EXOGENOUS CORTICOSTERONE ADMINISTRATION VS. AN ENVIRONMENTAL STRESS MODEL: APPROACH BEHAVIORS IN *PASSER DOMESTICUS*

By

Kirsten L. Bjornson

A thesis submitted to the faculty of Radford University
in partial fulfillment of the requirements for the degree of
Master of Arts in the Department of Psychology

May 2014

Dr. Jason Davis — Date: 7/29/2014
Thesis Co-Advisor

Dr. Pamela Jackson — Date: 7/29/2014
Thesis Co-Advisor

Dr. Jeffrey Willner — Date: 7/28/2014
Committee Member

Abstract

Stress responses to an immediate threat are thought to be beneficial for survival. The stress hormone, corticosterone (CORT), mediates bodily functions to better cope with a stressor. However, the behavioral effects of CORT are somewhat poorly understood. Previous studies have observed body condition and behavioral effects with repeated acute CORT administration over a long period of time.

Moreover, Cooper, Ross, Foltz, Moore, & Davis (2013) found that house sparrows (*Passer domesticus)* exhibited neophobic behaviors when exposed to the color red. In this thesis project, I presented house sparrows with a red stimulus directly after a single acute treatment of CORT with the expectation that CORT-treated birds will display more hesitant and anxious behaviors than those without CORT treatment. Additionally, few studies have compared whether exogenous CORT administration alone produces the same behavioral responses as a physical stressor. To investigate this, I compared the CORT-treated (DMSO+CORT) and DMSO birds to those birds in a "capture and restraint" group. I expected there to be no behavioral differences between DMSO+CORT and "Bagged" (DMSO+Bagged) birds, while there would be behavioral differences between DMSO birds and DMSO+Bagged birds. Finally, I compared Unhandled birds to the DMSO, DMSO+CORT, and DMSO+Bagged conditions to determine whether there were handling effects with the expectation that there would be behavioral differences between the Unhandled and handled birds.

The results from this study revealed that Unhandled, DMSO+CORT, and DMSO+Bagged groups did not differ from the DMSO control, with CORT levels of all groups being higher than baseline. However, there was a significant difference between

the DMSO+CORT and DMSO+Bagged treatment groups, suggesting that physiologically increasing stress was more effective than the environmental stress model. Baseline CORT levels were shown to be predictive of how often the birds approached the food bowl, feeding duration, and movement behaviors. The fact that baseline CORT levels are more predictive of approach and movement behaviors compared to the post-treatment, stressed CORT levels suggests that the house sparrow's natural state may be more relevant in predicting stress behaviors rather than the context of the stressful situation. While the results did not support the hypotheses, this study introduced a new method for furthering our knowledge of how CORT and environmental stressors influence the stress response.

Kirsten L. Bjornson, M.A.
Department of Psychology, 2014
Radford University

Acknowledgements

I would like to express the deepest appreciation to my thesis advisor Dr. Jason Davis for accepting me as an advisee from outside his department. Dr. Davis has provided endless guidance and encouragement towards the completion of this research project. Because of him, I have gained lasting knowledge and an unforgettable experience. I extend thanks to my committee members Dr. Pamela Jackson and Dr. Jeffrey Willner for their insightful comments which have further developed this thesis.

In addition, I would like to thank members of the Ecophysiology Lab, specifically Dylan McDaniel, Fionna Surette, Laken Cooper, and Neil McDonald for their time and assistance that helped make this project possible. Finally, I would like to thank the Experimental Psychology department and my cohort for instruction and support in the aspects of completing a research project. Thank you all, I am extremely grateful.

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Introduction

Studying animal behavior sheds light on how both humans and non-humans interact with the environment. For instance, imagine a woman walking down a dark alley in the middle of the night. Suddenly, a robber surprises her from behind. He threatens her, steals her purse, and runs away. This encounter leaves the woman shaken. A few minutes later, she returns to her house. As the woman is about to call the police, she finds a spider is sitting on her telephone.

Imagine a similar situation in which the woman walks through the alley during the day and does not come across the robber. When she returns home, she notices a spider on her table. While her reaction to these sequential stressors or even the single stressor may seem obvious, the physiologic mechanisms involved in stress are more complex than most people recognize. Moreover, physiological change also influences behavior. As these concepts are not fully understood, I am interested in learning how physiologically and physically inducing stress consecutively can shape behavior in house sparrows, a common species used in the study of stress.

The Theory of Stress

As we become more aware that stress may cause both psychological and physical damage, a greater emphasis has been placed on understanding why this occurs. Endocrinologist Hans Seyle first coined the term "stress" as a synonym for "general adaptation syndrome" to explain why a particular event or stimulus disrupts physiological homeostasis. General adaptation syndrome consists of three stages: (1) the body reacts to an initial stimulus, otherwise known as the stressor; (2) the body attempts to return to homeostasis by activating physiological systems; and (3) when constantly exposed to a

stressor, fewer physiological resources are available to return to homeostasis, causing the body to become more vulnerable to additional stressors and disease (Breedlove, Rosenzweig, & Watson, 2010).

Seyle's "general adaptation syndrome" became a foundation for Bruce McEwen's more novel theory to explain stress: allostasis. "Allostasis" is the ability of the physiological mechanisms to "achieve stability through change" when presented with a stressor (McEwen & Wingfield, 2002). A prime example from Robert Sapolsky's *Why Zebra's Don't Have Ulcers* (2004) describes the stressful situation of a wild zebra exposed to a lion. Undisturbed, the zebra is involved in behaviors such as grazing, mating, and grooming. However, exposure to the lion causes the physiological mechanisms involved in those behaviors to reallocate to more pertinent systematic functions in order to more efficiently escape from the lion. After the danger passes, the body systems recuperate from the stressful event and shift back to the normal physiologic state. Thus, instead of *only* attempting to maintain a constant state as in homeostasis, allostasis asserts that physiological mechanisms are adaptable to cope with a stressor and everyday tasks that are necessary for survival (McEwen, 1998).

The zebra and lion scenario is an example of an acute, or immediate stress. In the presence of acute stress, the body reallocates energy to power physiological mechanisms needed for immediate survival. Following the acute stress, there is a recovery period during which those mechanisms return to their original state. This normal, unstressed state is known as "baseline." When the physiological mechanisms and behavioral patterns return to baseline levels, the individual will then have enough energy to conduct

the normal homeostasis of daily survival tasks and prepare itself for another stressor (Sapolsky, 2004).

If exposed to multiple acute stressors, the body needs more energy to cope. This imbalance of the physiological mediators of allostasis is called an allostatic load (McEwen, 1998). When the body develops an allostatic load, there tends to be a lack of energy resources and thus it will not be as prepared to respond to an additional stressor (Sapolsky, 2004). For example, Busch et al. (2008) administered a repeated acute stressor to induce chronic, or prolonged, stress. The chronic stressor stimulated behavioral responses such as increased feeding over three weeks. While increased feeding is a behavioral response expected to compensate for the allostatic load, it is of interest to explore how a "lighter" allostatic load such as only two acute stressors will influence behavior. In other words, an acute stressor immediately followed by an additional acute stressor will require less energy and therefore elicits different behaviors compared to multiple acute stressors over a period of time.

Physiologically administering an acute stressor prior to a second stressor has seldom been observed. Moreover, behavioral stress responses to acute stressors in this time frame and administration have also infrequently been examined and require further investigation.

The standard method to induce acute stress in birds is the "capture and restraint" technique (Wingfield et al. 1992, 1994; Canoine, Hayden, Rowe, & Goymann, 2002; Baugh, van Oers, Naguib, & Hau, 2013). This technique involves placing a bird in a cloth bag for 30 minutes. After executing the "capture and restraint" technique, physiological and behavioral stress responses can be observed (Wingfield et al., 1992,

1994). Few studies have compared whether the "capture and restraint" technique produces similar stress responses as other natural physiological and/or environmental stressors such as evading predators (Canoine, et al., 2002; Pakkala, Norris, & Newman, 2013). For example, Pakkala, et al. (2013) found that wild-caught rock pigeons exposed to a predator had physiological stress levels that were over twice as high as those exposed to the "capture and restraint" technique. It is imperative to establish whether this method is accurately representing the stressor it is compared to because the "capture and restraint" method is so frequently used to stimulate baseline compared to another stressor.

Neuroendocrine Aspects of the Stress Response

A variety of hormones are produced when an animal is presented with a stressor. Corticosterone (CORT) is the primary hormone regulating energy management and resource allocation to cope with emergency situations. It is classified as a glucocorticoid due to its steroid structure, its ability to regulate sugar, and its synthesis by the zona fasciculata layer of the adrenal glands. The hypothalamus and pituitary regulate the adrenal glands' production of CORT within three minutes of the perceived stressor. Together, these structures make up the hypothalamic-pituitary-adrenal (HPA) axis and operate a relatively slow-acting cascade to regulate CORT production (Wingfield et al., 1982; Sapolsky, 2004; Cockrem, 2013).

After a stressor has been perceived, the hypothalamus releases corticotropin releasing factor (CRF) to stimulate the release of adrenocorticotropic hormone (ACTH) from the anterior pituitary gland. This, in turn, triggers the adrenal cortex to convert cholesterol into CORT. Once synthesized, the adrenal gland secretes the hormone into the bloodstream, activating a variety of cell receptors throughout the body and so

modifying gene expression. Because the HPA is a negative feedback loop system, CORT will continue to be produced until enough of the hormone inhibits further release of CRF from the hypothalamus (Breedlove, Rosenzweig, & Watson, 2010).

Examples of CORT effects include increased metabolism, respiration, and energy mobilization, along with decreased reproductive and developmental activity. Furthermore, CORT can increase or decrease immune and inflammatory responses. In addition, CORT can mediate a variety of behavioral responses to better defend against a perceived threat (McEwen & Lasley, 2002; Kovacs & Ojeda, 2012).

The Stress Response to Novel Stimuli

Exposure to novel stimuli has been known to induce physiological stress (Cooper, et al., 2013; Lendvai, Bokony, & Chastel, 2011). Examples of novel stimuli used in research studies on neophobia include food, feeders, plastic containers, gloves, rope, and a variety of other inanimate objects (Coleman, & Mellgren, 1994; Mettke-Hofmann, Rowe, Hayden, & Canoine, 2006; Visalberghi, Janson, & Agostini, 2003; Mettke-Hofmann, Winkler, Hamel, & Greenberg, 2013).

Greenberg (1990, 2003) discusses why animals are more likely to consider novel items as stressors through two hypotheses. The neophobia threshold hypotheses states that animals become familiar with what resources are necessary for survival and prefer familiarity as opposed to the unfamiliarity of a novel item. In addition, the dangerous niche hypothesis predicts that the experience of living in a dangerous environment causes the individual to develop cautious behaviors towards any stimuli that might present a threat, such as a novel item. When the novel item is perceived as a stressor, a common behavioral response is neophobia (Barnett, 1958; Mitchell, 1976; Greenberg, 1992;

Sunnucks, 1998; Webster & Lefebvre, 2000; Fox & Millam, 2006; Lendvai, et al., 2011). Neophobia is hesitation, avoidance, or caution due to the fear of novel stimuli. As latency to approach novel stimuli is easily quantifiable, this neophobic technique is often used to measure stress responses.

The type of novel stimulus can potentially influence differences in the stress behavioral responses (Bowers, Bilbo, Dhabhar, & Nelson, 2008; Canoine, et al., 2002; Mettke-Hofmann, et al., 2006). One method that has been considered to induce stress is the presentation of the color red (Khan et al., 2011; Cooper, et al., 2013). Because red is not a stimulus that is typically encountered in the natural environment, it could potentially fulfill both of Greenberg's (1990, 2003) hypotheses of unfamiliarity and/or perceptions of intimidation.

Cooper, et al., (2013) observed that the presentation of a novel red stimulus increased CORT levels in house sparrows, which in turn caused hesitant and apathetic behaviors. As these behaviors are common neophobic responses, the red novel stimulus was hypothesized to be a neophobic stressor for house sparrows.

Previous studies have found that experimentally increasing CORT may increase behavioral activity, but that birds with higher corticosterone behave more neophobically when exposed to a stressful situation (Breuner, Greenberg, & Wingfield, 1998 Cooper, et al., 2013; Lendvai, et al., 2011). Specifically, when Breuner, et al. (1998) repeatedly increased CORT acutely, Gambel's white-crowned sparrows increased perch hopping. However, when house sparrows were presented with a novel stimulus, CORT levels increased and hovering frequency decreased (Lendavai, et al., 2011). Given these results, it is of particular interest to study how behaviors in response to a novel stimulus may be

modified when the subject is already in a stressed state before encountering the novel situation.

Handling Effects of Stress

While controlling for all potential stress confounds is a difficult task, studies found handling animal subjects to have a significant influence on the stress response. The effects of stress due to handling have been observed in numerous animals, including species of birds, rats, and amphibians, to name a few (Hull, Cockrem, Bridges, Candy, & Davidson, 2007; Meaney, Aitken, Bodnoff, Iny, Tatarewicz, & Sapolsky, 1985; Narayan, Cockrem, & Hero, 2013). Specifically, high CORT levels were detected directly after three minutes of handling caught birds from the wild (Romero & Reed, 2005). High breathing rates and more submissive temperaments were also observed 30 days after the birds were handled 12 times over a 45 day period compared to birds that were only handled four times in the sample period (van Oers & Carere, 2007). It is of interest to observe behavioral and CORT effects after multiple handling times over only a couple of days. Taking into consideration the concept of handling effects will provide further explanation for the cause of stress behaviors in the current study.

Project Summary and Hypotheses

Like the example presented earlier, I raise a similar scenario that is more applicable to my research questions: an encounter between a bird and a hawk. First, the hawk will be perceived as a threat. This encounter will increase the bird's CORT levels, providing the necessary upsurge of energy for an escape attempt. In this instance, the bird escapes to its nest and discovers a small brightly colored worm has been placed

there. It appears to be food, but the bird has never encountered this species of worm before.

Similarly, imagine if the bird took a different route to its nest. Instead of encountering the hawk, the bird returns to the nest without an increase of CORT, and further without exhausting energy into an escape attempt. However, the bird still finds the novel, brightly colored worm. I am interested in replicating these scenarios in a lab setting to compare how a bird will react to a double stressor versus a stressor without an initial increase of CORT.

Repeatedly and acutely increasing CORT has produced different behavioral stress responses depending on the amount and timing of the treatment (Busch et al., 2008; Breuner, et al., 1998; Lohmus, Sundstrom, & Moore, 2006). The response to acutely increasing CORT as a one-time treatment followed by an environmental stressor has not been observed before. By manipulating CORT in this manner I intend to induce a hormonal change similar to the bird and hawk scenario.

Moreover, the neophobic response to the color red is a novel hypothesis in itself (Cooper, et al., (2013). It is for this reason that I would like to replicate Cooper, et al.'s (2013) red stimulus method to induce stress. With the novel stimulus as a post-stressor to the CORT manipulation, I hope to reenact the hypothetical bird and worm example and discover the bird's behavioral response to this double-stressor.

The discussed research has led me to hypothesize that with one pre-exposure acute increase in CORT, house sparrows that are exposed to a neophobic stimulus will exhibit more cautious behavior but higher behavioral measures of anxiety than those who do not have CORT. Specifically, I predict that the CORT-treated house sparrows will

have different durations in time to approach food, duration of feeding times, and significant movement frequencies than untreated house sparrows.

I am also interested in discovering whether the "capture and restraint" method will produce a similar stress response compared to a physiologically induced stressor. It is for this reason that I hypothesize that increasing exogenous CORT in house sparrow will not be significantly behaviorally different from house sparrows actually exposed to a highly stressful situation, specifically being held in a cloth bag.

Additionally, it is of interest to learn whether handling will influence CORT levels and behavioral responses to the various stressors presented in this study. Specifically, I hypothesize that the handled birds will have higher CORT levels and display different approach and movement behaviors compared to birds that were not handled. From these comparisons, I hope to increase our understanding of the role of CORT, and glucocorticoid hormones in general, in the modulation of neophobic responses.

Methods

Subjects and Housing

Forty adult house sparrows were captured in the Southwest Virginia area through passive mist netting techniques. This is the minimal number used in similar studies of stress reactivity and includes sufficient animals that we believe the results will be broadly applicable and statistically powerful (Cockrem, Barrett, Candy, & Potter, 2009).

Upon capture, birds were released if they appeared to be of poor health. Health was determined by measuring body weight, wing span, body fat, and tarsus (the leg bone) length. Captured birds were marked with colored darvic leg bands for identification and initially housed at the Selu aviary in (4.5 m x 2.36 m x 2.45 m) cages for at least two weeks to acclimate to captive conditions. Following this time period, subject birds were moved, in small groups (two) to single-housing cages (58.8 cm x 38.8 cm x 35 cm) on the Radford University campus two days prior to the study. During the transfer, birds were in transport cages (59.5 cm x 40.8 cm x 30.0 cm) covered with a blanket with availability to food and water. The transfer took no more than 30 minutes. When housed in the Curie facilities, though kept in individual cages, birds were within sight and hearing distance of conspecifics at all times and were never in complete isolation.

House sparrow care included providing seed, nutritional supplement (Quicko seed), mealworms, and water. Each day, seed was filled to the top of the bowl and fresh tap water was replaced. Along with feeding and watering, cage upkeep and subject health as previously described were also consistently maintained. Whenever the birds were taken out of the travel cage or single-housing for cage upkeep, transfer, blood sample collection, or treatment, they were retrieved quickly to avoid any additional stress

caused by handling. To more efficiently grab the birds, lights were turned off for vision impairment, allowing the researcher with night vision goggles to grab the birds.

Laboratory Setting

The single-housing cage was split into two sections with a white foam board to prevent the two house sparrows from seeing each other. The behavioral tracking program that was used tracks animals according to the light contrasts of the animal compared to its background. To better track the dark bird against the black bars of the cage, the floor of the cage was also covered with the white foam board. The bars of the ceiling were removed and replaced with plexiglass to record behaviors from a top view. A heating lamp was placed above each side of the cage and was only on during recording to better visualize the animal as described above.

The room where the birds were housed included a large window for natural sunlight exposure. However, to reduce overheating, a black screen was placed over the window. Along with natural lighting, the room ceiling lights were set on a timer for 12 hours of light and 12 hours of darkness (10 AM-10 PM) because an appropriate amount of light exposure is necessary to uphold proper circadian rhythms. In the laboratory room, another single-housing cage was directly across from the birds of this experiment, usually containing either two males or a male and a female house sparrow. Having multiple birds in the experimental room was desired for a more natural environment.

Cage upkeep included cleaning the white foam board on the cage floor. This entailed quickly relocating the birds to the adjoining room, taking them out of the original cage, and housing them in a separate single-housing cage for no more than 15 minutes for

cage upkeep to occur. After cleaning, the birds were returned to their original cage. Treatment and plasma collection also occurred in the adjoining room.

Capture

Adult house sparrows were captured through the use of passive mist netting, and then placed in Radford University's Selu Conservatory aviary. Passive mist netting involved laying seed on the ground below the net, encouraging birds to dive to the ground and essentially into the net. The trained researchers immediately retrieved the bird from the net, handling with extreme care so as not to injure the bird. Capturing continued throughout the duration of the study until 41 house sparrows were successfully housed.

Treatment Groups

House sparrows were randomly assigned to one of the four treatment groups: DMSO+CORT, DMSO, DMSO+Bagged, or Unhandled. The DMSO+CORT treatment group included one topical application of 20µg of CORT diluted into 20µl of dimethylsulfoxide (DMSO) to 10 house sparrows. The DMSO group had a similar treatment with a 20µl topical application of dimethylsulfoxide (DMSO) to 12 house sparrows, but without the CORT application. The DMSO+Bagged treatment group included 9 birds placed into a cloth bag for 30 minutes. The birds of the DMSO+Bagged treatment group received the same topical application as the DMSO group with 20µl of dimethylsulfoxide (DMSO) only, prior to being placed in the bag.

Additionally, seven house sparrows were included in the Unhandled treatment group. The Unhandled house sparrows were not treated with DMSO or CORT, nor were they placed in a bag. Moreover, baseline CORT plasma levels were not collected, nor were they removed from their cage for cage upkeep during their time in the lab.

Unhandled house sparrows were only handled for the stressed bleeding sample after the recording as described in the *Procedure* section below.

Behavioral Scoring

All behaviors were video-recorded throughout the experiment, and analyzed using Noldus EthoVision in combination with manual coding. The software accounted for the latency time to approach the food bowl, duration of feeding times, and flight and significant movement frequencies throughout the single-housing cage. I drew a perimeter directly surrounding the food bowl to represent the zone in which the house sparrow must enter to indicate an "approach." The latency to initially approach the food bowl was calculated in seconds. If the bird did enter the food bowl zone, feeding was represented as the cumulative amount of time it spent in that food bowl zone. If the bird did not enter the food bowl zone, feeding durations were not accounted as it is not possible to feed if it does not enter the food bowl zone in the first place.

Finally, a flight was considered as a take-off from either the ground or wall of the cage to another area of the cage. Other significant movements include hopping, climbing, or pacing along the floor or wall of the cage more than two "steps." Examples of movements that were not accounted for were if the bird was flapping its wings, fluffing its body, or preening itself.

Because behavioral tests are interpreted by the observer, the true animal behaviors might not be fully captured. To control for this bias, ensuring that the behavioral interpretations are consistent, inter-rater reliability methods are normally used (Kaufman & Rosenthal, 2009). However, the Noldus EthoVision is a state-of-the-art behavioral recording system that promotes reliability by more accurately quantifying repeated

behavior and reducing observer variance (Noldus, Spink, & Regelenbosch, 2001). The EthoVision software accounted for coding behaviors listed above and was managed by me to ensure that the software was recording correctly, coding for the correct behaviors, and functioning properly in general.

Blood Sampling and Corticosterone Assay

House sparrows of the DMSO+CORT, DMSO, or DMSO+Bagged treatment groups used in a given experimental set were taken from the aviary and acclimated to the lab room for 38-45 hours before an initial baseline blood sample was taken. This was compared to a second blood sample that was collected after the behavioral measures. However, only the post-behavioral blood sample was collected from the Unhandled treatment group.

All blood samples were collected within 3 minutes of approaching the birds to obtain an unstressed measure of CORT (Romero & Reed, 2005). A needle was used to puncture the brachial vein located on the underside of the wing. A cotton ball was pressed against the wound directly after the blood was collected into hematocrit tubes and the bird was placed back into housing. Following replacement of the birds into housing, the blood samples were centrifuged to separate the plasma from red blood cells. Depending on the effectiveness of the brachial vein puncture, 3-50µl of plasma was obtained and stored in a -80°C freezing unit until assay. CORT levels were determined with an Enzo Life Sciences' Corticosterone ELISA (enzyme-linked immunosorbant assay) kit. The ELISA allowed the plasma sample to be combined with specific antibodies to create a biochemical reaction, causing the solution to change colors. A

spectrometer plate read was used to identify the amount of color in the solution, representing the amount of CORT present in the sample.

Procedure

For logistic feasibility, all procedures were initiated upon sets of 1-2 birds per trial. The study birds were retrieved from the aviary with a hand net. Initial weight was collected by placing the bird in a nylon sock and clipping the scale to the sock. Birds typically weighed between 20 and 27 grams. Once placed in the travel cage, they were transported to the Curie facility where each bird was entered into one of four experimental conditions: DMSO+CORT, DMSO, DMSO+Bagged, or Unhandled. Birds of all four conditions began with a 38 – 45 hour acclimation period to single housing. After the acclimation period, baseline CORT measures were obtained from the DMSO+CORT, DMSO and DMSO+Bagged groups at 8:30 AM. Twenty-three hours later, birds from these groups received 20µl of DMSO spread manually onto the back of the neck, above the jugular vein. Depending on the treatment group, this was either DMSO alone for the DMSO and DMSO+Bagged conditions or DMSO and CORT (4µg per 1µl) for the DMSO+CORT condition. The Unhandled treatment group was not bled for a baseline sample nor treated with CORT and/or DMSO. Without this baseline blood sample collection or treatment, the Unhandled treatment group had a longer acclimation period of 62-69 hours before behaviors were recorded.

Following the topical application of those in the DMSO+Bagged group, the birds were immediately placed in a cloth bag for 30 minutes and then released into single housing where they remained for an additional 30 minutes. Instead of being placed in a bag, birds in the DMSO+CORT or DMSO groups were placed into the single housing

cage for a 60 minute drug absorption period. When the birds were returned into the single housing cages, both the camera and the lights were turned on so that the Noldus Ethovision program could be set up for recording. As the Unhandled birds were not removed for treatment and replaced back into the single housing, the researcher entered the lab room to turn on the camera and lights 20 minutes before recording.

At 8 AM, the researcher placed the novel stimulus directly in front of the outside cage wall where the food and water bowls are attached. The novel stimulus is a red-painted Elmer's foam board that covers an entire wall. Birds from all treatment groups were exposed to the red novel stimulus for 30 minutes while behavior was recorded. A blood sample was collected directly after behavioral recording at 8:30 AM. Timeline is shown in Figure 1. Later that day, birds were returned to the aviary and final weight was obtained in the same procedure as the initial weight.

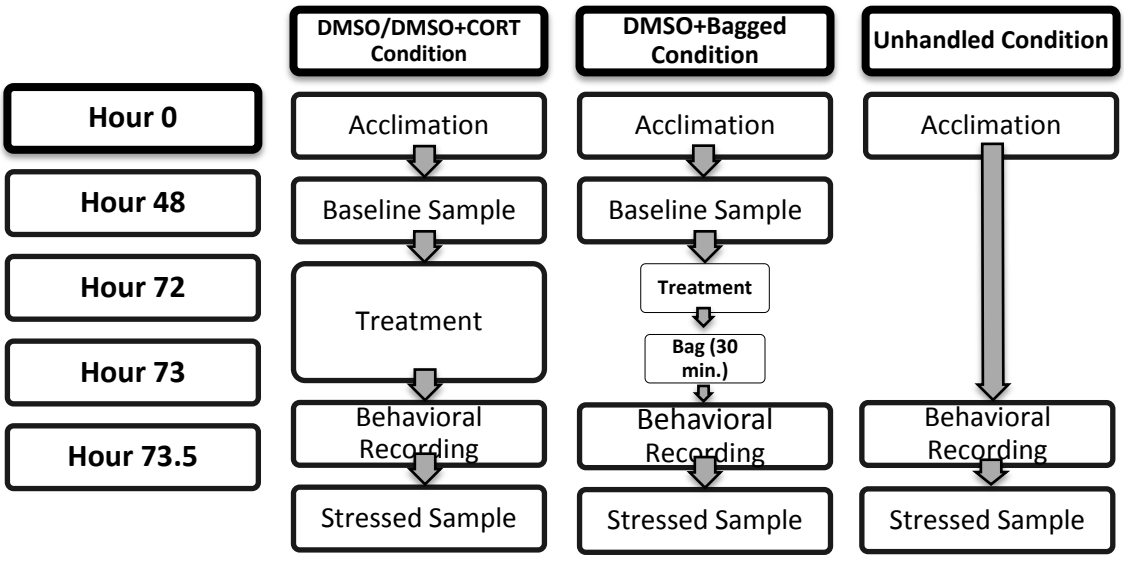

Figure 1: Procedure Timeline

Results

The aim of this study was to determine how the effects of increasing CORT in comparison to the "capture and restraint" response influences behavior after being presented with an additional, novel stimulus. The effects of CORT were observed by comparing four groups: DMSO, DMSO+CORT, DMSO+Bagged, and the Unhandled groups. Dependent measures included food and water approach behaviors, movement frequencies, and CORT levels. It was predicted that there would be significant differences in CORT levels and behaviors between DMSO+CORT and DMSO. Differences in CORT levels and behaviors were also expected to exist between Unhandled birds and the other three handled treatment groups: DMSO+CORT, DMSO, and the DMSO+Bagged groups. Finally, DMSO+CORT birds were not predicted to have CORT level or behavioral differences from the DMSO+Bagged birds. Weight and sex were also taken into account in order to establish whether these variables influenced the physiological or behavioral effects.

Multiple data points were not collected due to unforeseen circumstances, making overall comparison of all of the variables between the groups impossible. For example, there were many birds that did not require a baseline blood sample or it was unsuccessfully collected. If the baseline variable was included in the analysis, all birds with the missing baseline sample would be removed from the entire analysis, lowering the sample size. Thus, instead of analyzing multivariate tests of all of the variables, univariate or multivariate tests with various combinations of variables were used to provide the most accurate representation of the data. Descriptive statistics of all the variables are shown in Table 1.

Table 1: Descriptive Statistics

Variable	N	Mean	SD	Range
Treatment	40			
Food Approach	34			
Water Bowl Approach	34			
Latency to Food	25	332.25 sec.	541.74	1.10 to 1772.24 sec.
Latency to Water	23	188.59 sec.	366.11	.33 to 1588.82 sec.
Food Bowl Frequency	25	94.44	125.84	1 to 505
Water Bowl Frequency	23	113.61	131.06	1 to 468
Cumulative Feeding Duration	25	68.64 sec.	124.20	.03 to 571.13 sec.
Cumulative Drinking Duration	23	45.41 sec.	99.18	.17 to 426.48 sec.
Flight Frequency	34	381.01	245.80	1 to 886
Initial Weight	39	25.73 g.	1.82	20.5 to 30 g.
Final Weight	33	23.38 g.	2.27	19.5 to 27 g
Change in Weight	35	2.16 g.	1.66	-.50 to 6.50 g.
Baseline CORT	30	1260.43 pg.	1730.18	34 to 9573 pg.
Stressed CORT	31	2768.32 pg.	3244.42	314 to 13978 pg.

Effects of Treatment

Comparisons of all of the treatment groups, along with specific comparisons of treatment groups, determined whether there was an effect of treatment on CORT levels. A repeated measures ANOVA showed that while there was not a significant interaction between baseline and stressed CORT levels for the DMSO+CORT, DMSO, and DMSO+Bagged groups, $F(2, 21)= .61$, $p= .553$, $\eta^2= .055$, or a significant main effect between the three groups, $F(2, 21)= 1.40$, $p= .269$, *partial* $\eta^2= .117$, there was a trend for the main effect of baseline and stressed CORT levels , $F(1, 21)= 3.95$, $p= .06$, *partial* $\eta^2= .158$ (Figure 2).

Repeated ANOVAs for the specific comparisons of the DMSO+CORT and DMSO predictions showed that there was not a significant main effect between baseline and stressed CORT levels, $F(1, 13)= 2.97$, $p= .108$, *partial* $\eta^2= .186$, nor was there a significant interaction between the 2 treatment groups and the change in CORT, $F(1, 13)= .02$, $p= .898$, *partial* $\eta^2= .001$ (Figure 2). However, there was a marginal significant main effect between DMSO+CORT and DMSO groups, $F(1, 13)=.30$, $p= .60$, *partial* $\eta^2= .022$.

When comparing DMSO+CORT and DMSO+Bagged groups, there was not a significant main effect between DMSO+CORT and DMSO+Bagged groups, $F(1, 13) = 1.32$, $p= .272$, *partial* $\eta^2=.092$. However, there was a significant main effect between baseline and stressed CORT levels, $F(1, 13)= 11.47$, $p= .005$, *partial* $\eta^2= .469$, and a significant interaction between the difference of CORT levels and the DMSO+CORT and DMSO+Bagged groups , $F(1, 13)= 5.92$, $p= .030$, *partial* $\eta^2= .313$ (Figure 2).

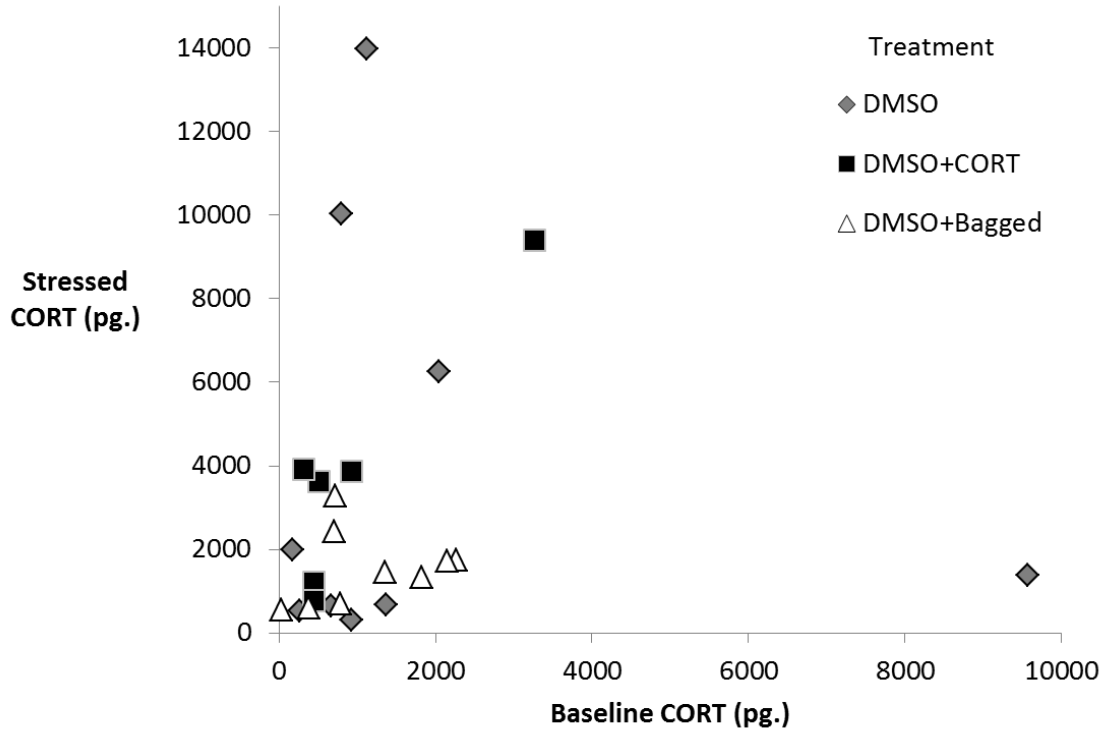

Figure 2: Baseline CORT and Stressed CORT

Baseline CORT levels pre-treatment, in relation to stressed CORT levels, post-treatment. No significant differences were found between treatment groups. However, significant differences were found between DMSO+CORT, and DMSO+Bagged groups.

In comparing means without treatment, a paired samples t-test revealed no differences for baseline and stressed CORT levels between DMSO+CORT and DMSO groups, $t(23)= -1.94$, $p=.065$, between DMSO+CORT and DMSO+Bagged groups, $t(14)= 1.86$, $p=.084$, between Unhandled and handled groups, $t(29)= -.636$, $p=.530$, nor was baseline a significant predictor of the stressed CORT levels, $\beta= .06$, $t(23) = .29$, $p=.776$.

Stressed CORT levels were also compared between the 3 handled groups, DMSO+CORT, DMSO, and DMSO+Bagged groups, and the unhandled group, $t(29)= -.64$, $p=.530$. The stressed CORT levels were also not correlated to weight, approach or movement behaviors (Figure 3).

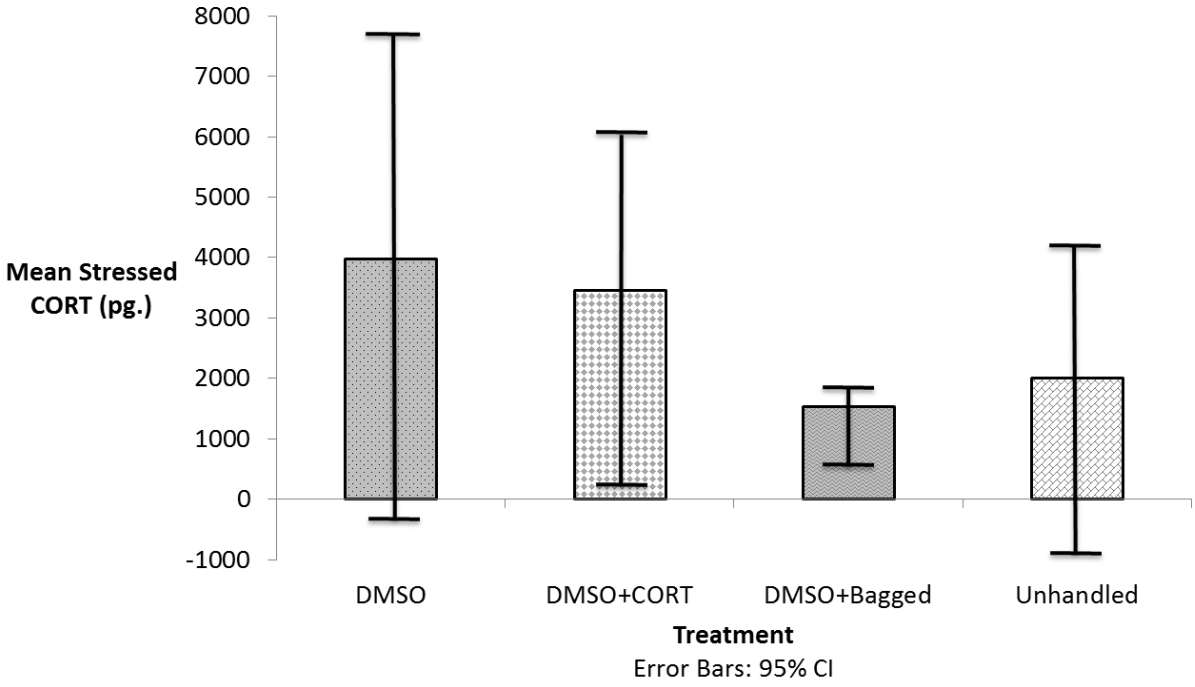

Figure 3: Means of Stressed CORT Levels

Multiple regression analyses revealed relationships between CORT levels, approach behaviors, movement, and weight variables, regardless of treatment. While most of the variables were not predictive of each other, regressions analyses showed baseline CORT to be a significant predictor of food bowl approach frequency, feeding duration, and movement. The higher the baseline CORT levels, the more often the birds approached the food bowl, $\beta = .57$, $t(18) = 2.85$, $p = .011$, with baseline CORT levels explaining a significant proportion of the variance in food bowl frequencies $R^2 = .189$, $F(1, 18) = 8.14$, $p = .011$ (Figure 4). The higher the baseline CORT levels, the longer the birds cumulatively fed over 30 minutes, $\beta = .45$, $t(18) = 2.06$, $p = .055$, with baseline CORT levels explaining a significant proportion of variance in feeding duration times, $R^2 = .152$, $F(1, 18) = 4.23$, $p = .055$ (Figure 5). Finally, the higher the baseline CORT levels, the more the birds moved, $\beta = .43$, $t(25) = 2.15$, $p = .041$, with baseline CORT levels explaining a significant proportion of movement variance $R^2 = .162$, $F(1, 25) = 4.65$, $p = .041$ (Figure 6).

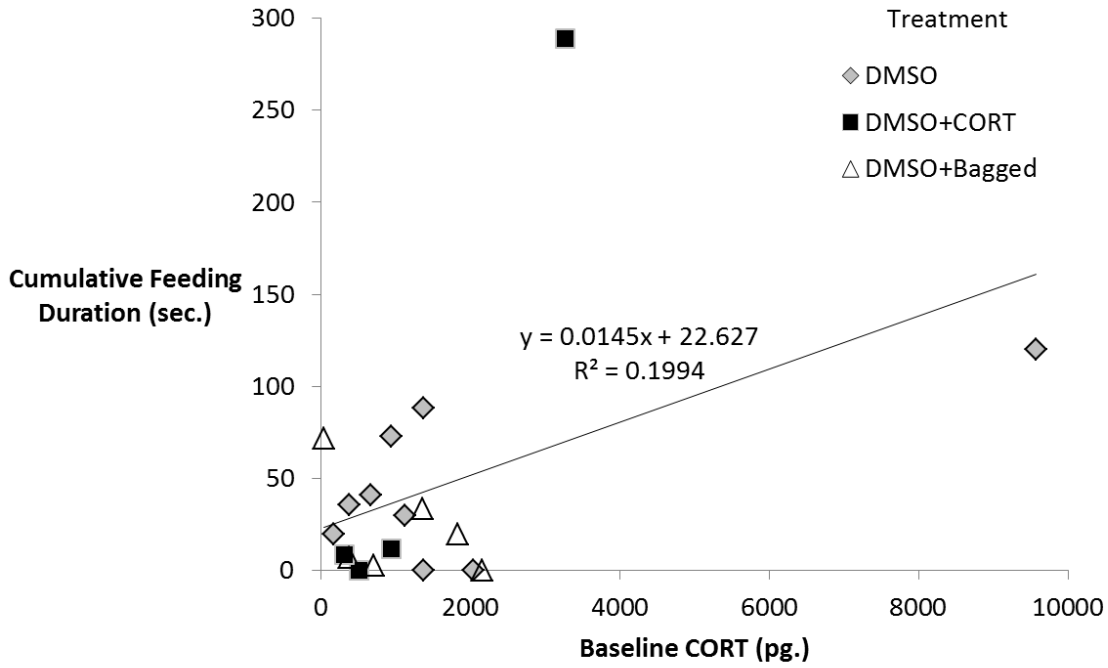

Figure 4: Baseline CORT Levels and Cumulative Feeding Duration

Baseline CORT levels (pg.) pre-treatment significantly predicts cumulative feeding duration time (sec.). Specifically, it was found that the higher the baseline CORT levels, the longer the birds fed.

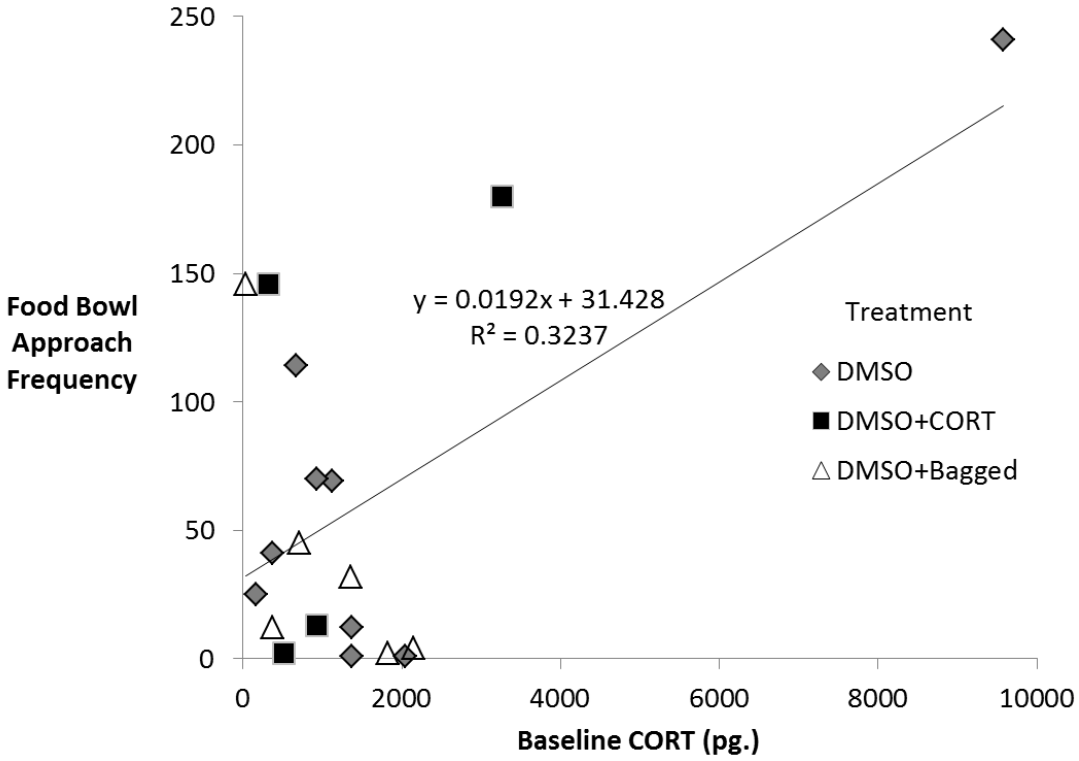

Figure 5: Baseline CORT levels and Food Bowl Approach Frequency

Baseline CORT (pg.) levels were predictive of the frequency to approach the food bowl regardless of treatment.

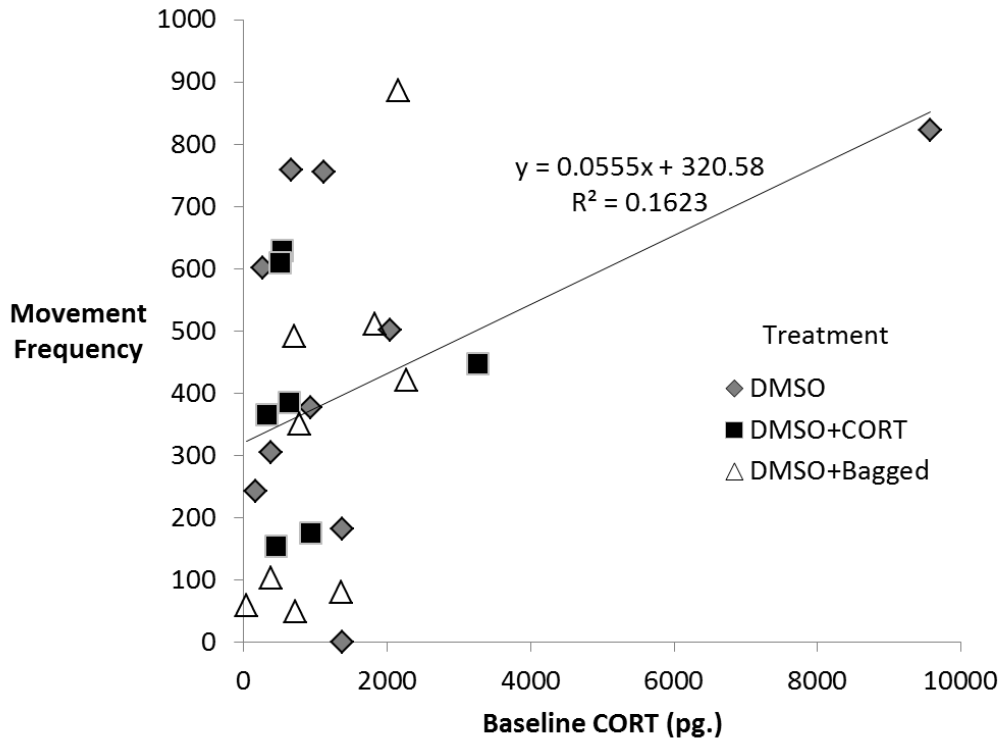

Figure 6: Baseline CORT and Movement Frequency

Baseline CORT levels (pg.) were predictive of movement behaviors regardless of treatment.

Behavioral Measures

Food and water bowl approach behaviors. The percentage of birds that approached the food bowl had a marginally significant difference between DMSO+CORT and DMSO, X^2 (1, N= 18) = 3.55, p= .06, η^2= .44, but did not differ between the DMSO+CORT and DMSO+Bagged groups, X^2 (1, N= 17) = .49, p= .486, η^2= .17. Similarly, there was no difference between DMSO+CORT and DMSO birds in approaches to the water bowl, X^2 (1, N= 18) = 1.8, p= .18, η^2= .32, nor was there a difference between DMSO+CORT and DMSO+Bagged groups, X^2 (1, N= 17) = .49, p= .486, η^2= .17. Finally, there was no difference between the Unhandled and handled groups in the percentage of birds that approached the food bowl, X^2 (1, N= 34) = .67, p= .412, η^2= .14, but there was a marginally significant difference in the percentage of birds that approached the water bowl, X^2 (1, N= 34) = .06, p= .81, η^2= .04.

Additionally, there were no significant multivariate effects across all treatment groups in the latency to approach the food bowl, latency to approach the water bowl, frequency of approaching the food, frequency of approaching the water bowl, feeding duration, or drinking duration, Wilk's λ= .365, F(3, 18)= .885, p= .568, *partial* η^2= .29. Individual ANOVAs were run for each variable for every comparison described in the hypotheses. No significant differences were found, as was expected since the multivariate effect provided null significance. However, there was a significant difference in the mean of food bowl frequency between the Unhandled group and the 3 other handled groups, F(1, 24) = 7.01, p= .014 (Figure 7).

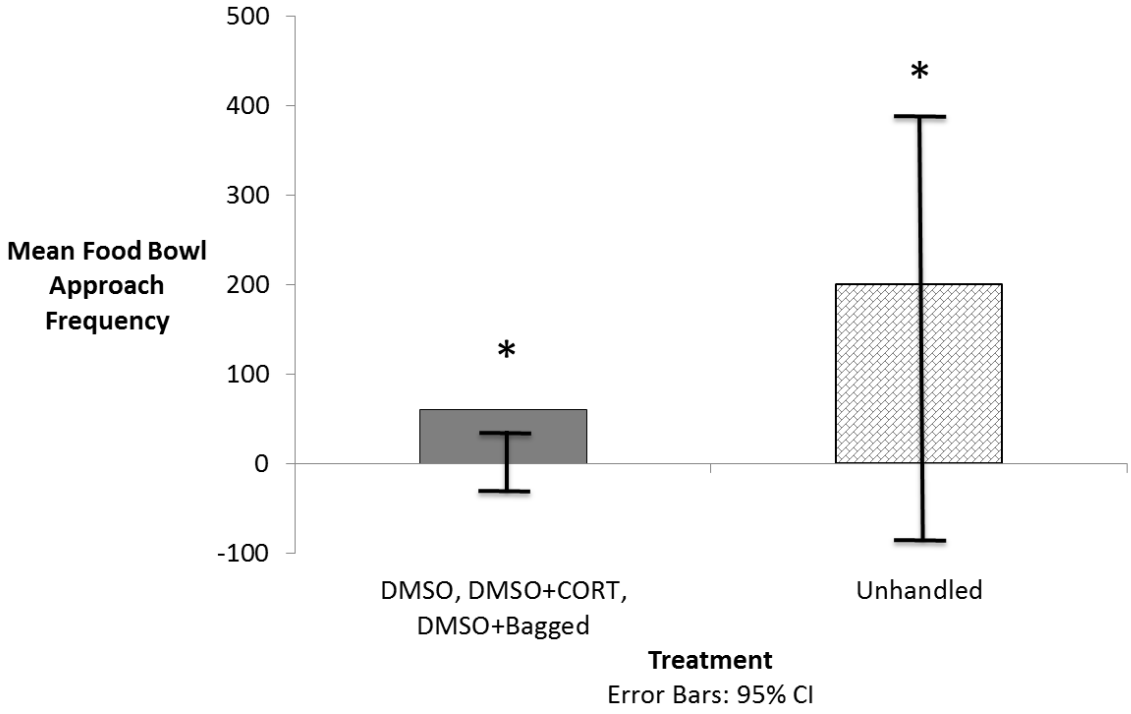

Figure 7: Mean Food Bowl Approach Frequency

Multiple regression analyses determined relationships between the variables regardless of treatment conditions. Analyses showed that frequency of approaching the food bowl was a significant predictor of cumulative duration of time feeding, β= .71, $t(24)$= 4.89, p <.001, with food bowl frequency explaining a significant proportion of feeding duration variance, R^2= .509, $F(1, 24)$ = 23.89, p<.001 (Figure 8), and also final weight, β= -.415, $t(21)$= -2.04, p=.055, with food bowl frequency explaining a significant proportion of final weight, R^2= .173, $F(1, 21)$ = 4.17, p=.055 (Figure 9). Feeding duration was also found to be a significant predictor of final weight, β= -.43, $t(21)$= -2.13, p=.046, with food bowl frequency explaining a significant proportion of feeding duration variance, R^2= .185, $F(1, 21)$ = 4.55, p=.046 (Figure 10).

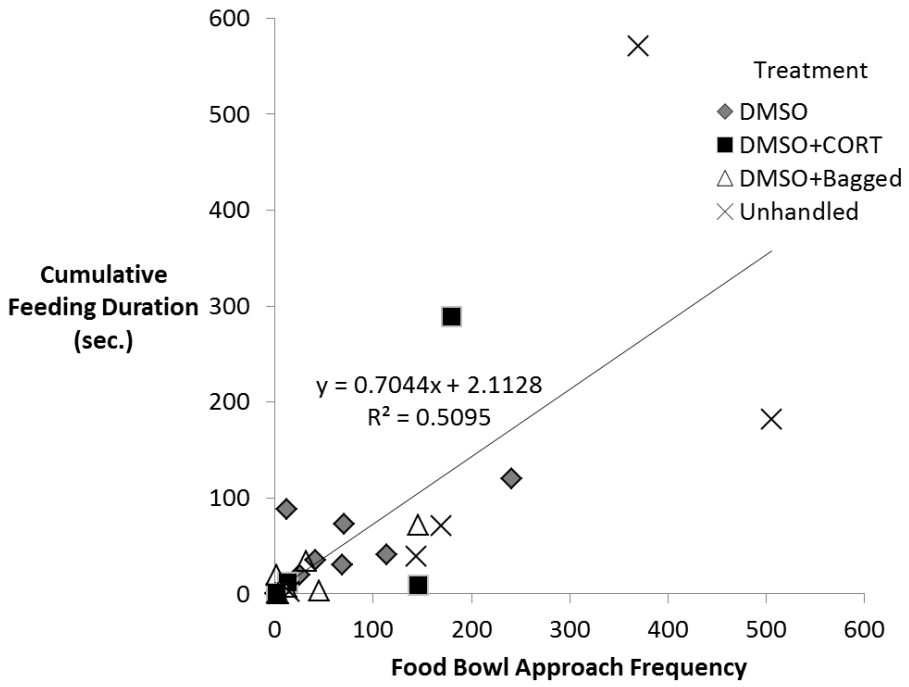

Figure 8: Food Bowl Approach Frequency and Cumulative Feeding Duration

A strong relationship between food bowl approach frequency and cumulative feeding duration was found with food bowl frequency significantly predicting feeding duration. The more often the birds approached the food bowl, the longer amount of total time they spent feeding.

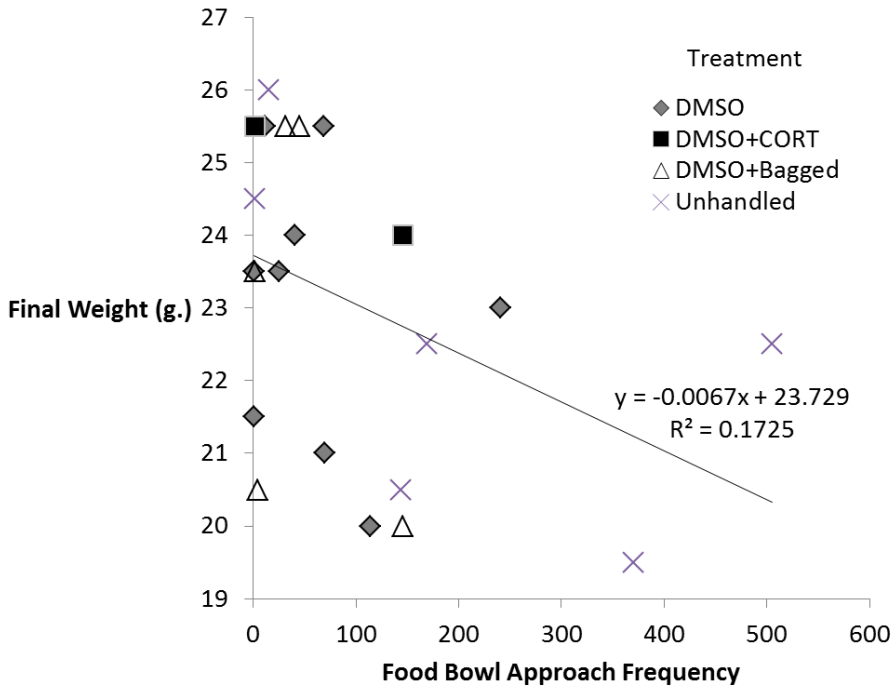

Figure 9: Food Bowl Approach Frequency and Final Weight

A weak negative correlation between frequency to approach the food bowl and final weight was found with food bowl approach significantly predicting final weight. The more the birds approached the food bowl, the less the birds weighed post-treatment.

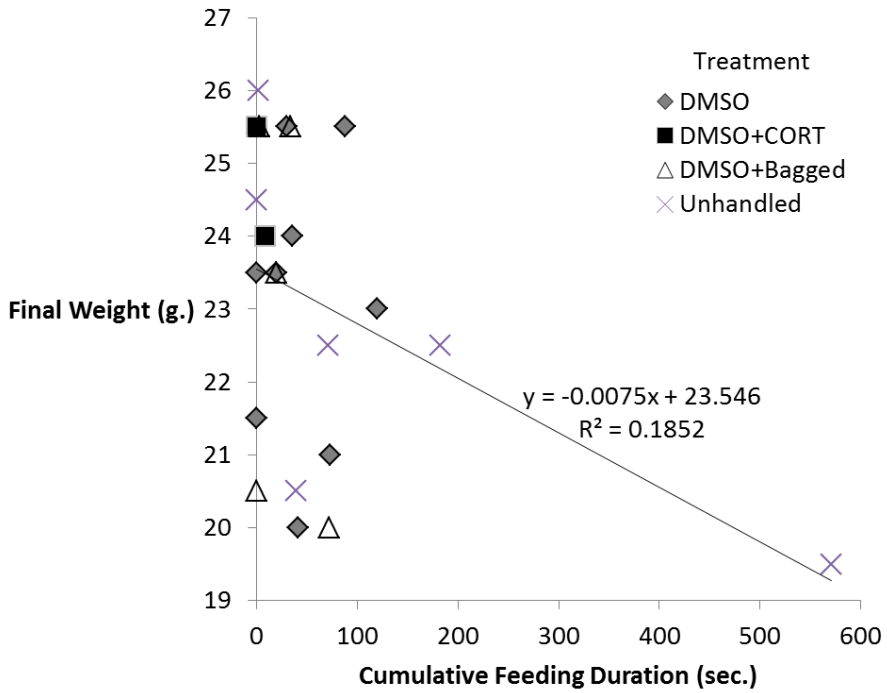

Figure 10: Cumulative Feeding Duration and Final Weight

A weak negative correlation between cumulative feeding duration and final weight was found with cumulative feeding duration significantly predicting final weight. The more the birds cumulatively fed, the less the birds weighed post-treatment.

Frequency of approaching the water bowl was a significant predictor of cumulative duration of time drinking, β= .57, $t(22)$= 3.18, p = .004, with water bowl frequency explaining a significant proportion of drinking duration variance, R^2= .325, $F(1, 22)$ = 10.12, p =.004 (Figure 11). Additionally, initial weight was found to be a significant predictor of food bowl frequency, β= -.496, $t(24)$= -2.74, p=.012, with initial weight explaining a significant proportion of food bowl frequency variance, R^2= .213, $F(1, 24)$ = 7.50, p=.012 (Figure 12), feeding duration, β= -.510, $t(24)$= -2.85, p=.009, and initial weight explaining a significant proportion of feeding duration, R^2= .26, $F(1, 24)$ = 8.10, p=.009 (Figure 13).

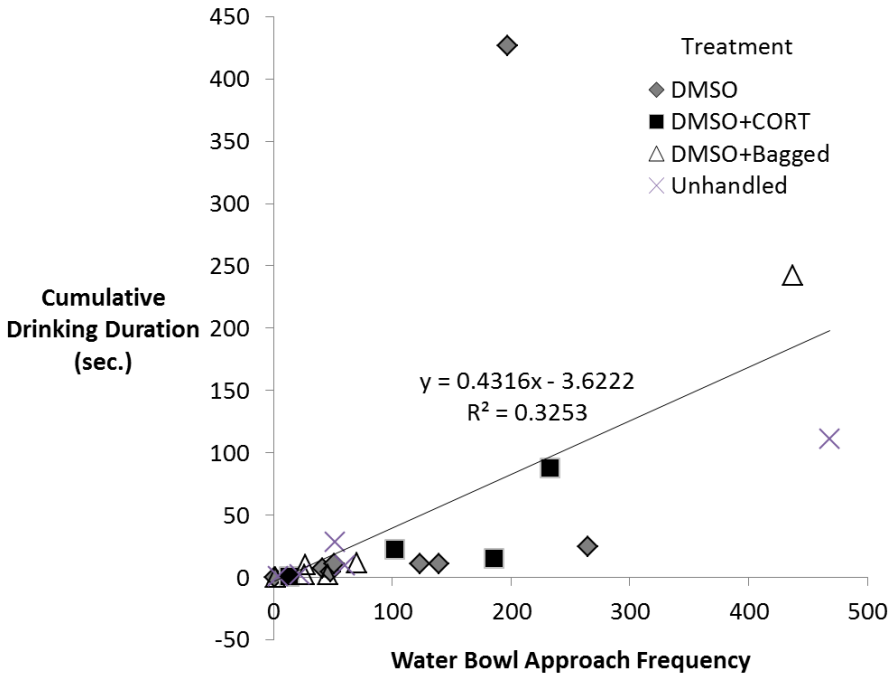

Figure 11: Water Bowl Approach Frequency and Cumulative Drinking Duration

A moderate relationship between water bowl frequency approach and cumulative drinking duration was found with water bowl frequency significantly predicting drinking duration. The more often the birds approached the water bowl, the longer amount of total time they spent drinking.

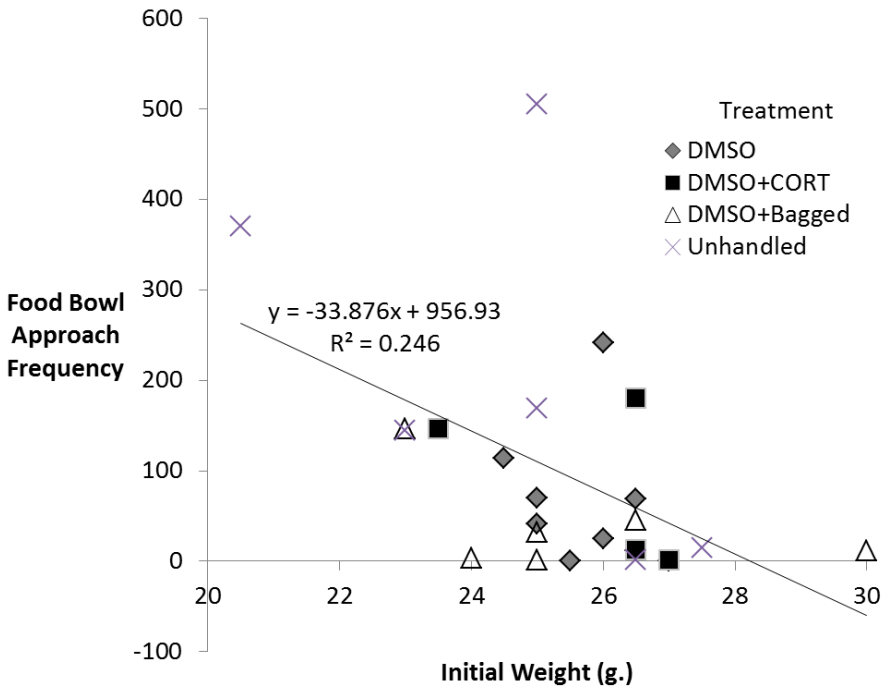

Figure 12: Initial Weight and Food Bowl Approach Frequency

A weak negative correlation between initial weight and food bowl frequency approach was found with initial weight significantly predicting food bowl frequency. The higher the initial weight of the birds, the less frequently they approached the food bowl.

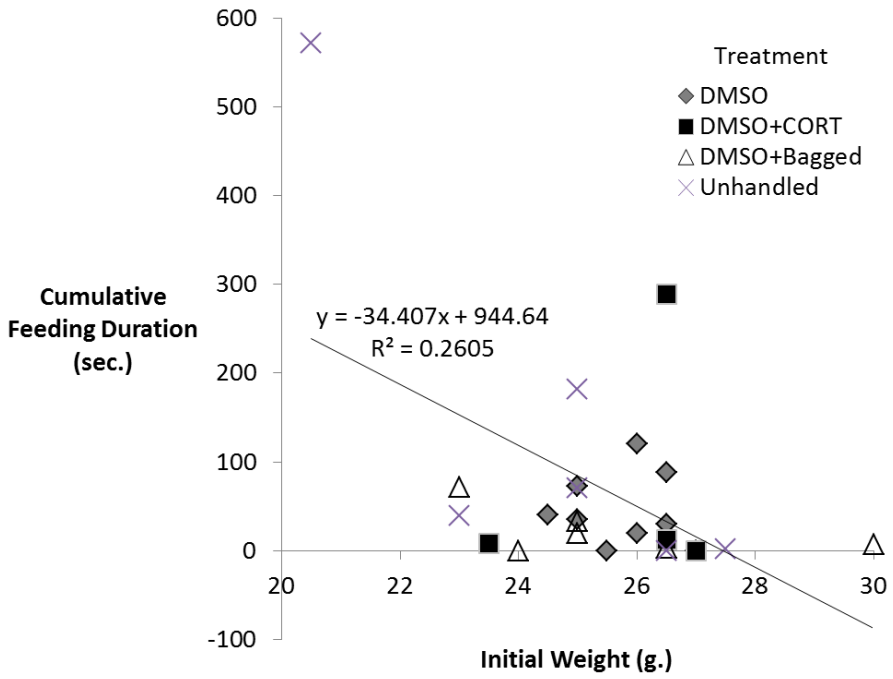

Figure 13: Initial Weight and Cumulative Feeding Duration

A weak negative correlation between initial weight and cumulative feeding duration was found with initial weight significantly predicting feeding duration. The more the birds weighed pre-treatment, the less frequently the birds fed.

Finally, there was a moderately negative correlation between latency of approaching the food bowl and frequency of approaching the food bowl for all groups, $r(25) = -.35$, $p = .041$ (Figure 14), while latency of approaching the food bowl was also a significant predictor of drinking duration, $\beta = .477$, $t(21) = 2.43$, $p = .025$, with food bowl latency explaining a significant proportion of drinking duration variance, $R^2 = .228$, $F(1, 21) = 5.90$, $p = .025$ (Figure 15).

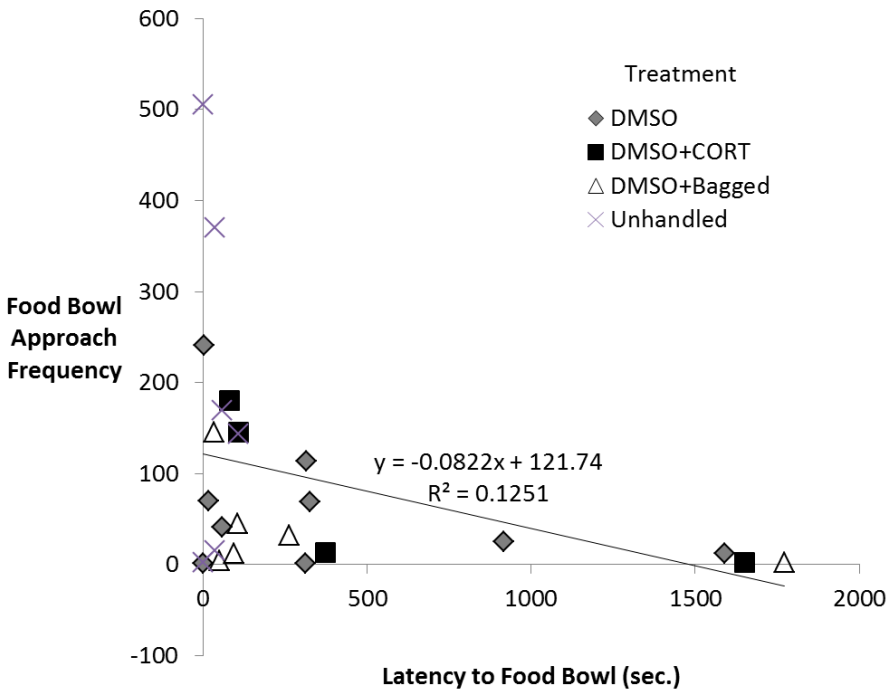

Figure 14: Latency to Food Bowl and Food Bowl Approach Frequency

A weak negative relationship between the latency to approach the food bowl (sec.) and food bowl approach frequency was found with latency to food bowl significantly predicting food bowl frequency. The longer it took the bird to initially approach the food bowl, the less frequently they approached the food bowl.

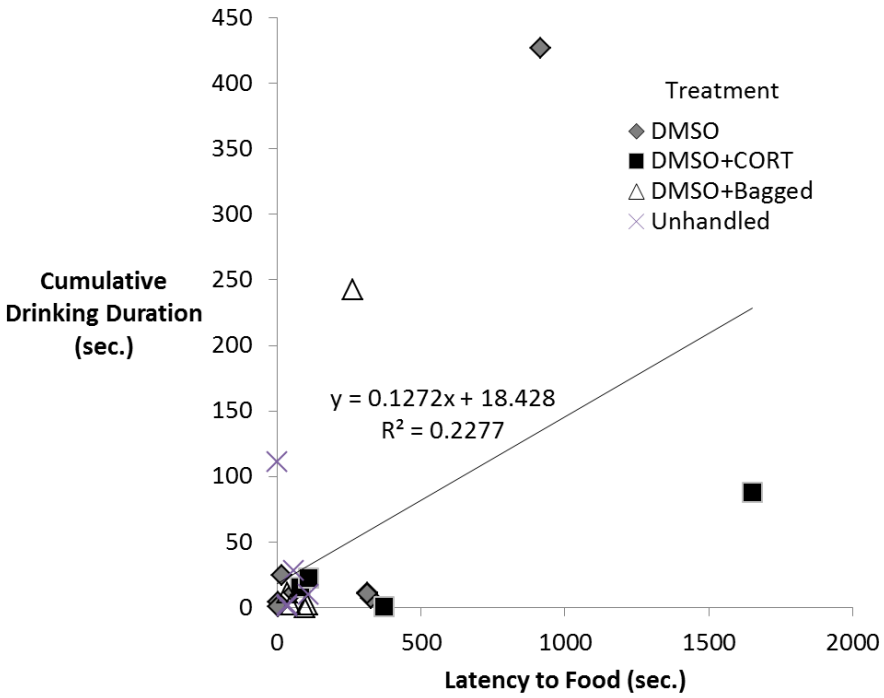

Figure 15: Latency to Food and Cumulative Drinking Duration

A weak relationship between latency to approach the food bowl and cumulative drinking duration was found with latency to the food bowl significantly predicting drinking duration. The longer it took to initially approach the food bowl, the more water they drank.

Movement behaviors. Univariate ANOVAs were conducted in order to best capture the entire sample size for movement behaviors. Results revealed that there was no main effect of DMSO+CORT on movement behaviors compared to DMSO, $F(1, 16)= 1.28$, $p= .274$ nor was a main effect found between DMSO+CORT and DMSO+Bagged groups, $F(1, 15)= .00$, $p= .985$. Multiple regressions and correlations revealed no relationships with the other behavioral measures.

Weight and Sex

In comparing treatment and the change of initial to final weight, there was a significant main effect of weight, $F(1, 28)= 46.58$, $p<.001$, *partial* $\eta^2= .625$, and a significant main effect in the difference between groups, $F(1, 28)= 3.39$ $p= .032$, *partial* $\eta^2= .267$, but no interaction between the treatment groups and weight, $F(1, 28)= 1.45$ $p= .249$, *partial* $\eta^2= .135$. Repeated ANOVA tests also showed that while there was a main effect between initial and final weights in comparing DMSO+CORT and DMSO groups, $F(1, 16)= 20.01$, $p<.001$, *partial* $\eta^2= .147$, the interaction was not significantly different, $F(1, 16)= 2.75$ $p= .117$, *partial* $\eta^2= .147$, nor was there a main effect between treatment groups, $F(1, 16)= 3.49$ $p= .081$, *partial* $\eta^2= .178$.

In comparing initial and final weight with the DMSO+CORT and DMSO+Bagged groups, there was a significant main effect in final weight and initial weight, $F(1, 12)= 9.26$, $p= .010$, *partial* $\eta^2= .436$, a significant main effect in the difference between groups, $F(1, 12)= 8.14$, $p= .015$, *partial* $\eta^2= .404$ but there was not a significant interaction, $F(1, 12)= .172$, $p= .685$, *partial* $\eta^2= .01$. Additionally, in comparing the Unhandled group with the handled birds, there was significant main effect of initial weight and final weight, $F(1, 31)= 35.62$, $p<.001$, *partial* $\eta^2= .535$, but there

was not a significant main effect between the Unhandled and handled birds, $F(1, 31)= 2.34$ $p= .137$, *partial* $\eta^2= .070$, nor was there a significant interaction, $F (1, 31)= .003$ $p= .956$, *partial* $\eta^2= .000$.

As there appeared to be significant differences between initial and final weight without treatment, a paired samples T-Test revealed final weights to be significantly lower than initial weights, $t(32)= 7.38$, $p<.001$. Additionally, regression analyses revealed that initial weight was a significant predictor of final weight, $\beta= .67$, $t(32) = 5.06$, $p < .001$, and that initial weight explained a significant proportion of variance of final weight, $R^2= .45$, $F(1, 32) = 25.62$, $p < .001$ (Figure 16). As mentioned previously, initial weight was significantly predictive of food bowl approach frequency and feeding duration. It was also stated that food bowl frequency and feeding duration were also significantly predictive of final weight.

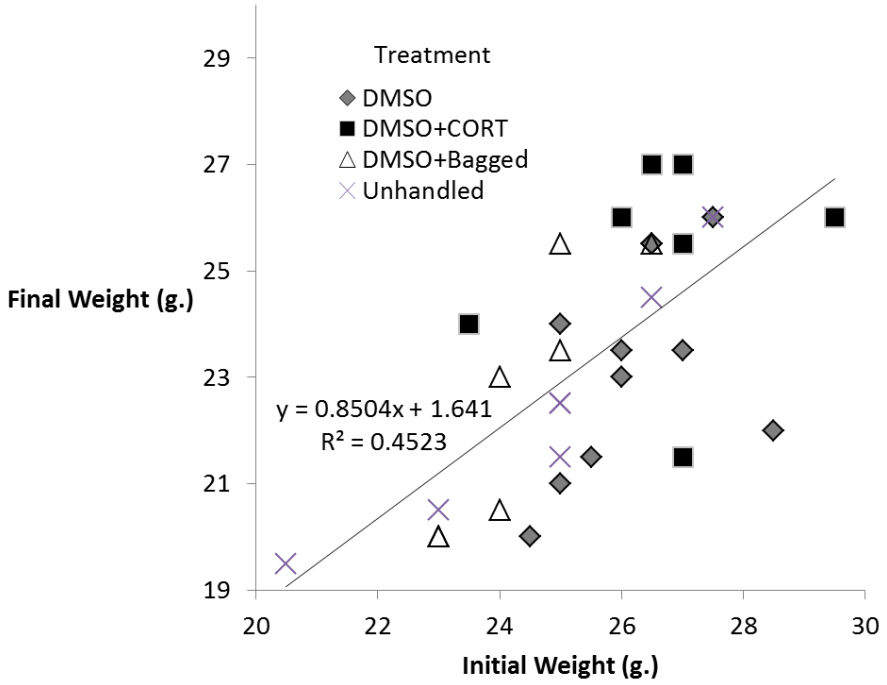

Figure 16: Initial Weight and Final Weight

A moderate positive correlation between initial weight and final weight was found with initial weight significantly predicting final weight. The more the birds initially weighed pre-treatment, the more the birds weighed post-treatment.

Univariate ANOVAs and repeated ANOVAs were used to determine if there were sex differences among behavioral measures, CORT levels, or change in weight. There were no significant sex differences. However, there was a main effect of initial and final weight, F (1, 30) = 50.57, p<.001, *partial* η^2= .628, a main effect between males and females, F (1, 30) = 6.46, p= .026, *partial* η^2= .177, but there was no interaction between sex and weight, F (1, 30) = .506 p= .483, *partial* η^2= .017.

Discussion

Previous literature provided reasoning for the proposed hypotheses that administering exogenous CORT would increase anxious and cautious behaviors (Busch 2008, Lohmus, et al., 2006). Additionally, the "capture and restraint" technique has been consistently used as model for a natural stressor (Wingfield et al. 1992, 1994; Jayne, Dudley, Greene, Moore, & Davis, 2012). It was of interest in this study to compare whether anxious and cautious behaviors of the "capture and restraint" technique would be similar to exogenously increasing CORT. Many of the results did not come out as expected. Despite this, there were many interesting conclusions to be drawn.

Was Treatment Efficacious?

We hypothesized that with an increase of exogenous CORT, birds would have increased CORT levels compared to those who only received DMSO. While there appeared to be a trend, the DMSO+CORT and DMSO groups were not significantly different. This suggests that CORT treatment was not efficacious.

Additionally, we hypothesized that birds given DMSO and then placed in a bag would have similar CORT levels to those of the DMSO+CORT condition. Results did not support this hypothesis. The fact that there was a significant difference in stressed CORT levels between the DMSO+CORT and DMSO+Bagged groups demonstrate that administering exogenous CORT may have been more efficacious in increasing CORT levels than presenting the birds with an actual stressor. To my knowledge, previous studies observing the relationship between CORT levels of birds that have had exogenous increases in CORT and those who have been placed in a cloth bag do not exist.

Finally, we also hypothesized that those of the Unhandled group would have lower CORT levels compared to all of the groups that were handled: DMSO, DMSO+CORT, and DMSO+Bagged. Results did not support this hypothesis. Considering that all four groups had different treatment conditions, and there were no statistical differences between them, it is of interest why this occurred. It should be noted that there was a moderately significant main effect in the difference between the baseline bleed and the stressed, post-treatment bleed, showing that there is a trend of the three handled groups increasing their CORT levels from pre- to post-treatment. However, the fact that there was not a significant difference in stressed CORT levels between all four groups, yet all had high CORT levels, suggests that despite treatment, something was causing all birds to have higher post-treatment CORT levels compared to baseline.

Did the time of bleeding affect CORT levels? As mentioned previously, CORT administration with DMSO has been found to increase baseline CORT levels in prior studies (Busch et al., 2008; Lohmus, et al., 2006). Furthermore, the "capture and restraint" technique is used as a standard method to induce stress. Typically, the stressed sample is taken immediately after being in the bag for 30 minutes and has been found on countless occasions to increase CORT levels (Wingfield et al. 1992, 1994; Canoine, et al., 2002; Jayne, et al., 2012; Baugh, et al., 2013).

In our study, the DMSO+Bagged birds were also held in a bag for 30 minutes. The stressed plasma sample was collected one hour after the birds were released from the bag (see Figure 1), as opposed to immediately afterwards. The reasoning behind this later CORT sample collection was to allow 60 minutes of DMSO absorption for all birds, along with providing an adequate amount of exposure time to the novel stimulus (30

minutes). It is possible that the birds' CORT levels plateaued, causing the lower response rate compared to the DMSO+CORT group. In fact, Canoine, et al. (2002) found that CORT plateaued 20 minutes after being released from the bag. This may explain why post-treatment CORT levels of the DMSO+Bagged birds are lower than levels of the DMSO+CORT birds.

How did the CORT administration timeframe influence treatment? Other members of our lab conducted a CORT manipulation study using similar methodologies to those described here. However, instead of receiving a one-time acute treatment of CORT, birds received multiple acute CORT treatments over a period of three days, along with a combination of lipopolysaccharide (LPS), a toxin that elicits the immune response. The heterophil: lymphocyte (H:L) ratio is a ratio of how many white blood cells are in the blood and is an indicator of immune function (Cīrule, Krama, Vrublevska, Rantala, & Krams, 2012). Significant differences were found between the H:L in the DMSO-administered, CORT-administered, and CORT with LPS administration groups, signifying that the HPA axis is activated in response to these physiologically-induced acute stressors.

Busch et al. (2008) used a similar method of DMSO and CORT application once or three times a day on white-crowned sparrows, and found a significant decrease in body conditions. Additionally, the concept that the timeframe might be a factor is consistent with Adams, Farnworth, Rickett, Parker, and Cockrem (2011), who found behavioral differences such as average frequencies of alert behaviors, locomotion, and alert calls varied depending upon the number of days that wild blackbirds were held captive. These studies support the possibility that it was the timeframe within the current study that

caused the lack of differences between CORT-administered groups and DMSO-administered groups in that it was too acute of a timeframe to see differences.

How does CORT dose and administration timeframe influence behavior? Lohmus, et al. (2006) found that noninvasively, repeatedly increasing immediate levels of CORT (4µg CORT per 20µg DMSO) of red-eyed vireos generally increased food bowl visits, specifically 3, 5, and 6 hours after the hourly consumption of CORT-filled mealworms. Additionally, previous studies in our lab showed that after 15 minutes of exposure to the same red wall used as a novel stimulus in the current study, increased CORT levels were significantly related to the latency of approaching the food bowl (Cooper, et al., 2013). Busch et al. (2008) found that frequent acute administrations of CORT only increased feeding over time. Moreover, Astheimer, Buttemer, and Wingfield (1992) found that after days of administering the CORT implants, feeding only increased after a day of fasting compared to the control group.

All of the listed studies include changes of feeding behavior in response to either exogenously increasing CORT, or increasing CORT with a novel stimulus. However, the timing and dosage of CORT administration vary across these studies, along with the current study, making it difficult to determine the effects CORT has on feeding and food bowl approach. It is of interest to standardize CORT dose and administration methods to better compare stress behaviors.

What are other possible mechanisms influencing the stress response? While the CORT released from the HPA plays a role in coping with stress, other mechanisms influence the response, as well. When a stressor is perceived, the sympathetic nervous system causes release of epinephrine from the adrenal medulla and norepinephrine from

sympathetic nerves (Axelrod & Reisine, 1984). One of the functions of these catecholamine hormones is to stimulate the anterior pituitary, activating the HPA. In turn, CORT regulates the enzymes that synthesize epinephrine in the adrenal medulla, along with enhancing catecholamine actions such as strengthening their affinity and ability to bind to receptors in the cardiovascular system (Sapolsky, Romero, & Munck, 2000). It can thus be recognized that the HPA and sympathetic nervous systems are intertwined. As the sympathetic nervous system was not taken into account in the present study, it is of interest to consider it in future studies to determine how or whether epinephrine and norepinephrine influenced the stress behaviors in relation to CORT.

Another mechanism directly involved with CORT regulation includes its affinity and the availability of the receptor that it binds to. During a stressful event, CORT binds to the Type II, glucocorticoid receptor (GR). Alternatively, when the body is undergoing "normal," everyday activities, CORT has a higher affinity to the Type I, mineralocorticoid receptor (MR) (Greenberg, Carr, & Summers, 2002). Gesing, Bilang-Bleuel, Droste, Linthorst, Holsboer, and Reul (2001) found that rats in a forced swim test had a significant increase in MR receptors. This increase in MR receptors was also found to inhibit HPA activity. However, applying an MR antagonist increased CORT levels 24 hours after the forced swim test. These results present additional questions regarding the involvement of the MR. It is interesting to consider when and for how long the house sparrows of the current study were actually stressed, whether the timeframe influenced the number of receptors, and if the receptors themselves influenced the stress response.

How did the novel stimulus influence treatment? One of the goals of the study was to understand how an environmental or physiological stressor may influence the

stress response as a result of a neophobic stimulus (the red wall). However, it is possible that the red wall might have been so much of a stressor that all of the birds exposed to it experienced increased CORT levels. Previous work in our lab has shown that the color red produces neophobic behaviors (Cooper, et al., 2013). In this study, it is impossible to know whether the red wall actually caused neophobic behaviors as there was not a control for the red wall itself.

This possibility of the red wall influencing the increase in CORT levels is emphasized with the inclusion of the Unhandled group. As the Unhandled group was exposed to the novel stimulus in the same manner as the handled groups, showing similar CORT levels post-manipulation, it signifies that handling or treatment did not influence those high CORT levels.

How did DMSO and/or handling influence treatment? Considering the possibility that the novel stimulus was not the cause for the similar increases in CORT levels, another explanation is that DMSO was an ineffective vehicle for transferring the CORT into the bloodstream. This possibility is unlikely as Busch et al. (2008) has effectively raised CORT levels using this method. It is also possible that the DMSO, itself, was a stressful experience because of the sensations it generates. Thus, the DMSO application and simply touching the bird may have been so stressful that it caused high CORT levels for all the groups, regardless of the CORT or Bagged treatments.

In addition to the confound of handling birds for the treatment application, the DMSO, DMSO+CORT, and DMSO+Bagged birds were also handled during cage maintenance. It was necessary to remove the birds out of the cage so that the floor of the cage may be cleaned. While these handling situations do not provide explanations for

why the Unhandled group has similar CORT levels to the handled groups, they may provide an explanation for why neither the DMSO+CORT nor the DMSO+Bagged groups were different from the DMSO group.

How did human presence influence the stress response? Along with the possibility of handling affecting the increase in CORT, another potential stressful event during the experimental procedure was the experimenter's presence in the room. On the morning of treatment day, the arena in the Noldus EthoVision needed to be prepared. To do this, the lamp above the cage needed to be switched on prior to the recording. After the birds had been treated and returned to the recording room, the camera and lamp were switched on. Thus, the light was on while the red wall was put alongside the cage and the birds were disturbed throughout the process.

Previous studies in our lab that did not use EthoVision had the light off in the room until the red wall was already in front of the cage and the researcher was out of the room (Cooper, et al., 2013), meaning that the birds were minimally disturbed during the process. The sight of a predator or human intruder can cause birds to produce cautious behaviors (Cockrem and Silverin, 2002), and subsequently increase CORT (Silverin, 1998; Nephew, Kahn, & Romero, 2003). As mentioned earlier, predator sightings have been shown to increase CORT to levels that are more than twice as high as the levels produced from the standard "capture and restraint" method (Pakkala, et al., 2013). It is possible that the presence of a researcher could have provided an additional stressor, increasing CORT levels and possible cautious behaviors.

Did acclimation time to the lab room influence treatment? The lab environment itself is another potential cause for increased CORT levels. Dickens,

Delehanty, and, Romero (2009) found that capture, captivity, and transport reduces baseline CORT levels and alters the stress response in chukar partridges. Of the house sparrows used in my study, the handled birds had two days to acclimate to the room after coming from the aviary, and the Unhandled birds had three. While all the birds had the same amount of traveling and acclimation time (besides the Unhandled birds), it is possible that traveling from the aviary to the lab or the fact that they did not have enough time to acclimate to the laboratory environment created additional stressors for the birds. Previous studies in our lab did not require traveling because the experiments were conducted at the aviary (Cooper, et al., 2013). Thus, traveling to the lab could have been a stressful situation, increasing CORT levels for all groups.

Did Treatment Influence Behavior?

Because CORT treatment did not work as expected in this study, it is difficult to draw conclusions about whether there were differences in behaviors between the groups. We did not predict there to be behavioral differences between the DMSO+CORT and DMSO+Bagged groups. While the results supported these predictions in that there were no behavioral differences between the groups, the fact that there was a difference between CORT levels questions whether there should have been behavioral differences, as well.

Was there a difference in approach behaviors between unhandled and handled birds? We expected significant behavioral differences between the Unhandled and handled birds. Most of the results do not support this prediction. However, the Unhandled birds approached the food bowl significantly more than the handled birds. The cumulative number of visits to the bowls was taken into account in this analysis,

demonstrating that this behavioral difference was specific to feeding, and not simply an artifact of increased movement.

Additionally, it was found that food bowl frequency was predictive of feeding duration and water bowl frequency was predictive of drinking duration, suggesting that the birds are actually feeding or drinking when they approached the food or water bowls. Thus, it can be concluded that Unhandled birds were actually feeding more because they approached the food bowl more often than handled birds, most likely because they were less stressed than handled birds, though this was not reflected in corticosterone levels. This may imply that mechanisms other than corticosterone play an important role in modulation of feeding behavior in response to handling stress.

It is possible that the Unhandled birds were actually less stressed than the handled birds as we predicted even though it is not represented in the results (possibly due to low sample size). Further, it is conceivable that handled birds, being more stressed, would be more cautious to approach the food bowl and were not as concerned with feeding. Because CORT has suppressive actions on appetite during stress, it is possible that the high CORT levels overrode the natural inclination to feed. (Anderson, 2006; Kovacs, et al., 2012).Thus, the handled birds might have been too stressed to feed.

Are baseline CORT levels indicative of behavioral syndromes? In our study, the underlying baseline CORT levels appear to consistently predict behaviors, in that higher baseline CORT levels were statistically predictive of more active behaviors (food bowl approach frequency, feeding duration, and movement frequency) regardless of intervening treatment. Baseline represents the circulating CORT levels during routine activities that adapt in response to a stressor (Bonier, Martin, Moore, & Wingfield, 2009).

The fact that baseline CORT levels were more predictive of behaviors in a stressed state than stressed CORT levels implies that there is something about the bird's originating state that may always predict behaviors, regardless of the stressful situation.

To understand animal behavior, scientific research typically focuses on studying the commonalities between individuals. While studying consistencies is relevant, behavioral and physiological variation is also common within a species, yet neglected in non-human research. Studying the underlying mechanisms in individual variation can provide a greater understanding of the species as a whole (Groothuis & Carere, 2005).

Animal behavioral profiles, or the personality of animals, are behavioral or physiological individual differences that are consistent over time. One type of behavioral profile to describe how animals respond to everyday challenges is coping styles. Coping styles are further described as having proactive or reactive characteristics. A proactive personality includes having an aggressive and bold behavioral style, less fearful, and a fight or flight behavioral response to threats. Alternatively, reactive personalities are described as having a non-aggressive and cautious behavioral style, more fearful, and a freeze or hide behavioral response to threats (Koolhaas, Korte, De Boer, Van Der Vegt, Van Reenen, Hopster, & Blokhuis, 1999; Cockrem, 2013).

It has been shown that the magnitude of change in CORT levels from baseline to an acute stressed situation may also be indicative of an individual's behavioral profile. Specifically, Ellis, Jackson, & Boyce (2006) predicted that proactive individuals would have a lower reactivity of the HPA axis compared to reactive individuals. This hypothesis has been supported in a number of cases. For instance, mice that reacted aggressively in a stressful situation were found to have a lower HPA reactivity as

opposed to mice that responded non-aggressively in the stressful situation (Korte, Meijer, de Kloet, Buwalda, Keijser, Sluyter, & Bohus, 1996). Additionally, the HPA reactivity in low feather pecker chicks were significantly higher during resting conditions than high feather peckers (Korte, Beuving, Ruesink, & Blokhuis, 1997) and basal CORT levels were lower in pigs that had more escape attempts than pigs that attempted to escape less (Hessing, Hagelsø, Schouten, Wiepkema, & van Beek, 1994).

While neuroendocrine research in behavioral profiles has mostly focused on HPA reactivity to stressful situations, it would also be very beneficial to consider whether baseline CORT levels are predictive of how an individual copes with an acute stressor. Baugh et al. (2013) found that slower great tit explorers in a novel environment exhibited greater elevation in CORT levels after capture and higher CORT concentrations during the stressful event. This result indicates that birds with higher elevated CORT levels and high stressed CORT levels may have had differences in baseline CORT levels, with the proactive birds having a higher baseline and smaller elevation of CORT levels and the reactive birds having a lower baseline and a higher elevation of CORT levels. Thus, it is possible that differences in baseline levels may be indicative of proactive or reactive personalities. The results from Baugh et al. (2013) are comparable with the current study in that less active house sparrows had lower baseline CORT levels. Birds with lower baseline levels would have required a greater elevation in CORT post-treatment and novel stimulus exposure compared to those birds with higher baseline CORT levels, thus suggesting that the birds with lower baseline levels that were also less active had more reactive personalities.

Indicators of proactive and reactive personality can provide valuable information regarding how an individual can cope in the environment. For instance, if a bird has high baseline levels and a relatively low response to stress, it may indicate that the bird has proactive tendencies. These proactive tendencies can include being less fearful and less sensitive to immediate changes in the environment. In contrast, birds with lower baseline levels with a relative high response to stress may indicate that the birds have a reactive personality, being more fearful, more successful in changing or unpredictable conditions, and more able to cope to environmental changes.

What other approach behaviors were correlated? In addition to baseline CORT levels being predictive of the behavioral measures, there were several other significantly correlated behavioral measures. As mentioned above, drinking duration and feeding duration were included in the analyses to assure that the bowl approach frequencies were indicative of drinking and feeding. The regression analyses supported these comparisons.

How did weight and sex influence behaviors? For all birds, initial weight was higher than final weight. Results showed that weight was associated with food bowl approach frequency and cumulative duration of feeding time. Specifically, the less the birds initially weighed, the more frequently they approached the food bowl and the longer the birds fed. Additionally, the longer the birds fed and the more times they approached the food bowl, the lower their weight was post-treatment. While weight was significantly predictive of these behaviors, they were only weakly correlated. It will be of interest to explore how a greater sample size would strengthen or weaken the relationship, because multiple studies show how CORT influences metabolism and fitness (Astheimer, et al.,

1992; Breuner, Patterson, & Hahn, 2008). However, among studies observing CORT and body mass, there have been discrepancies in whether weight has increased, decreased, or stayed the same, and is further complicated by differences in dose and timeframe of CORT administration (Busch, et al., 2008; Lõhmus, et al., 2006; Davies, Rodriguez, Sweazea, & Deviche, 2013). Future studies should include how body mass is related to CORT for a better understanding of how CORT influences body condition during an acute stress.

Finally, it should be recognized that there were not any differences in sex among the CORT, behavioral, and weight measures.

Future Directions and Conclusions

This study provided valuable insight and presents future questions regarding stress and the HPA axis. For instance, baseline CORT being more predictive of stress behaviors than stressed CORT levels suggests the possibility that individual differences and personality need to be taken into account. Future directions for using this methodology include treating and bleeding the birds in a different timeframe and frequency that is more consistent with previous literature than what has been done in the current study. Additionally, to assure that the red wall was being perceived as a stressor, it would be ideal to have another condition such as being presented with a white wall. It would also be interesting to consider other mechanisms that may be influencing stressful behaviors. Future studies may include considering the H:L ratio, heart rate, and GR and MR receptor levels. Finally, incorporating a larger sample size than the current study would also be beneficial. The current study had multiple missing values due to unforeseen complications. With a greater sample size, better environmental control, and

taking into account the above listed variables we can perhaps gain an understanding of how multiple stressors influence the stress response.

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