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Analyzing Physiological Stress Response Using Dermal Swabs in Plethodon montanus

John Tester

ABSTRACT

Upon exposure to environmental stressors, amphibians such as *Plethodon montanus* will release corticosterone (CORT) thus causing a behavioral and physiological response to cope with the stress. Currently, there are several invasive ways of collecting CORT in salamanders. However, these techniques typically require euthanasia of the organism. We hypothesized that exposure of *P. montanus* to stressful handling conditions will result in elevations of CORT that can be detected through dermal swabbing. To test this, two experiments were conducted which involved swabbing the dorsal side of the trunk before and immediately after exposing *P. montanus* to two different environmental stressors. The first experiment involved placing *P. montanus* into a behavioral chamber for twelve hours while the second experiment involved restraining *P. montanus* in a plastic bag with a damp paper towel for ten minutes. While both experiments indicated an elevation in CORT after the respective treatments, between-replicate variability were high, and the differences were not statistically significant. The results of the first experiment did reveal a stress response when *P. montanus* were taken out of the chamber. The second experiment's results also suggested that *P. montanus* did have an acute stress response when restrained. Future studies could replicate this research, but with a larger sample size and see if the results are congruent with the data obtained in this study.

ACKNOWLEDGEMENTS

I would first like to thank Dr. Joseph Bidwell for allowing me to be involved in his laboratory and to conduct my research. Along with him, I would also like to thank PhD candidate Trevor Chapman and graduate student Anna Grace Grizzard for all their help and support, whether it be helping collect *P. montanus* in the field or helping me set up my assay kits. Finally, I would like to thank Dr. Darrell Moore and Mr. Daniel Hedden for being my thesis readers.

INTRODUCTION

The East Tennessee region is a major habitat location for salamanders in the family Plethodontidae. Within their natural habitat, they are typically found underneath rocks, moss, logs, and bark in moist, cool forests (Tennessee Wildlife Resource Agency, n.d.). These low-energy lifestyle salamanders play a vital role in their ecosystem by eating insects and being a food source for larger animals. However, they are facing different environmental stressors like habitat loss, disease outbreaks, pollution, and climate change (Brook *et al*, 2008). Upon exposure to these different stressors, salamanders will release the glucocorticoid hormone corticosterone (CORT). Upon releasing this hormone, the individual will mediate both a behavioral and physiological response for dealing with the external stressor (Fonner, 2015). As such, a way to evaluate the physiological stress that these threats induce in the salamanders is to measure levels of glucocorticoid stress hormones, which are common to all vertebrates (Gifford, 2016).

The physiological response to these different stressors can suppress an individual's immune system, ultimately increasing their susceptibility to pathogens, promote severe protein loss, disrupt secondary cell messengers, and suppress their growth (Santymire, 2018). This can have a detrimental effect on the organism, as well as the respective ecosystem they reside in. Field-base monitoring of physiological stress hormones (glucocorticoids) in salamanders, such as *Plethodon montanus*, can help determine the sub-lethal effects of these different environmental stressors, thus providing early alerts when populations are chronically stressed (Narayan *et al*, 2019). One common problem with this however is that most methods of determining glucocorticoid stress hormones are typically fatal or involve euthanasia of the organism. This is why utilizing a non-invasive method of collecting glucocorticoid stress hormones is beneficial,

since it will allow for a deeper understanding of stress hormone response in *P. montanus* without the drawback of euthanasia or fatality.

A method commonly used to determine the level of these hormones in salamanders involves sampling blood plasma (Romero, 2004). Unfortunately, this approach is typically fatal for Plethodontid salamanders due to their small body size. Other methods of directly measuring stress hormones involve gathering sufficient volumes of faecal material or urine samples. Both of these methods are not well adapted to field studies that require sampling a large number of amphibians across multiple sites (Narayan *et al,* 2013). Another method of collection is a whole-body analysis, which requires euthanasia of the Plethodontid salamanders (Glennemeier and Denver, 2002; Barria *et al*., 2011). However, a recent study conducted by Santymire in 2018 revealed that dermal swabs may be a non-invasive and effective way to obtain the hormone samples in amphibians (Santymire, 2018). "This method of swabbing revealed that the salamanders had no long-lasting negative effects after being swabbed" (T. Chapman, personal communication, March 14, 2021). Santymire's study involved terrestrial, semi-aquatic, and fully aquatic amphibians, none of which were Plethodontids. Our study will focus solely on Plethodontids due to their conservation importance.

This dermal swab method was tested by a previous East Tennessee State University student and she determined that in *P. montanus*, swabbing the dorsal side of the trunk resulted in less variation in glucocorticoids than swabbing the lateral or ventral sides of the body. She also determined that using the Cayman Chemical Cortisol ELISA kits (Ann Arbor, MI) were just as effective as the inhouse kits that Santymire et al. (2018) used in their study. We sought to extend this work by applying the dermal swabbing method under field conditions after the animals had been exposed to stressful conditions associated with behavioral experimentation and normal field

collection . We hypothesized that in both experiments, the dermal swabs taken after the handling would yield a significantly higher concentration of glucocorticoid than before *P. montanus* were handled.

METHODS

Collection

P. montanus were captured off the Appalachian Trail leading to Round Bald from Carvers Gap, which is located at Roan Mountain State Park. We followed IUCAC protocol under East Tennessee State University P210302. The collection period started on August 18th, 2020 and went to September 23rd, 2020. Both experiments were conducted on site. We tried to avoid cross contamination by wearing a new set of latex gloves when capturing each individual *P. montanus,* as well as using different dermal swabs for each individual.

In the first experiment, each individual salamander was swabbed along the dorsal side of the back, approximately two and a half centimeters from the base of the head to the start of the tail using a Puritan sterile polyester tipped applicator dermal swab (Guilford, ME). The swab was then placed into a 1.5 mL plastic vial containing seventy percent ethanol and immediately put on ice. Following this, the swabbed salamander was placed into a plastic bag containing leaf litter. After collection, the vial labeled “Pre”, along with *P. montanus,* were taken to the mobile laboratory located at the base of Roan Mountain State Park. *P. montanus* were then held in a behavioral arena chamber that ran for twelve hours. The “Pre” vials were taken to the laboratory at East Tennessee State University and stored in a freezer at -5°C. After allocating for this twelve-hour period, each salamander was swabbed again as described above, except the vial the swab was placed in was labeled “Post”. Once these “Post” vials were collected, they were taken

to the laboratory at East Tennessee State University and stored in a freezer at -5°C. This process was repeated seven more times, which totaled for eight "Pre" swabs and eight "Post" swabs.

The second experiment involved capturing *P. montanus* in the field and immediately swabbing them approximately two and a half centimeters from the base of the head to the start of the tail upon collection. Once captured, the Puritan sterile polyester tipped applicator dermal swab (Guilford, ME) was placed into a 1.5 mL "Pre" vial containing seventy percent ethanol. The salamander was immediately placed into a plastic bag containing a damp paper towel and restrained inside for ten minutes. Following this ten-minute period, a second swab was obtained and placed into a vial labeled "Post" and put on ice. This process was repeated four times, totaling five "Pre" swabs and five "Post" swabs. Once the swabs were collected, all samples were taken to the laboratory at East Tennessee State University and stored in the same manner as experiment one. All salamanders used in both experiments were safely released back into the field after the "Post" swabs were obtained.

Cortisol Kit

Three Cortisol ELISA kits from Cayman Chemical (Ann Harbor, MI) were used in this experiment to collect corticosterone levels in all of our samples. These kits were stored at -20°C, which is the recommended temperature for storage provided by Cayman Chemical Company (Ann Harbor, MI). The kits were used before January 20th, 2022, which is the expiration date indicated on the outside of the box. Each kit came with: Goat Anti-mouse igG Coated 96 strip well plate and cover sheet, Cortisol ELISA monoclonal Antibody, Cortisol Ache Tracer, Cortisol ELSIA Standard, ELISA Buffer Concentrate (10x), Wash Buffer Concentrate (400x), Polysorbate 20, Ellman's reagent, Elisa Tracer Dye, and Elisa Antiserum Dye. Refer to *Appendix* for kit protocol preparation and set up. In conjunction, a Synergy HT plate reader from

BioTek Instruments was used to read the plates. This plate reader measured absorbance at 420 nm for all the plates used, which is within the recommended absorbance range provided by Cayman Chemical Company (Ann Harbor, MI). Once the plates were run through the plate reader, each well's absorbance was put into a excel sheet and presented as raw data. Once this raw data was obtained, it could then be put into the Cayman Chemical analysis tool (Ann Harbor, MI), which provided a standard curve and the concentration of CORT for each pre and post swab.

Statistical Analysis

CORT levels on the pre and post swabs derived from individual salamanders were analyzed on Excel using a paired T-Test at $\alpha=0.05$.

RESULTS

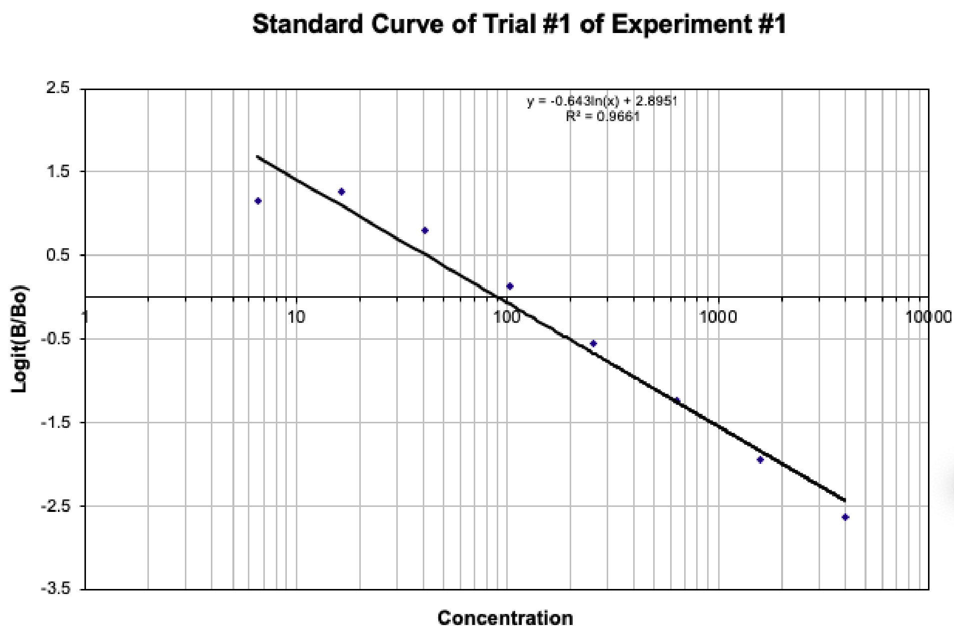

Figure 1: Standard curve of trial #1 of experiment #1 plotting $\%B/B_0$ against concentration of CORT.

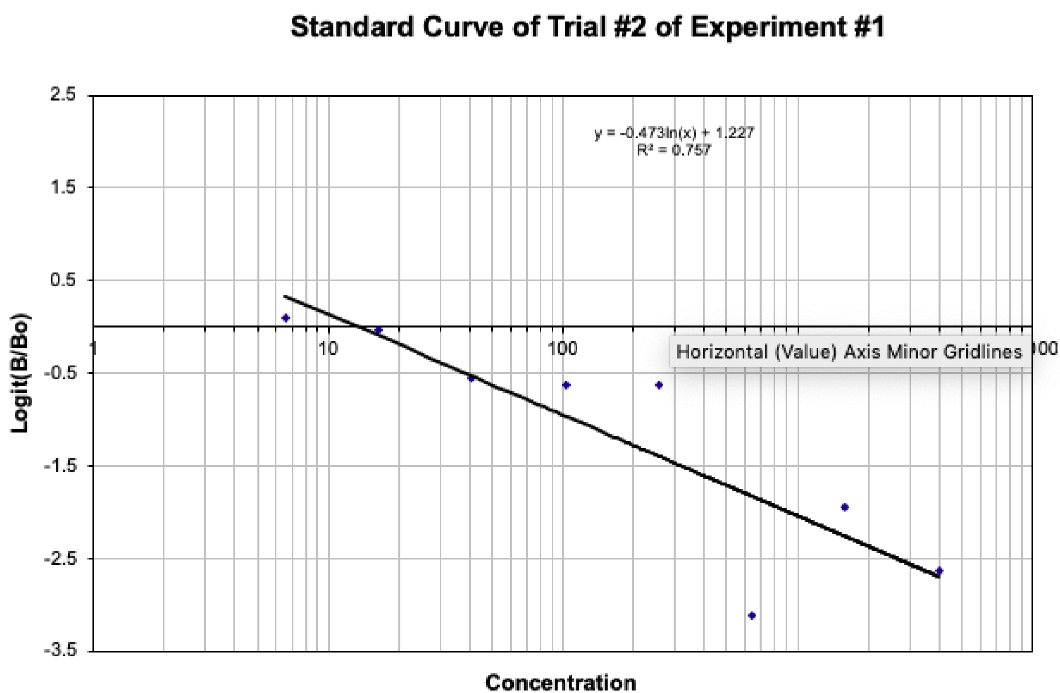

Figure 2: Standard curve of trial #2 of experiment #1 plotting $\%B/B_0$ against concentration of CORT.

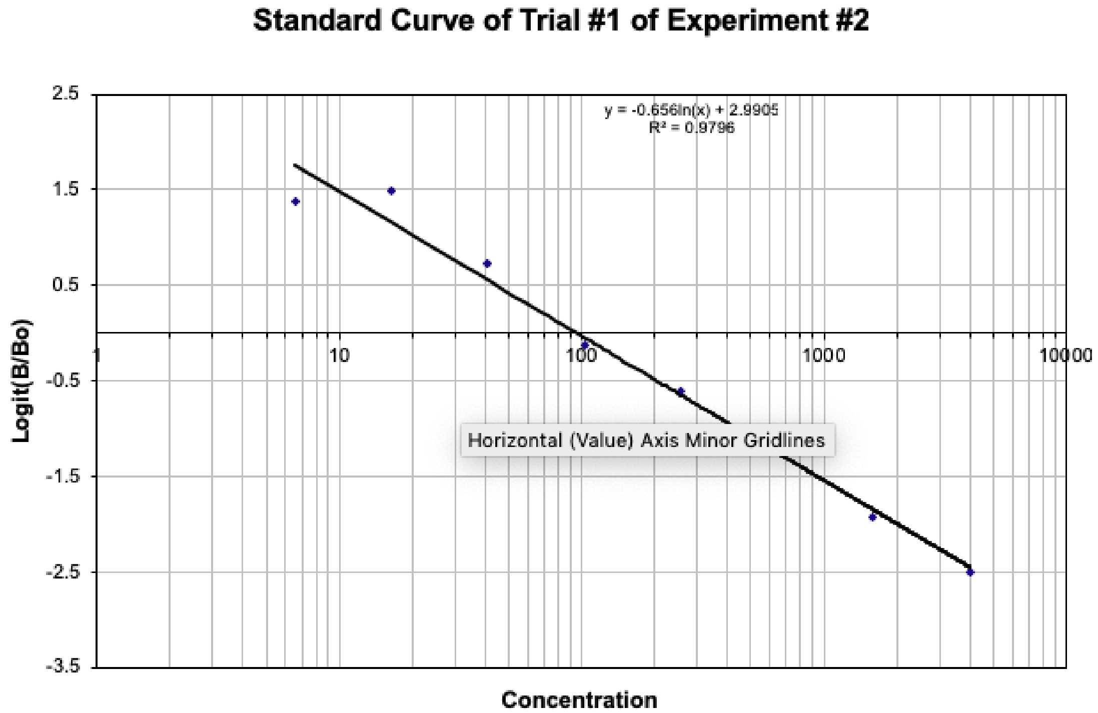

Figure 3: Standard curve of trial #1 of experiment #2 plotting %B/B_0 against concentration of CORT.

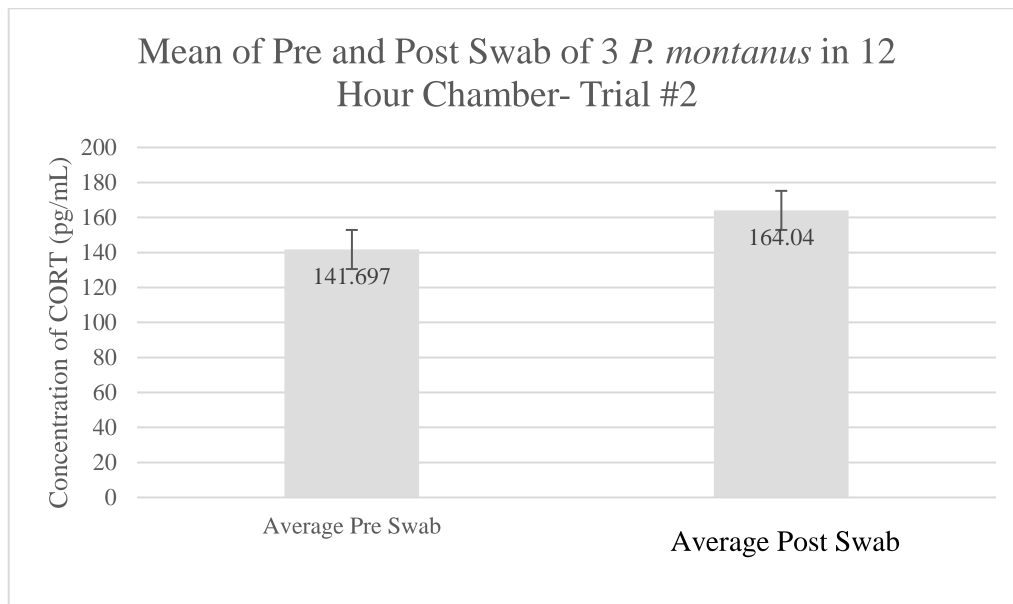

Figure 4: Bar graph depicting average pre-swab and average post swab after 3 *P. montanus* were subjected to the 12-hour chamber. Standard error bars have been added to both measurements.

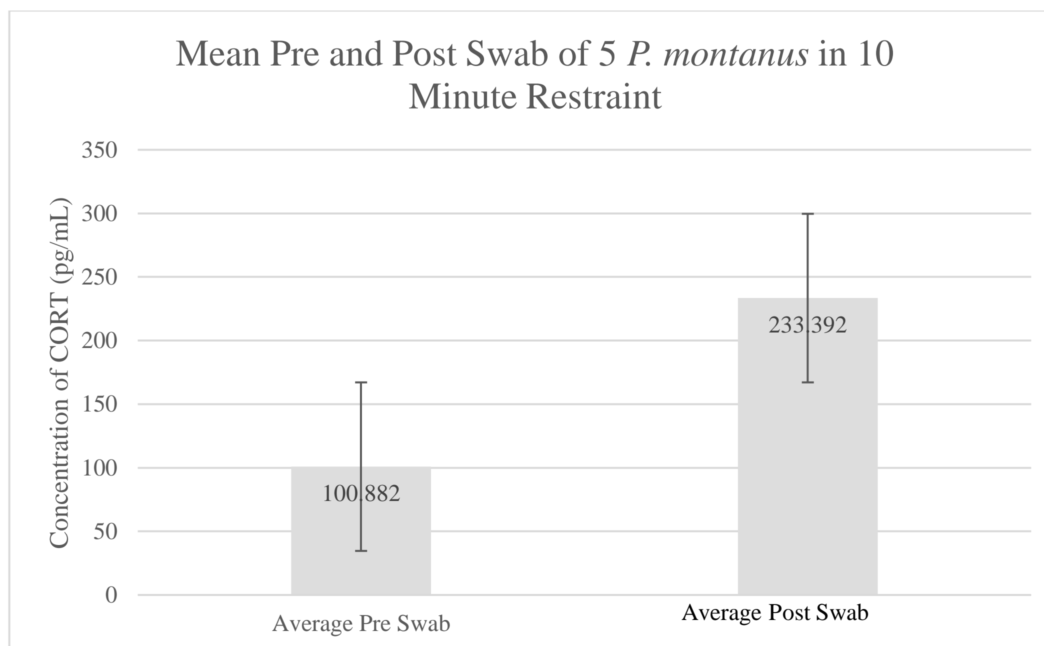

Figure 5: Bar graph depicting average pre-swab and average post swab for *P. montanus* after being subjected to the 10-minute restraint. Standard error bars have been added to both measurements.

The standard curves, as shown in *Figure 1-3*, indicated a good association between concentration and the absorbance of each well. *Figure 1* showed a strong relationship with an R^2 =0.9661. *Figure 2* did not reveal as strong of a relationship since it obtained an R^2=0.7570, while the R^2 for the plot in *Figure 3* was 0.9796.

The first trial of experiment one resulted in no significant difference of CORT between the "Pre" and "Post" swabs for *P. montanus* held in the behavioral chamber for twelve hours. While setting up the Cayman Chemical ELISA kit (Ann Harbor, MI), human error may have occurred which caused the data to be skewed. At the time of setting up these assay kits, my previous experience with single and multi-channel micropipettes was limited. Therefore, the error might have occurred during pipetting.

For the second trial of the first experiment, the average pre-swab of three *P. montanus* individuals was 141.697 pg/mL, while that for the post-swab was 164.04 pg/mL (*Figure 4*). This difference was not significant (p=0.368). As indicated by the standard error bars, there was a significant amount of variability in the second trial, which most likely led to our inability to find a significance difference in CORT levels.

Similarly, the first trial of the second experiment revealed elevated CORT levels when *P. montanus* were subjected to a ten-minute restraint in a plastic Ziplock bag. (*Figure 5).* In this trial, the average pre-swab for 5 *P. montanus* individuals was 100.882 pg/mL, and the average post swab was 233.392 pg/mL. Once again, this difference was not significant (p=0.146) as indicated by the standard error bars, there was a significant amount of variability in the second trial which led to our inability to find a significance difference in CORT levels.

DISCUSSION

After conducting both experiments, we found that exposure of *P. montanus* to stressful handling conditions did result in elevations of CORT that was detected through dermal swabbing, as shown in *Figure 4* and *Figure 5*; however, the difference was not significant. An interesting finding was that the ten-minute restraint, *Figure 5*, showed a much higher elevation of CORT than the behavioral chamber did, *Figure 4.* One challenge faced with the dermal swabbing technique was that *P. montanus* would sometimes be very active, making it hard to follow our swabbing protocol. This was seen in trial two of experiment one, an individual “Post” swab was not as accurate as the others due to the *P. montanus* moving so much. This outlier lowered the “Post” swab mean average. This is why trial two of experiment one had a higher p-value, p=0.368, and variation than what was seen in experiment two, p=0.146. While the results of both

experiments did yield a p-value outside the statistically significant range, this could be due to the low sample size found in both trials of experiment one and experiment two.

While not significant, our results do generally agree with those of Santymire et al. (2018). They conducted a pre-stress swab and then after five minutes of being restrained in the hand, gathered post-stress swabs at intervals of 15, 30, 45, 60, 90, and 120 minutes. Their results revealed a significant increase of CORT in the post-stressor swabs from multiple species of amphibians across varying-life stages (Santymire, 2018). These results support the notion that using the dermal swab technique after exposing *P. montanus* to stressful environments would yield a significantly higher concentration of glucocorticoid than before *P. montanus* was exposed.

Another study conducted by Scheun *et al* (2019) aimed to validate dermal secretions as a robust matrix for monitoring CORT alterations in the edible bullfrog (*Pyxicephalus edulis*) when subjected to being handled (Scheun *et al*, 2019). They used enzyme immunoassay kits (EIAs) to quantify CORT from dermal secretions in *P. edulis* after a handling event. Their study found that the handling event would result in an increase of CORT in the dermal secretions of the edible bullfrog (*Pyxicephalus edulis),* thus offering further support for the use of dermal secretions as an important matrix for monitoring physiological stress in amphibians (Scheun *et al*, 2019). These results support the notion that obtaining CORT from dermal swabs in amphibians, such as *P. montanus*, can help monitor physiological stress response.

Another study conducted by Narayan et al. (2013) found that short-term CORT stress responses are repeatable in free-living amphibians (Narayan *et al*, 2013). Their study consisted of placing male cane toads (*Rhinella marina)* into plastics bags, similar to that of our experiment, for five minutes and collecting CORT from urine samples over eight hours. Their results showed

that all the male cane toads (*Rhinella marina)* did express a CORT stress response over the eight-hour period to the standard capture and handling protocol and was highly repeatable (Narayan *et al*, 2013). This study revealed that a non-invasive technique of obtaining short-term corticosterone stress responses can be repeatable in free-living amphibians (Narayan *et al*, 2013). This relates to this study since it shows that the non-invasive technique, such as dermal swabbing, can be highly repeatable among free-living amphibians, such as *P. montanus.*

The results obtained in this study, while not significant , were interesting since this dermal swabbing could provide a non-invasive method to obtain CORT when *P. montanus* are in stressful handling conditions, as opposed to the more invasive methods such as whole-body analysis (Glennemeier and Denver, 2002; Barria *et al*., 2011) or blood plasma sampling (Romero, 2004). This would provide a much faster way to obtain CORT samples in the field as well as allow for a much better understanding of the physiological response in *P. montanus*. The idea that basic field handling of amphibians could induce a stress response is also important to consider in future studies with the animals since elevated levels of CORT could affect behavior and other physiological variables.

The biggest limitation of this study was sample size. Experiment one had a total of eight *P. montanus* while experiment two only had a total of five *P. montanus*. There were several times we would travel up to the site and would not find any *P. montanus* in the field. This is not uncommon however since that is a common hardship of field work.

Due to the broadness of this topic, it allows for future studies to be conducted dealing with *P. montanus*. One potentially could replicate this study; however, obtain a much larger sample size of *P. montanus* and see if the results are congruent with this research. By obtaining a larger sample size, this could potentially lower the variance and bring the p-value closer to a

statistically significant value. Another potential study could be to analyze seasonal variation from collected *P. montanus*. Since the collection period of this research occurred during the fall, one could collect during the spring and see if there is a seasonal variance in CORT in *P. montanus.*

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APPENDIX

96 strip well plate

Each 96 strip well plate Contained: 2 blanks, 2 non-specific binding wells (NSB), 2 maximum binding wells (B_0), 8 standards (S#), samples in 2 dilutions run in duplicates, and a total activity (TA) well.

Buffer preparation

-1 vial of (10x) buffer concentrate and 90 mL Ultrapure water.

-Can be stored 4 °C and will be stable for 2 months.

Wash Buffer preparation

-1 vial (5 mL) of (400x) concentrate and 2 L of Ultrapure water with 1 mL of Polysorbate 20.

Standard (Bulk) preparation

-Equilibrate micropipette tip in ethanol.

-Transfer 100 µL of Cortisol Standard and 900 µL Ultrapure water into a test tube.

Elisa Standard preparation

-Test tubes labeled #1-#8.

-900 µL buffer in #1.

-600 µL buffer in #2-8.

-100 µL bulk standard in #1.

-Serially dilute by moving 400 µL from #1 to #2.

-Move 400 µL from#2 to #3.

-Repeat for test tubes #4-#8.

-Should not be stored for more than 24 hours.

Tracer preparation

-100 dtn Tracer and 6 mL buffer.

Antibody preparation

-100 dtn Tracer and 6 mL buffer.

Sample preparation

-Swabs shaken on mixer for 5 minutes.

-Swab removed and 500 mL moved to new test tube.

-Dried in water bath at 60 °C.

-500 µL buffer (2 different dilutions).

-Tubes vortexed then sonicated for 20 minutes.

-Samples shaken for 30 minutes.

-Stored at 5 °C.

Assay set up

Using different tips for each reagent and not exposing pipette tip to reagents in well, add:

-100 µL buffer to NSB wells.

-50 µL buffer to B_0 wells.

-50 µL from #8 standard to S8 wells.

-50 µL from #7 standard to S7 wells.

-50 µL from #6 standard to S6 wells.

-50 µL from #5 standard to S5 wells.

-50 µL from #4 standard to S4 wells.

-50 µL from #3 standard to S3 wells.

-50 µL from #2 standard to S2 wells.

-50 µL from #1 standard to S1 wells.

-Add 50 µL of sample per well.

-Add 50 µL of tracer to each well except TA and blanks.

-Add 50 µL of antibody to each well except TA, NSB, and blanks.

-Cover plate with plastic film and incubate overnight at 4°C.

Development of plate

-Use the same day as prepared.

-Reconstitute Ellman's Reagent immediately before use.

-100 dtn and 20 mL Ultrapure water.

-Empty wells and rinse five times with buffer (wash).

-Add 200 µL Ellman's Reagent to each well.

-Add 5 µL of tracer to TA well.

-Cover plate with plastic film.

-Place on shaker in a dark room to develop for 120 minutes.

Reading plate

-Wipe bottom of the plate with dry wipe.

-Read the plate at 405nm, 410nm, 415nm, and 420nm absorbance level.

-Absorbance should be checked until B_0 has reached a minimum of 0.3 A.U.

-If the reading is larger than 2.0 A.U., wash buffer again and add fresh Ellman's Reagent and develop again.