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The use of carbon dioxide (CO2) as an alternative euthanasia method for goat kids

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The use of carbon dioxide (CO_2) as an alternative euthanasia method for goat kids

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ABSTRACT

The dairy industry is faced with the challenge of euthanizing unwanted male offspring in addition to other sick or injured neonates. Carbon dioxide (CO2) may be a potential alternative to current methods. The goat kid served as a model in approach-avoidance and conditioned place aversion paradigms. A preference test box was custom-made with two connected chambers; one chamber held an ambient atmosphere (control) and one maintained a static CO2 concentration (treatment). Kids were allotted 5-minutes in the control chamber before a sliding door was opened, after which kids were given 10-minutes access to the treatment chamber. The objective of the first study was to determine the ability of kids to move from the control to the treatment chamber to access a milk reward, and the effect of an olfactory or visual stimulus on learning. All kids (n=24) exhibited learning, and latencies to enter, touch the milk bottle, and suckle decreased over day ($P<0.0001$). Milk consumption increased over days ($P<0.0001$), and startle, bottle engagement, and lying behavior did not differ between days ($P>0.05$). The presence of an olfactory stimulus (peppermint oil) did not affect learning, and the visual stimulus (plastic curtain) did not prevent learning. The second study examined kids' tolerance of 10%, 20%, and 30% CO2. Kids (n=12) were randomly assigned 10% or 20% as the first treatment, and were systematically tested with all kids receiving 30% as the last treatment. A 2-day washout (ambient CO2) period occurred between each gas treatment. 10 kids tolerated 10% CO2, while one kid exited the treatment chamber after consuming his full ration, and 1 kid lost posture at 289s. At 20% and 30%, posture loss ranged from 83s to 271s. One kid exited before losing posture at 20%, then re-entered the chamber and became recumbent. Kids did not show avoidance behavior to any CO2

concentration, and did not appear to develop a conditioned aversion. The results of this study show promising results for CO2 as a euthanasia method in goat kids. Further research is required to confirm its suitability, and determine its potential for other ruminant species.

CHAPTER 1.

INTRODUCTION

Immanuel Kant stated in 1798 “The first time [man] said to the sheep 'Nature did not give thee the pelt thou wearest for thyself, but for me,' stripped him of it, and put it on himself, he perceived a prerogative, that he, by virtue of his nature, was above all animals, which he now considered...as the means and instruments left to his will for the accomplishment of his purposes at pleasure.” Kant further postulates that our treatment of animals is a reflection on our own morality, and that “any action whereby we may torment animals, or let them suffer distress or otherwise treat them without love, is demeaning to ourselves” (Korsgaard 2004). This philosophy is manifested in the evolving animal protection laws throughout history. Specifically, the passage of the Humane Methods of Slaughter Act by the United States in 1958 drew to attention the idea of a “humane death” during processing, and it outlined the steps necessary to achieve this concept (Becker 2008). Euthanasia as defined by the Merriam-Webster dictionary is “the act or practice of killing or permitting the death of hopelessly sick or injured individuals (as persons or domestic animals) in a relatively painless way for reasons of mercy” (Merriam-Webster 2015b). The American Veterinary Medical Association (AVMA) uses “euthanasia” as a term that describes the ending of an animal's life in a way that minimizes or eliminates pain (Leary, et al. 2013 p 6). Although there is some debate about the use of the word 'euthanasia' when referring to animals that are killed for human use, and while neither the disposal of experimental animals nor the slaughter of food animals falls under the definition of euthanasia, it remains the moral obligation of

humans to provide sensitive and responsible care in all human-animal relationships (Pavlovic 2011).

1.1 Euthanasia Needs

In 1963 the AVMA formed the Panel on Euthanasia (POE), a committee of experts tasked with providing guidelines to veterinarians about current and potential methods for euthanasia of dogs, cats, and small mammals. Since its formation in 1963, the POE has expanded its publication to include a multitude of animals such as food and laboratory animals, and wildlife. The guidelines in the POE publication list acceptable, acceptable with conditions, and unacceptable methods for euthanizing animals. Methods considered acceptable consistently provide a humane death when used as the sole euthanasia technique, while methods that are acceptable with conditions require certain conditions to be met to be considered a humane death. These conditions may have a greater risk for operator error or worker safety, little scientific documentation, or require a secondary method to ensure a death. Acceptable with conditions methods are considered to be as humane as acceptable methods when all required criteria are met. Unacceptable methods are techniques that are known or have significant potential to cause human or animal pain and suffering under any conditions (Leary, et al. 2013 p 10-11). These guidelines go in to detail about the appropriate steps for each acceptable, or acceptable with conditions, method to ensure that personnel who are training, or being trained, are provided with detailed and accurate information about the appropriate euthanasia of an animal. Furthermore, the guidelines published by the POE also include a focus on possible psychological and welfare implications in humans and animals concerning euthanasia methods (Leary, et al. 2013 p 5). When creating an on-farm

critical endpoint and euthanasia protocol, these guidelines provide the necessary information for collaboration between veterinary and farm personnel to establish the most appropriate animal care plan.

The Animal Health and Welfare panel of the European Food Safety Authority (EFSA) has published a similar document outlining the acceptable stunning methods for animals during processing. The "Welfare Aspects of Animal Stunning and Killing Methods" (EFSA 2013) contains requirements that the European Union developed to address humane slaughter and animal welfare in processing plants. This document states that stunning/killing methods for livestock processing must ensure either immediate and unequivocal unconsciousness and loss of sensibility, or a non-aversive, pain and distress-free induction to unconsciousness and insensibility. The guidelines go on to state that the duration of unconsciousness must be significantly longer than the total time required to ensure death of an animal. Akin to the euthanasia guidelines published by the AVMA, the EFSA document provides detailed descriptions and recommendations of methods for animal slaughter.

Both of these committees are tasked with providing easily accessible guidelines for the euthanasia of animals. These guidelines are crucial to promoting positive universal welfare of animals, particularly in their last moments of life. In order to provide comprehensive information, constant research must be conducted to assess and re-evaluate current methods and investigate the use of future methods. By regularly updating these guidelines, the committees are able to provide the information necessary for producers, veterinarians and animal caretakers to implement the most practical and humane euthanasia practices for their circumstances.

Euthanasia is a standard practice in most sectors of the animal industry today. Many veterinarians must regularly perform the euthanasia of a variety of animals including companion animals, livestock, wildlife, and laboratory animals. Similarly, animal caretakers such as food production employees are often tasked with appropriate euthanasia of their animals. Throughout the lifetime of a food animal operation, it is inevitable that there will be numerous situations where an ill or injured animal must be euthanized without veterinary assistance. While ill and otherwise moribund animals are not an uncommon occurrence, farms typically do not employ a veterinarian for daily visits. Subsequently, on-farm euthanasia performed by employees may be necessary due to the severity of the illness or injury, a lack of immediate veterinary personnel, a lack of physical means to immediately transport the animal to a veterinarian, or a myriad of other reasons. As such, it is necessary that farms work with their veterinarian to develop a detailed plan for critical endpoints and on-farm euthanasia methods (Turner & Doonan 2010). Defining euthanasia end-points for animals has been a source of controversy in regards to the welfare of the animal. The culling of an otherwise healthy animal due to productivity concerns is considered by some to be inhumane, and poor in terms of animal welfare. For example, even in the face of disease, some individuals maintain that euthanizing otherwise healthy animals as a control measure is wrong (Anthony 2004, Mepham 2001). Similarly, culling animals for lack of performance or profitability is a controversial, albeit common, practice in today's food industry. The argument against culling for productivity is based on the argument of longevity. The focus of the longevity argument is that the quantity of life is an independent, and fundamental, attribute of animal welfare. The reasoning is that in order to respect the individuality of an animal

and consequently not jeopardize its welfare, one must not interfere with its natural lifespan (Bruijnis, et al. 2013). However, despite the controversy of critical end points it remains that proper, revised, and well-defined guidelines for euthanasia have the potential to improve animal welfare worldwide.

1.1.1 Euthanasia needs in the dairy industry

On-farm euthanasia of dairy animals may occur for a multitude of different reasons, with illness and injury to an animal being the most well recognized although not the sole reasons for on-farm euthanasia. The critical end-points mentioned previously are important guidelines that caretakers must follow when it comes to the difficult decision to euthanize an animal. In order to keep economic balance within an operation, animals must produce a positive net yield in relation to their input cost, and an animal may be culled from the herd for illness and injury, as well as poor performance, poor temperament, and lack of potential profitability. The argument of longevity is especially pertinent in the dairy industry due to the relatively long lifespan of a dairy cow compared to a beef steer or finisher pig. While the lives of meat animals will ultimately be shortened in order to produce the end product, a dairy cow is expected to produce in a herd for several years. As such, there is support to further improve the lifespan of animals in the dairy industry.

A current issue associated with dairy animal welfare and longevity is unwanted male offspring born in to the industry every year that hold less production value than their female counterparts. According to the USDA semi-annual cattle report, as of July 2014, there are approximately 9.3 million milk cows currently in production in the United States. It can be concluded that the majority of these cows had calves by January 2015,

and out of these calves 4.6 million were retained as replacement heifers (USDA 2015a). This leaves approximately 4.7 million dairy calves that must be either put in to a meat finishing program or euthanized. There have been efforts to decrease the number of dairy bulls that are killed on-farm for economic reasons. For example, the percentage of calves euthanized on-farm in the UK has decreased from 21% to 12% from 2006 to 2012; 84,817 dairy calves were euthanized on-farm in 2006 while in 2012, 54,670 calves were euthanized on-farm. The numbers for on-farm euthanasia of dairy calves in the UK also reflect the 30,000 calves housed and likely euthanized on bovine tuberculosis positive farms (Compassion in World Farming 2013). This change has been driven mainly by the increasing profitability of dairy bull calves, and there has been an upward trend to finish unwanted dairy calves for food and other goods production instead of euthanizing the calves soon after birth (Leaders, et al. 2008, Maas & Robinson 2007). Furthermore, approximately 8% of the cattle harvested in the US are Holstein steers, and approximately 650,000 special-fed Holstein veal calves are produced each year (Schaefer 2005, Slayton 2002). However, despite the more positive outlook for dairy bull calves compared to previous years, on-farm euthanasia remains a regular occurrence.

Although the push for decreasing on-farm euthanasia of healthy calves has arguably improved welfare conditions on the premise of longevity, there are potential complications involved in raising bull calves for veal or beef. Potential complications include transportation stress, dietary stress, illness and weakened immune systems (Leaders, et al. 2008). Under inferior conditions, these stressors may quickly diminish the welfare of the calves to an unacceptable level. In North America, veal mortality rates range from 2.5% to 8.8% with respiratory and digestive illness being the leading causes

(Leaders, et al. 2008). A 2011 study of the mortality and post-slaughter wastage of New Zealand veal calves found that the processing plant had a pre-slaughter mortality of 0.7%. The primary cause of pre-slaughter mortality in New Zealand veal calves was found to be digestive tract issues, and the second leading cause was umbilical infections. Furthermore, 74% of a sample of veal calves from 3 European countries suffered from lesions in the abomasum (Thomas & Jordaan 2013). Dietary stress such as low rumen development and high incidences of rumen plaque formation is prevalent in veal calves due to their traditional ration that contains little to no roughage (Suárez, et al. 2007). This issue is exacerbated in the U.S. which, unlike the EU, does not require roughage to be fed to veal calves (Leaders, et al. 2008). Furthermore, transportation issues such as inadequate space allotment, food and water deprivation, long-distance travel, and seasonal differences have all been associated with decreased calf welfare during transport (Cave, et al. 2005, Jongman & Butler 2014). Although these issues do not negate the importance of the veal or dairy beef industry, it is important for producers to assess whether on-farm euthanasia may be a more humane option for a weak or otherwise unthrifty calf.

1.1.2 Euthanasia needs of small ruminants

Similar to dairy calves, euthanasia of unwanted kids and lambs is necessary when the animals are morbid, under developed, or unsellable (Rietveld 2003b). In 2014 the sheep industry had a lamb mortality rate of 10.6% which represents 365,000 lambs lost or euthanized (USDA 2015b). In 2009 the goat industry had a kid death loss of 175,000, excluding predators and unknown causes (NASS 2010). These numbers indicate

that approximately 540,000 sheep and goats under 1 year old die or are euthanized annually.

Dairy goat producers in particular face a unique challenge regarding the future of unwanted male offspring. In areas where the major demographic has little demand for goat meat, such as the US and the UK, male dairy goats are typically euthanized shortly after birth (Humane Slaughter Association 2008, Turner & Doonan 2010). One solution is the practice of providing euthanized kids as meat for zoos. It is recommended that whole carcasses be fed to captive carnivores to more appropriately emulate the wild diet of the species (Colahan, et al. 2012). This includes any hide, hair, bones, viscera, and gut contents. To mitigate potential health concerns, zoos must be discerning about their food source supplies. Any potential food source must be investigated for any possible deleterious attributes including pharmaceutical residues or signs of disease (Colahan, et al. 2012). To decrease the risk for disease or a foreign body to be present within the carcass, some zoos prefer, or even require, an unblemished whole carcass for feeding. This precaution immediately excludes animals with any visible lesions or lacerations, and subsequently restricts the on-farm euthanasia options for small ruminant producers who intend to sell their unwanted offspring as a food source for zoos.

1.2 Current Euthanasia Guidelines for Ruminants

1.2.1 Acceptable methods

In order to be considered an acceptable euthanasia method by an American Veterinary Medical Association (AVMA), any technique must be assessed by the following criteria: "(1) the ability to induce loss of consciousness and death with a

minimum of pain and distress; (2) time required to induce loss of consciousness; (3) reliability; (4) safety of personnel; (5) irreversibility; (6) compatibility with intended animal use and purpose; (7) documented emotional effect on observers or operators; (8) compatibility with subsequent evaluation, examination, or use of tissue; (9) drug availability and human abuse potential; (10) compatibility with species, age, and health status; (11) ability to maintain equipment in proper working order; (12) safety for predators or scavengers should the animal's remains be consumed; (13) legal requirements; and (14) environmental impacts of the method or disposition of the animal's remains" (Leary, et al. 2013 p 10-11). According to the Ontario Ministry of Agriculture and Food, there are three approved methods of euthanasia for ruminants: overdose by barbiturate, euthanasia by gunshot, and penetrating and non-penetrating captive bolt (Rietveld 2003a). The POE lists these methods, along with electrocution, as acceptable with conditions for the euthanasia of bovids and small ruminants (Leary, et al. 2013 p 51-56). Gunshot is currently the most common method for on-farm euthanasia in cattle, while captive-bolt is the most common pre-slaughter stunning method (Humane Slaughter Assocation 2013, Shearer, et al. 2013). Although gunshot is effective when performed correctly, the margin for error leads to welfare concerns. Additionally, these methods may be aesthetically displeasing for the personnel involved (Woods, et al. 2010). Captive-bolt presents similar advantages and disadvantages as gunshot, but also has a high initial cost due to the expensive equipment. Issues with electrocution include consistency of the electric shock relating to dehydration and cattle size, and significant danger to the operator if the equipment is inappropriate or faulty. Barbiturates are currently the only accepted form of euthanasia by the AVMA, and this method is

generally preferred because it is perceived as the most peaceful and humane (Leary, et al. 2013 p 27). However issues with cost and carcass disposal generally inhibit the practicality of barbiturate overdose as a primary on-farm euthanasia method (Leary, et al. 2013 p 51-56).

1.2.2 Unacceptable methods

Unacceptable methods of euthanasia for ruminants include manually applied blunt force trauma to the head, injection of chemical agents in to or exsanguination of conscious animals, injection of potassium chloride or magnesium sulfate with sedation relying solely on α-2-agonists, air embolism, electrocution using a 120-volt electrical cord, and drowning (American Association of Bovine Practicioners 2013). Ultimately, this restricts the euthanasia option for neonatal ruminants to the same methods approved for adults. The limited number of approved methods foreshadows possible welfare issues concerning the euthanasia of neonatal ruminant. Methods that require advanced technology and skill such as electrocution and barbiturate overdose are impractical, leading some producers to blunt force trauma, a traditional choice and the most cost efficient option.

These factors highlight the need for research in to alternative euthanasia methods in lieu of the traditional forms approved for ruminants. Inhaled euthanasia agents offer advantages where the previously mentioned methods fall short. They are a top choice for euthanasia by many swine and poultry producers, and are highly utilized in research settings (Matthis 2005, Raj, et al. 2004, Webster, et al. 1996). The POE states that inhaled agents are approved for use in most birds and mammals, but does not cite any specific research for the use of inhalant gases in ruminants. Although there is no

published research concerning the effects of using gas euthanasia in ruminants, it has been reported that carbon dioxide has been used for the euthanasia of goat kids and lambs by producers in several countries (Woods, et al. 2010). Due to this method currently being implemented without any guidelines, it is clear that further studies are needed in order to conclude whether inhalant agents are both humane and effective in ruminant species. These studies will allow us to determine whether this alternative method has potential to be considered acceptable by the AVMA.

1.3 The Potential of Carbon Dioxide

1.3.1 Homeostasis and carbon dioxide

Carbon dioxide has been used as a euthanasia agent for decades as animal caretakers capitalize on advantages in both employee safety as well as cost (Leary, et al. 2013 p 24-26). The lengthy history of CO_2 as a euthanasia method has provided a vast amount of information on the physiological responses of the body to high levels of CO_2. The normal level of CO_2 in atmospheric air is 0.04%. During homeostatic conditions, mammals maintain an arterial pressure of 35-45 mmHg CO_2 ($PaCO_2$). As CO_2 is produced as a waste product from tissues, it binds to the hemoglobin as blood travels through the tissue. As the CO_2-rich blood flows through the lungs, the CO_2 is released from the hemoglobin in favor of oxygen molecules from inspired air. This gas exchange in the lungs and other tissues is powered by the Bohr Effect phenomenon. When blood enters an environment that is high in CO_2 such as the tissues, the pH of the blood decreases as the CO_2 reacts with water in the blood to create carbonic acid. This drop in pH triggers hemoglobin to release the oxygen molecules that had bound to the proteins. As the hemoglobin releases oxygen molecules, its affinity for CO_2 molecules increases.

The CO_2-laden blood then travels through the venous system to the lungs. The environment within the lungs has much lower levels of CO_2 compared to other tissues in the body. The pH level of the blood begins to rise once it reaches the lungs, which encourages the dissociation of CO_2 molecules from hemoglobin. Additionally, the high levels of CO_2 within the blood coupled with the low levels of CO_2 in the lungs creates a concentration gradient that facilitates the diffusion of CO_2 molecules in to the alveoli (Shepard, et al. 1981). As the hemoglobin releases the bound CO_2 molecules, the increased pH of the blood promotes the binding of oxygen molecules that are present in the alveoli from inspired air. The cycle is then repeated as oxygenated blood is delivered to tissues and CO_2 molecules are expelled from the alveoli by exhalation.

1.3.2 Hypercapnia and carbon dioxide

As atmospheric CO_2 levels rise above the normal concentration of 0.04%, the body begins to experience a state of hypercapnia. Hypercapnia is defined as the presence of excessive CO_2 in the bloodstream. Hypercapnia can be induced by ailments such as sleep apnea and lung disease, however direct exposure to elevated atmospheric levels of CO_2 is the approach used for euthanasia. The extent of the hypercapnic state that occurs during CO_2 exposure is positively correlated with the level of CO_2 in the atmosphere. Additionally, as the level of CO_2 in the atmosphere increases, the amount of available oxygen decreases inducing both hypercapnia and hypoxia. Hypoxia occurs when there is a deficiency in the amount of oxygen being transported to tissues within the body. Hypoxia takes place due to the diminished binding affinity of oxygen to hemoglobin, as well as atmospheric air (29g/mol) being displaced by rising amounts of CO_2 (44g/mol) which further depletes oxygen sources. As the animal inspires high concentrations of

CO_2, excess carbonic acid (H_2CO_3) begins to form in the blood as the CO_2 molecules react with water. This leads to an imbalance of the normal ratio between H_2CO_3 and bicarbonate (HCO_3). The normal $1H_2CO_3$:$20HCO_3$ ratio acts as a buffer for homeostatic levels of H_2CO_3. When this ratio is skewed in favor of H_2CO_3, respiratory acidosis ensues. The increased levels of H_2CO_3 then cause acidemia which inhibits the Bohr effect and leads to tissue hypoxia, erratic cardiac activity, and reduced myocardial contractility (Hall & McShane 2013). Eventually, if there is no increase in available oxygen, heart rate decreases, hypotension and vascular collapse occurs, and death follows (Smith & Harrap 1997).

1.3.3 Induction of unconsciousness

The time needed to reach unconsciousness largely depends on the concentration of CO_2 being used. In an atmosphere containing 100% CO_2, rats reached insensibility within 25 seconds (Reed, et al. 2009). When placed in to a chamber containing 50% CO_2 coupled with a 20% box volume exchange(bve)/min flow rate, weaned pigs experienced a loss of posture at 35 seconds, while weaned pigs lost posture at 143 seconds when placed in to an ambient chamber with a 20% bve/min flow rate (Sadler, et al. 2014). Somatosensory potentials were lost in pigs exposed to 80-90% CO_2 within 17 to 25 seconds (Raj, et al. 1997). Six week old broiler chickens lost posture in 172 seconds in an atmosphere of 15.7% CO_2 (Gerritzen, et al. 2004).

While the latency to unconsciousness is more prolonged than the immediate physical methods, CO_2 does offer the benefit of anesthetic and analgesic effects. The depressant effect of CO_2-induced hypercapnia on the central nervous system has been well documented in many species. Human patients experience deep anesthesia after being

298 exposed to levels of 15%-20% CO_2 for up to 30 minutes (Morris 2002). When exposed to
299 80% CO_2 in ambient air for 120 seconds rats were deeply anesthetized for 77 seconds,
300 and after being exposed to 80% CO_2 in pure oxygen for 30 seconds, guinea pigs were
301 anesthetized for 50 seconds (Kohler, et al. 1999). Similarly, it was found that rats
302 experienced a deep level of anesthesia for 1 to 3 minutes after 30 seconds exposure to
303 70% CO_2 and exhibited antinociception to thermal and mechanical pain stimuli for up to
304 60 minutes (Mischler, et al. 1994). Withdrawal responses and pain behaviors in
305 moderately hypercapnic rats ($PaCO_2$: 40 ± 8 to 90 ± 9mmHg) were also reduced
306 (Fukuda, et al. 2006, Gamble & Milne 1990). However, contradictory results have been
307 observed in pigs anesthetized with CO_2 prior to castration. Pigs showed increased pain
308 behaviors when treated with CO_2 compared to pigs castrated without any pain mitigation
309 (Sutherland, et al. 2012). This difference may be attributed to the increased pain of
310 castration compared to mechanical pressure, and the production of pro-inflammatory
311 cytokines in response to CO2 exposure (Abolhassani, et al. 2009). It is possible that
312 exposure to CO_2 causes increased inflammation at injury sites, subsequently increasing
313 pain sensation after neural transmission returns to normal levels. It is also possible that
314 the response to CO_2 is not conserved across species. Rats were observed to show
315 significantly less distress and pain behaviors when placed in an environment with an air
316 replacement rate of 10% CO_2/min versus an environment with an air replacement rate of
317 50% argon/min. Rats exposed to CO_2 had significantly slower heart rates as compared to
318 rats exposed to argon. Furthermore, there were no observed incidents of gasping or
319 seizures prior to unconsciousness, unlike rats in 100% argon environments (Burkholder,

et al. 2010). This difference in pain behavior may be elicited by CO_2's ability to directly suppress the central nervous system.

1.3.4 Induction of anesthesia and analgesia

CO_2 is able to induce anesthesia by depressing the reactivity of both respiratory and non-respiratory neurons, an effect that is more pronounced with higher CO_2 concentrations (Lipski 1986). As peripheral and central chemoreceptors are stimulated by the decreasing pH of their environment during hypercapnia, stress-related mechanisms trigger the release of opioids (Fukuda, et al. 2006, Gamble & Milne 1990, Grönroos & Pertovaara 1994). Exogenous, as well as endogenous, opioids are linked to the depression of ventilatory rates, a lessened "need to breathe", and other sedative effects which may further support the tie between anesthesia and hypercapnia (Kimura & Haji 2014, Pattinson, et al. 2007, Zhang, et al. 2007). *In vitro* studies of the analgesic properties of CO_2 showed that under conditions of severe acidosis (pH ~6.7), rat spinal cords exhibited nociceptive responses that were similar to spinal cords treated with the analgesic dexmedetomidine (Otsuguro, et al. 2007). Although this strong analgesic response to hypercapnia may not extrapolate uniformly to the *in vivo* model, the release of endogenous opioids during hypercapnic stress may play a role in the observed reduction of pain response in the rodent model. The more robust anesthetic effect of hypercapnia is likely augmented by significantly increased levels of extracellular adenosine in cerebral fluid. Adenosine acts as an agonist for the G-protein coupled receptor A1, which when bound, has a significant inhibitory effect on neuronal transmission (Dulla, et al. 2005, Dunwiddie & Masino 2001, Eisenach, et al. 2004, Otsuguro, et al. 2007).

1.3.5 The potential for pain and distress

Although the ability of CO_2 to produce analgesia and anesthesia is well researched, it is not a euthanasia method that is completely devoid of causing distress and pain. Breathing CO_2 of varying levels is known to produce feelings of anxiety, fear, and pain. Air hunger is reported to begin in humans around 8% CO_2 (Liotti, et al. 2001). Subjects described breathing 50% to 100% CO_2 as very unpleasant and painful, respectively and experienced symptoms of panic during exposure to levels of 35% CO_2 (Danneman, et al. 1997, Van den Hout & Griez 1984). Higher concentrations of CO_2 were associated with more intense reactions of fear and pain (Danneman, et al. 1997, Vowles, et al. 2006). Studies using animal models have produced similar results. Rats begin to actively avoid CO_2 concentrations at 15%, and will forgo a meal in order to leave the environment before the CO_2 rendered them unable to exit. (Kirkden, et al. 2005, Niel & Weary 2007). Broiler chickens exhibited disruption in feeding as well as withdrawal behavior at concentrations of 40% CO_2 and above (McKeegan, et al. 2006). Furthermore, elevated levels of substance P were observed in neonatal pigs exposed to 100% CO_2 which suggests that exposure to this concentration of CO_2 is a painful and stressing experience (Sutherland, et al. 2012). The sense of fear and distress invoked by exposure to CO_2 is triggered by peripheral as well as central chemoreceptors. The carotid body, a peripheral chemoreceptor, is sensitive to changes in $PaCO_2$ within the blood and blood pH. Central chemoreceptors are located within the hindbrain and relay signals regarding pH and CO_2 detection in the brain (Nattie & Li 2009). Peripheral and central chemoreceptors exhibit a synergistic relationship within the body for detecting pH and CO_2 levels (Blain, et al. 2010). As the body moves away from homeostasis, these

366 chemoreceptors signal the central nervous system (CNS) to increase ventilation to restore
367 appropriate $PaCO_2$ levels (Blain, et al. 2010, Burnstock 2009, Nattie & Li 2009, Nurse &
368 Piskuric 2013). As these chemoreceptors are activated the ensuing feeling of
369 breathlessness urges the individual to escape the noxious environment.

370 Although analgesic effects have been observed after exposure to CO_2, these
371 effects do not appear to be powerful enough to negate pain caused by direct exposure to
372 the gas during the induction of anesthesia. The pain pathway is an innate protective
373 mechanism responsible for processing information about potential hazards to survival.
374 Pain receptors, otherwise known as nociceptors, are present throughout the body to alert
375 an animal to dangerous changes in temperature, pressure, chemical reactions related to
376 injury, and other noxious stimuli (Dubin & Patapoutian 2010, McKeegan 2004).
377 Nociceptors are located throughout the respiratory tract, and are likely reactive to pain
378 associated with tissue damage (Widdicombe 1982). In the nasal trigeminal system of the
379 domestic hen, there are 40 nociceptors that are stimulated by the presence of ammonia
380 (McKeegan 2004). These nociceptors serve to alert the animal of noxious chemicals
381 present in the environment, and the resulting sensation of pain then drives the animal to
382 escape from the hazardous environment. Upon inhalation of CO_2, carbonic acid is formed
383 as the molecule binds with water present in the respiratory tract. While the amount of
384 acid formed is negligible in ambient conditions, higher concentrations of CO_2 result in
385 comparatively large amounts of acid being formed on mucous membranes. This activates
386 the nociceptors within the respiratory tract and leads to the sensation of burning that
387 many describe when exposed to CO_2. There are also vagal afferent nerves are delegated
388 to detecting hazards via signals from visceral organs. Vagal bronchopulmonary C-fibers

are located within the lungs and are able to elicit pain signals in response to both endogenous and exogenous stimuli (Kollarik, et al. 2010). These fibers are mildly stimulated by the inhalation of 30% CO_2 for 5 to 8 breaths (Lin, et al. 2005). During euthanasia, it is likely that these nerves continue to be increasingly stimulated leading to increased sensations of pain.

Neonatal animals present a unique obstacle due to their innate resistance to hypercapnia. Fetal hemoglobin has a higher affinity for oxygen and a lower affinity for CO_2, than adult hemoglobin (Bauer, et al. 1975). Fetal hemoglobin begins to dissipate after birth, but it is still at detectable levels for up to 48 days in goat kids (Johnson, et al. 2002). Additionally, fetal circulation involves the shunting of oxygenated blood to the left atrium through the foramen ovale, a cardiac valve that allows blood to bypass the lungs. This valve begins to close soon after birth, however 7 days were required for complete closure of the foramen ovale in neonatal rats (Cole-Jeffrey, et al. 2012). Due to the low affinity and the shunting mechanism, the hemoglobin of the neonate is less likely to bind CO2 in the lungs, and it is possible that inhalation of CO2 would not cause a rapid increase in $PaCO_2$. Consequently, CO2 would not be as effective in neonates as it is in adults and may lead to unnecessary pain and distress. These physiologic differences may account for the increased exposure time to needed to euthanize neonatal rodents with CO2, although responses may differ by species based on evidence that neonatal pigs succumb to CO2 exposure faster than their older conspecifics (Pritchett, et al. 2005, Pritchett-Corning 2009, Sadler, et al. 2014).

1.4 Determining Stimulus Aversion and Corresponding Behaviors

1.4.1 The limbic system

The brain of a lamb, kid, calf, or similar animal consists of the first brain, known as the "R complex" or the reptilian brain, and the second brain which is better known as the "limbic" system. The reptilian brain elicits responses to basic survival needs such as hunger, thirst, and sexual drive, while the limbic system controls the more complex emotional responses of alertness, fear, and pain (Lubbe & Kenner 2009, Roxo, et al. 2011). Within the limbic system lies the amygdala, which is recognized as the center for developing emotional responses to stimuli. These responses are important for the survival of all animals. Olfactory, gustatory, visceral and other signals from sensory modalities travel via a series of signal cascades to higher order cortices and then to the amygdala. Efferent nerves run from the amygdala through the stria terminalis to the hypothalamus, and through the amygdalofugal pathway to the ventral striatum. The ventral striatum then projects to the basal ganglia where voluntary responses to emotional events are processed (McDonald 1998, Wright). During responses to a novel stimuli of either positive or negative valence, there is a significant increase in norepinephrine production in the prefrontal cortex, which then modulates the release of dopamine from the ventral striatum. This dopaminergic response to stimuli is essential to shaping behavioral responses to novel situations, and also to conditioning a reliable behavioral response such as a place preference (Schultz 2010, Ventura, et al. 2007). Through this series of reactions, the limbic structure provides an animal the ability to progressively assess a situation or stimulus. That information is then compounded in to a memory and available for the animal to draw upon in a future situation. This allows animals to be able

435 to reasonably anticipate the results of their future actions (Karli 1967). Based on the
436 consequences from reacting to a novel stimulus, an animal is reinforced to either repeat
437 their behavior or avoid the stimulus next time it is encountered. Through this learning
438 process animals are able to identify which behaviors are pertinent to survival, and will
439 begin to perform these behaviors with increasing reliability. When animals experience a
440 negative outcome associated with a stimulus, they are significantly more likely to avoid
441 that stimulus in the future.

442 **1.4.2 Conditioned place preferences and aversions**

443 In order to assess whether an animal finds a situation beneficial or aversive, its
444 behavior during the situation must be titrated against its normal behavior. Aversion has
445 been defined as "'a tendency to extinguish a behavior or to avoid a thing or situation and
446 especially a usually pleasurable one because it is or has been associated with a noxious
447 stimulus" (Merriam-Webster 2015a). Preference is defined as the "strength of motivation
448 to obtain or avoid one resource or stimulus and the strength of motivation to obtain or
449 avoid another" (Kirkden & Pajor 2006). Conditioned place preference and conditioned
450 place aversion paradigms are tools that can be used to evaluate the level of preference or
451 aversion an animal exhibits in response to a stimulus. Conditioned place preference tests
452 involve the pairing of an unconditioned stimulus, such as a test box, with a rewarding
453 stimulus such as food or cocaine. The animal will then begin to associate the previously
454 unconditioned stimulus with the reward. The unconditioned stimulus is now a
455 conditioned stimulus, and the animal will begin to seek access to the stimulus with an
456 expectation of reward (Prus, et al. 2009). Conditioned place aversion tests use the same
457 model, however the unconditioned stimulus is paired with something unpleasant such as

a shock or a dose of lithium chloride. The animal is then conditioned to associate the stimulus with an unpleasant event and will seek to avoid the stimulus. Conditioned place preference and aversion tests are the most direct measures of animal welfare because it's possible to evaluate the price an animal is willing to pay to either gain access to or avoid a certain situation (Dawkins 1990). These tests allow for the free choice of an animal, and it can be assumed that animals will choose the situation which they believe will benefit them the most. For example, lambs are able to discriminate between a novel, harmful foodstuff and a familiar, safe foodstuff. When presented with the choice between a familiar, safe feed and a novel, harmful feed lambs consistently decrease their intake of the novel food item over time. By nature, lambs graze on a variety of substances and as the lambs consumed both feeds and became ill, the consumption of the novel food reliably decreased. This behavior is consistent when lambs are presented two novel food groups as well. As the lambs sample both of the novel foods and begin to become ill, they decrease their consumption of the novel food that least resembled their initial, and safe, diet (Burritt & Provenza 1989). Similar results have been demonstrated in cannulated cattle, and rats (Lane, et al. 1990, Rozin & Kalat 1971). Alternatively, when animals experience a positive outcome related to a specific stimulus, they undergo appetitive associative learning and are subsequently more likely to repeat their actions in the future. This learning pathway is represented by the lambs consuming more of the familiar, safe foodstuff. It can also be represented by a mouse traversing a maze with increasing rapidity to obtain a reward. Similarly, it has been demonstrated by goats decreasing the time necessary to solve a visual puzzle in order to obtain drinking water. Goats are first able to associate a specific visual cue with access to drinking water. They are then able

discriminate between various shapes on a screen, and pick the correct shape that is associated with a reward. Furthermore, they are able to solve these problems at increasing speed. The number of attempts needed to reach a level of 46% correct became significantly fewer in subsequent puzzles compared to the first puzzle. This indicates that goats were learning to repeat a beneficial behavior (Langbein, et al. 2008, Langbein, et al. 2007). Heifers will exhibit fear behaviors in response to a visual stimulus that has been conditioned to be an indicator of an electric shock. The sight of the stimulus evokes memories of an electric shock (Veissier, et al. 1989). All of these examples exhibit the ability for animals to experience associative learning in response to environmental stimuli.

1.4.3 The effect of fear on the learning process

While the limbic system contains the food and reward center, it is also responsible for processing fear. Fear is a necessary emotion for survival; it allows animals to perceive and react to potential threats. "Fearfulness" in terms of animal behavior is defined as the likeliness of an individual animal to respond to a variety of potentially threatening situations. Any situation that is novel to an animal can be considered a "potentially threatening" situation, and the fearfulness of an animal to novelty can be measured by their reactivity in a new environment or during a new experience (Boissy 1995). While assessing a novel situation, animals must draw information from their memory and compare previous learning experiences to the new environment. Subtle similarities to a previous situation that was either positive or negative will influence an animal's response to a new stimuli. Fear-inducing stimuli can be classified in to five categories: dangers that are incorporated in the history of the

504 species, dangers associated with novelty, learned dangers, danger from conspecifics, and
505 fear from high intensity stimuli (Boissy 1995). Fear behaviors have been shown to
506 decrease with repeated exposure to a novel situation. After repeated exposure, an animal
507 begins to expect the consequences of a behavior.

508 The predicted error theory of learning postulates that learning occurs due to the
509 discrepancy between what an animal predicts of an unconditioned stimulus, and what
510 actually occurs during contact with the unconditioned stimulus. The surprise from the
511 predicted error facilitates learning and memory formation and an animal distinguishes a
512 new predicted value of the stimulus (Terao, et al. 2015). A novel object or environment
513 would likely have a low predicted value and may initially instigate a fear response related
514 to self-preservation. However, learning via predicted error will form a memory associated
515 with novel stimuli, and the animal may react differently towards the next novel stimulus.
516 This phenomenon can be seen in domestic chicks that were allowed access to enrichment
517 items. Chicks that were allowed access to enrichment items were less likely to exhibit
518 fear responses to novel objects or environments. In this case, the perceived danger of
519 novelty was reduced due to prediction error learning associated with enrichment (Jones &
520 Waddington 1992).

521 Although there is a physiological difference between the states of fear and
522 anxiety, they are considered to be similar motivators of the behavioral response of an
523 animal. When an animal is experiencing fear, it is responding to what it perceives as a
524 potential threat to its safety. The emotional state of fearfulness may elicit a range of
525 activities including fight or flight responses such as attacking, fleeing, or even complete
526 movement inhibition. Vocalizations, pheromone release, facial expressions, and

piloerection are also considered indicators of fear (Boissy 1995). The intensity of a response is often correlated with the intensity of the stimulus. For example, when exposed to a 3-second electric shock prior to food access, food-deprived rats will increase their consumption of the food. However, when the rats are exposed to an electric shock for 30 seconds, food consumption was significantly decreased (Strongman, et al. 1970). These observations suggest that animals experiencing very intense levels of fear or anxiety will exhibit movement inhibition, which includes the cessation of activities that are pertinent to survival such as feeding. If an animal is introduced to a novel environment with a food substance readily available and the animal does not eat, it is reasonable to conclude that the animal is in a state of intense fear and is experiencing inhibition of movement. Through an understanding of the psychological effects of fear and anxiety, it is possible to use an animal's response to a stressful stimulus as an accurate measurement of their emotional state.

1.5 Associative Learning in Neonates

Understanding the ability and the process of learning in neonates is crucial to gathering accurate data from their performance in conditioned place preference and conditioned place aversion paradigms. The associative learning process is vital in neonates for forming maternal connections and preferences. After birth, mammalian mothers tend to orient themselves or their offspring in a way that encourages tactile contact and nursing. In neonates, teat-seeking behaviors such as repetitive head and oral movements, are innate reflexes that are reinforced with the reward of milk. Similarly, behaviors exhibited by the mother to encourage suckling are soon associated with the reception of milk. When these sensory stimuli are coupled with the act of nursing, the

highly excitable pleasure center of the brain is activated. This initiates the release of dopamine, which in turn creates a rapid learning event and a strong association between a previously unconditioned behavior and the salient food reward. This learning process is pertinent to the survival of the offspring as the mother's milk is the sole source of nutrients in the early stages of life (Nowak 2006). The necessity of associative learning in neonates is illustrated by the response of lambs who were denied teat access up to 12 hours. Initially, teat-seeking behavior was high, representing the typical repetitive head and oral movements that are associated with teat-seeking behaviors in mammals. Although lambs received sensory information from their mother such as olfactory and tactile stimulation, they were not able to associate the salient stimulus of maternal contact with the positive outcome of nutritional support. Subsequently, teat-seeking behavior began decreasing significantly at 2 hours post-birth. After lambs were allowed teat access, teat-seeking behavior increased over time. The increase in teat-seeking behavior can be attributed to accidental contact with the newly uncovered teats. During contact with the teat, which is acting as a novel stimulus, there is a release of dopamine in to the brain. While the novelty of the stimulus promotes dopamine release, contact with the teat is a rewarding experience for the lamb. Lambs have an innate need to suckle that is separate from hunger, and contact with a teat would be a salient positive stimulus in relation to that behavioral need. Teat-seeking then becomes a lucrative action as the reward of food activates the amygdalofugal pathway and the lamb experiences learning (Alexander & Williams 1966). The lamb continues to increase this behavior as the reward maintains or surpasses its “predicted error”. Dopamine release in response to a reward follows prediction error coding. If a reward surpasses the originally expected value, there

is an increase in dopamine released. If a reward matches the expected value there is no change in the amount of dopamine released, and if the reward value is less than expected there will be a decrease in the dopamine released (Schultz 2010). Considering the information available about the cognitive ability of neonates, it is evident that using conditioned place preference and aversion paradigms are a useful tool in many species of immature animals.

1.6 Assessing the Tolerance of Neonatal Goats to CO_2

Although there is currently no literature supporting the problem-solving or associative learning abilities of neonatal goats, previous research provides supporting evidence that mature goats are capable of solving problems and neonates are capable of associative learning. Additionally, goats are especially sensitive to pain and will not tolerate painful procedures (Galatos 2011). This suggests that if a goat finds a situation unpleasant, it is likely to show some degree of avoidance behavior when presented again with the same stimulus. Subsequently, the use of a conditioned place aversion paradigm will be a relevant, feasible, and accurate test to determine the aversion of neonatal goats to CO_2.

1.6.1 Conditioned place paradigms

In order to properly evaluate the aversion that goat kids may develop to CO_2, it is necessary to determine what reward must be used as a cost comparison. Although there is a general lack of research considering what goats might perceive as valuable, it is possible to estimate what stimuli may be most relevant. Goats are gregarious creatures by nature. When isolated, juvenile goats will perform stress behaviors such as rearing and

595 vocalizing which suggests that goats prefer to be nearer to their penmates (Price & Thos
596 1980). While a kid's desire to be with conspecifics is valuable, research has also shown
597 that goats may not be as gregarious as other species, such as sheep. It has been observed
598 that goats do not have as high of a flocking tendency as sheep do, which suggests that
599 kids may need a higher value stimulus to enter the unconditioned environment (Lyons, et
600 al. 1993). An alternative reward would be access to a milk ration. As mentioned
601 previously, neonatal animals have a high drive to reach food, and are able to quickly
602 make the association of a specific stimulus with food. When goat kids are presented with
603 a milk ration while inside the unconditioned environment, they will begin to associate
604 that environment with food and the unconditioned environment will become a
605 conditioned stimulus. Once the kids consistently choose to be in the conditioned
606 environment, it will be possible to apply the CO_2 treatment and determine if exposure to
607 the gas is an aversive experience.

608 **1.6.2 Gas concentrations and flow rates**

609 One of the biggest components for proper euthanasia using gas is finding the
610 most appropriate concentration and gas flow rate. The most appropriate concentration and
611 gas flow rate will produce minimal levels of distress and pain during application. Finding
612 the least aversive method for administration of inhalant agents has been a continuous
613 topic of research, and new findings are refining the way the gas euthanasia is utilized.
614 When using CO_2 as a euthanasia agent, animals should be placed in an ambient container,
615 and the gas should be gradually introduced in to the environment. Rats showed lesser
616 amounts of distress during displacement with 100% CO_2 at a flow rate of 10% per minute
617 compared to 100% argon gas at a displacement rate of 50% per minute (Burkholder, et al.

2010). Rats exposed to CO_2 at a 20% per minute displacement rate lost consciousness before nociceptor activation and the onset of gasping and seizures (Hawkins, et al. 2006). According to the AVMA euthanasia guidelines, CO_2 flow rates should range from 10-30% volume displacement per minute (Leary, et al. 2013 p 24-26). The gradual fill method is recommended for the utilization of CO_2 due to evidence that nociceptors are activated at concentrations of 50% and above. Additionally, animals may remain conscious for up to 15 seconds in high levels of CO_2. For this reason, placing an animal in to a pre-filled chamber of 100% CO_2 is unacceptable (Hawkins, et al. 2006, Leary, et al. 2013, Wise, et al. 2003). Some literature has suggested that poultry are not as sensitive to CO_2, however 9 birds exhibited withdrawal behavior at 55% CO_2 which suggests that localized nociceptors were activated (McKeegan, et al. 2006). While different species may produce a wide range of responses to CO_2, the sensitive nociceptive pathway seems to be well conserved. Therefore, the use of concentrations near the common localized threshold (≤50%) will likely produce positive results during aversion testing of species where the pain threshold is unknown.

1.7 Research Objectives

Researching and refining any current or potential methods of on-farm euthanasia will ensure that the most humane and efficient methods will be readily available to all ruminants. Due to the fact that no current literature addresses the use of gas euthanasia for ruminants on-farm, it is imperative that this option is investigated for producers. In order to properly execute a conditioned place aversion model, it is first necessary to determine whether kids have the ability to problem solve and accomplish associative learning. Once these results are established, it will be possible to introduce

641 CO_2 and assess the resulting changes in behavior. The contents of this thesis will cover
642 the ability of neonatal goats to maneuver a novel environment in order to access a reward,
643 their ability to retain memory, and their response to a benign, novel odor. Additionally,
644 goat kids will be exposed to varying levels of CO_2 in order to evaluate their initial
645 response to the gas, as well as any avoidance behaviors exhibited due to conditioned
646 aversion. The use of neonatal goats for this study will provide beneficial information for
647 the direct application to goats, and also work as a model for extrapolation to other
648 ruminant species.

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CHAPTER 2.

ASSESMENT OF LEARNING ABILITY AND THE EFFECT OF FEAR IN NEONATAL GOATS

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2.1 Abstract

A key component in evaluating inhalant euthanasia is determining the aversiveness of the gas by conditioned place preference and aversion paradigms. The objective of this study was to determine the learning ability of goat kids, and if the presence of a visual obstacle or novel stimulus hinders learning or disrupts previous learning. A test box was custom built with two chambers connected by a sliding door. One chamber was vacant while the other held a milk reward. Twenty-four kids were enrolled in the study. Kids were given 5 minutes acclimation in the control chamber before the sliding door was open. Kids were then given 5 minutes to travel through the doorway voluntarily, after which kids were physically assisted. Kids were allotted 10 minutes in the treatment chamber. Twelve kids were tested for 5 days, and then re-tested with a novel odor after a short break. The remaining 12 kids were tested for 5 days with a transparent, plastic curtain placed in the doorway. Vocalizations occurring in the control chamber, and after the sliding door opened, decreased over day for all kids ($P < 0.0001$). Latency to enter, bottle touch and suckle all decreased over day for all kids ($P < 0.0001$). Milk consumption increased from day 1 to day 5 for all kids ($P < 0.0001$). Startle, bottle

engagement and lying behavior did not differ between days (P >0.05). These results suggest kids are able to learn with and without the presence of visual obstacles, and novel odor does not disrupt learning.

Keywords: conditioned, fear, goat, kid, learning, neonate

2.2 Introduction

Two common models used during euthanasia research are the conditioned place preference (CPP) and conditioned place aversion (CPA) paradigms. These models are important when investigating the level of pain or distress an animal might feel during exposure to a novel stimulus. The CPP and CPA paradigms follow the classical conditioning process where a potent stimulus is paired with an unconditioned stimulus. Depending on the nature of the salient stimulus, the test subject will begin to associate the unconditioned stimulus with positive or negative emotions (Prus et al. 2009). These tests are especially useful during euthanasia studies because it is possible to assess an animal's perception of the situation. Using a CPP test followed by a CPA test allows for the comparison of something that has a high value, i.e. food, enrichment items, or drugs, to something that has the potential to be aversive. This model is especially useful because it is possible to grade the level of aversion an animal has to the stimulus. By asking the animal how much it is willing to "pay" to access the high value stimulus, or forgo to avoid a noxious environment, it is possible to observe the most direct measure of how the animal experiences the situation (Dawkins 1990). Being able to collect this data is particularly relevant during euthanasia studies, as the potential for fear and distress is a critical factor in determining suitability of the methodology.

Considering the number of neonatal animals that must be euthanized on-farm, the use of neonatal animals during euthanasia research is imperative. One concern for the use of neonates is that cognitive development is just beginning, which suggests that they may not be not as capable as the mature animal to experience associative learning and

memory formation. If this were accurate, it would be difficult to properly apply a CPP or CPA test to gather information. However, neonates of many different species have shown the ability to process environmental cues and compound memories to facilitate learning (Alexander & Williams 1966, Barr & Rossi 1992, Boissy & Bouissou 1988, Liu et al. 2014, Nowak 2006).

The ability to research neonatal reactions directly is exceptionally pertinent to the dairy industry, where events of neonatal euthanasia greatly outweigh the euthanasia of mature animals (USDA 2007). An influx of male offspring that hold little to no value for the producers contributes to the disproportionate amount of neonatal euthanasia on dairy operations. When confronted with male offspring that will not be retained for breeding stock, producers must choose to either finish the animal for meat production or humanely end its life. Many dairy bull calves end up in the veal industry which has long been the center of several controversial welfare issues (Suárez et al. 2007, Thomas & Jordaan 2013). Other calves are exported for finishing which presents welfare issues that occur during transportation (Cave et al. 2005, Jongman & Butler 2014). For these reasons among others, producers may choose to euthanize their unwanted bull calves (Compassion in World Farming 2013). The dairy goat industry has a similar, but perhaps more dire, situation. The probability of unwanted buck kids being born is increased due to multiple births, and the market for goat meat in the United States and the United Kingdom is minimal (Harwood 2010, Liu et al. 2013). Subsequently this leads to an extensive number of kids euthanized by producers (Humane Slaughter Association 2008).

As such, further euthanasia studies focusing on the needs of these industries will provide a direct benefit to the welfare of these species.

Literature concerning the learning ability of domestic goats is limited, however there is literature supporting the ability of goats to solve problems and draw on past experiences to drive decision-making (Langbein et al. 2007). Goats have displayed the ability to solve visual puzzles to achieve a reward, and to recall these memories when presented with the same problems several days later (Langbein et al. 2008). Goats have also displayed an ability to discern between benign and harmful food sources. Goats who were naïve to the intoxication of the poisonous Brazilian plant *Ipomoea carnea* were averted from eating the plant after its pairing with lithium chloride (Burritt & Provenza 1989, Oliveira Júnior et al. 2014).

Although the learning ability of mature goats has been documented, data regarding the learning ability of neonatal kids is insufficient. While kids must be able to learn and associate maternal cues, little research has been done investigating the responses of kids to conditioned place preference tests. Consequently, it is necessary to gather this primary data regarding CPP before utilizing this model in future studies. Possible obstacles that may hinder the abilities of kids to achieve a conditioned place preference include the behavioral and physiological effects of fear. Acute stress has been shown to inhibit CPP (Bali et al. 2015, García-Pardo et al. 2014), and the acute stress of being isolated and placed in a novel environment may affect the potential for CPP in kids. Additionally, acute stress may have a strong impact on kid behavior due to their sensitive nature (Galatos 2011). However, literature has also supported that acute stress enhances a

98 salient reward and facilitates conditioned place preference (Brielmaier et al. 2012, Der-
99 Avakian et al. 2005). In consideration of the potential benefit for future euthanasia
100 studies, the evidence supporting the learning ability of kids merits further investigation.

101 The overall objective of this study was to determine if fear influences the ability
102 of kids to problem solve to reliably access a food reward. We hypothesized that trial and
103 error learning would occur, such that the kids would learn the task to obtain a food
104 reward and performance would improve over multiple tests. We also hypothesized that
105 fear attenuates learning, such that fearful kids would display reduced performance when
106 compared to non-fearful kids. A secondary objective was to determine the effects of a
107 novel stimulus on previous learning. We hypothesized the presence of a novel stimulus
108 would not affect the performance of the previously learned task for non-fearful kids.

109 **2.3 Materials and Methods**

110 The protocol for this experiment was approved by the Iowa State University
111 (ISU) Institutional Animal Care and Use Committee

112 **2.3.1 Experimental design**

113 A conditioned place preference paradigm was utilized to test the learning
114 abilities of the test subjects. This experiment was a repeated measures design with each
115 test subject acting as its own control and each kid was tested individually. Kids were
116 tested over 5 consecutive days of initial testing, and tests were conducted between the
117 hours of 1:00pm and 5:30pm after a period of feed deprivation which ranged between 5
118 to 9.5 hours. Kids were randomly assigned a testing time point, and were consistently

tested at that time point daily. The experiment was run over 2 trials with twelve kids assigned to each trial. In trial 1, kids received a 3 to 6 day break after the initial testing period, and were re-tested once with a novel stimulus (peppermint oil odor) present. In trial 2, the initial testing was modified to require kids to push through a clear plastic curtain to access the treatment chamber.

2.3.2 Experimental equipment

A preference testing box (Figure 2.1) was custom designed with two connecting chambers, defined as "control" and "treatment", separated by a sliding door. This testing box was designed for the purpose of evaluating the aversiveness of inhalant euthanasia gases. Fans were installed in the walls of the box and a continuous flow of ambient air was introduced in to the system via air inlets attached to the bottom structure of the box. The inside dimensions of each chamber measured 61 cm width x 61 cm length x 91 cm height. The side panels of the box were made of opaque, hard plastic. In the control chamber, plastic gloves were fitted on each side panel to facilitate handling of the animal when required during the test. These gloves were retracted from the box when not in use. To enable viewing, clear plastic was used for the doors which were located on the lateral ends and the top of the box. The floor was covered with rubber floor mats in both chambers to provide traction. To attract kids to the treatment chamber, two milk bottle holders were installed and contained 472mL milk bottles that were identical to those used during daily feeding.

In trial 1, a container filled with cotton swabs saturated with peppermint oil extract was placed in the conditioned air inlet for the novel stimulus test. In trial 2, the

doorway separating the chambers was fitted with a plastic curtain (58.4 cm length) made of 10 transparent PVC strips (2.5 cm width) in addition to the sliding door.

2.3.3 Animal husbandry and enrollment

A total of 24 mixed breed neonatal dairy kids (3 females, 21 males) were enrolled, sourced from three commercial herds in the Midwest USA. Kids were of various breeds including Toggenburg, LaMancha, Alpine-Sannen cross, and Nubian. Kids were collected and enrolled from March through October 2014. Kids were removed from the dam after birth and bottle-fed prior to enrollment. Kids were acquired between 1-7 days of age to ensure adequate consumption of colostrum, and the mean body weight upon arrival was 4 ± 0.2 kg. None of the male kids were castrated and no kids were disbudded. All kids were ear tagged for identification prior to arrival at ISU.

Kids were housed in 3 climate-controlled rooms at ISU Laboratory Animal Research (LAR) buildings, with a 12-hour light cycle from 6:00am to 6:00pm. Kids were housed in a 9.3 m^2 room that was divided equally into 5 pens to facilitate individual feeding. Pens were separated using spindle barriers with 5 cm separation between bars that allowed nose-to-nose contact for social interaction. Each pen contained one heat lamp, one plastic tub for climbing, and straw bedding for comfort.

Body temperatures were recorded daily using a hand held thermometer (Mabis Healthcare Inc. Waukegan, IL) and body weights were recorded weekly using a handheld scale that was accurate to 0.01kg (Pure Fishing, Inc Columbia, SC). All kids received daily milk rations equal to 18% of their body weight in grams. Advance milk replacer

(Milk Specialties Global Eden Prairie, MN) was fed using standard 472mL graduated lamb milk bottles equipped with Pritchard teats (Pritchard teats, Riverton, New Zealand). Nine kids were fed 3 times daily during all days of acclimation and testing. Fifteen kids were fed approximately every 4 hours for the first 4 days due to health concerns, and then 3 times daily.

Upon arrival at LAR, all kids received at least 3 days of acclimation, during which no experimental procedures were performed. Kids were observed for any health issues; the acclimation period was extended for kids that exhibited clinical signs of illness until these signs were no longer present. No kids developed clinical signs of illness during testing. In addition, kids were required to reach a behavioral start criteria based on suckling motivation before enrollment in testing. Kids were considered successful in meeting this criteria if during 4 out of 5 consecutive feedings they actively found and sucked on the nipple within two minutes of the bottle being placed in the bottle holder.

2.3.4 Testing procedure

Each kid was carried individually from their home pen to the testing room, and placed in the control chamber. Kids were provided with 5 minutes to acclimate to the box, after which the sliding door was opened providing access to the treatment chamber. Kids were given 5 minutes to voluntarily pass through the doorway, after which they were gently assisted through the doorway using the attached rubber gloves. Once in the treatment chamber, kids were given 10 minutes access to the entire testing box, during which they could move freely between treatment and control chambers. After testing concluded, kids were removed from the box and carried back to their home pen.

An indoor temperature monitor (AcuRite Lake Geneva, WI) was placed within the control chamber to record the relative humidity (%) and temperature (C°) of the test box prior to each test. This environmental data was recorded by the observer for each individual kid immediately prior to entry. The mean relative humidity inside the box was 36.8% and ranged from 16% to 69%. The mean temperature was 22.2° and ranged from 13.9° to 32.2°. The monitor was removed as each kid was placed in to the box, and replaced after each test concluded. Between tests the box was cleaned with a disinfectant (Accel, Virox Technologies Inc., Ontario, Canada).

2.3.5 Behavioral observations

Data was collected via live observation and video recording. Live observation was gathered by two observers. One observer (observer 1) was positioned on the right side of the box out of view of the test subject. The second observer (observer 2) was positioned in front of the treatment chamber so that the kid was visible to facilitate the recording of direct behavior observations. A black fabric curtain (2.1 m length x 0.9 m wide) and lighting placement was used to ensure that observer 2 was obstructed from the kid's view.

2.3.6 Live observations

Behaviors that were recorded via live observation were selected due to the difficulties associated with reliably discerning these behaviors on video (Table 2.1). The latency to enter the treatment chamber was measured using a timer (National Presto IND. Inc., Eau Claire, WI) after the sliding door was opened until both ears of the kid crossed the doorway form the control to the treatment chamber. When assistance was needed,

latency to enter was recorded as 5 minutes. Vocalizations were collected as a counted event and separated in to 3 categories: control, transition, and treatment. The amount of milk consumed from each bottle was recorded after each test.

2.3.7 Video observations

Video data was collected using a Noldus Portable Lab (Noldus Information Technology, Wageningen, NL). Four color Panasonic cameras (WV-CP484, Kadoma, Japan) were positioned to provide views from top and lateral doors of the control and treatment chambers (Figure 2.2). The recordings from these cameras were captured onto a PC using HandiAvi (v4.3, Anderson's Azcendant Software, Tempe, AZ) at 30 frames/s. Prior to each test, identifying information was presented on a dry erase board to the camera to identify the date, animal ID, test day, and trial number.

Behavior data was collected from videos by one trained observer who was blinded to the animal ID, date and test day. Behavioral data was recorded using Observer (v10.1.548, Noldus Information Technology, Wageningen, NL). A neutral individual performed the blinding procedures for the video recordings from all tests. The blinding procedures involved cutting the video recordings to remove identification presented at the beginning of each video, assigning a random number to each video segment and sorting for the purpose of providing a random sequence in which videos were to be scored. Seven videos were selected at random and duplicated within this sequence for the purpose of determining intra-observer reliability.

Prior to data collection, the observer was trained to use the Observer program by repeatedly scoring 2 videos and ethogram from an unrelated study until reaching a reliability score of $k \geq 0.90$ as calculated by the Observer program. After reaching this level of competence, data collection began using the current videos and ethogram (Table 2.2). Intra-observer reliability averaged $k = 0.91$.

2.3.8 Statistical analysis

Behaviors were evaluated based on duration or count data. Behaviors involving count outcomes e.g. vocalizations, were analyzed using a mixed effect Poisson regression model. Elimination behaviors were analyzed as binary outcomes by a mixed effect logistic regression model. Behaviors involving latencies e.g. latency to enter treatment chamber, were analyzed using mixed effect Cox models, and total milk consumption was analyzed by a linear mixed model. All linear mixed model analysis was fitted with the GLIMMIX procedure (SAS Inst. Inc., Cary, NC), while Cox model analysis was fitted with the PHREG procedure. Each kid was an experimental unit. The fixed effects for data analysis were: day, startle score, breed, sex, and time of day. Startle score was used as an indicator of fear and was determined by the sum of each kid's startle and escape attempts. The random effect was individual kid identification. The degrees of freedom was determined by the Kenward-Rogers method, and a P-value of ≤ 0.05 was considered significant. A Tukey-Kramer adjustment was utilized for multiple comparisons.

2.4 Results

2.4.1 Learning

All kids (n=24) completed the start criteria and were enrolled in the study. On day 1, the majority of kids (n=16 [66.6%]) needed assistance to enter the treatment chamber. Once kids entered the treatment chamber voluntarily, no assistance was needed for the remainder of the trial. Eleven kids required assistance on day 2, 5 kids (20.8%) required assistance on day 3 and only 1 (4.2%) kid required assistance on day 4. However, by day 5 all kids learned to enter the treatment chamber without assistance and consumed milk.

The average latencies to enter, first bottle touch and suckle for day, food deprivation period, breed, and sex are depicted in Table 2.3. The average latency to enter, first bottle touch, and suckle decreased over day ($P < 0.01$). The latency to enter for kids receiving 9 hours of feed deprivation was 3.5 seconds slower than kids with 9.5 hours ($P = 0.02$). However, differences in latency to enter were not observed between any of the other feed deprivation periods ($P > 0.1$). Toggenburg kids had a significantly shorter latency to enter than Nubian kids ($P < 0.01$). However, only 2 Nubian kids were enrolled compared to 9 Toggenburg kids. There was a trend for an association between latency to enter and individual kids ($P = 0.08$), and there was a significant association between individual kid and latency to suckle ($P < 0.01$). There were no other associations between time of day, kid, breed, or sex and these measures ($P > 0.1$).

The duration of bottle engagement and total milk consumed for day, food deprivation period, breed, and sex are depicted in Table 2.3. The total duration of bottle engagement did not change over time ($P > 0.1$). With the exception of 1 male kid, all kids consumed milk from both bottles on at least 1 test day. During 5 tests, kids consumed all the milk from one bottle and continued to suck air; these tests were terminated due to animal welfare concerns. Milk consumption increased over test day ($P < 0.01$), but time of day, breed and sex were not significant factors ($P > 0.1$).

2.4.2 Fear-related behaviors

The number of vocalizations, startles, rears, and duration of lying for day, feed deprivation period, breed, and sex are presented in Table 2.4. No tests were terminated early due to concerns of injurious or otherwise extreme fear behavior. Escape attempts were rare events, occurring only a total of 7 times over all days. Nine kids reared on day 1 (median 20, range 5 to 34), and on day 5 a total of 5 kids reared (median 5, range 2 to 9). Occurrences of elimination ranged from 11 to 18 events per day, with a mean of < 1 event per kid per day. Lying behavior did not differ between days ($P > 0.1$).

Vocalization was exhibited by all kids on all test days. Control vocalizations decreased over test days ($P < 0.01$), with days 4 ($P < 0.01$) and 5 ($P < 0.01$) differing significantly from day 1. Transition vocalizations decreased over test days, with day 1 significantly different from all other test days ($P < 0.01$). Time of day, kid, breed and sex were not associated with control or transition vocalizations ($P > 0.1$). Thirteen kids (54%) vocalized in the treatment chamber on day 1 (median 8, range 0 to 117), and on day 5 a total of 7 kids (29%) vocalized in the treatment chamber (median 16, range 0 to 36).

Startle score for each kid was determined by the cumulative number of startle events of an individual kid over all test days. Startle scores were positively skewed with a minimum of 0 and a maximum of 24 (Figure 2.4). The number of startle events did not differ over time ($P > 0.1$). Female kids had higher startle scores than males ($P = 0.05$), although this was likely due to the small sample of female kids. Startle score was not associated with control or transition vocalizations ($P > 0.1$; Figure 2.5), and was not associated with latencies to enter, bottle touch and suckle ($P > 0.1$; Figure 2.6). Startle score was not associated with the amount of time spent engaging the milk bottles or milk consumption, and startle score was not associated with elimination behavior or lying ($P > 0.1$).

During Trial 1 (novel stimulus test), 1 male Alpine cross kid made an escape attempt on both days, while 1 male Toggenburg made a single escape attempt on day 6. Elimination was observed in 4 kids on day 5 and 2 kids on day 6. The number of vocalizations, startles, and rears during trial 1 are presented in Table 2.5. Vocalizations did not differ between days 5 and 6, and there were no associations with time of day, kid, breed or sex ($P > 0.1$). The number of startles was significantly higher on day 6 ($P < 0.01$), the number of rears and duration of lying did not differ ($P > 0.1$).

All kids in trial 1 (n=12) entered the treatment chamber in the presence of the novel stimulus on day 6. The average latency to enter was longer on day 5 than day 6 by 2.9 seconds ($P < 0.01$; Table 2.6). Kids with 8.5 hours feed deprivation had the shortest latency to enter ($P =0.04$). There was an association between latency to enter and individual kids ($P = 0.03$), but not for breed or sex ($P > 0.1$). The latency to first bottle

touch did not differ between days and was not associated with time of day, kid, breed or sex ($P > 0.1$). Latency to suckle was significantly longer on day 5 than day 6 ($P < 0.01$). There was a trend for an association between latency to suckle and feed deprivation ($P = 0.07$), but not breed or sex ($P > 0.1$). There was no difference in milk consumption between day 5 and day 6 ($P = 0.1$; Table 2.6).

All kids in trial 2 (n=12) crossed through the curtain to access the treatment chamber within 5 days. On day 1, all kids needed assistance to cross the curtain to the treatment chamber, and all the kids that needed assistance on day 3 or 4 were all enrolled in this trial. The median latency to enter the treatment chamber was 178.5 seconds and ranged from 1 seconds to 300 seconds (Figure 2.7). The median latency to the first bottle touch was 2 seconds for both trials, and to suckle was 5 seconds for both trials.

During trial 2, 1 male Alpine cross made a single escape attempt on day 2, and 1 male Alpine cross and 1 male LaMancha made a single escape attempt on day 5. Elimination was observed in 5 kids on day 1 and 9 kids on day 5. Fear behaviors for trial 2 are presented in Table 2.7. Control vocalizations averaged 117.6 ± 19 on day 1, and 102.6 ± 13.8 on day 5. Mean transition vocalizations were 72.7 ± 12 on day 1, and 2 ± 0.8 on day 5. Mean treatment vocalizations were 6.7 ± 4.1 on day 1, and 3 ± 0.8 on day 5. The average number of startles on day 1 was 0.7 ± 0.2 on day 1, and 0.8 ± 0.2 on day 5. No rears occurred on day 1, and 1.5 ± 0.7 rears occurred on day 5.

2.5 Discussion

Live and video data were successfully recorded for all kids on all test days. The observer charged with collecting all vocalizations and latency to enter data was a rotation of various trained lab members and employees, and the data was consistent between observers. Fifteen of the 24 kids exhibited signs of illness including fever and moderate to severe diarrhea upon arrival at LAR. Four kids received saline solution, administered either intravenously or subcutaneously, due to severe dehydration. Five kids showed signs of severe diarrhea and received treatment with Naxcel until symptoms improved; 4 kids required 3 days of treatment and 1 kid required 5. One kid received treatment with Banamine upon arrival for a temperature above 104°. Testing occurred simultaneously with Naxcel treatment, and it is possible that the performance of these 5 kids was impaired by illness (Dilger & Johnson 2010). The performance of these kids may have also been impaired in relation to the other 7 kids from trial 2 due to a change in curtain resistance between the groups.

Data collected by direct observation was done so to ensure that small movements or angle-dependent behaviors that would not be visible on video were recorded accurately. Data collected by video observation included behaviors that occurred in fast succession, prolonged duration, or were not visible to either observer. The collected behaviors were either considered to be associated with fear, learning, or normal expressions of the domestic goat. Behaviors were categorized based on previous research of domestic goats and other species. Behaviors associated with fear included vocalization and rearing (Lyons et al. 1993, Price & Thos 1980, Siebert et al. 2011).

Learning behaviors such as the latency to enter the treatment chamber and latency to suckle focused on the amount of time needed to react to a previously encountered stimulus (Langbein et al. 2008, Langbein et al. 2007, Ruediger et al. 2012).

The choice of a conditioned place preference model was appropriate in determining the capability and motivation of kids to access a milk reward in the presence of exogenous stress. During CPP testing for trials 1 and 2, the milk reward successfully acted as a stimulus to motivate kids to move through the test box. The approach-avoidance model was successful in evaluating the motivation of kids to access a known food reward in the presence of a perceived hazard. Overall, kids were able to navigate the test box and engage the milk bottles with increasing efficiency over test days. All 24 kids presented signs of learning as the latencies to enter the treatment chamber, first bottle touch and latency to suckle decreased from test days 1 to day 5. In trial 1, the novel peppermint odor did not appear to be a potent negative stimulus in relation to kids' motivation to reach the milk bottles. In trial 2, the curtain did appear to act as a potent negative stimulus in relation to kids' motivation in reaching the milk bottles. The fact that latencies to first bottle touch and suckle did not differ between trials indicates that the visual obstacle of the curtain only impaired performance related to gaining access to the treatment chamber and not locating the milk bottles. The results from this study indicate that kids are able to solve a simple problem to access a food reward despite various stressors.

The learning process appeared to be uniform between all 24 kids. The average time for all latencies decreased across test days as kids were able to recall memories from

previous days and were motivated to access the milk. All kids learned to travel through the test box by trial and error learning. This learning style may be conserved in domestic goats and other ruminants evidenced by the trial and error grazing pattern that many ruminants employ to test the palatability and safety of novel plants (Burritt & Provenza 1989). The expression of fear behavior was not uniform across individual kids nor was it uniform between sexes. Startle score was likely influenced by individual genetics and experiences. Although female kids demonstrated higher startle scores than males, this interpretation should be taken cautiously because only 3 female kids were enrolled out of 24.

2.5.1 Interpretation of learning performance

The learning outcomes of this study coincide with other instances of learning present in the current literature. As the kid is placed in to a novel environment its responses are initially driven by the parasympathetic nervous system and based on the desire to survive. During this period of stress, it is difficult to approach a task using cognitive skill instead of purely stimulus reaction. On test day 1, only 8 of 24 kids voluntarily entered the treatment chamber. The unwillingness of most kids to travel through the doorway, even when no curtain was present, shows that the motivation to explore a novel environment was minimal compared to the perceived danger of the test chamber. These findings are in line with studies that also found stress to be a mitigating factor in problem solving and motivation (Doyle et al. 2014, Langbein et al. 2006, Welp et al. 2004). Similar to the outcomes in these studies, the latency to enter the treatment chamber decreased on subsequent test days. As these kids were guided to the milk they

398 became highly motivated to access the reward, and the predicted outcome of approaching
399 the treatment chamber changed. This indicates that with repeated exposure, the perceived
400 threat of the test chamber declined and kids were able to react to the stimulus in a more
401 discerning manner.

402 Through repeated exposure to the environment and trial-and-error learning, kids
403 were able to evolve their strategies from random searching to localized searching to
404 direct and purposeful movement towards the treatment chamber (Langbein et al. 2007,
405 Ruediger et al. 2012). Kids exhibited similar performance in regards to latency to first
406 bottle touch and latency to suckle, although these latencies were greatly reduced
407 compared to latency to enter the treatment chamber. This may be due to the strong teat
408 seeking behavior exhibited by neonates and the exceptionally potent visual stimulus of
409 the milk bottle (Alexander & Williams 1966, Tanaka et al. 1998). The kid that never
410 consumed milk from both bottles within a single test was likely satiated with 472mL of
411 milk, and exploratory behavior had a higher value than did the excess milk (Ferreira et al.
412 2006). The results of this study provides additional data to support the idea that neonatal
413 animals are capable of associative learning and problem solving (Dilger & Johnson 2010,
414 Drake et al. 2011, Rohde & Gonyou , Webb et al. 2015).

415 **2.5.2 Interpretation of fear indicators**

416 In this study, the overall fear level of kids was relatively low and was likely due
417 to the regular handling of kids through husbandry and testing procedures. Prolonged
418 instances of handling early in life may make animals less reactive to novel situations
419 (Boissy & Bouissou 1988, Oliveira et al. 2015, Stamatakis et al. 2008). Escaping from an

environment perceived as threatening is a relatively well conserved behavior, and the minimal number of escape attempts observed represents this tempered fear level (Barnard et al. 2015, Chojnacki et al. 2014, Sadler et al. 2014). The vocalizations recorded may have been due to the stress of social isolation or frustration of an inability to reach the milk (Manteuffel, et al. 2004, Price & Thos 1980, Siebert et al. 2011). It did appear that vocalizations were an appropriate measure of fear in this study based on their significant decrease over time. In contrast to previous data, and in support of the current hypothesis, kids did appear to become habituated to the test box (Siebert et al. 2011). This is supported by the decreasing amount of vocalizations across test days.

Habituation to the novel environment is likely due to the positive association between the test box and milk access. Additionally, wild goats tend to be out of sight of their conspecifics while feeding, a characteristic that may facilitate the habituation of domestic kids to social isolation (Price & Thos 1980). In contrast to vocalizations, rearing behavior did not follow the expected pattern of reduction after habituation. It is possible that rearing behavior was related to social isolation initially, but then was based in investigatory behavior or searching strategy. It is also possible that rearing behavior remained consistent due to olfactory evidence of conspecifics that lingered inside the box between tests (Siebert et al. 2011).

Although startle behavior did not decrease over time, there was a wide range of expression between kids. However, there was no relevance of startle score in the statistical model. This indicates two possibilities: the number of startles exhibited by a

kid is not an appropriate measure of fear, or a fearful personality type does not have an inhibiting effect on a kid's learning ability.

2.5.3 Association of fear and learning

Acute stress, such as social isolation, has the ability to inhibit learning (Bali et al. 2015, Doyle et al. 2014, Frisone et al. 2002, Langbein et al. 2006, Passecker et al. 2014, Welp et al. 2004). The lack of successful performance on the initial test days may be caused by the acute stress of social isolation coupled with the stress a novel environment. Additionally, the curtain in trial 2 presented a visual, as well as tactile, obstacle and likely incited feelings of fear. The perceived threat of the curtain may have inhibited kids from employing the appropriate search strategies to facilitate learning.

The concept of "learned helplessness" is a coping mechanism that manifests during uncontrollable stress. Instead of attempting to escape or mitigate the stress, the animal does nothing and endures the stressor (Chourbaji et al. 2005). Fearful animals may be more prone to experience learned helplessness, and subsequently more likely to endure a stressor rather than be proactive about the situation. However there was no change in lying behavior, a passive coping mechanism, over test days which indicates that learned helplessness does not fully explain the kids' early lackluster performance (Siebert et al. 2011).

Although startle score was unexpectedly irrelevant, it is possible that kids with higher startle scores are more fearful but also associated with the 'avoider' personality type observed in goats. The main characteristic of the avoider personality is reactivity,

and goats with this personality type will move quickly away from agonistic and nonagonistic conspecifics. It is logical to expect these goats may show more fearful behaviors, such as a startle or withdrawal response. However, avoider goats were able to solve a novel T-maze faster than their aggressive penmates (Pascual-Alonso et al. 2013). This suggests that although acute stress has an inhibiting effect of kids' learning, a more fearful personality type does not have the same effect. Further research concerning fearfulness and personality types would provide a more in-depth understanding of individual differences in learning between kids.

2.5.4 Potential for future use

The results of this study provide supporting evidence regarding the problem solving capabilities of the domestic kid, as well as the relationship between fear and learning. This knowledge is beneficial for future use in research as well as practical settings. In the practical setting, information concerning markers for a kid's personality that are associated with their ability to learn may assist producers in neonatal husbandry decisions. Additionally, the considerable fear of the novel visual stimulus may provide information to aid in future goat husbandry decisions or training. The results of this study are also relevant for future research of the learning ability of neonates, and presents novel data that supports the use of kids as a practical animal model. This study supports previous data concerning the inhibiting effect acute stress has on learning, but does not support the idea that individual fearfulness has an effect on learning ability. These results provide a strong foundation for the use of the kid in conditioned place preference,

approach-avoidance, and other learning based paradigms, and can be used to guide future studies that use the kid as an animal model.

2.6 Animal Welfare Implications

The basis for this study was to confirm the ability of kids to learn and form associations in the conditioned place preference paradigm. Kids proved to be adept at employing learning strategies to access the food reward. Furthermore, kids were highly motivated by the reward and able to overcome a fear-inducing obstacle to gain access. The lack of response to the novel odor suggests that visual and tactile, rather than olfactory, stimuli are more inclined to elicit kid fear responses. The kid has shown the ability to overcome the necessary obstacles that may impede an investigation of the aversiveness of carbon dioxide (CO_2) and as such, would be an advantageous animal model to assess the value of CO_2 as a euthanasia agent in ruminants. By employing conditioned place and approach-avoidance paradigms, it will be possible to evaluate the perception of CO_2 by kids and subsequently judge the merit of further investigation in to the topic.

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CHAPTER 2 TABLES

Table 2.1 Ethogram used for kid behavior collected during live observation during preference testing.

Measure	Behavior Category	Variable type	Description
Latency to enter	Learning	Latency	Both ears of the kid break the plane of the treatment chamber from the control chamber.
Latency to first bottle touch	Learning	Latency	The time from entry in to the treatment chamber to first deliberate touch of any part of the bottle using the nose, mouth or head
Latency to suckle	Learning	Latency	Time from entry in to the treatment chamber to active consumption of milk from the bottle
Use of both bottles	Learning	Binary	The kid suckled from both bottles (yes/no)
Milk consumption	Learning	Count	The total amount of milk consumed from both bottles during the 10-minute treatment period
Elimination	Fear	Binomial	Any act of urination or defecation within the control or treatment chambers of the box (yes/no).
Control vocalization	Fear	Count	Vocalizations that occur in the control chamber of the box before the sliding door is opened.
Transition vocalization	Fear	Count	Vocalizations that occur in the control chamber of the box after the sliding door is opened, but before the kid enters the treatment chamber.
Treatment vocalization	Fear	Count	Vocalizations that occur in either chamber after the kid has entered the treatment chamber.

Table 2.2 Ethogram used for kid behavior collected during video observation during preference testing.

Measure	Behavior Category	Variable type	Description
Bottle engage	Normal	Continuous	Any interaction with the bottle including oral contact, nursing, and butting.
Escape attempt	Fear	Count	Coordinated jump towards the top of the box, all 4 hooves leave ground.
Rear	Fear	Count	Weight-bearing on hind limbs only.
Lying	Normal	Continuous	Weight-bearing on no limbs.
Startle	Fear	Count	Lateral jump or fast withdrawal.
Startle Score	Fear	Count	Cumulative number of startles over all days per kid.

687 **Table 2.3** Raw means ± SE for latencies to enter treatment chamber (s), first bottle touch
688 (s), and suckle (s), total milk consumed (mL), and duration of bottle engage (s) for all
689 kids (n=24) by day, feed deprivation period (d.p.), breed, and sex during preference
690 testing**.**

Test Day	Enter	Bottle Touch	Suckle	Milk Consumed	Bottle Engage
1	235.2 ± 23.2^{A}	45.8 ± 24.9^{A}	70.4 ± 24.7^{A}	440.6 ± 26.6^{A}	443.3 ± 26.8^{A}
2	173.4 ± 26.7^{B}	3.9 ± 1.1^{C}	33.6 ± 24.7^{C}	485 ± 32.5^{A}	443.3 ± 26.1^{A}
3	92.5 ± 23.9^{C}	8.2 ± 5.9^{C}	12.9 ± 6.4^{C}	535.3 ± 23.7^{B}	435.6 ± 21.2^{A}
4	39.8 ± 14.5^{C}	2.83 ± 3.6^{C}	7.4 ± 1.6^{C}	573.7 ± 26.6^{B}	431.2 ± 19.6^{A}
5	12.3 ± 5.9^{C}	1.8 ± 0.2^{C}	4.2 ± 0.4^{C}	612.2 ± 26.6^{C}	426.7 ± 21.1^{A}
d.p.					
5	146.8 ± 64.1^{A}	7.2 ± 6.2^{A}	14.2 ± 9.3^{A}	621 ± 38.4^{A}	442.8 ± 21^{A}
5.5	161 ± 65.7^{A}	5 ± 3.8^{A}	39.6 ± 31.4^{A}	656.5 ± 59.1^{A}	452 ± 25.2^{A}
6	138.8 ± 61.1^{A}	4.6 ± 2^{A}	13.2 ± 4.2^{A}	455.4 ± 17.7^{A}	385.9 ± 38.8^{A}
6.5	83.6 ± 30.3^{A}	56.2 ± 39.9^{A}	99.3 ± 53.5^{A}	473.2 ± 53.2^{A}	406.9 ± 44.3^{A}
7	87.1 ± 26.1^{A}	10.9 ± 7^{A}	20.2 ± 8.7^{A}	538.2 ± 23.7^{A}	482.9 ± 18.9^{A}
7.5	103.6 ± 29.2^{A}	6.1 ± 2.1^{A}	17.2 ± 5.7^{A}	600.3 ± 20.7^{A}	479.1 ± 26.1^{A}
8	82.1 ± 26.5^{A}	4.1 ± 1.1^{A}	8.5 ± 1.6^{A}	482 ± 41.4^{A}	436.6 ± 23.6^{A}
8.5	88.1 ± 34.3^{A}	2.5 ± 0.6^{A}	9.1 ± 3.97^{A}	544.2 ± 35.5^{A}	422.8 ± 19^{A}
9	239.9 ± 31.7^{B}	4.1 ± 1.7^{A}	11.9 ± 3.4^{A}	485 ± 26.6^{A}	376.2 ± 37.1^{A}
9.5	122.4 ± 72.5^{A}	14.4 ± 13.2^{A}	16.8 ± 12.8^{A}	452.5 ± 56.2 ^{A}	347.1 ± 37.8^{A}

691

692

693

694

695 **Table 2.3 continued**

Breed					
Alpine	105.9 ± 41^{A}	9.7 ± 7.2^{A}	17.7 ± 10.1^{A}	564.9 ± 38.4^{A}	421.7 ± 25.3^{A}
LaMancha	192.7 ± 80.9^{A}	2.3 ± 1.1^{A}	12.9 ± 14.7^{A}	511.6 ± 59.1^{A}	359 ± 57.8^{A}
Nubian	210.9 ± 88.2^{C}	3.3 ± 1.8^{A}	10.5 ± 7.9^{A}	411.1 ± 91.7^{A}	443 ± 70.8^{A}
Toggenburg	66.3 ± 33.2^{A}	21.1 ± 30.3^{A}	42.2 ± 41.7^{A}	520.5 ± 56.2^{A}	475.9 ± 44^{A}
Sex					
Male	112.3 ± 28.3^{A}	13.1 ± 13.4^{A}	27.3 ± 18.7^{A}	529.4 ± 32.5^{A}	431.2 ± 23.9^{A}
Female	98.7 ± 71.9^{A}	8.3 ± 5.9^{A}	14.3 ± 8.7^{A}	529.4 ± 50.3^{A}	469.5 ± 71.7^{A}

^{A} indicates P > 0.1; ^{B} indicates P < 0.05; ^{C} indicates P < 0.01

696

697 **Table 2.4** Raw means ± SE of fear behaviors for all kids (n=24) by day, feed deprivation
698 period (d.p.), breed, and sex during preference testing.

	Vocalizations[a]					
Test day	**Control**	**Transition**	**Treatment**	**Startle**	**Rear**	**Lying**
1	125.9 ± 12.7^{A}	46.4 ± 9.3^{A}	15.2 ± 5.9	1.2 ± 0.4^{A}	7.5 ± 2.4	25.4 ± 16.3^{A}
2	107.9 ± 9.6^{A}	27.4 ± 7.1^{C}	6.9 ± 3.6	1.9 ± 0.4^{A}	4.8 ± 0.9	18.9 ± 12^{A}
3	101.5 ± 8.6^{A}	17.2 ± 5.3^{C}	6 ± 3	1.7 ± 0.5^{A}	1.9 ± 0.9	6.8 ± 4.5^{A}
4	94.2 ± 8.3^{C}	9.9 ± 4.2^{C}	3.4 ± 1.5	0.8 ± 0.2^{A}	3.5 ± 1.9	3.3 ± 3^{A}
5	91.3 ± 7.7^{C}	1.5 ± 0.4^{C}	5.1 ± 2.1	0.8 ± 0.15^{A}	1.1 ± 0.5	0.5 ± 0.5^{A}
d.p.						
5	135.6 ± 23.5^{A}	33.4 ± 13.7^{A}	0 ± 0	1.4 ± 0.7^{A}	9.8 ± 1.2	0 ± 0^{A}
5.5	92.2 ± 10.5^{A}	14.2 ± 6.6^{A}	0 ± 0	3.2 ± 1.2^{A}	1.8 ± 1.8	27 ± 21.3^{A}
6	105.8 ± 12.1^{A}	24.6 ± 11.4^{A}	1.6 ± 1.6	0.4 ± 0.2^{A}	0 ± 0	17.1 ± 17.1^{A}
6.5	110.7 ± 9.9^{A}	6.7 ± 3.2^{A}	12.8 ± 3.8	0.9 ± 0.3^{A}	1.3 ± 0.6	40 ± 20.4^{A}

699

Table 2.4 continued

7	88.7 ± 11.4^{A}	8.6 ± 2.8^{A}	0.2 ± 0.1	0.8 ± 0.2^{A}	1.3 ± 0.7	0.4 ± 0.4^{A}
7.5	114.7 ± 13.1^{A}	20.9 ± 8.6^{A}	18.8 ± 6.3	2.3 ± 0.7^{A}	3.3 ± 1.3	0 ± 0^{A}
8	92.2 ± 7.1^{A}	22.3 ± 8.2^{A}	1.7 ± 1.3	0.5 ± 0.2^{A}	5.2 ± 2.2	2.6 ± 2.6^{A}
8.5	119.7 ± 16.4^{A}	22.8 ± 11.2^{A}	15.8 ± 7.1	1 ± 0.3^{A}	9.3 ± 3.6	29.1 ± 24^{A}
9	111.1 ± 13.4^{B}	46.6 ± 12.1^{A}	2.3 ± 2	1.3 ± 0.5^{A}	0.8 ± 0.8	0 ± 0^{A}
9.5	70.8 ± 4.8^{A}	30.2 ± 23.7^{A}	1.6 ± 1.4	3 ± 1.3^{A}	6.2 ± 1.8	0 ± 0^{A}
Breed						
Alpine	84.2 ± 12.8^{A}	18.2 ± 9.3^{A}	4.6 ± 5.8	1.62 ± 0.7^{A}	3.2 ± 1.4	14.8 ± 17.3^{A}
LaMancha	165.5 ± 22.5^{A}	54.3 ± 30.3^{A}	8.3 ± 8.5	1.9 ± 1.6^{A}	11.9 ± 7.7	0 ± 0^{A}
Nubian	103.3 ± 22.9^{A}	45.4 ± 26^{A}	2.6 ± 5.7	0.7 ± 0.6^{A}	0 ± 0	5.2 ± 11.6^{A}
Toggenburg	106.1 ± 14.3^{A}	6.2 ± 3.5^{A}	11.1 ± 6.3	0.8 ± 0.3^{A}	2.5 ± 2.3	11.6 ± 15.9^{A}
Sex						
Male	106.3 ± 10.8^{A}	20.8 ± 7.3^{A}	6.4 ± 3.1	1.1 ± 0.4	3.3 ± 1.5	12.5 ± 10.7^{A}
Female	89 ± 16.8^{A}	18.3 ± 18.3^{A}	13.6 ± 18.7	2.3 ± 1.5^{B}	6.7 ± 6.7	0 ± 0^{A}

^{A} indicates $P > 0.1$; ^{B} indicates $P < 0.05$; ^{C} indicates $P < 0.01$

[a] Control vocalizations are vocalizations that occurred within the control chamber of the preference testing box, transition vocalizations occurred after the sliding door was opened but before entry to the treatment chamber, and treatment vocalizations occurred after the kid had crossed through the doorway.

Table 2.5 Raw means ± SE of fear behaviors for kids (n=12) in trial 1 (novel peppermint odor) by test day, feed deprivation period (d.p.), breed, and sex during novel stimulus test.

	Vocalizations*					
Test day[a]	**Control**	**Transition**	**Treatment**	**Startle**	**Rear**	**Lying**
5	80 ± 5.7^{A}	1 ± 0.3	7.3 ± 3	0.6 ± 0.3^{A}	7.4 ± 3.1^{A}	1 ± 0.9^{A}
6	83.5 ± 9.4^{A}	0.2 ± 0.1	3.9 ± 1.7	1.2 ± 0.4^{C}	14.6 ± 3.6^{b}	3 ± 2.4^{A}
d.p.						
6.5	93.8 ± 9.1^{A}	0.8 ± 0.4	9.7 ± 5	0.3 ± 0.3^{A}	10.8 ± 4.9^{A}	1.9 ± 1.8^{A}
7	82.5 ± 13.5^{A}	0.8 ± 0.5	0 ± 0	0.8 ± 0.3^{A}	8.5 ± 2.8^{A}	4.6 ± 4.6^{A}
7.5	61.2 ± 10.2^{A}	0.2 ± 0.2	7.8 ± 3.5	1.7 ± 0.7^{b}	8 ± 4.6^{A}	0 ± 0^{A}
8	80.8 ± 3.6^{A}	0.5 ± 0.3	1.3 ± 0.9	0.8 ± 0.5^{A}	16 ± 7.9^{A}	2.2 ± 2.2^{A}
8.5	107 ± 8^{A}	0.5 ± 0.5	12 ± 2	0.5 ± 0.5^{A}	18 ± 18^{A}	0 ± 0^{A}
Breed						
Alpine	60.3 ± 10.6^{A}	0 ± 0	2.7 ± 3.8	0.8 ± 0.8^{A}	17.2 ± 4.6^{A}	4.7 ± 6.5^{A}
Toggenburg	88.9 ± 7.9^{A}	0.8 ± 0.3	6.6 ± 2.8	0.9 ± 0.4^{A}	8.9 ± 4.2^{A}	1.1 ± 1.1^{A}
Sex						
Male	84 ± 8.8^{A}	0.7 ± 0.3	5.7 ± 2.8	0.7 ± 0.3^{A}	8.8 ± 3.5^{A}	0 ± 0^{A}
Female	70.8 ± 10.3^{A}	0 ± 0	5 ± 5.3	1.8 ± 0.9^{A}	22.3 ± 7.1^{A}	2.4 ± 2.1^{A}

^{A} indicates P > 0.1; ^{b} indicates P < 0.1; ^{B} indicates P < 0.05; ^{C} indicates P < 0.01

[a] Day 5 testing was ambient conditions, day 6 testing introduced peppermint oil

* Control vocalizations are vocalizations that occurred within the control chamber of the preference testing box, transition vocalizations occurred after the sliding door was opened but before entry to the treatment chamber, and treatment vocalizations occurred after the kid had crossed through the doorway.

Table 2.6 Raw means ± SE for latencies to enter the treatment chamber (s), first bottle touch (s) and latency to suckle (s), and total milk consumed (mL) for kids (n=12) by test day, feed deprivation period (d.p.), breed, and sex in trial 1 (novel peppermint odor) during preference testing**.**

Test day[a]	Entry	Bottle Touch	Suckle	Milk Consumed
5	5.7 ± 1.1	1.8 ± 0.3^{A}	4.3 ± 0.7	632.9 ± 26.6^{A}
6	2.8 ± 0.4^{C}	1.6 ± 0.2^{A}	2.9 ± 0.5^{C}	683.1 ± 29.6^{A}
d.p.				
6.5	6.5 ± 1.8^{A}	2 ± 0.4^{A}	4.2 ± 1.2^{A}	677.2 ± 35.5^{A}
7	3.5 ± 1.4^{A}	2.2 ± 0.5^{A}	4 ± 0.7^{A}	677.2 ± 23.7^{A}
7.5	3.5 ± 0.7^{A}	1.5 ± 0.3^{A}	3.2 ± 0.9^{A}	683.1 ± 50.3^{A}
8	4 ± 0.4^{A}	1.3 ± 0.3^{A}	4 ± 1.2^{A}	520.5 ± 26.6^{A}
8.5	2.5 ± 0.5^{B}	1 ± 0^{A}	1.5 ± 0.5^{b}	735.3 ± 29.6^{A}
Breed				
Alpine	4.7 ± 3.1^{A}	1.7 ± 0.5^{A}	3.5 ± 1^{A}	21.3 ± 0.9^{A}
Toggenburg	4.1 ± 0.6^{A}	1.7 ± 0.3^{A}	3.7 ± 0.7^{A}	22.6 ± 1.2^{A}
Sex				
Male	4.3 ± 1.1^{A}	1.7 ± 0.3^{A}	3.6 ± 0.7^{A}	22.8 ± 1.1^{A}
Female	4.3 ± 1.1^{A}	1.8 ± 0.7^{A}	3.8 ± 1.7^{A}	19.9 ± 1^{A}

^{A} indicates P > 0.1; ^{b} indicates P = 0.07; ^{B} indicates P < 0.05; ^{C} indicates P < 0.01
[a] Day 5 testing was ambient conditions, day 6 testing introduced peppermint oil

714

715 **Table 2.7** Raw means ± SE of fear behaviors for kids (n=12) by day in trial 2 (visual
716 stimulus test) during preference testing.

	Vocalizations*				
Test day	**Control**	**Transition**	**Treatment**	**Startle**	**Rear**
1	117.6 ± 19	72.7 ± 12	6.7 ± 4.1	0.7 ± 0.2	0 ± 0
2	111 ± 14.2	48.3 ± 11.2	0.8 ± 0.7	2.3 ± 0.8	6.4 ± 1.4
3	114.5 ± 13.5	30.2 ± 9.2	3.1 ± 2	1.7 ± 0.7	2.9 ± 1.6
4	99.6 ± 13	17.2 ± 7.9	0.3 ± 0.3	0.8 ± 0.3	2.5 ± 2.2
5	102.6 ± 13.8	2 ± 0.8	3 ± 0.8	0.8 ± 0.2	1.5 ± 0.7

* Control vocalizations are vocalizations that occurred within the control chamber of the preference testing box, transition vocalizations occurred after the sliding door was opened but before entry to the treatment chamber, and treatment vocalizations occurred after the kid had crossed through the doorway.

CHAPTER 2 FIGURES

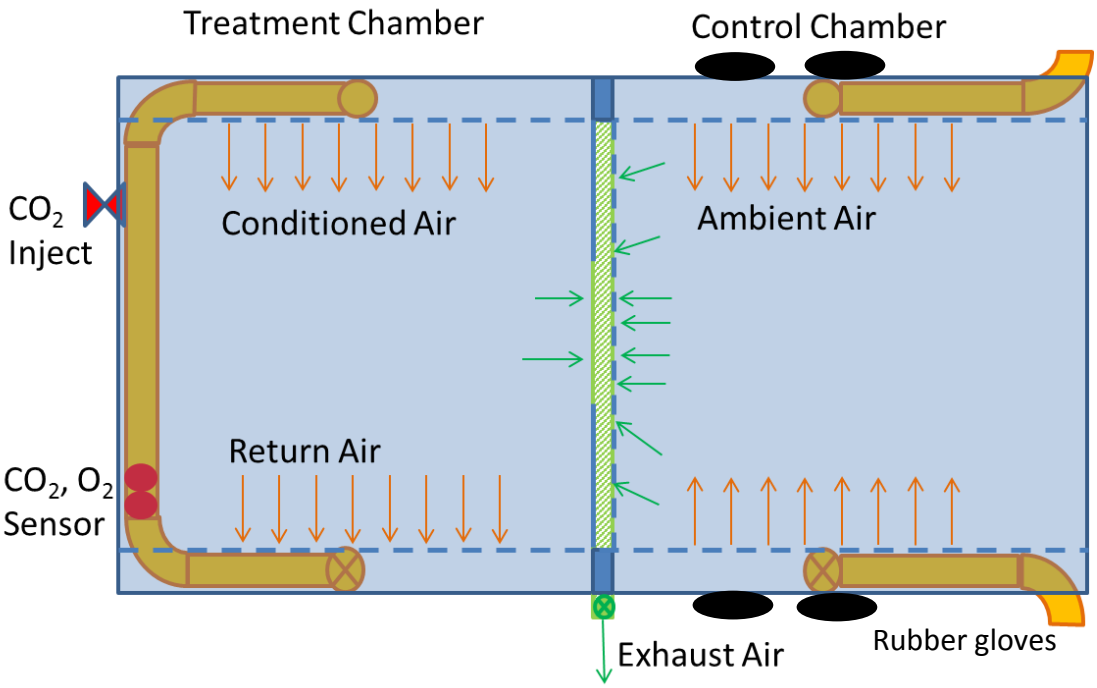
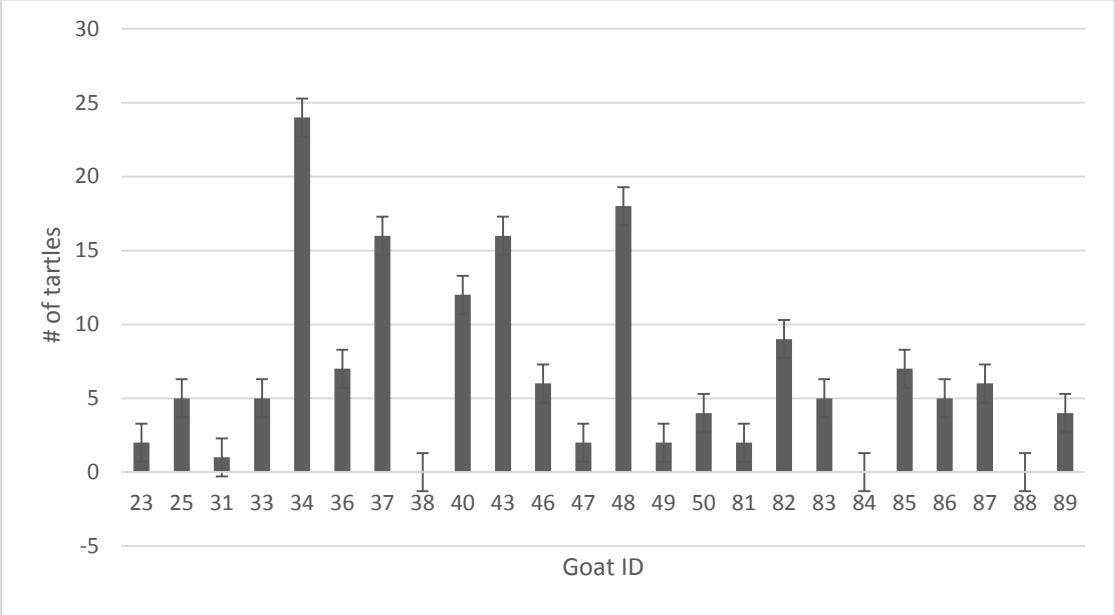


Figure 2.1 Blueprint demonstrating dual (control and treatment) chamber preference testing box used for conditioned place preference testing of kids (n=24).

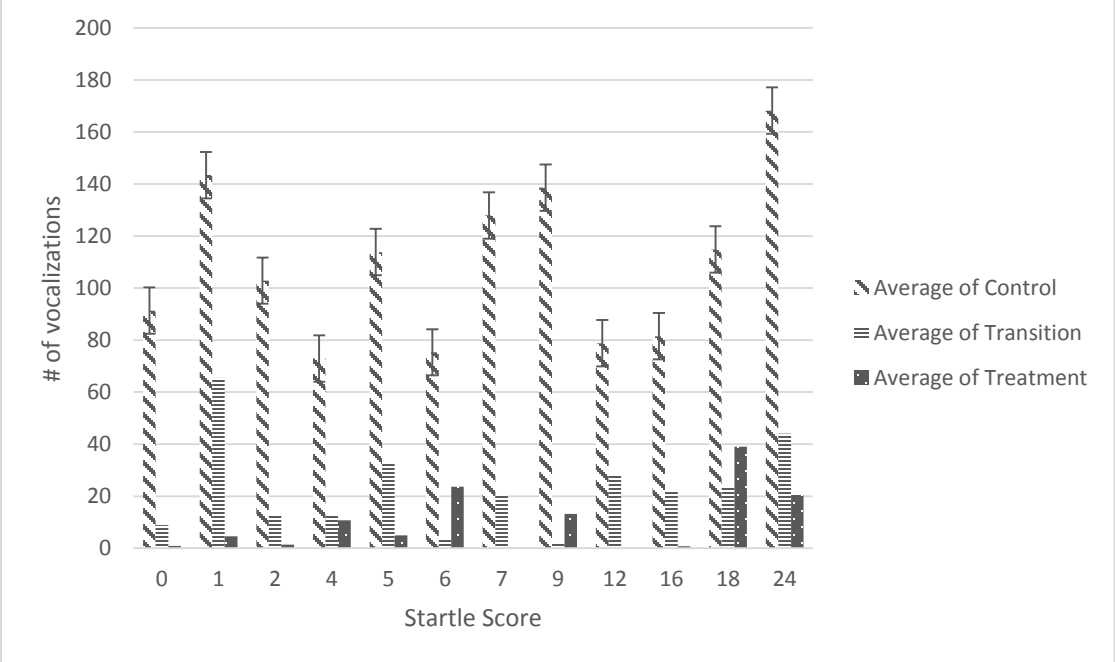
721

Figure 2.2 Test room setup showing kid in preference testing box, observer to right of test box, exhaust system, camera position, lighting placement and black curtains used for obscuring the observer from kid's view during preference testing of kids (n=24).

722



723 **Figure 2.3** Total number of startles exhibited by each kid (n=24) during preference testing. Startle score represents the cumulative number of startles by each kid over all test days.



724
725 **Figure 2.4** Average number of vocalizations (±SE) for all kids (n=24) in the control
726 chamber, transition period, and treatment chamber by number of startles displayed during
727 preference testing.

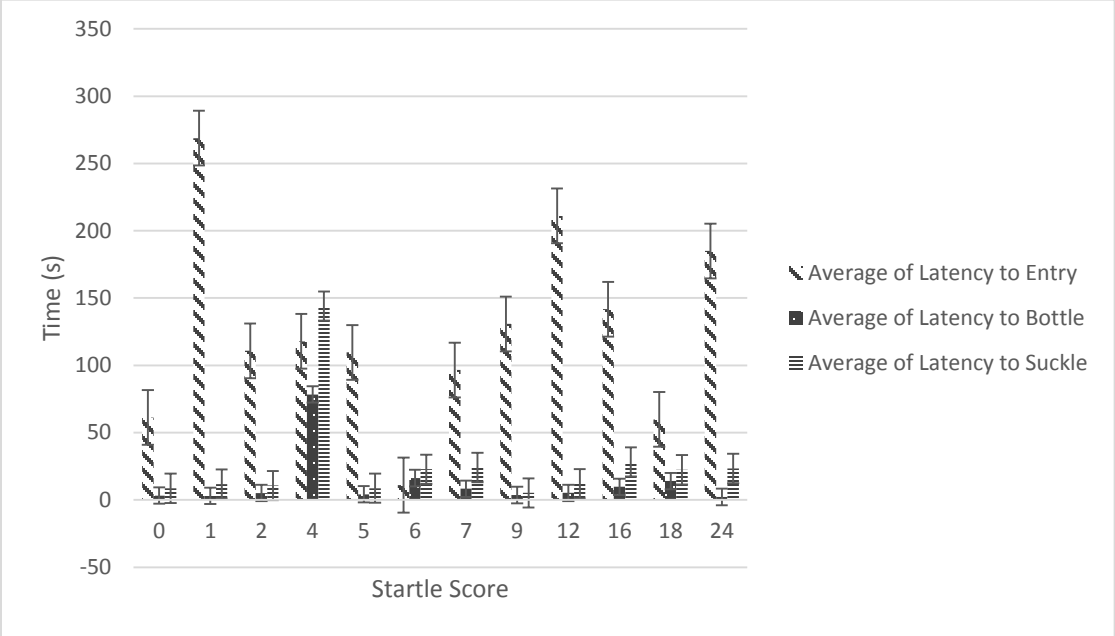


Figure 2.5 Average latencies (±SE) to enter treatment chamber, bottle touch and suckle for all kids (n=24) by number of startles displayed during preference testing.

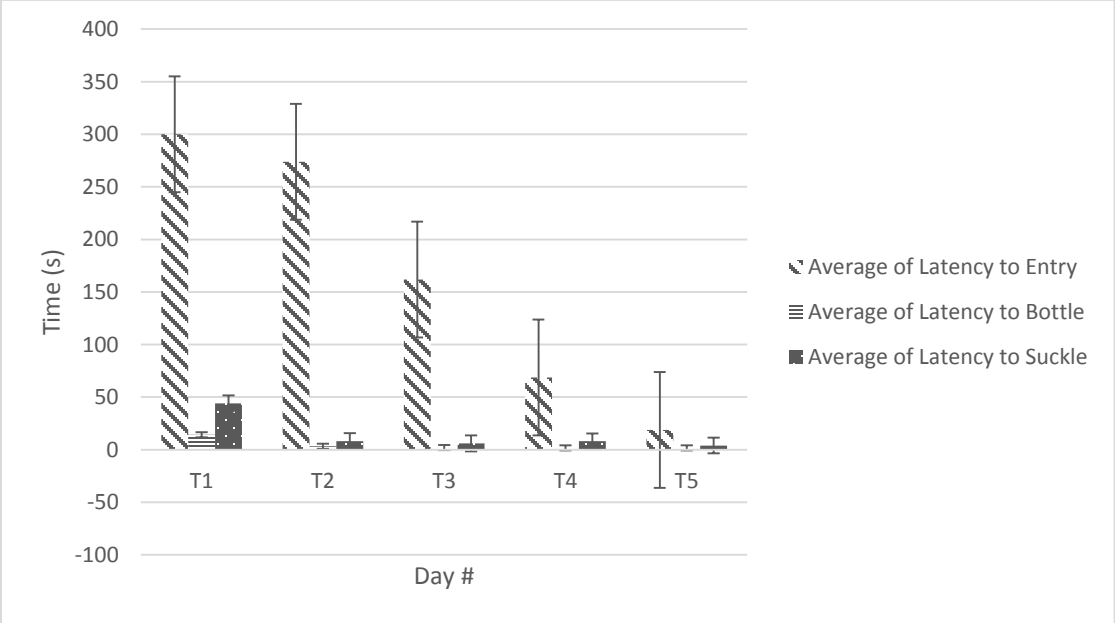


Figure 2.6 Average latencies (±SE) to enter treatment chamber, bottle touch and suckle during preference testing for kids in trial 2 (n=12) by day.

CHAPTER 3.

RESPONSES OF NEONATAL GOATS (KIDS) TO DIFFERENT CONCENTRATIONS OF CARBON DIOXIDE GAS

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This chapter will be prepared for the *Journal of Dairy Science*

3.1 ABSTRACT

The objective of this study was to analyze the suitability of carbon dioxide (CO_2) as an agent for euthanasia of kids. A test box was custom designed to maintain two connected chambers at static atmospheric concentrations. One chamber was held at ambient conditions (control), and the opposite was held at a predetermined CO_2 concentration (treatment). A total of 12 mixed breed dairy kids (11 males, 1 female) were enrolled in the study. Kids were individually trained for at least 5 consecutive days to travel from the control chamber to the treatment chamber under ambient conditions. Two milk bottles were present in the treatment chamber to provide a milk reward (32oz) for the kids. Kids were then tested at each of 3 gas levels: 10%, 20%, or 30%, while the control chamber was maintained <1% CO_2. During testing, kids were placed in the control chamber for 5 minutes, after which the sliding door was opened to provide access to the treatment chamber. After entering the treatment chamber, kids were provided with 10-minutes access to the treatment chamber and were then removed and returned to the home pen. Kids were randomly assigned 10% or 20% as the first treatment and were systematically tested, with all kids receiving 30% on the third treatment day. Kids received a 2-day washout period (ambient CO_2) between each gas treatment. 10 kids tolerated 10% CO_2 for 10 minutes. One kid exited the treatment chamber at 8.5 minutes after consuming his full ration, and 1 kid lost posture at 289s. At 20% and 30%, all kids became ataxic and posture loss ranged

from 83s to 271s. One kid exited the treatment chamber before losing posture at 20%, and then re-entered the chamber and became recumbent. All kids continued to consume milk prior to and during ataxia, and re-entered the treatment chamber on wash out days. Kids did not show any avoidance behavior to any CO_2 concentration, and did not appear to develop a conditioned aversion to the treatment chamber. The results of this study support CO_2 as a method for kid euthanasia and justify further research on the concept.

Keywords: carbon dioxide, euthanasia, goat, kid

3.2 INTRODUCTION

The euthanasia of an animal may happen on farm for a multitude of reasons including disease affliction, lameness, failure to conceive and other productivity issues. In 2006, 68% of Wisconsin dairy farmers stated that there had been at least one case of on-farm euthanasia within the past 3 years (Hoe and Ruegg, 2006). Due to the common occurrence of on-farm euthanasia and the need to provide euthanasia to ill and injured animals in a timely manner, many producers and caretakers must perform the procedure themselves due to unavailable or inaccessible veterinary care. This emphasizes the importance of producers working closely with veterinarians to determine both the necessary critical endpoints for animals as well as proper application of a euthanasia method (Turner and Doonan, 2010).

Carbon dioxide (CO_2) is an inhalant euthanasia agent that is regularly used for swine euthanasia, and during stunning of swine and poultry in processing facilities (OIE, 2014). Carbon dioxide is an inexpensive and effective method of euthanasia for small mammals when coupled with proper training and adequate equipment (Leary et al., 2013). Within the swine industry, CO_2 is perceived by many as more peaceful than the common method, blunt force trauma, used for pigs weighing less than 6 kg. Additionally, many caretakers agreed that holding the pig while performing euthanasia was an unpleasant experience (Matthis, 2005). Subsequently, CO_2 may be a preferred method for some animal caretakers who agree that a gentle, albeit longer, death is preferred to a quick, more distressing death (Matthis, 2005, Hawkins et al., 2006).

Carbon dioxide causes death by inducing hypercapnia and hypoxia. During hypercapnia, the pH of the blood in the lungs is too acidic to effectively bind oxygen, and

subsequently insufficient levels of oxygen are delivered to the tissues. This leads to tissue hypoxia, erratic cardiac activity, and reduced myocardial function which results in decreased heart rate, hypotension, vascular collapse and eventually death (Hall and McShane, 2013).

The time to loss of consciousness during CO_2 exposure varies greatly depending on the concentration used. In atmospheres of 100% CO_2 rats became insensible within 25 seconds (Reed et al., 2009). Similarly, in 80-90% CO_2, finishing pigs (40 ± 6 kg) lost somatosensory potentials within 17 to 25 seconds (Raj et al., 1997). Broiler chickens lost posture within 172 seconds in a 15.7% atmosphere and weaned pigs lost posture at 143 seconds during a 20% CO_2/minute box volume replacement rate (Gerritzen et al., 2004, Sadler et al., 2014a). While this induction time is relatively fast, there is still the potential for distress and pain during the animals' conscious period. In humans, air hunger begins at 8%, while 50% to 100% CO_2 levels have been described as 'unpleasant' to 'painful' (Van den Hout and Griez, 1984, Danneman et al., 1997, Liotti et al., 2001). Additionally, elevated levels of substance P were observed in neonatal pigs exposed to 100% CO_2 which suggests that exposure to this concentration of CO_2 is a painful and stressing experience (Sutherland et al., 2012). Inhaling elevated levels of CO_2 has the potential to cause acute pain by directly activating nociceptors located within the respiratory tract. For example, in the nasal trigeminal system of the domestic hen, there are at least 40 nociceptors sensitive to ammonia, and 5 of these were also activated by CO_2 (McKeegan, 2004). Furthermore, vagal bronchopulmonary C-fibers located within the lungs show sensitivity to CO_2, are able to elicit pain signals in response to both endogenous and exogenous stimuli, and show mild stimulation by the inhalation of 5 to 8 breaths at 30% CO_2 (Lin et al., 2005, Kollarik et al., 2010).

Although CO_2 has the potential to cause distress and pain, these factors may be attenuated by its ability to generate analgesia and anesthesia during induction of unconsciousness. Exposure to CO_2 depresses the reactivity of both respiratory and non-respiratory neurons (Lipski, 1986). During hypercapnia, stress-induced opioids are released (Gamble and Milne, 1990, Grönroos and Pertovaara, 1994, Fukuda et al., 2006), and these opioids are linked with the depression of ventilatory response, a lessened "need to breath" and other sedative effects that induce anesthesia and analgesia (Pattinson et al., 2007, Zhang et al., 2007, Kimura and Haji, 2014). Severely hypercapnic (pH ~6.7) rat spinal cords were shown to exhibit the same amount of nociception as spinal cords treated with the analgesic dexmedetomidine and guinea pigs exhibited deep anesthesia for 50 seconds after being exposed to 80% CO_2 for 30 seconds (Kohler et al., 1999, Otsuguro et al., 2007). Mildly hypercapnic rats ($PaCO_2$: 40 ± 8 to 90 ± 9mmHg) exhibited reduced withdrawal and pain responses (Gamble and Milne, 1990, Fukuda et al., 2006). Elevated levels of extracellular adenosine are also observed in hypercapnic cerebral fluid, and act as an agonist for the G-protein coupled receptor A1 which produces a significant inhibitory effect on neuronal transmission (Dunwiddie and Masino, 2001, Eisenach et al., 2004, Dulla et al., 2005, Otsuguro et al., 2007).

Some caretakers perceive CO_2 as a relatively hands-off method that is peaceful and safe, and there is merit in evaluating it as a euthanasia method for ruminants (Matthis, 2005). In order to be considered suitable for euthanasia, the method in question must not induce unnecessary or unavoidable pain or distress (Leary et al., 2013 p 10, OIE, 2014). Some of the most useful tools to evaluate the novel use of an inhalant euthanasia method are the conditioned place aversion and approach-avoidance paradigms (Dawkins, 1990). These tests directly ask the animal the question of whether they are willing to enter an environment filled with this inhalant,

and whether experiencing the inhalant was a strongly aversive event. The ultimate goal of this study was to determine if the presence of CO_2 within a previously conditioned environment was sufficiently irritating for neonatal kids to forgo a salient food reward, and if so, the concentration of CO_2 at which this occurs. A second objective was to determine whether experiencing CO_2 produced a conditioned place aversion by the neonatal kid.

3.3 MATERIALS AND METHODS

The protocol for this experiment was approved by the Iowa State University (ISU) Institutional Animal Care and Use Committee

3.3.1 Experimental design.

A conditioned place preference model was utilized to condition the kids to the test box, after which an approach-avoidance paradigm was used to assess the level of aversion kids developed to CO_2. This experiment was a repeated measures design with each test subject acting as its own control and each kid tested individually. Testing occurred between 1:00pm and 5:30pm after a period of feed deprivation of 5 to 9.5 hours. Kids were randomly assigned a time point for testing, and were consistently tested at that time point daily. The experiment was split into 2 phases: training and testing. During training, kids were tested for a minimum of 5 consecutive days with ambient air conditions in both control and treatment chambers (< 0.04% CO_2). Testing consisted of 3 gas treatment days, and 2 or 3 ambient washout days after each gas day. During gas treatments, the control chamber was maintained < 1% CO_2 while the treatment chamber was prefilled to a specified concentration. Gas treatments consisted of 1 gas type (CO_2 100%) and 3 concentrations (10%, 20% & 30%). Kids received all concentration treatments. Kids were randomly assigned either 10% or 20% CO_2 as the first gas concentration treatment,

and then systematically received the next treatment of either 10% or 20%. All kids received 30% CO_2 as the final treatment.

3.3.2 Experimental equipment.

A preference testing box (Figure 3.1) was custom designed with two connecting chambers separated by a sliding door. A gas sink approximately 5cm wide was located within the doorway to maintain the separate concentrations. A plastic curtain made of transparent PVC strips was also fitted to the doorway to aid in maintaining separate atmospheres. One chamber was held at ambient conditions (control chamber) and the opposite chamber was held at a designated CO_2 concentration (treatment chamber). Fans were installed in the walls of both chambers to promote air flow. A continuous flow of ambient air was introduced in to the control chamber via air inlets attached to the bottom structure of the box. Carbon dioxide was introduced in to the treatment chamber via similar air inlets attached to the bottom structure of the treatment side. The left air inlet at the bottom of the treatment chamber was equipped with a gas inlet to facilitate CO_2 delivery. The inside dimensions of each chamber measured 61 cm width x 61 cm height x 61 cm length. The side panels of the box were made of opaque, hard plastic. In the control chamber, plastic gloves were fitted on each side panel to facilitate handling of the animal when required during the test. These gloves were retracted from the box when not in use. To enable viewing, clear plastic was used for the doors which were located on the lateral ends and the top of the box. The floor was covered with rubber floor mats in both chambers to provide traction. To attract kids to the treatment chamber, two milk bottle holders were installed and contained 472mL milk bottles that were identical to those used during daily feeding.

Carbon dioxide was administered using a compressed cylinder of CO_2 (99% pure) purchased from ISU Chemistry Stores (Ames, IA, 50010). The gas was delivered to the

treatment chamber through a 9.5 mm hose. CO_2 gas levels were controlled using a gas regulator (Euthanex Corp., Palmer, PA) to maintain static gas concentrations throughout each treatment. Gas flow rate was 12 L/min at 10%, 30 L/min at 20%, and 55 L/min at 30%. Exhaust was funneled from the gas sink into the test room ventilation system.

3.3.3 Animal husbandry and enrollment.

A total of 12 mixed neonatal dairy goat kids (1 female, 11 males) were enrolled in this study, sourced from two commercial herds in the Midwest USA. Kids were of various breeds including Alpine-Saneen cross, LaMancha, and Nubian. Kids were collected and enrolled from May to October of 2014. Kids were removed from the dam after birth and bottle-fed prior to enrollment. Kids were acquired between 1-7 days of age to ensure adequate consumption of colostrum, and the mean body weight upon arrival was 3.7 ± 0.2 kg. None of the male kids were castrated and no kids were disbudded. All kids were ear tagged for identification prior to arrival at ISU.

Kids were housed in 3 climate-controlled rooms at ISU Laboratory Animal Research (LAR) buildings, with a 12-hour light cycle from 6:00am to 6:00pm. Kids were housed in a 9.3 m^2 room that was divided equally into 5 pens to facilitate individual feeding. Pens were separated using spindle barriers with 5 cm separation between bars that allowed nose-to-nose contact for social interaction. Each pen contained one heat lamp, one plastic tub for climbing, and straw bedding for comfort.

Body temperatures were recorded daily using a hand held thermometer (Mabis Healthcare Inc. Waukegan, IL) and body weights were recorded weekly using a handheld scale that was accurate to 0.01kg (Pure Fishing, Inc Columbia, SC). All kids received daily milk

rations equal to 18% of their body weight in grams. Advance milk replacer (Milk Specialties Global Eden Prairie, MN) was fed using standard 472mL graduated lamb milk bottles equipped with Pritchard teats (Pritchard teats, Riverton, New Zealand). Kids were fed approximately every 4 hours during acclimation and then 3 times over a 24-hour period after enrollment.

Upon arrival at LAR, all kids received at least 3 days of acclimation, during which no experimental procedures were performed. Kids were observed for any health issues; the acclimation period was extended for kids that exhibited clinical signs of illness until these signs were no longer present. In addition, kids were required to reach a behavioral start criterion based on suckling motivation before enrollment in testing. Kids were considered successful in meeting this criterion if they actively found and sucked on the nipple within two minutes of the bottle being placed in the bottle holder during 4 consecutive feedings in the home pen.

3.3.4 Testing procedure.

During training, each kid was carried individually from the home pen to the testing room, and placed in to the control chamber. Kids were provided with 5 minutes to acclimate to the box, after which the sliding door was opened providing access to the treatment chamber. Kids were given 5 minutes to voluntarily pass through the doorway, after which they were gently assisted through the doorway using the attached rubber gloves. Once in the treatment chamber, kids were given 10 minutes access to the entire testing box, during which they could move freely between treatment and control chambers. After testing concluded, kids were removed from the box and carried back to the home pen. In order to advance to testing, kids had to be trained for a minimum of 5 days and meet a criterion of entering the treatment chamber unassisted on 2 consecutive days immediately prior to testing. This criterion ensured that kids were exhibiting

strong motivation to enter the treatment chamber. Training days continued until this criterion was met.

During testing, washout days followed the same protocol as training days. On gas days, kids that did not enter the treatment chamber voluntarily were not assisted through the doorway; they were removed from the box and returned to the home pen. Kids that entered the treatment chamber on gas days were removed after either loss of posture or the 10-minute time limit.

An indoor temperature monitor (AcuRite Lake Geneva, WI) was placed within the control chamber to record the relative humidity (%) and temperature (C°) of the test box prior to each test. This environmental data was recorded by the observer for each individual kid immediately prior to entry. The monitor was removed as each kid was placed in to the box, and replaced after each test period concluded. Between tests the box was cleaned with a disinfectant (Accel, Virox Technologies Inc., Ontario, Canada).

3.3.5 Modification to study design.

Seven of the 12 kids were tested with a plastic PVC curtain that was designed as 10 small (2.5 cm) strips that were 58.4 cm long, and provided little resistance. During testing, several kids would linger in the doorway causing the curtain to remain open, impairing maintenance of a static < 1% CO_2 concentration in the control chamber when maintaining 30% concentration of CO_2 inside the treatment chamber. Subsequently, CO_2 levels during the 30% test ranged from 14% to 24.7% for 3 kids. A new curtain was designed for testing the last 5 kids. The material used was identical to the material used for the first curtain. The curtain consisted of 2 larger (12.7 cm) strips that were 58.4 cm long, and provided more resistance than the first curtain. One of the 5 kids required 1 additional training day with the new curtain in order to

reach the criteria to advance to testing. Carbon dioxide concentrations ranged from 28% to 31.5% with the new curtain without sacrificing control conditions.

3.3.6 Behavioral observations.

Data was collected via live observation and video recording. Live observation was gathered by two observers. One observer (observer 1) was positioned on the right side of the box out of the test subject's view. The second observer (observer 2) was positioned in front of the treatment chamber so that the kid was visible to facilitate the recording of direct behavior observations. A black fabric curtain (2.1 m length x 0.9 m wide) and lighting placement was used to ensure that observer 2 was obstructed from the kid's view.

3.3.7 Live observations.

Behaviors that were recorded via live observation were selected due to the difficulties associated with reliably discerning these behaviors on video (Table 3.1). The latency to enter the treatment chamber was measured using a timer (National Presto IND. Inc., Eau Claire, WI) after the sliding door opened until both ears of the kid crossed the doorway from the control to the treatment chamber. When assistance was needed, latency to enter was recorded as 5 minutes. Vocalizations were collected as a counted event and separated in to 3 categories: control, transition, and treatment. The amount of milk consumed from each bottle was recorded after each test.

3.3.8 Video observations.

Video data was collected using a Noldus Portable Lab (Noldus Information Technology, Wageningen, NL). Four color Panasonic cameras (WV-CP484, Kadoma, Japan) were positioned to provide views from top and lateral doors of control and treatment chambers

(Figure 3.2). The recordings from these cameras were captured onto a PC using HandiAvi (v4.3, Anderson's Azcendant Software, Tempe, AZ) at 30 frames/s. Prior to each test, identifying information was presented on a dry erase board to the camera to identify the date, animal ID, test day, and trial number.

Behavior data was collected from videos by one trained observer who was blinded to the animal ID, date and test day. Behavioral data was recorded using Observer (v10.1.548, Noldus Information Technology, Wageningen, NL). A neutral individual performed the blinding procedures for the video recordings from all tests. The blinding procedures involved cutting the video recordings to remove identification presented at the beginning of each video, assigning a random number to each video segment and sorting for the purpose of providing a random sequence in which videos were to be scored. Four videos were selected at random and duplicated within this sequence for the purpose of determining intra-observer reliability.

Prior to data collection, the observer was trained to use the Observer program by repeatedly scoring 2 videos and ethogram from an unrelated study until reaching a reliability score of $k \geq 0.90$ as calculated by the Observer program. After reaching this level of competence, data collection began using the current videos and ethogram (Table 3.2). Intra-observer reliability averaged $k = 0.91$.

3.3.9 Statistical analysis.

Means and standard errors were calculated using raw data in Excel (version 2013, Redmond, WA). A linear mixed effect model was utilized for discrete data such as vocalizations, head shakes, startle, rear, and escape attempt, as well as continuous data such as bottle engage, ataxia, and open mouth breathing. A survival analysis regression was used to analyze all latency

data. Elimination was categorized as binary data and was analyzed using a logistic regression model. Data was categorized in to 3 subsets: the day immediately prior to gas testing (baseline), gas testing, the day immediately after gas testing (washout). Means were then compared within and across subsets to observe changes in behavior. Each kid was an experimental unit. Gas concentration, day number and startle score were analyzed as fixed effects while the individual goat identification was a random effect.

3.4 RESULTS

All kids (n=12) completed the criteria of voluntarily entering the treatment chamber for 2 consecutive days immediately prior to gas testing. No kids became ill or seriously injured during testing. All kids entered the treatment chamber voluntarily during all gas treatments, and all kids lost posture within the treatment chamber at least once. Four kids were excluded from the 30% data set due to insufficient concentrations of CO_2 during testing.

3.4.1 Avoidance and aversion.

All means (±SE) of latencies to enter the treatment chamber, first bottle touch, and suckle for all test days are presented in Table 3.3. The mean latency to enter on all baseline days was 2.1 ± 0.3 seconds (range 1 to 5 seconds). The mean latency to enter was 2.4 ± 0.8 seconds (range 1 to 10 seconds) at 10%, 2.7 ± 0.8 seconds (range 1 to 11 seconds) at 20%, and 1.6 ± 0.5 seconds (range 1 to 5 seconds) at 30%. Mean latency to enter was 2.52 ± 0.8 seconds (range 1 to 15 seconds) for all washout days. The mean latency to enter was slightly longer on Gas day 2 (G2) compared to Gas day 1 (G1) at 10%, but this was not observed in either 20% or 30% treatment. One kid had a latency to enter of 2 seconds on G1 at 20%, and then 10 seconds on G2 at 10%. Another kid had a latency to enter of 11s on G1 at 20%. During washout, there were two

outliers (12 seconds and 15 seconds). Excluding these outliers, the range for latency to enter was 1 to 4 seconds for both 10% and 20% CO_2 and 1 to 4 seconds for washout days. When the outlying kids are excluded, the latency to enter is similar across all days and treatments.

Latency to first bottle touch ranged from 1 to 3 seconds on all days. The mean latency to suckle was 3.9 ± 0.4 seconds (range 1 to 7 seconds) on baseline days, 4.3 ± 1 seconds (range 1 to 13 seconds) at 10%, 3.9 ± 0.6 seconds (range 1 to 9 seconds) at 20%, 4.3 ± 0.5 seconds at 30% (range 2 to 8 seconds), and 4.7 ± 1.1 seconds (range 1 to 19 seconds) during washout days. One kid had a latency to suckle of 4 seconds on G1 at 20%, and 13 seconds on G2 at 10%. One kid had a latency to suckle of 18 seconds on Washout day 1 (W1), and different kid 19 seconds on Washout day 3 (W3). Excluding these outliers, the range for latency to suckle for 10% and washout is 1 to 9 seconds and 1 to 6 seconds, respectively. The shortest mean latency to suckle was on G2 at 20%, there were no other discernable differences between treatments or days.

3.4.2 Gas responses.

Latency and duration to ataxia and time to loss of posture outcomes for each concentration is depicted in Table 3.4. Only 2 kids displayed ataxia during 10%, while all kids became ataxic in 20% and 30% CO_2, with the exception of 1 kid that did not display ataxia but lost posture in 30%. The mean latency to ataxia for 10% was 381.4 ± 139.2 seconds, 105.6 ± 16.3 seconds for 20%, and 80.4 ± 11.3 seconds for 30%. The average duration of ataxia for kids in 10% CO_2 was 50.2 ± 31.5 seconds, 26.1 ± 4.9 seconds in 20%, and 13.8 ± 9.3 seconds in 30%. One kid lost posture in 10% CO_2 at 289 seconds. All kids lost posture in 20% and 30% concentrations. The mean latency to loss of posture was 191.4 ± 18.8 seconds and 117.3 ± 10.2 seconds for 20% and 30%, respectively. One kid exited the treatment chamber during both 10% and 20%. The kid exited 10% with 90 seconds left in the test and did not return to the treatment

chamber. After exiting the treatment chamber at 20%, the kid re-entered the treatment chamber and remained until loss of posture. Righting response occurred in 10 /12 kids during 20% (mean 2.3 ± 0.9) and 6/8 kids during 30% (mean 1.3 ± 0.4). The kid that lost posture at 10% required 1 second to recover, while the mean latency to recovery was 26.3 ± 4.5 seconds for 20%, and 25.8 ± 3.7 seconds for 30%.

During 10%, the majority of kids (9/12) exhibited head shaking in the control chamber (range 0 to 25 head shakes), while only 4 kids exhibited head shaking (range 0 to 4 head shakes) in the treatment chamber. Only 4 kids exhibited control chamber head shaking during 20%, and even fewer kids (2/12) exhibited head shaking in the treatment chamber. Head shakes ranged from 0 to 3 for 20%. During 30%, no kids exhibited control chamber head shaking, while 2 kids displayed 1 head shake each in the treatment chamber.

Pauses in nursing behavior were a common occurrence for all goats, on all days. The average rate of nurse pause behavior was 1.3 ± 0.2 on baseline and washout days, and 0.5 ± 0.2 for all gas days, with rates for 20% and 30% being the lowest. During 10% CO_2 treatment, kids spent an average of 328.5 ± 33.4 seconds engaging with the bottle. Out of this time, kids exhibited open mouth breathing (OMB) for 121.6 ± 34 seconds. At 20% CO_2, kids spent an average of 96.9 ± 13 seconds engaging with the bottle, and experienced OMB for 22.1 ± 6.4 seconds. At 30% CO_2, 66.4 ± 8.8 seconds were spent engaging with the bottle, with OMB occurring simultaneously for 19.6 ± 9.6 seconds.

3.4.3 Fear-related behaviors.

The mean numbers for control, transition, and treatment vocalizations for all test days are presented in Table 3.5. Mean control vocalizations were 98.3 ± 17.8 on baseline days (range

19 to 191 vocalizations), 87.2 ± 14.2 at 10% (range 36 to 162 vocalizations), 83.9 ± 17 at 20%, 84.4 ± 19.6 at 30% (range 15 to 177 vocalizations), and 98.3 ± 18.5 (range 29 to 182 vocalizations) on washout days. There was no perceivable difference between control vocalizations across treatments or days. Transition vocalizations ranged from 0 to 3 across all days and treatments, with only 9 instances of vocalization occurring over all days. Two kids vocalized during the transition period at 20%, 1 kid at 10%, and no kids vocalized during the transition period at 30%.

Mean treatment vocalizations were 2.2 ± 3 (range 0 to 45 vocalizations) on baseline days, 2.8 ± 1.5 (range 0 to 14 vocalizations) at 10%, 9.1 ± 2.3 at 20% (range 0 to 24 vocalizations), 5.9 ± 3.1 (range 0 to 25 vocalizations) at 30%, and 2.3 ± 3.1 (range 0 to 43 vocalizations) on washout days. The majority of kids (10/12) did not vocalize during baseline days, and one of these kids also vocalized during washout days while all other kids did not vocalize. The majority of kids (8/12) did not vocalize during 10%, while only 2 kids did not vocalize during 20%. Almost half of the kids (3/8) did not vocalize at 30%.

The average numbers for all rears and startles for all test days are presented in Table 3.6. Rears occurring in the control chamber (control rears) were exhibited by 8 of 12 kids during 10% and ranged from 0 to 38 rears, while rears occurring in the treatment chamber (treatment rears) were exhibited by 3 kids and ranged from 0 to 3 rears at that concentration. Seven of 12 kids exhibited control rears (range 0 to 17 rears), and 1 kid reared once while in the treatment chamber during 20%. The majority of kids (7/8) exhibited control rears (range 1 to 15 rears) during 30%, while no treatment rears occurred. This is similar to the amount of rears occurring on baseline and washout days, with only 7 occurrences of treatment rears over all baseline days (range 0 to 5 rears) and 8 bouts of treatment rears over all washout days (range 0 to 11 rears).

The majority of kids (10/12) displayed elimination behavior at 10%, while only 4 kids and 3 kids showed elimination during 20% and 30%, respectively. Elimination behavior was common during baseline, but decreased over time as illustrated by 8 of 12 kids displaying elimination behavior on B1 while only 2 of 8 kids showed elimination behavior on B3. Similarly, 7 of 12 kids eliminated during W1, while only 1 of 8 kids eliminated during W3. No escape attempts were observed during any gas treatment, although 1 kid made a single escape attempt on B1 and W1.

3.4.4 Fearfulness and aversion.

Startle score for each kid was determined by the cumulative number of startle events of an individual kid over all test days. The median startle score for all kids was 5 (range 0 to 14). Five kids had a startle score below the median (low startle), and 5 kids had a startle score above the median (high startle). The mean latency to enter for each treatment in terms of startle score is depicted in Figure 3.3. The mean latency to enter on baseline days for low startle kids was 1.2 ± 0.5 seconds, and the mean latency to enter was 1.4 ± 0.4 seconds for 10%, 1.4 ± 0.2 seconds for 20%, 1 ± 0 seconds for 30%, and 1.4 ± 0.3 seconds on washout days. For high startle kids, the mean latency to enter was 2.5 ± 0.5 seconds on baseline days, 4 ± 1.6 seconds for 10%, 4.2 ± 1.8 seconds for 20%, 2.3 ± 0.9 seconds for 30%, and 3.1 ± 1.2 seconds for washout days.

Mean treatment vocalizations for each treatment in terms of startle score is depicted in Figure 3.4. Low startle kids did not vocalize in the treatment chamber during baseline days, and mean treatment vocalizations were 0.6 ± 0.6 for 10%, 10.8 ± 3.3 for 20% and 9.5 ± 5.7 for 30%. Kids did not vocalize on washout days. Mean treatment vocalizations for high startle kids were 4.5 ± 5.5 on baseline days, 3.4 ± 2.7 for 10%, 6.2 ± 2.5 for 20%, 2.3 ± 1.7 for 30%, and 4.8 ± 5.8 on washout days. The kid that exhibited escape attempts had a startle score of 5. Low startle kids

did not rear during any gas treatment. Three high startle kids reared in the treatment chamber during 10% (range 1 to 3 rears), 1 high startle kid reared once in the treatment chamber during 20%, and no high startle kids reared in the treatment chamber during 30%.

The mean latency to loss of posture for each treatment in terms of startle score is depicted in Figure 3.5. The mean latency to loss of posture for low startle kids was 239.4 ± 15.4s for 20% and 135.5 ± 14.5.1s for 30%. The mean latency to loss of posture for high startle kids was 133 ± 22.3s for 20% and 98.5 ± 6.6s for 30%.

3.5 DISCUSSION

Data collected by direct observation was done so to ensure that small movements or angle-dependent behaviors that would not be visible on video were recorded accurately. Data collected by video observation included behaviors that occurred in fast succession, prolonged duration, or were not visible to either observer. Live and video data were successfully recorded for all kids on all test days. The observer charged with collecting all vocalizations and latency to enter data was a rotation of various trained lab members and employees, and the data was consistent between observers. Upon arrival at LAR, all kids exhibited clinical signs of illness including lethargy and diarrhea. All kids required tube feeding at least once and experienced moderate to severe diarrhea. Four kids received saline solution, administered either intravenously or subcutaneously, due to severe dehydration. Five kids showed signs of severe diarrhea and received treatment with Naxcel until symptoms improved; 4 kids required 3 days of treatment and 1 kid required 5. One kid received treatment with Banamine upon arrival for a temperature above 104°. Training occurred simultaneously with Naxcel treatment, but not testing. Due to the response uniformity across all 12 kids, it does not appear that treatment had an effect on

behavior. It is unlikely that the illness of the kids during training had any effect on responses during testing (Sadler et al., 2014c).

Overall, the results from this study suggest that kids did not form a conditioned place aversion after exposure to CO_2, and there did not appear to be an innate avoidance response to any of the CO_2 concentrations. All kids voluntarily entered the treatment chamber on all days. The majority (10/12) of kids entered the treatment chamber within ≤5 seconds, and the mean latency to enter the treatment chamber was within 3 seconds difference between all baseline and gas days. Contrary to expectations, the lack of a distinguishable difference of latency to enter between baseline days and any gas treatment indicates that the presence of CO_2 did not elicit a high enough fear response in kids to affect a previously conditioned behavior. Furthermore, there is no evidence that kids developed a conditioned aversion to the treatment chamber after exposure to any level of CO_2. The majority (10/12) of kids entered the treatment chamber <5 seconds on all washout days, and the mean latency to enter the treatment chamber was within 3 seconds difference between all days, suggesting that the physiological effects of CO_2 exposure were not salient enough to condition fear of the treatment chamber for most kids. Similarly, behaviors stayed consistent across baseline days, which provides more evidence regarding the lack of a lasting carryover effect. The mean latencies to bottle touch and suckle were similar between all days, further supporting that CO_2 presence at any concentration did not disrupt appetitive or consummatory behavior in kids. These results are inconsistent with the results of other studies, where mice and rats are willing to forgo a reward in order to escape CO_2 concentrations above 15% (Niel and Weary, 2007, Makowska et al., 2009). However, pigs have shown similar responses, and were willing to enter a chamber containing 30% CO_2 to retrieve a food reward (Raj and Gregory, 1995).

3.5.1 Avoidance and aversion.

The response to CO_2 appeared to be relatively uniform among all kids, and all concentrations of CO_2 appeared to be similarly tolerated. Three kids exhibited behaviors that could be interpreted as avoidance or aversion behavior. One kid required 11 seconds to enter the treatment chamber at 20% during G1, and required 12 seconds to enter the treatment chamber on the following washout day, suggesting that the kid may have recognized the noxious environment and recalled the negative experience on the following test day. However, if this had been a direct product of exposure to 20%, the same response would have likely been observed at 30% as well. Instead, this behavior was not replicated on any of the following gas treatments or washout days suggesting that these prolonged entry times were due to chance. Another kid required 10 seconds to enter 10% CO_2 on G2. This would suggest that the kid was able to differentiate between ambient and gas days, and was able to associate the negative effects that occurred at 20% on G1 with the presence of 10% on G2. Once again however, this behavior was not observed at 30% indicating that this response was likely not associated with self-preservation. Lastly, a kid required 15 seconds to enter the treatment chamber on W1 after exposure to 10% on G1, however this behavior was not repeated on any other day. Although similar results have shown that pigs will delay entry in to CO_2 atmospheres, rats exhibit decreased tolerance to an anesthetic gas from repeated exposure, and dairy calves are reluctant to approach an aversive stimulus, the inconsistent performance of these behaviors in the current study, an interpretation of these behaviors being associated with CO_2 is not supported (Pajor et al., 2003, Dalmau et al., 2010, Wong et al., 2013, Bertolus et al., 2015).

A fourth kid exited the treatment chamber on both G1 and G2, suggesting that 10% and 20% CO_2 may have been aversive. However, this behavior was also exhibited on the ambient

days Baseline 1 (B1), Baseline 2 (B2), Baseline 3 (B3), W1 and Washout day 2 (W2). Additionally, after exiting the treatment chamber at 20%, the kid re-entered the treatment chamber in this same test and remained in the chamber until recumbency. This behavior has also been previously observed in pigs, although the response is not uniform across the species (Raj and Gregory, 1995). When this behavior was exhibited on days when ataxia was not present, it is possible that the kid was satiated and exploratory behavior became more appealing than food, a behavior that has also been seen in rats (Ferreira et al., 2006).

The results from this study supported the hypothesis that kids would tolerate 10% CO_2 without displaying avoidance or fear behaviors, supporting the current evidence that 10% CO_2 is generally well tolerated, a trait also observed in broilers and rats (McKeegan et al., 2006, Niel and Weary, 2007, Niel et al., 2008, Burkholder et al., 2010). It has been well documented that rats and mice will leave CO_2 atmospheres before they are unable to do so, and this knowledge was used as the basis for the expectation that at 20% CO_2, kids would enter the treatment chamber but leave before losing posture (Kirkden et al., 2005, Niel and Weary, 2007, Niel et al., 2008, Makowska et al., 2009). However, none of the kids fulfilled the expectation of exiting the treatment chamber (or remaining on the control side after exiting in the case of 1 kid) before recumbency occurred. The hypothesis that kids would refrain from entering the treatment chamber on Gas day 3 (G3) was based on data that describes 30% CO_2 as capable of inducing feelings of panic and acute pain in humans (Van den Hout and Griez, 1984, Lin et al., 2005). This expectation was incorrect, and all kids entered the treatment chamber in ≤5 seconds. These results are similar to behavior observed in pigs exposed to 30% CO_2. Finishing pigs were willing to enter the chamber for a reward, although the majority of pigs will withdraw their head before loss of posture occurs and will not re-enter (Raj and Gregory, 1995). The difference of findings

in the current study compared to data in other species may be due to the ontogeny of the goat; a creature that is well adapted to live in high altitudes. It is likely that goats are more adept at handling hypercapnia and subsequently less perceptive of elevated CO_2 concentrations (Noyd et al., 2013).

The majority of pigs will exit a 30% CO_2 environment before losing posture, and similar outcomes have also been observed in mice and rats at even lower levels (Raj and Gregory, 1995, Makowska et al., 2009, Wong et al., 2013, Moody and Weary, 2014). Unlike these species however, all kids lost posture at 20% and 30% CO_2. This may be associated with the lack of avoidance or aversion behavior observed throughout the trial; kids did not find exposure to CO_2 to be a strongly aversive experience. This may also have been due to an inability of the kids to recognize the doorway as the escape route from the treatment chamber. Although two kids demonstrated the ability to pass from the treatment chamber back to the control chamber, it is possible that the other kids did not learn this task. However, both of these kids displayed the ability to pass freely between chambers before the last gas day and both kids became recumbent on G3, suggesting that there was little no to effort by these kids to escape before recumbency.

There are two factors that may have had an effect on kids' responses to CO_2. It is possible that kids were affected by the relatively fast-acting, depressant effects of CO_2 on the nervous system (Gamble and Milne, 1990, Kohler et al., 1999, Fukuda et al., 2006). Due to the rapid onset of incoordination and CNS depression, kids may have not had enough cognitive function to exit the treatment chamber. It is also possible that the lack of response to CO_2 was confounded by the extremely potent stimulus of the milk bottle, as food has been shown to decrease pain perception in human (Zmarzty et al., 1997). Furthermore, pain intensity can be

attenuated by attentional shifts and changes in focus (Gentle, 2001, Villemure et al., 2003). Subsequently, it is possible that in a less attractive environment, kids might have displayed a negative reaction to CO_2.

3.5.2 Gas responses.

Kids remained engaged with the bottle during the initial physiologic effects of hypercapnia, evidenced by the kids exhibiting OMB while simultaneously engaging with the bottle. OMB has been classified as a powerful indicator of stress, due to its link to air hunger and breathlessness (Beausoleil and Mellor, 2015). The focus on the bottle is indicative of the high motivation of kids to suckle even during potential harm, a behavior that has been observed in mice who are willing to endure a shock to access food (Latagliata et al., 2010).

Head shaking is a common reaction to the presence of CO_2 at concentrations above 10% in poultry (McKeegan et al., 2006), and generally a result of respiratory tract irritation from the formation of carbonic acid. Head shaking and nursing pause behavior was expected to increase during gas treatment due to respiratory irritation or disruption, however the occurrence of neither head shakes nor nursing pauses appeared to be associated with the presence of CO_2. It is possible that these outcomes were confounded by external factors such as ear tags increasing the number of head shakes, and social isolation stress influencing kids to pause often during feeding in an attempt to locate conspecifics. The shortened latency to ataxia during 30% indicates that depressant effects occurred most quickly during this treatment, an effect that is also supported by the decreased duration of ataxia, and reduced latency to loss of posture. The occurrence of righting responses were similar between 20% and 30%, however this may be due to the removal of kids from the box soon after loss of posture. The similarity of latency to regain

posture across treatments indicate that the reversibility of unconsciousness is not affected by the concentration used.

3.5.3 Fear behaviors.

It is likely that pain was experienced by all kids during exposure to all CO_2 concentrations. This is supported by the increase in treatment vocalizations during gas treatment days compared to both baseline and washout days, which agrees with previous literature reporting vocalizations as an indicator of stress in kids (Price and Thos, 1980, Lyons et al., 1993, Siebert et al., 2011).An increase in vocalizations is also interpreted as a distress response to CO_2 in rats (Niel and Weary, 2006). All baseline vocalizations and washout vocalizations were similar, indicating that frequency of vocalization was an appropriate measure to assess the effects of CO_2. Treatment vocalizations during 10% testing were similar to treatment vocalizations on baseline and washout days, further supporting the relatively benign nature of 10% CO_2. Treatment vocalizations were highest during 20% CO_2, and 30% vocalizations were close to midway between the numbers for 10% and 20%. Although 30% CO_2 had fewer vocalizations than 20% CO_2, the difference in vocalization frequency can likely be attributed to the shortened latency to ataxia and unconsciousness during 30%.

Elimination, escape attempts and rearing behaviors did not appear to be associated with the presence of CO_2. Elimination behavior in kids was low across all days, and did not increase or decrease consistently. Escape attempts are generally observed when an animal is in an environment that is perceived as threatening, and escape attempts are exhibited in pigs and rodents during gas exposure (Niel and Weary, 2006, Llonch et al., 2012, Chojnacki et al., 2014, Sadler et al., 2014b, Barnard et al., 2015). The lack of escape attempts during gas treatments may be an indicator of a lack of fear in response to CO_2, but it may also be associated with the ataxia

that accompanies CO_2 exposure. Although control rears were higher on G1 and W1 compared to B1 during 10% treatment, the numbers were inflated due to excessive rearing exhibited by 1 kid on both days. Control and treatment rears remained consistent across all other days, and rearing behavior decreased in the treatment chamber suggesting that the behavior is linked more closely with investigation and searching strategy than fear.

3.5.4 Fearfulness and aversion.

Fearfulness is a necessary characteristic for the survival of wild animals, and although it is reduced through domestication, it is still observed in most species. Startle behavior was observed in varying degrees, ranging from kids that displayed no startle response to unpredicted stimuli to kids that exhibited startle responses to predicted and unpredicted stimuli alike. As expected, high startle kids vocalized more than low startle kids on both baseline and washout day. However, high startle kids vocalized less during gas testing. This indicates several possibilities: fearful kids are not more sensitive to CO_2 than non-fearful kids, fearful kids are not more sensitive to CO_2 but they are more sensitive to social isolation than non-fearful kids, or startle score is not an appropriate measure of fear levels in kids. High startle kids did exhibit rearing behavior in the treatment chamber as opposed to low startle kids which may suggest an increased level of distress, however no rearing behavior in the previous study appeared to be associated with distress or fear (Chapter 2). Although high startle kids did take slightly longer to enter the treatment chamber during gas treatment, the difference was miniscule and does not provide robust data in support of the effect of fearfulness on response to CO_2.

3.6 CONCLUSIONS

The data from this study suggest that CO_2 is a suitable euthanasia method for neonatal ruminants, and further research should be done to confirm the results found in this study. Kids did not show an unconditioned avoidance to any level of CO_2 during the approach-avoidance paradigm, and did not show a conditioned place aversion to the treatment chamber after exposure to CO_2. This interpretation is supported by previous research which demonstrates the ability of kids to form conditioned place preferences and recall experiences from repeated exposure to a stimulus to facilitate problem solving. The results of this study suggest that out of the 3 concentrations utilized, 30% CO_2 would be the most humane and efficient option. The rapid depressant effects of 30% CO_2 minimized the amount of time that kids were able to clearly perceive pain or fear and also induced the shortest latency to loss of posture. The responses of fearful and non-fearful kids were similar across all treatments, indicating this method would be appropriate for all kids. These outcomes may be confounded by the milk bottle being too powerful of a stimulus, and attentional shifts affecting pain perception. It would be beneficial to investigate the concentration of CO_2 at which kids are willing to forgo the bottle, in order to establish at what point, if any, CO_2 is aversive to kids and also to confirm that kids are capable of avoiding a noxious gas environment. Recent research by our lab (unpublished) has shown that some kids will choose to forfeit the milk reward at 90% CO_2. Additionally, it will be necessary to assess whether a less valuable stimulus, such as social interaction, would produce similar responses to CO_2. These results would confirm whether the outcomes observed in the current study are due to pain attenuation from food consumption or attentional shifts, a circumstance that would likely not occur in practice. Overall, the current study presents favorable results for

implementing CO_2 as a euthanasia method for kids, and provides a basis for further research in to the use of alternative euthanasia methods for neonatal ruminants.

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CHAPTER 3 TABLES

Table 3.2 Ethogram used for kid behavior collected during live observation during preference testing.

Measure	Behavior Category	Variable type	Description
Latency to enter	Learning	Latency	Both ears of the kid break the plane of the treatment chamber from the control chamber.
Latency to first bottle touch	Learning	Latency	The time from entry in to the treatment chamber to first deliberate touch of any part of the bottle using the nose, mouth or head
Latency to suckle	Learning	Latency	The time from entry in to the treatment chamber to active consumption of milk from the bottle
Elimination	Fear	Binomial	Any act of urination or defecation within the control or treatment chambers of the box (yes/no).
Control vocalization	Fear	Count	Vocalizations that occur in the control chamber of the box before the sliding door is opened.
Transition vocalization	Fear	Count	Vocalizations that occur in the control chamber of the box after the sliding door is opened, but before the kid enters the treatment chamber.
Treatment vocalization	Fear	Count	Vocalizations that occur in either chamber after the kid has entered the treatment chamber.
Loss of Posture	Fear	Latency	Lateral or sternal recumbency. Only occurs after ataxia on treatment side. Goat is not weight bearing on any limbs and no muscle tension in the neck is present.
Regain Posture	Fear	Latency	Goat is weight bearing on all four limbs in the recovery pen.

Table 3.2 Ethogram used for kid behavior collected during video observation of preference testing.

Measure	Behavior Category	Variable type	Description
Rear	Fear	Count	Weight-bearing on hind limbs only.
Startle	Fear	Count	Lateral jump or fast withdrawal.
Startle Score	Fear		Cumulative number of startles over all days per kid.
Escape Attempt	Fear	Count	Coordinated jump towards the top of the box, all 4 hooves leave ground.
Bottle Engage	Normal	Continuous	Any interaction with the bottle including oral contact, nursing, and butting.
Open Mouth Breathing	Gas Response	Continuous	Mouth is visibly open and labored breathing is apparent by exaggerated flank movements. Goat is weight bearing on at least 2 limbs.
Righting Response	Gas Response	Count	Only occurs after loss of posture. Goats may be sternal or lateral. Coordinated movements are made to return to standing or sternal (respectively). Includes lifting head during lateral recumbency.
Ataxia	Gas Response	Continuous/ Latency	Minor to severely uncoordinated limb movements while the kid is still weight bearing on at least 2 limbs.
Head Shake	Gas Response	Count	Head shakes with enough force to move both ears.
Nurse Pause	Gas Response	Count	Kid disengages and is not touching the bottle briefly (< 5s) before returning to bottle engage behavior (see above).
Re-entry	Normal	Count	Both ears break plane going from treatment to control box.

Table 3.3 Raw means ± SE for latency to enter treatment chamber (s), first bottle touch (s), and suckle (s) for all kids (n=12) by %CO2 concentration and test day.

	10%			20%		
Day*	**Enter**	**Bottle**	**Suckle**	**Enter**	**Bottle**	**Suckle**
B1	2.8 ± 0.3	2 ± 0.3	4.3 ± 0.4	2.6 ± 0.7	1.2 ± 0.2	3.8 ± 0.7
G1	1.5 ± 0.3	1.5 ± 0.2	4 ± 1.2	3.8 ± 1.5	1.5 ± 0.2	4.5 ± 1.2
W1	4.5 ± 2.1	1.3 ± 0.2	5.8 ± 2.5	4 ± 1.7	1.2 ± 0.2	3.7 ± 0.8
B2	2 ± 0.4	1.3 ± 0.2	3.8 ± 0.9	2.5 ± 0.6	1.5 ± 0.2	3.3 ± 0.5
G2	3.33 ± 0.2	1.3 ± 0.2	4.3 ± 0.5	1.5 ± 1.4	1.5 ± 0.2	3.3 ± 1.9
W2	1.7 ± 0.5	1.7 ± 0.3	4.7 ± 1.3	1.6 ± 0.2	1.3 ± 0.2	3.3 ± 0.8
30%						
B3	1.4 ± 0.3	1.4 ± 0.2	4.1 ± 0.2			
G3	1.6 ± 0.5	1.3 ± 0.2	4.5 ± 0.7			
W3	1.4 ± 0.2	1.9 ± 0.2	6.4 ± 1.9			

* B1-B3 indicate the day immediately prior to gas exposure (baseline); G1-G3 indicate gas exposure day; W1-W3 indicate the day immediately after gas exposure (washout).

Table 3.4 Raw means ± SE for latency to ataxia (s), duration of ataxia (s), and latency to loss of posture (s) for all kids (n=12) by %CO2 concentration.

Gas %	Latency to Ataxia	Duration to Ataxia	Time to Loss of Posture
10%	381 ± 139.2	8.4 ± 6.8	289*
20%	105.6 ± 16.3	26.1 ± 4.9	191.4 ± 18.8
30%	80.4 ± 11.3	13.8 ± 9.3	117 ± 10.2

* only 1 kid lost posture during 10% CO2.

Table 3.5 Raw means ± SE for control chamber, transition period, and treatment chamber vocalizations for all kids (n=12) by %CO2 concentration and test day.

	10%			**20%**		
Day*	**Control[A]**	**Transition[B]**	**Treatment[C]**	**Control**	**Transition**	**Treatment**
B1	105.7 ± 15.2	0.5 ± 0.5	0.8 ± 0.5	88.3 ± 22.7	0 ± 0	0 ± 0
G1	103.2 ± 20.9	0 ± 0	5.5 ± 2.6	71.8 ± 22.6	0.3 ± 0.3	10 ± 3.5
W1	89.5 ± 18	1 ± 0.5	0 ± 0	82.7 ± 21.2	0.3 ± 0.3	0 ± 0
B2	75.7 ± 16.7	0.5 ± 0.5	0 ± 0	114.8 ± 20.2	0 ± 0	7.5 ± 7.5
G2	71.7 ± 18.7	0.2 ± 0.2	0 ± 0	96 ± 26.4	0.2 ± 0.2	8.2 ± 3.3
W2	76.5 ± 21.4	0 ± 0	0 ± 0	119.7 ± 22.4	0 ± 0	7.2 ± 7.2
	30%					
B3	104.8 ± 23.9	0 ± 0	2.5 ± 2.5			
G3	99.4 ± 24	0 ± 0	5.9 ± 3.1			
W3	116.9 ± 21.2	0 ± 0	3.6 ± 3.6			

* B1-B3 indicate the day immediately prior to gas exposure (baseline); G1-G3 indicate gas exposure day; W1-W3 indicate the day immediately after gas exposure (washout).

[A] Control vocalizations are vocalizations that occurred within the control chamber of the preference testing box

[B] Transition vocalizations occurred after the sliding door was opened but before entry to the treatment chamber

[C] Treatment vocalizations occurred after the kid had crossed through the doorway.

Table 3.6 Raw means ± SE for startles and rears occurring in the control chamber (CS and CR, respectively), and startles and rears occurring in the treatment chamber (TS and TR, respectively) for all kids (n=12) by %CO2 concentration and test day.

	10%				**20%**			
Day*	CS	TS	CR	TR	CS	TS	CR	TR
B1	0.7 ± 0.2	0.2 ± 0.2	2.5 ± 1.7	0.3 ± 0.3	0.3 ± 0.2	0.2 ± 0.2	5.7 ± 3.5	0 ± 0
G1	0.3 ± 0.2	0 ± 0	8 ± 6.1	0.3 ± 0.2	0.2 ± 0.2	0.5 ± 0.5	4.8 ± 2.5	0 ± 0
W1	0.2 ± 0.2	0 ± 0	8.2 ± 4.8	0.2 ± 0.2	1 ± 0.4	0.2 ± 0.2	4.7 ± 2.8	1 ± 0.5
B2	0.3 ± 0.2	0 ± 0	5.5 ± 3.2	1.7 ± 1.1	0.3 ± 0.2	0.2 ± 0.2	7 ± 3.8	0 ± 0
G2	0.8 ± 0.3	0 ± 0	4 ± 1.2	0.5 ± 0.5	0.2 ± 0.2	0 ± 0	6 ± 2.7	0.2 ± 0.2
W2	0.3 ± 0.2	0 ± 0	5 ± 2	0 ± 0	0.7 ± 0.3	0.7 ± 0.5	10.2 ± 2.5	2 ± 1.8
	30%							
B3	0.5 ± 0.3	0 ± 0	9.3 ± 2.7	0.8 ± 0.3				
G3	0.5 ± 0.2	0 ± 0	5.4 ± 1.8	0 ± 0				
W3	0.6 ± 0.3	0 ± 0	9 ± 2.7	0.3 ± 0.2				

* B1-B3 indicate the day immediately prior to gas exposure (baseline); G1-G3 indicate gas exposure day; W1-W3 indicate the day immediately after gas exposure (washout).

CHAPTER 3 FIGURES

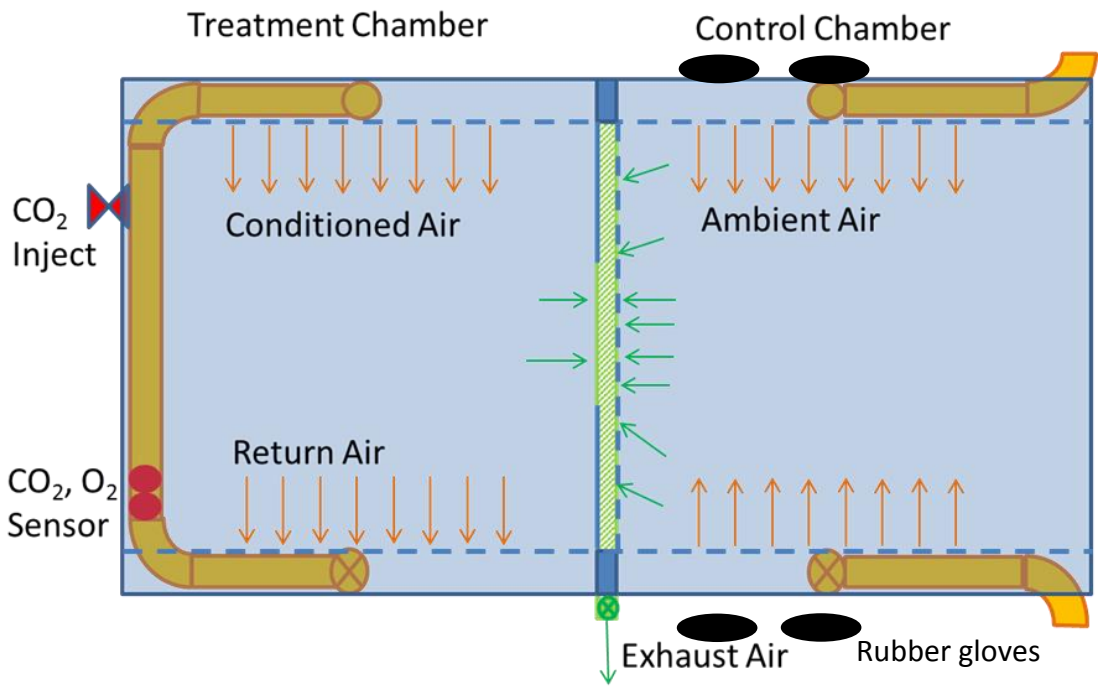

Figure 3.1 Blueprint demonstrating dual (control and treatment) chamber preference testing box used for conditioned place preference testing of kids (n=12).

Figure 3.2 Test room setup showing kid in preference testing box, observer to right of test box, exhaust system, camera position, lighting placement and black curtains used for obscuring the observer from kid's view during preference testing of kids (n=12).

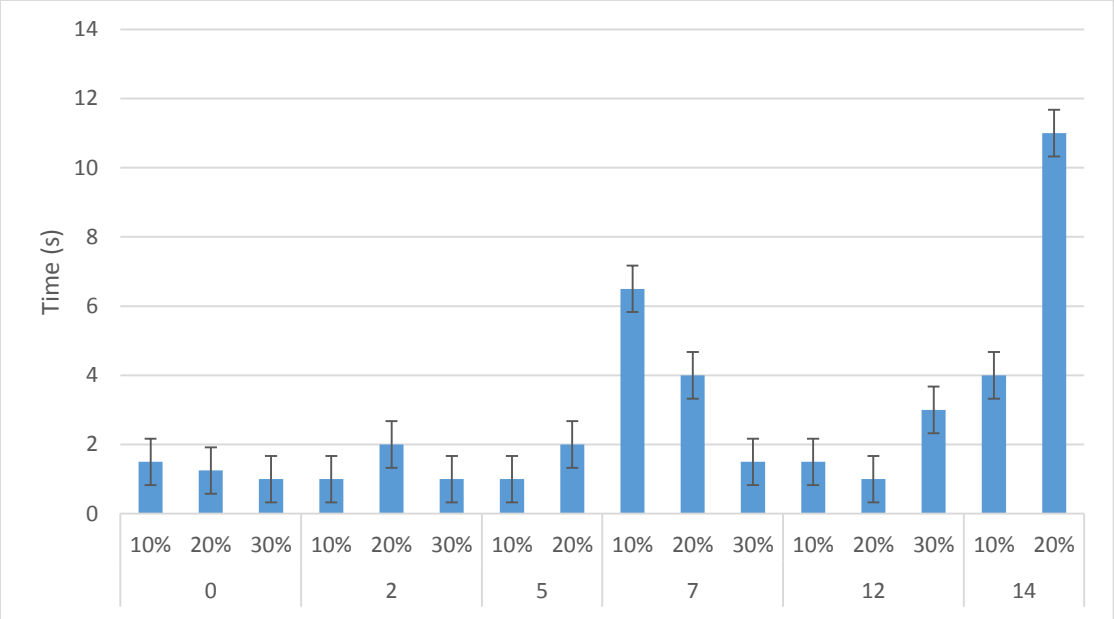

Figure 3.3 Average latency of kids (n=12) to enter (+ SE) treatment chamber for each %CO2 concentration by startle score. Top row of X-axis defines %CO2 concentration, and bottom row of the X-axis represents the number of startles a kid displayed during testing.

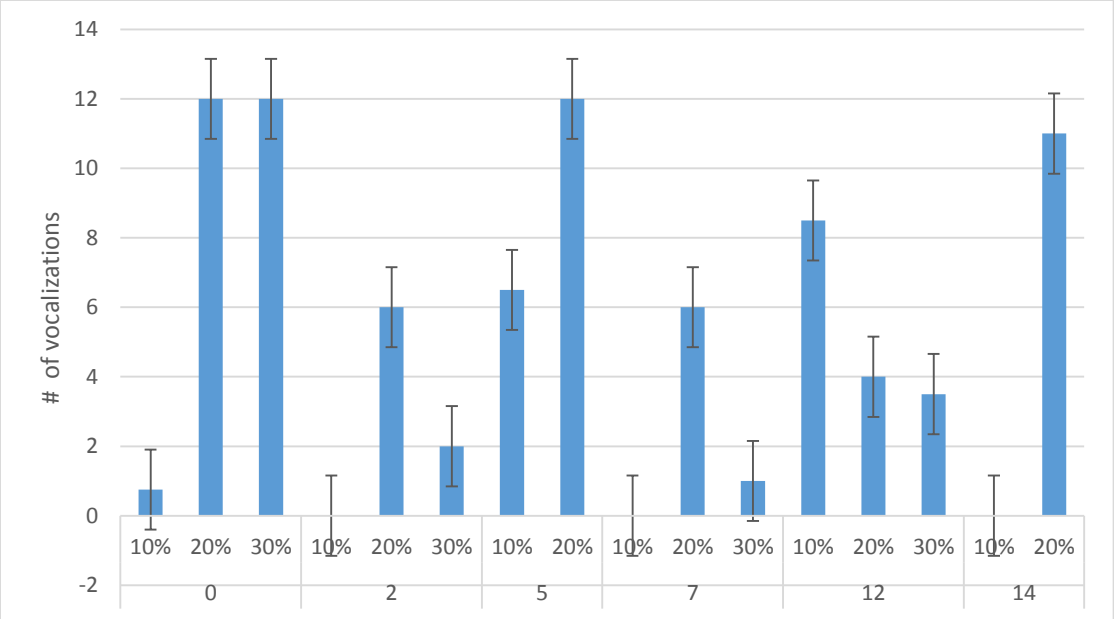

Figure 3.4 Average number of vocalizations (±SE) of kids (n=12) in the treatment chamber for each %CO2 concentration by startle score. Top row of X-axis defines %CO2 concentration, and bottom row of the X-axis represents the number of startles a kid displayed during testing.

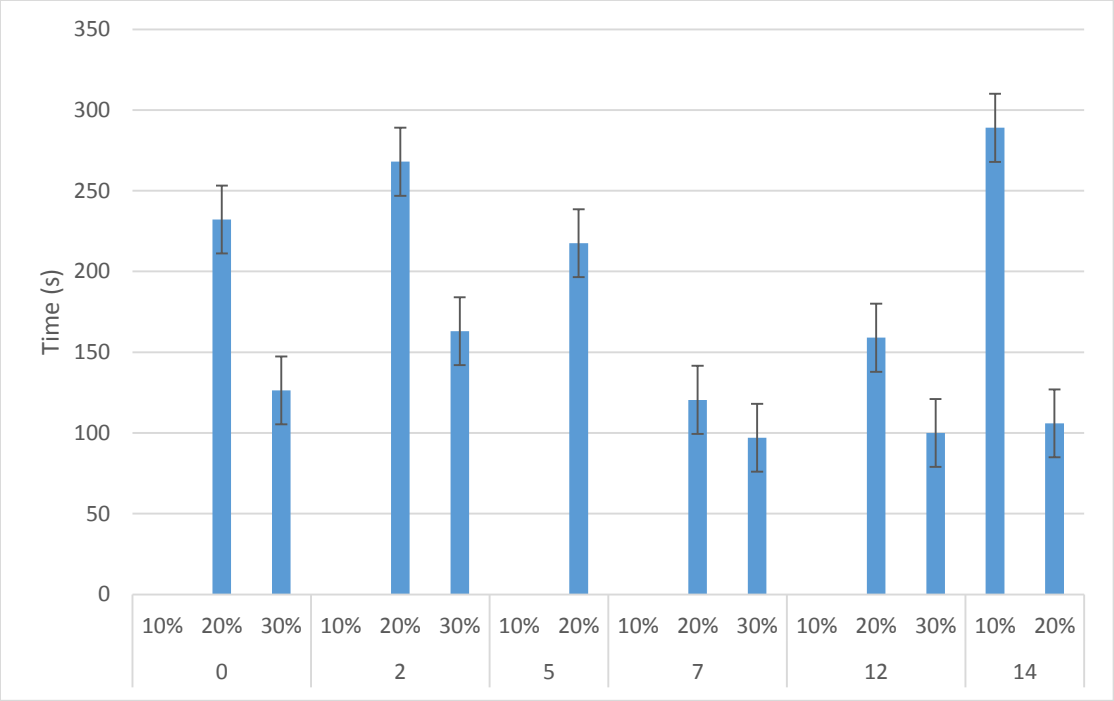

Figure 3.5 Average time to loss of posture (±SE) of kids (n=12) for each %CO2 concentration by startle score. Top row of X-axis defines %CO2 concentration, and bottom row of the X-axis represents the number of startles a kid displayed during testing.

CHAPTER 4.

GENERAL DISCUSSION

4.1 Conclusions

The domestication of animals accompanies a strong moral obligation to act in the best interest of the animal, sometimes ahead of even our own. The responsibility of upholding an animal's welfare includes the burden of ending its suffering by euthanasia when necessary. By nature, death is free of neither fear nor pain. As such, it is imperative to continuously work toward the goal of providing the most peaceful death possible to the animals in our care. Additionally, it should be noted that many euthanasia methods pose risks to those who must administer them, making human safety just as pertinent in the quest for suitable methods. The food animal industry is in the unique position of needing a euthanasia method that is humane, safe for both the worker and the animal, reliable, and cost efficient. This is especially true for the dairy industry, where many unvalued male offspring are euthanized at birth in addition to normal operation losses. The current euthanasia methods that are approved by the AVMA for ruminants pose disadvantages such as cost, including upfront costs and costs associated with carcass disposal, and worker safety. Carbon dioxide is a euthanasia method that is currently used in both swine and poultry producers, and may offer benefits as an option for dairy operations. CO2 provides a relatively fast-acting, hands off method that is safe and cost efficient when properly maintained equipment. This method could provide producers with the ability to euthanize more animals simultaneously, increase worker moral by using a hands-off method, and administer a consistent technique with little room for error.

One of the primary tools in determining the suitability of an inhalant euthanasia agent is the conditioned place preference and conditioned place aversion paradigms. These test models allow for a direct observation of an animal's perception of a stimulus. Due to the lack of documentation concerning the learning ability of the neonatal goat, it was not clear whether kids were capable of developing a conditioned place preference or aversion. Subsequently, the first step in evaluating CO2 was to determine whether the kid was an appropriate model. The results from this study concluded that kids were able to be conditioned to cross through a doorway to gain access to a milk reward. Kids proved to be adept at solving a simple problem, and were able to travel through the test box with increasing rapidity with repeated exposure. Kids were also able to retain learned behaviors and did not show any decreased performance after a several day interlude. Furthermore, kids demonstrated the ability to learn and successfully complete the task despite the presence of a visual and tactile, or olfactory, obstacle. The success of all kids regarding these obstacles supported the theory that kids would be able to move through a plastic curtain, a necessary component for atmospheric separation, and would be willing to enter an environment with a novel odor present. The importance of fearfulness in the kid as also evaluated, and the findings suggest that more fearful kid do not have decreased performance compared to non-fearful kids. Ultimately, the results support the use of the kid as an animal model in further research, and a suitable model for the evaluation of the effects of CO2 in ruminants.

The evaluation of CO2 as an acceptable euthanasia agent was based on unconditioned avoidance of the gas concentration on initial exposure, the extent of physiological gas-related responses, the presence of fear and pain behaviors exhibited during exposure and signs of conditioned place aversion on the day following exposure. The three concentrations used were

chosen based on literature concerning the reactions of other species to these concentrations, and on practical constraints associated with the preference testing equipment. The literature reported that 10% CO2 is generally considered non-aversive, 20% is aversive, and 30% has been described as painful (Makowska, et al. 2009, Niel & Weary 2007, Van den Hout & Griez 1984). As expected, kids did not show any avoidance behaviors during exposure to 10%, and the latency to enter the treatment chamber was very similar on the gas day compared to the baseline day. Surprisingly, kids did not show any avoidance behaviors during exposure to 20% or 30% concentrations. The latencies to enter, bottle touch, and suckle were similar across all test days, which suggests that none of these concentrations were potent enough to cause intense pain or fear reactions. Similarly, there were no consistent signs of conditioned place aversion on the washout days following gas testing. Although kids did not appear to recognize the CO2 as a potentially harmful stimulus, the increase in vocalizations during testing on gas days compared to baseline and washout days suggests that they did experience distress associated with fear, pain or both during exposure. Vocalizations observed during 20% and 30% treatments were similar across kids. Signs of CNS depression occurred more quickly, and the time to loss of consciousness was numerically reduced during 30% compared to 20%. Furthermore, vocalizations did not increase compared to 20% and there were no increases in avoidance behavior or conditioned aversion exhibited by any kids during 30% exposure. Thus, 30% CO2 may be the most humane and effective concentration for use in kids.

4.2 Future Research

Although the results of this study are promising, it also leaves several questions to be investigated further. During the assessment of kids' learning ability, it was not determined if they were able to develop conditioned place aversion. Young rodents have a heightened response to

fear conditioning compared to mature rodents; a trait that may also be present in kids of the age group (<4 weeks) in this study (Hefner & Holmes 2007). While it is reasonable to assume that if a kid can form a conditioned place preference, it can also form an aversion, there is no current literature supporting the latter. Evidence of the ability for kids to avoid a known danger would further support the concept that 30% CO2 is not highly aversive. Similarly, it must be investigated at what concentration of CO2 kids are willing to forgo a milk reward. An alternative approach would be to investigate whether kids would hesitate to enter an environment of 30% CO2 to access a less valuable reward than the milk bottle. This could be accomplished by offering reinstatement with conspecifics instead of a bottle to mitigate the drive to suckle, or the milk could be made less valuable by sating kids prior to testing. The answers to these questions are imperative to advancing the use of CO2 as a euthanasia agent in ruminants. The results from the current study merit continuing the investigation in to the use of CO2 as a euthanasia agent for ruminants. Through future studies and development of the concept, more knowledge will be available to producers, researchers, and veterinarians, the positive impact of which would affect ruminant welfare worldwide.

4.3 References

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