DEVELOPMENT OF NOVEL EUTHANASIA AND DEPOPULATION METHODS FOR NEONATAL POULTRY AND CAGED LAYING HENS

A Thesis

by

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ABSTRACT

Rapid depopulation of infected poultry is the primary US strategy to contain and eradicate reportable diseases. Two experiments were conducted to develop a compressed air foam based depopulation method for caged layer hens. The hypothesis of the first experiment was that a compressed air foam (CAF) system may be used as an alternative means to carbon dioxide (CO_2) inhalation for depopulating caged layer hens. In order to assess the stress response (corticosterone, CORT), young and spent hens were subjected to five treatments: normal handling (NEG control), CO_2 added to a chamber, CO_2 pre-charged chamber, CAF in cages, and CAF in a chamber. The times to cessation of movement (COM) were determined using spent hens, which were randomly assigned to three treatments: CAF in cages, CO_2 added to a chamber, and aspirated foam. Serum CORT levels of hens subjected to foam treatments were similar to CO_2 inhalation except that of spent hens in the CAF in a chamber group. Times to COM of spent hens subjected to CAF in cages and aspirated foam were significantly longer as compared to CO_2 in a chamber treatment. These data suggest that applying CAF in cages is a viable alternative for layer hen depopulation during a reportable disease outbreak.

The second experiment posited that infusion of gases such as CO_2 and nitrogen (N_2) into the CAF would reduce physiological stress and shorten time to cessation of movement of spent hens. There were six treatments in this experiment: a negative control, CO_2 inhalation, N_2 inhalation, CAF with air (CAF), CAF with 50% CO_2 (CAF CO_2), and CAF with 100% N_2 (CAF N_2). Serum CORT and serotonin levels as well as

time to COM were measured. The addition of CO_2 in CAF significantly reduced the foam quality as compared to the addition of N_2. The addition of gases into the foam did not result in significant improvements in the CORT and serotonin levels of spent hens as compared to foam with air. The time to COM of spent hens in the CAF N_2 treatment was significantly shorter than CAF and CAF CO_2 treatments, but longer than the gas inhalation treatments. The findings suggest that the addition of N_2 into foam is advantageous in terms of shortening time to death and foam quality than infusion of CO_2 for mass depopulation of caged layers.

Public concern on the use of maceration as a method of euthanasia of male layer chicks has resulted in negative publicity of the egg industry. We hypothesized that gas inhalation and low atmospheric pressure stunning (LAPS) are viable and humane alternatives for chick euthanasia. The study consisted of seven treatments: breathing air (NEG), 25% CO_2, 50% CO_2, 75% CO_2, 90% CO_2, 100% nitrogen (N_2), and LAPS. A custom made vacuum system was used to reduce air pressure inside the chamber from 101.3 kPa to 15.3 kPa for the LAPS treatment. Serum CORT and serotonin levels as well as latencies to loss of posture and motionlessness of day of hatch chicks were evaluated. The 25% and 50% CO_2 treatments were discontinued as the majority of the chicks recovered. The chicks in the NEG group had significantly higher levels of CORT but lower concentration of serotonin than the other four euthanasia treatments. The latencies to loss of posture and motionlessness of chicks exposed to 75% and 90% CO_2 were significantly shorter than the LAPS and N_2 inhalation treatments. These data suggest that LAPS and gas inhalation can be viable alternatives to maceration.

DEDICATION

I dedicate this thesis to my parents. All of my accomplishments are the results of your love, support, and encouragement. I am proud to be your son. Thank you.

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NOMENCLATURE

5-HT	Serotonin
ACTH	Adreno-Corticotropic Hormone
ADR	Adrenaline
AI	Avian Influenza
ANOVA	Analysis of Variance
APHIS	Animal and Plant Health Inspection Service
Ar	Argon
AVMA	American Veterinary Medical Association
CAF CO_2	Compressed Air Foam with Carbon Dioxide
CAF N_2	Compressed Air Foam with Nitrogen
CAF	Compressed Air Foam
CAFS	Compressed Air Foam System
CNS	Central Nervous System
CO_2	Carbon Dioxide
COM	Cessation of Movement
CORT	Corticosterone
CRF	Corticotropin-Releasing Factor
ELISA	Enzyme Linked Immuno-Sorbent Assay
END	Exotic Newcastle Disease
EU	European Union

GAMS	General Air Mixture System
HA	Hemagglutinin
HPA	Hypothalamo-Pituitary-Adrenal
HPAI	Highly Pathogenic Avian Influenza
LAPS	Low Atmospheric Pressure Stunning
LSD	Least Significant Difference
MAK	Modified Atmosphere Killing
N_2	Nitrogen
NEG	Negative
OIE	World Organization for Animal Health
pO_2	Partial Pressure of Oxygen
PVN	Paraventricular Nucleus
RNA	Ribonucleic Acid
SAM	Sympathetic-Adrenal-Medullary
SEM	Standard Error of Mean
SNS	Sympathetic Nervous System
USDA	United States Department of Agriculture
VSD	Ventilation Shutdown

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

The Economic Impact of Poultry Industry

Poultry farming has transcended into a global industry, providing inexpensive animal protein to billions of people. Besides addressing the growing demands for meat and eggs, the poultry industry contributes to the economy. The U.S. poultry industry employed 1.682 million people, paid $96.7 billion in wages, resulted in $441.2 billion in economic activity and provided $34.0 billion in taxes to the government in 2016 [1]. The chicken industry, turkey industry and egg industry together make up the U.S. poultry industry. The demand for chicken meat and eggs are expected to increase by 121% and 65% from 2005 to 2050 respectively [2]. Along with this opportunity for potential growth, the poultry industry faces challenges such as disease epidemics, welfare issues, feed costs, food safety, use of antibiotics and others [3].

Shell egg and processed egg product companies make up the U.S. table-egg industry [4]. Table or market type eggs, breaking eggs, and hatching eggs are products of the shell egg sector. The processed egg industries supply liquid, frozen and dried egg products. Within the table egg industry, 85% of production is from caged layer hens and the rest comes cage free (aviary, free range, and pasture) flocks [5]. As of July 1, 2017, the population of the table egg producing hens was 310 million in the U.S [6]. The commercial egg industry was responsible for 128,000 jobs, $7.2 billion in wages, $30.7 billion in economic activity and $2.2 billion in government revenue [7].

Male Layers- Current Situation and Practices

Genetic selection of chickens for meat or eggs was pivotal for the development of modern poultry industry. The specialization significantly improved productivity as specific breeds of chickens were developed. Broiler breeds are raised solely for meat purposes while layer hen breeds for table eggs. There is an equal likelihood of male and female chick to hatch from an egg (50:50) [8]. Unlike in broiler industry, only female chicks are useful for commercial egg industry. Modern male layer chickens lack genetic potential for faster growth, better feed conversion, carcass characteristics and yield poor meat quality compared to broilers [9]. Hence, male layer chicks are immediately euthanized in hatcheries. For instance, 7.3 billion laying hens were raised globally in 2015 [10] and an equal number of male layer chicks were euthanized.

Maceration (or instantaneous mechanical destruction) is the preferred method for euthanizing day-old male layer chicks in the U.S. [11]. Gas inhalation using carbon dioxide (CO_2), nitrogen (N_2) is another method being used in European countries. Despite these methods being approved by the American Veterinary Medical Association (AVMA) for poultry euthanasia, the public concerns on male layer chick euthanasia [12] has presented one such ethical challenge to the poultry industry [13]. The U.S. egg industry is looking for viable alternatives to maceration for chick euthanasia.

Reportable Diseases of Poultry

Reportable diseases are a significant threat to the poultry industry. Poultry industries in the U.S lose 20% of the gross worth of production due to diseases

[14]. The National List of Reportable Animal Diseases for the U.S. was developed by the Animal and Plant Health Inspection Service (APHIS) Veterinary Service [15]. The reportable diseases of poultry according to the list are mentioned below:

- Highly pathogenic avian influenza (HPAI)
- Low pathogenic avian influenza (LPAI; H5 or H7 subtypes)
- Exotic Newcastle disease (END)
- Turkey rhinotracheitis
- Avian infectious bronchitis
- Avian infectious laryngotracheitis
- Duck viral hepatitis
- Fowl typhoid (*Salmonella gallinarum*)
- Infectious bursal disease (Gumboro disease)
- Mycoplasmosis (*M. gallisepticum*)
- Avian chlamydiosis (psittacosis and ornithosis, *Chlamydia psittaci*)
- Pullorum disease (*Salmonella pullorum*)
- Mycoplasmosis (*M. synoviae*)

Highly Pathogenic and Low Pathogenic Avian Influenza

These are viral diseases caused by influenza type A viruses of *Orthomyxoviridae* family. These viruses have segmented negative-sense single stranded RNA [16]. The viral surface proteins hemagglutinin (HA) and neuraminidase determine the subtypes of the avian influenza virus. Each subtype has one of the 18 HA and 11 NA glycoproteins [17]. Based on virulence of the avian influenza viruses the World Organization for Animal Health (OIE) has classified these viruses into low and highly pathogenic strains [16]. The recent 2014-2015 outbreak of HPAI in the U.S. resulted in loss of 50.4 million commercial layer hens and turkeys combined across 15 states. The total economy wide loses was estimated to be $3.3 billion [18,19].

Exotic Newcastle Disease (END)

Avulavirus of the *Paramyxoviridae* family is the causative agent of exotic Newcastle disease. These viruses have single-stranded, nonsegmented, negative sense RNA [20]. The disease can result in 100% mortality in unvaccinated poultry flocks [20]. The most recent outbreak of END was in 2002. It started from California and quickly spread to nearby states Arizona, Nevada and Texas. The epidemic affected 3.16 million birds and cost the federal government $180 million to eradicate the virus [21].

Reportable Diseases Control Strategies

Multiple methods are essential to control outbreaks of reportable diseases in poultry [22]. Factors such as species affected, housing and management practices, stocking density, pathogenicity, and economic value determine the appropriate disease eradication strategies. However, the key components of such strategies are similar and overlapping across different diseases (**Figure 1)**.

Education

Contract growers, integrators, farm workers, and industry should be informed on ways of transmission of viruses such as influenza virus or bacteria like *Salmonella gallinarum* in poultry flocks and activities that increase risks of introducing the disease (backyard flocks) into farms. They should also be trained on appropriate and effective biosecurity procedures (shower in and shower out, footbaths, cleaning and disinfection procedures), and risks to human health [22].

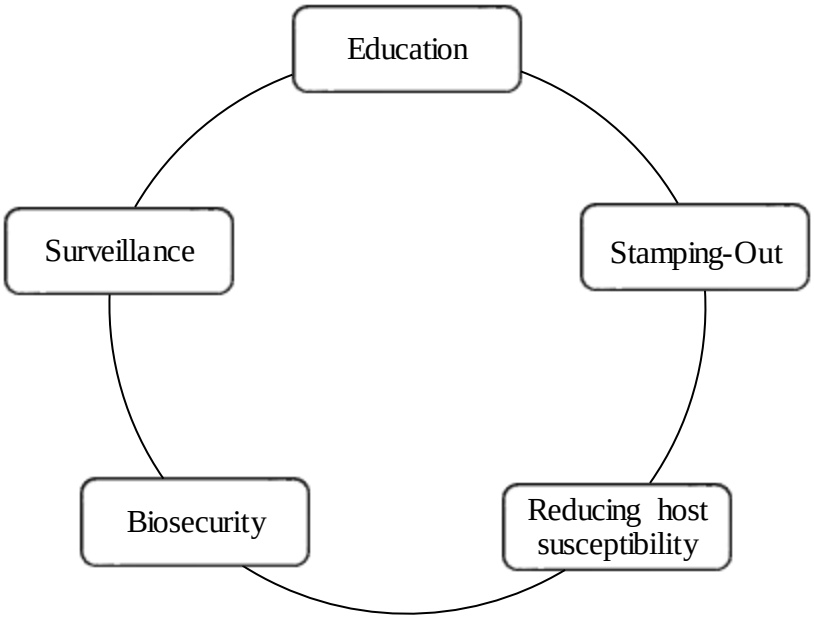

Figure 1: Avian influenza control strategies.

Biosecurity

Biosecurity is one of the most effective ways to prevent diseases from entering poultry flocks. Poultry companies should implement effective structural and operational biosecurity procedures. The goal of a good biosecurity program is to prevent entry of pathogens into farms either through vectors or fomites [23]. Effective quarantine measures should be in place to achieve biosecurity in farms. Movement of people, vehicles, live birds, eggs, feed, and other supplies in and out of the farm should be controlled [22]. Likewise, proper cleaning and disinfection programs should be in place at poultry farm such as before placement of new flocks [24].

Diagnostics and Surveillance

Rapid diagnosis of the index cases of diseases like HPAI and END is vital for launching an immediate response, including emergency killing of infected flocks. Identification of infected flocks helps in limiting the spread of the pathogen by immediately eliminating them. Regular monitoring of flock health through serological tests such as enzyme linked immunoassays and plate agglutination tests should be conducted in areas where those diseases are epidemic [22]. Since dabbling ducks like mallards are reservoirs of influenza viruses [25], surveillance programs to monitor and detect HPAI in wild birds are helpful in enhancing biosecurity and emergency disease responses [23].

Reducing Host Susceptibility

Vaccination programs should be followed under the guidance of field veterinarians. Effective vaccinations can prevent diseases like END, salmonellosis, infectious laryngotracheitis, infectious bursal diseases, and others. Homologous HA vaccines have been found to reduce host susceptibility to avian influenza viruses [22]. Use of dietary supplements and immunomodulators such as minerals can be helpful for enhancing the immune response of poultry to different pathogens [26].

Stamping-out

The OIE defines stamping-out as the killing of animals infected and suspected of infection in the flock [27]. Rapid elimination of an infected poultry flock is the primary control and eradication strategy of the United States for HPAI [28]. Poultry flocks diagnosed with the disease as well as birds within a 3 km radius of the index case are to

be killed [29]. Stamping-out acts by stopping the replication of viruses. Euthanasia and mass depopulation methods are used for stamping-out poultry flocks infected with reportable diseases.

Euthanasia and its Mechanisms

The word euthanasia is derived from two Greek words 'eu' means good and 'thanatos' means death. In other words, euthanasia means a good death. The American Veterinary Medical Association [11] defines euthanasia as death of animals in a way that causes minimum or no pain and distress to the animals. Euthanasia is carried out to reduce pain or suffering of animals infected with chronic and life threatening diseases. Food producing animals at the end of their production life such as spent layers and breeders are culled and euthanized. Male layers and culled chicks are also euthanized in hatcheries as these lack economic value. The methods used should result in a rapid and humane death in animals. The mechanisms of euthanasia are described below.

Depression of Cortical Neural System

Euthanasia agents act directly on neurons and disrupt functioning of cerebral cortex. Animals lose consciousness and show signs similar to those under anesthesia. Ultimately, animals suffer from respiratory arrest and loss of brain function.

Hypoxia

Hypoxia means a deficiency of oxygen. Animals exposed to high concentrations of gases such as CO_2, N_2, and Ar suffer from a lack of oxygen. The brain cells die due to oxygen deficiency which leads to loss of consciousness and death.

Physical Disruption of Brain Activity

Devices like captive bolt, pithing rod, and bullets directly destroy brain tissue. The respiratory and cardiac center immediately fails and animals ultimately die from injuries to the brain.

Methods of Poultry Euthanasia

Different methods of poultry euthanasia result in death by following any one of the above mentioned mechanisms. The AVMA euthanasia guidelines [11] has enlisted gas inhalation, decapitation, cervical dislocation, captive bolt, electrocution, gunshot, manual blunt force trauma, and injectable agents as methods of euthanizing poultry. The methods used depend upon the species, the numbers of birds, and reasons for euthanasia.

Injectable Anesthetics

Drugs such as barbiturates and barbiturate derivatives are injected intravenously at higher doses. These substances result in death by depressing the cortical neural system. These agents are administered only under the supervision of a veterinarian. The method is not suitable for euthanizing poultry for meat purposes as tissues get contaminated by the anesthetic agents [11].

Inhalant Agents

Carbon dioxide, N_2, and Ar cause death of birds by hypoxia. Gases used for euthanasia should be in a pure form, without contaminants. Duration of exposure and flow rates are important factors to consider for humane death of poultry as birds can recover from these gases [30]. Some common gases used for euthanasia of poultry are

described below.

Carbon dioxide

The AVMA guidelines on euthanasia as well as humane slaughter mention CO_2 to be widely used for euthanizing animals including poultry [11, 31]. Raj and Gregory [32] observed that broilers subjected to 55% CO_2 died within 2 minutes. It is recommended to use CO_2 at high concentrations such as 80-90% for euthanizing recently hatched chicks since chicks are exposed to higher concentrations of CO_2 during embryogenesis [11, 33]. The exposure to CO_2 inhalation decreases intracellular pH resulting in respiratory acidosis and anesthesia [34]. Continuous exposure to CO_2 leads to death of the animal from hypercapnic hypoxia [35]. Compressed CO_2 gas tanks can be used for euthanizing poultry in closed setups such as modified atmospheric killing (MAK) carts, CO_2 stunning chambers, and polyethylene tents [36, 37]. Liquid CO_2 containers can be used for whole house gassing of poultry [38].

Nitrogen and Argon

Inert gases such as N_2 and Ar result in death of birds by displacing oxygen in air, causing anoxia. While using these gases the residual oxygen concentration should be less than 2% for a humane death of poultry [11, 39]. Birds subjected to inert gases in the CO_2 mixtures were found to demonstrate least aversive signs as compared to CO_2 in air [40]. However, birds exposed to these anoxic gases undergo severe clonic and tonic convulsions as demonstrated by wing flapping and leg paddling after loss of posture [41]. These convulsions occur once the birds have become unconscious due to brain damage and hence they are not distressed [42].

Physical Methods

Cervical dislocation

Birds are killed by separating the cervical vertebrae from the cranium, either manually or mechanically, without crushing the spinal cord [11]. Birds rapidly become unconscious and display convulsive movements such as wing flapping and leg paddling [43]. Manual cervical dislocation is preferred for euthanizing small numbers of poultry. If performed manually, a maximum of 70 birds (up to 3 kg) are allowed to be killed per person per day by cervical dislocation in the European Union [44]. Manual cervical dislocation should not be used for birds larger than 3 kg as the procedure becomes arduous.

Decapitation

Severing the cranium from the body is known as decapitation. This method is used for collecting intact brain tissues from small birds (less than 200 g). Sharp instruments such as guillotines, blades rapidly sever the head from the neck inducing unconsciousness [11]. However, this method is not feasible for industry application as a large number of birds with different body weights have to be killed.

Captive bolt

Non-penetrating and penetrating captive bolts are used for killing individual turkeys, broiler breeders, ducks, hens, and geese [11, 45]. Birds should be restrained before discharging the captive bolt. The site for application of a captive bolt is the frontal bone in the middle between the ears and the eyes, above the cerebral cortex [45]. Birds should be monitored for any signs of recovery after they have been stunned using

captive bolt.

Maceration

The AVMA has approved the use of maceration for killing chicks up to 72 hours old, pipped eggs, and embryonated eggs [11]. The rotating blades of macerators result in an instantaneous death of chicks by physically disrupting their brain tissues [46]. The method is widely used for euthanizing recently hatched chicks in the U.S. because large numbers of chicks can be killed quickly and efficiently.

Poultry Depopulation

Depopulation, on the other hand, is an emergency measure for killing a large number of animals giving as much consideration to their welfare as possible [11]. Euthanasia methods can be used for depopulation, but not all depopulation methods meet guidelines for euthanasia. The circumstances that necessitate mass depopulation of poultry are natural disasters such as floods and tornadoes and during outbreaks of reportable diseases [47]. Elimination of poultry flocks infected with diseases like HPAI prevents the spread of the virus and reduces suffering of sick birds. Gas inhalation, electrocution, ventilation shutdown, and water-based foam are approved poultry depopulation methods [29, 48].

Gas Inhalation

Gases such as CO_2, Ar, and N_2 have been used for mass depopulation of poultry [41]. Birds are exposed to these gases following different procedures, such as whole house gassing, containerized gassing, polyethylene tent, and free standing panel

enclosures [48].

Whole House Gassing

A large number of birds can be killed in a short period of time with this method. Liquid CO_2 tanks can be used as gas sources. Poultry houses should be sealed to prevent escape of gas so that the required gas concentration can be achieved. Similarly, ventilation systems of the houses are turned off [48]. The amount of gas required depends upon the dimensions of poultry houses. However, it is estimated that the volume of CO_2 needed to reach fatal concentrations should be 1.5 times the volume of the house [49]. In addition, both of compressed CO_2 gas tanks and dry ice are other potential sources of CO_2. Turner et al. [38] observed that laying hens subjected to whole house gassing lost consciousness within 2 minutes of achieving an 18-20% CO_2 concentration. However, factors such as need for specialized equipment, prolonged duration to reach fatal gas concentrations, and different designs of poultry houses limit the application of this method [48].

Polyethylene Tent

Instead of using the whole house, gases such as CO_2 are applied to only a portion of the house. In broiler houses, polyethylene sheets (plastic sheets) are pulled from each sidewall of the house towards the opposite side. The outer edges of each plastic sheet are secured 2-3 feet deep in litter. Thus, birds are covered by two overlapping layers of plastic sheets [48, 49]. The front and back ends of the plastic are also closed. Valves of compressed CO_2 gas cylinders are opened releasing gas within a polyethylene tent [36].

Containerized Gassing System

Unlike whole housing and polyethylene tent methods, containerized gassing system has a closed set up which allows for easily controlling and monitoring gas concentration. Modified atmospheric killing cart is an example of containerized gassing system [50]. In this method, birds are manually caught and placed inside the containers. The containers are then filled with gases such as CO_2 and N_2 from compressed air tanks. It is a useful alternative to whole house gassing, especially if houses have structural damages or are naturally ventilated. The need for handling of birds as well as exposure to infective materials and gases in this method present safety risks to the personnel involved [37].

Free Standing Panel Enclosures

Metal or wooden panels are used to construct enclosed chambers inside poultry houses. Such enclosures can vary in dimensions depending upon number and size of the birds. Usually the panels are 4 ft. in height [48]. Once birds are driven into enclosed chamber, the top of the chamber is closed by plastic sheets. Compressed CO_2 tanks are turned on and the chamber is filled with CO_2. It is suitable for floor reared poultry only [48].

Electrocution

Each mobile electrocution unit consists of a water bath and a shackling line similar to electrical stunning system at broiler slaughter plants. It was used for poultry depopulation in the Netherlands during the 2003 HPAI outbreak [51]. Birds hoisted on shackling line moves through a water bath stunner where they are subjected to a high

current resulting in rapid death. The method is suitable for caged layer hens as well as floor reared poultry. However, handling and shackling makes this method stressful to birds.

Ventilation Shutdown (VSD)

Poultry flocks infected with reportable diseases like HPAI are subjected to cessation of natural or mechanical ventilation of air into poultry houses [52]. Poultry houses are sealed using plastic or boards. However, supplemental heat may be required for rapid increase in temperature inside the house. The temperature in the poultry house is expected to rise above 104 ºF within 30 minutes and is maintained for 3 hours. This leads to death of birds by hyperthermia [52]. Birds do have access to drinking water. Ventilation shutdown is implemented on case by case basis such as if other depopulation methods are not available or will not be available on time to meet the USDA-APHIS 24 hour depopulation goal [53].

Foam Depopulation

Foam is a mixture of air, water and foam concentrate. Specialized equipment is used to produce the finished foam. Benson and colleagues [47] of the University of Delaware developed the method of killing poultry using water-based foam in response to the 2004 Delmarva Avian Influenza (AI) event. The method works by forming a blanket of foam around birds which occludes the respiratory tracts. Birds suffer from hypoxia which leads to loss of consciousness followed by terminal convulsions, brain death and altered terminal cardiac activity [54]. The AVMA and the USDA-Animal and Plant Health Inspection Service (APHIS) have conditionally permitted the use of water based

foam for the depopulation of floor reared poultry [47, 55].

Expansion ratio refers to the volume of foam formed to total volume of soap and water solution. The expansion ratios of low, medium, and high expansion foam are less than 20:1, 20:1 to 200:1 and more than 200:1, respectively [56]. A low expansion foam is wetter than medium and high expansion foam. Medium and high expansion foam are more viscous than low expansion foam [57].

Some studies have focused on infusing gases like N_2, CO_2 into foam for poultry depopulation. Raj et al. [58] reported that laying hens exposed to dry foam infused with N_2 died (within seconds) due to inhalation of pure N_2 in the foam while birds exposed to foam with air survived for 5 minutes (till the end of the test period). McKeegan et al. [59] showed that high expansion foam containing N_2, CO_2 or air resulted in death of birds by anoxia. The birds exposed to foam with N_2, CO_2 demonstrated similar behavioral responses such as headshakes, loss of posture, wing flapping except gasping in the case of CO_2 infused foam [59].

The advantages of foam based depopulation methods are reduction in human exposure to birds infected with zoonotic diseases like HPAI, alternative to gas inhalation methods, and useful for poultry houses that cannot be effectively sealed [47, 59]. The methods available for eliminating caged laying hens at times of disease epidemics and natural disasters are limited. Unlike floor pens, layer cage houses present a different challenge for foam depopulation due to high stocking densities (100,000 or more layers per house), mesh cage floors preventing foam build up, and are multi-tier buildings (5-10 tiers of cages) limiting access to foam [60]. Foam produced using aspirated nozzles or

high expansion generators are not suitable for use in commercial cage layer operations.

Compressed air foam system (CAFS)

A CAFS produces finished foam by mixing of compressed air with foam solution in a mixing chamber and firefighting hoses [61]. The finished foam thus produced is known as compressed air foam (CAF). The finished foam is discharged using firefighting hoses. The components of a typical CAFS unit are (1) a water tank, (2) a foam concentrate tank, (3) a gasoline engine, (4) a water pump, (5) a foam proportioner, and (6) an air compressor [56].

Contrary to CAFS, general air mixture system (GAMS) is more traditional method of foam generation [62] in which foam is produced within the nozzle by drawing air into an aqueous foam solution [63]. The foam thus produced is commonly known as aspirated foam. An aspirated foam drains faster than CAF [63]. On the other hand, CAF has uniformly distributed bubble size which results in lower drainage rate [64]. It enhances stability of CAF [62, 65]. In a CAFS unit, the proportion of foam concentrate into aqueous foam solution can be altered using a foam proportioner. Other factors like flow rates of foam water solution and compressed air can be adjusted to alter foam characteristics such as the expansion ratio [57, 65]. A CAFS unit allows production of different kinds of foam as desired. Other advantages of CAFS come from its flexibility. In a CAFS unit, being a closed system, gases such as CO_2 and N_2 can replace compressed air to make a foam infused with gases.

Low Atmospheric Pressure Stunning (LAPS)

The method of stunning chickens by a gradual reduction of air pressure and a

subsequent decrease in partial pressure of oxygen (pO_2) using a vacuum pump is known as LAPS [66-68]. When the threshold of a minimum pO_2 is reached diffusion of oxygen from alveoli (in lungs) into blood (in capillaries) ceases. The animal suffers from hypobaric hypoxia and loses consciousness [69]. Purswell et al. [70] reported 100% death of broilers subjected to negative air pressure of 17.8 kPa. In the same study, Purswell and colleagues determined the maximum air pressure for 99.99% mortality of broilers to be 19.4 kPa. In the studies by McKeegan et al. [67]; Mackie and McKeegan [69]; Martin et al. [71] the total time period for each LAPS cycle was 280 s. The AVMA Guidelines for Humane Slaughter of Animals [68] has mentioned LAPS as a means of killing broiler chickens. The USDA has provided "no objection" status to LAPS for its application in broiler slaughter plants in the U.S. [67].

Poultry Welfare

Hans Selye [72] developed the "general adaptation syndrome" model to explain the response of an organism to nocuous agents. He later termed it "stress" [73]. Moberg [74] defines stress as a biological response of an individual to challenges to its homeostasis. Welfare is a state with no stress or a low level of stress [75].

Methods used for poultry euthanasia and depopulation should present minimum challenges to their welfare [11]. Birds should lose consciousness as quickly as possible without unnecessary fear, aversion, pain, and distress [76]. During emergencies such as an outbreak of HPAI, natural disasters like hurricanes and floods, the goal is to rapidly exterminate flocks to minimize their suffering and contain the disease. While doing so,

measures are taken giving due consideration to the welfare of birds as feasible.

However, procedures like handling and catching are stressful to poultry [77, 78]. Similarly, procedures such as exposure of poultry to gases or foam, and shutting off ventilation are likely to stress to these birds. Stressed animals exhibit behavioral and physiological responses to stressors [79, 80]. Assessment of such stress responses is vital for evaluating welfare of animals [81]. There are multiple indicators of stress in animals, such as biochemical, immunological, neuroendocrine parameters, and behavioral tests and observations [74, 82]. Evaluation of a single parameter does not provide a complete and accurate description of the welfare of birds [75, 83]. Assessment of physiological and behavioral responses are keys to understand bird welfare.

Physiological Responses to Stress

The physiological responses of animal to stressors are mediated mainly by two mechanisms: (1) sympathetic-adrenalmedullary (SAM) axis or "Fight or Flight" response (2) hypothalamo-pituitary-adrenal (HPA) axis [84, 85]. These two mechanisms have complementary actions for retaining or restoring homeostasis during stress [86]. The SAM axis acts by releasing catecholamines while effects of the HPA axis are mediated by glucocorticoids [75]. In addition to glucocorticoids and catecholamines, effects of stressors on serotonin (5-HT) and the serotonergic system have been reported [87, 88]. Leonard [89] in his review on the interaction between 5-HT and the HPA axis reported that stressors like restraining and electric shocks increase serotonin levels in brain.

Sympathetic-adrenalmeduallary (SAM) axis

Walter Cannon [90] summed up the phenomenon of increased secretion of adrenaline during fear, pain, rage and asphyxia as the "fight or flight" response. Such responses are mediated by the release of catecholamines (adrenaline, noradrenaline, dopamine) from chromaffin cells of the adrenal medulla and adrenergic neurons of the central and sympathetic nervous systems (CNS and SNS) [85]. Noradrenaline (or norepinephrine) is a precursor to adrenaline (ADR or epinephrine) synthesis and a neurotransmitter in the SNS [91]. Hypoglycemia (low blood sugar levels), glucopenia (condition of hypoglycemia), immobilization (shackling, restraining), and emotional stressors like fear and anxiety increase levels of ADR in the peripheral blood. On the other hand, physical stressors like cold, pain, immobilization results in rapid release of NA [91]. The measurement of concentration of catecholamines is an indicator of stress in animals. However, rapid secretion of hormones and their short half-life of 10-30 s followed by immediate metabolism and elimination affect their measurement in plasma and serum [92].

Hypothalamo-pituitary-adrenal (HPA) axis

The anatomic structures that comprise the HPA axis are paraventricular nucleus (PVN) of hypothalamus, anterior lobe of pituitary gland and adrenal gland [84]. Corticotropin-releasing factor (CRF) is the major regulator of the HPA axis. When animals are under stress CRF is released from the PVN of hypothalamus and through blood circulation reaches anterior lobe of pituitary gland. Adreno-corticotropic hormone (ACTH) is released by the pituitary gland which is carried by systemic circulation to the

adrenal cortex. The binding of ACTH to its receptors in the cortex of the adrenal gland induces synthesis and release of glucocorticoids. Cortisol (predominant in humans) and corticosterone (common in rodents, birds and reptiles) are the most common glucocorticoids [93].

Corticosterone

An assessment of the concentration of corticosterone (CORT) in the peripheral circulation is a method to evaluate the stress response [75]. In poultry, CORT is the major glucocorticoid released in response to stressors. Scanes [75] has reviewed literature on the responses of CORT to different stressors in poultry. The stressors such as light source [94], heat and cold [95], immobilization [96], fasting [97], molting [98], and shackling [99] have been reported to increase concentrations of CORT in peripheral circulation. Altholtz et al. [100] observed a higher CORT levels in the rats, euthanized by a 70%:30% CO_2:O_2 mixture compared to isoflurane administration. The elevated CORT level is vital for coping with stress by inducing glucose synthesis, lipolysis, and protein degradation [75].

Serotonin

Serotonin or 5-hydroxytryptamine (5-HT), is found in both plants and animals [101] and functions as a neurotransmitter, a hormone, and a mitogen [102, 103]. Serotonin, a biogenic monoamine, has roles in central nervous, digestive, and cardiovascular systems [104]. Only 2% of total body's 5-HT is found in the CNS [105]. The effects of the remaining 98% of body's serotonin are outside the CNS. Vasoconstriction, vasodilation, gastrointestinal motility, platelet aggregation,

hemostasis, regulation of heart rate, and glucose and lipid metabolism are some of the effects of 5-HT in peripheral tissues [105, 106]. Serotonin in the brain has behavioral effects such as appetite, mood, memory, fear, anxiety, anger, and addiction. The physiological processes like circadian rhythms, respiratory drive, motor control, body temperature, and CNS vascular tone are also affected by 5-HT in CNS [106].

Behavioral Responses

Different studies have reported behavioral responses such as head shaking, gasping, ataxia, loss of posture, and motionlessness in poultry subjected to euthanasia and depopulation methods [38, 107-109]. Head shakes, gasping, and depressed respiration are indicators of respiratory distress [40]. The formation of carbonic acid upon exposure of mucous membrane to CO_2 results in aversive responses in animals [110]. In addition, birds have intrapulmonary chemoreceptors sensitive to CO_2 [111]. Therefore, head shaking, gasping, and sneezing are more common in birds exposed to CO_2 [112,113]. On the other hand, inert gases like N_2 or Ar elicited fewer head shakes in birds [108]. Further exposure to these gases results in ataxia or loss of muscle coordination. Ataxia is shown by animals prior to loss of posture [114]. Signs like dizziness, staggering, swaying of body and/or head, attempts to stand or sit indicate ataxia [69]. Loss of posture is an indicator of unconsciousness in poultry depopulation trials [107]. Finally, after the end of convulsive movements the animals are in a state of motionlessness. Motionlessness is the visible absence of respiratory movement in an animal [69, 115].

Loss of posture and onset of terminal convulsions cannot be evaluated in birds immersed in water based foam [116]. The clonic and tonic convulsions are followed by relaxation and death [117]. The termination of these neuromuscular spasms or cessation of movement is an indicator of brain death [118]. Accelerometers can detect changes in velocity as a result of motion. Dawson et al. [116,119] demonstrated that accelerometers can detect cessation of movement (COM) in broilers subjected to foam depopulation. In case of gas inhalation and LAPS, loss of posture and motionlessness can be determined by visual observations. Video recording is a useful tool for measuring latencies to these behaviors. Vizzier-Thaxton et al. [66] observed the average time from the first movement to loss of posture to be 64.9 s in broilers subjected to LAPS. Behavioral responses of broilers in LAPS were different from birds exposed to the control atmospheric stunning [66]. Male broiler chickens during LAPS displayed a consistent pattern of behavioral signs: ataxia, loss of posture, clonic and tonic convulsions, leg paddling, and motionlessness [69, 71].

Outbreaks of reportable diseases and the growing public sentiment on methods and practices used in the poultry industry such as euthanasia are key challenges faced by the poultry companies today. The solutions to these issues can only come from scientific and rationale studies on alternative and innovative methods. The study aimed to develop novel methods for industry applications for the purposes of euthanizing day-old male layer chicks and depopulating caged layer hens. The experiments were designed to meet specific objectives of developing compressed air foam system for caged layer hen depopulation, infusing gases into the foam to reduce physiological stress and shorten

time to death, and evaluating the physiological and behavioral responses of male layer chicks to gas inhalation and LAPS methods of euthanasia.

CHAPTER II

DEPOPULATION OF CAGED LAYER HENS WITH A COMPRESSED AIR FOAM SYSTEM

Description of Problem

The U.S. poultry industry has faced disease outbreaks and natural disasters that require flocks to be destroyed. Natural disasters such as hurricanes and floods cause damage to poultry houses, feed mills, roads, and power lines leading to the emergency killing of flocks to prevent further suffering [47]. Reportable poultry diseases such as highly pathogenic avian influenza (HPAI), exotic Newcastle disease (END), avian infectious laryngotracheitis, avian infectious bronchitis, and mycoplasmosis are also threats to the poultry industry [15]. The last reported US END outbreak was in 2002-2003 in California which resulted in the loss of 3.16 million birds and $161 million [21]. Most recently, the U.S. poultry industry lost 43 million layer and pullet hens, and 7.4 million turkeys during the 2014-2015 HPAI outbreak [120]. The layers and turkeys lost alone were worth $1.6 billion, and the overall economic losses were estimated to be $3.3 billion [18, 19].

Euthanasia is the act of terminating the life of an animal in a way that involves minimum pain and distress [11]. Euthanasia, meaning a good death, is distinct from depopulation. Depopulation is an emergency measure used to rapidly kill animals with as much consideration given to their welfare as possible [11, 55]. Elimination of poultry infected or at risk of infection from HPAI is a primary US animal health policy to

control and eradicate the virus [28]. Therefore, birds within a three kilometer radius of an infected zone are killed and disposed of. Methods used for mass depopulation of poultry depend upon the species, housing type, age of bird, ambient temperature and available carcass disposal methods [47].

The AVMA has approved carbon dioxide (CO_2) inhalation as a means of euthanizing poultry [11]. Carbon dioxide is an analgesic and anesthetic gas [112, 121]. Exposure to CO_2 inhalation induces hypercapnic hypoxia in birds which results in rapid unconsciousness and ultimately leads to death [35]. However, mammals and birds show aversive responses to CO_2 inhalation [108]. Humans exposed to CO_2 concentrations between 40 and 55% experienced painful sensations [122] due to acidification of respiratory mucosa upon exposure to CO_2 [110]. Birds experience respiratory distress such as gasping (breathlessness) and deep breathing while remaining conscious [59]. Birds exposed to liquid CO_2 may also suffer from cold stress [123]. The use of CO_2 inhalation may not be suitable for some types of poultry houses due to differences in construction. The method requires effective sealing of poultry houses, special equipment, and the rapid flow of a large volume of gas over the birds [47, 59]. Application of CO_2 also presents a safety risk to human personnel involved [59].

Water-based foam is a suitable depopulation alternative to CO_2 inhalation of floor reared poultry [55]. Foam is a collection of air-filled bubbles derived from a solution of detergents and water [47, 62]. Foam has been widely used by firefighting departments for extinguishing fires [65, 124]. Water-based foam depopulation was developed in response to the 2004 Delmarva AI event [47]. Broilers and turkeys are

immersed in foam which blocks the respiratory tract resulting in hypoxia, leading to a loss of consciousness, convulsions, cerebral death, and cessation of cardiac activity [54]. However, depopulation of caged layer hens poses a different challenge. Foam developed for the floor reared poultry is a wet foam that drains quickly through mesh cage floors making it unfit for cage layer houses. Furthermore, commercial cage farms have high stocking densities (100,000 or more layers per house) and are multi-tier buildings (5-10 tiers of cages) which limit access to foam [60]. The outbreak of a disease, like avian influenza, in a caged layer facility would be the worst case scenario; as a large number of birds would have to be depopulated rapidly, safely and humanely.

The methods used for mass depopulation should be efficient and give due consideration to personnel safety and bird welfare. Birds subjected to euthanasia and depopulation methods suffer stress until they lose consciousness. It is vital for a depopulation method to result in a quick death to minimize suffering and contain the spread of viruses. Birds undergo clonic and tonic convulsions upon a loss of consciousness during euthanasia [116, 125]. Termination of such neuromuscular spasms (or cessation of movement) is an indicator of brain death [118]. Birds engulfed by foam cannot be visually evaluated for behavioral changes such as loss of posture, onset and cessation of convulsions. Accelerometers are sensors that measure changes in velocity due to movement [126]. Dawson and colleagues [116, 119] determined time to cessation of movement of broilers subjected to water-based foam depopulation based on their accelerometer data. Physiological responses of birds to stress are mediated by the limbic hypothalamo-pituitary-adrenocortical (HPA) axis and sympathetic-adrenalmedullary

(SAM) axis [84, 85]. The effects of HPA axis are mediated by release of glucocorticoids, like corticosterone, in response to stress [84, 127]. Serum corticosterone is a common physiological parameter used to assess welfare in birds [128-131]

Benson and colleagues [47] reported the use of compressed air foam (CAF) for broiler depopulation. A CAF system uses energy to make foam from the compressed air. A CAF system consists of a centrifugal water pump, a foam concentrate proportioner, an air compressor and a mixing chamber [61, 62]. In a CAF system, compressed air agitates a foam solution in a mixing chamber to produce a thick foam [61]. The ratio of aqueous foam solution and compressed air can be adjusted in a CAF to produce a drier or wetter foam [64, 65]. Ideal foam for cage operations (conventional, colony or enriched colony) would be one that has a longer dewatering time and at the same time has a small bubble size, since such foam would persist long enough in cages depriving birds of oxygen ultimately leading to death from mechanical hypoxia [132]. Compressed air foam has a uniform bubble size and better stability than air-aspirated foams [64].

We hypothesized that a compressed air foam is a rapid and humane means for caged layer hen depopulation. The specific objectives of this study were to determine and compare serum corticosterone levels and time to cessation of movement of birds subjected to CAF, CO_2 inhalation, and negative (NEG) control treatments. Hens subjected to CAF and CO_2 treatments were necropsied to evaluate signs of trauma or presence of foam in the respiratory tract.

Materials and Methods

Experimental Animals

Lohmann LSL young and spent hens were obtained from an egg integrator and housed in the Texas A&M University Poultry Science Research, Teaching, and Extension Center layer barn prior to trials. The young hens were 20 weeks of age while the spent hens were 76-95 weeks old. All birds were cared for under an approved TAMU Institutional Animal Care and Use Committee protocol.

Experiments

Based on the objectives of the study, two separate experiments were carried out. The objective of experiment 1 was to evaluate corticosterone (CORT) levels while that of experiment 2 was to determine time to cessation of movement (COM). Young and spent hens were the test subjects in experiment 1 while only spent hens were used in experiment 2. Birds in experiment 1 were subjected to five treatments: NEG control, CO_2 added to a chamber after bird placement, pre-charged CO_2 chamber, CAF in cages, and CAF in a chamber. Twelve young hens and thirteen spent hens were subjected to each of the five treatments. Experiment 2 consisted of three treatments: CO_2 added to a chamber, CAF in cages, and aspirated foam in a floor pen. The number of spent hens randomly assigned to each of the three treatments in experiment 2 was 16. The concentration of CO_2 in both experiments was 100%. A 55-gallon (208 liters) chamber was used for application of treatments in both experiments. Hens in the NEG control group were handled normally and placed in cages. Compressed air foam was filled into the cages of hens assigned to the CAF in cages group. In experiment 2, hens allocated to

the aspirated foam treatment were placed in a floor pen of the dimensions of 2.44 m × 2.44 m. The conventional cages used in the CAF in cages treatment, in both trials, were positioned 0.1524 m above a plywood platform to simulate a layer house manure belt.

Foam Production

The CAF unit (Rowe CAF LLC, Washington, AR) consisted of a 0.03 cubic meter per second rotary screw air compressor (Vanair Inc., Michigan City, IN), a 29.42 kW (40 HP) gasoline engine (Kohler, WI), a 9.46 liter per second centrifugal water pump (Hale Products, Inc., Ocala, FL), and a foam concentrate proportioner (0.1% - 10%; FoamPro, Kingston,NY). Water for the CAF was supplied from a 1135.62 liters tank. A 37.85 liters foam cell contained the foam concentrate necessary for the experiment. A Class A foam concentrate (ICL Performance Products, Rancho Cucamonga, CA) was used at 3.5% in the CAF and 1% in the aspirated foam. Foam concentrate was injected by the proportioner into the water manifold of the CAF unit. A separate air manifold supplied compressed air to the mixing chamber. These three constituents of foam were agitated in the mixing chamber of the CAF unit. The flow of air and water into the mixing chamber was adjusted to produce a foam of the desired consistency and thickness. The finished CAF was released from a 3.81 cm CAF unit through a 15 m long and 3.81 cm internal diameter firefighting hose attached to a 6.4 cm wide and 6 m long suction hose. An aspirated nozzle (Spumifer American Company, Ridgefield Park, NJ) was used to produce the finished foam which is commonly used for depopulation of floor reared broilers and turkeys.

Experiment 1

Serum CORT levels of young and spent hens were evaluated in this trial. A total of 65 spent hens and 60 young hens were used. Blood samples were collected from the right jugular vein of live birds in the NEG control group and by severing the femoral artery of recently killed birds in the rest of the treatment groups. Blood was allowed to clot for 1 hour at room temperature and transported on ice to the laboratory. The blood was stored at 4°C for 24 hours. Serum was collected by centrifuging blood at 1000 × g for 15 min. Serum corticosterone was evaluated by competitive ELISA kit ADI-901-097 (ENZO Life Sciences, Farmingdale, NY) according to the instructions from manual. Samples were run in duplicate. Absorbance was measured for each sample using a plate reader at 405 nm (BioTek Instruments, Inc., VT). A four-parameter logistic regression model was used for curve fitting to interpolate the CORT levels from absorbance readings for each individual experimental subject. Intra-assay variability was 2.8% and inter-assay variability was 7.2%.

Experiment 2

The trial was conducted to determine time to cessation of movement of laying hens subjected to each depopulation treatment. Accelerometer data loggers (HOBO Pendant G, Onset Computer Corporation, MA) were attached to the shanks of each bird by zip ties to measure changes in the movement as hens were exposed to the treatments. The accelerometers were programmed to start logging data every second. The end point of convulsive movements was determined as the point where a flat line (no signal) was recorded. The time-interval between application of treatment until loss of body

movement was determined for each hen. Forty-eight spent hens were used in total in this trial.

Postmortem Examination

Gross necropsy was performed on spent hens subjected to foam or CO_2 treatments in experiment 1. Respiratory tracts were visually evaluated for signs of physical injury or presence of foam.

Statistical Analysis

In experiment 1, samples with concentrations outside the range of the standard curve were removed from the study. Therefore, in case of pullet hens 4 samples were removed from CO_2 in cage group and one sample was omitted from negative control group. In case of spent hens all treatments had 13 samples each except CAF in a chamber group which had 12 samples. So, statistical analyses were performed on CORT values of serum samples from a total of 55 young and 64 spent hens. Statistical analyses of CORT and time to COM data were done by one-way ANOVA using the PROC ANOVA procedure (SAS 9.4, Cary, NC). Means deemed significant were further evaluated using Fisher's LSD post-hoc test. The tests were carried out at the 5% level of significance ($\alpha = 0.05$).

Results and Discussion

Serum Corticosterone Concentrations

The mean serum CORT concentration of young hens subjected to the NEG control, CO_2 added to a chamber, CO_2 pre-charged chamber, CAF in cages, and CAF in

a chamber treatments were 3.8 ng/mL, 4.8 ng/mL, 2.9 ng/mL, 3.4 ng/mL, and 7.02 ng/mL respectively (**Figure 2**). There were no statistically significant differences in serum CORT concentration of young hens subjected to the five treatments ($P = 0.569$). The stress responses of the young hens to gas inhalation and compressed air foam treatments were comparable to the NEG control group. Unlike the young hens, a statistically significant difference was observed in the CORT concentrations of spent hens among the treatment groups. Birds in the CAF in a chamber group had significantly higher CORT levels ($P = 0.0005$) than hens in the remaining four treatment groups. The mean serum CORT concentrations of spent hens assigned to CAF in a chamber group was 27.1 ng/mL while that of the NEG control, CO_2 added to a chamber, CO_2 pre-charged chamber, and CAF in cages were 5.0 ng/mL, 5.5 ng/mL, 2.6 ng/mL, and 6.8 ng/mL respectively.

Scanes [75] reported that stressors like heat, cold, floor space, restraining, catching, shackling, feed restriction, and nutrient deficiency elevates plasma CORT in poultry. Exposure to CO_2 causes pain or distress in animals [133]. Results of the CORT experiment suggest that poultry subjected to CAF might also elicit stress response due to fear and anxiety. The results of the CORT assay trial demonstrates that CAF in a chamber method was significantly more stressful for spent hens than the CO_2 inhalation, CAF in cages, and NEG control treatments. However, the serum CORT levels of young hens as well as spent hens subjected to CAF in cages were similar to the NEG control and CO_2 inhalation treatments. Benson and colleagues [47] reported that serum CORT levels of broilers had no statistically significant difference among foam alone, foam with

CO_2, and the CO_2 polyethylene tent method.

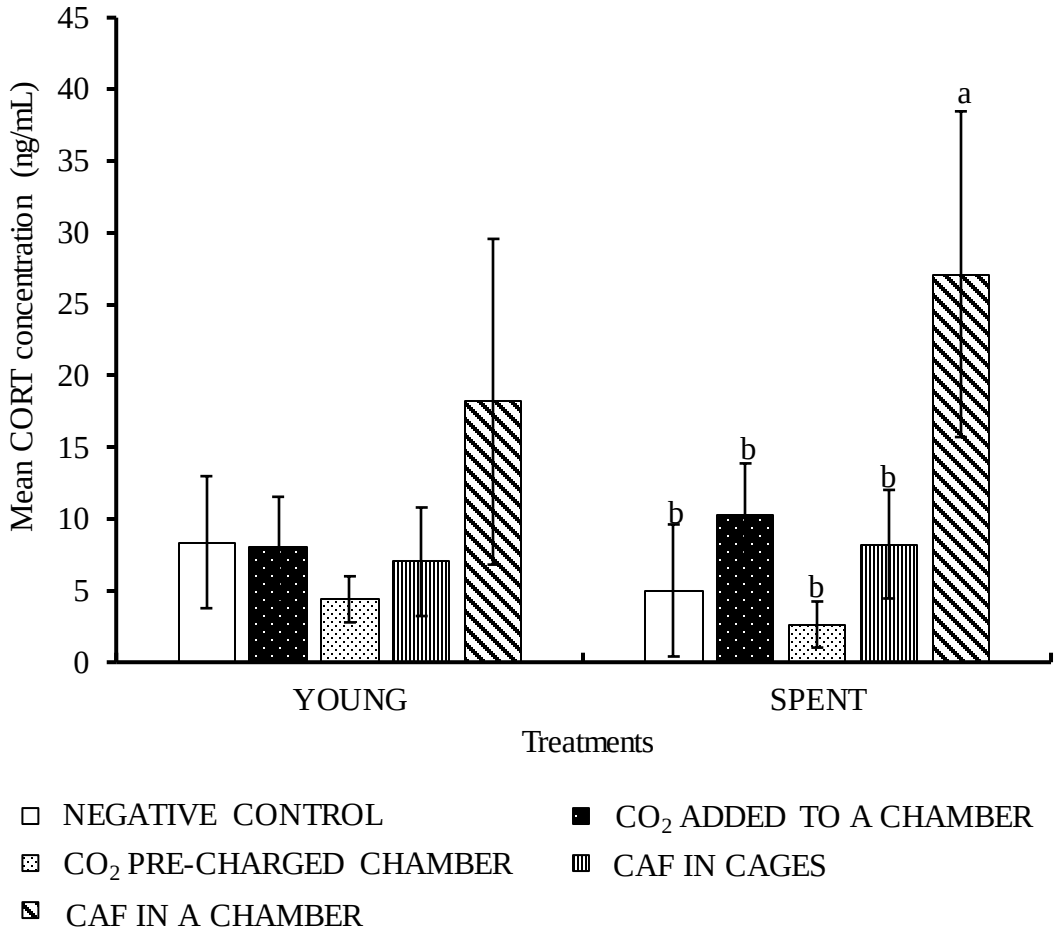

Figure 2. Mean serum corticosterone levels of young and spent hens.
The CORT concentrations were measured in duplicates and expressed in ng/mL. Bars (mean ± SEM) with different superscripts (a, b) are statistically significantly different by Fisher's LSD test ($P < 0.05$). Young and spent hen trials were conducted separately. Number of young and spent hens per treatment were 12 and 13 respectively.

Time to Cessation of Movement

Birds undergo terminal convulsive movements after the onset of unconsciousness until they become motionless [116]. The termination of clonic and tonic phase of convulsions is known as cessation of movement. The times to COM of spent hens subjected to the CAF in cages, CO_2 added to a chamber and aspirated foam in floor pens were determined based on accelerometer readings (**Figure 3**). The average time to COM was 90 s for birds treated by carbon dioxide in a chamber, 195 s for birds in CAF in cages, and 192 s for hens subjected to aspirated foam. A statistically significant difference in mean time to COM was observed among the three treatments ($P < 0.05$). A post hoc Fisher's LSD test revealed that cessation time of hens subjected to carbon dioxide added to a chamber was significantly shorter than that of hens in the CAF in cages and aspirated foam groups. Based on electrocardiography (ECG) readings, Benson et al. [47] reported that time to cessation of cardiac activity of broilers subjected to foam with CO_2, foam without CO_2, and the polyethylene tent method were 73 s, 64 s, and 139 s respectively.

In our study, the times to cessation of movement of spent hens assigned to CAF in cages and aspirated foam treatments were within the ranges reported in previous studies. Dawson et al. [116] reported the time to cessation of movement of broilers subjected to water-based foam to be in range from 25 s to 179 s. In our study, spent hens depopulated using carbon dioxide gas took significantly less time to become motionless than hens subjected to the aspirated foam and CAF in cages. The data suggest that hens in the CO_2 added to a chamber group lost consciousness in a shorter time period. Mean

cessation times of spent hens belonging to the CAF in cages and aspirated foam treatments were not significantly different. Wing flapping and struggling of the hens may have agitated foam bubbles creating air pockets in foam treatments. The time taken for foam to fill the cages and form a blanket of foam around the birds was longer than filling of the chamber with CO_2, which may suggest a second reason for increased cessation times in both of the foam treatments.

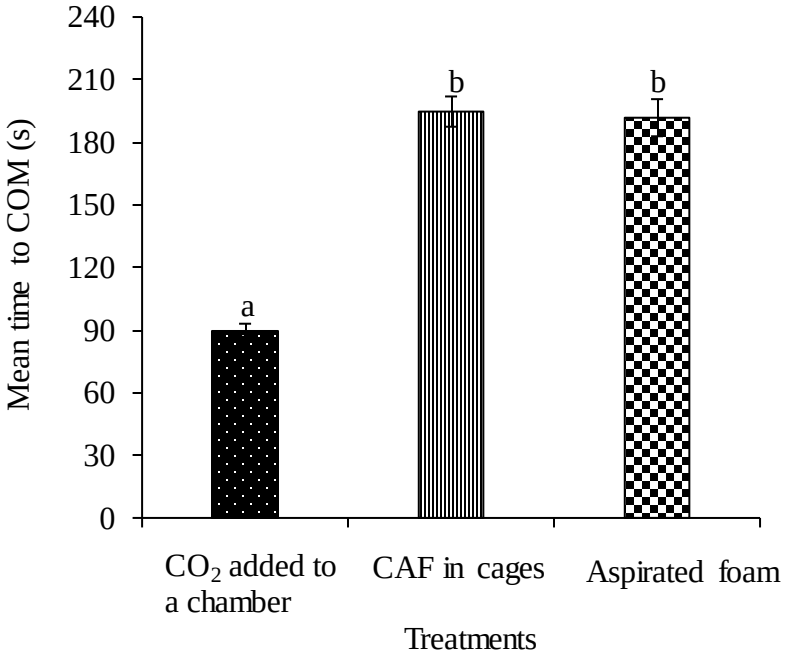

Figure 3. Average cessation times of spent hens.
Time to cessation of movement is also known as cessation times. The treatments were CO_2 added to a chamber, CAF in cages, and aspirated foam treatments. Bars (mean ± SEM) with different superscripts (a, b) have statistically significant difference by Fisher's LSD test ($P < 0.05$). Sixteen spent hens per treatment were used in this trial.

Gross Necropsy Findings

In the CORT trial, gross necropsy of hens subjected to CAF and CO_2 treatments was performed. The trachea, syrinx, and bronchi were evaluated for signs of hemorrhage, presence of foam, and the presence of blood. Foam was present within the first 5 cm of the upper trachea of all hens in the foam treatments. There were no signs of trauma or injury in the airways of hens since no blood or hemorrhages were found on necropsy. The hens randomly assigned to the CO_2 added to a chamber, and CO_2 pre-charged chamber groups had no signs of hemorrhage or injury to the respiratory tract. Birds exposed to CO_2 treatments died from hypercapnic hypoxia. The cause of death of all hens subjected to the CAF treatments was due to occlusion of airways by foam leading to hypoxia. Benson et al. [47] reported that broilers subjected to foam died due to mechanical hypoxia. In the same study, Benson and colleagues showed that foam was present in the trachea and lungs of broiler chickens. Blood was found in the syrinx, bronchi, and lungs upon histological examination. Raj et al. [58] conducted a trial to kill end-of-lay hens using a dry nitrogen foam in which they observed foam bubbles around the larynx and upper part of trachea. McKeegan et al. [59] in their study on gas filled high expansion foam observed small foam bubbles in trachea and tracheal openings of broilers exposed to high expansion nitrogen foam.

The CORT and COM trials data suggests that CAF applied in cages is a viable alternative to CO_2 inhalation for mass depopulation. Use of CAF for depopulation does not require manual handling of live birds. Unlike CO_2 inhalation, CAF does not present safety risks to the personnel involved. In addition, a CAF can be used as a means for

cleaning and disinfection of infected premises after completion of depopulation and disposal of the carcasses [135]. The paper is the first peer reviewed manuscript on the application of CAF as a means of depopulation of caged layer hens.

CHAPTER III

CARBON DIOXIDE AND NITROGEN INFUSED COMPRESSED AIR FOAM FOR DEPOPULATION OF CAGED LAYING HENS

Description of Problem

The U.S. poultry industry lost 50.4 million birds (layers, turkeys, and backyard flocks) in 15 states during the 2014-15 highly pathogenic avian influenza (HPAI) outbreak [120]. The overall economic loss was estimated to be $3.3 billion [18]. Besides HPAI, outbreaks of other reportable diseases like exotic Newcastle disease (END), infectious laryngotracheitis, mycoplasmosis, and Marek's disease have occurred in the past and pose significant risks to the industry [15]. The 2002-2003 California END outbreak resulted in the loss of 3.16 million birds and cost $180 million in federal money to remediate [136]. Protecting poultry from reportable diseases is still a major challenge facing the industry today [137].

Euthanasia and depopulation methods are followed to eliminate animals infected or suspected of infection after confirmation of a reportable disease. The AVMA defines euthanasia as an act of killing animals in a way that causes no or minimum pain and suffering. Depopulation, on the other hand, refers to an emergency measure to rapidly eliminate animals with as much consideration given to their welfare as possible [11]. These methods are vital for controlling the multiplication and spread of a reportable disease. The Animal and Plant Health Inspection Service (APHIS) depopulation goal during a HPAI outbreak is to kill infected poultry within 24 hours of a presumptive

diagnosis of a case [29]. The timing of depopulation is important to contain the disease, prevent further cases of infection, eradicate the pathogen, and facilitate business continuity [28].

Current depopulation methods are broadly categorized as gas inhalation and foam based methods [11, 29]. The most commonly used gas for mass depopulation during disease outbreaks is carbon dioxide. Carbon dioxide (CO_2) has been widely used as a means of euthanizing laboratory animals and stunning broilers, pigs and turkeys in slaughter plants [138, 139,140]. It is an analgesic and anesthetic gas [121] which causes rapid loss of consciousness by decreasing intracellular pH [34]. Chickens exposed to 45-50% CO_2 died within 2 to 5 minutes of exposure [141]. Water-based foam has been approved as a means for depopulation of floor reared poultry by the [55]. Foam is a collection of air filled bubbles produced from a solution of water and foam concentrate (detergents). Benson et al. [47] developed water-based foam as a method of depopulation in response to the Delmarva AI event in 2004. Poultry houses are flooded with the foam which forms a thick blanket around birds. Birds die due to mechanical hypoxia as a result of an obstruction of airflow [37, 47]. The advantages of this method are minimum safety risks, limited human contact with infected birds, no requirement for tight sealing of poultry houses, reduction in dusts and aerosols, and rapid depopulation. Ventilation shutdown was recently implemented as a method of depopulation by the USDA-APHIS to meet the 24 hour depopulation goal [29]. During ventilation shutdown the birds in poultry houses are deprived of natural or mechanical ventilation with or without increasing the temperature. The birds ultimately die from hyperthermia.

However, these methods have limitations and associated risks to use in commercial cage layer farms. The use of CO_2 is not suitable for all kinds of poultry houses as it requires effective sealing, needs special equipment, and has safety risks for the personnel involved [47, 59]. Chickens demonstrate aversive signs to CO_2 inhalation [142] as they possess intrapulmonary chemoreceptors for the gas [40, 111]. Water-based foam is not suitable for commercial caged layer operations. Caged layer houses present a different challenge for foam depopulation due to high stocking densities (100,000 or more layers per house), mesh cage floors that prevent foam build up, and multi-tier buildings (5-10 tiers of cages) which limit access to foam [60]. It is essential to develop alternative methods to rapidly and humanely depopulate caged layer hens during disease outbreaks. Ventilation shutdown is used only when all other methods are found to be inadequate to contain the spread of a pathogen [52, 53]. However, ventilation shutdown presents significant challenges to bird welfare.

A study in our laboratory found that CAF can be used as an alternative method for depopulation of caged layer hens (unpublished data). A CAF system is a widely used fire extinguishing method which makes use of foam concentrate, water, and compressed air to make a finished foam [61, 62, 65]. The foam and water solution is mixed inside a mixing chamber with compressed air in a CAF system [61]. The ratio of aqueous foam solution and compressed air can be changed as desired to produce a drier or wetter foam [64]. It is important that foam used for depopulation in cage operations (conventional, colony or enriched colony) has a longer dewatering time and a small bubble size. Such characteristics would allow foam to persist long enough in cages, depriving hens of

oxygen and ultimately causing their death from hypoxia [132]. Compressed air foam has a longer drainage time and uniform bubble size compared to aspirated foam [63]. Gases such as CO_2 or N_2 can be used instead of air to make CAF since a CAF unit is a closed system. Benson et al. [47] reported addition of CO_2 into the finished CAF using a gas injection nozzle for floor reared poultry depopulation. However, the concentration of CO_2 in the foam was 1% or less as reported by Benson and colleagues [47]. In our study, CO_2 or N_2 was agitated with the aqueous foam solution in the mixing chamber of the CAF system to make the finished foam.

We hypothesized that the addition of 40-50% CO_2 in air or 100% N_2 to make CAF would reduce physiological stress and shorten time to cessation of movement. The aim of the study was to evaluate the efficacy of CAF infused with CO_2 or N_2 to depopulate caged layer hens. The specific objectives were to develop CAF with CO_2 or N_2, to evaluate physiological responses of laying hens subjected to the treatments, and to determine time to cessation of movement of hens to estimate time to death.

Materials and Methods

Test Subjects

A total of 192 Lohman LSL spent hens of 90 weeks of age, were obtained from an egg integrator. These hens were housed at a layer barn in the Texas A&M University, Poultry Science Research, Teaching and Extension Center. The hens were supplied with clean drinking water and a diet that met industry recommendations. These birds were cared for following an approved Institutional Animal Care and Use Committee protocol.

Experimental Design

Spent hens were subjected to six treatments. The treatments were a negative control (NEG), 50% CO_2 in air (CO_2), 100% N_2 (N_2), CAF with air (CAF), CAF with 50% CO_2 (CAF CO_2), and CAF with 100% N_2 (CAF N_2). Four spent hens were chosen at random and assigned to each treatment. A total of 24 spent hens were used in each replication. The trial was replicated 8 times over a period of 4 months. Hens in the NEG group were placed inside the cage for the same duration as other treatments before removing them for blood collection.

Foam Production

The components of the CAFS unit (Rowe CAFS LLC, Hope, AR) were a 0.03 m^3/s rotary screw air compressor (Vanair Inc., Michigan City, IN), a 29.42 kW (40 HP) gasoline engine (Kohler, Kohler, WI), a 0.01 m^3/s centrifugal water pump (Hale Products, Inc., Ocala, FL), and a foam proportioning unit (0.1% - 10%) (FoamPro, Kingston, NY). The foam proportioning unit injected Class A foam concentrate (ICL Performance Products, Rancho Cucamonga, CA) into the water manifold of the CAFS unit to make a 3.5% foam water solution. An 1135.6 liters water tank installed on the trailer bed supplied water for producing foam. A separate air manifold supplied compressed air to the mixing chamber from the air compressor. The three constituents, air, water, and foam concentrate, were agitated in the mixing chamber of the CAFS unit. Foam of a desired consistency and thickness was produced by adjusting the flow of aqueous foam solution. A 6.4 cm wide and 6 m long suction hose connected to a 3.8 cm CAF system through a 15 m of firefighting hose of the same diameter was used to

deliver CAF to the spent hens.

CAF Infused with Gases

Liquid CO_2 and N_2 tanks delivered respective gases to produce CAF CO_2 and CAF N_2 foam. The liquid gases were heated using a 480 volt vaporizer set at 65 °C (Thermax Inc., MA) before flowing through mass flow controllers (Alicat, Tucson, AZ). In the CAF CO_2 treatment, compressed air from the air compressor was first diverted through two consecutive water/oil separators, a desiccant dryer and finally a particulate filter before flowing through the mass flow controller. The flow rates of CO_2 and compressed air were same, 0.008 m^3/s each, to obtain a gas mixture of equal parts of CO_2 and air. The gas mixture was then agitated with the foam water solution in the mixing chamber to make CAF CO_2 foam. The mixing tank was completely emptied each time before another gas was filled in. In the case of CAF infused with N_2, 100% N_2 gas was mixed with the foam water solution in a mixing chamber to make CAF N_2 foam. The flow rate of N_2 gas was adjusted at 0.02 m^3/s. A 25 kVA diesel engine generator (Multiquip Inc., Carson, CA) supplied power necessary for the vaporizer and mass flow controller. An infrared CO_2 analyzer was used to measure the concentration of the gas in the finished foam (Servomex, Crowborough, UK).

Gas Inhalation Treatments

Thick polyethylene was fixed to the sides of a cage to make a closed chamber for the 50% CO_2 and 100% N_2 treatments. The gases were introduced into the chamber using the same hoses used for application of the foam treatments.

Measurement of Expansion Ratio and CO_2 Concentration

Foam samples were collected in 125 liter containers. The foam was allowed to dewater overnight and the aqueous foam solution at the bottom of the container was measured using a graduated cylinder. The same procedure was followed for all three kinds of foam samples, CAF, CAF CO_2, and CAF N_2. The expansion ratio was calculated as the ratio of the volume of the finished foam to the volume of aqueous foam solution.

Foam samples from CAF CO_2 treatment were collected in 3.8 liter zip-lock bags. These bags were sprayed with a 10% anti-foam solution (Sigma-Aldrich, St. Louis, MO) to allow foam bubbles to rupture releasing CO_2 for measurement of the gas concentration in the samples. The infrared CO_2 analyzer was calibrated using 80% CO_2 prior to the measurements. A 20-gauge needle, connected through a delivery hose to the analyzer, was inserted into the top of each sample bag headspace for measurement of the CO_2 levels. Four samples were measured in each replication.

Assessment of Stress Hormones

Blood samples were collected from each individual bird postmortem by severing the femoral artery, except in the NEG group. Blood was collected from the jugular vein of birds in the NEG group. The blood samples were allowed to clot overnight before being centrifuged at 1000 × g for 10 minutes at 4° C to collect serum. Serum corticosterone (CORT) and serotonin (5-HT) levels were determined using competitive ELISA assay kits ADI-901-097 and ADI-900-175, respectively (Enzo Life Sciences, Farmingdale, NY) according to the manufacturer's directions. Three spent hens

subjected to the CAF treatment and one exposed to CAF CO_2 treatment had survived. Two blood samples of hens in the negative control and one sample in the CAF treatment group did not yield enough serum. Therefore, out of total 192 spent hens used in the study, only 185 serum samples were used in ELISA. In order to assess the 5-HT levels, sixteen serum samples from each treatment group were used except CAF CO_2 treatment (only 15 samples due to availability). Hence, the total number of samples for 5-HT assays was 95. Serum samples were run in duplicates for the CORT and 5-HT assays. The intra-assay and inter-assay variability of the corticosterone assay were 2.25% and 8.3% respectively. The intra-assay and inter-assay variability of the 5-HT assay were 2.3% and 5.7% respectively.

Determination of Cessation of Movement

In each of the six treatments, accelerometers were attached to the shank of each bird individually before placing them into a cage. However, data from spent hens in the NEG group were not used for statistical analysis. Time to cessation of movement (COM) was determined based on the accelerometer readings. The time to COM was calculated as the difference in time from the application of treatment to cessation of convulsive movements as indicated by a flat line in the accelerometer readings. Three spent hens in the CAF treatment and one in the CAF CO_2 treatment had survived the process. In addition, accelerometers fell off the shank of three hens exposed to CO_2 treatments and one off the hen subjected to CAF. Therefore, data was collected from 152 spent hens only.

Statistical Analysis

All data collected on CORT and 5-HT concentrations as well as time to COM from accelerometers were compiled in a spreadsheet (MS-EXCEL, Microsoft, Santa Rosa, California). Tukey's boxplot method was followed to identify four outliers from CAF CO_2 and one each from CAF and N_2 treatment groups. These outliers were removed and CORT levels of 179 samples were used in the statistical analysis. Statistical analysis of the CORT data was done using Welch's ANOVA following the PROC ANOVA procedure (SAS 9.4, Cary, NC) since these data violated homogeneity of variance assumption. For all other data on expansion ratio, 5-HT levels, and time to COM were analyzed using traditional one-way analysis of variance following PROC ANOVA procedure. Means deemed significant were further analyzed using Fisher's LSD test. All statistical tests were conducted at 5% significance level.

Results and Discussion

Foam Quality Parameters

Expansion ratios of all three kinds of foam and concentration of CO_2 in the CAF CO_2 foam were determined (**Figure 4**). The addition of CO_2 in the foam, CAF CO_2, significantly decreased the expansion ratio of the finished foam compared to CAF and CAF N_2 ($P = 0.004$). The mean expansion ratios of CAF, CAF CO_2, and CAF N_2 were measured to be 111:1, 66:1, and 111:1 respectively. The average CO_2 concentration achieved in the CAF CO_2 foam across the eight replications was measured at 43%. However, the mass flow meter was set to obtain a 50/50 blend of CO_2 and air. This

discrepancy could be due to sample contamination and intact foam, which did not release enough gas for measurement from the headspace.

Expansion ratio is one major factor affecting foam viscosity [57]. Low-expansion foams have lower viscosity [143] and hence, they drain faster. In commercial layer operations, foam should be stable in cages for considerable period to cause death of birds by mechanical hypoxia. The probable mechanism for a decrease in the expansion ratio of CAF CO_2 foam is the reduction of pH of foam solution due to the formation of carbonic acid. Carbon dioxide gas when reacts with water forms carbonic acid, H_2CO_3. Preliminary works on measurement of pH of compressed air foam with and without CO_2 had determined the values to be 5.8 and 8.1 respectively (unpublished data).

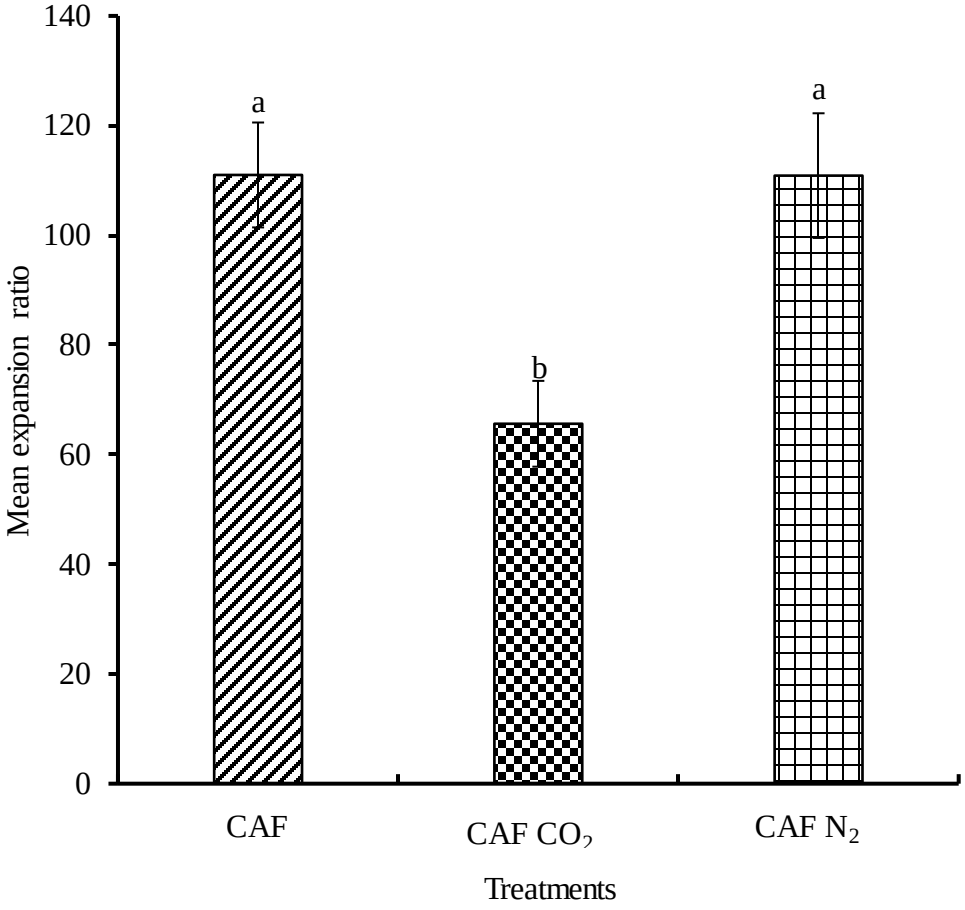

Figure 4. Mean expansion ratios of the three types of foam.
The three foam treatments were CAF with air, CAF with CO_2, and CAF with N_2. Expansion ratio is the ratio of volume of finished foam to volume of aqueous foam solution. Bars (mean ± SEM) with different superscripts (a, b) are significantly different by Fisher's LSD test ($P < 0.05$). The number of samples per treatment was 8.

Serum Corticosterone

Corticosterone is the predominant glucocorticoid released from the adrenal cortex in rodents, birds, and reptiles [93]. Once released into the peripheral circulation,

CORT binds to the intracellular glucocorticoid receptors. Glucose synthesis, lipolysis, and protein degradation are some of the effects of CORT to cope with stressors [84].

The mean CORT levels of spent hens subjected to NEG, CO_2, N_2, CAF, CAF CO_2, and CAF N_2 treatments were 12.1 ng/mL, 8.4 ng/mL, 8.5 ng/mL, 8.4 ng/mL, 8.0 ng/mL, and 6.8 ng/mL respectively (**Figure 5**). The CORT values of spent hens in all six treatment groups had no significant differences ($P = 0.1249$). The CORT level of spent hens subjected to the NEG group was numerically higher than the rest of the treatment groups. On the other hand, the spent hens in the CAF N_2 group had numerically the lowest CORT concentration among all six treatments. The three foam treatments CAF, CAF CO_2, and CAF N_2 did not differ among themselves ($P > 0.05$). The infusion of gases into CAF did not bring significant changes in the CORT concentration of spent hens as compared to the CAF treatment. The CORT concentration of spent hens subjected to foam treatments (CAF, CAF CO_2, and CAF N_2) did not differ significantly with that of the birds in gas inhalation treatments, CO_2 and N_2 ($P > 0.05$). These findings indicate that foam treatments are similar to the AVMA approved poultry euthanasia method of gas inhalation. Benson et al. [47] observed no significant differences in serum CORT levels of broilers among foam, foam with CO_2, and CO_2 polyethylene tent treatments. A study in our lab reported no significant differences in the serum CORT concentrations of young hens subjected to the negative control, CO_2 inhalation, and CAF treatments (Chapter II findings).

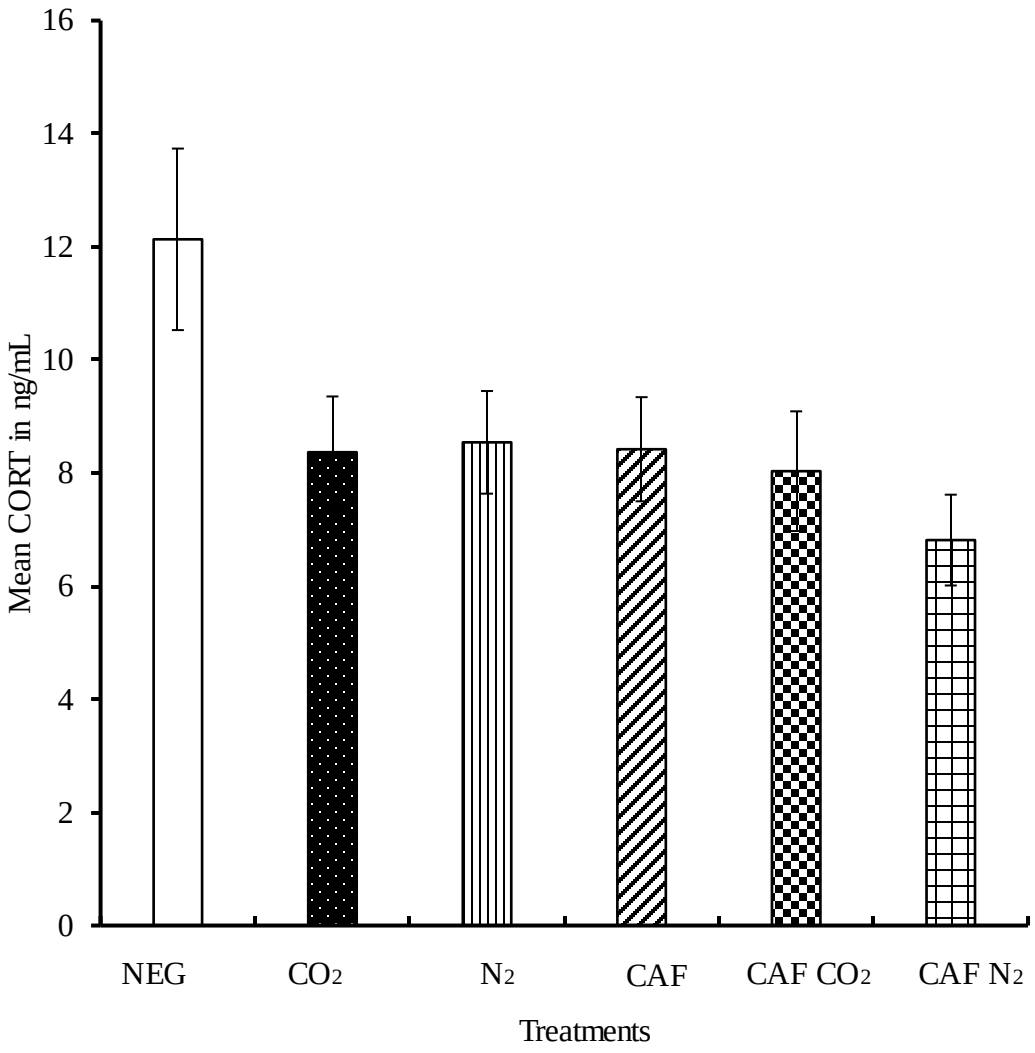

Figure 5. Mean serum corticosterone levels of spent hens.
The CORT concentrations were measured in duplicates and expressed in ng/mL. Bars (mean ± SEM) with no superscripts are not significantly different by Fisher's LSD test ($P > 0.05$). The total number of samples per treatment was 32.

Serum Serotonin

Serotonin, in birds, affects appetite, responses to fear, anxiety, and other stressors [144, 145]. The serotonergic system in central nervous system has been demonstrated to

be affected by handling and social separation in White Leghorn chicks [146].

The mean serum 5-HT concentration of the hens in NEG, CO_2, N_2, CAF, CAF CO_2, and CAF N_2 were 6.3 µg/mL, 8.8 µg/mL, 7.9 µg/mL, 10.1 µg/mL, 11.0 µg/mL, and 11.7 µg/mL respectively (**Figure 6**). The serum 5-HT levels of the spent hens differed significantly among the six treatments ($P = 0.0010$). The hens in the NEG group had significantly lower 5-HT levels as compared to CAF, CAF CO_2, and CAF N_2. However, foam treatments where gases were infused CAF CO_2 and CAF N_2 did not differ significantly with CAF in terms of mean 5-HT concentration. The 5-HT concentration of spent hens killed by AVMA approved euthanasia method of CO_2 inhalation was similar to CAF and CAF CO_2 treatments but significantly lower than CAF N_2 group. Birds in the N_2 inhalation treatment had similar 5-HT levels to CAF but significantly lower than CAF CO_2, and CAF N_2 treatments. Higher levels of whole blood 5-HT was found to be associated with positive mood in male volunteers [147] while higher concentration of corticosterone indicates higher stress levels [75]. The spent hens in the CAF N_2 treatment seemed to have lower anxiety and fear response as indicated by lower 5-HT levels than birds in the NEG, CO_2, N_2 treatment groups.

Uitdehaag et al. [148] suggested that peripheral 5-HT levels could be indicative of brain 5-HT activity in laying hens. Correlations between brain 5-HT and blood 5-HT were reported to be in range from 0.34 to 0.57 [148]. Uitdehaag et al. [148] reported mean blood 5-HT levels of Rhode Island Red and White Leghorn hens in pure groups (birds of the same breed) after 5 minutes of manual restraint to be 11 µg/mL and 7.8 µg/mL respectively. The 5-HT concentration of spent hens in our study varied from 6.5

µg/mL (NEG) to 11.7 µg/mL (CAF N_2). In this study, spent hens in the NEG group had the highest CORT concentration (numerically) but the lowest 5-HT levels. In contrast, birds subjected to CAF N_2 had the lowest CORT levels (numerically), but the highest 5-HT levels. Other studies have reported similar relationship between corticosteroids and brain 5-HT levels. Inoue and Koyama [149] observed that acute corticosterone administration decreased 5-HT in the hippocampus of rats. Similarly, Karten et al. [150] reported that chronic exposure to corticosteroid reduces 5-HT responses in hippocampus of rats.

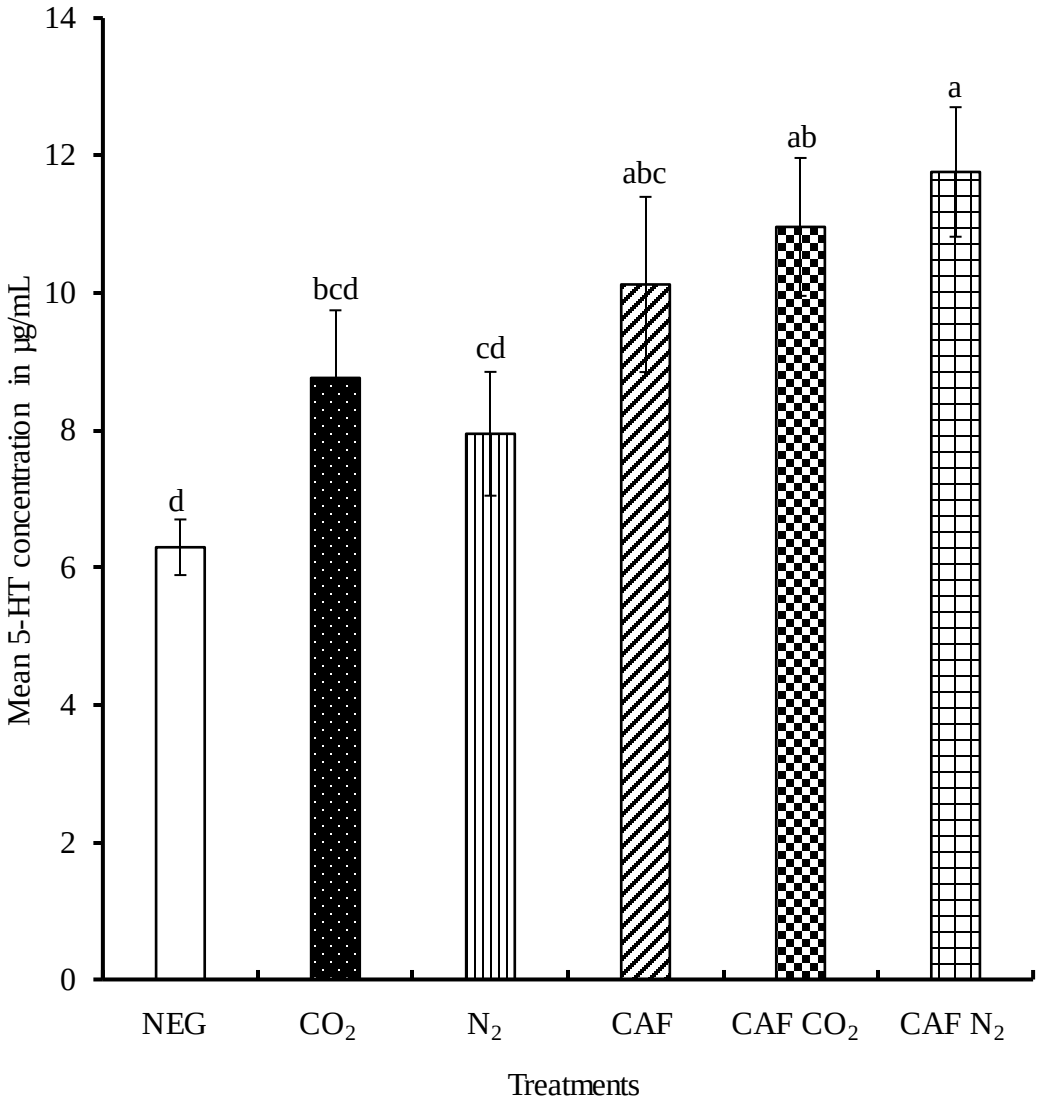

Figure 6. Mean serum serotonin levels of spent hens.
The 5-HT concentrations were measured in duplicates and expressed in µg/mL. Bars (mean ± SEM) with different superscripts (a-d) are significantly different by Fisher's LSD test ($P < 0.05$). The total number of samples per treatment was 16.

Time to Cessation of Movement

Animals subjected to euthanasia lose body posture, which is followed by the

onset of clonic and tonic convulsions [151]. The cessation of convulsive movements is an indicator of brain death [118].

The times to COM of the spent hens to the five treatments (except NEG) were derived from the accelerometer readings logged from each hen. The spent hens in the CO_2, N_2, CAF, CAF CO_2, and CAF N_2 treatments took 63 s, 73 s, 180 s, 167 s, and 132 s to demonstrate COM, respectively (**Figure 7**). The time to COM differed significantly among the five treatment groups ($P = < 0.0001$). Spent hens exposed to the AVMA approved euthanasia methods of CO_2 and N_2 inhalation had significantly shorter time to COM than the birds exposed to rest of the treatments. These two methods resulted in faster death as indicated by the shortest time to COM. A previous study in our lab reported that spent hens subjected to CAF in cages took longer time to die than the hens exposed to CO_2 in a chamber (unpublished data). Birds subjected to CAF N_2 treatment took significantly shorter time to the COM than the birds in the CAF and CAF CO_2 treatments. Compressed air foam with N_2 had better foam quality than CAF CO_2. The foam bubbles contained N_2 in CAF N_2 while CAF had air. Therefore, the poor foam quality of CAF CO_2 and the presence of air in the CAF might have led to delayed termination of convulsive movements in spent hens subjected to these treatments.

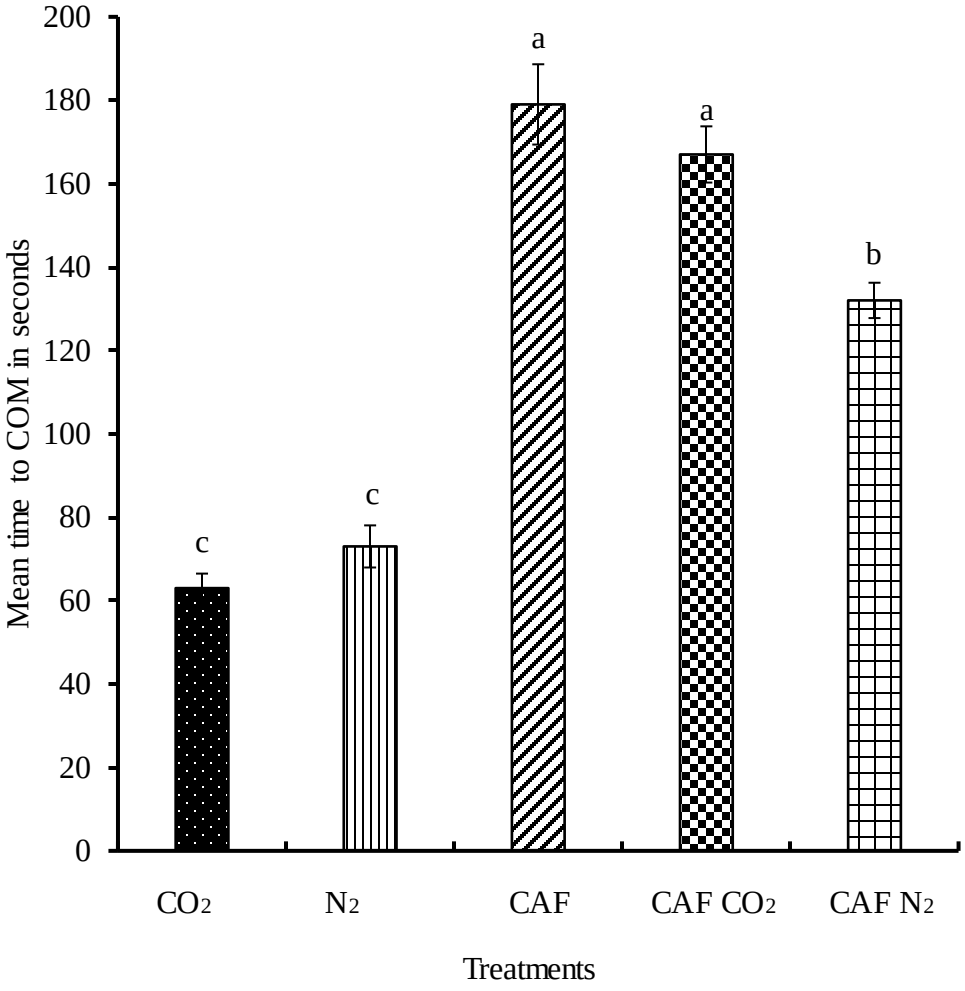

Figure 7. Mean time to cessation of movement of spent hens.
The time was expressed in s. Bars (mean ± SEM) with different superscripts (a-c) are significantly different by Fisher's LSD test ($P < 0.05$). The total number of samples per treatment was 32.

The study was successful in developing compressed air foam infused with CO_2 or N_2. The data suggests that foam with N_2 is advantageous than foam with CO_2 by improving foam quality and reducing time to death of caged laying hens during

depopulation. Future studies should focus on replicating the process in a commercial layer facility.

CHAPTER IV

EVALUATION OF ALTERNATIVE EUTHANASIA METHODS OF NEONATAL CHICKENS

Description of Problem

Genetic selection of chickens for either eggs or meat has benefitted the poultry industry and its consumers [152]. The broiler industry raises both males and females for meat, while the layer industry rears females only for eggs. The global laying flock reached 7.3 billion in 2015 producing 70.8 million metric tons of eggs [10]. In the US alone, 317 million laying hens produced 88.4 billion eggs in 2016 [153]. For each laying hen that is hatched, a male chick will also be produced. However, male layers do not have any economic value. These birds do not produce eggs, grow slowly, have a poor feed conversion ratio, incur high fattening costs to producers, and yield inferior quality meat [154, 155]. Therefore, recently hatched male layer chicks are euthanized after being sexed in hatcheries. In the EU that number is around 280 million every year [12].

The AVMA has approved maceration and carbon dioxide (CO_2) gassing as acceptable methods for euthanasia of chicks up to 72 hours of age [11]. Maceration results in instantaneous death of chicks, poults, and embryonated eggs by physical disruption of the brain [11]. The method requires special equipment, called macerators, which have rotating blades for rapid fragmentation and death. Carbon dioxide inhalation quickly reduces intracellular pH resulting in respiratory acidosis and anesthesia [34]. Continued exposure to the gas leads to hypercapnic hypoxia, respiratory depression, and

ultimately death [35]. Raj and Whittington [156] reported that day-old chicks subjected to 90% CO_2 in the air died within two minutes of exposure. The Council Directive 93/119/EC guidelines of the EU on animal protection at the time of slaughter or killing requires chicks to be exposed to the highest possible concentration of CO_2 dispensed from a 100% source and requires that the gas concentration be maintained until the death of the chicks [157]. Nitrogen (N_2) and argon (Ar) are tasteless, odorless gases that displace oxygen from breathing air resulting in loss of consciousness from anoxia and finally lead to death in birds [158]. Residual oxygen concentration should be maintained below 2% while using these anoxic gases for euthanasia [111, 156]. Raj and Whittington [156] reported that recovery rates of chicks exposed to mixtures of 20-40% CO_2 in Ar with 5% residual oxygen concentration ranged from 15-100%.

An alternative method for poultry stunning by reduction in atmospheric pressure has been developed [31]. The method is known as low atmospheric pressure stunning (LAPS) [66, 70]. The process induces unconsciousness in subjects by decreasing in air pressure inside a chamber, subsequently decreasing partial oxygen pressure and ultimately causing hypoxia [41, 66, 71]. The LAPS method received 'no objection' status from the USDA in 2010 [31, 71]. Purswell et al. [70] stated that during LAPS the anatomy of avian respiratory system makes it unlikely for gases to be trapped in body cavities in chickens unless the trachea is blocked.

The methods of euthanasia by gas inhalation, maceration, and LAPS have welfare concerns to address. Inhalation of CO_2 gas at concentrations between 40-55% cause painful sensations in humans [122]. Hens when exposed to CO_2 gas concentrations

of 40-50% can also feel pain or discomfort [108]. Similarly, Raj et al. [159] reported that 47% CO_2 gas in air is aversive to laying hens. Chicken neonates are exposed to high CO_2 concentrations, as much as 14%, in eggs before hatching [33] and therefore, require higher concentrations of CO_2 and longer exposure times as compared to adult chickens for euthanasia [11]. Birds exposed to inert gases and do not exhibit aversiveness upon initial exposure to such gases [111, 158]. Nevertheless, birds subjected to inert gases have shown severe wing flapping and convulsions [41]. Exposure to a reduced air pressure of 17.8 kPa for two minutes was lethal to broilers [70]. However, the requirement for day-old chicks and poults may be different. Therefore, scientific studies on potential applications of LAPS for euthanizing recently hatched chicks and poults are needed.

Scientific studies evaluating the current euthanasia procedures of day-old chicks are limited [160]. Raj and Whittington's 1995 paper titled "Euthanasia of day-old chicks with carbon dioxide and argon" is the single research article found in the scientific record on day-old chick euthanasia. Studies on behavioral and physiological responses of day-old male layers to these different euthanasia methods have not been published to the author's knowledge scientific literature yet. The poultry industry is also looking for humane and viable alternative methods to maceration for euthanasia of neonatal chickens. The aim of study was to develop viable alternative methods to maceration for chick euthanasia. The specific objectives of the study were to evaluate physiological responses of day-old male layers to gas inhalation and LAPS by measuring serum corticosterone (CORT) and serotonin (5-HT) concentrations and to assess behavioral

responses of chicks such as latencies to loss of posture and motionlessness.

Materials and Methods

Experimental Design

The study evaluated and compared alternative methods to maceration. The treatments included a negative control (breathing air), 25% CO_2, 50% CO_2, 75% CO_2, 90% CO_2, 100% N_2, and LAPS. Since it was practically unfeasible to achieve 100% CO_2 inside the chamber, we decided to use 90% CO_2 as one of our treatments instead of 100%. In each treatment, a batch of 10 day of hatch Hy-Line W-36 male chicks were used. Each treatment was replicated on 10 different days. The treatments- 25% CO_2 and 50% CO_2 were discontinued after the first replication due to a high number of survivors. A total of 520 male layer chicks were used during the entire study. The chicks were provided with clean drinking water. A 250 watt heating lamp was used to maintain optimal temperature during the trial. All birds were cared for under an approved Institutional Animal Care and Use Committee protocol.

Experimental Chamber

A custom built vacuum system mounted on a cart was procured from a manufacturer (Laco Technologies, UT). It included a horizontal cylindrical vacuum chamber, 0.45 m internal diameter and 0.5 m length, with a clear acrylic lid, a vacuum pump of flow rate 0.003 m^3/s, and a vacuum controller unit. The vacuum chamber was also used as the experimental chamber for all 7 treatments. A thermocouple measured the temperature of the chamber during each treatment.

Treatment Application

A batch of 10 chicks were exposed to each treatment gas or reduction in air pressure until the desired concentration or final air pressure was achieved inside the chamber. The chicks were then held inside the chamber for an additional five minutes. Gas tanks containing 100% CO_2, 100% N_2, and breathing air were procured from a local supplier for the study. A uniform gas delivery pressure of 103.4 kPa was used in all gas treatments. A variable area flow meter (Cole-Parmer, Vernon Hills, IL) was used to control the flow rates of the gases into the chamber. Flow rates of all CO_2 treatments were set at 0.0007 m^3/s while that of 100% N_2 and negative control (air) were 0.001 m^3/s. Carbon dioxide (100%) was delivered into the chamber, on the chicks, until the desired concentration of CO_2 was achieved inside the chamber. An infrared CO_2 sensor (Servomex, Crowborough, UK) was used to measure and confirm the gas concentration. Nitrogen gas flowed into the chamber until the oxygen concentration reached below 2%. The oxygen concentration inside the chamber was measured using an electrochemical sensor (Sper Scientific, Tucson, AZ). In the LAPS treatment, the air pressure inside the chamber was decreased gradually which subsequently reduced the partial pressure of oxygen. Purswell et al. [70] estimated that a negative atmospheric pressure of 19.4 kPa would result in 99.9% broiler mortality. However, our laboratory found that chicks survived the negative air pressures of 17.9 kPa and 19.4 kPa. Further preliminary trials determined that a negative air pressure of 15.3 kPa would result in 100% mortality of the chicks. Therefore, air pressure inside the chamber was reduced from 101.3 kPa to 15.3 kPa in the LAPS treatment.

Recovery

Chicks were observed for any signs of recovery after the end of 5 minutes of holding time. Death was ascertained in each chick by observing corneal and pedal reflexes.

Stress Physiology

Blood samples were collected from each chick by cardiac puncture after death had been verified in all treatments except negative control. In the negative control group, chicks were decapitated within 30 seconds of removal from the experimental chamber at the end of the duration of treatment. The blood samples were allowed to clot overnight at 4 °C. The next day serum was collected from the blood samples by centrifuging at 500 × *g* at 4 °C. Competitive ELISA kits ADI-901-097 and ADI-900-175 were used to measure the concentration of CORT and 5-HT, respectively (Enzo Life Sciences, Farmingdale, NY) following the instructions from kit manuals. All samples were run in duplicates. The intra-assay and inter-assay variability were 1.9% and 7.2% respectively for corticosterone assay. The intra-assay and inter-assay variability for 5-HT assay were 1.6% and 5.8% respectively.

Behavioral Observations

Treatments were videotaped for evaluation of behavioral responses of the chicks using a digital video camera (Sony, USA). The parameters measured were latencies to loss of posture and motionlessness.

Statistical Analysis

Serum CORT and 5-HT concentrations were interpolated from a standard curve

using a curve fitting software (Gen 5.0, Bio-Tek Instruments, Winooski, VT). The behavioral data were summarized in a spreadsheet (MS-EXCEL, Microsoft). Statistical analyses were done using a one-way analysis of variance following PROC ANOVA procedures (SAS 9.4, Cary, NC). Means deemed significant were further analyzed using Fisher's LSD post hoc test. The statistical tests were carried out at 5% significance level. Two CORT values from 90% CO_2 and one from 100% N_2 were identified as outliers by Tukey's boxplot method and removed from study.

Results and Discussion

Day-old male chicks exposed to the 25% CO_2 and 50% CO_2 treatments recovered after the end of a five minute holding period. All 10 chicks in the 25% CO_2 group recovered and only two chicks, out of ten, died in the 50% CO_2 group. Therefore, the two treatments were discontinued after the first replication. The results described are of five treatments- negative control (air), 75% CO_2, 90% CO_2, 100% N_2, and LAPS.

Serum Corticosterone

The average CORT levels of the chicks subjected to the negative control, 75% CO_2, 90% CO_2, 100% N_2 and LAPS were 12.9 ng/mL, 6.3 ng/mL, 6.8 ng/mL, 7.4 ng/mL, and 7.8 ng/mL respectively (**Figure 8**). The chicks in the negative control treatment had significantly higher serum CORT concentrations than that of the rest of the treatments ($P = < 0.0001$). However, the serum CORT concentrations of the chicks subjected to the gas inhalation methods, namely 75% CO_2, 90% CO_2, and 100% N_2 were similar to the LAPS treatment ($P > 0.05$). The chicks in the negative control group were

alive until they were decapitated for blood collection at the end of the treatment. The noise of flow of gases into the chamber and handling of chicks prior to decapitation for blood collection are probable reasons for such higher CORT levels in the chicks.

Carbon dioxide causes pain and discomfort to the animal due to the formation of carbonic acid in the mucous membrane [110]. Kaye et al. [161] reported that hypercapnia due to CO_2 stimulated sympathetic and hypothalamo-pituitary-adrenal axis. In our study, the chicks in 75% and 90% CO_2 groups might have lost consciousness before the potentially painful CO_2 level was attained inside the chamber. Therefore, the serum CORT levels were statistically lower as compared to negative control group. Exposure to 100% N_2 did not elevate CORT levels in the chicks as compared to the negative control. Birds lack intrapulmonary chemoreceptors sensitive to N_2 [111] and do not demonstrate aversive responses in the beginning. In addition, normal breathing air has 78% N_2 by volume and hence, animals are continuously being exposed to higher levels of N_2. In LAPS, the chicks experienced a reduction in air pressure and subsequently the partial pressure of oxygen, which led to failure in diffusion of oxygen into blood circulation and chicks died from anoxia. The mechanism of death from LAPS is similar to gas inhalation methods. The CORT data also suggests that LAPS method is similar to CO_2 and N_2 inhalation in terms of stress response. Broilers subjected to LAPS had significantly lower CORT levels as compared to electrical stunning [66]. Shackling during electrical stunning might be the reason for higher CORT concentration than LAPS.

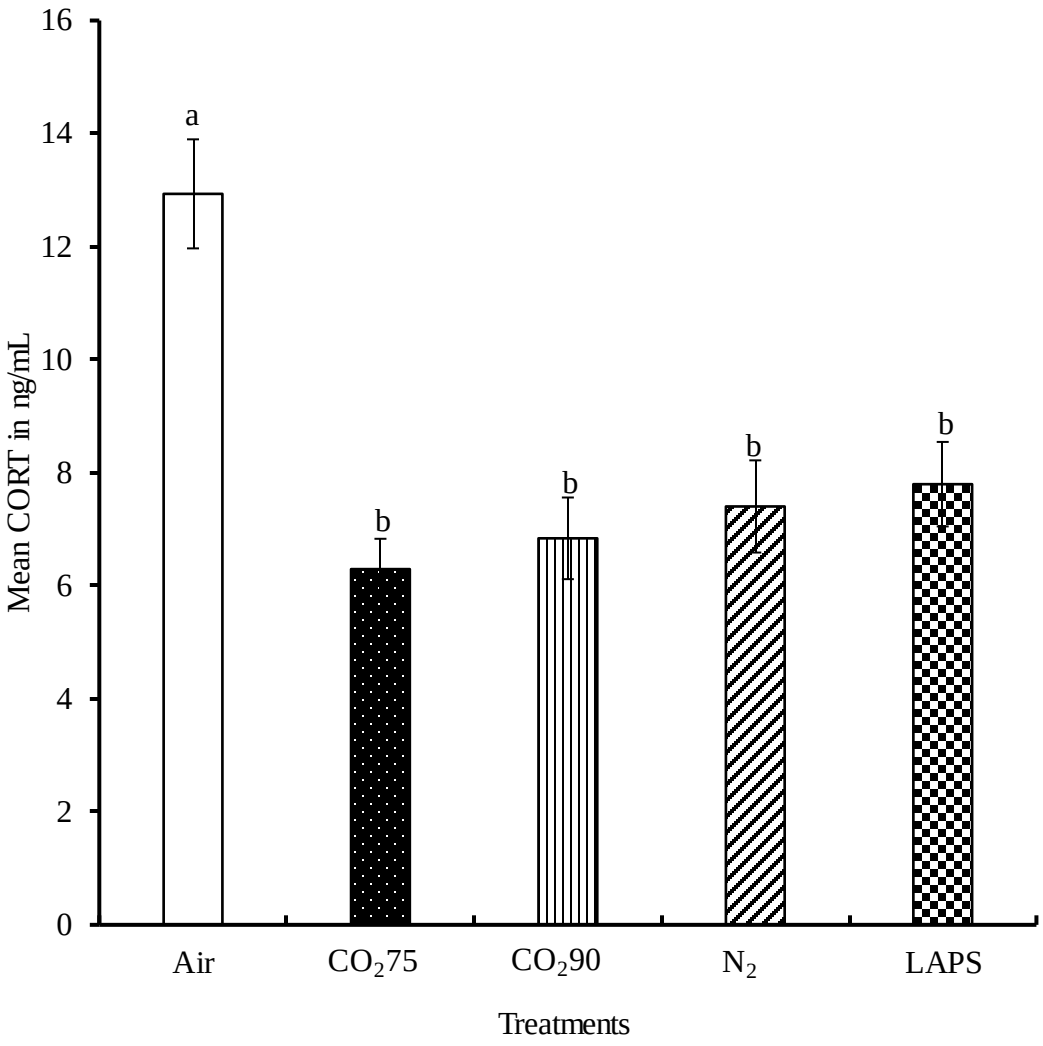

Figure 8. Mean serum corticosterone levels of male layer chicks.
The CORT concentrations were measured in duplicates and expressed in ng/mL. Bars (mean ± SEM) with different superscripts (a, b) are significantly different by Fisher's LSD test ($P < 0.05$). Number of samples per treatment was 100.

Serum Serotonin

The 5-HT concentration in peripheral blood of male layer chicks subjected to the five treatments were evaluated and compared. The mean serum 5-HT levels of chicks in

the negative control, 75% CO_2, 90% CO_2, and 100% N_2, and LAPS treatments were 3.5 µg/mL, 6.3 µg/mL, 5.9 µg/mL, 5.9 µg/mL, and 6.2 µg/mL respectively (**Figure 9**). Chicks in the negative control group had significantly lower concentration of serum 5-HT than rest of the treatments ($P = < 0.0001$). No significant differences were found in 5-HT levels among 75% CO_2, 90% CO_2, 100% N_2, and LAPS.

Serotonin has multiple functions in the central nervous system and in peripheral tissues and organ systems. The 5-HT in the brain affects behavioral and neuropsychological processes such as mood, perception, memory, anger, aggression, fear, stress responses, appetite, behavior, and circadian rhythm [106]. Peripherally, 5-HT is vital in platelet aggregation, vasoconstriction, vasodilation, and intestinal motility [103]. In humans, decrease in brain 5-HT levels and anxiety disorders are related [162]. Patients with depression have reduced whole blood [163, 164] and platelet [165] 5-HT levels. In the present study, chicks in the negative control group had significantly higher CORT levels but lower 5-HT levels. This finding suggests that the chicks in the negative control group were stressed and suffered from anxiety. Studies in our lab on layer hen depopulation also had similar findings. Spent hens with higher CORT levels were found to have lower 5-HT levels (unpublished results). Williams et al. [147] reported that higher blood 5-HT levels were positively associated with better mood. The chicks in 75% CO_2, 90% CO_2, 100% N_2, and LAPS treatments had significantly higher 5-HT but lower CORT levels as compared to the negative control. The data suggests that gas inhalation and LAPS treatments were less stressful to the chicks. Fear and anxiety responses were minimal with the euthanasia treatments as compared to the air treatment.

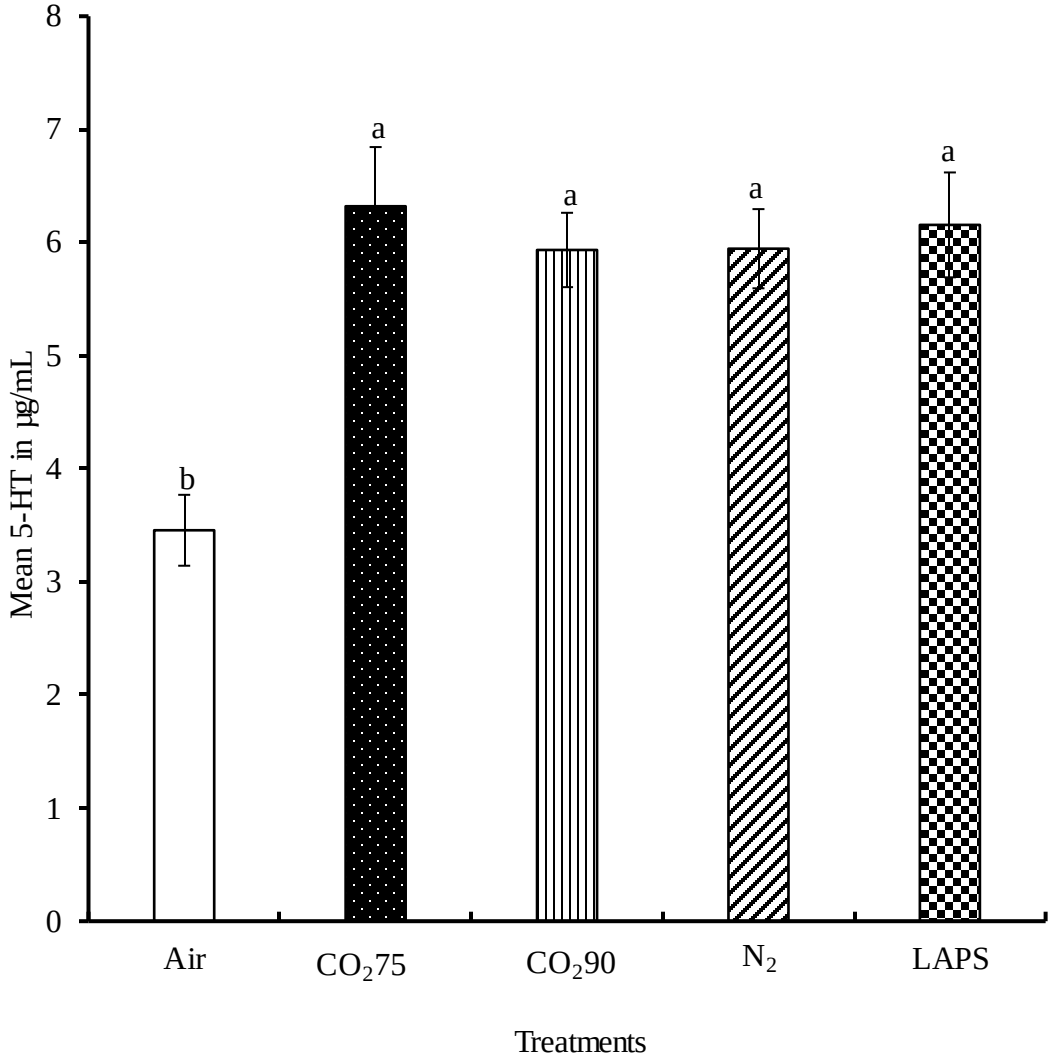

Figure 9. Mean serum serotonin levels of male layer chicks.
The 5-HT concentrations were measured in duplicates and expressed in µg/mL. Bars (mean ± SEM) with different superscripts are significantly different by Fisher's LSD test ($P < 0.05$). Number of samples per treatment was 20.

Behavioral Responses

Behavioral responses of the chicks to the euthanasia treatments were video recorded. Latencies until all chicks exhibited loss of posture or motionlessness were

determined for all treatment groups except the negative control.

Loss of posture is a behavioral indicator of loss of consciousness [151]. It is manifested by the inability to maintain neck tension and body balance [107,151].In the present study, the latencies to loss of posture of chicks subjected to 75% CO_2, 90% CO_2, 100% N_2, and LAPS were 43 s, 42 s, 148 s, and 58 s respectively (**Figure 10**). Significant differences were observed in latencies to loss of posture among four euthanasia treatments. The chicks subjected to 100% N_2 took the longest time to lose body posture compared to rest of the treatments ($P < 0.05$). The chicks exposed to the 75% CO_2 and 90% CO_2 lost body posture significantly faster than those chicks in LAPS or the 100% N_2 groups. The chicks subjected to LAPS lost posture earlier than chicks in 100% N_2 but later as compared to CO_2 inhalation treatments ($P < 0.05$). The findings of our study also demonstrate that CO_2 is faster than N_2 to induce loss of posture in the male layer chicks. Poole and Fletcher (1995) reported that time to loss of posture of broilers exposed to CO_2 was significantly shorter than N_2. Gerritzen et al. [113] showed that the time to loss of posture of broilers subjected to multistage CO_2 stunning ranged from 80 s to 93 s. Unlike N_2, exposure to CO_2 leads to reduction of intracellular pH [166]. Martoft et al. [34] reported that exposure to higher concentration of CO_2 leads to a rapid induction of anesthesia mediated by a decrease in the intracellular pH of brain. The anesthetic [167] and analgesic effect of CO_2 [121] may be the probable mechanism for rapid loss of posture. Nitrogen inhalation results death by anoxia [11]. Unlike in CO_2 inhalation, chicks in the LAPS did not experience the anesthetic effect. Therefore, chicks in the LAPS method took significantly longer time than CO_2 treatments.

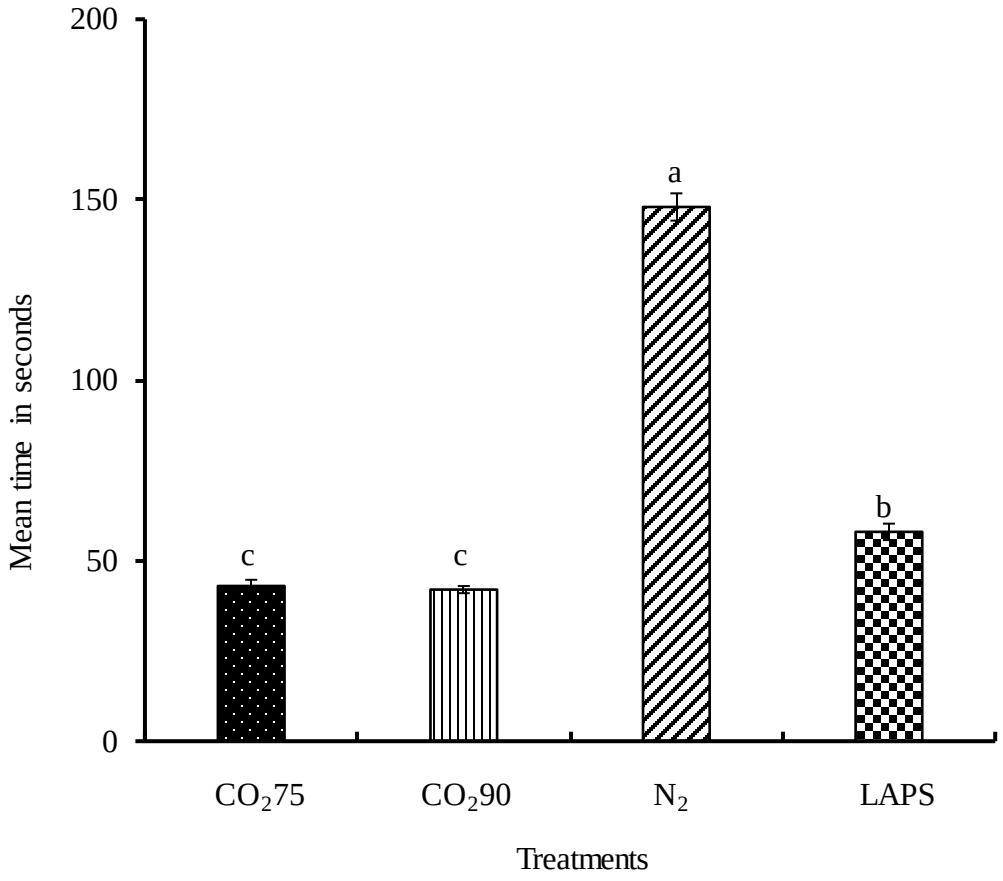

Figure 10. Mean latencies to loss of posture of male layer chicks.
The latencies are expressed in s. Bars (mean ± SEM) with different superscripts are significantly different by Fisher's LSD test ($P < 0.05$). Number of samples per treatment was 10.

The cessation of visible movements including respiratory motion is a state of motionlessness [115]. Latencies to motionlessness were determined for chicks in each replication in all treatment groups except for the negative control (**Figure 11**). In our study, chicks exposed to 75% and 90% CO_2 took 114 s and 113 s to reach the state of motionlessness. The male layer chicks subjected to 100% N_2 and LAPS were motionlessness after 250 s and 134 s respectively. The latencies to motionlessness of the

chicks followed a similar pattern to latencies to loss of posture. The chicks in the 100% N_2 treatment took significantly longer time to be motionlessness as compared to the rest of the treatments. The chicks in the LAPS group took longer time than 75% CO_2 and 90% CO_2 groups but shorter time than 100% N_2 to be motionlessness ($P < 0.05$). In the present study, the treatments that induced rapid loss of posture were the ones in which the chicks demonstrated motionlessness faster. The maintenance of concentration of the gas was also vital for preventing any recovery and finally leading them to death. In the present study, the chicks subjected to CO_2 treatments were motionlessness faster than 100% N_2. A similar result was reported by Gerritzen et al. [107]. They reported that broilers (2 weeks old) exposed to 100% CO_2 were motionlessness significantly faster than other treatments including 50% N_2 + 50% CO_2 and 30% O_2 + 30% N_2 + 40% CO_2 gas mixtures. Broilers stunned by LAPS took on average 199.4 s to be motionlessness with a range of 158.2 s to 245.6 s [69]. In our study, the latency to motionlessness of chicks subjected to LAPS ranged from 115 s to 151 s.

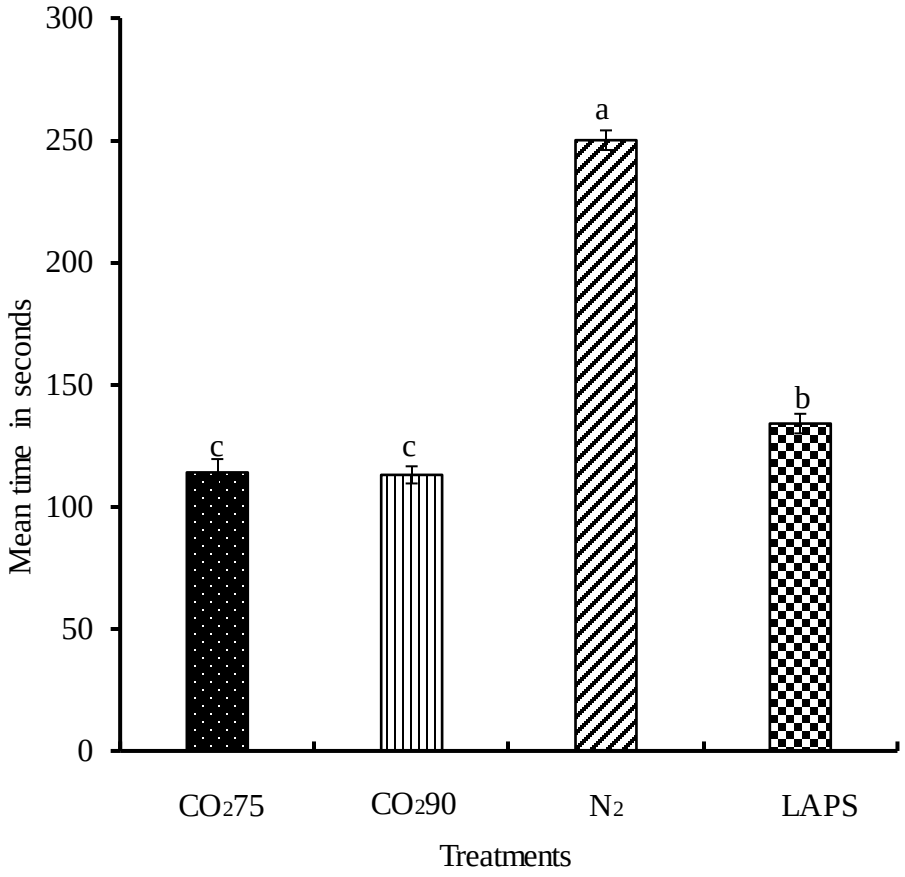

Figure 11. Mean latencies to motionlessness of male layer chicks.
The latencies are expressed in s. Bars (mean ± SEM) with different superscripts are significantly different by Fisher's LSD test ($P < 0.05$). Number of samples per treatment was 10.

The research findings demonstrate that LAPS can be as effective as CO_2 or N_2 inhalation for euthanizing day-old male layer chicks. The euthanasia treatments, both, gas inhalation and LAPS are similar in terms of physiological stress responses. However, the use of CO_2 results in a faster onset of unconsciousness and leads to death earlier than other methods.

CHAPTER V

CONCLUSIONS

Reportable diseases are a constant threat to the poultry industry. Elimination of infected poultry flocks is a key strategy to prevent such diseases from becoming an epidemic. The current methods of depopulation available for the commercial layer industry have limited usage. The layer industry needs alternative methods for rapid and humane depopulation of diseased birds.

In the first study, we evaluated the efficacy of a compressed air foam system for killing layer hens in caged houses. The corticosterone levels of hens subjected to compressed air foam in cages and the AVMA approved carbon dioxide inhalation treatments were similar to that of birds in the NEG control. The time to cessation of movement of hens exposed to CO_2 was shorter than foam treatments. The presence of foam in the upper trachea of hens confirmed that the cause of death was due to mechanical hypoxia. This research work in our laboratory established compressed air foam as a viable alternative method for caged layer hen depopulation during reportable disease outbreaks or natural disasters.

The second study aimed at enhancing the efficacy of compressed air foam by infusing CO_2 or N_2 into the foam. We were able to produce a viable compressed air foam infused with CO_2 or N_2. Compressed air foam with N_2 had better foam quality than foam with CO_2. Stress responses of spent hens were similar among compressed air foam with and without gas treatments. The time to cessation of movement of spent hens subjected

to compressed air foam with N_2 was faster as compared to foam with air or CO_2, but slower than CO_2 or N_2 inhalation. The data suggests that N_2 could be infused into compressed air foam for shortening time to death of spent hens.

The third study dealt with male layer chick euthanasia. The issue of euthanasia of male layer chicks in hatcheries has brought negative publicity to the layer industry. Maceration is the common method used by hatcheries for euthanizing recently hatched male layer chicks in the U.S. The layer industry needs alternative methods to maceration as public view this method as inhumane and aesthetically unpleasant.

The work in our lab focused on assessing CO_2, N_2, and low atmospheric pressure stunning as alternative methods for euthanizing day old male layer chicks. The study showed that male layer chicks can be euthanized using at least 75% CO_2 in air or a 100% N_2. Low atmospheric pressure stunning can be used for chick euthanasia provided that the negative air pressure is at least 15.3 kPa. There was no significant differences between CO_2 or N_2 inhalation and LAPS methods based on corticosterone and serotonin levels. Carbon dioxide treatments were significantly faster than the LAPS and N_2 inhalation based on latencies to loss of posture and motionlessness. Future studies should evaluate the efficacy of these alternative methods through on-field trials.

Thus, the study demonstrated that compressed air foam with air can be used for mass depopulation of caged layer hens. The addition of N2 into the foam reduces the time to death and such foam has better quality as compared to CO2 infusion into foam. Low atmospheric pressure stunning or at least 75% CO2 or 100% N2 can be viable alternatives to maceration for male layer chick euthanasia.

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