ASSESSMENT OF DISTRESS ASSOCIATED WITH CARBON DIOXIDE EUTHANASIA IN LABORATORY RATS

by

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B.Sc., Simon Fraser University, 2000

A THESIS SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

in

THE FACULTY OF GRADUATE STUDIES

(Animal Science)

The University of British Columbia

July 2006

Abstract

Carbon dioxide (CO_2) gas is the most widely used euthanasia agent for laboratory rodents. However, it has the potential to cause both pain and dyspnea, an unpleasant sensation of breathless, while animals are still conscious. The aims of this dissertation were to determine whether gradual-fill CO_2 euthanasia causes distress in laboratory rats, and to examine potential sources of distress, including pain, dyspnea and novelty. The first study examined the behavioural responses of rats during gradual-fill CO_2 euthanasia. Rats showed increased exploratory behaviours and escape behaviours during CO_2 euthanasia, suggesting that this procedure does cause distress. The second and third studies used approach-avoidance testing to investigate aversion to CO_2 in rats, by examining their willingness to enter a test cage containing CO_2 for access to an attractive food reward. Rats were found to avoid CO_2 concentrations that are sufficient to cause unconsciousness. Specifically, when tested with static concentrations of CO_2 rats showed avoidance at 15% CO_2 and greater, and when tested with gradually increasing concentrations of CO_2 at flow rates ranging from 3 to 27% per minute, rats showed avoidance at 13 to 16% CO_2. This avoidance indicates that rats are at least moderately averse to CO_2 concentrations occurring during gradual-fill CO_2 euthanasia, and that forced exposure likely causes distress. Concentrations of CO_2 that were associated with behavioural responses and aversion were not consistent with previous data on pain due to CO_2. However, similar concentrations have been shown to cause dyspnea in humans. The final study examined the role of novelty in rats' responses to CO_2, and found that novelty was not a major source of distress during gradual-fill CO_2 euthanasia. In summary, these studies suggest that gradual-fill CO_2 euthanasia causes distress in rats, and that this distress is likely due to dyspnea. Further research is necessary to examine the effects of CO_2 on other rodent species such as mice, and to identify alternative methods of euthanasia that cause unconsciousness without distress.

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List of Abbreviations

ACTH	adrenocorticotropic hormone
CNS	Central nervous system
CO_2	Carbon dioxide
CRH	Corticotrophin-releasing hormone
CSF	Cerebral spinal fluid
EEG	Electroencephalogram
HPA	Hypothalamic-pituitary-adrenal axis
O_2	Oxygen
SAM	Sympathetic-adrenergic-medullary axis
SEP	Somatosensory-evoked potential
USV	Ultrasonic vocalization

Acknowledgements

I am grateful to my supervisor, Dan Weary, for his endless enthusiasm and encouragement. Dan has allowed me tremendous freedom to pursue research that I am passionate about. He has also given me every opportunity to learn about all aspects of academia, from writing grants to teaching and training students. I am especially thankful for his constant barrage of questions and criticisms, which have been crucial to my growth as an independent and critical researcher. I have thoroughly enjoyed my disagreements with Dan, and I hope that future colleagues are as forthcoming with their opinions.

I would like to thank my committee members, David Fraser, Jim Love and Ken Craig for their insight into my thesis area, and for their help with editing. Dave has been a fount of knowledge on both animal welfare and grammar, and I have learned vast amounts from him during my time in the Animal Welfare Program. I am grateful to Jim and Ken for their scepticism, which has made me think carefully about appropriate interpretation and presentation of my data.

I would also like to thank a number of people who have made contributions to the studies in this thesis. First and foremost, I want to thank Cassandra Tucker, with whom I have spent countless hours discussing research ideas, experimental design and statistical analysis. I would also like to thank Richard Kirkden, for his insights on animal motivation. Gilles Galzi, Sylvia Leung and Jurgen Pehlke have been incredibly helpful with the day to day running of the lab. Murray Hodgson provided technical knowledge on ultrasound, and very generously lent me equipment for the ultrasound recordings in Chapter 2. Sarah Stewart, Marianne Pfaffinger and Gary Lee provided assistance with day to day lab duties and with data collection (Sarah, Chapters 4 and 5; Marianne, Chapters 3 and 5; Gary, Chapter 5). Mitja Sedlbauer, Nicole Fenwick, and Amanda Grout provided assistance with animal care.

I am also thankful to the many members of the Animal Welfare Program for their support and friendship. Lunchtime discussions and presentations have been a high point in my time as a graduate student.

I am eternally indebted to my family and friends for their unwavering support. A special thank you goes to my husband, Colin, who has helped me to stay sane throughout this process and has done far more than his fair share of the dog-walking these past few months.

Finally, completion of this work would not have been possible without the generous financial support that I received through the National Sciences and Engineering Research Council and the UBC Faculty of Land and Food Systems (Elizabeth Roxann Howland Fellowship, Leonard S. Klink Memorial Fellowship, Beryl March Travel Award and Charles River Laboratories Scholarship).

Co-Authorship Statement

Chapter 2:
I was responsible for identifying the research area, experimental design, data collection, statistical analyses, and writing the manuscript. Dan Weary contributed to the experimental design and to the interpretation and presentation of the manuscript.

Chapter 3:
I was responsible for identifying the research area, experimental design, data collection, statistical analyses, and writing the manuscript. Dan Weary contributed to the experimental design and to the interpretation and presentation of the manuscript.

Chapter 4:
I was responsible for a portion of the data collection, and for identifying the research area, experimental design, statistical analyses, and writing the manuscript. Sarah Stewart contributed to the experimental design and data collection. Dan Weary contributed to the experimental design and to the interpretation and presentation of the manuscript.

Chapter 5:
I was responsible for identifying the research area, experimental design, data collection, statistical analyses, and writing the manuscript. Dan Weary contributed to the experimental design, and to the interpretation and presentation of the manuscript.

CHAPTER 1: General Introduction

1.1 Introduction

In the industrialized countries, animals are routinely bred and used in scientific experimentation that serves to advance basic biological knowledge, advance human and veterinary medicine, and ensure the safety of humans, animals and the environment through regulatory testing. Rodents are the most widely used species in this research, and in Canada alone approximately one million mice and rats are used in research each year (CCAC, 2006)

The vast majority of research and breeding animals are eventually killed, either for experimental purposes or to reduce surplus stock. During an experiment, animals might be killed in order to alleviate pain and suffering due to an experimental manipulation or to allow for tissue collection and analysis. The term euthanasia is commonly used to refer to the killing of research animals. Euthanasia is derived from Greek for 'good death', which suggests a process which does not involve pain or distress (Blackmore, 1993). Although in practice it may not be possible to develop a procedure for the killing of animals that is completely devoid of stress, the goal is clearly to minimize any pain and distress associated with the procedure.

A number of different euthanasia methods are currently approved for killing laboratory rodents in Canada (CCAC, 1993). Euthanasia guidelines developed by the Canadian Council on Animal Care are generally in line with the suggested euthanasia methods of other western countries such as those of the USA (AVMA, 2001), the UK (UK Home Office, 1997), countries of the European Union (Close et al., 1997) and Australia and New Zealand (ANZCCART, 1993). These methods include physical techniques, injectable anaesthetics, and anaesthetic and non-anaesthetic gases. It is recognized that some of these methods have the potential to result in both pain and distress, but the chosen method does not depend solely on a reduction of animal

suffering. It also depends on pragmatic concerns such as the purpose of the killing and the constraints of time, money, and safety to humans, other animals and the environment.

The Canadian Council on Animal Care (CCAC), the body that governs animal research in Canada, stipulates in their Ethics of Animal Investigation guidelines (1989, p.1) that research animals "must not be subjected to unnecessary pain or distress" and "if pain or distress is necessary concomitant to the study, it must be minimized both in intensity and duration". Therefore, where multiple methods are available for achieving the same aims, the method that causes the least pain and distress should be used. Furthermore, where a painful or distressful procedure is required to meet the aims of the study, efforts must be made to mitigate these effects. Although these guidelines allow for pragmatic concerns to be addressed, they require that animal pain and distress be minimized to the greatest extent possible within these confines. In order to meet this requirement, we must first determine which methods cause pain and distress, and then determine how these states can be minimized.

To assess whether killing methods cause pain and distress we can examine three lines of evidence: 1) human self-report data to determine what subjective states are potentially associated with exposure, 2) physiological responses to observe the method's potential for activating nociceptors or neuro-endocrine stress responses, and 3) behavioural data to assess whether the method results in behaviours indicative of pain or distress, and to determine the level of aversion associated with the stimulus. Although it may not be possible to completely prevent pain and distress, it should be possible to identify those procedures that result in the least pain and distress, and thus provide a basis for recommendations that can be used by animal caregivers.

1.2 Carbon Dioxide Euthanasia

Carbon dioxide (CO_2) is the most widely used euthanasia agent for laboratory rodents. In CO_2 euthanasia of rodents, the animal is either placed into a chamber that is pre-filled with CO_2 at a concentration of greater than 70%, or the animal is placed in an empty chamber that is then gradually filled with CO_2. Although some previous work has examined whether CO_2 euthanasia causes distress in rats, the results to date have been inconclusive.

1.2.1 Mode of action

Although CO_2 is categorized as a non-anaesthetic gas, it does have anaesthetic properties. In the rat, it causes a decrease in brain excitability at concentrations as low as 5%, light anaesthesia beginning at 25%, and deeper anaesthesia at approximately 40% (reviewed by Woodbury et al., 1958). Humans show electroencephalogram (EEG) depression and are unable to carry out simple commands when breathing 20% CO_2 (Meyer et al., 1966). Death can occur at concentrations of approximately 30% CO_2 and higher (Danneman et al., 1997; Sharp et al., 2006), likely due to depression of brain centers responsible for circulation and respiration; the exact CO_2 concentration necessary for death depends on the duration of exposure (Danneman et al, 1997).

During metabolism, the body uses oxygen (O_2) and produces CO_2 and these two gases are constantly exchanged by the respiratory system. Air contains approximately 0.03% CO_2 and 20.9% O_2 (and 78% nitrogen), but due to metabolism, the tissues contain higher levels of CO_2 and lower levels of O_2. This results in a gas concentration gradient between the air and the blood at the lungs that allows for the uptake of O_2 into the blood and release of CO_2 into the air, and between the blood and the tissues in the rest of the body that allows for the release of O_2 to the tissues and the uptake of CO_2 into the blood. Because of this gradient, CO_2 generally occurs

only at low concentrations (<6%) in the body. Total CO_2 content in the body consists of carbamino compounds, CO_2, bicarbonate ions (HCO_3^-) and carbonic acid (H_2CO_3). The last three exist in the following equilibrium:

$$CO_2 + H_2O \leftrightarrow H_2CO_3 \leftrightarrow H^+ + HCO_3^-$$

As the concentration of CO_2 in the air is increased, it builds up in the tissues and shifts this equilibrium to the right, producing hydrogen ions and reducing pH. Although the body has adaptations that increase the removal of CO_2 at the lungs under normal conditions, the concentrations of CO_2 used in euthanasia are far beyond the body's compensatory mechanisms. Furthermore, the blood brain barrier is very permeable to CO_2, and the CSF has little buffering capacity. This results in a greater drop in pH in the CSF than in the blood and other tissues (Brodie & Woodbury, 1958), and it is thought that this reduction in pH causes CO_2 narcosis and death (e.g. Brodie & Woodbury 1958; Eisele et al., 1967; Martoft et al. 2003; Meyer et al., 1961; Woodbury et al., 1958). Specifically, these changes in CSF pH are thought to reduce neuron excitability. For example, in vitro studies have demonstrated that a reduction in extracellular pH due to exposure 20% CO_2 reduces the activity of neurons in the hippocampus via a pH dependent mechanism (e.g. Dulla et al., 2005; Hsu et al., 2000; Lee et al., 1996; Velisek, 1998). The exact mechanisms for these effects on neuron functioning are not currently known, but pH effects are thought to be mediated by alterations in cell ion gradients (e.g. Gifford et al., 1990; Pasternack et al., 1992; Tang et al., 1990; Tombaugh and Somjen, 1996), possibly through reconfiguration of relevant proteins associated with cell ion channels, receptors or enzymes (Somero, 1986).

1.2.2 Time-course of CO_2 euthanasia in rats

A number of studies have observed the time course of CO_2 euthanasia in rats, and this information provides an indication of the duration of exposure and the maximum concentrations of CO_2 that occur while the animal is still conscious[1]. These results have been obtained for pre-fill and gradual-fill CO_2 euthanasia of rats. Authors differ in their criteria for loss of consciousness, but the majority of experiments use onset of ataxia to indicate the initial depression of the central nervous system (CNS), and complete loss of posture to indicate loss of consciousness. Some studies examining species other than rats have used EEGs (Raj and Gregory, 1994; Raj et al., 1997; Raj et al., 1998; Martoft et al., 2002), and somatosensory-evoked potentials (SEPs) (Raj and Gregory, 1994; Raj et al., 1997; Martoft et al., 2002) to gain a more accurate estimate of time to unconsciousness, and there appears to be a slight time lag between loss of posture and changes in EEGs and SEPs (Coenen et al., 2000; Raj et al., 1992). Hewett et al. (1993) found that during gradual-fill CO_2 exposure at a flow rate of 20% per minute, rats showed immobility after 72 s and loss of righting reflex after 109 s. However, immobility was only defined as inactivity so the delay between complete loss of posture and loss of righting reflex would have been shorter. In fact, Coenen et al. (1995) found that complete loss of posture in rats was well correlated with onset of a fully aberrant EEG, and that the delay was less than 10 s during gradual-fill CO_2 exposure. For the sake of simplicity, ataxia and loss of posture will be used to summarize the duration data for each of the different CO_2 methods, with the assumption that these measures represent the onset of loss of consciousness and that complete insensibility likely follows shortly afterwards.

[1] The term 'conscious' is used here according to what Block (1995) has referred to as "phenomenal consciousness". This refers to awareness and the ability to experience events, but does not imply being able to report on this experience or of being self-conscious. 'Conscious' is used throughout this dissertation to indicate when the animal is awake and therefore is presumably capable of experiencing negative affective states.

During pre-fill euthanasia in rats with CO_2 concentrations greater than 70%, time to loss of posture generally ranges from 7 s to 20 s (Blackshaw et al., 1988; Kohler et al., 1999; Hewett et al., 1993; Danneman et al., 1997; Coenen et al., 1995; Britt, 1987), and ataxia is not generally observed because of the speed of collapse. With a fast flow rate (>50% of the chamber volume being added per minute), ataxia occurs at 13 s to 18 s and loss of posture at 26 s to 48 s (Coenen et al., 1995; Smith and Harrap, 1997). With a medium flow rate (15% to 50% of the chamber volume added per minute) ataxia ranges from 42 s to 120 s, and loss of posture ranges from 90 s to 120 s (Hornett and Haynes, 1984; Hackbarth et al., 1999; Smith and Harrap, 1997; Danneman et al., 1997). Slow fill rates (<15% of the chamber volume added per minute) take much longer, with ataxia occurring at 120 s to 180 s and loss of posture occurring at 120 s to 240 s (Hornett and Haynes, 1984).

Little is known about the concentrations of CO_2 that are required to cause loss of consciousness during gradual-fill CO_2 euthanasia. With a medium CO_2 flow rate, rats have been found to lose consciousness at concentrations as low as 40% (Smith and Harrap, 1997). The majority of studies examining gradual-fill CO_2 exposure have not monitored CO_2 concentrations, so it is not clear whether flow rate has an effect on the CO_2 concentration needed to cause loss of consciousness in rats. However, results in mice suggest that a medium flow rate of 30% per minute is associated with a lower concentration of CO_2 at loss of consciousness than a fast flow rate of 60% per minute (Ambrose et al., 2000). While the cause of this effect is unknown, the longer duration of CO_2 exposure with slow fill rates may allow more time for pH adjustments to occur in the blood and CSF.

In summary, pre-fill CO_2 exposure takes less time to cause loss of consciousness than gradual-fill exposure, but animals are exposed to higher CO_2 concentrations during the period of consciousness. With gradual-fill CO_2 exposure, faster flow rates cause a shorter duration to loss

of consciousness, but may be associated with higher CO_2 concentrations at the time of loss of consciousness.

1.2.3 Contexts of CO_2 use

Carbon dioxide is recommended for euthanasia of laboratory rodents in many western countries such as Canada (CCAC, 1993) the USA (AVMA, 2000), the UK (UK Home Office, 1997), the countries of the European Union (Close et al., 1997) and Australia and New Zealand (ANZCCART, 1993). The recommended method of delivery varies, but in general, gradual induction is recommended over pre-fill. Although some recommendations stipulate a flow rate for gradual induction, anecdotal reports suggest that few facilities monitor flow rate.

Carbon dioxide is also used for euthanasia of injured wildlife in rehabilitation centres and for stunning or killing of farmed mink, pigs, poultry, and fish (via water bath). Recommendations for use of CO_2 with these species differ from those applicable to laboratory animals in that they often require pre-filling or rapidly filling the chamber rather than gradual fill. For example, the UK Welfare of Animals (Slaughter or Killing) Regulation (1995) stipulates that for the stunning of pigs, the chamber must reach a concentration of at least 70% in less than 30 seconds, and for the killing of mink, the animal must be placed into a chamber containing 100% CO_2 (UK Home Office, 1995). In contrast, the UK Code of Practice for killing of experimental animals recommends exposure to a gradual increase in CO_2 (UK Home Office, 1997). However, there has been no research to justify the use of different delivery methods for different species.

1.2.4 Advantages of CO_2 euthanasia

There are a number of reasons for the widespread use of CO_2 euthanasia of laboratory animals. Anecdotal reports suggest that there is a general perception within the scientific

community that the effects of CO_2 on experimental results are known, and that unknown effects on experimental results may be introduced by changing to other procedures. Initial experiments on the use of CO_2 for killing occurred in the late 1800's (reviewed by Hill and Flack, 1908), and there is therefore a long history of CO_2 use. There have also been a number of studies conducted to determine the effects of CO_2 on specific metabolites and tissues, in order to ensure that experimental results are not affected (e.g. Fawell et al., 1972; Pecaut et al., 2000; Bergersweeney et al., 1994). In situations where a confounding effect on tissue measures is expected, such as during toxicological research, other euthanasia methods like decapitation and cervical dislocation are often employed.

CO_2 also has a number of advantages for the operator in comparison to other methods. Because it can be delivered in a chamber, it involves little handling of animals and can be used to quickly euthanize large numbers of animals at a time. In comparison to physical methods and injectable anaesthetics, it requires considerably less time. This method also requires little direct interaction with animals and results in few effects that may be disturbing to the operator. As a respiratory gas, CO_2 is also safer for the operator than other gas euthanasia agents such as carbon monoxide, chloroform, ether and gas anaesthetics, which can pose significant health risks.

CO_2 also has cost advantages in comparison to other methods. Decapitation and cervical dislocation require minimal equipment, but have considerable costs in terms of employee time. Other physical methods such as microwave irradiation require the purchase of expensive equipment. Anaesthetics tend to be much more expensive than CO_2. There are also indirect costs for anaesthetic methods, such as the purchase of needles (for injectable anaesthetics), and anaesthetic machines and scavenging systems (for gaseous anaesthetics).

With respect to the welfare of animals CO_2 provides many benefits in comparison to other euthanasia methods. Most importantly, CO_2 is easy to deliver properly with little training.

Physical methods, injectable anaesthetics and inert gases have the potential to be administered incorrectly and to result in considerable pain and distress for the animal. Gases in general are also advantageous because they require minimal handling of the animal. Laboratory rodents are not generally habituated to regular handling and restraint, and these procedures can therefore cause considerable distress (e.g. Sharp et al., 2002, 2003). Furthermore, injections into the peritoneal cavity can be painful, and injectable anaesthetics can be irritating, possibly stimulating visceral nociceptors. Although CO_2 has many advantages over other euthanasia methods, there has been ongoing debate as to whether it causes pain and distress in laboratory rodents.

1.3 Animal Distress and Carbon Dioxide

1.3.1 What is animal distress?

Animal welfare is generally considered to be enhanced by good health, positive affect, and the ability to perform natural behaviours, and reduced by poor health, negative affect and inadequate outlets for behaviour (Fraser et al., 1997). With a procedure such as CO_2 euthanasia, the main concern is with the affective state of the animal.

In describing everyday usage, The Canadian Oxford Dictionary (2000) defines distress as "severe trouble, anxiety, sorrow" (p.275), indicating a negative emotional state. Selye (1975) was the first to define distress scientifically, and he used this term to refer to biological responses to negative stressors, where stressor refers to an actual or perceived threat to homeostasis. With specific reference to animal welfare, the term distress has been defined in a number of different ways; however, in general there is agreement that 'distress' refers to a negative state that develops when an organism is unable to adapt to a stressor (e.g. Kitchen et al., 1987; Moberg, 2000; NRC, 2003; Rowan et al., 1998). This definition, therefore, acts as an

umbrella term that encompasses negative affect associated with more specific negative states such as pain, discomfort and fear.

1.3.2 Potential mechanisms for CO_2 distress

There are three main mechanisms by which CO_2 might cause distress in rats. The rationale for the first two mechanisms that are discussed is largely dependent on human experiences during CO_2 exposure. If humans report pain or distress during CO_2 exposure, then following the logic of Dawkins (1980) it can be hypothesized that CO_2 has the potential for similar sensations in animals with similar anatomy and physiology. Although humans have higher order cognitive processing that can affect the quality of a sensation, the general perception of a stimulus as appetitive or aversive is likely conserved between humans and other mammals. However, it is important to recognize that sensations may also differ between different species, since each is adapted to a particular niche.

1.3.2.1 Pain induced by carbonic acid formation

The most widely discussed mechanism for distress during CO_2 euthanasia is that CO_2 can form carbonic acid on nasal mucous membranes, and this stimulates trigeminal nociceptors and causes pain (e.g. Leach et al., 2002a). This can be extended by considering that CO_2 also has the potential to affect the cornea and conjunctiva of the eyes, and possibly chemoreceptors in the lower respiratory tract that are sensitive to other irritants, although this has not been fully examined.

A number of studies have assessed the potential for CO_2 to activate nociceptors and evoke pain in humans when applied to the nasal mucosa, cornea and conjunctiva. Negative nasal mucosal potentials have been recorded from the nasal septum and used as a non-invasive method for measurement of trigeminal nociceptor activation. Negative mucosal potentials "are

thought to be the result of summating receptor potentials of chemosensitive nociceptors of the trigeminal nerve" (Thurauf et al., 1993, p.293). These potentials have been recorded in response to CO_2 concentrations over 45%, and have been found to increase with CO_2 concentration and application duration (Thurauf, 1993). Human self-report data indicate that CO_2 is detectable at the nasal mucosa at concentrations of only 20%, and that it becomes overtly painful at concentrations above approximately 50% (Anton et al., 1992; Thurauf et al., 2002). However, the concentration that is perceived as painful does vary, with the pain threshold ranging from 32.5% to 55% depending on the individual (Anton et al., 1992). Danneman et al. (1997) had subjects rate the noxiousness of CO_2 at concentrations ranging from 50% to 100% when inhaled via the nose, and found that the majority of subjects rated 50% CO_2 as unpleasant or uncomfortable, and 100% CO_2 as painful. Furthermore, at each concentration some subjects felt they could not take a complete breath, and the words tingling, prickling and burning were used to describe the sensation at all concentrations.

In rats, CO_2 has been shown to activate dorsal horn neurons that receive input from trigeminal nociceptors in the nasal mucosa (Anton et al., 1991; Peppel and Anton, 1993), suggesting that CO_2 also has the potential to cause pain in rats. Peppel and Anton (1993) found that rat nociceptors respond to CO_2 concentrations that are similar to those previously reported for human nociceptors. CO_2 concentrations below 37% were subthreshold for the majority of rat neurons, and the CO_2 nociception threshold ranged from 37% to 50% CO_2 for the majority of neurons. Furthermore, the graded response tended to increase in a linear fashion until saturation was reached at approximately 87% CO_2.

Another method of assessing the potential for a substance to elicit pain at the upper respiratory tract is through its ability to elicit changes in cardiorespiratory rhythms. Inhaled irritants are known to induce a reflex apnea and heart rate reduction, and these responses are thought to reduce transfer of harmful substances into the body (Widdicombe, 1986). In rats,

100% CO_2 elicits this apnea and bradycardia, but CO_2 concentrations of 10, 25 and 50% do not (Yavari et al., 1996). The threshold necessary for eliciting cardiorespiratory alterations was not determined, but this data suggests that CO_2 concentrations less than 50% are not irritating to the nasal mucosa in rats.

The effect of CO_2 on pain and activation of nociceptors in the cornea and conjunctiva appears to be similar to its effect at the nasal mucosa. Feng and Simpson (2003) examined pain thresholds in humans in response to CO_2 application to the cornea and conjunctiva and found that they occurred at 31% and 54%, respectively. Participants again characterized the sensation at threshold as burning or stinging. In another study examining the effect of CO_2 on the cornea, subjects reported mild stinging at a mean CO_2 concentration of 33.5%, and overt pain at 47.5% (Chen et al., 1995). Chen et al. also examined the responses of cat corneal nociceptors to CO_2 application and found that they responded at a mean threshold of 40% CO_2, which is similar to the pain responses observed in humans. Activation of rat corneal nociceptors by CO_2 has also been demonstrated (Hirata et al., 1999), although the threshold level of CO_2 needed to elicit nociceptor activation was not identified.

This evidence suggests that CO_2 has the potential to cause pain in rats through stimulation of nociceptors in the nasal mucosa and cornea. Pain is likely to occur with pre-fill methods in which a conscious animal is exposed to high concentrations of CO_2 (>70%). During gradual fill CO_2 euthanasia animals reach unconsciousness at about 40% (Smith and Harrap, 1997), so there is some potential for low levels of pain to occur around the time of loss of consciousness.

1.3.2.2 Non-pain discomfort associated with hypercapnia and hypoxia

Distress during CO_2 euthanasia could also occur through the effects of hypercapnia (elevated blood CO_2) and hypoxia (reduced blood O_2), which have been shown to cause dyspnea

in humans. Dyspnea is an unpleasant sensation of breathlessness which is sometimes accompanied by other negative sensations such as headache, flush, restlessness, heart pounding, drowsiness, and dizziness (e.g. Moosavi et al., 2003). With mild increases in inspired CO_2 and decreases in inspired O_2, increased ventilation results in a reduction or elimination of dyspnea (Banzett et al., 1996; Fowler, 1957; Shea et al., 1996), but there are limits to this compensatory mechanism such that dyspnea still occurs during spontaneous breathing with moderate hypercapnia and hypoxia (Shea et al., 1996).

Numerous studies have demonstrated that hypercapnia results in dyspnea in humans during spontaneous breathing (e.g. Banzett et al., 1996; Shea et al., 1996). Most studies examining dyspnea due to hypercapnia have monitored end-tidal partial pressures (P_{ET}) of CO_2 (concentration of CO_2 breathed out) rather than inspired levels of CO_2, and it is therefore difficult to determine the inspired concentrations of CO_2 that result in different levels of dyspnea. However, some studies have examined inspired CO_2 levels and can provide insight into the CO_2 concentrations necessary to elicit dyspnea. Liotti et al. (2001) had subjects rate their sensations of dyspnea on a 100 point scale during exposure to 8% CO_2 in O_2, and dyspnea levels were rated as either 73 or 55 depending on whether the gas mixture was delivered using a facemask or a mouthpiece, respectively. Dripps and Comroe (1947) found that approximately 30% of study participants reported dyspnea when exposed to either 7.6 or 10.4% CO_2 in O_2.

In the late 1800's and early 1900's researchers often used themselves as experimental subjects in the investigation of respiratory physiology (summarized by Hill and Flack, 1908). These studies provide insight into the concentrations of CO_2 that result in moderate to severe dyspnea during spontaneous breathing. According to Hill and Flack (1908), Greenwood found that he was able to breathe a mixture of 15.3% CO_2 and 14.5% O_2 with marked dyspnea, but that higher levels of CO_2 resulted in closure of the glottis and prevented inhalation. Haldane and Smith (1892) found that when breathing 18.6% CO_2 in air, they developed profound dyspnea in

one to two minutes that was accompanied by throbbing in the head and mental dullness. Together with the results from modern studies, these results indicate that during spontaneous breathing, dyspnea and other negative sensations can occur with CO_2 concentrations as low as 8%, and that severe dyspnea occurs at CO_2 concentrations greater than approximately 15%.

Hypoxia also appears to have the potential to cause dyspnea in humans, but to a lesser extent than hypercapnia. Moosavi et al. (2003) found that during constrained breathing, participants reported onset of dyspnea when P_{ETO2} decreased below approximately 60 Torr (P_{ETO2} levels were 108 Torr during baseline when breathing air). However, at the study's ethical P_{ETO2} limit of 40 Torr (the lowest inspired O_2 tested was 7%) the majority of participants rated their dyspnea as less than 40 on a visual analogue scale which ranged from 0 (no dyspnea) to 100 (extreme dyspnea). This indicates that O_2 concentrations of less than 7% are needed to evoke moderate sensations of dyspnea in humans during constrained breathing. The potential for dyspnea due to hypoxia alone in spontaneously breathing humans has not been fully investigated. Hypoxia-induced loss of consciousness occurs occasionally in pilots as a result of cabin pressurization failure (Cable, 2003), suggesting that spontaneously breathing humans do not experience dyspnea prior to loss of consciousness with hypoxia. However, hypoxia has a synergistic effect on ventilatory responses to hypercapnia (e.g. Nielson and Smith, 1952), and augments sensations of dyspnea due to hypercapnia in humans (Banzett et al., 1996; Masuda et al, 2001). These results indicate that hypoxia alone is unlikely to cause sensations of dyspnea during free breathing, but that it may increase dyspnea due to hypercapnia. Thus, if dyspnea occurs during CO_2 euthanasia, hypoxia may be a contributing factor.

To date, no models have been developed for the assessment of dyspnea in conscious animals, so it is not known whether hypercapnia and hypoxia cause dyspnea in rats. Dyspnea in rats may be indicated by increases in breathing depth and frequency. The term dyspnea is sometimes used in the veterinary literature to refer directly to these breathing changes (e.g.

Hornett and Haynes, 1984), but 'dyspnea' actually refers to a sensation of breathlessness rather than the physical changes in breathing that sometimes accompany this sensation. While increases in breathing (sometimes referred to as gasping or laboured breathing) have been observed in rats during CO_2 euthanasia, it is not clear whether they occur before or after loss of consciousness, and whether they are more severe with pre-fill or gradual-fill exposure (Coenen et al., 1995; Hornett and Haynes, 1984; Iwarsson & Rehbinder, 1993; Smith and Harrap, 1997). Furthermore, human medical studies suggest that breathing alterations and sensations of dyspnea do not always occur concurrently (Lush et al., 1988), which brings into question the usefulness of this measure for assessing dyspnea in rats.

While there is no evidence confirming that dyspnea occurs in rats, it is reasonable to assume that such a basic response to changes in air composition and blood gas levels would be conserved between species. If so, both pre-fill (<6% O_2, >70% CO_2) and gradual-fill CO_2 euthanasia (<12% O_2, >40% CO_2) have the potential to cause strong sensations of dyspnea in rats, mainly due to hypercapnia.

1.3.2.3 Fear due to novelty

Novelty has been suggested to induce an approach-avoidance conflict in rats, resulting from an interaction between exploratory motivation and fear (Montgomery, 1955). CO_2 could therefore cause distress in rats by acting as a novel stimulus that elicits fear. Odour perception occurs as a result of both olfactory and trigeminal stimulation (e.g. Cain and Murphy, 1980). While CO_2 is thought to stimulate mainly trigeminal neurons, humans perceive an odour quality when asked to describe sensations occurring with CO_2 inhalation (Cain and Murphy, 1980). Rats can detect CO_2 at concentrations between 0.04 and 1.7% (Youngentob, 1991), which is far below the level required to stimulate trigeminal neurons (Peppel and Anton, 1993). The exact quality of CO_2 that rats are perceiving at these low levels is unknown, but a large portion of the

rat brain is devoted to odour detection so rats may be more sensitive to the odour quality associated with CO_2 than humans. Wallace and Rosen (2000) demonstrated that exposure of rats to novel odours, such as butyric acid (similar to rancid butter) and isoamyl acetate (similar to banana), causes avoidance, reduces grooming time and increases freezing time, suggesting that novel odours can elicit fear in rats. Thus, CO_2 may cause distress in rats because it is acting as a novel stimulus that elicits fear.

1.4 Assessment of Distress During CO_2 Euthanasia

In the assessment of animals' subjective states it is not possible to obtain verbal reports of their experience, and it is therefore necessary to use other measures. There are two types of measures that can be used to assess whether a procedure such as CO_2 euthanasia causes pain and distress in animals: 1) physiological measures of stress, and 2) behavioural measures of distress and aversion.

1.4.1 Physiological assessment of distress

The term stress generally refers to a biological response to an actual or perceived threat to homeostasis (e.g. Moberg, 2000), and involves both behavioural and physiological responses that act to maintain homeostasis. Physiological responses, such as activation of the sympathetic-adrenergic-medullary axis (SAM) and the hypothalamic-pituitary-adrenal axis (HPA), occur in response to both physiological and psychological stressors and serve to redirect body systems towards coping with the stressor. Activation of the SAM axis occurs almost immediately in response to a stressor. This system activates the body by releasing epinephrine and norepinephrine, increasing glucose metabolism, respiration, heart rate and blood pressure, and reducing functions that are not of immediate necessity (summarized by Toates, 1995).

Activation of the HPA axis results in release of corticotrophin-releasing hormone (CRH) and other secretagogues from the hypothalamus, that lead to the release of adrenocorticotropic hormone (ACTH) from the pituitary and then cortisol or corticosterone (in the rat) from the adrenal cortex. This system causes a number of effects including mobilization of energy, suppression of the immune system, and release of endorphins to modulate pain responses (summarized by Toates, 1995). The presence or absence of activation of the SAM and HPA axes can provide information as to whether a stimulus acts as a stressor, but as will be discussed later, their usefulness for assessing distress is limited.

Consistent activation of the SAM axis in response to reduced O_2 and elevated CO_2 has not been observed in rats. Borovsky et al. (1998) found that a 30 s exposure to 100% CO_2 or 100% nitrogen was sufficient to increase plasma norepinephrine levels in rats, and Fukuda et al. (1989) found that 100% CO_2 increases cardiac sympathetic activity. However, other studies using lower CO_2 concentrations and higher O_2 concentrations have failed to observe activation of the SAM axis. Hodges et al. (2002) found that when rats were exposed to either 12% O_2 in air or 7% CO_2 in O_2, ventilation increased, but heart rate and blood pressure did not. Similarly, Raff and Roarty (1988) found that blood pressure was not affected by low O_2 (10% O_2), high CO_2 (4% or 8% CO_2) or a combination of low O_2 and high CO_2 (7% O_2 and either 4% or 8% CO_2). Three studies have examined SAM variables during CO_2 euthanasia in rats. With pre-fill exposure, all studies found that heart rate (Coenen et al., 1995; Sharp et al., 2006; Smith and Harrap, 1997) and blood pressure (Sharp, 2006; Smith and Harrap, 1997) decreased immediately. With gradual-fill exposure, Coenen et al. (1995) found that heart rate decreased, but Smith and Harrap (1997) found that heart rate and blood pressure initially increased before declining. The immediate decline in heart rate with pre-fill euthanasia can probably be explained by rapid depression of the CNS. Also, irritant stimuli, including CO_2 at high concentrations, are known to cause bradycardia via stimulation of trigeminal nociceptors (Yavari et al., 1996).

Unfortunately the results for gradual fill are difficult to interpret because neither study used handled controls or acclimatized the animals to the chamber. It is therefore unclear how these variables relate to resting values, and whether activation of the SAM axis occurred as a result of handling.

Increased HPA axis activity during moderate hypoxia and hypercapnia has also been examined in rats. Marotta et al. (1976) found that prolonged exposure to low O_2 (10% O_2 in nitrogen) or high CO_2 (10% CO_2, 20% O_2, in nitrogen) resulted in corticosterone levels almost eight times those observed in control animals. Similarly, Raff and Roarty (1988) measured ACTH after exposure to low O_2 (10% O_2), high CO_2 (4% CO_2 or 8% CO_2) or a combination of low O_2 and high CO_2 (7% O_2 and either 4% or 8% CO_2), and found that it was elevated with 8% CO_2 and with the combination mixtures. Although one study has specifically examined ACTH and corticosterone levels during gradual-fill CO_2 euthanasia and found no increase (Hackbarth et al., 1999), the results were likely affected by problems with experimental design. Not only were the sample sizes extremely small for analysis of these variables (N = 4), but decapitation was performed 30 s, 75 s, and 120 s into the procedure. This time frame does not allow for increases in these hormones to occur in the blood stream (Terlouw et al., 1997), thus no effect could be expected. The two previous studies indicate that gradual-fill CO_2 euthanasia could result in activation of the HPA axis.

There are, however, three major limitations with the use of physiological stress measures in the assessment of distress associated with CO_2 euthanasia. Firstly, SAM and HPA activation do not imply that the stressor or the response is negative or positive for the animal. In fact, physiological stress responses are associated with neutral and positive events such as exercise and mating. Thus a stress response does not necessarily mean that an animal's welfare is compromised (Dawkins, 1998). However, by examining whether the animal finds the stressor

appetitive or aversive, it is often possible to determine whether the stressor is perceived in a negative or positive manner.

Secondly, SAM and HPA activation occur in response to both physiological and psychological stressors, such that the actual effects of the stimulus on the subjective state of the animal are difficult to determine. In the case of the HPA axis, it appears that there are at least two types of CNS pathways that can elicit release of CRH. Herman and Cullinan (1997) suggest that there are direct pathways for immediate physiological threats, and indirect pathways via higher brain circuits for stimuli that require interpretation with respect to previous experience. HPA responses to what the authors label "processive" stressors like restraint and novelty appear to be associated with relay through higher brain areas such as the forebrain and limbic system nuclei, whereas responses to "systemic" stressors like hypoxia and ether exposure are more likely directly relayed from brainstem nuclei (reviewed by Herman and Cullinan, 1997; Herman et al., 1996). This suggests that HPA responses to CO_2 euthanasia could occur without perception by higher brain centres. If so, they would be a poor measure of psychological distress associated with this procedure. In fact, it has been shown that hypothalamic vasopressin-containing neurons, which secrete the CRH secretogogue vasopressin, produce similar increases in c-Fos (indicating activation of the neuron) in response to CO_2 exposure in both awake and anesthetized rats (Kc et al., 2002). Although these measures can provide a gauge of how the stimulus affects the animal physiologically, and thus the potential for causing distress, they do not necessarily allow one to assess the psychological state of the animal.

Finally, because CO_2 euthanasia occurs relatively quickly, it is not possible to measure HPA responses to the portion of the procedure that occurs while the animal is still conscious. In pre-fill and gradual CO_2 euthanasia, rats become unconscious in approximately 15 s and 90 s respectively, and in terms of distress we are concerned only with what the animal experiences during this time. Changes in the SAM occur within seconds and can be measured during this

procedure, but evidence of activation of the HPA axis does not appear this quickly and the state of the animal at the time of loss of consciousness cannot be preserved. By observing this system after prolonged exposure or after the animal regains consciousness, we are no longer observing what occurred during that limited period of time. For example, nociceptive CO_2 concentrations might occur after loss of consciousness, and this might have an effect on HPA variables that are not associated with the animal's conscious experience.

1.4.2 Behavioural assessment of distress

Unlike physiological measures, behavioural measures can be easily monitored during the course of CO_2 exposure, and the types of behaviours performed can provide us with information about how the animal perceives the stimulus. During the euthanasia process we can observe whether the animal exhibits behaviours that are indicative of distress such as escape behaviours, distress vocalizations and behaviours associated with pain or discomfort. In tests of aversion we can observe whether the animal avoids CO_2 exposure, and whether it will pay a cost to avoid exposure. We can also use other behavioural tests to determine whether CO_2 exposure elicits fear in animals.

1.4.2.1 Behaviour during CO_2 exposure

During exposure to an aversive or painful gas, animals might show both general and species-specific signs of distress. General signs might include behaviours associated with escape, such as increased activity, exploration, and concentrated activity at known exits. In the rat this might be indicated by increased locomotor activity and rearing, as well as wall-climbing, investigation of chamber surfaces and pushing, scratching and biting at potential exits. Although an increase in activity indicates that the animal is responding to the stimulus, an increase in actual escape behaviours, such as pushing, scratching and biting at potential exits, is important

to show that the stimulus is perceived as negative and is not simply eliciting exploration. Rats have also been shown to respond to aversive stimuli by freezing (e.g. Barnett, 1975; Dielenberg and McGregor, 2001), which differs from simple inactivity in terms of increased muscle tone and a lack of head movements. Although this seems contradictory to the increase in activity described above, it is widely recognized that animals, including rats, can respond proactively or reactively when confronted with a stressor (reviewed by Koolhas et al., 1999). Therefore distress could be indicated by either an increase in activity and escape behaviour or by freezing behaviour. In assessment of either behavioural response, it is important to compare it to stable baseline or control data to ensure that the response is in fact due to stimulus exposure, and not to some other factor such as handling or exposure to a novel environment.

Distress can also be indicated by the production of vocalizations. Vocalizations produced during distress might serve a number of purposes, such as to warn conspecifics of danger, to elicit a care-giving response or to assist in regaining contact with conspecifics (reviewed by Klump and Shalter, 1984, reproduced in Hauser, 1996). Rats have been shown to produce ultrasonic vocalizations (USVs) in a variety of contexts, and these calls may be an indicator of affective state (Knutson et al., 2002). USVs in the 50 kHz and 20 kHz ranges tend to occur in response to appetitive and aversive stimuli respectively. For example, USVs in the 20-30 kHz range have been observed in response to painful stimulation (Dinh et al., 1999; Colpaert et al., 1987; Jourdan et al., 1995, 1998), exposure to predators (Blanchard et al., 1991), drug withdrawal (Vivian and Miczek, 1991), fighting with conspecifics (Thomas et al., 1983), acoustic startle (Kaltwasser, 1990) active avoidance learning (Cuomo et al., 1992) and touching by an unfamiliar human (Brudzynski and Ociepa, 1992). While these calls appear to be relatively non-specific and their signalling context is not fully understood, they do have an association with situations that appear to be negative.

The occurrence of behaviours associated with pain or with activation of a stress response may also indicate that CO_2 euthanasia causes distress. Pain-related behaviours might include shaking of the head or pawing at the nose and eyes. Urination and defecation are also sometimes used as measures of distress because they are associated with activation of the autonomic nervous system during the stress response (reviewed by Vingerhoets et al., 1985). Although urination and defecation can be indicators of distress, their absence does not necessarily indicate a lack of distress because they can be affected by recent feeding and elimination behaviour.

A number of studies have observed the behaviour of rats during CO_2 euthanasia, but often conclusions are drawn on the basis of opinion rather than objective data. Responses to CO_2 have been characterized by some as "apprehensive" (Hornett and Haynes, 1984), or "abnormal", "excited" and "agitated" (Coenen et al., 1995), without precise definitions or descriptions of behavioural measures. Hackbarth et al. (1999) suggest that they saw no signs of "fear" in rats during CO_2 euthanasia. However, no data are provided to support this assertion, even though they describe a number of behavioural variables that were measured.

Other studies have used objective measures of behaviour, but the usefulness of these measures is complicated by small sample sizes or a lack of appropriate distress criteria, controls or acclimatization prior to gas exposure. Blackshaw et al. (1988) compared rats' responses during exposure to either air or pre-fill CO_2, and measured movement, time spent stationary and wall touches and climbs during the first 10 s of exposure. During CO_2 exposure, rats exhibited less overall activity, but more wall climbing. The decrease in activity is difficult to interpret because it may be due to the animal freezing or the anaesthetic effects of CO_2. However, the increased incidence of wall climbing does suggest increased exploration. Wall climbing was even greater during exposure to ether and chloroform, which are known irritants, suggesting that this behaviour was indicative of some level of distress, but also that the response was not

maximal. However, the results of this study must be interpreted with caution because only three animals per group were observed.

Smith and Harrap (1997) recorded the behavioural responses of rats to both rapid-fill and gradual-fill euthanasia. They observed wall climbing in only one animal and did not observe any other escape behaviours, but they did observe circling of the chamber by the majority of animals in both groups and immediate urination by all rats in the rapid fill group. The circling behaviour was not quantified so it is not known whether it differed between groups, and this study did not use controls or acclimatization, so it is unclear how much of the effect was due to handling and novelty. However, urination by all rats in one group and none in the other does suggest distress in the rapid fill group.

Finally, Britt (1987) examined the responses of two strains of rats during baseline and during exposure to either pre-fill or gradual-fill CO_2 euthanasia. However, interpretation of this data is difficult because statistical analyses were not performed and information on variability was not provided. Pre-fill euthanasia was only completed with one rat. During gradual-fill exposure, Sprague Dawley rats showed increases in activity and climbing, while Lister Hooded rats showed a decrease in activity, no change in climbing and an increase in backward movements. Increased rearing and moving indicate increased exploration, and the reduction in activity in the second strain is difficult to interpret because of the narcotic effects of CO_2. The presence of backward movements, which are not generally seen in rats, may indicate that the animals were trying to remove themselves from the stimulus. Both strains also showed increases in shaking, urination and defecation. Increased shaking indicates that contrary to previous suggestions, gradual-fill exposure may result in pain prior to loss of consciousness, and increased urination and defecation suggest activation of the autonomic nervous system. However, shaking was not defined, making the occurrence of this behaviour difficult to

interpret. Although ultrasonic data were recorded, no vocalizations were detected for either strain.

In summary, the majority of research on the behavioural responses of rats to CO_2 exposure has been poorly designed and the results are difficult to interpret. While some studies have found no evidence of distress during CO_2 exposure, others have found behaviours that suggest distress and possibly pain. Well-controlled research is necessary to clarify these discrepancies.

1.4.2.2 Aversion Testing

Aversion testing can be used to examine whether an animal will avoid a stimulus, as well as the strength of aversion to that stimulus. Firstly, we can observe whether an animal will remove itself from a stimulus if given the opportunity, and how quickly it does so. Secondly, we can test the strength of aversion to the stimulus by having the animal work to avoid the stimulus, or by comparing the stimulus to another stimulus of known value using either approach-avoidance testing or avoidance-avoidance testing. In approach-avoidance conflict, an attractive stimulus is paired with an aversive stimulus, and the animal must determine whether it is willing to accept the aversive stimulus in order to gain access to the attractive one. In avoidance-avoidance testing, the animal must choose between exposure to one of two aversive stimuli. By comparing the test stimulus to other stimuli of known value, it is possible to rate how aversive the test stimulus is (Rushen, 1996).

Preference studies have been used to determine whether rats find different concentrations of CO_2 aversive, and how these rank against argon-induced hypoxia (<2% O_2) and gaseous anaesthetics. Leach et al. (2002 a,b) tested rats in a preference system consisting of two chambers connected by a tunnel, where one chamber contained the test gas and the other contained air. During control sessions with air, rats withdrew from the test chamber in

approximately 15 s, whereas during testing with CO_2 at concentrations greater than 25.5%, rats withdrew from the test chamber in approximately 1 s. Furthermore, during the 180 s test session, the time spent in the CO_2 chamber was only 1 to 2 s, while the time spent in this chamber during control sessions was 35 to 50 s. Time to exit and time spent in the chamber were significantly greater for severe hypoxia and anaesthetic gases, suggesting that hypoxia and anaesthetic gases are less aversive than CO_2. These results indicate that rats are able to quickly detect CO_2 at concentrations greater than 25.5% and that they find these concentrations aversive. However, because there was no cost to leaving the chamber, these results do not indicate the strength of aversion to CO_2. Furthermore, these results only show the response to moderate, static concentrations of CO_2, and responses to gradual-fill exposure might differ. It is thought that anaesthesia begins to occur at low levels of CO_2, and animals may therefore be partially anesthetized when moderate concentrations of CO_2 occur during gradual-fill exposure.

While the strength of aversion to CO_2 has not yet been examined in rats, approach-avoidance testing has been used to examine CO_2 aversion in other species. Studies with poultry have found that a large proportion of birds will enter a chamber containing CO_2 at concentrations greater than 60% in order to gain access to food or social contact (Gerritzen, et al., 2000; Raj, 1996; Webster & Fletcher, 2004). Interestingly, Raj (1996) found that birds that entered a chamber containing 75% CO_2 did so while exhibiting symptoms of pain and distress such as gasping, head shaking and vocalizing. Aversion to CO_2 has also been examined in pigs, and while they will generally tolerate 30% CO_2 in order to gain access to a food reward, they will not tolerate exposure to 90% CO_2, even after a 24-h period of food deprivation (Raj and Gregory, 1995). Mink have been shown to work to obtain access to novel objects (Mason et al., 2001), but will avoid a chamber containing a novel object when it contains100% CO_2 (Cooper et al., 1998). The fact that pigs and mink are willing to forgo access to desired items to avoid high concentrations of CO_2 suggests that it is aversive. However, responses to moderate CO_2

concentrations that are sufficient to induce loss of consciousness have not been examined. Similar studies are needed to determine whether rats exhibit significant aversion to CO_2 exposure.

1.4.2.3 Tests of 'anxiety'

Researchers have used the Vogel conflict test to determine whether CO_2 causes what they have termed 'anxiety' in rats. For the Vogel conflict test, rats are given limited access to water, and during specified periods they can drink but only with concomitant exposure to mild electric shock. It is predicted that when an anxiety-provoking stimulus is delivered prior to water access, drinking will be reduced. Cuccheddu et al. (1995) observed the effect of a 10-minute exposure to gradual fill with a 35% CO_2 / 65% O_2 mixture, and found a 40% reduction in drinking. This result was similar to the effect of dosing with an anxiogenic compound. It was also determined that this suppression could be eliminated by dosing the rats with anxiolytics prior to CO_2 exposure. Although this study uses an indirect method of measurement, it does indicate that exposure to an increasing concentration of CO_2 causes anxiety in rats.

1.4.2.4 Summary of Behavioural Measures:

Most studies have not found behaviours that are indicative of distress during exposure of rats to CO_2, either using the pre-fill or gradual-fill methods. However, the results of behavioural tests with rats suggest that they are averse to concentrations of CO_2 that are sufficient to cause loss of consciousness. Aversion to gradual-fill CO_2 exposure has not yet been examined, and the strength of CO_2 aversion in rats is still unknown.

1.5 Objectives

Although pre-fill CO_2 euthanasia of rats is relatively fast, human self-report data and data on nociceptor stimulation suggest that it has a high potential for pain in rats. It also has a high potential for non-pain distress due to dyspnea. The potential for distress during gradual-fill CO_2 euthanasia is less clear because loss of consciousness occurs at approximately 40% CO_2 (Smith and Harrap, 1997). In general, 40% CO_2 is not sufficient to cause pain in humans, or to stimulate nociceptors in rat nasal mucosa. However, it is sufficient to cause dyspnea in humans, and it is possible that rats also experience this sensation during exposure to increased levels of CO_2. The majority of studies examining responses to CO_2 exposure have not observed clear behavioural signs of distress in rats, but the studies to date have been poorly executed. Behavioural testing has determined that CO_2 exposure causes aversion in rats and other species, but the strength of rats' aversion to CO_2 and the effects of gradual-fill exposure have not yet been determined.

From the information currently available it is not possible to determine conclusively whether either method of CO_2 euthanasia causes distress in rats. However, pre-fill CO_2 exposure has a high potential for causing pain, so I decided to focus my dissertation research on gradual-fill CO_2 euthanasia. The two main objectives of my thesis were: 1) to determine whether gradual-fill CO_2 euthanasia causes distress in laboratory rats, by examining behavioural responses during euthanasia, and aversion during approach-avoidance testing, and 2) to determine whether pain, dyspnea and novelty are likely sources of distress during gradual-fill CO_2 euthanasia.

In my first study (Chapter 2) I performed a detailed analysis of behavioural responses of rats during gradual-fill CO_2 euthanasia in order to determine whether they show behavioural signs of distress. I also examined rats' responses to a reduction in O_2 concentrations to

determine whether hypoxia played a role in their responses to CO_2. In my second study (Chapter 3), I used approach-avoidance testing to determine whether rats find CO_2 more aversive than a valuable food reward, and to determine which concentrations of CO_2 rats find aversive when tested with either static or gradually increasing CO_2. I also examined rat aversion to argon-induced hypoxia, which has been suggested as an alternative gas euthanasia method. In my third study (Chapter 4), I expanded my investigation of rat aversion to gradually increasing concentrations of CO_2 by determining whether CO_2 flow rate affects rat aversion to CO_2. Within the first three studies I also examined the CO_2 concentrations that resulted in behavioural signs of distress and aversion, and compared these values with those from previous studies on the potential for CO_2 to cause pain and dyspnea. In doing this I was able to assess whether pain and dyspnea were likely causes of this distress and aversion. In my final study (Chapter 5), I investigated whether novelty was a cause of distress and aversion during gradual-fill CO_2 exposure.

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CHAPTER 2: Behavioural responses of rats to gradual-fill carbon dioxide euthanasia and reduced oxygen concentrations[2]

2.1 Introduction

Methods commonly used for euthanasia of small laboratory rodents include physical techniques, injectable anaesthetics, and exposure to anaesthetic and non-anaesthetic gases. Exposure to CO_2 is one of the most widely recommended euthanasia methods for rats (ANZCCART, 1993; AVMA, 2000; Canadian Council on Animal Care, 1993; Close et al., 1997; UK Home Office, 1997). Rats are either placed into a chamber pre-filled with gas or the gas is gradually introduced into an air-filled chamber, and this results in narcosis due to the properties of CO_2 followed by death. Both methods are relatively easy to perform, inexpensive, safe for laboratory workers, and involve little handling and restraint of animals. Ideally, a euthanasia method should also result in a quick death with minimal pain and distress before loss of consciousness, but it is not clear whether CO_2 euthanasia meets these last criteria.

CO_2 forms carbonic acid when it comes into contact with moisture. It begins to stimulate nociceptors in rat nasal mucosa at CO_2 concentrations above 25%, and the threshold for the majority of nociceptors is between 37% and 50% CO_2 (Anton et al., 1991; Peppel & Anton, 1993). In humans, CO_2 is detectable and begins to become painful at the cornea, conjunctiva and the nasal mucosa at concentrations between 30% and 54% (Anton et al., 1992; Chen et al, 1995; Feng & Simpson, 2003). High CO_2 concentrations can also cause dyspnea, or shortness of breath, which includes the sensations of both air hunger and increased breathing effort (Lansing et al., 2000). At low levels of CO_2 dyspnea can be overcome by ventilatory adjustments (Shea et al, 1996), but spontaneously breathing humans report this sensation at CO_2 concentrations of only 8% (Dripps & Comroe, 1947; Liotto et al., 2001) and severity increases with increasing

[2] A version of this chapter has been published. Niel, L., Weary, D.M., 2006. Behavioural responses of rats to gradual-fill carbon dioxide euthanasia and reduced oxygen concentrations. Appl. Anim. Behav. Sci. (in press).

CO_2 concentrations (Banzett et al., 1996). Addition of CO_2 to a chamber causes a reduction in O_2 levels with displacement of air, which may also cause dyspnea. The O_2 concentration in ambient air is 20.9%, and humans report dyspnea at O_2 levels of less than 8% when compensatory breathing is constrained (Moosavi et al., 2003). This sensation was alleviated with spontaneous breathing, but O_2 concentrations less than 7% were not examined. During pre-fill CO_2 euthanasia rats are exposed to CO_2 concentrations above 70%, so it seems likely that rats experience both pain and dyspnea using this method. However, during gradual-fill CO_2 euthanasia rats typically lose consciousness at CO_2 concentrations below 40% (Smith & Harrap, 1997) and so may avoid some of these negative sensations.

If CO_2 does cause pain or dyspnea in rats, 'distress' behaviours would be expected during exposure. These could include behaviours associated with pain such as head-shaking and rubbing the nose and eyes, behaviours associated with gas avoidance such as increased exploration and escape attempts, and general distress behaviours such as increases in particular vocalizations. Behavioural studies on rats during CO_2 euthanasia have generally examined responses to different methods of CO_2 delivery, without comparison to a control session. In these studies, some authors have reported a lack of distress behaviours during pre-fill (Smith and Harrap, 1997) and gradual-fill CO_2 exposure (Hackbarth et al., 2000; Hornett & Haynes, 1984; Smith & Harrap, 1997). Other studies have reported 'agitation and asphyxiation' during both pre-fill and gradual-fill exposure (Coenen et al., 1995), and 'mild to moderate stress' during pre-fill exposure (Iwarsson & Rehbinder, 1993). However, these authors provide few detailed behavioural descriptions or data to support their conclusions. Three studies have taken objective behavioural measures during both CO_2 and air exposure. Blackshaw et al. (1988) found that pre-fill CO_2 exposure caused a decrease in activity. Leach et al. (2002) examined behavioural responses and aversion of rats to static CO_2 concentrations greater than 25.5%, and found that rats avoided CO_2 exposure, and that it caused increased face-washing. Britt (1987)

examined responses to gradual-fill exposure and found that changes in activity and wall-climbing varied with strain, but that shaking always increased.

If dyspnea does occur during CO_2 euthanasia, some of this effect may be due to reduced O_2 levels. No studies to date have measured the responses of rats to O_2 reduction at the levels that occur during gradual-fill euthanasia. However, studies using subjective assessments of distress have claimed that O_2 supplementation decreases responses of rats during pre-fill (Iwarsson & Rehbinder, 1993) and gradual-fill (Coenen et al., 1995) CO_2 exposure.

The aims of the current study were to determine whether rats show behavioural signs of distress during gradual-fill CO_2 euthanasia and during an equivalent reduction in O_2 level caused by displacing air with argon. We predicted that distress during exposure would be accompanied by increased exploration, escape attempts and vocalization, and that pain at the mucosal membranes and cornea would be accompanied by head shaking and face washing.

2.2 Materials and Methods

2.2.1 Subjects

Sixteen 400-500 g, mature, male Sprague Dawley rats were obtained as surplus stock (i.e. animals already slated for euthanasia) from the UBC Rodent Breeding Unit. Animals were group-housed at 21°C under a 12:12-hr light-dark cycle, and given ad libitum access to food (Lab Diet 5001, PMI Nutrition International, Indiana, USA) and tap water. All testing was conducted during the light portion of the light-dark cycle.

2.2.2 Experimental Apparatus

The euthanasia chamber was a 20 L polypropylene cage 20.5 cm high, 45.5 cm long and 24 cm wide at the top (Lab Products Inc.), fitted with a Plexiglas lid. The lid had a gas inlet

centered at one end, two air outlets positioned at the opposite end, and a gas sampling tube inserted at the center of the chamber to a depth of half the chamber height. The air outlets were covered with mesh to prevent the rats from pushing their noses outside the chamber. The back and sides of the chamber were covered with black paper so that the animals could not see the person conducting the experiment.

Argon, an inert gas, was used to displace air in the O_2-reduction treatment group. Carbon dioxide and argon were delivered to the chamber from compressed gas cylinders (Praxair, Richmond, B.C.), while room air was delivered via an air compressor situated in an adjoining room. The treatment gases were passed through a copper coil in a room temperature water bath to regulate the temperature of the gas before it entered the chamber. Preliminary tests indicated that the chamber temperature did not drop during the filling process. Flow rates of the gases were measured by a variable area flowmeter (Model VSB-66-BV, Dwyer Instruments, Inc., Michigan), and measured flow rates for CO_2 and argon were adjusted for density by the correction factors 0.812 and 0.852 respectively. Gas concentrations in the chamber were monitored during the experiment via a gas sampling tube using a Mocon LF700D O_2 analyzer. It was assumed that any decrease in O_2 was directly related to a decrease in air and a corresponding increase in the treatment gas. Therefore the following formula was used to calculate the concentration of CO_2 at specific time points (t = x) during the filling process:

$$[CO_{2\,(t=x)}] = 100 - (100 * ([O_{2\,(t=x)}] / [O_{2\,(t=0)}]))$$

2.2.3 Apparatus Testing

Before starting the animal experiments, gas concentrations were measured in different areas of the empty chamber during the CO_2 filling process. The chamber was divided into twelve sectors by partitioning the chamber into three segments in the x-plane (length), two segments in the y-plane (width) and two segments in the z-plane (height). The gas sampling tube

was placed in the center of the sector to be tested, and CO_2 was added at a rate of 3.5 L/min. The O_2 concentration was recorded every 5 s for 10 min. Each sector was tested three times. The sectors were tested in random order to account for minor fluctuations in environmental parameters.

2.2.4 Experimental Procedure

Animals were randomly allocated to the CO_2 or O_2 reduction (using argon) treatment groups (n = 8 for both). For both groups, animals were first tested with air exposure and then with the treatment gas on the following day. On both testing days, each animal was individually placed into the euthanasia chamber for a 15-min period of acclimatization during which air was added to the chamber at a rate of 3.5 L/min. The length of the acclimatization period was based on preliminary observations showing how long it took animals to cease exploration and become inactive after entry into the testing apparatus. After acclimatization, air flow ceased and either air, CO_2 or argon flow was started at a rate of 3.5 L/min. This rate corresponded to 17.25% of the chamber volume being added per minute. Although air flow ceased during treatment gas exposure, the air compressor remained on throughout the experiment in order to control for noise effects. CO_2-treated animals remained in the chamber and were monitored until death but argon-treated animals were removed from the chamber at the end of the 105-s observation period. Preliminary observations showed that CO_2-treated animals ceased all purposeful movement within this period, so any relevant effects of O_2 deprivation would be present during this time. The level of O_2 reduction resulting from argon addition was not sufficient to cause unconsciousness or death, and was used only to simulate reductions in O_2 levels that occur when a chamber is filled with CO_2.

2.2.5 Behavioural Analysis

The euthanasia chamber and O_2 meter readout were video recorded during the experimental procedure. Each animal was scored continuously during the last 105 s of the acclimatization period (baseline) and the first 105 s after gas flow began (exposure) for pre-defined behaviours thought to relate to pain and distress (Table 2.1). The time until complete recumbency and cessation of breathing was also recorded. Recumbency was defined as a loss of posture and muscle tone. The time until onset of ataxia was not recorded as it could not be accurately assessed in all animals.

Sound data were collected with a ¼''condenser microphone (Bruel and Kjaer, Type 4135), connected to a preamplifier (Bruel and Kjaer, Type 2619) and a measuring amplifier (Bruel and Kjaer, Type 2636). The signal was recorded directly to a high-capacity hard disk at a rate of 250 kHz using a 330 kHz PCI-DAS1200/JR data acquisition card (Computerboards, Inc.) and CBDisk 1.4 software (Engineering Design, Belmont, MA). The microphone end was suspended 0.5 cm into the euthanasia chamber through one of the air outlets.

Sound was recorded during the last 105 s of the acclimatization period (baseline) and the first 105 s after gas flow began (exposure). Sound analysis was with SIGNAL 4.0 (Engineering Design, Belmont, MA). Calls were identified by their form and as being distinct from ambient noise. Sounds of less than 5 ms duration were difficult to distinguish from ambient noise and were discarded from the analysis. Suspected vocalizations were played back in a frequency range audible to humans by slowing the recordings by a factor of 0.05 to 0.1. Whistle-like sounds were accepted as vocalizations while clicks and other mechanical sounds were discarded. Rats produce whistle-like USVs by pushing air through a 1 to 2 mm hole formed via the tight opposition of the two vocal cords (Sanders et al., 2001). It is possible that rats use another production mechanism to produce clicks, but in this study it was not possible to adequately distinguish vocal clicks from those resulting from movement in the cage.

Vocalizations were subjected to spectrographic analysis to determine call duration, peak frequency, maximum frequency and minimum frequency.

2.2.6 Statistical Analysis

The behavioural data were non-normal with unequal variances and could not be corrected through the use of transformations, so non-parametric statistics were used for analysis. Head-shaking was not observed in any animals and face-washing was observed only in two rats during baseline and one rat during CO_2 exposure, so these behaviours were not included in the analysis. CO_2 and reduced O_2 exposures were not conducted concurrently, therefore direct comparisons between the treatments were not performed. The Wilcoxon Signed Ranks Test was used to compare the change in behaviour from baseline during air exposure with the change from baseline during either CO_2 or reduced O_2 exposure. All behavioural data are presented as medians with 25% and 75% interquartile ranges. Vocalization parameters and time to recumbency and death are presented as means ± standard deviations.

2.3 Results

CO_2 concentrations in the empty chamber rose asymptotically during the filling process, reaching 87% after 600 s (Fig. 2.1). Concentrations of CO_2 tended to be greater at the bottom of the chamber than the top, with a peak differential of 7% (bottom at 9.4%, top at 2%) after 20 s. After this time, the concentrations began to converge. There were no concentration differences in the y-plane during filling, and the x-plane CO_2 concentration differential between the positions closest and furthest from the gas inlet was less than 2% throughout the filling process. During the actual experiment gas samples were taken at a depth of half the chamber height, and

CO_2 and argon concentrations consistently fell within the range of values found during preliminary testing.

During the baseline period rats were generally inactive, but all behavioural measures increased after CO_2 flow began (Fig. 2.2). Rearing and touching of the nose to the lid started to increase within the first 15 s. Activity and rearing peaked between 15 s and 60 s, and touching of the nose to the lid, escape behaviours and vocalizations peaked between 60 s and 90 s after CO_2 began. O_2 and CO_2 concentrations reached approximately 20% and 5% after 15 s, 17% and 20% after 60 s, and 15% and 28% after 90 s. Complete recumbency occurred on average 106 ± 12 s after flow initiation, at O_2 and CO_2 concentrations of approximately 14% and 33%. Rats did not stop breathing until 443 ± 14 s into the procedure, at O_2 and CO_2 concentrations of approximately 4% and 80%.

When compared to air exposure, rats exposed to CO_2 were more active, and showed significant increases in the frequency of rearing, escape behaviours, vocalizations and in the time spent with the nose contacting the chamber lid (Table 2.2). However, there was considerable variation in behavioural response among animals, as reflected by the large interquartile ranges for several behaviours. For example, during the CO_2 exposure period one rat performed 34 escape behaviours, while two others performed none.

Vocalizations during CO_2 exposure consisted of pure tones and calls with frequency modulation, and these did not fall into obvious categories. Calls varied in length and frequency, ranging from 5 to 150 ms and 8.6 to 102.1 kHz. On average the call duration was 33 ± 28 ms, the average frequency range was 22 ± 19 kHz and the peak frequency was 44 ± 20 kHz. Due to the small number of vocalizations produced during air and argon exposure, call parameters were not compared between treatments.

Rats that were exposed to reduced O_2 concentrations using argon exhibited only a small increase in the time spent with the nose contacting the chamber lid and no increases in any other

variables in comparison with air exposure (Table 2.3). Rats did not show any signs of ataxia or recumbency during the 105-s observational period for this treatment.

2.4 Discussion

In contrast to several previous studies on gradual-fill CO_2 euthanasia (Hackbarth et al., 2000; Hornett & Haynes, 1984; Smith & Harrap, 1997), we found that this procedure does cause behavioural signs of distress in rats. Not only did the rats exhibit general signs of exploration such as increased locomotion, rearing, and touching the nose to the lid, they also showed escape behaviours and vocalizations. This behavioural response began within the first 15 s after the start of gas flow, demonstrating that rats respond to even low (approx. 5%) concentrations of CO_2. We did not observe increases in head-shaking or face-washing during exposure, suggesting that animals did not experience pain during the time when they were capable of mounting a behavioural response. However, it is also possible that the measured behaviours were not appropriate indicators of upper respiratory pain in rats.

Our results are consistent with the subjective assessments of 'agitation' during gradual-fill reported by Coenen et al. (1995), and with the increase in activity reported by Britt (1987) for Sprague Dawley rats. Interestingly, Britt reported a decrease in activity for Lister Hooded rats in the same study, indicating that strain may be an important factor in response differences. Other studies have reported few behavioural changes in Wistar (Blackshaw et al., 1988; Hornett & Haynes, 1984) and F-344 rats (Hackbarth et al., 2000) during CO_2 exposure. In our study, we found considerable variation in response among individuals, with some animals displaying numerous escape attempts and others showing little response during the procedure. It is unclear whether this variation indicates a difference in the level of distress resulting from the procedure,

or a difference in how animals respond to distress. A lack of behavioural response does not necessarily indicate that the rats perceive the procedure as innocuous.

While Leach et al. (2002) have demonstrated that rats will avoid static CO_2 concentrations of 25.5% and greater, this is the first study to show increased escape attempts by rats during CO_2 euthanasia. The design of previous euthanasia experiments may have discouraged expression of escape behaviours. The rats in our study had access to the chamber lid and time to explore it thoroughly before CO_2 exposure. Rats in other studies may not have had access to the chamber lid and in most other experiments rats were unfamiliar with the chamber at the time of exposure, which may have inhibited escape attempts.

Only one other study has attempted to measure vocalizations during CO_2 exposure, and no calls were detected (Britt, 1987). The author did not provide details on the sound collection apparatus, so the sensitivity and frequency range of the equipment is unknown. We found that vocalizations were present at low levels during baseline and argon exposure, but increased during CO_2 exposure. The majority of studies on rat USVs have focused on what have been described as the 22 kHz (ranging from approximately 20-30 kHz and 300 – 3000 ms) and 50 kHz (ranging from approximately 30-70 kHz and < 80 ms) calls (reviewed in Knutson et al., 2002). Calls in the current study appear to be consistent with those that have been described as 50 kHz calls, and no 22 kHz calls were observed. Calls that have been grouped under the 50 kHz label in previous studies have actually varied considerably in length, frequency and shape, and few studies have provided detailed call descriptions. This makes it difficult to determine whether these calls are consistent across studies, and whether they are indicative of similar states. Although the 50 kHz calls have been observed during positive contexts such as anticipation of reward (Burgdorf et al., 2000; Knutson et al., 1998; Knutson et al., 1999), they have also been observed during intermale aggression (Sales, 1972; Thomas et al., 1983) and exposure to anesthetized conspecifics (Blanchard et al., 1993). These occurrences during

potentially negative contexts suggest that the calls may also be associated with distress. However, the CO_2-exposed animals were more active and spent more time near the lid in the vicinity of the microphone, and this may have improved our ability to detect calls during this condition. The increase in calls could also have been a by-product of increases in breathing frequency and depth that occur during hypercapnia, but the effects of breathing changes on USV production has not previously been examined.

The densities of the gases used in the current study were higher than air and may also have affected USV characteristics. Roberts (1975) found that a reduction in gas density results in an increase in fundamental frequency and a decrease in amplitude of USVs, and it is therefore likely that an increase in gas density would have the opposite effect. However, this effect is due to changes in the speed of sound in gases with different densities, and the effect of helium is much greater than the effects of either CO_2 or argon. The speed of sound in air, CO_2 and argon at 21°C is approximately 343, 269 and 320 m/s respectively, whereas the speed of sound in helium is approximately 1000 m/s. Furthermore, the gases in the current experiment were mixed with air in relatively low concentrations at the time when rats were responding. While this may have had a minor effect on USV characteristics, it is unlikely that density alone was responsible for the increased number of calls detected during CO_2 exposure.

Britt's (1987) conclusion that rats experience distress during CO_2 exposure was based partly on the occurrence of shaking, but this behaviour was not seen in our study. Britt (1987) did not state the flow rates used, but higher flow rates tend to result in a higher concentration of CO_2 in the chamber at the time of loss of consciousness (Ambrose et al., 2000) and increase the likelihood that rats experience pain. Previous studies have also found increases in urination and defection during exposure to CO_2 (Britt, 1987; Smith & Harrap, 1997). We did not record urination and defecation as they often occurred when the animal was initially placed in the

chamber, and the likelihood of future events is strongly related to the time since these last occurred.

The potential for animals to experience distress during euthanasia is limited to the period of consciousness, and in this experiment we have assumed that complete loss of consciousness occurred when the animals became fully recumbent. Recumbency in rats during CO_2 exposure is associated with a loss of pedal and corneal reflexes (Hornett and Haynes, 1984) and with a drop in heart rate and onset of an aberrant EEG (Coenen et al., 1995). However, some researchers have found a delay between collapse and loss of reflexes (Danneman et al., 1997; Hewett et al., 1993), so it is possible that the depth of unconsciousness varies at the time of recumbency. We found that during gradual-fill CO_2 exposure, rats became recumbent after an average of 106 s, and this finding is consistent with other studies using similar flow rates (Hewett et al., 1993; Hornett and Haynes, 1984; Danneman et al., 1997). An assessment of ataxia would have provided an indication of the time that loss of consciousness began to occur, but we were unable to accurately detect ataxia in the current study because a loss of muscle coordination can only be detected when particular postures and movements are initially present. For example, when a rat is crouched and stationary a reduction in muscle coordination may not be obvious. In the current experiment, some rats exhibited recumbency without obvious ataxia beforehand.

Two possible reasons for the rats' behavioural response to CO_2 are pain from carbonic acid formation at the mucosa and cornea, and dyspnea from hypercapnia and hypoxia. We found that the O_2 and CO_2 concentrations in the chamber at the time of recumbency were approximately 14% and 33%. However, the O_2 and CO_2 concentrations were approximately 20% and 5% when animals started to respond, and 15% and 28% when all behaviours had peaked and were declining. Physiological data suggest that the CO_2 threshold for the majority of nociceptors in rat nasal mucosa is between 37 and 50% (Peppel & Anton, 1993). Human self-report data indicate that CO_2 is painful if applied to the nasal mucosa at concentrations above

47%, although this value ranges from 32.5% to 55% depending on the individual (Anton et al., 1992). For the cornea, humans report stinging at 33% and overt pain at 47% CO_2 (Chen et al, 1995). Receptors found in the larynx, trachea, and bronchi are also sensitive to inhaled irritants (reviewed in Widdicombe, 2001), but responses to CO_2 have not been fully investigated for these receptors. However, Danneman et al. (1997) had human subjects inhale CO_2 into the lower airways and found that only 7 of the 40 subjects reported that 50% CO_2 was overtly painful, suggesting that pain in the lower airways is not occurring at a markedly lower CO_2 concentration than has been demonstrated for the nasal mucosa. Although it is possible that rats in this study experienced some pain before losing consciousness, the CO_2 concentration during the period of maximal response was much lower than the probable pain threshold, and the rats did not exhibit increases in head-shaking and face-washing. Head-shaking and face-washing have been observed in rats during exposure to moderate to high CO_2 concentrations (Britt, 1987; Leach et al., 2002), and face-washing has also been observed in rats during exposure to irritating compounds such as chloroform (Blacksaw et al., 1988). Hence, pain is unlikely to be the cause of the rats' responses in the current study.

In contrast, the concentrations of CO_2 in the chamber during the period of maximal response have been found to cause sensations of dyspnea in spontaneously breathing humans (Dripps & Comroe, 1947; Liotti et al., 2001), and may cause similar effect in rodents. In humans, hypercapnia is also associated with other negative physical symptoms such as headache, flush, restlessness, heart pounding, drowsiness and dizziness (Moosavi et al., 2003). It therefore seems likely that the distress response to gradual-fill CO_2 exposure is due to dyspnea and other symptoms of hypercapnia rather than pain. Furthermore, Dripps and Comroe (1947) found that humans vary in their response to hypercapnia, a finding consistent with the variability in the rats' responses to CO_2 exposure in the current study. Previous studies have assessed breathing in rats during CO_2 exposure and found that it causes changes described as "laboured

breathing" (Iwarsson & Rehbinder, 1993), "gasping and asphyxia" (Coenen et al., 1995) and "gasping or laboured breathing" (Smith & Harrap, 1997) prior to loss of consciousness, and these changes might be indicative of the sensation of dyspnea. In the current study we did not assess changes in breathing because we could not make an accurate assessment from video. Ideally breathing could be assessed objectively by measuring its frequency and depth, but this was beyond the scope of the current study. However, human medical studies suggest that laboured breathing is poorly correlated with the sensation of dyspnea (Lush et al., 1988), so it is not clear that breathing measures are useful as an indicator of dyspnea during CO_2 exposure.

Our results indicate that O_2 reduction alone causes only minimal distress in rats over the range of O_2 concentrations that we examined. Although rats exhibited a slight increase in touching the nose to the lid during argon treatment, the increase in this behaviour was much less than that seen in CO_2-exposed animals and there were no other behavioural changes. This result is consistent with human self-report data suggesting that O_2 levels in the range seen during this experiment do not cause dyspnea during spontaneous breathing (Moosavi et al., 2003). However, hypercapnia and hypoxia are known to have synergistic effects on ventilatory responses (Nielson & Smith, 1952), such that the response to increased CO_2 may be potentiated by a level of O_2 reduction that has no effect on its own. Masuda et al. (2001) found that increasing levels of hypoxia augment the effect of hypercapnia on dyspnea scores in humans. Assuming that these results are applicable to rats, it would appear that hypoxia during CO_2 euthanasia may increase dyspnea, and this is supported by results showing that O_2 supplementation reduces the adverse effects of CO_2 exposure on rats (Coenen et al., 1995). It is important to note that these results have no bearing on distress associated with argon when used for euthanasia. Rats can survive for greater than 20 minutes when exposed to O_2 concentrations of 4.9% in argon (Altland et al., 1968), and our O_2 concentrations were only reduced to 14%. For timely unconsciousness and death of pigs and poultry with argon, concentrations of 90% or

greater have been used to reduce O_2 levels below 2% (e.g., Raj, 1999; Raj et al., 1998), and gradual fill exposure has not been investigated.

Our study indicates that gradual-fill CO_2 euthanasia causes distress in rats, and the concentrations involved suggest that this distress is due to dyspnea rather than pain. The lack of consistency between experiments suggests that further research is necessary to examine whether there are strain differences in sensitivity or responsiveness to CO_2. Further research is also necessary to determine the extent of the distress caused by CO_2 exposure, and to determine whether other gas euthanasia agents cause less distress. The variability in behavioural responses to CO_2 suggests that tests of motivation might be a better approach. For example, approach-avoidance testing could be used to examine whether rats will forgo an attractive reward of known value to avoid exposure to CO_2 and other gas euthanasia agents.

Table 2.1. Descriptions of rat behaviours recorded during baseline and during exposure to CO_2 or reduced O_2 concentrations.

Behaviour	Description
Activity	Movement that results in the back feet crossing a line that divides the length of the chamber in half (event).
Rear	Raising of the upper body while standing on the two back feet. Includes wall climbing. Climbing on the air sampling tube while chewing it and rearing during grooming were excluded (event).
Nose to lid	Time spent with the nose in contact with the chamber lid (state).
Escape behaviours:	
Scratch at lid	A rapid movement of the front paw from the lid through at least a 90^{o} downward angle (event).
Push at lid	A push at the chamber lid using the nose or front paw evidenced by body and lid movement (event).
Head shake	Rapid rotation of the head about the axis (event).
Face washing	Placement of one or both paws to the nose. Performance during grooming was excluded (event).

Table 2.2. Difference from baseline for each of the five behavioural responses of rats during air and CO_2 exposure (n = 8 rats). Data are presented as medians with 25th and 75th percentiles, and statistical comparisons were made with Wilcoxon Signed Ranks Test (T; based on N values >0).

Behaviour	Air **Med** (25th, 75th)	CO_2 **Med** (25th, 75th)	**T (N)**	***P*-value**
Activity (no.)	**0.0** (-0.5, 0.5)	**3.5** (2.0, 4.5)	**0 (8)**	**<0.005**
Rears (no.)	**0.0** (-2.5, 1.0)	**10.5** (8.0,13.0)	**0 (8)**	**<0.005**
Nose to lid (s)	**0.0** (-15, 6.5)	**23.0** (9.0,25.5)	**3 (7)**	**<0.05**
Escape behaviours (no.)	**0.0** (0.0, 0.0)	**4.0** (0.5,10.5)	**0 (7)**	**<0.05**
Vocalizations (no.)	**-0.5** (-1.0, 0.0)	**6.0** (-0.5,13.0)	**1.5 (7)**	**<0.05**

Table 2.3. Difference from baseline for each of the five behavioural responses of rats during air and exposure to reduced O_2 concentrations (n = 8 rats). Data are presented as medians with 25th and 75th percentiles, and statistical comparisons were made with the Wilcoxon Signed Ranks Test (T; based on N values >0).

Behaviour	Air **Med** (25^{th}, 75^{th})	Reduced O_2 **Med** (25^{th}, 75^{th})	**T (N)**	***P*-value**
Activity (no.)	**0.0** (-0.5, 0.5)	**0.0** (0.0, 1.5)	**5 (8)**	**NS**
Rears (no.)	**-0.5** (-2.5, 0.0)	**3.0** (0.0, 6.0)	**4 (7)**	**NS**
Nose to lid (s)	**0.0** (-3.5, 0.0)	**2.5** (1.0, 8.0)	**1.5 (8)**	**<0.05**
Escape behaviours (no.)	**0.0** (0.0, 0.0)	**0.0** (0.0, 0.0)	-	-
Vocalizations (no.)	**0.0** (-0.5, 0.0)	**0.0** (0.0, 0.5)	**5 (7)**	**NS**

NS signifies $P > 0.05$

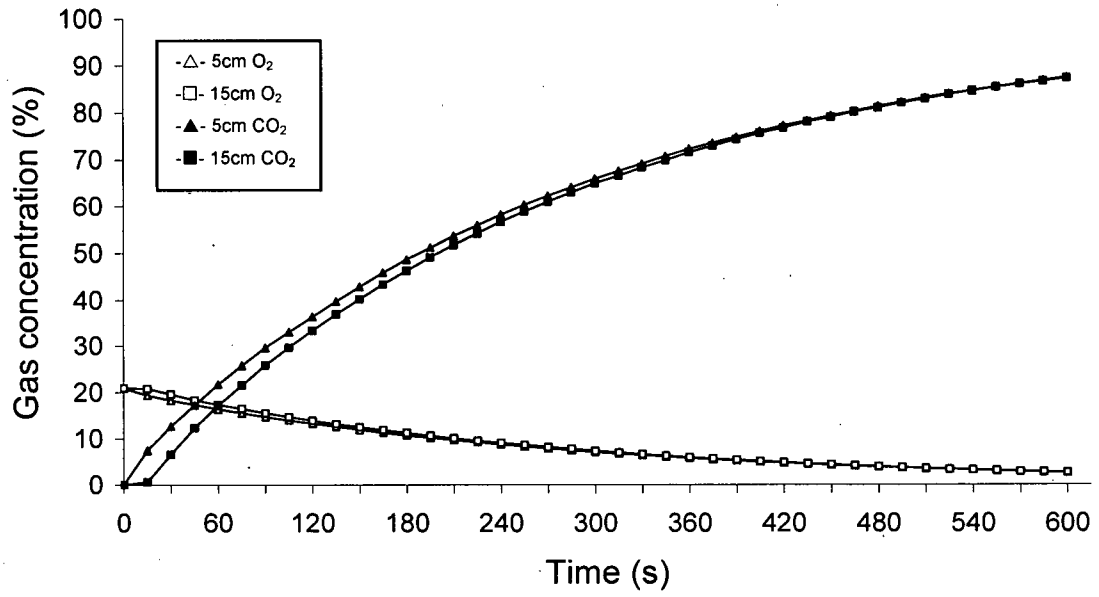

Figure 2.1. Average concentrations of O_2 (open markers) and CO_2 (filled markers) in the chamber during the first 600 s of the filling process. Concentrations were taken 5 cm (triangles) and 15 cm (squares) from the chamber bottom.

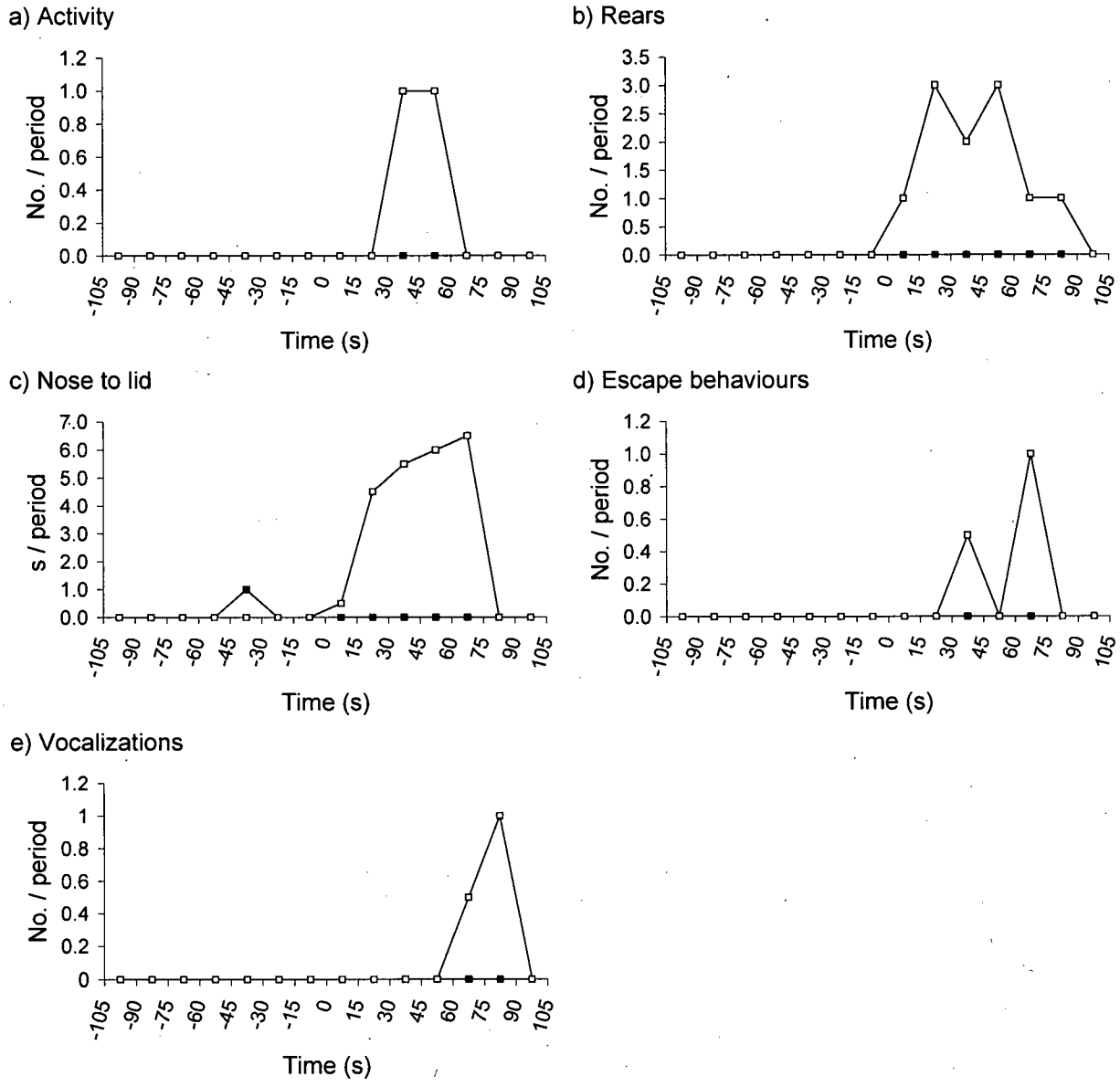

Figure 2.2. Responses by rats during the baseline period and then during exposure to either air (filled squares) or CO_2 (open squares) starting at t = 0. Median values per 15 s period are shown for a) activity, b) rears , c) nose to lid, d) escape behaviours, and e) vocalizations (n = 8 rats).

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CHAPTER 3: Rats avoid exposure to carbon dioxide and argon[3]

3.1 Introduction

Small laboratory rodents are euthanized using a number of methods, including physical techniques, injectable anaesthetics, and exposure to volatile anaesthetics and other gases. One of the most common methods is exposure to CO_2. Animals are exposed to either a gradually increasing concentration of CO_2 or a pre-filled chamber, and this causes unconsciousness followed by death.

Ideally, a euthanasia method should result in a quick death with minimal pain and distress. In humans CO_2 is known to cause dyspnea, a sensation of breathlessness, at concentrations of 8% (Dripps & Comroe, 1947; Liotti et al., 2001). At CO_2 concentrations ranging from 30% to 54%, humans also experience pain at the cornea (Chen et al., 1995; Feng & Simpson, 2003), conjunctiva (Feng & Simpson, 2003) and nasal mucosa (Anton et al., 1992; Thurauf et al., 2002). In rats, the threshold for the majority of nociceptors in the nasal mucosa is between 37% and 50% CO_2 (Anton et al., 1991; Peppel & Anton, 1993). Some studies have reported no behavioural evidence of distress in rats during pre-fill (Blackshaw et al., 1988; Smith & Harrap, 1997) and gradual-fill CO_2 exposure (Hackbarth et al., 2000; Hornett & Haynes, 1984; Smith & Harrap, 1997). However, others have suggested that CO_2 causes behavioural signs of distress (Britt, 1987; Coenen et al., 1995; Iwarsson & Rehbinder, 1993; Chapter 2). This variability between experiments suggests that simply monitoring behavioural responses during exposure may be inadequate as a method for assessing the rat's perception of CO_2.

[3] A version of this chapter has been accepted for publication. Niel, L., Weary, D.M., 2006. Rats avoid exposure to carbon dioxide and argon. Appl. Anim. Behav. Sci. (accepted).

Aversion to CO_2 exposure has also been examined using preference testing. Leach et al. (2002) found that rats moved to an air filled chamber when exposed to moderate CO_2 concentrations. However, only static concentrations between 25.5% and 50.8% were tested. Rats' responses to lower concentrations and to gradual-fill exposure have not been examined, and to date no studies have addressed the strength of aversion to CO_2.

Another form of preference testing, the approach-avoidance test, has been used to examine aversion to CO_2 in mink (Cooper et al., 1998), pigs (Raj & Gregory, 1995) and poultry (Raj,1996; Gerritzen et al., 2000; Webster & Fletcher, 2004). During this procedure, entry into the chamber is voluntary and animals are motivated to enter and remain for a reward. If animals avoid the chamber, even when it contains something that they are trained and motivated to obtain such as a food reward, this indicates that they find the conditions of the test cage aversive. Approach-avoidance testing has not previously been used to examine aversion to CO_2 in rats.

The aim of this study was to use approach-avoidance testing to characterize rats' aversion to static and gradual-fill CO_2 exposure. We also examined whether rats exhibit aversion to 90% argon in air, which causes death by reducing O_2 levels to 2% and has been proposed as an alternative method of gas euthanasia for rats (Leach et al., 2002).

3.2 Materials and Methods

3.2.1 Subjects and Housing

The subjects were 10 male Wistar rats, 400 to 500g, obtained from the UBC Animal Care Centre Rodent Breeding Unit as surplus supply stock and destined for euthanasia. Animal rooms were kept at 21 ± 1 °C under a 12:12-hr light-dark cycle, and rats were given ad libitum

access to food (Lab Diet 5001, PMI Nutrition International, Richmond, USA) and tap water. All testing was conducted during the light portion of the light-dark cycle.

Rats were singly housed in the testing apparatus, consisting of two transparent cages connected by an opaque tunnel made of black, ribbed, PVC tubing with a diameter of 10 cm and sloped so that one cage was 27 cm higher than the other. The 'home' cage measured 48 x 38 x 20 cm, and contained food, water, bedding, an opaque nestbox and a Nylabone dog chew. The secondary cage measured 45 x 24 x 20 cm and contained bedding. The bottom cage was always used for testing because the test gases were denser than air. During preliminary testing we determined that the test gases were restricted to the bottom cage and the lower portion of the tunnel. To determine the effect of cage familiarity, half of the rats were tested in the home cage and the other half were tested in the secondary cage. This was accomplished by positioning the home cage on the bottom for half of the rats, and the secondary cage on the bottom for the other half of the rats. However, many rats spent a portion of their time in the secondary cage, so were familiar with both cages.

3.2.2 Testing Procedure

During experimental testing, each animal and its testing apparatus were transferred individually to a test room. At this time, the nest box was removed and the wire lid on the test cage was replaced with a plexiglas lid that featured two air outlets positioned at the end closest to the tunnel, a gas inlet at the far end of the test cage, and a gas sampling tube inserted at the center of the test cage. The air outlets were covered with mesh to prevent the rats from pushing their noses outside the chamber. The experimenter was concealed behind a blind during testing. The testing apparatus and O_2 meter readout were video recorded during testing.

Air, CO_2 and argon were delivered to the test cage from compressed gas cylinders (Praxair, Richmond, B.C.). The treatment gases were passed through a copper coil in a room

temperature water bath to regulate gas temperature before entering the test cage. Flow rates of gases were measured using a variable area flow meter (Dwyer Instruments VSB-66-BV), and measured flow rates for CO_2 were adjusted for density using a correction factor of 0.812. O_2 concentrations in the test cage were monitored during the experiment using a Mocon LF700D O_2 analyzer, and were used to calculate CO_2 concentrations at specific time points (t = x) with the formula: $CO_{2\,(t=x)} = 100 - (100 * ([O_{2\,(t=x)}] / [O_{2\,(t=0)}]))$.

Testing of the apparatus was completed to ensure that CO_2 concentrations were uniform throughout the test cage during gradual-fill CO_2 addition. During CO_2 addition at a rate of 17% of the test cage volume per minute, gas concentrations were monitored at a depth of half the test cage height at five different sites. There were no obvious trends for lower CO_2 concentrations at the end of the chamber closest to the tunnel, and CO_2 concentrations at the different sites in the chamber varied by less than 3%.

Before beginning the experiment, rats were trained to perform the approach-avoidance task. Rats were trained for 10 days with air only and this was followed by 9 days of training where air and different concentrations of CO_2 were alternated to familiarize the animals with gas exposure and remove any effects of novelty. For this final stage of training all rats were exposed to air, gradual-fill CO_2 at 17% of the test cage volume per minute and static CO_2 concentrations of 5, 10, 15 and 20%. Animals were not exposed to argon prior to testing because we were concerned that this would affect performance in general. In preliminary testing one rat that was exposed to argon appeared to have difficulty determining which cage contained air and refused to run the task the following day. Furthermore, argon was not expected to evoke a novelty response because it is an odourless and non-irritating gas.

During both training and experimental sessions, rats were first locked into the top cage for 5 min to allow time for addition of treatment gases and food rewards to the test cage. Following lock removal they were able to enter the lower test cage for a food reward of 20

Honey Nut Cheerios™ (General Mills, Inc., Minnesota). For static exposure, the test cage was pre-filled with either CO_2 or argon. For gradual fill exposure, CO_2 flow into the test cage was initiated when the rat started eating the food reward. The session ended 300 s after lock removal, and animals were allowed to exit and re-enter the test cage during this period. At the end of the session, the remaining reward items were removed and the rat was returned to the holding room.

On the final day of training, all rats were run with air in the test cage and performed the task correctly, consuming at least 19 reward items each during the first entry into the test cage.

The experiment consisted of three test periods. The aim of Part 1 was to determine which static concentrations of CO_2 rats find aversive by filling the test cage with air or static CO_2 at concentrations of 5, 10, 15 and 20%. Each rat was tested once with each condition over a 5-d period. Treatment order was allocated according to a Latin square and counterbalanced according to home cage positioning (high vs. low). We recorded the total number of reward items eaten over the entire test session as well as the eating and dwelling times for the first entry (the maximum exposure time tolerated), and predicted that these variables would decline with increasing CO_2 concentration. We also recorded attempted entries into the test cage as a measure of motivation to enter the test cage with each treatment. Finally, we predicted that if a treatment was aversive, it would increase the time taken to enter the test cage on the following day. Therefore, we recorded the latency to enter the tube leading to the test cage following lock removal.

The aim of Part 2 was to determine what concentration of CO_2 rats find aversive during gradual-fill exposure, in order to compare it to the static fill data from Part 1. Rats were given a single exposure to gradual-fill CO_2 at a rate of 17% of the test cage volume per minute. We were specifically interested in the gas concentrations when the rats stopped eating and left the test cage so the O_2 concentration was the only variable recorded. This test took place the day after the end of testing for Part 1.

The aim of Part 3 was to evaluate rats' responses to a static concentration (90%) of argon gas in air (2% O_2), and compare this with air exposure. This test took place the day after testing with gradual-fill CO_2 exposure. As described above, some adverse reactions to argon were observed during pilot testing, and due to the potential for carryover effects following argon exposure, rats were exposed to air on the first day and argon on the second day. Variables recorded were identical to Part 1 except the treatments were not counterbalanced, so the latency to enter the tube leading to the test cage was not recorded.

3.2.3 Statistical Analysis

One animal did not learn the task and was removed from the experiment. The data for Part 1 and Part 3 were non-normal with unequal variances and could not be corrected through the use of transformations, so non-parametric statistics were used for analysis. An initial evaluation of the data indicated there were no differences for any variables between rats tested in the home cage versus the secondary cage, therefore the data were pooled for further analysis. For Part 1, the Friedman's test was used to compare differences in dependant variables across static CO_2 concentrations of 0, 5, 10, 15 and 20%. For Part 2, descriptive statistics are presented only. For Part 3, the Wilcoxon Signed Ranks test was used to compare differences in dependent variables for air and argon exposure.

3.3 Results

3.3.1 Part 1 – Exposure to static concentrations of CO_2

All 9 rats entered the test cage at 0, 5, 10 and 15% CO_2. At 20% CO_2 one rat refused to enter the test cage. At 0, 5 and 10% CO_2 rats ate all of the reward items provided, but the number eaten declined with 15 and 20% CO_2 (Fig. 3.1a; $P < 0.005$). At 15% one animal refused

to eat. At 20% CO_2 only two animals ate, and each consumed only one or two reward items. Considering only the first entry into the test cage, the eating and dwelling times were reduced at the highest CO_2 concentrations (for both: $P < 0.005$). From 0% to 10% CO_2 these variables declined only slightly (Fig. 3.1b), but there was increased variability in response at 10% CO_2. At 15% there was a steep drop in eating and dwelling times (median 32 s and 46 s, respectively), and at 20% CO_2 these values had dropped again (median 2 s and 5 s, respectively). The total number of times that the rats attempted to enter the test cage during the entire session differed across the five CO_2 concentrations (Fig. 3.1c; $P < 0.005$). At 0 and 5% CO_2 rats entered the test cage only once and remained for the majority of the session, but at higher concentrations rats showed an increasing numbers of entries. The time taken to enter the tube leading to the test cage was not affected by the treatment on the previous day ($P > 0.1$). On average rats took 2.3 ± 2 s to enter the tube.

3.3.2 Part 2 – Gradual-fill CO_2 exposure

During gradual-fill CO_2 exposure, rats stopped eating and left the test cage at CO_2 concentrations of 17.3 ± 2.1% (mean ± standard deviation) and 18.4 ± 2.0% respectively.

3.3.3 Part 3 – Argon exposure

During argon exposure, three rats refused to enter the test cage, no rats ate, and the median dwelling time was 3 s (Table 3.1). The median number of entries during argon exposure was two. In contrast, during air exposure rats ate all of the reward items and spent almost the entire session in the test cage. The number of reward items eaten, and the eating and dwelling times were all significantly greater during the session with air ($P < 0.005$).

3.4 Discussion

The ultimate aim of gas euthanasia is to deliver gases in such a way as to render the animal unconscious without causing distress. In the current study rats tolerated extended exposure to 5% and 10% CO_2, but this was not sufficient to cause unconsciousness. The rats were unwilling to tolerate extended exposure to 15% and 20% CO_2, and concentrations greater than 30% are necessary to cause loss of consciousness (Chapin & Edgar, 1963; Chapter 2). Nociceptors in rat nasal mucosa begin to respond to CO_2 concentrations of approximately 25% (Anton et al., 1991; Peppel & Anton, 1993), so pain is unlikely to be the cause of CO_2 aversion at the two highest concentrations that we tested. Dyspnea due to hypercapnia more likely accounts for our results. Some humans report dyspnea at CO_2 levels of only 8% (Dripps & Comroe, 1947; Liotti et al., 2001), and this sensation increases in severity with higher CO_2 concentrations. Dyspnea can also be accompanied by other negative physical symptoms such as headache, flush, restlessness, heart pounding, drowsiness and dizziness (Moosavi et al., 2003). However, humans vary in their tolerance to hypercapnia (Dripps & Comroe, 1947). Similar differences in tolerance in rats might explain the variability in eating and dwelling times observed at 10% CO_2 in the current study. The variability at 10% CO_2 and the steep drop in eating and dwelling times at 15% CO_2 suggests that the onset of severe dyspnea in rats may occur with 10% to 15% CO_2.

It has been suggested that gradual-fill CO_2 exposure results in a slow onset of unconsciousness without distress, but in the current study rats left the test cage when the CO_2 concentration reached on average 18.4%. This CO_2 concentration is consistent with rats' avoidance of static CO_2 concentrations, suggesting that gradual-fill exposure does not cause a gradual loss of consciousness without causing aversion. In other research we have found that rats do not become recumbent until a CO_2 concentration of approximately 30% is reached, about

105 s after gas flow is initiated at a flow rate of 17% per minute (Chapter 2). The depth of unconsciousness at the onset of recumbency is unknown since some studies report a short delay before a loss of reflexes occurs (Danneman et al., 1997; Hewett et al., 1993). This suggests that some awareness of aversive CO_2 concentrations might persist for a short period after onset of recumbency. In the present study, a concentration of 18% was reached after 60 s, suggesting that during gradual-fill CO_2 exposure, rats are exposed to aversive CO_2 concentrations for at least 45 s before losing consciousness. Increased flow rates would expose rats to higher CO_2 concentrations, but likely for a shorter duration before unconsciousness. The net effects on the rats of this conflict between intensity and duration are unknown.

For static CO_2 exposure, the number of reward items eaten, and eating and dwelling times show similar trends, but eating and dwelling times appear to be more sensitive measures, at least at lower concentrations of CO_2. While eating and dwelling times indicate the maximum time rats are willing to tolerate exposure, the number of reward items eaten provides a measure of the rats' activity in the test cage over the entire testing procedure and is affected by re-entries. Furthermore, rats can consume the same amount in a shorter exposure period by increasing eating speed. Although rats ate the same amount with air and 5% CO_2, at 5% CO_2 the median time spent eating was slightly reduced without an increase in entries, suggesting that they simply ate more quickly.

The responses of rats in the current study may not be indicative of how rats would respond if exposed to CO_2 for the first time. In order to perform within-animal comparisons it was necessary to remove the effects of novelty by familiarizing animals with all CO_2 concentrations and delivery methods prior to testing. Rats tended to be less tolerant of static CO_2 during training, suggesting that novelty made exposure more aversive. Thus, by removing the effects of novelty we have obtained a better indication of aversion to the properties of the gas itself. Animals were not trained with argon, but it is an inert gas with no perceptible odour, so a

novelty response to odour would not be expected. Increases in inspired CO_2 and reductions in inspired O_2 both cause an initial increase in ventilation rate and depth (Lumb, 2000), which could also contribute to novelty. However, because hypercapnia and hypoxia have similar effects on ventilation, training with CO_2 would likely also have been effective for argon exposure.

Our results demonstrate that approach-avoidance testing can provide a sensitive and objective method for examining rats' aversion to gas euthanasia agents. The majority of previous studies examining distress associated with CO_2 have recorded behavioural responses during pre-fill or gradual-fill euthanasia, and the results have been variable. Some studies have reported no behavioural evidence of distress during CO_2 exposure (Hornett & Haynes, 1984; Smith & Harrap, 1997; Hackbarth et al., 2000). However, others have conducted subjective assessments of distress (Iwarsson & Rehbinder, 1993; Coenen et al., 1995) and taken objective behavioural measures (Britt, 1987; Chapter 2) and concluded that indications of distress were present. Some variability among experiments may be due to differences in the way behavioural responses were interpreted. For example, Britt (1987) reported that Sprague Dawley rats exhibited increased activity and Lister Hooded rats exhibited decreased activity, yet both responses were interpreted as indicative of 'discomfort'. The current methodology provides a measure of aversion that is less open to subjective interpretation.

Preference testing has previously been used to determine whether rats will avoid exposure to 25.5, 34.9 and 50.8% CO_2 (Leach et al., 2002). This previous study reported consistent avoidance at all three CO_2 concentrations, but imposed no cost to leaving the chamber, making it difficult to assess the strength of aversion to CO_2. With the current approach-avoidance design, rats had to forgo a food reward to avoid CO_2 exposure. We can therefore conclude that the rats' motivation to avoid CO_2 at concentrations above 15% is stronger than their motivation to obtain a palatable food reward when fed ad libitum. Although

the strength of rats' motivation for sweet foods when fed ad libitum has not specifically been investigated in the current study, there is evidence to suggest that it ranges from moderate to high. In the present study rats were quick to enter the cage following lock removal, and consumed the entire food reward during all sessions with air. Furthermore, previous studies have shown that motivation for sucrose when fed ad libitum is as much as 50-75% of the motivation for sucrose when food deprived. McGregor et al. (1999) trained rats to lick a sipper tube for access to 8.6% sucrose, and during a 45 minute test session with a progressive ratio schedule of reward it was found that ad libitum fed rats licked approximately 1500 times, while food deprived rats licked approximately 2000 times. The maximum number of licks for a single reward was approximately 55 for ad libitum rats and 70 for food-deprived rats. In another study rats were trained to bar press for access to sucrose, and it was found that ad libitum fed rats bar pressed approximately 55% as much as food-deprived rats did for access to 4% and 16% sucrose (Collier and Bolles, 1968). These results suggest that rats in this experiment were well motivated by the food reward, and thus indicates that the rats' motivation to avoid exposure to CO_2 concentrations of 15% and greater was at least as moderate. The strength of rats' aversion to CO_2 could be more accurately assessed in future studies by increasing hunger levels to ensure that motivation for the food reward is high.

Approach-avoidance testing has also been used to examine aversion to CO_2 in other species. The majority of these studies have examined moderate to high CO_2 concentrations because gas stunning regulations for farm animals generally require pre-fill exposure (e.g., European Union, 1993). Cooper et al. (1998) found that mink will avoid a chamber containing more than 80% CO_2, even when it contains a novel object that they are motivated to obtain. Pigs have been found to avoid a food reward when it is paired with 90% CO_2, even following food deprivation, but will tolerate moderate durations of exposure to 30% CO_2 (Raj & Gregory, 1995). Previous studies with poultry have found that turkeys and chickens will enter CO_2

concentrations greater than 60% for a reward of food or social contact, and will lose consciousness before they are able to exit the chamber (Raj, 1996; Gerritzen et al., 2000; Webster & Fletcher, 2004). This suggests that poultry have a greater tolerance for CO_2 exposure, but it is not clear whether this interspecific variability demonstrates a difference in gas perception or in motivation to obtain the reward. The birds were found to exhibit behavioural and physiological signs of distress during exposure such as hyperventilation, coughing and head shaking, suggesting that the gas was likely detectable and unpleasant.

CO_2 euthanasia is widely used because it is easy to perform, inexpensive, safe for laboratory workers, and involves little handling and restraint for animals. Another gas euthanasia agent which meets these criteria is argon gas, which causes unconsciousness and death by O_2 displacement. In pigs and poultry, unconsciousness and death occur at argon concentrations greater than 90%, which lowers O_2 concentrations below 2% (e.g., Raj, 1999; Raj & Tserveni-Gousi, 2000). During approach-avoidance testing, pigs, turkeys and chickens have been found to enter and remain in lethal concentrations of argon for a food reward (Raj & Gregory, 1995; Raj, 1996; Webster & Fletcher, 2004). During preference testing, rats and mice have been found to tolerate argon exposure for longer than CO_2 exposure, but to exit before loss of consciousness for both gases (Leach et al., 2002). This suggests some level of aversion with both CO_2 and argon. In the current study we found that when the test cage contained 90% argon, some rats refused to enter and others exited immediately. This indicates that rats can detect argon-induced hypoxia and that they find lethal argon concentrations aversive. Argon is an odourless and non-irritating gas, so this aversion is not likely to be due to the properties of argon. However, low levels of inspired O_2 results in hypoxia, and this could cause a sensation of dyspnea. The O_2 concentration in ambient air is 20.9%, and Moosavi et al. (2003) found that humans report dyspnea at less than 8% O_2 when breathing is constrained. This sensation was alleviated with spontaneous breathing, but O_2 concentrations less than 7% were not examined.

However, human pilots have been found to lose consciousness after cabin depressurization without apparent efforts at correction, indicating that humans may not experience dyspnea with hypoxia levels that are sufficient to cause loss of consciousness (Cable, 2003). The design of the current study does not allow for direct comparisons between responses to CO_2 and argon, but it appears that rats are not willing to tolerate exposure to either gas for sufficient periods to cause unconsciousness.

In humans, there is a delay to onset of dyspnea after a change in inspired levels of CO_2 or O_2. This delay is due to the time necessary for changes in blood CO_2 and O_2 levels to reach the peripheral and central chemoreceptors, which is about 5 to 15 s in humans (reviewed by Cunningham et al., 1986), and for hypoxia and hypercapnia to reach levels that are sufficient to evoke dyspnea. Banzett (1996) calculated the half-time for development of a stable level of dyspnea in humans to be approximately 32 s. In the current study, the median latency to leave with 20% CO_2 and with 90% argon exposure was only 5 s and 3 s, respectively, and this response was much quicker than the time taken for dyspnea to develop in humans. However, the circulatory delay is only 2 s in rats (Lagneaux, 1986), and the dynamics of dyspnea in rats is unknown, so dyspnea cannot be ruled out as a potential source of aversion during both CO_2 and argon exposure in the current study.

Our results suggest that pre-fill and gradual-fill CO_2 exposure, and 90% argon exposure, cause aversion in rats. They also indicate that approach-avoidance testing is a sensitive and objective method for assessing aversion to gas euthanasia methods in rats. Further work is needed to assess how aversion to CO_2 compares with other gas and non-gas methods of euthanasia, so that the most humane methods of euthanasia can be implemented.

Table 3.1. Median (with 25th and 75th percentiles) number of reward items eaten during the entire session and eating and dwelling times during the first entry with either air or argon in the test cage (n = 9 rats).

	Type of gas		Wilcoxon Signed Ranks test	
	Air	Argon	S	*P*
Reward items eaten (no.)	**20** (20, 20)	**0** (0, 0)	**22.5**	**<0.005**
Eating time (s)	**261** (252, 281)	**0** (0, 0)	**22.5**	**<0.005**
Dwelling time (s)	**296** (296, 297)	**3** (3, 6)	**22.5**	**<0.005**

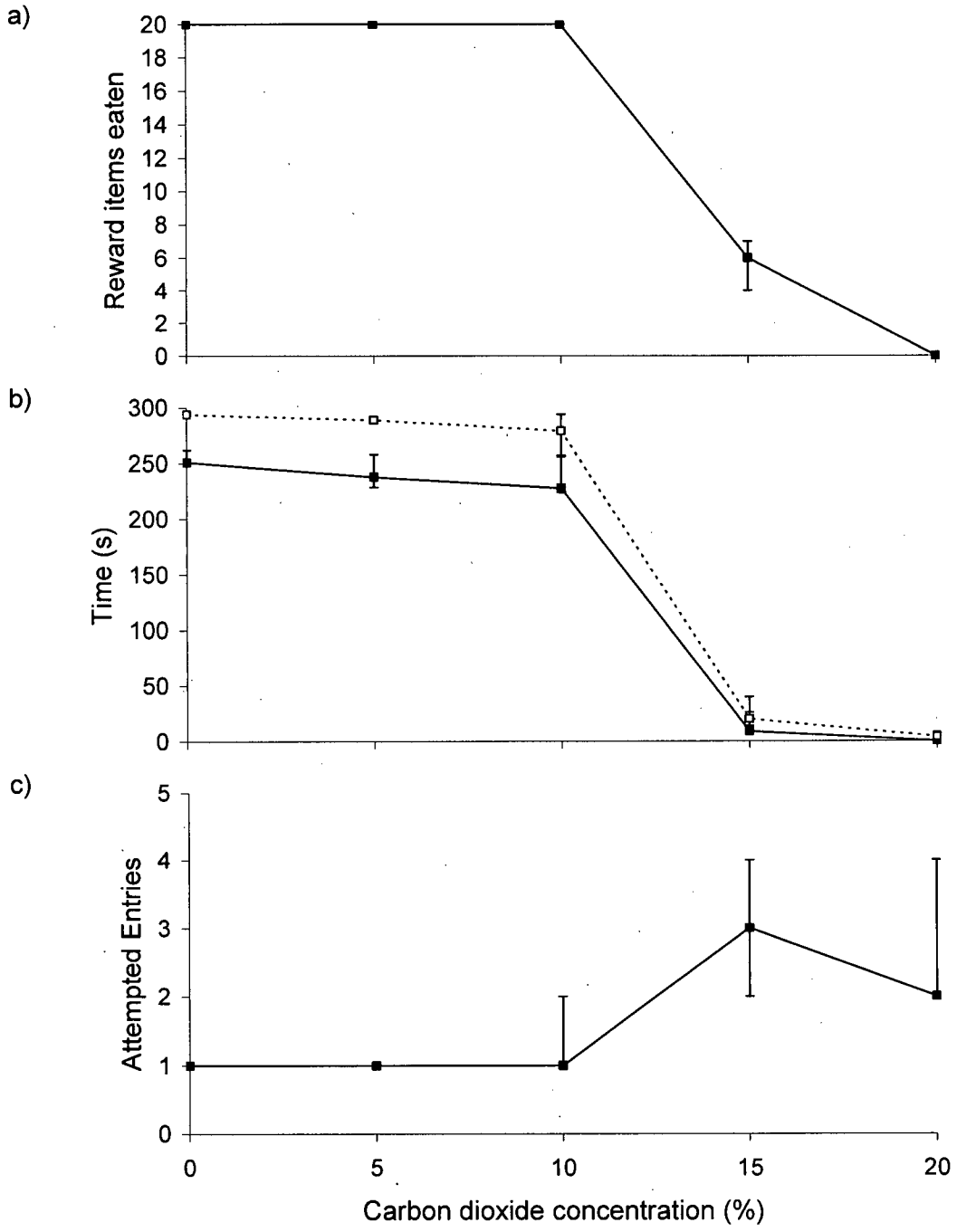

Figure 3.1. Median (± interquartile ranges) a) number of reward items eaten, b) eating time (filled squares) and dwelling time (open squares) for the first entry, and c) number of attempted entries into the test cage during sessions with 0, 5, 10, 15 and 20% CO_2 (n = 9 rats).

3.5 References

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CHAPTER 4: Effect of flow rate on aversion to gradual-fill carbon dioxide euthanasia in rats[4]

4.1 Introduction

Laboratory rats are commonly euthanized using CO_2, which induces unconsciousness followed by death. Animals are either placed into a chamber that has been pre-filled with CO_2, or the chamber containing the animals is gradually filled with CO_2 until death is confirmed. However, the term 'gradual' encompasses a large range of flow rates, and optimal flow rates for minimizing distress have not yet been identified.

Ideally, a method of euthanasia should induce death quickly without causing pain or distress. CO_2 concentrations of greater than 8% are known to cause dyspnea, which is an unpleasant sensation of breathlessness, in humans (Dripps & Comroe, 1947; Liotto et al., 2001), and may cause similar sensations in animals. CO_2 also forms carbonic acid on the mucous membranes and is known to cause pain in humans at concentrations greater than 30 to 50% (Anton et al., 1992; Chen et al., 1995; Feng & Simpson, 2003; Thurauf et al., 2002). Nociceptors in the nasal mucosa and cornea of rats are also stimulated by CO_2 (Peppel and Anton, 1993; Hirata et al., 1999), suggesting CO_2 likely also causes pain in rats. During gradual-fill CO_2 euthanasia, faster flow rates cause loss of consciousness and death more quickly (Hornett & Haynes, 1984; Coenen et al., 1995). However, with faster flow rates animals lose consciousness at higher CO_2 concentrations (Ambrose et al., 2000), likely because loss of consciousness during CO_2 exposure is dependent on pH changes in the cerebral spinal fluid and slow fill rates allow more time for these pH changes to occur. This exposure to higher CO_2 concentrations before loss of consciousness may increase the severity of any pain and dyspnea that occur. Thus, the potential for distress may vary with flow rate; high flow rates will expose

[4] A version of this chapter has been accepted for publication. Niel, L., Stewart, S.A., Weary, D.M. 2006. Effect of flow rate on aversion to gradual-fill carbon dioxide euthanasia in rats. Appl. Anim. Behav. Sci. (accepted).

animals to gas concentrations with a high potential for causing distress for a short period, while low flow rates will cause prolonged exposure to gas concentrations with a low to moderate potential for causing distress. It has been suggested anecdotally that a slowly increasing CO_2 concentration allows for gradual onset of unconsciousness, without the animal experiencing aversive CO_2 concentrations. The interaction between flow rate and maximum CO_2 concentration before unconsciousness indicates that this may be plausible, such that a range of slow flow rates may allow for euthanasia without causing distress.

Rats' reactions to gradual-fill CO_2 euthanasia have been assessed using both behavioural responses to exposure and preference testing. While some studies have reported a lack of behavioural response to gradual-fill CO_2 euthanasia (Hackbarth et al., 2000; Hornett & Haynes, 1984; Smith & Harrap, 1997), others have reported behavioural responses that suggest distress (Britt, 1987; Coenen et al., 1995; Chapter 2). Two studies have specifically examined the effect of flow rate during gradual-fill CO_2 exposure in rats. Hornett and Haynes (1984) did not find an effect of flow rate on behavioural responses, but Coenen et al. (1995) found that a rate of 125% of the chamber volume per minute caused greater gasping than 14% of the chamber volume per minute. This increase in gasping suggests a potential for dyspnea with the faster flow rate. In Chapter 3 we found that rats show aversion to gradual-fill CO_2 exposure at a rate of 17% per minute, but the effects of flow rate were not examined.

The aim of this study was to use approach-avoidance testing to determine whether aversion to gradual-fill euthanasia varies with flow rate.

4.2 Materials and Methods

4.2.1 Subjects and Housing

The subjects were eight 8-month old, male Wistar rats destined for euthanasia as surplus stock from the UBC Rodent Breeding Unit. Animal rooms were kept at 21 ± 1 °C under a 12:12-hr light-dark cycle, and rats were given ad libitum access to food (Lab Diet 5001, PMI Nutrition International, Richmond, USA) and tap water. All testing was conducted during the light portion of the light-dark cycle.

Rats were singly housed in the testing apparatus, consisting of two transparent cages connected by a sloped, opaque tunnel. The top or 'home' cage measured 48 x 38 x 20 cm, and contained food, water, bedding, an opaque nestbox and a Nylabone dog chew. The bottom or 'test' cage measured 45 x 24 x 20 cm and contained bedding. The home cage was 27 cm higher than the test cage, and the connecting tunnel was made of black, ribbed, PVC tubing with a diameter of 10 cm.

4.2.2 Testing Procedure

During experimental testing, each animal and its testing apparatus were transferred individually to a separate room. At this time, the wire lid on the test cage was replaced with a plexiglas lid fitted with a gas inlet in the center, two air outlets (1.5 cm in diameter) positioned at the end closest to the tunnel, and a gas sampling tube inserted at the far end of the test cage. The air outlets were covered with mesh to prevent the animals from pushing their noses outside the test cage. A partition was placed behind the experimental set-up to conceal the experimenter during testing.

Because the test cage opened directly into the tunnel, the total volume (24 L) was calculated to include the volume of the test cage plus the volume of the portion of the tunnel that was at the same height as the test cage. Preliminary testing was conducted to examine variability

in CO_2 concentrations in the test cage during the filling process. CO_2 concentrations were monitored at a depth of half the test cage height at 5 different sites with flow rates ranging from 5% to 28% of the test cage volume per minute, and were found to vary by less than 3.5% with any given flow rate. Furthermore, there was no obvious trend for lower CO_2 concentrations at end of the test cage closest to the tunnel. Because CO_2 concentrations tend to be greater near the bottom of the chamber than at the top (Britt, 1987; Chapter 2), measurements during the experiment were taken 10 cm above the site of reward delivery.

Air and CO_2 were delivered to the test cage from compressed gas cylinders (Praxair, Richmond, B.C.). The treatment gases were passed through a copper coil in a room temperature water bath to regulate the temperature of the gas before it entered the test cage. Flow rates of gases were measured with a variable area flowmeter (Model VSB-66-BV, Dwyer Instruments, Inc.), and measured CO_2 flow rates were adjusted for density using a correction factor of 0.812.

Rats were trained to enter the lower cage for a food reward, and had previously been tested with exposure to static and gradually increasing concentrations of CO_2 for a separate experiment. During experimental sessions, rats were first locked in the top cage for 2 min. After the lock was removed the rats were able to enter the lower test cage for a food reward of 20 Honey Nut Cheerios™ (General Mills, Inc., Minnesota). As soon as the rat entered the test cage and started eating the Cheerios™, either air or CO_2 flow was initiated at a pre-determined rate. Rats could remain in the test cage for a maximum of 300 s from the time that gas flow began, after which the test session was ended. If the rat entered the home cage during this period the test session was stopped. At the end of the session, the remaining reward items were removed and counted, and the rat was returned to the holding room.

The testing apparatus and O_2 meter readout were video recorded during testing. We also recorded the total number of reward items eaten over the entire test session as well as the

latency to stop eating and the latency to leave the test cage after gas flow had begun. Gas concentrations in the test cage were monitored during the experiment via the gas sampling tube using a Mocon LF700D O_2 analyzer. The O_2 concentration was recorded and used to calculate CO_2 concentrations at each of these times (t = x) with the formula: $CO_{2\,(t=x)} = 100 - (100 * ([O_{2\,(t=x)}] / [O_{2\,(t=0)}]))$.

Rats were tested in two replicates of eight test sessions, and in each replicate rats were tested on five days with CO_2 and on three control days with air. For both replicates, rats were tested with five different CO_2 flow rates: 3, 7, 14, 20, and 27% of the test cage volume per minute. In the first replicate, a flow rate of 21% per minute was used for all three test sessions with air. In the second replicate, flow rates of 4, 17 and 33% per minute were used for the three test sessions with air. Treatment order for CO_2 and air was balanced across rats and days by an 8 x 8 Latin square. Only flow rates less than 30% per minute were examined because the results of Ambrose et al. (2000) suggest that faster flow rates result in potentially painful CO_2 concentrations before animals lose consciousness.

4.2.3 Statistical Analysis

Data were averaged within rat and CO_2 flow rate for the two replicates, resulting in 40 observations for the analysis of CO_2 flow rate (8 rats and 5 flow rates). For the analysis of air flow rate, the three flow rates from the second replicate were examined, resulting in 24 observations (8 rats and 3 flow rates). Dependent variables were analyzed using a mixed model (SAS v9.1) that included rat (7 d.f.) as a random effect, and tested for linear and quadratic effects of flow rate (1 d.f. for each) against an error term with 30 d.f. for the test of CO_2 flow rate and 14 d.f. for the test of air flow rate. Latency to leave the test cage was not tested in the air flow analysis because all animals remained in the test cage for the entire testing period.

4.3 Results

During test sessions with air, rats ate on average (± SE) 19.3 ± 0.3 reward items out of 20, and finished eating 270 ± 6 s after entering the test cage. All rats remained in the test cage for the entire 300 s testing period for all air sessions. Changes in air flow rate did not affect the latency to stop eating (linear: $F_{1,14} = 1.74$, $P > 0.1$; quadratic: $F_{1,14} = 0.19$, $P > 0.1$) or the number of reward items eaten (linear: $F_{1,14} = 0.29$, $P > 0.1$; quadratic: $F_{1,14} = 0.07$, $P > 0.1$).

In contrast, the number of reward items eaten, the latency to stop eating, and the latency to leave the test cage decreased with increasing CO_2 flow rates (Fig. 4.1 a, b). Both the linear and curvilinear effects were significant for the number of reward items eaten (linear: $F_{1,30} = 67.21$, $P < 0.001$; quadratic: $F_{1,30} = 5.02$, $P < 0.05$), the latency to stop eating (linear: $F_{1,30} = 128.36$, $P < 0.001$; quadratic: $F_{1,30} = 10.91$, $P < 0.01$), and the latency to leave the test cage (linear: $F_{1,30} = 171.24$, $P < 0.001$; quadratic: $F_{1,30} = 11.84$, $P < 0.01$).

The rats did not remain in the test cage for long enough to lose consciousness at any of the flow rates, but there was a curvilinear relationship between CO_2 flow rate and the CO_2 concentration at the time rats stopped eating ($F_{1,30} = 5.65$, $P < 0.05$) and left the test cage ($F_{1,30} = 9.02$, $P < 0.01$). Rats stopped eating and left the test cage at lower CO_2 concentrations with the lowest and highest flow rates (Fig. 4.1 c). Rats left the test cage at the highest CO_2 concentration (15.9% CO_2) when tested at the 14% per minute flow rate. However, the CO_2 concentration when rats left the test cage varied considerably across rats. When averaged within rat across all days for all flow rates, the average CO_2 concentration when rats left the test cage ranged from 11.1% to 18.6%. The maximum and minimum CO_2 concentrations tolerated before rats left the test cage ranged from 4.8% to 25.3%. Variability was also observed for individual rats; for example, one rat left the test cage at 4.8% CO_2 on one day and at 21.5% CO_2 on a different day.

4.4 Discussion

It has been suggested anecdotally that slow CO_2 fill rates can result in loss of consciousness in rats before aversive CO_2 concentrations occur. However, rats in the current study left the test cage before losing consciousness for all test sessions with CO_2. This result demonstrates that rats are averse to gradual-fill CO_2 exposure with flow rates ranging from 3% to 27% of the test cage volume per minute. Flow rate had no effect on any variables during test sessions with air, indicating that it was CO_2 exposure that resulted in aversion rather than sound or air currents associated with changes in gas flow dynamics.

In the current study, the latency to leave the test cage decreased with increasing flow rate, such that on average rats left the test cage when CO_2 concentrations were between 13.0% and 15.9%. In a previous study examining aversion to CO_2, rats showed aversion to static CO_2 at concentrations of 15%, and to a gradually increasing concentration of CO_2 at approximately 18% (Chapter 3). These results indicate that there is a threshold CO_2 concentration that rats find aversive, and that it is relatively consistent regardless of flow rate. This concentration of CO_2 is unlikely to cause pain in rats. The majority of receptors in the nasal mucosa respond to CO_2 concentrations between 37 and 50% CO_2 (Anton et al., 1991; Peppel & Anton, 1993). Furthermore, painful stimulation of the nasal mucosa is known to elicit apnea and bradycardia, and this response is not observed in rats at CO_2 concentrations ranging from 10% to 50% (Yavari et al., 1996). However, CO_2 concentrations as low as 8% have been associated with a sensation of dyspnea, or breathlessness, in humans (Dripps & Comroe, 1947; Liotti et al., 2001), and this sensation may occur in rats. We therefore suggest that dyspnea is more likely to be the cause of aversion in the current study.

The CO_2 concentration at leaving time varied with flow rate, with rats tolerating slightly higher CO_2 concentrations at intermediate flow rates. At low flow rates, rats are likely leaving at

lower CO_2 concentrations because the extended period of exposure to lower concentrations reduces their overall tolerance for CO_2. Sensations of dyspnea due to hypercapnia are likely mediated by central and peripheral chemoreceptors (American Thoracic Society, 1999), which are sensitive to reductions in the pH of blood and cerebral spinal fluid. Extended exposure would allow greater time for these adjustments, such that the maximum tolerance is reached at a lower concentration. Reduced tolerance at high flow rates indicates that CO_2 detection mechanisms might be sensitive to not only absolute CO_2 concentration, but also to the rate at which CO_2 is increasing.

Only flow rates less than 30% per minute were examined in the current study because the results of Ambrose et al. (2000) suggest that high flow rates result in CO_2 concentrations that are sufficient to cause pain before unconsciousness in mice. Ambrose et al. (2000) found that a flow rate of 60% of the chamber volume per minute resulted in CO_2 concentrations above 50% in the 10 s or so before mice lost consciousness, while at 30% per minute mice became unconscious at CO_2 concentrations under 50%. Thus, although faster flow rates result in a shorter duration of exposure before loss of consciousness, slower flow rates may prevent exposure to CO_2 levels that are sufficient to cause pain. While flow rates greater than 30% per minute were not examined, the CO_2 concentration at which rats left the test cage varied in a parabolic manner, suggesting that rats would leave at lower concentrations with flow rates above those tested in the current study.

The approach-avoidance test used in the current study indicates that the maximum CO_2 concentration tolerated varies with flow rate, but this study provides little information on the level of distress that rats would have experienced had they been forced to remain in the test cage until death. This distress would be dependent both on the duration of the period between onset of aversion and unconsciousness, and the strength of aversion to the CO_2 concentrations occurring during this period. Previous studies have attempted to examine behavioural responses

during the entire euthanasia procedure as a means of assessing distress. While some studies have found evidence to suggest a distress response (Britt, 1987; Coenen et al., 1995; Chapter 2), others have reported no effect (Hackbarth et al., 2000; Hornett & Haynes, 1984; Smith & Harrap, 1997). Some of these studies have specifically compared behavioural responses during gradual-fill CO_2 exposure at different flow rates. Hornett and Haynes (1984) examined flow rates ranging from 6 to 40% per minute with rats and while adverse reactions were not reported for any flow rate, a rate of 19.5% per minute was recommended based on a subjective assessment of the procedure and time to unconsciousness and death. Time to unconsciousness was approximately 4 min at 6% per minute, but was reduced to approximately 2 min for flow rates of 13 to 40% per minute. Coenen et al. (1995) compared flow rates of 14% and 125% per minute and found that both treatments resulted a similar period of 'excitation and agitation', but that gasping was slightly higher in the fast fill group, suggesting increased dyspnea. However, the time to recumbency, aberrant EEG and abnormal ECG were significantly longer in the 14% per minute group.

In conclusion, rats show avoidance during exposure to gradual-fill CO_2 exposure with flow rates ranging from 3% to 27% per minute. The CO_2 concentration at the time rats left the test cage varied with flow rate, indicating that a flow rate of 14% per minute is optimal in terms of initial aversion. However, forced exposure to CO_2 beyond this initial aversion is likely to result in distress with all flow rates, therefore further research is needed to develop better methods of euthanasia for laboratory rats.

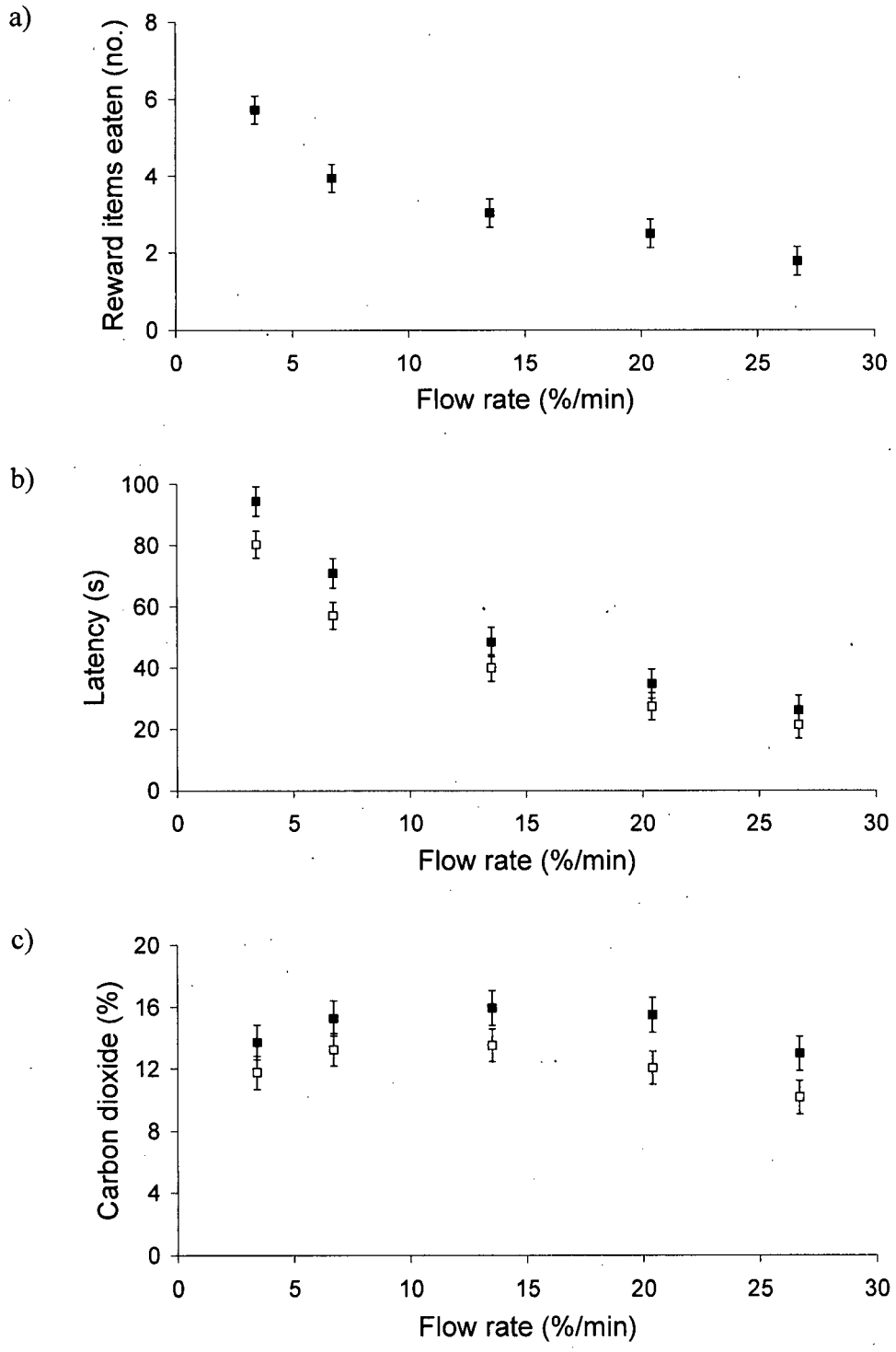

Figure 4.1. Least squares mean (± SEM) a) number of reward items eaten, b) latency to stop eating (open) and to leave the test cage (filled), and c) CO_2 concentration at the time when rats stopped eating (open) and left the test cage (filled) during test sessions with CO_2 flow rates of 3, 7, 14, 20 and 27% of the test cage volume per minute (n = 8 rats).

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CHAPTER 5: Effects of novelty on rat responses to CO_2 exposure[5]

5.1 Introduction

Carbon dioxide is widely used for killing laboratory rodents, but recent evidence suggests that exposure to CO_2 may cause distress and aversion before loss of consciousness in rats. Exposure of rats to a gradually increasing concentration of CO_2 has been shown to result in escape behaviours, such as pushing and scratching at the chamber lid, and increased exploration (Chapter 2). Furthermore, Leach et al. (2002) found that rats avoid CO_2 concentrations of 25.5% and greater, and, as described in Chapters 3 and 4, rats will forgo a palatable food reward in order to avoid CO_2 concentrations of 15% and greater.

Both dyspnea (an unpleasant sensation of breathlessness) and pain have been suggested as potential causes of distress and aversion during CO_2 euthanasia. CO_2 concentrations of greater than 30% are necessary to cause loss of posture in rats (Chapter 2; Smith and Harrap, 1997), and loss of posture indicates the approximate onset of unconsciousness (e.g. Coenen et al., 1995). CO_2 forms carbonic acid when it comes into contact with moisture at the mucosal membranes, and starts to cause pain in humans at concentrations of 30 to 50% (Anton et al., 1992; Chen et al., 1995; Feng & Simpson, 2003; Thurauf et al., 2002). Rats have nociceptors in the nasal mucosa that respond to CO_2 at similar concentrations (Anton et al., 1991; Peppel and Anton, 1993), and so rats may experience pain at moderate CO_2 concentrations. CO_2 also causes dyspnea in humans at concentrations of only 8% (Dripps & Comroe, 1947; Liotti et al., 2001). In previous studies on CO_2 euthanasia, rats have shown behavioural signs of distress and aversion during exposure to relatively low concentrations of CO_2 (Chapters 2, 3 and 4). These

[5] A version of this chapter has been submitted for publication. Niel, L., Weary, D.M. 2006. Effects of novelty on rat responses to CO_2 exposure. Appl. Anim. Behav. Sci. (submitted).

responses occurred at CO_2 concentrations that were lower than nociceptive thresholds, therefore dyspnea is a more likely cause of distress and aversion than pain.

However, another potential source of distress during CO_2 exposure is novelty. Novelty has been suggested to induce an approach-avoidance conflict in rats, resulting from an interaction between exploratory motivation and fear (Montgomery, 1955). Wallace and Rosen (2000) demonstrated that exposure of rats to novel odours, such as butyric acid (similar to rancid butter) and isoamyl acetate (similar to banana), causes avoidance, reduces grooming time and increases freezing time, and these responses suggest that novel odours can elicit fear. Odour perception occurs as a result of stimulation of both olfactory and trigeminal neurons, with the latter contributing mainly to pungency (Cain and Murphy, 1980). CO_2 is thought to stimulate mainly trigeminal neurons in the nasal mucosa, but because odour perception occurs as a result of both olfactory and trigeminal input, humans perceive an odour quality when asked to describe sensations occurring with CO_2 inhalation (Cain and Murphy, 1980). Rats can detect CO_2 at concentrations between 0.04 and 1.7% (Youngentob, 1991), which is below the levels required to stimulate trigeminal neurons in rat nasal mucosa (Peppel and Anton, 1993). The exact quality of CO_2 that rats are responding to is unknown, but they have a sensitive olfactory system and may have an enhanced ability to detect the odour quality of CO_2.

In order to test whether distress and aversion associated with CO_2 exposure is due in part to novelty, we used two different experimental approaches. For the first set of experiments, we used an approach-avoidance test to examine gas aversion in rats, by pairing gas exposure with a food reward. In Experiment 1 we examined rat aversion to gradual-fill CO_2 exposure. Rats were tested with repeated CO_2 exposure to document whether their responses would show habituation. In Experiment 2 we used the same methodology to examine how a novel odour affects rat performance on the approach-avoidance test. We used peppermint as the novel odour, and this stimulus was not expected to produce pain or respiratory stimulation. For both

experiments, we compared rat responses to the first exposure and subsequent exposures to determine whether each condition was aversive and whether novelty was a source of aversion, using the following logic. We reasoned that: 1. a lack of aversion would be indicated by similar eating and dwelling times with air and the treatment gas, 2. aversion due to novelty would result in animals eating less and leaving earlier on the initial exposure, but that this reaction would decrease on subsequent exposures, and 3. aversion without an effect of novelty would result in the animals eating less and leaving earlier on all exposures.

For the second experimental approach, we examined the behavioural responses of rats during exposure to a gradually increasing concentration of either CO_2 or peppermint odour (Experiment 3). We predicted that behaviours that were due to novelty would be present with both treatments, but that those occurring as a result of other factors, such as pain and dyspnea, would occur only in the CO_2 treatment group.

5.2 Materials and Methods

5.2.1 Subjects, Housing, and Equipment

Rats were obtained as surplus stock (i.e. animals already slated for euthanasia) from the UBC Rodent Breeding Unit, and housed at 21°C under a 12:12-hr light-dark cycle with ad libitum access to food (Lab Diet 5001, PMI Nutrition International, Indiana, USA) and tap water. All testing was conducted during the light portion of the light-dark cycle.

CO_2 and air were delivered from compressed gas cylinders (Praxair, Richmond, B.C.). For some treatments, air was scented with peppermint odour by routing the air flow through a 266 mL chamber containing three cotton balls soaked in 2 mL of peppermint extract (Canada Safeway Ltd., Calgary). The treatment gases were passed through a copper coil in a room temperature water bath to regulate the temperature of the gas before it entered the chamber.

Flow rates of the gases were measured by a variable area flowmeter (Model VSB-66-BV, Dwyer Instruments, Inc., Michigan), and measured CO_2 flow rates were adjusted for density with a correction factor of 0.812. Gas concentrations in the chamber were monitored during the experiment via a gas sampling tube using a Mocon LF700D O_2 analyzer, and the following formula was used to calculate the concentration of CO_2 at specific time points ($t = x$) during the filling process: $[CO_{2\,(t=x)}] = 100 - (100 * ([O_{2\,(t=x)}] / [O_{2\,(t=0)}]))$.

5.2.2 Experiments 1 & 2: Approach-avoidance Testing

We used an approach-avoidance test to examine rats' avoidance of a gradual addition of either CO_2 (Experiment 1) or air with peppermint odour (Experiment 2). The peppermint was not expected to cause pain or respiratory stimulation.

Rats were singly housed in the testing apparatus consisting of two transparent cages connected by an opaque tunnel made of black, ribbed, PVC tubing with a diameter of 10 cm and sloped so that one cage was 27 cm higher than the other. The 'home' cage measured 48 x 38 x 20 cm, and contained food, water, bedding, an opaque nestbox and a Nylabone dog chew. The secondary cage measured 45 x 24 x 20 cm and contained bedding. The lower cage was always used for testing because the test gases were denser than air. During preliminary testing of the experimental apparatus we determined that the test gases remained in the lower cage and the lower portion of the tunnel. For Experiment 1, half of the rats were tested in the home cage and the other half were tested in the secondary cage for the purposes of a separate experiment (Chapter 3). For Experiment 2, all rats were tested in the secondary cage.

During experimental testing, each animal and its testing apparatus were transferred individually to a test room. At this time, the nest box was removed from the home cage and the wire lid on the test cage was replaced with a plexiglas lid that featured two air outlets positioned

at the end closest to the tunnel, a gas inlet at the far end of the cage, and a gas sampling tube inserted at the center to a depth of 10 cm above the cage floor. The air outlets were covered with mesh to prevent the rats from pushing their noses outside the test cage. The experimenter was concealed behind a blind during testing. The testing apparatus and O_2 meter readout were video recorded during testing.

During both training and experimental sessions, rats were first locked into the upper cage for 2 min. The lock was then removed and they were able to enter the lower test cage for a food reward of 20 Honey Nut Cheerios™ (General Mills, Inc., Minnesota). When the rat started eating the food reward, gas flow (air, air with peppermint or CO_2) into the test cage was started at a rate of 17% of the cage volume per minute. The session ended 300 s after lock removal. The animals were allowed to exit and re-enter the test cage throughout the test period. At the end of the session, the remaining reward items were removed and the rat was returned to the holding room.

For Experiment 1 the subjects were nine 5-month old, male Wistar rats. Initially they were trained for 9 days to perform the approach-avoidance task with air flowing into the test cage. This training was followed by 17 days of testing. The rats were first tested over five consecutive days, with air on day 1, gradual-fill CO_2 on days 2, 3, and 4, and air again on day 5. From days 6 to 15, the rats were tested two times with air and with static CO_2 concentrations of 5, 10, 15 and 20% for a separate experiment (Chapter 2). These data are not included in the current study, but served to provide the rats with further experience of CO_2 exposure. Rats were then tested again with gradual-fill CO_2 and air on days 16 and 17, respectively.

For Experiment 2 the subjects were seven 13-month old, male Wistar rats. Before this experiment, they had performed in another approach-avoidance experiment, and had considerable experience with air or CO_2 flowing into the test cage. However they had not previously been tested with a novel odour such as peppermint. The rats were tested over five

consecutive days, with air on day 1, air with peppermint odour on days 2, 3, and 4, and air again on day 5.

For Experiments 1 and 2, we recorded the total number of reward items eaten during each test session, the latency to stop eating and the latency to leave the test cage after gas flow started. For Experiment 1 we also recorded the O_2 concentrations when the rat stopped eating and when it left the test cage, and used these values to calculate CO_2 concentrations as described above.

5.2.3 Behavioural Responses: Experiment 3

In this experiment we examined the behavioural responses of rats during the gradual addition of either CO_2 or air with peppermint odour.

The exposure chamber was a 20 L polypropylene cage measuring 20.5 x 45.5 x 24 cm (Lab Products Inc.), fitted with a Plexiglas lid. The lid had a gas inlet centered at the end, two air outlets positioned at the opposite end, and a gas sampling tube inserted at the center of the chamber to a depth of half the chamber height. The air outlets were covered with mesh to prevent the rats from pushing their noses outside the chamber. The back and sides of the chamber were covered with black paper so that the animals could not see the person conducting the experiment.

The subjects were thirty-two 4 to 6-month-old, male Wistar rats. Animals were randomly allocated to the CO_2 or air with peppermint odour treatment groups (n = 16 for both). The rats were individually placed into the euthanasia chamber for a 27-min period of acclimatization, during which air was added to the chamber at a rate of 17% of the chamber volume per minute. After acclimatization, air flow was stopped and either CO_2 or air with peppermint odour was started at a rate of 17% of the chamber volume per minute. CO_2-treated animals remained in the chamber and were monitored until death, but animals treated with peppermint odour were

removed from the chamber at the end of the 135-s observation period. Preliminary observations showed that CO_2-treated animals ceased all purposeful movement within this period, so any relevant effects of peppermint exposure would be present during this time.

The euthanasia chamber and O_2 meter readout were video recorded during the experimental procedure. Each animal was scored continuously during the last 135 s of the acclimatization period (baseline) and for 135 s after gas flow began (exposure) for pre-defined behaviours thought to relate to distress (Table 1). In a previous study these behaviours were found to increase during gradual-fill CO_2 exposure (Chapter 2).

5.2.4 Statistical Analyses

5.2.4.1 Experiment 1 - Approach-avoidance Testing with CO_2

Dependent variables were analyzed for the first three days of CO_2 exposure with a mixed model (SAS v9.1) which included rat as a random effect (8 d.f), and tested for a linear effect of order (1 d.f.) against an error term with 17 d.f. For those variables where no statistical differences were found across the first three days of CO_2 exposure (all except number of reward items eaten), data were averaged within rat and the average response was then compared to the response on the final day of CO_2 testing (Day 16) with a mixed model which included rat as a random effect (8 d.f.) and examined the effect of order (1 d.f.) against an error term with 8 d.f. Number of reward items eaten was compared between the third and final CO_2 test sessions with a similar mixed model.

5.2.4.2 Experiment 2 - Approach-avoidance Testing with Peppermint Odour

Only 1 of the 7 rats ate fewer than 20 reward items during testing with air or peppermint odour, so statistical analyses were not performed with this variable. The remaining dependent variables were compared across the three days of testing with peppermint odour with a mixed

model which included rat as a random effect (6 d.f) and tested for a linear effect of order (1 d.f.) against an error term with 13 d.f. Rats showed similar responses to peppermint odour exposure over the three days of testing, so data were averaged within rat for the two days of testing with air and for the three days of testing with peppermint odour. Dependent variables were then compared with a mixed model which included rat as a random effect (6 d.f) and examined the effect of gas treatment (1 d.f.) against an error term with 6 d.f.

5.2.4.3 Experiment 3 - Behavioural Responses

The number of times the rat reared and the time spent with the nose in contact with the chamber lid were analyzed with a mixed model which included rat as a random effect (30 d.f.) and examined the effect of period (1 d.f.), gas treatment (1.d.f) and the interaction between period and gas (1 d.f.) against an error term with 30 d.f. Escape behaviours and activity (recorded as side changes) were not observed in all animals, so the number of animals which showed increases in these behaviors during exposure was compared between gas treatments by a G-test with a William's correction (described in Sokal and Rolf, 1995).

5.3 Results

5.3.1 Experiment 1 - Approach-avoidance testing with CO_2

During approach-avoidance testing on the three control days with air (Days 1, 5 and 17), rats consumed all 20 reward items, and, on average (mean ± S.E.), they stopped eating and left the test cage after 266 ± 4 s and 288 ± 2 s, respectively. In contrast, on the four CO_2 test days (Days 2, 3, 4 and 16), rats consumed an average of only 2.7 ± 0.2 reward items, and their latencies to stop eating and leave the test cage dropped to 30 ± 2 s and 40 ± 2 s, respectively.

Across the first three days of testing with CO_2, there was no change in the latencies to stop eating or leave the test cage, or in the CO_2 concentration at which rats stopped eating and left the test cage (Fig. 5.1 a, b; $P > 0.1$ for all). However, on the final day of testing rats showed a 52% increase in latency to stop eating ($F_{1,8} = 18.99$, $P < 0.005$) and a 25% increase in latency to leave the test cage ($F_{1,8} = 7.75$, $P < 0.05$), resulting in higher CO_2 concentrations at these time points (stop eating: $F_{1,8} = 31.9$, $P < 0.001$; leave test cage: $F_{1,8} = 15.2$, $P < 0.01$).

The number of reward items eaten showed a linear increase over the first three days of testing with CO_2 (Fig. 5.1 c; $F_{1,17} = 5.45$, $P < 0.05$). The number of reward items eaten also increased from the third to the final test session ($F_{1,8} = 25.54$, $P < 0.001$).

5.3.2 Experiment 2 - Approach-avoidance testing with peppermint odour

Rats' performance on the approach-avoidance task was similar on the two control days with air (Days 1 and 5), and on the three days of testing with peppermint odour (Days 2, 3 and 4). During testing with both air and peppermint odour, six of the seven rats consumed all 20 food reward items on each test day. The seventh rat ate for the entire test period each day, but only consumed 17 to 20 of the reward items due to a slow eating rate. In comparison to air exposure, peppermint exposure did not affect the latency for rats to stop eating (232 vs. 235 ± 14 s; $F_{1,6} = 0.66$, $P > 0.1$) or leave the test cage (274 vs. 283 ± 6 s; $F_{1,6} = 2.06$, $P > 0.1$).

Rats' performance was also consistent across the three days of testing with peppermint odour. They consumed a similar number of reward items, and there was no difference in their latency to stop eating ($F_{1,13} = 0.33$, $P > 0.1$) or to leave the test cage ($F_{1,13} = 0.44$, $P > 0.1$).

5.3.3 Experiment 3 - Behavioural responses

During the 135-s baseline period, rats from both the CO_2 and the peppermint odour treatment groups reared about four times and spent about 12 to 14 s with the nose touching the

chamber lid (Fig. 5.2). After initiation of either CO_2 or peppermint odour, rats showed increases in both rearing ($F_{1,30} = 14.13$, $P < 0.001$), and time spent with the nose touching the chamber lid ($F_{1,30} = 8.11$, $P < 0.01$). While the increase in response for these behaviours was numerically larger with CO_2 than with peppermint odour, we found neither an effect of gas treatment nor an interaction between period and gas treatment ($P > 0.1$).

During exposure to either CO_2 or peppermint odour, less than half of the rats showed increases in activity in comparison to baseline. Furthermore, the number of rats that showed an increase did not differ between the CO_2 (6 of 16 rats) and peppermint odour (7 of 16 rats) treatment groups (G = 0.12, $P > 0.1$).

During the baseline period, escape behaviours were only performed by one rat. This rat was from the peppermint odour treatment group, and it did not perform escape behaviours during exposure to peppermint odour. Increases in escape behaviours were observed in 1 of 16 rats during peppermint exposure, but were observed in 10 of 16 rats during CO_2 exposure ($G = 11.89$, $P < 0.001$). The number of escape behaviours performed by rats during CO_2 exposure ranged from 1 to 21.

5.4 Discussion

Previous studies have found that when rats are exposed to novel stimuli such as novel environments (Dubovicky et al., 1999; Montgomery, 1955) and objects (Zangrossi and File, 1994), they show signs of habituation by the second exposure. In contrast, the rats in Experiment 1 did not show any reduction in their avoidance of gradual-fill CO_2 exposure over the first three days of approach-avoidance testing. They ate faster on the second and third days of testing, but showed similar latencies to stop eating and leave the test cage. Because the response to CO_2 differed from that observed with other simple, novel stimuli, it is unlikely that

the rats' response was due to novelty. This conclusion is supported by the results of Experiment 2, in which novel peppermint odour had no effect on rats' performance during approach-avoidance testing. Hence, exposure to a novel odour is not sufficient to deter rats from this type of task. Furthermore, by the final day of approach-avoidance testing with CO_2, rats were still showing consistent aversion to CO_2 concentrations below those needed to cause unconsciousness. This result indicates that rats do not habituate to gradual-fill CO_2 exposure, and suggests that aversion to CO_2 is due mainly to factors other than novelty.

Rats in the current study initially left the test cage when CO_2 concentrations reached 14%, but this increased to 18% on the final day of testing. In previous approach-avoidance studies on CO_2 aversion in rats, the rats were tested for aversion to CO_2 after being familiarized with CO_2 exposure (Chapters 3 and 4). The current results suggest that this likely resulted in a modest underestimation of the CO_2 concentrations that rats find aversive on initial exposure.

One potential explanation for this increased tolerance for CO_2 with repeated exposure is that the rats learned to tolerate the unpleasant sensations associated with CO_2 exposure, or that they learned strategies, such as breatholding, that allowed them to remain in the test cage for longer. Alternatively, the rats may have developed an increased physiological tolerance for CO_2. Previous studies have found that chronic exposure to elevated CO_2 can result in a reduced ventilatory response to hypercapnia through acid-base adjustments (Lai et al., 1981) or alterations in chemoreceptor activity (Mitchell and Johnson, 2003). Chronic exposure to low levels of CO_2 (<3%) has also been shown to increase the level of hypercapnia needed to cause dyspnea during acute CO_2 exposure in humans (Bloch-Salisbury et al., 1996). However, in comparison to other studies in which this increased tolerance has been demonstrated, rats in the current study were exposed to CO_2 for only short periods (less than 5min per day), whereas changes in dyspnea tolerance in humans developed only after multiple days of chronic CO_2 exposure (Bloch-Salisbury et al., 1996).

Only one study has looked at the effect of short, daily CO_2 exposures on ventilatory responses to an acute CO_2 challenge. Waters and Tinworth (2001) examined changes in the ventilatory responses of piglets to a challenge with an inspired gas mixture of 9% O_2 and 6% CO_2 after seven days of acute, cyclic exposure to this same gas mixture. Cyclic exposure consisted of one 48-min session per day in which fresh air and exposure to the gas mixture were alternated every 4 min. Following seven days of cyclic exposure, the piglets showed a smaller increase in breathing rate and volume in response to the challenge in comparison to controls. However, it is not known whether the relatively short periods of CO_2 exposure that occurred during the current study were sufficient to cause such changes, and whether these changes would have altered the rats' aversion to CO_2 exposure.

Behavioural changes of rats during forced exposure to an unfamiliar stimulus, such as peppermint odour, might be due to novelty, which would decrease with repeated exposure, or due to intrinsic aversion to the stimulus, which would remain constant with repeated exposure. While humans do not generally find peppermint odour intrinsically unpleasant, no previous studies have examined aversion to peppermint odour in rats. In Experiment 2 we found that rats showed no sign of aversion to peppermint odour at the concentrations used in the current study, even on the first exposure. This result suggests that rats' aversion to this stimulus was less than their motivation to obtain the reward items. Not only did rats consume a similar number of reward items in a similar amount of time with both air and peppermint odour, but during peppermint odour exposure they remained in the test cage for an average of 48 s after they stopped eating. The rats' willingness to remain in the test cage even after the reward was finished suggests that rats do not find peppermint odour aversive, and that any behavioural responses observed during forced exposure were not due to aversion.

In a previous study we found that exposure to a gradually increasing concentration of CO_2 caused an increase in both exploratory and escape behaviours in rats (Chapter 2). One of

the aims of the current study was to determine whether these responses were due to novelty or other aversive properties of CO_2. In Experiment 3, exposure to either CO_2 or a novel odour in gradually increasing concentrations resulted in increased activity, rearing, and time spent with the nose touching the chamber lid. However, more rats performed escape behaviours during CO_2 exposure than during exposure to peppermint odour. This indicates that exploratory behaviours during gradual-fill CO_2 exposure could be due to novelty, but that escape behaviours are mainly due to other properties of CO_2. Exploratory behaviours are difficult to interpret in terms of animal distress, but escape behaviours presumably indicate that the animal would exit the exposure chamber if given the opportunity. These results therefore suggest that distress during gradual-fill CO_2 exposure is not due to novelty. However, one animal did perform escape behaviours in response to peppermint odour exposure, so exposure to a novel stimulus may also contribute to distress during CO_2 exposure. Previous studies have found that freezing in response to a novel odour depends on the odour that is used and its intensity (Wallace and Rosen, 2000), so we cannot rule out the possibility that another odour stimulus might elicit a similar behavioural response to that seen during CO_2 exposure.

Of the three potential causes of distress and aversion during CO_2 exposure - novelty, pain and dyspnea - the current study indicates that novelty likely contributes to the responses of rats during gradual-fill CO_2 exposure, but it is not the main cause of distress and aversion. Moreover, previous studies have found that behavioral responses and aversion to a gradually increasing concentration of CO_2 occur at concentrations that are below the threshold for pain at the eyes and the nasal mucosa (Chapters 2, 3 and 4). Therefore, the most likely cause of distress and aversion during gradual-fill CO_2 exposure is dyspnea, which occurs in humans at CO_2 concentrations of only 8% (Dripps & Comroe, 1947; Liotti et al., 2001). Further research is needed to determine whether dyspnea does occur in rats in response to CO_2 exposure, and if so whether it is possible to mitigate sensations of dyspnea during CO_2 euthanasia.

Table 5.1. Descriptions of rat behaviours recorded during baseline and during exposure to CO_2 or peppermint odour (Experiment 3).

Behaviour	Description
Activity	Movement that results in the back feet crossing a line that divides the length of the chamber in half (event).
Rear	Raising the upper body while standing on the back feet. Includes wall climbing. Climbing on the air sampling tube while chewing it and rearing during grooming were excluded (event).
Nose to lid	Time spent with the nose in contact with the chamber lid (state).
Escape behaviours:	
Scratch at lid	A rapid movement of the front paw from the lid through at least a 90° downward angle (event).
Push at lid	A push at the chamber lid using the nose or front paw evidenced by body and lid movement (event).

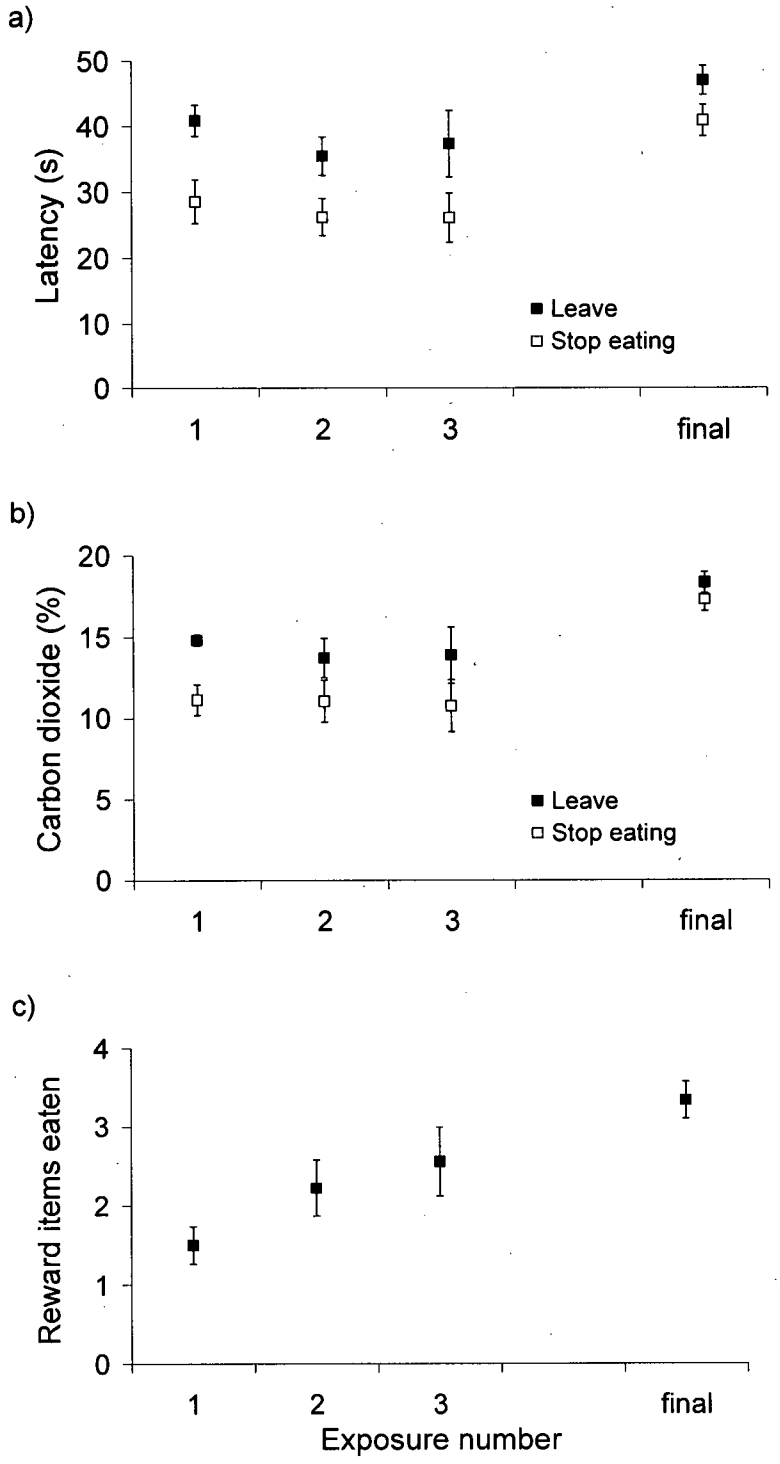

Figure 5.1. Approach-avoidance responses to CO_2 for the first three days and for the final day (Day 16) of exposure (Experiment 1). Mean (± SEM) (a) latency to stop eating and leave the test cage, (b) CO_2 concentration when rats stopped eating and left the test cage, and (c) number of reward items eaten ($n = 9$ rats).

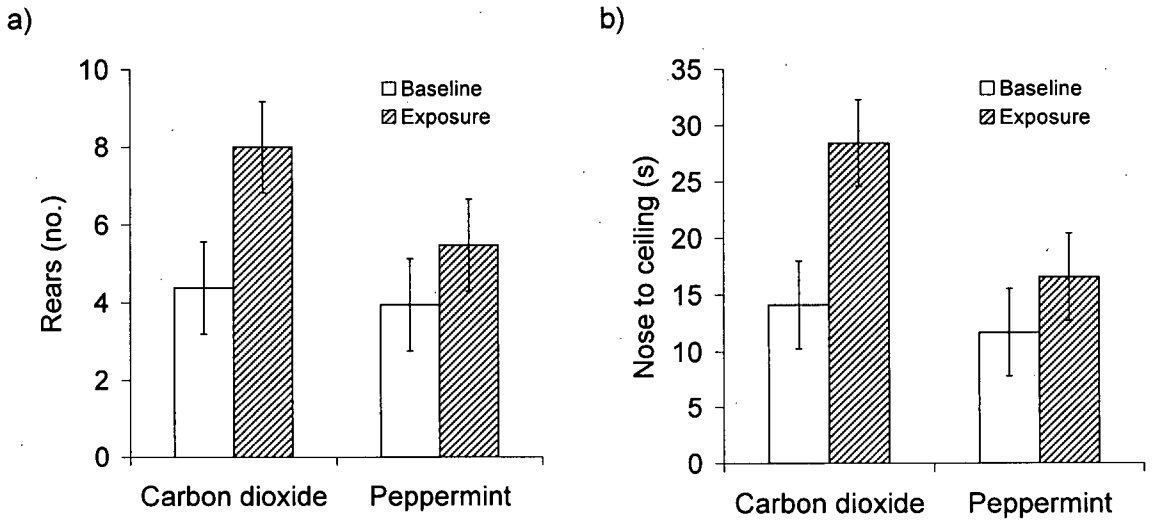

Figure 5.2. Behavioural responses of rats to CO_2 euthanasia and peppermint odour exposure (Experiment 3). Least squares mean (± SEM) (a) number of rears, and (b) time spent with the nose in contact with the test cage lid during baseline and during exposure to either CO_2 (n = 16 rats) or air with peppermint odour (n = 16 rats).

5.5 References

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CHAPTER 6: General Discussion

The term euthanasia refers to a good death, which implies a lack of distress. However, due to the potential for CO_2 to cause pain and dyspnea, there has been considerable debate as to whether CO_2 euthanasia can produce death without distress. The two main objectives of my thesis were: 1) to determine whether gradual-fill CO_2 euthanasia causes distress in laboratory rats by examining behavioural responses during euthanasia, and aversion during approach-avoidance testing, and 2) to determine whether pain, dyspnea and novelty are likely sources of distress during this procedure.

6.1 Distress in rats during CO_2 euthanasia

As discussed in Chapter 1, the conclusions of previous research on distress in rats during CO_2 euthanasia have been highly variable. Some studies have found behavioural responses to CO_2 exposure that may be indicative of distress (Britt, 1986; Coenen et al., 1995; Iwarsson and Rehbinder, 1993), but others have not noted these effects (Blackshaw et al., 1988; Hackbarth et al., 2000; Hornett and Haynes, 1984; Smith and Harrap, 1997). In a more recent study, Leach et al. (2002) found that CO_2 causes avoidance in rats, suggesting that CO_2 exposure is aversive. The results of my thesis build on this previous research, and provide further evidence that gradual-fill CO_2 exposure does cause distress in laboratory rats.

Chapters 2 and 5 demonstrate that two widely used strains of laboratory rats, Wistars and Sprague-Dawleys, exhibit behaviours that are indicative of distress during gradual-fill CO_2 euthanasia. During CO_2 exposure, both strains showed evidence of increased exploration, including increased activity, rearing, and time spent with the nose touching the chamber lid. More importantly, rats in both studies showed scratching and pushing at the chamber lid, behaviours that suggest the rats were trying to escape from the chamber. Increased exploratory

behaviour has also been observed in some previous studies (e.g. Britt, 1986), and while these behaviours indicate increased arousal they do not necessarily suggest distress. However, escape behaviours strongly suggest that the animals would avoid CO_2 exposure if given the opportunity, and that forced CO_2 exposure causes some level of distress.

Chapters 3, 4, and 5 demonstrate that rats will avoid CO_2 concentrations necessary to cause unconsciousness, even when this requires that they give up a valuable food reward. In Chapter 3, I found that rats tolerated extended exposure to static CO_2 concentrations of 5 and 10%, but that the latency to leave the test cage dropped dramatically at 15% CO_2. Rats left the test cage at about this same concentration when exposed to a gradual-fill procedure. In Chapter 4, I found that the flow rate used during the gradual-fill procedure had only a small effect on avoidance; rats left the test cage at CO_2 concentrations between 13 and 16% with flow rates ranging from 3 to 27% of the test cage volume per minute. In Chapter 5, I found that rats are averse to CO_2 regardless of habituation, although tolerance does increase slightly with repeated exposure. This increased tolerance suggests that the aversive concentrations reported in Chapters 3 and 4 would likely have been about 4% lower on initial contact. Together, these results indicate that rats are averse to CO_2 concentrations greater than approximately 15% regardless of how CO_2 is delivered. From these results, I conclude that forced exposure to higher concentrations likely results in distress.

6.2 Sources of distress

In Chapter 1, I identified three potential sources of distress during CO_2 euthanasia: pain, dyspnea and novelty. The occurrence of pain and dyspnea were not assessed directly in the current thesis. However, I determined the CO_2 concentrations that elicited behavioural responses and aversion in Chapters 2, 3, and 4, and compared these data with previous research on

nociceptor activation in rats, and pain and dyspnea in humans. The potential for CO_2 to elicit distress as a result of novelty was examined directly in Chapter 5.

6.2.1 Pain

As discussed in Chapter 1, CO_2 is known to cause pain at the nasal mucosa and cornea in humans at concentrations of 30 to 50%, and this concentration range is similar to that required to stimulate nociceptors in rat nasal mucosa (Anton et al., 1992; Chen et al., 1995; Danneman et al., 1997; Feng and Simpson, 2003; Peppel and Anton, 1993; Thurauf et al., 2002). In Chapter 2, I found that rats started to show behavioural responses to gradual-fill CO_2 euthanasia at CO_2 concentrations of only 5% CO_2, and this response had peaked and was declining at CO_2 concentrations of only 28%. Similarly, in Chapters 3 and 4 I found that rats avoided CO_2 concentrations greater than approximately 15%, and the maximum CO_2 concentration tolerated by a single rat during gradual-fill exposure was only 25%. These results suggest that pain does not account for the behavioural responses of rats during gradual-fill CO_2 euthanasia or for their aversion to CO_2. Furthermore, in Chapter 2 I did not observe an increase in pain-related behaviours, such as head-shaking and face-washing, during CO_2 exposure, suggesting that the rats did not experience pain while they were able to mount a behavioural response. However, it is possible that rats experience pain around the time of loss of posture.

6.2.2 Dyspnea

As discussed in Chapter 1, spontaneously breathing humans report dyspnea with CO_2 concentrations of only 8% (Dripps and Comroe, 1947; Liotti et al., 2001), and severe dyspnea has been reported with CO_2 concentrations of 15% to 20% CO_2 (reviewed by Hill and Flack, 1908). While conclusive evidence of dyspnea in rats is not available, the CO_2 concentrations that elicit dyspnea in humans are consistent with the concentrations that elicited a behavioural

responses and aversion in laboratory rats, suggesting that dyspnea is a likely cause of distress during CO_2 exposure.

Humans generally show a delay between a change in inspired CO_2 and the onset of dyspnea, but very little delay was observed in rats. During approach-avoidance testing with 20% CO_2, one rat refused to enter the test cage, and the latency to leave the test cage for the remaining rats ranged from 2 to 16 s. If we assume a maximum of 2 s of exposure in the tunnel leading to the test cage, this indicates that avoidance occurred after only 2 s of exposure for the rat that did not enter the test cage, and 18 s of exposure for the rat with the longest latency to leave the test cage.

Part of the delay before onset of dyspnea is due to the time taken for inspired CO_2 to increase blood CO_2 levels, and stimulate peripheral and central chemoreceptors in the carotid bodies and the medulla, respectively. For humans, the delay between a change in inspired CO_2 and the onset of increased ventilation is 5 to 15 s depending on whether peripheral or central chemoreceptors are driving the response (reviewed by Cunningham et al., 1986). While responses to hypercapnia tend to occur mainly via the central chemoreceptors, for which there is a longer delay, the peripheral chemoreceptors also contribute. Blood CO_2 concentrations continue to increase towards inspired levels, resulting in increased ventilation and dyspnea if hypercapnia is sufficient. Banzett (1996) calculated that a step change in inspired CO_2 against a hypoxic background results in a logarithmic increase in dyspneic sensations in humans, with a half-time of approximately 32 s for development of a stable dyspnea rating. The time to onset of dyspnea was not reported, but the half-time provides an indication of how long it took for moderate levels of dyspnea to develop under the conditions that were used. Similarly, Haldane and Smith (reviewed by Hill and Flack, 1908) found that there was a delay of 1 to 2 min until severe dyspnea set in during inhalation of 18.6% CO_2. However, rats may differ from humans in the time taken to develop dyspnea. Lagneaux (1986) found that rats show only a 2 s lag in their

ventilatory response to inspiration of 1.5% CO_2, suggesting a much shorter circulatory delay in rats. Furthermore, the increase in ventilation occurred much more quickly with 1.5% CO_2 than with 0.5 or 1% CO_2, indicating that the time for development of ventilation, and likely dyspnea, is dependent on CO_2 concentration. These results suggest that the distress and aversion observed in this thesis could have been due to dyspnea.

6.2.3 Novelty

The results of Chapter 5 illustrate the role of novelty on rat responses to gas exposure. While novelty results in increased exploratory behaviours and may contribute to distress due to fear, it does not account for performance of escape behaviours or avoidance of CO_2. These results suggest that novelty is not a major source of distress during gradual-fill CO_2 euthanasia.

6.2.4 Alternative hypotheses

One alternative explanation for behavioural signs of distress and aversion in rats during CO_2 exposure is that they could detect the onset of unconsciousness, and that this sensation of diminished consciousness either evoked an innate escape response or was perceived as unpleasant. However, pigs, poultry and humans have been shown to lose consciousness during exposure to low O_2 concentrations without demonstrating obvious attempts at avoidance (Cable, 2003; Raj & Gregory, 1995; Raj, 1996; Webster & Fletcher, 2004), suggesting that diminished consciousness on its own does not evoke escape responses or cause unpleasant sensations in other species. Furthermore, recent results from our research group suggest that avoidance of CO_2 during gradual-fill exposure is not well correlated with ataxia, an initial indicator of diminished consciousness (Kirkden, unpublished data). Leach et al. (2002a) also found that rats avoided 25.5% CO_2 after only 1.1 s, even though ataxia took 30 s to occur at this concentration of CO_2.

Avoidance of CO_2 during approach-avoidance testing could also have been influenced by the effects of CO_2 on taste. The approach-avoidance task that I used is dependent on rats being highly motivated to obtain a food reward, and alterations in taste could affect this motivation. CO_2 forms carbonic acid when combined with water, as would occur at the oral mucosa, and acids are known to stimulate taste buds to produce a sour taste (DeSimone et al., 2001). CO_2 is widely described to have a sour taste, but no information is available on the concentration of CO_2 that would be necessary to evoke this taste in rats. However, rats appeared highly motivated to eat right until they left the test cage, and would often grab one or two reward items to take with them. Furthermore, this hypothesis cannot account for the escape behaviours that rats performed during gradual-fill CO_2 euthanasia.

6.2.5 Conclusions on sources of distress and aversion

The most likely source of distress and aversion during gradual-fill CO_2 exposure appears to be dyspnea. While there is some question as to whether this sensation could develop within the time it took for rats to exhibit aversion to CO_2, the other hypotheses presented do not fully account for the responses that were observed. In order to determine more conclusively whether CO_2 causes dyspnea in rats, further studies are needed. In humans, dyspnea due to experimentally-induced hypercapnia and due to disease has been shown to be relieved by inhalation of aerosolized furosemide (Minowa et al., 2002; Nishino et al., 2000; Ong et al., 2004; Shimoyama and Shimoyama, 2002). Furosemide is labelled for use as a diuretic, and the mechanism for its effects on dyspnea is not fully understood. It has been found to increase the activity of pulmonary stretch receptors (Sudo et al., 2000), which may feed back into systems responsible for the generation of dyspnea. This effect on pulmonary stretch receptors is specific to aerosol delivery and does not occur after systemic delivery, suggesting that this is a localized effect at the level of the respiratory epithelium. An experiment to test the effects of furosemide

on tolerance to CO_2 would provide more direct evidence of the role of dyspnea in rat aversion to CO_2.

6.3 Critique of methods

To examine distress associated with CO_2 euthanasia in rats I used two different methods: 1) behavioural assessment of rat responses during CO_2 euthanasia, and 2) approach-avoidance testing with static and gradually increasing concentrations of CO_2. While previous studies have assessed the behaviour of rats during CO_2 euthanasia and found variable results, I improved upon these studies by developing a well defined list of pain and distress behaviours, using an adequate sample size for statistics, acclimatizing the rats before gas exposure, and comparing behavioural responses to both baseline levels and responses during air exposure.

Preference testing had also been used to examine rats' avoidance of CO_2 (Leach et al., 2002a), but I improved upon this methodology by examining the strength of rats' motivation to avoid CO_2 using approach-avoidance testing. While this method had been used to examine gas aversion in other species (e.g. Cooper et al.,1998; Gerritzen et al., 2000; Raj, 1996; Raj & Gregory, 1995; Webster & Fletcher, 2004), it had not previously been developed for use with rats.

Both of the assessment methods that I used provided useful and complementary information about the potential for CO_2 to elicit distress. Rats performed escape behaviours during CO_2 euthanasia, and left the test cage before loss of consciousness during approach-avoidance testing. Both of these responses suggest that gradual-fill CO_2 exposure was unpleasant and that forced exposure likely causes distress. However, rats' behavioural responses during CO_2 euthanasia were not compared to a known aversive stimulus, so it is difficult to determine the level of distress that is indicated by the level of responses that I observed. In fact,

one rat showed escape behaviours during exposure to peppermint odour, a stimulus that was found to be non-aversive during approach-avoidance testing. This finding suggests that escape behaviours may not always indicate a high level of distress. Approach-avoidance testing provides a better indication of the severity of distress caused by CO_2 exposure, because motivation to avoid CO_2 is compared against motivation to obtain a food reward. As discussed in Chapter 3, rats' motivation to obtain sweet foods when fed ad libitum is moderate to high; therefore, approach-avoidance testing indicates that their motivation to avoid CO_2 is at least moderate. An even better indication of strength of aversion to CO_2 can be gained by food-depriving rats for different periods of time prior to testing to ensure that their motivation for the food reward is high. Using this procedure, Kirkden et al. (2005) found that even after food deprivation for up to 24 h, rats showed much the same aversion to gradual-fill CO_2 exposure. This result suggests that rats find this exposure highly aversive.

Approach-avoidance testing appears to be a more sensitive measure of aversion to CO_2 in rats than simple behavioural responses. Although rats showed many behavioural changes during CO_2 euthanasia, the majority of the behaviours observed are difficult to interpret. Only escape behaviours provide a clear indication of aversion. Behavioural responses to CO_2 euthanasia were also highly variable between rats, with some animals showing little or no response. While this could be interpreted to indicate that some rats do not find this procedure distressing, rats always avoided CO_2 concentrations of approximately 15% and higher during approach-avoidance testing. This indicates that CO_2 was likely aversive to all of the rats during CO_2 euthanasia, but that the behavioural responses that I measured are variable and likely poor measures of distress during forced gas exposure. In particular, it is interesting that escape behaviours were not observed in all animals, because the results of my thesis and that of Kirkden et al. (2005) suggest that rats are highly motivated to avoid CO_2. It is possible that some rats did not perceive a potential to exit the exposure chamber and therefore did not try to

escape. While there is the possibility that rats were performing relevant behaviours that I did not detect, the fact that some rats remained completely motionless throughout the exposure suggests this not to be the case. However, this raises the possibility that individual rats were coping with the forced exposure in different ways. Individuals can respond either proactively or reactively when confronted with a stressor (reviewed by Koolhas et al., 1999). Hence, another explanation for the variability in my results is that rats were coping in different manners, with some showing freezing and others showing escape behaviours.

In experiments where I examined the behavioural responses of rats during CO_2 euthanasia, animals were tested in a novel environment. During exposure to a predator odour, rats have been found to show behaviours associated with fear when tested in a novel environment, but not when tested in a familiar environment (Morrow et al., 2002). This finding suggests that fear in rats is enhanced by a novel environment. It is possible that I may have obtained different results if animals were tested in a familiar environment. However, rats were tested in a familiar environment during approach-avoidance testing and still avoided CO_2 concentrations greater than 15%, indicating that the overall conclusions of the thesis are valid.

The main limitation of approach-avoidance testing is that it provides information only about the rats' initial perception of CO_2, and does not address any effect that might occur with continued exposure until loss of consciousness. From the time-course of CO_2 euthanasia with a medium flow rate, it appears that there is a period of at least 45 s between the onset of aversion and loss of consciousness. Distress during the entire procedure could be assessed by comparing gradual-fill CO_2 exposure until loss of consciousness with a known aversive stimulus. One way to examine this would be to compare the willingness of animals to re-enter the exposure chamber after exposure to each of the stimuli. For example, Jongman et al. (2000) used this method to compare pigs' aversion to CO_2 and to an electric shock delivered from a prod, and found that pigs would more readily re-enter the exposure chamber after exposure to 90% CO_2

than after exposure to electric shock. Another method of comparing two aversive stimuli is to use avoidance-avoidance testing, where animals must choose between two aversive stimuli. The stimulus that is chosen is assumed to be the less aversive of the two. For example, Rushen (1986) used a Y-maze to compare aversion to different handling techniques in sheep.

Another limitation of the experimental design that I used for approach-avoidance testing was that I had to habituate the rats to CO_2 exposure prior to testing. This resulted in rats tolerating slightly higher CO_2 concentrations than would be observed on an initial exposure, as would occur during euthanasia. However, it was necessary to habituate the rats to avoid a drift in response to CO_2 throughout the experiment. Moreover, I was able to estimate the magnitude of this effect in Chapter 5.

6.3 Future directions

6.3.1 Other rodent species

The results of my thesis suggest that gradual-fill CO_2 euthanasia causes distress in rats, but we do not know whether this is also true for other rodent species such as mice. Previous studies on distress during CO_2 euthanasia in mice have been inconclusive (Ambrose et al., 2000; Blackshaw et al, 1988; Britt, 1986; Iwarsson and Rehbinder, 1993). Like the rat studies, these studies suffer from problems with experimental design, including a lack of appropriate sample sizes, control groups and acclimatization before gas exposure. Leach et al. (2002) demonstrated that mice will avoid CO_2 during a preference test, but Godbey (personal communication) found that mice that are provided with a food reward during gradual-fill CO_2 euthanasia will lose consciousness while eating. This latter result suggests that mice might be less averse to CO_2 than rats, but well controlled studies are needed to determine if this is the case.

6.3.2 Alternative euthanasia methods

Gradual-fill CO_2 euthanasia appears to cause distress in rats, suggesting that this method should be replaced with other methods that are more humane. However, few studies have examined whether the other methods that are currently available cause distress in rats, and if so, whether they are better or worse than CO_2 euthanasia.

One animal welfare benefit of CO_2 euthanasia is that it involves minimal handling and restraint of the animals. Aside from CO_2 euthanasia, the two major classes of gas euthanasia agents are inhalant anaesthetics and inert gases, such as argon and nitrogen, which are used to displace O_2 and cause severe hypoxia. Inhalant anaesthetics are not known to be painful or to cause dyspnea, but do have a pungent odour that rats may find unpleasant. Using simple preference testing, Leach et al. (2002b) demonstrated that rats will avoid exposure to inhalant anaesthetics before losing consciousness, but further research is needed to examine the strength of this aversion.

As discussed in Chapter 1, hypoxia can cause dyspnea in humans, but does not appear to do so during spontaneous breathing (Cable, 2003; Moosavi et al., 2003). Furthermore, pigs and poultry will enter a chamber containing 90% argon in air (2% O_2) to access food and conspecifics, and will remain for long enough to lose consciousness (Raj & Gregory, 1995; Raj, 1996; Webster & Fletcher, 2004). This suggests that it might be possible to kill rats with hypoxia without causing distress. However, in contrast to these results with other species, Hornett and Haynes (1984) examined euthanasia by hypoxia with a gradually increasing concentration of nitrogen and found that it caused "panic" (p.99) in rats. Furthermore, Leach et al. (2002a) found that rats avoid 90% argon in air before losing consciousness in a simple preference test. In Chapter 3, I expanded on this work be examining rats' aversion to 90% argon in air using approach-avoidance testing, and found that they would forgo a palatable food reward in order to avoid argon exposure. In fact, the median latency to leave with argon was

only 3 s, which was similar to that seen for 20% CO_2. As an inert gas, argon is not thought to have a perceptible smell, so the most likely explanation for this avoidance is dyspnea. It is not clear why rats behave differently from other species, but it is possible that they are more sensitive to hypoxia as a result of burrow-dwelling adaptations. Enhanced sensitivity to hypoxia and hypercapnia would be useful in fossorial species for avoidance of gas irregularities that can occur underground. While *Rattus norvegicus* is not fossorial, this trait may be conserved among rodents. Ventilation in fossorial species is actually known to be less responsive to hypoxia and hypercapnia than in non-fossorial species, likely in order to tolerate the elevated CO_2 (1 to 9.5%) and reduced O_2 (6 to 20%) concentrations that occur in closed burrows (reviewed by Tenney and Boggs, 1986). However, the causes and dynamics of dyspnea are still poorly understood, and it is possible that these species experience a sharp rise in dyspneic sensations at levels of hypoxia and hypercapnia that are dangerous to survival.

Carbon monoxide gas is another potential alternative euthanasia agent for laboratory rodents. Carbon monoxide is an odourless and non-irritating gas that causes death by direct toxic effects on cells, and by competing for O_2 binding sites on haemoglobin and preventing sufficient delivery of O_2 to body tissues (reviewed by Kao and Nanagas, 2005). It has been suggested that carbon monoxide poisoning causes death without distress (Close et al., 1996). However, humans suffering from carbon monoxide poisoning report symptoms such as headache, nausea, chest pain, dyspnea, and elevated heart rate and breathing rate, which suggests that it does have the potential to cause distress (reviewed by Kao and Nanagas, 2005). Carbon monoxide has not been examined as a euthanasia agent for rodents. However, Chalifoux and Dallaire (1993) found that carbon monoxide caused vocalizations and signs of agitation in some dogs before loss of consciousness. One major problem with the use of carbon monoxide in the laboratory is that it is an odourless, non-irritating gas that is potentially dangerous to human

health, and its use would therefore require precautions to ensure human safety. Further research examining the effects of carbon monoxide on rodents is necessary.

Commonly used non-gas euthanasia methods for laboratory rats include the physical methods, such as decapitation and cervical dislocation, and injectable anaesthetics. If done properly, the physical methods are quick, and the main animal welfare concern is with handling and restraint. However, the CCAC ranks physical methods as only conditionally acceptable because considerable animal pain can occur if these methods are performed improperly. Injection of anaesthetics in rats also requires restraint and some pain.

It appears that all of the commonly used euthanasia methods for laboratory rats involve factors that might cause distress before loss of consciousness. While my thesis suggests that CO_2 exposure also causes distress I cannot say whether this distress is more or less severe than that which occurs with these other procedures. Only one study to date has compared aversion to CO_2 in rats against other gas euthanasia methods. Leach et al. (2002a, b) found that rats would remain in inhalant anaesthetics and argon for longer than they would remain in CO_2, but they avoided all of the agents before loss of consciousness. This result indicates that all of the agents are aversive on initial exposure, and that further research is necessary to determine which agent causes unconsciousness with the least level of distress. One next step would be to compare rats' aversion to these different gas euthanasia agents using the aversion testing methods described above in 6.3. Rats would be exposed to each agent until unconsciousness and then removed. Their willingness to re-enter compartments associated with each agent would then be compared so that the agents can be ranked according to the level of aversion that they cause. These methods could also be used to examine whether rats find gas euthanasia agents or handling and injection more aversive. The agent or method that caused the least aversion would be assumed to cause the least distress before unconsciousness. However, with this methodology it is

assumed that rats remember the exposure; therefore, it is important to first ensure that none of the agents affects memory.

6.4 Conclusions

Over the past 10 years, there has been increasing concern about the use of CO_2 as a euthanasia agent for laboratory rodents. The Humane Society of the United States has called for a ban on the use of CO_2 for euthanasia of conscious rodents based on animal welfare concerns (Conlee et al., 2005), and recent developments have seen regulatory agencies, such as the European Food Safety Authority, suggest that CO_2 should not be used as a sole euthanasia agent for conscious animals (EFSA, 2005). The results of my thesis suggest that gradual-fill CO_2 euthanasia causes distress in laboratory rats. I have demonstrated that rats perform escape behaviours during gradual-fill CO_2 euthanasia, and that they are at least moderately averse to CO_2 concentrations below those necessary to cause loss of consciousness, regardless of the delivery method. These results suggest that laboratory rats should not be euthanized with CO_2. However, previous research indicates that rats also show aversion to other euthanasia agents, so it is not clear that any of the agents currently available can induce unconsciousness without distress. Further research is needed to determine which euthanasia agents cause the least amount of distress in rats and other rodent species, and to identify alternative methods that can cause death without distress.

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