	20% CO_2 (n = #pigs)	30% CO_2 (n = #pigs)
Ataxia	N/A (n = 0)	40.2 ± 8.1^a (n = 5)	40.2 ± 9.1^a (n = 4)
Loss of posture	N/A (n = 0)	154.6 ± 23.7^a (n = 5)	74.2 ± 12.7^b (n = 4)
Neuromuscular excitation	N/A (n = 0)	193.2 ± 25.4^a (n = 5)	75.7 ± 9.9^b (n = 4)

---

[1] Data represent pigs that lost posture and/or lost consciousness (Total pigs = 9)

[2] Different superscripts within a row indicate significant differences ($P < 0.05$)

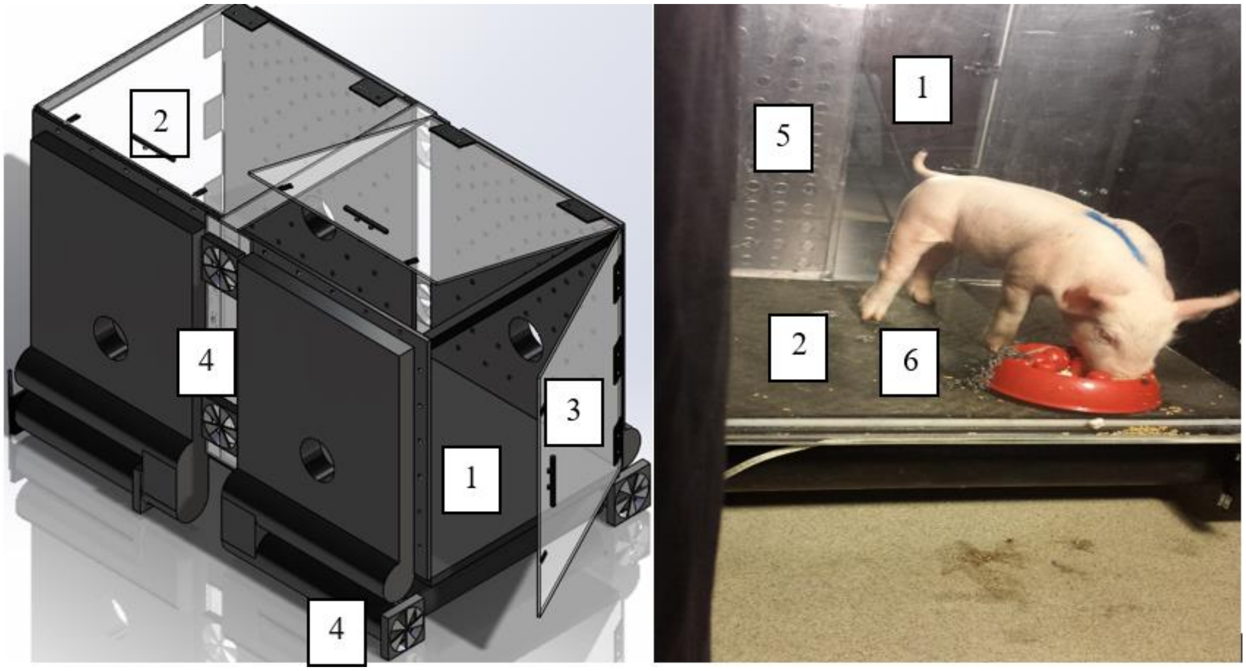

Figure 2.1 (Left) Isometric view of custom-built preference box where pigs were trained and tested in CO_2 aversion study. (Right) Picture of a pig eating feed rewards during the experiment. The numbers in the figure represent following: 1: Control chamber 2: Control chamber 3: Door 4: Fans 5: Sliding door 6: Rubber mat

CHAPTER 3

AVERSION TO ARGON INDUCED HYPOXIA IN PIGS[1]

(This chapter is prepared as a manuscript for submission to **Animal Welfare**)

[1] The author would like to thank the USDA Agriculture and Food Research Initiative (Grant No. 2012-67021-19363) for the financial support to this project. The authors would also like to acknowledge the invaluable technical support from the following people: Rebecca Parsons, Isabelle Withrock, Kassi Miller, Kesley Grossman, Katie Tapper, and Laboratory Animal Resources personnel.

3.1 Abstract

The objective of this study was to assess weaned pigs' aversion to hypoxia induced by argon gas displacement using approach avoidance and conditioned place avoidance paradigms. A custom built preference box (61cm x 61cm x 76cm) was designed with 2 identical chambers, control chamber (CC) and treatment chamber (TC) separated by a sliding door and an exhaust sink. Twelve healthy crossbred weaned pigs (4.4 ± 0.2 kg) were individually trained for 5 consecutive days to enter TC when the sliding door was opened to interact with food rewards followed by 10 minutes during which they could freely move between the chambers. The same methods were used during the testing phase with TC maintained at one of the two O_2 levels: at 6% and 2%. Test concluded when loss of consciousness (LOC) occurred or after 10 min. Pigs experienced the assigned O_2 on gas day (G), preceded by ambient conditions on one baseline day (B) and on one washout day (W) during 2 rounds. We hypothesized pigs would display avoidance at lowest O_2 and when avoidance or LOC occurred, conditioned place avoidance would be observed on W for that round. Behaviors were collected using live observations and video recordings. Aversion outcomes included latencies to enter, leave, and re-enter TC. Pigs left TC faster but re-entered TC slower on G. Latency to leave TC was faster at 2% O_2. Carry over effects of O_2 was not evident on any of W. In conclusion, hypoxia at both O_2 concentrations was mildly aversive to pigs compared to ambient conditions, however the relative degree of aversion did not provoke avoidance behavior and conditioned place avoidance was not observed.

Keywords: animal welfare, approach-avoidance, aversion, conditioned place avoidance, hypoxia, pigs

3.2 Introduction

Millions of young pigs (*Sus scrofa domesticus*) are euthanized annually in the US due to poor health and grave prognosis (Sadler, 2013). Euthanasia means "good death" and should induce loss of consciousness (LOC) and death with minimum pain and distress (Leary et al. 2013, p10). Responses of animals before LOC is crucial to appraise possible pain and distress during the euthanasia process (Sadler et al., 2014).

Cognitive studies are effective tools to ask conscious animals' how they perceive a stimulus or a situation. Approach- avoidance (AA) paradigm measures strength of aversion when a positive stimulus is paired with a negative experience and there is a motivational conflict (Kirkden et al., 2008). Conditioned place avoidance (CPA) is another paradigm to assess animals' response when re-exposed to a neutral environment previously associated with a negative stimulus (Dixon et al., 2013).

Carbon dioxide (CO_2) is the most commonly used euthanasia agent for young pigs and usually high CO_2 concentration (80-90%) is used (Leary et al. 2013 pp10, AASV & NPB 2016).

High CO_2 produces rapid LOC (Velarde et al., 2007; Sadler et al., 2014), however serious concerns are raised about pain and distress associated with CO_2 in animals (Conlee et al., 2005) including pigs (Raj and Gregory, 1995), poultry (McKeegan et al., 2006), rodents (Makowska et al., 2009), including human (Danneman et al., 1997). Argon gas has been proposed as an alternative to overcomes the adverse effects of CO_2 (Raj and Gregory, 1995; Mota-Rojas et al., 2012). Argon gas displaces oxygen and produces hypoxia which may be less aversive than hypercapnic hypoxia produced by CO_2 (Leach et al., 2004). Nonetheless, problem with argon gas also exists as it takes longer to euthanize animals (Raj and Gregory, 1995; Rault et al., 2013; Sadler et al., 2014) which can cause unnecessary suffering to animals (Leary et al., 2013).

It is, therefore very difficult to weigh which is more important from an animal welfare standpoint-quick LOC with more pain and distress or a prolonged LOC with less pain and distress. To overcome this dilemma, two-stage euthanasia method has been suggested in various guidelines (Leary et al., 2013; Pig Code of Practice Scientific Committee, 2014). In this method, animals are first rendered unconscious with anesthetics and once unconsciousness is achieved, higher concentration of CO_2 is used to ensure death. The focus in this method is to ensure less suffering to animals prior to loss of consciousness (LOC). This has led to a question whether argon gas could be a suitable inhalant for the two – step method to render LOC in pigs. Very little is known about weaned pigs' aversion to hypoxia induced by argon gas.

The central objective of this study was to determine pigs' aversion to argon induced hypoxia using AA and CPA paradigms. The first objective was to determine proximate avoidance associated with hypoxia using an AA paradigm. We hypothesized pigs would not avoid argon induced hypoxia. The second objective was to determine learned aversion to hypoxia using a CPA paradigm. Our hypothesis was pigs would not avoid a chamber previously associated with hypoxic environment and/or where LOP was experienced.

3.3 Materials and methods

The protocol for this experiment was approved by Institutional Animal Care and Use Committee (IACUC), Iowa State University.

3.3.1 Animals, housing and feeding

Twelve healthy crossbred (7 *Duroc* and 5 *Landrace X Yorkshire*) newly weaned pigs were enrolled in the study. A clinical examination was performed, including general health assessment, weight and identification numbers before enrollment. All pigs were females with an initial average weight of 4.4 ± 0.2 kg and 21 day old. Pigs were group housed in two identical temperature controlled rooms in the Laboratory Animal Research facility at Iowa State University. The average room temperature was set between 27 – 29 ºC and relative humidity was between 60 – 65%. Six pigs were assigned to each room, balanced by weight. Each room contained a single pen (3.04 m x 3.65 m) housing all 6 pigs (1.84 m^2/pig) with concrete flooring and a single rubber mat (61 cm x 61 cm). A heat lamp was provided as a supplemental heat source for the first 5 days. Environmental enrichment included a rubber boot and a metal chain attached to either a toy or a ball. Pigs were provided with *ad libitum* access to water through a nipple drinker. A commercial starter diet (Heartland Co-op, Prairie City, IA) was scattered on the rubber mat. To facilitate behavioral observations, pigs were dorsally identified using a livestock marker (All Weather Twist Stick Livestock marker, LA-CO, Elk Grove, IL) that was reapplied each afternoon during testing.

3.3.2 Experimental room and preference chamber

All testing was conducted in an experimental room located in the same building where the pigs were housed. Testing was conducted in a custom-built preference box that consisted of two identical chambers (61cm x 61cm x 76 cm) separated by a sliding door (Fig. 1a; Fig. 1b). Each chamber was equipped a hinged door through which pigs could be placed into or removed from the chamber. The floor in each chamber was fitted with a commercially available non-slip rubber mat. Box doors and top panels were made of Plexiglas to facilitate behavior observations. Opaque side walls were fitted with gloves for guiding pigs through the sliding door, and walls contained perforations to evenly distribute gases.

The ventilation system of each chamber was independently controlled to achieve a desired O2 concentration within treatment chamber (TC). The TC ventilation was operated as a recirculating positive pressure system: two small fans pulled air from the right sidewall of the chamber where argon gas was injected and mixed into the air stream prior to being returned to the chamber through the left sidewall, thereby reducing O_2 concentration. The control chamber (CC) was operated as a positive pressure system: two small fans introduced fresh air to the left and right sidewalls, air escaped the chamber through designed outlets on the ceiling and front wall. A negative pressure exhaust sink was located between the two chambers to evacuate excess argon and maintain separate TC and CC environments.

Argon gas was injected into the treatment side through a pipe that was fitted to compressed argon gas cylinders (industrial grade, 99% pure), fitted with volumetric high output heated two-stage argon regulator (Euthanex, Allentown, USA). A real time oxygen (O_2) sensor (TR25OZ Oxygen sensor, CO2Meter Inc, FL, USA) connected to an automatic data recording system (GASLAB, CO2Meter Inc, FL, US). A versatile oxygen sensor (Rosemount Oxygen Analyzer, Model 755A, TN, USA) was installed to monitor O2 levels displaced by argon gas

3.3.3 Experimental design

A repeated measure design was utilized, with 12 newly weaned pigs assigned to receive each of two concentrations of O_2 , 6% (moderate) and <2% (severe) displaced by argon gas in two rounds. Sample size was based on a similar experiment conducted by Wahi et al., 2011 for conditioned place preference with suckling pigs. Each pig was an experimental unit and represented its own control. Pig responses to treatment O_2 concentrations on Gas days (Day 0) were compared to responses on atmospheric air on baseline days (Day-1) and wash out days (Day+1). Pigs were tested in the same order on all test days and order was assigned using a random number generator. Pigs were assigned randomly to receive either 6% or <2% O_2 during Round 1 and were systematically assigned to receive the alternate treatment in Round 2.

3.3.4 Training procedures

Pigs were acclimatized to the research environment for 3 days in their home pens, followed by training within the home pen to desensitize them to social isolation and to familiarize them with food rewards. A 2.2 m x 1.3 m spindle bar panels training pen was assembled in each room, which allowed pigs to see and touch pen-mates. The first home pen training stage included all pigs simultaneously. All pigs were encouraged to enter the pen, which included a slow feeding bowl (Brake-fast, LLC, VA) containing starter diet, few mini marshmallows and raisins, and a Kong-rubber dog toy (The Kong Company, Golden, CO) smeared with strawberry fruit spread (Great Value, Batesville, IL) and attached to a spindle panel with a chain. Pigs were allowed a 6-min food interaction reward period. The second stage followed the previous training, but was done on an individual pig basis. The third stage introduced visual social isolation, which was achieved by affixing all training pen walls with solid plastic panels. Starting with the second stage, each pig had to meet a performance standard (snout contact with at least one food reward within 2 min after pen entry) to graduate to the next stage of home pen training. Pigs that failed to meet this standard continued with the same training on subsequent days until the performance criteria was met. Preference box training in the experimental room began once home pen training was completed.

All pigs were trained individually in the preference box for 4 days. To encourage pigs to cross through the sliding door, food rewards previously experienced in the home pen training were provided in the TC. In addition, strawberry jam was smeared on the mat. The sliding door separating the chambers was closed at the start of the training session. The pigs were individually carried to the experimental room and placed into the CC. After 2 min, the sliding door was opened which provided the pig with access to both chambers. The pig was gently guided through the sliding door into TC using the gloves if it did not enter the TC within 5 min after the sliding door was opened. Once the pig entered the TC, a 10-min time period was provided during which it could freely move about the chambers. The pig was removed and returned to the home pen after the test. The preference box was cleaned and disinfected with Accel (ACCDISC1G-US, Virox Technologies Inc, ON, Canada) between tests and food rewards were replenished. On day 4 of training, pigs were deprived of food for 5-6 h prior to the training session to increase foraging motivation and was included in all subsequent training and test sessions. On the same training day, a plastic curtain with a slit in the middle was added on the TC side of the sliding door to facilitate stable O_2 concentrations in the TC.

3.3.5 Testing procedures

First day of testing was baseline day that followed day 4 of training day. During the testing phase, each pig was tested on B, G and W over two rounds for 6 days. The procedures on B and W were identical to those described for preference box training. Prior to placing each pig in the CC on G days, the TC was prefilled and stabilized at the designated O_2 treatment concentration. If

LOC occurred, the pig was removed from the TC and placed on a rubber mat in a recovery pen located in the testing room. For ethical reasons and injury concerns, removal criteria were later modified to include situations where loss of posture (LOP) and neuromuscular excitation (NME) occurred prior to LOC. During recovery, affected pigs were observed for a minimum of 5 min after return to consciousness and standing posture. Pigs were then provided with a tablespoon of starter diet and returned to the home pen after normal foraging (rooting or eating) behavior occurred.

3.3.6 Behavior data collection

Behavior during testing was recorded using direct observations and video recordings. Direct observations were collected by two observers. One observer sat beside the preference box and out of the test pig's view. The second observer sat approximately 0.5 m from the TC door from which the pig was visible. A black fabric curtain (2.1 m x 0.9 m) and lighting placement were used to obscure this observer from the test pig.

Continuous video was recorded using four color digital video cameras (Panasonic, Model WV-CP-484, Matsushita Co. Ltd., Kadoma, Japan) which were positioned to provide overhead and lateral views of CC and TC. The cameras were fed into a multiplexer using Noldus Portable Lab (Noldus Information Technology, Wageningen, The Netherlands) that enabled capture of a dual recording at 30 frames/s onto a computer using HandyAVI software (version 4.3 D, Anderson's AZcendant Software, Tempe, AZ)

Data collected as direct observation included latency to enter TC, latency to leave TC, latency to re-enter TC, vocalization and elimination. Latency to enter, leave and re – enter TC were recorded using a digital timer (National Presto IND. Inc., Eau Claire, WI) and were collected as measures of avoidance. Vocalization was collected as counts using a manual counter (Great Star Tally counter). Vocalizations were separated into 2 categories – those that occurred before the sliding door was opened (VB) when the pig could see but not access feed rewards, and those that occurred after the sliding door was opened (VA) when the pig could freely move between chambers. Elimination (defecation and urination) was collected as binary data (yes/no). Both vocalization and elimination were collected as measures of distress.

Data collected by video recordings included open mouth breathing (OMB), ataxia, escape attempts, loss of posture (LOP), righting response (RR) and neuromuscular excitation (NME) were collected as measures of induction of loss of consciousness (LOC). Data were collected from videos by a trained observer, blinded to the pig's ID, date and test day, using Observer (version 10.1.548; Noldus Information Technology). A neutral individual performed the blinding procedures for the video recordings from all tests. The blinding procedures involved cutting the video recordings to remove identification presented at the beginning of each video, assigning a random number to each video segment and sorting for the purpose of providing a random sequence in which videos were to be scored. Seven videos were selected at random and duplicated within this sequence for the purpose of determining intra-observer reliability. Prior to data collection, the observer was trained to use the Observer XT program by repeatedly scoring 2 videos and an ethogram from an unrelated CO_2 study until reaching an inter and intra-reliability score of $k \geq 0.90$ as calculated by the Observer program. After reaching the desired level of competence, data collection began using blinded videos and ethogram (Table 3.1).

3.3.7 Statistical analysis

For analysis, behavioral data were assessed as latency, duration or frequency wherever appropriate. Latency to enter, latency to leave and latency to re-enter TC were calculated relative to the time the sliding door was opened. Because pigs moved in and out of TC more than once, measures of avoidance were analyzed for the first event only. Likewise, latencies for measures of induction of loss of consciousness were calculated relative to the time of last entry to TC for those pigs that displayed LOP and/or LOC. Frequency of entries into TC (first entry after sliding door opened + other consecutive entries) was calculated as total number of entries to TC during testing. Duration was calculated as total time recorded over all bouts during testing.

Data were evaluated for normality using PROC UNIVARIATE (SAS Inst. Inc., Cary, NC). For analysis of day effects, fixed effects of day (B, G and W) was included. For analysis of hypoxia effects, fixed effects of O_2 (6% and 2%) concentration on behaviors during G was included. Pig was included as a random effect in both models. Latencies for behaviors indicating avoidance was analyzed using PROC GLIMMIX (SAS Inst. Inc., Cary, NC) with an autoregressive covariance structure and gamma distribution. Frequency of entries into TC, vocalizations were analyzed using PROC GLIMMIX with a Poisson distribution. Elimination data were analyzed using PROC FREQ (SAS Inst. Inc., Cary, NC). The Chi-square test for equal proportions was used to determine treatment differences.

Mean differences were considered significant at $P < 0.05$, whereas $0.1 > P \geq 0.05$ was considered to represent a tendency for a difference. When significant differences were detected, Tukey-Kramer adjustments were used for multiple comparisons. Least square means estimates and standard errors (SE) are reported in the results and corresponding tables.

3.4 Results

All 12 pigs successfully completed home pen training within 4 days. All 12 pigs entered treatment chamber (TC) on all days.

3.4.1 Behaviors indicating avoidance

Latency to enter TC did not change by Day ($P = 0.91$) (Table 3.2). Within G, no significant difference was observed for latency to enter TC between two O_2 treatments ($P = 0.72$).

Some pigs left TC after they experienced hypoxia in TC. Pigs leaving TC included 22/24 on B, 17/24 on G and 20/24 on W. Latency to leave TC differed significantly by day (*P* < 0.0001), with pigs leaving TC 2 times faster on G than B and W (Table 3.2). Within G, 11/17 and 6/17 pigs left TC at 6% and 2% O_2 respectively. Latency to leave TC differed significantly with O_2 treatments (*P* = 0.033), with pigs leaving TC 2 times faster at 2% O_2.

Pigs that left TC, re-entered on all days (Table 3.2). Pigs re-entering TC included 21/22 on B, 17/17 on G and 18/20 on W. Latency to re-enter TC differed by Day (*P* = 0.0001), with pigs re-entering TC 2 times faster on G than B and W. Latency to re-enter was not affected by O_2 treatment (*P* = 0.105).

Frequency to enter TC differed significantly by Day (*P* = 0.003) with pigs entering TC twice more on G than B and W (B = 3.61 ± 0.49, G = 6.43 ± 0.82 and W = 3.74 ± 0.50). Frequency to enter TC was not affected by O_2 treatment (*P* = 0.22).

3.4.2 Behaviors indicating distress and LOC

Some pigs defecated during testing. Defecation altered significantly by Day (*P* = 0.024). More pigs defecated on G followed by W and B (G =15.28%, W = 6.94% and B = 4.17%). Defecation did not differ by O_2 concentration (*P* = 0.68). Urination did not differ by Day (*P* = 0.11) or O_2 treatment (*P* = 0.20).

Some pigs vocalized during testing. Pigs' vocalizations in CC before the sliding door was opened (VB) differed significantly by Day (*P* = 0.0019) with pigs vocalizing 3 times more on B than W (B = 27.90 ± 4.64, G = 14.09 ± 3.31 and W = 8.66 ± 2.58). VB did not differ by O_2 treatments (*P* = 0.66). Pigs' vocalizations after the sliding door was opened (VA) differed significantly by Day (*P* = 0.03) with pigs vocalizing twice more on G than W (B = 16.24 ± 4.07, G = 26.98 ± 5.25 and W = 10.25 ± 3.32). VA did not differ by O_2 treatments (*P* = 0.71).

Open mouth breathing (OMB) was difficult to score reliably due to camera placement, and for this reason data was not analyzed further.

Some pigs displayed ataxia but left TC prior to LOP (Table 3.3). Seven of 12 pigs at 6% O_2 and 11/12 pigs at 2% O_2 were ataxic. At 6% O_2, ataxic pigs were able to move between until test lasted hence, it was difficult to assess the duration of ataxia. At 2% O_2, average ataxia duration was 59.4 ± 11.6 s (Range 8.3 – 119.1 s) and this constitute those pigs that displayed LOP.

None of the pigs displayed LOP and/or LOC at 6% O_2 (Table 3.3). At 2% O_2, 7/12 pigs displayed LOP and of those 5 displayed LOC. Righting response (RR) was observed in 6 of 7 pigs that lost posture at 2% O_2 (Table 3.3). Neuromuscular excitation (NME) was observed in 1 pig that displayed LOP at 2% O_2.

3.5 Discussion

The main aim of the current study was to determine pigs' responses to argon induced hypoxia during exposure to and induction of LOC using AA and CPA paradigms. Our results indicate argon induced hypoxia produces mild aversion to pigs but the degree of aversion is not pronounced to provoke marked conditioned place avoidance.

All pigs entered TC on all testing days within 2 s suggesting pigs learned to enter TC that was designed to attract them with feed rewards (Rault et al., 2013). In addition, pigs were feed restricted and therefore, food motivated (Lawrence et al., 1988) which may be the reason for short latency to enter TC to retrieve feed rewards. Some pigs that entered TC, left the chamber on all days even on B and W (ambient conditions) which was not expected. Reason for leaving TC on non-gas days could be pigs' inherent foraging or explorative behavior (de Jonge et al., 2008) or interest in the feed rewards diminished with time. Our first hypothesis was pigs would not avoid argon induced hypoxia. Contrary to the hypothesis, 70% pigs left TC on G indicating pigs find hypoxia aversive. Pigs left TC 2.3 times faster on G compared to ambient conditions on B and W which indicates pigs could detect low concentrations of O_2 and have also figured out to move in and out of the chamber at will. In a similar approach-avoidance paradigm, rodents detected low O_2: mice around 8.3-9.3% O_2 received with 4 flow rates (Makowska et al., 2009) and rats around 7.7-8.8% O_2 received with 5 different flow rates (Makowska et al., 2008) and all rodents left cage before losing consciousness in less than 2 min in both cases. Rodents are burrowing animals and are sensitive to changes in oxygen levels which is why they left the cage with O_2 levels above 7% (Makowska and Weary, 2009) unlike pigs in the current study that left TC after 189 s when exposed to 6% O_2. Our results are in contrast with Raj and Gregory (1995) where pigs did not detect hypoxia induced by 90% argon and stayed in the gas chamber until they lost consciousness. The pigs used in the current study were 3 weeks of age and were tested for 10 min. Young pigs have higher metabolic rate with high demand for oxygen until 5 weeks of age (Mount and Rowell, 1960) suggesting higher oxygen demand may have driven those pigs to leave TC in approximately 3 min. Raj and Gregory (1995) used adult pigs and tested for 3 min in hypoxic conditions, possibility of detection of hypoxia exists with longer exposure. Within G, shorter latency to leave TC at 2% O_2 indicates severe hypoxia is aversive to pigs compared to 6% O_2. Interestingly, 100% pigs that left, re-entered TC on G and with a shorter latency compared to B. In addition, pigs re-entered TC 6 times on average on G. This suggests proximate hypoxia may be mildly aversive to pigs prompting them to leave faster, however the aversion may not be strong enough for them to completely avoid re-entering TC.

In the current study, pigs were able to detect hypoxia at 6% O_2. Given a free choice hens can detect 10% O_2 (Raj and Gregory, 1991). None displayed LOP and/or LOC at 6% O_2. Similar result was reported when pigs were exposed to 5% O_2 (Raj and Gregory, 1996), however in that study pigs were lowered in a well (dip lift system) containing predetermined O_2 levels for a minute. At 2% O_2, 60% displayed LOP, of which 71% displayed LOC. Latency to LOP was 86 seconds which is similar to previous study looking into the effects of stocking density during euthanasia using argon gas, in which weaned pigs lost posture around 75 seconds (Fiedler et al., 2016). However, the present study is different in the way that pigs had choice to move across chambers. In contrast, rats and mice stayed in gas chamber until LOC suggesting rats are more sensitive to hypoxia than pigs (Makowska et al., 2008). Distress behaviors like ataxia, open mouth breathing, righting response, potential escape attempts that occur prior to loss of posture are part of the pigs' physiological response to hypoxia. Similar behaviors have been used in various studies to determine the effects of hypoxia, for example in poultry (Lambooij et al., 1999; Gerritzen et al., 2004), in rats (Leach et al., 2004), in pigs (Sadler et al., 2014). In the present experiment, almost half of the pigs that were exposed to 2% O_2 during first exposure showed all distress behaviors except neuromuscular excitation (only 1 pig showed) indicating events prior to LOP at 2% O_2 is aversive. Out of 11 pigs that showed ataxia, 5 left TC which means pigs avoid any adverse situation whenever they have access. In accordance with our second hypothesis, none of the pigs that experienced LOP and/or LOC displayed conditioned place avoidance on any of W which is similar to what Raja and Gregory (1995) observed with pigs. One reason suggests hypoxia at 2% O_2 could be tolerable to all pigs even those that displayed LOP and/or LOC or pigs could not remember previous gas exposure due to LOC (Raj and Gregory, 1995). Conditioned place avoidance has been observed in pigs for different handling experiences (Wahi et al., 2011).

According to AVMA POE, pigs should be exposed to <2% O_2 for humane euthanasia for >7 min. In the present study, majority of pigs at 2% O_2 showed some degree of aversion, however, the aversion was not strong enough for them to completely avoid the chamber as evident by their frequent entry in the chamber on same gas day for those pigs that did not display LOP or on W for those pigs that displayed LOP and/or LOC. O_2 at 2% on the other hand was not effective for anesthesia or euthanasia purpose as none of the pigs displayed LOP lost or showed a strong aversion to that concentration.

3.6 Animal welfare implications

Hypoxia induced by argon gas displacement is mildly aversive to pigs. The current study did not provide a conclusive evidence that argon induced hypoxia would be a reliable option for anesthesia/euthanasia. Though hypoxia induces LOC with mild aversion, the latency to LOC is longer and could ensue poor welfare. From animal welfare point of view, the distress behavior associated with hypoxia may be less severe than commonly used CO_2 ethical dilemma to choose between argon and CO_2 still exists. Further research is needed to refine current euthanasia methods and find a better alternative.

3.7 References

AASV, and NPB. 2016. On-Farm Euthanasia of Swine Recommendations for the Producer.

Aitken, P. G., and S. J. Schiff. 1986. Selective neuronal vulnerability to hypoxia in vitro. Neurosci. Lett. 67:92–96.

Anton, F., I. Euchner, and H. O. Handwerker. 1992. Psychophysical examination of pain induced by defined CO2 pulses applied to the nasal mucosa. Pain 49:53–60.

Beausoleil, N., and D. Mellor. 2015. Introducing breathlessness as a significant animal welfare issue. N. Z. Vet. J. 63:44–51.

Benson, E. R., R. L. Alphin, M. K. Rankin, M. P. Caputo, C. A. Kinney, and A. L. Johnson. 2012. Evaluation of EEG based determination of unconsciousness vs. loss of posture in broilers. Res. Vet. Sci. 93:960–964.

Boissy, A. 1995. Fear and Fearfulness in Animals. Q. Rev. Biol. 70:165–191.

Boissy, A., G. Manteuffel, M. B. Jensen, R. O. Moe, B. Spruijt, L. J. Keeling, C. Winckler, B. Forkman, I. Dimitrov, J. Langbein, M. Bakken, I. Veissier, and A. Aubert. 2007. Assessment of positive emotions in animals to improve their welfare. Physiol. Behav. 92:375–397.

Bromm, B. 2001. Brain images of pain. News Physiol. Sci. 16:244–9. Available from: http://www.ncbi.nlm.nih.gov/pubmed/11572930

Casey-Trott, T. M., S. T. Millman, P. V. Turner, S. G. Nykamp, P. C. Lawlis, and T. M. Widowski. 2014. Effectiveness of a nonpenetrating captive bolt for euthanasia of 3 kg to 9 kg pigs. J. Anim. Sci. 92:5166–5174.

Chen, X., J. Gallar, M. A. Pozo, M. Baeza, and C. Belmonte. 1995. CO2 stimulation of the Cornea: A Comparison Between Human Sensation and Nerve Activity in Polymodal Nociceptive Afferents of the Cat. Eur. J. Neurosci. 7:1154–1163.

Conlee, K. M., M. L. Stephens, A. N. Rowan, and L. A. King. 2005. Carbon dioxide for euthanasia: concerns regarding pain and distress, with special reference to mice and rats. Lab. Anim. 39:137–61.

Coryell, M. W., A. E. Ziemann, P. J. Westmoreland, J. M. Haenfler, Z. Kurjakovic, X. Zha, M. Price, M. K. Schnizler, and J. A. Wemmie. 2007. Targeting ASIC1a Reduces Innate Fear and Alters Neuronal Activity in the Fear Circuit. Biol. Pshychiatry 62:1140–1148.

Dalmau, A., P. Rodríguez, P. Llonch, and A. Velarde. 2010. Stunning pigs with different gas mixtures: aversion in pigs. Anim. Welf. 19:325–333.

Damasio, A., and G. B. Carvalho. 2013. The nature of feelings: evolutionary and neurobiological origins. Nat. Rev. Neurosci. 14:143–152.

Danneman, P. J., S. Stein, and S. O. Walshaw. 1997. Humane and practical implications of using carbon dioxide mixed with oxygen for anesthesia or euthanasia of rats. Lab. Anim. Sci. 47:376–385.

Davis, M. 1992. The Role of the Amygdala in Fear and Anxiety. Annu. Rev. Neurosci. 15:353–375.

Dawkins, M. S. 1988. Behavioural deprivation: A central problem in animal welfare. Appl. Anim. Behav. Sci. 20:209–225.

Dawkins, M. S. 1990. From an animal’s point of view: Motivation, fitness, and animal welfare. Behav. Brain Sci. 13:1–9.

Desire, L., A. Boissy, and I. Veissier. 2002. Emotions in farm animals:: a new approach to animal welfare in applied ethology. Behav. Processes 60:165–180.

Dixon, L. M., V. Sandilands, M. Bateson, S. Brocklehurst, B. J. Tolkamp, and R. B. D’Eath. 2013. Conditioned place preference or aversion as animal welfare assessment tools: Limitations in their application. Appl. Anim. Behav. Sci. 148:164–176.

Dodman, N. H. 1977. Observation on the use of the Wernberg dip-lift carbon dioxide apparatus for pre-slaughter anesthesia of pigs. Br. Vet. J. 133:71–80.

Dripps, R. D., and J. H. Comroe. 1947. The respiratory and circulatory response of normal man to inhalation of 7.6 and 10.4 percent CO2 with a compariosn of the maximal ventilation produced by severe muscular exercise, inhalation of CO2 and maximal voluntary hyperventilation. Am. J. Physiol. 149:43–51.

Dubin, A. E., and A. Patapoutian. 2010. Nociceptors: the sensors of the pain pathway. J. Clin. Invest. 120:3760–72. Available from: http://www.ncbi.nlm.nih.gov/pubmed/21041958

Dulla, C. G., P. Dobelis, T. Pearson, B. G. Frenguelli, K. J. Staley, and S. A. Masino. 2005. Adenosine and ATP link PCO2 to cortical excitability via pH. Neuron 48:1011–23.

Duncan, I. J. H. 2005. Science-based assessment of animal welfare: farm animals. Rev. sci. tech. Off. int. Epiz 24:483–492.

EFSA. 2004. Opinion of the Scientific Panel on Animal Health and Welfare (AHAW) on a request from the Commission related to welfare aspects of the main systems of stunning and killing the main commercial species of animals. EFSA J. 45:1–29.

Erickson, H. H., J. P. Goff, E. E. Uemura, and W. O. Reece, eds. 2015. Dukes' Physiology of Domestic Animals. 13th editi. John Wiley & Sons, New York.

Feng, Y., and T. L. Simpson. 2003. Nociceptive Sensation and Sensitivity Evoked from Human Cornea and Conjunctiva Stimulated by CO2. Invest. Ophthalmol. Vis. Sci. 44:529–532.

Fiedler, K. J., R. L. Parsons, L. J. Sadler, and S. T. Millman. 2014. Effects of stocking rate on measures of efficacy and welfare during carbon dioxide gas euthanasia of young pigs. Anim. Welf. 23:309–321.

Fiedler, K., R. Parsons, L. Sadler, and S. Millman. 2016. Effects of stocking rate on measures of efficacy and welfare during argon gas euthanasia of weaned pigs. Anim. Welf. 25:83–89.
Fraser, D. 2009. Can We Measure Distress in Animals? Ethol. Nonhum. Anim.:1–10. Available from: http://animalstudiesrepository.org/acwp_ena/7

Fraser, D., D. M. Weary, E. A. Pajor, and B. N. Milligan. 1997. A Scientific Conception of Animal Welfare that Reflects Ethical Concerns. Anim. Welf. 6:187–205.

Gerritzen, M. a, B. Lambooij, H. Reimert, a Stegeman, and B. Spruijt. 2004. On-farm euthanasia of broiler chickens: effects of different gas mixtures on behavior and brain activity. Poult. Sci. 83:1294–1301.

Gigliotti, F. 2010. Mechanisms of dyspnea in healthy subjects. Multidiscip. Respir. Med. 5:195.

Hall, C. S. 1934. Emotional behavior in the rat I. Defecation and urination as measures of individual differences in emtionality. J. Comp. Psychol. 18:385–403.

Hall, J. E., and A. C. Guyton. 2011. Guyton and Hall textbook of medical physiology. 12th ed. Saunders/Elsevier, Philadelphia, PA.

Hemsworth, P. H., D. J. Mellor, G. M. Cronin, and A. J. Tilbrook. 2015. Scientific assessment of animal welfare. N. Z. Vet. J. 63:24–30.

Hsu, K.-S., Y.-C. Liang, and C.-C. Huang. 2000. Influence of an extracellular acidosis on excitatory synaptic transmission and long-term potentiation in the CA1 region of rat hippocampal slices. J. Neurosci. Res. 62:403–415.

Ito, R., and A. C. H. Lee. 2016. The role of the hippocampus in approach-avoidance conflict decision-making: Evidence from rodent and human studies. Behav. Brain Res. 313:345–357.

Jones, J. B., C. M. Wathes, and A. J. F. Webster. 1998. Operant responses of pigs to atmospheric ammonia. Appl. Anim. Behav. Sci. 58:35–47.

de Jonge, F. H., S.-L. Tilly, A. M. Baars, and B. M. Spruijt. 2008. On the rewarding nature of appetitive feeding behaviour in pigs (Sus scrofa): Do domesticated pigs contrafreeload? Appl. Anim. Behav. Sci. 114:359–372.

Jongman, E. C., J. L. Barnett, and P. H. Hemsworth. 2000. The aversiveness of carbon dioxide stunning in pigs and a comparison of the CO2 stunner crate vs. the V-restrainer. Appl. Anim. Behav. Sci. 67:67–76.

Kirkden, R. D., L. Niel, G. Lee, I. J. Makowska, M. J. Pfaffinger, and D. M. Weary. 2008. The validity of using an approach-avoidance test to measure the strength of aversion to carbon dioxide in rats. Appl. Anim. Behav. Sci. 114:216–234.

Kirkden, R. D., and E. A. Pajor. 2006. Using preference, motivation and aversion tests to ask scientific questions about animals' feelings. Appl. Anim. Behav. Sci. 100:29–47.

Lambooij, E., M. . Gerritzen, B. Engel, S. J. . Hillebrand, J. Lankhaar, and C. Pieterse. 1999. Behavioural responses during exposure of broiler chickens to different gas mixtures. Appl. Anim. Behav. Sci. 62:255–265.

Lansing, R. W., B. S. H. Im, J. I. Thwing, A. T. R. Legeda, and R. B. Banzett. 2000. The Perception of Respiratory Work and Effort Can Be Independent of the Perception of Air Hunger. Am. J. Respir. Crit. Care Med. 162:1690–1696.

Lawrence, A. B., M. C. Appleby, and H. A. Macleod. 1988. Measuring hunger in the pig using operant conditioning: The effect of food restriction. Anim. Prod. 47:131–137.

Leach, M. C., V. A. Bowell, T. F. Allan, and D. B. Morton. 2002. Aversion to gaseous euthanasia agents in rats and mice. Comp. Med. 52:249–257.

Leach, M. C., V. A. Bowell, T. F. Allan, and D. B. Morton. 2004. Measurement of aversion to determine humane methods of anaesthesia and euthanasia. Anim. Welf. 13:77–86.

Leary, S., W. Underwood, R. Anthony, S. Cartner, D. Corey, T. Grandin, C. Greenacre, S. Gwaltney-Brant, M. A. McCrackin, R. Meyer, D. Miller, J. Shearer, R. Yanong, G. C. Golab, and E. Patterson-kane. 2013. AVMA Guidelines for the Euthanasia of Animals: 2013 Edition. American Veterinary Medical Association, Schaumburg, IL.

LeDoux, J. E. 1995. Emotion: Clues from the Brain. Annu. Rev. Psychol. 46:209–235.

Liotti, M., S. Brannan, G. Egan, R. Shade, L. Madden, B. Abplanalp, R. Robillard, J. Lancaster, F. E. Zamarripa, P. T. Fox, and D. Denton. 2001. Brain responses associated with consciousness of breathlessness (air hunger). Proc. Natl. Acad. Sci. U. S. A. 98:2035–2040.

Llonch, P., P. Rodríguez, M. Gispert, A. Dalmau, X. Manteca, and A. Velarde. 2012. Stunning pigs with nitrogen and carbon dioxide mixtures: effects on animal welfare and meat quality. Animal 6:668–75.

Makowska, I. J., L. Niel, R. D. Kirkden, and D. M. Weary. 2008. Rats show aversion to argon-induced hypoxia. Appl. Anim. Behav. Sci. 114:572–581.

Makowska, I. J., L. Vickers, J. Mancell, and D. M. Weary. 2009. Evaluating methods of gas euthanasia for laboratory mice. Appl. Anim. Behav. Sci. 121:230–235.

Makowska, I. J., and D. M. Weary. 2009. Rat aversion to induction with inhalant anaesthetics. Appl. Anim. Behav. Sci. 119:229–235.

Marchant-Forde, J. N., D. C. Lay, K. A. McMunn, H. W. Cheng, E. A. Pajor, and R. M. Marchant-Forde. 2009. Postnatal piglet husbandry practices and well-being: the effects of alternative techniques delivered separately. J. Anim. Sci. 87:1479–92.

Martínez-Miró, S., F. Tecles, M. Ramón, D. Escribano, F. Hernández, J. Madrid, J. Orengo, S. Martínez-Subiela, X. Manteca, and J. J. Cerón. 2016. Causes, consequences and biomarkers of stress in swine: an update. BMC Vet. Res. 12:171. Available from: http://www.ncbi.nlm.nih.gov/pubmed/27543093

Martoft, L., L. Lomholt, C. Kolthoff, B. E. Rodriguez, E. W. Jensen, P. F. Jorgensen, H. D. Pedersen, and A. Forslid. 2002. Effects of CO2 anaesthesia on central nervous system activity in swine. Lab. Anim. 36:115–126.

Mathur, P., B. Lau, and S. Guo. 2011. Conditioned place preference behavior in zebrafish. Nat. Protoc. 6:338–345.

McKeegan, D. E. F., J. McIntyre, T. G. M. Demmers, C. M. Wathes, and R. B. Jones. 2006. Behavioural responses of broiler chickens during acute exposure to gaseous stimulation. Appl. Anim. Behav. Sci. 99:271–286.

Mellor, D. 2016. Updating Animal Welfare Thinking: Moving beyond the “Five Freedoms” towards “A Life Worth Living.” Animals 6:21.

Mendl, M., and E. Paul. 2004. Consciousness, emotion and animal welfare: insights from cognitive science. Anim. Welf. 13:17–25.

Merriam-Webster. 2017. Merriam-Webster Online Dict. Accessed 2017-04-25. Available from: https://www.merriam-webster.com/dictionary/aversion

Meyer, R. E. 2015. Physiologic Measures of Animal Stress during Transitional States of Consciousness. Animals 5:702–716.

Meyer, R. E., D. Acvaa, J. T. Whitley, W. E. M. Morrow, L. F. Stikeleather, C. L. Baird, J. M. Rice, B. V Halbert, D. K. Styles, and C. S. Whisnant. 2013. Effect of physical and inhaled euthanasia methods on hormonal measures of stress in pigs. J. Swine Heal. Prod. 21:261–269.

Millman, S. T. 2013. Behavioral Responses of Cattle to Pain and Implications for Diagnosis, Management, and Animal Welfare. Vet. Clin. North Am. Food Anim. Pract. 29:47–58.

Millot, S., M. Cerqueira, M. F. Castanheira, Ø. Øverli, C. I. M. Martins, and R. F. Oliveira. 2014. Use of conditioned place preference/avoidance tests to assess affective states in fish. Appl. Anim. Behav. Sci. 154:104–111.

Moosavi, S. H., E. Golestanian, A. P. Binks, R. W. Lansing, R. Brown, and R. B. Banzett. 2003. Hypoxic and hypercapnic drives to breathe generate equivalent levels of air hunger in humans. J. Appl. Physiol. 94:141–54.

Mormede, P., S. Andanson, B. Auperin, B. Beerda, D. Guenmene, J. Malmkvist, X. Manteca, G. Manteuffel, P. Prunet, C. G. Van Rennen, S. Richard, and I. Veissier. 2007. Exploration of the hypothalamic-pituitary-adrenal function as a tool to evaluate animal welfare. Physiol. Behav. 92:317–339.

Mota-Rojas, D., D. Bolanos-Lopez, M. Concepcion-Mendez, J. Ramirez-Telles, P. Roldan-Santiago, S. Flores-Peinado, and P. Mora-Medina. 2012. Stunning Swine with CO2 Gas: Controversies Related to Animal Welfare. Int. J. Pharmacol. 8:141–151.

Mount, L. E., and J. G. Rowell. 1960. Body size, body temperature and age in relation to the metabolic rate of the pig in the first five weeks after birth. J. Physiol. 154:408–416.

Murphy, E., R. E. Nordquist, and F. J. van der Staay. 2014. A review of behavioural methods to study emotion and mood in pigs, Sus scrofa. Appl. Anim. Behav. Sci. 159:9–28.

Nattie, E. 1999. CO2, brainstem chemoreceptors and breathing. Prog. Neurobiol. 59:299–331.

Nattie, E. 2006. Why do we have both peripheral and central chemoreceptors? J. Appl. Physiol. 100:9–10.

Neubauer, J. A., J. E. Melton, and N. H. Edelman. 1990. Modulation of respiration during brain hypoxia. J. Appl. Physiol. 68:441–51.

Niel, L., and D. M. Weary. 2006. Behavioural responses of rats to gradual-fill carbon dioxide euthanasia and reduced oxygen concentrations. Appl. Anim. Behav. Sci. 100:295–308.

Niel, L., and D. M. Weary. 2007. Rats avoid exposure to carbon dioxide and argon. Appl. Anim. Behav. Sci. 107:100–109.

O'Connor, A., R. Anthony, L. Bergamasco, J. Coetzee, S. Gould, A. K. Johnson, L. A. Karriker, J. N. Marchant-Forde, G. S. Martineau, J. McKean, S. T. Millman, S. Niekamp, E. A. Pajor, K. Rutherford, M. Sprague, M. Sutherland, E. von Borell, and R. S. Dzikamunhenga. 2014. Pain management in the neonatal piglet during routine management procedures. Part 2: grading the quality of evidence and the strength of recommendations. Anim. Health Res. Rev. 15:39–62.

OECD. 2000. Guidance Document on the Recognition, Assessment, and Use of Clinical Signs as Humane Endpoints for Experimental Animals used in Safety Evaluation. Organisation for Economic Co-operation and Development Office, Paris, France.

OIE. 2017. Terrestrial Animal Health Code Chapter 7.5 Slaughter of Animals Article 7.5.7. :1–18. Available from: http://www.oie.int/index.php?id=169&L=0&htmfile=chapitre_aw_slaughter.htm
Paul, E. S., E. J. Harding, and M. Mendl. 2005. Measuring emotional processes in animals: the utility of a cognitive approach. Neurosci. Biobehav. Rev. 29:469–491.

Peppel, P., and F. Anton. 1993. Responses of rat medullary dorsal horn neurons following intranasal noxious chemical stimulation: effects of stimulus intensity, duration, and interstimulus interval. J. Neurophysiol. 70:2260–75.

Pig Code of Practice Scientific Committee. 2014. The Code of Practice For the Care and Handlings of Pigs: Review of scientific research on priority issues. Ottawa, Ontario. Available from: http://www.nfacc.ca/pdfs/codes/pig_code_of_practice.pdf

Prabhakar, N. R., and G. L. Semenza. 2015. Oxygen Sensing and Homeostasis. Physiology 30:340–8.

Raj, A. B. M. 1999. Behaviour of pigs exposed to mixtures of gases and the time required to stun and kill them: welfare implications. Vet. Rec. 144:165–168.

Raj, A. B. M., and N. G. Gregory. 1991. Preferential feeding behaviour of hens in different gaseous atmospheres. Br. Poult. Sci. 32:57–65.

Raj, A. B. M., and N. G. Gregory. 1996. Welfare implications of the gas stunning of pigs 2. Stress of induction of anaesthesia. Anim. Welf. 5:71–78.

Raj, A. B. M., S. P. Johnson, S. B. Wotton, and J. L. M. Instry. 1997. Welfare Implications of Gas Sturming Pigs: 3. the Time to Loss of Somatosensory Evoked Potentials and Spontaneous Electrocorticogram of Pigs During Exposure to Gases. Vet. J. 153:329–339.

Raj, A., and N. Gregory. 1995. Welfare implications of the gas stunning of pigs 1. Determination of aversion to the initial inhalation of carbon dioxide or argon. Anim. Welf. 4:273–280.

Rault, J. L., K. A. Mcmunn, J. N. Marchant-Forde, and D. C. J. Lay. 2013. Gas alternatives to carbon dioxide for euthanasia: A piglet perspective. J. Anim. Sci. 91:1874–1883.

Rushen, J. 1996. Using aversion learning techniques to assess the mental state, suffering, and welfare of farm animals. J. Anim. Sci. 74:1990–5.

Sadler, L. J. 2013. Effects of flow rate, gas type and disease status on the welfare of sucking and weaned pigs during gas euthanasia. PhD Diss. Iowa State University, Ames, IA.

Sadler, L. J., C. D. Hagen, C. Wang, T. M. Widowski, A. K. Johnson, and S. T. Millman. 2014. Effects of flow rate and gas mixture on the welfare of weaned and neonate pigs during gas euthanasia. J. Anim. Sci. 92:793–805.

Stalder, K. 2016. 2010-2015 Summary Analysis of U.S. Pork Industry Productivity. Des Moines, IA. Available from: http://www.pork.org/wp-content/uploads/2016/08/2016-pork-industry-productivity-summary.pdf

Sutherland, M. A. 2011. The use of different gases and gas combinations to humanely euthanize young suckling pigs. National Pork Board, Des Moines , IA.

Sutherland, M. A., B. L. Davis, T. A. Brooks, and J. F. Coetzee. 2012. The physiological and behavioral response of pigs castrated with and without anesthesia or analgesia. J. Anim. Sci. 90:2211–21.

Sutherland, M., P. Bryer, and B. Backus. 2017. The effect of age and method of gas delivery on carbon dioxide euthanasia of pigs. Anim. Welf. 26:293–299.

USDA, N. A. S. S. (NASS). 2017. Quarterly Hogs and Pigs (March 2017). Available from: http://usda.mannlib.cornell.edu/usda/current/HogsPigs/HogsPigs-03-30-2017.pdf

Velarde, A., J. Cruz, M. Gispert, D. Carrión, J. L. Ruiz de la Torre, A. Diestre, and X. Manteca. 2007. Aversion to carbon dioxide stunning in pigs: Effect of carbon dioxide concentration and halothane genotype. Anim. Welf. 16:513–522.

Wahi, P., T. Widowski, and S. Yue Cottee. 2011. I'm not going there! using conditioned place preference to assess the aversiveness of restraint and blood sampling in piglets. In: J. N. Marchant-Forde and E. A. Pajor, editors. Proceedings of the 45th Congress of the International Society for Applied Ethology (ISAE). Wageningen Academic Publishers The Netherlands, Indianapolis, Indiana USA. p. 9.

White, R. G., J. A. DeShazer, C. J. Tressler, G. M. Borcher, S. Davey, A. Waninge, A. M. Parkhurst, M. J. Milanuk, and E. T. Clemens. 1995. Vocalization and physiological response of pigs during castration with or without a local anesthetic. J. Anim. Sci. 73:381–386.

Widdicombe, J. G. 1982. Pulmonary and respiratory tract receptors. J. Exp. Biol. 100:41–57.

Ziemann, A. E., J. E. Allen, N. S. Dahdaleh, I. I. Drebot, M. W. Coryell, A. M. Wunsch, C. M. Lynch, F. M. Faraci, M. A. Howard, M. J. Welsh, J. A. Wemmie, and J. A. Wemmie. 2009. The amygdala is a chemosensor that detects carbon dioxide and acidosis to elicit fear behavior. Cell 139:1012–21.

Table 3.1 Ethogram used during live and video observations.

Behavior	Definition
Enter ^{D}	Pig's ears and forelegs cross plastic curtain and pig enters treatment chamber
Leave ^{D}	Pig's ears and forelegs cross plastic curtain and pig enters control chamber
Elimination ^{D}	Urination or defecation in the control or treatment chamber of the preference box
Vocalizations ^{D}	High pitched squeals that occur in control chamber before the sliding door was opened
Open mouth breathing^{V,1}	First point at which pig begins breathing rapidly through continuous open mouth (panting)
Ataxia ^{V,1}	An apparent loss of co-ordination during voluntary movement such as stumbling, dropped hocks or crossed leg stance
Loss of posture^{V,2}	Pig slumps down after ataxia into lateral or sternal recumbency, may follow attempts to right itself, but is unsuccessful
Righting response^{V,1}	Apparent attempt to restore standing, sitting, or sternal posture from sitting or recumbent position that was unsuccessful in maintaining the posture
Neuromuscular excitation^{V,1}	Period of seemingly involuntary and unproductive muscular activity including tonic rigidity or clonic movements such as thrashing, paddling, kicking or lordosis
Loss of consciousness ^{V}	Pig has lost posture; shows no righting reflex and neck tension

1 Sadler et al, 2014; 2 Fiedler et al, 2014; L Live observations V Video recordings

Table 3.2 Mean (± SEM) latencies (s) of pigs' avoidance behaviors by Day, O_2 treatment and Round from the argon induced hypoxia aversion study[1].

	Avoidance behaviors		
Day[2,D]	**Latency to enter TC**	**Latency to leave TC**	**Latency to re-enter TC**
B	2.0 (± 0.4)^{a} (n=24)	364.8 (± 34.3)^{a} (n=22)	376.3 (± 33.9)^{a} (n=21)
G	1.7 (± 0.3)^{a} (n=24)	156.7 (± 16.8)^{b} (n=17)	206.9 (± 20.7)^{b} (n=17)
W	1.9 (± 0.4)^{a} (n=24)	370.9 (± 36.6)^{a} (n=20)	383.4 (± 38.4)^{a} (n=18)
O_2 treatment[3,O]			
6%	1.8 (± 0.3)^{c} (n=12)	189.5 (± 32.3)^{c} (n=11)	239.8 (± 40.7)^{c} (n=11)
2%	1.6 (± 0.3)^{c} (n=12)	96.6 (± 22.3)^{d} (n=6)	146.6 (± 33.7)^{c} (n=6)

[1] Different superscripts within a column indicate significant differences ($P < 0.05$). Superscripts *a, b* denote differences within Day; superscripts *c, d* denote differences within O_2 treatments; superscripts *e, f* denote differences within Round.

[2] Day included baseline (B) on which outcomes were collected under ambient conditions during the day prior to testing, gas (G) on which outcomes were collected under CO_2 conditions during testing day, washout (W) on which outcomes were collected under ambient conditions during the day after testing.

[D] Effect of Day – Pigs' average latency to enter was analyzed for all pigs (n = number of pigs) that entered treatment chamber (TC) over 2 rounds on each B, G and W. Average latency to leave TC was analyzed for all pigs that left TC over 2 rounds on each B, G and W. Average latency to re-enter TC was analyzed for all pigs that re-entered TC over 2 rounds on each B, G and W.

[3] O_2 treatment included 6% and 2% O_2. O_2 treatment was randomly assigned to pigs on G. Pigs experienced assigned O_2 treatment in TC.

[O] Effect of O_2 treatment – Pigs' average latency to enter was analyzed for all pigs (n = number of pigs) that entered TC at 2 O_2 treatments. Average latency to leave TC was analyzed for all pigs that left TC at 2 O_2 treatments. Average latency to re-enter TC was analyzed for all pigs that re-entered TC at 2 O_2 treatments.

Table 3.3 Summary of behaviors (Mean ± SE) associated with argon induced hypoxia for 12 pigs enrolled in the study[1].

Behaviors	**O_2 treatment**	
Latency[2] (s)	**6% O_2**	**2% O_2**
Ataxia	106.3 (± 27.0) (Range 35.7-240.7) (n=7)	34.9 (± 8.9) (Range 12.5-93.3) (n = 11)
Loss of posture (LOP)	N/A	85.9 (± 19.3) (Range 16.5-172.8) (n = 7)
Righting response (RR)	N/A	84.4 (± 22.1) (Range 18.4-173.0) (n = 6)
Neuromuscular excitation (NME)	N/A	18.1 (n = 1)
Loss of consciousness (LOC)	N/A	205.3 ± 35.7 (Range 93.2-303.1) (n = 5)

[1] Behaviors indicating induction of loss of consciousness produced due to hypoxia at 6% O_2 and 2% O_2.

[2] Latency was calculated relative to the time of last entry to TC for those pigs that lost posture and/or loss consciousness

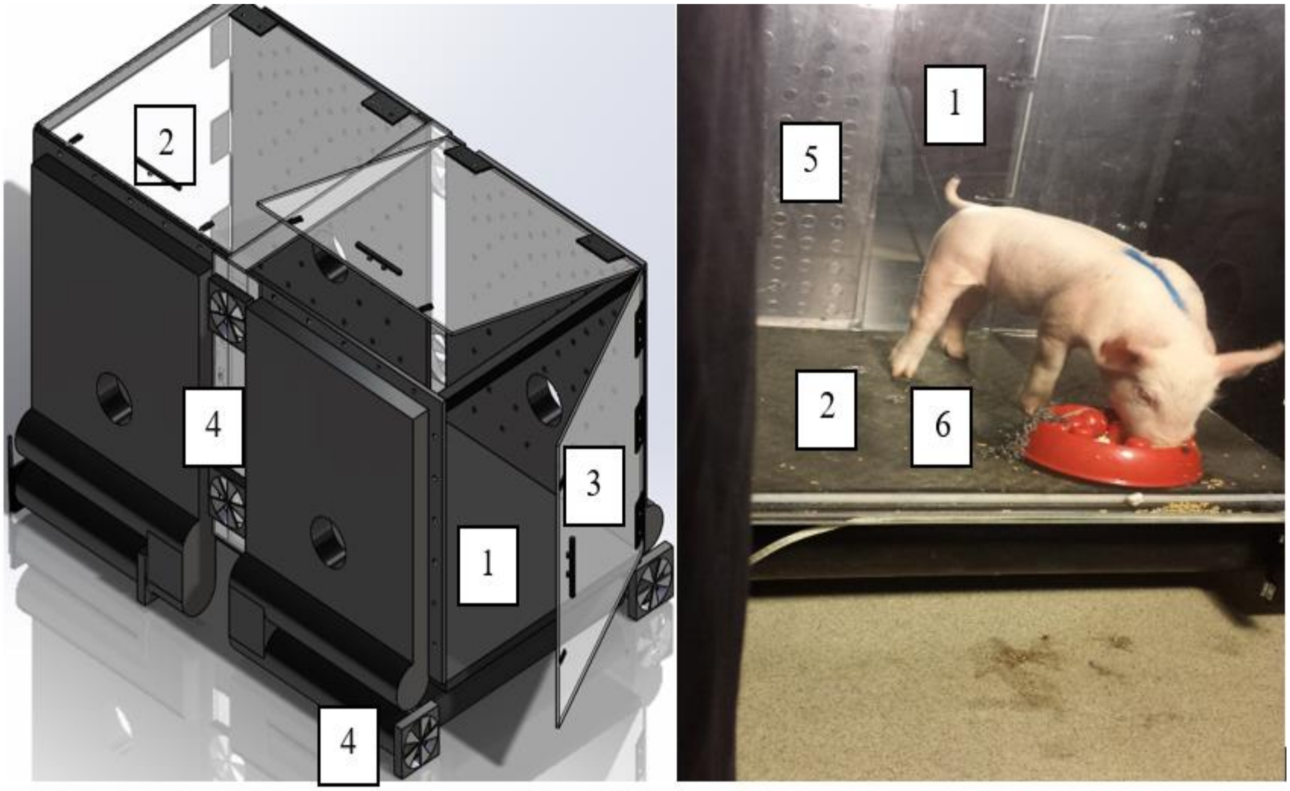

Figure 3.1. Isometric view of custom-built preference box where pigs were trained and tested in CO_2 aversion study (Left). Picture of a pig eating feed rewards during the experiment. The numbers in the figure represent following: 1: Control chamber 2: Control chamber 3: Door 4: Fans 5: Sliding door 6: Rubber mat (Right)

CHAPTER 4 GENERAL DISCUSSION

The first objective for this thesis was to assess pig's aversion to exposure to and to induction of loss of consciousness with CO_2 using approach-avoidance and conditioned place avoidance paradigms. The second objective for this thesis was to investigate pigs' aversion to exposure to and to induction of loss of consciousness with argon induced hypoxia using approach-avoidance and conditioned place avoidance paradigms. The objective of this last chapter is two-fold – a) to critically review the results of the two experimental chapters and b) to identify some of the challenges and limitations of the two studies, and to suggest possible solutions for future extension of this work.

4.1. Aversion assessment

Number of weaned pigs that require on-farm euthanasia is significant and in practice, CO_2 is considered the most suitable euthanasia method. We addressed the adverse effects of high CO_2 in Chapter 2 by testing pigs' responses to low CO_2 concentrations. This is the first study to assess weaned pigs' aversion to low CO_2 using approach-avoidance and conditioned place avoidance paradigms. These two paradigms were used in adult pigs to investigate aversion to CO_2 and argon (Raj and Gregory, 1995). However, in that study treatment gases were confounded with day. Moreover, pigs were exposed to gas while feeding in a wooden trough which may not ensure a consistent concentration throughout the box. In our study, low CO_2 concentrations included 10%, 20% and 30%. These concentrations have been found to produce mild to moderate aversion in pigs (Raj and Gregory, 1996) and rodents (Krohn et al., 2003; Niel and Weary, 2007). The overall result from this study indicated that low CO_2 was mildly aversive to pigs which is similar to what Raj and Gregory (1996) found. However, the forced immersion method used in their study was not equipped to investigate pigs' inherent responses to an unpleasant experience (CO_2 exposure) compared to the current study. In addition, the avoidance behaviors used in our study (latency to enter, leave and re-enter the treatment chamber) provided an objective assessment of initial gas exposure as indicated by Leach et al. (2002, 2004). Furthermore, the degree of aversion associated with low CO_2 was not pronounced as conditioned place avoidance was not observed on any washout days by naïve pigs or pigs that displayed loss of posture and or loss of consciousness during previous exposure. This is in contrary to Dalmau et al. (2010) and Raj and Gregory (1995) who reported following an aversive gas experience, pigs either took longer or completely avoided to enter the stunning unit or the box respectively. Carbon dioxide at 10% is not beneficial for induction of loss of consciousness as none of the pigs displayed loss of consciousness. Mildly aversive 30% CO_2 may be effective for inducing loss of consciousness within 1.5 minute, however occurrence of muscular excitation was concerning ethically. It was beyond the scope of the current study to confirm reliably whether or not pigs were unconscious during muscular excitation. As an addition to the previous knowledge, the current study introduced the use of cognitive tests to determine the weaned pigs' perceived aversion to low CO_2 with the aid of a customized preference box. This study also evaluated the possible merits of using low CO_2 in a 2-step euthanasia method. One of the limitations of this study was round was confounded with CO_2 concentrations, which was done to overcome the practical constraint associated with the preference box.

Argon as a recommended alternative to high CO_2 (Leary et al., 2013) was used as the treatment gas in Chapter 3. The overall result suggested argon induced hypoxia was mildly aversive to weaned pigs, however the aversion was not strong enough as conditioned placed avoidance was not observed in any pigs on any of the washout days. Our results coincide with Raj and Gregory (1996) who tested 2% and 5% O_2 in adult pigs and did not find behaviors associated with aversion. However, our study was more effectively designed to 'ask' pigs how they feel during the exposure compared to immersion study by Raj and Gregory (1996). The AVMA (2013) suggest hypoxia above 6% O_2 should not be used. Raj and Gregory (1996) used 5% O_2 and none of the pigs displayed loss of posture within a minute exposure in a stunning unit. We do not think a minute exposure is enough time, as hypoxia generally takes longer to induce loss of consciousness. In this regard, our study confirmed hypoxia at 6% should be avoided for euthanasia purpose. Time to loss of consciousness at <2% O_2 was 85 sec which was similar to Fiedler et al. (2016) and Sutherland (2011) but differed with Raj and Gregory (1996) and Dalmau et al. (2010). In Dalmau et al. (2010) pigs were individually exposed to gas for 3 times during the 9-day trial. The authors found loss of posture occurred earlier in the third session compared to the previous two sessions. The repeated exposure may have caused fast respiratory depression resulting in early time to loss of consciousness. One of 7 pigs displayed muscular excitation immediately after loss of posture, however the pig was not unconscious which is in accordance to what Sutherland (2011) reported. Dalmau et al., (2010) reported 70% pigs did not show any muscle jerks when exposed to 90% argon, while the rest exhibited muscle jerks before, during and after loss of posture. According to Raj and Gregory (1997), physical activity occurred after the onset of slow waves in electrocorticogram in pigs exposed to 90% argon. Muscle excitation may be distressing for conscious pigs, however there is a lack of consistent results to confirm the conscious state of animals in the previous studies. Due to ethical concerns our study required pigs to remove once neuromuscular excitation occurred. Advanced technologies like telemetry which has been used in poultry (McKeegen et al., 2013) may be useful in such situations. To add to the current knowledge of argon induced aversion in pigs, our study reported weaned pigs' mild aversion to <2% hypoxia during proximate exposure as well as during induction of loss of consciousness.

4.2.Challenges/future directions

In the present study, food rewards (pleasant) were paired with test gases (unpleasant). The situation created a motivational conflict in pigs as whether to stay or to avoid the chamber and sacrifice the positive incentive. Pigs' stay in the treatment chamber was important in our study to determine how they perceive the gas during the initial exposure as well as when they lost posture. We were aware that pigs would voluntarily move between chambers once the sliding door opened. The frequent movement would have been difficult to assess pigs' motivation to leave the treatment chamber during gas exposure. To better assess pigs' motivation, we tested pigs' responses on a gas day and compared with a preceding baseline day and a following washout day. On baseline and washout day, pigs experienced ambient air in the treatment chamber. Main challenge was to keep pigs occupied to the food rewards so that they could stay longer in the treatment chamber. Pigs were attracted to food rewards briefly and in intervals. Through preliminary testing, we found pigs were fond of strawberry jam, may be due to its sweet taste or semi-solid consistency. Hence, we smeared jam on the rubber mat near the treatment chamber door, on the ridges of feeding bowl and on the Kong, which was proved to be effective. In future studies, a high value feed reward or an environmental enrichment should be introduced for pigs in experiments of similar nature. Conditioned place avoidance test relies on previous experiences, and based on that experience animals display avoidance behavior on the subsequent aversive exposures. Therefore, the test may not be beneficial in studies where animals possibly undergo memory loss. In the current studies, both CO_2 and argon induced hypoxia produced some degree of loss of consciousness, hence there is a possibility that pigs did not remember the previous gas exposure. In future, other robust cognitive tests should be used for better assessment of behavioral responses.

In summary, CO_2 and argon induced hypoxia are mildly aversive to pigs and similar to previous studies we found relatively rapid loss of consciousness with CO_2 but a lower degree of aversion with hypoxia. From animal welfare point of view, argon inducing <2% hypoxia has merits over 30% CO_2 as a 2-step euthanasia method for young pigs. Further research is required to reliably assess pigs’ conscious state during muscular excitation.

REFERENCES

AASV, and NPB. 2016. On-Farm Euthanasia of Swine Recommendations for the Producer.

Aitken, P. G., and S. J. Schiff. 1986. Selective neuronal vulnerability to hypoxia in vitro. Neurosci. Lett. 67:92–96.

Anton, F., I. Euchner, and H. O. Handwerker. 1992. Psychophysical examination of pain induced by defined CO2 pulses applied to the nasal mucosa. Pain 49:53–60.

Beausoleil, N., and D. Mellor. 2015. Introducing breathlessness as a significant animal welfare issue. N. Z. Vet. J. 63:44–51.

Benson, E. R., R. L. Alphin, M. K. Rankin, M. P. Caputo, C. A. Kinney, and A. L. Johnson. 2012. Evaluation of EEG based determination of unconsciousness vs. loss of posture in broilers. Res. Vet. Sci. 93:960–964.

Boissy, A. 1995. Fear and Fearfulness in Animals. Q. Rev. Biol. 70:165–191.

Boissy, A., G. Manteuffel, M. B. Jensen, R. O. Moe, B. Spruijt, L. J. Keeling, C. Winckler, B. Forkman, I. Dimitrov, J. Langbein, M. Bakken, I. Veissier, and A. Aubert. 2007. Assessment of positive emotions in animals to improve their welfare. Physiol. Behav. 92:375–397.

Bromm, B. 2001. Brain images of pain. News Physiol. Sci. 16:244–9. Available from: http://www.ncbi.nlm.nih.gov/pubmed/11572930

Casey-Trott, T. M., S. T. Millman, P. V. Turner, S. G. Nykamp, P. C. Lawlis, and T. M. Widowski. 2014. Effectiveness of a nonpenetrating captive bolt for euthanasia of 3 kg to 9 kg pigs. J. Anim. Sci. 92:5166–5174.

Chen, X., J. Gallar, M. A. Pozo, M. Baeza, and C. Belmonte. 1995. CO2 stimulation of the Cornea: A Comparison Between Human Sensation and Nerve Activity in Polymodal Nociceptive Afferents of the Cat. Eur. J. Neurosci. 7:1154–1163.

Conlee, K. M., M. L. Stephens, A. N. Rowan, and L. A. King. 2005. Carbon dioxide for euthanasia: concerns regarding pain and distress, with special reference to mice and rats. Lab. Anim. 39:137–61.

Coryell, M. W., A. E. Ziemann, P. J. Westmoreland, J. M. Haenfler, Z. Kurjakovic, X. Zha, M. Price, M. K. Schnizler, and J. A. Wemmie. 2007. Targeting ASIC1a Reduces Innate Fear and Alters Neuronal Activity in the Fear Circuit. Biol. Pshychiatry 62:1140–1148.

Dalmau, A., P. Rodríguez, P. Llonch, and A. Velarde. 2010. Stunning pigs with different gas mixtures: aversion in pigs. Anim. Welf. 19:325–333.

Damasio, A., and G. B. Carvalho. 2013. The nature of feelings: evolutionary and neurobiological origins. Nat. Rev. Neurosci. 14:143–152.

Danneman, P. J., S. Stein, and S. O. Walshaw. 1997. Humane and practical implications of using carbon dioxide mixed with oxygen for anesthesia or euthanasia of rats. Lab. Anim. Sci. 47:376–385.

Davis, M. 1992. The Role of the Amygdala in Fear and Anxiety. Annu. Rev. Neurosci. 15:353–375.

Dawkins, M. S. 1988. Behavioural deprivation: A central problem in animal welfare. Appl. Anim. Behav. Sci. 20:209–225.

Dawkins, M. S. 1990. From an animal’s point of view: Motivation, fitness, and animal welfare. Behav. Brain Sci. 13:1–9.

Desire, L., A. Boissy, and I. Veissier. 2002. Emotions in farm animals:: a new approach to animal welfare in applied ethology. Behav. Processes 60:165–180.

Dixon, L. M., V. Sandilands, M. Bateson, S. Brocklehurst, B. J. Tolkamp, and R. B. D’Eath. 2013. Conditioned place preference or aversion as animal welfare assessment tools: Limitations in their application. Appl. Anim. Behav. Sci. 148:164–176.

Dodman, N. H. 1977. Observation on the use of the Wernberg dip-lift carbon dioxide apparatus for pre-slaughter anesthesia of pigs. Br. Vet. J. 133:71–80.

Dripps, R. D., and J. H. Comroe. 1947. The respiratory and circulatory response of normal man to inhalation of 7.6 and 10.4 percent CO2 with a compariosn of the maximal ventilation produced by severe muscular exercise, inhalation of CO2 and maximal voluntary hyperventilation. Am. J. Physiol. 149:43–51.

Dubin, A. E., and A. Patapoutian. 2010. Nociceptors: the sensors of the pain pathway. J. Clin. Invest. 120:3760–72. Available from: http://www.ncbi.nlm.nih.gov/pubmed/21041958

Dulla, C. G., P. Dobelis, T. Pearson, B. G. Frenguelli, K. J. Staley, and S. A. Masino. 2005. Adenosine and ATP link PCO2 to cortical excitability via pH. Neuron 48:1011–23.

Duncan, I. J. H. 2005. Science-based assessment of animal welfare: farm animals. Rev. sci. tech. Off. int. Epiz 24:483–492.

EFSA. 2004. Opinion of the Scientific Panel on Animal Health and Welfare (AHAW) on a request from the Commission related to welfare aspects of the main systems of stunning and killing the main commercial species of animals. EFSA J. 45:1–29.

Erickson, H. H., J. P. Goff, E. E. Uemura, and W. O. Reece, eds. 2015. Dukes’ Physiology of Domestic Animals. 13th editi. John Wiley & Sons, New York.

Feng, Y., and T. L. Simpson. 2003. Nociceptive Sensation and Sensitivity Evoked from Human Cornea and Conjunctiva Stimulated by CO2. Invest. Ophthalmol. Vis. Sci. 44:529–532.

Fiedler, K. J., R. L. Parsons, L. J. Sadler, and S. T. Millman. 2014. Effects of stocking rate on measures of efficacy and welfare during carbon dioxide gas euthanasia of young pigs. Anim. Welf. 23:309–321.

Fiedler, K., R. Parsons, L. Sadler, and S. Millman. 2016. Effects of stocking rate on measures of efficacy and welfare during argon gas euthanasia of weaned pigs. Anim. Welf. 25:83–89.

Fraser, D. 2009. Can We Measure Distress in Animals? Ethol. Nonhum. Anim.:1–10. Available from: http://animalstudiesrepository.org/acwp_ena/7

Fraser, D., D. M. Weary, E. A. Pajor, and B. N. Milligan. 1997. A Scientific Conception of Animal Welfare that Reflects Ethical Concerns. Anim. Welf. 6:187–205.

Gerritzen, M. a, B. Lambooij, H. Reimert, a Stegeman, and B. Spruijt. 2004. On-farm euthanasia of broiler chickens: effects of different gas mixtures on behavior and brain activity. Poult. Sci. 83:1294–1301.

Gigliotti, F. 2010. Mechanisms of dyspnea in healthy subjects. Multidiscip. Respir. Med. 5:195.
Hall, C. S. 1934. Emotional behavior in the rat I. Defecation and urination as measures of individual differences in emtionality. J. Comp. Psychol. 18:385–403.

Hall, J. E., and A. C. Guyton. 2011. Guyton and Hall textbook of medical physiology. 12th ed. Saunders/Elsevier, Philadelphia, PA.

Hemsworth, P. H., D. J. Mellor, G. M. Cronin, and A. J. Tilbrook. 2015. Scientific assessment of animal welfare. N. Z. Vet. J. 63:24–30.

Hsu, K.-S., Y.-C. Liang, and C.-C. Huang. 2000. Influence of an extracellular acidosis on excitatory synaptic transmission and long-term potentiation in the CA1 region of rat hippocampal slices. J. Neurosci. Res. 62:403–415.

Ito, R., and A. C. H. Lee. 2016. The role of the hippocampus in approach-avoidance conflict decision-making: Evidence from rodent and human studies. Behav. Brain Res. 313:345–357.
Jones, J. B., C. M. Wathes, and A. J. F. Webster. 1998. Operant responses of pigs to atmospheric ammonia. Appl. Anim. Behav. Sci. 58:35–47.

de Jonge, F. H., S.-L. Tilly, A. M. Baars, and B. M. Spruijt. 2008. On the rewarding nature of appetitive feeding behaviour in pigs (Sus scrofa): Do domesticated pigs contrafreeload? Appl. Anim. Behav. Sci. 114:359–372.

Jongman, E. C., J. L. Barnett, and P. H. Hemsworth. 2000. The aversiveness of carbon dioxide stunning in pigs and a comparison of the CO2 stunner crate vs. the V-restrainer. Appl. Anim. Behav. Sci. 67:67–76.

Kirkden, R. D., L. Niel, G. Lee, I. J. Makowska, M. J. Pfaffinger, and D. M. Weary. 2008. The validity of using an approach-avoidance test to measure the strength of aversion to carbon dioxide in rats. Appl. Anim. Behav. Sci. 114:216–234.

Kirkden, R. D., and E. A. Pajor. 2006. Using preference, motivation and aversion tests to ask scientific questions about animals' feelings. Appl. Anim. Behav. Sci. 100:29–47.

Krohn, T. C., A. K. Hansen, and N. Dragsted. 2003. The impact of low levels of carbon dioxide on rats. Lab. Anim. 37:94–99.

Lambooij, E., M. . Gerritzen, B. Engel, S. J. . Hillebrand, J. Lankhaar, and C. Pieterse. 1999. Behavioural responses during exposure of broiler chickens to different gas mixtures. Appl. Anim. Behav. Sci. 62:255–265.

Lansing, R. W., B. S. H. Im, J. I. Thwing, A. T. R. Legeda, and R. B. Banzett. 2000. The Perception of Respiratory Work and Effort Can Be Independent of the Perception of Air Hunger. Am. J. Respir. Crit. Care Med. 162:1690–1696.

Lawrence, A. B., M. C. Appleby, and H. A. Macleod. 1988. Measuring hunger in the pig using operant conditioning: The effect of food restriction. Anim. Prod. 47:131–137.

Leach, M. C., V. A. Bowell, T. F. Allan, and D. B. Morton. 2002. Aversion to gaseous euthanasia agents in rats and mice. Comp. Med. 52:249–257.

Leach, M. C., V. A. Bowell, T. F. Allan, and D. B. Morton. 2004. Measurement of aversion to determine humane methods of anaesthesia and euthanasia. Anim. Welf. 13:77–86.

Leary, S., W. Underwood, R. Anthony, S. Cartner, D. Corey, T. Grandin, C. Greenacre, S. Gwaltney-Brant, M. A. McCrackin, R. Meyer, D. Miller, J. Shearer, R. Yanong, G. C. Golab, and E. Patterson-kane. 2013. AVMA Guidelines for the Euthanasia of Animals: 2013 Edition. American Veterinary Medical Association, Schaumburg, IL.

LeDoux, J. E. 1995. Emotion: Clues from the Brain. Annu. Rev. Psychol. 46:209–235.

Liotti, M., S. Brannan, G. Egan, R. Shade, L. Madden, B. Abplanalp, R. Robillard, J. Lancaster, F. E. Zamarripa, P. T. Fox, and D. Denton. 2001. Brain responses associated with consciousness of breathlessness (air hunger). Proc. Natl. Acad. Sci. U. S. A. 98:2035–2040.

Llonch, P., P. Rodríguez, M. Gispert, A. Dalmau, X. Manteca, and A. Velarde. 2012. Stunning pigs with nitrogen and carbon dioxide mixtures: effects on animal welfare and meat quality. Animal 6:668–75.

Makowska, I. J., L. Niel, R. D. Kirkden, and D. M. Weary. 2008. Rats show aversion to argon-induced hypoxia. Appl. Anim. Behav. Sci. 114:572–581.

Makowska, I. J., L. Vickers, J. Mancell, and D. M. Weary. 2009. Evaluating methods of gas euthanasia for laboratory mice. Appl. Anim. Behav. Sci. 121:230–235.

Makowska, I. J., and D. M. Weary. 2009. Rat aversion to induction with inhalant anaesthetics. Appl. Anim. Behav. Sci. 119:229–235.

Marchant-Forde, J. N., D. C. Lay, K. A. McMunn, H. W. Cheng, E. A. Pajor, and R. M. Marchant-Forde. 2009. Postnatal piglet husbandry practices and well-being: the effects of alternative techniques delivered separately. J. Anim. Sci. 87:1479–92.

Martínez-Miró, S., F. Tecles, M. Ramón, D. Escribano, F. Hernández, J. Madrid, J. Orengo, S. Martínez-Subiela, X. Manteca, and J. J. Cerón. 2016. Causes, consequences and biomarkers of stress in swine: an update. BMC Vet. Res. 12:171. Available from: http://www.ncbi.nlm.nih.gov/pubmed/27543093

Martoft, L., L. Lomholt, C. Kolthoff, B. E. Rodriguez, E. W. Jensen, P. F. Jorgensen, H. D. Pedersen, and A. Forslid. 2002. Effects of CO2 anaesthesia on central nervous system activity in swine. Lab. Anim. 36:115–126.

Mathur, P., B. Lau, and S. Guo. 2011. Conditioned place preference behavior in zebrafish. Nat. Protoc. 6:338–345.

McKeegan, D. E. F., J. McIntyre, T. G. M. Demmers, C. M. Wathes, and R. B. Jones. 2006. Behavioural responses of broiler chickens during acute exposure to gaseous stimulation. Appl. Anim. Behav. Sci. 99:271–286.

McKeegan, D. E. F., D. A. Sandercock, and M. A. Gerritzen. 2013. Physiological responses to low atmospheric pressure stunning (LAPS) and their implications for welfare. Poult. Sci.:858–868.
Niel, L., and D. M. Weary. 2007. Rats avoid exposure to carbon dioxide and argon. Appl. Anim. Behav. Sci. 107:100–109.

Mellor, D. 2016. Updating Animal Welfare Thinking: Moving beyond the “Five Freedoms” towards “A Life Worth Living.” Animals 6:21.

Mendl, M., and E. Paul. 2004. Consciousness, emotion and animal welfare: insights from cognitive science. Anim. Welf. 13:17–25.

Merriam-Webster. 2017. Merriam-Webster Online Dict. Accessed 2017-04-25. Available from: https://www.merriam-webster.com/dictionary/aversion

Meyer, R. E. 2015. Physiologic Measures of Animal Stress during Transitional States of Consciousness. Animals 5:702–716.

Meyer, R. E., D. Acvaa, J. T. Whitley, W. E. M. Morrow, L. F. Stikeleather, C. L. Baird, J. M. Rice, B. V Halbert, D. K. Styles, and C. S. Whisnant. 2013. Effect of physical and inhaled euthanasia methods on hormonal measures of stress in pigs. J. Swine Heal. Prod. 21:261–269.

Millman, S. T. 2013. Behavioral Responses of Cattle to Pain and Implications for Diagnosis, Management, and Animal Welfare. Vet. Clin. North Am. Food Anim. Pract. 29:47–58.

Millot, S., M. Cerqueira, M. F. Castanheira, Ø. Øverli, C. I. M. Martins, and R. F. Oliveira. 2014. Use of conditioned place preference/avoidance tests to assess affective states in fish. Appl. Anim. Behav. Sci. 154:104–111.

Moosavi, S. H., E. Golestanian, A. P. Binks, R. W. Lansing, R. Brown, and R. B. Banzett. 2003. Hypoxic and hypercapnic drives to breathe generate equivalent levels of air hunger in humans. J. Appl. Physiol. 94:141–54.

Mormede, P., S. Andanson, B. Auperin, B. Beerda, D. Guenmene, J. Malmkvist, X. Manteca, G. Manteuffel, P. Prunet, C. G. Van Rennen, S. Richard, and I. Veissier. 2007. Exploration of the hypothalamic-pituitary-adrenal function as a tool to evaluate animal welfare. Physiol. Behav. 92:317–339.

Mota-Rojas, D., D. Bolanos-Lopez, M. Concepcion-Mendez, J. Ramirez-Telles, P. Roldan-Santiago, S. Flores-Peinado, and P. Mora-Medina. 2012. Stunning Swine with CO2 Gas: Controversies Related to Animal Welfare. Int. J. Pharmacol. 8:141–151.

Mount, L. E., and J. G. Rowell. 1960. Body size, body temperature and age in relation to the metabolic rate of the pig in the first five weeks after birth. J. Physiol. 154:408–416.

Murphy, E., R. E. Nordquist, and F. J. van der Staay. 2014. A review of behavioural methods to study emotion and mood in pigs, Sus scrofa. Appl. Anim. Behav. Sci. 159:9–28.

Nattie, E. 1999. CO2, brainstem chemoreceptors and breathing. Prog. Neurobiol. 59:299–331.

Nattie, E. 2006. Why do we have both peripheral and central chemoreceptors? J. Appl. Physiol. 100:9–10.

Neubauer, J. A., J. E. Melton, and N. H. Edelman. 1990. Modulation of respiration during brain hypoxia. J. Appl. Physiol. 68:441–51.

Niel, L., and D. M. Weary. 2006. Behavioural responses of rats to gradual-fill carbon dioxide euthanasia and reduced oxygen concentrations. Appl. Anim. Behav. Sci. 100:295–308.

Niel, L., and D. M. Weary. 2007. Rats avoid exposure to carbon dioxide and argon. Appl. Anim. Behav. Sci. 107:100–109.

O’Connor, A., R. Anthony, L. Bergamasco, J. Coetzee, S. Gould, A. K. Johnson, L. A. Karriker, J. N. Marchant-Forde, G. S. Martineau, J. McKean, S. T. Millman, S. Niekamp, E. A. Pajor, K.

Rutherford, M. Sprague, M. Sutherland, E. von Borell, and R. S. Dzikamunhenga. 2014. Pain management in the neonatal piglet during routine management procedures. Part 2: grading the quality of evidence and the strength of recommendations. Anim. Health Res. Rev. 15:39–62.

OECD. 2000. Guidance Document on the Recognition, Assessment, and Use of Clinical Signs as Humane Endpoints for Experimental Animals used in Safety Evaluation. Organisation for Economic Co-operation and Development Office, Paris, France.

OIE. 2017. Terrestrial Animal Health Code Chapter 7.5 Slaughter of Animals Article 7.5.7. :1–18. Available from: http://www.oie.int/index.php?id=169&L=0&htmfile=chapitre_aw_slaughter.htm
Paul, E. S., E. J. Harding, and M. Mendl. 2005. Measuring emotional processes in animals: the utility of a cognitive approach. Neurosci. Biobehav. Rev. 29:469–491.

Peppel, P., and F. Anton. 1993. Responses of rat medullary dorsal horn neurons following intranasal noxious chemical stimulation: effects of stimulus intensity, duration, and interstimulus interval. J. Neurophysiol. 70:2260–75.

Pig Code of Practice Scientific Committee. 2014. The Code of Practice For the Care and Handlings of Pigs: Review of scientific research on priority issues. Ottawa, Ontario. Available from: http://www.nfacc.ca/pdfs/codes/pig_code_of_practice.pdf

Prabhakar, N. R., and G. L. Semenza. 2015. Oxygen Sensing and Homeostasis. Physiology 30:340–8.

Raj, A. B. M., and N. G. Gregory. 1991. Preferential feeding behaviour of hens in different gaseous atmospheres. Br. Poult. Sci. 32:57–65.

Raj, A., and N. Gregory. 1995. Welfare implications of the gas stunning of pigs 1. Determination of aversion to the initial inhalation of carbon dioxide or argon. Anim. Welf. 4:273–280

Raj, A. B. M., and N. G. Gregory. 1996. Welfare implications of the gas stunning of pigs 2. Stress of induction of anaesthesia. Anim. Welf. 5:71–78.

Raj, A. B. M., S. P. Johnson, S. B. Wotton, and J. L. M. Instry. 1997. Welfare Implications of Gas Sturming Pigs: 3. the Time to Loss of Somatosensory Evoked Potentials and Spontaneous Electrocorticogram of Pigs During Exposure to Gases. Vet. J. 153:329–339.

Raj, A. B. M. 1999. Behaviour of pigs exposed to mixtures of gases and the time required to stun and kill them: welfare implications. Vet. Rec. 144:165–168.

Rault, J. L., K. A. Mcmunn, J. N. Marchant-Forde, and D. C. J. Lay. 2013. Gas alternatives to carbon dioxide for euthanasia: A piglet perspective. J. Anim. Sci. 91:1874–1883.

Rushen, J. 1996. Using aversion learning techniques to assess the mental state, suffering, and welfare of farm animals. J. Anim. Sci. 74:1990–5.

Sadler, L. J. 2013. Effects of flow rate, gas type and disease status on the welfare of sucking and weaned pigs during gas euthanasia. PhD Diss. Iowa State University, Ames, IA.

Sadler, L. J., C. D. Hagen, C. Wang, T. M. Widowski, A. K. Johnson, and S. T. Millman. 2014. Effects of flow rate and gas mixture on the welfare of weaned and neonate pigs during gas euthanasia. J. Anim. Sci. 92:793–805.

Stalder, K. 2016. 2010-2015 Summary Analysis of U.S. Pork Industry Productivity. Des Moines, IA. Available from: http://www.pork.org/wp-content/uploads/2016/08/2016-pork-industry-productivity-summary.pdf

Sutherland, M. A. 2011. The use of different gases and gas combinations to humanely euthanize young suckling pigs. National Pork Board, Des Moines , IA.

Sutherland, M. A., B. L. Davis, T. A. Brooks, and J. F. Coetzee. 2012. The physiological and behavioral response of pigs castrated with and without anesthesia or analgesia. J. Anim. Sci. 90:2211–21.

Sutherland, M., P. Bryer, and B. Backus. 2017. The effect of age and method of gas delivery on carbon dioxide euthanasia of pigs. Anim. Welf. 26:293–299.

USDA, N. A. S. S. (NASS). 2017. Quarterly Hogs and Pigs (March 2017). Available from: http://usda.mannlib.cornell.edu/usda/current/HogsPigs/HogsPigs-03-30-2017.pdf

Velarde, A., J. Cruz, M. Gispert, D. Carrión, J. L. Ruiz de la Torre, A. Diestre, and X. Manteca. 2007. Aversion to carbon dioxide stunning in pigs: Effect of carbon dioxide concentration and halothane genotype. Anim. Welf. 16:513–522.

Wahi, P., T. Widowski, and S. Yue Cottee. 2011. I'm not going there! using conditioned place preference to assess the aversiveness of restraint and blood sampling in piglets. In: J. N. Marchant-Forde and E. A. Pajor, editors. Proceedings of the 45th Congress of the International Society for Applied Ethology (ISAE). Wageningen Academic Publishers The Netherlands, Indianapolis, Indiana USA. p. 9.

White, R. G., J. A. DeShazer, C. J. Tressler, G. M. Borcher, S. Davey, A. Waninge, A. M. Parkhurst, M. J. Milanuk, and E. T. Clemens. 1995. Vocalization and physiological response of pigs during castration with or without a local anesthetic. J. Anim. Sci. 73:381–386.

Widdicombe, J. G. 1982. Pulmonary and respiratory tract receptors. J. Exp. Biol. 100:41–57.

Ziemann, A. E., J. E. Allen, N. S. Dahdaleh, I. I. Drebot, M. W. Coryell, A. M. Wunsch, C. M. Lynch, F. M. Faraci, M. A. Howard, M. J. Welsh, J. A. Wemmie, and J. A. Wemmie. 2009. The amygdala is a chemosensor that detects carbon dioxide and acidosis to elicit fear behavior. Cell 139:1012–21.