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Application of wireless electroencephalogram (EEG) to measure stress in ducks

Abstract
Animal welfare and public perception of animal welfare is guiding animal agriculture, practices and policies. Producers are faced with the challenge of improving production efficiency and meeting environmental and animal welfare restrictions. Many of the current measures of assessment of animal welfare are qualitative rather than quantitative. The objective of this study is to develop and evaluate quantitative measures to directly measure stress through evaluation of brain activity using electroencephalography (EEG). Two experiments were performed to develop and validate EEG as a tool for assessing poultry welfare. In Experiments 1 and 2, White Pekin ducks were treated with known stressors including auditory, mild electric stimuli, and changes in the microenvironment (i.e. exposure to ammonia). In Experiment 1, 16 (5-10 wk) White Pekin ducks were implanted with EEG transmitters and sensing electrodes positioned on the surface of the telencephalon. Each bird was individually placed in a controlled chamber and treated with one of the above stressors while being monitored during a 15 minute (900 s) trial. In Experiment 2, 8 (5-10 wk) White Pekin ducks were treated the same as in Experiment 1 but with an extended observation time of 45 minutes (2700 s). After treatment with one of the three stressors, auditory, mild electrical stimuli, or ammonia, EEG files were analyzed both on a raw and frequency domain basis for identifiable signs. EEG analysis for Experiment 1 showed no differences between time periods for all frequencies. Experiment 2 ECG results showed no differences between time periods for all treatments. Experiment 2 corticosterone results showed differences between pre-treatment and post-treatment; however, there were no differences between treatments and no differences between treatments and control.

Keywords
Electroencephalogram, EEG, stress, broiler, duck, animal welfare

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Application of wireless electroencephalogram (EEG) to measure stress in ducks

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Abstract. *Animal welfare and public perception of animal welfare is guiding animal agriculture, practices and policies. Producers are faced with the challenge of improving production efficiency and meeting environmental and animal welfare restrictions. Many of the current measures of assessment of animal welfare are qualitative rather than quantitative. The objective of this study is to develop and evaluate quantitative measures to directly measure stress through evaluation of brain activity using electroencephalography (EEG). Two experiments were performed to develop and validate EEG as a tool for assessing poultry welfare. In Experiments 1 and 2, White Pekin ducks*

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were treated with known stressors including auditory, mild electric stimuli, and changes in the microenvironment (i.e. exposure to ammonia). In Experiment 1, 16 (5-10 wk) White Pekin ducks were implanted with EEG transmitters and sensing electrodes positioned on the surface of the telencephalon. Each bird was individually placed in a controlled chamber and treated with one of the above stressors while being monitored during a 15 minute (900 s) trial. In Experiment 2, 8 (5-10 wk) White Pekin ducks were treated the same as in Experiment 1 but with an extended observation time of 45 minutes (2700 s). After treatment with one of the three stressors, auditory, mild electrical stimuli, or ammonia, EEG files were analyzed both on a raw and frequency domain basis for identifiable signs. EEG analysis for Experiment 1 showed no differences between time periods for all frequencies. Experiment 2 ECG results showed no differences between time periods for all treatments. Experiment 2 corticosterone results showed differences between pre-treatment and post-treatment; however, there were no differences between treatments and no differences between treatments and control.

Keywords. Electroencephalogram, EEG, stress, broiler, duck, animal welfare

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Introduction

Animal welfare and public perception of animal welfare is guiding animal agriculture. One of the major problems with assessment of animal welfare is that many of the current measures are qualitative rather than quantitative. Assessment of welfare components of animal husbandry procedures commonly relies on behavior and interpretation of the results can be questioned (Jongman et al., 2000). Qualitative assessment raises potential questions about bias and interpretation. A potential quantitative assessment tool for animal welfare is 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 the electrical activity is recorded by a computer (Purves, et al., 2001). EEG has been used to monitor brain activity of humans and a number of different 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; Gerritzen et al., 2006; Raj, 1998). Radiotelemetery 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). In these applications, the EEG was recorded from the device's paired sensing electrodes positioned on the surface of the telencephalon, the electrode leads being passed under the skin and held in place with the use of dental acrylic (Savory et al., 2006).

EEG has been used in studies of agricultural animals to evaluate emergency depopulation and stunning methods for slaughter. Literature on the use of EEG in chickens is limited (Hunter et al., 2000). 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. Gerritzen et al. (2006) studied the susceptibility of ducks and turkeys to atmospheric stunning using EEG to determine the point of unconsciousness. Several researchers have employed use of high amplitude, low frequency activity in the theta and delta waves in other studies for hens and broilers to determine the point of unconsciousness (Raj et al, 1992; Raj, 1998, Gerritzen et al., 2004). 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). Further work has shown that suppression of the alpha and beta waves, together with the occurrence of the theta and delta waves, are typical for loss of consciousness in other species such as rats and broilers (Forslid, et al., 1986; Raj, 1998). The changes that are seen in the frequency, or the number of occurrences of a repeating event per unit time, specifically the suppression of alpha and beta and the occurrence of the theta and delta are indicative of the loss of consciousness.

While EEG is typically used to evaluate unconsciousness, it has also been used to evaluate response to stimuli, such as pain, in both traditional and non-traditional food animals. The use of frequency spectral analysis of EEG as an animal welfare assessment of pain in food animals is a relatively new technique. Although animals are unable to verbalize pain levels, Ong et al. (1997) correlated EEG and behavioral changes of sheep in response to mild electric shock. 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. Based on these and similar studies, EEG should have applications for assessing pain or stress in poultry.

In the current study, ammonia was chosen as a stressor due to previous publications indicating that ammonia has a stressful effect on poultry. Jones et al. (2005) demonstrated that broilers

chose to spend greater amounts of time in chambers with lower levels of ammonia. The study concluded that broilers found ammonia levels above 10 ppm aversive. McKeegan et al. (2002a) showed an activation of nasal nociceptors, or pain receptors, in hens during an exposure to ammonia. McKeegan et al. (2002b) also showed that 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. In an EEG study with humans, van Toller et al. (1993) 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.

The final stimulus chosen for the current study was auditory. A study by de Boer et al. (1988), demonstrated that rats exposed 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 cessation of the stimulus. Gross (1990) showed an increase in the heterophil/lymphocyte ratio of chickens after exposure to a stressful auditory stimulus of 104 db. The heterophil/lymphocyte ratio is often used to measure the effects of stress (Gross, 1990), but for the current study plasma corticosterone was measured because it rises more quickly after a stressful stimulus. Given that rats showed increases in plasma corticosterone levels and corticosterone can be correlated with stress, it seemed likely that EEG could be used to evaluate auditory stress.

The objective of this study is to evaluate the suitability of the implantable EEG transmitter for determining quantitative trends in brain activity associated with stress 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. The hypothesis of these two experiments was that an increase would be seen in the relative alpha or relative beta frequencies and a decrease would be seen in the relative delta or relative theta frequencies during the application of stressful stimuli. It was assumed that relative delta/theta would decrease during a stressful period because relative delta/theta tend to increase during unconsciousness , when an animal is not responding to stimuli (Lambooij et al., 2002; Lopes, 1983; Raj et al, 1992; Raj, 1998, Gerritzen et al., 2004; Alphin et al., 2010). Plasma corticosterone levels were used in Experiment 2 as a standard measure of evaluating stress (Harvey et al., 1980; Klingbeil, 1985; de Boer et al., 1988) against which any changes seen in the relative frequency bands of the EEG were measured.

Methods and Materials

General Procedures and Instrumentation

Two experiments using similar procedures were used to collect electroencephalogram (EEG) and electrocardiogram (ECG) data from ducks exposed to stimuli intended to create stress. In each experiment, 25 straight run White Pekin ducks were obtained from a commercial hatchery and raised in cohorts of 4 ducks following standard care and conditions. Ducks were raised from 1 d and instrumented with a surgically implanted EEG sensor at approximately 5 wk of age, once birds reached the minimum size of 2000 g for surgery. Ducks that were to receive surgery were randomly selected, food withheld for approximately 8 h and water withheld for approximately 2-6 h before surgery. Each duck was anesthetized using 5% isoflurane (IsoSol; Vedco, Inc., St. Joseph, MO) at induction with 3% isoflurane for maintenance of anesthesia. Three channel wireless biopotential transmitters (PhysioTel model F50-EEE, Data Sciences International St. Paul, MN) were surgically implanted in the back of the neck of each duck. 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). Two leads were implanted in the complexus muscle just below the base of the skull for electromyography (EMG). The ducks were given 0.4 mg/kg carprofen and 0.1 mg/kg penicillin injected subcutaneously after the procedure and then allowed to recover for at least 24 h. The surgical procedure was based on Savory and Kostal (1997, 2006) and Alphin et al. (2010).

Signals from the wireless transmitter were recorded by 4 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 files and EMG files were analyzed in DSI NeuroScore software. The raw EEG files were analyzed in Neuroscore by adding labeled markers over 2 s epochs indicating specific time periods: pre-treatment, stimulus, and no stimulus. 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 mean EEG, mean EMG, alpha (8-12 Hz), beta (16-24 Hz), delta (0.5-4 Hz), theta (4-8 Hz), and sigma (12-16 Hz) values and markers were exported on a 2 s basis from Neuroscore to Excel (Microsoft Corp., Redmond, WA) and charted.

Birds were placed in a (0.81 m x 0.80 m x 0.65 m) clear acrylic observation chamber. The observation chamber included two regions: a 0.81 m x 0.56 m x 0.65 m region for the birds and a 0.81 m x 0.24 m x 0.65 m region for heating ammonium hydroxide to create ammonia gas. The ammonium hydroxide was heated on a hot plate 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 region with the bird. Birds were not able to move between regions. An external control system was used to activate ventilation between the ammonia region and bird chamber and to vent to the outside.

Stimuli included auditory, mild electric shock and changes in the microenvironment (50 ppm NH_3). Stimuli were applied individually and only one stimulus was used per treatment based on a randomization table in Excel (Microsoft Corp., Redmond, VA). For auditory stimuli, an 88 dB alarm was applied for specific durations throughout the stimulus period(s). For mild electric 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 to apply a single shock of 60 mA (~1 sec) for a specific number of times in the stimulus period depending on the experiment. For the environmental stimuli, ~50 ppm NH_3 was continuously applied for the entire stimulus period. Ammonia concentration in the chamber was monitored using a ToxiRAE II Ammonia Sensor (RAE systems, San Jose, CA.)

At the conclusion of the study, the birds were euthanized and the transmitters removed. All testing was performed under the approval and guidelines of the University of Delaware Agricultural Animal Care and Use Committee and followed the guidelines laid out by the Federation of Animal Science Societies (Federation for Animal Science Societies, 2010).

Experiment 1

In Experiment 1, a trial time of 15 min (900s) was broken into the following time periods: pre-treatment (first 180 s), stimulus 1 (180 s), no stimulus 1 (180 s), stimulus 2 (180 s), and no stimulus 2 (final 180 s). Birds were instrumented and placed in the observation chamber as described above and were treated with one of the three stimuli. For auditory stimuli, the 88 dB alarm was applied continuously throughout both the stimulus 1 and stimulus 2 time periods. For mild electric shock, a single shock of 60 mA was applied once every 30 seconds during the stimulus 1 and stimulus 2 time periods. For environmental stimuli, ~50 ppm NH_3 was applied

continuously throughout stimulus 1 and stimulus 2 time periods. Ventilation of the chamber was performed immediately following each stimulus period using external controls. Each individual bird was exposed to multiple treatments during the five week period in which each bird was used.

Statistical analysis was performed using the Wilcoxon Two-Sample Test in JMP (SAS Institute Inc., Cary, NC). For statistical analysis of EEG data, the mean of each relative frequency for each period was averaged within each treatment. Stimulus 1 and 2 periods were combined and charted as "Stimulus". The No Stimulus 1 and 2 periods were combined and charted as "No Stimulus". All tests were conducted at the 5% (α=0.05) significance level.

Experiment 2

In Experiment 2, a trial time of 45 min (2700 s) was broken into the following time periods: pre-treatment (first 1800s), stimulus (600 s), and no stimulus (final 300 s). Birds were placed in the observation chamber as described above and were treated with one of the three stimuli. For auditory stimuli, the 88 dB alarm was applied for 12 sec per minute during the stimulus period. For mild electric shock, a single shock of 60 mA was applied once per minute for a total of 10 shocks. The birds wore the shock collar regardless of whether they were to receive the electric shock treatment so there were no differences in instrumentation between treatments. For environmental stimuli, ~50 ppm NH_3 was applied continuously throughout the stimulus period. Ventilation of the chamber was performed immediately following the stimulus period using external controls. Each bird was exposed to multiple treatments during the five week period in which an individual bird was used.

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 processed through BIOPAC Systems, Inc. MP30A acquisition unit. Analysis of the ECG signals was conducted using BIOPAC BSL Pro. For analysis, ECG files were broken into seven regions and the average heart rate in beats per minute (bpm) determined. The regions for analysis included the first 30 s of pre-treatment, the last 30 s of pre-treatment, the first 30 s of stimuli, the middle 30 s of stimuli, the last 30 s of stimuli, the first 30 s after stimuli was removed and the last 30 s of the no stimuli period.

On the day of treatment, 1 mL blood was collected from each duck at the same time each morning to serve as a pre-stimulus corticosterone level. Upon entering the room, researchers collected all the blood samples within three minutes to avoid the influence of handling stress (Littin and Cockrem, 2001). Once the treatment was completed, a 1 mL blood sample was collected to serve as a post-stimulus corticosterone level. Blood was placed in an EDTA lined tube and centrifuged to obtain plasma for corticosterone analysis using an Enzo Life Sciences (Farmingdale, NY) ELISA kit.

Statistical analysis was performed for the corticosterone and heart rate data using the Wilcoxon Two-Sample test, Kruskal Wallis Test, and Students T-Test in SAS (SAS Institute Inc., Cary, NC). For statistical analysis of heart rate data, the mean bpm for each period was averaged within each treatment. All tests were conducted at the 5% (α=0.05) significance level. No statistical analysis was performed on the EEG data due to a limited number of samples.

Results and Discussion

Experiment 1

The results of Experiment 1 demonstrated there are no differences between time periods in each of the relative frequency bands (alpha, beta, delta, sigma, and theta) between the pre-treatment, stimulus, and no stimulus periods for all treatments (Fig. 1, 2, and 3). In all tests, relative delta was the dominant frequency.

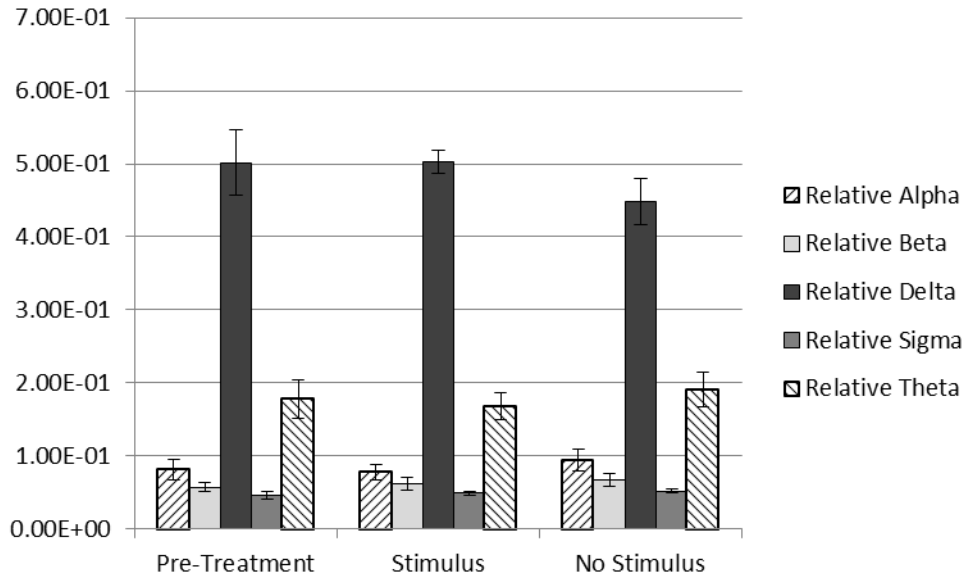

Figure 1. Mean relative EEG frequencies of White Pekin ducks by treatment period for auditory stimulus. Error bars represent S. E. M. (n=9).

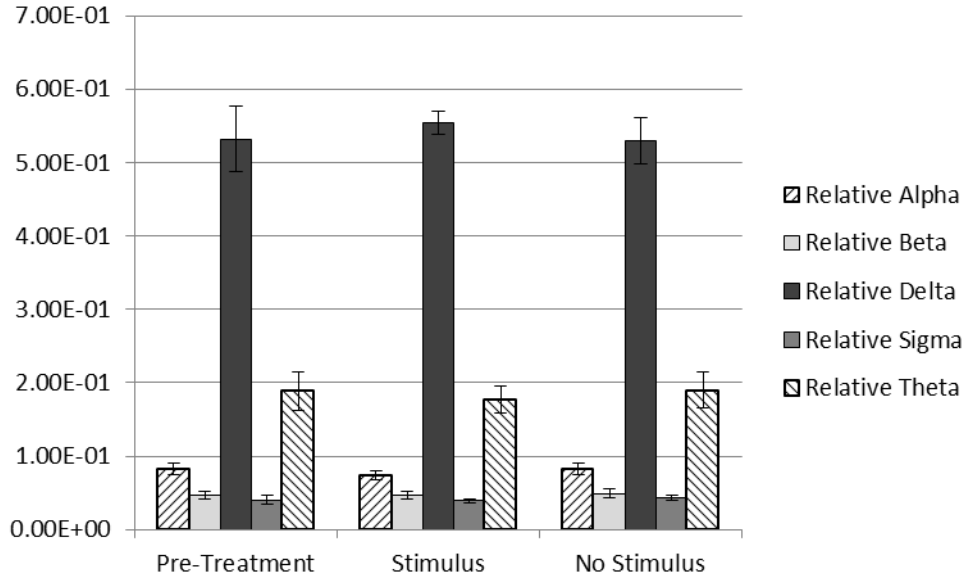

Figure 2. Mean relative EEG frequencies of White Pekin ducks by treatment period for mild electric shock stimulus. Error bars represent S. E. M. (n=20).

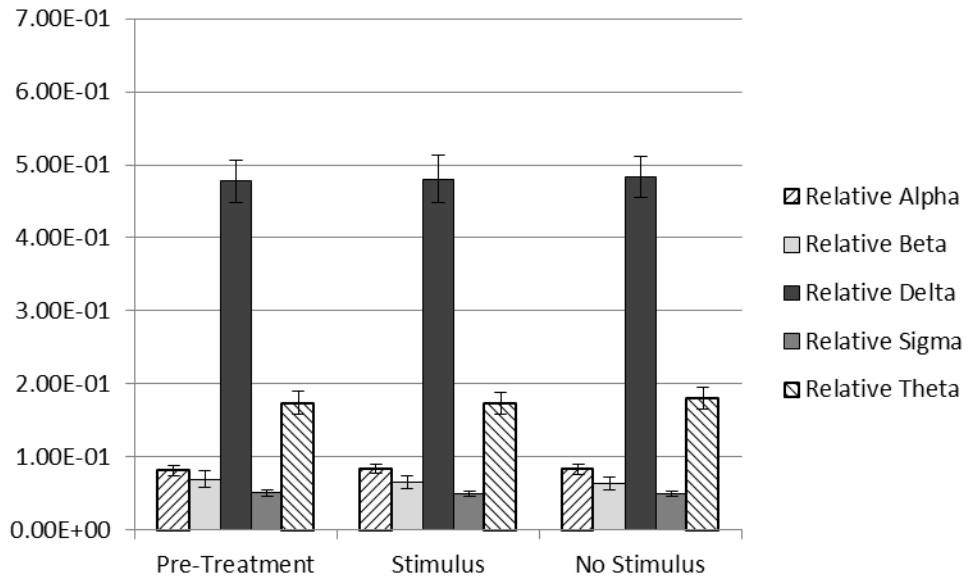

Figure 3. Mean relative EEG frequencies of White Pekin ducks by treatment period for ammonia stimulus. Error bars represent S. E. M. (n=14).

Experiment 2

The figures below show preliminary EEG data for auditory, electric shock, and ammonia stimuli with longer time periods as compared to Experiment 1 (Fig. 4, 5, and 6). A longer pre-treatment period was used in Experiment 2 to allow the birds to acclimate themselves to the chamber before treatment. Due to ambient electromagnetic noise, a number of EEG data files were corrupted. In addition, in Experiment 2, a control treatment in which the birds remained in the observation chamber for the same length of time as the stimuli treatments was added.

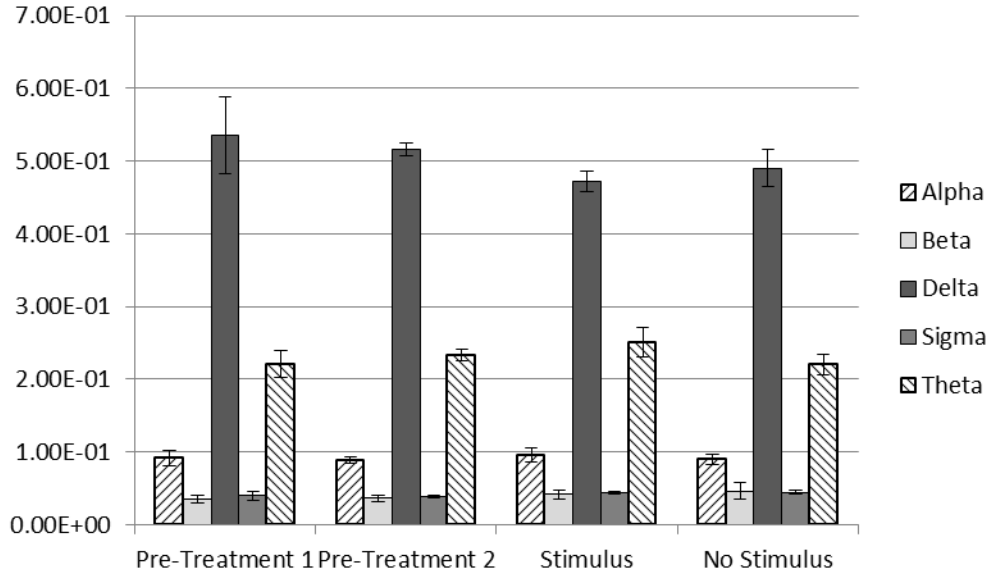

Figure 4. Mean relative EEG frequencies of White Pekin ducks by treatment period for auditory stimulus. Error bars represent S. E. M. (n=3). As with Experiment 1, the differences between

treatments and control were not significant. In Experiment 2, sample counts were not significant for statistical analysis.

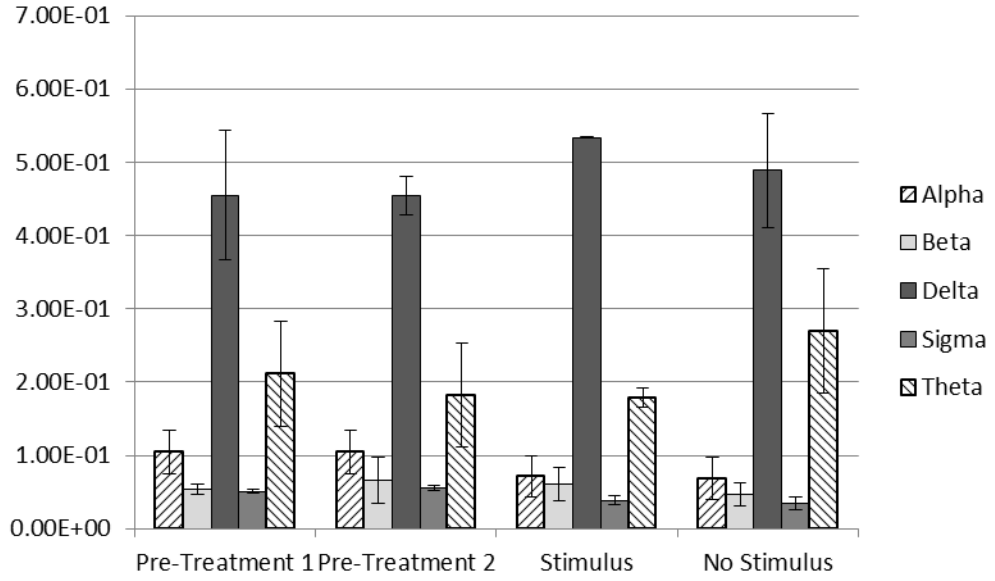

Figure 5. Mean relative EEG frequencies of White Pekin ducks by treatment period for mild electric shock stimulus. Error bars represent S. E. M. (n=2).

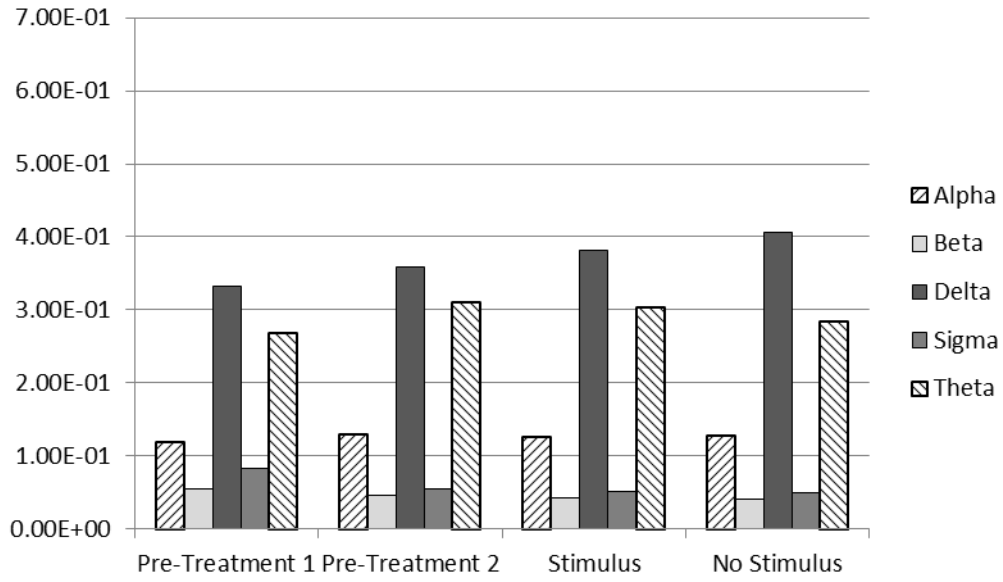

Figure 6. Mean relative EEG frequencies of White Pekin ducks by treatment period for ammonia stimulus. (n=1).

A total of 21 pre-treatment and post-treatment blood sample sets were collected. The results are shown in Table 1. Post-treatment corticosterone levels were higher than pre-treatment corticosterone levels, indicating a rise in stress during the observation period. Pre-treatment corticosterone levels were not distinguishable by treatment, which was expected. Stimuli specific post-treatment corticosterone levels were also not distinguishable from the control, which indicates that the observation process caused greater stress than the individual stimuli.

Table 1. Pre-treatment and post-treatment blood corticosterone[1]

Treatment	Pre-Treatment	Post-Treatment
Ammonia	1093 ± 206	3030 ± 240 *
Shock	934 ± 271	2751 ± 400 *
Sound	858 ± 117	2492 ± 370 *
Control	744 ± 92	2654 ± 307 *

*Indicates a difference of means between columns ($P < 0.05$).

[1] Data presented as mean ± standard error of the mean (X ± SEM).

ECG files were broken into seven regions for analysis and the difference in heart rate evaluated. A total of 18 valid observations were used. In each case, the treatments could not be separated from the control. In the case of shock, there appeared to be a rise in heart rate with additional shock stimuli, however, the results were not significant. There were no differences within each treatment between time periods or between treatments (Fig. 7, 8, 9).

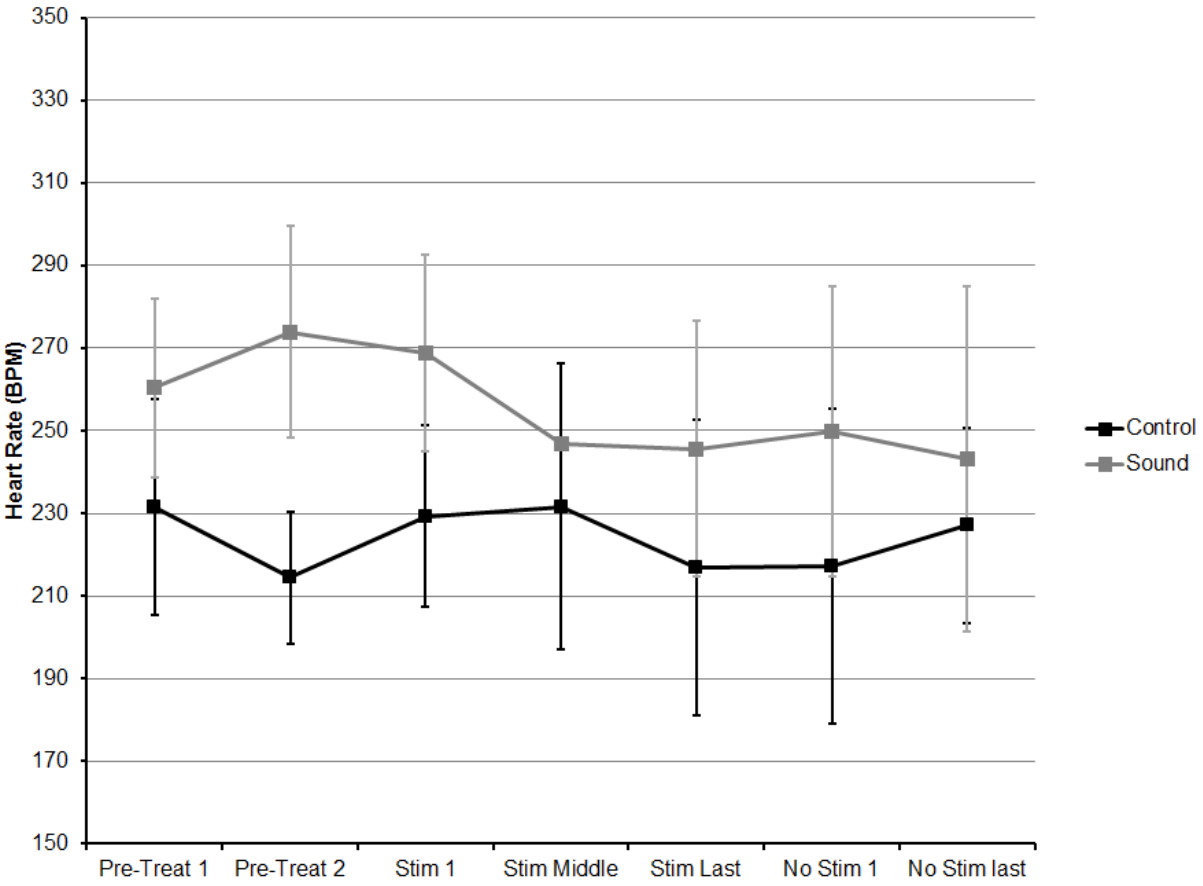

Figure 7. Heart rate in bpm was recorded over 30 s intervals during pre-treatment, stimuli, and post-treatment for control and auditory treatments. Error bars represent S.E.M. (Auditory: n=4, Control: n=5)

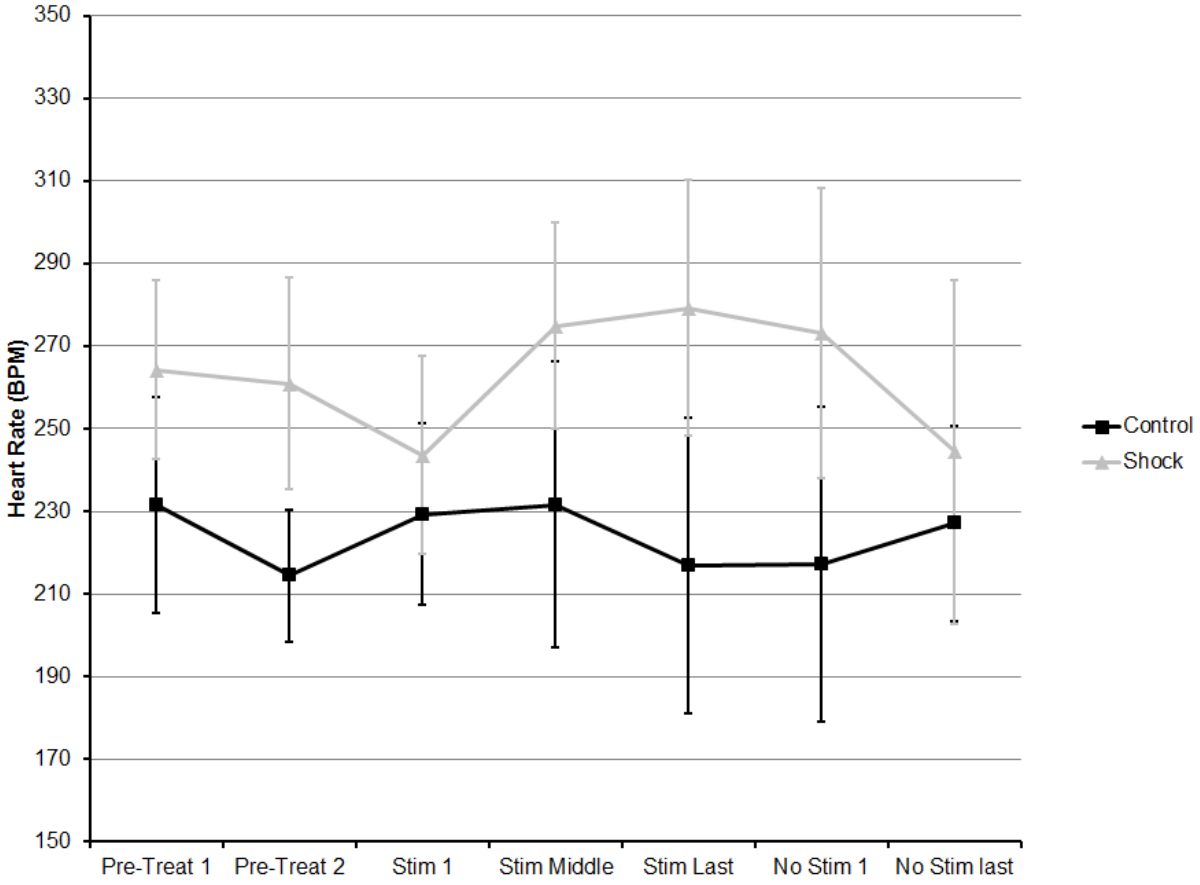

Figure 8. Heart rate in bpm was recorded during pre-treatment, stimuli, and post-treatment for control and shock treatments. Error bars represent S.E.M. (Shock: n=4, Control: n=5)

Based on the results of Experiment 1, it was determined a longer observation time and additional recording parameters would be needed. The pre-treatment, stimulus, and no stimulus periods were extended in Experiment 2. The pre-treatment was extended to 30 minutes to allow the ducks to acclimate to the new surroundings prior to the beginning of the stimulus period. The stimulus period was extended to 10 minutes to allow for ample time to react to the stimulus applied. The no stimulus period was extended to 5 minutes to allow for proper ventilation after completion of the ammonia stimulus. The additional recording parameters were ECG and corticosterone analysis. ECG was added to measure the effect of the stimulus on the heart rate while the corticosterone was added to measure the effect of the stimulus on the hormonal stress response.

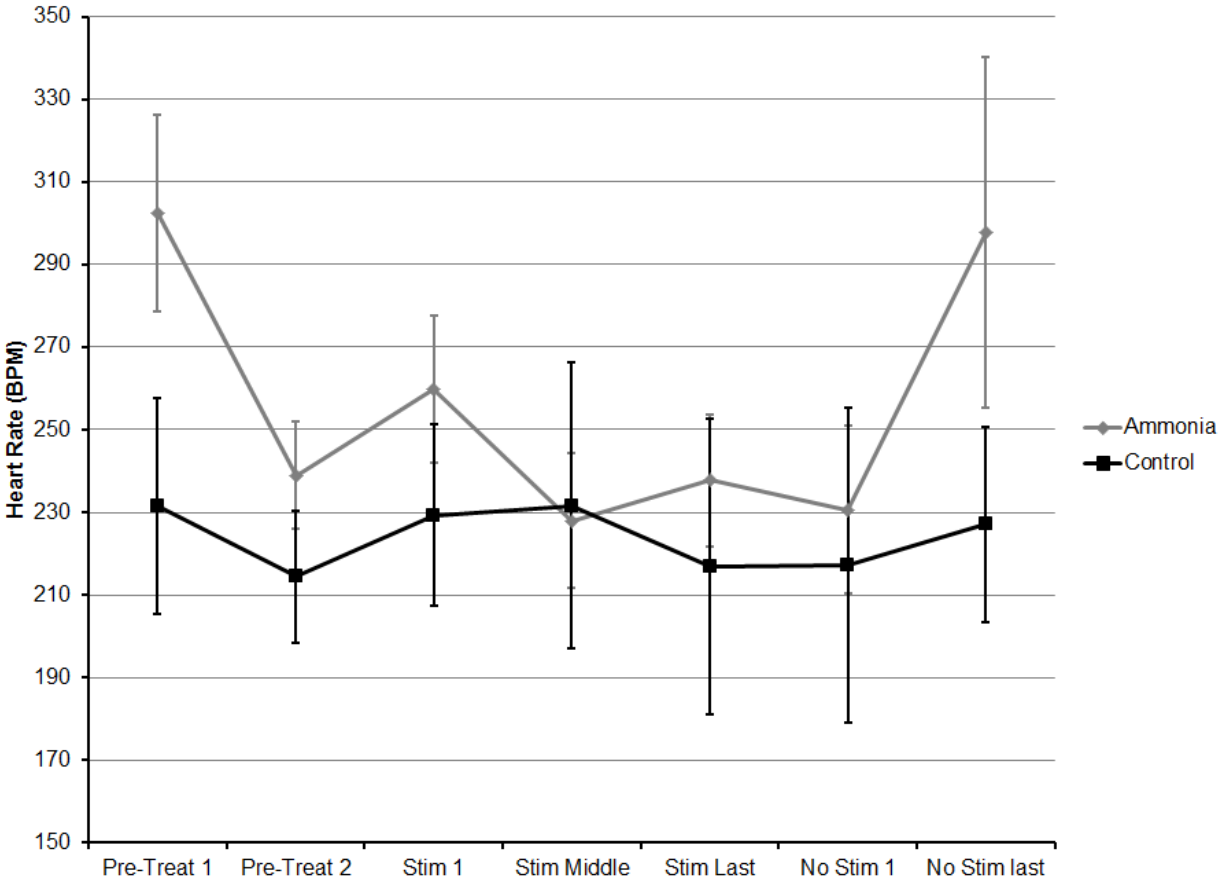

Figure 9. Heart rate in bpm was recorded during pre-treatment, stimuli, and post-treatment for control and ammonia treatments. Error bars represent S.E.M. (Ammonia: n=4, Control: n=5).

Artifacts are a concern in monitoring EEG and ECG patterns, particularly in conscious, free-moving subjects. Remote monitoring of EEG, ECG and behavior was recently used during a controlled atmosphere stunning test using broilers (Coenen et al., 2009). 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 which can be verified by comparing EEG, ECG, EMG, and motion cessation results (Alphin et al., 2010; Coenen et al., 2009). 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). When EEG, EMG, and ECG are used in combination, it is possible to eliminate areas of the recording that are tainted by motion artifact. This practice allows for a more accurate analysis of the recorded EEG signal (Amy L. Johnson, DVM, Department of Clinical Studies, University of Pennsylvania, personal communication). In previous trials performed (unpublished data), broilers and leghorns were treated to a protocol similar to Experiment 1. In these studies, leghorns continued to move during the entire monitoring and stimuli period, creating problems with motion artifacts. During EEG analysis, these muscle movements created artifacts that prevented separation of brain activity from muscle activity throughout the entire recording, rendering such files unusable. Broiler chickens showed the opposite trend and were basically unresponsive to stimuli.

Although the ECG could be used to determine heart rate and to identify areas of motion artifact, the ECG cables and electric shock collar placed restrictions on natural bird activity. Movement itself can affect heart rate, which in addition to the restriction caused by the cables may have confounded the results. A study by Crowther et al. (2003) determined that the heart rate of ostriches during transport was significantly lower when the birds were sitting as opposed to standing. In the current experiments, the ducks are free-moving in the observation chamber, and therefore sitting, standing, and walking were typically all observed throughout the entire trial regardless of treatment. These expressions of activity may account for the variable heart rates observed which may be why no differences were seen between control and the treatments.

Motion artifact was less of an issue for broilers, since broilers were generally unresponsive to stimuli. Based on the layer and broiler results, ducks were then used to determine if there would be a response to the stimulus unlike that of broilers and leghorns. It appears, however, that based on the plasma corticosterone results ducks are experiencing a stress response regardless of treatment type or even treatment presence. This stress response, since it is independent of treatment, may be caused either by being placed in the observation chamber or by the presence of the ECG leads and harness. Klingbeil (1984) demonstrated that binding the legs of mallard ducks while allowing the wings to remain free increased corticosterone levels. Although the restrictions experienced in the current experiment were not as severe, the ECG leads had to be secured to the ducks' legs and wing using a flexible wrap which may have had an impact on the birds' natural movements and behavior.

Conclusion

Experiment 1 EEG results demonstrated there were no differences between time periods in each of the relative frequency bands (alpha, beta, delta, sigma, and theta) between the pre-treatment, stimulus, and no stimulus periods for all treatments. Experiment 2 corticosterone results showed there was no difference in pre-treatments between the controls. A difference in corticosterone levels was seen between pre-treatment and post-treatment in all treatments. There was, however, no difference between treatments indicating that the observation process caused greater stress than the individual stimuli. Experiment 2 ECG results showed no differences between time periods within each treatment and between the treatments. There appeared to be an increase in the relative delta frequency during the stimulus period for Experiment 2 EEG mild electric shock which looks promising for future studies. Although this contradicts our hypothesis, further testing may result in consistent repeatable results. At the time this article was written, additional data was being collected for all treatments to increase sample size and reduce variability.

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