ALTERNATIVES TO CARBON DIOXIDE EUTHANASIA FOR LABORATORY RATS

by

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Abstract

The most commonly used method of euthanasia of laboratory rodents is exposure to carbon dioxide (CO_2), but recent studies have shown that rodents find this gas aversive. The aim of my thesis was to evaluate rat aversion to inhalant agents that could be used as humane alternatives to CO_2. The first study used approach-avoidance testing to examine rat responses to argon-induced hypoxia when argon was introduced at flow rates of 40-239% of the test cage volume per min. Rats never remained in the test cage long enough to lose consciousness when tested with argon. They consumed fewer reward items, stopped eating sooner, and left the test cage more quickly than when tested with air. Rats stopped eating and left the test cage when the oxygen (O_2) concentration had dropped to about 7.7 and 6.8%, respectively, but these O_2 concentrations are too high to cause unconsciousness. Although humans exposed to hypoxia report only subtle symptoms that include cognitive impairments and light headedness, rats are burrowing rodents and could therefore be more sensitive to these effects. I conclude that argon is not a humane alternative to CO_2. The second study used approach-avoidance testing to evaluate rat responses to different concentrations of the inhalant anaesthetics halothane and isoflurane introduced with vaporizers or from soaked cotton balls. On the first day of exposure to anaesthetics, most rats remained in the test cage until they were ataxic and showing difficulty returning to the home cage. On subsequent days of testing most rats left the test cage within seconds, but if given the option, all promptly returned and stayed until they were ataxic, indicating that the learned aversion is transient. Rats were likely sedated by the time they chose to leave, suggesting that forced exposure from the onset of aversion until loss of consciousness is less of a welfare concern than forced exposure to non-sedating agents. I suggest that the use of inhalant anaesthetics for inducing unconsciousness prior to euthanasia is a more humane method than the commonly used CO_2.

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CHAPTER 1: General introduction

1.1. Introduction

In the Western world, the use of animals for scientific purposes began around 350 BC (Wood, 1931) and is now widespread. Millions of animals are bred and used for fundamental and medical research, regulatory testing, the development of products, and education and training. In Canada, over 2.5 million animals were used for scientific purposes in 2006, with mice and rats representing nearly 50% of the total number of animals used (CCAC, 2007). Rodents are commonly chosen because of their small size, low purchase and maintenance costs, and high reproductive rate.

Virtually all animals used in research are killed at the end of a study or to collect tissue samples, and many more are killed to reduce surplus breeding stock. The term generally used when referring to the killing of laboratory animals is 'euthanasia', which is derived from Greek and signifies 'good death' – presumably, death free from pain and distress (Blackmore, 1993). In Canada, animal experimentation is regulated by the Canadian Council on Animal Care (CCAC). Policy guidelines established by this regulatory body stipulate that methods used to cause death must be "painless, must minimize fear and anxiety, be reliable, reproducible, irreversible, simple, safe and rapid" (CCAC, 1993). Similar regulations have been established in the United States (United States Department of Agriculture, 1985), the European Union (Council of the European Communities, 1986), and Australia and New Zealand (Reilly and Rose, 2001). In Canada, accepted methods of euthanasia of rodents include physical methods such as cervical dislocation or decapitation, injectable agents such as barbiturates, and inhalant agents such as carbon dioxide (CO_2), inert gases, and inhalant anaesthetics (CCAC, 1993). Although some of these methods are more humane than others, the choice of method depends not only on the degree of suffering caused to the animal, but also on the purpose of killing and

human safety. Cost and convenience should not take precedence over animal welfare (CCAC, 1989).

1.2. Carbon dioxide

Currently the most widely used method of killing laboratory rodents is exposure to CO_2. This gas can be administered in two ways: with the pre-fill method, a cage is filled with at least 70% CO_2 before animals are placed in it; with the gradual-fill method, CO_2 is administered to a cage already containing animals until the concentration reaches lethal levels.

1.2.1 Mode of action

During metabolism, the body uses O_2 from the air and produces CO_2. Excess CO_2 is released from tissues into the blood and from the blood into the lungs where it is expired. 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:

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

When the concentration of CO_2 in air is increased (from 0.03% in ambient air to up to 100% in a euthanasia chamber), CO_2 is delivered to tissues more quickly than it can be eliminated and therefore starts accumulating in the body. This accumulation of CO_2 shifts the equilibrium to the right, producing hydrogen ions which lead to pH reduction in the tissues. It is thought that this reduction in pH is what causes CO_2 narcosis and death (e.g. Brodie and Woodbury, 1958; Eisele et al., 1967; Martoft et al., 2002).

1.2.2. Advantages of CO_2

Exposure to inhalant agents as a method of euthanasia presents advantages over physical methods and injections. From the animal welfare perspective, exposing animals to a lethal gas in

a chamber (preferably the animal's home cage) minimizes distress because it involves little to no handling and restraint. Laboratory rodents are generally not habituated to regular handling and restraint, so these procedures can cause considerable distress (Sharp et al., 2002, 2003). Moreover, injections into the peritoneal cavity can be painful, and injectable anaesthetics can be irritating (Niel, 2006). Cervical dislocation requires high technical competence, and if done incorrectly could cause considerable pain. Exposure to inhalant agents is also advantageous from the operator's perspective. This method requires little training, and it saves time since many animals can be euthanized at once. CO_2 specifically has the advantage of being non-explosive and non-flammable, relatively inexpensive, and generally safe to use. Moreover, CO_2 does not accumulate in tissues and does not cause cell distortion (CCAC, 1993); these are important considerations if carcasses are fed to birds of prey or reptiles, or if the animals are killed for tissue samples.

1.2.3. Distress and pain during CO_2 euthanasia

Despite the many advantages associated with CO_2 euthanasia, recent evidence suggests that this method causes considerable distress and pain in rodents. Several studies in humans indicate that breathing even low levels of CO_2 causes dyspnea, which is a "highly distressing" urge to breathe (Hawkins et al., 2006). In one study, subjects were asked to rate their perception of dyspnea on a 100 point scale while breathing 8% CO_2 in O_2. These subjects reported dyspnea levels of 55 and 73 when CO_2 was administered with a mouthpiece or a facemask, respectively (Liotti et al., 2001). Another study found that approximately 30% of subjects experienced dyspnea when breathing 7.6 or 10.4% CO_2 in O_2 (Dripps and Comroe, 1947). Further evidence of dyspnea in humans comes from studies that researchers performed on themselves in the late 1800s and early 1900s (summarized by Hill and Flack, 1908). As described by Hill and Flack, Greenwood reported severe dyspnea while breathing a mixture of 15.3% CO_2 and 14.5% O_2, and an inability to breathe with higher CO_2 levels due to the closure of the glottis. Haldane and

Smith developed severe dyspnea, throbbing in the head and mental dullness within 1-2 min of breathing 18.6% CO_2 in air. Taken together, these studies indicate that in humans, dyspnea begins at approximately 8% CO_2 and becomes severe around 15% CO_2.

Several behavioural studies with rats indicate that these animals may also experience dyspnea when exposed to CO_2. Rats exposed to static CO_2 concentrations in an approach-avoidance test remained in the test cage and consumed all reward items at 0, 5, and 10% CO_2 but many rats refused to enter the cage or left quickly when it contained 15 or 20% CO_2 (Niel and Weary, 2007). When exposed to gradual-fill CO_2, rats chose to leave the test cage at the cost of abandoning their food reward when CO_2 concentrations exceeded about 17 % (Niel and Weary, 2007; Niel et al., 2008). When rats were not given the option to leave the chamber during gradual-fill CO_2, they exhibited escape behaviours, vocalizations and nose-to-lid contact beginning at 10% CO_2 and these behaviours peaked at 20% (Niel and Weary, 2006). These aversion thresholds are consistent with human thresholds for dyspnea, suggesting that dyspnea is a cause of this aversion.

In addition to dyspnea with low CO_2 concentrations, CO_2 also causes pain with higher concentrations. When CO_2 at high concentrations comes into contact with moisture, it is converted into carbonic acid. The formation of carbonic acid results in the activation of nociceptors causing a sensation of burning pain in human nasal mucosa, conjunctiva, and cornea. Human self-report data indicate that CO_2 is detectable at the nasal mucosa at concentrations of 20% and that it becomes painful at concentrations ranging from 32.5 to 55%, depending on the individual (Anton et al., 1992; Thurauf et al., 2002). Physiological recordings from nociceptors in the nasal mucosa have shown that these nociceptors are activated at concentrations greater than 45% (Thurauf et al., 1993). Pain thresholds for the conjunctiva and cornea are approximately 50% and 30% CO_2, respectively (Chen et al., 1995; Feng and Simpson, 2003).

In rats, CO_2 has been shown to activate dorsal horn neurons that receive input from nociceptors in the nasal mucosa at concentrations ranging from 37 to 50% (Anton et al., 1991; Peppel and Anton, 1993), indicating that rats have the potential to experience pain when exposed to high CO_2 concentrations. Activation of rat corneal nociceptors has also been demonstrated, although the threshold level that elicited activation was not reported (Hirata et al., 1999). Inhaled irritants are also known to cause bradycardia (heart rate reduction), likely evolved to reduce transfer of harmful substances into the body (Widdicombe, 1986). Bradycardia was shown to occur in rats exposed to gradual-fill CO_2 when the concentration reached about 47% (Hawkins et al., 2006).

1.2.4. Summary

Evidence in humans indicates that exposure to low levels of CO_2 (>8%) causes dyspnea, while exposure to higher levels (>30%) causes a burning sensation in the nasal mucosa, conjunctiva and cornea. Physiological and behavioural data from rats suggest that these animals may also experience dyspnea and pain at similar CO_2 concentrations. When rats are euthanized using the pre-fill method, conscious animals are exposed to CO_2 concentrations greater than 70%, suggesting that they experience both dyspnea and pain during the procedure. When the gradual-fill method is used, rats lose consciousness when CO_2 concentration reaches about 40% (Smith and Harrap, 1997), suggesting that they feel dyspnea, and may start to feel low levels of pain around the time of loss of consciousness. Although humans likely possess higher order cognitive processes than rats and this may affect the quality of dyspnea and pain sensations, the general perception of a stimulus as pleasant or unpleasant is likely conserved among mammalian species with similar anatomy and physiology (Dawkins, 1980).

1.3. The search for alternatives

There is strong evidence to suggest that CO_2 euthanasia is aversive to rodents, and therefore does not satisfy CCAC's guidelines on euthanasia. In search for humane alternatives, scientists often suggest the use of argon (e.g. Raj and Gregory, 1991; Gerritzen et al., 2000; Young, 2006), although very little is known of the distress associated with exposure. Inhalant anaesthetics could also be a good alternative since they are commonly used to induce unconsciousness in animal surgeries.

1.3.1 Argon

Argon is an inert gas that is odourless, colourless and tasteless and as such does not cause painful irritation (Leach et al., 2004). This gas is also safe and easy to administer since it is heavier than air. When argon is introduced into a chamber, it displaces air. As air is displaced, O_2 concentration is reduced and this leads to hypoxia, unconsciousness and death.

1.3.1.1. Mode of action

Respiration is stimulated either by a decrease in the partial arterial pressure of O_2 (Pa_{O2}) or an increase in the partial arterial pressure of CO_2 (Pa_{CO2}), and these changes in O_2 and CO_2 pressures are monitored by peripheral chemoreceptors in the arteries. Breathing low levels of O_2 (hypoxic atmosphere) translates into low Pa_{O2}, which causes ventilation to increase. After ventilation reaches a peak, it begins to decline, reaching a plateau level still above resting ventilation.

At the molecular level, exposure to hypoxic atmospheres causes a shift from aerobic pathways to anaerobic pathways that trigger a complex series of cellular changes, leading first to reduced cellular function and ultimately cell death. In simplified form, the contrasting pathways are:

AEROBIC PATHWAY	ANAEROBIC PATHWAY
Glucose	Glucose
⇩	⇩
Pyruvic acid	Pyruvic acid
⇩	⇩
$CO_2 + H_2O + 38$ ATP	Lactic acid + 2 ATP

The anaerobic pathway produces lactic acid, which is comprised of hydrogen (H+) and lactate ions. The blood-brain barrier is relatively impermeable to charged ions, so H+ and lactate ions are retained within the neurones of the hypoxic brain, causing intracellular acidosis (Lumb, 2005).

In addition to acidosis, lack of high-energy compounds and other direct effects of hypoxia also contribute to cell death (Martin et al., 1994). ATP (adenosine tri-phosphate) is the main biological source of energy. The anaerobic pathway produces considerably less ATP than the aerobic pathway, and this causes depletion of these high-energy compounds. Hypoxia directly increases potassium conductance, causing potassium to leak out of the cell and sodium to enter the cell. Lack of ATP accelerates this process by causing failure of the sodium-potassium-ATPase pump; as a result, sodium and potassium channels probably remain open, allowing free passage of ions across the cell membrane. Free passage of ions depolarizes the cell membrane, which causes the release of calcium from the mitochondria and the endoplasmic reticulum, leading to an increase of intracellular calcium. This increase of calcium is harmful, causing the activation of various enzymes and proteases. Membrane depolarization by potassium leakage and derangement of calcium channel function prevent normal synaptic transmission and lead to cellular destruction.

1.3.1.2. Aversion to argon-induced hypoxia

Argon-induced hypoxia is only slightly aversive or not aversive in birds. During approach-avoidance testing, all 12 turkeys (Raj, 1996) and all 12 hens (Webster and Fletcher,

2004) tested entered a feeding chamber containing > 90% argon, and in each case 11 individuals died before leaving. Similarly, Gerritzen et al. (2000) found no evidence that broiler chickens could detect or avoid atmospheres containing > 90% argon, and these birds gradually became unconscious without showing any signs of distress. Behavioural data show that broilers (Lambooij et al., 1999; Gerritzen et al., 2000; McKeegan et al., 2006; but see Coenen et al., 2000) and turkeys (Raj, 1996) killed with argon exhibit less head shaking and less gasping than those killed with CO_2. Pigs also entered a feeding box filled with 90% argon and withdrew their heads only after losing balance, but all promptly resumed feeding after regaining a steady gait (Raj and Gregory, 1995). Diving mammals such as mink will also enter a chamber containing 90% argon but they never remain long enough to lose consciousness, suggesting that they are not averse to argon *per se* but are averse to the resulting hypoxia. The amount of time they spend in argon atmosphere is similar to dive duration (Raj and Mason, 1999).

Little is known about the effects of argon on burrowing rodents. Niel and Weary (2007) reported that in an approach-avoidance task rats either refused to enter, or immediately left a cage containing 90% argon. Leach et al. (2002a, 2004) showed that rats and mice, which were free to enter and leave chambers containing various gases matched for the time they took to induce ataxia, would spend more time in a chamber containing argon than one containing CO_2, but less time than if it contained air.

1.3.2. Inhalant anaesthetics

Inhalant anaesthetics are commonly used to induce unconsciousness in humans and animals undergoing surgery, and these agents could also be used to induce unconsciousness in animals prior to euthanasia. Among the most commonly used volatile liquid anaesthetics in animal anaesthesia are the fluorinated hydrocarbons halothane and isoflurane (Flecknell, 1996). Both drugs are non-flammable, but waste gases escaping from the chamber must be scavenged to prevent exposure by the operator. Exposure to anaesthetic waste by healthcare professionals and

veterinarians has been linked to increased incidences of neurologic and reproductive dysfunction and neoplasia (Smith and Bolon, 2002).

1.3.2.1. Mode of action

Inhalant anaesthetics act through rapid chemical depression of the nervous system, leading to a loss of sensation in the body (Blackmore, 1993; Kohn et al., 1997). Anaesthetics typically induce (in this order) analgesia, amnesia, loss of consciousness, inhibition of sensory and autonomic reflexes, and skeletal muscle relaxation (Trevor and White, 2006). Depth of anaesthesia depends on the concentration of anaesthetic in the brain, and this requires the transfer of the anaesthetic from the lungs to the blood, and from the blood to the brain. The rate at which a given concentration of anaesthetic in the brain is reached depends on the solubility of the anaesthetic (i.e. its affinity for the blood compared to air), its concentration in inspired air, rate and depth of pulmonary ventilation, pulmonary blood flow, and the partial pressure gradient of the anaesthetic between arterial and mixed venous blood. Halothane has higher solubility than isoflurane (blood/gas coefficient of 2.4 vs. 1.4) so its onset of action is slower than isoflurane. Increases in the inspired anaesthetic concentration will increase the rate of induction of anaesthesia by increasing the rate of transfer into the blood. Increased ventilation increases the speed of induction of anaesthesia, while increased blood flow exposes a larger volume of blood to the anaesthetic, so anaesthetic concentration in the blood rises more slowly and induction is slower.

Inhaled anaesthetics depress the activity of neurons in many regions of the brain (Trevor and White, 2006). The most likely targets of anaesthetics are ion channels, which regulate the flow of ions across the cell membrane (Franks and Lieb, 1994, 1998; Narahashi et al., 1998; Campagna et al., 2003). The behavioural and physiological actions of anaesthetics are linked to ion channels that regulate the electrical activity of cells. Neurotransmitter receptors, such as nicotinic acetylcholine and $GABA_A$ receptors, are particularly sensitive to the actions of

anaesthetics. A working hypothesis is that inhalant anaesthetics enhance inhibitory post-synaptic channel activity (e.g. $GABA_A$ receptors), and inhibit excitatory synaptic activity (e.g. nicotinic acetylcholine receptors; Campagna et al., 2003). For example, anaesthetics increase the sensitivity of receptors to GABA, and this increases the $GABA_A$-receptor mediated inhibition of neurotransmitter transmission (e.g. Jones and Harrison, 1993). Inhaled anaesthetics also decrease the duration of opening of nicotinic acetylcholine receptors, and this decreases the excitatory effects of acetylcholine (an excitatory neurotransmitter; e.g. Violet et al., 1997).

The neuropharmacologic basis for the sequential progression from analgesia and amnesia to unconsciousness and muscle relaxation appears to be differential sensitivity of specific neurons or neuronal pathways to the anaesthetic drugs. For example, neurons in the substantia gelatinosa of the dorsal horn of the spinal cord are very sensitive to relatively low anaesthetic concentrations. Interaction with neurons in this region interrupts sensory transmission in the spinothalamic tract, including transmission of pain stimuli. This is why the first effects of exposure to anaesthetics are analgesia and conscious sedation (Trevor and White, 2006).

1.3.2.2. Aversion to induction with inhalant anaesthetics

Although inhalant anaesthetics are commonly used in surgeries, little research has been done to assess any distress associated with induction. A series of studies on New Zealand White rabbits revealed that these animals are strongly averse to induction with halothane and isoflurane (Flecknell et al., 1996; Flecknell et al., 1999; Hedenqvist et al., 2001). During induction, rabbits initially ceased respiration for periods of 30-180 s, and then breathed intermittently between periods of breath-holding. Most animals made violent attempts to escape and pawed at their nose and face.

Few studies have been conducted with rodents. Young (2006) compared rats' behavioural reactions to gradual-fill halothane, CO_2, and a mixture of CO_2/O_2. She found that,

unlike rats exposed to CO_2 or a mixture of CO_2/O_2, those exposed to halothane did not exhibit aversive behaviours such as drawn in abdomen, gasping, motionlessness and rapid body movements. Grooming, which is associated with normal behaviour, was observed only in the halothane group. All groups exhibited defecation and urination, a reaction sometimes associated with stress. Leach et al. (2002b, 2004) compared total dwelling times of female rats and mice in chambers that contained air, inhalant anaesthetics, or CO_2 at low, medium and high concentrations. They found that both species remained in a chamber for shorter periods when it contained at least medium concentrations of one of the experimental agents than when it contained air. CO_2 was by far the most aversive, and of the anaesthetic gases, rats stayed the longest with halothane and shortest with isoflurane.

1.4. Aims

Humane killing is a CCAC requirement, and arguably a moral responsibility of those who use animals in research (Leach et al., 2004). There is now strong evidence that the most common method of euthanasia, exposure to CO_2, is aversive to laboratory rats. Although several alternatives have been put forward, no experiments have assessed rat aversion to exposure. We should not move away from one aversive method to another that may be just as aversive if not more – scientific studies are needed to determine which, if any, agents are less aversive than CO_2.

The aim of my thesis was to use approach-avoidance testing to evaluate rat responses to two classes of agents often suggested as possible alternatives to CO_2: the inert gas argon (Chapter 2), and the inhalant anaesthetics halothane and isoflurane (Chapter 3). Approach-avoidance testing is a form of aversion testing in which an animal is simultaneously exposed to a positive stimulus and a negative stimulus, and must decide whether it is willing to tolerate the negative stimulus to gain access to the positive. In my experiments, rats had to choose between avoidance of argon, halothane or isoflurane and access to a palatable sweet food reward.

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CHAPTER 2: Rats show aversion to argon-induced hypoxia[1]

2.1. Introduction

Of all laboratory procedures, euthanasia is the most common as virtually all animals are killed on completion of a study or to reduce surplus stock. Guidelines in Canada and regulations in the United States, the European Union, and Australia and New Zealand state that euthanasia must be relatively quick and painless (Canadian Council on Animal Care, 1993; United States Department of Agriculture, 1985; Council of the European Communities, 1986; Reilly and Rose, 2001). Current methods of killing small laboratory rodents include physical techniques such as cervical dislocation or decapitation, overdose of injectable anaesthetics, and exposure to volatile anaesthetics and other gases. The advantage of inhalant anaesthetics and gases is that they involve minimal handling and minimal chance of operator error.

When carbon dioxide (CO_2) is used for killing, animals are either placed in a chamber that has been pre-filled with a lethal concentration of the gas, or the gas is administered into a chamber containing animals until the concentration reaches lethal levels. Despite the wide use of CO_2, several studies have shown that rodents find this gas aversive. Rats are unwilling to tolerate extended exposure to CO_2 concentrations as low as 15% (Leach et al., 2002a, b, 2004; Niel and Weary, 2007), but exposure to 30% CO_2 is necessary to induce loss of consciousness (Chapin and Edgar, 1963; Niel and Weary, 2006). One study that tested rats' preference for different atmospheres over a period of 2 days revealed that rats clearly preferred cages without CO_2 or with 1% CO_2 to cages with 3% CO_2 (Krohn et al., 2003). When exposed to gradually increasing concentrations, rats left the test cage at mean concentrations of 18.4% CO_2 (Niel and Weary,

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2007) and 15.9% CO_2 (Niel et al., 2008) even though they had a sweet food incentive to stay, indicating that aversion to CO_2 is greater than motivation to consume sweet food items. Avoidance of CO_2 has also been found in mice (Leach et al., 2002a, b; 2004), mink (Cooper et al., 1998), broiler chickens (McKeegan et al., 2006) and pigs (Raj and Gregory, 1995). Furthermore, physiological and behavioural signs of distress such as gasping, head shaking and vocalizations have been observed in rats (Britt, 1987; Smith and Harrap, 1997; Niel and Weary, 2006), broiler chickens (Lambooij et al., 1999; Coenen et al., 2000; Gerritzen et al., 2000) and pigs (Raj and Gregory, 1996; Raj, 1999) exposed to high concentrations of this gas. Exposure to CO_2 concentrations greater than 37-50% causes the formation of carbonic acid on nasal mucous membranes, and this stimulates trigeminal nociceptors and causes pain (e.g. Peppel and Anton, 1993; Leach et al., 2002a). Dyspnea, a discomfort caused by the urge to breathe, occurs in humans at CO_2 concentrations greater than approximately 8% and becomes severe at 15% (Hill and Flack, 1908). Dyspnea could also be a cause of distress in rodents exposed to this gas (Niel and Weary, 2006, 2007).

Argon is often suggested as an alternative to CO_2 because it is tasteless and odourless (Raj and Gregory, 1991; Gerritzen et al., 2000). Moreover, this gas is safe and can be administered easily. Argon acts by displacing air; air contains 20.9% oxygen (O_2), so O_2 concentration is reduced as air is displaced. Reduced O_2 levels cause hypoxia, leading to unconsciousness and death.

Birds (e.g., hens: Webster and Fletcher, 2004; turkeys: Raj, 1996; broilers: Gerritzen et al., 2000), and some terrestrial mammals (e.g., pigs: Raj and Gregory, 1995) will freely enter a chamber containing > 90% argon and most will lose consciousness before they are able to exit. Behavioural data show that broilers (Lambooij et al., 1999; Gerritzen et al., 2000; McKeegan et al., 2006; but see Coenen et al., 2000) and turkeys (Raj, 1996) killed with argon exhibit less head shaking and less gasping than those killed with CO_2. Diving mammals such as mink (Raj and Mason, 1999) will also enter a chamber containing 90% argon but they never remain long

enough to lose consciousness, suggesting that they are not averse to argon *per se* but are averse to the resulting hypoxia. The amount of time they spend in argon atmosphere is similar to dive duration (Raj and Mason, 1999). Little is known about the effects of argon on burrowing rodents. Niel and Weary (2007) reported that in an approach-avoidance task rats either refused to enter, or immediately left a cage containing 90% argon. Leach et al. (2002a, 2004) showed that rats and mice, free to enter and leave chambers containing various gases, would spend more time in a chamber containing argon than one containing CO_2, but less time than if it contained air.

To our knowledge, no one has investigated the effects of gradual-fill argon exposure in rats, or indeed in any other animals. The aim of the present study was to use approach-avoidance testing to evaluate rat responses to argon-induced hypoxia when argon was introduced over a range of flow rates. In the present study, rats' aversion to argon-induced hypoxia was tested against motivation to consume sweet foods. There is evidence that motivation for sweet foods in rats is at least moderate, even if they are not food deprived (Collier and Bolles, 1968; McGregor et al., 1999).

2.2. Materials and methods

2.2.1. Subjects and housing

This experiment was run in two phases: Phase 1 tested low flow rates (40, 66, 93, and 120% of the test cage volume per min) and Phase 2 tested higher flow rates (120, 159, 199, and 239% of the test cage volume per min). Phase 1 was run with eight, 10-month-old male Wistar rats purchased from the University of British Columbia's Animal Care Centre Rodent Breeding Unit as surplus stock and destined for euthanasia. Phase 2 was run a month later with the same subjects, less one that refused to perform the task after the first day of testing in this phase. Rats were given *ad libitum* access to food (Lab Diet 5001, PMI Nutrition International, Richmond, USA) and tap water. Animal rooms were kept at an average (± standard deviation) temperature

of 22.7 ± 0.4°C and an average relative humidity of 29 ± 4%. Rats were housed under a 12-h light:12-h dark cycle with all testing done during the light phase (08:00 – 20:00).

Subjects were single-housed in an apparatus consisting of two transparent polycarbonate cages (Lab Products Inc., Seaford, DE, USA), one shelved 33 cm higher than the other and connected by an opaque ribbed PVC tube that was 10-cm in diameter. The top cage (48 cm x 38 cm x 20 cm) contained food, tap water, bedding (Aspen Chip, Northeastern Products Corp., New York, USA), an opaque nest box, and a Nylabone dog chew (Nylabone® Original Flavor, Nylabone Products, Neptune, NJ, USA), while the bottom cage (45 cm x 24 cm x 20 cm) contained bedding.

2.2.2 Experimental Apparatus

For testing sessions, rats were brought separately into the procedures room in their top cage. The PVC tube remained connected to the top cage, but the bottom cage was replaced by a smaller cage (28 cm x 17 cm x 12 cm) that contained bedding. This test cage was fitted with a Plexiglas lid with a gas inlet in the centre, a sampling tube at the far end of the cage, and two air outlets (1.8 cm in diameter) covered with mesh at the end closest to the tube.

Air and argon were delivered to the test cage from compressed gas cylinders (Praxair, Richmond, BC, Canada). 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. Preliminary tests indicated that the cage temperature did not drop during the filling process. Gas flow rates were measured with a variable area flow meter (Model VSB-66-BV, Dwyer Instruments, Inc., Michigan City, IN, USA for Phase 1; and Model VFB-67, Dwyer Instruments, Inc., Michigan City, IN, USA for Phase 2), and observed argon flow rates were multiplied by a correction factor of 0.85 to adjust for density and obtain the true flow rate.

2.2.3. Training

Rats were trained to enter the bottom cage for a reward of 20 Honey Nut Cheerios® (General Mills Canada Corporation, Mississauga, ON, Canada). Our subjects had previously been used in two experiments that tested aversion to CO_2 gas with the same experimental procedure. The rats were re-trained with air prior to each phase of this experiment with the range of flow rates used in this experiment.

2.2.4. Testing Procedure

Each phase of the experiment ran for 8 days, with alternating days of argon and air. A constant flow rate of 63% of the test cage volume per min was used on air days in Phase 1, while in Phase 2 the same set of flow rates was used with air as with argon. Treatment order for flow rates was balanced across rats and days using a double 4x4 Latin square (in Phase 2, one line was removed to account for the missing rat).

After rats were individually brought into the procedures room and their bottom cage replaced, they were allowed 120 s for exploration, then locked in their top cage and given a reward item. After 120 s the lock was removed and animals were free to access the bottom cage once more for their reward of 20 reward items. Trials began as soon as rats entered the test cage and started eating, at which time air or argon was turned on at the pre-determined flow 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 rats left the test cage before the 300 s elapsed, they were not allowed to re-enter; the test session ended and the remaining reward items were removed and counted.

2.2.5. Data Collection

Trials were video recorded using a Panasonic CCTV camera (Model WV-BP330, Laguna, Philippines). From the videos we measured the latency to stop eating, the latency to leave the test cage, the O_2 concentration at which rats stopped eating, and the O_2 concentration at which they left the test cage. Latency to stop eating was from the moment gas was turned on until last contact between a reward item and the paws. Latency to leave was from the moment gas was turned on until the moment the ears crossed into the tube. O_2 concentrations during the experiment were monitored through a gas sampling tube using a Mocon LF700D (Japan) O_2 analyser. The sampling tube was situated approximately 1 cm above the rats' head when they were eating. We also recorded the number of reward items left and the number of reward items that rats retrieved and brought to the upper cage at the end of each trial.

2.2.6. Statistical Analysis

Dependent variables were analysed using a mixed model (SAS v9.1) that included rat (7 d.f. in Phase 1, 6 d.f. in Phase 2) as a random effect, and tested for linear and quadratic effects of flow rate (1 d.f. for each) against an error term with 21 d.f. for the test of argon flow rate in Phase 1 and 18 d.f. in Phase 2, and 18 d.f. for the test of air flow rate in Phase 2. Latency to leave the test cage was not tested in the air flow analysis because, in all tests except one, animals remained in the test cage until they had eaten all the reward items or until the 300 s were up.

2.3. Results

In Phase 1, rats ate all 20 reward items in all but one out of 32 control trials with air; in the one exception the rat still had one item left at the end of the 300-s session. On average (± standard deviation), rats finished eating 252 ± 22 s after air was turned on. In Phase 2, the air control was presented at a range of flow rates but changes in air flow did not affect the number of

reward items eaten (linear: $F_{1,18} = 0.46$, $P = 0.51$; quadratic: $F_{1,18} = 0.74$, $P = 0.40$) or the latency to stop eating (linear: $F_{1,18} = 0.00$, $P = 0.95$; quadratic: $F_{1,18} = 0.45$, $P = 0.51$). In 26 out of 28 air trials, rats ate all 20 reward items. In one trial the rat had one item left at the end of the 300-s session and in another trial a rat left the test cage after eating only one reward item. On average (± standard deviation), rats finished eating 232 ± 51 s after air was turned on.

When tested with argon, rats never remained in the test cage long enough to lose consciousness. They consumed fewer reward items, stopped eating sooner, and left the test cage more quickly than when tested with air. Rats ate for an average (± standard deviation) of 104 ± 31 s when tested at the lowest argon flow rate, and this time decreased with increasing flow rates (Fig. 1a). The number of reward items eaten (Fig. 1b) also decreased with increasing argon flow rates. In Phase 1, both the linear and the quadratic effects were significant for the latency to stop eating (Fig. 1a; linear: $F_{1,21} = 196.76$, $P < 0.0001$; quadratic: $F_{1,21} = 21.77$, $P = 0.0001$), the latency to leave the test cage (Fig. 1a; linear: $F_{1,21} = 254.56$, $P < 0.0001$; quadratic: $F_{1,21} = 31.25$, $P < 0.0001$), and the number of reward items eaten (Fig. 1b; linear: $F_{121} = 170.02$, $P < 0.0001$; quadratic: $F_{1,21} = 18.10$, $P = 0.004$). There was also a significant linear effect of flow rate on the O_2 concentration at which rats stopped eating (Fig. 1c; linear: $F_{1,21} = 12.71$, $P = 0.0018$; quadratic: $F_{1,21} = 0.21$, $P = 0.65$) and the concentration at which they left the test cage (Fig. 1c; linear: $F_{1,21} = 14.80$, $P = 0.0009$; quadratic: $F_{1,21} = 0.01$, $P = 0.92$) in this phase.

In Phase 2, the linear effect of flow rate was significant for the latency to stop eating (Fig. 1a; linear: $F_{1,18} = 34.34$, $P < 0.0001$; quadratic: $F_{1,18} = 2.01$, $P = 0.17$), the latency to leave the test cage (Fig. 1a; linear: $F_{1,18} = 66.18$, $P < 0.0001$; quadratic: $F_{1,18} = 2.44$, $P = 0.14$), and the number of reward items eaten (Fig. 1b; linear: $F_{1,18} = 23.72$, $P = 0.0001$; quadratic: $F_{1,18} = 1.22$, $P = 0.28$). Effects were not significant for the O_2 concentration at which rats stopped eating (Fig. 1c; linear: $F_{1,18} = 0.05$, $P = 0.83$; quadratic: $F_{1,18} = 1.52$, $P = 0.23$) or the concentration at which they left the test cage (Fig. 1c; linear: $F_{1,18} = 0.38$, $P = 0.55$; quadratic: $F_{1,18} = 0.81$, $P = 0.38$). The average

(±standard deviation) O_2 concentrations at which rats stopped eating and left the test cage were 7.7 ± 1.4% and 6.8 ± 1.2%, respectively.

Before leaving the test cage at the end of an argon trial, rats would sometimes retrieve one to three reward items and take them to the top cage. In Phase 1, the number of items retrieved increased linearly with successive test days, from an average (± least square standard error) of 0.0 ± 0.3 on day 1 to 1.1 ± 0.3 on day 4 ($F_{1,21}$ = 12.08, P = 0.002) . There was no difference in this measure for Phase 2 ($F_{1,18}$ = 0.04, P = 0.85). The O_2 concentration at which rats stopped eating (Fig. 2; $F_{1,21}$ = 21.56, P = 0.0001) and left the test cage (Fig. 2; $F_{1,21}$ = 11.53, P = 0.002) increased linearly with successive days of testing in Phase 1. This effect was not present in Phase 2 (Fig. 2; stop eating: $F_{1,18}$ = 0, P = 0.96; left the test cage: $F_{1,18}$ = 0.7, P = 0.41).

Both phases of the experiment tested argon at a flow rate of 120% of the test cage volume per min. At this flow rate there was no difference between the two phases in the latency to stop eating ($F_{1,6}$ = 0.02, P = 0.90), the latency to leave the test cage ($F_{1,6}$ = 0.00, P = 1.00), the number of reward items eaten ($F_{1,6}$ = 0.78, P = 0.41), the O_2 concentration at which rats stopped eating ($F_{1,6}$ = 0.11, P = 0.75) and the concentration at which they left the test cage ($F_{1,6}$ = 0.01, P = 0.93).

2.4. Discussion

When tested with argon, rats never remained in the test cage long enough to lose consciousness. Argon is an inert gas that is odourless and non-irritant, so rats' aversion is likely due to the resulting hypoxia. Sound or air currents associated with gas entry were not the cause of aversion since only changes in the flow rate of argon, and not air, had an effect on the variables tested.

The results of the current study indicate that argon-induced hypoxia is sufficiently aversive to rats to override the motivation to consume a preferred food reward. Rats are burrowing rodents and likely evolved the ability to detect hypoxia, but how they do this is not

clear. Feelings of dyspnea are not likely. Chonan et al. (1998) found that human subjects exposed to sustained hypoxia reported some "difficulty of breathing" at ventilatory peak, but no information was given about different qualities or dimensions of the breathing sensation. In another study, subjects reported no dyspnea even when breathing 7% O_2 (Moosavi et al., 2003). However, humans exposed to hypoxia usually experience symptoms that include headache, dizziness and visual changes, but these symptoms are very subtle and it appears as though only people who have been trained to recognize them in a hypobaric chamber are able to do so (Cable, 2003). It is possible that rats are more sensitive to headaches, dizziness and visual changes associated with low O_2 and that these symptoms caused aversion in this study.

Rats are able to detect small differences in O_2 concentration in inspired air (Arieli, 1990). Within 1 s of exposure, rats correctly identified which of two atmospheres had a higher O_2 concentration even when these differed by as little as 4%, and this discrimination was possible between normoxic and hyperoxic atmospheres (e.g. 21% versus 30% O_2), between hypoxic and normoxic atmospheres (e.g. 13-17% versus 21% O_2), and between two hypoxic atmospheres (e.g. 9% versus 3% O_2). These results suggest that rats possess a mechanism for detecting O_2 concentrations independent of the symptoms caused by hypoxia. Arieli (1990) suggests that because O_2 is an active molecule, rats may be able to detect O_2 content through olfaction or O_2 receptors in the airways.

Hypoxia triggers increased ventilation when the partial arterial pressure of O_2 (PaO_2) is reduced, and ventilatory suppression if PaO_2 levels are further reduced (Hayashi and Fukuda, 2000). In conscious rats exposed to hypoxia, it appears that respiratory frequency and ventilation per minute start to plateau at about 8% O_2 (Mizusawa et al., 1995). In our study rats stopped eating at an average O_2 concentration of 7.7%, suggesting that they did so around the onset of ventilatory depression.

The O_2 concentration at which rats stopped eating and left the test cage decreased over the lower range of flow rates tested in Phase 1, but showed no further decrease over the higher

flow rates tested in Phase 2. Therefore, rats' aversion to argon-induced hypoxia seems to decrease with increasing argon flow rates to a threshold of approximately 6.8-7.7% O_2 with flow rates equal to or above 120% of the test cage volume per min. Rats were sufficiently alert at the time of leaving the test cage to be able to learn to retrieve progressively more reward items with successive days of testing in Phase 1.

Rats show aversion to O_2 concentrations of 6.8-7.7% when the cage is filled gradually, but what concentrations are required to cause unconsciousness and death? When O_2 is gradually replaced by nitrogen (N_2), the lethal inspired pressure of O_2 (PO_2) is approximately 30 mm Hg (Hall, 1966; Morrison and Rosenmann, 1975). At 6.8-7.7% O_2, the PO_2 is between 51.6 and 64.2 mm Hg (Altland et al., 1968). However, rats are more resistant to hypoxia in N_2 than in argon, perhaps due to differences in the densities of the two gases (Altland et al., 1968; Sharp et al., 2006). Niel (2002; unpublished data) measured responses of two rats during forced exposure to argon-induced hypoxia when argon was introduced at 20% of the test cage volume per min. At this flow rate, rats became ataxic after approximately 240 s, when O_2 decreased to 7.4 % for one rat, and 7.7% for the other. Recumbency was measured only for one of the rats, and it occurred after about 540 s when O_2 was 2.7%. Most other studies have recorded time to death and unconsciousness with static O_2 concentrations. Sharp et al. (2006) showed that rats placed in 0% O_2 in argon lost consciousness after 54 s. Altland et al. (1968) found that all 24 rats tested survived more than 160 min of exposure to 6.6% O_2 in argon, but 2 out of 24 rats (8%) died within 10 min and 21 out of 24 rats (88%) died within 1 h of exposure to 4.9% O_2 in argon.

In Phase 1, rats stopped eating and left the test cage at higher O_2 concentrations with successive days of testing. In Phase 2, one rat refused to run the task after the first day of testing with argon and was withdrawn from the study. Another rat in this phase left the test cage after eating just one reward item during the air trial following the first day of testing with argon. Niel (2006) also found that a rat refused to run a similar approach-avoidance task the day following exposure to 90% argon, a response that was not observed following exposure to various

concentrations of CO_2. Taken together, these results suggest that exposure to hypoxia increases the strength of avoidance in the approach-avoidance test during subsequent sessions. These results further suggest that animals may have learned to avoid the effects of hypoxia. Raj and Gregory (1991) reported that hens exposed to reduced O_2 atmospheres over the course of 3 days spent on average less time in the atmosphere than did birds exposed to it once. Learning to avoid hypoxia provides further evidence that animals found its effects aversive.

Nishino et al. (1986) reported that the swallowing reflex was inhibited in cats exposed to hypoxia. However, these cats were anaesthetized, paralysed and artificially ventilated, and recordings were taken from sectioned and desheathed laryngeal nerves. The effects of anaesthesia may be problematic, as anaesthetics themselves tend to inhibit the swallowing reflex. In other studies on terrestrial mammals (pigs: Raj and Gregory, 1995) and birds (hens: Webster and Fletcher, 2004; turkeys: Raj, 1996), animals were exposed to argon while feeding and lost consciousness in the chamber without any report of swallowing reflex inhibition. A study using non-sedated burrowing mice (Khurana and Thach, 1996) found that the swallowing reflex was not inhibited by hypoxia. We therefore assume that rats in our study did not experience swallowing reflex inhibition and did not leave the test cage simply because they were unable to swallow in hypoxic atmospheres.

It is possible that animals find recovery from hypoxia aversive, but not hypoxia *per se*. If this was the case we would expect animals to remain in the chamber during the first exposure to hypoxic atmospheres, but then avoid subsequent exposures after experiencing recovery from hypoxia. In the present study, as well as in Niel and Weary (2007), rats avoided the hypoxic atmosphere upon first exposure, suggesting they find exposure to hypoxia *per se* aversive.

2.5. Conclusion

Our study demonstrates that rats show aversion to argon-induced hypoxia over a range of flow rates, and the effects of hypoxia become aversive at approximately 7.7% O_2. These results

suggest that argon is not a suitable alternative to CO_2 for the euthanasia of rats. Further research is now required to test other alternatives, such as exposure to anaesthetic gases.

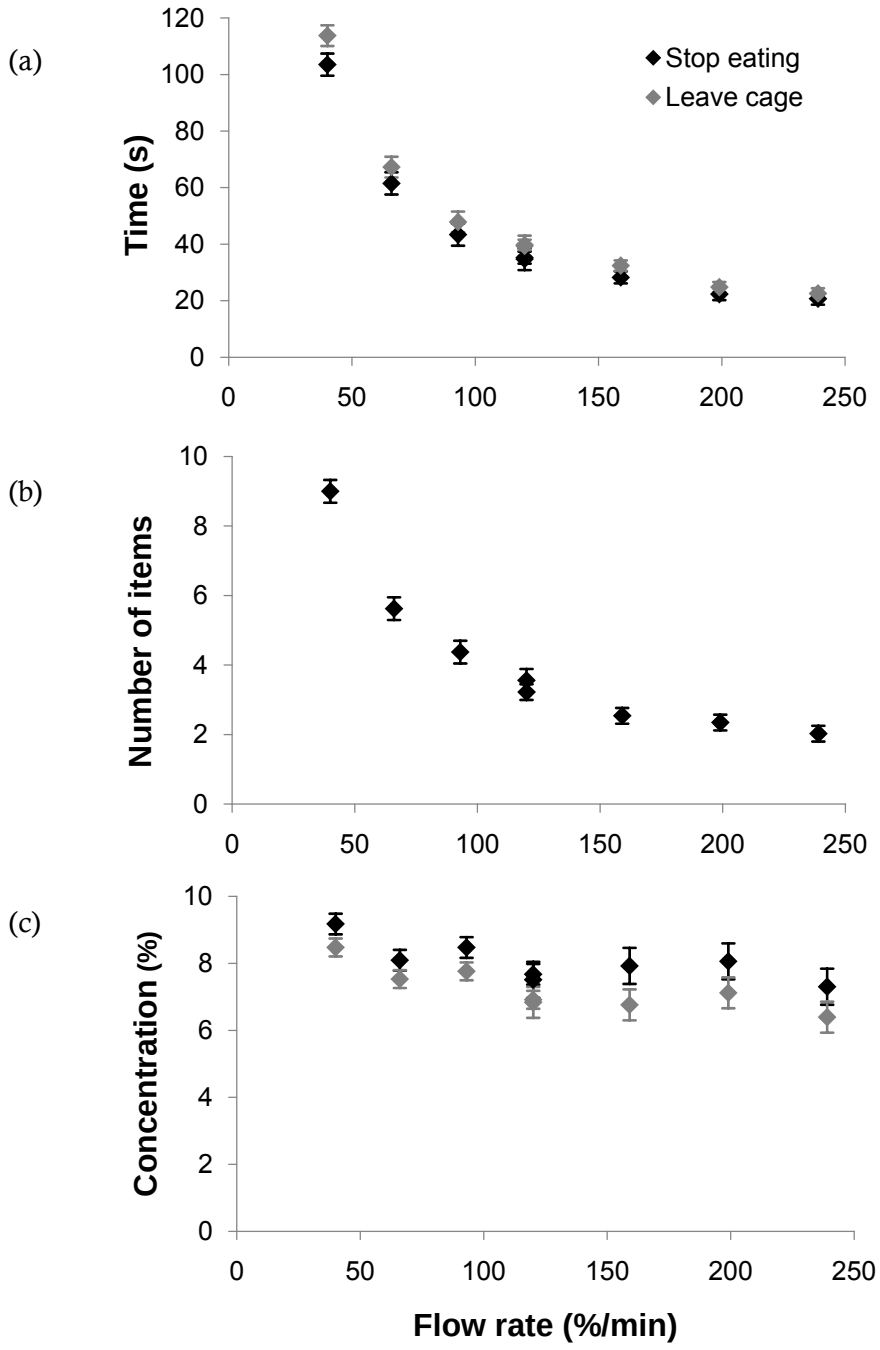

Figure 2.1. Least square means (± S.E.M.) for (a) latency for rats to stop eating and leave the test cage, (b) number of reward items eaten, and (c) O_2 concentration at which rats stopped eating and left the test cage during sessions with argon at flow rates of 40, 66, 93 and 120% of the test cage volume per min in Phase 1 (n = 8) and 120, 159, 199 and 239% in Phase 2 (n = 7).

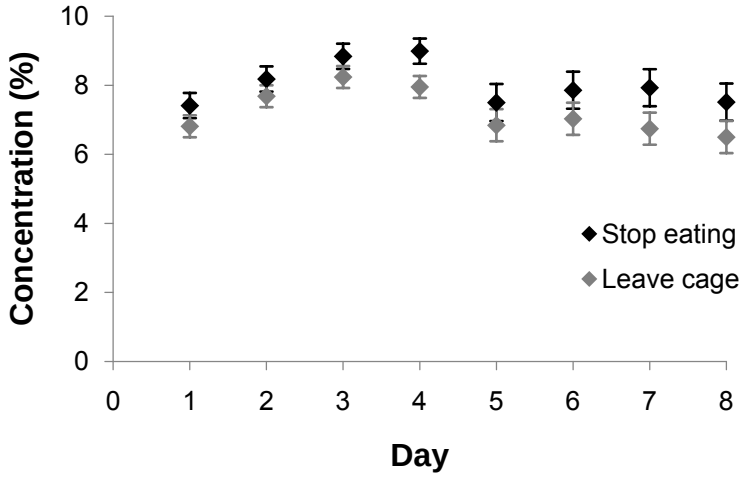

Figure 2.2. Least square means (± S.E.M.) for O_2 concentration at which rats stopped eating and left the test cage according to day of testing in Phase 1 (days 1-4; *n* = 8) and Phase 2 (days 5-8; *n* = 7) during sessions with argon.

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CHAPTER 3: Inhalant anaesthetics:

an alternative to CO_2 euthanasia in rats[2]

3.1. Introduction

In the word euthanasia, the Greek prefix *eu* provides a positive connotation and *thanatos* means death. Euthanasia, then, literally means *good death* – a designation that likely does not apply to the common practice of carbon dioxide (CO_2) killing of laboratory rodents, since several studies have now demonstrated that rodents find this gas aversive. Low concentrations of CO_2 likely cause dyspnea, which is an unpleasant urge to breathe, while higher concentrations cause pain.

CO_2 can be administered by pre-filling a chamber with a lethal concentration of CO_2 before animals are placed in it, or by gradually increasing the CO_2 concentration to lethal levels in a chamber already containing animals. In humans, dyspnea begins at concentrations greater than 8% CO_2 (Liotti et al., 2001) and becomes severe at 15% (Hill and Flack, 1908). Rats exposed to static CO_2 concentrations in an approach-avoidance test remained in the test cage and consumed all reward items at 0, 5, and 10% CO_2 but many rats refused to enter the cage or left quickly when it contained 15 or 20% CO_2 (Niel and Weary, 2007). When exposed to gradual-fill CO_2, rats chose to leave the test cage at the cost of abandoning their food reward when CO_2 concentrations exceeded about 17 % (Niel and Weary, 2007; Niel et al., 2008). These aversion thresholds are consistent with human thresholds for dyspnea, suggesting that dyspnea is a cause of this aversion. At higher concentrations, CO_2 is converted to carbonic acid when it comes into contact with moisture. This formation of carbonic acid results in a burning sensation in the

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cornea, conjunctiva and nasal mucosa of humans exposed to concentrations in excess of 30 to 50% CO_2 (Anton et al., 1992; Thurauf et al., 1993; Chen et al., 1995; Feng and Simpson, 2003). Similarly, the threshold for most rat nociceptors in the nasal mucosa is 37-50% CO_2 (Peppel and Anton, 1993). When CO_2 is used for killing with the pre-fill method, CO_2 concentrations in the cage are greater than 70%, suggesting that rats experience pain and dyspnea during the procedure. With the gradual-fill method, rats typically lose consciousness at CO_2 concentrations below 40% (Smith and Harrap, 1997), suggesting that they experience dyspnea but not pain. Aversion to CO_2 has also been documented in other species, such as mice (Leach et al., 2002a, b; 2004), mink (Cooper et al., 1998), broiler chickens (McKeegan et al., 2006) and pigs (Raj and Gregory, 1995).

Argon is an inert gas that acts by displacing oxygen (O_2), and this leads to hypoxia and death. Several authors have suggested that argon could be a suitable alternative to CO_2 (Raj and Gregory, 1991; Gerritzen et al., 2000; Young, 2006) because it is odourless, non-irritant and safe to administer. Although not aversive or only slightly aversive in birds (e.g., hens: Webster and Fletcher, 2004; turkeys: Raj, 1996; broilers: Gerritzen et al., 2000) and some mammals (e.g., pigs: Raj and Gregory, 1995), argon-induced hypoxia is aversive to rats. When the chamber is pre-filled, animals either refuse to enter or leave quickly (Leach et al., 2002a, 2004; Niel and Weary, 2007), and when the chamber is filled gradually, rats always leave prior to loss of consciousness, when O_2 is reduced to approximately 7% (Makowska et al., 2008).

Inhalant anaesthetics are commonly used to induce unconsciousness in animals undergoing surgery, and these agents can also be used to induce unconsciousness in animals prior to euthanasia. Inhalant anaesthetics act through rapid chemical depression of the nervous system, leading to a loss of sensation in the body (Blackmore, 1993; Kohn et al., 1997). Among the most commonly used volatile liquid anaesthetics for animals are the fluorinated hydrocarbons halothane and isoflurane (Flecknell, 1996). Halothane is said to have a relatively

pleasant odour, while isoflurane is said to have a somewhat pungent odour and to cause respiratory irritation (Yentis et al., 1996; Gallacher and Hutton, 2002).

Although inhalant anaesthetics are commonly used in veterinary anaesthesia, little research has been done to assess any distress associated with induction. A series of studies on New Zealand White rabbits revealed that these animals are strongly averse to both halothane and isoflurane induction, as evidenced by breath holding and violent attempts to escape during induction (Flecknell et al., 1996; Flecknell et al., 1999; Hedenqvist et al., 2001).

Few studies have assessed induction with rodents. Young (2006) found that unlike rats exposed to CO_2 or a mixture of CO_2/O_2, those exposed to halothane did not exhibit aversive behaviours such as gasping and rapid body movements. Leach et al. (2002b, 2004) found that rats and mice remained in chambers that contained at least moderate concentrations of halothane, isoflurane or enflurane for shorter periods than when they contained air, but longer than when they contained CO_2. However, Leach et al. provided animals with no incentive to remain in the chambers, so dwelling times even with air were usually under a minute. Several authors claimed that halothane anaesthesia had amnesic effects, since rats' performance on a task was impaired if the task was learned shortly before halothane anaesthesia (e.g. Penrod and Boice, 1971; Angel et al., 1972). However, by performing carefully controlled experiments, Alexinsky and Chapouthier (1979), Schmaltz (1979) and Gisquet-Verrier (1981) concluded that this impairment was more likely explained by rats' aversion to halothane rather than by amnesic effects of halothane.

The aim of the present study was to use approach-avoidance testing to evaluate rat responses to induction with the inhalant anaesthetics halothane and isoflurane. We ran two experiments to test rat responses to each of two existing methods of drug delivery: through a vaporizer in Experiment 1 and from soaked cotton balls in Experiment 2.

3.2. Materials and methods

3.2.1. Experiment 1

3.2.1.1. Subjects and housing

We purchased 24 male Wistar rats from the Rodent Breeding Unit at the University of British Columbia's Animal Care Centre. These rats were surplus and destined for euthanasia. Rats were pair-housed, and one individual from each pair was marked by clipping a small patch of hair on the lower back. Eight rats from different pairs were used in the main experiment while the remaining 16 were used for preliminary testing. Subjects weighed 500- 670 g (mean ± standard deviation: 608 ± 58 g) at the beginning of the study and 552- 794 g (680 ± 79 g) at the end of the study. Rats were given *ad libitum* access to food (Lab Diet 5001, PMI Nutrition International, Richmond, USA) and tap water. Temperature and relative humidity during the study averaged (± standard deviation) 21.5 ± 0.9 °C and 52 ± 7%, respectively. All testing was done during the light phase of a 12-h light: 12-h dark cycle, with lights on at 08:00.

Rats were housed in an apparatus that consisted of two polycarbonate cages (Lab Products Inc., Seaford, DE, USA) connected by a sloped, opaque, ribbed PVC tube that was 10-cm in diameter, such that one cage was 33 cm higher than the other. The top cage was larger (48 cm x 38 cm x 20 cm) and contained food, tap water, bedding (Aspen Chip, Northeastern Products Corp., Warrensburg, NY, USA), an opaque nest box, and a Nylabone dog chew (Nylabone® Original Flavor, Nylabone Products, Neptune, NJ, USA), while the bottom cage was smaller (45 cm x 24 cm x 20 cm) and contained only bedding.

3.2.1.2. Experimental apparatus

Prior to the experiment, rats were trained to go up and down the ribbed tube for a food reward (Honey Nut Cheerios®, General Mills Canada Corporation, Mississauga, ON, Canada)

at the sound of fingers being dragged along this tube. Before a trial, both rats of a pair were called to the top cage and given a reward item using this method. The tube between the two cages was then disconnected, the nest box and water bottle were removed, and the non-experimental rat was picked up and placed in the bottom cage. The top cage containing the experimental rat was moved into a fume hood in the procedures room and connected to a smaller bottom cage (28 cm x 17 cm x 12 cm) that contained bedding. This test cage was fitted with a Plexiglas lid with a gas inlet in the centre and two air outlets (1.8 cm in diameter) covered with mesh at the end closest to the tube. The test cage was disinfected (Quatricide® PV, Pharmacal Research Laboratories, Inc., Waterbury, CT, USA) and filled with fresh bedding between rats.

Oxygen was delivered to the test cage from a compressed gas cylinder (Praxair, Richmond, BC, Canada). The temperature of the gas was regulated by passing it through a copper coil in a room temperature water bath. Gas flow was controlled using a flow meter (Model GL-616, Porter Instruments Company, Hatfield, PA, USA) that was attached to a table-top anaesthetic machine (ARVS, Langley, BC, Canada). Oxygen was delivered alone (as a control) or as a carrier for halothane (Halocarbon Laboratories, River Edge, NJ, USA) or isoflurane (Baxter Corporation, Mississauga, ON, Canada). These anaesthetics were delivered to the test cage from Fluotec 4 and Isotec 4 vaporizers (Ohmeda, Steeton, West Yorkshire, England), respectively.

3.2.1.3. Preliminary testing

Slightly higher concentrations are required for halothane than isoflurane to achieve similar times to recumbency (loss of the righting reflex). To facilitate comparisons between the two drugs, we measured the time until rats (n = 4) became recumbent with each of four concentrations of halothane (2, 2.5, 3.25 and 5%) and isoflurane (1.25, 2, 2.5 and 3.75%) to ensure that the concentrations were well matched. A period of at least 20 h was allowed between

exposures. Times to recumbency with these concentrations averaged (± standard deviation) 158 ± 55 s, 138 ± 7 s, 114 ± 3 s, and 88 ± 16 s for halothane and 153 ± 14 s, 135 ± 14 s, 111 ± 8 s, and 79 ± 18 s for isoflurane. The overall average across the two drugs was 155, 136, 113, and 83 s.

3.2.1.4. Testing procedure

We ran two replicates of the experiment, each testing the four concentrations of halothane (2, 2.5, 3.25 and 5%) and four concentrations of isoflurane (1.25, 2, 2.5 and 3.75%) in O_2 delivered at a flow rate of 63% of the test cage volume per minute. Treatment order for drugs was balanced across rats and days using an 8x8 Latin square. Every second day rats were run with pure O_2 as a control and to avoid extinction, so each replicate ran for 16 days.

Once the testing apparatus was in place in the fume hood, rats were allowed 120 s for exploration before being locked in their top cage and given a reward item. After 120 s the lock was removed and animals could access the bottom cage to obtain 20 reward items. Trials began as soon as rats entered the test cage and started eating the reward items, at which time O_2 was turned on either alone, or together with the pre-determined concentration of halothane or isoflurane. Trials ended when rats returned to the top cage or after 300 s, whichever occurred first. Rats were not allowed to re-enter the test cage after they returned to the top cage.

3.2.1.5. Data collection

Trials were video recorded with a Panasonic CCTV camera (Model WV-BP330, Laguna, Philippines) and scored for the latency to leave the test cage (from the time gas was turned on until the time the ears crossed into the tube), and the number of reward items eaten. Latency to leave the test cage was not scored for the O_2 control trials since rats remained in the cage until we enticed them to leave (i.e. > the 300 s maximum). We also recorded whether rats were ataxic at the end of a trial, with ataxia defined as loss of muscular coordination. Finally, we calculated

the difference between the time at which rats left the test cage and the time at which recumbency was expected to occur (i.e. 155, 136, 113, and 83 s for the four concentrations). During euthanasia, welfare may be compromised during the period between onset of aversion (when rats would choose to leave the cage if allowed) until unconsciousness, so we wanted to get an indication of how long this period would be with the various concentrations of inhalant anaesthetics we used.

3.2.1.6. Statistical analysis

Rats behaved differently on the first day of exposure to anaesthetics than on subsequent days. Descriptive statistics are presented for day 1. Inferential statistics are based on the results from days 2-16. Dependent variables were analysed using a mixed model (SAS v9.1) that included rat (7 d.f.) as a random effect, and tested for effects of drug, treatment, and drug by treatment interaction (1 d.f. each) against an error term with 53 d.f.

3.2.2. Experiment 2

The aim of Experiment 2 was to test rat responses to halothane and isoflurane delivered in soaked cotton balls. The results of Experiment 1 indicated that upon re-exposure to inhalant anaesthetics, most rats avoided halothane and isoflurane even though they had never experienced that particular anaesthetic before. Since these anaesthetics have different smells, we hypothesized that a single exposure to an anaesthetic conditioned an aversion to all novel smells, including novel anaesthetics. We tested this hypothesis by first exposing rats to isoflurane and then to either a novel odour (peppermint extract) or a novel anaesthetic (halothane). We also assessed whether this learned aversion was transient by allowing the rats to leave and re-enter the test cage at will.

3.2.2.1. Subjects and housing

We purchased 20 surplus male Wistar rats from the Rodent Breeding Unit at the University of British Columbia's Animal Care Centre. Twelve rats were used in the main study and the remaining eight were used for preliminary testing. Rats weighed 350- 436 g (mean ± standard deviation: 386 ± 28 g) at the beginning of the study and 367- 465 g (407 ± 31 g) at the end of the study. Temperature and relative humidity averaged (± standard deviation) 21.4 ± 0.6 °C and 51 ± 3%, respectively. Housing conditions and husbandry were the same as in Experiment 1.

3.2.2.2. Experimental apparatus

The experimental apparatus was identical to that described for Experiment 1, except that anaesthetics were delivered from soaked cotton balls instead of vaporizers. We placed two cotton balls (Safeway Limited, Calgary, AB, Canada) inside a cylindrical plastic container (diameter: 3.5 cm; height: 2.2 cm) with an open end that we covered with mesh. Balls were placed into the container to prevent direct contact with the liquid anaesthetics, as these are topical irritants (Canadian Council on Animal Care, 1993). Liquid halothane (5 mL), isoflurane (7 mL), peppermint extract (5 mL) or water (5 mL) was poured directly onto the cotton balls in the container. This container was placed mid-length and close to one wall of the test cage.

3.2.2.3. Preliminary testing

We measured the time until recumbency with the amount of halothane and isoflurane selected for this study. These amounts were 7 mL for halothane and 5 ml for isoflurane, and the time each took to induce recumbency (mean ± standard deviation; $n = 4$)) was 205 ± 5 s and 168± 23 s, respectively.

3.2.2.4. Testing procedure

The experiment ran for 5 days, with 3 experimental days interspersed with two control (5 mL of water) days to avoid extinction. On the first experimental day all rats were exposed to 5 mL of isoflurane. On the following experimental day, half of the rats were exposed to 7 mL of halothane, a novel anaesthetic, and half were exposed to 5 mL of peppermint extract (Safeway Limited, Calgary, AB, Canada), a novel non-anaesthetic odour. To assign rats to receive halothane or peppermint extract, we paired rats such that the two rats that had stayed in the test cage the longest when tested with isoflurane formed one pair, the next two formed another pair, and so on. We then randomly assigned one rat from each pair to be tested with halothane and the other with peppermint extract. On the third experimental day, rats that had previously been tested with halothane were tested with peppermint extract and vice versa.

Before each trial rats were allowed 120 s for exploration of the apparatus and were then locked in their top cage and given a reward item. Rats remained locked in the top cage for 120 s, during which time we placed 20 reward items in the test cage, added the soaked cotton balls, and closed the Plexiglas lid. When the lock was removed rats could access the test cage to obtain their reward items. Unlike in the previous experiment, rats were allowed to re-enter the test cage after returning to the top cage. Because the eating rate of these rats was slower than for those used in Experiment 1, trial length was 390 s (vs. 300 s in Experiment 1) to ensure all rats had time to eat all the reward items during a session. Trials ended after 390 s, or if rats returned to the top cage when they were already ataxic, if they returned to the top cage for more that 90 s, or if they failed to enter the test cage within 90 s of lock removal.

3.2.2.5. Data collection

In this experiment, the treatments were present within the test cage before rats were allowed to enter. For this reason, we considered the moment a rat's nose first emerged into the test cage as equivalent to the moment gas was turned on in Experiment 1. Trials were video

recorded and scored for the latency to leave the test cage (from the moment a rat's nose first emerged into the test cage until the time the ears crossed into the tube), the number of reward items eaten, and whether rats were ataxic at the end of the trial. Latency to leave the test cage could not be scored for the water control and the peppermint extract trials since all rats remained in the cage until we enticed them to leave (i.e. > the 390 s maximum). Because rats were free to re-enter the test cage, we also recorded the number of times rats went to the top cage during a trial, the time at which the first trip occurred, as well as the amount of time they remained in the test cage and the number of reward items eaten after they returned. We also scored the latency to start eating (time from when the rat's nose emerged into the test cage until it started eating) taken as an indication of hesitancy to start eating.

3.2.2.6. Statistical analysis

We compared responses to the anaesthetic and non-anaesthetic novel odours (halothane vs. peppermint) and the two non-anaesthetic treatments (water vs. peppermint) using specified contrasts in a mixed model (SAS v9.1) that included rat as a random effect. We did not test differences between the two anaesthetics because this comparison was confounded with order, and because the amounts of anaesthetics were not matched for the time they took to induce recumbency.

3.3. Results

3.3.1. Experiment 1

Rats ate all 20 reward items in 126 out of 128 O_2 control trials. In two trials, one of the items was lost in the bedding and the rat left the test cage after eating 19 items. All rats remained during the entire trial.

On the first day of exposure to anaesthetics, rats remained in the test cage for an average (± standard deviation) of 64 ± 28 s and left the cage 57 ± 27 s before expected recumbency. The average number of reward items eaten was 4.3 ± 1.8. Six of the eight rats were ataxic before leaving the test cage, losing balance within the test cage and showing difficulty going up the tube to the top cage.

On days 2-16, most rats left the test cage very quickly after anaesthetic exposure. Ataxia was observed in 19 out of 120 trials; 12 of these were with one rat that consistently remained in the test cage for an average of 50 ± 28 s, left 65 ± 34 s before expected recumbency, and consumed 5.2 ± 3 reward items. Of the remaining seven trials where ataxia was observed, all were with isoflurane; four of these were with the lowest concentration of isoflurane, two with the second lowest concentration and one with the third lowest concentration. In the remaining 101 trials where ataxia was not observed, rats remained in the test cage for an average of 8 ± 6 s and consumed on average 0.8 ± 0.7 reward items.

On days 2-16, rats remained in the test cage longer when exposed to isoflurane than to halothane (Fig. 1a; $F_{1, 53} = 4.04$, $P < 0.05$), and longer with lower concentrations of each drug (Fig. 1a; $F_{1, 53} = 4.15$, $P < 0.05$). However, rats remained in the cage closer to the time of expected recumbency when exposed to higher concentrations of each drug (Fig. 1a; $F_{1, 53} = 55.12$, $P < 0.0001$). Rats ate more reward items when exposed to isoflurane (Fig. 1b; $F_{1, 53} = 7.33$, $P = 0.009$) and to lower concentrations of each drug (Fig. 1b; $F_{1, 53} = 5.37$, $P = 0.024$).

3.3.2. Experiment 2

All rats entered the test cage and remained for the entire trial when exposed to the water control. Rats ate all 20 reward items in 19 out of 24 of these trials; in four trials they ate 19 items and in one trial a rat ate 18. When exposed to peppermint extract, all rats entered the test cage and remained for the entire trial. Every rat ate all 20 reward items. The number of reward items

eaten and the number of trips to the upper cage did not differ between the water control and the peppermint extract trials.

On the first day of exposure to isoflurane, 3 out of 12 rats did not enter the test cage. For the nine rats that did, the mean (± standard deviation) latency to leave the test cage was 129 ± 31 s and the mean number of reward items eaten was 6.8 ± 2.1. Eight of the nine rats stayed until ataxic, leaving once they had lost balance and fallen over. Some rats had difficulty going up the tube due to ataxia.

When exposed to halothane, 3 out of 12 rats did not enter the test cage. Two of these three rats were the same ones that had refused to enter the cage with isoflurane. Of the nine rats that entered the test cage, four went to the top cage once or twice during their trial. These rats went to the top cage seconds after entering but quickly came back and stayed for an average of 91 ± 31 s and ate 4.3 ± 2.2 reward items. The other five rats that did not leave the test cage during their trial remained in the cage for an average of 125 ± 26 s and consumed 7.1 ± 3.0 reward items. All nine rats were ataxic at the end of their trial, leaving after having lost balance and showing difficulty going up the tube. Rats ate fewer reward items ($F_{1, 19} = 438.96$, $P < 0.0001$) and made more trips to the top cage during a trial ($F_{1, 19} = 12.35$, $P = 0.0023$) when exposed to halothane in comparison with the other novel odour, peppermint.

In the halothane trials, latency to start eating was about 10 s but two rats had latencies of 94 and 97 s, respectively. These rats were excluded from the analysis of latency to start eating because their values were extreme outliers and including them told a story that was not representative of the majority of the data. Latency to start eating did not differ between the halothane and the peppermint extract trials (Fig. 2; $F_{1, 17} = 0.04$, $P = 0.85$), but latency was longer in the peppermint trials than the water control trials ($F_{1, 17} = 16.9$, $P = 0.0007$).

3.4. Discussion

On the first day of exposure to halothane or isoflurane, most rats remained in the test cage long enough to become ataxic, whether delivery was through a vaporizer or from soaked cotton balls, indicating that either method can be used to induce unconsciousness in rats.

Results of Experiment 1 showed that rats remained in the test cage longer when exposed to isoflurane than halothane, indicating that isoflurane is less aversive than halothane. Rats also remained in the test cage longer with lower concentrations of each drug, but closer to the time of predicted recumbency with the higher concentrations; higher concentrations minimize the time between onset of aversion and loss of consciousness.

Little is known of the physiological changes or the subjective experience during induction with inhalant anaesthetics. Research in cats demonstrates slight central nervous system excitation during induction with halothane or isoflurane, followed by progressive depression with deeper anaesthesia. Responses to an auditory stimulus are reduced during the period of induction with halothane (Winters et al., 1967; Mori et al., 1968; Ogawa et al., 1992). Human clinical observations show that during induction, patients initially experience analgesia followed by amnesia (Trevor and White, 2006). One study showed that individuals performing a visual search task while breathing subanaesthetic concentrations of isoflurane had reduced cognitive performance demonstrated by longer reaction times and increased error rates. These individuals reported that the details of the search task became blurred and described their state during the task as "slightly intoxicated" (Heinke and Schwarzbauer, 2001).

This literature suggests that rats in our study were already partially sedated when they left the test cage. We suggest that forced exposure from this point until loss of consciousness is less of a welfare concern than forced exposure to agents that do not cause sedation. CO_2 is not considered to be an anaesthetic gas, but it does possess anaesthetic properties; however, its mechanism of action is different from that of conventional inhalant anaesthetics (Brosnan et al., 2007). Reduced brain pH appears to be the critical factor in CO_2 anaesthesia (e.g. Meyer et al.,

1961). In the rat, CO_2 decreases brain excitability at concentrations as low as 5%, induces light anaesthesia beginning at 25%, and deeper anaesthesia at approximately 40% (reviewed by Woodbury et al., 1958). In humans, breathing up to 6% CO_2 has no effects on manual dexterity or ability to perform arithmetic; in fact, Case and Haldane (1941) reported an improvement in performance. At 6-7%, there is very little mental impairment or deterioration of manual skill, though all subjects find exposure distressing (Case and Haldane, 1941). Seevers (1944) and Smith and Harrap (1997) found no signs of ataxia in rats exposed to static CO_2 concentrations below 20%, and Kirkden (unpublished data) found that rats show no signs of ataxia at CO_2 concentrations sufficient to cause aversion during approach-avoidance testing. The above evidence suggests that rats are less sedated at the onset of aversion to CO_2 compared to inhalant anaesthetics, and that exposure to inhalant anaesthetics beyond the point of aversion is likely less of a welfare concern than exposure to CO_2. This suggestion is consistent with the results of Young (2006), who found that rats exhibited few signs of stress, and more normal behaviours such as grooming, when exposed to inhalant anaesthetics than when exposed to CO_2.

Exposure to inhalant anaesthetics was still aversive; some rats refused to enter when the anaesthetic was already in the cage, and all rats that entered eventually left the test cage at the cost of abandoning their food reward. Refusal to enter was likely not due to neophobia, since these rats entered the cage when it contained another novel odour, peppermint. Niel and Weary (2007) showed that some rats also refused to enter the test cage in a similar approach-avoidance test when it contained more than 10 or 15% CO_2.

Although most rats remained in the test cage until ataxic, none remained long enough to become unconscious. It has been suggested that rats avoid anything that produces a state change, whether this change is negative or positive (Gamzu, 1977; Hunt and Amit, 1987; Parker, 2003). Rats may have left the test cage after sensing the signs of ataxia or sedation. These physiological changes *per se* may not be aversive, but experiencing this novel state unexpectedly may cause fear. This novel state may also be unpleasant if rats are unaware of what caused it. This

interpretation is supported by human clinical evidence, where subjects who did not know what drug they were receiving described the effects of amphetamine, morphine and heroin as unpleasant (Lasagna et al., 1955).

It is also possible that rats were leaving the test cage because of airway irritation. Isoflurane, and to a lesser degree halothane, are respiratory irritants causing coughing, bronchoconstriction, laryngospasm and mucous secretion in humans, dogs, cats and rabbits (Doi and Ikeda, 1993; Yentis et al., 1996; Mutoh et al., 2001; Gallagher and Hutton, 2002). In humans, irritation can occur even with doses of isoflurane insufficient to produce anaesthesia or sedation (Goodwin et al., 2005). In Experiment 1, rats remained in the test cage longer with isoflurane than with halothane, suggesting two possibilities: airway irritation was not the cause for leaving the test cage, or that rats avoided isoflurane because of airway irritation but avoided halothane for some other reason. Another possibility is that rats were leaving the test cage because of difficulty to swallow the reward items, since anaesthetics are known to inhibit the swallowing reflex (Nishino, 1993). However, in humans (Cleaton-Jones, 1976; Nishino et al., 1987) and cats (Nishino et al., 1984; Ochiai et al., 1989), swallowing inhibition begins at higher levels of sedation than levels avoided by the rats in this study, suggesting that difficulty swallowing was not the reason rats chose to leave the test cage. From a welfare perspective, it may not matter why rats find the exposure aversive – their preference was to leave, and forced exposure beyond the point of aversion is likely unpleasant.

When rats were re-exposed to inhalant anaesthetics in both experiments, most left the test cage seconds after entering, indicating learned aversion. In Experiment 1 rats were not allowed to re-enter the test cage. In Experiment 2 rats were allowed to re-enter the test cage; all promptly returned, indicating that the aversion was transient. However, this transient learned aversion may be a welfare concern during euthanasia of rats with prior experience with inhalant anaesthetics (e.g. during surgery). In humans, the amount of control one has over a situation plays an important role in mediating stress (Bandura, 1982). Rats clearly found the re-exposure

aversive, choosing to leave the cage for at least a few seconds before deciding to return; it is possible that rats that cannot leave would experience stress.

Learned aversion to halothane and isoflurane may be associated with recovery from these anaesthetics. In humans, postoperative nausea and vomiting is one of the most common complaints following anaesthesia (Ku and Ong, 2003; Warren and King, 2008) with an incidence of vomiting of 20-30% with volatile anaesthetics (Watcha and White, 1992). Although rats cannot vomit due to the anatomy of their peripheral musculature, there is strong evidence that they experience nausea (Davis et al., 1986; Parker, 1998; Parker et al., 2003). Rats' gastric vagal afferents respond to physical and chemical stimulation in a manner similar to what precedes vomiting in the ferret (Parker et al., 2003), and they display conditioned rejection reactions to a wide range of stimuli that produce vomiting in species capable of emesis (Davis et al., 1986; Parker, 1998). 'Emergence delirium' or 'post-operative agitation' is another well-documented clinical phenomenon associated with up to 80% of recoveries from anaesthetics in humans. This condition is characterized by alterations in orientation and mental status, and is associated with confusion, disorientation and irritability (Scott and Gold, 2006; da Silva et al., 2008). Animals may also be predisposed to emergence delirium when isoflurane is used, due to the rapid recovery associated with this drug (Muir et al., 2000; Arai et al., 2004). Upon re-exposure, rats may have associated some cue with nausea or delirium felt shortly after first exposure, thus initially choosing to avoid another exposure.

Upon re-exposure, many rats avoided halothane and isoflurane even though they had never experienced that particular drug before. These anaesthetics have different smells, so we hypothesized that rats may have developed a general aversion to novel smells following first exposure. This hypothesis was not supported in Experiment 2, as all animals entered the test cage, ate all reward items and made no trips to the upper cage when exposed to the novel odour of peppermint; these responses were similar to those during the water control trials. However, latency to start eating in the peppermint trials was significantly longer than in the water control

trials and similar to the halothane trials, indicating that rats were aware of the peppermint. Upon re-exposure to an anaesthetic, rats may have recognized one of the constituents that both drugs have in common, such as the halogenating agents fluorine or chlorine that are used to ensure non-flammability and increase potency (Eger, 2004).

3.5. Conclusion

These results indicate that most rats will tolerate exposure to halothane and isoflurane until the point of ataxia, whether the anaesthetics are delivered through a vaporizer or soaked cotton balls. Rats were likely sedated by the time they chose to leave, suggesting that forced exposure from the onset of aversion until loss of consciousness is less of a welfare concern than forced exposure to other non-sedating agents. Most rats showed aversion to the drugs upon re-exposure, but this aversion is transient as animals that were given the opportunity returned to the chamber and remained until ataxic. We suggest that the use of inhalant anaesthetics for inducing unconsciousness prior to euthanasia is a more humane method than the commonly used CO_2.

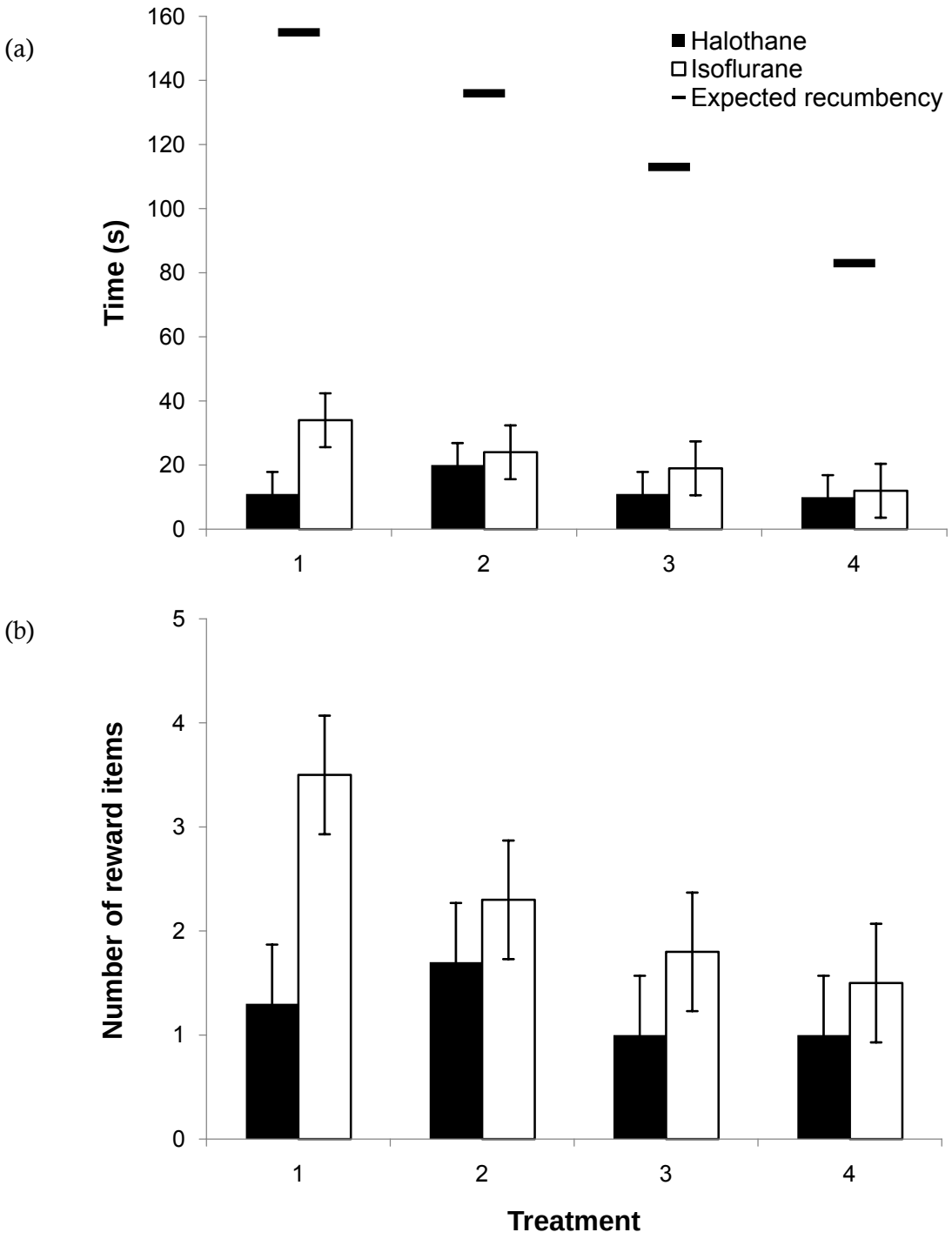

Figure 3.1. Least square means (± S.E.M.) of rats (n = 8) for (a) latency to leave the test cage (columns) and time at which rats are expected to become recumbent (horizontal bars), and (b) the number of reward items eaten on days 2-16 in response to four concentrations of halothane and isoflurane.

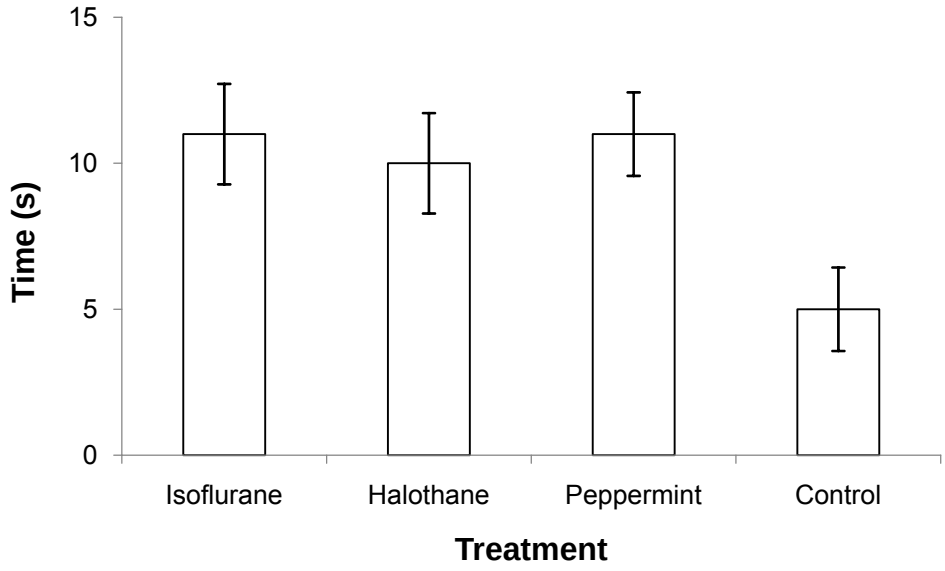

Figure 3.2. Least square means (± S.E.M.) for the latency to start eating when exposed to isoflurane (*n* = 9), halothane (*n* = 7), peppermint extract (*n* = 12) and water as a control (*n* = 12).

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CHAPTER 4: General discussion

4.1. Brief summary

Carbon dioxide (CO_2) is the most widely used agent for euthanasia of laboratory rodents, used on an estimated tens of millions of laboratory rodents worldwide each year (Conlee et al., 2005). Recent studies have shown that this method causes distress (likley due to dyspnea) and pain in rodents, and therefore fails to satisfy the guidelines on euthanasia established by the Canadian Council on Animal Care and other regulatory bodies internationally. There is a pressing need to find humane alternatives to CO_2.

The aim of my thesis was to test whether argon and inhalant anaesthetics are humane alternatives to CO_2 euthanasia for rats. To do this, I used approach-avoidance testing, a method that has been used to test aversion to CO_2 and argon in many species including rodents. In approach-avoidance testing, motivation to avoid gas exposure is compared against motivation to obtain a food reward, in this case Honey Nut Cheerios® (35% sugar). Operant conditioning studies have shown that rats are moderately motivated to consume sweet food items when fed *ad libitum* (Collier and Bolles, 1968; McGregor et al., 1999). This indicates that if rats choose to forgo their food reward to avoid gas exposure, then their motivation to avoid gas exposure is at least moderate.

Rats never remained in the test cage long enough to lose consciousness during gradual-fill argon exposure. This observation indicates that rats find argon-induced hypoxia at least moderately aversive. Although humans (Cable, 2003), pigs (Raj and Gregory, 1995) and poultry (Raj, 1996; Gerritzen et al., 2000; Webster and Fletcher, 2004) cannot detect hypoxia, rats are burrowing rodents and likely evolved the ability to detect low O_2 levels. During gradual-fill with argon, rats left the test cage when O_2 concentration had decreased to approximately 6.8%, but lower O_2 levels are needed to induce unconsciousness and death.

When tested with the inhalant anaesthetics halothane and isoflurane, rats also failed to remain in the test cage long enough to lose consciousness, but most were already ataxic when they chose to leave. Evidence suggests that rats are partially sedated at that point, indicating that if they were forced to remain in the test cage, their experience from this point until unconsciousness may be less aversive than with non-sedating agents. Therefore, exposure to anaesthetics may be a humane alternative to CO_2.

4.2. Critique of the method

When testing rat aversion to argon-induced hypoxia, I selected a wide range of flow rates with the idea that if the test cage filled very slowly, the onset of hypoxia may be so subtle that rats may not detect it; or alternatively, if the test cage filled very quickly, the onset of hypoxia may be so rapid that rats may not have time to detect it before losing consciousness. In practice, the lowest flow rate I was able to test was 40% of the test cage volume per min, which is a moderate flow rate. I was not able to test lower flow rates because I was constrained by the minimum time rats remained in the test cage during control trials – I needed the argon concentration to reach lethal levels (>90%) within the time it took rats to consume all reward items. It is possible that with lower flow rates, rats may not sense the onset of hypoxia. However, with lower flow rates death may take a very long time to occur, and this would not satisfy the criterion of euthanasia as "relatively quick". Flow rates higher than 239% of the test cage volume per minute (maximum flow rate tested) were not tested because I feared that the noise and wind caused by the gas flow might itself become aversive.

Although exposure to inhalant agents as a method of euthanasia presents many advantages over physical methods and injectables, this method may not be devoid of stress even if an ideal agent (one rats would not be able to detect as noxious) was found. This is because rats are generally neophobic, and bringing them into a procedures room and introducing gas currents into their cage may be stressful. When rats were first trained to perform the approach-avoidance

task with control gases such as air or O_2, most were reluctant to sit in the bottom cage for a prolonged period of time, and often carried reward items for consumption in the tube. Rats showed these signs of fear when first exposed to the task, and with every increment in flow rate. This fear was more pronounced and took considerably more training sessions to dissipate with single-housed rats (Chapter 2) than pair-housed rats (Chapter 3). Single-housed rats are known to be more responsive to stressors than pair-housed rats (e.g. Sharp et al., 2003). By training rats, I ensured that the aversion they showed to the euthanasia agents was caused by the agents themselves and not by fear of novelty (e.g. gas currents). It is likely that during actual euthanasia, rats may experience stress much earlier in the course of exposure than during these approach-avoidance tests due to the novelty of the experience, especially if high flow rates are used.

Even if rats are not fearful of being brought into the procedures room and being exposed to gas currents, they may experience stress sooner during forced exposure than during approach-avoidance testing. During approach-avoidance testing, rats had learned how to leave the test cage. It is possible that the presence of a known escape route increased the rats' willingness to be exposed to the euthanasia agents. During approach-avoidance testing, rats likely sensed a change in their environment soon after gas delivery began, but had learned that they could leave the cage if they experienced aversive effects. During forced exposure, sensing a change in the environment may cause a desire to escape, and if an exit is not found this may cause distress before the gas levels *per se* become aversive. During forced exposure to gradual-fill CO_2, Niel and Weary (2006) found that rearing, touching of the nose to the lid, and activity increased from baseline during the first 15 s of exposure, although after 15 s CO_2 concentration in the cage was only 5%. These same authors (2007) found that during approach-avoidance testing, rats would enter and remain in a cage that contained 5% CO_2, and if the cage was filled gradually, rats remained in the cage until the concentration reached about 18.4% CO_2. This suggests that although during approach-avoidance testing rats may not experience any adverse effects until a

certain threshold concentration is achieved in the cage, during actual euthanasia rats may experience distress before that threshold concentration is reached.

Finally, in both of my studies I compared the time or gas concentration at the onset of rats' aversion with the time or gas concentration at which animals would lose consciousness. I think this measure is important, because it gives an indication of the amount of time rats would potentially suffer during euthanasia. The time it takes animals to lose consciousness is measured using animals that are subjected to forced exposure to an agent. As described above, these animals are likely stressed by the manipulations alone, and exhibit escape behaviours at gas concentrations below those they would avoid during approach-avoidance testing. Stress and heightened activity cause heart rate and ventilation to increase, and this results in quicker uptake of the drugs. Stress also has hormonal effects that likely modify the sensitivity of animals to toxic materials in inhalation chambers (Larsen et al., 2000). As a result, it is likely that during forced exposure to euthanasia agents rats lose consciousness more quickly and at lower inspired gas concentrations than they would if they were calmer, as during approach-avoidance testing (Drew, 1982, cited by Larsen et al., 2000). For example, during approach-avoidance testing with gradual-fill carbon monoxide, rats chose to leave the test cage at inspired gas concentrations above those that induced recumbency in rats during forced exposure (Makowska and Weary, unpublished data).

4.3. Future directions

Rats have shown aversion to every inhalant euthanasia agent tested to date, and in practice it may not be possible to develop a procedure for killing rats that is completely devoid of stress. However, the goal is clearly to minimize any pain and distress associated with the procedure. In tests of aversion to CO_2, argon and inhalant anaesthetics, it was possible to establish the amount of time rats were willing to tolerate gas exposure, and the approximate amount of time rats would have to spend in the chamber from the onset of this aversion until

unconsciousness during euthanasia. However, quality of exposure is likely just as important as duration, so it cannot be assumed that the method minimizing the time to unconsciousness or the time between onset of aversion and unconsciousness is the most humane option. Similarly, it may be wrong to assume that a sedated animal will find exposure beyond the point of aversion less aversive than a non-sedated animal (e.g. isoflurane vs. argon). In order to determine which euthanasia agent rats perceive as least aversive, it is necessary to assess rats' subjective experience from beginning of gas exposure until unconsciousness with the various agents used to euthanize laboratory rats.

One experimental approach to this issue would be to repeatedly subject rats to the euthanasia agents (e.g. CO_2, argon and isoflurane) up to the point of unconsciousness and then allow recovery. If rats learn to associate entrance into the euthanasia chamber with gas exposure, then any responses they show can be associated with their expectation of another exposure. In this experiment, we could record behavioural and physiological signs of stress, including defecation / urination, freezing and rapid breathing (Young, 2006), and high plasma cortisol levels (Moberg, 2000), when animals are returned to the euthanasia chamber. Half of the animals from each group could receive an anxiolytic before testing to identify which of the observed responses are associated with anxiety *per se*. The prediction is that animals associating the chamber with an aversive experience would exhibit more signs of stress than animals associating it with a less aversive experience, and that animals that received an anxiolytic would exhibit fewer signs of stress than animals that did not.

This first study could be complemented by two additional studies that could take advantage of the innovative new approaches to assessing subjective states in animals. Specifically, these studies could assess the long-term effects of repeated exposure to an aversive procedure. The first study could record whether treatment groups differ in their consumption of water containing an anxiolytic. Earlier work has shown that animals in pain will self-administer analgesics, but to my knowledge only one other study to date has used self-administration of

anxiolytics as a measure of the subject's anxiety (Sherwin and Olsson, 2004). My prediction is that animals exposed to an aversive gas would be more anxious, and therefore consume more water containing an anxiolytic than animals exposed to a less aversive gas. The second study could borrow from research on 'cognitive bias' in humans that has recently been applied to questions in animal welfare. These studies have shown that people and animals that are anxious or depressed tend to interpret ambiguous stimuli more negatively than healthy controls (Harding et al., 2004; Bateson and Matheson, 2007). Rats could be trained to perform an operant response (e.g. lever press) to a tone that is paired with a positive event (e.g. arrival of food) and avoid the response to another tone paired with a negative event (e.g. noise). During the test phase rats could be presented with un-reinforced ambiguous tones that are intermediate between the two training stimuli. The prediction is that rats repeatedly exposed to an aversive gas would be more likely to interpret the ambiguous tones negatively than those exposed to a less aversive gas. These studies would be the first to recommend a euthanasia agent based on direct comparisons between the various available agents.

4.4. Conclusion

In order to conform with regulations and satisfy our moral obligation to research animals, the method we use for euthanizing laboratory rodents should minimize pain and distress. The most commonly used method of euthanasia, exposure to CO_2, is known to be aversive to rodents but continues to be used because there are no proven humane alternatives. The aim of my thesis was to test rat aversion to argon and inhalant anaesthetics to assess whether these agents were humane alternatives to CO_2. Although many scientists hypothesized that argon could be humane (Raj and Gregory, 1991; Gerritzen et al., 2000; Young, 2006), the results from Chapter 2 indicate that rats are avoid argon-induced hypoxia. Results from Chapter 3 show that the inhalant anaesthetic isoflurane appears to be the most humane agent tested to date, since rats were already partially sedated at the onset of aversion. A study that would assess

rats' subjective experience from beginning of gas exposure until unconsciousness with the various euthanasia agents would be useful in confirming this suggestion.

4.5. References

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