Cover Sheet
Final Report Project BRU007: March 30, 2017
Submited to:
John R. Glisson, DVM, MAM, PhD
U.S. Poultry & Egg Association
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Tucker, GA 30084-7303

Title: Evaluating hen behavior and physiological stressors during VSD for the development of humane methodologies for mass depopulation during a disease outbreak

Principal Investigator: Kenneth E. Anderson, PhD

Research Organization(s) and Address:
North Carolina State University
Prestage Dept. of Poultry Science
Scott Hall, Rm 237
2711 Founders Drive
Raleigh, NC 27695-7608

Email: kanderso@ncsu.edu
Office: (919) 515-5527
Fax: (919) 515-7070

Co-investigators:

Dr. Kimberly Livingston	North Carolina State University, CALS Prestage Dept. of Poultry Science
Dr. Sanjay Shah	North Carolina State University, CALS Biological and Agricultural Engineering
Dr. Mike Martin	North Carolina State University, CVM Population Health and Pathobiology, Poultry Health Management Team

Collaborator:

Dr. Wallace Berry	Auburn University Poultry Science Dept.

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Industry Summary

In 2015, the egg industry was hit with the worst highly pathogenic avian influenza outbreak in U.S. poultry history. Timely depopulation was identified as a critical measure to contain the outbreak. Current depopulation methods, including CO_2 kill carts, CO_2 injection and fire-fighting foam, were quickly overwhelmed and prolonged the suffering of infected birds.

The overall goal of this project was to evaluate welfare parameters of ventilation shut down (VSD) for depopulating laying hens in caged systems through monitoring environmental parameters, behavior and stress physiology. The specific objectives were to 1) determine the time to brain death from VSD using electroencephalograms (EEG), and 2) examine the effectiveness of VSD in a multi-level, commercial cage setting.

This project provided the means to develop and evaluate VSD and other depopulation methods including VSD combined with heat (VSDH) and CO_2 (VSDCO). The first aspect of this project was to develop an environmental profile of an individual hen housed in a cage layer facility. Environmental conditions included building volume (3.4 ft^3/hen) and temperature, relative humidity (RH) and CO_2 recordings to understand the dynamics within the environment for each method. This allowed for the determination of the duration to time of death (TOD). The environmental temperature, CO_2 and RH were similar at the start of each test at 89 ºF, 0.22 percent and 31.6 percent, respectively. At the end of the tests the environmental temperature was highest for VSDH at 107 ºF. VSDCO had the highest level of CO_2 at 31.5 percent. RH increased in VSD and VSDH to 62.5 and 66.0 percent, respectively. The high level of RH appears to have contributed to the diminished ability to reduce core body temperature (CBT). At TOD the CBT was highest for VSD (113.1 ºF) and VSDH (115.3 ºF) while VSDCO CBT was (105.8 ºF). The time to reach TOD was longest in VSD at 91 minutes followed by VSDH at 54 minutes and VSDCO with the shortest TOD at 12 minutes. Comparing EEGs and behavior profiles demonstrated that VSD hens spent 82 percent of the time unconscious while the VSDH and VSDCO hens were unconscious 56 percent and 65 percent of the time, respectively. It was also observed that the stress indicator of heat shock protein 70 (HSP70), for hens exposed to stressors such as heat, humidity or CO_2, declined in the VSD and VSDH environments from time 0 to TOD possibly due to the duration of the unconscious state of the hens.

A field scale up of the process to evaluate the effectiveness of VSD, VSDH and VSDCO in a multi-tier cage system was conducted using white leghorns housed in 2-tier stair step cage system at industry densities (72 in^2/hen). The environment was a force ventilated negative static pressure room sealed to prevent air exchange of any type. An inner chamber around the cages emulated the building volume per hen found in the industry. The data collected included CO_2, RH, environmental temperature profiles, CBT and HSP70. We found that VSD by itself did not result in 100 percent euthanasia of the flock with 4 percent of the hens surviving. When we added heat or CO_2 to the VSD system we accomplished 100 percent mortality. The duration to TOD was no different between VSDH and VSDCO. Based upon these field studies, VSDH and VSDCO appear to be the most humane methods of depopulating large numbers of caged he

NC STATE UNIVERSITY

College of Agriculture and Life Sciences
Prestage Department of Poultry Science

http://harvest.cals.ncsu.edu/

Campus Box 7608
2711 Founders Drive, Scott Hall
Raleigh, NC 27695-7608
P: (919) 515-2626

Scientific Report

Materials and Methods:

The project was approved by the North Carolina State University's IACUC and monitored by animal welfare specialists, North Carolina State University's attending veterinarian and other veterinarians within NCSU's School of Veterinary Medicine. All animals were maintained on North Carolina State University property for the duration of the study. In Phase 3, since we were dealing with a potentially lethal environment the NCSU IACUC Approval, was contingent upon Environmental Health and Safety Training for "Self-Contained Breathing Apparatus" (SCBA) required that training for entry into potentially lethal environment training be conducted for at least 4 of the research group. In order to conduct each trial in Phase 3 we had to have at least 2 individuals present during the trial who were certified for SCBA entry into a potentially lethal environment. To monitor the hens activities in Phase 3, 4 remote video cameras were mounted inside the inner chamber so the hens could be monitored without having to dress out in SCBA Gear and enter the room. The cameras were mounted on both the upper and lower tiers on opposite sides of the cage row so that all of the locations could be observed simultaneously. In phase 3 the behaviors were not recorded but those behaviors observed in Phase 1 were the guidance for the lethality of the process.

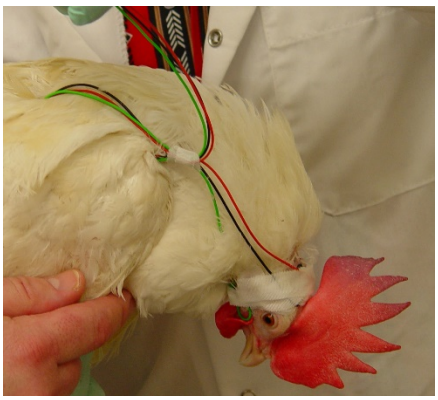
Phase 1:
A series of 4 replicates were conducted with 4 ventilation shut down (VSD) treatments. These treatments were ventilation shut down (VSD), VSD + Heat (VSDH), VSD + CO_2 (VSDCO), and VSD + Heat + CO_2 (VSDHCO) using a total of 16 white commercial leghorn chickens approximately 69 weeks of age. The hens were randomly chosen for the study from a small flock maintained at the NC State University research facility. The hens were housed in single bird conventional cages and had no procedures beyond the protocol for at least one week prior to being tested. Before electrode placement, an elastic hobble was placed on the hen's shanks above the dew claw (Figure 1). This hobble allowed the hen to stand and walk but did not allow it to raise its leg towards its head, which could cause removal of the EEG leads. At least one day prior to sampling, each hen had the feathers removed from the external occipital crest on each side of the comb and at the base of the neck five minutes after application of a 2% topical lidocaine gel. On the day of testing, three monopolar 32-gauge needle electrodes (AD Instruments, Grand Junction, CO) were inserted subcutaneously three minutes after application of lidocaine to the occipital crest area and base of the neck. The red and black electrodes were inserted behind the chicken's comb along the external occipital crest of the cerebral cranium on either side of the brain, while the green (ground) electrode was inserted between the cerebellar fossa of the lower mandible between the waddles in the neck (Figure 2) (Chamberlain, 1943). All electrode tips were secured to the head using ¼" surgical adhesive tape passing the tape around

each electrode leaving adhesive ‘wings’ and then attaching the adhesive ‘wings’ to the hen after electrode insertion. The electrode leads were then taped together, run under the bird’s wing and looped around the head of the humerus at the scapular joint to reduce entanglement and eliminate dislocation of the electrodes (Figure 3). The bird was then placed in an individual treatment/observation chamber to adjust 1-2 minutes prior to inducing varied physical stressors. This adjustment period allowed for the visual identification of movement artifacts in the EEG activity due to walking, preening, and/or wing flapping.

Figure 1. A hobbled leghorn hen

Figure 2. Red and black electrode placement along the external occipital crest of the cerebral cranium on each side of the comb.

Figure 3. Electrode leads looped around the wing to reduce entanglement and eliminate dislocation of the electrodes.



Individual Treatment/Observation Chambers

Individual treatment/observation chambers were utilized to induce a variety of environmental stressors, including ventilation shutdown (VSD), supplemental heat, and increased carbon

dioxide (CO_2) levels, singly or in combination. Each 3.4 ft^3 cube-shaped chamber was made of 0.25 in clear Plexiglas and covered with a 1-in closed cell foil-backed Styrofoam on one side to reduce chamber heat loss; the remaining front side was not covered to allow observation of the hen. Fresh air was brought into the chamber through a 1-in hole close to the bottom of a vertical face and the stale air was exhausted using a 12VDC fan (Model: OD3010-12HB01A, Orion Fans, China) installed on the face close to the ceiling of the chamber. With an airflow rate of 5 cfm (at 0-in of water column), the fan provided the hot weather ventilation rate for layers recommended by Midwest Plan Service (1990). During the study, when one or more of the environmental stressors were applied to the bird, the fan was turned off and both the air inlet and fan outlet were plugged with rubber stoppers. The heat source was a 12-in long 75-watt incandescent lamp (Model: 75T8C, Bulbrite, Moonachie, NJ) placed in an aluminum reflector mounted on the ceiling of the chamber. In order to allow the hen to maintain a normal posture and grip, a 1-in x 2-in welded wire raised floor was placed in the chamber.

Electroencephalogram

Differential recording electrodes, insulated except at the tips, were attached to a pre-amplifier which transferred the EEG signals to a laptop based recording system. The EEG data was recorded at a standard EEG frequency band of 10–50 Hz, and sampled at 100 Hz/channel using Powerlab 8SP equipped with Lab Chart 4 software (AD Instruments, Grand Junction, CO). Then the digital filter was set at a band-pass frequency of 10 Hz (low) to 30 Hz (high) and the transition width was set at auto adjust and the mV range was set at -0.04 to 0.04 mV. The settings were selected to facilitate the interpretation for overall brainwave activity in a chicken. EEG activity in 0.5 sec intervals was recorded from the point the ground lead was inserted up to a maximum of 2.5 hrs post-placement in an individual treatment/observation chamber.

Validation Electroencephalogram

In order to ensure that the observed electrical activity was neural (EEG) and not muscular (EMG), a validation test was conducted using a strobe light for photic stimulation (Bullock et. al., 1990; A'kos Szabo´ et. al., 2016). The EEG equipment was attached to a flock mate of the three hens described above and placed in an individual treatment/observation chamber within a completely darkened room. A 25-Hz strobe light was turned on four times for 20-second over a ten-minute period. EEGs and behavior data was collected concurrently.

Behavior Observations

Video cameras were positioned in front of each individual treatment/observation chamber in order to clearly collect behavior data (GoPro Hero 4, GoPro, San Mateo, CA).

Table 1. Descriptions of the recorded behaviors

Behavior	Description
Conscious	
Headshake	Rapid shaking or lateral movement of the head
Mandibulation	Repetitive tasting movement with bill
Standing	Legs extended, fully upright
Wing flapping	A bout of continuous, rapid wing flapping
Crouch	Legs are folded under bird with body positioned on top
Unconscious	

Panting	Deeper than normal inspiration through the open mouth, generally accompanied by movement of the tongue and bill
Respiratory disruption	Deep open bill breathing with prolonged inspiration or prolonged open bill gasping, or both, combined with difficulty inhaling
Loss of Posture	Loss of balance or posture, or both (lateral recumbency)

Recording began from the start of treatment until brainwave activity was below 0.01 mV and time of death was called. Behavioral actions as described by Webster and Fletcher (2001) and Coenen et. al (2009) were recorded from the video tapes using a modified scanning technique based on Anderson et al (1989) using a 10 sec observational interval by a trained observer. The behavioral actions were then broken down into two categories—conscious and unconscious—as listed in **Table 1**. Then the behaviors were summarized over 1 min interval to parallel the EEG brainwave activity.

Thermal environment

Four temperature and RH sensors (Lascar Electronics, Salisbury, UK; Model: EL-USB-2-LCD; Accuracy: ±0.5 °C and ±3%) were used to measure temperature and RH 4 cm from the top of each chamber. Temperatures, and RH, were recorded every minute.

Carbon dioxide (CO_2) concentrations

Chamber CO_2 concentrations were monitored and recorded using four ISU infrared monitors portable monitor units (PMU's) at 1-min intervals, covering spatial variations. Carbon dioxide concentrations were measured with IR sensors (CO_2Meter, Inc., Ormond Beach, FL) with their sampling ports collocated with the temperature and RH sensors. Two 0-100% CO_2 monitors (Model: CM-0003; Accuracy: ±70 ppm±5% of measured value) measured CO_2, on top of the top tier while two 0-5% CO_2 monitors (Model: CM-0056; Accuracy: ±70 ppm±5% of measured value) measured CO_2, on top of the bottom tier. The CO_2 monitors were mounted on the French door so that CO_2 levels could be monitored from outside the room; the sampled air was released back into the room, close to the floor. Prior to this test as well as subsequent tests, all CO_2 sensors were calibrated at ambient concentrations using a more-accurate handheld CO_2 meter following the procedure recommended by the supplier. CO_2 concentrations were recorded every minute which were set up in the same fashion as the temperature and RH probes.

Core body temperature

Core body temperature was measured at start of each trial for each of the chambered hens. The CBT was measured immediately after the hen's panting ceased and pronounced dead by the veterinarians present as close to TOD as possible.

Phase 2:

This aspect of the study was to examine the humane aspects and effectiveness of ventilation shut down (VSD) for depopulating laying hens in cage systems by measuring stress physiology via heat shock protein 70 (HSP70) and blood chemistry (BC). In this study the individual treatment/observation chambers as described in Phase 1 were utilized to induce a uniform environmental stressors, including the 3 treatments of ventilation shutdown (VSD), supplemental heat (VSDH), and increased carbon dioxide (VSDCO) levels.

The Four individual treatment/observation chambers were set up identically for VSD, VSDH, and VSDCO treatments. For each treatment, 2 trials were conducted for each treatment. Five hens were selected from the NCSU flock for each trial. The hens were placed 1/chamber and the ventilation fans were started. The 5th hen served as the time 0 sample. At time 0, the Chambers were sealed, and the environmental conditions imposed. The sampling sequence was time-based, derived from the established time of death (TOD) therefor, hens were sampled at time 0 to establish the baseline for HSP70 and BC, then hens were sampled at ¼, ½, and ¾ point of the duration to TOD and as close as possible to TOD. The total hens sampled were 30 (5 per treatment).

At each sampling point, the hen was removed from the chamber, blood was collected for blood chemistry analysis within 60 sec. The hen was restrained on its side and bled via the brachial vein. 1.5 to 2 ml were collected in a 3 ml syringe then transferred to BD Vacutainer tube with Lithium Heparin the heparinized blood was then placed on the *i*-STAT® diagnostic system (CG8) for blood chemistry analysis. The hen was then euthanized by trained individuals via cervical dislocation, then the brain tissue was collected, snap frozen in Liquid Nitrogen for transport to the laboratory and placed in -80°C for HSP70 analysis.

Phase 3:

At the start of each experiment, 144 end-of-lay white or brown egg laying hens were obtained from a local commercial egg laying company. All hens were maintained in two multi-tiered, full stair step conventional cage systems for 5-7 days prior to treatment application to acclimate to the environment. Three birds were added to each cage upon arrival. The cage systems were kept in a 29.5 ft x 15 ft x 8 ft room at the Poultry Entomology Research Unit at NC State University. On the south end of the room, an 8 ft x 7 ft roll door was flanked by one 12 in (470 cfm) fan and one 24 in (2,500 cfm) fan. The north end contained a set of 5.83 ft x 6.5 ft French doors and an intake vent. A 60,000 BTU propane heater was located in the northeast corner. Each cage was 12 in x 18 in (72 in^2 per bird) and provided 12 in feeder space and 2 nipple drinkers. Birds were maintained on the NC State University Layer diet. Feed and water were provided ad libidum.

Thermal environment

Four temperature and RH sensors (Lascar Electronics, Salisbury, UK; Model: EL-USB-2-LCD; Accuracy: ±0.5 °C and ±3%) were used to measure temperature and RH on top of the lower tier (two, mid-lengths of cages 1 and 2, Fig. 1) and on top of the upper tier (two, mid-lengths of cages 1 and 2, Fig. 1). Temperatures, and RH, were recorded every minute which were set up in the same fashion as the temperature and RH probes.

Carbon dioxide (CO_2) concentrations

Chamber CO_2 concentrations were monitored as in Phase 1and recorded using four ISU portable monitor units (PMU's) at 1-min intervals. with their sampling ports collocated with the temperature and RH sensors. Two 0-100% CO_2 monitors measured CO_2, on top of the top tier while two 0-5% CO_2 monitors measured CO_2, on top of the bottom tier. The CO_2 monitors were mounted on the French door so that CO_2 levels could be monitored from outside the room; the sampled air was released back into the room, close to the floor. Prior to this test as well as

subsequent tests, all CO_2 sensors were calibrated at ambient concentrations using a more-accurate handheld CO_2 meter following the procedure recommended by the supplier. CO_2 concentrations were recorded every minute which were set up in the same fashion as the temperature and RH probes.

Core body temperature

Core body temperature was measured in a random sample of hens at start of each trial 5 from the upper level and 5 from the lower cage levels. At the end of each trial the CBT was measured as soon after the hen's panting ceased and pronounced dead TOD by the veterinarians present as possible. The CBT was measured to represent an equal number of hens in the top tier and bottom tier of the cage row.

Pilot study

A pilot study was conducted to determine facility parameters and their impact on the process of ventilation shutdown. A false wall was built to allow for evacuation of potentially dangerous levels of CO_2 as requested by EHS. This reduced the room's dimensions to 19.5 ft x 15 ft x 8 ft, a reduction of 1,200 ft^3 from the original room size. The false wall was constructed of 2x4 studs covered with 2 in GreenGuard® XPS Extruded Polystyrene Insulation Board (Kingspan, Atlanta, GA). An evacuation panel was cut in the center of the false wall to allow for the quick release of gases to outside of the building. Temperature and RH probes as well as the intake pumps for four CO_2 monitors were placed on each level of the cage system in the back and front of the room to collect environmental data. Outside environmental conditions were obtained via The Weather Channel. The CO_2 monitors were placed by the entry so that levels could be monitored from outside the room. GoPro cameras (GoPro, San Mateo, California) were positioned on the cages to allow for determination of loss of posture and discourage entry of the room until all birds were observed to be euthanized.

To begin sealing the room, the ventilation was turned off and the seams of the false wall, evacuation panel, and intake vent were taped with a ½ foam adhesive to prevent any airflow or leakage. The temperature and RH probes, CO_2 monitors, and cameras were turned on. Feed was left in the feeding troughs and the water was kept on. The French doors were then shut and sealed with 2 in adhesive around the entire door frame. Once the doors were completely sealed, environmental and behavior monitoring began.

***Experiment 1-Ventilation Shutdown* (VSD)**

Information gathered from the pilot study led to the development of chamber around the cage system to decrease the cubic foot per bird from 14.2 ft^3 to as close to the industry standard 4.14 ft^3 for white laying hens. The 15 ft x 7.5 ft x 5.3 ft chamber was constructed of 2x4 studs and 10 ml polyethylene plastic. The walls of the chamber attached to the false wall completely enclosing the cage system.

To seal the chamber, walls were attached to the false wall and to each other using screws. Then the plastic ceiling was screwed into place, completely sealing the cage system. The room was

then sealed, the ventilation was turned off and the seams of the false wall, evacuation panel, and intake vent were taped with a ½ foam adhesive to prevent any airflow or leakage. The temperature and RH probes, CO_2 monitors, and cameras were turned on. Feed was left in the feeding troughs and the water was turned off. The French doors were then shut and sealed with 2 in adhesive around the entire door frame. Once the doors were completely sealed, environmental and behavior monitoring began.

The goal was to achieve a heated temperature of 40 to 41°C (104-106 F) which was similar to the starting core body temperature of the hens. A 60,000 BTU propane heater was located in the northeast corner (LB White, Onalaska, WI) located outside the inner chamber. This heater was used to emulate commercial surroundings ensuring that surrounding air temperature was equal to the air temperature within the chamber. This minimized the heat transfer across the 10 mil plastic chamber and more accurately represented what the hens would experience in a larger commercial facility where they would be surrounded by additional hens.

Experiment 2- Ventilation Shutdown + Heat **(VSDH)**

This experiment was conducted in a manner identical to Experiment 1 except that supplemental heat was provided inside the enclosure. Two 1-kW convective heaters were at opposite ends of the enclosure, directly beneath the top tier of cages and they blew hot air towards the middle. The goal was to achieve a heated temperature of 40 to 41°C which was similar to the starting core body temperature of the hens. A 60,000 BTU propane heater was located in the northeast corner (LB White, Onalaska, WI) located outside the inner chamber was used to ensure the surrounding air temperature was equal to the air temperature within the chamber. This minimized the heat transfer across the 10 mil plastic chamber and more accurately represented what the hens would experience in a larger commercial facility.

Experiment 3- Ventilation Shutdown + CO_2 **(VSDCO)**

Prior to sealing the room as described in Experiment 1, the day prior to the CO_2 trial distribution hoses for the CO_2 gas were put in place. Heaters were used to warm the regulators to ensure the carbon dioxide was released through two ¼ in. inside diameter flexible tubes at the opposite end of the enclosure from the partition at floor level. A small computer fan (100 cfm) located at 5 ft height installed in the partition was used to evacuate air from the enclosure as the CO_2 displaced the existing air to ensure that the enclosure did not get over-pressurized which could have resulted in leakage. One CO_2 sensor was used to monitor CO_2 concentration at the fan outlet for 9 min based on the fan airflow rate and enclosure volume. After 9 min, the CO_2 sensor's sampling inlet was switched from the fan outlet to the enclosure sampling location.

Statistical analysis

Phase 1: Prior to analysis, the EEG data mV of the EEG Wavelength was summarized over 10 sec. intervals to consolidate the 102 EEG data points. For each of the EEG data sets (graphs) were transformed by taking the absolute value of the integral, to mitigate the baseline noise, relative to the baseline at each 10 second interval. Each value was then subjected to the Hyperbolic Arcsin to emphasize the lower mV readings were filtered (The integral gets rid and

accounts for the inherent "noise"). Data was analyzed with GLM with full factorial effects for CO2 and heat were fit to each of several response variables and all pairwise comparisons used Tukey's HSD. The transformed EEG analysis used the integral area under the curve calculated using the Trapezoid method; using a NPARM analysis.

The behavior data was collected by a trained observer and recorded as the principle behaviors performed during the 10 sec interval. These were summarized as a frequency of behaviors performed as a conscious (voluntary) or unconscious (involuntary) behavior. The summarized EEG brainwave activity over the same time intervals was then overlaid with the birds observed behavioral frequencies. Then a correlation analysis examining the relationship between EEG brainwave activity (mV) and conscious (voluntary) or unconscious (involuntary) behavior was accomplished using Pearson Linear Correlation Analysis in SAS JMP-PRO® 12.2.0 (SAS Institute, 1989).

Blood chemistry was analyzed using the *i*-STAT® diagnostic system. The relative expression of HSP70 mRNA in total brain samples was measured using RT-PCR and reported as HSP70/18S. HSP70 data were transformed (HSP70/18S)^-1 and analyzed using a one-way ANOVA. The mean comparisons for all pairs used Tukey-Kramer HSD Confidence Quantile (q*=3.76082; alpha=0.05). The blood chemistry was analyzed using GLM with means separated using Students-T Test.

Results and Discussion:

Phase 1:

Shown in Table 1, the chamber conditions of temperature, % CO_2, and % RH were all similar for all of the treatments at the start averaging 89 ºF, 0.22 % and 31.6 %, respectively. Surprisingly, at the end of each of the trials the chamber temperatures for VSD, VSDCO and VSDHCO were not significantly different having similar ending temperatures. Theoretically, it was expected that the VSD chamber would have had a significantly higher temperature at the end due to the body heat production of the hen being conducted to the chamber environment during the VSD process. The room was kept at about 88-90 F so heat loss from the chamber would be lower compared with VSDH where the thermal gradient between the inside and outside would be higher.

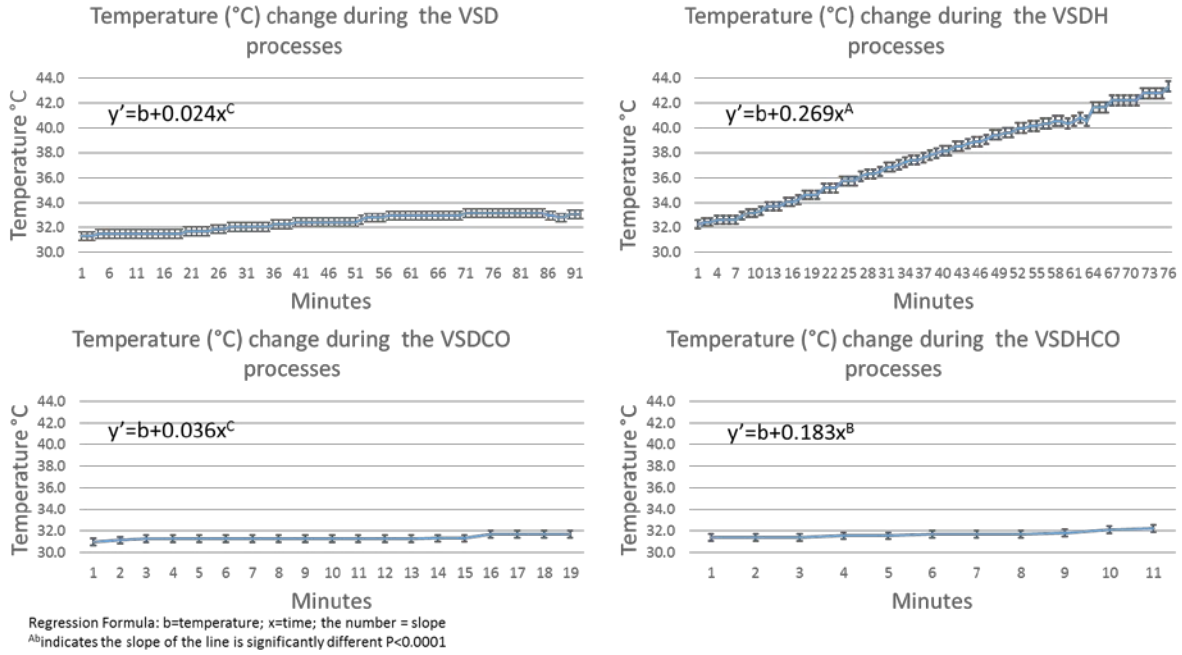
Table 1. Temperature, CO_2 and Relative Humidity in the Chambers

Treatment	Temperature (ºF)		CO_2 Concentration (%)		Relative Humidity (%)	
	Start	End	Start	End	Start	End
SD	88.5	91.5^{B}	0.09	2.61^{B}	29.50	62.50^{A}
VSDH	90.0	107.0^{A}	0.27	2.36^{B}	34.50	66.00^{A}
VSDCO	88.0	88.3^{B}	0.22	34.41^{A}	32.75	30.38^{B}
VSDHCO	88.5	91.3^{B}	0.33	31.52^{A}	30.00	34.38^{B}
SEM	±0.7	±1.0	±0.01	±1.81	±4.39	±1.81
p-value	0.2008	<0.0001	0.1159	<0.0001	0.8332	0.0004

^{AB}Means significantly different

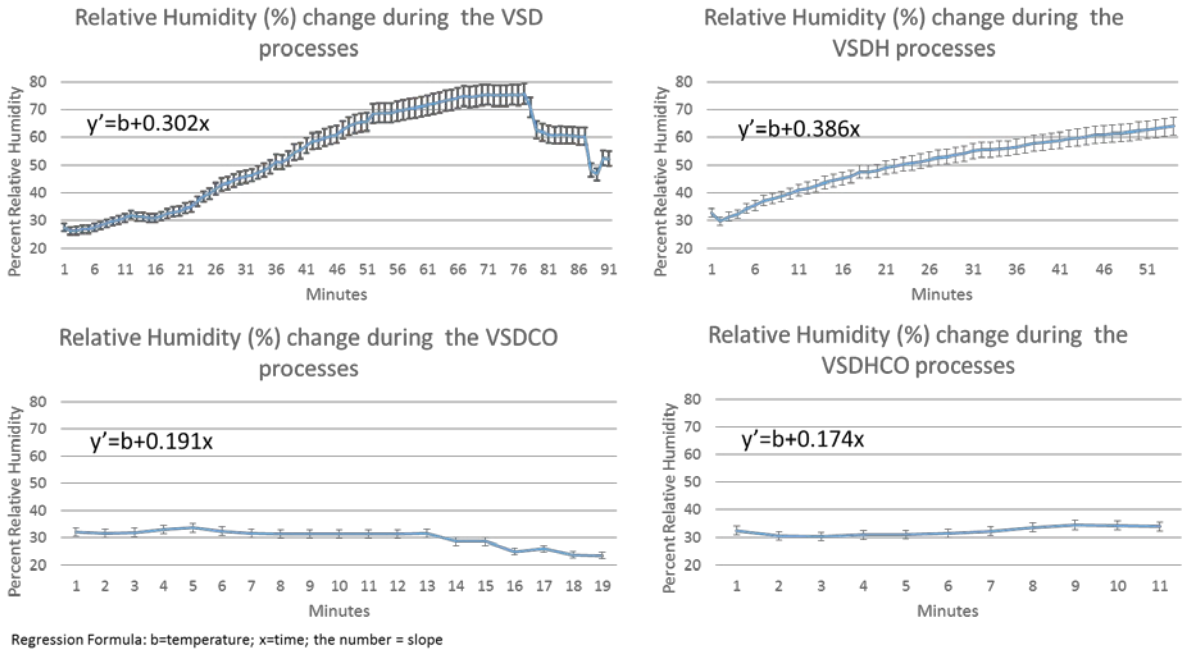
However, Figure 1 shows the temperature curves for the various VSD treatments and surprisingly the slope of the temperature profiles doe VSD and VSDCO were no different. Logically the VSDH temperature curve had the steepest ($P<0.0001$) slope for any of the treatments with the VSDHCO being intermediate.

Figure 1. Effect of VSD, VSDH, VSDCO and VSDHCO on temperature changes during the processes to TOD.



Regression Formula: b=temperature; x=time; the number = slope
[Ab]indicates the slope of the line is significantly different P<0.0001

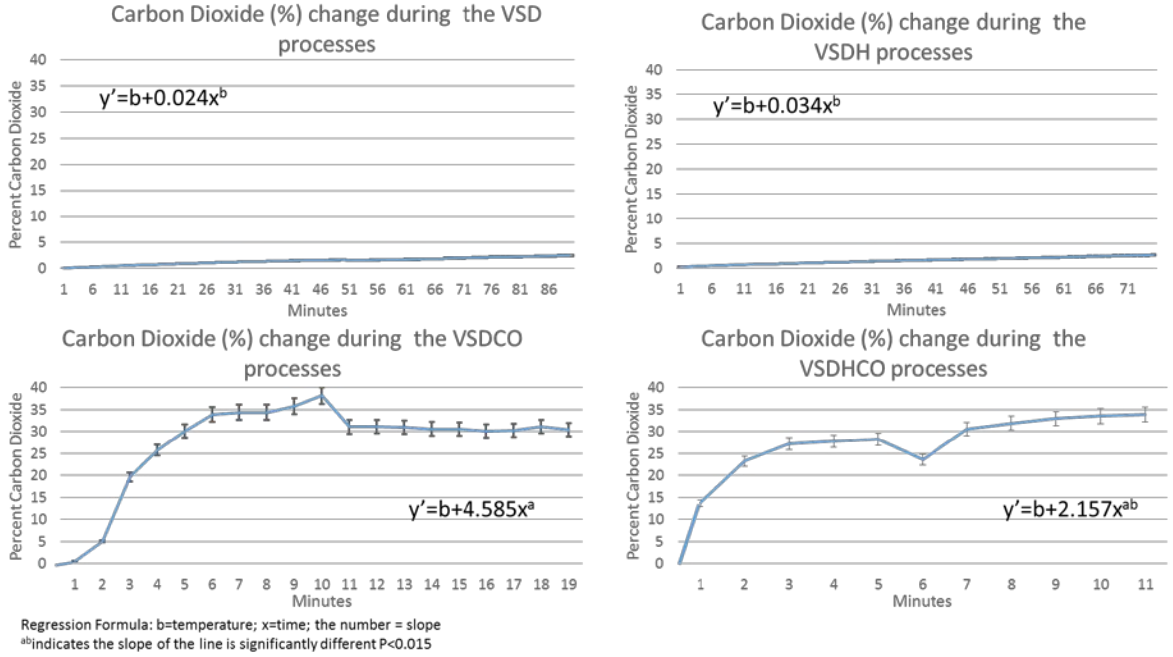
Figure 2. Effect of VSD, VSDH, VSDCO and VSDHCO on percent relative humidity changes during the processes to the average TOD for the hens.



Regression Formula: b=temperature; x=time; the number = slope

Since temperature did not appear to be the primary contributor to hyperthermia in the VSD treatment as the primary component related to the TOD as was the case. The hyperthermia appeared to be caused by a combination of higher temperature and sufficient high RH that combined to produce very high heat stress. Of course the high RH reduced the bird's ability to lose latent heat while the high chamber temp impeded the bird's ability to lose sensible heat. However, the VSDH temperature was higher ($P<0.0001$) at 107 ºF which facilitated the VSDH process in less time (Table 2). Indications are that other factors contributed to the inability of the hen to dissipate core body heat in the VSD process, possibly related to the increase in RH% not allowing for evaporative heat loss as shown in Figure 2. The potential is that if the RH% becomes too high the hen will not be able to dissipate body heat through evaporation in a warm environment (Table 2). Even though the slopes related to RH percentage changes were not significantly different between the 4 VSD methods from the start to TOD, however, the RH % increased ($P<0.0004$) in the VSD and VSDH by more than 30% from an average of 31.6% to 62.5 and 66.0 %, respectively (Table 1). In the VSD trials the RH% dropped as the hens neared TOD.

Figure 3. Effect of VSD, VSDH, VSDCO and VSDHCO on percent carbon dioxide changes during the processes to the average TOD for the hens.



Regression Formula: b=temperature; x=time; the number = slope

[ab]indicates the slope of the line is significantly different $P<0.015$

It was interesting that in the VSD and VSDH environments the CO_2 levels of 2.61 and 2.36 %, respectively, basically resulted in increased panting in the hens. Whereas Chamber % CO_2 was logically elevated in the VSDCO, and VSDHCO where CO_2 was injected into the chambers, therefore had the highest ($P<0.0001$) ending CO_2 % at 34.41 and 31.52 %, respectively.

The core body temperature (CBT) of the hens as shown in Table 2, at the start of all of the trials, the hens temperatures were not different, ranging from 105.1 to 106.4 ºF. However, at time of death (TOD) the CBT was highest ($P<0.0001$) for VSD (113.1 ºF) and VSDH (115.3 ºF) this would be the result of Hyperthermia as the primary cause of death in these two treatment groups.

While in the VSDCO and VSDHCO treatment groups the CBT at death was 105.8 ºF for both the CO_2 treatments, this would be consistent with the death of the hens by asphyxiation rather than Hyperthermia.

Table 2, shows that the duration to reach TOD was longest (P<0.0001) for the VSD at 5,497 s (91.6 min) which could have also contributed to the elevated CBT in those hens rather than for VSDCO, and VSDHCO being the shortest times at 690 s (11.5 min) and 552 s (9.2 min) respectively. The hens TOD for the VSDH treatment were intermediate at 3,202 s (53.4 min). The hens EEG waves in the 0 to 0.01 mV were determined to indicate unconsciousness in the hens by the behavior observations in relation to the EEG. Even though the VSD treatment had the greatest duration to TOD the hens was in the unconscious state 82% of the time that hen's EEG mV in the less than 0.01 mV range. The VSDH hens EEG indicated that they spent 56% of the time in an unconscious state and both VSDCO and VSDHCO spent 65% of the time in an unconscious state. This would indicate with the work in the chamber that even though the VSD process was extended the actual time in which the hen could perceive any distress was actually shorter than the VSDH hens. In the chamber setting the CO_2 treatments did have the shortest period of time in a conscious state.

Table 2. Effect of VSD Treatment Groups on Core Body Temperature, Time of Death (TOD) and the Strength of the EEG Wave (mV)

Treatment	Core Body Temp (°F)		TOD	Percent of time within eacg EEG mV Range			
	Start	End	(sec)	0 - 0.01 mV	0.01-0.03 mV	0.03-0.05 mV	>0.05 mV
VSD	105.5	113.1^{A}	5497^{A}	82	15	5	7
VSDH	105.7	115.3^{A}	3202^{AB}	56	31	19	13
VSDCO	106.4	105.8^{B}	690^{B}	65	16	2	7
VSDHCO	105.1	105.8^{B}	552^{B}	65	11	4	1
SEM	±0.5	±0.5	±737	±16	±6	±9	±7

^{ABC}Means significantly different P<0.0001

Figure 4 shows the complete composite curves of the transformed EEGs for each of the four treatments used in Phase 1. The most striking aspect associated with this Figure is associated with the first 300 sec (5 min) of time in which the hens were in the chambers and exposed to the different treatments. The highlighted section is the primary point of EEG wave strength where the hens appeared to be the most active and appeared to be conscious and aware of their surroundings and the environment. After this initial 5 min. period the hens were typically in lateral recumbence, quiet, and panting appearing to be relying on autonomic behavior for survival in an unconscious state. This pattern was supported by the EEGs which were below the 0.01 mV threshold for unconsciousness. After this point there were short bursts of activity (Seconds) where the hen would wing flap or jump then immediately return to lateral recumbence and panting. These bursts were accompanied by EEG spikes above the 0.01mV level indicating the hen may be conscious. As each of the processes continued toward the end nearing the TOD, the incidence of behavior bursts and EEG spikes decreased and the hens tended to remain in a single position.

Figure 4. Complete Composite Transformed EEG graph for TOD of all treatments averaged over 4 trials n=16

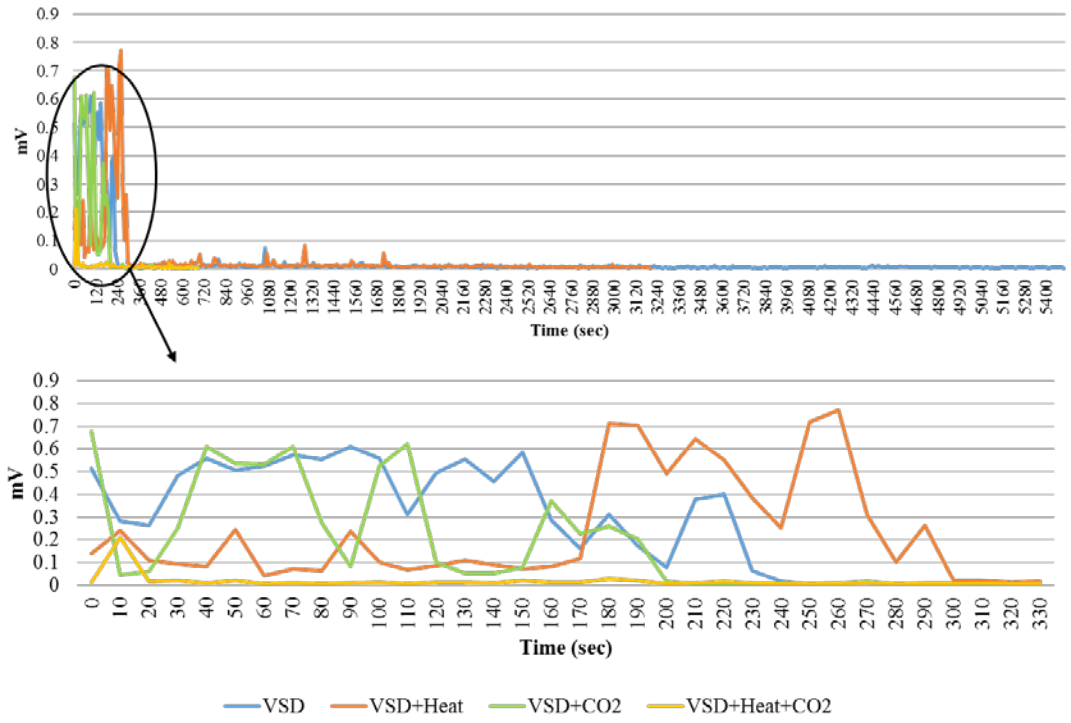


Figure 5, illustrates that the analysis of the EEG graphs for each of the VSD methods were not significantly different. There were no significant differences between the slopes of the transformed EEG curves that are shown in Figure 4. This is primarily due to the variation between the hens used throughout the study and their individual responses to the various treatments. The variation between hens will be the barrier to an effective process with 100% effectiveness as required by the AVMA in any process.

Figure 5. Analysis comparing the slopes of the Transformed EEG graphs for VSD, VSDH, VSDCO, and VSDHCO.

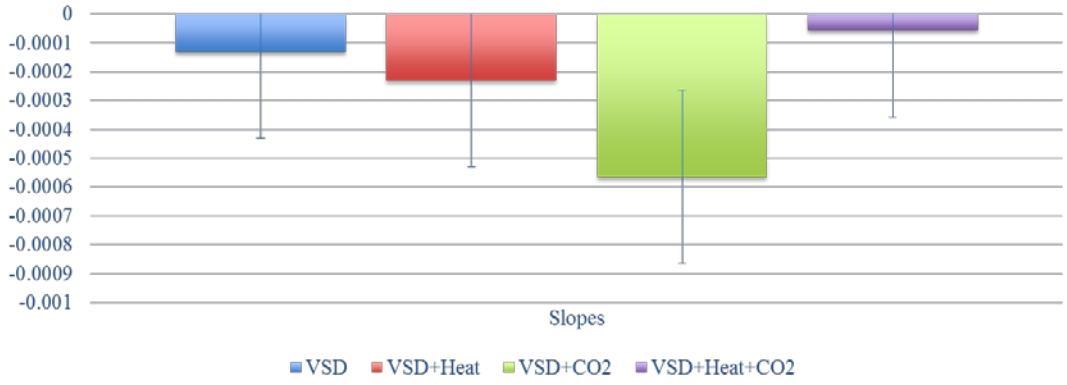
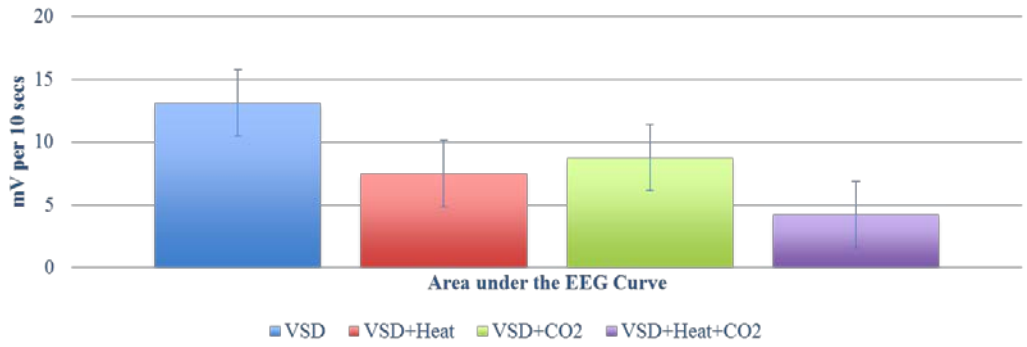


Figure 6. Comparison of the Integral area under the EEG graph calculated using the trapezoid method



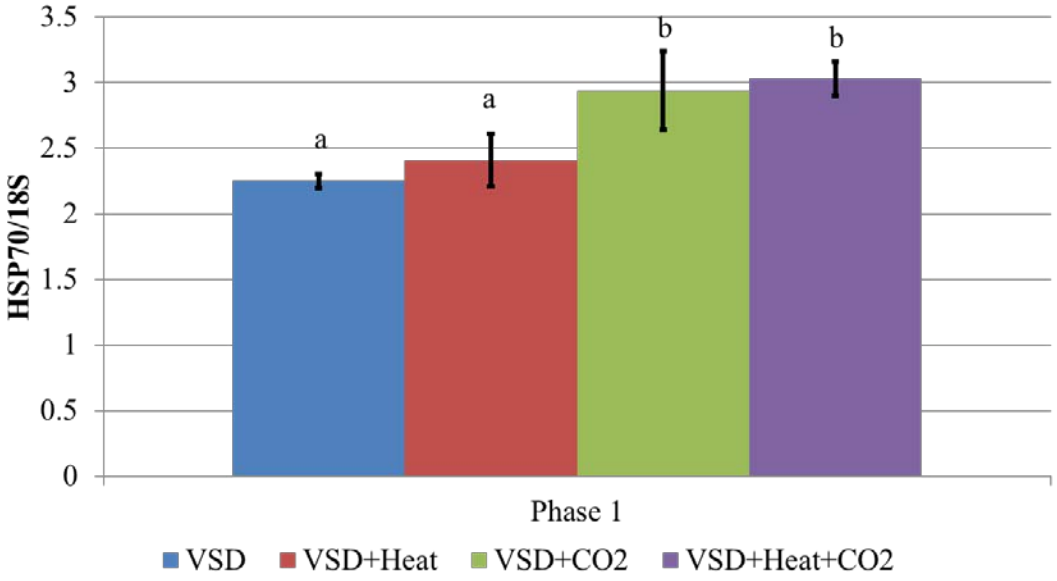
The slopes of the EEG Graphs did not have different slopes, so to verify that the curves were not different the integral area under the EEG curves for each of the hens in the 4 treatments were calculated. The analysis shown in Figure 6 indicated that areas were not significantly different (P=0.2007). This analysis reduced the amount of variation between the hens within a treatment which would allow us to potentially identify a difference between the 4 methods if it was present.

Table 3. Pearson Linear Correlation Coefficients associated with the behavior observations as they relate to the EEG Wave Strength (mV)

Treatment	Conscious	Confidence Interval			Behaviors
	<0.01 mV	Lower 95%	Upper 95%	P Value	n=
VSD	0.337	0.278	0.394	<0.0001	900
VSDH	0.309	0.221	.0392	<0.0001	431
VSDCO	0.244	-0.001	0.463	0.0513	64
VSDHCO	0.290	0.013	0.526	0.0409	50
Treatment	**Unconscious**	**Confidence Interval**			**Behaviors**
	>0.01 mV	**Lower 95%**	**Upper 95%**	**P Value**	**n=**
VSD	-0.372	-0.427	-0.314	<0.0001	900
VSDH	-0.345	-0.426	-0.259	<0.0001	431
VSDCO	-0.022	-0.264	0.225	0.8629	64
VSDHCO	-0.282	-0.520	-0.004	0.0470	50

Table 3 highlights the correlation between the EEG wave strength and their positive relationship to the conscious behaviors which were observed watching the digital videos. As conscious behaviors increased in frequency the strength of the EEG Wave also increased significantly for the VSD, VSDH and VSDHCO. This relationship was marginal for the VSDCO method, which may have been related to the speed at which the chicken succumbed to the treatment. The unconscious behaviors were negatively correlated to the EEG wave strength. In this case as the unconscious behaviors became more frequent the weaker the EEG Wave became and were closely associated with the loss of posture (lateral recumbency) and panting/gasping of the hen as it neared TOD.

Figure 7. The level of heat shock protein 70 (HSP70) by treatment in brain tissue of laying hens in environmental chambers examining the physiological and behavioral parameters associated with VSD, VSDH, VSDCO and VSDHCO



[ab](P<0.05)

The HSP70 levels were significantly higher in both of the VSDCO and VSDHCO treatments that included CO_2 in the process shown in Figure 7. This was surprising since the chamber temperatures, RH%, and the duration to TOD was significantly shorter in both of these treatments. It was expected that the HSP70 levels would have been higher in the VSD and VSDH treatment groups due to the duration of the hens in the chambers and the increase in RH% and temperatures. However, in this case the indication appears to be that the amount of time the hens are in the unconscious state has an impact on the formation of the HSP70 levels.

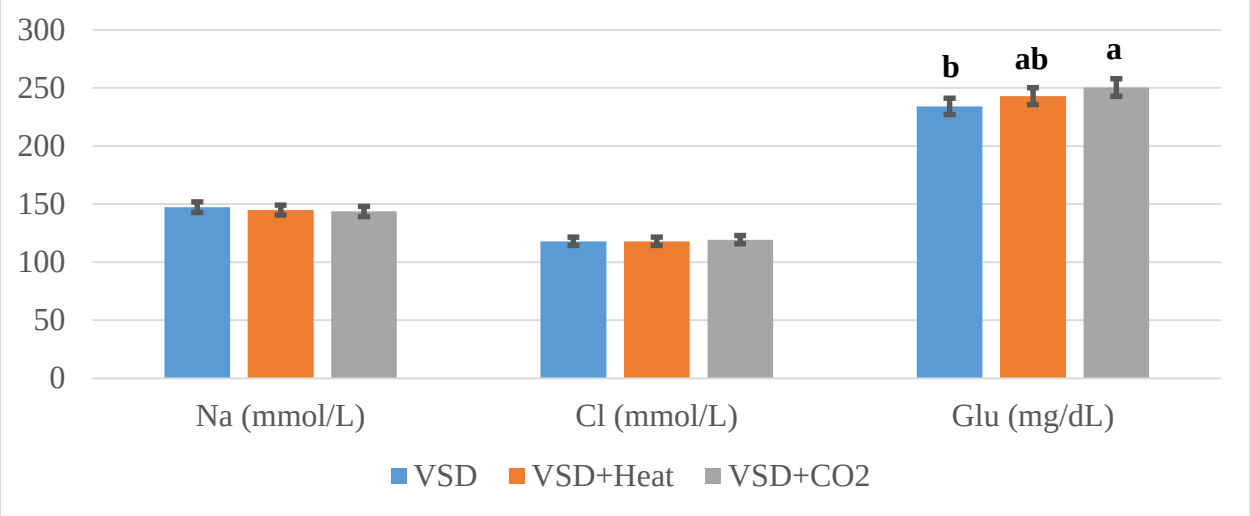
Conclusions Phase 1:

1. The conclusion remains that core body temperature associated with VSD methods impacted the TOD. The time to TOD was longest for VSD followed by VSDH in the chambers.
2. The time to TOD was not different between VSD vs. VSDH treatments, however, the TOD was shortest in both VSDCO and VSDHCO
3. Hens in VSD environment spent the greatest percentage of time (82%) in the unconscious mV range (0 to 0.01 mv)
4. Surprisingly the hens in VSDH environment spent the greatest percentage of time in the conscious mV range (>0.01 mV).

Phase 2:
In Phase 2 the emphasis was on the monitoring of Blood Chemistry and HSP70 of the hens at 5 specific points within each of the treatment groups based upon time exposure to the environment. The VSDCO and VSDHCO were found to be statistically equivalent based on the Phase 1 treatment analysis, therefore, it was determined to only work with VSDCO treatment group from this point forward in the project. In phase 2, the 3 ventilation shut down methods examined in this phase included VSD, VSDH, and VSDCO.

In Figure 8, three components of the blood chemistry are shown. In this study the Na nor the Cl changed between any of the treatments. This is similar to the findings of Glowinska et al. (2010) who showed no change in these levels over a 24 hr heat stress period. However, we did have a Glucose response with the VSDCO having the highest overall Glu response. It has been shown that prolonged Hyperthermia will suppress Glu response in hens. With the VSD and the VSDH the core body temperature was significantly higher than the VSDCO treatment which may have contributed to this suppression in the blood. It was surprising of the speed at which Glu increased in the VSDCO treatment group since this was the shortest time to TOD of any of the groups. Koelkebeck et al (1995) showed an increase in plasma Glu in laying hens subjected to heat stress and CO2 infusion. However, it can be concluded that just handling of birds causes stress which increases blood Glu.

Figure 8. Phase 2, Blood chemistry for VSD, VSDH, and VSDCO for Sodium, Chloride, and Glucose



In Figure 9, additional blood parameters are shown including PO_2 where the levels were suppressed in both VSD and VSDH where the chamber temperature was highest and the core body temp. This was similar to Glowinska et al (2010). The increased PO2 in the VSDCO group can be explained by the Bohr Effect, whereby high CO2 levels can an increase in PO2. The other

blood parameters were not influences by the VSD method as similarly reported by Arad et al (1983).

Figure 9. Phase 2, Blood chemistry for VSD, VSDH, and VSDCO for Total CO_2 in serum (TCO2), Partial pressure of CO_2 (PCO2), Amount of O_2 dissolved (PO_2), Bi-carbonate Buffer (HCO_3), and Oxygen saturation (SO_2)

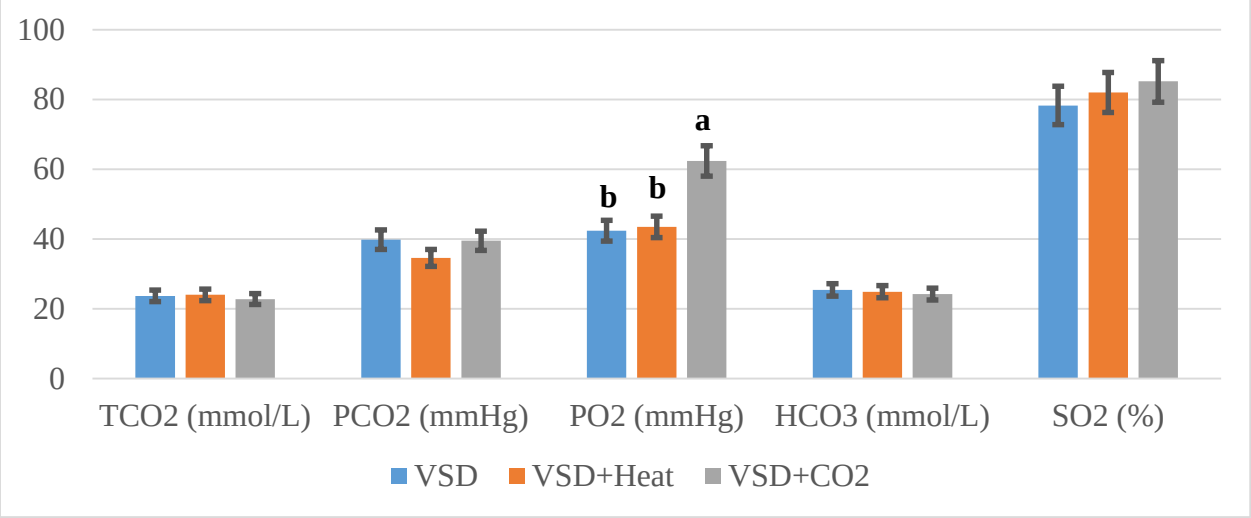
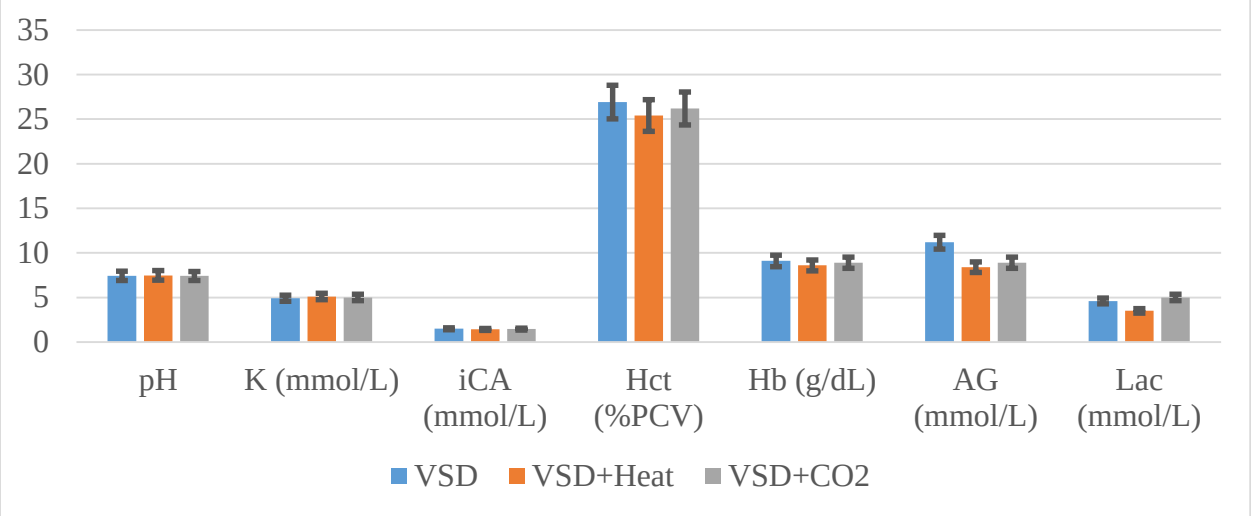


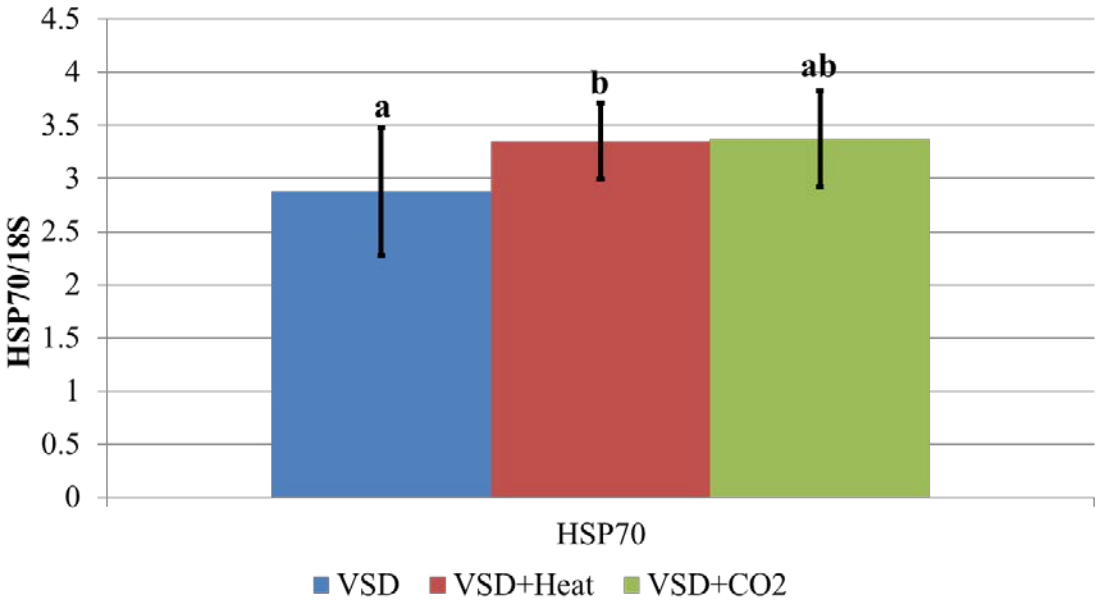
Figure 10, addition blood parameters were evaluated including pH, K, iCA, Hct, Hb, AG, and lactate. There were no differences observed among the treatments. Koelkebeck et al (1994) reported an increase in blood pH and lactate with heat stress, however similar results were not observed in this study.

Figure 10. Phase 2, Blood chemistry for VSD, VSDH, and VSDCO for pH, Potassium (K), Ionized Calcium (iCA), Hematocrit (Hct), Hemoglobin (Hb), Anion gap (AG), and Lactate (Lac)



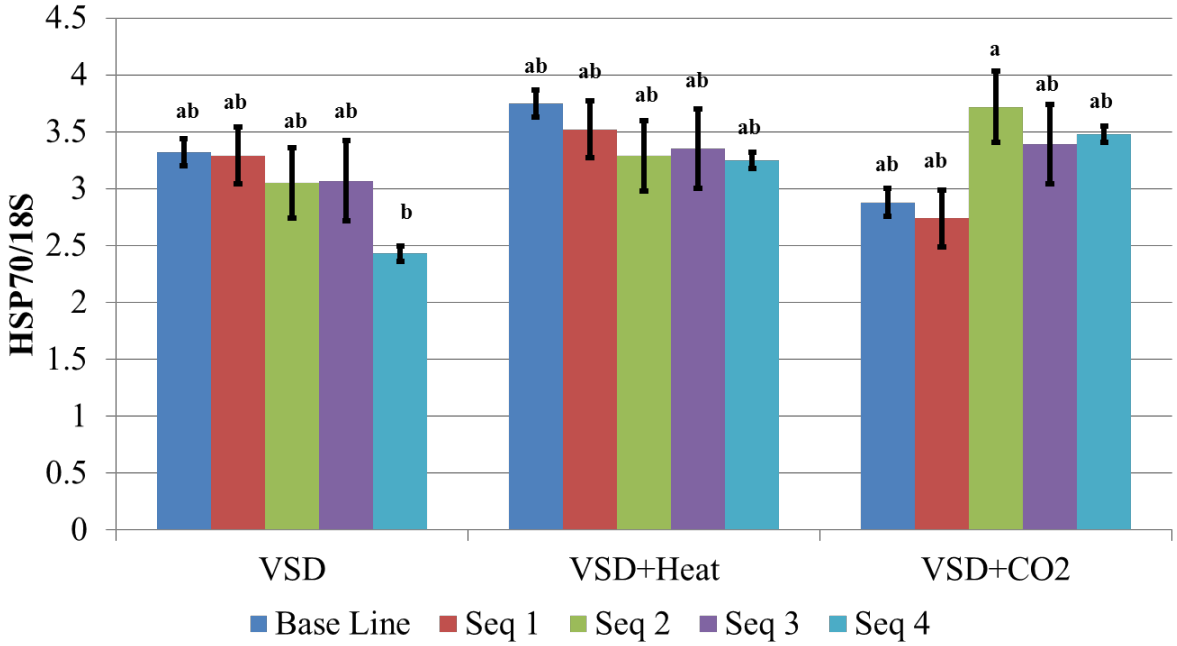
In Figure 11, the relative expression of HSP70 decreased in the VSD and VSD + Heat environments from time 0 to TOD; however, the highest levels were observed with in VSD + Heat and VSD + CO_2 treatments at 3.35 and 3.37 HSP70/18S, respectively. Blood PO_2 at 18.9 mmHg and Glucose 250.3 mg/dL higher in the VSD+CO_2 than the VSD treatment. The HSP70 and BC differences may have been related to the speed to TOD than in the other methods. The hen's similar physiological responses to VSD, VSDH, or VSDCO methods other than the duration of the processes appear to indicate no definitive differences. This would indicate equivalency between the methods as being humane poultry flock depopulation methods.

Figure 11. Expression of HSP70 in VSD, VSDH, and VSDCO treatment groups



[ab](P<0.05)

Figure 12. Expression of HSP70 during the progression from the start of VSD, VSDH, and VSDCO through to the TOD



[ab](P<0.05)

Baseline=time 0, Seq 1= ¼ time to TOD, Seq 2 = ½ time to TOD, Seq 3 = ¾ time to TOD, and Seq 4 = time of TOD;

The sampling sequence of the hens experiencing the 3 VSD processes is interesting in the trends which are shown and the differences between the methods. In the VSD and VSDH the HSP70 levels decreased as the hens progressed to TOD. In the VSD treatment the hens nearest TOD had the lowest level of HSP70. Conversely, in the VSDCO treatment group the hens had increasing HSP70 as they neared TOD. IN the VSDCO treatment the hens had the highest HSP70 levels at what appears to be the transition point Seq 2, between conscious and unconsciousness, which occurred at a CO_2 concentration of approximately 10%.

Conclusions Phase 2:

1. The conclusion remains that blood chemistry associated with VSD and associated methods methods appeared to be impacted by the CB and duration of exposure until the TOD was reached.
2. The HSP70 values were similar within all of the treatment used, however the Seq 2 hen had the highest HSP700 level in the VSDCO group.

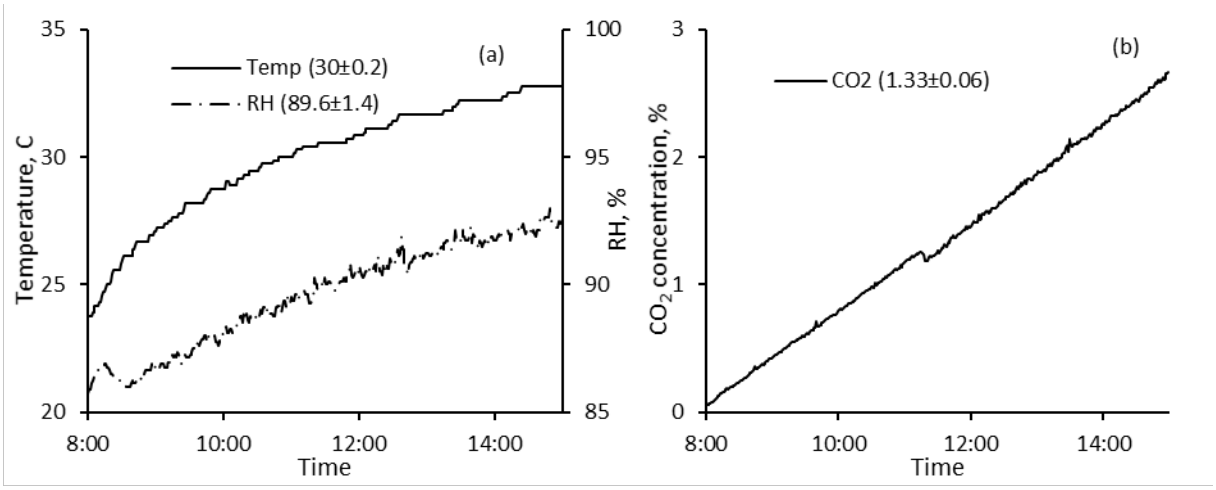
Phase 3:

In this phase the VSD processes were scaled up to a room in order to evaluate the influence of environmental changes and to discover the problems associated with scaling up a process from the chamber containing a single hen at a time where all of the variables could be controlled precisely to a room size trial, which had conventional ventilation and 2 level stair step cages. We conducted 5 trails each utilizing 144 hens housed at the UEP cage density (72 sq in/hen).

Two pilot trials were conducted to discover additional problems associated with translating chamber data to a large scale in this case a room.

After 7 hours, the VSD Pilot 1 treatment was stopped by opening the access panel and resuming ventilation. Because the four temperature readings were close to one-another (as were the four RH readings), average temperature and average RH were calculated for each minute (Fig. 13 (a)). Of the four CO_2 sensors, three sensors output very similar concentrations whereas one, whose sampling line fell to the floor during study, yielded slightly different and variable values than the other sensors after its sampling line was disturbed. Therefore, for the duration when the sampling lines remained undisturbed (8:00 – 11:18), average CO_2 concentration (Fig. 13(b)) was calculated based on four sensors; thereafter, concentrations were calculated based on three undisturbed sensors. There was no evidence of stratification of any of the environmental parameters probably because vertical separation between the sensors was <0.4 m. During this period, ambient average air temperature was 30°C (range: 23.1° C to 32.8°C) while ambient average RH was 89.6 % (range: 85.9% to 92.3%)

Figure 13. Changes in (a) air temperature and RH and (b) CO_2 concentrations during the pilot study (8:00 – 15:00, 29 June 2016). Environmental parameters were measured every minute and their trends are based on the average of four sensors except for CO_2 which is based on four sensors from 8:00 – 11:18 and three sensors thereafter. The legend shows mean±SD.



Over the 7-h period, average temperature (n = 4) increased from 24 C to 33 C while average RH (n = 4) increased linearly from 86% to 92 % (Fig. 13 (a)). While RH decreases as temperature, provided no moisture is added to the system, RH increased even though temperature increased. This was because as the temperature increased, the birds' ability to lose sensible heat decreased, they sought to maintain homeothermy by losing latent heat, resulting in increased RH in the room. Because the birds continued to receive drinking water during the study, they were able to continue losing body heat through evaporation and defecation, delaying the onset of hyperthermia which might have otherwise resulted in higher mortality if drinking water had been

disconnected. Average chamber CO_2 concentration increased linearly from ~0.06% (550 ppm) to 2.67% (Fig. 13(b)) as the birds continued to exhale CO_2.

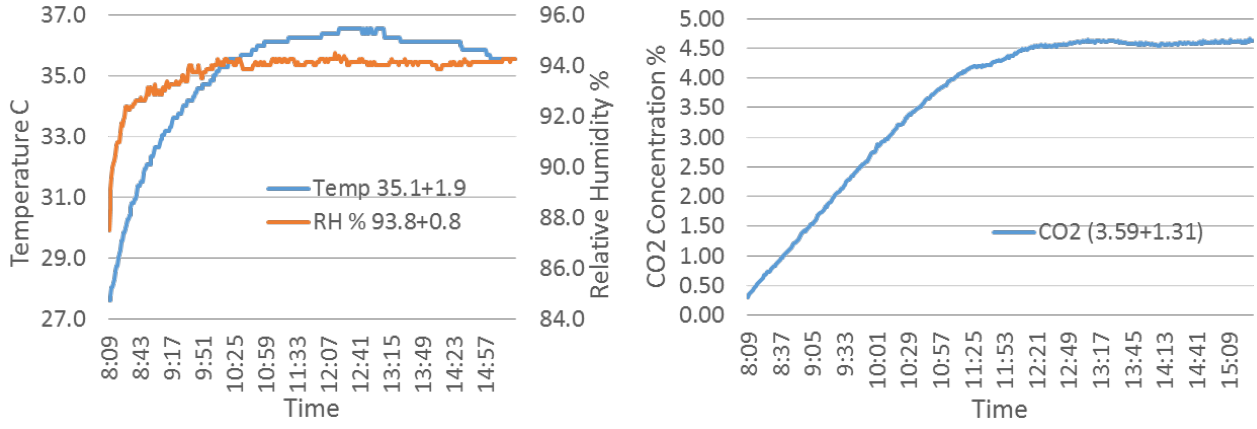
Table 4. Hen (Gallus domesticus) and Environmental parameters pre and post for pilot trials using the VSD process

Treatment	Pre-test				
	Body wt	Core body temp	House volume	Room temp	Room RH
	(g)	°F (°C)	(ft^3/hen)	°F (°C)	(%)
VSD pilot 1	1939	105.1 (40.6)	14.2	73.5 (23.1)	86.0
VSD pilot 2	1646	104.7 (40.4)	4.1	74.0 (23.3)	73.0
	Post-test				
	Core body temp	Room temp°F	Room RH	Room CO_2	Survivor
	°F (°C)	°F (°C)	(%)	(%)	(%)
VSD pilot 1	106.8 (41.6)[1]	86.4 (30.2)	89.6	2.9	100
VSD pilot 2	107.8 (42.1)[1]	93.7 (34.3)	89.1	3.9	43
	102.8 (39.3)[2]				

[1]Core body temperature of surviving hens
[2]Core body temperature of dead hens

In the VSD Pilot 1, we had heavier hens which translated to increase BTUs generated per hen with a room temperature increase of only12.9°F. However, due to the room volume/hen and the access to water throughout the pilot trial the core body temperature (CBT) failed to reach lethality (Table 4).

Figure 14. Changes in (a) air temperature and RH and (b) CO_2 concentrations during the pilot study (8:00 – 15:30, 29 July 2016). Environmental parameters were measured every minute and their trends are based on the average of four sensors except for CO_2 which is based on four sensors from 8:00 – 11:18 and three sensors thereafter. The legend shows mean±SD.



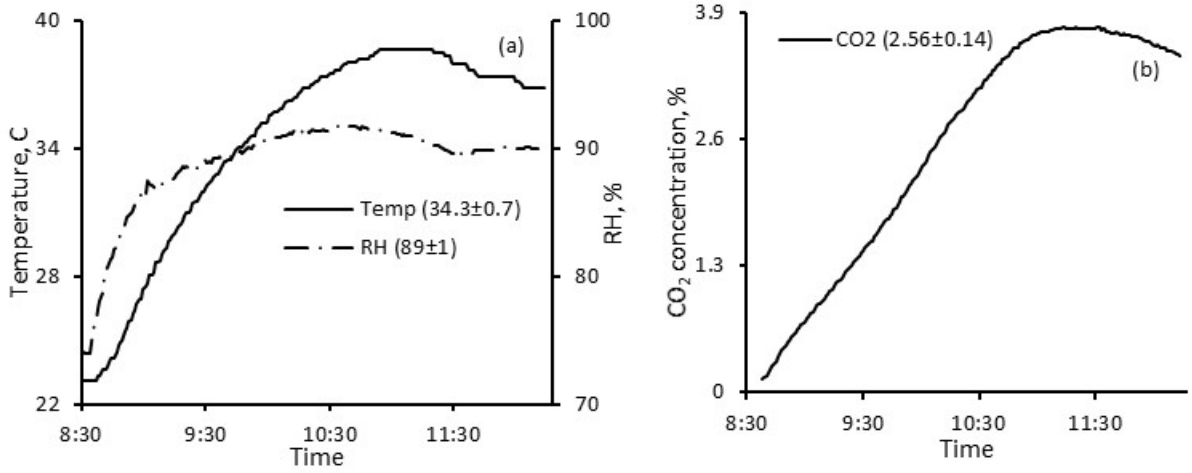
We determined in phase 1 that the hen typically has an allowance of 3.4 ft^3 of space in a conventional laying facility. In the preliminary trial we determined that the dynamics of the process are altered based upon the Cubic Ft of space allowed to each hen. Two components were attributed to the failure of Pilot 1 and the partial success of Pilot 2. The amount of cubic space allocated per bird appeared to have an effect on VSD. The room was measure to allow 14.2 ft^3 space per bird, which we initially did not believe would have an impact on the process. Second, feed and water were not removed from the system. The water access allowed the birds to drink ad libitum, thereby affording them the ability to sustain a lowered core body temperature since water is a natural heat sink for chickens via conductance. After modifications to address the problems as determined a secondary run of this process we determined that mimicking the cubic space allowances/hen in the industry as closely as possible is important to evaluate the process. In the second VSD Pilot 2, we constructed a chamber immediately surrounding the cage row using a 2x4 frame covered with 10 mil plastic. The water lines were drained to prevent water being used metabolically to lower core body temperature. The effectiveness of the VSD procedure in pilot 2 was improved to 57% mortality (Table 4). However, we also found that the heat transfer across the 10 mil plastic walls of the inner chamber was too great to allow for the increase in temperature to universal hyperthermia among the hens (Figure 14). The surviving hens in Pilot 2 reached a CBT of only 107.8℉. Those hens that died had cooled due to the duration of time where the Pilot 2 was ended. The dead hens only had CBT of 102.8℉ (Table 4).

Experiment 1- Ventilation Shutdown

In order to simulate current industry housing standards a method is in development to replicate this in the animal room being used for this process including matching heat transfer. During this 3.75-h experiment, due to very little vertical separation, and hence, stratification, the four temperature (also RH) sensors tracked one-another closely as did the four CO_2 sensors (Fig 15). Ambient average air temperature was 34.3°C (range: 23.1°C to 38.6°C) while ambient average RH was 89.0 % (range: 74.3% to 91.3%) during this period (Reference).

Ventilation shutdown resulted in rapid rise in temperature from 23°C to a maximum of about 39° C in <2.5 h where it stabilized for about 30 min before declining to ~37°C at the end of the study (Fig. 14(a)). The drop in temperature at the end is due to the decrease in metabolic heat due to the mortalities causing the drop in BTUs produced. This is a natural disadvantage to VSD alone and could be a problem in large scale depopulation during cooler seasons. The temperature rise in this trial was much more rapid and maximum temperature achieved was much higher than the pilot study due to the smaller chamber and denial of water to the birds during the study. Since the birds did not have access to water, their ability to maintain homeothermy using evaporative cooling was limited, as is evident from the RH trend (Fig. 15(b)). RH increased rapidly from 74% at the start of the study to 87% in about 35 min as the birds sought to maintain homeothermy by increasing evaporative heat loss as sensible heat loss declined with rise in air temperature (Fig. 15 (b)). Thereafter, increase in RH was slower though it rose to a maximum of 92% before declining slowly as mortalities increased.

Figure 15. Changes in (a) air temperature and RH and (b) CO_2 concentrations during the VSD study (8:30 – 12:15, 29 June 2016). Environmental parameters were measured every minute and their trends are based on the average of four sensors except for CO_2 which is based on four sensors from 8:00 – 11:18 and three sensors thereafter. The legend shows mean±SD.



Carbon dioxide concentration increased linearly and peaked at 2.56% (Fig. 14(b)) and peaked around the same time as temperature (Fig. 15(a)). Both temperature and CO_2 concentrations had similar trends and remained at their highest levels for about 30 min before starting to decline, as heat and CO_2 production declined from the hyperthermic birds and the mortalities began to cool. Even with the improved VSD conditions the AVMA performance standard of 100% lethality was not achieved as shown in Table 5, with 2.8% of the hens surviving the process.

Table 5. Hen and Environmental parameters pre and post for trials using VSD, VSDH and VSDCO processes

Treatment	Pre-test				
	Body wt	Core body temp	House volume	Room temp	Room RH
	(g)	°F (°C)	(ft^3/hen)	°F (°C)	(%)
VSD	1509	105.8 (41.0)	4.1	73.6 (23.1)	74.0
VSDH	1637	105.1 (40.6)	4.1	74.3 (23.5)	82.8
VSDCO	1712	105.4 (40.8)	4.1	78.4 (25.8)	84.9
	Post-test				
	Core body temp	Room temp°F	Room RH	Room CO_2	Survivor
	°F (°C)	°F (°C)	(%)	(%)	(%)
VSD	111.7 (44.3)[1] 109.3 (42.9)[2]	98.2 (36.8)	90.0	3.7	2.8
VSDH	112.3 (44.6)	111.2(44.0)	73.0	1.9	0
VSDCO	99.4 (37.4)	83.3 (28.5)	88.9	40.8	0

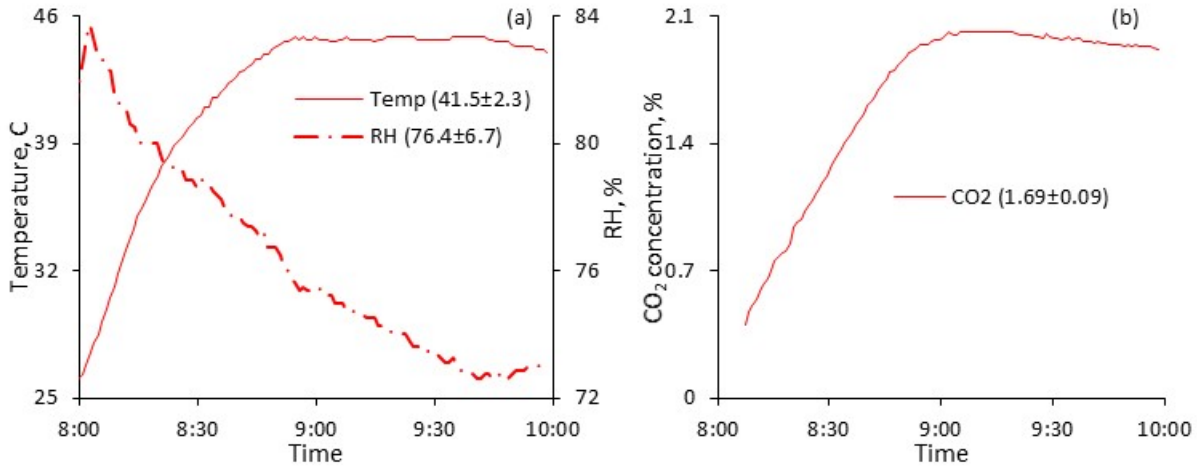
[1]Core body temperature of surviving hens
[2]Core body temperature of dead hens

This was surprising with a CBT of 109.3°F in the survivors, here again the key was the gradual cooling in the room which occurred as the majority of the hens died.

Experiment 2-Ventilation Shutdown + Heat

In this experiment 2, VSDH, during the first 1.25-h of the study, one temperature-RH sensor measured about 5.5°C higher while its RH value was 16% lower than the average of the three other temperature-RH sensors. This could have been because that temperature-RH sensor, placed on top of the lower tier, might have been due to closer to the heater. However, during the remainder of the study, measurements of all four sensors were similar; therefore, all readings of all four sensors were averaged (Fig. 16(a)). Among the four CO_2 sensors, two sensors showed unexplicable fluctuations during the first 20 min; thereafter, all four sensors showed similar values and trends. One of CO_2 sensors (5%) that showed initial fluctuations, measured slightly higher CO_2 concentrations, by 0.18 %, than the other three CO_2 sensors for reasons that are unclear. Average CO_2 concentrations for the first 20 min were calculated based on the two properly-functioning sensors while the outputs of all four sensors were used to calculate average CO2 concentrations for the rest of experiment (Fig. 16(b)). It may be noted that the CO_2 sensors were turned on 8 min after the start of the experiment. Ambient average air temperature was 41.5° C (range: 23.5°C to 44.0°C) while ambient average RH was 76.4 % (range: 82.8% to 73.0%) during this period (Reference).

Figure 16. Changes in (a) air temperature and RH and (b) CO_2 concentrations during the VSD + heat study (8:00 – 10:00, 4 August 2016). Environmental parameters were measured every minute and their trends are based on the average of four sensors. The legend shows mean±SD.



Addition of heat during VSD resulted in chamber temperature increasing from 26°C to a maximum of 45°C in <1 h (Fig. 16 (a)). By comparison, use of VSD alone, resulted in a lower temperature maximum (39°C) and the rate of temperature increase was much lower (Fig. 15(a)). However, it should also be noted that this study was done under warmer ambient conditions than VSD only. In the latter half of the study, chamber temperature declined by 1°C (Fig. 16(a)) probably because of the birds' core temperatures had likely exceeded the upper critical limit and heat loss from those hyperthermic birds declined. As would be expected, as temperature increased and with no drinking water access, RH in the chamber declined rapidly from a high of 84% to a minimum of 73%.

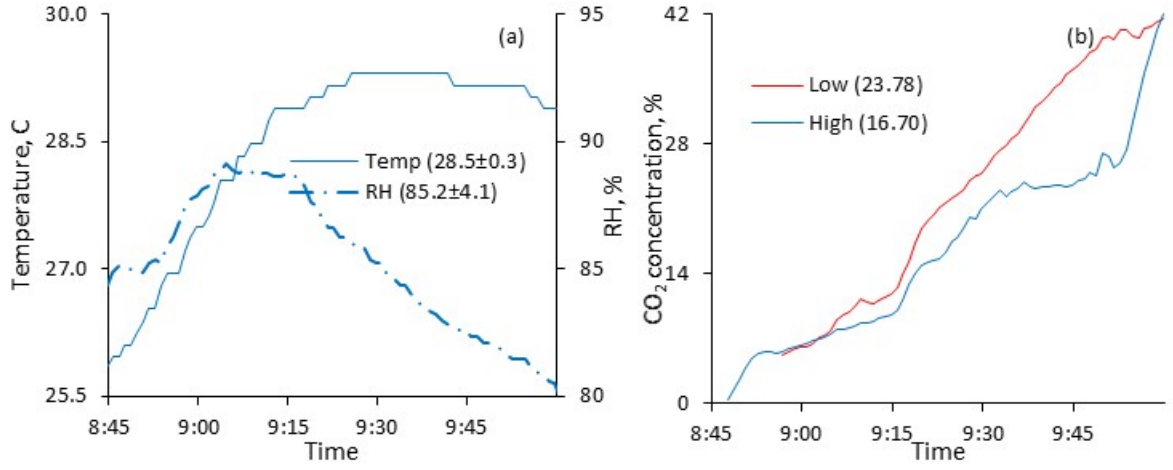
Average CO_2 concentration in the chamber increased linearly from about 0.65% (8 min after the start of the experiment) to a maximum of ~2% in ~1 h and then declined slowly to 1.9% by the end of the study (Fig. 16(b)). Changes in temperature and CO_2 concentrations were similar to one-another (Fig. 16) and also to the trends observed in Experiment 1 (Fig. 13). Gradual decline in temperature and CO_2 concentrations in the latter half of the study could be attributed to reduced heat and CO_2 production from the hyperthermic birds.

VSDH was 100% effective in meeting the AVMA performance standard. The added heat into the process resulted in the room temperature reaching 44.0°C (Table 5). The core body temperatures of the hens which died averaged 44.6°C. This occurred rapidly which resulted in hyperthermia in the hens without the characteristic drop in environmental temperatures seen in the VSD process as the metabolic heat declines as hens die which eliminates survivors.

Experiment 3-Ventilation Shutdown + CO2

In this 75-min experiment, all four temperature (and RH) sensors had similar values and were thus, averaged, as shown in Fig. 4(a). Temperature increased from 26 C to 29 C in <45 min and declined gradually thereafter, whereas RH declined from 85% at the start of study to 80% at the end (Fig. 17(b)). In this experiment CO_2 was the main cause for inducing mortality, temperature and RH trends are of secondary importance.

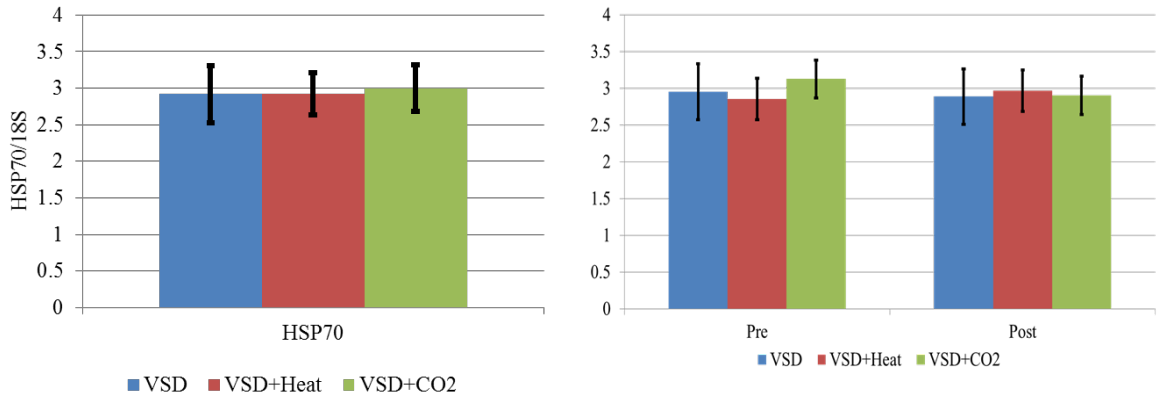
Figure 17. Changes in (a) air temperature and RH and (b) CO_2 concentrations during the VSD + CO_2 study (8:45 – 10:00, 1 September 2016). Environmental parameters were measured every minute and their trends are based on the average of four sensors for temperature and RH and two sensors for CO_2. The legend in Fig. 17(a) shows mean±SD where it shows mean values for the low and high CO_2 sensors.



Carbon dioxide concentrations with the Low sensor were discarded for the first 9 min since this sensor was used to measure CO_2 concentrations in exhaust of the computer fan used to exhaust the room of air as it was being filled up with CO_2. CO_2 concentrations in the room increased rapidly and linearly to 41% by the end of the experiment (Fig. 17b)). The High sensor that was located <0.4 m above the Low sensor recorded lower concentrations because it took time for the

chamber to fill up with CO_2 which is 50% heavier than air. Over the study period, excluding the 9 minutes of readings recorded by the Low sensor, the mean±SD of the two sensors was 19.01±3.75%.

Figure 18. Effect of VSD, VSDH, and VSDCO on overall HSP70 levels and the relative change between the hens Pre and Post during the two sensors for CO_2.



Relative expression of HSP70 was lower in the VSD and VSD + Heat environments and the highest levels were observed with in VSD + CO_2 treatments but they were not significantly different. There were minor differences in HSP70 between the pretreatment hens and the post treatment hens, here again there were no significant differences between any of the treatments in phase 3 related to HSP70. The hen's similar physiological responses to VSD, VSDH, or VSDCO methods other than the duration of the processes appear to indicate no definitive differences. This would indicate equivalency between the methods as being humane poultry flock depopulation methods.

Data from these experiments support the idea that ventilation shut down with the addition of supplemental materials, like heat and CO_2, may be a more humane way to euthanize a flock that exposure to ventilation shut down alone. And based upon the time associated with VSDH and VSDCO they are equivalent

Conclusions Phase 3:

1. The conclusion remains that core body temperature associated with VSD methods impacted the TOD. The time to TOD was longest for VSD followed by VSDH and VSDCO.
2. Survivor ship in the VSD does not meet the AVMA standard
3. Surprisingly the speed of the process and hen survivorship was similar for VSDH and VSDCO environment

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K. E. Anderson, J.N. Petitte, K.A. Livingston, S. Shah, M. Martin, K. Eberle, and R. Malheiros. 2017. Effect of ventilation shut down (VSD) on changes in heat shock protein 70 and blood chemistry throughout depopulation. Poultry Sci. Suppl. 96

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Eberle, Krista, Martin, Michael, Shah, Sanjay, Malheiros, Ramon, Livingston, Kim and Anderson, Kenneth. 2017. A non-invasive electroencephalogram methodology for evaluating environmental stressors on unanesthetized chickens. Poultry Science (In Review)

Research cited in DRAFT FOR COMMENT: The AVMA Guidelines for the Depopulation of Animals (2017)

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4595 This Response Guide states that the temperature of the house must be raised to
4596 104°F or higher as quickly as possible and preferably within 30 minutes, maintaining a
4597 temperature of between 104°F and 110°F for a minimum of three hours. Recent research
4598 conducted at North Carolina State University and the USDA Response Guidance indicates

https://www.aphis.usda.gov/animal_health/emergency_management/downloads/hpai/ventilationshutdownpolicy.pdf
11 HPAI Response Guide, Using Ventilation Shutdown to Control HPAI, January 15, 2016, http://minnesotaturkey.com/wp-content/uploads/2015/03/USDA-NEW-Using-VSD-1.15.2016_V2.pdf
220
4599 that VSD alone may not achieve this outcome and that supplemental heat may be needed to
4600 achieve this standard. While the USDA guidelines do not recommend the addition of
4601 supplemental CO2, the NCSU research demonstrated that VSD with the addition of
4602 supplemental heat, CO2 or heat plus CO2 were equally beneficial in decreasing time to
4603 100% mortality. VSD with the addition of heat ensures the temperature standard is met. The obvious goal is 100% mortality in as short a time as possible.13 4604
4605 Future research may provide additional information to inform decision-making
4606 surrounding VSD. Until then, the following categorizations will apply in these Guidelines:
4607 1. VSD plus heat, VSD plus CO2 and VSD plus heat and CO2 meet the classification category
4608 of “Allowed in Constrained Circumstances.”
4609 2. VSD alone is categorized as “Not Recommended.”