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Response of Leukocyte Profiles to Corticosterone Manipulation in the Prairie Rattlesnake (Crotalus viridus)

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Abstract

Prior work has shown that when animals are stressed they have altered leukocyte profiles. The use of leukocyte profiles could provide an inexpensive and efficient method for determining stress. Here we present a validation of the use of leukocyte profiles for showing induced stress in Prairie Rattlesnakes (*Crotalus viridus*). Snakes (N=19) with masses ranging from 682g to 137g were used in a repeated measures design to examine the effect of hormone manipulation on leukocyte profiles. During each trial snakes were dosed with either corticosterone and sesame oil (1.1µg/g) or only sesame oil. Prior to dosing, baseline blood samples were collected to analyze a pre-dosing leukocyte count. Immediately after blood draw, snakes were placed into small chambers that would facilitate dosing located in an undisturbed room; 24 hours after baseline samples animals were dosed. The dosing mixture was left to be absorbed for 24 hours and a final blood sample was taken. Whole blood from each snake sample was used to create blood smears. Blood smears were stained with Wright-Geimsa stain. Slides were scored to determine the ratio of heterophils to lymphocytes. We found a significant affect of corticosterone on heterophil:lymphocyte ratios. Results of this study will be used in conjunction with plasma corticosterone assays to validate the use of leukocytes profiles for determining stress levels in pitvipers.

INTRODUCTION

One of the most vital aspects of life is the maintenance of homeostasis. When homeostasis is perturbed, an organism must adjust crucial factors in order to address the effector. The effects of stress on immune response and reproduction can be either beneficial or antagonistic (Lucas and French 2012). Organisms are sometimes forced to prioritize one physiological need over another. To achieve this prioritization the reallocation of resources may be necessary to ensure survival (French et. al. 2007). An animal undergoing chronic stress may have to alter the allocation to competing energy demands such as maintenance, growth and reproduction in favor of diverting energy to immune response. Corticosterone release can arbitrate trade-offs of physiological resources, making it an obvious candidate to study the impacts of stress on energy budgets (French et. al. 2007).

Corticosterone is a steroid hormone that shows a multifaceted affect on vertebrates. Corticosterone is released from the adrenal glands as glucocorticoid and its main function is the initiation of immune and stress responses. One of the body's physiological responses to stress is to upsurge the levels of plasma corticosterone (Davis and Maerz 2008). Acute upsurges of corticosterone prepare the body for a fight-or-flight response by raising blood pressure and temporarily inhibiting the release of insulin (thereby temporarily raising plasma glucose levels). Once the organism is able to overcome the stressful situation then the corticosterone concentrations decrease back down to baseline levels. Concentrations of corticosterone in plasma can be detected by enzyme-linked

immunosorbent assay (ELISA) and radioimmunoassay (RIA) (Abelson et. al. 2005). Alternatively, chronic stress can be measured by observances of body condition, yearly reproductive output, or immune function. Malnourishment or long-term starvation (poor body condition) can be an adequate indicator of perceived stress (Moore and Jessop 2003). An increase in skin lesions and other skin abnormalities can be an indicator that an animal is chronically stressed (French et. al. 2006). Animals that exhibit these physical qualities are often suffering from long-term depressions in immune function due to the observed stressor(s) (Morici et. al. 1997).

An alternative way of measuring the level of physiological stress levels in animals is through leukocyte profiling. Leukocyte profiling involves assessing the abundance of leukocytes (white blood cells) in blood samples. Typically five types of white blood cells are used for leukocyte counts but the most commonly used for indicating stress are neutrophils (heterophils in reptiles) and lymphocytes. Changes in the abundance of cell types are indicative of changes in stress levels; where increases in heterophils and decreases in lymphocytes signify increased stress levels (Davis 2008). Although, determination of corticosterone levels is an efficient tool to measure stress, leukocyte profiling is relatively inexpensive and easier to manipulate. For example, once blood is drawn from the animal only a light microscope is needed to analyze leukocytes. Analysis of corticosterone is expensive and involves investment in expensive equipment or sub-contracting blood to other labs for quantification. Corticosterone up-regulation also occurs within minutes while leukocyte mobilization can take hours; therefore leukocyte profiling may

allow us to better identify a stressor because the results are less impacted by handling stress (Davis et. al. 2008). The use of leukocyte profiling to understand chronic stress in animals can be done in both laboratory settings and in the field (Davis 2008).

Leukocyte profiling studies have been conducted in a variety of different taxa such as birds, mammals, salamanders and lizards (Davis et. al. 2008). As a technique, leukocyte profiling is easy to verify and can be quickly adapted to other taxa (Davis et. al. 2008). Much like how the adiposity of an animal can tell us whether the animal is able to allocate enough time and energy to feeding, the profiling of leukocytes and corticosterone levels may tell us the condition of their habitat.

Our research will use the Prairie Rattlesnake (*Crotalus viridus viridus*) to demonstrate the affects of corticosterone on leukocyte profiles. Pitvipers are informative models for the determination of environmental stress because they are significantly susceptible to changes throughout their ecosystems, while also maintaining relatively small habitat ranges, which make pitvipers ideal indicator organisms (Beaupre and Douglas 2009). Pitvipers have relatively small home ranges making them sensitive to environmental perturbations. A pitviper's relatively immediate response to environmental changes and consistent diet make them a model lab population species (Beaupre and Douglas 2012). Snakes have also been found to be good indicator species for community level responses. Due to their narrow range of predictability they function well as proxies for other organisms, which makes them idyllic organisms to study (Beaupre and Douglas 2009).

Our study is attempting to test whether or not leukocyte profile analysis can indicate a stress response in Prairie Rattlesnakes. The work conducted could aid in determining aspects of the snakes' physiological health. Chronic stress causes a decrease in snake immune response that often results in lesions or risk of pathogenic illness, such as hibernation sickness (Moore et. al. 2003). Long- term environmental decline may result in chronic stress and potentially a difference in reproduction (Moore and Jessop 2003). Determination of stress through corticosterone assay and leukocyte profile analysis may allow one to determine whether or not an organism is thriving in its environment. An increase in corticosterone levels or white blood cell presence would indicate that the organism is undergoing stress, possibly related to its environment. Therefore, an animal with high corticosterone levels or an increased H:L ratio, relative to a baseline, is likely stressed in its environment and forced to prioritize resources towards immune function which may have a negative influence on growth, reproduction and survival. Our work attempts to test whether or not leukocyte profile analyses are an efficient and accurate method of determining stress in pitvipers. We will use corticosterone, a stress hormone, and white blood cell counts as indicators of stress in a pitviper.

METHODS

Animal Maintenance

The snakes were maintained in individual cages, kept on a 12:12 cycle (light: dark), at 25˚C, given water *ad libitum*, and fed (at minimum) every four weeks. For our study, 19 snakes were used (eleven females and eight males). The snakes ranged in size from 138.7g to 682.8g.

During the course of the study we examined the leukocyte response of each animal to being dosed with either sesame seed oil or sesame seed oil and corticosterone. Corticosterone is a steroid hormone that can be applied topically and can pass through the skin into the blood stream (French et. al. 2007). Each trial lasted a total of three days and prior to dosing animals were fasted for at least 14 days. Between trials animals were allowed to rest for at least one month before being used again.

During each trial, snakes were randomly assigned to either treatment or control. Each snake received both a treatment and control dosage throughout two different trials.

Blood Sampling

On day one, the snakes were individually pulled from their cages to have their blood drawn and initial mass recorded. A timer was started when the snakes were first removed from their storage room and the handling time until completion of blood draw must be less than or equal to four minutes in order to avoid handling stress. After four minutes, the transportation or handling of the animal will cause a measurable increase in the hormonal stress response that can be detected through

leukocyte profile analysis (Davis et. al. 2008). Using tongs, snakes were placed into a plexi-glass squeeze-box to safely restrain them long enough for blood to be drawn. Blood was drawn from the caudal vein, which lies posterior to the cloaca (Bush and Smeller 1978). Approximately 0.5 to 0.6 ml of blood was drawn and placed into micro-centrifuge vials that were coated with EDTA, and placed on ice until smears could be made. After blood was drawn, each snake was weighed and put into a mock metabolic chamber. The chambers were stored on shelves and shielded by dark plastic to avoid any additional stress or arousal when we enter the room. Each chamber was connected to an air pump to simulate open flow respirometry (with a flow of ~500 ml/min). The chambers also allowed for quick dosing of the animals. The snakes were left undisturbed for at least twenty-four hours until dosing.

Whole blood was used for preparing slides (see below). The remaining blood samples were then micro-centrifuged to allow for plasma removal. The centrifuge was set on 13000 rpm for 20 minutes. After centrifugation, the plasma was drawn and placed in a separate micro-centrifuge vial and frozen. Plasma samples will be sent to an external lab for corticosterone analysis.

Dosing

Twenty- four hours after the initial blood draw animals were dosed with either a mixture of corticosterone and sesame seed oil (treatment) or only sesame oil (control) (Meylan et. al. 2009). To make both the control and treatment mixtures, two vials of 20 ml of sesame seed oil were pipetted out. The treatment solution was made up of 0.1g of corticosterone (Sigma) dissolved in 20 ml of oil. The hormone solution was shaken with a vortexer to ensure that the corticosterone dissolved.

Each snake, regardless of treatment type, was given a dose of 1.1 µl/g. To administer the control or treatment dose a luer lock syringe with a 1/16" hose barb and a section of 1/16" tubing was used. A micropipette was used to fill the tubing with the exact dose that was to be spread on the snake. The dose was applied approximately 24 hours after each snake's blood was drawn. The tubes connecting the snake chambers to the air pump were removed and the dosing tube was placed through the port on the mock metabolic chamber. Care was taken to ensure the tip of the tubing was touching the snake so that the snake will come into direct contact with the dosed material. Once the tubing was on top of the snake, the syringe was slowly emptied. The luer lock syringes were fully emptied into the tube three times to ensure adequate removal of the oil from the tubing. Even if the oil or oil mixture was not directly applied to an animal, the small size of the chamber and the snake's movements ensured that the animal would come into contact with the dosed material. After dosing was completed, the chambered snake was returned back to the shelving and reattached to the air pump. A final blood sample was taken 24 hours after dosing for comparison to the initial blood sample.

Slide Prep

Whole blood was used to make blood smear slides for leukocyte profiling. Each blood sample was gently mixed and placed onto slides as a single drop ranging from 40 to 80 microliters. The drop of blood was carefully pushed across the slide with an extra slide in order to create blood smears that were only one cell layer thick. At least two slides for each animal were made to prevent loss of data due to staining errors. Wright-Giemsa stain (1.0 ml) was then applied to each slide using a

pipette. The stain was left on the slide for four minutes and then 2.0 ml of distilled water was dropped evenly over the surface of the slide. The distilled water was given eight minutes to set. The remaining stain was then washed off the slides using distilled water. The slides were left upright to dry overnight.

Slide Scoring

Although multiple copies of each slide were made, only one slide from each snake was scored. A Leica DM750 light microscope and Leica Acquire program (version 2.0) were used to analyze the slides. The magnification used was the 100x/1.25 oil emersion. A slide cover was placed over the slide and one drop of oil was placed onto the center of the cover. Non-overlapping fields were counted and assessed until 100 leukocytes were found (Davis and Maerz 2008). The leukocytes that were counted included monocytes, eosinophils, basophils, lymphocytes and neutrophils (Davis 2008). Image J (NIH) was used to keep a running tally of each detected cell type.

Data Analysis

A repeated measures design was used for this study. We used a Kruskal-Wallis Non-Parametric ANOVA to test for differences between two variables. The variables compared were the ratio of lymphocytes to heterophils before and after dosing. Analysis was conducted using R software (R development Core Team 2014). The assumptions of a parametric ANOVA are that the data are normally distributed, variance is homogenous, and samples are independent. Our data did not meet the assumptions of a parametric ANOVA due to our data being based on ranked counts and the abundance of zeros in the data set. A non-parametric ANOVA is less robust

than parametric ANOVAs because they are not confined by assumptions of normality. Other data analysis methods, like chi-square, would have been appropriate if the data were normally distributed and had fewer zeros.

RESULTS

In total, 152 slides were scored. Figure 1 (Appendix) shows the increase in H:L ratios between pre- and post-treatment groups. The X- axis shows the H:L ratio between heterophils: lymphocytes and the Y-axis shows the frequency of the H:L ratios found in each treatment. The high number of zeros in the control group result from the absence of heterophils in the sample in relation to number of lymphocytes. In the treatment group we saw an increase in heterophils and decrease in lymphocytes resulting in the increase in H:L ratio. The results of the Kruskal- Wallis Non-Parametric ANOVA showed a significant effect from the application of corticosterone on H:L ratios between the control and treatment ($H = 6.6825$, p-value $= 0.009736$, $df= 1$). Table 1 (Appendix) provides the mean, SD, and range for all cell types counted for both the pre-and post-dosing leukocyte counts.

DISCUSSION

Graphic examination (Figure 1) indicates an increase in the ratio of lymphocytes to heterophils from the pre- treatment to post-treatment blood draw. Coupled with the results of the Kruskal- Wallis Non-Parametric ANOVA, which showed a significant effect from the application of corticosterone. We can say that the manipulation of leukocyte profiles appears to be an accurate measurement for

determining stress in pitvipers. Despite the lack of the quantification of plasma corticosterone levels we still found a significant effect of manipulating corticosterone on H:L ratios. Improvements to the study include increasing the sample size, having balanced sex ratios and a wider array of animal sizes. Discussion of all of the work conducted may have to be re-evaluated once the corticosterone plasma assays are received from the external lab. The validation of this technique for other taxa, (especially reptiles and amphibians) will allow for accurate and cost effective determination of chronic stress.

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APPENDIX

Figure 1: A graphical examination indicating the difference (post-treatment – pre-treatment) in H:L ratios between the control and treatment groups. The X- axis shows the H:L ratio between heterophils: lymphocytes and the Y-axis shows the frequency of the H:L ratios found in each treatment. The high number of zeros in the control group result from the absence of heterophils in the sample in relation to number of lymphocytes. Negative values occurred because of the presence of more leukocytes in the pre-treatment than the post-treatment. In the treatment group we saw an increase in heterophils and decrease in lymphocytes resulting in the increase in H:L ratio.

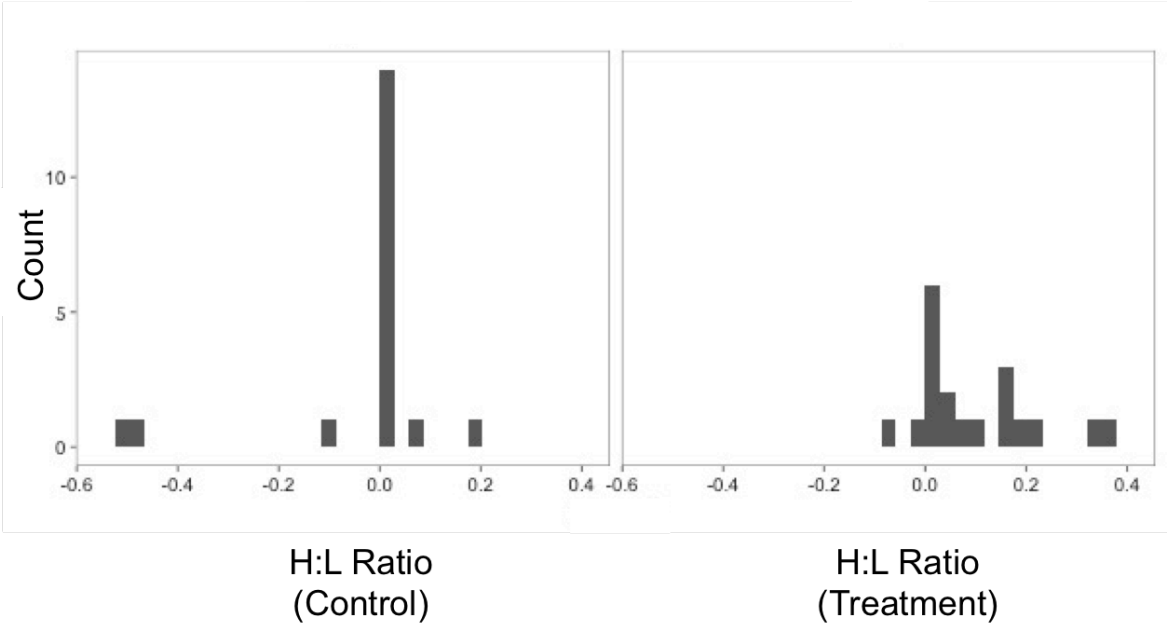

Table 1: Summary of pre and post-dosing leukocyte profile analyses and the comparison of treatment and control. Columns are the five different types of leukocytes recorded. Rows are the mean, standard deviation and range (maximum- minimum) of each leukocyte type.

Pre-Dosing		Monocytes	Eosinophils	Basophils	Lymphocytes	Heterophils
Mean	Treatment	18.89	0	0.56	78.74	1.94
	Control	22.05	0	0.83	70.53	3.22
Standard Deviation	Treatment	15.6	0	1.04	16.84	3.65
	Control	22.93	0	1.82	27.57	7.21
Post Dosing						
Mean	Treatment	17.53	0	0.06	76.48	6.28
	Control	12.84	0.05	0.17	85.79	1.33
Standard Deviation	Treatment	20.59	0	0.24	25.05	6.05
	Control	14.78	0.23	0.38	16.81	2.38