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Using Glycosylated Hemoglobin and Heat Shock Protein 70 as Thermal Biomarkers in North American Pikas (*Ochotona princeps*)

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May 2015

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Abstract

Pikas (*Ochotona princeps*), high altitude lagomorphs, are potentially one of the first mammals to be directly affected by global warming. Population decline has been observed in pika populations in Nevada and California. The cause of their decline is unknown but several studies suggest that heat stress, especially at lower altitudes, is a contributing factor. In Montana, populations are potentially stressed in the same way. One hypothesis is that direct thermal stress is causing population decline. This study looks at heat shock protein 70 (HSP70) and glycosylated hemoglobin levels in high and low altitude pika populations as biomarkers for thermal stress. Pikas were trapped from Gold Creek (1962m) and Vista Point (2832m) Montana. Blood was extracted and separated into plasma and packed blood cells. ELISA high sensitivity tests were used for HSP70 quantification. Chromatography techniques, using m-animophenylboronic beaded gels, were used to measure the percent of glycosylated hemoglobin. A strong positive correlation was observed between HSP70 levels and weekly average temperatures prior to capture. Due to the inconsistent nature of the glycosylated hemoglobin results, these assays are unable to support oxidative stress as a factor caused by direct thermal stress. Future work includes attaining a larger sample size, locating more trapping sites, further temperature data collection, and refining techniques to assay for glycosylated hemoglobin before definitive conclusions can be made.

Introduction

Background

High altitudes provide a stressful environment for some species. Low barometric pressure, high wind, lack of shelter, or high UV exposure are a few of the harsh conditions that could create this kind of stressful environment. A native lagomorph species, North American pikas (*Ochotona princeps*) may be struggling with these stressful conditions (Beever *et al.*, 2003, Sheafor, 2003). Pikas live in talus fields above tree line throughout a variety of altitudes, anywhere from 900m to 3700m (Beever *et al.*, 2010). Food can be scarce and environmental conditions can be harsh. Adaptations to this environment may require key physiological characteristics that are necessary to sustain life.

Pikas living in these conditions struggle to maintain cool body temperature, especially during summer months (Beever *et al.*, 2003). To aid in internal cooling, pikas rely on cooler ambient temperatures, high altitudes, and shelter from the sun by seeking shelter below rocks (Beever *et al.*, 2010, Feder and Hofmann, 1999). Although shelter can provide protection, pikas are still forced to emerge from beneath the talus fields to forage for food (Beever *et al.*, 2010). This emersion from the rocks exposes them to various elements, specifically UV rays, sunlight, and heat (Blasiak *et al.*, 2014, Feder and Hofmann, 1999). The more time pikas spend in direct sunlight, the more they struggle to keep cool (Beever *et al.*, 2003, Feder and Hofmann, 1999).

Climate Change

Global increase in temperature may be directly impacting pikas (Beever *et al.*, 2010). Global temperatures have increased 0.6°C in the past decade (Krajick, 2004). Recent data suggest a strong correlation between seasonal temperature data and pika behaviors such as earlier mating, changes in eating habits, and altered daily activity periods (Beever *et al.*, 2010). In

addition, pika populations in the Great Basin (1994-1999 and 2003-2007) and California (2015) have declined according to previous work (Beever *et al.*, 2003, Stewart *et al.*, 2015). Therefore, warmer climates may be negatively affecting pika populations (Beever *et al.*, 2010).

Typically, higher elevations have cooler climates. The montane habitats where pikas live tend to follow a -0.5°C change in temperature with every 100m of altitude gain (Krajick, 2004). Due to this temperature gradient, pikas may be forced to move to higher altitudes in search of less heat stressed environments; this phenomenon is known as the escalator effect (Tobin, 2013). However, high altitude environments are limited in size (Krajick, 2004). Warmer temperatures may force lower elevation pika populations to deal with stressfully high ambient temperatures.

Warmer ambient conditions may cause North American pikas to change behavior or decrease in population size for three possible reasons (Beever *et al.*, 2003). First, decreasing levels of winter snowfall may create less snowpack in the winter that would otherwise give pikas more insulation. This may contribute to increased heat stress or acute cold stress (Beever *et al.*, 2010, Tobin, 2013). Second, changing climate conditions drastically change plant communities, thus negatively impact pika food sources. For example, pikas rely on specific plants that have high levels of phenolic compounds to prevent bacterial growth in their hay pile stores, which contributes to longer food storage in winter months (Dearing, 1997). A change in such food may cause a change in specific pika populations' range and size (Beever *et al.*, 2003, Rodhouse *et al.*, 2010). Lastly, direct thermal stress is a potential physiological explanation for the recent decline in pika populations, both in size and in location range (Beever *et al.*, 2003, Ostling *et al.*, 2007).

Ambient temperatures up to 25.5°C may cause pika deaths (Rodhouse *et al.*, 2010). Pika populations struggle more in warmer conditions found at low altitudes, rather than in cooler high altitude climates as observed in the Great Basin (Beever *et al.*, 2010). The present study

investigates the physiological effects of high and low altitudes on North American pika populations and how the ambient temperatures in those environments might be affecting physiological stress levels.

Physiological Research

Heat shock proteins (HSP) are chaperone molecules that aid in refolding cellular proteins under stressful conditions. Without HSPs, proteins may denature, thus killing the cells and potentially the organism (Haley-Vincent, 2008). Within a cell, more than one type of HSP may work together to complete the refolding process (Haley-Vicente, 2008). One specific HSP molecule commonly found in mammals is HSP70 (Haley-Vicente, 2008).

HSP70 is inducible by heat stress, oxidative stress, and UV exposure (Haley-Vicente, 2008). HSP70 is effective only at certain temperatures; if a cell is over heated beyond the point where HSPs can be effective in refolding proteins, the cell may expire through apoptosis (Pirkkala *et al.*, 2001). In a cell that is heat shocked, the heat shock cognate (HSC; cognate associated with HSP70) is up regulated and becomes active to its inducible form of HSP70 (Haley-Vicente, 2008). When HSP70 is induced by thermal stress, it is regulated by Heat Transcription Factor-1(HSF1) allowing DNA binding (Pirkkala *et al.*, 2001). Therefore, mammals exposed to high ambient temperatures are expected to have high HSP70 levels.

In addition to inducing HSP production, thermal stress can induce oxidative stress (Krajick, 2004). Oxidative stress is an imbalance of oxidants and antioxidants in animal cells (Blasiak *et al.*, 2014). An excess of free oxidative radicals causes an increased chance of harmful chain reactions in cells (Jones, 2008; Blasiak *et al.*, 2014). The chain reactions commonly target polyunsaturated fats, lipids, proteins, or DNA, and can directly disrupt the cell membranes (Jones, 2008). Oxidative stress may be induced by the presence of one free radical,

which may cause a chain reaction and damage between 200 and 400 macromolecules before it is terminated (Jones, 2008). Termination typically occurs when two radicals react with one another, but is rare at typical oxygen radical concentrations (Jones, 2008). The intermediate species of the oxygen radicals is not only harmful to animal tissue, but could also be fatal (Grune and Berger, 2007).

Increased glycosylation in blood suggests that more oxygen-free radicals are present, indicating more oxidative stress (Calabrese *et al.*, 2007). Glycosylation occurs when a protein is bound with reactive agents such as sugars or amino groups (Dunn *et al.*, 1989). Because oxidative stress can be difficult to quantify, glycosylated hemoglobin can aid in measuring oxidative stress levels (Grune and Berger *et al.*, 2007). Oxidative stress might show evidence indicating thermal stress, making it a good biomarker to use with pikas.

Current Study

At higher altitudes, pikas are living in cooler, less thermally stressful conditions (Beever *et al.* 2010). At lower altitudes, higher temperatures may cause more oxidative and thermal stress to pika populations. Alternatively, at higher altitudes, hypoxic environments or higher UV radiation may play a role in high altitude stress (Blasiak *et al.*, 2014). This study involves collecting blood samples from pikas and golden-mantled ground squirrels (*Callospermophilus lateralis*) for comparison between two species of mammals. Ultimately, we are investigating thermal stress differences between high and low altitudes, and temperature differences in Montana pika populations.

In Montana, pikas may be experiencing thermal stresses at low elevations. Populations in the Gold Creek, and Red Lodge areas are being studied to determine if the levels of HSP70 and glycosylated hemoglobin can act as biomarkers for thermal stress. I hypothesize that pikas from

high altitude environments are exposed to lower temperatures and, therefore, will have lower levels of HSP70 and glycosylated hemoglobin in the blood, when compared to pikas from lower altitudes that are exposed to higher temperatures. Secondly, I hypothesize that as temperatures progressively increase throughout June, July, and August, HSP70 levels and glycosylated hemoglobin levels in pika blood, should show a positive correlation with environmental temperature, at the same altitude.

Methods

Field sites in Montana were chosen based on pika density, site accessibility, and elevation. Prior knowledge about pika populations and locations were obtained from previous work on this project (Allen, 2014, Nearpass, 2014) and from GIS files from the April edition of the Craighead Institute in Bozeman, Montana. Low altitude sites were in the Gold Creek area (1962m), and high altitude sites were at Vista Point on the Beartooth pass (2832m).

Field Work

Pikas were captured in Tomahawk® live traps (16"x5.5"x5.5") set approximately 20m apart in talus fields containing a high density of pikas. The traps were set on flat ground or rocks and camouflaged with leaves, sticks, and rocks. Pikas were baited with apples by rubbing apple pieces and juices on the rocks surrounding the traps. A small apple piece was placed in the back of the trap. Once set, the traps were checked hourly. When the traps were not in use, they were locked open to familiarize the pikas to the traps in their environment.

Upon capture of a pika, the trap was placed in the shade and covered to minimize stress. Isoflurane (USP produced for Butler Schien®), an aesthetic inhalant, was administered by dripping small amounts onto a cotton ball and placing the cotton by the pikas nose until active behavior slowed. The dosage was closely monitored and adjusted according the pika's alertness.

Anesthesia was administered to minimize stress. The pika was kept under anesthesia for the remainder of the handling process. Blood samples were collected in order to assay for HSP70 and glycosylated hemoglobin levels. Stress of capture will not affect HSP70 or glycosylated hemoglobin levels if samples are collected within an hour of capture (Feder and Hofmann, 1999). Weight (g) of the captured pika was taken using a 300g field scale (PESOLA®).

Blood was collected by clipping a toenail slightly past the cuticle—enough to allow the pika to bleed. Micro-Cal heparinized hematocrit capillary tubes (I.D. 0.5-0.6mm O.D. 1.4-1.75mm and length of 75mm: Kimble Chase®) were used to collect blood. Approximately 15-20 capillary tubes were collected from each animal for a total 30µl blood sample. To stop bleeding and facilitate blood clotting, baking flour was placed on the pika's nail. The collected whole blood was extracted from the capillary tubes into an Eppendorf® microcentrifuge tube. The blood was centrifuged on site at 3,500 rpm for 3.5 minutes, using a centrifuge powered by a car adaptor. Plasma was collected with a 1.0mL syringe and a 23G needle. The plasma extractions were performed an average of two to three times to ensure maximum plasma recovery. Each plasma sample was placed in its own microcentrifuge tube. Both plasma and packed cell samples were stored on dry ice until return to the laboratory. Pikas were released in the same area in which they were caught. GPS coordinates and weather conditions were recorded at the time of each capture. All samples were stored at -80° C until tests were performed.

On occasion, golden-mantled ground squirrels were trapped at low altitude sites in areas where squirrel and pika habitats overlap. Squirrel blood samples were collected using the protocols described above.

Laboratory Protocols

Blood samples from laboratory mice (*Mus musculus*) were used for data comparison purposes and to ensure the accuracy of the protocols. To induce HSP70 production, lab mice were housed in plastic bins heated by a water bath (41° C- 43° C) for two to six hours (Feder and Hofmann, 1999). Mice were euthanized and blood was extracted via cardiac puncture (Allen, 2013). Cells and plasma were separated by centrifugation and kept at -80° C until analyzed.

To assay for HSP70 levels in plasma, a high sensitivity ELISA kit was used (Enzo® catalog# ADI-EKS-715). Manufacturer protocols were followed. Samples from pikas were extracted in the field and frozen at -80° C. Blood plasma was slowly thawed from -80° C to 4° C, and mixed in a 1:4 µl sample size to assay buffer ratio.

A 25µl plasma sample was pipetted into a separate Eppendorf tube and mixed with 100µL of assay buffer. For each sample, 100µL of sample/buffer solution was pipetted into the designated wells. The plate was sealed, placed on a shaker, and left to incubate at room temperature for two hours. The contents of the wells were emptied and washed four times with 400µL of wash buffer. After the wash process, all samples received 100µL of antibody solution. The plate was then sealed, placed on a shaker, and left to incubate at room temperature for one hour. The same wash procedure (as described above) was performed after the one hour incubation period. Following the wash process, all samples received 100µL of conjugate solution. The plate was again sealed, placed on a shaker, and left to incubate at room temperature for one hour. The same wash procedure was performed in the manner described above. All samples received 100µL of substrate solution following the wash procedure. The plate was sealed, placed on a shaker, and left to incubate at room temperature for 30 minutes. Following

this final 30 minute incubation period, all samples received 100 μ L of stop solution. The data were read by a microplate reader (Finstruments®) at 450nm.

Glycosylated hemoglobin levels were measured using chromatography techniques. Protocols were modified from Yue *et al.*, (1982) using m-aminophenylboronic acid affinity chromatography. To quantify the percent of glycosylation, 1mL of beaded m-aminophenylboronic acid-agarose gel was placed in the bottom of a 10mL chromatography column. The gel was pre-equilibrated with 5mL of 50mM phosphate buffer (pH 9.2). A 25 μ L sample of packed blood cells from the desired sample was mixed with 50 μ L of ultrapure deionized water to create a testable hemolysate. A 50 μ L sample of hemolysate was placed into the column. The column was washed with five separate 1mL aliquots of 50mM phosphate buffer (pH 9.2) and retained. The glycosylated hemoglobin was then eluted by washing the column again with five separate 1ml aliquots of 50mM phosphate buffer (pH 9.2) containing a 0.2M concentration of sorbitol. A 1mL from each elusion was mixed with 1mL of Drabkins reagent to convert all hemoglobin to met-hemoglobin (changes from Fe²⁺ to Fe³⁺).

Absorbance values were collected at 540nm after all bubbles were removed from the sample and the absorbance reading stayed consistent. A spectrophotometer was used to read the absorbencies at a wavelength of 540nm. The non-glycosylated solution, which was eluted first, was considered absorbance one, and the second elusion, being the glycosylated hemoglobin, was considered absorbance two. The percent of glycosylation was generated from the equation:

$$GHb(\%) = \frac{\text{absorbance 2}}{\text{absorbance 1} + \text{absorbance 2}} \times 100 \text{ (Yue } et al., 1982).$$

To regenerate the m-aminophenylboronic acid-agarose gel for another trial the column was washed with 5mL of 50mM phosphate buffer pH 9.2 with sorbitol. Then it was washed with 5mL of 1M borate buffer (pH 9.8) containing 1.0M NaCl. Then it was washed with 5mL of 1M

borate buffer (pH 9.8). The column was then let to incubate at room temperature for 15 minutes. The column was then washed with 5mL of ultrapure deionized water. Then it was washed with 5mL of 2.0M NaCl solution. Then it was washed with 5mL of 50mM phosphate buffer (pH 9.2). All washes were eludes with approximately 60mmHg of pressure applied to the column supplied by a squeeze bulb.

Results

Overall, fifteen pikas were captured for analysis. Seven samples were obtained from the low altitude site (Gold Creek, MT, 1962m) and eight samples from the high altitude site (Vista Point, MT, 2832m). For some pika samples, not enough blood was extracted to test both HSP70 and glycosylated hemoglobin levels. Temperature data was obtained from GIS files found on at www.prism.oregonstate.edu. Monthly temperature averages throughout the summer, were consistently lower at Vista Point (higher altitude) than at Gold Creek (lower altitude). In addition, there was a higher frequency of days above 15° C at Gold Creek than Vista Point (Figures 1 and 2).

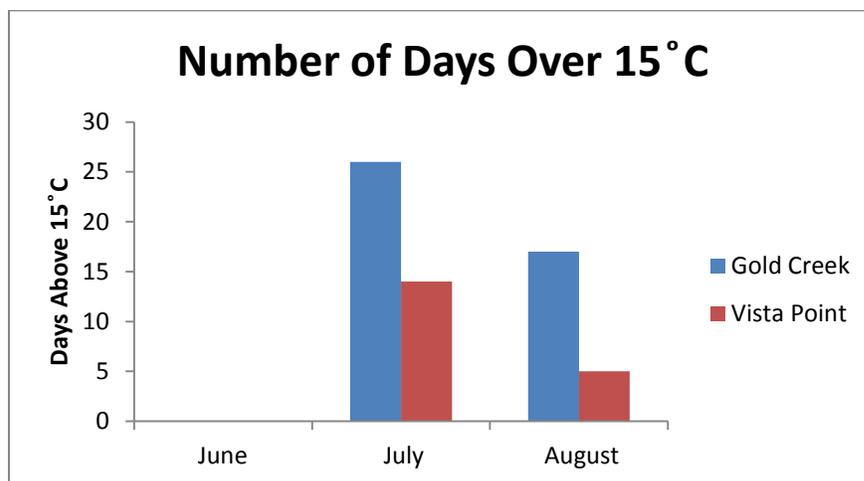


Figure 1. The number of days above 15°C in June, July and August (2014). June did not have any days that were above 15°C. Gold Creek showed a higher frequency of days above 15°C in July and August.

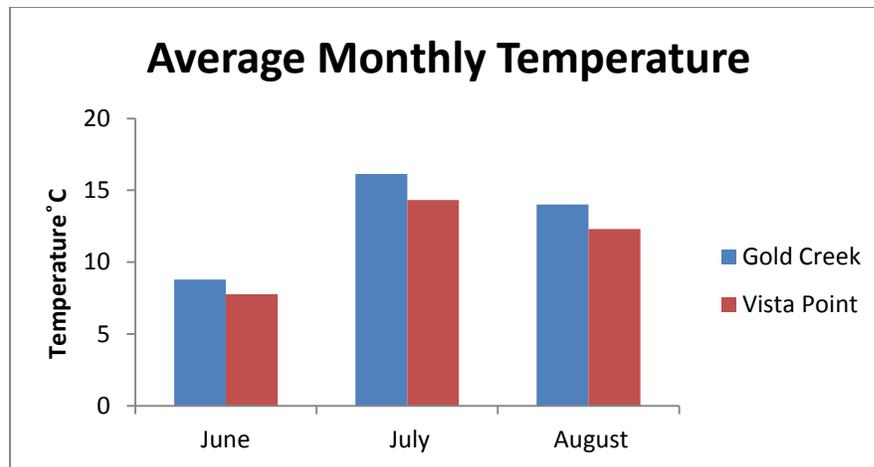


Figure 2. The average monthly temperatures in June, July, and August (2014).

HSP70

The HSP70 ELISA test compared ten different pika samples: four from low altitude and six from high altitude (Figure 3). Data were analyzed using a student t-test (Table 1). When comparing high and low altitude pika samples, a p-value of 0.178 ($\alpha=0.05$ for all tests) was generated indicating no significant difference between high altitude and low altitude HSP70 levels. Low altitude pikas were compared to golden-mantel ground squirrels from the same site. The p-value generated was 0.059 showing no significant difference. When compared, non-heat shocked mice and heat shocked mice, showed a p-value of 0.049. Both the pikas at high altitude and low altitude showed a significant difference for greater amounts of heat shock protein than in the non-heat shocked lab mice (p-value=0.036 for high altitude, and p-value=0.0006 for the low altitude comparisons). When comparing the high and low altitude pikas to the laboratory heat shocked mouse, no significant difference was identified. High altitude and heat shocked mouse comparisons produced a p-value of 0.108, and low altitude and heat shocked mouse comparisons produced a p-value of 0.087 (Table 1).

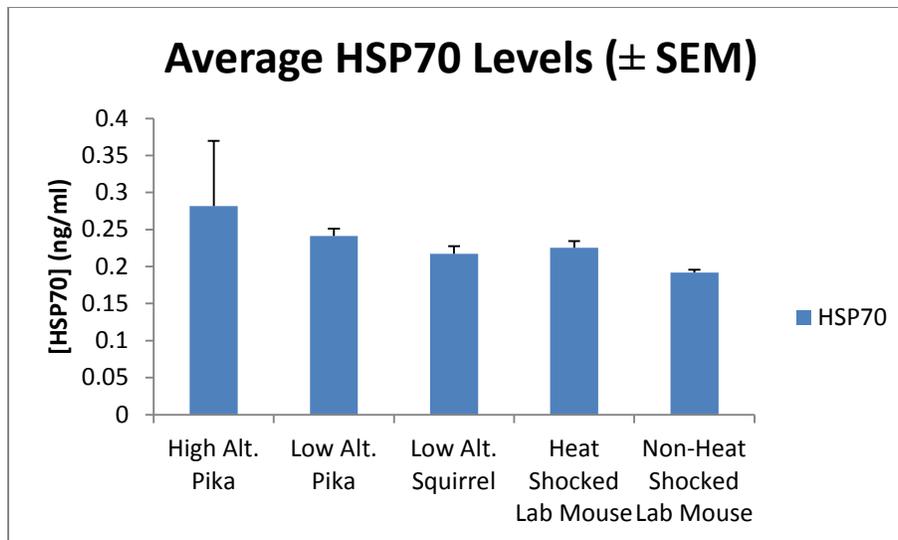


Figure 3: The relationship between pikas, golden mantle-ground squirrel, and laboratory mice at various environmental and laboratory parameters. Student t-test analysis indicated no significant difference between high and low altitude pikas.

Table 1: The p-values of compared species from student t-test analysis. Showing significance at the .05 Level, 95% confidence. Green indicates a significance difference, and red indicates no significance difference.

	High Altitude Pikas	Low Altitude Pikas	Golden-Mantel	Non-HS Mouse	HS Mouse
High Altitude Pikas	_____	.178	N/A	.036	.108
Low Altitude Pikas	_____	_____	.059	.0006	.087
Non-HS Mouse	_____	_____	_____	_____	.049

Both high and low altitude populations illustrated an increase in HSP70 levels as temperature increased on day of capture. The low altitude populations resulted in an R^2 value of 0.45 ($y=0.0101x + 0.0699$) and the high altitude resulted in an R^2 value of 0.18 ($y=0.0165x + 0.0149$). The R^2 values for the previous days' average temperature was $R^2=0.54$ ($y=0.0045x + 0.167$). When plotting HSP70 concentrations against the average temperature of the week prior to capture, the R^2 value was 0.95 ($y=0.0163x + 0.0271$) (Figure 4). There was no positive correlation seen at high altitudes for the prior day or for the prior week (Figure 5).

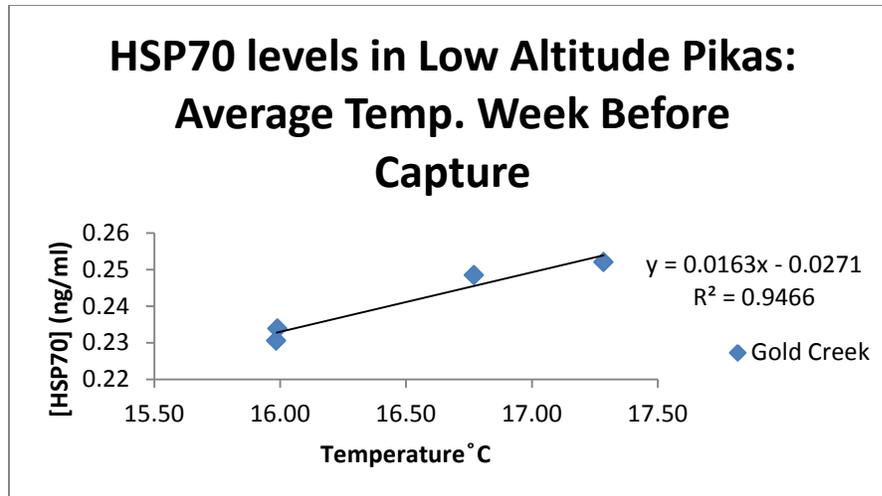


Figure 4. HSP70 levels compared to the average temperature of the prior seven days to the capture at low altitudes. A strong positive correlation is indicated.

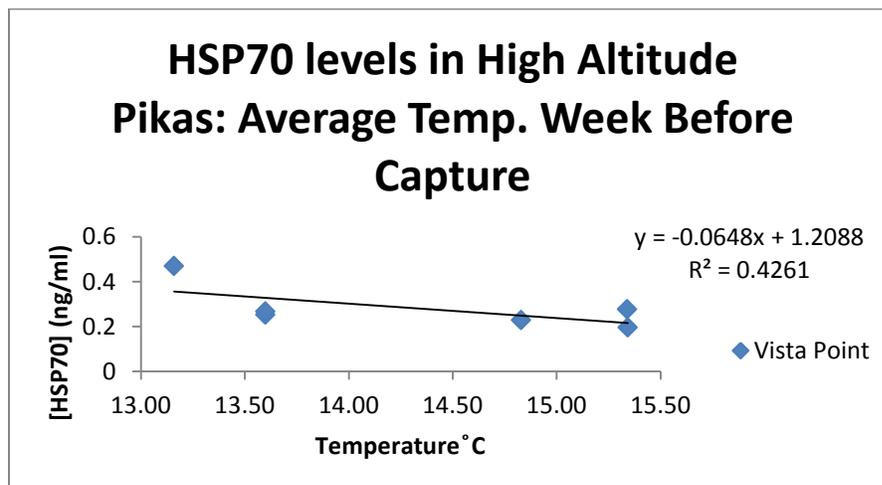


Figure 5. HSP70 levels compared to the average temperature of the prior seven days to the capture at high altitudes. No correlation is suggested.

Glycosylated Hemoglobin

Glycosylated hemoglobin levels were compared between four pikas at low altitudes and seven at high altitudes. Data were analyzed using a student t-test. There was no significant difference between high and low altitude pika populations, with a p-value 0.475 (Figure 6).

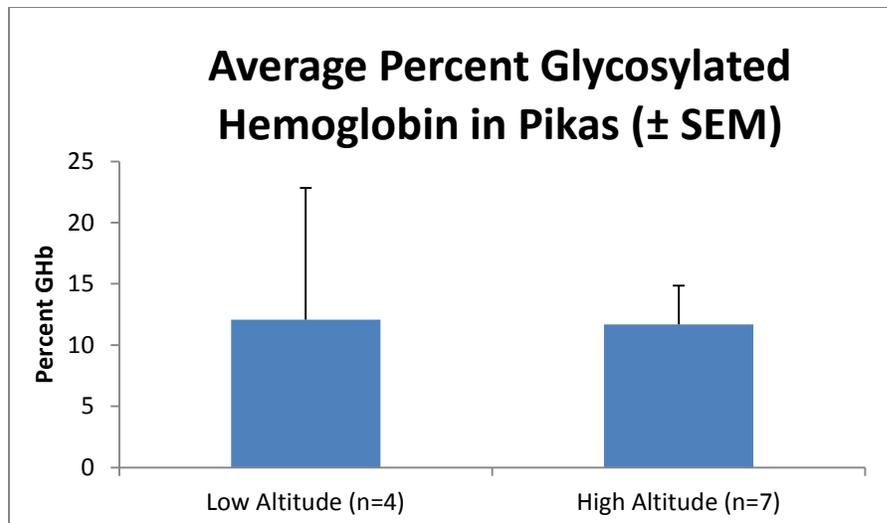


Figure 6. The relationship between high and low altitude pika populations. Although there is slightly higher averages in lower populations there is no significant difference between the two.

When comparing glycosylated hemoglobin levels to temperature data at low altitudes, R^2 values and slopes did not indicate any correlation. There was no relationship between the average weekly temperatures prior to capture and the levels of glycosylated hemoglobin (Figures 7 and 8).

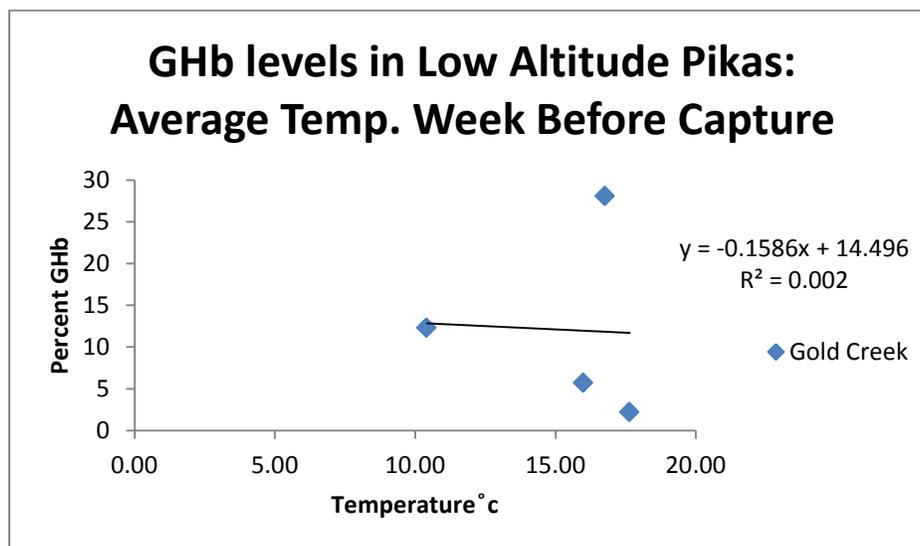


Figure 7. The glycosylated hemoglobin levels of low altitude pikas compared to the average weekly temperature prior to capture. No correlation between the two is indicated.

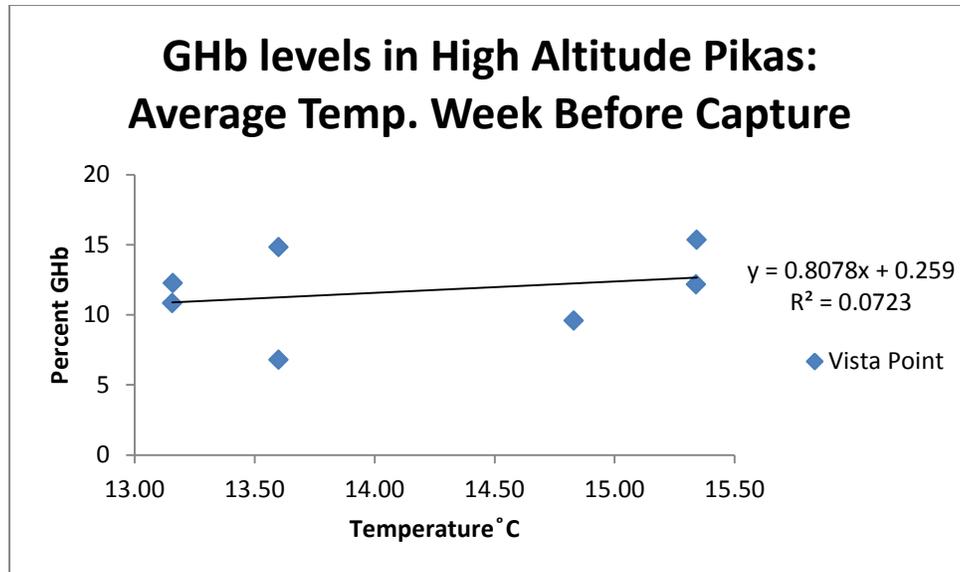


Figure 8. The glycosylated hemoglobin levels of high altitude pikas compared to the average weekly temperature prior to capture. No correlation between the two is indicated.

Discussion

HSP70

The significant difference between HSP70 in the heat shocked mice and the non-heat shocked mice indicated that this test is an accurate measurement of direct thermal stress. When comparing levels of HSP70 between high and low altitude pikas, there was no significant difference, indicating a similar heat stress throughout all altitudes of pika populations. This may be because all pikas, regardless of the altitude, are being stressed, or HSP70 levels could be normally higher in lagomorphs. When comparing HSP70 levels to temperature data, there is a strong correlation between the weekly temperatures at low altitudes and HSP70 production. Because this correlation was not seen at high altitudes, this indicates that, at low altitudes, there is the potential for greater amounts of direct thermal stress (Figures 4 and 5). This positive correlation at low altitudes could also indicate an environment less conducive for pikas to escape the heat. The weekly temperature data showed a stronger R^2 value (Figure 4) than the day of capture temperature data. This may indicate that a prolonged time in a stressful environment

induces a higher level of HSP70 to build up in pikas before HSP70 is able to degrade. Thus, pikas may be unable to adequately lower HSP70 levels before more stress is experienced.

Exposure to lower temperatures may result in the degradation of HSP70 and a lower thermal stress level for the pikas. Staying beneath talus, or cooler night temperatures may allow HSP70 to degrade at a quicker rate as well. More research should be done by collecting sub-talus, and day- night temperature data.

Glycosylated Hemoglobin

There was no correlation between glycosylated hemoglobin and temperature. Pikas either may not be experiencing thermal stress, or there may not be enough thermal stress to detect an adequate amount of glycosylated hemoglobin. Higher temperatures, such as in the Great Basin (Beever et al. 2010), may cause enough thermal stress to induce a measurable amount of oxidative stress in pikas. To strengthen this argument, the methods of collection and testing may need to be refined by different assays such as a glycosylated hemoglobin high sensitivity ELISA kit, or latex-enhanced immunoturbidimetric assay (Lakshmy and Gupta, 2009). Although the chromatography techniques showed consistent results, they may not be sensitive enough to measure thermally induced stress at these temperatures.

Temperature

Of the summer months, the temperatures of June, July and August showed what was expected: the higher altitudes tend to have lower temperatures and the lower altitudes tend to have higher temperatures. This may be evidence for lower altitude environments being more prone to direct thermal stress. Because the frequency of days above 15°C per month were much higher at lower altitudes (Figure 1) and the overall monthly temperatures were also higher at

lower altitudes (Figure 2), these data support that pikas at lower altitudes may be subjected to greater thermal stress.

It may be of interest to investigate the differences between day and night temperatures at high and low altitudes. If there is a difference between the day and night temperatures at low altitudes when compared to the differences between day and night temperatures at high altitudes, then this may affect pikas ability to lower stress levels. Another temperature difference that may be relevant is the temperature of sub-talus, sheltered areas. If there are differences in the shelter temperatures between high and low altitude populations then this may also affect pikas ability to lower stress levels.

Conclusion

Prior research suggests that pika populations may be in more danger in higher temperature environments, especially as global temperatures continue to rise (Nearpass, 2014). However, these data need to be supported by a larger sample size to further support this claim. HSP70 is supported as being an adequate biomarker for identifying direct thermal stress. Glycosylated hemoglobin may be a viable marker for thermal stress in pikas; however more research must be done to verify its effectiveness. Overall, more pika samples are needed to strengthen arguments for both HSP70 and glycosylated hemoglobin levels and as biomarkers to make definite conclusions about their usefulness. For the future, different sites should be examined that have higher temperatures or lower elevation. Additional temperature analysis should be done to indicate what environments may be more conducive to lowering stress levels in pikas.

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