APPLICATION OF WIRELESS ELECTROENCEPHALOGRAM TO MEASURE STRESS IN WHITE PEKIN DUCKS

by

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A thesis submitted to the Faculty of the University of Delaware in partial fulfillment of the requirements for the degree of Master of Science in Animal Science

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ABSTRACT

Stress in poultry can produce many undesirable effects on bird health and overall production performance including decreased immune function and feed efficiency (Horvath-Papp, 2008, 426). The objective of this study is to develop and evaluate an additional potential measure to quantitatively assess stress through evaluation of brain activity using electroencephalography (EEG). In three experiments, White Pekin ducks (5-11 weeks old) were implanted with EEG transmitters and treated with potential stressors. In Experiment 1, 16 straight-run ducks were treated for 15 minutes in a controlled environmental chamber and treated with three known stressors: auditory, mild electric shock, and ammonia. In Experiment 2, 24 straight-run ducks were treated the same as in Experiment 1 but with an extended trial time of 45 minutes. Electrocardiograms (ECGs) and blood corticosterone were also analyzed for Experiment 2 as a standard measure of stress against which any changes seen in the relative frequency bands of the EEG were measured. In Experiment 3, 8 male ducks were treated the same as in Experiment 2 but in their respective holding pen rather than a controlled chamber. Mild electric shock was compared to no shock and control trials. EEG analysis for Experiment 1 showed no differences between time periods for all frequencies for all treatments. EEG analysis for Experiment 2 showed no differences between time periods for all

frequencies for auditory and ammonia stimuli; however, a significant rise in the relative delta frequency and a significant decrease in the relative alpha frequency was seen during the stimulus period for mild electric shock stimuli. ECG results for Experiment 2 showed no differences for auditory and control trials. Mild electric shock heart rate increased during the middle and last 30 seconds of the stimulus period and ammonia heart rate decreased during the middle and last 30 seconds of the stimulus period. Corticosterone results for Experiment 2 showed a significant difference between pre-treatment and post-treatment; however, there were no differences between treatments or between treatments and control. EEG results for Experiment 3 showed no differences between time periods for all frequencies for mild electric shock, no shock, and control trials. Corticosterone results for Experiment 3 showed no difference between pre-treatment and post-treatment levels for no shock trials. There was a significant decrease in post-treatment corticosterone levels for control trials and a significant increase in post-treatment levels for shock trials when compared to pre-treatment levels. The post-treatment corticosterone levels for mild electric shock were significantly higher than control post-treatment levels. Based on the results of all experiments, EEG is currently not a viable measurement of stress in commercial poultry.

Chapter 1

INTRODUCTION

Stress in animals is an important welfare concern that many producers must face. Stress in poultry can produce many undesirable effects on bird health and production, which can lead to an increased susceptibility to disease and decreased production efficiency (Horvath-Papp, 2008, 426). Several qualitative and quantitative measures including behavior observations and blood parameters are available to evaluate poultry stress. A potential quantitative assessment tool for animal welfare is the electroencephalogram (EEG). EEG represents the voltage recorded between electrodes applied to the scalp or implanted surgically. The sensors or electrodes are attached to the head and a computer records the electrical activity. A potential benefit of the EEG is that after implantation of the wireless EEG transmitter, birds can be evaluated without significantly impacting the behavior and activity of the bird. It is hypothesized that EEG may have the capability to help researchers not only identify a stress response in the EEG brain waves, but also help discern between stressors based on patterns that may emerge in the brain waves.

EEG has been used to monitor brain activity of humans and a number of different animal species. EEG has been used in connection with animal welfare studies to determine the time to unconsciousness and brain death during euthanasia or

depopulation (Alphin et al., 2010,757-762; Gerritzen et al., 2006, 1055-1061; Raj 1998, 1815-1819). Radiotelemetry systems have been used to allow the real time measurement of heart rate, blood pressure, body temperature and telencephalic EEG to be used as an indicator of bird welfare parameters (Savory and Kostal, 1997, 963-969). In these applications, the EEG was recorded from the device's paired sensing electrodes positioned on the surface of the telencephalon with leads being passed under the skin and held in place with the use of dental acrylic (Savory et al., 2006, 599-606).

Though EEG has been used in studies of agriculture animals to evaluate emergency depopulation and stunning methods for slaughter, literature specifically pertaining to EEG in chickens is limited. When EEG has been used with chickens and other poultry species, it largely has been used as an indicator of general integrity of the nervous system or as a measure of specific brain states (Hunter et al., 2000, 23-28). Gerritzen et al. (2006) studied the susceptibility of ducks and turkeys to atmospheric stunning using EEG to determine the point of unconsciousness. The time to unconsciousness is an important welfare assessment as this is when the bird is no longer sensitive to pain (American Veterinary Medical Association, 2013, 102). Researchers studying unconsciousness have used several techniques to analyze EEG. A common analysis technique is the time to loss of somatosensory evoked potentials (SEPs). Raj (1998, 686-695) showed that loss of SEPs along with changes in the EEG is indicative of loss of consciousness in broilers. Additionally, several wave

frequencies can be analyzed via the EEG. In chickens, alpha (8-12 Hz), beta (16-24 Hz), and sigma (12-16 Hz) waves are low amplitude, high frequency waves while theta (4-8 Hz) and delta (0.5-4 Hz) waves are high amplitude, low frequency waves (Benson et al., 2012a, 884-890; Benson et al., 2012b, 960-964; Alphin et al., 2010, 757-762). Several researchers have employed the use of high amplitude, low frequency (HALF) activity in the theta and delta waves for hens and broilers to determine the point of unconsciousness (Benson et al., 2012a, 884-890; Benson et al., 2012b, 960-964; Raj et al., 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 occurrence of theta and delta waves occurred at approximately the same time as loss of posture, indicating that the complete loss of posture is a sign of unconsciousness (Gerritzen et al., 2004, 1294-1301). A behavioral indicator of loss of consciousness is loss of posture, or when the bird is "unable to maintain a sitting position and neck tension" (Gerritzen et al., 2004, 1294-1301). Further work has shown that the presence of HALF patterns in the EEG is typical for loss of consciousness in other species such as rats (Forslid et al., 1986, 281-287). The changes that are seen in the frequency, or the number of occurrences of a repeating event per unit of time, specifically the suppression of alpha and beta waves and the occurrence of theta and delta waves are indicative of loss of consciousness.

A common welfare concern in poultry production is the effect stress may have on poultry. Birds that are under stress are more susceptible to disease due to the

hormonal changes evoked by the stress response. There is also an impact on profitability due to a decrease in growth and feed conversion efficiency (Horvath-Papp, 2008, 426). If a stimulus is perceived as a threat to survival or is impending, there is an increase in adrenal activity via the hypothalamic-pituitary-adrenal (HPA) axis (Klingbeil, 1985, 10-19). This adrenal stress response leads to an increase in circulating corticosterone, the primary glucocorticoid in poultry (Sturkie, P.D. ed., Avian Physiology, 1986, 516; Singh et al., 2009, 1346-1351). Corticosterone levels rise fairly quickly after stimuli and are considered a good indicator of stress in poultry (Davis et al., 760-772; Rettenbacher et al., 2004, 704-711). Handling stress can therefore have an effect on corticosterone levels and must be considered when collecting samples. Researchers draw blood samples in less than 5 minutes to help mitigate this issue and obtain valid corticosterone levels (Vleck et al., 2000, 392-400). Another method of stress evaluation in poultry is the analysis of the heterophil to lymphocyte (H/L) ratio. Heterophils and lymphocytes are two leukocytes present in avian blood (Campbell and Ellis, 2007, 3). Stressors can increase the number of heterophils and decrease the number of lymphocytes in the blood. This method of stress analysis is more applicable to long-term stressors since the leukocyte changes are much slower (up to 20 hours) in response to a stressor than corticosterone (Vleck et al., 2000, 392-400).

Several stressors have been identified in literature as stressors capable of inducing a stress response in poultry production; mild electric shock, ammonia, and

auditory. Mild electric shock is an acute and measurable stimulus and was chosen for all three Experiments based on studies involving stress responses to shock, pain, and EEG. While EEG is typically used to evaluate unconsciousness, it has also been used to evaluate responses to a stressful stimulus such as pain, in both traditional and non-traditional food animals. Although animals are unable to verbalize pain levels, Ong et al. (1997, 189-193) was able to make a correlation between EEG changes and behavioral changes of sheep in response to mild electric shock. Ong et al. showed that in the following four seconds after the shock, there was an overall increase in EEG power. They concluded that EEG changes were a good measure of acute pain in sheep. Jongman et al. (2000) used frequency spectral changes in EEG to evaluate the perceived pain of castration, mulesing, and docking in lambs. Jongman et al. showed there was an overall decrease in the EEG immediately following the stress presentation. While the results of this study were in contrast with Ong et al., it was another instance in which EEG changes were correlated with behavioral changes in response to stressful stimulus presentation. Gentle and Hunter (1990, 95-101) showed a high amplitude, low frequency EEG pattern during immobility after progressive removal of feathers in chickens. Gentle and Hunter concluded that feather removal was a painful experience that could be categorized as a welfare concern. McFarlane and Curtis (1989, 522-527) conducted a seven-day study using Hubbard chicks. Throughout the trial, the intensity of the electric shock gradually increased and the length of each exposure was random. It was concluded that there was a significant

increase in the H/L ratio after the seven-day electric shock exposure. Based on these and similar studies, it is possible that EEG could be used to measure stress in poultry.

Ammonia is a common stressor present in production facilities and is a concern for poultry producers. The current acceptable level of ammonia in production houses is 25 parts per million (ppm); however, it is not uncommon for levels to exceed 50 ppm or even 100 ppm (Anderson et al., 1964, 369-379; Saif, Y.M., 1997, 11, 1245). These levels for extended periods of time can have many undesirable effects such as keratoconjunctivitis, respiratory infections and inhibit growth and performance (Saif, Y.M., 1997, 11, 1245). Jones et al. (2005, 293-308) demonstrated that broilers chose to spend greater amounts of time in chambers with lower levels of ammonia and found ammonia levels above 10 ppm aversive. McKeegan et al. (2002a, 1033-1035) showed an activation of nasal nociceptors, or pain receptors, in hens during an exposure to ammonia. McKeegan et al. (2002b, 101-111) also showed avian receptors function similarly to mammals and are more fine-tuned than originally thought. It was also shown that ammonia triggers both olfactory and trigeminal receptors indicating hens not only smell ammonia but also experience pain at exposure to a median threshold of 3.75 ppm. McFarlane and Curtis (1989, 522-527) showed a significant increase in the H/L ratio of Hubbard chicks following an exposure of 125 ppm ammonia for seven days. In an EEG study with humans, van Toller et al. (1993, 1-16) demonstrated that there is a rise in the alpha frequency during an exposure to

ammonia. Based on these studies, it may be possible to use EEG to monitor the effects of ammonia on poultry.

Finally, a study by de Boer et al. (1988, 273-280) demonstrated that rats subjected to a stressful auditory stimulus of white noise at 100 dbA for 10 minutes showed a significant increase in plasma corticosterone levels. The corticosterone levels peaked approximately 10 minutes after the removal of the stimulus. Gross (1990, 759-761) showed an increase in the H/L ratio of chickens after exposure to a stressful auditory stimulus of 104 db. While there is little to no research involving auditory changes correlated with EEG changes in poultry, sound is a potential stressor that is present in production and should be explored.

The objective of the current study is to evaluate the suitability of using EEG for determining quantitative trends in brain activity associated with common stressors (mild electric shock, auditory, ammonia) in poultry. White Pekin ducks were chosen for this study because they are a meat-type floor-reared production bird of suitable temperament and availability. Relative frequency-based EEG analysis was used in all three experiments based on the technology used for EEG recording. Plasma corticosterone levels are used in both Experiments 2 and 3 as a standard measure of evaluating stress (Harvey et al., 1980, 161-171; Klingbeil, 1985, 10-19; de Boer et al., 1988; 273-280) against which any changes seen in the relative frequency bands of the EEG were measured. Table 1 summarizes the three experiments performed. It is the author's hypothesis that EEG may have the capability to help researchers not only

identify a stress response in the EEG brain waves, but also help discern between stressors based on patterns that may emerge in the brain waves.

Table 1 Summary of materials and methods of three experiments performed using White Pekin ducks.

	Experiment 1	**Experiment 2**	**Experiment 3**
Year	2011	2012	2013
# of Ducks (instrumented)	16	24	8
Stimuli	Sound Ammonia Shock	Sound Ammonia Shock Control	Shock No Shock Control
Trial Length	15 minutes	45 minutes	45 minutes
ECG?	NO	YES	NO
Corticosterone?	NO	YES	YES
Chamber	YES	YES	NO

Chapter 2

EXPERIMENT 1

Materials and Methods

Subject Selection and Surgical Procedure

Sixteen straight-run White Pekin ducks were obtained from a commercial hatchery (Metzer Farms, Salinas Valley, CA) and raised from 1 day of age in cohorts of 8 ducks. Standard care and conditions followed the approved University of Delaware AACUC Protocol Number (33) 12-10-10R. EEG transmitters (PhysioTel model F50-EEE, Data Sciences International St. Paul, MN) were surgically implanted at approximately 5 weeks of age, once birds reached the minimum size of 2000 g for surgery following the surgical procedure outlined in Savory and Kostal (1997, 963-969). Ducks were randomly selected, with food and water withheld for approximately 8 hours and 2-6 hours prior to surgery, respectively. Each duck was anesthetized using 5% isoflurane (IsoSol; Medco, Inc., St. Joseph, MO) at induction with 3% isoflurane for maintenance of anesthesia. Three leads were placed on the meninges covering the telencephalon through 0.9 mm holes that were drilled into the parietal bone; two holes on the right side of the midline and one on the left, using a high speed microdrill (model 18000 17, Fine Science Tools, Foster City, CA). Furthermore, two

leads were implanted in the complexus muscle just below the base of the skull for electromyography (EMG) to measure muscle movement. The ducks were given 0.4 mg/kg carprofen and 0.1 units/kg penicillin injected subcutaneously immediately prior to the procedure to allow the medications time to take affect. The birds were then given 5 days for recovery.

Electroencephalogram (EEG) Collection and Analysis

Signals from the wireless transmitter were recorded by four wireless telemetry receivers (model RMC-1, DSI) and the signals from the receivers were passed through a signal conditioner (model DSI Matrix, DSI). Brain activity was monitored and recorded using DSI Dataquest A.R.T. Acquisition software. EEG and EMG files were analyzed in DSI NeuroScore software. The raw EEG files were analyzed in NeuroScore by adding labeled markers over 2 second epochs indicating specific time periods: pre-treatment, stimulus, vent (for ammonia), and no stimulus. An epoch is a marker placed over an area of the total EEG in which each frequency is averaged to result in one value for each frequency. The markers were placed based on visual analysis of the EEG signal using the EMG signal as a reference to eliminate motion artifacts, which appear as high amplitude spikes in both the EEG and EMG channels. The number of epochs placed throughout each time period was variable based on each individual trial. Files that had significant artifact or interference that greatly reduced the number of epochs for any one of the time periods were excluded. The mean EEG,

mean EMG, alpha, beta, delta, theta, and sigma values and markers were exported on a 2 second epoch basis from NeuroScore to Excel (Microsoft Corp., Redmond, VA) and charted. Frequencies were converted to relative frequencies by dividing each frequency by the total EEG power for analysis.

A trial time of 15 minutes (900 seconds) was broken into time periods for analysis dependent on the stimulus used. Mild electric shock and auditory time periods were as follows: 3 minute pre-treatment, 3 minute stimulus, 3 minute no stimulus, 3 minute stimulus, and 3 minute no stimulus. The two stimulus periods and two non-stimulus periods were respectively combined for analysis. Ammonia trials were broken into the following time periods: 3 minute pre-treatment, 3 minute stimulus, 3 minute ventilation, 3 minute no stimulus, and 3 minute stimulus. Similar to the mild electric shock and auditory, the stimulus periods were combined and the ventilation and non-stimulus periods were combined for analysis. Birds were raised in cohorts of eight ducks in a common holding pen and each bird was individually placed in a separate, clear acrylic observation chamber (0.81 m x 0.80 m x 0.65 m) (Figure 1) for each trial. The observation chamber was covered with opaque brown paper to prevent the experimental bird from viewing researcher movements during the trial. Stimuli were applied individually and only one type of stimulus was used per treatment based on a randomization performed in Excel (Microsoft Corp., Redmond, VA). For the auditory stimulus, an 88 dB alarm (SpectrAlert, System Sensor, St. Charles, IL) was applied continuously for the duration of each three minute stimulus

period. For mild electric shock stimuli, an electric dog-training collar (SportDog Brand SD-400, Knoxville, TN) was fitted to a harness and positioned on the sternum of the duck (Figure 2) to apply a single shock of 60 mA (~1 second) every 30 seconds during the three minute stimulus periods. This shock level was chosen based on the reactions of previous experiments using broilers (E. Pritchett, M. Caputo, C. Kinney, E. Benson, and R. Alphin, Application of wireless EEG to measure stress in poultry, 2011).

For the environmental stimulus, approximately 50 ppm ammonia was continuously applied for the duration of each stimulus period. The observation chamber included two regions: a 0.81 m x 0.56 m x 0.65 m region for the bird (Figure 1c – right side) and a 0.81 m x 0.24 m x 0.65 m region for heating the ammonium hydroxide (NH_4OH) to produce ammonia gas (NH_3) (Figure 1c – left side). The ammonium hydroxide was heated on a hot plate (heated to reach the boiling point of 37.7°C) located in the ammonia region of the chamber. Once the ammonia gas was produced, a fan between the two regions pulled the ammonia into the chamber with the bird. Birds were not able to move between regions. An internal control system was used to activate ventilation between the ammonia chamber and the bird chamber and to vent to outside the building during the ventilation period. Ammonia concentration in the chamber was monitored using a ToxiRAE II Ammonia Sensor (RAW Systems, San Jose, CA).

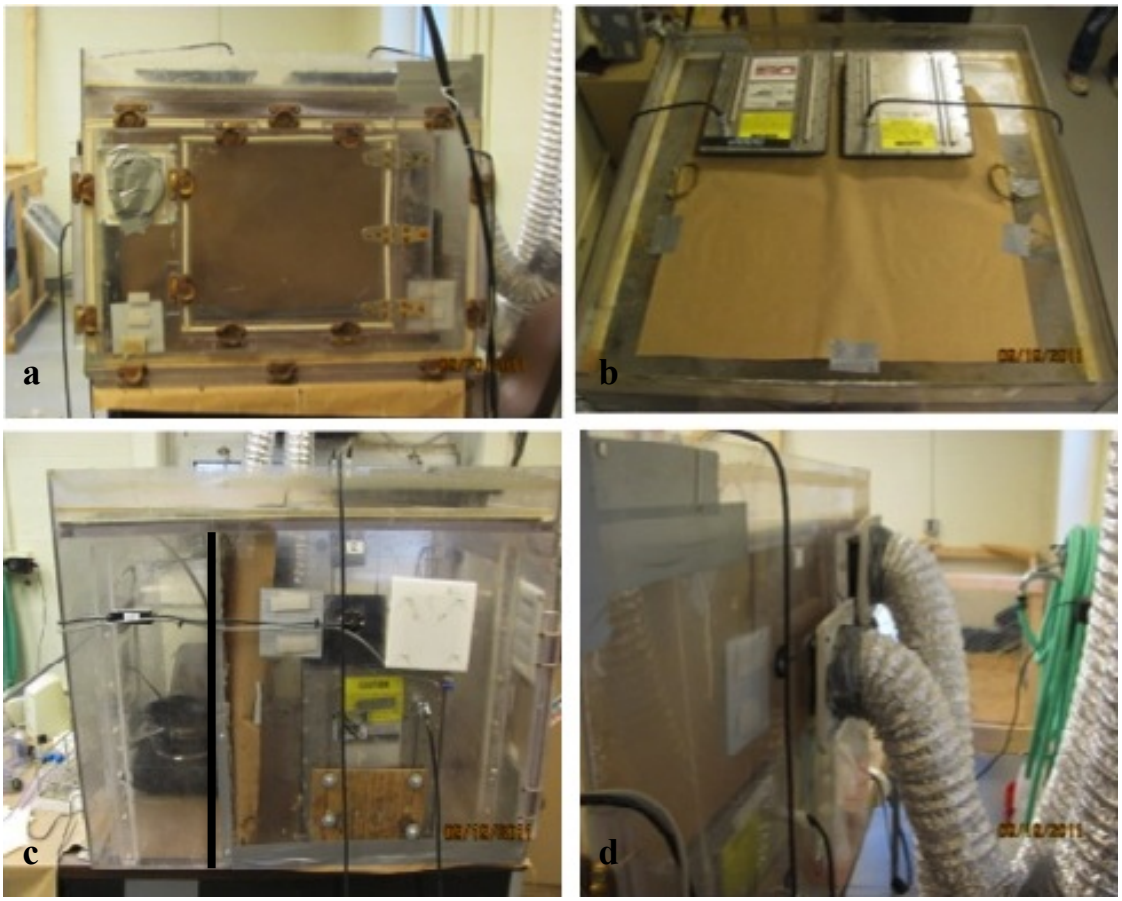

Figure 1 Observation chamber used for presentation of three stimuli: auditory, mild electric shock, and ammonia. a) Front View. b) Top View with two receiver plates. c) Side View, left = ammonia chamber, right = bird chamber. Bird chamber contains one receiver plate d) Exhaust Fans for ventilation of ammonia and one receiver plate.

Figure 2 White Pekin duck instrumented with an electric dog-training collar positioned over the sternum.

Statistical Analysis

Statistical analysis was performed for the EEG data using ANOVA and Student's T-Test in the statistical software JMP (Cary, NC). All tests were conducted at the 5% ($\alpha = 0.05$) significance level.

Results

The EEG results of Experiment 1 demonstrated there were no differences in each of the relative frequency bands (alpha, beta, delta, theta, and sigma) between the pre-treatment, stimulus, and no stimulus/vent periods for any of the stimuli (auditory,

mild electric shock, or ammonia) (Figures 3-5). In all trials, relative delta was the most prominent frequency, followed by relative theta.

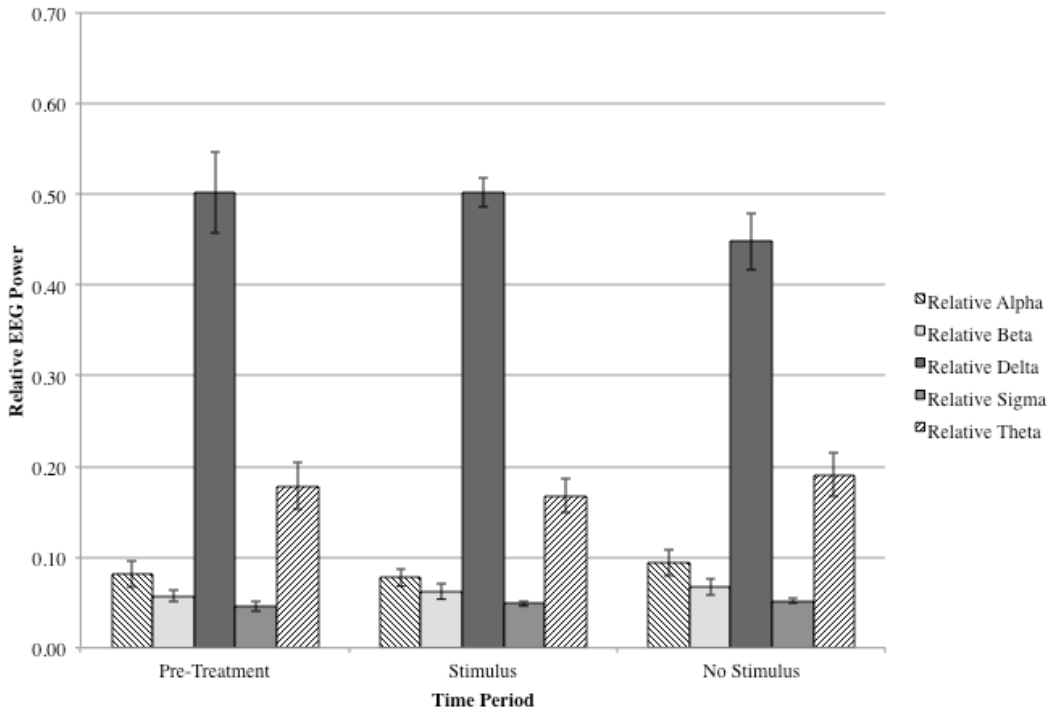

Figure 3 Mean relative frequencies of White Pekin ducks by treatment period for auditory stimulus. Error bars represent standard error of the mean (n=9).

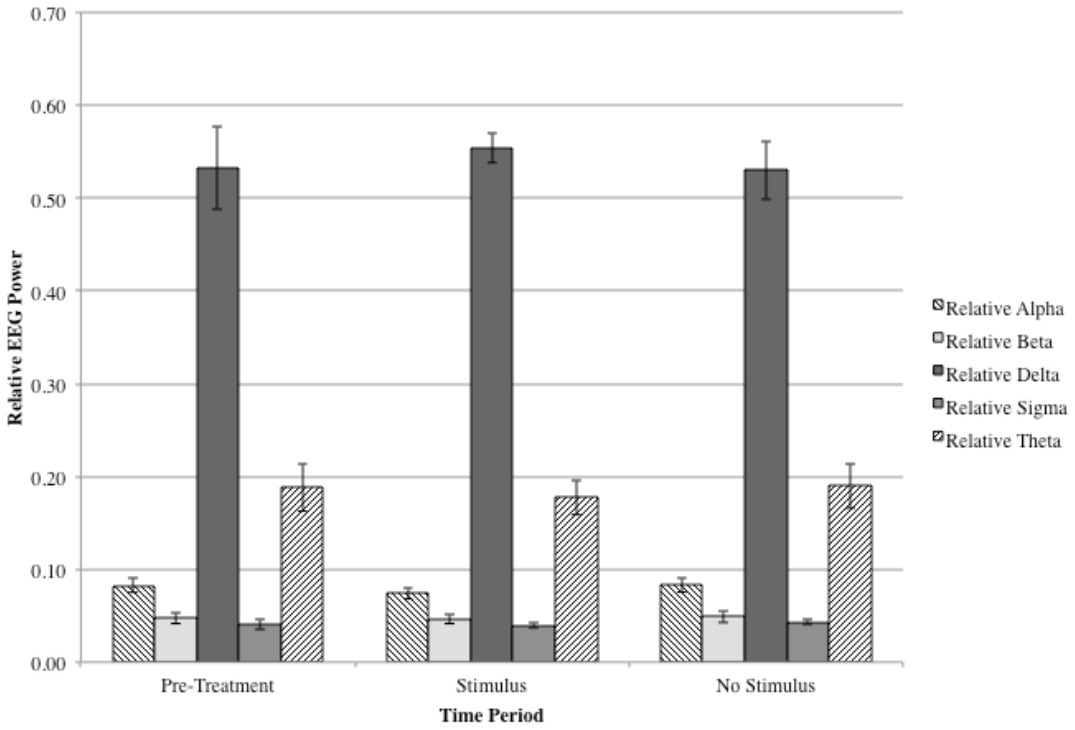

Figure 4 Mean relative EEG frequencies of White Pekin ducks by treatment period for mild electric shock stimulus. Error bars represent standard error of the mean (n=20).

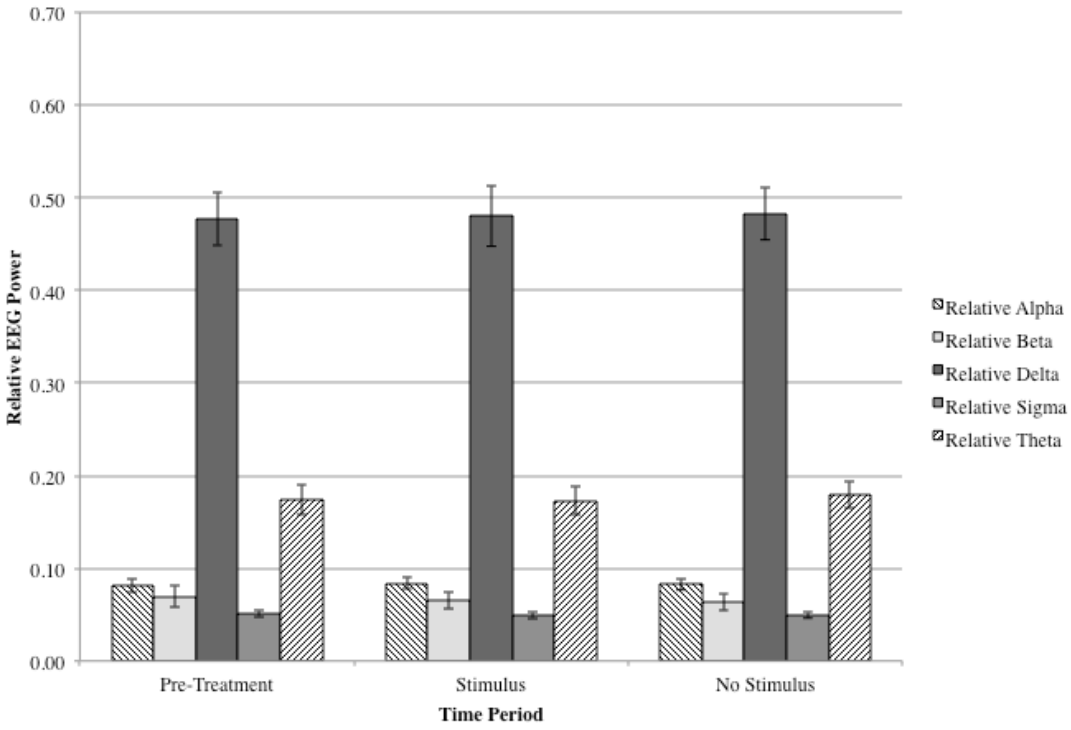

Figure 5 Mean relative EEG frequencies of White Pekin ducks by treatment period for ammonia stimulus. Error bars represent standard error of the mean (n=14).

Discussion

Previous studies performed to assess stimuli for protocol development initially used broilers with an observation time of 15 minutes. During the treatment, broilers displayed very little behavioral reaction to all stimuli presented (data not shown). White Pekin ducks were then used due to their availability and suitable temperament. After analysis of the EEG data in the present experiment -Experiment 1, it was

determined that the 15 minute trial was potentially not a sufficient recording time to see a discernable difference between time periods. The EEG files also contained considerable motion artifacts. These artifacts could be due to the fact that the birds did not have ample time to acclimate to the observation chamber and were unable to reach a relaxed state. The lack of a relaxed state during the treatments led researchers to believe the EEG results could have been impacted due to the birds potentially being under a stressed state throughout the entirety of the trial. Based on these results, it was determined that an extended recording time and analysis of additional parameters such as electrocardiography (ECG) and corticosterone levels for corroborative detection of stress would be incorporated into Experiment 2. ECG and corticosterone were added to confirm a stress presence.

Chapter 3

EXPERIMENT 2

Materials and Methods

Subject Selection and Surgical Procedures

For each of the three replications, twenty-five straight-run White Pekin ducks were obtained from a commercial hatchery (Metzer Farms, Salinas Valley, CA) and raised from 1 day of age in cohorts of 8 ducks. Standard care and conditions followed the approved University of Delaware AACUC Protocol Number (33) 12-03-12R. EEG transmitters (PhysioTel model F50-EEE, Data Sciences International St. Paul, MN) were sterilized following published protocol from DSI after completion of Experiment 1 and after completion of each of the three replications for Experiment 2. Once sterilized, EEG transmitters were surgically implanted in 8 birds for each replication (total of 24 implanted birds) at approximately 5 weeks of age, once birds reached the minimum size of 2000 g for surgery following the surgical procedure outlined in Savory and Kostal (1997, 963-969). Surgery and all necessary preparations and recovery procedures followed the protocol outlined in the Materials and Methods section of Experiment 1 in Chapter 2 (pg 18).

Electroencephalogram (EEG) and Electrocardiogram (ECG) Collection and Analysis

All equipment and monitoring procedures for EEG are found in the Materials and Methods section of Experiment 1 in Chapter 2 (pg 19). The raw EEG files were analyzed in NeuroScore by adding labeled markers over 2 second epochs indicating specific time periods: pre-treatment 1, pre-treatment 2, stimulus, and no stimulus.

A trial time of 45 minutes (2700 seconds) was broken into the following time periods: pre-treatment (first 1800 seconds), stimulus (600 seconds), and no stimulus (final 300 seconds). For each trial, individual birds were placed in the observation chamber used for Experiment 1. Stimuli included auditory, mild electric shock, and changes in the environment (50 ppm NH_3). Stimuli were applied individually and only one type of stimulus was used per treatment based on a randomization performed in Excel (Microsoft Corp., Redmond, VA). For the auditory stimulus, an 88 dB alarm (SpectrAlert, System Sensor, St. Charles, IL) was applied for 12 seconds per minute for the 10 minute stimulus period. For mild electric shock, the same equipment and procedure was used as in Experiment 1; however, a single shock was applied once per minute throughout the 10 minute stimulus period. All birds wore the shock collar, regardless of treatment, to eliminate the differences due to physical restraint by the collar harness.

For the environmental stimulus, approximately 50 ppm NH_3 was continuously applied for the entire stimulus period as described in Experiment 1 Materials and Methods in Chapter 2 (pg 20). The no stimulus period served as the ventilation

period. All birds underwent one control trial of 45 minutes without any stimulus presentation prior to receiving any other treatments to establish baseline EEG activity and stress levels.

To measure electrical cardiac activity, each duck was instrumented with ECG electrodes and leads (BIOPAC Systems, Inc., Goleta, CA) placed on a previously plucked area on each leg and underneath the right wing. ECG signals were recorded using BIOPAC Student Lab (BSL) software and was processed through BIOPAC Systems, Inc. MP30A acquisition unit. Analysis of the ECG signals was conducted using BIOPAC BSL Pro. ECG files were analyzed and broken into seven regions and the average heart rate in beats per minute (bpm) determined. The regions for analysis included the first and last 30 seconds of the pre-treatment, the first, middle, and last 30 seconds of the stimulus period, and the first and last 30 seconds of the no stimulus period.

Corticosterone Measurement

Plasma corticosterone levels were determined for all birds as a standard measurement of stress to correlate with any changes seen in the EEG. One milliliter (mL) of blood was drawn from the dorsal metatarsal vein prior to surgery (baseline), at 8:30 am on the day of treatment (pre-treatment), and immediately after completion of each trial (post-treatment). Blood samples were collected in under five minutes from the moment the researchers entered the room (pre-treatment) or from the completion

of the trial (post-treatment) to minimize the influence of handling stress (Vleck et al., 2000, 392-400). Blood was placed in EDTA-lined tubes and centrifuged to obtain plasma for corticosterone analysis using an Enzo Life Science (Farmingdale, NY) ELISA kit.

Statistical Analysis

Statistical analysis was performed for the corticosterone and heart rate data using the Wilcoxon Two-Sample test and Each Pair test, Kruskal-Wallis Test, and the Student's T-Test in JMP (Cary, NC). For statistical analysis of heart rate data specifically, the mean heart rate for each period was averaged within each treatment. EEG data was analyzed using ANOVA and the Student's T-Test in JMP (Cary, NC). All tests were conducted at the 5% ($\alpha = 0.05$) significance level.

Results

Of the 98 original EEG files, six were excluded due to electrical interference during recording. The valid EEG results are summarized below. As in Experiment 1, relative delta was the most prominent frequency detected, followed by relative theta in all trials. EEG analysis showed there were no significant differences between the pre-treatment, stimulus, and no stimulus periods for auditory and ammonia stimuli in each of the relative frequency bands (alpha, beta, delta, theta, and sigma). There was a significant difference during the stimulus period in the relative delta frequency when compared to pre-treatment periods ($p = 0.0022$) or periods without stimulus ($p =$

0.0072) for mild electric shock stimuli. Also during the stimulus period for mild electric shock there was a significant decrease in the relative alpha frequency when compared to pre-treatment periods ($p = 0.0449$) or periods without stimulus ($p = 0.0189$). The figures below show EEG data for auditory, mild electric shock, and ammonia stimuli (Figures 6, 7, and 8).

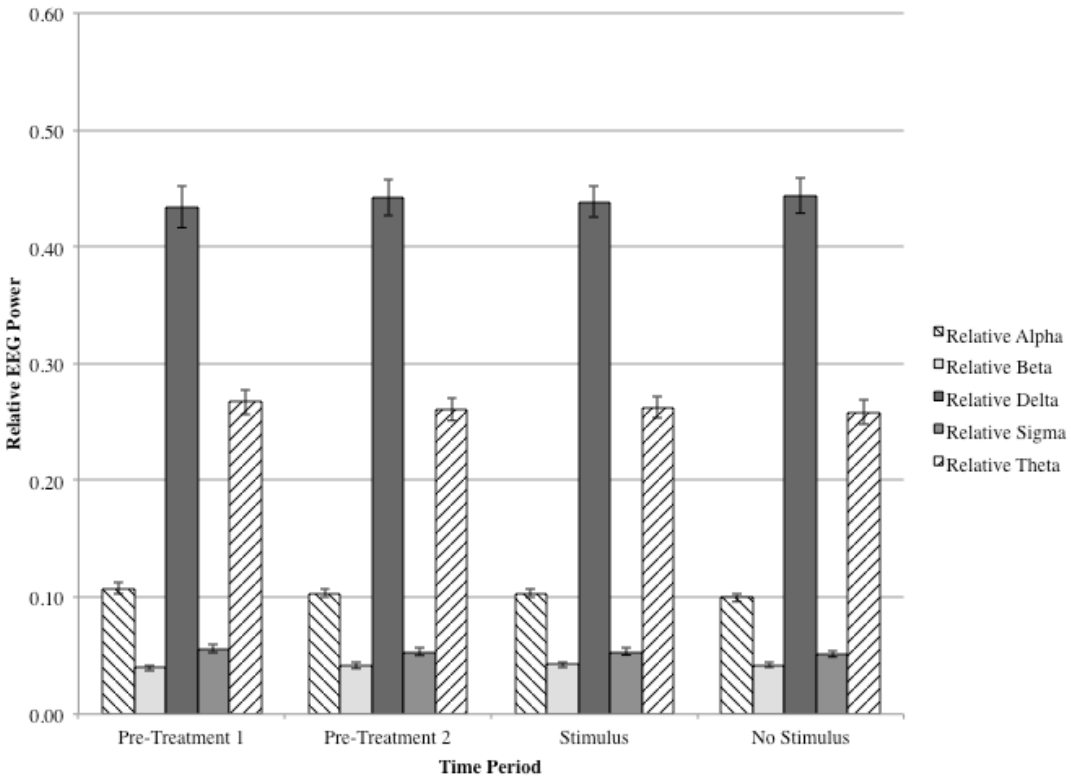

Figure 6 Mean relative EEG frequencies of White Pekin ducks by treatment period for auditory stimulus. Error bars represent standard error of the mean (n=18).

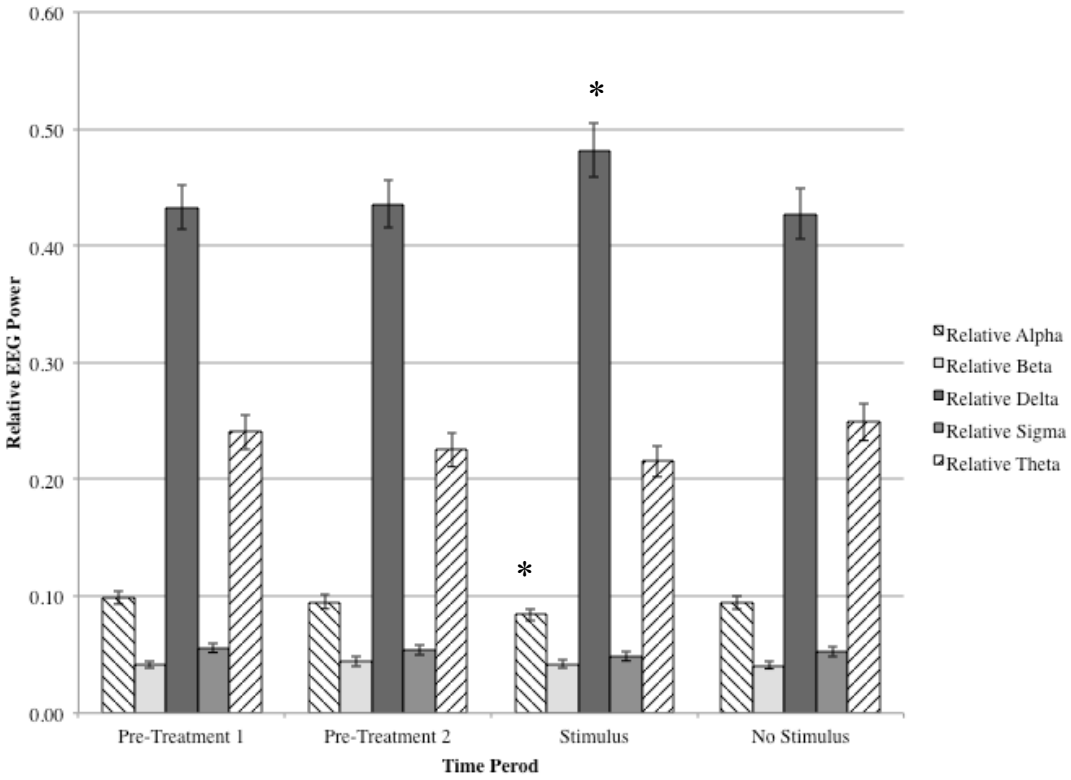

Figure 7 Mean relative EEG frequencies of White Pekin ducks by treatment period for mild electric shock stimulus. Error bars represent standard error of the mean (n=18). Relative frequencies with asterisk show significance between time periods ($p < 0.05$).

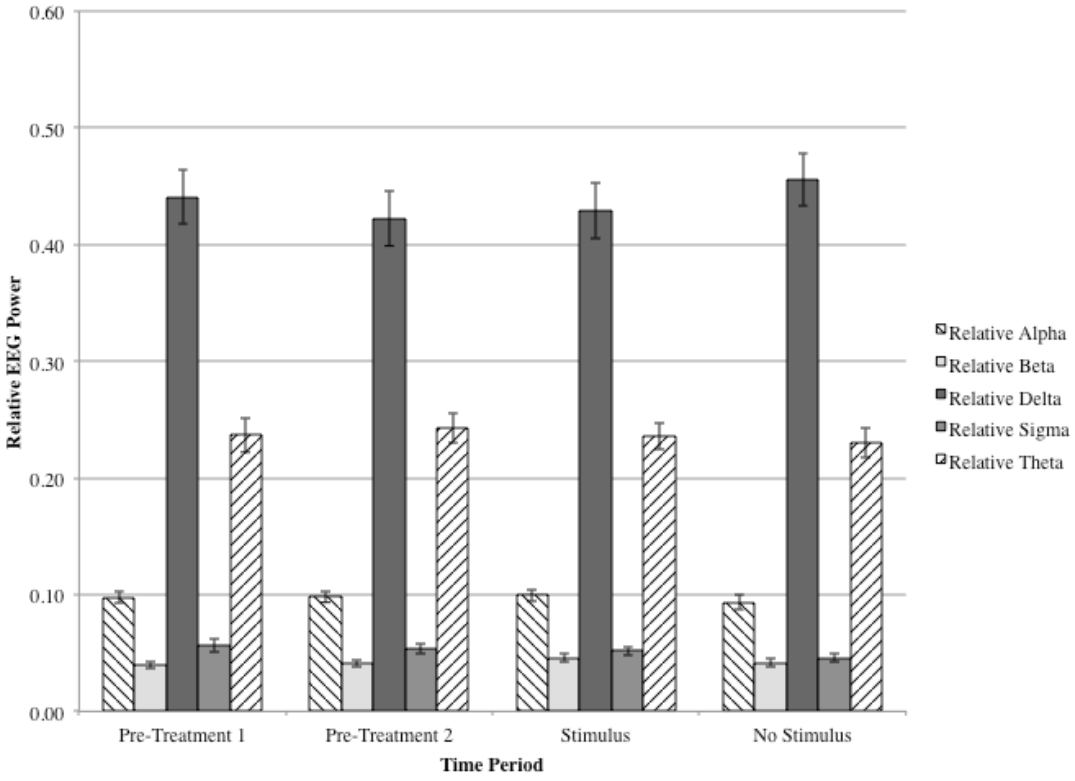

Figure 8 Mean relative EEG frequencies of White Pekin ducks by treatment period for ammonia stimulus. Error bars represent standard error of the mean (n=19).

The relative delta frequency was compared between each stimulus (auditory, mild electric shock, and ammonia) and the control trials since it was the most prominent frequency in all trials. When compared to control trials, there was no difference between time periods for auditory and ammonia. Mild electric shock

stimuli showed an increase in the relative delta frequency over that of the control trial during the stimulus period ($p = 0.0059$) and no difference between the two during the pre-treatment ($p = 0.0566$), no stimulus period ($p = 0.7262$). The figures below show EEG relative delta frequency comparisons between control trials and each stimulus (auditory, mild electric shock, and ammonia, respectively) by time period (Figures 9, 10, and 11).

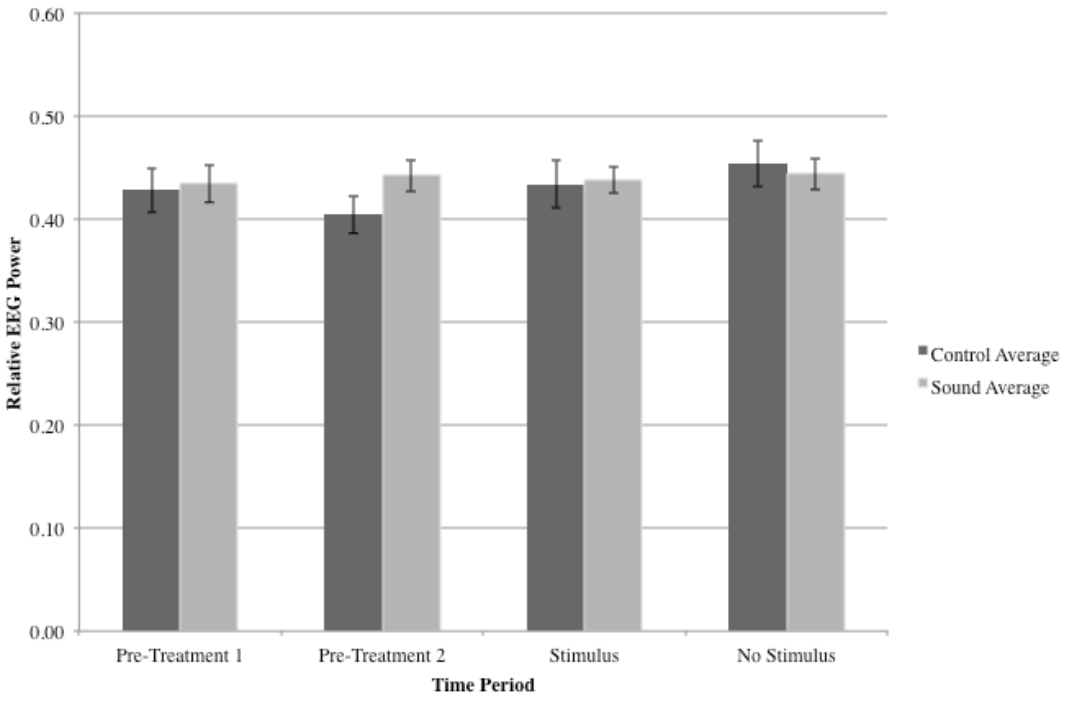

Figure 9 Mean relative delta frequency for auditory (sound) vs control trials. Error bars represent standard error of the mean (auditory n=18, control n=16).

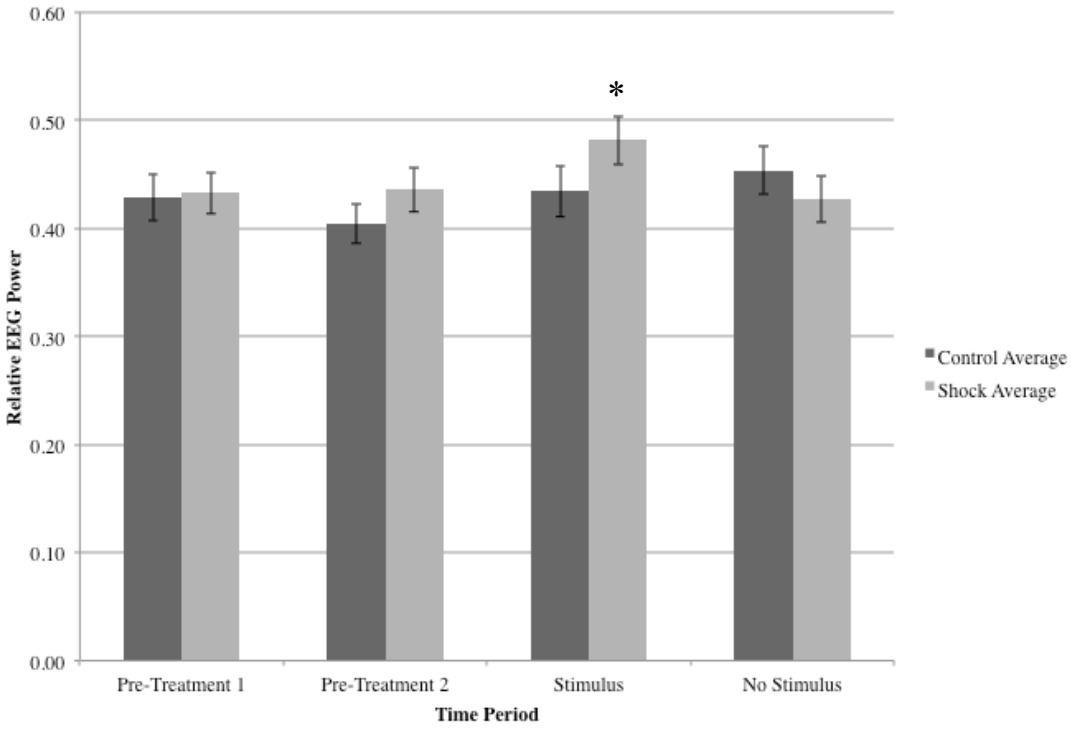

Figure 10 Mean relative delta frequency for mild electric shock vs control trials. Error bars represent standard error of the mean (shock n=18, control n=16). Treatments with asterisk are significant within each time period ($p < 0.05$).

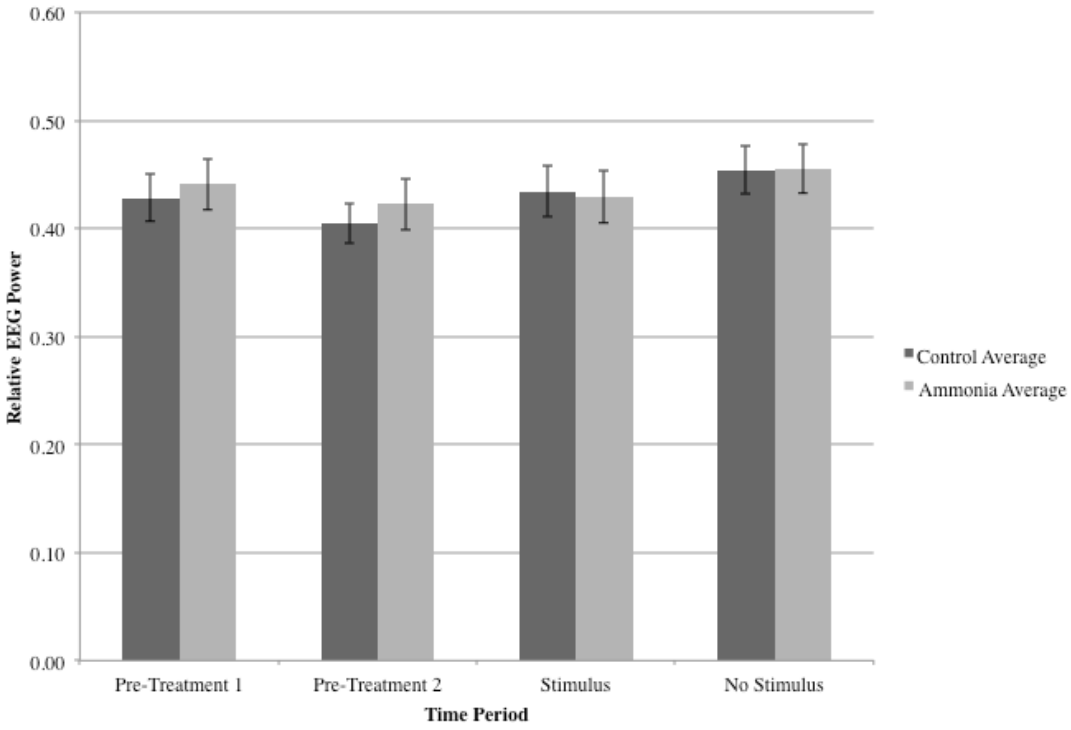

Figure 11 Mean relative delta frequency for ammonia vs control trials. Error bars represent standard error of the mean (ammonia n=19, control n=16).

Relative alpha for mild electric shock was compared to control trials due to the significant decrease during the stimulus period (Figure 12). Mild electric shock stimuli showed a decrease in the relative alpha frequency under that of the control trial during the stimulus period ($p = 0.0146$) and no difference between the two during the pre-treatment ($p = 0.1184$), no stimulus period ($p = 0.7767$).

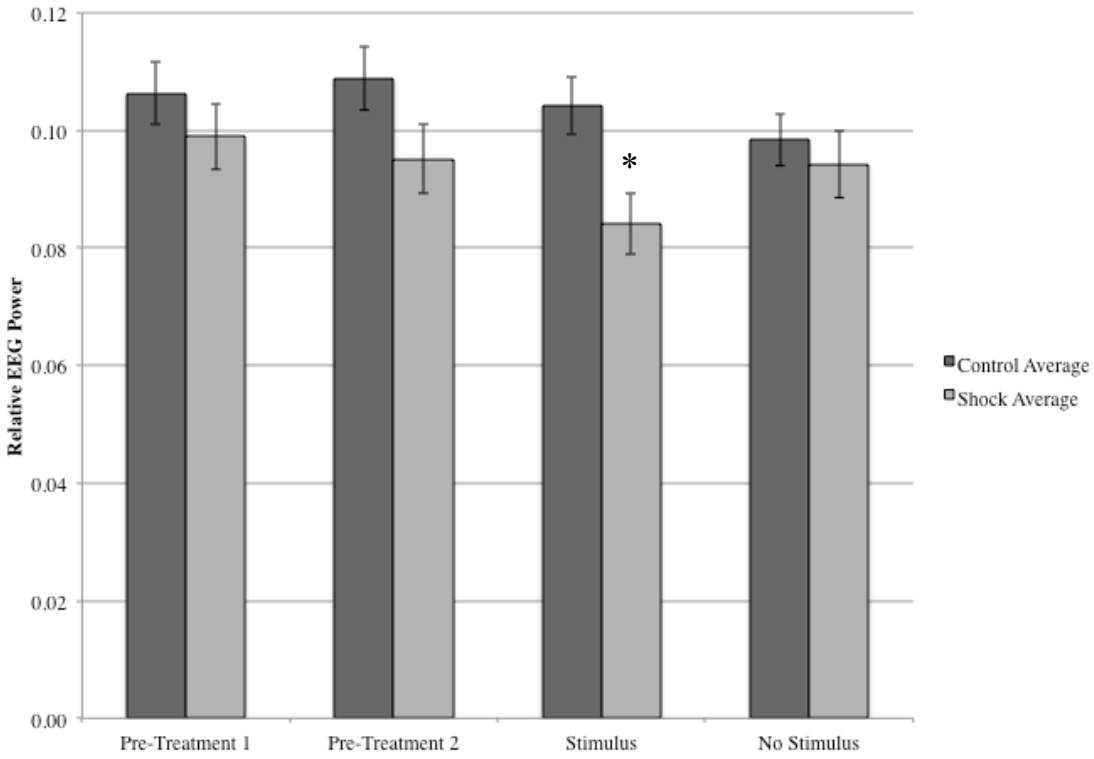

Figure 12 Mean relative alpha frequency for mild electric shock vs control trials. Error bars represent standard error of the mean (shock n=18, control n=16). Treatments with asterisk are significant within each time period ($p < 0.05$).

A total of 186 blood samples were collected and the results are shown in Table 3 and Figure 13. Post-treatment corticosterone levels were higher than pre-treatment corticosterone levels, indicating a rise in a stress during the observation period. Pre-treatment corticosterone levels were not distinguishable by treatment or when compared to pre-surgery baseline levels, as expected. Stimuli specific post-treatment

corticosterone levels were also not distinguishable from the control, which indicated the experimental process caused greater stress than the individual stimuli.

Table 2 Pre-treatment and post-treatment blood corticosterone in pg/ml. Data presented as mean ± standard error of the mean (X ± SEM). Significant differences in means within a row are indicated by unique superscript ($p < 0.05$).

Treatment	**ALL Pre-Treatment**	**Post-Treatment**
Ammonia	1064.8 ± 92.9^{a}	2191.9 ± 245.9^{b}
Shock		2159.8 ± 210.1^{b}
Sound		2175.8 ± 213.7^{b}
Control		2309.1 ± 226.3^{b}

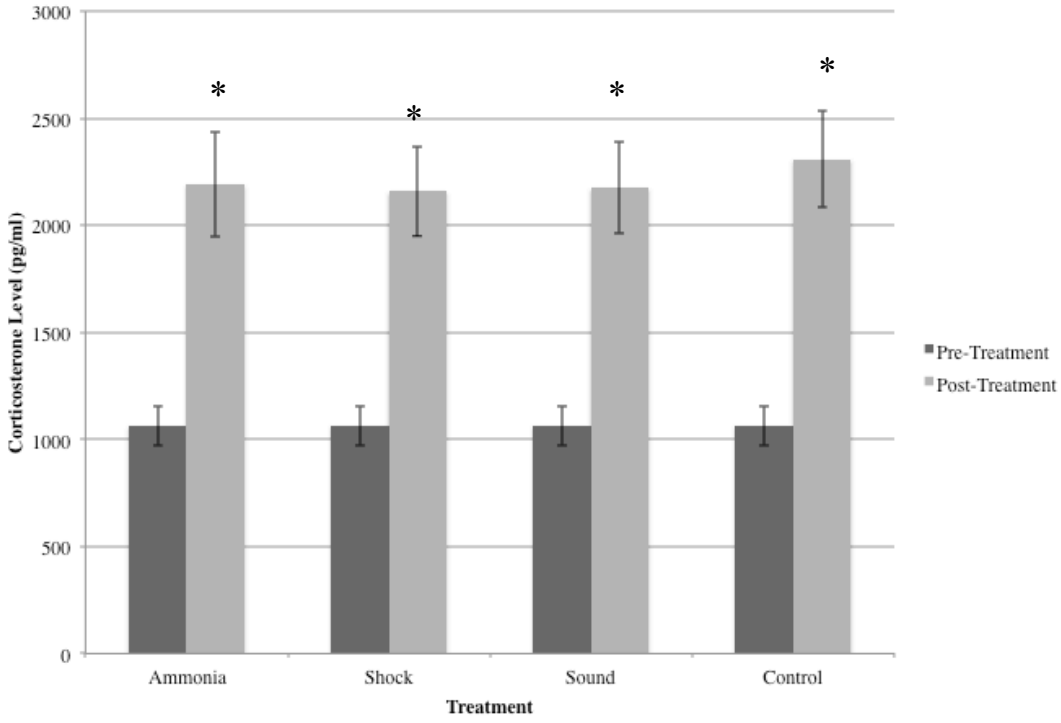

Figure 13 Pre-treatment and post-treatment corticosterone levels in pg/ml by treatment. Error bars represent standard error of the mean. Corticosterone post-treatment levels with asterisks differ from pre-treatment levels ($p < 0.05$).

ECG files were broken into seven regions for analysis and the difference in heart rate evaluated. There was an increase in heart rate during the middle and last 30 seconds of the stimulus period for mild electric shock (pre-treatment vs middle 30 seconds $p = 0.0419$; pre-treatment vs last 30 seconds $p = 0.0040$). There was a decrease in heart rate during the middle and last 30 seconds of the stimulus period for ammonia (pre-treatment vs middle 30 seconds $p = 0.0018$; pre-treatment vs last 30

seconds p = 0.0282). There were no significant differences between time periods for auditory and control treatments. The results are shown in Figure 14 below.

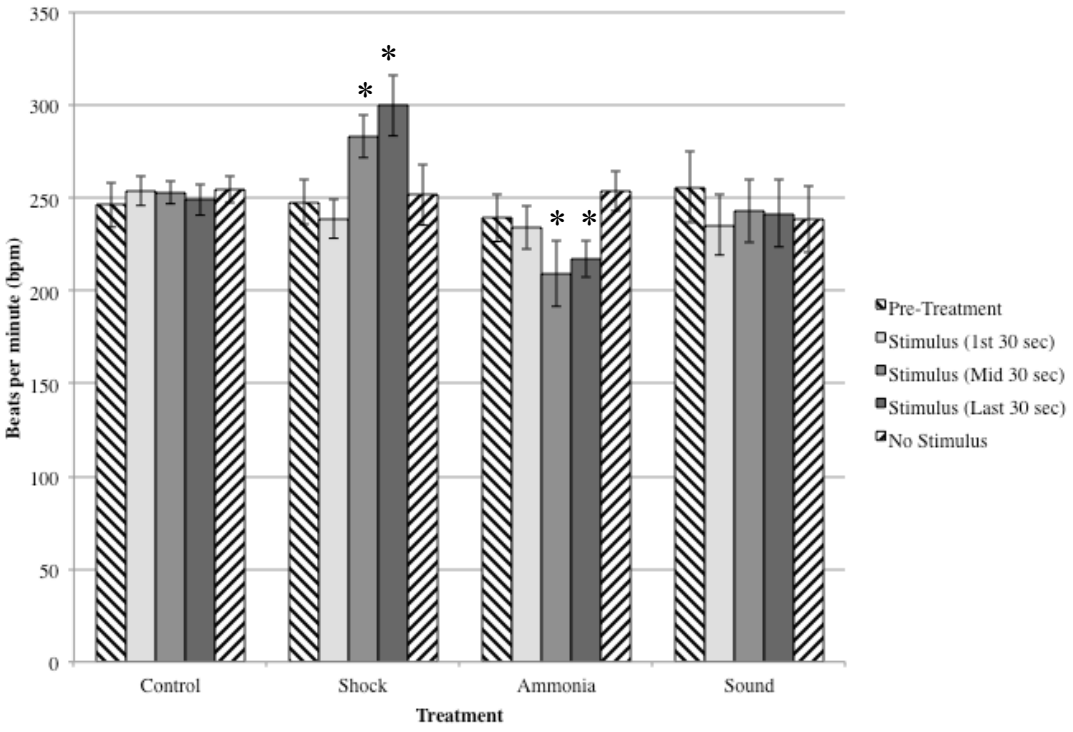

Figure 14 Heart rate in beats per minute was recorded over 30 second intervals during pre-treatment, stimulus, and no stimulus for control, mild electric shock, and ammonia treatments. Error bars represent standard error of the mean. (Control n=9, Shock n=14, Ammonia n=14, Sound n=14). Time periods with asterisks differ from remaining time periods within each treatment ($p < 0.05$).

Discussion

For Experiment 2, the observation time was extended from 15 minutes to 45 minutes. Experiment 1 EEG results contained significant motion artifact and it was determined the 15 minute recording time was not long enough to allow for acclimation to the chamber. For the extended 45 minute trials in Experiment 2, the 30 minute pre-treatment allowed time for acclimation to the observation chamber. The 10 minute stimulus period allowed for multiple stress presentations and time to react to the stimulus applied. The 5 minute no stimulus period allowed for ventilation after completion of the ammonia stimulus. Based on observations throughout each trial and on EEG analysis, it was determined this extended trial time successfully allowed for the birds to reach a relaxed state prior to entering the stimulus period. Based on the results of the corticosterone levels however, it was determined that while the birds seem to have reached a relaxed state, there was a significant rise in corticosterone levels for all treatments, including control trials.

Artifacts are a concern when monitoring EEG and ECG patterns, particularly in conscious, free-moving subjects. Remote monitoring of EEG, ECG, and behavior was 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. Artifacts can be caused by physical movements of the birds, struggling, wing flaps and clonic convulsions (no seizure activity was observed during this study) which can be verified by comparing EEG, ECG, EMG,

and motion cessation results (Alphin et al., 2010, 757-762; Coenen et al., 2009, 10-19; Caputo et al., 2012, 3057-3064). 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). When EEG, EMG, and ECG are used in combination, it is possible to eliminate areas of the recording that are impacted by motion artifact. This practice allows for a more accurate analysis of the recorded EEG signal (Amy Johnson, DVM, Department of Clinical Studies, University of Pennsylvania, personal communication).

Although the ECG could be used to determine heart rate and to identify areas of motion artifact, the ECG cables used in this experiment appeared to place restrictions on natural bird activity. When analyzing the control trial ECG data, it was seen that the overall heart rate was often higher than the heart rate of the three treatments with the exception of the certain time periods of the mild electric shock and ammonia trials. Movement itself can affect heart rate, which in addition to the restriction caused by the cables, may have confounded the results and led to some degree of variability. A study by Crowther et al. (2003, 365-370) determined that the heart rate of ostriches subjected to transport were significantly lower when the birds were sitting as opposed to standing. In the current experiment, the ducks are free moving in the observation chamber, and therefore sitting, standing and walking are typically observed throughout the entire trial regardless of treatment. These expressions of activity may account for the variable heart rates observed. It is unclear if any of the stimuli changed the frequency of the sitting, standing and walking

behaviors observed. For mild electric shock, it was not surprising to see an increase in heart rate during the stimulus period. Researchers hypothesized the increase would be evident during the first, middle, and last 30 seconds; however, there was no significant rise during the first 30 seconds of the stimulus period. This could be due to the fact that the birds had only received one shock and the effects on heart rate may not have been evident at this point. During the ammonia trials, it was interesting to note the decrease in heart rate during the middle and last 30 seconds of the stimulus period. Researchers were not certain as to why this pattern emerged but there is a potential it could be due to a decreased level of oxygen in the chamber or due to the birds decreased respiration once the presence of ammonia was noticed. Further testing should be done to determine the cause of the lowered heart rate during the middle and last 30 seconds of the stimulus period for the ammonia treatments.

Heterophil to lymphocyte (H/L) ratios were collected for Experiment 2 but did not result in a discernible pattern for analysis (data not shown). These results are most likely due to the fact that H/L ratios are better suited to monitor long-term stressors rather than acute stressors.

Based on the results of the plasma corticosterone, it appears that the ducks were experiencing a stress response regardless of treatment type or even treatment presence. This stress response, which is independent of treatment, may be caused by being placed in the observation chamber, by the presence of the ECG leads, or a combination of both. In a study involving rats and electric shock, a significant

increase in plasma corticosterone, similar to the increase after a shock presentation, was observed when subjects were placed in the experimental cage without a shock presentation (Friedman et al., 1967, 323-328). Based on corticosterone results of Experiment 2, Experiment 3 was conducted with the stress treatment applied in a natural "home" environment to attempt to remove the ambient stress encountered during the experimental process.

Chapter 4

EXPERIMENT 3

Materials and Methods

Subject Selection and Surgical Procedure

Thirty male White Pekin ducks were obtained from a commercial hatchery (Metzer Farms, Salinas Valley, CA) and raised from 1 day of age. Standard care and conditions followed the approved University of Delaware AACUC Protocol Number (33) 12-03-12R. EEG transmitters (PhysioTel model F50-EEE, Data Sciences International St. Paul, MN) were sterilized and surgically implanted following the protocol outlined in the Materials and Methods section of Experiment 2 in Chapter 3 (pg 28). Researchers administered pain medication and antibiotics to all experimental birds once per week for three weeks post-surgery following the dosage information in the Materials and Methods section of Experiment 1 in Chapter 2 (pg19). EEG monitoring and analysis followed the protocol outlined in the Materials and Methods section of Experiment 2 in Chapter 3 (pg 29).

The trial time of 45 minutes (2700 seconds) was broken into the following time periods: pre-treatment (first 1800 seconds), stimulus (600 seconds), and no stimulus (final 300 seconds). When the birds reached three weeks of age, 10 bird pairs

were randomly chosen based on a randomization R-script in JMP (Cary, NC) and placed in an enclosed pen (0.61 m x 0.91 m x 0.81 m) from three weeks to completion of the experiment (Figure 15).

Figure 15 Individual pens for each bird pair. Each row contains 5 pens. a) View of all pens b) Left side view c) End pen d) Inside pens

Birds were raised in a common holding pen with shavings for the first three weeks to prevent leg splaying. The birds were grouped in pairs to reduce general stress apparent as increased corticosterone from Experiment 2 and because White

Pekin ducks are sociable animals and do not perform well in isolation (FASS, 2010). Once the pairs were established, both ducks were within the same pen and neighboring pairs were visible in adjacent pens. Within the 8 experimental pairs, only one of the two birds was instrumented with an EEG sensor and used for EEG and corticosterone monitoring. The remaining bird in each experimental pair (companion) did not receive stimuli, was not monitored for EEG, but was evaluated for corticosterone levels. Two extra pairs of ducks were raised as replacements in case of loss during surgery. Each pen was equipped with PolyMax poultry flooring (0.22 m square openings, FarmTek, Dyersville, IA), independent stainless steel feed bins (0.58 m x 0.12 m x 0.09 m), automatic fill waterers (Kuhl Corporation, Cup-Q, Flemington, NJ, modified with a Kerick float valve, Grainger, Lake Forest, IL) and separated from the next with wire (0.03 m x 0.02 m) to allow for visibility but prevent the birds from freely moving from pen to pen. Four "mock receivers" (0.30 m x 0.30 m black plywood) were built and two were placed on the outside and top of each pen to acclimate the birds to the EEG receivers. All testing occurred individually in each of the 8 pens containing the instrumented ducks. Unlike Experiments 1 and 2, there was no movement of the instrumented duck to an observation chamber for testing.

In Experiments 1 and 2, only mild electric shock showed separation from control. As a result, in Experiment 3 only mild electric shock was applied as a stimulus and compared to no shock trials. Mild electric shock or no shock was applied individually and chosen based on a randomization performed in the statistical software

JMP (Cary, NC). Similar to Experiments 1 and 2, an electric dog-training collar (SportDog Brand SD-400, Knoxville, TN) was fitted to a harness but was positioned on the back of the duck. The shock collar placement was moved from the sternum as in Experiments 1 and 2 to the back to allow the birds to lie down and display normal bird behavior. The shock collar was placed on birds to be tested the day prior of testing to prevent handling before treatments. The shock (~60 mA) was applied once (~1 second) per minute for the 10 minute stimulus period. No shock trials have no stimulus treatment during the stimulus period and were randomly dispersed between the shock treatments to avoid an anticipatory response. Birds to receive a no shock trial also wore the collar to prevent association of the shock with placement of the collar. All birds underwent one control trial of 45 minutes without a stimulus presentation prior to receiving any mild electric shock or no shock treatments for comparison purposes. Birds did not wear the shock collar for the control trial.

In Experiment 2, the ECG cables may have led to variability in the heart rate data. For this reason and because of the cage configuration of Experiment 3, ECG readings were not collected. Removal of ECG recordings also eliminated the stress of attaching cables and the impact the cables have on the movement of the birds. Instead, plasma corticosterone alone was collected from all birds as a standard measurement of stress to correlate with EEG results. One milliliter (1 mL) of blood was drawn from the metatarsal vein prior to surgery (baseline), at 8:30 am the day prior to treatment (pre-treatment), and immediately after completion of each trial (post-treatment).

Blood collection and processing followed the protocol outlined in the Materials and Methods section of Experiment 2 in Chapter 3 (pg 30).

Statistical Analysis

Statistical analysis was performed on the EEG data using ANOVA and Student's T-Test and the corticosterone data was analyzed using Wilcoxon Each Pair test in the statistical software JMP (Cary, NC). All tests were conducted at the 5% ($\alpha = 0.05$) significance level.

Results

The EEG results of Experiment 3 demonstrated there were no differences between time periods in each of the relative frequency bands (alpha, beta, delta, theta, and sigma) between pre-treatment, stimulus, and no stimulus periods for mild electric shock, no shock, and control trials. In all trials, relative delta was the most prominent frequency, followed by relative theta. The figures below show the EEG data for mild electric shock, no shock, and comparisons between shock and no shock trials with control trials (Figures 16, 17, 18, and 19, respectively).

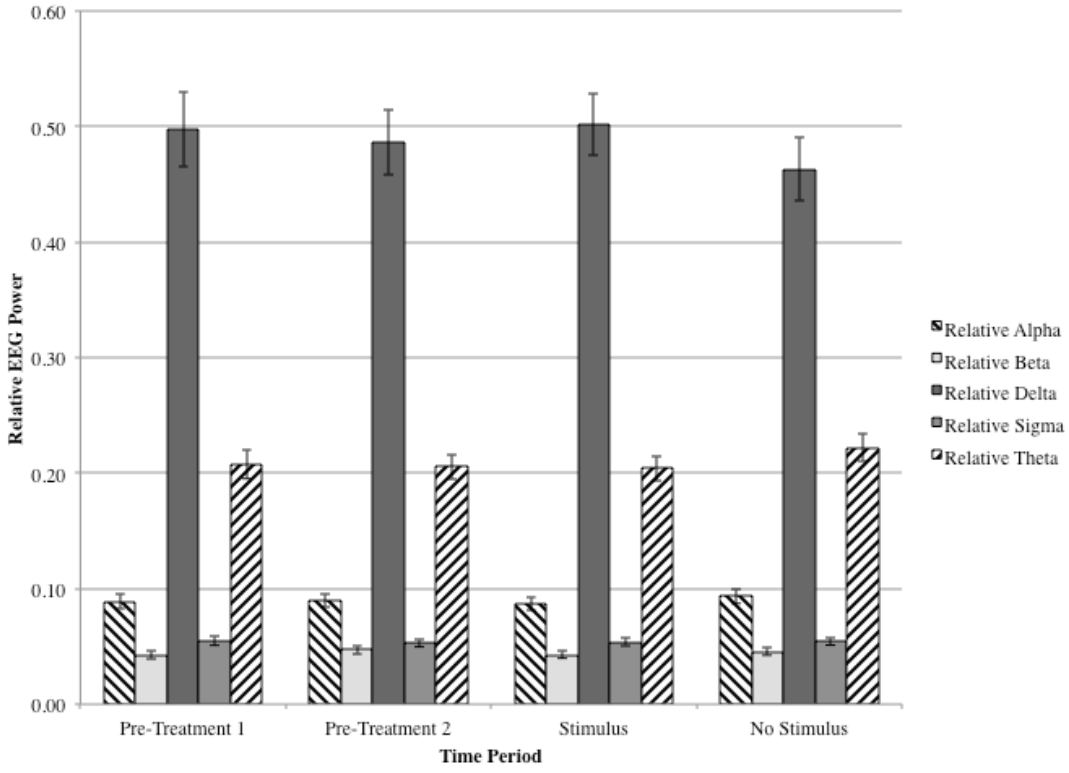

Figure 16 Mean relative EEG frequencies of White Pekin ducks by time period for mild electric shock stimulus. Error bars represent standard error of the mean (n=17).

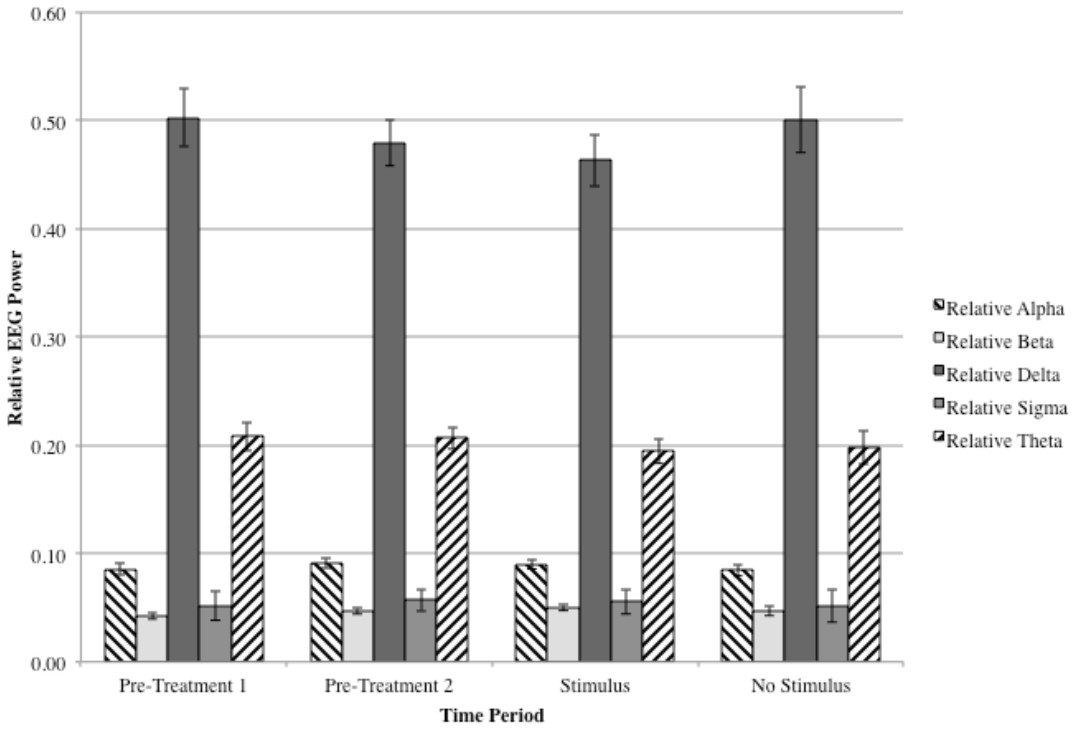

Figure 17 Mean relative EEG frequencies of White Pekin ducks by time period for no shock trials. Error bars represent standard error of the mean (n=17).

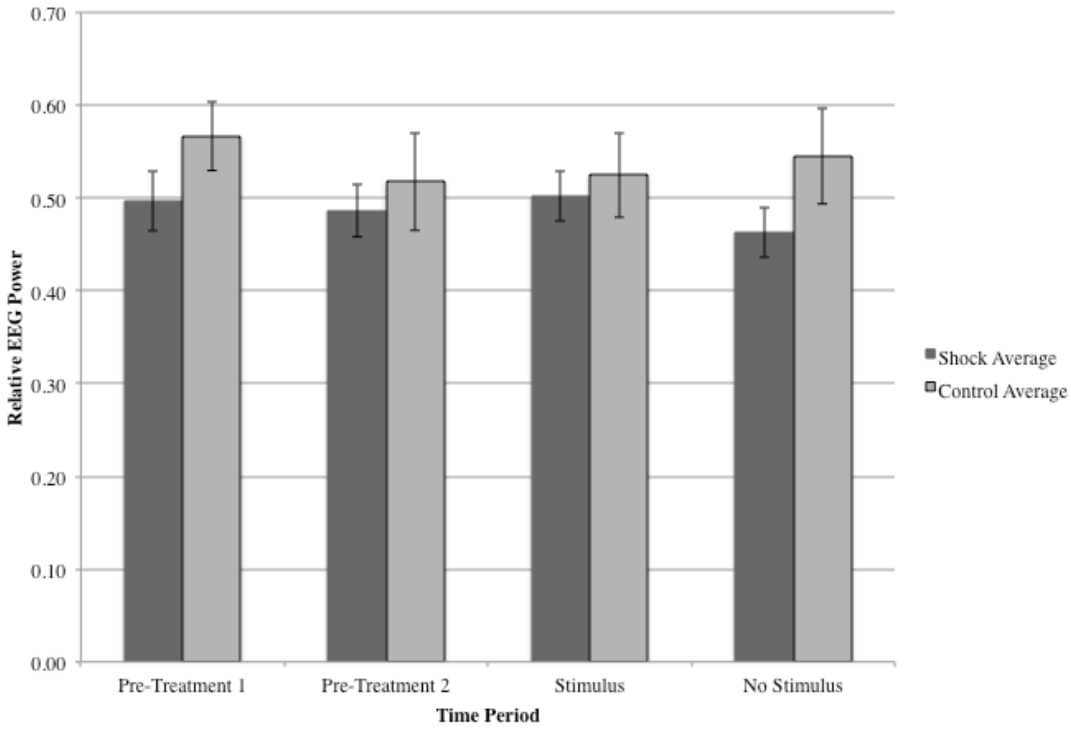

Figure 18 Mean relative delta frequencies for shock vs control trials. Error bars represent standard error of the mean (shock n=17, control n=9).

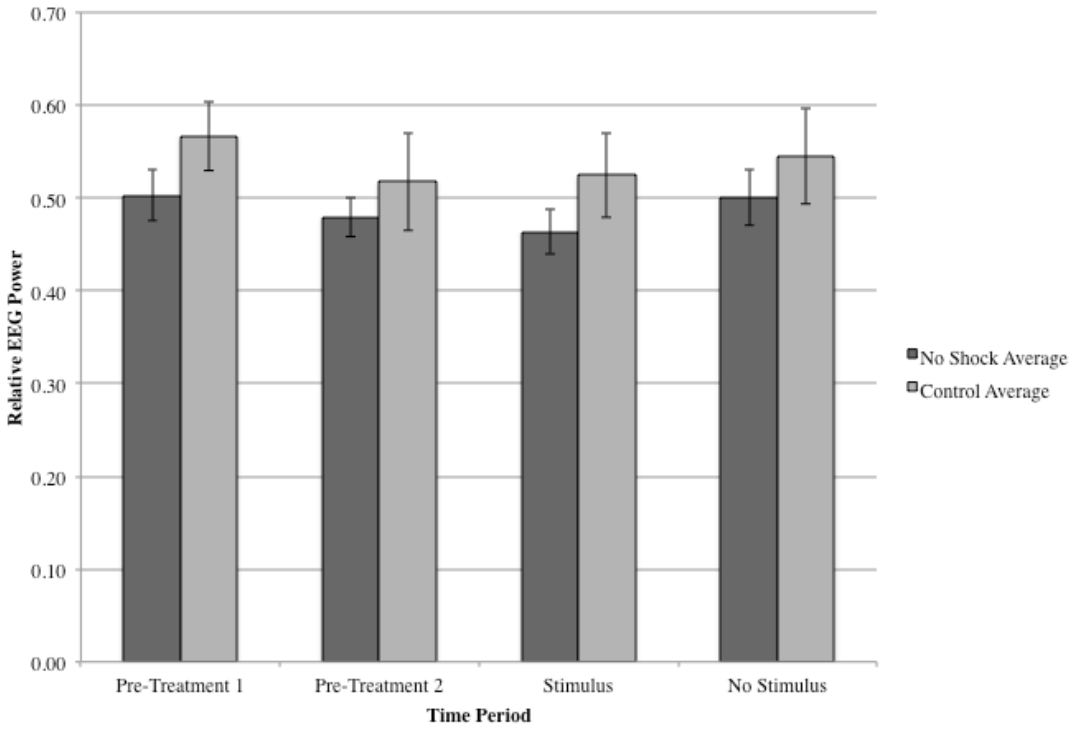

Figure 19 Mean relative delta frequencies for no shock vs control trials. Error bars represent standard error of the mean (no shock n=17, control n=9).

A total of 147 blood samples were collected from both experimental and companion birds. Two post-treatment mild electric shock values were excluded due to a lack of response to shock presentation, potentially due to shock collar malfunction. Two post-treatment no shock values were also excluded due to extremely high activity of the experimental bird throughout the trials. The results are shown in Tables 2, 3, and 4. Table 3 compares the pre-treatment and post-treatment corticosterone values for the experimental birds. All pre-treatment values were combined for analysis due

to no significant difference between treatments. There was no significant difference between the pre-treatment and post-treatment levels for no shock ($p = 0.6372$). For control, the post-treatment corticosterone level was lower ($p = 0.0345$). Mild electric shock post-treatment levels were higher than pre-treatment levels ($p = 0.0308$). Companion pretreatment and post-treatment levels were compared and no significant differences were found. Figure 20 shows the pre-treatment and post-treatment values for the experimental birds by treatment. Table 4 compares experimental post-treatment levels for shock and no shock to that of control trials. There was no significant difference between post-treatment control levels and post-treatment no shock levels ($p = 0.1358$). It was determined that post-treatment shock levels were significantly higher than post-treatment control corticosterone levels ($p = 0.0014$). Companion post-treatment comparisons led to no significant differences. Table 5 compares experimental and companion pre-treatment levels and experimental and companion post-treatment levels. The companion pre-treatment levels were lower than experimental birds ($p = 0.05$). There was no significant difference between experimental post-treatment control ($p = 0.7911$) and no shock ($p = 0.1002$) values when compared to companion post-treatment corticosterone values. Experimental post-treatment shock values were higher than companions ($p = 0.0016$). Baseline levels were not distinguishable from the pre-treatment levels, which was expected.

Table 3 Pre-treatment and Post-treatment corticosterone levels for Experimental birds. Data presented in mean corticosterone levels in pg/ml ± standard error of the mean (X ± SEM). Means within a row with different superscripts differ ($p < 0.05$).

EXPERIMENTAL BIRDS	
All Pre-Treatment	**Control Post-Treatment**
2378.8 ± 418.7^{a} n=36	830.8 ± 253.7^{b} n=9
	Shock Post-Treatment
	4689.1 ± 1284.6^{b} n=14
	No Shock Post-Treatment
	2054.1 ± 844.5^{a} n=8

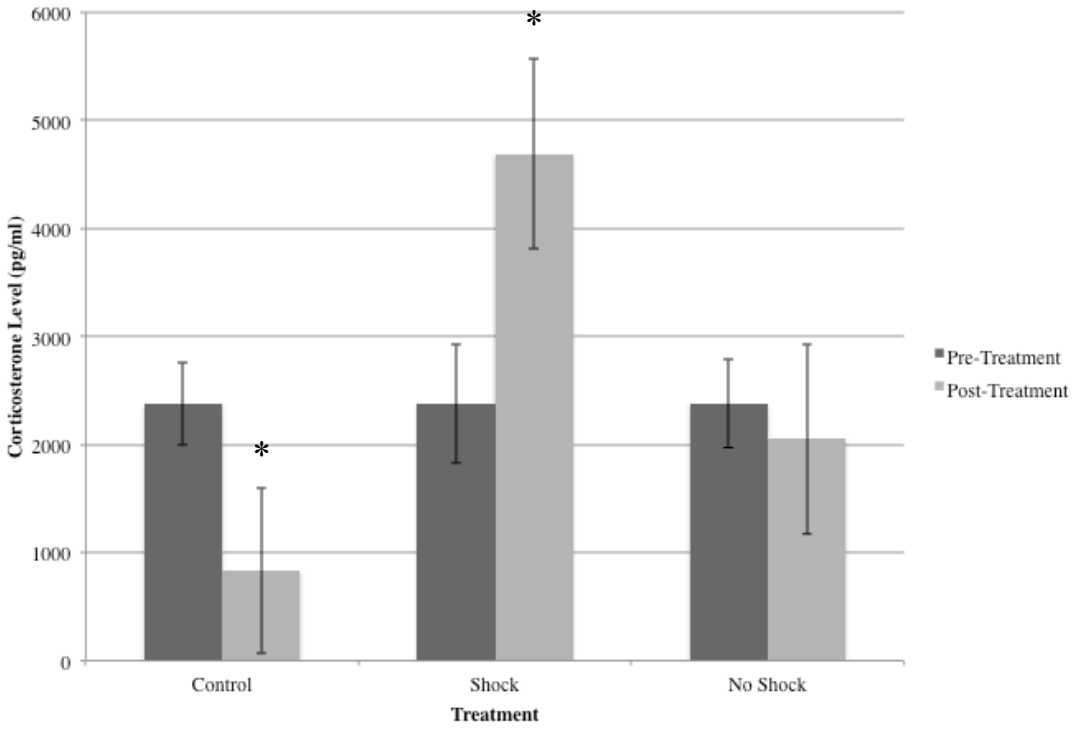

Figure 20 Pre-treatment and Post-treatment corticosterone levels in pg/ml for experimental birds by treatment. Error bars represent standard error of the mean (Pre-treatment n=36, Post-treatment control n=9, shock n=14, no shock n=8). Corticosterone post-treatment levels with asterisks differ from pre-treatment values ($p < 0.05$).

Table 4 Post-treatment shock and no shock corticosterone levels for Experimental birds as compared to control levels. Data presented in mean corticosterone levels in pg/ml ± standard error of the mean (X ± SEM). Means within a row with different superscripts differ ($p < 0.05$).

<table>
<tr><th colspan="2">EXPERIMENTAL BIRDS</th></tr>
<tr><th>Control Post-Treatment</th><th>Shock Post-Treatment</th></tr>
<tr><td rowspan="3">830.8 ± 253.7[a]
n=9</td><td>4689.1 ± 1284.6[b]
n=14</td></tr>
<tr><td>No Shock Post-Treatment</td></tr>
<tr><td>2054.1 ± 844.5[a]
n=8</td></tr>
<tr><th>Shock Post-Treatment</th><th>No Shock Post-Treatment</th></tr>
<tr><td>4689.1 ± 1284.6[a]
n=14</td><td>2054.1 ± 844.5[b]
n=8</td></tr>
</table>

Table 5 Experimental bird corticosterone levels as compared to companion bird levels. Data presented in mean corticosterone levels in pg/ml ± standard error of the mean (X ± SEM). Means within a row with different superscripts differ ($p < 0.05$).

EXPERIMENTAL BIRDS	COMPANION BIRDS
All Pre-Treatment	**All Pre-Treatment**
2378.8 ± 418.7^{a} n=36	1796.17 ± 534.2^{b} n=34
Control Post-Treatment	**Control Post-Treatment**
830.8 ± 253.7^{a} n=9	1008.4 ± 383.7^{a} n=7
Shock Post-Treatment	**Shock Post-Treatment**
4689.1 ± 1284.6^{a} n=14	1000.6 ± 155.9^{b} n=15
No Shock Post-Treatment	**No Shock Post-Treatment**
2054.1 ± 844.5^{a} n=8	879.9 ± 285.9^{a} n=10

Discussion

Experiment 2 EEG results showed a significant increase in the relative delta frequency during the stimulus period for the mild electric shock stimulus. This was not replicated in Experiment 3 EEG results. Removal of the experimental chamber and ECG leads was hoped to not only replicate these EEG results but also potentially increase the separation between treatments. It was determined that EEG is currently

not a viable option for stress measurement in commercial poultry. It is interesting to note that the relative delta frequency was the most prominent frequency throughout all experiments. Further testing should be done to determine why relative delta was the most prominent frequency considering it is a low amplitude frequency most often associated with unconscious states.

Pre-treatment and post-treatment corticosterone level results were as expected with only mild electric shock resulting in a significant increase in post-treatment levels. It is also important to note that shock post-treatment values were significantly higher than control post-treatment values ($p = 0.0028$). The significant increase from pre-treatment to post-treatment corticosterone levels in only mild electric shock led researchers to believe the shock presentation was in fact resulting in a stress response in the birds. When experimental bird values were compared with companion bird values, the mild electric shock post-treatment values for the experimental birds were significantly higher than companion post-treatment values ($p = 0.0016$). There was also no significant difference between the pre-treatment values, post-treatment control values, and post-treatment no shock values between experimental and companion birds. This result indicates there were no ambient stressors present in the environment (similar to that of the chamber in Experiment 2) that could have impacted the corticosterone levels. It is interesting to note the overall increase in corticosterone pre-treatment levels from Experiment 2 to Experiment 3. For Experiment 2, the combined pre-treatment cotricosterone level was 1064.8 ± 92.9 pg/ml whereas for

Experiment 3, the combined pre-treatment corticosterone level for experimental birds was 2378.8 ± 418.7. Because the pre-treatment corticosterone levels are prior to any treatment, these levels should be comparable between experiments. This variability could be due to differing bird numbers from Experiment 2 (24 birds) and Experiment 3 (8 birds). Another potential explanation for the difference between corticosterone levels is the change in preparation for the pre-treatment blood draws between Experiments 2 and 3. For Experiment 2, all testing and blood draws were performed in a room inside a building whereas for Experiment 3, all testing and blood draws were done in a small building (brooder house). When researchers arrived for the pre-treatment draws for Experiment 3, it is possible the birds could hear movement outside the building and this resulted in a stress response that was present in the corticosterone. This could also explain the significant decrease in corticosterone levels for the control post-treatment values in Experiment 3. Once the researchers completed the pre-treatment draws and began control trials, there was very little movement, which allowed the birds to reach a relaxed state.

Chapter 5

CONCLUSION

The electroencephalogram is a very powerful recording tool; however, based on the results of the three experiments, it has been determined that the currently available EEG technology is not suitable for stress detection in commercial poultry. Experiment 1 had a recording time of 15 minutes utilizing several stressors: mild electric shock, sound, and ammonia. No identifiable changes or patterns in the EEG were detected, leading to the conclusion that the brief recording time did not allow for relaxation and could not accurately capture any changes the stress may be inducing in the birds' brain waves. Experiment 2 was designed to address the issue of recording length by extending the recording time as well as comparing stress results with control trials, or normal brain activity. Corticosterone and ECG were also measured to determine that a stress response was present to corroborate any changes seen in the EEG. While an increase in the delta frequency was seen in mild electric shock trials during the stimulus period, no significant changes were seen for ammonia or sound. Based on these results, it was determined that EEG would not be a good indicator of a long-term, more subtle stressor such as ammonia. This further reduces the practicality of the EEG as a measurement of stress considering the high prevalence of ammonia in production facilities. It was also determined in Experiment 2 that the experimental

process, or placing the birds in the experimental chamber, in itself led to a significant stress response that could have been masking any changes in the EEG induced by the stressors applied. Experiment 3 was designed to address this issue as well as to replicate the results seen for mild electric shock. The results seen in Experiment 2 for mild electric shock (a significant increase in the relative delta frequency during the stimulus period) was not replicated in Experiment 3. When taken together, the results of the three experiments indicate that the EEG technology used is not a viable option for stress detection in commercial poultry. While the unpredictability of the EEG is an issue, there were also several other challenges associated with EEG technology that should be addressed.

Transmitters must be surgically implanted in the test subjects to eliminate the possibility of lost leads over a long-term study. This surgery requires trained professionals, anesthesia, necessary medications (pain and antibiotics), and a sterile environment to ensure the transmitter is implanted properly. Once the surgery is complete, it is recommended the bird remain in a clean environment; however, this is not always possible. Placing the bird with the other birds leads to potential problems such as wound picking and infection. Surgical implantation of the transmitter followed the protocol outlined in Savory and Kostal (2006, 599-606) and has been used in the research group with broilers (Alphin et al., 2010, 757-62), turkeys (Rankin, 2010), layers, and ducks (Caputo et a., 2012, 3057-64). In this specific research with ducks, researchers found this protocol was more suitable for poultry other than

waterfowl for several reasons. Maintenance of anesthesia simply using isoflurane (a volatile agent) was difficult in ducks due to physiological adaptations of waterfowl. Breath holding and tolerance of lower oxygen partial pressures led to an insufficient depth of anesthesia. Researchers found this adaptation was more prominent as the birds grew larger. It is recommended that waterfowl be intubated and utilize intermittent positive-pressure ventilation (IPPV) to maintain anesthesia or use of an injectable agent. It is also recommended that surgeons monitor intubation tubes carefully due to increased saliva production, which may block the intubation tube (Avian Medicine, 2009, 284-285).

In Experiment 3, infection was suspected to be an issue for several of the experimental birds. While the specific infection was never confirmed, generalized malaise (sickness), fluid build-up around transmitter site, and a marked change in bird behavior led to the depopulation of two birds during the experiment. Researchers administered additional doses of antibiotics each week for 3 weeks post surgery to help with any present infections. Infection can be a serious issue once the surgery is complete, especially in waterfowl. Preening is a common practice of birds in which the feathers are groomed. Birds will clean dust and dirt and orient feathers in the proper position to maintain good health. In addition to cleaning the feathers, most birds also have a preen gland that secretes a protective oil. It was noticed in this study that the birds were utilizing the back of the head to distribute this oil over the body,

which is a major concern and could have been the source of the infection. Further studies should be done to determine ways to mitigate this issue.

Another problem with the current EEG technology is electromagnetic interference. The EEG technology used in this experiment had several instances of interference. Video monitoring was initially used to capture behavior during trials in Experiment 2; however, it was found that the camera was causing an interference pattern in the EEG and video recording was eliminated. Experiment 3 also showed that an interference reaction was being seen in the EEG in response to the shocks applied via the shock collar for the mild electric shock stimulus. It is important to note that during the EEG analysis for all experiments, the moment in which a shock was applied was not included in the analysis due to the presence of artifact. The extreme movement in response to the shock led to an area in which the EEG data file could not be evaluated. This could potentially eliminate the concern for interference from the shock collar during the mild electric shock trials. Further testing should be done utilizing non-electrical acute stressors to eliminate the issue of interference.

Finally, the current EEG technology has a very limited range. A production facility could not implant the transmitter in a test subject and monitor the EEG while the bird moved freely within the house. It was found in the testing that the transmitter needed to be within 30 cm of a receiver plate. If the bird is outside this range, it leads to a reduction of the signal strength and a reduction in overall EEG quality.

In conclusion, the EEG technology used in this study is currently not a suitable method of stress determination in poultry. The EEG equipment used in this experiment can be very useful when determining brain death and unconsciousness in poultry because these brain states are dramatic changes from normal brain behavior and are now easily recognized. It is possible the EEG technology used is not capable of detecting subtle changes in brain waves in response to a stressful stimulus. Researchers currently have limited understanding of the avian brain and further work should be done in this area. It is also possible that maturation of the White Pekin brain throughout the aging process would result in changes in EEG patterns similar to the differing results seen in Ong et al. (1997, 189-93), and Jongman et al. (2000, 339-43) between lambs and sheep. Placement of EEG leads for these experiments was on the meninges of the telencephalon. It is possible that placing the leads on different areas of the brain may also result in differing EEG patterns that could be more useful for stress studies. Researchers briefly analyzed the numerical electromyography output as a measurement of stress detection and saw no observable patterns; however, further work should be conducted to determine if EMG output could be used for stress detection. Another area of interest is the learning effect that may be present once a bird has received a shock. Further studies should be done to determine if there is a learning effect present and what affect this might have on stress studies. Future studies would also benefit from implantable telemetry devices capable of measuring

other parameters such as heart rate and blood pressure to help researchers obtain a more comprehensive view of the animal throughout testing.

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Appendix

AACUC APPROVAL LETTERS

UNIVERSITY OF DELAWARE

COLLEGE OF AGRICULTURE AND NATURAL RESOURCES

AGRICULTURAL ANIMAL CARE AND USE COMMITTEE

Application for Use of Agricultural Animals

In Teaching or Research

AACUC Protocol Number: Benson (33) 12-10-10 R

TITLE OF PROJECT: Development and validation of EEG analysis as a tool to evaluate welfare in poultry

INSTRUCTOR/PRINCIPAL INVESTIGATOR: Eric Benson

_____ 01/05/2011

New or Three Year Review (mark one)

NEW X THREE YEAR

If this is a 3 year renewal, what is the assigned existing protocol number? _____

(This section for Committee use only)

Application Approved (date): 1/6/2011

Application Rejected (date): _____

Reason for Rejection: _____

_____ 1/6/2011

Signature, Animal Care and Use Committee Date

UNIVERSITY OF DELAWARE

COLLEGE OF AGRICULTURE AND NATURAL RESOURCES

AGRICULTURAL ANIMAL CARE AND USE COMMITTEE

Application for Use of Agricultural Animals

In Teaching or Research

AACUC Protocol Number: (33) 12-03-12R

TITLE OF PROJECT: Comparison of blood based stress indicators to EEG based stress analysis for evaluation of poultry welfare

INSTRUCTOR/PRINCIPAL INVESTIGATOR

Eric Benson _____ 2/15/2013
Printed Name **Signature** **Date**

(This section for Committee use only)

Application Approved (date) 2-7-13 ✓

Application Rejected (date) _____

Reason for Rejection _____

Robert M Dyer 2/16/2013
Signature, Animal Care and Use Committee **Date**