Biochemical Markers for Thermal Stress in North American Pikas (*Ochotona princeps)*

Submitted in partial fulfillment of the requirements for graduation with honors from the Department of Natural Sciences at Carroll College, Helena, MT

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April, 2014

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April 1, 2014

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Abstract

North American pikas (*Ochotona princeps)* are a high altitude keystone species that are indicative of that ecosystem's condition. Over the last twenty years, numerous populations of pikas have declined or disappeared. Because pikas are exceptionally sensitive to high ambient temperatures, it has been suggested that these declines are due to thermal stress imposed by climate change. Thermal stress has been shown to cause oxidative stress through an increased cellular concentration of oxygen radicals. Therefore, levels of oxidative stress markers are strong indicators of thermal stress. In order to quantify the degree of thermal stress placed on pika populations, fecal and plasma samples were assayed for a number of biochemical markers over a range of altitudes and ambient temperatures. Fecal corticosterone levels were assayed as a marker for long-term stress. Oxidative stress markers, including thiobarbituric acid reactive substances (TBARS), 8-hydroxy-2-deoxy guanosine (8-OH-dG) and glutathione peroxidase (GPX), were used to assess disparities in oxidative stress among pika populations. Results show low elevation pikas to have higher corticosterone levels in early summer when compared to late summer but no other correlations have been found. More sampling and testing are required to determine if other relationships exist.

Introduction

North American pikas (*Ochotona princeps)* are a keystone species that are indicative of ecosystem conditions and the well being of other high altitude species (Beever et al., 2003). Over the last 20 years studies have shown that pikas are vulnerable to heat stress with the disappearance of populations in Utah, Nevada, and Idaho (Beever et al., 2003, Rodhouse et al., 2010), Franken and Hik 2004). Furthermore, studies have

shown dramatic decreases in pika populations in historically heavily populated areas (Rodhouse et al., 2010). Locally, there has also been a population decline in Montana (Beever et al., 2003).

The habitat of the North American pika is comprised of talus fields in high altitude ecosystems (Sheafor 2003). Physiological and behavioral limitation of this environment include a decreased level of available oxygen and the restricted capability of long-range travel or migration, due to discontinuous habitable terrain (Sheafor 2003). To survive in an environment with less oxygen, pikas have evolved an extremely high metabolism by altering many enzymatic pathways for prime efficiency and sustainability (Sheafor 2003). Consequently, these same adaptations of increased metabolism may make pikas more susceptible to toxic metabolic byproducts. Therefore, discontinuous habitats may result in pikas being unable to evade regional climate changes when conditions become adverse for extended periods of time, which may leave them exposed to thermal stress.

Exposure to thermal stress causes oxidative stress (Jones 2008). Oxidative stress occurs when oxygen radicals bind to cellular components resulting in macromolecule damage and the alteration of cell signaling pathways (Jones 2008) and occurs when organisms produce reactive oxygen species (ROS) at a higher rate than the body can detoxify them (Grune and Berger 2007). ROS's are oxygen atoms that possess unpaired electrons that are highly reactive and allow the atom to freely bind to cellular components such as hemoglobin, lipids, proteins, and DNA (Jones 2008). Once bound to substances within the cell, the oxygen radical alters the functionality of these components and modifies them into a dysfunctional form (Jones 2008). Even under ideal conditions the

cell will produce limited quantities of oxygen radicals as a byproduct of normal cellular metabolism (Jones 2008). The problem arises when stresses, including thermal stress, cause the rate of cellular metabolism to rise creating higher concentrations of oxygen radicals within the organism and therefore resulting in oxidative stress. This effect is compounded in organisms, such as the pika, that already possess an elevated metabolism. This increased concentration of oxygen radicals exceeds the organism's ability to adequately neutralize the radicals before they can bind to cellular components causing harm (Jones 2008). Although organisms possess regulatory mechanisms to protect against such strain, when exposed to adverse conditions for extended periods of time the organism fails to maintain equilibrium (Jones 2008). Defenses include scavenging enzymes, most notably superoxidase dismutase and scavenging chemicals known as micronutrients, such as vitamin C and vitamin E (Jones 2008).

Since climate change may occur more rapidly than populations can adapt, physiological stress should be more prevalent in individuals that live in marginal biomes (Beever et al., 2008). However, the majority of previous studies on pikas have come from high altitude alpine environments (Rodhouse et al., 2010). In order to fully understand potential factors affecting pikas a thorough examination of populations over a spectrum of elevations is required. By measuring oxidative stress markers and general stress hormones in plasma and fecal samples over an elevation gradient, a better understanding of how climate affects pikas might be made. Increased global temperatures caused by climate change, may be a driving force behind the recession of pikas (Beaver et al., 2003). Increased temperature may amplify potential complications of thermal and oxidative stress (Giuseppina et al., 2011).

Objectives:

Glycosylated hemoglobin is a viable marker of oxidative stress in humans (Cahill et al., 2013) and is an ideal test because it can be measured by the BayarA1CNow® Selfcheck kit. Therefore, my first objective was to measure the percentage of glycosylated hemoglobin as the primary protein marker.

Glutathione peroxidase (GPX) is a good indicator of oxidative stress because it is a scavenging enzyme responsible for defending cellular components from oxidative stress damage by degrading any components of the cell affected by oxidative stress (Forstrom et al., 1978). This assay is a good indicator because laboratory protocols for detecting GPX are very accurate (Grune and Berger 2007). My second objective was to measure GPX levels in plasma. GPX was measured using Glutathione Peroxidase Assay Kit from Cayman Chemical, item number 703102.

The mRNA sequence coding for the GPX enzyme is called 8-hydroxyguanosine (Wu et al., 2004). The presence of 8-hydroxyguanosine would infer that GPX is being produced; therefore suggesting oxidative stress had occurred. My third objective was to measure plasma levels of 8-hydroxyguanosine. It was measured using the DNA/RNA Oxidative Damage enzyme immunoassay (EIA) Kit from Cayman Chemical item number 589320.

Malondialdehyde (MDA) is a lipid marker found in plasma and is commonly associated with organisms experiencing oxidative stress. (Goulart et al. 2005). My fourth objective was to measure levels of MDA. It was measured using the TBARS

(Thiobarbituric Acid Reactive Substances) Assay Kit from Cayman Chemical, item number 10009055.

Corticosterone is a long-term stress hormone released by the cortex of the adrenal glands (Vázquez-Palacios et al., 2001). Mammals under stressful conditions for extended periods of time will express increased levels of corticosterone (Vázquez-Palacios et al., 2001). This test is most ideal because it uses fecal samples. The benefits to this include being able to collect samples with out trapping a pika and not causing additional stress. My fifth objective was to measure corticosterone levels. It was measured using the Corticosterone EIA kit from Cayman Chemical, item number 500655.

The final objective was to gain knowledge of possible trapping sites in Montana over a gradation of altitudes, to refine trapping and processing techniques, and find viable assays that could be used for future studies.

In the present study I hypothesize that thermal stress and oxidative stress may be leading factors in the reduction of pika populations. By using a combination of biological markers including proteins, nucleotides, hormones, enzymes and lipids in conjunction with one another, a better understanding of thermal stress within pika populations might be gained.

Materials and Methods

Locations:

Information on pika locations was obtained from Global Information Systems (GIS) file from the April Craighead of Craighead Institute in Bozeman, Montana and based on the previous knowledge of Dr. Brandon A. Sheafor and Stuart W. Allyn.

Trapping:

Fifteen Havahart® 1088 Collapsible Animal Cage Trap's sized 24x8x8 were used along with thirty Sherman Folding Vole Traps. Bait consisted on slices of Fuji Apples. Traps were set up by 4:00 PM and checked periodically until dark when they were closed. The following morning traps were reopened at daybreak and left open until early afternoon.

Processing:

Fecal samples were collected from latrines and from droppings in the traps. Captured pikas were transferred from the trap into a pillowcase to make the specimen easier to handle and process. Pikas were then sedated with the anesthetic isoflurane. Anesthetics were beneficial in making pikas easier to handle and by decreasing stress from the trapping and processing procedures and were obtained via Matt Blanford of Apex Veterinary Clinic. Anesthetics were administered by soaking part of cotton ball with isoflurane and placing the swab over the pika's mouth until respiration had slowed and physical resistance had decreased to a point where the specimens were easy to handle. Anesthetics were reapplied as deemed necessary.

Once sedated, pikas were weighed and a toenail was clipped with toenail clippers. Blood from the toenail was collected in heparinized capillary tubes and sealed. Toenail clips provided a quick and effective way of collecting blood samples. Once enough blood had been extracted, baking flour was used to clot the bleeding. After bleeding had stopped and the pika had recovered from the anesthetic, it was returned to the area it had been trapped and released. Some whole blood was set-aside for Stuart Allyn's project

and some was used in the A1C test. The remainder of the blood was centrifuged and serum was separated.

Sample Preservation:

Blood and fecal samples were placed on dry ice until they were returned to the laboratory and placed in a -80°C freezer. Fecal samples were lyophilized to remove any water. An 80% solution of methanol was used to extract corticosterone. The methanol was then evaporated under a stream of nitrogen gas and samples were reconstituted in EIA Buffer from Cayman Chemical item number 400060.

Data Collection:

Spectroscopy was used to assess samples in all tests except the BayarA1CNow® Selfcheck. The protocol included in the BayarA1CNow® Selfcheck, Glutathione Peroxidase Assay, DNA/RNA Oxidative Damage EIA, TBARS Assay, and Corticosterone EIA was followed.

Controls:

Laboratory rats were used as a control. The heat shocked rat was placed in an incubator at 39 degrees Celcius with all necessary provisions for a period of 7 days before blood was extracted. The normal mouse remained at room temperature in the week before blood was extracted.

Results

Data were analyzed by using a T-test to determine whether molecular results were influenced by high verses low elevation. A T-test was also used to detect differences between early and late samples. Early samples were defined as any sample collected

from May 1st through July 15th, conversely late samples were those collected from July 16th through August 30th. Low elevation sites were defined as those below 2,399 meters above sea level and any site at 2,400 meters or higher were defined as a high elevation site.

MDA assay results used for these T -tests can be found in Table 1. A T-test showed there was no significant difference of MDA levels between high and low elevations ($p= 0.29$). Due to the unusually high value found in the Elkhorn Peak sample, another T-test without this sample was run to determine if this sample had skewed the results. However, no effect of elevation was detected even after the Elkhorn Peak value had been omitted ($p=0.30$). It was also found that there was no significant difference between early and late samples ($p=0.26$). This again was observed when the Elkhorn Peak sample had been omitted ($p=0.34$).

(Table 1).

T-tests were used to assess Corticosterone levels in fecal samples. Tests showed no significant difference between high and low elevation ($p=0.32$). A test of early high elevation samples verses late high elevation samples was not possible because no data for early high elevation samples were collected. A test of late samples between high and low elevations showed no significant difference between the two ($p=0.33$). Low elevation samples collected early and late showed a significant difference ($p=0.03$, Fig1). Tests also showed a significant difference between low elevation and mice samples ($p<0.05$, $p=0.04$) and no difference between high elevation and mice samples ($p>0.05$, $p=0.09$). Corticosterone assay results used for T-tests can be found in Table 2.

The BayarA1CNow® Selfcheck kit proved to be an unreliable the field assessment and was unable to produce any viable results.

Table 1. MDA levels as related to sites by date.

Sample	Malondialdehyde (MDA) Levels Elevation (m)	MDA ng/ml
High Elevation		
Early		
Red Lodge	2,846	18.22
Late		
Red Lodge 1	2,846	9.89
Red Lodge 2	2,846	19.37
Elkhorn Peak*	2,814	174.3
Low Elevation		
Early		
Lincoln	1,989	13.05
Late		
Dear Lodge	2,088	25.69
Controls		
Normal Rat	1,230	10.75
Heat Shock Rat	1,230	28.28

Note: The Elkhorn Peak sample with * appeared in poor condition at time of capture. Special considerations were used in evaluating this data point.

Figure 1.

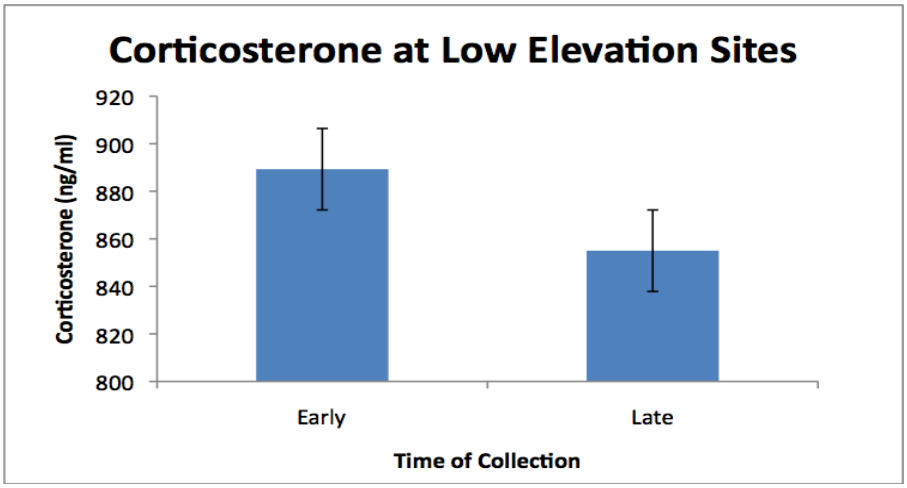

Fig 1. Columns show mean levels of Corticosterone found in fecal samples. Error bars depict standard error.

Table 2. Corticosterone levels related to sites by date.

Sample	Corticosterone Levels Elevation (m)	ng/ml
High Elevation		
Late		
Elkhorn	2,814	857.1
Red Lodge #1	2,846	926.5
Red Lodge #2	2,846	818.3
Low Elevation		
Early		
Deer Lodge #1	2,088	880.5
Deer Lodge #2	2,088	897.8
Lincoln #1	1,989	921.0
Lincoln #2	1,989	897.8
Late		
Deer Lodge#1	2,088	820.7
Deer Lodge#2	2,088	887.2
Deer Lodge #3	2,088	857.1
Controls		
Mouse1	1,230	916.9
Mouse 2	1,230	918.3

Discussion

MDA results show no variation in stress exposure when analyzed over an elevation gradient. Due to the limited number of samples it is difficult to fully assess differences between high and low elevation. The sample from Elkhorn Peak could be an exceptionally valuable sample. At the time of collection this pika appeared to be in poor condition. While the cause of this condition is unknown, it appears that when organisms are subjected to circumstances causing stress, be it pathogens, predation, climate based, or other, there may be a visible change in their physical appearance, as well as a dramatic physiological response occurring on the molecular level (Jones 2008). Our mice samples obtained in the lab as a control suggests that heat may cause fluctuation in MDA levels. Also, low elevation samples, while not statistically significant, may suggest an increase over the summer. More samples are needed to thoroughly understand this relationship. We conclude that MDA may be a viable method to measure oxidative stress in pikas. But my observations, which may in fact be true, are based on one sample.

Measuring fecal corticosterone levels was shown to be a viable method in Striped Field Mice (*Apodemus agrarius*: Wang et al., 2011). Although the Wang et al. study demonstrated that fecal samples are a practical method in a laboratory setting, their findings may not translate into the field because of the additional environmental factors influencing corticosterone degradation. The decision to collect fecal sample as a means of obtaining more data was beneficial, although it is now clear that measuring corticosterone levels in fecal matter may not be the best approach to accurately quantifying this hormone in a field setting. It is possible that the freshness or age of the

sample could influence corticosterone levels through degradation. Other factors that may influence hormonal degradation might include sunlight exposure, temperature, and moisture. All of these variables fluctuate dramatically between each latrine, possibly introducing undetectable variables, causing too much inconsistency to accurately determine any sort of relationship in regards to elevation or time. My data may suggest that pikas are under higher stress levels early in the summer months. This contradicts my initial prediction. It could be that other factors such as limited vegetation growth during the spring, breeding related stresses, or other alternative stresses have a more pronounced influence early in the summer and that they might mask any stress definitively caused by thermal forces.

Besides the environmental variables that may have influenced the out come of this study, trapping and processing techniques dramatically affected our productivity and efficiency in the field. Due to the preference of pikas for high elevation habitats, the process of scouting out viable and potentially productive sites at lower altitudes was a time consuming task. High elevation provided another challenge because access to these sites was impeded due to snow pack. Both of these factors consumed vast amounts of time in the early portion of the trapping season and therefore resulted in fewer samples.

There was also a large learning curve to understanding where to place traps, how to set them up properly, and how to properly camouflage them to optimize the chances of catching a pika. This was evident when analyzing the number of late samples compared to the early samples. It is clear that as we spent more time in the field our trapping and processing techniques became refined and our efficiency increased. This was also aided by implementing the Sherman vole traps. These traps were smaller in size allowing them

to be placed in areas more conducive to pika activity and made them easier to conceal. The Sherman traps also have solid aluminum walls and flooring whereas the Havahart® traps, have one inch by one inch steel mesh. This attribute of the Sherman traps may simulate a pikas naturally space constricted habitat. Their reduced size and weight also allowed us to transport more traps into trapping sites.

The BayarA1CNow® Selfcheck kit was unreliable in a field setting. Samples were tested immediately in the field because this test required fresh whole blood. Two potential causes of this unreliability include environmental variables such as temperature and wind or transportation to and from the trapping sites, resulting in inconsistent results. It is also possible that the BayarA1CNow® Selfcheck kit is not compatible with pika blood. The latter is less likely because hemoglobin is a highly conserved protein (Weed et al. 1963) and the assay worked in a lab setting when mouse blood was used.

Thus, I conclude that the MDA assay could be a useful test with larger sample sizes. Using this assay in future studies could provide direct insight to oxidative stress within pika populations. Corticosterone assays might still be applicable to this study if the assay were to be run on plasma samples instead of fecal samples. Glycosylated hemoglobin appears to be an unreliable measure of oxidative stress. Knowing which tests are most effective at measuring oxidative stress is beneficial because it allows further studies to focus efforts on collecting plasma samples, instead of fecal samples. Also knowing which areas are best to trap will be beneficial to beginning trapping earlier in the summer which could result in a more complete data set. Further studies must be done to fully assess the effects of oxidative stress in pikas.

Acknowledgements

This work was made possible through the efforts of many people. I would like to thanks Dr. Brandon A. Sheafor, my thesis advisor, and Stuart Allyn for their continual support, guidance, and encouragement. Thanks to Dr. G. F. Shields for his help in the writing process as well as Dr. Grant Hokit and Dr. Ron Wilde for taking on the role of faculty readers. This project was funded by The Murdock Charitable Trust and The Cargill Conservation Grant.

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