THE IMPOSSIBLE SALAMANDER: ABERRANT COLORATION AS A RESULT OF METAL TOXICITY, CRYPSIS, OR LIGHT EXPOSURE?

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ABSTRACT

THE IMPOSSIBLE SALAMANDER: ABERRANT COLORATION AS A RESULT OF METAL TOXICITY, CRYPSIS, OR LIGHT EXPOSURE?

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The Buck Creek Serpentine Barrens in Clay County, North Carolina is an unusual habitat comprised of a pine savannah with endemic plant species underlain by serpentinite rock. The area is drained by a stream with a mostly open canopy. Most *Desmognathus monticola* (Seal Salamanders) living in the Barrens stream have bright yellow patches on their skin, although this population is genetically similar to normally-colored populations of *D. monticola.* My research explored whether this unusual coloration was due to: 1) trace metals from the serpentinite rock, 2) phenotypic plasticity for crypsis against the lightly colored serpentine rock on the stream bottom, or 3) excessive light exposure and decreased shade. I found that metals were unlikely to cause the yellow coloration because the metals of concern were at low concentrations in the stream water. Moreover, there were no differences in white blood cell counts or liver mass between Barrens and non-Barrens (control) populations, suggesting that there was no ongoing immunological response to or accumulation of toxic metals. The Barrens salamanders did have less epithelial pigment than control salamanders. Salamanders from both Barrens and control populations became more yellow in every crypsis lab experiment, whether exposed to light, dark, yellow, or red backgrounds. Another experiment found that the yellow coloration of salamanders was affected by salamander population source, light level they were exposed to, and calcium

concentration, but luminosity (brightness) of the salamanders was not affected by any factors in that experiment. Understanding the environmental stimuli that induce this morphological change in these salamanders' integument, and if these morphological changes are unique to this population, will help to shed insight upon a unique part of North Carolina's landscape diversity.

PREFACE

The Buck Creek Serpentine Barrens in the Nantahala National Forest, North Carolina (Figure 1) is a unique habitat underlain by serpentinite rocks containing relatively high levels of naturally occurring trace metals (Worthington, 1964). It supports a sparse canopy of Pitch Pine (*Pinus rigida*), and a habitat for rare and unusual plants such as Serpentine Aster (*Aster depauperatus*) and Long-haired Barrens Chickweed (*Cerastium villosissinum*) (Mansberg and Wentworth, 1984). Past research has examined the accumulation of trace metals and toxicity tolerance of plants to trace metals (e.g., nickel, molybdenum, and lead) found in the Buck Creek Serpentine Barrens, and how metal toxicity resulted in stunted growth and sometimes death at this site (Pollard, 2017). USDA Forest Service Botanist Duke Rankin once stated in a presentation that metal concentrations in the tributary flowing through the Buck Creek Serpentine Barrens were too high for almost any organism to survive. A member of the audience, Kyle Pursel, replied that he had found salamanders in the stream. Rankin replied that this finding was "impossible." Pursel (*personal communication*) had found *Desmognathus monticola* (Seal Salamanders) and *Eurycea wilderae* (Blue Ridge Two-lined Salamander) in the stream but noted that the *D. monticola* exhibited a strange color pattern, while the *E. wilderae* exhibited normal coloration. The Seal Salamanders had bright yellow patches on their head, back, legs, and tail, and had a greenish tint beneath their eyes (Figure 2). The *E. wilderae* in the Barrens stream did not exhibit the same pattern, possibly because all adults of this species are naturally yellow. Amphibian experts at first thought that these unusually colored salamanders may be a new species but genetic evidence later confirmed that they were *Desmognathus monticola* (J. J. Apodaca, and D. Beamer, *personal communication*).

The mystery of these aberrantly colored salamanders was furthered by personal observations of unusual behaviors, as well as a scarcity of food in the Barrens stream. These Seal Salamanders have been observed basking in the bottom of stream pools during the day, as well as actively foraging during daylight hours (Harmon, *personal observation).* It is possible that this population is active during the day, unlike other Seal Salamander populations, due to the observed food shortage at the Buck Creek Serpentine Barrens (Harmon, *personal observation).* But, these altered behavioral characteristics may increase the risk of predation from water snakes and other predators (Harmon, *personal observation).*

The presence of these salamanders in the Barrens, and their unique traits raise a number of questions. Do these salamanders represent a Serpentine Barrens ecotype or color morph? Are these salamanders altering their skin color to camouflage themselves from predators? Or, is the coloration a morphological response to trace metal exposure? Do geochemical characteristics of the water, such as high calcium concentrations, contribute to this aberrant coloration? Is excessive light exposure, due to living in an environment with a sparse canopy, causing phototoxic stress and inducing this yellow coloration? Is this Serpentine Barrens population histologically and hematologically different than *D. monticola* not in contact with the serpentinite belt?

Overall, this study will increase our understanding of the unique community of the Serpentine Barrens, which contributes to North Carolina's landscape diversity. This study may also provide further insight into salamander integument morphology, a topic for which information is greatly lacking.

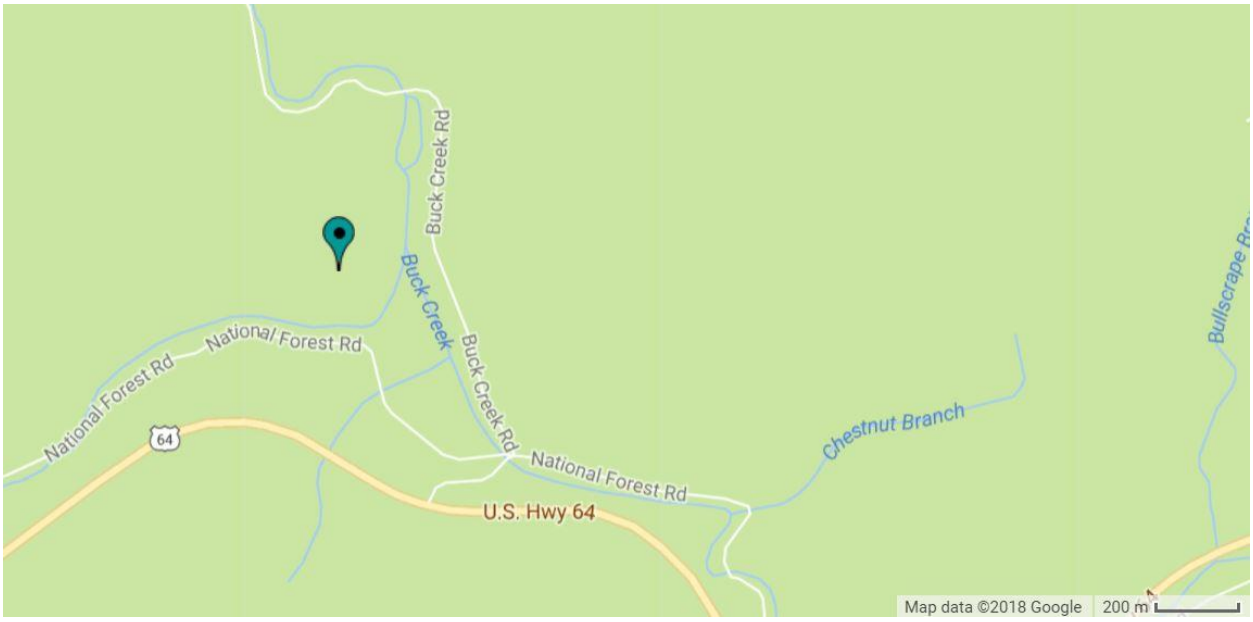


Figure 1. Site map of the Buck Creek Serpentine Barrens.

Figure 2. Observed aberrant coloration of Desmognathus monticola at the Buck Creek Serpentine Barrens, North Carolina.

CHAPTER ONE: HEMATOLOGICAL AND HISTOLOGICAL ASSESSMENT OF THE BUCK CREEK SERPENTINE BARRENS SEAL SALAMANDERS

Introduction

The Buck Creek Serpentine Barrens, Macon Co., North Carolina contain a tributary stream where trace metals naturally occur (Worthington, 1964; Figure 1). The Serpentine Barrens are a notably harsh environment for a Seal Salamander due to the lack of suitable terrestrial habitat, canopy cover, and food resources (Harmon, *personal observation*). Despite the seemingly unfavorable conditions of this habitat *Desmognathus monticola* (Seal Salamanders), *Eurycea wilderae* (Blue Ridge Two-lined Salamander), *Desmognathus ocoee* (Ocoee Salamander), and *Gyrinophilus porphyriticus* (Spring Salamander) larvae have been observed within this stream (Harmon, *personal observation*). Of the four species that have been found in the Barrens stream, all are colored normally except for *D. monticola*. The *D. monticola* found within the Buck Creek Serpentine Barrens are covered in bright yellow patches on their head, dorsum, appendages, and tail (Figure 2). One possibility is that the aberrant coloration of these salamanders is caused by the presence of trace metals in the stream.

The trace metals are emitted into the stream by weathering of the serpentinite rocks within the stream bed (Worthington, 1964). Some of the trace metals that can be found within the stream are aluminum, chromium, iron, magnesium, molybdenum, and nickel (Harmon, Appendix 1). The *D. monticola* that inhabit the Barrens stream are naturally exposed to these trace metals while in the water and while burrowed in the stream sediment. The trace metals in the water and the compounds of these metals are available for absorption into the body of the salamanders (Goyer & Clarkson, 1996). The trace metals in solution can be toxic to the salamanders that inhabit the Barrens stream and may explain the unusual coloration.

Aluminum is not typically found in a free or highly soluble state but can be consumed through dietary absorption (Goyer & Clarkson, 1996). Aluminum can cause behavioral alterations, which could explain the altered behaviors exhibited by this salamander population, such as basking in stream pools during the day as opposed to hiding under a cover object (Goyer & Clarkson, 1996; Harmon, *personal observation*). Chromium is a metal that precipitates and is deposited in the water, which can cause skin irritation and color change in invertebrates (Goyer & Clarkson, 1996). Excess iron can cause liver damage as well as mucosal cell damage in amphibians (Goyer & Clarkson, 1996). Magnesium is typically not easily absorbed by organisms but can cause central nervous system depression and decreased endocrine activity (Goyer & Clarkson, 1996). Magnesium could also explain the atypical behavior of the Barrens salamanders, or the decreased endocrine activity it causes could prevent the animals from properly detecting the stress stimuli of their habitat (Goyer & Clarkson, 1996). Molybdenum is easily absorbed into the tissues (such as blood), as well as organs made of tissues (such as the liver) (Goyer & Clarkson, 1996; Lefcort et al., 1998). Furthermore, this trace metal causes skin irritation and can induce color change due to this irritation (Goyer & Clarkson, 1996; Lefcort et al., 1998). Molybdenum has caused the induction of yellow coloration in plants, as well as macroinvertebrates, such as crayfish and mollusks (Lefcort et al., 1998). There is a molybdenum deposit within 3 km upstream of the Buck Creek Serpentine Barrens, which could contribute molybdenum to the Barrens stream and could be responsible for the aberrant coloration the resident population of *D. monticola*. Nickel is easily absorbed through the skin, and can induce contact dermatitis reactions, which could contribute to the patchy coloration of the *D. monticola* inhabiting the Barrens (Goyer & Clarkson, 1996).

The literature suggests that the salamanders' presumed frequent contact with these metals, and the stress that metal toxicity can induce, could cause histopathological and immunological changes in the liver, blood, and other tissues (Medina et al., 2016). The liver is a target organ in which trace metals tend to accumulate, which increases the size and mass of the liver (Medina et al., 2016). The immunological response to metal toxicity Results in tissue inflammation, such as inflammation of the skin and liver (Medina et al., 2016). Leukocyte profiles can be used to identify stress in amphibians, including toxicity stress or environmental stress (Davis et al., 2008). I expected the *D. monticola* from the Barrens to have enlarged livers due to trace metal accumulation, and to display tissue inflammation due to heavy metal toxicity. Furthermore, I expected to observe a high proportion of macrophages, agranular leukocytes, and thrombocytes relative to lymphocytes in the Barrens salamanders, compared to other salamander populations. I conducted histological sampling to search for signs of metal toxicity and a stress response in the salamanders. I also looked for the presence or absence of yellow pigment within the skin of the salamanders. I also wanted to determine if the Barrens salamanders were more yellow or less pigmented than the surrounding control populations.

Overall, I expected that trace metal accumulation would increase liver mass, that inflammation would occur due to trace metal exposure, and that the leukocyte profile of these salamanders would exhibit signs of stress. I predicted that the Barrens salamanders were aberrantly colored due to an accumulation of metals that induced yellow pigment expression.

In this chapter, I use hematology and histology to determine if the aberrant coloration of the *D. monticola* in the Buck Creek Serpentine Barrens is a result of metal toxicity, or other stressors in the Barrens environment. I observed the tissues and organs of this population to determine if there were any clinical signs of heavy metal toxicity, which could be indicated by

organomegaly and connective tissue inflammation (Medina et al., 2016). I predicted that if the Barrens salamanders had a greater proportion of lymphocytes, macrophages, non-granulated leukocytes, and thrombocytes than the control salamanders, then the aberrant coloration may be the result of an immunological response to the external stimuli of the Barrens environment (Davis et al., 2008). External stimuli have been suggested to alter coloration through a brightening reaction, which causes a color change downstream (Boyer & Swierk, 2017). For example, Anolis lizards are known to brighten their body coloration when exposed to an environmental stimulus (Boyer & Swierk, 2017). Examples of external stimuli include temperature, light, pollutants, or metals in the case of the Buck Creek Serpentine Barrens (Boyer & Swierk, 2017). Lastly, I was able to determine whether this aberrant coloration was an expression of pigment, or a lack of pigment.

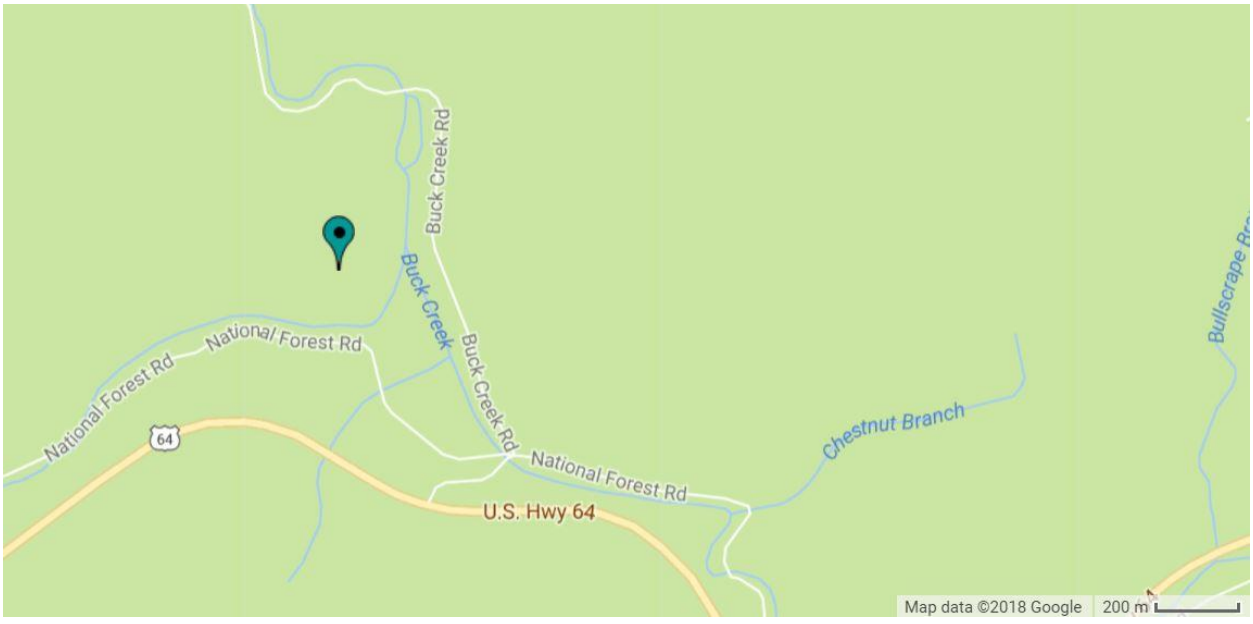


Figure 1. Map of the Buck Creek Serpentine Barrens, Macon County, North Carolina.

Figure 2. Observed aberrant coloration of Desmognathus monticola at the Buck Creek Serpentine Barrens, North Carolina.

Methods

Differential Counts of Lymphocytes

Blood collection from 10 individuals (5 non-Barrens individuals and 5 Barrens individuals) was conducted at the University of Tennessee at Knoxville under the supervision of Dr. Debra Miller and Dr. Rebecca Hardman. The non-Barrens (control) salamanders were collected from a stream near the Buck Creek Serpentine Barrens that did not come in contact with the serpentinite belt, and the Barrens salamanders were collected from the Buck Creek Serpentine Barrens stream. All salamanders were collected one day previous to blood collection.

Each salamander was euthanized using a 30% benzocaine gel, which was applied directly to the head. After 15 minutes, each individual was checked for movement, heart beats, and breathing. The euthanized individual was placed flat on its back on a sterile surface. A total volume of 1 mL of blood was collected from the heart using a syringe. The blood collected was used to create blood smear slides by dropping the collected blood onto a clean microscope slide and smearing the blood with a second clean microscope slide. The blood smears were air-dried and stained using VETONE™ Rapid Differential Stain Kit (180 mL). Following staining, the white blood cells were identified and counted at 1000x using oil immersion. The proportion of lymphocytes, macrophages, agranular leukocytes, and thrombocytes was determined after counting 50 leukocytes, and calculating the relative number of white blood cell types divided by the relative number of leukocytes (Davis & Durso, 2009). If the Barrens salamanders exhibit a high proportion of these types of white blood cells when compared to the control salamanders, these data would suggest a greater immunological response in the former.

Histopathological Assays

The same 10 individuals (5 non-Barrens individuals and 5 Barrens individuals) were used for histopathological assays. Each individual was weighed previous to blood collection, and a total mass (g) was recorded. Following blood collection, the liver was removed from each salamander and weighed (g). A full necropsy of each individual was then performed, and all parts of the organisms were fixed within cassettes. The cassettes were then cast in wax, and slides were made using a microtome. The histological slides were stained using Diff-Quik™. Each slide was observed for any signs of organ enlargement, tissue inflammation, and the amount of melanization. The dermal sections were from the head, dorsum, appendages, and tail of the salamanders. All transverse sections of the salamanders were counted by splitting the section into quadrants and determining a percent coverage for each part of the quadrant, then

determining the average melanin coverage per section per individual. This average was pooled with the melanization amount on all other skin sections from the same organism. Non-transverse sections were viewed 20 μm at a time, and the amount of areas missing melanin along an entire section of skin was counted. Then, the average coverage overall of any given individual from the control population and Barrens population was determined.

Statistical Analyses

A partitioned G-test of homogeneity was used to evaluate if there were any differences in while blood cell distribution among all individual salamanders, among the individuals within the two groups of salamanders, and between the control salamanders and Serpentine Barrens salamanders (R Core Team, 2016; Hervé, 2018). A significant difference ($p \leq 0.05$) between the two groups would indicate an immunological difference between the two populations.

Welch's t-test was used to evaluate if there was a difference in liver mass between the control salamanders and the Barrens salamanders. An ANCOVA was used to analyze the percent body mass that was liver mass amongst the two populations.

The average percent pigmentation of the control group versus the Barrens group was also compared using Welch's t-test to determine if there was a difference ($p \leq 0.05$) in pigmentation between the two groups.

Results

Differential Counts of White Blood Cells

There was a significant difference in the distribution of types of white blood cells among all individual salamanders ($p < 0.00001$, Table 1). Furthermore, there was significant variation amongst all Barrens individuals, as well as all control salamanders ($p < 0.00001$, Table 1). However, there was no significant difference in distribution of types of white blood cells of the Barrens salamanders versus the control salamander population ($p = 0.7899$, Table 1).

Table 1. Summary of partitioned G-test of homogeneity of types of white blood cells of all salamanders, all Barrens salamanders, all control salamanders, and between the Barrens and control salamanders.

Comparison of Salamander Source	**G**	**df**	***p*-value**
Among all salamanders	137.9	27	< 0.00001
Among Barrens salamanders	81.18	12	< 0.00001
Among control salamanders	55.72	12	< 0.00001
Between Barrens and Control groups	1.01	3	0.7988

Histopathological Assays

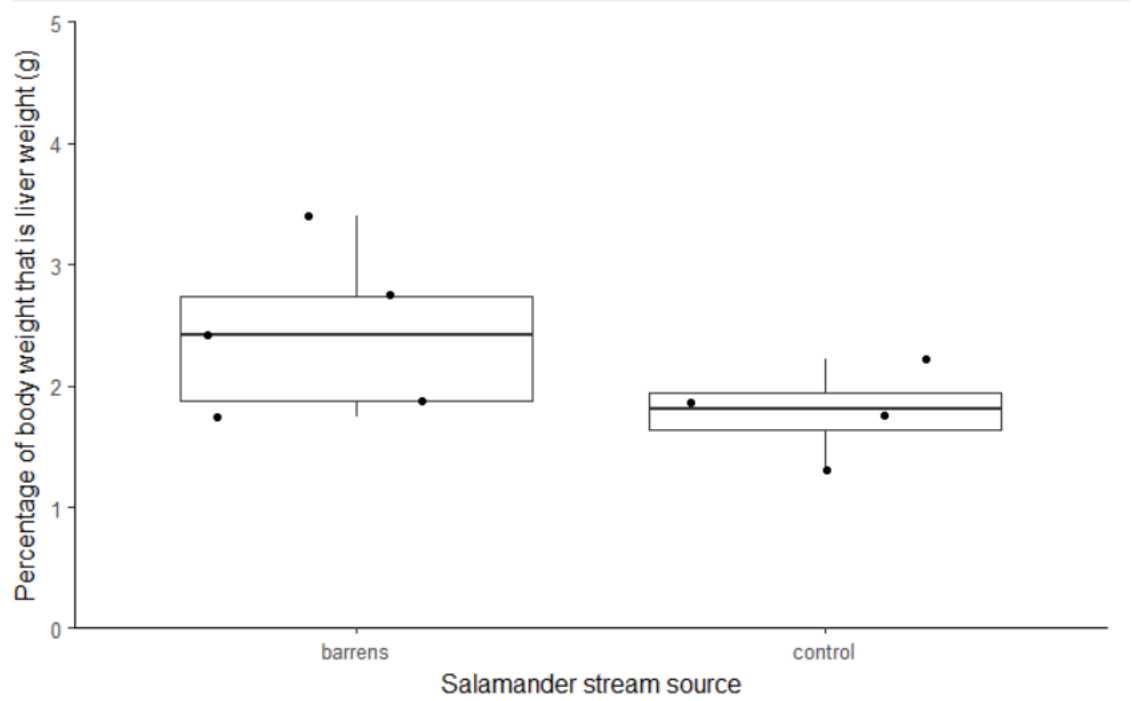
There was no significant difference in liver mass between the two groups of salamanders ($p = 0.4545$, Figure 3; Table 2). Furthermore, there was no evidence of other organs being enlarged, or of inflammation of connective tissue. Based upon visual inspection at 1000x, the

Serpentine Barrens salamanders also did not contain any carotenoids within their dermal sections, and appeared to be lacking melanin altogether in some areas of their skin.

The control salamanders had an average of 69% pigmentation of pooled dermal sections per individual, whereas the Barrens salamanders had an average of 40% pigmentation of pooled pigmentation of all dermal sections per individual (Figures 4 A & 4 B). The Serpentine Barrens salamanders had significantly less pigmented integument than the control salamanders ($p = 0.0001$, Figure 5).

Table 2. An ANCOVA of percent body mass that is liver mass for the Serpentine Barrens and control salamander populations.

	df	**Sum sq**	**Mean sq**	**F**	**P (>F)**
Salamander source	1	0.10550	0.10550	0.7437	0.4170
Log10(body mass [g])	1	0.27722	0.27225	1.9192	0.2085
Residuals	7	0.99296	0.14185		



/Figure 3. The average percentage of body mass (g) that is liver mass (g) of control and Serpentine Barrens salamanders.

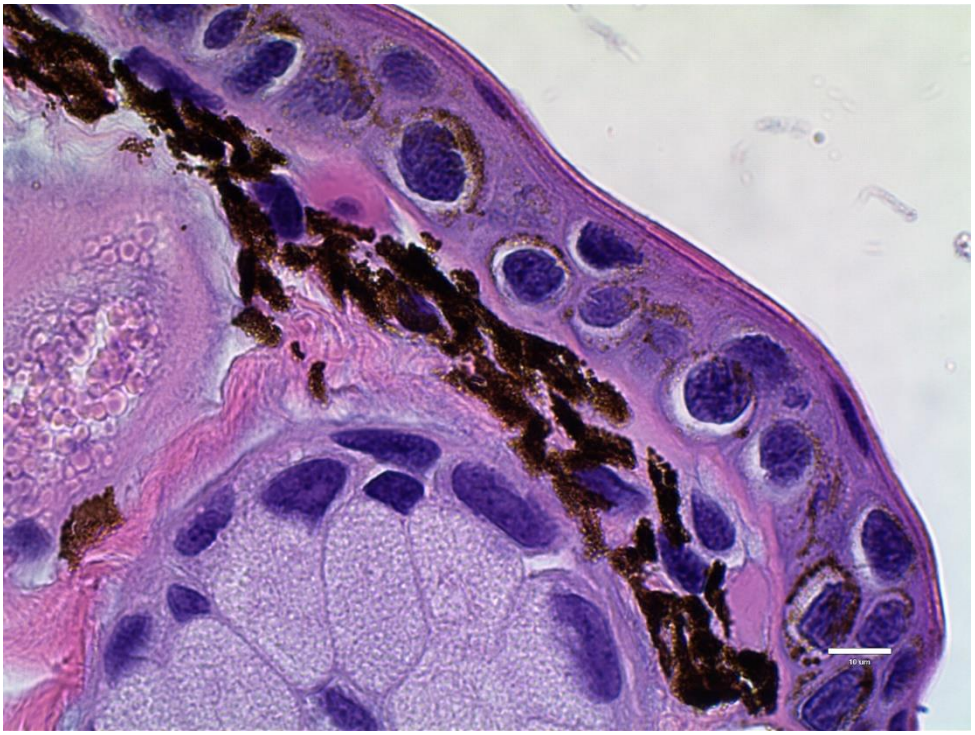


Figure 4 A. A histological section of the epidermis, dermis, subcutis, connective tissue, muscle, and mucous glands of a Desmognathus monticola from a non-Barrens (control) population. Note the lack of inflammation in the integument and connective tissue, and the contiguous layer of melanin.

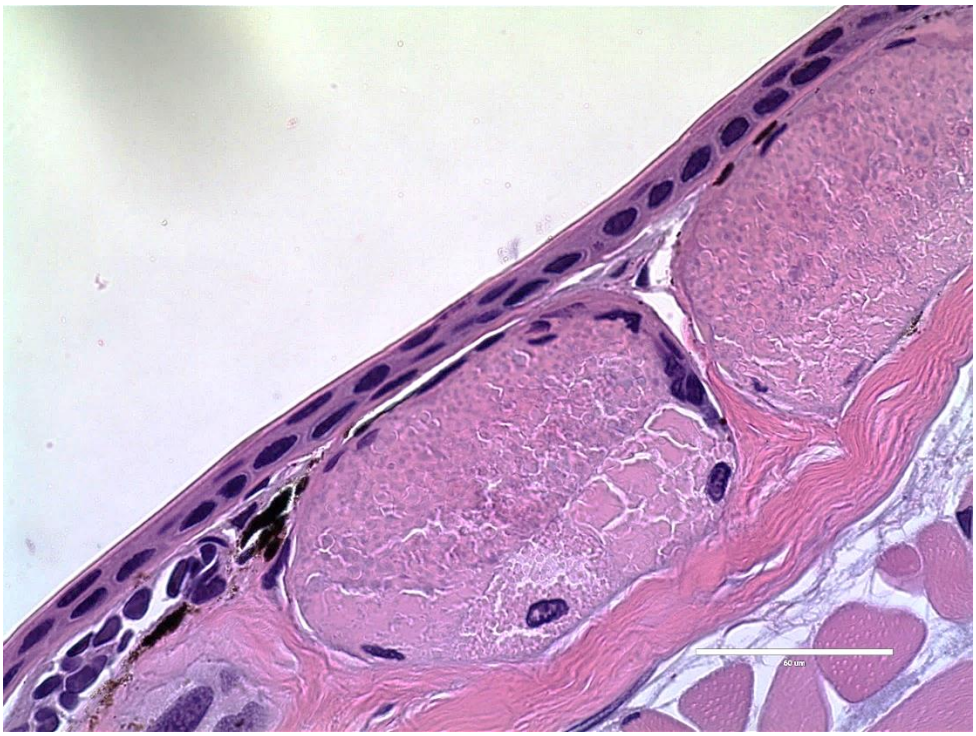


Figure 4 B. A histological section of the epidermis, dermis, subcutis, connective tissue, muscle, and mucous glands of a Desmognathus monticola from the Buck Creek Serpentine Barrens population. Note the lack of inflammation in the integument and connective tissue, as well as the large portions of integument lacking melanin.

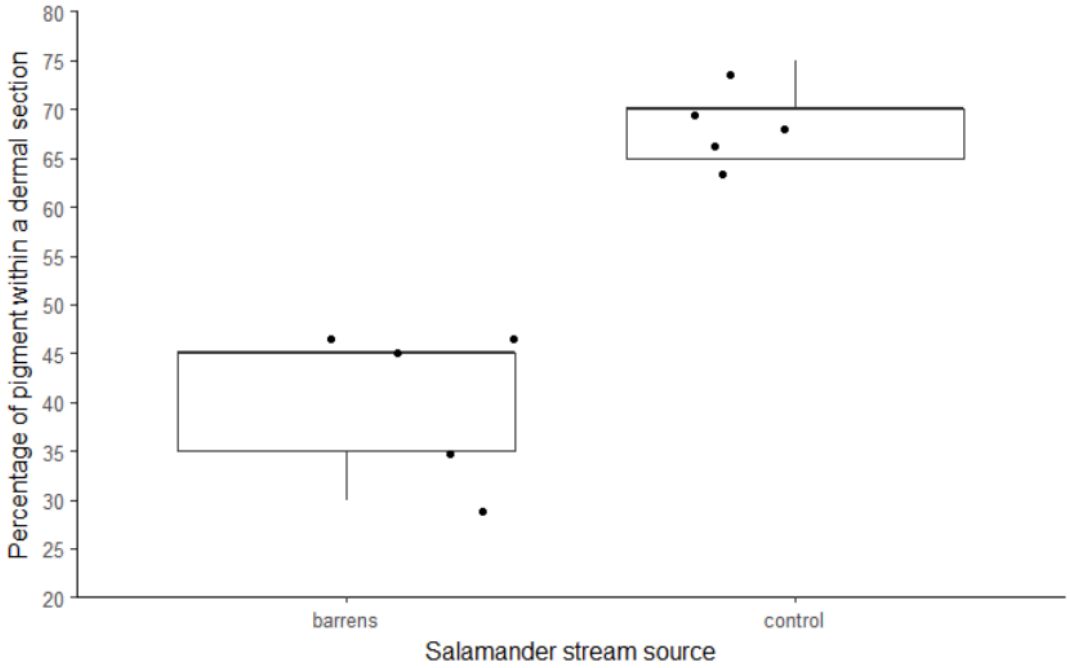


Figure 5. The average percentage of the dermis that is pigmented with melanin within a dermal section of a control salamander versus a Serpentine Barrens salamander.

Discussion

Due to the expectation of high concentrations of various trace metals in Serpentine Barrens, I was surprised to find that the salamanders did not exhibit signs of heavy metal toxicity or stress (Rajakaruna et al., 2009). High concentrations of trace metal accumulation have been recorded within plants growing near the Buck Creek Serpentine Barrens (Pollard, 2017). Perhaps the lack of metal accumulation and lack of stress response to trace metals may provide explanations of how this population can persist, despite the prediction that no organism could survive the harsh conditions of the Buck Creek Serpentine Barrens (Duke Rankin, *personal communication*). Could it be that the trace metals are not bioavailable to the salamanders due to being too tightly bound to the substrate (Goyer & Clarkson, 1996)? Are the trace metals that are present not easily assimilated into the body of an organism (Goyer & Clarkson, 1996)? Are these salamanders particularly adept at filtering metal ions (Haslam et al., 2014)? The lack of evidence for heavy metal toxicity, such as no increase in liver mass and lack of inflammation, was consistent with the Results of my analyses of trace metal concentrations in the Buck Creek Serpentine Barrens (Harmon, Appendix 1). Thus, maybe other habitat stressors or stimuli were responsible for the lack of pigment in the Barrens salamanders.

Before this study, it was assumed that the Barrens salamanders were expressing yellow coloration. The histology revealed that the Barrens salamanders were not producing yellow pigments, or carotenoids. Instead, the salamanders that were in contact with the Serpentine Barrens environment have patches of their dermis that appears to lack pigment altogether. Regarding the significant difference in pigmentation, there were more pigmented areas and a

higher density of melanophores observed within the control salamanders. The Serpentine Barrens salamanders had areas that seemed to be lacking melanophores, and the pigmented areas had a lower density of melanophores within the integument. The Barrens salamanders may exhibit a "color loss," as opposed to the previously hypothesized "color expression." The observed differences in integument pigmentation are most likely due to the different environmental conditions the control salamanders and the Buck Creek Serpentine Barrens salamanders are exposed to. *In vitro* stimulation of melanophores sampled from these salamanders would help to confirm these Results.

Two alternative explanations of the Barrens salamanders' aberrant coloration are 1) energy allocation, and 2) the migration of melanosomes when exposed to light (Van Buskirk, 2011; Blaney, 1952). The salamanders may lack melanin in large portions of their skin, because they allocate energy towards other functions to survive the impact of the trace metals of the Buck Creek Serpentine Barrens (Van Buskirk, 2011). Furthermore, the salamanders may not intake enough energy due to a lack of food, and therefore do not have the resources to produce pigment (Van Buskirk, 2011). The alternative explanation of the "missing" pigment may be that pigment is not absent but has moved and only appears to be missing. The phenomenon of melanosome aggregation within a melanophore occurs when an amphibian is exposed to an excess of light (Sköld et al., 2012). When the melanosomes aggregate there are patches of skin that appear pale under the microscope, but the integument itself appears coppery yellow to the human eye (Yamada & Fujii, 2002). An example of such a light exposed habitat with light substrate color is the Buck Creek Serpentine Barrens, where the salamanders have been observed foraging during the day and basking in the bottom of pools within the stream. I found mixed evidence for this hypothesis (Harmon, Chapter 3).

Overall, I cannot fully rule out stress at this time as a cause of the unusual coloration, because cortisol was not measured. However, the leukocyte profiles are considered good indicators of stress and stress hormone levels, and they did not indicate stress (Davis et al., 2008). It is important to note that responses to stresses and stimuli from an organism's environment can have interspecific and intraspecific variation (Davis et al., 2008). The variation amongst individuals increases biodiversity, and the Buck Creek Serpentine Barrens salamanders are an example of the landscape diversity of North Carolina. Gathering more information about this population will help to preserve North Carolina's organismal diversity and may help to preserve other aberrantly colored salamander populations.

CHAPTER TWO: CRYPSIS & BACKGROUND MATCHING OF BUCK CREEK SERPENTINE BARRENS SALAMANDERS

Introduction

Many organisms alter their color patterns through time as they adapt to their environmental surroundings (Sköld et al., 2012). One purpose of these altered color patterns is to evade detection by predators and prey, known as crypsis (Sköld et al., 2012; Segev, 2009). The ability for an organism to become better at matching their background is dependent upon cellular mechanisms (Rowe et al., 2013). These cellular mechanisms can respond rapidly (physiological color change), or color change may occur during the course of weeks, months, or years (morphological color change; Rowe et al., 2013). During long term stimulus (ex. excess light exposure, or trace metal exposure), the result can be morphological changes as a result of phenotypic plasticity (O'Hanlon et al., 2017). One of the environmental pressures to consider is substrate color, such as the yellowy substrate found at the Buck Creek Serpentine Barrens (Sköld et al., 2012). Salamanders at the Serpentine Barrens may match the light substrate to avoid predation, because these salamanders forage frequently for food, due to the lack of food (Harmon, *personal observation*). These salamanders could benefit from crypsis, in that it would camouflage them from predators during the daylight and at night from nocturnal predators.

The background color an organism lives on is perceived via the retina, and organisms with lateral eyes are most adept to perceive background color and reflectance (Moore & Lofts, 1976). If the organism perceives the background as dark, then the pars intermedia is stimulated to release melanocyte stimulating hormone (MSH), which causes the dispersion of melanosomes within melanophores, so that an organism will appear dark to match the dark substrate (Moore & Lofts, 1976). If the organism perceives the background as light, then the pars intermedia inhibits

the release of MSH or is stimulated to release melanocyte concentrating hormone (MCH), which causes the aggregation of melanosomes to the center of the melanophore. This aggregation allows the organism to appear light and blend with a light substrate (Moore & Lofts, 1976; Sköld et al., 2012).

In this chapter I describe an experiment in which I hypothesized that the Serpentine Barrens salamanders are covered in yellow patches on their skin as a result of background matching. These salamanders can mimic their background by using their lateral eyes to detect the amount of light reflected by their yellowy background (Mueller & Neuhauss, 2014). The background matching of the Barrens salamanders was likely an attempt to camouflage themselves from predators. Furthermore, the Barrens salamanders have been observed to lose their yellow coloration when kept in a lab, on a lab bench, within three weeks (K. Kozak and D. Beamer, *personal communication*). The objective of this experiment was to determine if loss of yellow coloration occurred regardless of background color when the Barrens salamanders are removed from the Barrens stream and placed on a colored background. Or, if Barrens salamanders retain their yellow coloration regardless of background color. This experiment also explored if control salamander populations in the same geographic locale are able to become yellow when exposed to a lighter background color. I hypothesized that control salamanders on a control background would maintain a darker coloration, and that Serpentine Barrens salamanders on a control background would lose their yellow coloration. I also hypothesized that control salamanders would become lighter when placed on the Serpentine Barrens background, and that Barrens salamanders would maintain their yellow coloration or become more yellow when placed on a Serpentine Barrens background. I expected that all salamanders, regardless of source, would become darker on a black background and lighter on a white background. I hypothesized

post hoc that no salamanders would produce yellow or red coloration to match the yellow and red backgrounds (no colored pigments aside from melanin were observed in the histological samples of these salamanders, Chapter 2).

Methods

Stream Bed Substrate Matching

A total of 20 salamanders from the Buck Creek Serpentine Barrens, and 20 salamanders from other (control) streams within the Little Tennessee River drainage in the Nantahala Mountains in Macon County were used for this experiment. All salamanders were collected within two weeks before the experiment began. Each salamander was placed within a plastic container (30 cm x 15 cm x 3.5 cm) ¼ filled with dechlorinated water from the Western Carolina University Water Treatment Plant. Half of the plastic containers housing salamanders from each group (n = 20) were wrapped with custom wrapping paper that was printed with a picture of Serpentine Barrens stream bed substrate, and the other half of the plastic containers were wrapped with wrapping paper that was printed with a picture of stream bed substrate from a control stream. The 40 containers were distributed among 2 lab benches (24 containers on one bench, and 16 containers on the other bench). The benches were divided into blocks with each treatment represented in each block. I used a 2 x 2 factorial treatment design deplored in a randomized complete block experiment design. The containers were rotated within the block three times per week to prevent treatment by block interactions. A 12-hour light: 12-hour dark cycle was scheduled for the lab using full-spectrum lighting. The salamanders were fed bloodworms twice per week and received a 50% water change twice per week. Over the course of three weeks, the salamanders were photographed (following the protocol described below) every third day.

Color Matching

The same protocol was used for this experiment as was used in the substrate matching experiment. There were four treatment backgrounds: black, white, red, and yellow, and there were 10 boxes wrapped in each color for a total of 40 containers. Each treatment group was comprised of 4 Barrens salamanders and 4 control salamanders, on each of the 4 background colors. The 40 containers were divided between two blocks, one block containing 16 containers and the other block containing 24 containers, with each combination of treatment and salamander source represented equally within each block. The containers were rotated three times per week within each block to decrease treatment by block interactions. The salamanders were fed Ocean Nutrition™ bloodworms for freshwater fish twice per week and received a 50% water change twice per week. A 12-hour light: 12-hour dark cycle was scheduled for the lab using full-spectrum lighting. Over the course of three weeks, the salamanders were photographed (following the protocol reported below) every week. I used the photos to determine if loss of the yellow coloration occurs regardless of background color, or if salamanders can lighten or darken their color to match a black/white background, or if salamanders can produce red or yellow coloration to match a yellow/red background. If so, then background matching may be responsible for the aberrant coloration of the *D. monticola* that inhabit the Barrens.

Photography Procedure

Changes in the yellow coloration and luminosity (which is brightness perceived by the human eye), in the laboratory experiments were observed, quantified, and documented. The salamanders were photographed once a week during the light exposure trials using a Cannon EOS 70D DSLR™ camera mounted in an ORtech Professional Lighting Photo Box Plus™. The box captured every photograph at the same distance. Every individual was photographed on a new sheet of black construction paper as the background. A white to gray color standard was

used to ensure trueness and consistency of color in each photograph. I used Adobe Photoshop CC 2018 (19.0) ™ to measure color on the areas of the body that exhibit the "piebald" pattern most frequently (i.e. the head and the dorsum). Four portions of the body were digitally excised (9.6 mm x 9.6 mm), one section of the top of the head, and three sections from the dorsum., The CYMK histogram was used to determine the portion of the pixels of the dark color versus light color (luminosity), as well as the number of pixels in an excised area that exhibit the yellow color. The change in the number of pixels that were dark versus light indicated how much a salamander's color darkened or lightened over time. I determined the portion of the pixels that did, and did not, exhibit the yellow pattern to quantify any changes in the yellow skin coloration throughout the exposure trials.

Statistical Analyses

The overall change in yellow coloration was determined by pooling the values of color change for the head and three sections of the dorsum for each individual. The initial amount and final amount of yellow coloration on each individual was quantified, and the change in yellow coloration was determined by subtracting the initial amount of yellow coloration from the final amount of yellow coloration. The overall change in luminosity for each salamander was determined using the same method. I used a linear mixed effects analysis to determine which factors affected the yellow coloration and luminosity of the salamanders for each background matching experiment ($p \leq = 0.05$) (R Core Team, 2016; Bates et al., 2015; Kuznetsova et al., 2016). The factors that were evaluated for the first crypsis experiment were: (1) salamander source (where the salamander was sampled from), (2) block (which lab bench), (3) stream bed substrate (photograph of control stream bed substrate or Barrens stream bed substrate), (4) the interaction between treatment and block, and (5) the interaction between block and stream bed substrate. The factors evaluated for the second crypsis experiment were: salamander source,

treatment (which background color), block (which area on lab bench), bench, the interaction between treatment and block, the interaction between treatment and bench, and the interaction between block and bench.

Results

In the stream bed substrate matching experiment, salamander coloration and luminosity were not significantly affected by any factors (Tables 3 & 4). Similarly, in the color matching experiment, there were no significant main effects or interactions (Table 5 & 6). Overall, all salamanders became significantly more yellow and increased significantly in luminosity regardless of background color in both experiments (Table 7; Table 8).

Table 3. Summary of ANOVA of change in yellow coloration of salamanders placed on pictures of the bottom of the Serpentine Barrens stream or the control stream.

Factor	df	Sum Sq	Mean Sq	F	P (>F)
Source	1	70.145	70.145	0.3307	0.5691
Treatment	1	171.49	171.49	0.8086	0.3751
Block	1	279.78	279.78	1.3192	0.259
Stream bed substrate	1	124.22	124.22	0.5857	0.4495
Treatment x Block	1	53.025	53.025	0.25	0.6204
Block x Stream bed substrate	1	611.43	611.43	2.8829	0.0989
Residuals	33	6998.9	212.09	NA	NA

Table 4. Summary of ANOVA of change in luminosity of salamanders placed on pictures of the bottom of the Serpentine Barrens stream or the control stream.

Factor	df	Sum Sq	Mean Sq	F value	P (>F)
Block	1	135.85	135.85	1.7753	0.1919
Treatment	1	70.326	70.326	0.9191	0.3447
Source	1	4.5532	4.5532	0.0595	0.8088
Block x Treatment	1	8.6264	8.6264	0.1127	0.7392
Treatment x Source	1	28.826	28.826	0.3767	0.5436
Block x Source	1	3.374	3.374	0.0441	0.835
Residuals	33	2525.2	76.52	NA	NA

Table 5. Summary of ANOVA of change in yellow coloration of salamanders placed on yellow, red, white, or black backgrounds.

Factor	df	Sum Sq	Mean Sq	F value	P (>F)
Treatment	3	1357.2	452.39	1.7667	0.1773
Block	1	501.38	501.38	1.958	0.1731
Bench	1	52.156	52.156	0.2037	0.6554
Treatment x Block	3	1096	365.32	1.4266	0.2567
Treatment x Bench	3	854.56	284.85	1.1124	0.3613
Block x Bench	1	16.87	16.87	0.0659	0.7994
Residuals	27	6913.9	256.07	NA	NA

Table 6. Summary of ANOVA of change in luminosity of salamanders placed on yellow, red, white, or black backgrounds.

Factor	df	Sum Sq	Mean Sq	F value	P (>F)
Treatment	3	259.28	86.427	1.4678	0.2455
Block	1	78.62	78.62	1.3352	0.258
Bench	1	38.523	38.523	0.6542	0.4257
Treatment x Block	3	292.36	97.454	1.655	0.2002
Treatment x Bench	3	205.56	68.52	1.1637	0.3418
Block x Bench	1	1.6802	1.6802	0.0285	0.8671
Residuals	27	1589.8	58.883	NA	NA

Table 7. Average change in yellow coloration and luminosity of salamanders used in background matching experiments.

Crypsis Experiment	Average Overall Change in Yellow Coloration	Average Overall Change in Luminosity
Stream bed substrate matching	-20.26	9.679
Color matching	-10.87	5.207

Table 8. A statistical analysis of average yellow coloration and luminosity changes of salamanders in background matching experiments using a one sample t-test.

Experiment	Measurement	t	df	p
Stream bed matching	Yellow Coloration	-18.001	40	<2.2e-16
Stream bed matching	Luminosity	14.878	40	<2.2e-16
Color matching	Yellow Coloration	-8.4758	40	9.06E-11
Color matching	Luminosity	8.4931	40	8.59E-11

Discussion

All salamanders in this study became lighter and more yellow but failed to match the background color in both experiments. For this chapter, I used "yellow coloration" as an operational measurement of the patches of the body that exhibited yellow coloration even though these were areas of the body lacking pigment (these salamanders did not produce yellow pigment, but had patches of skin void of melanin, Harmon, Chapter 2). I expected that background color would predict an individual's color change and luminosity, but this did not occur. From this study I conclude that the *D. monticola* within the Serpentine Barrens did not become lighter and more yellow in the Barrens in an attempt to match the background coloration of their habitat.

The Results of this study were unexpected, because background matching has been observed in fish and amphibians. Typically, organisms with lateral eyes can adapt to their background by detecting the ratio of visible light above and below the individual via dorsoventral retinal input, and by detecting the amount of light reflected by the background of the habitat via dorsal retinal input (Mueller & Neuhauss, 2014). These two inputs are interpreted by the brain, and the body is signaled to release melanin stimulating hormone (MSH), or melanin concentrating hormone (MCH), which determines the pigment dispersal within the dermis of an individual (Mueller & Neuhauss, 2014). In this case, the amphibians did not match their background color.

Spearman (1973) argued that many amphibians can change color but are not well adept at emulating background patterns, in that their color matching and pattern matching abilities are limited to a small range of pigment changes. Perhaps the color changes and increased luminosities in this study were due to the excess light exposure (since the salamanders were not

provided cover objects; as a tradeoff to survive in a harsh environment or to become cryptic (Van Buskirk, 2011). The increased color change and luminosity may have been an attempt to prevent damage from light radiation. Some organisms can undergo melanosome dispersion and aggregation to prevent UV-light damage, in that a lighter coloration could reflect more light to prevent more light absorption (Mueller & Neuhauss, 2014). Organisms, such as lizards, have been observed changing their coloration when short term environmental conditions are harsh (Cote et al., 2010). The stress of living in harsh conditions can increase oxidative stress and corticosterone levels, which can alter the expression of pigmentation (Cote et al., 2010). It was possible that the Serpentine Barrens salamanders altered their pigmentation as a stress response to the harsh conditions of their habitat. The Buck Creek Serpentine Barrens stream contained trace metals, was highly exposed to sunlight, and was lacking in food resources. Such speculations cannot be confirmed at this time, in that corticosterone levels were not measured before and after the experiment (however, stress levels were estimated via leukocyte profiles; Harmon, Chapter 1). Regardless of the lack of crypsis, this unique population has overcome the harsh conditions of the Barrens by altering the pigmentation of their bodies.

CHAPTER THREE: CAN LIGHT AND Ca^{2+} EXPOSURE CAUSE DEPIGMENTATION IN SEAL SALAMANDERS?

Introduction

Color change as a result of phenotypic plasticity has been observed in a variety of taxa, and often is the result of adaptation to environmental stimuli (Sköld et al., 2012). Furthermore, the coloration of ectotherms is a particularly plastic response and can be adjusted at the individual level via physiological and morphological changes (Sköld et al., 2012). The *Desmognathus monticola* present at the Buck Creek Serpentine Barrens, Macon County, North Carolina exhibit yellow "piebald" coloration caused by a lack of, or reduced dispersion of, melanin in the dermis (Aberrant coloration of *D. monticola*, Chapter 1). This coloration is unusual, in that *D. monticola* typically has a brown dorsum with dark markings, and a pale venter (Powell et al., 2016). These salamanders also have been found to express this yellow coloration in captivity under some conditions (observed in my background matching experiments after less than 3 weeks, Chapter 2). One common factor between the Serpentine Barrens and the laboratory was the greater exposure to light compared to typical Seal Salamander habitats. The long term, excessive light exposure at the Barrens stream was due to the surrounding pine savannah habitat which provided sparse canopy cover for the *D. monticola* living within the stream. The increased light exposure, coupled with the calcium concentrations within the stream (0.437 mg/, Harmon, Appendix 1), may have induced melanosome aggregation in the Barrens salamanders as an adaptation to intense light in their habitat to reflect light away from their bodies (Yamada & Fujii, 2002).

Physiologists are often interested in categorizing and quantifying the form and composition of pigment cells, and how these cells affect color change when stimulated (Moore &

Lofts, 1976). There are three primary types of chromatophores, which are cells that contain specific pigments, which include: melanophores (brown or black pigment cells), iridophores (pigment cells that are reflective), and xanthophores and erytrhophores (cells which can produce a range of yellows and reds; Moore & Lofts, 1976). Each of these chromatophore types respond to physiological agents, such as hormones, which can induce either physiological color change or morphological color change (Moore & Lofts, 1976). Morphological color change occurs over a long time (days to years) after stimulation and is a result of an altered amount of pigment contained within the chromatophores (Moore & Lofts, 1976). Physiological color change over a short period (hours to days), and is the result of "rapid intracellular displacement of pigments" (increase of yellow dorsal coloration was observed within three weeks, Chapter 3; Moore & Lofts, 1976).

Physiological color change can be quickly induced, but is a transitory response, which means the organism, when triggered by a stimulus (such as light), can revert to its original color after having reached an altered color condition (Moore & Lofts, 1976). The rapidity of this response is possible via melanosome (a melanin-containing vesicle) dispersion and aggregation within a melanophore (Meuller & Neuhauss, 2014). The movement of melanosomes within melanophores contributes to the hue of an organism's skin because the location of melanosomes within a melanophore determines the darkness or paleness of an organism's skin (Blaney, 1952). When melanosomes are dispersed throughout a melanophore an organism will appear darkly colored, and when melanosomes are centrally aggregated within a melanophore an organism will appear pale, or "coppery" colored (Blaney, 1952). The melanosomes are transported within the dermal pigment cell by microtubule tracks and actin filaments, which are controlled by different molecular motors (Meuller & Neuhauss, 2014). This body color changing phenomenon is plastic

in ectothermic organisms and the color change amount can vary dependent upon the individual (Sköld et al., 2012). However, the presence of extracellular Ca^{2+} increases the rate of melanosome aggregation within a melanophore, and this effect can be induced using $CaCl_2$ (Yamada & Fujii, 2002).

The objective of this experiment was to determine if light exposure can alter the skin coloration of *D. monticola* from the Barrens population, as well as in control salamanders from surrounding populations. I explored if this physiological process was expedited in the presence of excess calcium in the water. I hypothesized that all salamanders would be most yellow and most luminous when not shaded from the light (0% shade), and treated with 0.437 mg/L calcium solution (0.437 mg/L was the average calcium concentration of the Barrens stream; Harmon, *unpublished data*). I also hypothesized that all salamanders would be least yellow when most shaded from light exposure (90% shade), and were not treated with calcium solution (Van Buskirk, 2011).

Methods

Light Exposure in the Presence/Absence of Ca2+

A total of 18 salamanders were collected from the Buck Creek Serpentine Barrens, Macon Co., NC, along with 18 salamanders from populations from other streams within the Nantahala Mountains in Macon County, within the Little Tennessee River basin. I used a 2 x 2 x 3 factorial treatment design deplored in a randomized split-plot experimental design. The factors evaluated in this experiment were salamander source (control vs. Barrens), calcium exposure (calcium present, or not present), and shade amount (0%, 50%, or 90%). Each salamander was placed within a small plastic GladWare™ round 250 mL plastic container within a larger plastic container (30 cm x 15 cm x 3.5 cm). The 36 small containers were filled with dechlorinated tap water. I added calcium chloride (0.437 mg/L) to the water used to fill half of the small

GladWare™ round 250 mL plastic containers. The 18 large containers were distributed among three shelves (6 containers per shelf, with 2 small GladWare™ round 250 mL plastic containers per large container). Each shelf had a Grower's Supply Company 120 Volt full-spectrum grow light, to simulate sunlight, hanging 37 cm above each container. Each large container was covered with no shade cloth, an Agfabric Breathable Mesh™ 50% shade cloth (to simulate the amount of light exposure at the Serpentine Barrens), or a Shatex™ high density (140GSM) 90% shade cloth (to simulate the amount of light exposure at control sites found within more heavily shaded areas; Table 8). Within each large container, one salamander was treated with the calcium solution, and the other salamander was not. Each shelf contained 3 large containers with Serpentine Barrens salamanders and 3 larger containers with control salamanders. The shelves were also shrouded using a black cloth to ensure that no excess light entered into the enclosures from lighting in the lab. The salamanders were fed Ocean Nutrition™ bloodworms for freshwater fish twice a week and had a scheduled 12-light: 12-dark cycle. Water changes were performed three times per week and were 100% water changes to ensure that calcium ion levels remained as consistent as possible, while avoiding excessive stress to the study organisms. The experiment was conducted over the course of three weeks.

Photography Procedure

The salamanders were photographed once a week during the light exposure trials using a Cannon EOS 70D DSLR™ camera mounted in an ORtech Professional Lighting Photo Box Plus™. This arrangement insured that every photograph was taken at the same distance from the subject. Each individual was photographed on a new sheet of black construction paper as the background. A X-rite ColorChecker® was used to ensure trueness and consistency of color in each photograph. I used Adobe Photoshop CC 2018 (19.0) ™ to measure color on the areas of the body that exhibit the "piebald" pattern most frequently (i.e. the head and the dorsum). Four

portions of the body were digitally excised (9.6 mm x 9.6 mm), one from the base of the head and three sections from the dorsum. The CYMK histogram was used to determine the portion of the pixels of the dark color versus light color (luminosity), as well as the number of pixels in an excised area that exhibit the yellow color. The change in the number of pixels that were dark versus light indicated how much a salamander's color darkened or lightened over time. Determining the portion of the pixels that did, and did not, exhibit the yellow pattern quantified any changes in the yellow skin coloration throughout the exposure trials. The overall change in yellow coloration was determined by pooling the values of color change for the head and three sections of the dorsum for each individual.

Statistical Analysis

The change in yellow coloration was determined by subtracting the initial amount of yellow coloration from the final amount of yellow coloration. The overall change in luminosity for each salamander was determined using the same method. A linear mixed effects model was used, and the model factors were evaluated using an Type III SS, ANOVA with Satterthwaite approximation for degrees of freedom, to determine which factors impacted the yellow coloration and luminosity of the salamanders ($p \leq = 0.05$; R Core Team, 2016; Bates et al., 2015; Kuznetsova et al., 2016). The fixed effects that were evaluated in the model were: shade (0%, 50%, or 90%), calcium (no solution versus solution exposure), and source (which stream the salamander was sampled from), and the random factors were blocks.

Results

Overall, all salamanders became more yellow and increased in luminosity (Table 10). The overall change in yellow coloration was affected by the salamander source– the control salamanders had a greater increase in yellow; ($p = 0.0043$) – and a significant three-way interaction

of shade, calcium, and source (p = 0.0304; Table 11). There Serpentine Barrens salamanders overall change in yellow was affected by the salamander source as well (p = 0.0043) but did not have as great an increase in yellow coloration as the control salamanders (Table 11). The overall change in yellow coloration of the Barrens salamanders was also affected by a significant three-way interaction of shade, calcium, and source (p = 0.0304; Table 11). There were opposite responses observed at the 0% shade level for the control salamanders in terms of yellowness (Figure 6). At the 0% shade level with calcium, the control salamanders became more yellow in color, but without calcium the salamanders had a lesser increase in yellow (Figure 6). At the 50% shade level the control salamanders treated with calcium became less yellow over time than the control salamanders not treated with calcium (Figure 6). No factors significantly affected the overall change in luminosity of the salamanders, with the exception of a marginally significant salamander source effect (p = 0.0709; Table 12; Figure 7).

Table 9. Average UV photometer reading for 0 %, 50%, and 90% shade levels.

Shade level (%)	Mean UV	SD (± UV)
0	87	1.5
50	54	1.5
90	44	1.5

Table 10. Average change in yellow coloration and luminosity of salamanders when exposed to light and calcium over three weeks.

Measurement	Average Change	t	df	p
Yellow Coloration	39.995	-12.347	35	1.29e-14
Luminosity	19.4493	12.077	35	2.44e-14

Table 11. Summary of ANOVA of change in yellow coloration of salamanders from different sources (Serpentine Barrens or control streams) when exposed to three levels of shade, and with or without calcium addition.

Factor	Sum Sq	Mean Sq	NumDF	DenDF	F value	P (>F)
Shade	302.7	151.3	2	33	0.804	0.4561
Calcium	10.39	10.39	1	33	0.055	0.8157
Source	1770	1770	1	33	9.403	0.0043
Shade x Calcium	826.2	413.1	2	33	2.195	0.1274
Shade x Source	58.8	29.4	2	33	0.156	0.856
Calcium x Source	279.9	279.9	1	33	1.487	0.2313
Shade x Calcium x Source	1464	732.1	2	33	3.889	0.0304

Table 12. Summary of ANOVA of change in luminosity of salamanders from different sources (Serpentine Barrens or control streams) when exposed to three levels of shade, and with or without calcium addition.

Factor	Sum Sq	Mean Sq	NumDF	DenDF	F value	P (>F)
Shade	129.4	64.68	2	33	1.134	0.3338
Calcium	0.25	0.25	1	33	0.004	0.9478
Source	198.6	198.6	1	33	3.483	0.0709
Shade x Calcium	108.7	54.32	2	33	0.953	0.396
Shade x Source	60.64	30.32	2	33	0.532	0.5925
Calcium x Source	2.64	2.64	1	33	0.046	0.8309
Shade x Calcium x Source	244.1	122	2	33	2.14	0.1337

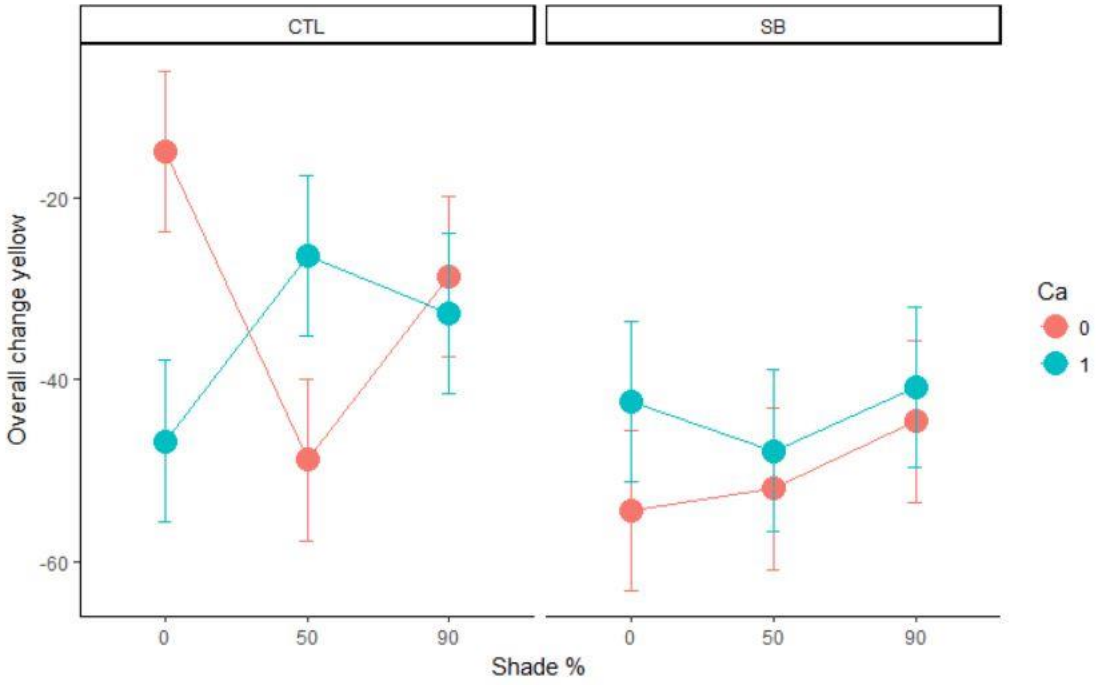


Figure 6. The overall change in yellow coloration of control salamanders (CTL) versus Serpentine Barrens Salamanders (SB) in relation to amount of shade (%) and calcium. The values that are closer to -60 indicate salamanders that became more yellow in coloration throughout the experiment, and the values closer to -20 indicate salamanders that did not change as much in yellow coloration throughout the experiment.

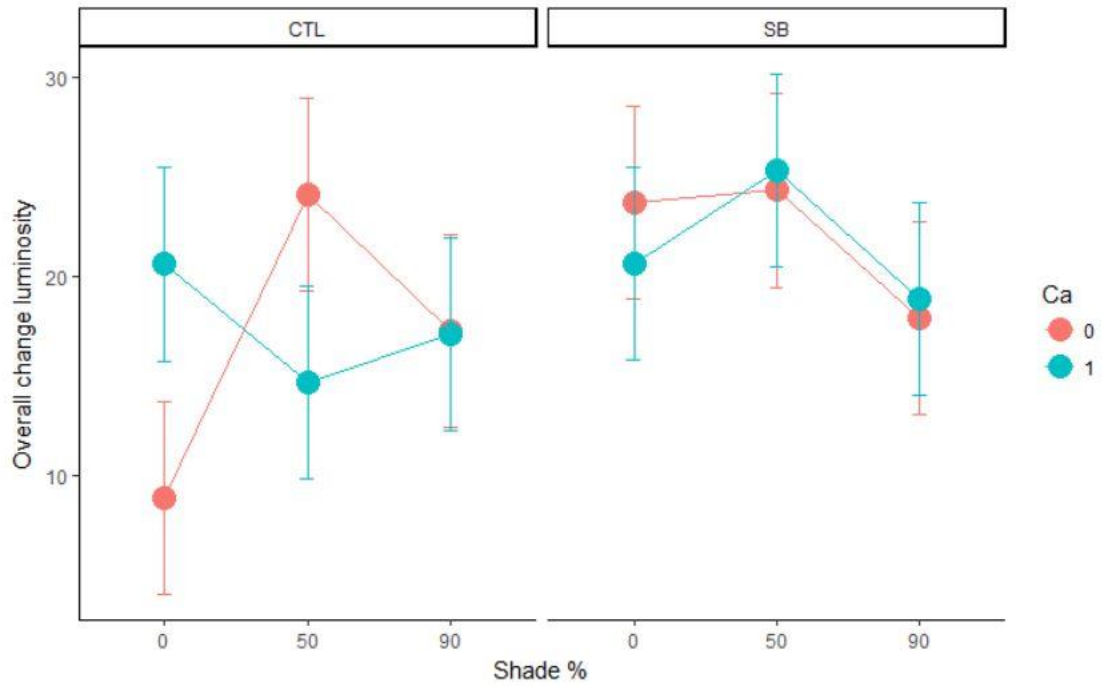


Figure 7. The overall change in luminosity of control salamanders (CTL) versus Serpentine Barrens Salamanders (SB) in relation to amount of shade (%) and calcium. The values that are closer to 30 indicate salamanders that became more luminous throughout the experiment, and the values closer to 10 indicate salamanders that did not change as much in luminosity throughout the experiment.

Discussion

The Results of this experiment were unexpected and did not support any of my hypotheses. For this chapter, the yellow patches on the body were measured operationally as "yellow coloration," and will be discussed as such (Harmon, Chapter 2, found that the "yellow" patches on the body were actually due to lack of melanin in parts of the dermis). Furthermore, the salamanders in this experiment became uniformly more yellow in coloration, rather than yellow in patches like the wild population, but this aspect was not measured. It was interesting that all salamanders became more yellow and brighter regardless of their starting coloration, with no consistent effect of shading. I expected only the salamanders in 0% shade, which were treated

with calcium, to become more yellow and more luminous. It is possible that all salamanders became yellower and lighter due to being exposed to consistent light for 12 hours a day over the course of 3 weeks, which is more consistent light exposure than these salamanders would experience in nature.

Neither light level nor calcium alone explained the variation in changes in yellow coloration or luminosity in this experiment. Changes in yellowness were affected by a complex three-way interaction between these two factors and salamander source. There were no linear responses to calcium in that calcium had different effects at different light levels. I expected calcium to facilitate the process of melanosome aggregation when the salamanders were exposed to light (Yamada & Fujii, 2002). But, calcium suppressed this reaction at some light levels. For example, it was puzzling that the control salamanders became more yellow at the 0% shade level with calcium, but not at the 50% shade level. One possible explanation of this unusual response is that all individuals had a long acclimation period in the lab. Long acclimation periods can confound an organism's physiological changes when used in an experiment, such as how that organism responds to stimuli (Obernier & Baldwin, 2006). In contrast, the Barrens salamanders had the opposite reaction to calcium at the 0% shade level (Figure 6). Two possible explanations of this opposite response are that the Barrens salamanders were already yellow and may have already had calcium in their system that was accumulated from the Barrens stream. If calcium was already present in the bodies of the Barrens salamanders, the amount of melanosome aggregation that occurred in the lab may have been less drastic compared to a control organism experiencing an influx of calcium (Meuller & Neuhass, 2014).

The Results of this experiment reflected the controversy in the literature concerning the phenomenon of melanosome aggregation and dispersion (Meuller & Neuhass, 2014; Yamada &

Fujii, 2002; Sköld et al., 2012). The literature surrounding this topic predicts various responses of how amphibian melanosomes interact with the stimuli of light and extracellular calcium. Melanosome aggregation can act as a "sunscreen," in that the development of pale patches on the body of an animal with thin skin can protect the organism from UV irradiation via light reflection (Meuller & Neuhass, 2014; Yamada & Fujii, 2002). Other literary sources suggest that melanosome dispersion is a more efficient way of avoiding the damage of excessive light exposure by allowing the organism to absorb more light efficiently (Sköld et al., 2012). Overall, this study found different responses to light exposure in relation to melanosomes aggregating or dispersing within melanophores in the dermis. Perhaps the responses of the salamanders in this study reflected the pros and cons of the melanosome migration response. This concept is not easily resolved based upon the current literature or my knowledge. Currently this phenomenon is not well understood, and there are simply things that remain enigmatic. More studies regarding how external stimuli can induce physiological color change in amphibians will help to identify the ecological tradeoffs of aberrant coloration as a stress response. Once there is a better understanding of the relationship between environmental stimuli and the movement of melanosomes, specific physiological pathways can be furthered explored. Understanding which stressors induce certain pathways and how those pathways cause color change could be used to identify habitat stressors via visual assessment of a population exhibiting aberrant coloration.

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APPENDICES

Appendix 1:

Methods

Heavy Metal Sampling

Water and sediment samples were collected and analyzed to determine the seasonal concentrations of heavy metals in the Buck Creek Serpentine Barrens stream. Once a month (September, 2016 to April, 2017), 5 water samples and 5 sediment samples were haphazardly collected from locations along the length of the stream. The water samples were collected using ultra-clean sampling protocols in 12 mL polyphyrene Whatman autovial 0.45 µm filters and filtered directly into a 15 mL plastic sample bottle. The water samples were placed into two plastic bags, and were placed inside of a cooler without ice to prevent any light from affecting the metals in the sample. The sediment samples were collected in 250 mL plastic sample bottles, using a plastic trowel (to prevent metal contamination). The trowel was washed in the water of the tributary stream, dug into the sediment, washed again, and then used to collect the sediment sample.

In the laboratory, the sediment samples were digested using aqua-regia, consisting of a mix of nitric acid and hydrochloric acid. The digested sediment was diluted to a volume of 100 mL in a volumetric flask. The sediment dilution was transferred into two 50 mL Falcon tubes for storage.

The samples were analyzed using Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES) to determine which trace metal elements were present, and how concentrated each metal was within the water and sediment of the Barrens stream each month. The water and sediment samples provided insight into the concentration of heavy metals that naturally occurred at the Serpentine Barrens.

Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES) Standard Procedure

The Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES) instrument was used during this study to analytically determine the trace metals that were found within the water and sediment of the Serpentine Barrens stream, as well as the concentration of those metals.

Metal concentrations

Analyte wavelength	**Al 351.308**	**Ca 342.3953**	**Cr 236.638**	**Fe 238.883**	**Mg 285.213**	**Mo 202.938**	**Ni 226.626**
concentration unit	**mg/L**	**mg/L**	**mg/L**	**mg/L**	**mg/L**	**mg/L**	**mg/L**
October	0.00001	0.00001	0.00400	0.00001	0.36700	0.09000	0.09000
	0.00001	0.00001	0.00500	0.16000	0.07700	1.0100	0.07000
	0.03000	0.00001	0.00400	0.00001	0.07400	0.03900	0.10100
	0.00001	0.00001	0.00500	0.00001	0.00001	0.09000	0.09000
	0.00001	0.00001	0.00400	0.00001	0.02100	0.09100	0.09100
November	0.04800	0.00001	0.00500	0.03100	10.1400	0.09050	0.00001
	0.05500	0.00001	0.00500	0.13600	10.430	0.09200	0.00001
	0.06000	0.00001	0.00500	0.10700	10.080	0.09000	0.00001
	0.04200	0.00001	0.00500	0.00200	10.620	0.09000	0.00001
	0.06000	0.00001	0.00100	0.10000	10.540	0.09050	0.00001
December	0.08650	0.00001	0.00450	0.12250	7.8910	0.09100	0.00001
	0.11000	0.00001	0.00500	0.24100	8.0740	0.09000	0.00001
	0.09800	0.00001	0.00550	0.18850	8.0270	0.09150	0.00001
	0.11450	0.00001	0.00450	0.11700	7.9250	0.09150	0.00001

	0.10600	0.00001	0.00500	0.18650	8.0130	0.09250	0.00001
January	0.16500	0.00001	0.00550	0.22250	7.2750	0.09050	0.00001
	0.14750	6.1980	0.00550	0.16500	8.0860	0.09000	0.00001
	0.08650	0.18030	0.00500	0.00900	7.4390	0.09000	0.00001
	0.18650	0.03200	0.00550	0.27450	6.5490	0.09000	0.00001
	0.07700	6.1050	0.00550	0.12250	8.0860	0.09100	0.00001
February	0.08700	6.25300	0.00450	0.00001	9.8920	0.09250	0.00001
	0.05700	0.00100	0.00450	0.00001	8.0980	0.08950	0.00001
	0.05500	0.02330	0.00500	0.00001	8.1830	0.09100	0.00001
	0.06300	0.03300	0.00500	0.00001	8.4835	0.09100	0.00001
	0.04550	0.00001	0.00450	0.00001	8.0010	0.09150	0.00001
March	0.06300	0.03300	0.00500	0.00001	8.4835	0.09100	0.00001
	0.10400	0.00001	0.00500	0.13450	7.8300	0.09100	0.00001
	0.24500	0.21900	0.00600	0.69500	8.1350	0.09650	0.00001
	0.07900	0.00001	0.00500	0.05000	8.0400	0.08950	0.00001
	0.17900	0.00001	0.00500	0.37100	7.0120	0.09100	0.00001
April	0.10050	0.02330	0.00550	0.15450	7.9850	0.08850	0.00001
	0.07000	0.07070	0.00550	0.03700	8.1720	0.09050	0.00001
	0.07450	0.03800	0.00450	0.01250	8.1630	0.07320	0.00001
	0.08300	0.08270	0.00450	0.06750	8.4580	0.09100	0.00001
	0.08550	0.16600	0.00500	0.05050	8.2030	0.09100	0.00001

Table 10. Trace metal concentrations within monthly water samples from the Buck Creek Serpentine Barrens, Macon Co., North Carolina.