CURRENT METHODS OF MOUSE EUTHANASIA: REFINEMENTS AND AVERSION

by

Carly Moody

B.Sc. (Hons.), The University of British Columbia, 2011

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in

THE FACULTY OF GRADUATE AND POSTDOCTORAL STUDIES

(Applied Animal Biology)

THE UNIVERSITY OF BRITISH COLUMBIA

(Vancouver)

November 2013

Abstract

Mice are routinely euthanized by gradual-fill carbon dioxide (CO_2) gas or with isoflurane; the aim of my thesis was to assess refinements to these procedures. The first study assessed the CO_2 method of euthanasia with the aim of minimizing the duration of dyspnea without exposing mice to painful concentrations (>40% CO_2). Various CO_2 flow rates (20, 30, 40, 50% cage vol/min) were used to examine the duration between the onset of dyspnea (identified by laboured breathing) and insensibility (identified by recumbency, loss of the righting reflex or loss of the pedal withdrawal reflex). The interval between the onset of dyspnea and loss of the righting reflex averaged 38.2 ± 2.4 s versus 59.2 ± 2.4 s, using 50% and 20% cage vol/min fill rates, respectively. Thus even at the highest flow rate tested mice experienced more than 30 s of dyspnea, suggesting other methods of euthanasia should be used when possible. The second study examined the same three measures of insensibility during the isoflurane method of euthanasia, with the aim of identifying when it is safe to switch to a high flow rate of CO_2, without subjecting conscious animals to painful concentrations. The results suggested that the onset of recumbency and loss of the righting reflex are not safe indicators of insensibility when using induction with isoflurane; continued induction with 5% isoflurane carried by 17% cage vol/min of oxygen for a minimum of 79 s after the appearance of recumbency is advised before switching to a high flow rate of CO_2. The final study in this thesis used a light-aversion test to examine mouse aversion to: 1) 20% gradual-fill CO_2, 2) 5% isoflurane administered using a vaporizer, and 3) 5% isoflurane administered using the drop-method. Mice chose to remain in the dark chamber longer when exposed to isoflurane administered using a vaporizer compared to both CO_2 and isoflurane drop. Mice were also more likely to become recumbent in the dark side

when exposed to the isoflurane vaporizer versus other methods. These results indicate that isoflurane delivered by a vaporizer is a humane refinement for the euthanasia of laboratory mice.

Preface

All research included in this thesis was approved by the University of British Columbia's Animal Care Committee (Certificate Number: A12-0254).

Chapter 2:

The study area was identified by Beverly Chua and study design was created by Beverly Chua, Carly Moody and Daniel Weary. This research was conducted by Beverly Chua and Carly Moody, while analysis of the research data was performed by Carly Moody and Daniel Weary. The manuscript was written by Carly Moody and modified by Beverly Chua, Marina von Keyserlingk, Shelly McErlane and Daniel Weary. A version of this manuscript has been submitted for publication:

Moody C., Chua B., Weary D. 2013. The effect of carbon dioxide flow rate on laboratory mice. Laboratory Animals.

Chapter 3:

Joanna Makowska identified the study area and Joanna Makowska, Carly Moody and Daniel Weary created the study design. Carly Moody conducted this research with the help of Devina Wong. Carly Moody and Daniel Weary performed data analysis. This manuscript was written by Carly Moody and modified by Joanna Makowska, Marina von Keyserlingk, Shelly McErlane and Daniel Weary. A version of this manuscript has been submitted for publication:

Moody C., Makowska J., Weary D. 2013. Testing three measures of mouse insensibility following induction with isoflurane for a more humane euthanasia. The Journal of the American Association for Laboratory Animal Science.

Chapter 4:

The research area was identified by Daniel Weary and Carly Moody and the study design was created by Carly Moody with the help of Devina Wong. Carly Moody conducted the research and data analysis was performed by Carly Moody and Daniel Weary. The manuscript was written by Carly Moody and modified by Joanna Makowska, Marina von Keyserlingk, Shelly McErlane and Daniel Weary. A version of this manuscript has been submitted for publication:
Moody C., Weary D. 2013. Mouse aversion to isoflurane versus carbon dioxide gas. Applied Animal Behaviour Science.

This thesis was written by Carly Moody and has been reviewed and modified by Drs. Daniel Weary, Marina von Keyserlingk and Shelly McErlane.

Table of Contents

List of Tables

List of Figures

Acknowledgements

I am extremely thankful to Dan Weary for believing in my abilities, for teaching me to think critically and always being there to help guide me through endless research questions and problems. Your enthusiasm for science and research is contagious! I also want to thank Joanna Makowska and Bev Chua who have been exceptional mentors, first introducing me to animal welfare science during my undergraduate experience and then guiding me through a master's degree, thank you.

I am grateful to Shelly McErlane for sitting on my committee in the midst of my degree, as well as Nina von Keyserlingk for always keeping her door open, providing advice and support.

My graduate experience would not have been the same without Devina Wong, whom I was lucky to have started and finished a master's degree along side. I also want to thank all of the students in the Animal Welfare Program for all the advice, laughs and endless intellectual discussions that have enlightened me.

A special thanks to my family (Mom, Dad, Drew, Alana and Alyssa) for their everlasting support and interest in my work. I'd especially like to thank Steve McMullen, who not only helped bring my projects to life with his electrical skills, but also gave his unconditional and constant support, reminding me to stay true to myself and always pursue what I love to do, no matter what.

This thesis is dedicated to the mice used in my studies and the millions of laboratory mice subject to euthanasia.

Chapter 1: Introduction

1.1 Background

In 2009, over 3.3 million animals were used in Canada for research purposes, and over one million of these were mice (CCAC, 2010). The fate of most laboratory mice is euthanasia, occurring at experimental endpoint or humane endpoint, and for those regarded as surplus or old breeding stock. The term euthanasia means 'good death' and has been defined by many regulatory bodies with guidelines on laboratory animal care. In Canada, laboratory mouse euthanasia is guided by recommendations from the American Veterinary Medical Association (AVMA) and the Canadian Council on Animal Care (CCAC). The CCAC (1993) states a humane euthanasia should be "death without signs of panic, pain or distress." and the AVMA (2013) states the goal of euthanasia is to "minimize pain, distress, and negative effect to the animals, the humaneness of the technique (i.e., how we bring about the death of animals) is also an important ethical issue".

The most common agents used to euthanize laboratory mice are the inhalant anaesthetics carbon dioxide (CO_2) gas and isoflurane. The AVMA (2013) guidelines states these agents are acceptable with conditions when it is impractical or difficult to practice physical restraint, with the recommendation that CO_2 should be administered by gradual-fill using a flow rate between 10-30% chamber volume per minute (vol/min), until the animal is unconscious, then increasing the flow rate to reduce the time to death. Isoflurane may be delivered using a vaporizer or the drop method, although no specific method of delivery has been recommended by either the AVMA or the CCAC. The CCAC (2010) recommends CO_2 gas as conditionally acceptable and isoflurane as an acceptable method for rodent euthanasia. Since an inhalant anaesthetic can take

a long time to kill a rodent, the guidelines suggest a secondary method is used to ensure death; in many cases, a high flow rate of CO_2 is used once the animal is insensible. Although isoflurane and CO_2 gas are both inhalant anaesthetics, they have different modes of action and different concerns when used as a euthanasia agent.

1.2 Carbon dioxide

CO_2 is commonly used for euthanasia because of its low cost, safety to personnel and easy administration to a large number of rodents at one time (Ambrose et al., 2000). CO_2 does not accumulate in body tissues and thus is often the method of choice for many animal studies in which analysis of tissues is required. When used for euthanasia, CO_2 is commonly delivered via a pressurized gas tank using a CO_2 flow meter. Anaesthetic induction with CO_2 is relatively short and narcosis may result soon thereafter (Martoft et al., 2003).

1.2.1 Conscious perceptions: pain

During the gradual-fill method of CO_2 euthanasia, CO_2 concentration builds up in the euthanasia chamber until the animal is rendered unconscious. At higher concentrations, CO_2 reacts with water, for example on mucosal surfaces, forming carbonic acid. This acidic product is painful in humans when CO_2 concentrations reach between 32.5-55% (Anton et al., 1992). Humans report an unpleasant burning sensation when breathing 50% CO_2 and concentrations >60% have been described as painful, piercing, or stabbing (Danneman et al. 1997). It is likely that rodents also experience pain when exposed to these concentrations, as innervation of the respiratory and nasal tissues and nerve responses to CO_2 are similar to humans (Peppel and Anton, 1993; Thurauf et al., 2002). In rats, CO_2 concentrations over 37% stimulate nociceptors

in the nasal mucosa (Anton et al., 1991) and concentrations >60% stimulate nociceptors in the cornea (Hirata et al., 1999). When using gradual-fill CO_2 for rodent euthanasia, a flow rate between 10-30% cage vol/min is used so that insensibility is reached before concentrations exceed 40%; thereby reducing the likelihood that conscious animals are exposed to painful concentrations of CO_2 (Smith and Harrap, 1997).

1.2.2 Conscious perceptions: distress

In the veterinary literature, the term dyspnea is normally used to refer to laboured breathing. However, in the human literature dyspnea is referred to as air hunger, comprising multiple dimensions including sensory and affective dimensions (Lansing et al., 2009). For example, dyspnea is defined by the American Thoracic Society as "a subjective experience of breathing discomfort that consists of qualitatively distinct sensations that vary in intensity" (ATC ad hoc Committee, 1999). In humans, CO_2 induced air hunger results in a conscious awareness of the urge to breath, evoked by hypercapnia (Lansing et al., 2009). Different types of dyspneic sensations have been identified resulting from pathological breathlessness, including air hunger, tightness and work (Lansing et al., 2009). Air hunger is driven by the fundamental biological drive to breathe and is the strong awareness for the desire to breathe (Lansing et al., 2009). CO_2 causes hypercapnia, which in turn results in secondary effects comprising the physical and emotional components of dyspnea. In both humans and animals CO_2 inhalation is used to create stress-related disorders such as panic (Gorman et al., 1984; Johnson et al., 2011) and anxiety (Bailey et al., 2005). Prolonged inhalation of 5-7.5% CO_2 is used to induce feelings of anxiety and fear in humans (Bailey et al., 2005), and higher concentrations (20-35%) have been used to study panic responses in humans (Griez and Van den Hout, 1982; Schmidt et al. 1997). Mice

exposed to rising concentrations of CO_2 show responses indicative of intense fear, a response consistent with a reduction in brain pH as sensed by the amygdala (Ziemann et al., 2009). CO_2 may also directly activate other brain structures surrounding the amygdala as these areas also possess pH and CO_2 sensing chemoreceptors (Wemmie, 2011).

The perception of dyspnea has at least two components: sensory and affective state (von Leupoldt et al., 2008). The sensory component comprises the intensity dimension associated with the effort to breath and the affective dimension is associated with a strong negative psychological influence. The negative affective dimension results from unpleasantness or distress that arises from dyspnea, leading to fear and anxiety (Carrieri-Kohlman et al., 1996). Distress may be defined as "…an aversive, negative state in which coping and adaptation processes fail to return an organism to physiological and/or psychological homeostasis" (NRC, 2008). Distress may result when a stressor, in this case CO_2 gas, threatens an animal's welfare with potential end of life effects (NRC, 2008). Dyspnea is a threatening and aversive stimulus initiating a fight or flight response as an adaptive response to avoid potential tissue damage or asphyxiation (von Leupoldt et al., 2009). Humans experiencing dyspnea use strong negative emotional statements, suggesting a negative affective state associated with the unpleasantness when experiencing hypercapnia elicited air hunger (Banzett et al., 1990).

1.2.3 Mode of action

When CO_2 enters the lungs it diffuses through the alveoli membranes into arterial blood, causing vasodilation thereby increasing the concentration of arterial CO_2 (hypercapnia) and reducing blood pH (Guais et al., 2011). Dyspnea occurs as a result of the body's attempt to eliminate excess CO_2. In the blood, CO_2 is partnered with haemoglobin and is carried as

dissolved CO_2, carbonic acid, bicarbonate ions, and as other CO_2 plasma protein compounds (Abolhassani et al., 2009). These permeate the blood brain barrier, causing a decrease in cerebral spinal fluid (CSF) pH in relation to blood pH (Lee et al., 1996; Martoft et al., 2003). The decreased pH of the CSF occurs more quickly than the decrease in arterial pH because the CSF contains fewer buffering components (Martoft et al., 2003). The decreased CSF pH affects the central nervous system by decreasing nerve cell function and cerebral electrical activity and as a result induces anaesthesia and analgesia (Eisele et al., 1967; Lee et al., 1996).

1.2.4 Aversion to CO_2

There has been a developing interest in the use of other inhalant anaesthetics for mouse euthanasia, as many studies have shown that both mice and rats find CO_2 aversive. Approach-avoidance testing has been used to test rodent motivation for a food reward against the avoidance of CO_2 gas exposure. When tested with gradual fill CO_2 at 17% chamber vol/min, rats left the chamber containing the food reward when the chamber concentration reached an average of 18% CO_2 (Niel and Weary, 2007); even when food deprived, rats still left a chamber containing a food reward at an average CO_2 concentration of 16% when using a 15% chamber vol/min gradual-fill flow rate of CO_2 (Kirkden et al., 2008). Behavioural changes seen in rats euthanized with 17% chamber vol/min of CO_2 include increased activity, rearing, touching the nose to the chamber lid, vocalizations, and escape behaviours, suggesting CO_2 may cause distress in rats (Niel and Weary, 2006). In an aversion study by Leach et al. (2002), CO_2 induced a high degree of aversion in both rats and mice.

One study providing evidence that CO_2 concentrations greater than 3% are aversive in rats was published by Krohn et al. (2003); rats avoided chambers with concentrations of 3 and 5%

CO_2. A study by Ziemann et al. (2009) used four paradigms to study CO_2 induced fear and aversion in mice. The results of this study indicated that 10% CO_2 elicited fear as shown by freezing behaviour. Concentrations greater than 5% caused mice to show more anxiety-like behaviour, for example spending less time in the centre of an open-field test. Mice also spent 90% of their time in a chamber with 2% CO_2 when given the choice between chambers containing 2 or 15% CO_2. CO_2 also acted as an unconditioned stimulus in mice and increased fear memory when paired with a foot shock. The Ziemann et al. (2009) study indicates that CO_2 produces fear and aversion in mice. Sensations of dyspnea likely play a part in this aversion, and mice are subject to concentrations that induce dyspnea before they are rendered unconsciousness. Minimizing the period during which animals experience this dyspnea-induced distress may refine this method of euthanasia.

1.3 Isoflurane

Isoflurane is a liquid halogenated hydrocarbon that is volatile at typical room temperature and pressure. Isoflurane has many attributes that make this liquid a suitable anaesthetic for use in veterinary medicine. Isoflurane is a potent muscle relaxant and has the largest safety margin of the inhalant anaesthetics (Stimpel and Gershey, 1991). It also allows insensibility to occur in a graded way (Alkire et al., 2008). However, these advantages as an anaesthetic may be disadvantages when it is used as a euthanasia agent, as it may result in a prolonged time to death. This slow kill time increases the need for a secondary method to complete the procedure, such as a high flow rate of CO_2. However, graded sensibility also means it may be difficult to assess when an animal can be humanely exposed to high concentrations of CO_2.

Isoflurane may be administered one of two ways: 1) by use of a vaporizer machine and a carrier gas (such as O_2 or air), or 2) by the drop method. Vaporizer machines deliver isoflurane gradually, thereby slowly increasing the isoflurane concentration within a chamber. This method of delivery is commonly used for anaesthetic induction and maintenance in animals. However, some may argue that a vaporizer is not necessary for euthanasia as the amount administered does not need to be controlled. The wide safety margin of isoflurane means it can take a long time to actually kill a rodent even when using the maximum settings on a vaporizer and carrier gas flow meter. In addition, vaporizer machines may be costly to purchase and maintain, therefore researchers may prefer using the drop method of administration, requiring almost no specialized equipment. In the drop method, liquid isoflurane is placed on an absorbable material, such as a piece of gauze, and placed into a euthanasia chamber. This method administers isoflurane much faster than a vaporizer, as the liquid is vaporized almost instantly when exposed to air. A barrier is required to prevent an animal from direct contact with the liquid isoflurane as this may cause skin irritation. For both systems, a scavenging system is required to avoid gas exposure to personnel; isoflurane has been linked with human neurological and reproductive impairment, as well as neoplasia (Smith and Bolon, 2002).

1.3.1 Mode of action

Isoflurane acts on the central nervous system by inhibiting neurotransmitter pathways (Campagna et al., 2003; Herring et al., 2009; Westphalen et al., 2013). The molecular action of isoflurane is not fully understood, but involves selective interactions with globular proteins and ion channels, which are proteins regulating ion flow across cytoplasmic membranes (Campagna et al., 2003). Ion channels sensitive to volatile inhalant anaesthetics include cysteine-loop

neurotransmitter receptors, which include $GABA_A$ (γ-aminobutyric acid type A), the brain's most numerous inhibitory neurotransmitter receptor (Campagna et al., 2003). In humans, lower concentrations of isoflurane are known to reduce task-induced brain activation in many cortical regions, leaving sub-cortical regions such as the visual and motor cortex unaffected (Heinke and Schwarbauer, 2001). The effective potency of isoflurane is dose-dependent, making it a useful anaesthetic for conscious sedation in humans. Induction with isoflurane first results in the absence of memory and verbal responsiveness, then absence of pain to a surgical incision, followed by deep anaesthesia blocking autonomic responses of pain. The minimum alveolar concentration (MAC) at which 50% of subjects show abolishment of purposeful movement in response to a supra-maximal noxious stimulus is generally used to measure standard anaesthetic potency. A study by Mogil et al. (2005) showed that baseline nociceptive sensitivity varies with mouse strain, thereby causing a variation in mice isoflurane MAC. This means that the concentration of isoflurane needed to induce insensibility may vary with mouse strain.

1.3.2 Aversion to isoflurane

Exposure to isoflurane gas is not known to cause pain, but it has a pungent odour and may cause eye and respiratory tract irritation, likely becoming more pronounced with increasing concentration (Cervin and Lindberg, 1998; Doi and Ikeda, 1993). Studies by Leach et al. (2002, 2004) measured aversion using initial withdrawal and total dwelling time in an apparatus consisting of two connecting chambers, one filled with air and one pre-filled with the test gas. These studies tested aversion to low pre-filled concentrations of various inhalational anaesthetics in mice and rats. Concentrations of various anaesthetics were matched to induce ataxia in 10, 20 and 30 s, with low, medium and high concentrations, respectively. The results suggest that mice

and rats find halothane least aversive, followed by isoflurane, enflurane, sevoflurane, desflurane and lastly, CO_2 (Leach et al., 2004). When exposed to the highest concentration of each agent, rats spent more than 20 times longer with halothane compared to CO_2 and mice spent over 10 times longer with enflurane than CO_2.

Approach-avoidance testing, comparing motivation for a reward versus that to avoid an aversive stimulus, has been used to assess mouse and rat aversion to various inhalant gases. In these studies, the testing apparatus consisted of two chambers connected by a tube, one chamber with a palatable sugary treat with gradual-fill of a test gas, and a second chamber filled with air. Using this paradigm, Makowska et al. (2009) compared mouse aversion to various inhalant anaesthetics, including CO_2, halothane and isoflurane. More mice stayed until recumbency when tested with isoflurane compared to halothane, and isoflurane showed the weakest aversion in mice of all the agents tested. A similar experiment in rats showed lower aversion to isoflurane compared to halothane, when gas concentrations were matched to produce similar times to recumbency (Makowska and Weary, 2009). These latter two studies are in conflict with Leach et al. (2002,2004), who suggested that rodents find isoflurane less aversive than halothane. However, these differences may be attributed to methodological differences, as Leach et al. (2002,2004) used a pre-filled chamber compared to the gradual-fill method used by Makowska et al. (2009) and Makowska and Weary (2009). When taking into consideration practical application, the gradual-fill method more closely imitates what rodents experience during euthanasia, suggesting that rodents prefer isoflurane over halothane when gradually-filled. Despite the differences described above, Leach et al. (2002,2004) and Makowska et al. (2009) agree that CO_2 was the most aversive of all the inhalant gases tested, suggesting that other gases should be used for euthanasia.

Aversion-avoidance testing has been used to assess rat aversion to isoflurane and CO_2 during initial and re-exposure (Wong et al., 2013). The light-dark paradigm was used to create a light-aversion test. Rats chose between remaining in a dark compartment (preferred during baseline testing) gradually filling with either isoflurane or CO_2, or escaping to an aversive brightly lit chamber. Rats often remained in the dark compartment until recumbency when the chamber was gradually filling with isoflurane but never did so with CO_2; this result indicates that initial exposure to isoflurane is less aversive than CO_2. However, rats also avoided isoflurane when re-exposed, suggesting that re-exposure is more aversive than initial exposure.

A study by Altholtz et al. (2006) compared physiological stress responses of rats exposed to gradually increasing 5% isoflurane in O_2 for 2 min or 70:30% CO_2:O_2 for 1.5 min, within an induction chamber to induce anaesthesia for blood collection. The CO_2 treated rats showed a higher increase in corticosterone compared to baseline than isoflurane treated animals, suggesting CO_2 is a more stressful stimuli when used for anaesthesia. A recent study examined ultrasonic vocalizations in female rats euthanized with gradual-fill CO_2 or 2.5% isoflurane with O_2, both delivered at 30% chamber vol/min (Chisholm et al., 2013). All rats exposed to CO_2 vocalized versus none of the rats tested with isoflurane, again suggesting that CO_2 exposure is less humane than isoflurane. Although there is mounting evidence that rats find exposure to CO_2 more aversive and distressing than to isoflurane, there is a lack of evidence showing this in mice.

1.4 Measuring insensibility

The term anaesthesia was coined by Oliver Wendell Holmes to indicate insensibility to surgical pain. This definition is quite general, as various concentrations of anaesthetics cause different graded reversible effects such as analgesia, sedation, muscle relaxation, euphoria and

hypnosis (Campagna et al., 2003). Determination of the distress period that may be experienced with the CO_2 or isoflurane method of mouse euthanasia depends on when insensibility occurs. Anaesthetic depth in mice can be assessed many ways, such as recumbency, loss of the righting reflex, loss of the pedal withdrawal reflex, and loss of the palpebral and corneal reflexes. A study by Coenen et al. (1995) suggested that when euthanizing rats with CO_2, loss of posture and muscle tone (i.e. recumbency) was correlated with the onset of an abnormal electroencephalogram pattern and the authors suggest this indicates loss of consciousness. However, loss of the righting reflex is commonly used to assess insensibility in many animal species, such as rats, mice, and goats (Antognini et al., 2005; Franks et al., 2008). It has been suggested that failure to respond to a verbal command in humans is correlated with loss of the righting reflex in rodents, both signifying loss of consciousness (Franks et al., 2008). The correlation between loss of consciousness and lack of ability to respond to a verbal command in humans has been recognized since the introduction of anaesthesia (Alkire et al., 2008). However, this definition may be problematic, as unconsciousness may not equate with unresponsiveness. For example, low dose ketamine causes decreased motivation to respond to verbal commands (Tucker et al., 1984), while at higher doses patients appear unresponsive, but neuro-imaging studies show complex changes in the brain suggesting a level of consciousness (Langsjo et al., 2005). It has also been suggested that awareness (appropriate response to a command) and memory may be lost at anaesthetic concentrations less than 50% of those needed to abolish movement (Antognini et al., 2005). A study by Zacny et al. (1994) testing sub-anaesthetic concentrations of isoflurane in humans resulted in subject reports of feeling confused, sedated and carefree. This may be expected, as isoflurane causes analgesia and sedation in a dose-dependent way (Antognini et al. 1997; Schlunzen et al 2006).

Veterinarians commonly use loss of the pedal withdrawal reflex to assess when a surgical plane of anaesthesia has been reached and therefore painful surgery may be performed. Many parameters are used to assess insensibility in animals, but the most common are recumbency, loss of the righting reflex and loss of the pedal withdrawal reflex. In animals, nociceptive reflexes such as loss of righting and loss of pedal withdrawal are used to assess pain, but it is not clear if these reflexes decrease with motor or pain-related neural activity, or both (Antognini et al., 2005). Given the lack of knowledge in this area, several indicators should be used when assessing unconsciousness in mice undergoing anaesthesia and euthanasia.

1.5 Thesis goals

Laboratory mice may experience distress before the point of insensibility when exposed to gradual-fill CO_2. Current recommended flow rates are meant to induce unconsciousness before concentrations become painful, but mice likely experience fear and anxiety associated with dyspnea at lower concentrations. When isoflurane is used during euthanasia, it is commonly followed by a high flow rate of CO_2 gas when insensibility is reached to avoid the time and expense necessary to kill an animal with isoflurane alone. Currently there is no scientific basis for recommending when the switch should be made to avoid pain associated with higher CO_2 concentrations. As well, there is a lack of evidence examining mouse aversion to CO_2 and isoflurane gas, and no assessment of the drop method of isoflurane administration.

The aim of my thesis was to fill the gaps in the literature described above by testing mouse aversion to CO_2 and isoflurane, as well as two methods of isoflurane administration. The overall goal was to gather science-based evidence for more humane methods of mouse euthanasia.

Chapter 2: The effect of carbon dioxide flow rate on laboratory mice

2.1 Introduction

At the end of a study carbon dioxide (CO_2) gas is commonly used to kill laboratory mice. Current guidelines suggest that when using this method, the euthanasia chamber should be filled gradually using a flow rate between 10-30% chamber vol/min of CO_2 (AVMA, 2013; CCAC, 2010). Use of flow rates lower than 30% chamber vol/min are thought to reduce the likelihood that CO_2 concentration in the chamber will exceed painful levels (>40%) before insensibility is reached (Ambrose et al., 2000).

Unfortunately, pain is not the only welfare concern associated with exposure to CO_2. Humans report sensations of dyspnea, defined as an experience of breathlessness or air hunger, at concentrations of CO_2 as low as 7% (Liotti et al., 2001) and this feeling has been described as distressing in many studies (Banzett et al., 1990; Lansing et al., 2000; O'Driscoll et al., 1999; von Leupoldt and Dahme, 2005). The experience of dyspnea has been used to induce fear and panic in humans using CO_2 concentrations between 7.5-35% (Bailey et al., 2005; Feinstein et al., 2013; Gorman et al., 1984; Pappens et al., 2012), exposure to these concentrations of CO_2 also causes fear responses in animals (Ziemann et al., 2009; Concas et al., 1993). In human literature, the negative emotional response resulting from dyspneic experiences results in aversion (Lansing et al., 2009; Steel and Shaver1992). Sensations of dyspnea may also explain why mice and rats are unwilling to tolerate exposure to CO_2 even at relatively low concentrations (<20%) (Kirkden et al., 2008; Leach et al., 2002; Makowska et al., 2009; Niel et al., 2008; Niel and Weary 2007; Wong et al. 2013). The term dyspnea contains both an affective and behavioural response in the human literature. In the veterinary literature, dyspnea more typically refers to laboured breathing

in animals. In this study, laboured breathing was directly assessed but we cannot be certain that this behaviour was associated with negative affect. However, on the basis of human evidence and the rodent research on aversion responses, we posit that this behavioural response is accompanied by a negative affective experience. On this basis, we argue that euthanasia methods that minimize the duration of laboured breathing should be considered more humane.

The aim of this study was to examine the effect of CO_2 flow rate on the interval from the onset of laboured breathing until loss of sensibility. This interval is the period during which the mice may consciously experience negative affect associated with dyspnea. There are at least three progressive measures of loss of sensibility during euthanasia: recumbency, loss of the righting reflex and loss of the pedal withdrawal reflex (Table 1). Loss of the pedal withdrawal reflex is an autonomic response of the hind limb that is assessed by pinching the hind paws (Whelan and Flecknell, 1992). This reflex is commonly used to determine a surgical plane of anaesthesia when an animal cannot experience pain and surgery may be performed (Whelan and Flecknell, 1992). Loss of the righting reflex is commonly assessed during rodent euthanasia by tilting the euthanasia box to roll the animal and examine self-righting behaviour (Thomas et al., 2012); this measure cannot be performed practically when enclosures are large and heavy, such as in some automated euthanasia systems. The onset of recumbency is the simplest measure, as only examination of posture and muscle tone is required. However, animals may be able to experience unpleasant sensations beyond this point.

We hypothesized that higher flow rates would minimize the experience of dyspnea as measured from the onset of laboured breathing to recumbency, loss of the righting reflex, and loss of the pedal withdrawal reflex, for mice euthanized using the gradual-fill method of CO_2 euthanasia.

2.2 Methods and materials

2.2.1 Animals and housing

We used 24 surplus naive female albino C57Bl/6J-Tyr mice at the University of British Columbia's Centre for Comparative Medicine, Vancouver, Canada. All mice were five months old during testing and weighed between 21.6 – 28.4 g. Mice were group housed in an OptiMICE®(Animal Care Systems, USA) cage system with autoclaved clean polycarbonate cages (Makrolon®, Animal Care Systems, USA) with dimensions 31.8 cm long x 27.9 cm wide front x 8.9 cm wide rear x 12.9 cm height. Each cage contained autoclaved ECOfresh™(Absorption Corporation, WA, USA) bedding, a nest box, a cotton nesting square (Ancare, USA), brown crinkle paper (Enviro-dri®, Shepherd Specialty Paper, USA) and free access to food (5001 PMI Lab Diet, Harlan Laboratories Inc., IN, USA) and filtered water. The average humidity and temperature during testing were 48% and 23ºC, respectively. Mice were kept under 12-h light: 12-h dark cycle; testing took place during the light phase (between 8:00-10:00) on two consecutive days. The University of British Columbia's Animal Care Committee approved all procedures used in this study.

2.2.2 Experimental apparatus

An Innocage® mouse disposable IVC transparent mouse cage (Universal Euro Type II Long, Innovive Inc., 37.3 cm L x 23.4 cm W x 14.0 cm H, with 205 cm^2 floor space) was used as the test cage. On one side of the cage a hole was cut to project a powdered surgical latex glove (Perry® Style 42®, Ansell, size 7) sealed with tape. This allowed one hand to be placed into the cage during testing. A non-slip pad (Shaw Floors, wood flooring underlayment) was cut to fit the bottom of the test cage to minimize slipping. A clear Plexiglas lid with a small hole in the middle

was placed on top of the cage to allow insertion of a tube to deliver CO_2. Before each trial, 500 ml of aspen-chip bedding was added to the test cage and removed after each euthanasia procedure. The tube used to deliver gas to the test cage was connected to a CO_2 tank (Praxair, Canada) and the flow was measured with a CO_2 flow meter (Western Medica, USA). A small hole near the base of the cage in the centre of the anterior wall of the test cage allowed insertion of the sampling tube connected to an oxygen (O_2) analyzer (Series 2000, Percent Oxygen Analyzer, Alpha Omega Instrument Corporation, USA).

2.2.3 Oxygen analyzer testing

Prior to experimental testing, the lag time of the O_2 analyzer was measured as the time from insertion of an anoxic sample until the reading on the analyzer began to decline. Repeat testing showed this delay to be 10 s. To assess variability in CO_2 concentration within the test cage, the sampling tube was placed in seven different areas of the cage while filling the cage with CO_2 at 20% cage vol/min. Oxygen concentrations were recorded every 15 s for 5 min. CO_2 concentration in the test cage was calculated: $[CO_{2\,(t=x)}] = 100 - (100 \times ([O_{2\,(t=x)}] / [O_{2\,(t=0)}]))$. Values for the left anterior corner were found to be most similar to the average readings for the test cage.

2.2.4 Experimental procedure

One researcher retrieved a mouse from the housing room while another cleaned the apparatus, added 500ml of bedding, a stainless steel 8.9 cm straight mosquito hemostat (Lawton, Germany), and placed the O_2 sampling tube through the cage hole into the left anterior corner of the test box. During the trials the O_2 analyzer readings were monitored and the gas flow was adjusted to

hold the CO_2 concentration in the test cage just below a 40% concentration; in this way conscious mice were never subjected to CO_2 concentrations associated with pain.

At the beginning of each trial a mouse was placed into the prepared test cage. The Plexiglas lid was placed on top of the cage and the CO_2 tube was projected through the cage lid. A researcher then placed one hand into the test cage glove and kept the hand motionless on the floor of the cage. Trials began with an onset of CO_2 into the cage; mice were randomly assigned to one of four flow rates: 20 (n=6), 30 (n=6), 40 (n=6), or 50 (n=6) percent cage vol/min. Once the mouse was recumbent the experimenter (blind to treatment) tested for loss of the righting reflex by placing the mouse on its back. Three mice attempted to escape the approaching hand and in these cases the researcher waited until the animal was recumbent again for 3 s, before retesting. Immediately after failure to self-right, loss of the pedal withdrawal reflex was tested then retested every 10 s using the hemostat (clamping down until reaching the first notch) to pinch alternating left and right hind paw inter-digital webbing. Loss of the pedal withdrawal reflex was signified by the absence of a response in 3 consecutive pinches. After loss of this reflex, gas flow was increased to 60% cage vol/min until the mouse was no longer breathing. The gas was then turned off and cervical dislocation was used to ensure death.

2.2.5 Data collection

Each trial was recorded using an HDC-TM41 Panasonic camera (Malaysia). Videos were scored for: onset of laboured breathing, onset of recumbency, loss of the righting reflex, and loss of the pedal withdrawal reflex (Table 1), with observers blind to treatment. We calculated the interval between onset of laboured breathing – onset of recumbency, onset of laboured breathing – loss of the righting reflex, and onset of laboured breathing – loss of the pedal withdrawal

reflex. Before the experiment, all behavioural scoring was practiced to establish inter-observer reliability. Video recordings of previous mouse euthanasia procedures were viewed and scored independently by two observers; all score times were consistent (± 2 s) between observers. One observation from the 30% treatment group was identified as an extreme outlier (more than 3 standard deviations above the mean) for loss of the pedal withdrawal reflex and was removed from the analysis.

Table 2.1 Definitions of mouse behaviours used in this study to assess dyspnea and various levels of insensibility

Behaviour	**Definition**
Onset of laboured breathing	Deep rapid breathing
Onset of recumbency	Head resting on cage floor, head and body motionless, loss of muscle tone
Loss of righting reflex	Unable to self right when placed on back
Loss of pedal withdrawal reflex	The first of three consecutive non-responses to alternating hind paw pinches

2.2.6 Statistical analysis

The effect of flow rate (3 d.f.) on behavioural responses was tested with a general linear model (Proc GLM in SAS v. 9.3) that included home cage as block (with 8 d.f.) and mouse body weight as a co-variate (1 d.f.). Below we report least-square means ± one standard error.

2.3 Results

The first sign of dyspnea (i.e. the onset of laboured breathing) occurred approximately 14 s after the start of gas flow; onset of dyspnea did not vary with gas flow ($F_{3,22}$=0.70, P=0.56; Table

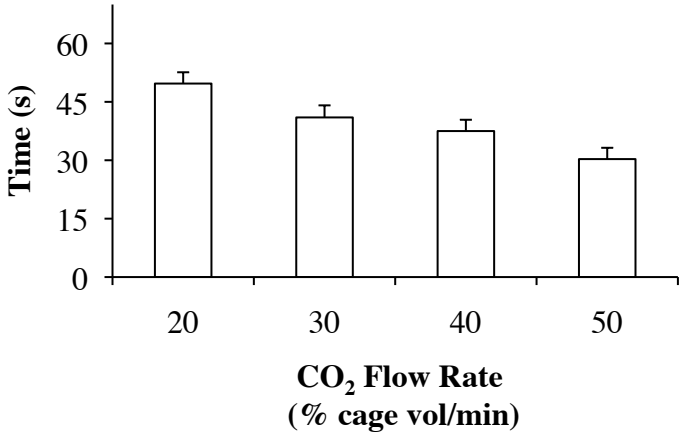
2.2). Our most conservative estimate of insensibility (loss of the pedal withdrawal reflex) occurred approximately 109 s after CO_2 began to flow into the cage, again with no effect of flow rate ($F_{3,22}$=0.66, P=0.59), but with considerable between-subject variation. The interval between the onset of laboured breathing and the pedal withdrawal reflex averaged approximately 95 s, and was also not affected by gas flow ($F_{3,22}$=0.57, P=0.64). Less conservative estimates of insensibility (recumbency and loss of the righting reflex) did vary with gas flow ($F_{3,22}$=7.12, P=0.0021 and $F_{3,22}$=11.68, P=0.0001, respectively). Mice became recumbent approximately 22 s sooner when exposed to 50% versus 20% cage vol/min flow rates of CO_2. Similarly, loss of the righting reflex occurred at approximately 51 s at the highest flow rate versus 75 s at the lowest flow rate. The interval between onset of laboured breathing and onset of recumbency was also lower for the higher flow rates ($F_{3,22}$=7.83, P=0.0013, Figure 2.1a), as was the interval between onset of laboured breathing and loss of the righting reflex ($F_{3,22}$=13.62, P<0.0001, Figure 2.1b).

Table 2.2 Mean (± S.E.) time of first sign of dyspnea and three measures of insensibility in mice euthanized using gradual-fill CO_2 at flow rates of 20, 30, 40, and 50% chamber vol/min

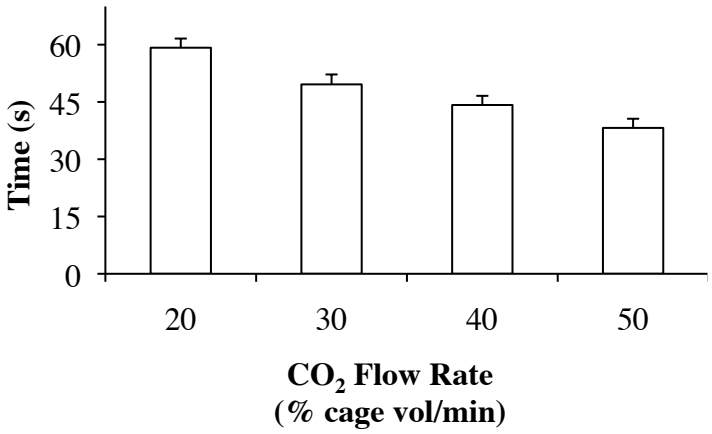
Behavioural parameter	**CO_2 flow rates (% cage vol/min)**			
	20	**30**	**40**	**50**
Dyspnea onset	15.5 ± 1.5	13.8 ± 1.6	12.8 ± 1.5	13.0 ± 1.5
Recumbency onset	65.2 ± 3.4	54.8 ± 3.8	50.3 ± 3.4	43.3 ± 3.4
Loss of righting reflex	74.7 ± 2.9	63.4 ± 3.2	57.0 ± 2.9	51.2 ± 2.9
Loss of pedal reflex	119.2 ± 10.0	98.8 ± 11.0	110.3 ± 10.0	106.2 ± 10.0

Figure 2.1 Mean (±S.E.) period during which mice exposed to gradual-fill of CO_2 gas may have consciously experienced dyspnea, in relation to the flow rate of CO_2 introduced into the chamber. Two periods are shown: (a) onset of laboured breathing until recumbency, and (b) onset of laboured breathing until loss of the righting reflex.

a)



b)



2.4 Discussion

The onset of laboured breathing occurred at a similar time for all the flow rates tested, suggesting that dyspnea may begin soon after exposure to even low concentrations of CO_2. Gas

flow rate had a stronger effect on the time until mice became recumbent and lost the righting reflex (and also on the intervals between the onset of laboured breathing and these measures); these results are consistent with the idea that loss of sensibility will occur more quickly when animals are exposed to higher cumulative dose of anaesthetic (Clark and Rosner, 1973).

Loss of the pedal withdrawal reflex did not vary with flow rate. However, this measure showed considerable variability among mice. Past studies suggest that use of a hemostat to assess loss of the pedal withdrawal reflex is the best indicator of a surgical depth of anaesthesia in mice (Arras et al., 2001; Buitrago et al., 2008). However, the previous studies used a locking pin on the hemostat for greater standardization of the pinch, whereas we used the first notch on the hemostat. The variation in this measure may indicate difficulty in applying the hemostat in a consistent manner or differences among animals in their response to the pinch. We suggest future work is needed to assess the repeatability of methods used to assess loss of the pedal withdrawal reflex.

We used the interval from onset of laboured breathing to: 1) recumbency, 2) loss of the righting reflex, and 3) loss of the pedal withdrawal reflex, to assess the time when mice may experience unpleasant sensations associated with dyspnea. Which of the three intervals are most relevant depends upon when mice undergoing gradual-fill CO_2 euthanasia, are no longer able to experience negative affect. From a welfare perspective, the best case would be that mice are unresponsive after the onset of recumbency, suggesting that they consciously experience between 30 - 50 s of dyspnea, depending on flow rate. However, the worst case is that they are able to experience negative affect associated with dyspnea up until loss of the pedal withdrawal reflex (i.e. 90 s or more) with no benefit from a faster flow rate.

A study by Ziemann et al. (2009) examined four paradigms to assess CO_2 as a fear-inducing stimulus in mice by examining CO_2 and: 1) freezing behaviour, 2) open–field test, 3) aversion, and 4) fear conditioning. A 10% concentration of CO_2 was found to cause more freezing behaviour and reduce time in an open-field test. As well, mice with the choice between a chamber with <2% CO_2 or 15% CO_2 spent >90% of their time in the chamber with the lower CO_2 concentration. In the fear conditioning test, mice subjected to 10% CO_2 before and while receiving foot shocks, showed significantly more freezing behaviour than those not subjected to CO_2 while receiving the foot shocks. When re-tested the following day without CO_2, mice again showed more freezing behaviour than those mice never subjected to CO_2. These results indicate that CO_2 gas, even at low concentrations, is both fear inducing and aversive in mice. A series of studies have now shown that rodents do not willingly tolerate exposure to even relatively low CO_2 concentrations (Kirkden et al., 2008; Leach et al., 2002; Makowska et al., 2009; Niel et al., 2008; Niel and Weary 2007; Wong et al., 2013). If CO_2 is used to kill mice, refinements to minimize distress during this procedure are important. Our study results indicate that a gradual-fill CO_2 flow rate of 50% cage vol/min reduced the period from the onset of laboured breathing until the onset of recumbency and loss of the righting reflex. When using this flow rate, a gas holding technique should be used to ensure that those painful CO_2 concentrations (>40%) are not reached until after insensibility occurs. This can be achieved by manually controlling the flow meter or by using a programmable automated euthanasia machine.

2.5 Conclusion

The results of this study indicate that sensations of dyspnea can be minimized when euthanizing mice with gradual-fill CO_2 by way of a flow rate of 50% chamber vol/min, provided

that the total concentration in the cage is held to below 40% to ensure mice are not exposed to high CO_2 concentrations associated with pain. Even when using this refinement, mice likely experience more than 30 s of dyspnea. We suggest that when possible other methods of euthanasia should be used.

Chapter 3: Testing three measures of mouse insensibility following induction with isoflurane or CO_2 for a more humane euthanasia

3.1 Introduction

Laboratory rodents are commonly euthanized via exposure to gradually increasing concentrations of carbon dioxide (CO_2) gas. However, mice and rats find CO_2 gas aversive, and are unwilling to tolerate exposure to CO_2 at concentrations sufficient to cause insensibility (Kirkden et al., 2008; Leach et al., 2002; Makowska et al., 2009; Niel et al., 2008; Niel and Weary, 2007). Isoflurane is less aversive to mice and rats than CO_2 (Makowska et al., 2009; Wong et al., 2013) and other inhalant anaesthetics (Makowska and Weary, 2009). Isoflurane is used to induce insensibility; once unconscious the gas can be switched to CO_2 to avoid the time and expense necessary to kill an animal with isoflurane alone. It is important that the mice are indeed insensible when the switch from isoflurane is made, because exposure to concentrations of CO_2 >15% are aversive (as cited above) and exposure to concentrations >40% causes pain due to the conversion of CO_2 into carbonic acid on mucosal surfaces (Anton et al., 2005; Thurauf et al., 2002).

One recent study reported that 5 of 10 mice euthanized with isoflurane followed by CO_2 regained consciousness after isoflurane was switched to CO_2 (Valentine et al., 2012); recovery during the procedure represents the worse case scenario as animals experience the negative effects of isoflurane induction and exposure to high concentrations of CO_2. One reason why animals may recover during this procedure is that depth of anaesthesia is insufficient. There has been no research on methods to establish the appropriate depth of isoflurane-induced anaesthesia

before exposure to high concentrations of CO_2. The aim of the current experiment was to evaluate three measures of insensibility and use these to establish when to switch from isoflurane to a high flow rate of CO_2 gas when euthanizing laboratory mice. For comparison, we also examined these same response measures for mice exposed to gradual-fill CO_2, as some users increase the flow rate of CO_2 when animals are thought to be insensible to decrease time to death.

3.2 Materials and methods

3.2.1 Animals and housing

Thirteen surplus C57Bl/6J male mice slated for euthanasia by The Centre for Disease Modeling at the University of British Columbia, Vancouver, Canada were used for the study. Mice were housed under a 12-h light: 12-h dark cycle, weighed between 28 and 34 g and were between 3-4 months old. Testing occurred between 12:30-15:00 during the light cycle. Mice were group-housed in ventilated polysulfone type IIL cages (EHRET, Germany) on a ventilated Bio A.S. IVC rack (Ehret, Germany) complete with corncob bedding (7087 Soft Cob Bedding, Harlan Teklad, USA), a transparent tinted polycarbonate mouse igloo (Bio-Serve, USA), brown crinkle paper (Enviro-dri, Shepherd Specialty Papers, USA) and one cotton nest square (Ancare, USA) per cage. All animals were given *ad libitum* access to food (irradiated Global Rodent Diet 2918, Harlan Teklad, USA) and reverse osmosis water in autoclaved 250 ml water bottles (Ehret, Germany). Average humidity and temperature during testing were 62% and 21.2°C, respectively. The University of British Columbia's Animal Care Committee approved all animal procedures used during this study.

3.2.2 Experimental apparatus

The test cage consisted of an Innocage® mouse disposable IVC transparent mouse cage (Universal Euro Type II Long, Innovive Inc., 37.3 cm L x 23.4 cm W x 14 cm H) in which we cut a hole in the side used to project a surgical nitrile glove (Kimtech Pure G3, 30.5 cm Size 7) sealed using tape. This allowed one hand to be placed into the cage during testing. A non-slip pad (Shaw Floors, wood flooring underlayment) was cut to fit the bottom of the test cage to minimize slipping, and 500ml of autoclaved bedding identical to that in the home cage, was added to the test cage between trials. A clear Plexiglas lid with a small hole in the middle was placed on top of the cage. The hole allowed a vinyl tube to be inserted into the cage to deliver either: 1) 20% cage vol/min of CO_2 gas (Praxair, Canada) with the flow measured by a CO_2 flow meter (Western Medica, USA) or, 2) 5% isoflurane (Baxter Corporation, Mississauga, Canada) via an Isotec 4 isoflurane vaporizer (Ohmeda, Steeton, West Yorkshire, England) using 2 l/min (17% cage vol/min) of O_2 (Praxair, Canada) as the carrier gas.

3.2.3 Experimental design

Mice were randomly assigned to isoflurane (n=7) or CO_2 (n=6) treatments. Mice were subject to the euthanasia procedure individually while all other mice were kept in the housing room. The experimental apparatus was wiped with PerCept™ (Diversey, USA) cleaning spray between trials. Mice were tested using three measures of insensibility (Table 3.1). The experimenter administering the tests of sensibility was blind to the treatment (isoflurane or CO_2) and could not see controls to the gas tanks or the vaporizer.

Table 3.1 Definitions for the behavioural insensibility indicators of mice applied to the isoflurane and CO_2 methods of euthanasia.

Behaviour	Definition
Recumbency onset	Head resting on cage floor, head and body motionless, loss of muscle tone
Loss of righting reflex	Unable to self right when placed on its back
Loss of pedal withdrawal reflex	The first of three consecutive non-responses to alternating hind paw pinches, applied every 10 s between the metatarsal and phalanges bone

3.2.4 Experimental Procedure

Mice were transferred individually from the housing room to the test room using a plastic transparent cage. Once a mouse was placed into the experimental apparatus, the cage lid was positioned on the cage, the blinded experimenter placed a hand into the glove that projected into the cage and gas flow began. The experimenter's hand remained motionless on the cage floor until the mouse became recumbent. The experimenter then moved the gloved hand towards the mouse; if no response was elicited the mouse was placed on its' back to test for loss of the righting reflex. Leg paddling, forward or lateral movement away from the hand was taken as evidence of an escape response. Leg paddling was also taken as evidence of purposeful movement during the loss of the righting reflex, even if the mouse failed to self-right. Once unable to self-right, loss of the pedal withdrawal reflex was tested using three consecutive toe pinches on alternating hind paws between metatarsal and phalange bones. A response at any stage (i.e. escape attempt during initial approach, righting or other purposeful movement during the righting reflex, or pedal withdrawal following any toe pinch) resulted in the experimenter waiting until the mouse satisfied that insensibility definition, followed by re-testing at the same stage. Once a mouse showed loss of the pedal withdrawal reflex, the treatment gas was turned off

and CO_2 gas at 60% chamber vol/min was introduced until last breath. All gas was turned off and the mouse left in the cage for an additional 3 min. The mouse was then removed from the cage and weighed. Cervical dislocation was performed as a secondary method of euthanasia. Each trial was video recorded using a Panasonic HDC-TM41 camera (Malasyia) and all responses were scored from video with the observer blind to treatment.

3.2.5 Statistical analysis

The effect of treatment on recumbency onset, loss of the righting reflex, loss of the pedal withdrawal reflex, time to last breath and time from the switch to 60% chamber vol/min flow rate of CO_2 until last breath was analyzed using a general linear model (SAS v. 9.3). The effects of treatment on the number of mice responding to different tests of insensibility were analyzed using a Fisher's exact test.

3.3 Results

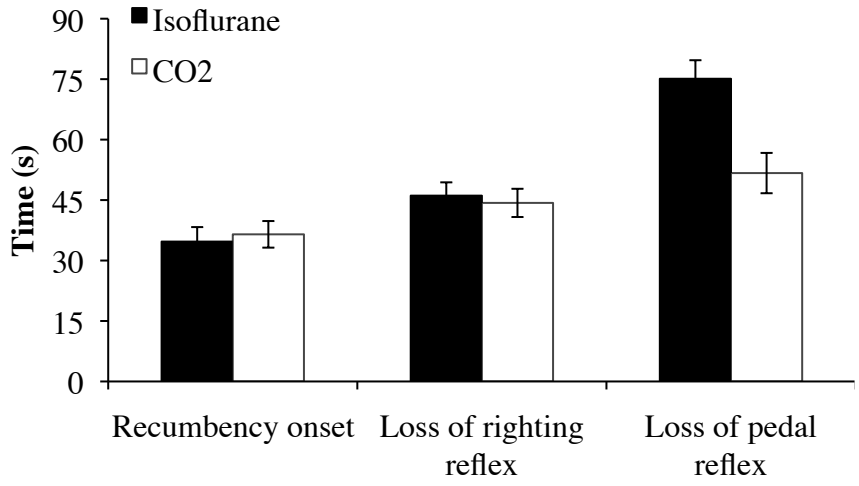
The time between start of gas flow and the first sign of insensibility (i.e. recumbency) did not differ between treatment ($F_{1,12}$=0.13, P=0.72; Figure 3.1), but once recumbent, mice exposed to isoflurane were more likely to respond to the approaching hand than mice exposed to CO_2 (7/7 versus 2/6 mice, respectively; P=0.02). Of those showing an escape response, six isoflurane and no CO_2 mice showed forward or lateral escape movements away from the hand (P=0.0005).

The time between the start of gas flow and loss of the righting reflex also did not differ between treatments ($F_{1,12}$=0.14, P=0.71; Figure 3.1), but when testing for loss of this response all isoflurane mice demonstrated purposeful movement versus none of the mice tested in the CO_2 treatment (P=0.0006).

The time until loss of the pedal withdrawal reflex was approximately 25 s longer when using 5% isoflurane versus CO_2 at a 20% chamber vol/min flow rate ($F_{1,12}$=12.0, P=0.005; Figure 3.1). All mice in the isoflurane treatment responded at least once to the toe pinch versus just one of the mice tested in the CO_2 treatment (P=0.005). The mean (± S.D.) time between the first sign of insensibility (recumbency) and the most cautious measure (loss of the pedal withdrawal reflex) averaged (±S.D.) 40.4 ± 12.9 s for isoflurane versus just 15.7 ± 10.9 s with CO_2.

The time from beginning of the procedure until last breath averaged 222.1 ± 5.4 s for isoflurane versus 114.8 ± 5.8 s for CO_2 ($F_{1,12}$=182.2, P< 0.0001). Time from the switch to 60% cage vol/min flow rate of CO_2 until last breath was 112.6 ± 3.9 s for isoflurane versus 25.0 ± 4.2 s for CO_2 ($F_{1,12}$=234.2, P<0.0001). No mouse from either treatment showed any sign of purposeful movement after the switch to a high flow rate of CO_2.

Figure 3.1. Least Squares Means (±S.E.) of recumbency onset, loss of the righting reflex, and loss of the pedal reflex for mice exposed to the isoflurane (n=7) and CO_2 (n=6) methods of euthanasia



3.4 Discussion

CO_2 and isoflurane have different modes of action. CO_2 affects the central nervous system by decreasing the pH of the cerebral spinal fluid (Eisele et al., 1967; Lee et al., 1996; Martoft et al., 2003); the decreased pH disrupts neuron function and decreases cerebral electrical activity (Lee et al., 1996; Woodbury et al., 1958) leading to anaesthesia and narcosis (Eisele et al., 2003; Maroft et al., 2003). Isoflurane inhibits neurotransmitter pathways in the central nervous system (Campagna et al., 2003; Herring et al., 2009; Westphalen et al., 2013). Isoflurane is known to have the largest safety margin of the inhalant anaesthetics (Stimpel and Gershey, 1991), which explains the longer induction time and time to last breath in the current study.

Recumbency and loss of the righting reflex are commonly used to indicate insensibility. For example, it has been suggested that loss of consciousness (failure to respond to a verbal command) in humans is related to loss of the righting reflex in rodents when using isoflurane anaesthesia (Franks, 2008). However, humans may understand verbal questions but be unable to respond to them, suggesting that verbal responsiveness may not be the best correlate of consciousness (Alkire et al., 2008; Owen et al., 2006; Veselis et al., 2002).

In our study mice were tested for loss of the righting reflex after recumbency onset. Even though recumbent, all mice in the isoflurane treatment and two mice in the CO_2 treatment attempted to escape from the approaching hand, indicating that they were still conscious. In addition, purposeful movement was exhibited by all of the isoflurane treatment mice when the experimenter checked for loss of the righting reflex. These differences suggest that the onset of recumbency and loss of the righting reflex may be more appropriate measures of loss of sensibility when using gradual-fill CO_2 than when using isoflurane. Indeed, isoflurane is used for conscious sedation in human procedures where the patient remains conscious, but may or may

not be able to respond to verbal commands (Rodrigo and Rosenquist, 1988; Sparacino et al., 1999).

Loss of the pedal withdrawal reflex was our most conservative measure of insensibility. This autonomic motor response of the hind limb is commonly used to determine when a surgical plane of anaesthesia has been reached (Arras et al., 1991; Buitrago et al., 2008; Whelan and Flecknell, 1992). A lighter plane of anaesthesia may be sufficient to prevent purposeful movement but insufficient to prevent the pedal withdrawal reflex (Antognini et al., 2005). For the purposes of the current study, the relevant question is at what point do mice no longer perceive the pain and aversion associated with exposure to CO_2? Minimum alveolar concentration sufficient to inhibit responses to pain vary considerably across mouse strains (Mogil et al., 2005; Sonner et al., 2000; Sonner et al., 1999), suggesting a wide safety margin is required to reduce the risk of suffering during euthanasia. On average (±S.D.), mice in the current study lost the pedal withdrawal reflex 40 ± 13 s and 16 ± 11 s after the onset of recumbency during the isoflurane and CO_2 treatments, respectively. After 79 and 49 s (i.e. mean + 3 S.D.) more than 99% of the mice would likely be at a surgical plane of anaesthesia before exposure to high concentrations of CO_2 when using the 5% isoflurane and 20% chamber vol/min gradual-fill CO_2 methods for euthanasia, respectively.

3.5 Conclusion and recommendation

Isoflurane induction is a humane alternative to gradual-fill CO_2 for euthanasia, but once rendered insensible, mice may be killed using exposure to high concentrations of CO_2 gas. When using isoflurane for induction, we recommend that upon the appearance of recumbency users wait a minimum of 79 s (when using 5% isoflurane and a 17% chamber vol/min fill rate with O_2

as the carrier gas) before switching to CO_2.

Chapter 4: Mouse aversion to isoflurane versus carbon dioxide gas

4.1 Introduction

Current laboratory mouse euthanasia guidelines recommend using an inhalant anaesthetic over carbon dioxide gas (CO_2) for rodent euthanasia (AVMA, 2013; CCAC, 2010). Current evidence suggests isoflurane is less aversive to mice and rats than CO_2 (Makowska and Weary 2009; Wong et al., 2013) and other inhalant anaesthetics (Makowska et al., 2009). Isoflurane is a volatile liquid halogenated hydrocarbon. Generally, one of two methods can be used to administer isoflurane for euthanasia: a vaporizer machine or the drop method. A scavenging system and a carrier gas is required when using an isoflurane vaporizer, and some animal users have argued that the use of a vaporizer is unnecessary for rodent euthanasia. In addition, vaporizer machines can be costly to purchase and maintain, likely reducing accessibility for some users. Alternatively, animal users may use the drop method, which involves placing liquid isoflurane on an absorbent material such as gauze, and placing this in a closed chamber. Despite the practicality of the drop method, to our knowledge no studies have compared aversion to the drop versus vaporizer methods of isoflurane administration. In addition, many laboratories still use the gradual-fill method of CO_2 for euthanasia. Thus we tested mouse aversion to isoflurane administered by a vaporizer, isoflurane administered via the drop method, and gradual-fill CO_2.

The light-dark paradigm is a conflict-based anxiety test originally developed by Crawley and Goodwin (1980) to test anti-anxiety medications on mice. This paradigm uses the innate unconditioned preference for dark versus light areas and fear of open spaces in mice. The light-dark apparatus is composed of three compartments, a large light chamber, a small dark chamber and a middle chamber separating the light and dark areas. Habituation to the apparatus changes

this novel environment into a familiar one, therefore producing a light aversion test instead of testing anxiety (Matynia et al., 2012). This paradigm has been previously used to test rat aversion to CO_2 versus isoflurane in rats (Wong et al., 2013).

Using the light-dark box paradigm, we tested mouse aversion to: 1) 20% gradual-fill chamber vol/min of CO_2, 2) 5% isoflurane administered using a vaporizer with a flow of oxygen (O_2) set at 4 l/min (40% chamber vol/min), 3) 5% isoflurane administered using the drop method. Mice were able to choose between remaining in the small dark chamber with a rising concentration of one of three treatments or escaping to a larger brightly lit chamber. Initial exposure aversion was examined for all treatments. In addition, re-exposure aversion was examined for the isoflurane vaporizer treatment; mice commonly undergo surgical procedures using an isoflurane vaporizer machine, so exposure to isoflurane during euthanasia may not be their first exposure.

In a pre-trial, we measured the rate at which isoflurane concentrations increased within a chamber when using the vaporizer and drop treatments; this allowed us to estimate the approximate concentrations that mice would likely be exposed to during the experiment.

4.2. Materials and methods

4.2.1 Pre-trial: rising isoflurane concentration in the vaporizer and drop conditions

An Innocage® mouse disposable IVC transparent mouse cage (Universal Euro Type II Long, Innovive Inc. USA, 37.3 cm L x 23.4 cm W x 14.0 cm H, with 205 cm^2 floor space) was used as the test cage. A Plexiglass lid with a centrally placed hole was placed on top of the cage during testing. A Capnomac Ultima™(Datex Ohmeda, Finland) capnograph was used to measure the rising concentration of isoflurane in the cage, via a PE/PVC sampling line (Datex Ohmeda,

Finland) inserted into a hole near the base of the anterior wall of the cage. Testing took place in Medical Block C at the University of British Columbia, Vancouver, Canada.

For the isoflurane vaporizer treatment, 5% isoflurane (Baxter Corporation, Canada) was administered via an Isotec 4 isoflurane vaporizer (Ohmeda, Steeton, England) using 4 l/min (33% chamber vol/min) of room air as the carrier gas. The isoflurane drop treatment used wire mesh (Activa, USA) to create a rectangular apparatus (11 cm x 7 cm x 3 cm), which held a piece of 5.1 cm x 5.1 cm gauze (Professional Preference, Canada) opened length wise to stretch the entire apparatus. The volume of isoflurane required to provide a 5% concentration in the chamber was determined to be 4.6 ml using the universal gas law (PV=nRT) and a room temperature of 22°C. The liquid isoflurane was drawn through a glass syringe and dropped onto the gauze within the wire mesh apparatus.

4.2.2 Experiment

4.2.2.1 Animals and housing

We used thirty male C57Bl/6J mice were housed at the University of British Columbia's Centre for Disease Modeling, Vancouver, Canada. All mice were group housed, weighed between 22.4-28.3 g, and were two months old at the time of testing. Mice were housed with a nest-box, brown crinkle paper (Enviro-dri, Shepherd Specialty Papers, USA), one cotton nest square (Ancare, USA), beta chip bedding (Nepco, Northeastern Products, USA), *ad libitum* access to food (Harlan 2918 Tekland Global Rodent Maintenance, USA), reverse osmosis chlorinated water, and kept under a reverse 12 h light:12 h dark cycle with light intensity ranging from 240-340 lux throughout the light phase. All animal procedures were approved by the University of British Columbia's Animal Care Committee.

4.2.2.2 Experimental set up and testing apparatus

All testing took place over four days in a darkened test room lit by a low-pressure sodium lamp undetectable to mice (McLennan and Taylor-Jeffs, 2004). Testing took place during the dark cycle between 10:00 and 17:00 h, at an average (±S.D.) room temperature of 20.6 ± 0.5 °C. All mice were placed in a biological safety cabinet within the test room one hour before testing started to allow acclimation.

The Plexiglas test box (67 cm x 20 cm x 25 cm) was divided into three compartments: 1) a light chamber (40 cm x 20 cm x 25 cm), 2) a dark chamber (20 cm x 20 cm x 25 cm), and 3) a middle buffer compartment (6 cm x 6 cm x 6 cm). The middle compartment connected the light and dark compartments, with an opening (6 cm x 6 cm x 6 cm) on either side to allow a mouse to pass between the light and dark compartments. These openings were covered with flexible, overlapping black plastic strips with vertical slits, to minimize gas exchange between the compartments while allowing the mice to pass through. Holes drilled into the side of the middle compartment helped vent any test gas entering this chamber. Black plastic was placed on all sides of the dark compartment, except the front where video recording took place. The experimenter sat away from the testing apparatus and was hidden by a blind. Fresh beta chip bedding was placed into the light (500 ml) and dark (300 ml) compartments between testing cages of mice. A Plexiglas lid with a hole centrally placed above both the light and dark compartments was custom made to fit the box. A lamp (Barometer work lamp, Ikea, China) with a 7-watt line voltage PAR20 LED light (Lightline, Canada) was used as the light source. The lamp head was directed overhead the light compartment to minimize light diffusion into the other compartments.

For isoflurane drop trials, wire mesh (Activa, USA) was molded to create a rectangular shape (11.5 cm x 3 cm x 24 cm) that held a piece of 10.2 cm x 10.2 cm gauze (Professional Preference, Canada) opened length wise to stretch the entire mesh apparatus and increase surface area. This apparatus was placed into the dark side against the end of the dark compartment.

4.2.2.3 Habituation

Mice were randomly assigned to one of three treatments: isoflurane vaporizer, isoflurane drop or CO_2 gas. All mice were habituated to the test apparatus three times each over six days. For the CO_2 treatment, 2 l/min of CO_2 was delivered to the light (10% chamber vol/min flow rate) and dark (20% chamber vol/min flow rate) compartment. For the isoflurane vaporizer treatment, 4 l/min of O_2 was delivered to the light (20% chamber vol/min flow rate) and dark (40% chamber vol/min flow rate) compartment. Separate gas lines for each compartment connected to the same O_2 tank (Praxair, Canada) with separate flow meters (Western Medica, USA). Mice assigned to the isoflurane drop treatment did not receive any gas flow and instead, holes in the Plexigas lid were covered.

At the start of each habituation trial, the light source above the light compartment was turned on and a lux meter (Traceable Dual-Range light meter, VWR International, Radnor, Pennsylvania, USA) was used to measure light intensity in the light and dark compartments. Measurements in the dark compartment did not exceed three lux and the darkest corner of the light compartment exceeded 700 lux. A Panasonic HDC-TM41 video camera (Malaysia) was turned on and a mouse placed into the light compartment to encourage exploration of the dark compartment. The trial ended after 20 min or when one full minute was spent in the dark side (our criterion for preference). The light-dark paradigm is based on mouse aversion to large bright

areas, and preference for darker smaller enclosures. Since the light source used was a LED light, it did not heat up over time. Past studies using the light-dark paradigm have shown that mice find 500 lux aversive (Costall et al., 1989; Matynia et al., 2012). Our light source was aversive to the mice in our study, shown by mouse preference for the dark compartment. Mice show higher frequencies of entries and exits compared to rats (Leach et al., 2002). In this study, mice frequently shuttled back and forth between compartments, spending more time in the dark compartment but rarely staying longer than a minute and a half. On this basis we used the criterion of one full minute in the dark compartment to signify preference.

The test apparatus was cleaned with 70% alcohol between cages of mice and then aired out and wiped with water to decrease any smell or novelty. The apparatus was not cleaned between mice that shared the same home cage (following Hascoet & Bourin, 1998).

4.2.2.4 Testing

Experimental testing followed the habituation procedure exactly, up until a mouse had spent one full minute in the dark side. The CO_2 trials, a gas line connected to a CO_2 tank (Praxair, Canada) and a CO_2 flow meter (Western Medica, USA), was turned to 2 l/min (20% compartment vol/min) and O_2 flow into the dark compartment was discontinued. For all isoflurane vaporizer treatments, isoflurane (Baxter Corporation, Mississauga, ON, Canada) was administered using a MSS isoflurane vaporizer machine (Highland Medical Equipment, USA) at 5% with 4 l/min (40% compartment vol/min) O_2 as the carrier gas. Again, the supplemental O_2 flow into the dark compartment was discontinued. For all isoflurane drop method trials, 3.7 ml (5% volume of isoflurane determined using the universal gas law: PV=nRT, with 20°C room temperature) was syringed onto the gauze in the wire mesh apparatus and the lid closed.

Trials ended when the mouse became recumbent in the dark compartment (Table 4.1), or when two minutes were spent continuously in the light compartment indicating aversion to the dark compartment. Mice that stayed in the dark chamber until recumbent were transferred to an empty cage, placed on a heating pad, and allowed to recover before being placed back into the home cage.

Of 30 mice included in the study, three failed to spend one full minute in the dark side therefore were removed from the study; this left a total of eight mice tested with CO_2 and nine mice with each isoflurane treatments.

Re-exposure occurred only for the isoflurane vaporizer treatment (n=9) one week after initial exposure. These trials followed the same procedure as the initial exposure trials for the isoflurane vaporizer treatment.

After completing these trials, all treatment mice were euthanized using 5% isoflurane gas with 4 l/min of O_2 as the carrier gas, followed by 50% chamber vol/min CO_2 to complete the euthanasia procedure. Mice were weighed and then under went cervical dislocation as a secondary method of euthanasia.

4.2.2.5 Data collection

Both the pre-trial and the experiment were video recorded using the video camera. Pre-trial videos were used to record capnograph readings for the concentration of isoflurane every 5 s. Experiment videos were scored for: latency to leave, recumbency, number of re-entries, and total time spent re-entering the dark compartment after initially leaving, for both initial and re-exposure trials. Only those animals that re-entered the dark compartment were used to analyze number of re-entries and total time spent re-entering the dark compartment.

Table 4.1 Behaviours used to assess mice in the light-dark box.

Parameter	Definition
Latency to leave	Initial time exposed to isoflurane or CO_2 before choosing to leave the dark compartment
Recumbency	Head resting on floor with loss of muscle tone for 5 seconds
Re-entries	Number of times a mouse chose to re-enter the dark side after first leaving
Total time	Time spent re-entering the dark compartment after initially leaving

4.2.3 Statistical analysis

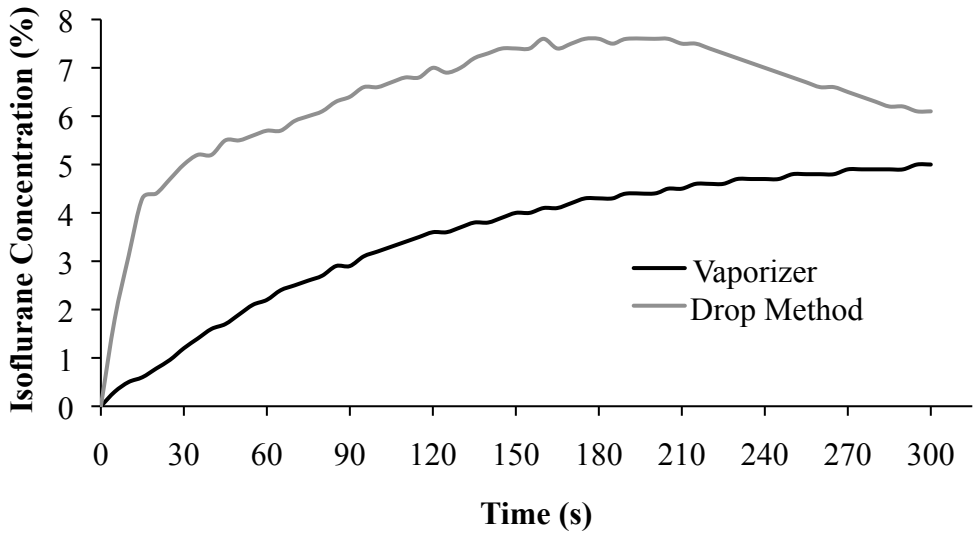
Latency to leave and total time spent re-entering the dark compartment were analyzed for both initial and re-exposure data using a mixed model (SAS v. 9.3) that included mouse as a random effect. A general linear model was used to examine treatment differences in latency to leave, number of re-entries and total time spent re-entering the dark compartment after first leaving. Mice that never left the dark compartment upon gas exposure were not included in the analysis for the number of re-visits or total time spent re-entering the dark compartment. For initial exposure trials, Fisher's exact test was used to compare treatments for the number of mice that became recumbent in the dark side. A Cochran-Mantel-Haenszel test was used to test differences in the number of mice that became recumbent in the dark side between initial versus re-exposure to isoflurane vaporizer. Means are reported ± S.E.M.

4.3 Results

4.3.1 Pre-trial

The drop method of isoflurane administration resulted in a faster increase in concentration compared to the isoflurane vaporizer method (Figure 4.1). The target concentration of 5% was achieved after 5 min when using the vaporizer versus 30 s when using the drop method. The vaporizer isoflurane concentration maximized at 7.5% and then levelled out to 5%, likely due to sampling line placement within the cage.

Figure 4.1 Rising concentration of 5% isoflurane administered by a vaporizer (using 4 l/min O_2 as the carrier gas), compared to the drop method as established in the pre-trial.



4.*3.2 Experiment*

4.3.2.1 Initial exposure

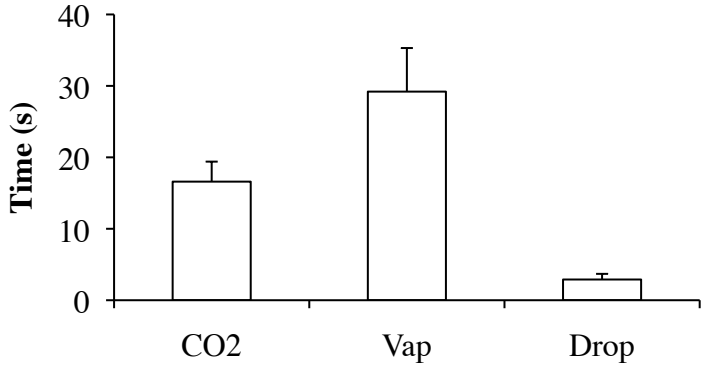
Latency to leave the dark compartment was greater for mice exposed to isoflurane by the vaporizer machine compared to the drop method ($F_{1,17}$=22.6, P<0.0001; Figure 4.2) and CO_2

($F_{1,16}$=4.9, P=0.04). Mice were also more likely to become recumbent in the dark compartment when isoflurane was delivered using a vaporizer machine versus the drop method ($F_{1,17}$=7.0, P=0.3; Table 4.2) or CO_2 ($F_{1,16}$=8.0, P=0.03). Of the five mice that became recumbent during isoflurane vaporizer exposure, two never left the dark chamber after the isoflurane was turned on and three became recumbent upon re-entry. With the drop method, two mice became recumbent in the dark compartment upon re-entry. None of the eight CO_2 treatment mice became recumbent.

Mice exposed to the isoflurane vaporizer treatment re-entered the dark compartment after initially leaving, more than mice exposed to either the drop method treatment ($F_{1,15}$=7.5, P=0.013) or the CO_2 treatment ($F_{1,14}$=8.5, P=0.008). The total time spent re-entering the dark compartment after initially leaving was greater in the vaporizer treatment versus both the drop ($F_{1,12}$=15.8, P=0.0012) and CO_2 treatments ($F_{1,11}$=19.7, P=0.0005).

Figure 4.2 Mean (± S.E.M) latency for: a) mice to leave the dark compartment after initial exposure to CO_2 (n=8), isoflurane vaporizer (n=9) and isoflurane drop (n=9), and b) total time spent re-entering the dark compartment after first leaving when exposed to CO_2 (n=5), isoflurane via the vaporizer (n=7) or the drop method (n=6).

a)



b)

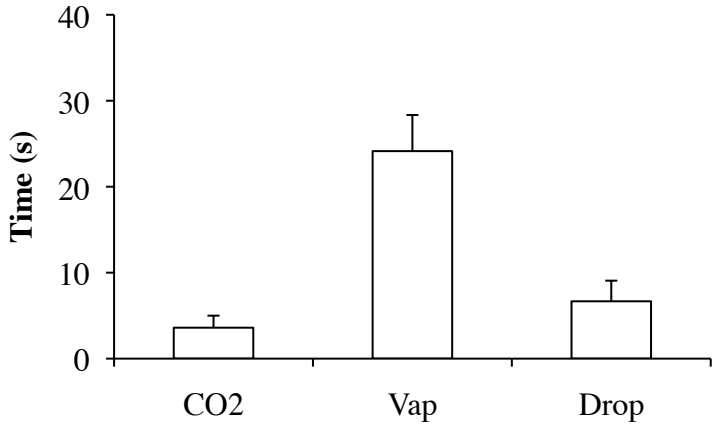


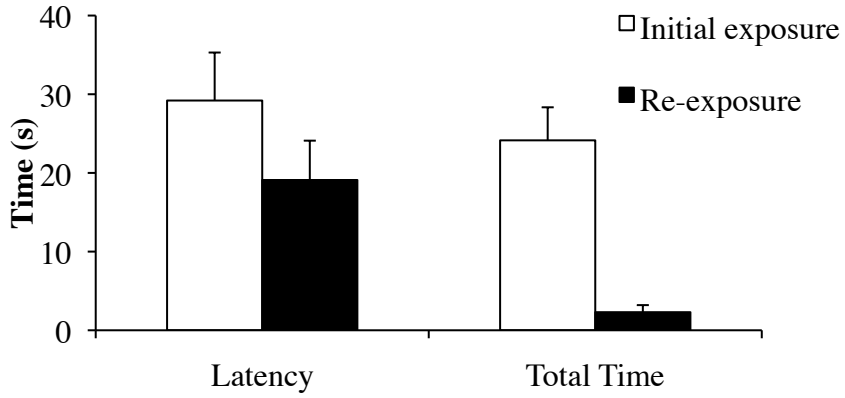
Table 4.2 The number of mice that became recumbent in the dark compartment relative to the number of mice in each treatment, and mean (±S.E.M) number of times mice re-entered the dark compartment.

	CO_2	Isoflurane Drop	Isoflurane Vaporizer Initial Exposure	Isoflurane Vaporizer Re-exposure
I. Recumbent	0/8	2/9	5/9	2/9
II. Re-entries	1.0 ± 0.3	1.2 ± 0.4	3.6 ± 1.0	1.0 ± 0.4

4.3.2.2 Re-exposure: isoflurane vaporizer treatment

The latency to leave the dark compartment decreased from a mean (± S.E.M) of 29.2 ± 6.1 s to 19.1 ± 5.0 s upon re-exposure to isoflurane ($F_{1,8}$= 3.7, P=0.09; Figure 4.3). Only two of nine mice became recumbent during re-exposure, compared to five of nine mice during initial exposure (Table 4.2). The two mice that became recumbent during re-exposure had also become recumbent during the initial exposure trials. The number of re-entries to the dark compartment decreased from a mean (±S.E.M) of 3.6 ± 1.0 during initial exposure to 1.0 ± 0.4 upon re-exposure ($F_{1,6}$=5.8, P=0.05). Total mean (±S.E.M.) time spent re-entering the dark compartment after first leaving, decreased from 24.1 ± 4.2 s during initial exposure to 2.3 ± 0.9 s during re-exposure ($F_{1,6}$=34.3, P=0.0011).

Figure 4.3 Mean (±S.E.M) latency during initial (n=8) and re-exposure (n=8) to the isoflurane vaporizer treatment, as well as total time spent re-entering the dark compartment after first leaving, during initial (n=7) and re-exposure (n=7).



4.4 Discussion

In the current study the vaporizer was at the highest setting possible (5%) combined with the highest flow rate of O_2 (4 l/min); these settings are chosen to minimize the time between onset of aversion and insensibility when using the vaporizer (Makowska et al., 2009). However, results of the pre-trial showed that isoflurane concentration rises much more quickly when using the drop method versus the vaporizer. This difference means that mice spending the same amount of time in the dark compartment would be exposed to a higher concentration of isoflurane in the drop versus vaporizer treatments. The latency to leave the dark compartment was 2.9 versus 29.2 s with the isoflurane drop and vaporizer treatments, respectively. At these times, the isoflurane concentration in the dark compartment would have been approximately 1 to 2%, suggesting that concentrations in excess of 1% are aversive to mice. A potential refinement of the drop method would be to use less isoflurane, resulting in a lower maximum concentration.

The number of re-entries to the dark side averaged one and four for the isoflurane drop and vaporizer treatments, respectively; this difference again may be attributed to the slower build up of isoflurane concentration using the vaporizer method. When mice chose to re-enter the dark side in the isoflurane drop treatment, the concentration of isoflurane was likely higher than at the equivalent time in the vaporizer treatment. This difference likely also explains the decreased total time re-entering and total time spent in the dark compartment with the drop treatment versus the vaporizer treatment.

The latency for mice to leave the dark compartment was longer for the vaporizer treatment versus gradual-fill CO_2. The concentration of CO_2 likely exceeded 10% when mice chose to leave the dark compartment, based upon a theoretical 20% gradual-fill CO_2 curve. Previous work has shown that mice and rats begin to avoid exposure to CO_2 at about this level (Kirkden et al., 2008, Leach et al., 2002, Makowska et al., 2009, Wong et al., 2013). The number of visits and total re-entry time was also less for mice exposed to CO_2 compared to vaporized isoflurane, suggesting that initial exposure to CO_2 is more aversive than initial exposure to vaporized isoflurane. None of the mice exposed to CO_2 chose to stay in the dark compartment until recumbency, compared to about half of the mice tested with the isoflurane vaporizer treatment. The results also indicate that mice find CO_2 more aversive than isoflurane.

Our study results suggest that re-exposure to isoflurane is more aversive than initial exposure. One study (Makowska et al., 2009) using an approach-avoidance paradigm failed to find evidence of learned aversion to isoflurane in mice, but a more recent study using the light-dark paradigm (Wong et al., 2013) found learned aversion in rats when re-exposed to isoflurane. Isoflurane administered by a vaporizer is commonly used for induction and maintenance of

anaesthesia in mice. Euthanasia with isoflurane may be more aversive for mice with previous exposure.

In agreement with current literature, our study results suggest that mice are willing to be exposed to concentrations that cause anaesthesia with isoflurane (delivered by a vaporizer) but not with CO_2 gas. Mice always chose to leave the dark compartment and enter the larger brightly lit compartment before CO_2 concentrations reached those that could render a mouse insensible. Although this study showed that isoflurane was less aversive than CO_2 in mice, it was still aversive as indicated by mice choosing to leave the dark compartment upon start of treatment. Isoflurane has a pungent odour (Flecknell, 2009) and is known to cause eye irritation and potential irritation to upper airway mucosa (Cervin and Lindberg, 1998; Doi and Ikeda, 1993); these effects are likely pronounced at higher concentrations, like those experienced in the drop method treatment in the current study.

4.5 Conclusion

Our results indicate that isoflurane administered by a vaporizer is a humane alternative to 20% chamber vol/min gradual-fill CO_2 for mouse euthanasia. In addition, mice chose to leave earlier and fewer mice became recumbent with isoflurane administered using the drop method compared to the vaporizer method, suggesting the drop method (as tested in the current study) should be avoided. Our results indicate that re-exposure to isoflurane administered with a vaporizer is more aversive than initial exposure, suggesting that this method should be avoided when animals have had previous exposure.

Chapter 5: Discussion and Conclusion

5.1 CO_2 gradual-fill method of mouse euthanasia

The use of CO_2 gas for mouse euthanasia is the most common method used, but over the last decade there have been questions concerning whether or not this method is humane. Several refinements have been suggested. For example, using gradual flow (with flow rates between 10-30% chamber vol/min) rather than a pre-filled chamber, to render rodents insensible before concentrations reach those associated with pain. Even with this refinement rodents likely experience both sensory and affective components of dyspnea when exposed to CO_2 (Lansing et al., 2009).

The evidence presented in Chapter 2 shows that CO_2 flow rate can affect the duration of dyspnea. To minimize this period, I recommend that researchers use a 50% chamber vol/min flow rate but hold the rising chamber concentration to just below 40% CO_2 so that mice are not subjected to concentrations associated with pain while conscious. However, even with this refinement, mice still must endure more than 30 s of dyspnea when euthanized with CO_2.

Others have also attempted to refine the induction period, testing various CO_2 gas mixtures to produce less aversion. Thomas et al. (2012) combined N_2O and CO_2 (60:20), resulting in mice losing the righting reflex about 10% faster than with CO_2 alone. The authors conclude this decreases the conscious period and therefore distress. However, mice have never been tested for aversion to a combination of CO_2 and N_2O or to N_2O alone. If we are to refine the induction period using gas mixtures, it is important to test animal aversion to the mixtures before they are used for euthanasia. Thomas et al. (2012) reported that the N_2O:CO_2 mixture induced a more severe state of hypoxia compared to gradual-fill CO_2 alone. Therefore it is possible this gas

mixture may be more aversive given that hypoxia has been shown to be aversive in rats (Makowska et al., 2008).

Other gas-mixtures that have been examined include supplementing CO_2 with N_2 (Thomas et al., 2012), O_2 (Coenen et al., 1995; Danneman et al., 1997; Kirkden et al., 2008; Pecaut et al., 2000), argon (Leach et al., 2002) and air (Hornett and Haynes, 1984). Argon-CO_2 mixtures were reported as more aversive to rats and mice than CO_2 alone (Leach et al., 2002). CO_2-O_2 mixtures were reported as less aversive than CO_2 alone (Kirkden et al., 2008), resulting in less distress related behaviours in mice (Coenen et al., 1995) but with an increased time to unconsciousness and death (Pecaut et al., 2000). Increased time to death is a concern as the experience of dyspnea may be prolonged. CO_2 is known to cause pulmonary oedema and haemorrhage, increasing in severity with rising concentrations of inhaled CO_2 (Danneman et al., 1997). A study by Ambrose et al. (2000) examined oedema and alveolar hardening caused by inflammation within the lungs (consolidation), in mice following euthanasia with 30% chamber vol/min CO_2 or CO_2-O_2 mixture (30:20%). Mice euthanized with the CO_2-O_2 mixture had more severe cases of oedema and alveolar consolidation versus those mice euthanized with CO_2 alone. Since the CO_2-O_2 mixture results in a longer period of consciousness, the authors report mice likely experienced not only hyperventilation but also a state of conscious drowning during the euthanasia procedure (Ambrose et al., 2000). Given this evidence reviewed above, I conclude that none of the gas mixtures tested to date provide a refinement over the CO_2 gradual-fill method of mouse euthanasia.

To my knowledge, no other refinements for CO_2 mouse euthanasia have been discussed in the literature. An abundance of literature indicates that CO_2 is aversive in mice and rats (Niel and Weary 2007; Niel and Weary, 2006; Leach et al., 2002; Wong et al., 2013; Ziemann et al. 2009)

likely due to dyspnea, resulting in an increased effort to breath. In human studies, CO_2 concentrations as low as 7% have caused dyspnea, reported as distressing and resulting in emotions such as fear and anxiety (Banzett et al., 1990; Lansing et al., 2000; O'Driscoll et al., 1999; Von Leupoldt and Dahme, 2005), while exposure to concentrations between 10-35% have shown to cause fear responses in mice and rats (Concas et al., 1993; Niel and Weary, 2006; Ziemann et al., 2009); as well, 10% CO_2 acts as an unconditioned fear stimulus and increases fear memory when paired with foot-shock in mice (Ziemann et al., 2009). Approach-avoidance studies conducted in rats show animals leaving a gradually filled CO_2 chamber when concentrations are on average 18% (Niel and Weary 2007) and 16% (Kirkden et al., 2008). It is likely that the CO_2 exposure induces fear, given that mice choose to avoid CO_2 at concentrations at similar concentrations that humans experience dyspnea. Unfortunately there is a lack of direct evidence supporting this point. I encourage research using a conditioned place aversion test, wherein mice are subjected to a chamber with CO_2 concentrations that induce dyspnea, and then later are tested for aversion behaviour when re-exposed to the chamber in absence of CO_2. I predict that the unconditioned stimulus of CO_2-induced dyspnea would turn a neutral environment, the chamber where exposure occurred, into a conditioned stimulus. If mice learned this association they would be less likely to return to the chamber re-tested in the absence of the test gas.

Although the proposed experiment described above would allow us to draw stronger conclusions regarding the welfare effects of exposure to CO_2, I suggest that the existing evidence still provides a basis for the conclusion that CO_2 does not provide a good death. I recommend that researchers move away from CO_2 for inducing anaesthesia.

5.2 Isoflurane followed by CO_2 for mouse euthanasia

Exposure to isoflurane is not painful, but may be irritating to the respiratory tract and eyes, in addition to having an unpleasant pungent odour (Cervin and Lindberg, 1998; Doi and Ikeda, 1994). Little work has been done to refine the method of mouse euthanasia using isoflurane. A study by Thomas et al. (2012) compared an isoflurane-O_2 (5:95) mixture versus an isoflurane-N_2O-O_2 (5:75:25) mixture. The authors found that the isoflurane-N_2O-O_2 mixture decreased time to loss of the righting reflex by 17%. However, these authors failed to describe any changes in behaviour during induction and it is unknown if mice find the gas mixture more aversive.

When using the isoflurane method of mouse euthanasia, many users are unsure how long an animal should be exposed to isoflurane before a sufficiently deep plane of anaesthesia has been reached, allowing a high flow rate of CO_2 (or some other form of secondary euthanasia) to begin. The results of Chapter 3 provide the first scientific basis for recommending when this switch can occur. When using 5% isoflurane with 2 l/min (17% chamber vol/min) of O_2, users should wait a minimum of 80 s after the appearance of recumbency to switch to a high flow rate of CO_2, thereby reducing the risk of exposing sentient mice to painful concentrations of CO_2. This recommendation would also likely apply to other secondary methods including cervical dislocation and guillotine, assuming little or no delay between removing the animal from the chamber and applying the secondary method.

More work is needed to further refine the isoflurane method of mouse euthanasia. For example, I suggest testing mouse aversion to various isoflurane concentrations, examining gas-mixtures that create a smoother induction by minimizing eye or respiratory irritation and assessing aversion to these mixtures.

5.3 Aversion to isoflurane and CO_2

The light-aversion results described in Chapter 4 provides the first direct evidence that mice find CO_2 more aversive than isoflurane, and that isoflurane administered via a vaporizer is less aversive compared to the drop method. Both methods of isoflurane administration delivered 5% isoflurane, but the different procedures resulted in different rates of accumulation within the dark compartment. Regardless of treatment, mice left when the dark compartment contained about 1-2% isoflurane, suggesting that mice find concentrations >1% aversive. Induction with the drop method was more aversive in mice than with the vaporizer method, likely due to the difference in rising isoflurane concentration. Future work is needed to assess mouse aversion to lower concentrations of isoflurane delivered via the drop method, as it is possible that lower but effective concentrations may be less aversive.

Another potential cause of isoflurane aversion is the experience of becoming increasingly sedated. Using the drop method this experience would occur much faster than with the vaporizer method, thereby causing the mice to leave the chamber much earlier.

A potential weakness of the light-aversion test is that during exposure to isoflurane, mice may have become too sedated to escape the gas-filling compartment even if they wanted to. However, no mice stayed until recumbency with CO_2, (which has a smaller safety margin), suggesting that induction with isoflurane is still less aversive than induction with CO_2.

Although mice showed a preference for the smaller dark compartment, they chose to remain in the large brightly lit chamber more often when exposed to 20% chamber vol/min gradual-fill CO_2 or isoflurane delivered with the drop method compared to when isoflurane was delivered using the vaporizer. These results are a strong indication of aversion, suggesting a negative experience was endured during agent-exposure. Collectively, the work described in this thesis

builds upon current welfare research that exposure to CO_2 is aversive and that this agent should not be used for euthanasia of conscious animals.

5.4 Conclusion

Animal researchers should provide the best death possible for the mice used in their research. The aim of my thesis was to identify refinements to the isoflurane and CO_2 methods of mouse euthanasia. My results, in accordance with other published research, indicate that the most humane method of rodent euthanasia is exposure to isoflurane delivered by a vaporizer machine. If using this method and a 2 l/min (17% chamber vol/min) flow rate of O_2, my results indicate that upon the appearance of recumbency, users should wait a minimum of 80 s before switching to CO_2 gas or using another secondary method. If using the CO_2 gradual-fill method for mouse euthanasia, my results suggest using a 50% chamber vol/min flow rate, while ensuring the concentration in the euthanasia chamber does not exceed 40% before loss of consciousness occurs, to reduce the duration of dyspnea. Isoflurane delivered by a vaporizer is a refinement, but even with this method not all mice stayed in the gas-filling dark compartment until recumbency. Therefore the search for more humane agents for laboratory mouse euthanasia should continue.

References

Abolhassani M, Guais A, Chaumet-Riffaud P, Sasco AJ, Schwartz L. 2009. Carbon dioxide inhalation causes pulmonary inflammation. Am. J. Physiol. Lung. Cell. Mol. Physiol. 296: L657-L665.

Alkire MT, Hudetz AG, Tononi G. 2008. Consciousness and anaesthesia. Science. 322: 786-880.

Altholtz LY, Fowler KA, Badura LL, Kovacs MS. 2006. Comparison of the stress response in rats to repeated isoflurane or CO_2:O_2 anaesthesia used for restraint during serial blood collection via the jugular vein. J. Am. Assoc. Lab. Anim. Sci. 45: 17-22.

Ambrose N, Wadham J, Morton D. Refinement in euthanasia. 2000. In: Balls M, van Zeller AM, Halder ME. (eds) Progress in the reduction, refinement and replacement of animal experimentation. Amsterdam: Elsevier Science. pp.1159-1169.

Antognini JF, Barter L, Carstens E. 2005. Movement as an index of anaesthetic depth in humans and experimental animals. J. Am. Assoc. Lab. Anim. Sci. 55: 43-418.

Antognini JF, Buonocore MH, Disbrow EA, Carstens E. 1997. Isoflurane anaesthesia blunts cerebral responses to noxious and innoculous stimuli: a fMRI study. Life Sci. 61: PL349-PL354.

Anton F, Euchner I, Handwerker HO. 1992. Psychophysical examination of pain induced by defined CO_2 pulses applied to the nasal mucosa. Pain. 49: 53-60.

Anton F, Peppel P, Euchner I, Handwerker HO. 1991. Controlled noxious chemical stimulation: response of rat trigeminal brainstem neurons to CO_2 pulses applied to the nasal mucosa. Neurosci. Lett. 123: 208-211.

Arras A, Autenried P, Rettich A, Spaeni D, Rulicke T. 2001. Optimization of intraperitoneal injection anaesthesia in mice: drugs, dosages, adverse effects, and anaesthesia depth. Comp. Med. 51: 443-456.

ATC (American Thoracic Committee) *ad hoc* Committee. 1999. Dyspnea mechanisms, assessment and management: a consensus statement. Am. J. Respir. Crit. Care Med. 159: 321-340.

AVMA (American Veterinary Medical Associated). 2013. Guidelines for the euthanasia of animals: 2013 edition, https://www.avma.org/KB/Policies/Documents/euthanasia.pdf (Accessed August 20, 2013).

Bailey JF, Argyropoulos SV, Kendrick AH, Nutt DJ. 2005. Behavioral and cardiovascular effects of 7.5% CO_2 in human volunteers. Depress. Anxiety. 21: 18-25.

Banzett RB, Lansing RW, Brown R. et al. 1990. 'Air hunger' from increased CO_2 persists after complete neuromuscular block in humans. Resp. Physiol. 81: 1-17.

Buitrago S, Martin TE, Tetens-Woodring J, Belicha-Villanueva A, Wilding GE. 2008. Safety and efficacy of various combinations of injectable anaesthetics in BALB/c mice. J. Am. Assoc. Lab. Anim. Sci. 47: 11-17.

Campagna JA, Miller KW, Forman SA. 2003. Mechanisms of actions of inhaled anaesthetics. N. Engl. J. Med. 348: 2110-2124.

Carrier-Kohlman V, Gormley JM, Douglas MK, Paul SM, Stulbarg MS. 1996. Differentiation between dyspnea and its affective components. West J. Nurs. Res. 18: 626-642.

CCAC (Canadian Council on Animal Care). 2010. CCAC guidelines on: euthanasia of animals used in science, http://www.ccac.ca/Documents/Standards/Guidelines/Euthanasia.pdf (Accessed August 20, 2013).

CCAC (Canadian Council on Animal Care). 1993. Guide to the care and use of experimental animals. Volume 1, 2nd Edition. (Eds) Olfert ED, Cross BM, McWilliam AA. Ottawa, CCAC. http://www.ccac.ca/Documents/Standards/Guidelines/Experimental_Animals_Vol1.pdf (Accessed September 14, 2013).

Cervin A, Linderberg S. 1998. Changes in mucociliary activity may be used to investigate the airway-irritating potency of volatile anaesthetics. Br. J. Anaesth. 80: 475-480.

Chisholm J, De Rentere D, Fernandez NJ, Krajacic A, Pang DSJ. 2013. Carbon dioxide, but not isoflurane, elicits ultrasonic vocalizations in female rats. Lab. Anim. 47: 324-327.

Clark DL, Rosner BS. 1973. Neurophysiologic effects of general anaesthetics: I. the electroencephalogram and sensory evoked responses in man. Anesthesiology. 35: 564-582.

Coenen AML, Drinkenburg WHIM, Hoederken R, van Luijelaar ELJM.1995. Carbon dioxide euthanasia in rats: oxygen supplementation minimizes signs of agitation and asphyxia. Lab Anim. 29: 262-268.

Concas A, Sanna E, Cuccheddu T, Paola M. 1993. Carbon dioxide inhalation, stress and anxiogenic drugs reduce the function of $GABA_A$ receptor complex in the rat brain. Prog. Neuropsychoph. 17: 651-661.

Costall B, Joanes BJ, Kelly ME, Naylor RJ, Tomkins DM. 1989. Exploration of mice in a black and white box: validation as a model of anxiety. Pharmacol. Biochem. Behav. 32: 777-785.

Crawley JN, Goodwin FK. 1980. Preliminary report of a simple animal behaviour for the anxiolytic effects of benzodiazepines. Pharmacol. Biochem. Behav. 13: 167-170.

Danneman PJ, Stein S, Walshaw SO. 1997. Humane and practical implications of using carbon dioxide mixed with oxygen for anaesthesia or euthanasia on rats. Lab. Anim. Sci. 47: 376-384.

Doi M, Ikeda K. 1993. Airway irritation produced by volatile anaesthetics during brief inhalation: comparison of halothane, enflurane, isoflurane and sevoflurane. Can. J. Anaesth. 40:122-126.

Eisele JH, Eger EI, Muallem M. 1967. Narcotic properties of carbon dioxide in the dog. Anesthesiology. 28: 856-865.

Feinstein JS, Buzza C, Hurlemann R. et al. 2013. Fear and panic in humans with bilateral amygdala damage. Nat. Neurosci.16: 270-272.

Flecknell P. 2009. Laboratory animal anaesthesia, third ed. Academic Press, UK. pp54.

Franks NP. 2008. General anaesthesia: from molecular targets to neuronal pathways of sleep and arousal. Nat. Rev. Neurosci. 9: 370-386.

Gorman JM, Askanazi J, Liebowitz MR et al. 1984. Response to hyperventilation in a group of patients with panic disorder. Am. J. Psychiatry. 141: 857-861.

Griez E, van den Hout MA. 1982. Effects of carbon dioxide-oxygen inhalations on subjective anxiety and some neurovegetative parameters. J. Behav. Ther. Exp. Psychiatry. 13: 27-32.

Guais A, Brand G, Jacquot L. et al. 2011.Toxicity of carbon dioxide: a review. Chem. Res. Toxicol. 24: 2061-2070.

Hascoet M, Bourin M. 1998. A new approach to the light/dark procedure in mice. Pharamcol. Biochem. Behav. 60: 645-653.

Heinke W, Schwarbauer C. 2001. Subanaesthetic isoflurane affects task-induced brain activation in a highly specific manner: a functional magnetic resonance imaging study. Anesthesiology. 94: 973-981.

Herring BE, Xie Z, Marks J, Fox AP. 2009. Isoflurane inhibits the neurotransmitter release machinery. J. Neurophysiol. 102: 1265-1273.

Hirata H, Hu JW, Bereiter DA. 1999. Responses by medullary dorsal horn neurons to corneal stimulation by CO_2 pulses in the rat. J. Neurophysiol. 82: 2092-2107.

Hornett TD, Hayes AR. 1984. Comparison of carbon dioxide/air mixture and nitrogen/air mixture for the euthanasia of rodents: design of a system for inhalation euthanasia. Anim. Technol. 35: 93-99.

Johnson PL, Fitz SD, Hollis JH, Moratalla R, Lightman SL, Shekhar A. et al. 2011. Induction of c-Fos in ‘panic/defence'-related brain circuits following brief hypercarbic gas exposure. J. Psychopharmacol. 25: 26-36.

Kirkden RD, Niel L, Lee G, Makowska IJ, Pfaffinger MJ, Weary DM. 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.

Krohn TC, Hansen AK, Dragsted N. 2003. The impact of low levels of carbon dioxide on rats. Lab. Anim. 37: 94-99.

Langsjo JW, Maksimow A, Salmi E. et al. 2005. S-ketamine anaesthesia increases cerebral blood flow in excess of the metabolic needs in humans. Anesthesiology. 103: 258-268.

Lansing RW, Graely RH, Banzett RB. 2009. The multiple dimensions of dyspnea: review and hypotheses. Resp. Physiol. Neurobi. 167: 53-60.

Lansing RW, Im BSH, Thwing JI, Legedza ATR, Banzett RB. 2000. The perception of respiratory work and effort can be independent of the perception of air hunger. Am. J. Resp. Crit. Care Med. 162: 1690-1696.

Leach MC, Bowell VA, Allan TF, Morton DB. 2002. Aversion to gaseous euthanasia agents in rats and mice. Comp. Med. 52: 249-257.

Lee J, Taira T, Pihlaja P, Ransom BR, Kaila K. 1996. Effects of CO_2 on excitatory transmission apparently caused by changes in intracellular pH in the rat hippocampal slice. Brain Res. 706: 210-16.

Liotti M, Brannan S, Egan G. et al. 2001. Brain responses associated with consciousness of breathlessness (air hunger). Proc. Nat. Acad. Sci. U.S.A. 98: 1035-2040.

Makowska IJ, Niel L, Kirkden R, Weary DM. 2008. Rats show aversion to argon-induced hypoxia. Appl. Anim. Behav. Sci. 114: 572-581.

Makowska IJ, Vickers L, Mancell J, Weary DM. 2009. Evaluating methods of gas euthanasia for laboratory mice. Appl. Anim. Behav. Sci. 121: 230-235.

Makowska IJ, Weary DM. 2009. Rat aversion to induction with inhalant anaesthetics. Appl. Anim. Behav. Sci. 119: 229-235.

Martoft L, Jørgenson HS, Foslid A, Pedersen HD, Jørgenson PF. 2003. CO induced acute respiratory acidosis and brain tissue intracellular pH: a 31P NMR study in swine. Lab. Anim. 37: 241–248.

Matynia A, Parikh S, Chen B. et al. 2012. Intrinsically photosensitive retinal ganglion cells are the primary but not exclusive circuit for light aversion. Exp. Eye Res. 105: 60-69.

McLennan IS, Taylor-Jeffs J. 2004. The use of sodium lamps to brightly illuminate mouse houses during their dark phases. Lab. Anim. 38: 384-92.

Mogil JS, Smith SB, O'Reilly MK, Plourde G. 2005. Influence of nociception and stress-induced antinociception on genetic variation in isoflurane anaesthetic potency amoung mouse strains. Anesthesiology. 103: 751-780.

Niel L, Stewart SA, Weary DM. 2008. Effect of flow rate on aversion to gradual-fill carbon dioxide exposure in rats. Appl. Anim. Behav. Sci. 109: 77-84.

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

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

National Research Council (US Committee on Recognition and Alleviation of Distress in Laboratory Animals). 2008. Stress and Distress: Definitions. In: Recognition and Alleviation of Distress in Laboratory Animals. Washington, USA: National Academies Press. http://www.ncbi.nlm.nih.gov/books/NBK4027/ (Accessed September 14, 2013).

O'Driscoll M, Corner J, Bailey C.1999. The experience of breathlessness in lung cancer. Eur. J. Cancer Care (Engl). 8: 37-43.

Owen AM, Coleman MR, Boly M, Davis MH, Laureys S, Pickard JD. 2006. Detecting awareness in the vegetative state. Science. 313: 1402.

Pappens M, De Peuter S, Vansteenwegen D, Van den Bergh O, Van Diest I. 2012. Psychophysiological responses to CO_2 inhalation. Int. J. Psychophysiol. 84: 45-50.

Pecaut MJ, Smith AL, Jones TA, Gridley DS. 2000. Modification of immunologic and hematologic variables by method of CO_2 euthanasia. Comp. Med. 50: 595-602.

Peppel P, Anton F. 1993. Responses of rat medullary dorsal horn neurons following intranasal noxious chemical stimulation: effects of stimulus intensity, duration, and interstimulus interval. J. Neurophysiol. 70: 2260-2275.

Rodrigo MRC, Rosenquist JB. 1988. Isoflurane for conscious sedation. Anaesthesia. 43: 369-375.

Schlunzen L, Cold GE, Rasmussen M, Vafaee MS. 2006. Effects of dose-dependent levels of isoflurane on cerebral blood flow in healthy subjects studied using positron emission tomography. Acta. Anaesthesiol. Scand. 50: 306-312.

Schmidt NB, Trakowski JH, Staab JP. 1997. Extinction of panicogenic effects of 35% CO_2 challenge in patients with panic disorder. J. Abnorm. Psychol. 106: 630-638.

Smith JC, Bolon B. 2002. Atmospheric waste isoflurane concentrations using conventional equipment and rat anaesthesia protocols. Contemp. Top. Lab. Anim. Sci. 41:10-17.

Smith W, Harrap SB. 1997. Behavioural and cardiovascular responses of rats to euthanasia using carbon dioxide gas. Lab. Anim. 31: 337-346.

Sonner JM, Gong D, Eger EI 2nd. 2000. Naturally occurring variability in anaesthetic potency among inbred mouse strains. Anesth. Analog. 91: 720-726.

Sonner JM, Gong D, Li J, Eger EI 2nd, Laster, MJ. 1999. Mouse strain modestly influences minimum alveolar anaesthetic concentration and convulsivity of inhaled compounds. Anesth. Analg. 89: 1030-1034.

Sparacino GM, Hoffman PM, Martin R, Lichtor JL, Zachny JP. 1993. Is isoflurane a suitable agent for conscious sedation? Anesth. Analg. 76: S415-S415.

Steel B, Shaver J. 1992. The dyspnea experience: nociceptive properties and a model for research and practice. Adv. Nurs. Sci. 15: 64-76.

Stimpel TM, Gershey EL. 1991. Selecting anaesthetic agents for human safety and animal recovery surgery. F.A.S.E.B. J. 5: 2099-2014.

Thomas AA, Flecknell PA, Golledge HDR. 2012. Combining nitrous oxide with carbon dioxide decrease the time to loss of consciousness during euthanasia in mice - refinement of animal welfare? PLOS ONE. 7: e32290.

Thurauf N, Gunther M, Pauli E, Kobal G. 2002. Sensitivity of the negative mucosal potential to the trigeminal target stimulus CO_2. Brain Res. 942: 27-86.

Tucker MR, Hann JR. Phillips CL. 1984. Subanaesthetic doses of ketamine, diazepam, and nitrous oxide for adult outpatient sedation. J. Oral Maxillofac. Surg. 42: 668-72.

Valentine H, Williams WO, Maurer KJ. 2012. Sedation or inhalant anaesthesia before euthanasia with CO_2 does not reduce behavioral or physiologic signs of pain and stress in mice. J. Am. Assoc. Lab. Anim. Sci. 51: 50-57.

Veselis RA, Reinsel RA, Feshchenko VA, Dnistrian AM. 2002. A neuroanatomical construct for the amnesic effects of propofol. Anesthesiology. 97: 329-337.

von Leupoldt A, Dahme B. 2005. Differentiation between the sensory and affective dimensions of dypsnea during resistive load breathing in normal subjects. Chest. 128: 3345-3349.

von Leupoldt A, Mertz C, Kegat S, Burmester S, Dahme B. 2006. The impact of emotions on the sensory and affective dimension of perceived dyspnea. Psychophysiology. 43: 382-386.

von Leupoldt A, Sommer T, Kegat S. et al. 2009. Dyspnea and pain share emotion-related brain network. NeuroImage. 48: 200-206.

von Leupoldt A, Sommer T, Kegat S. et al. 2008. The unpleasantness of perceived dyspnea is processed in the anterior insula and amygdala. Am. J. Respir. Crit. Care Med. 177: 1026-1032.

Wemmie JA. 2011. Neurobiology of panic and pH chemosensation in the brain. Dialogues Clin. Neurosci. 13: 475-483.

Westphalen RI, Desai KM, Hemmings Jr HC. 2013. Presynaptic inhibition of the release of multiple major central nervous system neurotransmitter types by the inhaled anaesthetic isoflurane. Br. J. Anaesth. 110: 592-599.

Whelan G, Flecknell P. 1992. The assessment of depth of anaesthesia in animals and man. Lab. Anim. 26: 153-162.

Wong D, Makowska IJ, Weary DM. 2013. Rat aversion to isoflurane versus carbon dioxide. Biol. Lett. 9: 20121000.

Woodbury DM, Rollins LT, Gardner MD. et al. 1958. Effects of carbon dioxide on brain excitability and electrolytes. Am. J. Physiol. 192: 79-90.

Zacny JP, Sparacino G ,Hoffmann PM, Martin R, Lichtor JL. 1994. The subjective, behavioral and cognitive effects of subanaesthetic concentrations of isoflurane and nitrous oxide in healthy volunteers. Psychopharmacology (Berl). 114: 409-41.

Ziemann AE, Allen JE, Dahdaleh NS. et al. 2009. The amygdala is a chemosensor that detects carbon dioxide and acidosis to elicit fear behavior. Cell. 135: 1012-1021.