COMPARISON OF WATER BASED FOAM AND INERT GAS EMERGENCY DEPOPULATION METHODS OF TURKEYS

by

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ABSTRACT

Current control strategies for avian influenza (AI) and other highly contagious poultry diseases include surveillance, quarantine, depopulation, disposal, and decontamination. Selection of the best method of emergency mass depopulation needs to maximize human health and safety while minimizing disease spread and animal welfare concerns. The method used must be compatible with species, age, housing type, and disposal options. Research has shown differences in gassing and foam depopulation procedures when comparing time to and consistency of time to brain death. An overall goal of this project was to find a way to evaluate the welfare of the poultry subjected to a depopulation treatment. During depopulation, the time to unconsciousness needs to be evaluated to determine when the birds are no longer aware of their surroundings or feeling any pain.

This study consisted of two experiments to evaluate the efficacy of mass depopulation methods. Experiment 1 was conducted as a proof of concept for the use of the alpha/delta (A/D) ratio in evaluating the time to loss of consciousness in poultry. Experiment 2 was conducted to evaluate the effectiveness of two mass depopulation methods on turkeys. The methods that were tested were carbon dioxide (CO_2) gassing and water based foam.

In Experiment 1, the use of the alpha/delta ratio was evaluated as a method to analyze poultry time to unconsciousness using layer hens. Experiment 1 tested the use of the alpha/delta ratio under controlled anesthesia and the resulting unconsciousness. This study was done as a proof of concept for application to subsequent studies. The results of this study indicate that there is a consistent

suppression pattern in the transition from consciousness to unconsciousness. The alpha/delta ratio was suppressed by the effects of the isoflurane as the bird began to lose consciousness. The layer hens were found to become unconscious an average of 278 seconds after the start of the treatment with a standard deviation of 113 seconds. This concept was then used to evaluate the time to loss of consciousness in turkeys during depopulation in Experiment 2.

The purpose of Experiment 2 was to evaluate the efficacy of two different treatments used for depopulating market age turkeys. This experiment was conducted using a randomized block design with commercial male turkeys exposed to one of two randomly selected depopulation treatments; either 100% CO_2 gas or water based foam with ambient air. The time to unconsciousness, terminal convulsions, brain death, and cardiac relaxation were recorded for each bird. The time to unconsciousness and brain death were evaluated using the EEG signals recorded from a wireless transmitter surgically implanted into the brain of the bird. Motion cessation was determined through analysis of data recorded from an accelerometer attached to the turkey's leg during depopulation. Cardiac relaxation was evaluated through analysis of the ECG data recorded via wired electrode pads attached to the wing and legs. Being able to determine the point of unconsciousness allows for better evaluation of the effectiveness of different depopulation methods. Critical times for physiological events were extracted from the EEG, ECG, and accelerometer data and were compiled in Excel and statistical analysis was performed using SAS. The data subsets were not normally distributed and thus a non-parametric statistical analysis was conducted on each data subset in SAS. A Wilcoxon Exact test was used to analyze the treatment-dependent data sets. All tests were conducted at the 5% ($\alpha = 0.05$) significance level.

There was a statistically significant difference in the time to brain death between the two methods. Water based foam was the fastest treatment with respect to brain death (μ = 190 sec). The CO_2 gas was significantly slower (μ = 242 sec). Water based foam resulted in faster (μ = 64 sec) time to unconsciousness than CO_2 gas (μ = 90 sec). The time to terminal convulsions of the birds showed that there was no statistically significant difference in the time to motion cessation for water based foam (μ= 166 sec) and CO_2 gassing (μ= 174 sec). The time difference for cardiac relaxation for water based foam (μ=208 sec) and the CO_2 gas (μ= 242 sec) are not statistically significant.

The results of this experiment show that water based foam is more effective at causing brain death than the CO_2 gas. Though not statistically significant, water based foam caused unconsciousness, cardiac relaxation and motion cessation faster than CO_2 gas. The times to brain death and unconsciousness for water based foam were also more consistent, with less variation from the mean compared to CO_2 gas. When comparing water based foam and CO_2 gas, there are other qualitative advantages to the use of the water based foam including responder safety and emotional welfare as well as compatibility with carcass composting. This information may also play a role in how agencies such as the USDA and organizations such as AVMA evaluate water based foam for mass emergency depopulation of poultry.

Chapter 1

REVIEW OF LITERATURE

The possibility of a highly pathogenic avian influenza virus (HPAIV), virulent Newcastle disease (VND) or other highly infectious disease outbreak is an ongoing concern for the poultry industry. Avian influenza epidemics in densely populated poultry areas have resulted in the loss of millions of birds throughout the world. There were outbreaks with 13 million dead birds in Italy in 1999-2000 (H7N1), 5 million dead birds in the United States in 2002 (H7N2), 30 million dead birds in the Netherlands in 2003 (H7N7), and 17 million depopulated in Canada in 2004 (Capua and Marangon 2007, 317-322). From late 2003 until early 2005, H5N1 Avian influenza spread across ten Asian countries resulting in the death and destruction of more than 150 million birds (World Health Organization 2010). During the 1995 outbreak in turkeys there were 178 farms that were infected resulting in an economic loss of approximately US $600,000 in one year in Minnesota (Halvorson et al. 2003, 36-46). An outbreak of virulent Newcastle disease (VND) in 2002 led to the loss of 3.16 million birds at a cost of $23 million in California, Nevada, Texas, and Arizona (Breitmeyer, Whiteford, and Shere 2003, 65-70). As there is currently no practical treatment for these diseases, the use of surveillance and strict biosecurity in the commercial poultry industry is used to minimize the occurrence. The United States Department of Agriculture Animal and Plant Health Inspection Service (USDA APHIS) has developed a protocol for the control of HPAIV in the United States. It states in part that: “When AI breaks occur in poultry, quarantine and depopulation of

all infected, exposed or potentially infected birds, followed by proper disposal of carcasses and the quarantining and rigorous disinfection of farms and surveillance around infected flocks are the preferred eradication options" (USDA APHIS 2007, 1-86).

Influenza viruses are members of the *Orthomyxoviridae* family. The family is a group of negative sense, single stranded RNA viruses that include the genera Influenza A, B, C, and Thogotovirus. Avian influenza virus is a member of the genus Influenza A, the only type to infect poultry, and is a major threat to the poultry industry. Within the nucleocapsid of the virus are 8 RNA segments that encode for a total of 10 proteins. This RNA is susceptible to high levels of mutation due to its dependence on the RNA polymerase for replication. Antigenic change is also possible through reassortment where two different AIVs can exchange RNA segments by infecting the same cell (Alexander 2000, 3-13). There are unique properties to the glycoprotein structures on the surface of the virus particles that allow distinction between them. Two of the identifiers are the hemagglutinin and neuraminidase proteins. Each virus has one hemagglutinin (H) and one neuraminidase (N) antigen, apparently in any combination (Capua and Alexander 2009, 842-846). All 16 hemagglutinin and 9 neuraminidase subtypes of influenza viruses are known to infect wild waterfowl and shorebirds, which accounts for an extensive reservoir of influenza viruses constantly circulating in wild bird populations. In these birds the viruses are endemic and primarily cause enteric infections.

The influenza A viruses that infect poultry can be divided into two groups based on the level of pathogenicity they cause in the birds; low pathogenic avian influenza virus (LPAIV) and highly pathogenic avian influenza virus (HPAIV). LPAI

can be asymptomatic or only cause minor effects in the birds to include ruffled feathers, drop in egg production, or mild effects on the respiratory system. HPAI can quickly cause severe disease that leads to internal hemorrhaging and high mortality. Only the subtypes H7 and H5 have been shown to mutate from LPAI to the extremely virulent viruses causing HPAI which can cause flock mortality levels as high as 100%. All the other avian influenza viruses (AIV) have been classified as LPAI. HPAIV are able to replicate systemically in the bird, which causes damage to vital organs and tissues causing extensive disease and subsequent death. All outbreaks of HPAI, including those in commercial poultry, have been caused by the subtypes H5 and H7. However, not all H5 and H7 viruses are highly pathogenic, but most are thought to have the potential to become so. HPAIVs possess a distinctive basic amino acid motif in the cleavage site of the H which is associated with their increased virulence (Swayne and Halvorson 2008, 153-184).

In surveillance studies, many LPAI viruses have been isolated from wild birds (Capua and Alexander 2009, 842-846). LPAI viruses have been shown to undergo a mutation event in which they can become highly pathogenic (HPAIV). The factors that cause a mutation from a LPAIV to a HPAIV are not known, but in some instances mutations seem to take place after introduction to poultry from wild birds, while in other instances it seems that the LPAIV has been circulating in poultry before mutating. It is currently impossible to predict when, or if this mutational event will occur (Capua and Alexander 2009, 842-846). The threat of a HPAIV outbreak occurring due to a mutation of LPAIV is of great concern to the poultry industry.

Recent commercial poultry surveillance programs in the United States for AI have detected nine H and six N subtypes of the AI virus and/or specific antibodies

in 16 states, most of which have been associated with live bird markets (LBM) (Senne 2007, 167-173). In recent history, there have been several LPAI outbreaks associated with commercial poultry. In May 2002, there were two flocks of commercial chickens that tested positive in Texas and were subsequently depopulated. This outbreak was linked to a LBM in Houston. In September of 2002, LPAIV H5N2 was isolated from a grandparent flock of turkey breeders in California. All birds on the farm were voluntarily depopulated by the owner. In March 2003, an outbreak of LPAIV H7N2 was confirmed in several multi-age, in-line table egg layer operations in Connecticut. This outbreak affected approximately 3.5 million layers and 1.2 million replacement pullets. The outbreak was controlled by a comprehensive vaccination program as well as extensive monitoring of the flocks. Also in 2003, a LPAIV H6N2 was isolated from several flocks of table egg layers in California. In 2004, two flocks of chickens in Delaware and one flock in Maryland were infected with a LPAIV H7N2 and were depopulated. Two flocks of birds in Pennsylvania were infected with a LPAIV H2N2 virus in 2004. In 2005, a LPAIV H7N2 was isolated from a duck production facility in New York. The facility was quarantined and disinfected. Also in 2005, a LPAIV H4N2 was isolated from a chicken flock in Pennsylvania, but no clinical disease was reported (Senne 2007, 167-173).

The HPAIV H5N1 is believed to have been circulating in Asia since at least 1996. The viruses have now been found in more than 40 countries in poultry, wild birds, or both (Capua and Alexander 2009, 842-846). It has been shown that the progenitor virus for subsequent outbreaks of H5N1 subtype was from an infection of commercial geese in the Guandong province in China in 1996 (Xu et al. 1999, 15-19).

The virus most likely stayed in the area, circulating in the domestic ducks raised in southern China, with some level of genetic variation occurring (Sims et al. 2005, 159).

The World Health Organization reported that the H5N1 HPAIV was initially confirmed in Southeast Asia in mid-2003 and from there spread to parts of Europe. There have been outbreaks reported in nine Asian countries to include: Republic of Korea, Viet Nam, Japan, Thailand, Cambodia, the Lao People's Democratic Republic, Indonesia, China and Malaysia. The virus is now considered endemic in Viet Nam, Cambodia, and other select parts of Asia. In late July 2005, the virus spread to affect poultry and wild birds in the Russian Federation and adjacent parts of Kazakhstan. It was also reported in wild birds in Mongolia. In October 2005, the virus had spread to Turkey, Romania, and Croatia and by December it had also been reported in domestic birds in Ukraine (World Health Organization 2010). Migratory birds continue to play a role in the spread of the disease, as those countries that lie in the flight pathways have a greater potential for an outbreak to occur. The disease is also being spread through the practice of LBM. People are taking birds to the market. The birds are then exposed to AI infected birds in the market become infected, and then the virus is spread when the birds are taken back by the owner or to a new location by the purchaser. These live bird markets also provide an environment for the reassortment of the AIV (Liu et al. 2003, 267-275). Birds sold within the LBM systems in Asia are at a very high risk for the transmission of the disease due to the co-mingling of different species of birds from different flocks (Yee, Carpenter, and Cardona 2009, 325-340). There have been similar studies in the LBMs in the United States, specifically New York City, which have shown that these markets play an important role in the distribution and genetic interaction of influenza viruses as well

(Liu et al. 2003, 267-275). The illegal trade and transport of infected poultry or exotic birds is also contributing to the spread of AI. In countries where the demand for poultry is high, poultry is transported illegally, despite the threat of H5N1 (Yee, Carpenter, and Cardona 2009, 325-340). The spread of AI into African countries has been speculated to be caused by the movement of infected poultry and poultry products in addition to the migration of wild birds (Sims and Narrod 2010, 50).

In North and South America, there have been three HPAI outbreaks between 2002 and 2005. In each case, the outbreaks were caused by LPAIV that had mutated to a HPAIV. There was an outbreak in Chile in 2002 of an H7N3 virus that was limited to a large broiler breeder operation and a turkey flock nearby. In 2004, there was an outbreak of H5N2 in Texas which was limited to one farm where chickens were raised to be sold in a nearby live market (Pelzel, McCluskey, and Scott 2006, 1869-1875). In the spring of 2004, the largest HPAI outbreak of H7N3 occurred in the Fraser Valley of British Columbia, Canada. The active outbreak lasted more than 90 days; 42 commercial poultry farms were identified as infected premises, and more than 17 million birds were culled (Bowes et al. 2004, 928-934). In each of these outbreaks, the virus was effectively controlled with the depopulation of all the birds on infected farms (Senne 2007, 167-173).

Although many of the outbreaks occur in chickens, there have been AI outbreaks associated with domestic turkey flocks, particularly in Europe. A LPAIV outbreak of an H7N3 virus on a turkey farm occurred in the Netherlands in 2002. The diagnosis was made after the flocks showed signs of decreased food intake, mild respiratory signs that led to respiratory distress, general depression, and a spike in mortality. Post mortem examination of the birds showed airsacculitis, pericarditis, and

sinusitis (Velkers et al. 2006, 403-405). Another outbreak of a HPAIV from an Asian lineage H5N1 was seen on a large commercial meat turkey site stocked with 159,000 birds in Great Britain. Suspicion of the disease was reported to the United Kingdom's equivalent to the USDA, the Department for Environment, Food and Rural Affairs (DEFRA) after there was deterioration in the health of seven to eight week old birds that led to a spike in mortality in one of the houses. Up to 90% morbidity was seen in the house as many of the birds were sitting down; showing minor head tremors and others were moribund. The flocks were very quiet, feed and water consumption was greatly decreased and the birds showed no interest in humans. Post mortem examination of the birds showed no specific changes that would indicate avian influenza, however, presence of the virus was confirmed by real-time reverse transcriptase polymerase chain reaction (rRT-PCR) which detected the presence of the influenza A matrix gene. A few of the birds showed minor airsacculitis, congested livers and enlarged or mottled spleens (Irvine et al. 2007, 100-101). There have been AI outbreaks in turkeys in North America, both in Canada and the United States. From 2006 to 2008, there were three detections of AI in turkeys in Canada. A LPAI H3N2 virus was isolated from two flocks of turkeys. This virus was seen in 2006 in a flock in Manitoba and again in 2007 in Ontario. Also, a LPAI H6N1 virus was isolated from a turkey breeder flock in Ontario in 2006 (Senne 2010, 179-186). In the United States from 2006-2008 virus or antibodies were detected to eight H (H1-H7 and H10) and eight N (N1, N2, N4, N5, and N7-N9) subtypes from 20 states. Three of these detections involved LPAI in commercial meat turkeys in Nebraska, Virginia, and West Virginia (Senne 2010, 179-186).

As previously mentioned, the possibility of a HPAIV, virulent Newcastle disease (VND) or other highly infectious disease outbreak is an ongoing concern for the poultry industry. The major steps involved in control during an AI outbreak include, surveillance, quarantine, depopulation, disposal, and disinfection (USDA APHIS 2007, 1-86). The quarantine of a farm is an immediate method of stopping the spread of the disease by restricting all movement of poultry, people and vehicles. Depopulation of the diseased flock minimizes animal suffering and stops virus replication and dissemination. Once the flock has been depopulated, the carcasses must be disposed of properly to prevent the spread of the disease to neighboring farms. The goal of disposal is elimination of these materials (dead birds, litter, feces) in a timely, safe, biosecure, aesthetically acceptable, and environmentally responsible manner. Disposal decisions should be made on a case-by-case basis depending on the individual premises circumstances. Specific disposal methods, including composting, burial (onsite or landfill), incineration, digestion, and rendering, should be assessed and applied as appropriate. For poultry, in-house composting is usually the preferred method of initial disposal/decontamination and helps prevent dispersal of the virus. However, besides composting being accomplished on the premises in the house, it can also be done in silage bags, on adjacent land, or at an approved central location (USDA APHIS 2007, 1-86). This is then followed by cleaning and disinfection of the farm using approved chemicals and procedures. Cleaning is one of the most important and cost effective steps in the disinfection process. If items cannot be adequately cleaned and disinfected, they should be disposed of by burial, incineration, or other appropriate means (USDA APHIS 2007, 1-86).

Depopulation or mass culling is done during the course of a HPAIV outbreak to prevent or mitigate the spread of the disease through elimination of infected or exposed domesticated birds and other susceptible livestock (USDA APHIS 2007, 1-86). The American Veterinary Medical Association (AVMA) has outlined the animal welfare standards for the management of animals during outbreaks. The 2007 AVMA Guidelines on Euthanasia devotes only one paragraph to mass euthanasia in which it states "Under unusual circumstances, such as disease eradication and natural disasters, euthanasia options may be limited. In these situations, the most appropriate technique that minimizes human and animal health concerns must be used" (AVMA, 2007). The guidelines indicate that the euthanasia technique should minimize distress or anxiety of the animal before it becomes unconscious, but recognize that the absence of pain or distress cannot always be avoided. Mass euthanasia in poultry and other livestock is only used in emergency situations such as disease outbreaks, bioterrorism, and natural disasters. The currently AVMA listed methods of mass euthanasia are (CO_2) gassing and physical methods such as gunshot and penetrating captive bolt. Conditionally accepted methods of euthanasia include nitrogen (N_2) and argon (Ar) gassing, cervical dislocation, and decapitation; however, other methods can be included on a species specific or context specific basis (AVMA, 2007). These methods cause death by 1) direct or indirect hypoxia, 2) direct depression of neurons, or 3) physical disruption of brain activity (AVMA, 2001). Other methods accepted by the AVMA include overdoses of barbiturates or inhalant anesthetics. Overdose of these agents in the feed or water can be used to sedate or kill poultry in houses or in the field. To maximize uptake of the agent, typically alpha-chloralose, the birds should be fasted for at least 24 hours. Field reports indicate that this procedure may

not be reliable (Balicer et al. 2007, 1601-1603). In Israel in 2006, birds were culled by administering organophosphate poison in the flock's drinking water after 24 hours of water deprivation. This process was not satisfactory since some birds survived and had to be manually slaughtered (Inbar 2008). The problems with using these methods, particularly those that require the birds to consume water or feed are that sick birds generally do not eat or drink. This can lead to a large number of the birds not being affected by the agent.

For avian influenza, the primary control measure is to depopulate the flocks that are infected or exposed to the infection. As defined by the AVMA, mass depopulation refers to the methods by which large numbers of animals must be destroyed quickly and efficiently with as much consideration given to the welfare of the animal as practicable, but where the circumstances and tasks facing those doing the depopulation are understood to be extenuating. In comparison, euthanasia involves the transitioning of an animal to death in a manner that is painless and as stress free as possible. The method of emergency mass depopulation used in disease situations needs to maximize human health and safety while minimizing the disease spread and animal welfare concerns. Selection of a method is dependent on the species, age, housing type and disposal options available. There is no single method that is suitable for all situations. Gassing of the birds is one of the methods that is currently accepted. This method is typically done with CO_2 gas and can be done as a whole house, partial house, or containerized system. Another method is the use of water based foam which was conditionally approved for floor reared poultry by the USDA and AVMA in 2006 (AVMA 2006, 1-4). The AVMA currently considers the destruction of poultry using water- based foam a method of mass depopulation and not

a form of euthanasia, but supports further research to evaluate whether water-based foam can be an accepted form of euthanasia (AVMA 2006, 1-4).

In the turkey industry, however, controlled marketing can be used. In controlled marketing, market age birds that are infected with a LPAIV have been held in the finishing unit for two to three weeks post-infection. During this time, they may be vaccinated for the virus. Once the flock is free of all signs of disease, the birds may be marketed (Halvorson et al. 2003, 36-46). Currently controlled marketing is permitted in flocks infected with LPAIVs other than H5 and H7.

The use of CO_2 gas is the most commonly used gassing procedure for the depopulation of poultry, and has been approved by the AVMA for the depopulation of poultry. The gas can be applied as a whole house treatment, partial house or in a containerized system. There are some important issues associated with the implementation of a gassing system. Carbon dioxide can be harmful to humans and therefore worker safety must be considered during any gassing operation.

Carbon dioxide is an acidic gas and has been found to be painful and cause irritation to the nasal mucosa, lips and forehead in humans when administered in concentrations greater than 65% (Hari et al. 1997, 145-151). Several investigators have suggested that inhalation of high concentrations of CO_2 may be distressing to animals, because the gas dissolves in moisture on the nasal mucosa. The resulting product, carbonic acid, may stimulate nociceptors in the nasal mucosa (AVMA, 2007). Carbon dioxide acts by directly affecting the respiratory system of the birds in contrast to other gases like argon (Ar) and nitrogen (N_2) that work by displacing the oxygen in the environment causing hypoxia (van den Bogaard,1985 in (Gerritzen et al. 2000,

928-933). CO_2 may cause a sense of breathlessness resulting in increased stress in poultry (Raj, Wotton, and Gregory 1992, 147-156); (Raj et al. 1998, 686-695). The use of CO_2 and other gases, such as argon, cause a rapid loss of brain function in chickens (Raj, Gregory, and Wotton 1991, 322-330); (Raj 1996, 592-593) and turkeys (Raj and Gregory 1993, 318-320); (Raj and Gregory 1994, 222-223); (Raj 1996, 592-593). Loss of posture has been used to indicate a loss of consciousness (Raj, Wotton, and Gregory 1992, 147-156); (Raj and Gregory 1995, 273-280). Gerritzen and others have reported that the combination of CO_2 gas and Ar gas (70% Ar and 30% CO_2) showed short induction periods until the onset of unconsciousness (Gerritzen et al. 2000, 928-933). They also reported that gasping and headshaking behavior was seen in treatment birds (broilers) as a response to the breathlessness and pungent odor of the gas (Gerritzen et al. 2000, 928-933). Gerritzen has reported that the CO_2 levels for large scale depopulation of broilers must exceed 30% in combination with a residual O_2 level below 13% and that these levels must be maintained for 30 min to ensure that death occurs in all the birds (Gerritzen et al. 2004, 1294-1301). This can add logistical complications if the house is older or has been damaged thus allowing CO_2 gas to escape before effective levels can be reached. A partial house CO_2 gassing procedure would require 12-18 x 90.72 kg (200 lb) bottles of CO_2 for a 15.2 m x 152.4 m (50 ft. x 500 ft.) broiler house. The temperature drop caused by CO_2 injection raises animal welfare concerns during whole house gassing procedures. The plume of vaporized liquid CO_2 can extend up to 10 m and can be as cold as -79° C, which is the sublimation temperature of the CO_2 at atmospheric pressure (Ryan, Sheehan, and Gaynor 2006). This temperature drop can lead to freezing of the birds that are in close proximity to the gas distribution site. During the application of the gas, the birds are

exposed to the gas and temperature drop before the gas reaches unconsciousness levels (20% CO_2), (Gerritzen et al. 2006, 39-42) or lethal levels (30% CO_2), (Gerritzen et al. 2004, 1294-1301).

In turkeys, Raj (Raj 1996, 592-593) reported that turkeys are able to sense the presence of high concentrations of CO_2 and when given a choice, will avoid the area. Carbon dioxide gas causes an unpleasant sensation during the inhalation of the gas and the birds exhibit gasping, vocalization and headshaking during the induction of unconsciousness (Raj 1996, 592-593). This behavior is in contrast to what is seen in the use of Ar gas. Argon is an inert gas with no taste or odor, was not detected by the birds and they exhibited no stress behaviors or signs of respiratory distress before they lost consciousness (Raj 1996, 592-593). Other sources, including Alphin et al. (2010), document that field use of Ar - CO_2 gas mixtures may take significantly longer and result in more variability than CO_2 gas (Alphin et al. 2010, 757-762). Gerritzen reported that a loss of posture (indicating a loss of consciousness) was seen in turkeys at 19.3% CO_2 and that the first observation that the animals noticed a change in the environment was at a CO_2 concentration of only 2% (Gerritzen et al. 2007, 179-185). Carbon dioxide is the main chemical stimulant of respiration and any increase in the concentration of CO_2 will increase the pCO_2 in the blood which is then detected by the "respiratory center" in the medulla oblongata and ultimately leads to an increased respiratory rate in an attempt to lower the pCO_2. Based on this knowledge, it is likely that the birds are being affected by the presence of the gas (Guyton and Hall 2000). Gerritzen observed that shortly after the onset of heavy breathing at 5 - 8% CO_2 concentration, turkeys began to shake their heads and gasping began at approximately 6.4 - 9.5% CO_2 concentration (Gerritzen et al. 2007, 179-185). The act of head

shaking and gasping has been described as an indication of an aversion to CO_2 and respiratory distress (Webster and Fletcher 2001, 1371-1377) or as an alerting response in an attempt to regain an alert state (Hughes 1983, 45-53).

In whole house gassing, the house is sealed, a manifold or injection lance is placed within the house, and large quantities of CO_2 gas are injected into the house. Sealing is used to reduce gas requirements and allow a lethal gas concentration to be reached. The houses are sealed with heavy duty builder's plastic sheeting to cover all the vents and openings in the house. This can be very labor intensive and will expose workers to the diseased birds. It is estimated that a well prepared team of three workers could successfully seal one large house (107 m x 18 m; 59 ft. x 351 ft.) in approximately three hours (Ryan, Sheehan, and Gaynor 2006).

For partial house gassing, the turkey industry has used a portable panel system for depopulation using gas. Groups of birds are driven into a temporary enclosure, covering it with a tarp and introducing the gas. This method could be used with batches of up to 5,500 turkeys at a time and required an average of 6 min 20 sec for all audible signs of activity to cease (Kingston et al. 2005, 730-738). A similar study was conducted using 4,200 ten week old turkeys. In this study, construction of the enclosure, using plywood sheets, tarps, rope and bungee cords took approximately seven people two hours to complete (Kingston et al. 2005, 730-738).

When dealing with an outbreak situation it is imperative to work to ensure the safety of the workers. This is a key aspect when determining the depopulation method to be used. Efforts must be made to limit the number of people that must be exposed to the birds as well as the gasses that are being used. The use of the

containerized systems presents another issue in that the birds in the house must be hand caught and placed into the containers. This means that the workers are being exposed to the infected chickens. Additionally, this presents a biosecurity risk because the birds are often carried outside of the house to be placed in the containers. During this time the virus can be spread through the air or to other surfaces as the birds shed the virus. The act of being caught is also an additional stress on the birds that could be avoided.

The use of water based foam was developed after experiences and challenges during the 2004 Delmarva LPAIV H7N2 outbreak. This process was developed for floor raised poultry and employs the use of foam generation equipment to cover the birds with a blanket of modified firefighting foam. The immersion in the foam causes a rapid blocking of the airway, causing mechanical hypoxia, resulting in the cessation of heart activity (Benson et al. 2007, 219-224). Testing the foam with and without CO_2 included in the bubbles compared to the CO_2 gas (polyethylene tent method) indicated that the foam with and without the CO_2 was faster to bird death than the CO_2 gas based on ECG readings. For heart activity cessation, determined using the ECG, foam with the CO_2 required 1 min 13 s, foam without CO_2 required 1 min 4 s and the CO_2 gas method required 2 min, 19 s. The differences in the foam times with and without the CO_2 present were not significant, indicating that the presence of the gas did not affect ECG cessation time (Benson et al. 2007, 219-224). Blood samples were also taken from the birds pre- treatment and immediately after death to measure stress levels based on corticosterone. The corticosterone levels were higher postmortem than those at pre-treatment, but there was no difference in the stress

levels, measured by corticosterone, between the gas and the foam treatments (Benson et al. 2007, 219-224).

For the study of depopulation procedures, a key aspect to evaluate is the time to loss of consciousness. When the bird has lost consciousness, it means that they are no longer aware of their surroundings and will be unable to feel pain. For the welfare of the animal, the goal is to have the animal become unconscious as quickly as possible and for death to soon follow. To monitor unconsciousness, the use of the electroencephalogram (EEG) has been used. The EEG represents the voltage recorded between two electrodes applied to the scalp or implanted surgically. The sensors or electrodes are attached to the head and the electrical activity is recorded by a computer (Purves et al. 2001). A radiotelemetry system was used to allow the real time measurement of heart rate, blood pressure, body temperature and telencephalic EEG to use as an indicator of bird welfare parameters (Savory and Kostal 1997, 963-969). EEG was recorded from the device's paired sensing electrodes positioned on the surface of the telencephalon, the electrode leads being passed under the skin and held in place with the use of dental acrylic (Savory, Kostal, and Nevison 2006, 599-606). Initial testing with wired EEG systems for foam depopulation raised signal quality concerns (Malone, personal communication). EEG results can be analyzed on a raw signal basis or frequency domain or by analyzing the presence of somatosensory evoked potentials (SEP). In the frequency domain analysis, the component or relative power of the signals for the brain waves alpha (8 – 13 Hz), beta (> 13 Hz), delta (< 4 Hz), and theta (4 – 8 Hz) are compared (Gerritzen et al. 2004, 1294-1301; Gerritzen et al. 2006, 1055-1061). The brain waves are based on the electrical impulses of the brain.

There are several different reported methods to analyzing EEG results. In ostriches after the use of electrical and mechanical stunning methods the delta and theta waves tending to an isoelectric line were seen on the EEG. One of the animals in the test, immediately after stunning, showed delta and theta waves as well as alpha and beta waves which changed to delta and theta waves within 25 seconds (Lambooij et al. 1999, 339-345). Several researchers have employed the use of high amplitude, low frequency activity in the delta and theta waves (Gerritzen et al. 2006, 1055-1061) for ducks and turkeys and in other studies for hens and broilers (Raj, Wotton, and Gregory 1992, 147-156); (Raj 1998, 1815-1819); (Gerritzen et al. 2004, 1294-1301). When looking at depth of anesthesia or unconsciousness in horses, it has been shown that as the depth of the anesthesia increases, there is a shift from the high frequency/low amplitude to low frequency/high amplitude patterns with delta and theta activity being the most pronounced (Otto and Short 1991, 362-371). Similar results have also been shown while monitoring the depth of anesthesia during surgical stimulation of humans and various animal species. Results of these studies showed that most anesthetic agents will cause similar EEG changes to include the replacement of fast alpha and beta rhythms by the slow delta and theta rhythms during increased depth of anesthesia (Otto 2008, 45-61). Other studies have tried to correlate the suppression of the alpha and beta waves and the loss of posture as these events tend to occur at approximately the same time indicating a loss of consciousness (Gerritzen et al. 2004, 1294-1301). Analysis of the alpha, beta, delta, and theta waves have indicated a complete loss of consciousness at the occurrence of suppression of the alpha and beta and an occurrence of the delta and theta waves. This has been shown for several different species (Matteson, Stinson, and Clark 1972, 2043-2049); (Rhesus Monkey); (Forslid

and others 1986, 281-287) (Rat); (Raj 1998, 1815-1819) (Broiler). In a study done by Leon-Carrion with humans, the use of the delta/alpha ratio was used to assess the level of recovery after neurorehabilitation in patients with acquired brain injuries (ABI). The high delta/alpha ratio in this study is equivalent to a low alpha/delta ratio. The results of the study showed the high delta/alpha ratio has a strong negative correlation with the functional outcome in patients with ABI after six months of neurorehabilitation. The lower the delta/alpha ratio, the higher the patients potential for recovery after rehabilitation, the lower the delta power and/or the higher the alpha power the better the patients outcome (Leon-Carrion et al. 2009, 1039-1045). Another method that has been used is to evaluate the presence of a SEP. The presence of a short or long SEP indicates different responses by the bird to stimulation. SEP are present in the animal during anesthesia, but their absence indicates further brain dysfunction (Raj 1998, 1815-1819). Unconsciousness was also defined using the EEG as the point where the EEG shows an isoelectric pattern, whereas death was defined as the point when birds showed an isoelectric EEG pattern with nonreversible properties, and this is always so when the heart rate is extremely low. In chickens, this was defined as less than 180 beats per minute (Coenen et al. 2000, 225-234).

EEG has also been used to determine the loss of brain function during stunning for slaughter of non-traditional food animals. Lambooij reported that after stunning, eels showed delta and theta waves. The authors concluded that consciousness and the possibility of pain perception can be excluded since the delta and theta waves tending to no brain activity were seen on EEG (Lambooij et al. 2007, 171-179). The occurrence of the delta and theta waves tending to no brain activity and no response to pain stimuli on both EEG and behavior supports the assumption that

the eels were unconscious and insensitive as gauged by analogy with similar EEG changes in humans (Lopes 1983, 3-12). Other work by Lambooij further indicated the presence of an unconscious state. Before being placed in ice water, eels were shown to exhibit on the EEG the presence of alpha and beta waves. These waves changed to delta and theta waves after placing the animals in ice water (Lambooij et al. 2002, 159-169). This study again showed the occurrence of delta and theta waves and no response to pain stimuli, both on the EEG and in the behavior of the eel, which supports the idea that the eels were unconscious and insensible as gauged by analogy with similar EEG changes in humans and laboratory animals (Lopes 1983, 3-12). These studies were also performed with common carp in which the appearance of delta and theta waves and spikes on the EEG indicated the loss of brain activity which was used as an index for unconsciousness and insensibility after percussive stunning (Lambooij et al. 2007, 171-179).

EEG has been used in several studies in agriculture both for depopulation purposes as well as to test stunning procedures for the slaughter of animals. Gerritzen used EEG to study the susceptibility of ducks and turkeys to severe hypercapnic hypoxia (Gerritzen et al. 2006, 39-42). High levels of CO_2 led to the occurrence of high amplitude, low frequency activity in the delta and theta waves which have been shown to indicate unconsciousness in broilers and hens (Raj, Wotton, and Gregory 1992, 147-156), (Raj 1998, 1815-1819); (Gerritzen et al. 2004, 1294-1301). The onset of the suppression of the alpha and beta waves and the loss of posture occurred at approximately the same time, indicating that the complete loss of posture is a sign of unconsciousness (Gerritzen et al. 2004, 1294-1301). The changes that are seen in the frequency, or the number of occurrences of a repeating event per unit time,

specifically the suppression of alpha and beta and the occurrence of the delta and theta are indicative of the loss of consciousness. The further suppression of the delta and theta waves will lead to an irreversible isoelectric EEG or brain death (Gerritzen et al. 2004, 1294-1301), (Gerritzen et al. 2006, 1055-1061). Strong suppression of the EEG to a near isoelectric form occurred at CO_2 levels of 43.4% for ducks and 41.8% for turkeys. Ducks and turkeys became unconscious at a CO_2 concentration of 20 - 25% in the air and were established using EEG, based on the loss of posture (Gerritzen et al. 2006, 1055-1061).

In a study to assess the use of CO_2 as a stunning gas for broilers, EEG suppression was used to determine the state of consciousness. EEG suppression occurred on average 40 s after the exposure to the CO_2 - O_2 mixture. To determine this, researchers monitored the presence of SEP using the EEG. SEPs are a series of waves that reflect the sequential activation of neural structures along different somatosensory pathways. The dorsal column-medial lemniscus pathway is the major substrate of the SEP. This pathway is responsible for transmitting fine touch, vibration and conscious proprioceptive information from the body to the cerebral cortex (Bradley 2000). The presence of short or long SEPs indicates different responses by birds to stimulations (Raj 1998, 1815-1819). Ernsting (1965) reported that under anoxic conditions, depression of activity in the brain extends progressively from the telencephalon to the diencephalon and then to the mesencephalon which supports the testing for the loss of SEPs (Ernsting 1965, 270-289). SEP can be induced by stimulating a peripheral mixed nerve with a low voltage electrical current. These potentials are present in an animal during anesthesia, but their absence indicates a brain dysfunction (Raj 1998, 1815-1819). In the case of turkeys, currents greater

than 250 mA are required per bird to abolish SEP following a stunning with a 50 Hz alternating current (Raj, Gregory, and Wotton 1991, 322-330). The results of a second stunning study (Raj and Gregory 1994, 222-223) showed that CO_2 - Ar mixture is more rapid than the anoxia alone in achieving loss of brain function in both chickens and turkeys. Turkey brains appeared to be relatively more tolerant to anoxia than the chicken brains.

In a study to determine the time to the loss of SEP and the time to the onset of a suppressed and isoelectric EEG when stunning turkeys with mixtures of CO_2, Ar, and N_2 in air, Raj and Gregory reported that the susceptibility of a turkey's brain to the toxic nature of the CO_2 - Ar mixture is similar to that seen in laying hens. However, the onset of the isoelectric EEG occurred more quickly in turkeys, which suggests that brain death ensues faster in the turkey when exposed to CO_2 - Ar mixture (Raj and Gregory 1993, 318-320). Remote monitoring of EEG, electrocardiogram (ECG) and behavior was recently used during a controlled atmosphere stunning test using broilers (Coenen et al. 2009, 10-19). The author reported artifacts in the EEG starting immediately after the birds were placed in the system. These were caused by physical movements of the birds, struggling, wing flaps, and clonic convulsions which were verified by comparing the EEG tracings with the behavioral recordings. ECG was also observed to have artifacts produced by the movement of the birds that coincided with those seen in the EEG (Coenen et al. 2009, 10-19). Unconsciousness was defined as the point where the EEG shows an isoelectric pattern, whereas death was defined as the point when birds showed an isoelectric EEG pattern with nonreversible properties, and this is always so when the heart rate is extremely low. In

chickens, this was defined as less than 180 beats per minute (Coenen et al. 2000, 225-234).

In a study done by Alphin et al (2010), foam with and without CO_2 infused into the bubbles were evaluated and compared with CO_2 gas using EEG as a depopulation method for broilers to determine if the addition of the CO_2 gas to the foam improved the speed and effectiveness of the foam. Results show that CO_2 gas takes approximately 2 min 14 s for EEG silence or brain death in broilers. If foam was an effective carrier for CO_2 gas, the time to EEG silence would need to include time for the bubbles to reach the bird, burst, release the gas, and time for the gas to affect the bird. If this was the case, the mean time to EEG silence for foam with CO_2 would include the release time and time for CO_2 to affect the bird. The results do not support this given that the mean time to EEG silence for foam with CO_2 (2 min) is less than for CO_2 gas alone (2 min 14 s). Based on the results of the study, the birds are being affected by the foam before the CO_2 is released from the bubbles to impact the birds. This indicates that for broilers, water based foam with CO_2 gas does not provide any material benefit over water based foam with ambient air (Alphin et al. 2010, 757-762). Some members of the international community feel that the use of water based foam is not humane due to the airway obstruction that it causes. As an alternative, there have been studies done to test the use of low water content (or dry) foam. The bubbles for this foam are made from surfactants similar to those used in hair shampoo. The results of a preliminary study done by Raj and others suggest that the use of nitrogen in the bubbles may be another option (Raj, Hickman, and Smith 2008, 722-723). The dry foam containing the nitrogen bursts as the test birds moved creating an acute anoxia adequate to quickly kill the birds. The residual oxygen levels

at bird height were less than 1%. The authors acknowledge, while these preliminary results are positive, further work needs to be done on this method to determine its potential for use in the field under emergency conditions (Raj, Hickman, and Smith 2008, 722-723).

The objective of this study was to evaluate the efficiency of depopulation methods for mature turkeys. In this study the two methods tested were CO_2 gassing, an approved and commonly used technique, and the use of water based foam, a conditionally approved method for floor raised poultry. The efficiency was evaluated using the electrocardiogram (ECG), the electroencephalogram (EEG) and the accelerometer to measure physical parameters of the birds during the depopulation. The EEG was used to determine the time to loss of consciousness in the birds. This study was conducted to parallel the challenges of an outbreak in a market age turkey flock.

Chapter 2

MATERIALS AND METHODS

Experiment 1:

The purpose of this experiment was to evaluate the use of the alpha/delta ratio to evaluate unconsciousness in poultry. During depopulation, the time to unconsciousness needs to be evaluated to determine when the birds are no longer aware of their surroundings or feeling any pain. The overall goal of this project was to find a way to evaluate the welfare of the poultry going through a depopulation treatment. Experiment 1 tested the use of the alpha/delta ratio under controlled anesthesia and the resulting unconsciousness. Using this alpha/delta ratio analysis may better evaluate the effects of the different depopulation methods performed on the birds. This study was done as a proof of concept for application to subsequent studies.

The birds used in this experiment were spent layer hens (> one year of age). A total of four birds were used for all testing. The birds were used to collect a total of three data sets. Each of the four birds was used in each data collection, for a total of 12 readings collected. All testing was performed under the approval and guidelines of the University of Delaware Agricultural Animal Care and Use Committee and followed the guidelines laid out by the Federation of Animal Science Societies (Federation for Animal Science Societies 1999).

Data Sciences International (DSI, St. Paul, MN) 3-channel PhysioTel Model F50-EEE wireless EEG transmitters were surgically implanted in the back of the neck of each bird 24 to 48 h before treatment. Birds were provided with 5% isoflurane at induction with 2% isoflurane for maintenance of general anesthesia during surgery and allowed to recover for 24 to 48 h prior to the anesthesia trial. Biopotential leads for EEG were implanted through the parietal bone to lay on the meninges through 0.9 mm holes drilled into the skull, using a Fine Science Tools (Foster City, CA) Model 18000-17 high-speed micro drill. There were three holes drilled into the parietal bone of the bird, avoiding the midline of the bone and all major blood vessels. Two recording leads and one ground lead were placed into the three holes and held in place with glue. The recording leads were placed on opposite sides of the midline. An additional two leads for EMG (muscle analysis) were implanted in the complexus muscles near the surgery site. The surgical procedure was based on Savory and Kostal (2006) and Savory and Kostal (1997).

Signals from the wireless transmitter were recorded by two DSI RMC-1 PhysioTel receivers. Two transmitters were placed opposite one another at the bottom of a 62.7-L (16.6 gal) chamber. The signals from the receivers were passed through a DSI Matrix. Brain activity was monitored and recorded using DSI Dataquest A.R.T. Acquisition software. EEG files were processed and analyzed in DSI NeuroScore.

The birds were placed in the anesthesia chamber and the lid sealed. The bird was allowed to calm and decrease activity prior to the start of any monitoring. The sensor output from the EEG sensor was monitored for a period of 15 minutes (900 s). Treatment with isoflurane (VEDco Inc, St. Joseph, MO) was applied 5 minutes (300 s) after sensor recording commenced. The isoflurane was applied to the bird at

2.5 L/min and at a concentration of 5% isoflurane. One minute after the start of the anesthesia treatment, the birds were stimulated with a blast from an air horn directed at the chamber. The air horn's audible stimuli were continued every 30 sec while the anesthesia was being applied. The isoflurane was applied to the bird until the bird no longer responded to the audible stimuli and had lost posture. At this point, the gas was allowed to run for another 2 - 4 min, after which the isoflurane was turned off, the lid removed from the top of the chamber, the computers re-set for another 15 min of monitoring and the oxygen allowed to flow into the chamber at a rate of 2.5 L/min. The birds were then monitored and recordings taken as they became conscious.

For unconsciousness, a frequency based quantitative approach was used. Frequency based EEG brain analysis breaks the signal down into different frequency regions: alpha (8-12 Hz), beta (16-24 Hz), delta (0.5-4 Hz), sigma (12-16 Hz), and theta (4-8 Hz). The sigma and beta frequencies are often included together. Neither the sigma nor beta waves were used for analysis in this project. The recorded signal was broken down into two different regions based on an analysis using recorded time as well as the EMG and EEG patterns: the area before the bird received treatment (first 5 min (300 sec) of the recording), and the post treatment period (period of time after the first 300 sec until the end of monitoring). The EEG trace was then labeled with markers to match these descriptions. The time periods that were selected for marking were two second epochs in which there was no artifact due to movement, as determined based on visual analysis of the EMG output and corresponding high-amplitude spikes in the EEG trace. The mean EEG signal, the mean EMG signal, the values for the alpha, beta, delta, theta and sigma waves, and markers were exported using NeuroScore. This signal information was then exported into Excel and charted.

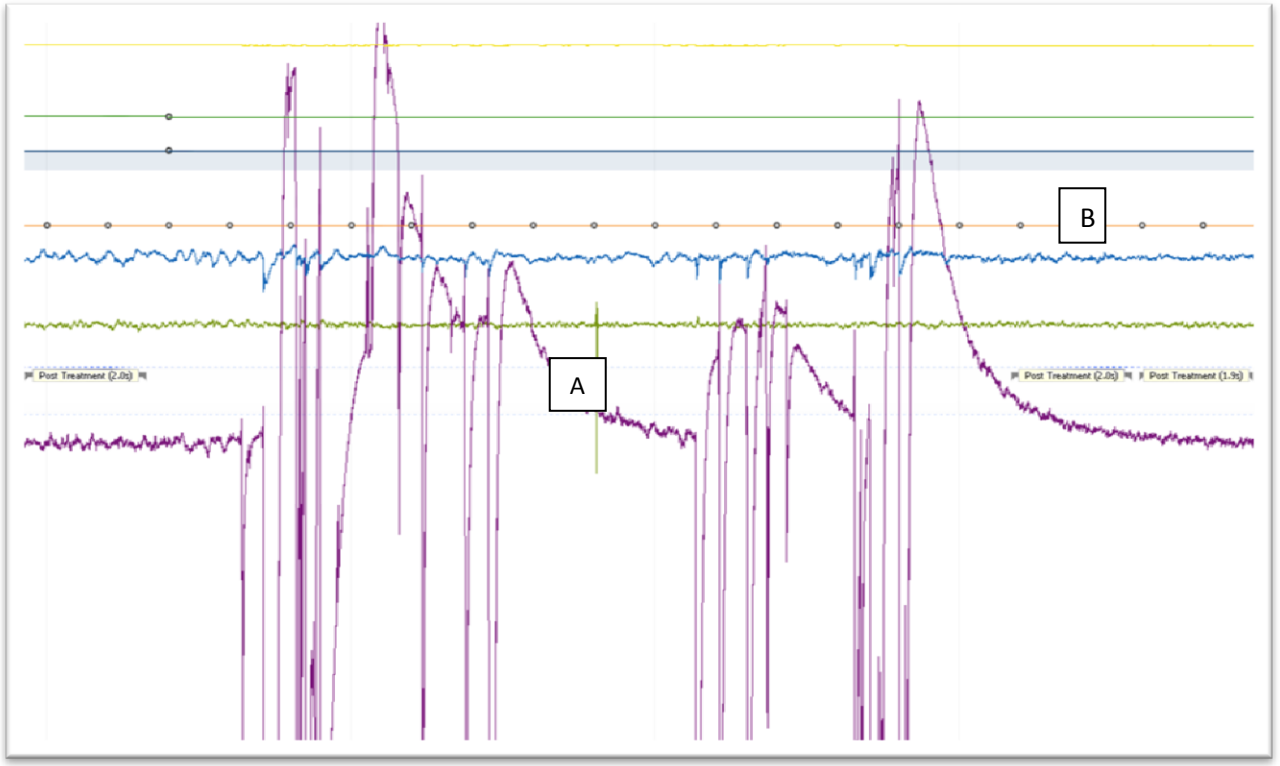

Figure 1: Raw signal used for placing markers. A (purple) is the EMG signal and B (green) is the EEG signal. EMG was used to help isolate motion artifacts in the signal so that artifact-free 2 second epochs could be selected from the EEG.

For the determination of unconsciousness in the birds, the relative power band ratio alpha/delta was used as shown in Equation 1. This was used to monitor a trend from high frequency brain wave activity to low frequency activity. During an unconsciousness period there is suppression in the alpha and beta waves and an occurrence of the delta and theta waves (Gerritzen, 2004, 1294-1301). The determination of the time to the loss of consciousness was based on the location of a localized minimum after treatment application in the plotting of the alpha/delta wave as shown in Figure 2.

$$A/DRatio = \frac{Alpha}{Delta} \tag{1}$$

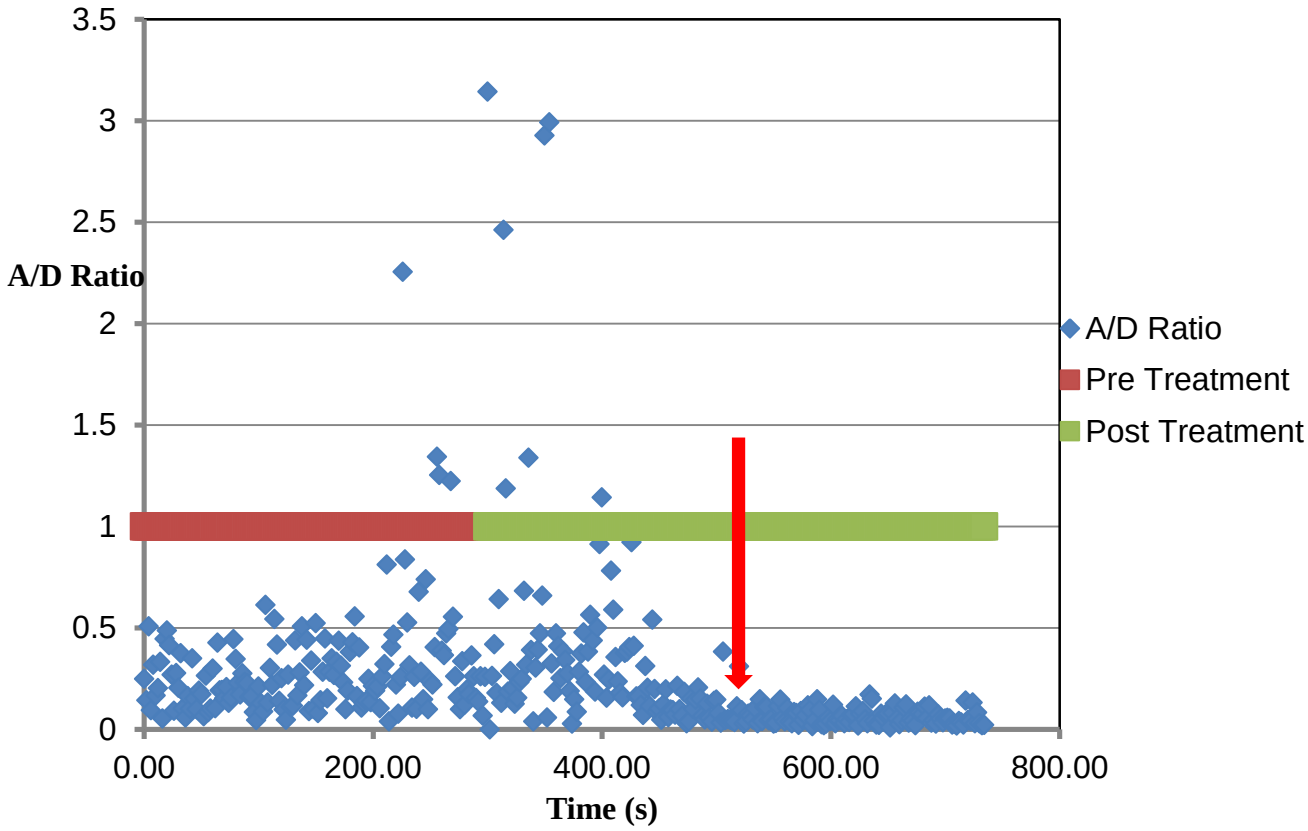

Figure 2: Charting of the AD ratio based on markers placed in the raw signal from Experiment 1. Point of unconsciousness is shown as the first localized minimum after treatment application. Layer 4 from 6/4/2010. Bird became unconscious at 522 sec. This time correlates to 222 sec after the start of the isoflurane treatment.

Experiment 2:

The purpose of this experiment was to evaluate the efficacy of depopulation treatments for use in depopulation procedures of market age turkeys. An experiment was conducted using a randomized block design with commercial male turkeys exposed to one of two randomly selected depopulation treatments; either 100% CO_2 gas or water based foam with ambient air. A total of 48 birds were depopulated. The same surgical procedure was used as in Experiment 1. The only modification to the procedure was during surgery the turkeys were provided with 5% isoflurane at induction with 3% isoflurane for maintenance of general anesthesia. The birds were allowed to recover for 24 to 48 h prior to depopulation.

Signals from the wireless transmitter were recorded by four DSI RMC-1 PhysioTel receivers. Two transmitters were placed opposite one another at the bottom of a 265-L (70 gal) chamber, and two others were placed opposite one another at approximately 0.9 m (3 ft.), the approximate height of an adult tom turkey head. The signals from the receivers were passed through a DSI Matrix. Brain activity was monitored and recorded using DSI Dataquest A.R.T. Acquisition software. EEG files were processed and analyzed in DSI NeuroScore.

Immediately before depopulation, ECG electrodes were attached to the birds to monitor heart activity. Prior to surgery, while the birds were anesthetized, locations on each leg and the right wing were plucked and prepared. Each bird was instrumented with ECG electrodes attached to each leg and the right wing. ECG signals were processed through BIOPAC Systems, Inc. MP30A acquisition unit and recorded using BIOPAC Student Lab (BSL) software. Analysis of the ECG signals was conducted using BIOPAC BSL Pro to review the recorded signals in detail and

find critical points. For the purposes of this study, ECG stabilization was the point at which the baseline voltage for the heart signal returns to ≈0 mV after the completion of terminal (tonic-clonic) convulsions.

To detect motion and cessation of motion, a PCB Piezotronics shear mode accelerometer was attached to the (left) leg immediately before treatment. Validation of the accelerometer procedure and comparison to EEG and ECG measurements were presented in Dawson et al. 2007 and Dawson et al., 2009. The Model 603C01 accelerometer had a sensitivity of 10.2 $mV \cdot s^2/m$ ± 10% (100 mV/g ± 10%) and was capable of operating over a range of ± 490 m/s^2 (50 g) of peak acceleration. For the purpose of this study, motion cessation was determined as the mean 0-V signal (flat line) occurring after terminal convulsions.

The output from the accelerometer was passed through a PCB Piezotronics single-channel signal conditioner, Model 480C02, connected to a National Instruments PCI-6036E data acquisition card. The conditioned signal was collected at 100 HZ in a custom-written virtual instrument (VI) developed in National Instrument's LabVIEW data acquisition and analysis software. The VI charted accelerometer activity during treatment, but a text file of the data points was also exported from the VI. The text files generated by the VI were processed through a custom program written in Visual Basic for Applications in Excel (VBA Excel) designed to reduce the signal frequency, if necessary, and add a relative time base for charting purposes.

A total of 48 male turkeys, 14 to 26 weeks of age were raised following standard care and conditions. Beginning at 15 weeks of age, four turkeys were

randomly selected per week for surgery and depopulation. A wireless EEG transmitter was surgically implanted as described above, 24 to 48 h prior to depopulation. Immediately before treatment, ECG electrodes and an accelerometer were securely attached to the bird. All birds were instrumented with all three sensing strategies (EEG, ECG, and motion). Each bird was treated with one depopulation treatment (CO_2 gas or water based foam).

The birds were placed in a 265-L (70 gal) treatment chamber. The treatment order was assigned randomly. The sensor output from all three sensors was simultaneously monitored for a period of 15 min (900 s). Treatment was applied 60 s after sensor recording commenced.

All testing was performed under the approval and guidelines of the University of Delaware Agricultural Animal Care and Use Committee and followed the guidelines laid out by the Federation of Animal Science Societies (Federation for Animal Science Societies 1999).

Gassing was conducted in an airtight chamber. The top of the chamber was covered with a 0.64 cm (¼ in.) sheet of transparent Plexiglas, allowing observation of the birds during gas stunning. CO_2 gas was introduced into the treatment chamber at a rate of 2265 L/min (80 ft^3/hr). The gas was applied continuously until the birds exhibited terminal convulsions, at which time the gas was turned off. The CO_2 gas levels as well as residual oxygen levels were monitored for all birds receiving the gas treatment using a Bacharach model 2820 (New Kensington, PA) CO_2 sensor and a Gas Alert Micro multigas meter from BW Technologies by Honeywell (Arlington, TX). The CO_2 level for the turkeys in the depopulation

chamber had a minimum level of 30% and a maximum level of 39.8% before equipment became saturated. This range was suitable for depopulating all the turkeys used in this experiment.

Water based foam with ambient air was created using a Spumifer (Ridgefield Park, NJ) AG-1 nozzle type foam depopulation system. This system draws air through the rear of the nozzle and combines it with a mixture of the foam concentrate and water. A 1% solution of Phos-Check (St. Louis, MO) MD-881 foam and water was premixed on the day of trial. A Darley (Itasca, IL) 2-1/2AGE 31 BS gasoline pump was used to supply the required pressure and flow. The Darley pump was driven by a 23 kW (31 hp) Briggs & Stratton (Milwaukee, WI) Vanguard gasoline engine providing a rated performance of 1136 L/min (300 gal/min) at 586 kPa (85 psi). This foam system meets the USDA APHIS conditional requirements for water based foam depopulation. Foam was applied until the 265-L chamber was full. No additional foam was added to make up lost volume due to bird motion. The expansion rate of the system was measured at 32.6:1, well within the 25:1 to 140:1 expansion rate criteria included in the USDA APHIS conditional requirements.

For unconsciousness, a frequency based quantitative approach was used. Frequency based EEG brain analysis breaks the signal down into different frequency regions: alpha, beta, delta, sigma, and theta. The recorded signal was broken down into four different regions based on an analysis using recorded time as well as the EMG and EEG patterns: the area before the bird received treatment (first 60 seconds of the recording), the post treatment period (period of time from the first 60 seconds to the first convulsion), the convulsion period (period of time after the first set of convulsions until the last convulsion) and the post convulsion period (the period after

the last convulsion). The EEG trace was then labeled with markers to match these descriptions. The time periods that were selected for marking were two second epochs in which there was no artifact due to movement, as determined based on visual analysis of the EMG output and corresponding high-amplitude spikes in the EEG trace. The mean EEG signal, the mean EMG signal, the values for the alpha, beta, delta, theta and sigma waves, and markers were exported using Neuroscore. This signal information was then exported into Excel and charted.

For the determination of unconsciousness in the birds, the relative power band ratio alpha/delta was used as shown in Equation 1. This was used to monitor a trend from high frequency brain wave activity to low frequency activity. During an unconsciousness period there is suppression in the alpha and beta waves and an occurrence of the delta and theta waves (Gerritzen, 2004, 1294-1301). The determination of the time to the loss of consciousness was based on the location of a localized minimum after the response to treatment in the plotting of the alpha/delta wave as shown in Figure 3. To determine the point of unconsciousness, there were four rules that were followed for an objective analysis: 1) the point of unconsciousness must occur after treatment application; 2) the loss of consciousness should occur before the convulsion phase; 3) generally, there is a rise in the signal after treatment application, believed to be a response from the birds to the treatment, then the signal begins to be suppressed; 4) when the suppression is maintained after treatment, that is the point of unconsciousness.

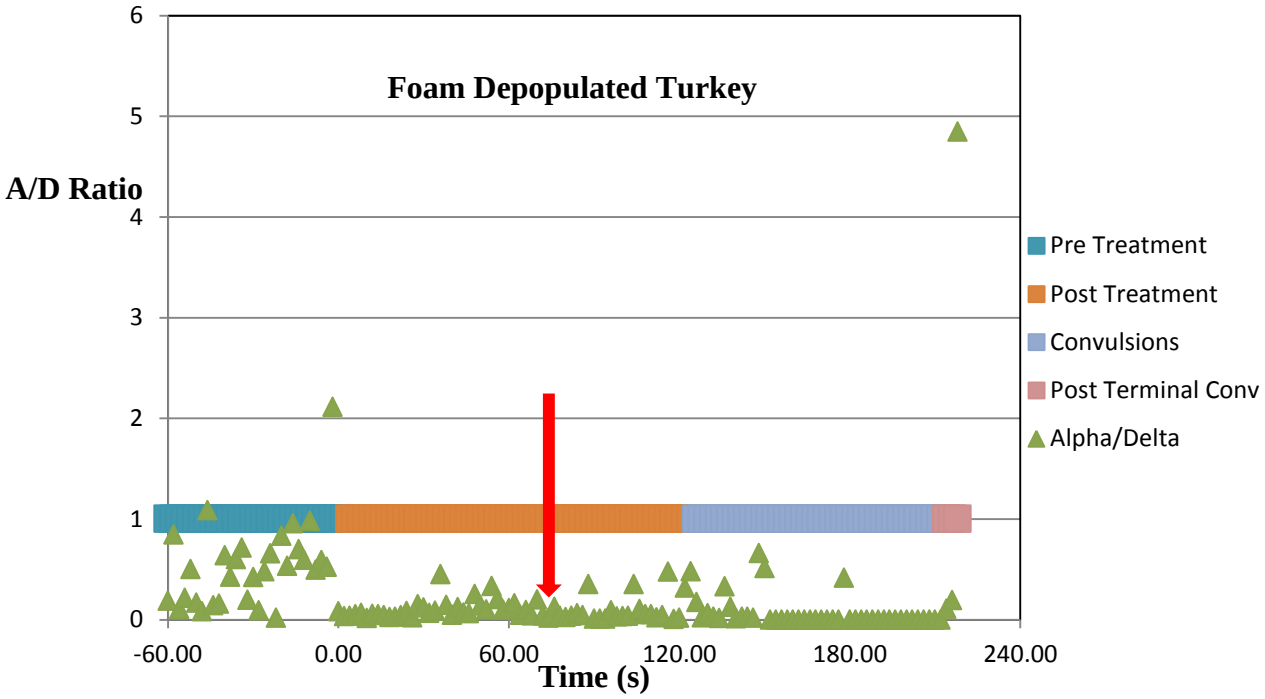

Figure 3: Charting of the AD ratio based on markers placed in the raw signal. Point of unconsciousness is shown as the first localized minimum after treatment application. Turkey 3 from 7/28/2009. Bird became unconscious at 72 seconds after the application of the foam treatment, as indicated by the arrow.

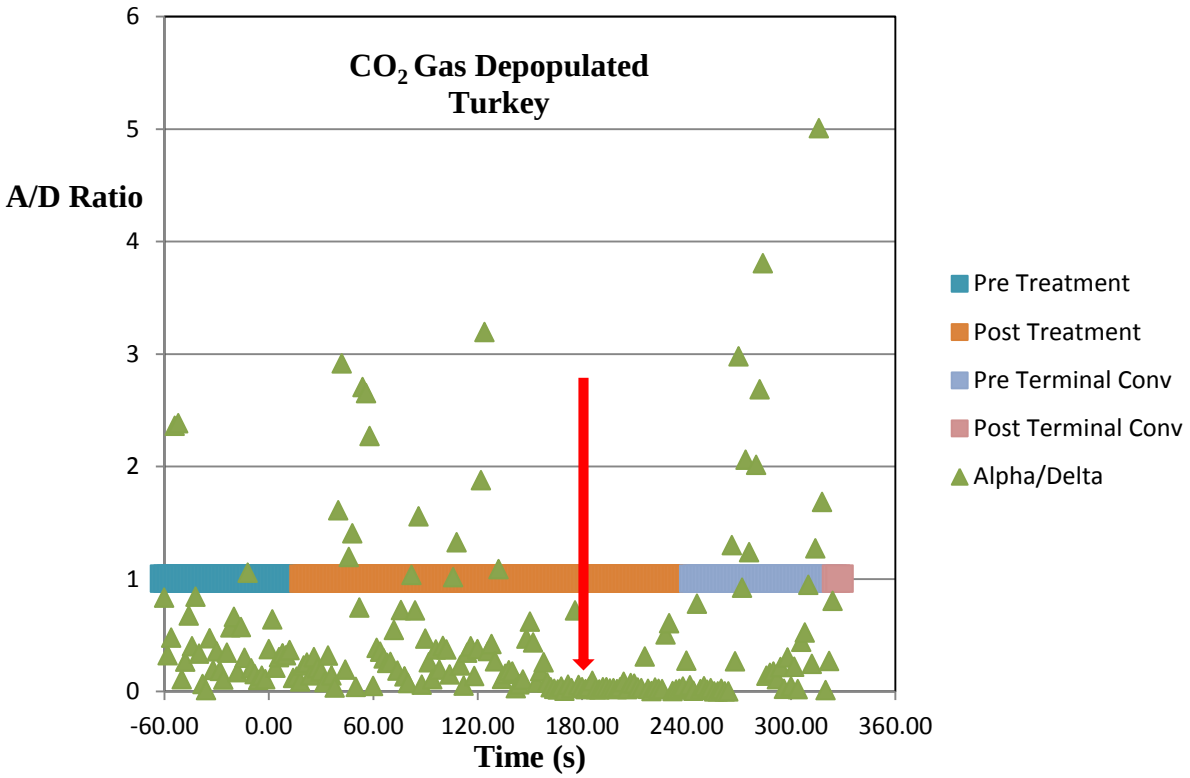

Figure 4: Charting of the AD ratio based on markers placed in the raw signal. Point of unconsciousness is shown as the first localized minimum after treatment application. Turkey 3 is from 6/19/2009. Bird became unconscious 178 seconds after the start of CO2 gas application as indicated by the arrow.

ECG was evaluated for cardiac relaxation (the time at which the heart relaxes), and was evaluated in BSL Pro monitoring software. This is point at which the heart is no longer functioning correctly and is not producing a distinguishable beat. Cardiac arrest was not seen within the fifteen minute experimental window.

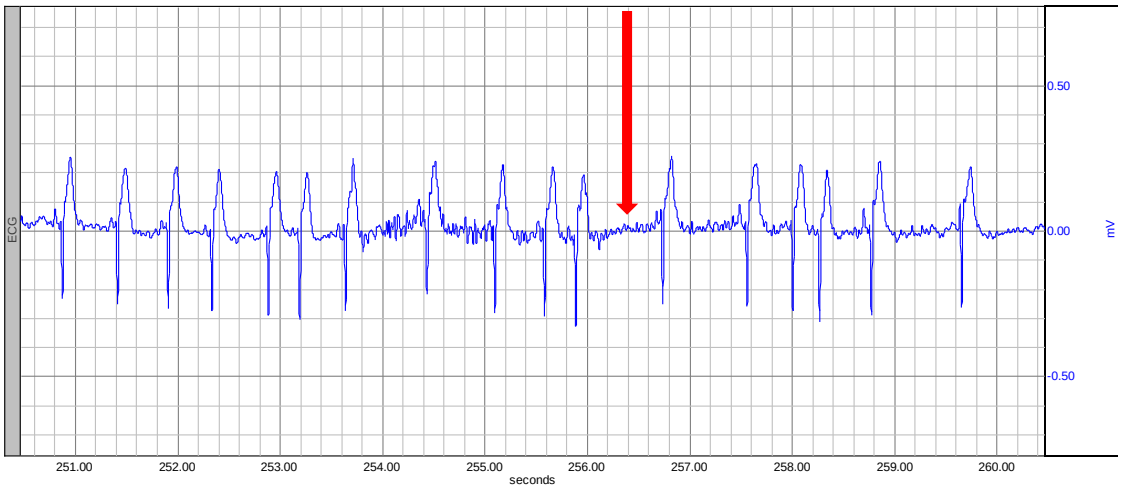

Figure 5 **Chart of the ECG signal to determine the point of ECG relaxation. The time to relaxation was 256 seconds as indicated by the arrow. This time correlates to 196 seconds after treatment application. This was a foam depopulated turkey from 7/28/2009.**

The accelerometer was used to determine the point of motion cessation in the bird. For depopulation, the cessation of motion is defined as the mean 0-V signal (flat line) occurring after convulsions.

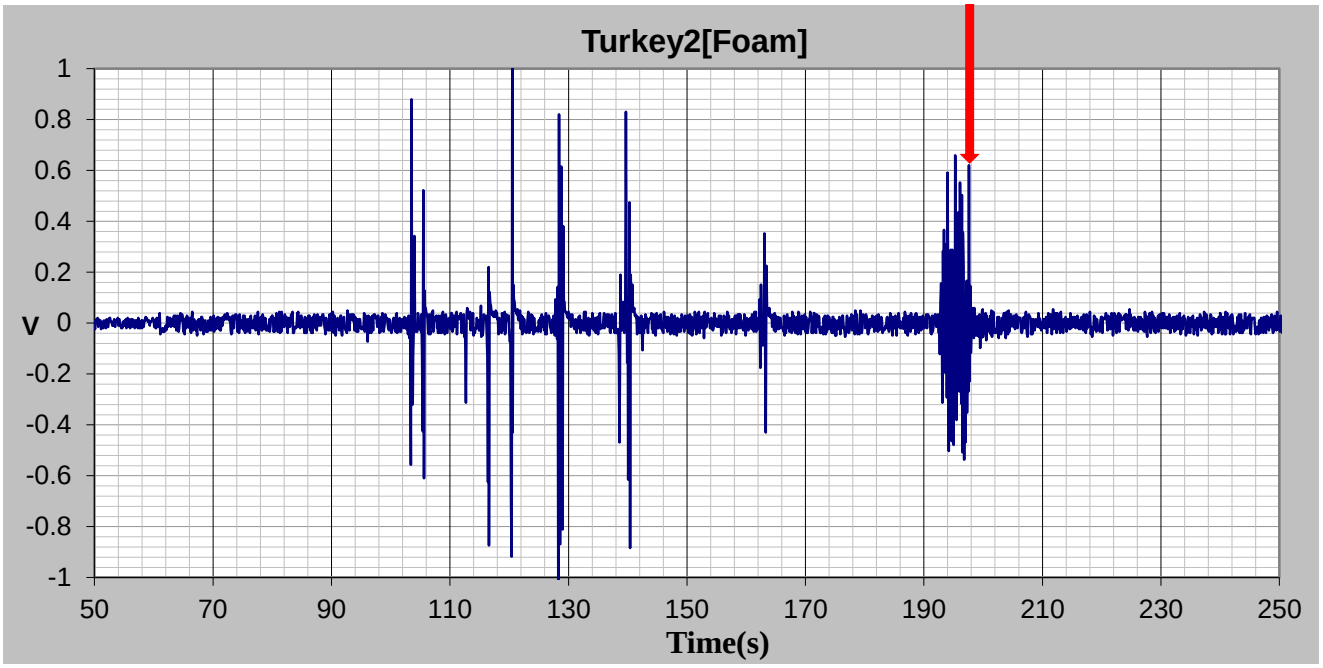

Figure 6: **The charting of the accelerometer data from 7/28/2009 Turkey 2. The point of motion cessation is determined to be the last spike seen on the chart. Motion cessation for this bird was determined to be 198 seconds. This correlates to a time of 138 seconds after treatment application.**

For data analysis, critical times for physiological events were extracted from the EEG, ECG, and accelerometer data as described above and were compiled in

Excel and statistical analysis was performed using SAS. The SAS data set was coded to extract sensor data valid for treatment analysis. The extracted treatment data was used to determine the distribution of analysis-specific data sets. The data subsets were not normally distributed and thus a non-parametric statistical analysis was conducted on each data subset in SAS. A Wilcoxon Exact test was used to analyze the treatment-dependent data sets. All tests were conducted at the 5% ($\alpha = 0.05$) significance level.

Chapter 3

RESULTS AND DISCUSSION

Experiment 1:

This experiment was conducted as a proof of concept that the alpha/delta ratio could be used to evaluate the time to unconsciousness in poultry. The results of this study indicate that there is a consistent suppression pattern in the transition from a conscious to an unconscious bird. This study was done as a controlled anesthesia procedure using spent layer hens from the University of Delaware flock. The alpha/delta ratio was suppressed by the effects of the isoflurane as the bird began to lose consciousness. After an initial response to the treatment by the bird, a suppression of the alpha/delta ratio was observed. The layer hens were found to go unconscious an average of 278 seconds after the start of the treatment with a standard deviation of 113 seconds. Representative charts of the time to loss of consciousness are shown in Figure 5. This concept was then used to evaluate the time to loss of consciousness in turkeys during depopulation in Experiment 2. The time to unconsciousness cannot be directly compared for Experiment 1 and 2 because of the differences in experimental procedure.

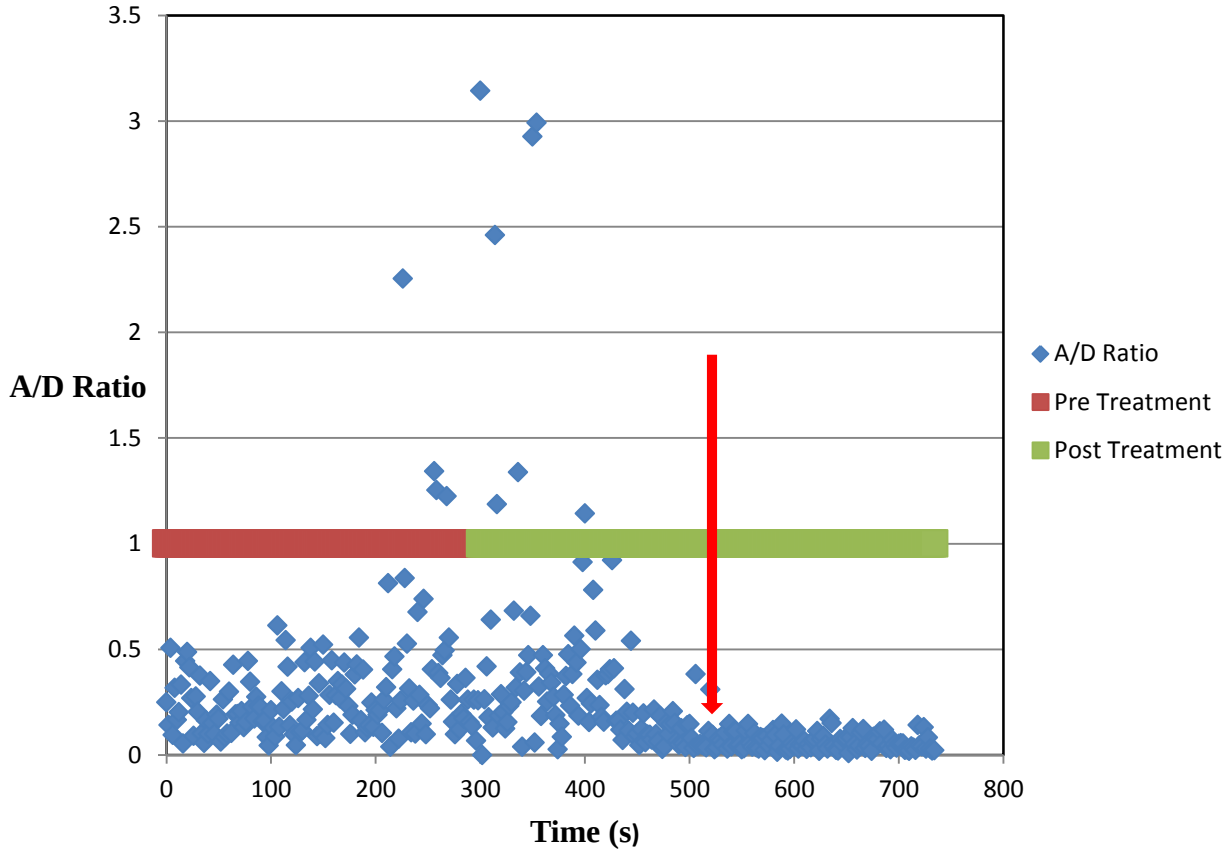

Figure 7: Charting of the AD ratio based on markers placed in the raw signal. Layer 1 from 6/4/2010. Point of unconsciousness is shown as the localized minimum after treatment application. This layer hen went unconscious at a time of 522 seconds on this chart. This correlates to a time of 222 seconds after the start of treatment due to subtraction of the 300 seconds (5 minute) baseline monitoring. The point of unconsciousness is indicated by the arrow.

Experiment 2:

The turkeys used in this study were Hybrid Converter commercial males, 14-26 weeks old. All birds in the study whether treated with the foam or the CO_2 gas were successfully depopulated. During the depopulation the birds were monitored with three instruments (EEG transmitter, accelerometer, and ECG electrodes) to determine the point of unconsciousness, brain death, terminal convulsions, and cardiac relaxation. All instruments readings were taken for 15 minutes, with the first 60 seconds being a baseline value that was used as a reference point in the analysis. The treatments were applied immediately following the 60 seconds baseline. The application of the foam was quick, taking less than 15 seconds to fill the depopulation chamber. The CO_2 gas was applied to the birds until terminal convulsions were observed.

There was a statistically significant difference in the time to brain death between the two methods. For this analysis, the gross signal was passed through a filter and analyzed for the point of silence. Brain death was determined to be the point at which the mean signal over 1 s period was stable (minimal to no change) about 0 μV. Water based foam was the fastest treatment with respect to brain death (μ=190 sec). The CO_2 gas was significantly slower (μ= 242 sec). Due to signal irregularities, some of the recordings were eliminated from analysis. This is reflected in the number of replicates for the time to brain death being less than the 24 birds per treatment that was described in the methods and materials section. Representative brain death traces from both treatments are shown in Figure 8.

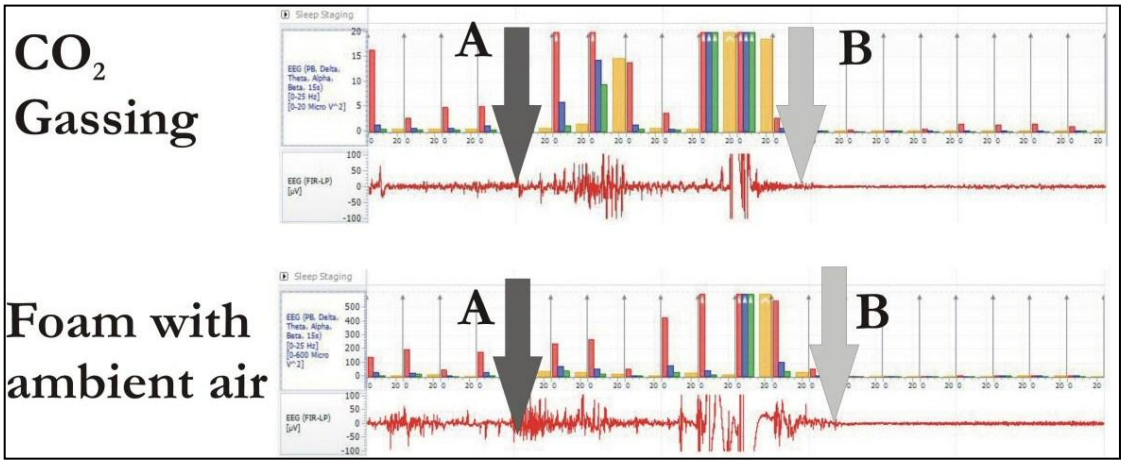

Figure 8: Representative filtered brain death results for CO_2 gassing and water based foam. Point A indicates start of treatment application and point B indicates brain death.

Table 1 Comparison of the mean and standard deviation of the brain death for the water based foam and CO_2 gassing depopulation

	Brain Death	
Treatment	Number of Birds (n)	Time(s)
Water Based Foam	17	190 + 42
CO_2 Gas	17	242 + 47

The time to unconsciousness, brain death, terminal convulsions, and cardiac relaxation were recorded for each bird. The time to unconsciousness and brain death were evaluated using the EEG signals recorded from a transmitter surgically implanted into the brain of the bird at the base of the skull. The determination of the time to unconsciousness is important because it shows the point at which the bird is no longer aware of its surroundings or feeling any pain. Being able to determine the point of unconsciousness allows for a more complete evaluation of the effectiveness of the two depopulation methods. From an animal welfare perspective, it would be ideal for the bird to become unconscious as quickly as possible and for death to soon follow to minimize any pain or discomfort. This information may also play a role in how agencies such as the USDA and organizations such as the AVMA evaluate water based foam for mass emergency depopulation of poultry. The time to brain death was also evaluated using the data from the EEG transmitter. The cardiac relaxation results

were determined through analysis of the ECG data. The motion cessation information was determined from an analysis of the accelerometer.

Water based foam resulted in faster (μ= 64 sec) time to unconsciousness than CO_2 gas (μ= 90 sec). The differences in time between the two treatments were not statistically significant. Representative unconsciousness charts for the two treatments are shown in Figures 9(a) and 10(a). Further analysis was then done on EEG output to remove all motion artifacts from the analysis using the EMG (muscle analysis). This is a standard procedure that is done in a clinical setting to allow for optimal evaluation. An artifact is any recorded electrical potential that does not originate in the brain. Muscle artifact, from the movement of the subject, can cause short potentials with sharp features (Redding 1984, 30-33). Representative unconsciousness charts with motion artifact removed for both treatments are show in Figures 9(b) and 10(b).

Table 2 Comparison of the mean and standard deviation of the time to unconsciousness for the water based foam and the CO_2 gas.

	Unconsciousness	
Treatment	Number of Birds(n)	Time (s)
Water Based Foam	10	64 $\pm$ 19
CO_2 Gas	14	90 $\pm$ 53

The time to terminal convulsions of the birds was determined through the use of an accelerometer. The results show that there was no statistically significant difference between the water based foam (μ=166 sec) and the CO_2 gas (μ=174 sec) resulting in similar times to motion cessation. This data was used to determine when the bird enters and finishes the terminal convulsion phase of death. The terminal convulsion phase is an irreversible point, where the birds are no longer conscious. Validation of the accelerometer procedure and comparison to EEG and ECG measurements were presented in Dawson et al. 2007 and Dawson et al. 2009. The results of these studies showed that motion cessation can be used to determine the end of the convulsive phase and as an estimator of the time to brain death (Dawson et al. 2007, 583-591; Dawson et al. 2009). Death occurs quickly once the bird reaches this point.

Table 3: Comparison of the mean and standard deviation of the time to motion cessation for the water based foam and CO_2 gassing depopulation.

	Motion Cessation	
Treatment	Number of Birds (n)	Time (s)
Water Based Foam	13	166 $\pm$ 59
CO_2 Gas	12	174 $\pm$ 50

The AVMA defines clinical death in animals as cardiac arrest. However, the study of poultry physiology shows that death occurs in phases. First, brain activity is suppressed, and then the response to external stimuli ceases. Convulsions occur once brain activity is irreversibly suppressed. (Raj, Wotton, and Gregory 1992, 147-156) The time differences for cardiac relaxation for water based foam (μ=200 sec) and the CO_2 gas (μ= 220 sec) were not statistically significant. Cardiac relaxation is the point at which the heart is no longer functioning correctly and is not producing a distinguishable beat. Cardiac arrest due to failure of the heart occurred in most of the birds outside the fifteen minute experimental recording period.

Table 4: Comparison of the mean and standard deviation of the time to cardiac relaxation for the water based foam and CO_2 gassing depopulation.

	Cardiac Relaxation	
Treatment	Number of Birds (n)	Time (s)
Water Based Foam	16	208 $\pm$ 46
CO_2 Gas	13	242 $\pm$ 30

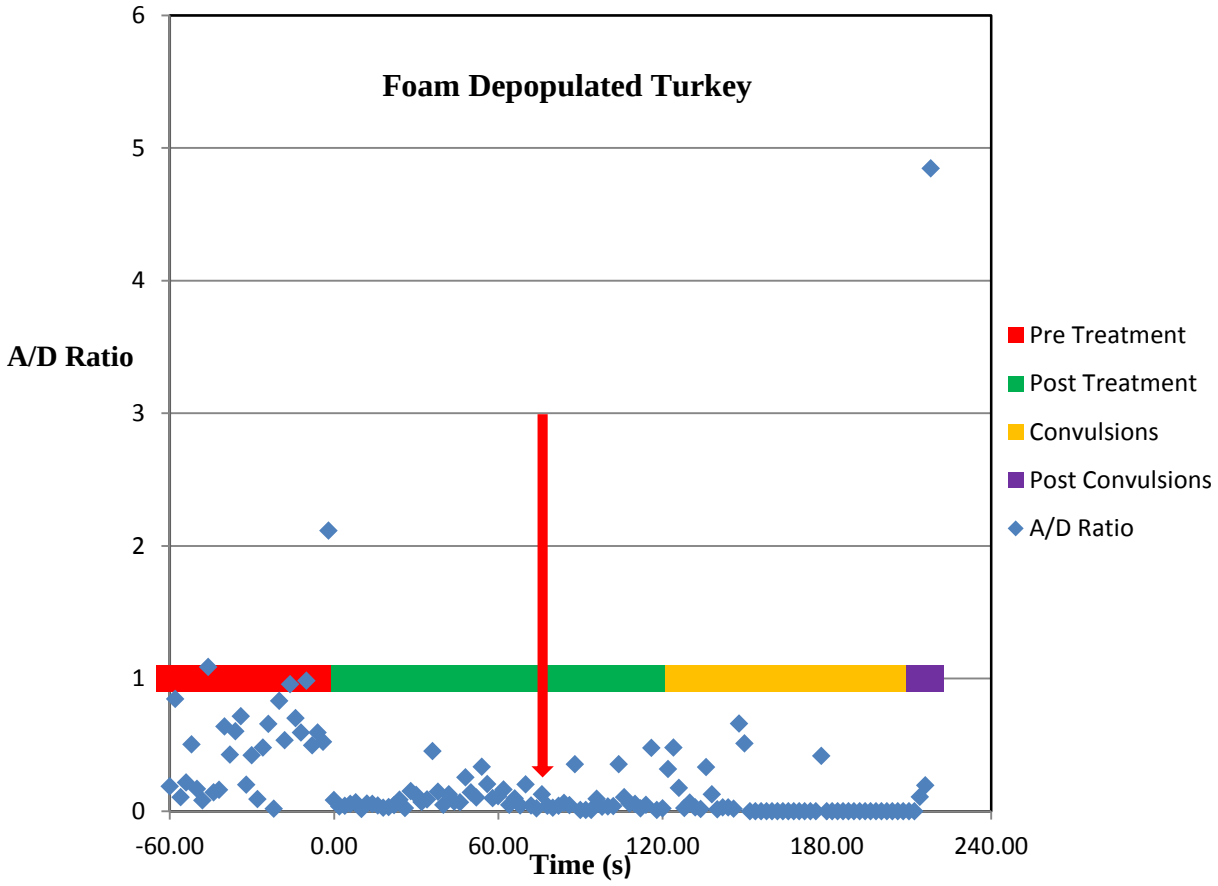

Figure 9(a): Charting of the A/D ratio based on markers placed in the raw signal. Point of unconsciousness is shown as the first localized minimum after treatment application. Turkey 3 from 7/28/2009. Bird became unconscious at 72 seconds after the application of the foam treatment, as indicated by the arrow.

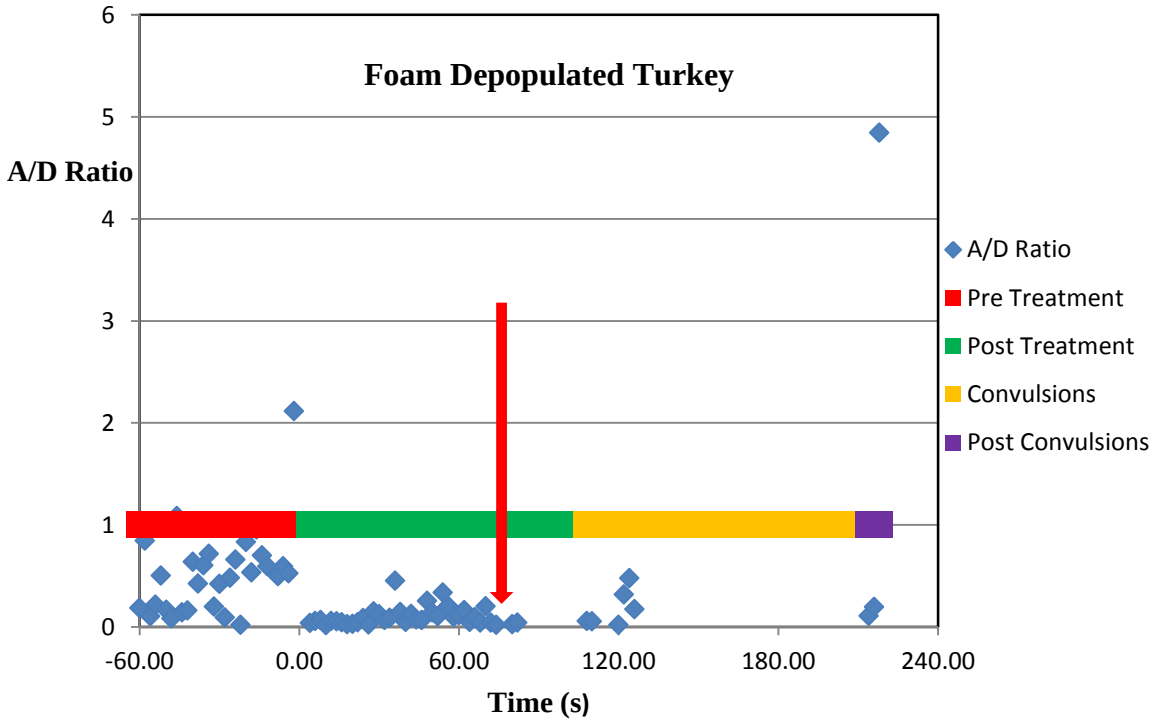

Figure 9(b): Charting of the A/D ratio based on markers placed in the raw signal with all motion artifacts removed from analysis. Point of unconsciousness is shown as the first localized minimum after treatment application. Turkey 3 from 7/28/2009. Bird became unconscious at 72 seconds after the application of the foam treatment, as indicated by the arrow.

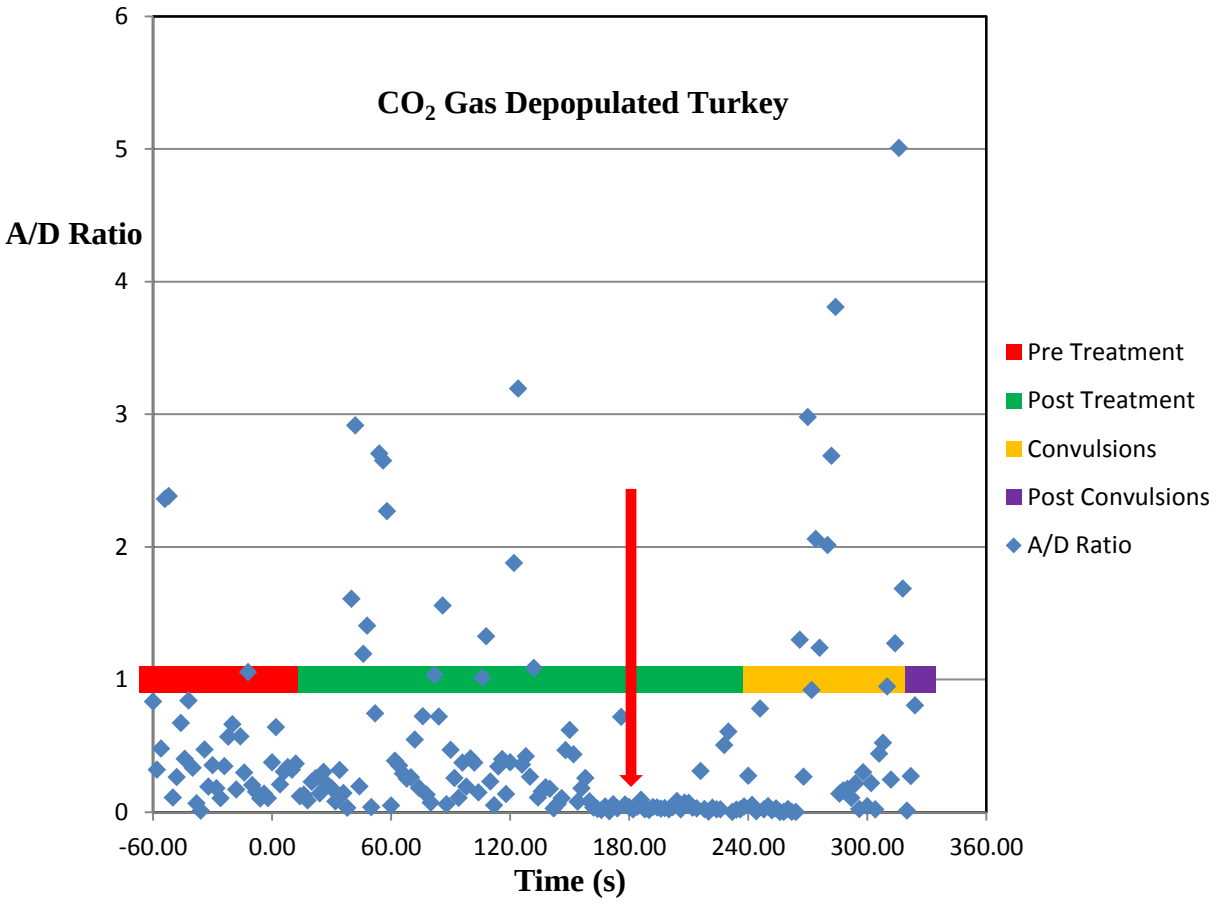

Figure 10(a): Charting of the A/D ratio based on markers placed in the raw signal. Point of unconsciousness is shown as the first localized minimum after treatment application. Turkey 3 is from 6/19/2009. Bird became unconscious 178 seconds after the start of CO_2 gas application as indicated by the arrow.

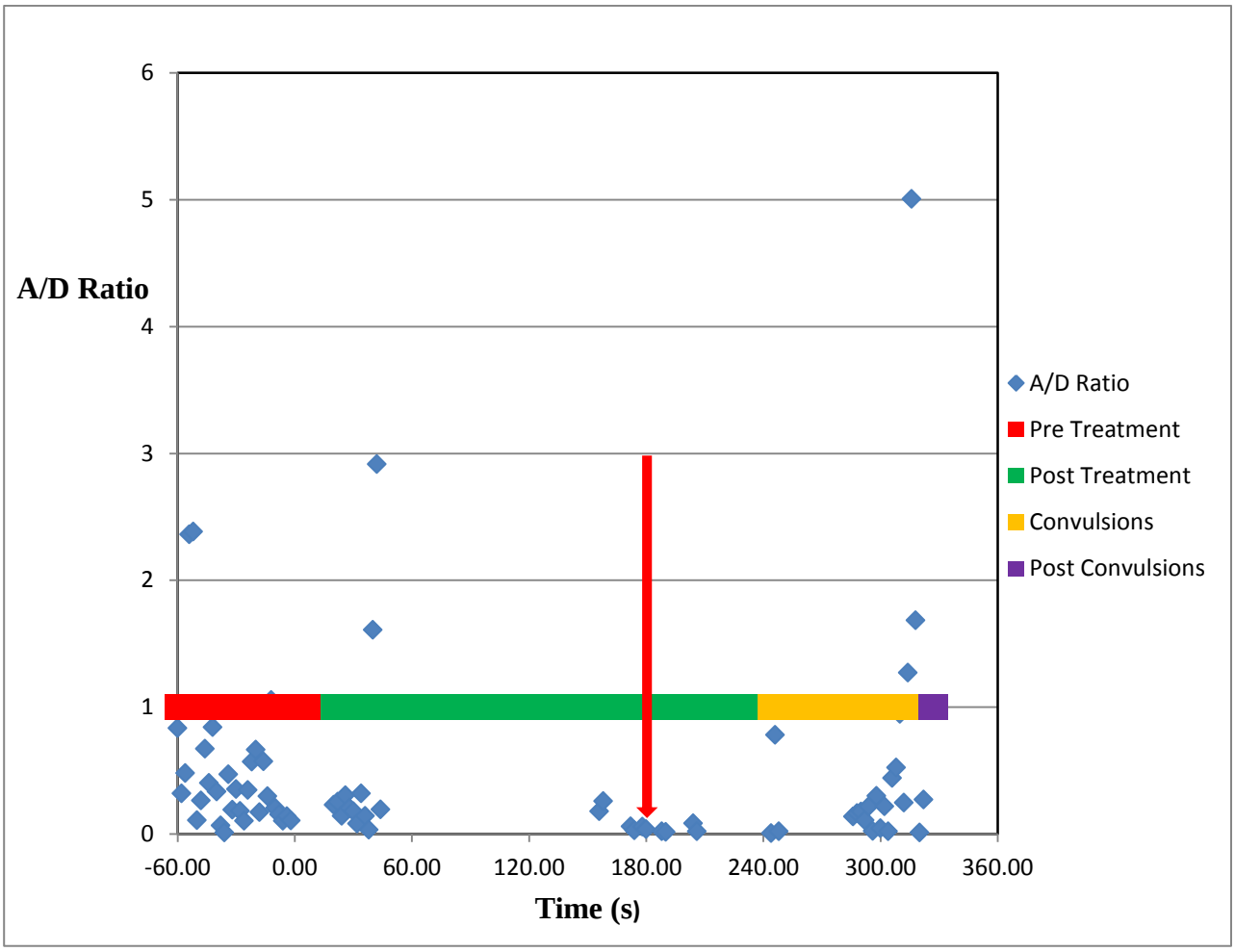

Figure 10(b): Charting of the A/D ratio based on markers placed in the raw signal with all motion artifacts removed from analysis. Point of unconsciousness is shown as the first localized minimum after treatment application. Turkey 3 is from 6/19/2009. Bird became unconscious 178 seconds after the start of CO_2 gas application as indicated by the arrow.

The results of this experiment show that water based foam is more effective at causing brain death than the CO_2 gas. Though not statistically significant, the water based foam caused unconsciousness, cardiac relaxation and motion cessation

faster than the CO_2 gas. The water based foam values for brain death and unconsciousness were also more consistent, with less variation from the mean compared to the CO_2 gas.

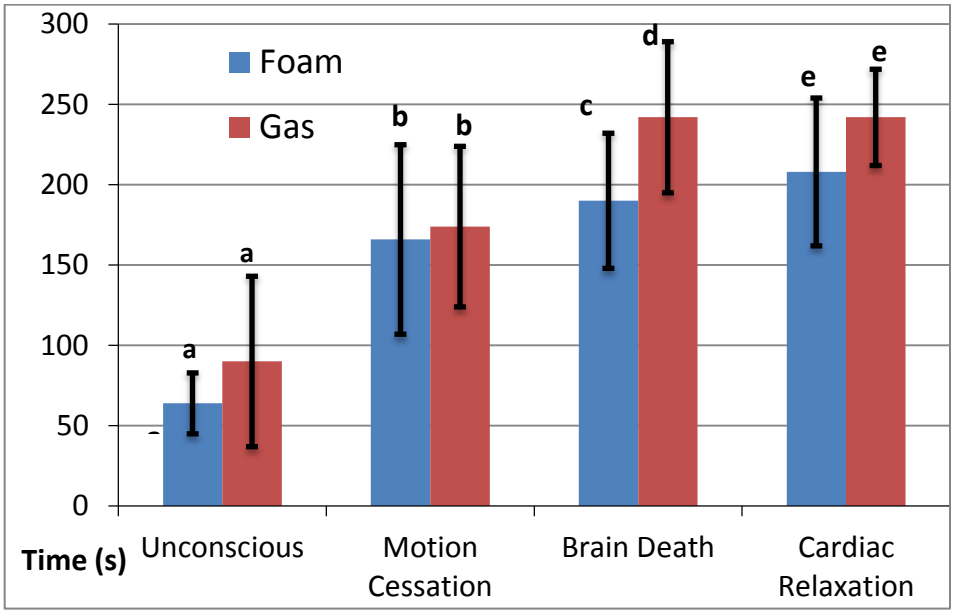

Figure 11: Summary chart of the physiological parameters evaluated in this study. The only parameter with a statistically significant difference between the two treatments was the time to brain death.

Mass emergency depopulation of poultry is something that many in the poultry industry hope to never be involved in. The reality is, many of them will. This situation can arise due to disease outbreak or a poultry house structural failure caused by a natural disaster (i.e. hurricane, tornado, etc.). In any case, responders need to be prepared and able to handle the situation. The use of the CO_2 gas has been successful and is widely used for the depopulation of poultry. The use of CO_2 gas can be done as a whole house, partial house or containerized system. However, there are some drawbacks to the use of CO_2 gas. The necessity to seal the house for the whole house and partial house methods to prevent escape of the virus particles and to allow the gas to reach a lethal level is one of the largest issues. This can be very time consuming and difficult if the house is older or entirely impossible if the house has been damaged by a storm or other natural disaster. The containerized method can increase the biosecurity risk because this method involves workers entering the house to catch the birds, potentially exposing the workers to the virus. Additionally, the birds are commonly taken outside the house to be placed in a container. This can lead to the spread of the virus particles outside the house. The CO_2 gas is also toxic to humans requiring that responders use a self-contained breathing apparatus (SCBA).

The use of water based foam was conditionally approved by the USDA-APHIS and AVMA under certain conditions to include: 1) animals infected with a potentially zoonotic disease, 2) animals infected with a rapidly spreading infectious disease that, in the opinion of state or federal regulatory officials, cannot be contained by conventional or currently accepted means of mass depopulation or, 3) animals housed in structurally unsound buildings which are hazardous for human entry(such as those damaged during a natural disaster) (AVMA, 2006, 1-4). The use of the water

based foam eliminates some of the logistical issues that the use of CO_2 gas presents. There is no need to seal the house and it is not harmful to the responders. Additionally it enhances the use of in-house composting which the USDA has outlined as a preferred approach to handling the carcasses left after a depopulation. Flory and Peer found that when used properly, the use of water based foam increased the depopulation compost pile moisture content to optimum levels and if foam had not been used, addition of water would have been necessary for effective composting (Flory and Peer 2010, 149-157). Foam also reduces the biosecurity risk and reduces the labor and exposure of the workers to the virus. Some of the disadvantages of the use of foam include the initial cost of the equipment and the large amount of water that are used to create the foam.

This study has shown that water based foam is a valid option for depopulation of mature turkeys. This information gives responders another option when deciding what method to use when handling an emergency response. This study has also demonstrated a method to evaluate the loss of consciousness in poultry. This may aid agencies such as the USDA and organizations such as the AVMA evaluate the use of water based foam for mass emergency depopulation of poultry. It may also have other applications for welfare studies of poultry.

Chapter 4

REFERENCES

Alexander, D. J. 2000. A review of avian influenza in different bird species. Veterinary Microbiol. 74:3-13.

Alphin, R. L., M. K. Rankin, K. J. Johnson, and E. R. Benson. 2010. Comparison of water-based foam and inert-gas mass emergency depopulation methods. Avian Dis. 54:757-62.

American Veterinary Medical Association. AVMA guidelines on euthanasia. 2007.

American Veterinary Medical Association (AVMA). 2006. Use of water-based foam for depopulation of poultry. Am Vet Med Assoc.:1-4.

American Veterinary Medical Association (AVMA). 2001. Report of the AVMA panel on depopulation. J. Am. Vet. Med. Assoc. 218:669-689.

Balicer, R. D., S. Reznikovich, E. Berman, M. Pirak, A. Inbar, S. Pokamunski, and I. Grotto. 2007. Multifocal avian influenza (H5N1) outbreak. Emerg. Infect. Dis. 13:1601-1603.

Benson, E., C. R. Pope, G. L. Van Wicklen, M. D. Dawson, G. W. Malone, and R. L. Alphin. 2007. Foam-based mass emergency depopulation of floor-reared meat-type poultry operations. Poultry Sci. 86:219-224.

Bowes, V. A., S. J. Ritchie, S. Byrne, K. Sojonky, J. J. Bidulka, and J. H. Robinson. 2004. Virus characterization, clinical presentation, and pathology associated with H7N3 avian influenza in British Columbia broiler breeder chickens in 2004. Avian Dis. 48:928-934.

Bradley, W. G. 2000. Neurology in clinical practice. 3rd ed. Vol. 1. Elsevier Science & Technology Books.

Breitmeyer, R. E., A. M. Whiteford, and J. A. Shere. 2003. California experience with exotic Newcastle disease: A state and federal regulatory perspective. Paper presented at Proceedings of the 107th Annual Meeting of the United States Animal Health Association, San Diego, CA.

Capua, I., and D. J. Alexander. 2009. Avian influenza infection in birds: A challenge and opportunity for the poultry veterinarian. Poultry Sci. 88:842-846.

Capua, I., and S. Marangon. 2007. The challenge of controlling notifiable avian influenza by means of vaccination. Avian Dis. 51:317-322.

Coenen, A., A. Smit, L. Zhonghya, and G. van Luijtelaar. 2000. Gas mixtures for anesthesia and euthanasia in broiler chickens. Worlds Poultry Sci. J. 56:225-234.

Coenen, A. M. L., J. Lankhaar, J. C. Lowe, and D. E. F. McKeegan. 2009. Remote monitoring of electroencephalogram, electrocardiogram, and behavior during controlled atmosphere stunning in broilers: Implications for welfare. Poultry Sci. 88:10-19.

Dawson, M. D., K. J. Johnson, E. R. Benson, R. L. Alphin, S. Seta, and G. W. Malone. 2009. Determining cessation of brain activity during depopulation or euthanasia of broilers using accelerometers. J. Appl. Poult. Res.18:135-142.

Dawson, M. D., M. E. Lombardi, E. R. Benson, R. L. Alphin, and G. W. Malone. 2007. Using accelerometers to determine the cessation of activity of broilers. J. Appl. Poult. Res. 16:583-591.

Ernsting, J. 1965. The effect of anoxia on the central nervous system. In A textbook on avian physiology., ed. J. A. Gilles, 270-289. London, England: Permagon Press.

Federation for Animal Science Societies. 1999. Guidelines for the care and use of agriculture animals in agricultural research and teaching. 1st rev. ed. Savoy, IL: Fed Anim. Sci. Soc.

Flory, Gary A., and Robert W. Peer. 2010. Verification of poultry carcass composting research through application during actual avian influenza outbreaks. Institute for Laboratory Animal Research Journal 51:149-157.

Forslid, A., M. Ingvar, I. Rosen, and D. H. Ingvar. 1986. Carbon dioxide narcosis: Influence of short-term high concentration carbon dioxide inhalation on EEG and cortical evoked responses in the rat. Acta Physiol. Scand. 127:281-287.

Gerritzen, M., A. Stegeman, B. Spruijt, B. Lambooij, and H. Reimert. 2007. A note on behaviour of poultry exposed to increasing carbon dioxide concentrations. Appl Anim Behav Sci 108:179-185.

Gerritzen, M. A., E. Lambooij, H. G. M. Reimert, B. M. Spruijt, and J. A. Stegeman. 2006. Susceptibility of duck and turkey to severe hypercapnic hypoxia. Poultry Sci. 85:1055-1061.

Gerritzen, M. A., E. Lambooij, J. A. Stegeman, and B. M. Spruijt. 2006. Slaughter of poultry during the epidemic of avian influenza in the Netherlands in 2003. Vet. Rec.: Journal of the British Veterinary Association 159:39-42.

Gerritzen, M. A, B. Lambooij, H. Reimert, A. Stegeman, and B. Spruijt. 2004. On-farm euthanasia of broiler chickens: Effects of different gas mixtures on behavior and brain activity. Poultry Sci. 83:1294-1301.

Gerritzen, M. A, E. Lambooij, S. J. Hillebrand, J. A. Lankhaar, and C. Pieterse. 2000. Behavioral responses of broilers to different gaseous atmospheres. Poultry Sci. 79:928-933.

Guyton, A. C., and J. E. Hall. 2000. Chapters 5 and 7. In Textbook of medical physiology. 10th ed. Philadelphia, PA, USA: W.B. Sauders Company.

Halvorson, D. A., D. D. Frame, K. A. J. Friendshuh, and D. P. Shaw. 2003. Outbreaks of low pathogenicity avian influenza in U.S.A. Avian Dis. 47:36-46.

Hari, P., K. Portin, B. Kettenmann, V. Jousmaki, and G. Kobal. 1997. Right-hemisphere preponderance of responses to painful CO_2 stimulation of the human nasal mucosa. Pain 72:145-151.

Hughes, B. 1983. Head shaking in fowls: The effect of environmental stimuli. Appl Anim Ethol 11:45-53.

Inbar, A. 2008. New technologies for mass depopulation and disposal of poultry in case of HPAI in Israel. BARD/MARD workshop on the evaluation of novel

technologies for reducing environmental spread and efficient eradication strategies for high pathogenicity avian influenza.

Irvine, R. M., J. Banks, B. Z. Londt, S. A. Lister, R. J. Manvell, L. Outtrim, C. Russell, et al. 2007. Outbreak of highly pathogenic avian influenza caused by Asian lineage H5N1 virus in turkeys in Great Britain in January 2007. The Vet. Rec. 161:100-101.

Kingston, S. K., S. Taylor, T. Holt, B. Porter-Spalding, M. E. Geib, C. A. Dussault, R. S. Zaidlicz, and N. H. Faltas. 2005. Evaluation of two methods for mass euthanasia of poultry in disease outbreaks. JAVMA- J. Am. Vet. Med. A. 227:730-738.

Lambooij, E., C. Pieterse, C. M. Potgieter, J. D. Snyman, and G. L. Nortje. 1999. Some neural and behavioural aspects of electrical and mechanical stunning in ostriches. Meat Sci. 52:339-345.

Lambooij, E., M. Pilarczyk, H. Bialowas, J. G. M. van den Boogaart, and J. W. van de Vis. 2007. Electrical and percussive stunning of the common carp (*Cyprinus carpio L.*): Neurological and behavioural assessment. Aquacult Eng 37:171-179.

Lambooij, E., J. W. Vis, R. J. Kloosterboer, and C. Pieterse. 2002. Welfare aspects of live chilling and freezing of farmed eel (*Anguilla anguilla L.*): Neurological and behavioural assessment. Aquaculture (Amsterdam, Netherlands) Aquaculture 210:159-169.

Leon-Carrion, J., J. F. Martin-Rodriguez, J. D.-Lopez, J. M. Barroso y Martin, and M. R. Dominguez-Morales. 2009. Delta-alpha ratio correlates with level of recovery after neurorehabilitation in patients with acquired brain injury. Clin. Neurophysiol. 120:1039-1045.

Liu, Ming, Shiqin He, David Walker, NanNan Zhou, Daniel R. Perez, Bing Mo, Fan Li, Xiaotian Huang, Robert G. Webster, and Richard J. Webby. 2003. The influenza virus gene pool in a poultry market in south central china. Virology 305:267-275.

Lopes, da Silva. 1983. The assessment of unconsciousness: General principles and practical aspects. Curr. T. Vet.:3-12.

Matteson, J. L., J. M. Stinson, and C. S. Clark. 1972. Electroencephalographic power-spectral changes coincident with the onset of carbon dioxide narcosis in rhesus monkey. Am. J. Vet. Res. 33:2043-2049.

Otto, K. A., and C. E. Short. 1991. Electroencephalographic power spectrum analysis as a monitor of anesthetic depth in horses. Vet Surg 20:362-371.

Otto, K. A. 2008. EEG power spectrum analysis for monitoring depth of anaesthesia during experimental surgery. Lab. Anim. 42:45-61.

Pelzel, A. M., B. J. McCluskey, and A. E. Scott. 2006. Review of the highly pathogenic avian influenza outbreak in Texas, 2004. JAVMA- J. Am. Vet. Med. A. 228:1869-1875.

Purves, D., G. J. Augustine, D. Fitzpatrick, L. Katz, A. S. Lamanta, J. O. McNamara, and S. M. Williams. 2001. Neuroscience. Sunderland, MA, USA: Sinauer Associates Inc.

Raj, A. B. M. 1996. Aversive reactions of turkeys to argon, carbon dioxide and a mixture of carbon dioxide and argon. Veterinary Record: Journal of the British Veterinary Association 138:592-593.

Raj, A. B. M., and N. G. Gregory. 1995. Welfare implications of the gas stunning of pigs. 1. determination of aversion to the initial inhalation of carbon dioxide or argon. Animal Welfare 4:273-280.

Raj, A. B. M., N. G. Gregory, and S. B. Wotton. 1991. Changes in the somatosensory evoked potentials and spontaneous electroencephalogram of hens during stunning in argon induced anoxia. British Veterinary Journal 147:322-330.

Raj, A. B. M., G. Hickman, and C. Smith. 2008. Novel method for killing poultry in houses with dry foam created using nitrogen. Vet. Rec.: Journal of the British Veterinary Association 162:722-723.

Raj, A. B. M., S. B. Wotton, and N. G. Gregory. 1992. Changes in the somatosensory evoked potentials and spontaneous electroencephalogram of hens during stunning with a carbon dioxide and argon mixture. Brit. Vet. J. 148:147-156.

Raj, A. B. M., S. B. Wotton, J. L. McKinstry, S. J. W. Hillebrand, and C. Pieterse. 1998. Changes in somatosensory evoked potentials and spontaneous electroencephalogram of broiler chicken during exposure to gas mixtures. Brit. Poultry Sci. 39:686-695.

Raj, M. 1998. Welfare during stunning and slaughter of poultry. Poultry Sci. 77:1815-1819.

Raj, M., and N. G. Gregory. 1994. An evaluation of humane gas stunning methods for turkeys. Vet. Rec.: Journal of the British Veterinary Association 135:222-223.

———. 1993. Time to loss of somatosensory evoked potentials and onset of changes in the spontaneous electroencephalogram of turkeys during gas stunning. Vet. Rec.: Journal of the British Veterinary Association 133:318-320.

Redding, R. W. 1984. Artifacts. In Atlas of electroencephalography in the dog and cat., 30-33. New York, NY, USA: Praeger Publishers.

Ryan, J. M., K. Sheehan, and S. Gaynor. 2006. Report on the real-time exercise in the "whole house" carbon dioxide gassing technique for the humane killing of poultry in a disease emergency. Department of Agriculture and Food, State Veterinary Service, Dublin, Ireland.

Savory, C. J., L. Kostal, and I. M. Nevison. 2006. Circadian variation in heart rate, blood pressure, body temperature and EEG of immature broiler breeder chickens in restricted-fed and ad libitum-fed states. Brit. Poultry Sci. 47:599-606.

Savory, C. J., and L. Kostal. 1997. Application of a radiotelemetry system for chronic measurement of blood pressure, heart rate, EEG, and activity in the chicken. Physiol. Behav. 61:963-969.

Senne, D. A. 2007. Avian influenza in north and South America, 2002-2005 [electronic resource]. Avian Dis. 51:167-73.

Senne, Dennis A. 2010. Avian influenza in North and South America, the Caribbean, and Australia, 2006–2008 [electronic resource]. Avian Dis. 54:179-186.

Sims, L., and C. Narrod. Understanding avian influenza. in Food and Agriculture Organization [database online]. 2010 Available from

http://www.fao.org/avianflu/documents/key_ai/key_book_preface.htm (accessed March 2010).

Sims, L. D., J. Domenech, C. Benigno, S. Kahn, A. Kamata, J. Lubroth, V. Martin, and P. Roeder. 2005. Origin and evolution of highly pathogenic H5N1 avian influenza in Asia. Vet. Rec. 157:159.

Swayne, D. E., and D. A. Halvorson. 2008. Influenza. In Diseases of poultry., eds. Y. M. Saif, A. M. Fadly, J. R. Glisson, L. R. McDougald, L. K. Nolan and D. E. Swayne. 12th ed., 153-184. Ames, Iowa, USA: Blackwell Publishing Professional.

United States Department of Agriculture, Animal and Plant Health Inspection Service (USDA-APHIS). 2007. Summary of the national highly pathogenic avian influenza (HPAI) response plan. 1-86.

Velkers, F. C., A. Bouma, M. G. R. Matthijs, G. Koch, S. T. Westendorp, and J. A. Stegeman. 2006. Outbreak of avian influenza H7N3 on a turkey farm in the Netherlands. Vet. Rec. 159:403-405.

Webster, A. B., and D. L. Fletcher. 2001. Reactions of laying hens and broilers to different gases used for stunning poultry. Poultry Sci. 80:1371-1377.

World Health Organization. Avian influenza. In World Health Organization [database online]. 20102010]. Available from http://www.who.int/csr/disease/avian_influenza/en (accessed February 5 2010).

Xu, Xiyan, K. Subbarao, N. J. Cox, and Y. Guo. 1999. Genetic characterization of the pathogenic influenza A/Goose/Guangdong/1/96 (H5N1) virus: Similarity of its hemagglutinin gene to those of H5N1 viruses from the 1997 outbreaks in Hong Kong. Virology 261:15-19.

Yee, K. S., T. E. Carpenter, and C. J. Cardona. 2009. Epidemiology of H5N1 avian influenza. Comp. Immunol. Microb. 32:325-340.