Assessing the Use of Sedation Prior to Carbon Dioxide Euthanasia of Mice

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ABSTRACT

Carbon dioxide (CO_2) administration is the most commonly used method of euthanasia of mice in research, yet questions remain regarding whether CO_2 euthanasia is associated with pain and stress. This study aims to characterize the level of pain and stress induced in mice during CO_2 euthanasia, and to determine if premedication with acepromazine or midazolam, or anesthetic induction with isoflurane, alters these levels during CO_2 euthanasia. Mice were assigned to one of six euthanasia groups: (control) CO_2 only at a flow rate that displaces 20% of the cage volume per minute (V/min); premedication with acepromazine (5 mg/kg), midazolam (5 mg/kg), or saline followed by 20% V/min CO_2; induction with 5% isoflurane followed by > 100% V/min CO_2; or 100% V/min CO_2 only. Behavioral measures of stress included ultrasonic sound recordings and analysis of video recordings, by an observer blinded to group identity, to assess increased respiratory effort, increased activity, and pain. Physiological parameters of stress were assessed by measuring plasma adrenocorticotropic hormone (ACTH) and corticosterone levels immediately post-euthanasia. Finally, we assessed the acute neuromolecular marker of pain and stress, *c-fos*, by quantitative PCR. The use of premedication with acepromazine or midazolam did not significantly alter behavioral indicators of stress but did significantly induce a higher level of *c-fos* expression in the brain compared to 20% V/min CO_2 alone. Furthermore, the use of isoflurane induction prior to CO_2 euthanasia significantly increased stress in the mice based on both behavioral and neuromolecular indicators. These data strongly indicate that in comparison to the other modalities analyzed in this study, 20% V/min CO_2 is a humane, rapid euthanasia method that is not associated with significant pain or stress in mice.

BIOGRAPHICAL SKETCH

Helen Valentine was born in Ormskirk, England and raised in various parts of England and the USA. She received a Bachelor of Arts in 2000 from New York University following completion of a double major in Biology and Psychology. She received a Doctorate of Veterinary Medicine from Cummings School of Veterinary Medicine at Tufts University in 2008. Following veterinary school, she completed a Laboratory Animal Medicine Residency at Cornell University within the Cornell Center for Animal Resources and Education. During her residency, she was accepted into the Cornell Graduate School in the Field of Comparative Biomedical Science from which she will be receiving a Master of Science in August 2011.

This is dedicated to the mice used in teaching and research.

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CHAPTER ONE:

INTRODUCTION

Carbon dioxide (CO_2) administration is a frequently used form of euthanasia in laboratory mice due to its ease of use, availability, low expense, and high level of personnel safety (3; 12). Furthermore, there is no need to handle or manipulate mice prior to euthanasia, thereby minimizing any additional handling stress. However, questions remain with respect to the level of pain and stress that mice experience during CO_2 euthanasia (12; 24; 41).

CO_2 stimulates receptors in the nasal mucosa (62). Pain associated with this stimulation is concentration dependent and studies in humans indicate that one full breath of CO_2 at concentrations ranging from 50 to 100% can induce pain (14). Human subjects have reported that exposure to 50% CO_2 induced tingling sensations and that it was associated with an unpleasant odor or taste; however, high CO_2 concentrations (100%) induced pain which was variably described as piercing or stabbing (14). Rodents, like humans, posses these receptors in the nasal mucosa (62). Rodent studies assessing the use of CO_2 as a euthanizing agent are less clear, and some suggest that CO_2 induces pain and stress while others indicate there are no signs of pain or stress (12; 14; 24).

Concerns over the use of CO_2 have led the Canadian Council for Animal Care (CCAC) to recommend the use of an inhalant pre-anesthetic followed by CO_2 for euthanasia of rodents (10). Additionally, the Morris Animal Foundation does not consider the use of CO_2 alone to be an appropriate method of euthanasia and will not fund projects that propose to use CO_2 for euthanasia without premedication (52). In some larger species, pre-medication prior to

euthanasia is standard practice; however, the potential benefit of this practice has not been prospectively analyzed in mice. Therefore, we tested the hypothesis that pre-anesthetics can alleviate pain and stress associated with CO_2 euthanasia. Specifically, we evaluated three pre-anesthetics to determine if their use before euthanasia would affect behavioral, physiological or neuromolecular indicators of pain and stress during CO_2 euthanasia. Additionally, we evaluated the effect of high (100% V/min) and low (20% V/min) flow rate of CO_2. Behavioral measures of pain and stress analyzed in this study included ultrasonic sound recordings and video recordings examined post-hoc by a blinded observer trained in assessing pain and stress in rodents. Physiological parameters of stress measured in this study included plasma adrenocorticotropic hormone (ACTH) and corticosterone levels. Neuromolecular assessment of pain and stress was evaluated from fresh brains utilizing quantitative PCR (qPCR) detection of *c-fos* mRNA, a well established immediate early brain marker of pain and stress (26; 33; 72).

CHAPTER TWO:

LITERATURE REVIEW

Existing Recommendations for the CO_2 Euthanasia of Rodents

According to the 2007 American Veterinary Medical Association (AVMA) Guidelines on Euthanasia, CO_2 euthanasia should only be performed using a compressed CO_2 gas cylinder. In addition, the optimal flow rate of CO_2 should displace at least 20% of the euthanasia chamber volume per minute (V/min) (1). The American College of Laboratory Animal Medicine has issued a report of rodent euthanasia that states a similar recommendation (3). Specifically, CO_2 should be added to existing room air in the euthanasia chamber at a fill rate of 20% of the chamber V/min in order to achieve a balanced gas mixture that will provide rapid unconsciousness with minimal distress to the animals.

In Canada, recommendations differ in regard to CO_2 euthanasia of rodents. According to the Canadian Council on Animal Care in Science (CCAC), CO_2 should not be used when other methods of euthanasia are practical for the species and the experiment. However, if CO_2 is used, whenever practical, animals should be anesthetized, preferably using an inhalant anesthetic, prior to CO_2 exposure. Furthermore, if CO_2 must be used without prior anesthesia, a gradual-fill rate of 20% to 30% of the chamber V/min should be used (10).

CO_2 as a Pain Stimulus

The controversy over the use of CO_2 for euthanasia stems from the ability of inhaled CO_2 to induce pain in humans (2; 14; 30; 38) and rodents (40; 62; 75). When CO_2 comes into contact

with moisture on the nasal or oral mucosa it forms carbonic acid which stimulates nociceptors in the mucosa and triggers pain via the trigeminal nerve (40). Rodents, like humans, have been shown to have these nociceptors in the nasal mucosa (62).

In human studies that assess CO_2 as a pain stimulus, specific concentrations of CO_2 are delivered directly to the nasal cavity through either a face mask or Teflon tube (2; 14; 30; 38). In one study, human volunteers were asked to rate the degree of discomfort associated with one full breath of 50 to 100% CO_2 mixed with oxygen (14). The volunteers reported that increasing concentrations of CO_2 were progressively more noxious, with 50% CO_2 being considered "highly unpleasant" bordering on "uncomfortable" and 100% CO_2 being rated as "painful". In another study, a series of stimuli (2s duration) that consisted of 5 to 100% CO_2 were applied directly to the nasal cavity via a Teflon tube at 5% increments in either an ascending or descending series (2). The average individual pain thresholds ranged from 32.5 to 55.0% CO_2 (mean 47.1%).

Rodent studies that use CO_2 as a pain stimulus also apply specific concentrations of CO_2 directly to the nasal cavity while the animal is under anesthesia (40; 62; 75). One such study in rats examined the activity of chemonociceptive medullary dorsal horn neurons in response to noxious chemical stimulation using pulses of CO_2 applied to the nasal mucosa (75). The response threshold of most of the neurons tested ranged from 37 to 50% CO_2, which corresponded closely with a human study that used a similar CO_2 stimulus delivery device in which a mean pain threshold of 47% was obtained (2; 75).

Nociceptor electrical responses have been recorded from the nasal respiratory epithelium in response to CO_2 exposure in both humans (76) and rats (75). Through the application of electrodes in the nasal septum of human volunteers, negative mucosal potentials (NMPs) were

recorded during stimulation of the trigeminal nerve by a number of noxious stimuli (isoamylacetate, acetaldehyde, CO_2), but not following stimulation of the olfactory nerve with hydrogen sulphide (76). The NMPs are thought to be a summation of chemosensitive nociceptor potentials of the trigeminal nerve. Both increasing CO_2 stimuli duration and concentration caused a significant increase in NMP amplitudes and area under the curve. In addition, NMPs were restricted to the site of stimulated receptors in that they were only recorded from the nostril that received the CO_2 and not the contralateral nostril. In a similar study conducted in rats, NMPs were recorded from the nasal respiratory mucosa following stimulation with CO_2 (75). Local administration of topical analgesia (capsaicin and lidocaine) eliminated the NMPs during CO_2 exposure, supporting the theory that NMPs are the result of chemosensitive nociceptor activation.

These studies have been used to support the finding that CO_2 euthanasia is not humane through the argument that similar concentrations to those used for euthanasia have been used to elicit pain (12; 41; 57; 58; 75). However, this argument fails to account for the method in which CO_2 is delivered in pain studies. Specifically, in the pain studies, set concentrations of CO_2 are applied directly to the nasal mucosa, whereas euthanasia guidelines in the USA and Canada recommend that rodents be euthanized using a gradual fill method of CO_2 exposure, thereby exposing the animals to a gradual increase in CO_2 concentration. Additionally, the average pain threshold for CO_2 concentration was between 32.5 to 55.0% in humans (2) and 37.0 to 50.0% in rats (based on nociceptor stimulation) (75). Using the recommended CO_2 fill rate of 20 – 30% chamber V/min allows the rodents to be partially sedated or unconscious prior to being exposed to pain-eliciting concentrations of CO_2 (1; 3; 10).

Approach – Avoidance Testing

Approach-avoidance tests are used to determine an animal's relative aversion to a particular stimulus, such as CO_2 exposure (35). A set level of food deprivation occurs prior to testing. During the test, food rewards are made available to the animal during exposure to gas stimuli. If the hungry animal is unwilling to tolerate the stimulus for food, the stimulus is considered aversive. This test is based on an assumption of additivity in that tolerance of an aversive stimulus should increase as the level of hunger increases (35).

The approach-avoidance test involves housing the animal in the test apparatus which consists of two transparent cages with wire lids that are connected by a sloped opaque tunnel (Figure 1) (58). The top home cage contains bedding, food and water and the bottom test cage contains just bedding. The animal has access to both cages. Prior to the experiment, the animal is trained to enter the test cage to receive a food reward. During the experiment, the test apparatus is moved to a fume hood and the wire lid on the test cage is replaced with an acrylic lid fitted with a gas inlet in the center, two air outlets at the end of the cage closest to the tunnel, and a gas sampling tube at the end of the cage opposite the tunnel (Figure 1). Initially, the animal is locked in the home cage. After the lock is removed, the animal is able to enter the test cage for a food reward, such as 20 pieces of sugared cereal. As soon as the animal enters the test cage and starts eating, either air or the test gas is initiated at a pre-determined rate. The test ends once the animal leaves the test cage, and percentage of gas in the cage (as determined through the gas sampling tube), time to exit the test cage, and amount of food eaten can be used as measures of aversion to the test gas (58).

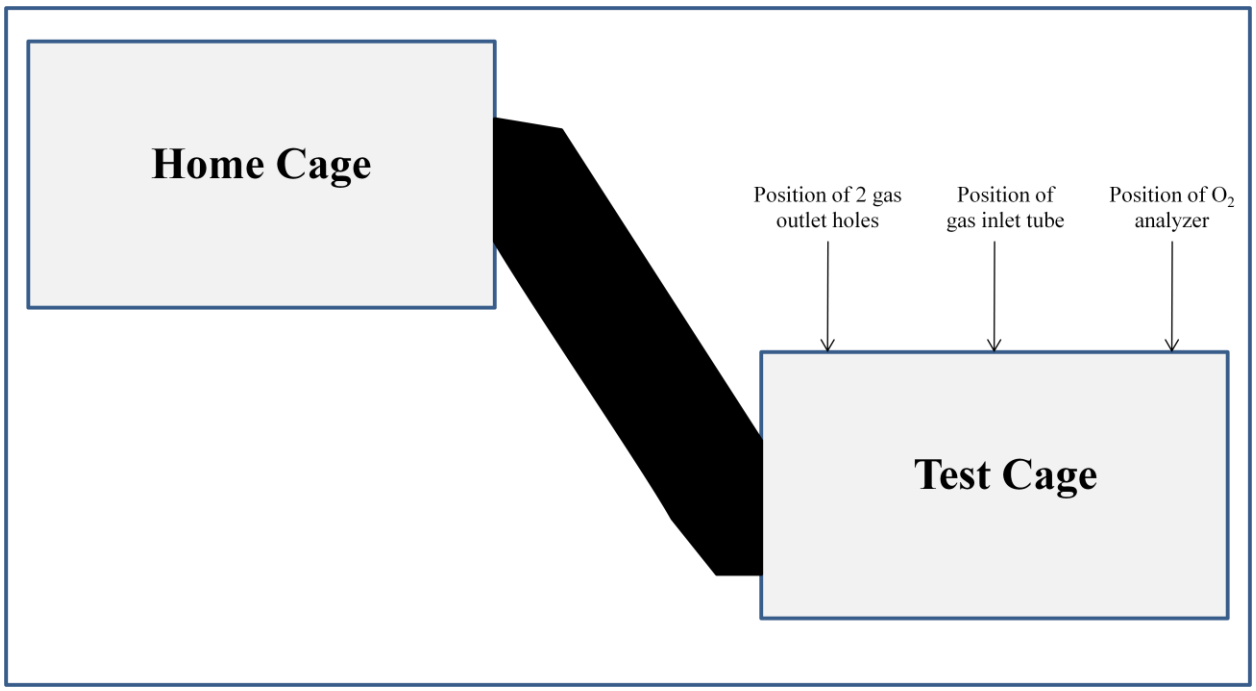

Figure 1: Approach – Avoidance Test Experimental Apparatus. See text for details.

Many studies that argue against the use of CO_2 euthanasia use an approach-avoidance test to measure how aversive CO_2 is to rodents (36; 45; 57; 58). In one such experiment, rat aversion to CO_2 was tested by examining rats' responses to static CO_2 concentrations of 5, 10, 15, and 20% (58). For static exposure, the test cage was pre-filled with the designated concentration of CO_2 before the rats were given access. At static CO_2 concentrations of 5 and 10%, the rats ate all 20 food reward items. There was a significant decrease in time spent in the test cage and number of food items eaten with 15 and 20% CO_2. At 20% CO_2 the majority of rats refused to eat and left the test cage immediately. When the rats were exposed to a gradual fill of CO_2 at a flow rate of 17% of the chamber V/min, they stopped eating and left the cage when the chamber CO_2 concentrations reached on average 17.3 ± 2.1% and 18.4 ± 2.0%, respectively (58). In a similar

study, mice exposed to a CO_2 flow rate of 18% of the chamber V/min left the test cage on average at a CO_2 concentration of 18.2 ± 2.0% (45).

However, a study that tested the validity of using an approach-avoidance test to measure the strength of aversion to CO_2 in rats found that when food deprivation prior to testing exceeded seven hours, hunger made the rats less willing to tolerate CO_2 exposure for food instead of motivating them to stay longer (35). The authors attributed these results to increased anxiety due to hunger and therefore cautioned the use of food deprivation to assess the strength of aversion to CO_2 (35). While these studies do use a gradual fill method of introducing the gas to the rodents, the test ends once the rodent chooses to leave the gas chamber and euthanasia is not a part of the study. These studies confirm that exposure to CO_2 is more aversive than a particular food item is attractive; however, aversion does not indicate pain or stress. In fact, other approach-avoidance studies use stimuli that are aversive to rodents but would not be considered painful or stressful, such as a brief puff of air (77). Furthermore, aversive responses in female mice have been demonstrated in mate preference tests in which mice have been shown to display aversive responses to odors of male mice that are infected with the murine louse, *Polyplax serrata*, which demonstrates that aversive behaviors do not only coincide with pain and/or stress (32).

Additionally, the CO_2 approach-avoidance studies fail to adequately account for the evolutionarily conserved avoidance response to hypoxia. Specifically, avoidance of a hypoxic environment can be demonstrated in organisms as primitive as nematodes (*Caenorhabditis elegans*), which lack the cognitive or anatomic features necessary to experience pain as it is currently defined (7). Hypoxia avoidance has also been demonstrated in fish that will not tolerate hypoxic conditions to reach a food source (28; 44) and in rats that will avoid carbon monoxide, which has not been found to be painful in humans, in an approach-avoidance test (46).

Argon as an Alternative to the use of Carbon Dioxide

Argon (Ar) is an odorless, inert, non-flammable, and non-explosive gas, which unlike CO_2, is non-irritating to the nasal mucosa and therefore has been suggested as an alternative to CO_2 euthanasia of rodents (1; 58). Mouse and rat aversion to Ar (93.7 to 99.2%) was compared to CO_2 (25.5 to 50.8%) and a CO_2/Ar mixture (15.5 to 59.2/5%) (41). The test apparatus consisted of one test chamber that was prefilled with a specific gas concentration and connected to either one regular air chamber (rats) or four regular air chambers, one on each side (mice). No food reward was provided in the test chamber and animals had free range of the test chamber and all regular air chambers. The animals were introduced into the test apparatus via a flap in one of the connecting tubes near the entrance to the test chamber. Each test lasted three minutes and initial withdrawal time from the test chamber and total dwelling time in the test chamber were measured as indicators of aversion for each of the gases. Results from this study suggested that Ar was less aversive to both mice and rats than either CO_2 alone or a CO_2/Ar mixture based on longer withdrawal times and total dwelling times within the test chamber.

As a follow up to the previous study, an approach – avoidance test was performed to test rat aversion to Ar compared to CO_2 when each gas was paired with a food reward (58). In this study, rats were tested with a gradually increasing concentration of CO_2 at a flow rate of 17% of the chamber V/min, slightly lower than current CO_2 euthanasia recommendations (1; 3; 10), and a static concentration of 90% Ar, which is a suggested replacement for CO_2 euthanasia (58). During CO_2 exposure, rats stopped eating and left the test chamber when CO_2 concentrations reached 17.3 ± 2.1% and 18.4 ± 2.0%, respectively. However, when the test chamber contained 90% Ar, no rats ate the food and they either refused to enter or left the test chamber immediately. Results of this study indicate that Ar is more aversive for rats when exposure is consistent with

euthanasia recommendations for each gas. These findings contradict the previous study (41) which did not take into account the gradual fill recommendation for CO_2 euthanasia.

Finally, euthanasia of rats using Ar at 50% chamber V/min was compared to euthanasia using CO_2 at 10% chamber V/min by assessing heart rate and behavior (9). The heart rate of rats exposed to Ar did not change, whereas the heart rate of rats exposed to 10% chamber V/min CO_2 declined significantly, likely due to the sedative properties of CO_2. Additionally, rats euthanized with Ar gasped and demonstrated seizure-like activity, both of which were not seen in the CO_2 euthanized group. The authors concluded that Ar as a sole euthanizing agent was highly aversive to rats, whereas 10% chamber V/min CO_2 did not result in any overt behavioral indicators of distress (9).

Overall, these findings suggest that Ar is not a suitable replacement for CO_2 euthanasia. Additionally, according the 2007 AVMA Guidelines on Euthanasia, rodent euthanasia using Ar is only conditionally acceptable when oxygen concentrations below 2% are achieved rapidly and the animals are heavily sedated or anesthetized, therefore other methods of euthanasia are preferable (1).

Isoflurane Anesthesia Prior to CO_2 Euthanasia

According to the CCAC euthanasia guidelines, whenever practical, animals should be anesthetized, preferably using an inhalant anesthetic, prior to CO_2 euthanasia (10). The two studies that were cited in support of this recommendation used approach-avoidance tests to assess rodent aversion to gas stimuli (41; 47). However, one study assessed rat and mouse aversion to pre-filled chambers of CO_2, CO_2/Ar mixture, or Ar without the presence of a food reward in the test chamber (41), while the other study compared rat aversion to gradual fill

halothane or isoflurane with a food reward in the test chamber (47). In the CO_2/Ar study, the authors concluded that induction with CO_2 either alone or in combination with Ar is likely to cause considerable distress in rodents before they lose consciousness (41). This finding was based upon significantly shorter initial withdrawal times and total dwelling times in the test chamber that was pre-filled with CO_2 at concentrations that ranged from 25.5 to 50.8% compared to the control of room air. The halothane/isoflurane study concluded that both inhalant anesthetics were aversive to rats (47). However, because some of the rats remained in the test cage long enough to become ataxic, the authors concluded that the rats were likely in a state of partial sedation at the time they chose to leave the test cage, therefore, continued forced exposure from the onset of aversion to unconsciousness may be more humane than forced exposure to CO_2. The two studies were not comparable in their design (pre-fill vs. gradual fill; no food reward vs. food reward), and neither study directly compared aversion of CO_2 to isoflurane.

In an approach-avoidance study that did assess both CO_2 and isoflurane, isoflurane was still found to be less aversive than CO_2 (45). On average, mice left the test cage when CO_2 concentrations were between 13.5 and 18.2%, and no mice remained long enough to become recumbent. Mice remained in the test cage longer with exposure to isoflurane and two out of six mice remained long enough to become recumbent.

A major drawback of all these studies is that they do not involve euthanizing the rodents or assess any direct or indirect indicators of pain or stress. Further studies that directly assess the use of isoflurane anesthesia prior to CO_2 euthanasia are needed to determine if this recommendation is justified as a more humane method of euthanasia over the use of CO_2 alone.

Behavioral Assessment of Pain and Distress

Pain is a perceived unpleasant sensory and emotional experience that results from actual or potential tissue damage (6). Pain can result from a physiologic stressor such as injury, surgery, or disease; it can result from an environmental stressor such as chemical or thermal; and it can be potentiated by a psychological stressor such as fear and anxiety (56). Distress is an aversive state which occurs when an animal is unable to fully adapt to a stressful situation and the stress manifests itself in the form of maladaptive behaviors (56). For example, in mice distressful maladaptive behaviors may include decreased food intake, lack of grooming, inappropriate social interactions, or poor reproductive performance. Distress can also manifest in physiologic conditions such as gastrointestinal ulceration, hypertension, and immunosuppression.

Stress refers to the effect of external or internal stressors on an animal's biologic equilibrium (56). External stressors may refer to a physical event such as restraint, or environmental factors such as noise, odor, temperature, or the presence of people or other species. Internal stressors may include physiologic factors such as injury or disease, or psychological factors such as fear, anxiety or loneliness. Not all stress is ultimately bad; in fact, it may initiate a response that has potential beneficial effects, such as moving on to a new habitat that has better food resources or less predation. Depending on how well an animal is able to cope, both pain and non-pain stressors may result in either stress or distress. An animal will use behavioral and/or physiological coping mechanisms, such as activation of the sympathetic nervous system and the hypothalamic-pituitary-adrenal axis, to adapt and counteract the disruption to its state of equilibrium. Making the distinction between stress and distress is an important consideration when evaluating animal welfare since an animal experiencing a brief episode of stress is very different from an animal in distress.

Determining whether an animal is experiencing pain or stress is a challenge due to differences in behavioral responses to noxious stimuli between and within species, and the lack of validation between a particular response and the underlying cause. Typically, a change from an individual animal's normal behavior is used as an indication of pain or stress. Examples of pain or stress related changes in behavior include: decreased locomotion, decreased grooming, indifference to surroundings, inappetence, and decreased fecal output (39). However, even these behaviors are not invariably indicative of pain or stress. To make things simpler, in assessing animal welfare, pain and stress are generally discussed together since either one can compromise welfare and need to be avoided. However, to complicate things further, normal behavior does not indicate a pain or stress-free state, especially in prey species which need to continue normal behavior as long as possible to avoid predation or disruption of the social hierarchy.

Assessing whether an animal is experiencing pain and/or stress is further complicated by the subjective nature of such observations. The controversy regarding the humaneness of CO_2 euthanasia centers on the difficulty in interpreting behavioral responses of animals during CO_2 exposure. Different observers draw different conclusions concerning the same animal behaviors, some conclude that no pain or stress was observed while others conclude that significant pain or stress was evident. Each study that uses behavioral observations to assess pain or stress has its own criteria of behavioral indicators. The behaviors assessed are not consistent across studies, thereby making comparison across studies difficult. In addition, an association between the behavior described and an underlying cause, such as pain or stress, may not have been validated, again making the interpretation of a particular behavior subjective.

In one study that evaluated CO_2 euthanasia of rats, assessment of behavior was based on the appearance of the rat's eyes, respiration, overall appearance, defecation, urination, abnormal

activity, locomotion and vocalization (24). The authors described three distinct phases of behavioral patterns during CO_2 euthanasia. In the initial period of gas exposure, rats showed tachypnea and a higher attention span, with no vocalization, urination or defecation. They also interpreted no signs of pain due to the lack of sneezing and tears. The second phase was characterized by continuation of tachypnea, but a lack of movement in which the rats sat quietly and showed reduced attention to their environment. In the final phase, the rats appeared unconscious with total muscle relaxation. While these behavioral parameters and their interpretation appear reasonable, there are no studies to validate their interpretation. While chromodacryorrhea has been associated with pain and stress in rats, the lack of porphyrin secretion does not verify an absence of pain or stress. In a rat restraint trial, in which rats were loosely wrapped with porous adhesive tape in such a way that both forelimbs were bound to the thorax, latency from restraint to chromodacryorrhea ranged from 3 to 40 minutes (25). This finding indicates that even if CO_2 exposure was enough of a noxious stimulus to cause chromodacryorrhea, the duration of exposure prior to death may be too short. Tachypnea is a biological response to hypoxia and therefore cannot invariably be attributed to pain or stress. Defecation and urination are such frequent occurrences in rodents that it is difficult to attribute either to pain or stress. Abnormal activity and locomotion are probably the best indicators a change from the animal's normal behavior, although this is subjective. Since ultrasonic recordings were not a part of this study design, it is unclear if ultrasonic vocalizations were present and undetected. Because of all the questions that can be raised by behavioral analyses, responses need to be interpreted in conjunction with more objective and quantifiable data. In the rat CO_2 euthanasia study (24), serum concentrations of glucose, ACTH and corticosterone were analyzed and were consistent with the authors' behavioral interpretation of no pain or distress.

Additionally, rats that were either sedated with acepromazine or anesthetized with pentobarbiturate prior to CO_2 euthanasia had similar hormone levels as the conscious rats, indicating no increase in pain or stress in the conscious animals during CO_2 euthanasia.

In a study comparing the behavioral and cardiovascular responses of rats to rapid induction or gradual fill CO_2 euthanasia, behavioral parameters that were assessed included: head raising, vocalization, escape, tail lashing, gait, circling, twitching, recumbency, urination/defecation, and respiration (71). Rats displayed inquisitive behaviors, followed by labored breathing and death. Two of the four rats exposed to rapid induction showed head raising behavior at the beginning of the procedure, but this was interpreted as disorientation due to anoxia, and was not considered an indicator of stress. There was no incidence of vocalization, escape attempts or tail lashing. Again there was no report of using ultrasonic recordings to capture any ultrasonic vocalizations. Gait, circling, twitching, recumbency, and respiration were assessed more to describe phases of behavior during CO_2 euthanasia rather than as measures to detect stress. The study included measures of pulse rate and blood pressure as objective measures, both rapidly dropped below control baseline levels, which was consistent with rapid central nervous system depression instead of stress which would be expected to raise both measures. The authors concluded that no overt behavioral signs of pain or distress were apparent in either group.

Behavioral measures of pain and stress are important components of any CO_2 euthanasia welfare study; after all, the animal behavior is what personnel performing the euthanasia witness. However, because those perceptions will vary from person-to-person, it is important to have quantifiable objective measures to support conclusions drawn about observed behaviors. Advances in utilizing behavioral measures may be possible with the use of a Force-Plate

Actometer (Bioanalytical Systems, Inc., West Lafayette, IN), which is essentially an enclosure above a force-plate that is able to quantify animal movements within the enclosure. This device can be used with both mice and rats and can quantify locomotor activity, rotation, startle, ataxia, and stereotypies, such as head bobbing and rearing, grooming, scratching, and whole body tremors (20; 78).

Ultrasonic Vocalization Analysis

Another alternative for quantifying a behavior involves the use of an ultrasonic sound recorder to capture and analyze rodent vocalizations. Rodents communicate with each other through a series of vocalizations that are either within the human audible range or in the ultrasonic range (> 20 kHz), which is above the human audible frequency range (63; 68). Audible sounds, as in most other mammals, are produced by vibrating structures in the larynx (68). Ultrasonic vocalizations (USVs) are produced by expired air against a closed glottis which creates a whistle-like mechanism in the larynx (66). USVs in rodents are speculated to have evolved because they provide an intra-species highly-directional method of communication over a short distance without alerting predators such as birds, many of which are unable to hear sounds above 25 kHz (60). Ultrasounds also have a high rate of attenuation and are easily deflected by small objects such as grass or twigs and even dust particles in the air, therefore even predators at a distance that have ultrasonic hearing abilities are not likely to be alerted by rodent USVs (60). Although ultrasounds do not carry well across distances, they are easily localized over short distances, for example, a young rodent pup cry easily enables the mother to locate the pup without alerting distance predators (60).

Three classes of USVs have been characterized in the laboratory rat (*Rattus norvegicus*) (8; 63; 68). Infant rats emit USVs in response to a number of distressful situations such as

separation from the dam, isolation from the nest, unfamiliar environment or odors, and thermal or tactile stimuli (8; 68). Juvenile and adult rats emit two distinctive USVs, a high-pitched and short "50 kHz vocalization" and a low-pitched and longer "22 kHz vocalization" (42; 63; 68). The 22 kHz vocalizations have frequencies between 18 and 32 kHz, last 300 to 4000 ms, and are associated with a number of aversive situations such as exposure to a predator, inescapable foot shocks, startling noises, and during male-on-male aggression and social defeat (42; 63). During 22 kHz vocalizations, rats may exhibit behaviors such as tense, motionless couching (freezing) and pronounced breathing, which reflect a negative affective state of the animal (63). The 50 kHz vocalizations have frequencies between 32 and 96 kHz, last 30 to 50 ms, and are associated with non-aversive situations such as during sexual behaviors, juvenile play, and pleasant manual tactile stimulation (tickling) by investigators (63). These vocalizations have not been recorded during freezing behaviors and are believed to indicate a positive affective state of the animal (63).

Since USVs are an objective quantifiable measure, many studies have been conducted to validate vocalization as an indicator of stress (8; 23; 37; 42; 51; 60; 63; 68). Because rats will often produce conditioned anticipatory stress vocalization upon re-exposure to an environment in which they previously experienced an aversive stimulus, USV has been used as a measure to test the anxiolytic potential of pharmacological agents (18; 51; 68). Anxiolytic benzodiazepines, such as chlordiazepoxide and diazepam which reduce anxiety in humans, have been shown to reduce the USVs induced by tail-holding stress in rat pups at doses that had little central nervous system depressant activity (21). Reduction of maternal separation induced USVs has been demonstrated in rats using anxiolytic drugs such as benzodiazepines and serotonergic agents (51).

While stress vocalizations are not well characterized in mice, numerous studies have successfully used maternal separation USVs in mouse pups to test the anxiolytic effects of benzodiazepines and serotonergic agents (5; 18; 54; 55). As seen in rats, mouse pups treated with midazolam (benzodiazepine) or allopregnanolone (a serotonergic agent) dose dependently emitted fewer USVs than vehicle-treated pups when separated from dam and littermates (18). These findings support the use of USVs as a measure of stress in infant mice as well as rats; however, continued research needs to be conducted to further characterize the USV patterns of adult mice.

Biochemical Indicators of Pain and Stress

Biochemical indicators of pain and stress are commonly included in CO_2 euthanasia welfare studies to provide quantifiable objective support to behavioral measures of pain and stress. Acute stress caused by pain or noxious stimuli results in activation of the hypothalamic-pituitary-adrenal (HPA) axis which is a major component of the neuroendocrine system that controls an animal's reaction to stress. The hypothalamus secretes corticotrophin-releasing factor, which in turn stimulates the pituitary to release ACTH, which stimulates the release of glucocorticoids from the adrenal cortex (53). Increase in levels of glucocorticoids is an adaptive mechanism that allows the animal to cope with stressors by mobilizing body reserves in preparation for the fight or flight response (53). ACTH and glucocorticoids reach their target organs via the blood stream which allows for the evaluation of their concentration in the peripheral circulation to be utilized as a quantifiable biochemical indicator of pain or stress across many species (27; 53; 59; 61). However, it is important to note that increases in ACTH and glucocorticoids do not invariably indicate pain or stress. Equivalent levels of glucocorticoids have been demonstrated in stallion plasma at all time points between 0 and 30 minutes following

sexual stimulation and stressors such as physical exercise, restraint via a twitch, and epinephrine administration (11).

In rodents, corticosterone is the primary glucocorticoid produced in response to pain or stress, and therefore, plasma ACTH and corticosterone concentrations are commonly assessed in rodent studies when animal welfare is in question (61). The stress of handling has been shown to double the baseline level of corticosterone in rats in as little as two minutes (43). However, because factors other than pain or stress can affect levels of these hormones, it is important to interpret their concentrations in conjunction with other parameters of pain and stress.

Molecular Marker of Pain or Distress

Neurons in the brain and spinal cord express the immediate early gene *c-fos* in a transient and rapidly induced manner in response to stimulation (15; 26; 74). While most research on *c-fos* has been conducted in rats, studies have also shown that noxious stimulation induces *c-fos* expression in mice, fish, insects, humans, and various other species (4; 16; 34; 65; 67). Various types of noxious stimulation, including chemical, mechanical and thermal, induce elevated expression of *c-fos* (26). For example, injection of dilute formalin into a hind-foot induces an increase in *c-fos* expression consistent with nociceptive behaviors of flinching and licking of the injected foot (17). Injection of dextromethorphan, but not saline, prior to formalin injection suppresses both the increase in *c-fos* expression and the nociceptive behaviors, confirming that *c-fos* elevation is due to nociception of the formalin and not formalin itself (17).

Acute restraint stress has also been shown to stimulate expression of *c-fos* in the rat cortex, hippocampus, hypothalamus, septum, and brainstem (50). Similar to plasma corticosterone concentration, habituation of *c-fos* responses occur with repeated exposure to the same stress. Specifically, *c-fos* expression becomes significantly smaller in animals restrained

once daily for four days, and nonexistent in animals restrained once daily for nine days. In addition, the habituation of *c-fos* expression is stressor specific: exposure of restraint-adapted rats to a novel stress, such as 20 minutes of swim time, produces an increase in *c-fos* expression comparable to rats exposed to swimming for the first time (50). Elevation of *c-fos* expression has also been shown with exposure of rats to an environment in which they had previously received foot shocks (4). The benzodiazepine, Diazepam, produces a dose-dependent decrease in conditioned stress-induced *c-fos* expression in most regions of the brain that would otherwise have shown increased *c-fos* expression (4).

Since both nociception and stress induce activation of *c-fos* and the half-life is relatively short, expression of *c-fos* closely mirrors the intensity and duration of the noxious stimulus (15; 26; 74). Therefore quantification of *c-fos* mRNA can be used as an additional indicator of pain and stress in mice.

CHAPTER THREE:

SEDATION PRIOR TO CARBON DIOXIDE EUTHANASIA

MATERIALS AND METHODS

Animals

Female CD-1 mice (n=10/group; age 8 - 11 weeks of age) were purchased from Charles River Laboratories and acclimated for a minimum of one week prior to experimental procedures. All mice were screened by the vendor and were deemed specific-pathogen free for all commonly tested bacterial and viral pathogens and parasites. All animal procedures were approved by Cornell's Institutional Animal Care and Use Committee.

Housing

Mice were housed in an AAALAC-accredited facility in groups of 2-3 in individually ventilated polycarbonate cages [11.5 in (l) x 6.5 in (w) x 5 in (h)], with autoclaved corncob bedding (7097A; Harlan Teklad, Fredrick, MD). Cages were maintained on a Micro-FLO/Micro-VENT Environmental Rack System (Allentown Caging Equipment Company, Allentown, NJ). The mice were maintained in a temperature (70 ± 2 ^{o}F) and humidity (30-70%) controlled room with a 14 hour light and 10 hour dark cycle. All mice had free access to food (7912; irradiated maintenance mouse diet, Harlan Teklad) and acidified reverse-osmosis water through an automated watering system (Edstrom, Waterford, WI). All cages were provided with sterile nestlets for enrichment.

Experimental Design

Mice were weighed the day prior to euthanasia and were randomly allocated into six euthanasia groups (n=10/group; Table 1). Group 1 (control) was euthanized by CO_2 alone. Group 2 received acepromazine (5mg/kg, 0.20 ml volume in sterile saline) via an intraperitoneal (IP) injection 10 minutes prior to euthanasia. Group 3 received midazolam (5mg/kg, 0.20 ml volume in sterile saline) IP 10 minutes prior to euthanasia. Group 4 received a 0.20 ml IP injection of saline 10 minutes prior to euthanasia (negative control for IP injection). Groups 1-4 were all euthanized with CO_2 at a flow rate of 20% chamber air displacement per minute (20% V/min; 1.2 L/min). Group 5 was anesthetized with 5% isoflurane at a flow rate of 1.2 L O_2 per minute until all mice in the cage were unconscious and then immediately euthanized with CO_2 at a flow rate of > 100% V/min (consistent with the CCAC recommendation). Group 6 was euthanized by CO_2 at flow rate of 100% V/min (6.1 L/min). Flow meter setting was calculated using the following formula: (% air displacement per min) × (volume of cage in L).

Table 1. Experimental Groups of Mice.

Group[1]	Premedication[2]	Displacement rate V/min
1	None	20%
2	5 mg/kg acepromazine 10 min prior to euthana	20%
3	5 mg/kg midazolam 10 min prior to euthanasia	20%
4	0.20 ml saline 10 min prior to euthanasia	20%
5	5% isoflurane immediately before euthanasia[3]	>100%
6	None	100%

[1]Each group included 10 mice.

[2]Premedications given in 0.20 ml volume per mouse.

[3]Flow rate of 1.2 L O_2/min until all mice were unconscious, then euthanized with >100% CO_2.

Behavioral measures of pain and stress.

Separate digital video recordings and sound recordings of each cage were collected for approximately two minutes immediately following administration of premedication (induction), two minutes immediately prior to gas administration (pre-euthanasia), and for the duration of gas administration (euthanasia). Video recordings were collected using a Sony digital video camera directed at the long axis of the cage. Each video recording was assigned a random number and blindly scored for increased respiratory effort (a marker of dyspnea), increased locomotion (a behavioral marker of stress and agitation), and presence of any behaviors indicative of pain on a scale of 0-3 (0 = none, 1 = mild, 2 = moderate, 3 = severe) by an individual that was unfamiliar with the study design or the group identity. Time to unconsciousness and time to death were recorded for each mouse starting from the onset of CO_2 exposure. Unconsciousness was defined as cessation of voluntary movement, and death was defined as complete cessation of breathing.

Ultrasonic sound recordings.

Sound recordings in the range of 0-120 kHz were collected for two minutes prior to gas administration (pre-euthanasia) and during gas administration (euthanasia) using an ultrasonic microphone (USG 116 to 200 UltraSoundGate Kit, Avisoft, Berlin, Germany) to capture vocal emissions made by the mice. Sound recordings were recorded until the time when mice lost consciousness. The microphone was directed into the cage through a hole in a platform that was placed on top of the cage after removing the bonnet and wire rack (Figure 2). An averaged power spectrogram of each cage recording was created using software provided by the manufacturer (SASLab Pro, version 4.3, Avisoft). Sonograms from two cages of each group were averaged and plotted on a graph comparing pre-euthanasia and euthanasia values.

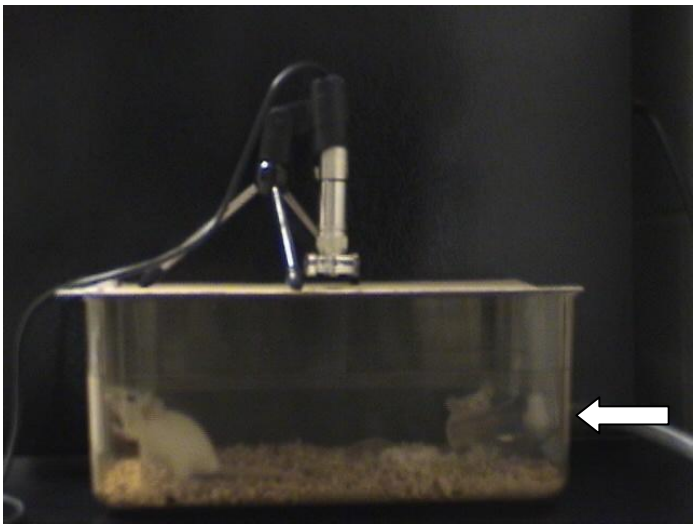

Figure 2: Euthanasia Set-Up. Animals remained in the home cage and CO_2 was administered through the lixit port (arrow) and an ultrasonic microphone was placed over the cage.

Physiological measures of pain and stress.

Immediately following euthanasia, mice were exsanguinated by cardiac puncture and blood was collected in EDTA tubes. Euthanasia of all groups was coordinated to occur at the same time (9-11 A.M.) in a random order to ensure no fluctuations in circadian rhythm and that the order of euthanasia was not a factor in the parameters analyzed. Plasma was isolated and stored at -20^{o}C until assayed for ACTH and corticosterone levels. A chemiluminescent enzyme-linked immunosorbent assay (ELISA) (Calbiotech, Spring Valley, CA) was used to measure plasma ACTH levels and a competitive ELISA (VWR, Radnor, PA) was used to measure plasma corticosterone levels.

Neuromolecular measure of pain and stress.

A coronal midsection of brain tissue, including the hypothalamus, was collected and processed for *c-fos* mRNA quantification. Total RNA was isolated from the samples using the

E.Z.N.A. Tissue RNA kit (Omega Bio-Tek, Inc, Norcross GA) and 1 μg of RNA was converted to cDNA (Quanta biosciences, Gaithersburg, MD 20877). Quantitative PCR was performed for *c-fos* and *Gapdh* mRNA expression utilizing SYBR/Lo Rox (Quanta biosciences, Gaithersburg, MD 20877) and run on an ABI Fast 7500 machine. Expression of *c-fos* was normalized to *Gapdh* (housekeeping gene) and analyzed using the $\Delta\Delta C_t$ method as described previously (49).

Statistics.

All statistical analyses were conducted using GraphPad Prism (version 5.04, GraphPad Software, San Diego, CA). All parametric data sets, including time measurements, ACTH, corticosterone, and *c-fos* mRNA expression were analyzed using a one-way ANOVA and Tukey's post-test. Behavior data were analyzed using a nonparametric Kruskal-Wallis test followed by Dunn's post-test. All data were considered significant at $P < 0.05$.

CHAPTER FOUR:

RESULTS

Time measurements.

Mice euthanized with 100% V/min CO_2 had the most rapid loss of consciousness (39.6 ± 1.9 s), which was significantly faster than the 20% V/min CO_2, saline, and isoflurane groups ($P < 0.05$; 137.2 ± 10.5 s; 112.9 ± 12.7 s; 122.9 ± 16.0 s, respectively; Figure 3). The 100% V/min CO_2 group also reached death (79.9 ± 4.2 s) significantly faster than all other groups, which ranged from 222.3 ± 22.5 to 385.2 ± 46.7 s ($P < 0.05$; Figure 4).

Premedication of mice with acepromazine or midazolam significantly decreased the time to unconsciousness (74.5 ± 8.3 s; 71.6 ± 6.6 s, respectively) compared to 20% V/min CO_2 alone ($P < 0.05$; 137.2 ± 10.5 s; Figure 3). However, premedication with midazolam significantly lengthened the time to death (385.2 ± 46.7 s) compared to 20% V/min CO_2 alone ($P < 0.05$; 234.6 ± 18.4 s; Figure 4). Induction with isoflurane did not reduce time to unconsciousness or death, but instead produced the unwanted side-effect of 5/10 mice recovering consciousness while the cage was being treated with >100% V/min CO_2. Induction with isoflurane required a longer CO_2 exposure time (131.1 ± 18.6 s), although not significantly, than the 100% V/min CO_2 group.

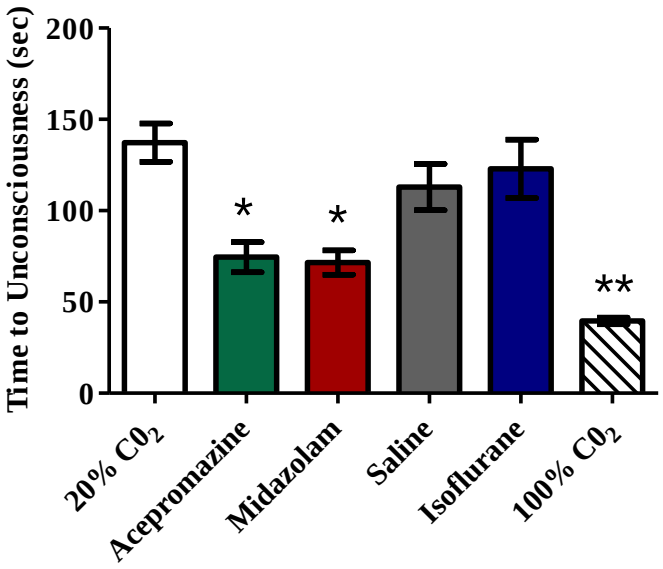

Figure 3: Time to Unconsciousness. Time (mean ± SEM) to unconsciousness was analyzed for each group. ***** indicates a significant decrease in time compared to 20% V/min CO_2 and isoflurane; and ****** indicates significantly shorter time compared to 20% V/min CO_2, saline, and isoflurane. *P < 0.05.*

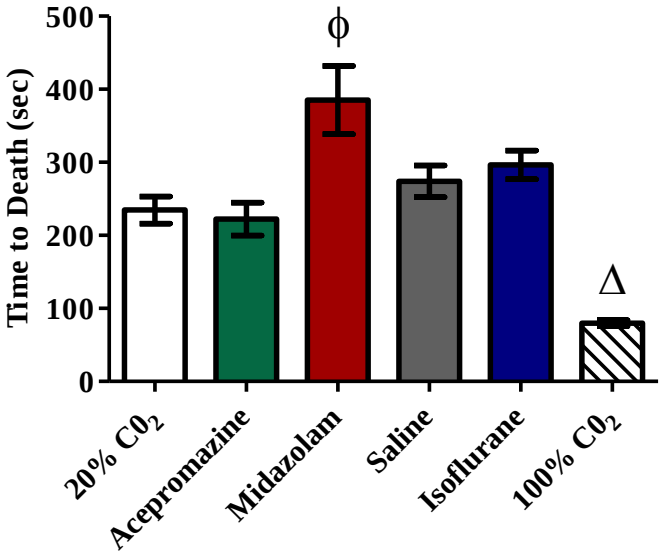

Figure 4: Time to Death. Time (mean ± SEM) to death was analyzed for each group. Despite a shorter time to unconsciousness, mice euthanized with midazolam experienced a significant increase in time to death compared to 20% V/min CO_2. **Φ** indicates a significant increase in time compared to 20% V/min CO_2, acepromazine, and saline; **Δ** indicates significant changes compared to all other groups. *P < 0.05.*

Behavior analysis

To determine if any of the euthanasia methods induced greater evidence of distress, blinded observations were conducted for the induction, pre-euthanasia and euthanasia periods. Groups of mice were compared based upon both treatment and time (induction, pre-euthanasia, euthanasia). Behavior scores at induction were compared between the acepromazine, midazolam, and saline treated groups. Induction with midazolam resulted in significantly higher agitation scores compared to induction with acepromazine ($P < 0.05$; 1.4 ± 0.3 and 0.4 ± 0.2, respectively) but did not differ significantly from the saline control (0.9 ± 0.1; Figure 5). Midazolam induction also resulted in significantly higher agitation scores compared to pre-euthanasia and euthanasia time points ($P < 0.05$; 0.3 ± 0.2 and 0 ± 0, respectively), and higher agitation scores, although not statistically significant, when compared to euthanasia with 20% V/min CO_2 alone. Only two mice received a mild (=1) pain score, one midazolam treated mouse during induction and one acepromazine treated mouse during pre-euthanasia (data not displayed).

During euthanasia, the isoflurane group received agitation scores (3.0 ± 0) that were significantly higher than the 20% V/min CO_2, acepromazine, midazolam, and saline groups ($P < 0.05$; 0.8 ± 0.1; 0.4 ± 0.2; 0 ± 0; 0.7 ± 0.2, respectively; Figure 5). Euthanasia caused a significant increase in dyspnea ($P < 0.05$) in all groups of mice compared to their respective pre-euthanasia scores, but dyspnea scores did not differ significantly between any of the euthanasia groups (Figure 6).

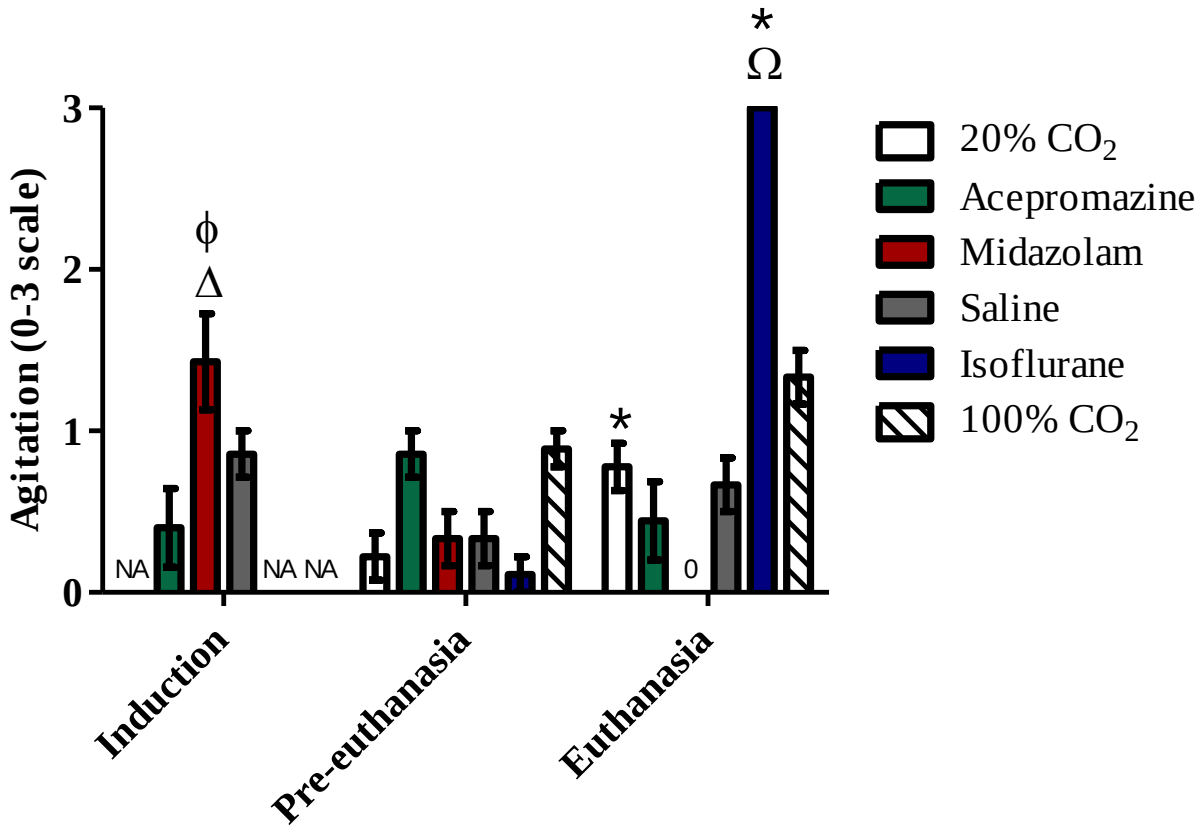

Figure 5: Behavioral Agitation Scores. A blinded observer examined videotapes taken at induction, pre-euthanasia and euthanasia and scored the level of agitation on a scale of 0-3 (0 = none, 1 = mild, 2 = moderate, 3 = severe). Mice anesthetized with isoflurane displayed the highest mean scores of agitation during euthanasia. The level of agitation produced during induction with midazolam was greater, thought not statistically significant, than the level of agitation produced during euthanasia with all other agents except isoflurane. **NA** indicates no applicable data; **Δ** indicates a significant increase in agitation compared to pre-euthanasia and euthanasia time points for that group; **Φ** indicates a significant increase in agitation compared to mice induced with acepromazine; ***** indicates a significant increase in agitation compared to respective pre-euthanasia scores; **Ω** indicates a significant increase in agitation at euthanasia compared to mice treated with 20% V/min CO_2, acepromazine, midazolam, or saline. $P < 0.05$.

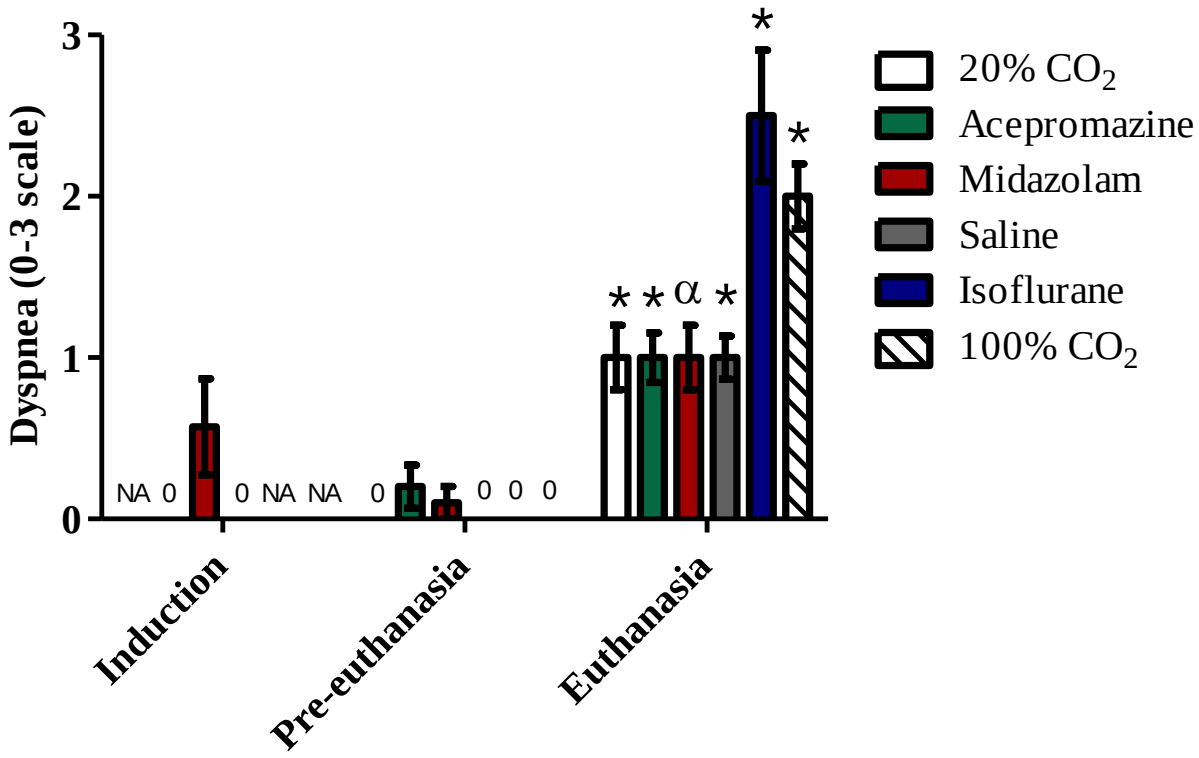

Figure 6: Behavioral Dyspnea Scores. A blinded observer examined videotapes taken at induction, pre-euthanasia and euthanasia and scored the level of dyspnea on a scale of 0-3 (0 = none, 1 = mild, 2 = moderate, 3 = severe). Regardless of treatment, all groups displayed significant dyspnea at the time of euthanasia compared to either pre-euthanasia or induction levels. Mice anesthetized with isoflurane displayed the highest mean scores of dyspnea during euthanasia. **NA** indicates no applicable data; ***** indicates a significant increase in dyspnea compared to respective pre-euthanasia and induction score (if applicable); **α** indicates a significant increase in dyspnea compared to respective pre-euthanasia score. $P < 0.05$.

Ultrasonic sound recordings.

Since mice can emit ultrasonic distress calls, ultrasonic sound recordings were taken to capture any altered vocalizations made during euthanasia. All pre-euthanasia and euthanasia sound spectrograms displayed sound peaks at 26.5 kHz that were not present in background noise control recordings in which no mice were present; therefore, this peak is presumed to be mouse vocalization (blue arrows, Figure 7). Mice euthanized with 20% V/min CO_2 or premedicated with acepromazine had euthanasia spectrograms that were identical to their respective pre-euthanasia spectrograms (Figure 7A and B). Mice premedicated with midazolam had an overall lower amplitude spectrogram during euthanasia for all data points greater than 7 kHz, except at the 26.5 kHz vocalization peak (Figure 7C). This finding is consistent with the marked decreased activity noted in this group, but demonstrates that vocalization was not altered. The saline premedicated group had a higher amplitude spectrogram during euthanasia for all data points greater than 18 kHz consistent with increased activity and increased vocalization (Figure 7D). Mice induced with isoflurane had a large increase in amplitude during euthanasia, which differed from the pre-euthanasia values for all data points greater than 8 kHz, including the 26.5 kHz vocalization peak (Figure 7E), consistent with the noted agitation in this group and increased vocalization. Mice euthanized with 100% V/min CO_2 had some fluctuations of higher amplitude during euthanasia (9-11 kHz, 13-18.6 kHz, 23-27 kHz, 28-32 kHz, and 33-37 kHz; some of which were similar to fluctuations seen in background noise control recordings of 100% V/min CO_2 in which no mice were present (black arrow, Figure 7F); however, there was an increase at the 26.5 kHz vocalization peak similar to that noted in isoflurane treated mice.

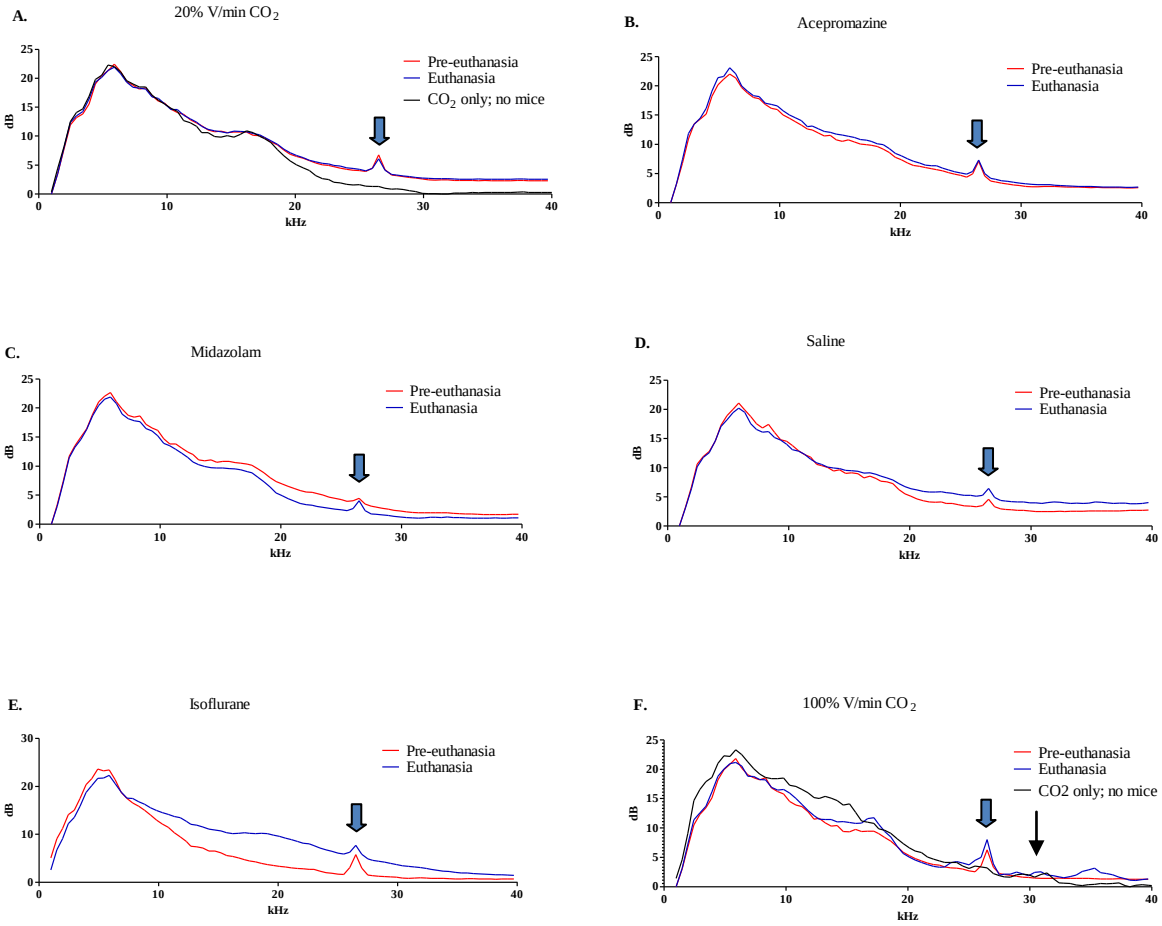

Figure 7: Ultrasonic Sound Spectorgrams. Pre-euthanasia recordings (red) were generated for two minutes prior to administration of gas. Euthanasia recordings (blue) were initiated at the beginning of gas administration and continued until all mice in the cage were unconscious. Each spectrogram represents the averaged value from two cages of mice from each group. All groups had a peak at 26.5 kHz (blue arrow) during both recordings which were absent when mice were not in the cage (black). This peak represents the only peak readily attributable to vocalization. Mice euthanized with 20% V/min CO_2 (A) or administered acepromazine (B) had identical baseline pre-euthanasia and euthanasia recordings. In contrast, mice treated with midazolam (C) had lower decibel (dB) recordings in the 10-25 kHz range during euthanasia consistent with the heavy sedation and decreased movement in the cage, but not a decrease at the 26.5 kHz vocalization peak. Mice administered saline (D), isoflurane (E), or 100% V/min CO_2 (F) had higher amplitude recordings during euthanasia at the vocalization peak consistent with increased vocalization at euthanasia. Isoflurane (E) euthanasia also produced a large degree of background noise (10-26.5 kHz range) consistent with increased movement and agitation noted in this group. 100% V/min CO_2 (F) produced fluctuations in dB level (black arrow) which were also present when 100% V/min CO_2 was provided to empty cages, indicating that these noises were likely the result of increased CO_2 flow and not the mice.

Physiological measures of pain and stress.

Plasma ACTH and corticosterone concentrations are commonly used to quantitatively assess stress. No significant differences in ACTH concentration were noted between any treatment groups (Figure 8A). In contrast, midazolam treated mice had significantly higher corticosterone concentrations (147.9 ± 37.0 ng/ml) than all other groups, which ranged from 26.7 ± 9.0 to 65.8 ± 3.5 ng/ml ($P < 0.05$; Figure 8B).

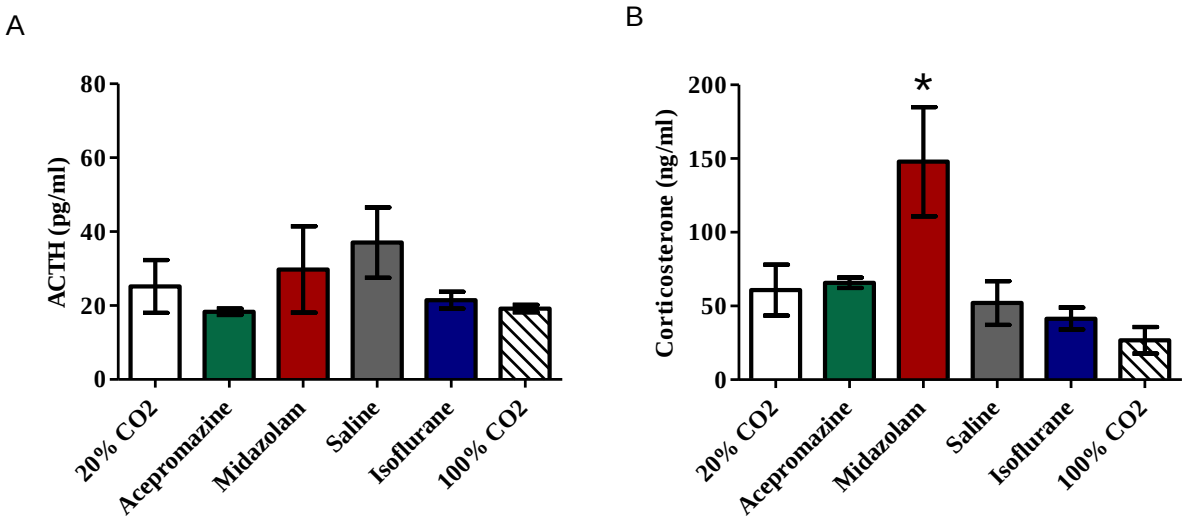

Figure 8: Plasma ACTH (A) and Corticosterone (B) Levels. (mean ± SEM) Mice that received midazolam displayed a significant increase in corticosterone (*) but not ACTH, which was suggestive of a hypoxic ACTH-independent activation of adrenal steroidogenesis. This finding was consistent with the increased time to death following unconsciousness in this group. *$P < 0.05$.*

Neuromolecular measure of pain and stress.

Increases in *c-fos* mRNA expression in the brain are a well established indicator of pain and stress in rodents (17; 26). Since *c-fos* is expressed rapidly in association with a painful or stressful event, assessment of *c-fos* expression was used to evaluate acute pain or stress that may have been too early to detect by plasma ACTH or corticosterone levels. All premedication groups and the isoflurane anesthetized group displayed significant ($P < 0.05$; 3- to 7-fold) increases in *c-fos* expression when compared to either 20% or 100% V/min CO_2 only groups (Figure 9). There was little to no difference in *c-fos* expression between the two CO_2 concentrations.

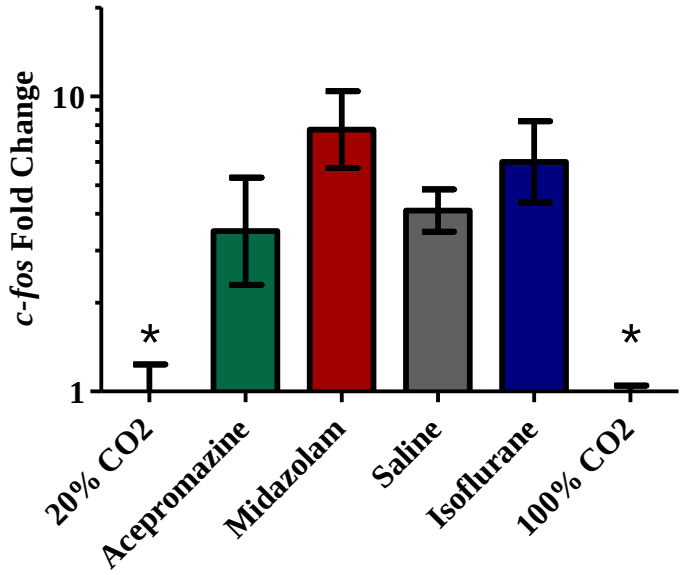

Figure 9: Relative *c-fos* Levels in the Brain. (mean ± SEM) Both 20% and 100% CO_2 treated groups expressed significantly less *c-fos* compared to all other groups (*). *$P < 0.05$.*

CHAPTER FIVE:

CONCLUSION

The hypothesis of the current study was that premedication with acepromazine, midazolam or isoflurane prior to CO_2 euthanasia would alleviate pain and stress compared to CO_2 administration alone in mice. The data described in this manuscript is in agreement with a similar study that assessed the use of sedation or anesthesia prior to CO_2 euthanasia of rats (24). Specifically, premedication of mice did not provide any advantage over the use of CO_2 alone, as it did not decrease behavioral, physiological, or neuromolecular markers of pain or stress. Furthermore, all parameters of pain or stress assessed were minimal in mice euthanized with CO_2 at a flow rate of 20% air displacement per minute. In addition, to our knowledge, this is the first study that provides direct quantifiable evidence that isoflurane induction is more stressful to mice than euthanasia with CO_2 alone at the flow rates of 20 or 100% V/min.

Numerous studies, primarily using rats, have attempted to determine if CO_2 is a humane and acceptable method of euthanasia, however there has been great variation in methodology. The major questions that remain over the use of CO_2 for euthanasia stem from the ability of CO_2 to induce pain in human (2; 14; 30; 38) and rodent studies (40; 62; 75). Unfortunately, the manner in which those studies were conducted differs greatly from the way that rodents are commonly euthanized with CO_2 in that specific concentrations of CO_2 were applied directly on mucosal surfaces (2; 14; 30; 38; 40; 62; 75). In a human study, the mean concentration of CO_2 that was reported to induce pain was 47.1% (2); similarly in a study utilizing rats, nociceptors in the nasal mucosa responded to concentrations of 37 to 50% CO_2, although no assessment of pain

was made (62). Human subjects reported that increasing concentrations of CO_2 were progressively more noxious, with 50% V/min CO_2 being considered "highly unpleasant" bordering on "uncomfortable" and 100% V/min CO_2 being rated as "painful" (14). In contrast to the conduct of those studies, our mice experienced a chamber filled with 100% CO_2 mixed with ambient air at a specified flow rate (20% V/min displacement). Mice generally lost consciousness ~ two minutes after initiation of CO_2 indicating that they lost consciousness prior to concentrations (~50%) which are reported to be painful in humans. Specifically, concentrations of CO_2 achieved in the cage after two minutes of treatment would reach ~ 40%. Thus, gradually filling the cage with CO_2 achieves unconsciousness prior to the dose that is reported to be painful in humans.

In CO_2 euthanasia studies, despite the same behaviors being described, some observers would describe the animals as experiencing no pain or stress while others would conclude that the animals experienced significant pain or stress (12; 14). To avoid basing recommendations on subjective data alone, objective measures of pain and stress have also been evaluated, including heart rate and blood pressure monitoring, plasma ACTH, corticosterone, and glucose level quantifications, and ultrasonic vocalizations, which supported the notion that CO_2 is a humane method of euthanasia in rodents (9; 24; 71).

Acepromazine was chosen for the current study because it is one of the most commonly used tranquilizers in veterinary medicine. There are species-specific differences in the reported effectiveness of acepromazine prior to euthanasia. Specifically, in dogs it reduces agitation (13), however, in rats no beneficial effect is noted (24). In the present study, the use of acepromazine did provide some beneficial effect in that it reduced time to unconsciousness, but this benefit was

negated by the marked increase in *c-fos* expression likely associated with the stress of handling and injection.

Midazolam was chosen for this study because it is a fast-acting anxiolytic with heavy sedative properties. Similar to acepromazine, midazolam significantly decreased the time to unconsciousness, however, there was a significant increase in time to death compared to all other euthanasia groups except isoflurane. The increased time to death following unconsciousness may be explained by the mechanism of action of midazolam, which increases the efficiency of gamma-aminobutyric acid (GABA), an inhibitory neurotransmitter in the brain. GABA is neuroprotective against ischemia during hypoxia (69; 79) and the protective effects of GABAergic drugs, such as midazolam, against neural hypoxic damage may have prolonged the time to death in these mice.

Induction with midazolam caused a significantly higher level of agitation compared to induction with acepromazine, and a higher level of agitation, albeit not statistically significant, than seen during euthanasia with 20% V/min CO_2 alone. This increase in agitation at induction is likely due to paradoxical excitement that is occasionally seen with sedative doses of benzodiazepines, such as midazolam. This benzodiazepine-induced excitation has been demonstrated in numerous species including humans and may include restlessness, agitation, violent behavior, and self-mutilation (22; 31; 48). The mechanism of action for this reaction is unknown.

Mice pretreated with midazolam had ACTH levels that were comparable to the other treatment groups; however, corticosterone levels were significantly increased. This finding is suggestive of a hypoxic ACTH-independent activation of adrenal steroidogenesis (64). Elevation of corticosterone only in the midazolam treated group was consistent with the extended time

period of hypoxia that the group was exposed to during the prolonged time to death. The *c-fos* levels in the midazolam treated group were the highest of all the groups, 7.7-fold higher than the control. Because of neuromolecular and behavioral indicators of stress being elevated in this group, and because of the marked increase in time to death, it does not appear to provide any benefit as a premedication for euthanasia. These data are similar to studies in rats which indicate that sedation with oral acepromazine or anesthesia with pentobarbital (IP injection) did not appreciably alter behavioral or biochemical parameters of pain in rats euthanized with CO_2 (24).

Similar to midazolam and acepromazine treatment, saline treated mice upregulated *c-fos* expression which is consistent with increased stress. These findings indicate that the stress of handling is more potent then the stress of CO_2 euthanasia itself. These findings imply that there are fundamental differences between mice and standard companion animals. That is, standard pre-euthanasia regimes that reduce stress in companion species appear deleterious in animals in which human contact and handling itself is viewed as a stressful event.

Anesthetic induction with isoflurane prior to CO_2 euthanasia was conducted in this study based on current recommendations within the CCAC guidelines on euthanasia of animals used in science. According to the CCAC, where practical, animals should be anesthetized, preferably with an inhalant anesthetic, prior to the use of CO_2 (10). The two studies that were cited in support of this recommendation used approach-avoidance tests to assess rodent aversion to gas stimuli (41; 47). However, one study assessed rat and mouse aversion to pre-filled chambers of CO_2, CO_2/Ar mixture, or Ar without the presence of a food reward in the test chamber (41), while the other study compared rat aversion to gradual fill halothane or isoflurane with a food reward in the test chamber (47). In the CO_2/Ar study, the authors concluded that induction with CO_2 either alone or in combination with Ar is likely to cause considerable distress in rodents

before they lose consciousness (41). This finding was based upon significantly shorter initial withdrawal times and total dwelling times in the test chamber that was pre-filled with CO_2 at concentrations that ranged from 25.5 to 50.8% compared to the control of room air. The halothane/isoflurane study concluded that both inhalant anesthetics were aversive to rats (47). However, because some of the rats remained in the test cage long enough to become ataxic, the authors concluded that the rats were likely in a state of conscious sedation at the time they chose to leave the test cage, therefore, continued forced exposure from the onset of aversion to unconsciousness may be more humane than forced exposure to CO_2. The two studies were not comparable in their design (pre-fill vs. gradual fill; no food reward vs. food reward), and neither study directly compared aversion of CO_2 to isoflurane. In addition, both of these studies did not involve euthanizing the rodents and did not assess any direct or indirect indicators of pain or stress.

In the present study, the hypothesis that isoflurane anesthesia prior to CO_2 euthanasia would decrease the level of pain or stress experienced with CO_2 alone was directly tested. Interestingly, isoflurane induction resulted in the need for a longer CO_2 exposure at greater than 100% V/min than the use of 100% V/min CO_2 alone. This increase in time to death during CO_2 exposure, despite the higher concentration of CO_2, is likely due to the hypothermic effect of general anesthesia which has been shown to be neuroprotective during hypoxia (19; 69).

Isoflurane was the highest scoring group for both dyspnea and agitation, which were significantly higher than pre-gas exposure scores, and produced significantly higher scores of agitation than the 20% V/min CO_2 group during euthanasia. The ultrasonic spectrogram also reflected elevated movement within the cage, indicative of agitation, and increased mouse vocalization at the 26.5 kHz peak, potentially indicative of stress induced vocalization.

Additionally, *c-fos* expression was increased significantly, 6-fold higher than the 20% V/min CO_2 group.

An additional negative effect of isoflurane is that some mice awoke after isoflurane induction and during the euthanasia phase. This finding highlights a weakness in the CCAC recommendation, in that, due to the rapid recovery from isoflurane mice may recover once the isoflurane is removed and CO_2 is initiated. Indeed, with newer gas anesthetics (eg., sevoflurane) this may become even more of an issue. Previous studies that recommended the use of isoflurane prior to CO_2 euthanasia (45; 47) do not account for the rapid recovery from isoflurane that occurs while the chamber is filling with CO_2, and since they were merely avoidance tests, they missed altogether the possibility of pain or stress associated with anesthetic induction. In short, isoflurane, in the current study, was the worst option with regard to minimizing animal pain or stress.

A group of mice euthanized with CO_2 at a flow rate of 100% air displacement per minute was included in this study because the current AVMA recommendation describes > 20% CO_2 V displacement per minute. Time to unconsciousness and time to death were significantly faster than the 20% V/min CO_2 control. However, dyspnea and agitation scores were higher than the control, although not significantly, and the ultrasonic spectrogram showed elevations in amplitude during the euthanasia recording that were not present in the pre-euthanasia recording. These behavioral responses suggest a trend toward increased pain or stress with increased flow rate; however, ACTH, corticosterone, and *c-fos* levels were comparable to the 20% V/min CO_2 control. Further, because mice were still conscious in the cage during the timeframe when the CO_2 concentration exceeded 50%, there is a possibility that mice experienced mucosal pain from

CO_2 at this flow rate. Based upon these data it may be worthwhile to evaluate higher CO_2 flow rates in future studies to determine the recommended upper flow rate limit for euthanasia.

Data which call into question the humane nature of rodent CO_2 euthanasia generally fall into two categories: those that rely on human studies indicating that CO_2 at high concentrations is painful, and those that rely on approach-avoidance tests. With regard to the former, our data indicate that with 20% V/min flow rates mice reach unconsciousness prior to the buildup of painful concentrations of CO_2. With regard to the latter, we suggest that approach-avoidance tests are not ideal tests to determine a humane euthanasia method. Specifically, aversion does not necessarily indicate pain or stress. In fact, there are several examples supporting this. Specifically, a brief puff of air induces aversion in mice (77) and urine collected from male mice infected with *Polyplax serrata* induces aversion in female mice (32). In these examples, non-conditioned avoidance/aversion responses are not necessarily indicators of pain or stress, but rather evolutionary survival cues (puff of air = predator, infected mouse urine = poor mate). Indeed, non-conditioned CO_2 avoidance is evolutionarily conserved (presumably to avoid hypoxic death) and can be demonstrated in organisms as diverse as nematodes (*Caenorhabditis elegans*), *Drosophilla*, fish, and in mice which can actually sense CO_2 concentrations at near atmospheric levels (i.e., far below those which would ever be reported as painful or unpleasant in humans) (7; 28; 29; 44; 73). Further, approach-avoidance tests fail to adequately distinguish between stressors, which are not necessarily negative, and distress, which is the inability to appropriately respond (either behaviorally or physiologically) to those stressors (56; 70). That is, just because an animal encounters an external stressor (e.g., puff of air, mouse urine, CO_2) and chooses to avoid it does not mean that distress is induced. Our data would indicate that from a

behavioral, physiological, and neuromolecular level, overt distress does not occur with CO_2 euthanasia at the 20% V/min flow rate tested.

In summary, the present study demonstrates that premedication with acepromazine or midazolam prior to CO_2 euthanasia did not improve euthanasia with respect to animal welfare, and based on neuromolecular markers, induced greater stress then CO_2 alone. Furthermore, the use of isoflurane induction prior to CO_2 euthanasia significantly increased behavioral and neuromolecular indicaters of pain and stress. These data show that in comparison to the other modalities analyzed in this study, 20% V/min CO_2 alone is a humane euthanasia method which is not associated with increases in behavioral, ultrasonic, physiologic or neuromolecular markers of pain or stress in mice.

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