The Effect of Corticosterone on Learning in Cuban Treefrog (*Osteopilus septentrionalis*) Tadpoles

Amelia Ayotte & Dr. Travis Wilcoxen

Senior Seminar

May 3, 2024

Abstract

Tadpoles can serve as model organisms for the developmental process of vertebrates that are in utero or in ovo at comparative developmental stages in their free-living larval state. The purpose of these experiments was to determine the effects of corticosteroid levels on learning in Cuban Treefrog (Osteopilus septentrionalis) tadpoles as a proxy for how elevated glucocorticoids during vertebrate development may influence learning. We completed three experiments. Experiment 1 explored differences in learning among individuals with acute and chronic elevations of corticosterone. Experiment 2 explored differences in learning among individuals exposed to high and low doses of exogenous corticosterone. Experiment 3 explored differences in learning among individuals with acute and chronic exposure to metyrapone, a glucocorticoid release blocker. For each experiment, groups of tadpoles were also distinguished between pseudoconditioned or true conditioned in the learning component of the experiment. After the conditioning period, learning behaviors were tested with a behavioral assay and data were analyzed through a two-way ANOVA. Overall, our findings indicate that having corticosterone reduced is detrimental for learning just like having increased levels of corticosterone or sustained low level elevations of corticosterone for too long negatively impacts learning.

Introduction

These experiments were designed to test the developmental effects of corticosterone on learning in tadpoles. Cuban Treefrog (*Osteopilus septentrionalis*) tadpoles were chosen for this experiment because their free-living state is developmentally similar to that of an early human fetus; and therefore, can serve as a model for glucocorticoid-learning dynamics associated with early development. For example, increased corticosteroids impact human birth timing by maturing the organs and getting the fetus ready to transition from an aquatic to a terrestrial environment (Crespi & Denver, 2005). The process is similar in the tadpoles. There have been many studies of the stress-response of amphibians after development. For example, one research study tested post-embryonic behavior in salamanders when exposed to stress in the embryonic stage of development. The researchers found that these organisms had lower activity levels and higher "shelter-seeking" tendencies than their non-stressed counterparts (Mathis et al, 2008).

The hormone used in these experiments was corticosterone (CORT). Corticosterone is a type of glucocorticoid hormone in amphibians. It is responsible for activating the sympathetic nervous system or what is better known as the "fight or flight" response. An outside stimulus is transmitted to the amygdala, which is responsible for processing emotions such as fear or arousal. The amygdala sends a signal to the hypothalamus which in turns causes the adrenal glands on top of the kidneys to release corticosterone (Thau et al., 2023). In amphibians, they have one gland comparable to the adrenal glands called the intrarenal gland. Glucocorticoids are known to increase available energy and regulate various behavioral processes (Romero, 2002). However, if an organism has too much glucocorticoids in their systems it can lead to muscle catabolism and decreased development (Sapolsky, 1993). For example, in Hyla regilla (Pacific Treefrog) increased corticosterone levels decreased growth (Belden et al, 2005). Corticosterone, specifically, is important for metamorphosis in amphibians (Belden et al, 2005). Corticosterone has also been found to alleviate stress-responses in amphibians. For example, in a study conducted by Gabor et al, it was found that, when exposed to contaminants that reduce immunity and growth, corticosterone is able to lessen the negative results (2018).

For the third experiment in this research, a corticosterone inhibitor hormone was utilized called metyrapone (MET). Metyrapone successfully inhibits corticosterone synthesis by hindering the conversion of 11-deoxycortisol to cortisol (Bader & Yehuda, 2016).

To test learning, the idea of classical conditioning was implemented. Classical conditioning is defined as the "process in which an automatic, conditioned response is paired with specific stimuli" (Rehman, 2023). This form of conditioning is often used to treat phobias, drug addictions, or other psychological disorders (Rehmen, 2023). We decided on this form of conditioning because it has been utilized successfully in previous studies. A similar method to a previous study tested various groups of tadpoles who were exposed to varying levels of threat. Fish were used as the predator instead of salamanders; however, the process will be similar. In the study, they found that the tadpoles exposed to higher levels of threat responded better to threat and retained that learning for a longer period of time (Ferrari, 2014). This helped us understand that tadpoles have an unconditioned response to the stress hormones of other injured tadpoles, making our research possible.

Using this knowledge, we wanted to test acute vs chronic learning, dosage-dependent learning, and blocked CORT learning. It was hypothesized that increased periods of exposure (chronic) to corticosterone, higher levels of corticosterone (high dosages), and increased periods of exposure (chronic) to metyrapone in pre-development tadpoles would result in hindered learning.

Methods & Materials

The experimental design and testing were consistent throughout all three experiments with slight differences to the amount of hormones administered during testing periods. To set up this experiment, a total of 150 tadpoles were used, split into 6 equal groups of 25 tadpoles each. The water used for the tanks had been taken from the sink and put aside to age for at least two days before adding the tadpoles. This aging process allows for chemicals such as chlorine and fluoride to dissipate from the water. The tadpoles were allowed a few days, once separated, to grow accustomed to their new environment, and they were fed weekly with spirulina algae discs.

For experiment 1, the groups were as follows: acute true conditioned, acute psuedoconditioned, chronic true conditioned, chronic psuedoconditioned, control true conditioned, and control psuedoconditioned. For experiment 2, the groups were as follows: high true conditioned, high psuedoconditioned, low true conditioned, low psuedoconditioned, control true conditioned, and true pseudoconditioned. The groups for experiment 3 were labeled the same as experiment 1 but were treated with MET rather than CORT.

The true conditioned groups were treated with both fish and injured tadpole scents, whereas the psuedoconditioned groups were only treated with the fish scent. The psuedoconditioned groups make it possible to rule out any innate behavior responses to the fish scent, which was created by having Creek Chubs (*Semotilus atromaculatus*) swim inside a 1 liter container of water for 30 minutes. To create the tadpole scent, a random selection of tadpoles were sacrificed and killed (30 tadpoles in 0.5 liters of water) so that they released chemokines associated with tissue injury and stress hormones. Conditioning periods lasted for 6 days leading up to the testing day. Ethanol was used as the control dose.

For experiments 1 and 3 subsequently, the acute groups were given 40 µL of corticosteroids / metyrapone only during the conditioned sessions. The chronic groups were given 40 µL of corticosteroids / metyrapone every day for 6 days leading up to and during the conditioned sessions. For experiment 2, the low groups were given 20 µL corticosteroids and 20 μL ethanol every day for 6 days leading up to and during the conditioning sessions. The high groups were given 40 μL corticosteroids every day for 6 days leading up to and during the conditioning sessions. All control groups did not receive any corticosteroids or metyrapone leading up to or during the conditioned sessions. No hormones or scents were given to any tadpoles on the actual test day.

For testing learning behavior in the tadpoles, each individual tadpole was separated into their own individual container and allowed to acclimate for 1-2 hours. On each container, there was a line along the bottom to separate the container in half. This line was used to measure the tadpole's learning behavior through counting the number of times the tadpole crossed the line before and after the fish scent was added. The difference of these values was recorded and evaluated to determine learning behavior. According to prior research findings, once exposed to a perceived threat, "decreased activity is a well-established antipredator response for tadpoles" (Ferrari, 2014). Tadpoles were observed in groups of four to increase efficiency. Tadpoles were observed for four minutes without any interference and the number of line crossings were recorded. Then, 2mL of fish scent were added to each container, and the tadpoles were observed for four additional minutes and their line crossings recorded. The difference in these values was measured and represented in Figure 1-3. A two-way ANOVA was used to conduct the statistical analyses.

Results

P-values of less than 0.05 are statistically significant.

There were significant differences in how the tadpoles responded to the fish scent stimulus among CORT groups ($F_{2, 144} = 14.41$, $p < 0.001$). Tadpoles in the control ($p = 0.034$)

and acute (p = 0.019) conditioned groups each demonstrated significantly less activity after exposure to the fish scent, compared to those in the pseudoconditioned groups. There was no significant difference in learning behavior within the chronic group.

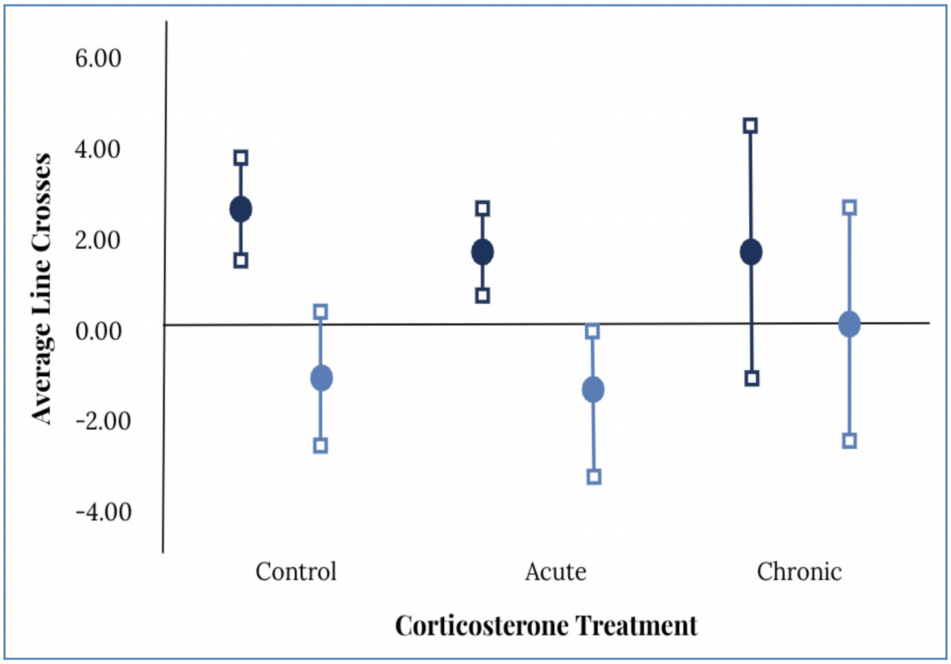

Figure 1: Mean line crossings of true conditioned compared to pseudoconditioned tadpoles when given control, acute, or chronic treatments of corticosterone for Experiment 1.

There was no significant difference among treatments in the high CORT group (p = 0.219). There were significant differences within the control group (p = 0.002) and the low CORT group (p = 0.034). The conditioned groups with no CORT (control) and low CORT each demonstrated significantly less activity after exposure to the fish scent, compared to those that were pseudoconditioned. There was no difference within the high CORT group.

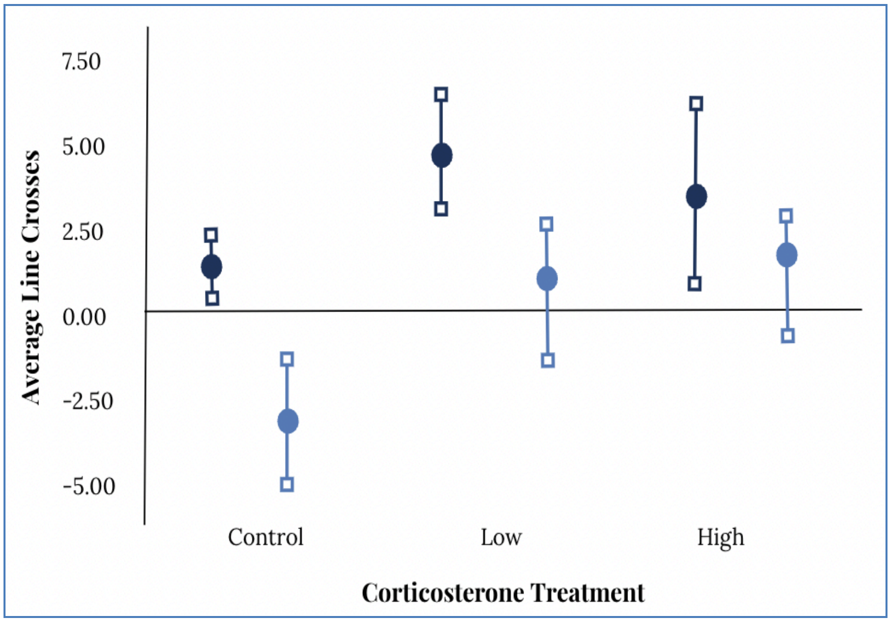

Figure 2: Mean line crossings of true conditioned compared to pseudoconditioned tadpoles when given control, low, or high treatments of corticosterone for Experiment 2.

The only significant difference was within the control group, where the no-metyrapone, conditioned tadpoles crossed significantly less after the stimulus ($p = 0.013$). There were no other differences ($p > 0.441$ in all cases).

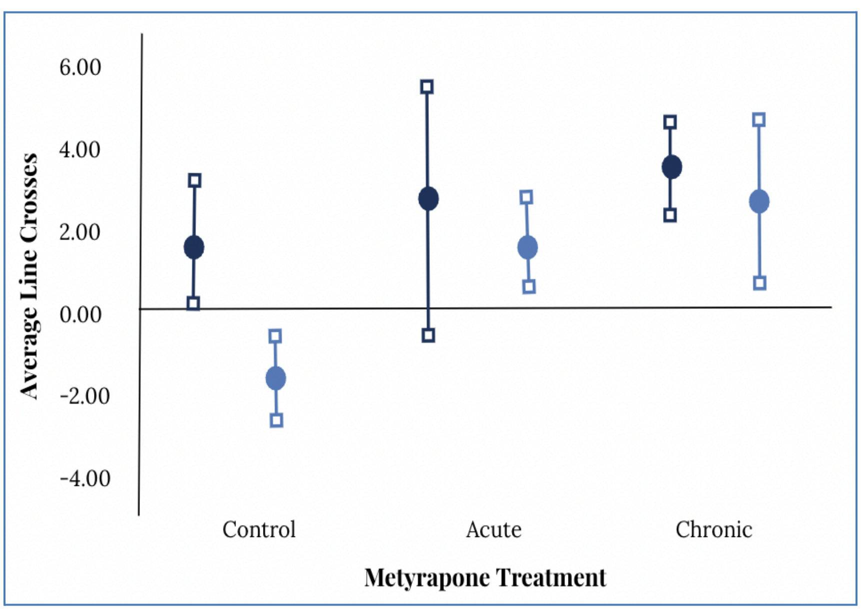

Figure 3: Mean line crossings of true conditioned compared to pseudoconditioned tadpoles when given control, acute, or chronic treatments of metyrapone for Experiment 3.

Discussion

The key findings of this research showed that there was no evidence of learning in the chronic group from Experiment 1, the high group from Experiment 2, or any CORT-blocked groups in Experiment 3. These results do suggest that learning via classical conditioning was disrupted. The acute group from Experiment 1 and low group from Experiment 2 showed the highest evidence of learning suggesting that learning is improved with lower doses of CORT or with shorter exposure periods. These findings support the first two hypotheses that increased periods of exposure (chronic) to corticosterone and higher levels of corticosterone (high dosages) would result in hindered learning. The final hypothesis that increased periods of exposure (chronic) to metyrapone in pre-development tadpoles would result in hindered learning was rejected due to there being no significant data supporting this conclusion.

The results of this experiment also suggest that without any experimental interference, the tadpoles have a natural learning ability to respond to stressors in their environment. For instance, this is demonstrated through the P=value of 0.034 in the control group of Experiment 1, indicating that the tadpoles had less activity, on average, after being treated with the fish scent than they did prior being treated with the scent, having successfully associated the smell of fish with the smell of injured or dying conspecifics. The acute group of Experiment 1 had similar findings. Their p-value was 0.019 demonstrated learning after being treated with the fish scent as well. However, the chronic group did not show evidence of learning behavior.

These findings can be compared to the findings from prior research studies. For instance, Sapolsky determined that increased levels of glucocorticoids can lead to a decrease in development (1993). This could explain why the tadpoles exposed to chronic stress did not respond to stress as well as their experimental counterparts. It could have been possible that due to the increased level of stress hormones, the chronic tadpoles did not develop at the same rate as the average tadpole. Further, the evidence from this experiment can support the findings from Ferrari's research. They found that tadpoles exposed to high levels of threat have a better learning response (Ferrari, 2014). This can be supported through the findings in the difference between the groups. In the first experiment, the acute group had a better learning response than that of the control group, most likely due to the help of the experimentally-administered hormone.

We were anticipating that the tadpoles would swim less in the latter half of the testing period; however, the majority of them swam more. It could be beneficial to repeat this experiment with environmentally-similar objects in the containers to see if the tadpoles would reproduce these results or seek shelter more often. It would also be interesting to look into long-term learning. To better the current experiment, using a different species of tadpoles could be beneficial as this specific species has been found to be resistant to stress (Wilcoxen et al. 2021). Using a more stress-resistant species may yield more conclusive results.

Overall, this experiment was able to expand on ideas and findings from previous experiments with similar research tactics and hypotheses. Further experimentations could help to develop more knowledge of the impact of corticosteroids and glucocorticoids on learning behavior.

Acknowledgments

I would like to thank Dr. Travis Wilcoxen for being a fantastic mentor and teacher throughout this process. His love for research and science has made this an incredible learning experience! I would also like to thank Millikin University, the Leighty-Tabor Research Scholarship, and Dr. Alicia Knudson for making this all possible through the scholarship and biology program.

References

Bader, H. N. & Yehuda, R. (2016). Neuroendocrine Aspects of Posttraumatic Stress Disorder. *ScienceDirect.* 4(3), 275-288. https://www.sciencedirect.com/science/article/abs/pii/B9780128035924000808

Belden, L. K., Moore, I. T., Wingfield, J. C., & Blaustein, A. R. (2005). Corticosterone and Growth in Pacific Treefrog (Hyla regilla) Tadpoles. Copeia, 2005(2), 424–430. http://www.jstor.org/stable/4098550

Crespi, E. J. & Denver, R. J. (2005). Ancient Origins of human developmental plasticity. A*merican Journal of Human Biology.* https://sites.lsa.umich.edu/denver-lab/wp-content/uploads/sites/67/2014/04/ajhb-17_44.pdf

Ferrari, M. (2014). Short-term environmental variation in predation risk leads to differential performance in predation-related cognitive function. *ScienceDirect*. https://www.sciencedirect.com/science/article/abs/pii/S0003347214002450

Ferrari, M., Manek, A. & Chivers, D. (2010) Temporal learning of predation risk by embryonic amphibians. *The Royal Society Publishing*, 6, 308-310. http://doi.org/10.1098/rsbl.2009.0798

Gabor, C. R., Knutie, S. A., Roznik, E. A., & Rohr, J. R. (2018). Are the adverse effects of stressors on amphibians mediated by their effects on stress hormones? *Oecologia*, 186(2), 393–404. https://www.jstor.org/stable/48718926

Mathis, A., Ferrari, M. C. O., Windel, N., Messier, F., & Chivers, D. P. (2008). Learning by embryos and the ghost of predation future. Proceedings: *Biological Sciences*, 275(1651), 2603–2607. http://www.jstor.org/stable/25249849

Rehman, I. (2023, August 14). Classical conditioning. StatPearls [Internet]. https://www.ncbi.nlm.nih.gov/books/NBK470326/

Romero, L.M. (2002) Seasonal changes in plasma glucocorticoid concentrations in free-living vertebrates. General and Comparative Endocrinol, 128,1-24. http://www.jstor.org/stable/4098550

Sapolsky, R. M. 1993. Neuroendocrinology of the stress-response. P. 287-324. *In*: Behavioral Endocrinology. J.B. Becker, S. M. Breedlove, and D. Crews (eds). MIT press, Cambridge, Massachusetts. http://www.jstor.org/stable/4098550

Thau, L., Gandhi, J., & Sandeep S. (2023). Physiology, Cortisol. *StatPearls*. https://www.ncbi.nlm.nih.gov/books/NBK538239/