

University of Nevada, Reno

**The Effects of Exogenous ACTH on Aggression in Mountain Chickadees**

A thesis submitted in partial fulfillment  
of the requirements for the degree of

Bachelor of Science in Biology and the Honors Program

by

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May 2018

**UNIVERSITY  
OF NEVADA  
RENO**

**THE HONORS PROGRAM**

We recommend that the thesis  
prepared under our supervision by

**MORGAN FERGUSON**

entitled

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**BACHELOR OF SCIENCE, BIOLOGY**

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May 2018

## Abstract

Aggression, especially male-male aggression, has been well documented in many taxa to be caused by an increase of the sex hormone, testosterone. However, in birds the link between testosterone and aggression is equivocal. Studies on this subject have produced contradictory results leading some to investigate other mechanisms that may be responsible for this increase in aggression. We examined aggressive behavior in Mountain Chickadees, *Poecile gambeli*, that were injected with either adrenocorticotropin hormone (ACTH), which causes an acute rise in corticosterone, or saline control solution. We found that ACTH birds were faster to return to the nest and would get closer to a speaker playing a male Mountain Chickadee song. However, we did not see any difference in number of songs sang between the groups. These results suggest that corticosterone levels and the HPA axis play a role in mounting an aggressive response to a perceived intruder.

## Acknowledgments

I would like to extend my gratitude to Dr. Ouyang for helping me throughout this whole experience. She has not only been an inspiration to me but a great mentor as well. Dr. Ouyang knew very little about me when I applied to be part of her lab, yet she took me in with open arms. She gave me an opportunity that others can only dream of and for that I am very grateful.

I also must thank Dr. Scott Davies who was very kind throughout this whole experience and allowed someone who had no experience in this field to join in on his research.

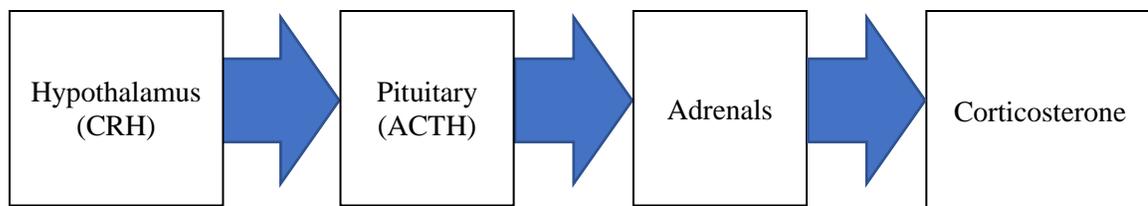
Finally, I would like to thank the Ouyang lab group in general. I enjoyed meeting all of them and they helped make this an enjoyable experience.

## Introduction

Biological organisms face many different and challenging fluctuations in their environment. Not only do they face these challenges, they must recognize and mount a response to cope with whatever challenge they are facing. Challenges organisms may face fall into two categories, abiotic and biotic. Abiotic challenges are those not associated with a living organism, such as the change between night and day. Biotic challenges, those associated with living organisms can include things such as interactions with other organisms (Hau et al., 2016). To cope with these different situations and challenges, organisms developed a system known as the endocrine system that secretes the principle mediators of these different cues, hormones. The endocrine system is a bodily system that is the primary chemical messenger in an organism and is composed of hormone secreting cells and tissues, hormones and hormone receptors (Johnstone et al., 2014). When an organism faces a challenging situation, the endocrine system will secrete hormones that ultimately change the behavior of the organism to not only cope with the situation, but also maintain homeostasis (Hau et al., 2016). Whether the challenge be regular and predictable or erratic and random, the endocrine system will mount a response that will alter the behavior of the organism allowing the organism to better cope. (Hau et al., 2016).

One of the most important parts of the endocrine system is the Hypothalamus-Pituitary-Adrenal axis, also known as the HPA axis. The HPA axis is activated by an abiotic or biotic challenge which in turn causes the production and release of glucocorticoids (GCs) into the organism's body allowing the organism to cope with the stressor (Lattin et al., 2012). Up regulation of the HPA axis works in a cascading fashion

where one product from one organ influences the next organ to produce another product. The cascade starts with the brain perceiving a stressor and the hypothalamus releasing corticotropin releasing hormone (CRH). CRH stimulates the pars distalis to make and secrete adrenocorticotropin hormone (ACTH). ACTH then stimulates the adrenals to make GCs (Hau et al., 2016, Figure 1). The final product of this cascade, GCs, then cause physiological or behavioral changes such as glucose provisioning and foraging (Hau et al., 2016) These behavioral and physiological changes in an organism allow the organism to better deal with the situation (Smith and Vale, 2006). While it is important to activate the HPA axis, it is equally as important to deactivate it. This is accomplished by a negative feedback loop where GCs will interact with the brain, thus stopping the production of GCs and avoiding the deleterious effects of prolonged high GC concentrations within the body (Romero, 2004).



**Figure 1.** The HPA axis. When faced with a stressful situation this axis is activated and mounts a response to help cope with the stress. This pathway incorporates parts of the brain and endocrine glands to ultimately produce glucocorticoids to respond to stressful situations. By using the concentrations of GCs secreted by this pathway, an organism's stress physiology can be quantified.

While all organism will mount a stress response that ends with GC production when faced with a stressor, the predominate GC changes depending on the species in question (Touma and Palme, 2005). For example, the primary GC secreted in mammals and teleost fish is cortisol. In birds, amphibians, and reptiles, the main GC secreted in a stress response is corticosterone (Hau et al., 2016). When at normal or baseline

concentrations in the body, GCs' main purpose is energy metabolism and maintaining homeostasis within the body. However, a spike in concentration when facing a stressor is thought to support the "emergency life history stage" (Wingfield et al. 1998). In supporting this life history stage, GCs will activate different activities in the body while simultaneously shutting down processes that are not required for immediate survival. This two-tier coping system allows the organism to focus all attention and bodily resources to coping with the stressor. When at high concentrations, GCs will start catabolizing energy stores and increasing foraging activity to provide more energy to increase survival. Simultaneously, GCs will inhibit activity within the body that is not completely essential for immediate survival including digestion, reproduction and growth. In turn, the energy saved from shutting down these processes temporarily can go toward survival (Hau et al., 2016).

One of the major stressors birds face is interaction and competition with others for resources. For males, this interaction, especially during breeding season, can lead to an aggressive response to ward off potential competitors. Most would presume that this aggressive behavior in males is mediated by the sex steroid testosterone (Wingfield et al., 1987). However, in birds, research on this topic produce some contradictory results. Wingfield et al. found that seasonally, testosterone and aggression correlated with birds like the house sparrows and European starlings where competition for nesting sites is very intense (1986). Yet, in another study with Lapland longspurs, the results differed. It was found that Lapland longspurs with testosterone implants did not show a significant difference in aggression from the controls, but testosterone implanted birds did show an increase in song (Hunt et al., 1997). However, outside of normal seasonal variation in

testosterone concentrations, a male bird's first response to another male is to show aggression and attack even though circulating testosterone levels may be low (Wingfield et al., 1987). Further adding to the disparities in the data, a study from 2012 found that House wrens did not exhibit a correlation between high testosterone and aggressive behavior (Cramer, 2012). Despite what was previously thought, new studies have begun to show that testosterone does play a role in aggressive behavior, but it may not be the major hormone causing the behavior.

A study conducted by Wingfield and Silverin used corticosterone injections to determine if cort increased aggression in Song sparrows. What they found was corticosterone injected sparrows were less aggressive overall when compared to controls (1987). However, the injected levels were chronically high and likely not to mimic short term ecologically relevant stressors, such as an aggressive interaction. For my research, I focused on determining whether GCs cause an increase in aggressive behavior. As male-male interaction is a major stressor in a bird's life, it makes sense to investigate the effects of GCs on aggressive behavior. My experimental design differs from Wingfield and Silverin in two ways. One, we injected the birds with ACTH rather than GCs to avoid the negative feedback loop associated with GCs. Second, our injections were meant to act as a more realistic acute stressor. In a real environment, a male will not continually secrete GCs because eventually the stressor, the competing male, will subside and there will no longer be a need to have a stress response. I predict that if male Mountain Chickadees are injected with ACTH, aggressive behavior will increase when compared to control injected male Mountain Chickadees.

## Materials and Methods

12 male Mountain Chickadees (*Poecile gambeli*), 6 controls and 6 experimental, were caught at Caughlin Ranch, Reno, NV, USA (39.4998 °N, 119.8591 °W) using a spring trap on day 8 post chick hatching. If the female was caught, she was bled, measured, banded, and released. Upon the male being caught, a blood sample was collected (50 µL max) to establish baseline cort levels. The male was then banded and measurements of body mass, tarus length, wing cord, CP width, and furcular fat score were taken and recorded. Depending on which group the male was part of, it received an injection into the pectoral muscle of either 50 µL of working ACTH solution or 50 µL of PBS control solution. After receiving an injection, males were released, and 30 minutes were allowed to pass to allow the ACTH to cause a rise in cort. During the 30-minute waiting period, distance flags and a speaker were set up for behavioral observation. Flags were placed at 2, 4, 8, and 16 m from the speaker to allow for distance determination during behavioral analysis.

Metrics were recorded on a Sony ICD-PX440 voice recorder (date, time of day, nest box number, etc) before a random male mountain chickadee song was played from an Arespark (model: AS 200) speaker. Behavior was recorded for 6 minutes with focus on the distance from the speaker, the number of songs sung by the male, and the return to nest time (latency). Distance from the speaker was recorded in five second intervals over the course of the 6 minutes. If during a five second interval, a male was 0-2 m from the speaker, it was given a 1 m distance rating. If 2-4 m it received a 3 m distance rating, 4-8 received a 6 m distance rating, 8-16 received a 12 m distance rating, and anything over 16

m from the speaker received a 24 m distance rating. If the male did not return to the site after 10 minutes of playback, the trial was aborted and noted. Once the trial was complete, the playback was stopped, and equipment was removed from the site.

To determine the cort levels in the Mountain Chickadee plasma samples, we used an Enzo Life Sciences competitive enzyme-linked immunosorbent assay (ELISA) kit in the lab. To measure the cort in the plasma samples, 3.75 $\mu$ l of each sample was added to a single well and diluted 20x using the assay buffer, which contained the steroid displacement reagent (SDR) at 0.5% of the volume of the plasma samples. The samples were run in singles and randomly assigned across and within two microplates; however, the paired 0 minute and 30-minute samples from a single day for each individual were included on the 21 same plate. A standard curve was run on each plate, which ranged from 32 pg/ml-20,000 pg/ml. A cort antibody and a cort conjugate were added to each well and the plates were then placed on the plate shaker for 2 hours. After the plates were shaken, the wells were washed 3x with 400 $\mu$ l of wash solution. Next, 200 $\mu$ l of an enzyme substrate (pnitrophenyl phosphate) was added to each well, then the plates were covered and incubated in a dark space for 1 hour. At the hour, 50 $\mu$ l of tri-sodium phosphate was added to each well to stop the enzymatic reaction. The plates were immediately read in a Fisher Scientific accuSkan FC microplate reader at 405nm. The assay sensitivity was 2.1pg/ml, with an intra-plate (2 plates) coefficient of variation of 10.3% and inter-plate coefficient of variation of 3.3%.

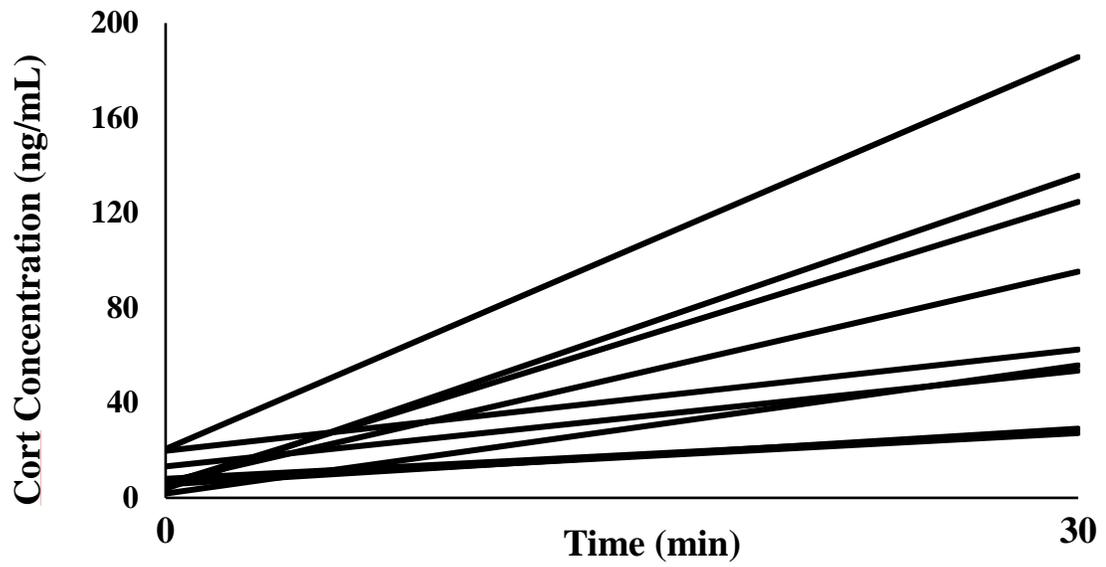
Statistical analysis was done using R (version 3.5.0) to determine significance in aggression between control and ACTH injected Mountain Chickadees. A linear model was used to model response to treatment. The linear model was used to determine if

significance differences existed between the two groups in latency, average distance from the speaker, and the amount of songs sang. The model controlled for the differences in days birds were captured. Significance level was taken at  $\alpha=0.05$ .

### Results

Before experimentation, validation tests were done to show that 30 minutes post ACTH injection, cort levels would rise. In the ten birds used for validation, baseline cort (t=0) levels had a mean of  $9.23 \pm 1.88$  ng/mL. 30 minutes post injection cort levels showed an increase in mean to  $79.75 \pm 15.57$  ng/mL.

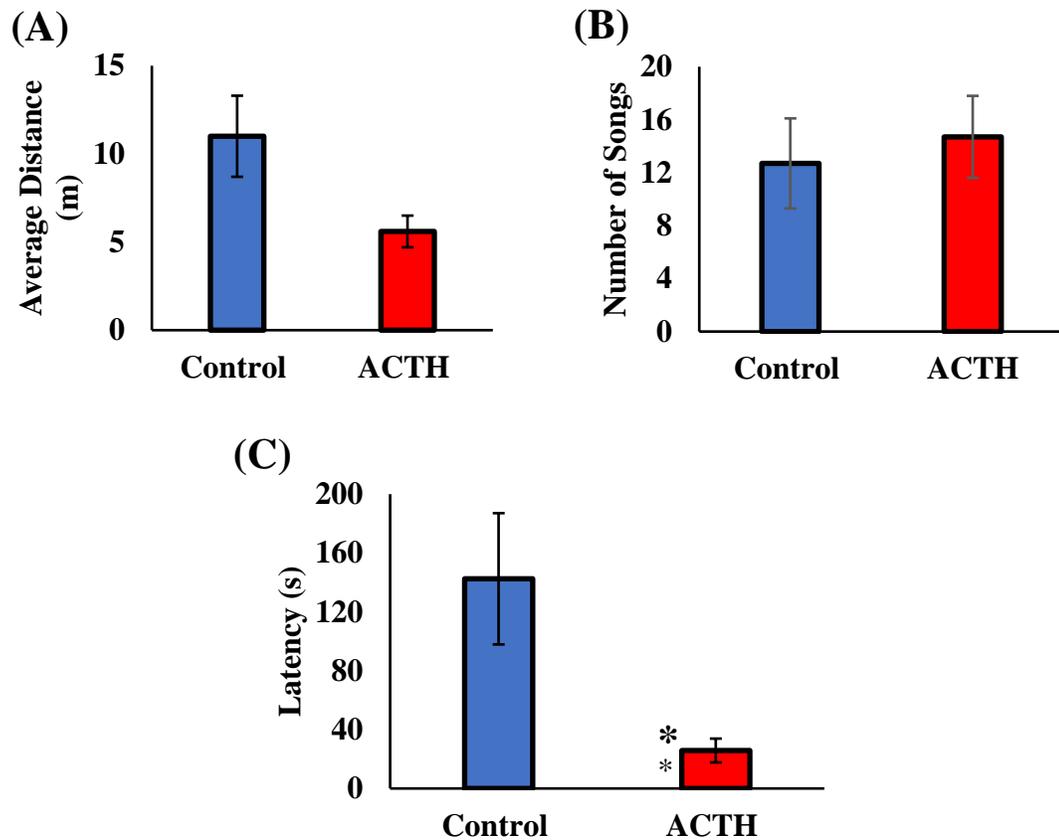
With respect to latency a significant difference was observed between the two groups. ACTH injected Chickadees took significantly less time to return to the nest following the start of the playback recording ( $p=0.0354$ , Table 1, Figure 2). Average distance was not significantly different between the two groups. However, it was close to being significant ( $p=0.064$ , Table 1, Figure 2). The number of songs sang back between the two groups did not show significant differences ( $p=0.7067$ , Table 1).



**Figure 2.** ACTH validation data. The figure shows the increase in cort from time 0 to 30 minutes post injection. All ten validation birds show an increase in cort concentration during the 30-minute waiting period.

**Table 1.** Model estimates to test effect of ACTH on aggression. Latency between the two groups was significantly different from one another. Number of songs did not show statistical difference between the group. While average distance did not show statistical significance, it was very close to being significant. All values calculated based on linear model created in R.

variable	estimate	s.e.	<i>t</i>	<i>p</i>
latency	-114.99	46.51	-2.47	0.0354
number of songs	1.69	4.35	0.39	0.7067
average distance	-5.29	2.51	-2.11	0.064



**Figure 3.** Bar graph of the means of the different factors used to determine aggression. Blue bars represent control groups while red indicate ACTH injected Chickadees. Panel A represents mean distance in meters the focal individual male was from the speaker during the six-minute trials. Panel B shows the average number of songs sung by individuals during the six-minute trials. Panel C shows latency or time taken to return to the nest in seconds. Of all three behaviors recorded only latency showed a statistical difference between the two groups.

## Discussion

Aggression is a crucial part of a bird's survival and reproductive success. By showing aggressive behaviors, a male will be able to protect his territory, his mate, and his offspring. While the reasons for being aggressive are well known, the mechanism behind what causes this aggressive response is not. Many have examined the role testosterone and the HPG axis play in aggression in birds as this is correlated with increased aggression in many organisms. However, in birds the results have been contradictory. Due to the contradictory results of these studies, we asked whether something else is responsible for controlling aggression. We hypothesized that the HPA axis played a role in controlling aggression and activating this axis by injecting ACTH into birds would cause an increase in aggressive behavior.

Our results suggest this axis may play a role in aggressive behavior. We found that latency, or return to nest time, was significantly reduced in ACTH injected Chickadees. It is possible that acute increases in circulating GCs cause the males to stay closer to territory or that they were more prone to respond when hearing an intruder's song. Our results contradict what has been reported in the literature. In most studies in which latency is used as a measure of aggression, results usually show that cort either causes an increase in latency time or has no effect at all. In a study on collard flycatchers a small correlation between latency and aggression was found but nothing of statistical significance (Garamszegi et al. 2012). An interesting fact about this study was the birds were not injected with cort at all. Instead, cort metabolites were determined from droppings in at natural concentrations. On the opposite end of the spectrum, Wingfield

and Silverin caused a chronic increase in cort by using implants and found that implanted individuals had a significantly increased latency time (1986). What our results suggest when compared to these two studies is that the amount of time exposed to high cort concentrations is very important for behavior. No spike in cort showed no correlation with latency. Too much for too long showed an increase in latency time while an acute spike in cort showed a significant decrease in latency time.

We also found that average distance ACTH injected birds were willing to come to the speaker was closer than controls despite not being significant. Our power to detect differences is small, so perhaps with increases in sample size, the average distance the focal male comes may change. However, our data showed no real difference in the number of songs sang between the two groups. While this failure to show a difference between number of songs sang may seem counterintuitive, it actually shows that there may be a connection between testosterone, corticosterone, and aggression.

Taken as a whole, these data, along with data from other studies, suggest a complex interplay between testosterone and corticosterone. Data from this study and from other studies suggests that some aggressive behaviors are controlled by corticosterone, while others are controlled by testosterone. Wingfield and Silverin found in a study on song sparrows that birds injected with corticosterone showed a significant reduction in circulating testosterone (1986). Then Ketterson et al. found that in bird's testosterone controls the vocal behavior of birds (1992). If these two claims hold true, they may help to explain why the data show no difference in number of songs.

Administration of ACTH would increase corticosterone concentration which could affect

testosterone levels accounting for the why there was no statistical difference between the number of songs sang by the two groups. Alternatively, but not mutually exclusive, singing and distance that an animal is to the intruder could be controlled separately by the HPG and HPA axes. Aggression is a suite of behaviors and is likely to be coordinated by various hormones. Overall, this study along with others have only begun to lay down the framework for understanding aggression in birds. Further studies should focus further on what affects the HPA axis has on aggression while also determining the complex interaction of cort and testosterone and their affects on aggression.

To some, studying the mechanism behind what causes aggression in birds may seem trivial or unimportant. However, this could not be farther from the truth. If we can gain a better understanding of what causes aggression and the mechanisms behind it, we can begin to better understand why birds may act in a certain way. Without understanding mechanisms behind behavioral changes, true understanding of an organism's complexities, evolutionary history, or how an organism fits within a niche cannot be fully gained. In studying and understanding mechanisms, the framework is laid for greater discovery. In knowing the mechanism behind what causes aggression in birds, the greater implications of a bird's endocrine evolution and how that affects its fitness can start to be investigated. Those are just a few examples of what can be discovered, but before we can gain an understanding of the bigger pictures, the building blocks of what determine different bodily processes must be understood fully.

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