COLORATION, CHRONIC STRESS, AND WATERBORNE HORMONE ANALYSIS OF THE ABERRANTLY COLORED POPULATION OF *DESMOGNATHUS MONTICOLA* FOUND AT THE BUCK CREEK SERPENTINE BARRENS

A thesis presented to the faculty of the Graduate School of
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ABSTRACT

Amphibians around the globe must survive physiological stress, whether it originates from chemical pollutants, food shortages, or predation. This physiological stress may cause changes in phenotypes as well as health and performance issues, leading to population declines and ultimately extinction. The Buck Creek Serpentine Barrens (BCSB) in the Nantahala National Forest, Clay County, North Carolina is a unique ecosystem with shallow ultramafic bedrock dominated by pitch pine trees and herbaceous grasses, unlike the surrounding hardwood forests. Seal salamanders (*Desmognathus monticola*) found at the BCSB often exhibit abnormal yellow patches on their heads, backs, legs, and tails, despite being genetically similar to nearby populations. Despite previous research, the cause of this population's unique appearance is not understood and was hypothesized to be associated with chronic stress exposure from malnutrition and exposure to harsh serpentine barrens conditions. Analysis of physiological stress requires studying corticosterone (CORT) concentrations, typically using plasma samples. In all but the largest salamander species, however, the collection of plasma samples is usually a terminal procedure. Waterborne hormone analysis is a nonlethal alternative method to plasma sampling that involved submerging sampled individuals into water baths to allow hormones to diffuse across their skin and gills into the water, which is then analyzed for CORT instead of plasma. However, this technique requires validation for each species. In this study, I validated waterborne hormone analysis for seal salamanders using paired water bath and plasma hormone samples. I then used waterborne hormone analysis to investigate whether the unique coloration of the BCSB seal salamander population was associated with high CORT concentrations. I observed no difference in average CORT concentration among the BCSB and neighboring populations, however. Although there were no differences in CORT among populations, I

detected significantly higher magnesium concentrations, iron concentrations and water temperatures, as well as decreased calcium concentrations, canopy cover, and macroinvertebrate abundance at the BCSB. These findings document the unusual conditions seal salamanders must overcome to survive at the BCSB. This work provides insight on the effects of serpentine barrens and landscape heterogeneity within the Southern Appalachian Mountains on seal salamander phenotypic diversity and demonstrates the use of a nonlethal method for quantifying CORT in *Desmognathus*.

INTRODUCTION:

Amphibians are the most threatened and declining vertebrate taxa, with 43.2% of amphibian species experiencing population declines (Stuart et al. 2004). Although the causes of amphibian species decline vary, the majority are associated with anthropogenic factors (Luedtke et al. 2023). Agriculture, timber production, pollution, and urban development are all leading causes of amphibian decline worldwide, with climate change, wildfires, diseases, and invasive species also contributing (Luedtke et al. 2023). These disturbance events adversely affect the habitat and ecosystem conditions in the environment, leading to population declines and loss of biodiversity. When these disturbance events occur, their effects can cause stress and influence amphibian physiology (Gabor et al. 2018; Tornabene et al. 2021). To fight against amphibian declines and protect species around the globe, scientists must gain a greater understanding of how stress affects amphibian physiology.

The vertebrate stress response follows two physiological pathways that have highly conserved cores, but highly variable functions across taxa in facilitating an individual's survival when exposed to harmful environmental factors (Romero and Gormally 2019). The catecholamine, or fight-or-flight, stress response occurs within seconds of a stressor arising (Woodley 2017). The catecholamine response is initiated by the sympathetic nervous system sending electrical signals via axons to the adrenal medulla (Romero and Gormally 2019). Once at the adrenal medulla, these electrical signals stimulate the production of the catecholamines, epinephrine and norepinephrine (Romero and Gormally 2019). These catecholamines then bind to various beta-adrenergic receptors across numerous tissues throughout the body, regulating the fight-or-flight stress response (Romero and Gormally 2019).

The glucocorticoid stress response is a result of the hypothalamus-pituitary-adrenal (-interrenal in amphibians; HPA/I) axis and occurs within minutes to hours of a stressor arising (Woodley 2017). The HPA/I axis is initiated when neurons in the hypothalamus stimulate the production of arginine vasopressin (AVP) and corticotropin-releasing hormone (CRH; Woodley 2017; Romero and Gormally 2019). AVP and CRH travel to the pituitary gland to prompt the production and release of adrenocorticotropic hormone (ACTH; Woodley 2017). ACTH then stimulates the production and release of glucocorticoids from the adrenal cortex (interrenal tissue in amphibians). Glucocorticoids circulate in the bloodstream at both baseline and stress-induced levels (Woodley 2017). When a stressor occurs, additional glucocorticoids are produced and superimposed on the baseline glucocorticoid pattern (Landys et al. 2006). Glucocorticoids within the bloodstream bind to mineralocorticoid and glucocorticoid receptors in a concentration dependent manner to influence gene expression and facilitate a stress response (Woodley 2017; Romero and Gormally 2019). These glucocorticoids can also exert negative feedback to both the pituitary and hypothalamus as well as stimulate increased glucocorticoid release (Romero and Gormally 2019).

Although stress responses are strongly conserved among vertebrate taxa, the primary glucocorticoid hormone varies across taxa. In humans and fishes, the primary glucocorticoid hormone is cortisol while the primary glucocorticoid hormone in rodents, amphibians, and many other nonmammalian vertebrates is typically corticosterone (CORT; (Woodley 2017)). However, the eastern hellbender (*Cryptobranchus alleganiensis*) uses cortisol as its primary stress hormone, as may other basal amphibian taxa (Hopkins et al. 2020). Additionally in amphibians, arginine vasotocin (AVT) is produced instead of AVP and may play a more prominent role in stimulating ACTH production than CRH (Okada et al. 2016).

In general across vertebrate taxa, acute, short-term exposure to a stressor evokes a beneficial stress response via the HPA/I axis by physiologically prioritizing immediate avoidance of predators and other dangers. This is achieved by stress-induced concentrations of glucocorticoids increasing the effects of the catecholamine stress response, glucose availability, arousal, escape behavior, learning, memory, and some immune responses (Woodley 2017), which are essential for immediate survival. Simultaneously, stress-induced glucocorticoids suppress growth, digestion, reproduction and other immune responses, which are more important for long-term survival and have little effect on immediate danger avoidance (Woodley 2017). Although short-term stimulation of the HPA/I axis evokes a beneficial stress response, long-term stimulation can cause a plethora of harmful effects. These harmful effects include muscle wasting, neuron death, immune suppression, and inhibition of growth and reproduction (Chrousos 2009; Woodley 2017).

In addition to causing beneficial or harmful stress responses, increased concentrations of CORT can facilitate color changes. Coloration observed in amphibians, reptiles, fishes, and birds is facilitated by pigment cells called chromatophores (Kindermann and Hero 2016). In amphibians, three types of chromatophores have been identified: melanophores, xanthophores, and iridophores (Bagnara et al. 1968; Kindermann and Hero 2016). These chromatophores are arranged in layers below the basal lamella of amphibians and are collectively called the dermal chromatophore unit (Bagnara et al. 1968). Xanthophores are the outermost pigment cell, iridophores are found directly beneath xanthophores and contain light-reflecting pigment, and melanophores are the deepest pigment, but have finger-like projections that fill the space between xanthophores and iridophores (Bagnara et al. 1968). These dermal chromatophore units are responsible for many amphibians' ability to undergo rapid color change (Bagnara et al.

1968). When an amphibian color change occurs, melanosome pigments move from the deep region of the melanophores to the finger-like projections, covering or partially-covering the xanthophores and iridophores (Bagnara et al. 1968). This migration of melanosome pigment obscures the xanthophores and iridophores below, changing the reflected color of the individual (Bagnara et al. 1968).

Although most research investigating amphibian and reptilian color change focuses on species' abilities to undergo rapid color change for camouflage or display, studies have also investigated the impacts of stress on coloration, with a few focusing on CORT. There are multiple reports of hormones along the HPA/I axis influencing the dermal chromatophore unit. In cultured bullfrog tadpole cells, melanophores expanded after being treated with ACTH while iridophores contracted, which are both also observed responses of the dermal chromatophore unit to melanocyte-stimulating hormone (Ide 1973). In squamates, increased CORT concentrations caused color changes in *Lacerta vivipara* (Fitze et al. 2009), *Ctenophorus decressii* (Lewis et al. 2017), and *Crotalus helleri* (Stepanek et al. 2019). Stress also caused the brightening of body coloration in water anoles (*Anolis aquaticus*), which may decrease their conspicuousness through disruptive camouflage (Boyer and Swierk 2017). However, stressed *Anolis carolinensis* lizards are inversely darker brown, while unstressed individuals are greener (Greenberg 2002). Because of the complex relationship between stress and coloration, additional studies investigating their dynamics are warranted for both reptiles and amphibians.

Despite chronic stress being detrimental to health and fitness, vertebrate populations can often be found in stressful environments. At the Buck Creek Serpentine Barrens (BCSB) in the Nantahala National Forest in Clay County, North Carolina, there is an aberrantly colored population of *Desmognathus monticola* (seal salamanders) residing in a stressful ecosystem.

Individuals within this population may display bright yellow patches on their head, back, legs, and/or tail (Figure 1; Harmon 2018). The BCSB ecosystem starkly contrasts with its surroundings (Mansberg and Wentworth 1984). Instead of being dominated by a dense hardwood Southern Appalachian cove forest, the BCSB is dominated by stunted *Pinus rigida*, shrubs, and grasses (Mansberg and Wentworth 1984). The unique ecosystem observed at the BCSB is due primarily to the serpentine barrens' unique soil. Serpentine soils are harshly infertile to most plants and are formed from the erosion of ultramafic rock, which is rock that contains 70% ferromagnesian (iron and magnesium) minerals (Brooks 1987). Serpentine soils also often contain high concentrations of metals, such as magnesium, nickel, chromium, and cobalt, and low concentrations of nutrients, such as calcium, nitrogen, phosphorus, and potassium (Brooks 1987). Due to its high porosity, serpentine soils also have moisture deficiencies even in regions with high rainfall (Brooks 1987), such as the Southern Appalachian Mountains. Previous studies investigating the abnormal population of seal salamanders found at the BCSB found no evidence that the unique coloration was due to genetic differences among populations (David Beamer, *personal communication*), increased light exposure, or the salamanders attempting to match the light colored rocks in the stream (Harmon 2018). However, the extreme environmental conditions at the BCSB may cause physiological stress to the native seal salamander population, resulting in an associated color change.

Figure 1. The aberrant coloration of *Desmognathus monticola* (Seal Salamander) found at the Buck Creek Serpentine Barrens. Unusual yellow spots are frequently present on the head, legs, back, and tail of individuals from this population.

A variety of conditions at the BCSB may cause significant stress to the local seal salamander population. Although many metals are essential for growth and health, all metals can be detrimental to life if in excess, causing lethal or sublethal effects that are additive with other metals (Cockerham et al. 1994; Francis 1994). Toxic metals can affect reproductive health, behavior, blood composition, and growth, and can make organisms more susceptible to disease, parasites, and carcinogenesis (Peterle 1991; Cockerham et al. 1994). The specific physiological effects of each metal on aquatic organisms are difficult to determine due to metal toxicity being influenced by concentration, stream flow rates, the size and nature of metal particulates, and other physical and chemical characteristics (Cockerham et al. 1994). However, magnesium toxicity in aquatic algae, macroinvertebrates, and fishes is largely due to magnesium being a calcium channel antagonist (van Dam et al. 2010). These competitive conditions between calcium and magnesium make aquatic species residing in calcium-poor waters, such as at serpentine barrens, highly susceptible to small elevations above background magnesium concentrations (van Dam et al. 2010). Although the harsh chemical conditions of the BCSB likely impact the local seal salamander population, it also can impact the aquatic

macroinvertebrate community, which is a primary food source for seal salamanders. With few aquatic macroinvertebrates, seal salamanders at the BCSB may face a perpetual food shortage, further contributing to stress. The differing soil conditions and plant community can also contribute to seal salamander stress at the BCSB. Warm water temperatures, low canopy cover, and scarce leaf packs for aquatic macroinvertebrates anecdotally observed at the BCSB are not typical of seal salamander habitat, which may also contribute to stress. Overall, the chemical and environmental conditions at the BCSB may cause severe stress to the local seal salamander population, causing their unique coloration.

Although there are a variety of potential stressors at the BCSB, quantifying stress in seal salamanders is not straightforward. Quantification of stress is traditionally conducted by analyzing blood plasma for CORT. However, blood sampling in plethodontid salamanders and other smaller amphibians often requires sacrificing specimens due to their small blood vessels and low blood volume (Wright 2001; Narayan et al. 2019). In response, a variety of non-invasive alternative methods for analyzing CORT, including waterborne, fecal, urine, and dermal sampling, have arisen. These non-invasive techniques allow for repeated sampling of individuals to estimate temporal patterns and larger sample sizes to account for variation among individuals (Narayan et al. 2019). However, non-invasive CORT sampling techniques have varying success rates and must be validated via an ACTH/physical stress challenge and/or a correlation study before use.

The most promising non-invasive CORT sampling technique for seal salamanders is waterborne hormone analysis, which is conducted by placing individuals into a water bath of predetermined volume for a set amount of time (ranging anywhere from fifteen minutes to two hours; Gabor et al. 2013). This technique relies on the passive diffusion of hormones into the

water bath from the skin, gills, urine, and feces of specimens, which can then be analyzed instead of plasma. Waterborne glucocorticoid analysis originated with the analysis of cortisol in fishes but has been validated for the analysis of CORT in various amphibian taxa, such as the common midwife toad (*Alytes obstetricans*), various aquifer *Eurycea* species (Gabor et al. 2013; 2016), and the Túngara frog (*Engystomops pustulosus;* Baugh et al. 2018). However, validation of waterborne hormone analysis failed in some amphibian taxa, such as *Ambystoma maculatum* (Millikin et al. 2019), which emphasizes the importance of validating the technique across species.

A variety of factors at the BCSB could be causing stress to the local seal salamander population, which in turn may be causing their abnormal coloration. In this study, I investigated environmental and ecological conditions at the BCSB and neighboring streams, including water chemistry, canopy coverage, and aquatic macroinvertebrate community assemblages to identify potential stressors for seal salamanders. I also validated waterborne hormone analysis for seal salamanders using paired waterborne and plasma CORT samples. I then used waterborne hormone analysis and color analysis to test whether the BCSB seal salamander population has a different baseline CORT concentration than neighboring seal salamander populations to determine if chronic stress exposure could be the cause of their unique coloration.

METHODS:

Study Sites

I originally selected four streams for this study within the Nantahala National Forest in western North Carolina. These were: the stream draining the BCSB (BC), the headwaters of Buck Creek near Deep Gap (DG), Muskrat Branch in the Shooting Creek watershed (SC), and Hurricane Creek in the Standing Indian area (HC). I used a fifth stream, Long Branch in the

Standing Indian area (LB) instead of HC when comparing CORT concentrations among the populations due to difficulty finding seal salamanders at HC. BC, DG, and SC are all located within Clay County, NC, and HC and LB are located in Macon County, NC. At each stream, I placed ten transects perpendicular to the stream approximately thirty meters apart for salamander, environmental and aquatic macroinvertebrate sampling.

Study System

The seal salamander is a medium-sized stream-dwelling salamander that is often abundant within its range. The species' natural range spans from northern Georgia to southern Pennsylvania along the Appalachian Mountains and adjacent Piedmont (Beane et al. 2010; Powell et al. 2016; Pyron et al. 2023), with an introduced invasive population inhabiting the Ozark Mountains in Arkansas (Bush et al. 2017). Seal salamanders primarily inhabit the banks and waters of small, cold mountain streams, seepages, and springs that flow through closed-canopy hardwood forests and can often be found in holes and under moist rocks (Petranka 1998; Lannoo 2005; Beane et al. 2010). Seal salamanders are commonly used as fish-bait, which may have caused the introduction of the species to new areas by anglers (Lannoo 2005). The species is active most of the year, except during the peak of winter, when they burrow deeper into the ground or stream bed to prevent freezing and desiccation. Seal salamanders feed predominantly on aquatic and terrestrial macroinvertebrates and will forage on stream banks and under terrestrial leaf litter as well as in the water (Beane et al. 2010). Seal salamanders have an aquatic larval stage that lasts 9-10 months, but it varies in length with elevation (Bruce 1989). Following metamorphosis, seal salamanders spend an additional two or more years developing as juveniles (Bruce 1989).

Seal salamanders have a dorsum that is highly variable in both patterning and coloration. The dorsum is usually buff-colored, greenish grey, or light brown (Petranka 1998; Beane et al. 2010). The dorsal patterning usually has well-defined light spots, a dark splotchy pattern, or a dark net-like pattern (Petranka 1998; Beane et al. 2010). Juveniles typically have 4-5 pairs of reddish-brown spots along their dorsum, distinguishing the species from *Desmognathus cheaha* which has 6-7 pairs of dorsal spots (Beane et al. 2010; Pyron et al. 2023). The venter of seal salamanders is pale white or translucent and can have light to no mottling (Petranka 1998; Beane et al. 2010). All collections of seal salamanders for this study were taken from public lands with the use of state permitting (Wildlife Collection License 23-SC01564) and Institutional Animal Care and Use Committee approval (WCU IACUC #2023-05-15-06). A maximum of five specimens were collected per site (transect).

Waterborne Hormone Analysis

I validated waterborne hormone analysis by collecting paired waterborne hormone and plasma samples from a total of eighteen seal salamanders. In July 2023, I transported the first four of the eighteen salamanders (masses ranged from 1.5 g to 9.0 g) to Auburn University to determine the viability of a nonlethal plasma sampling procedure. Before attempting nonlethal plasma sampling, I collected a waterborne hormone sample from each of the four salamanders by placing them into separate 75- or 200ml dechlorinated water baths for thirty minutes, with the volume of the water bath dependent on the size of each salamander. I used two water bath sizes to minimize the influence of confinement on stress, which would have varying impacts depending on the size of the salamander. I collected waterborne hormone samples from these four individuals to allow them to be included in the waterborne hormone validation. After collecting the waterborne hormone samples, I anesthetized each of the four salamanders via

immersion in 0.5g/L tricaine methanesulfonate (MS-222) buffered with sodium bicarbonate. I collected plasma samples by inserting a heparinized insulin syringe with a 28-gauge needle dorsally into the vertebral sinus of the tail and readjusted the needle until it was positioned between two caudal vertebrae near the base of the tail (Figure 2). Once in position, I collected blood by aspirating the syringe and via capillary action. I collected no more than 1% of the mass of each specimen in blood to prevent causing health issues (Wright 2001). I collected at least 15 µl of blood from each of the four individuals brought to Auburn University.

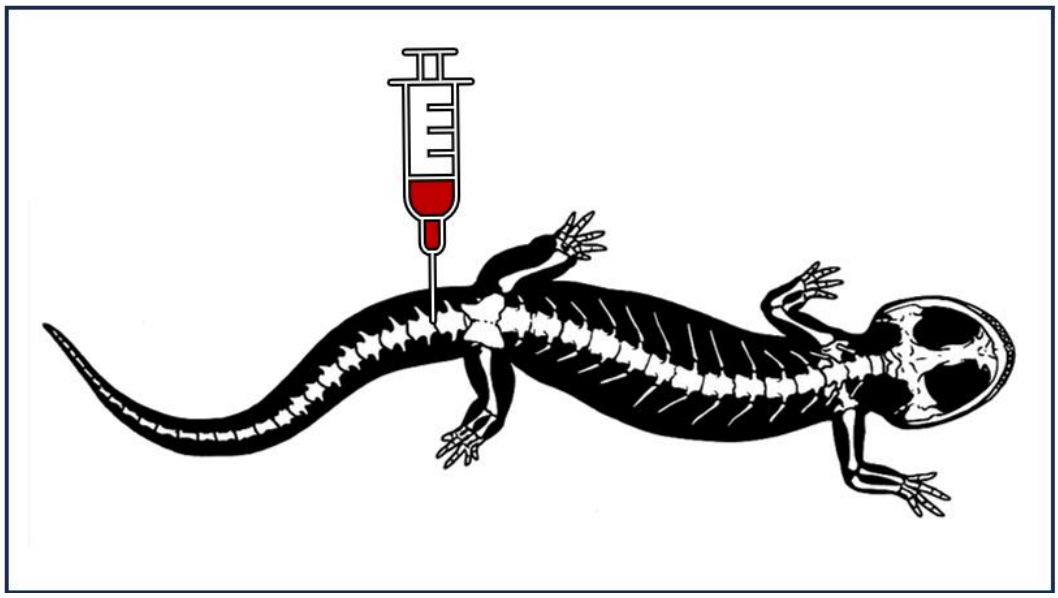

Figure 2. Positioning used for nonlethal blood collection from seal salamanders. I inserted heparinized insulin syringes dorsally into the vertebral sinus of the tail of each individual to collect blood.

After determining that nonlethal blood sampling was viable with no discernable ill effects, I collected blood and waterborne hormone samples in the field from 14 additional seal salamanders from September to November 2023. I caught all salamanders within two minutes of their initial discovery due to the increased likelihood of introducing stress during a prolonged capture pursuit. Immediately after capture, I collected a waterborne hormone sample from each

salamander by placing it into a 75- or 200ml dechlorinated water bath, with the size of the bath determined by the size of each salamander. After collecting a waterborne hormone sample, I collected a blood sample using the same protocol as described above. I used a drop of blood from each specimen to make a blood smear for heterophil/lymphocyte ratio analysis, which may be an indicator of stress (Goessling et al. 2015). The duration of blood sampling time was highly variable, ranging from 00:25:00 to 2:00:00 h per salamander. After sample collection, each salamander was released where it was caught after it recovered from anesthesia. I conducted all sampling between 11:00-15:00 h EST to standardize the effects of circadian rhythm on CORT concentrations. Blood and water bath samples were kept on ice until returning to the lab within 6 hours of their collection.

I extracted plasma from each blood sample immediately upon returning to the lab by centrifuging at 3000 x G for 1 minute. I pipetted the plasma off the blood pellet and stored the plasma at −20°C until analysis. I processed the waterborne hormone samples by filtering them through 0.45 µm filter paper. After filtration, I ran each waterborne hormone sample through Sep-Pak C18 solid phase extraction columns from Waters Inc., Milford, MA using a vacuum manifold. I first primed the C18 columns with two 2ml washes with methanol followed by two 2ml washes with DI water before running the samples on the columns. I froze the waterborne hormone samples on the C18 cartridges at −20°C until analysis. I removed the hormone samples from the C18 cartridges by first washing twice with 2ml of DI water and then pipetting 2ml of ethyl acetate into each cartridge twice. I then evaporated the ethyl acetate off the samples to allow for resuspension of the samples during analysis. I used enzyme-linked immunosorbent assay (ELISA) kits from Cayman Chemicals Inc., Ann Arbor, MI to analyze both waterborne and plasma hormone samples for CORT.

In May-June 2024, I collected eight salamanders each from BC, SC, DG, and LB for a total of thirty-two salamanders to compare baseline CORT concentrations and coloration among the four populations. I collected no additional individuals from HC due to a lack of active seal salamanders in the population. I collected waterborne hormone samples following the procedure described above. After hormone sampling, I photographed each salamander using a Canon (Melville, NY) EOS 30D digital camera equipped with an EF-S18-55mm f/3.5/3.6 II lens in a Duclus Photography Light Box with a Datacolor (Lawrenceville, NJ) Spyder Checkr and non-reflective white ruler from ThermoFisher Scientific (Waltham, MA) for color correction. After sampling, I transported all thirty-two salamanders to the laboratory for a twelve-week experiment investigating the link between coloration and stress, which was not included in this thesis, before ultimately releasing each individual where they were initially discovered,.

Color Analysis

I conducted color analysis of photos using Adobe Photoshop version 26.3 software (San Jose, CA). I first white-balanced each photo using the white balance tool. With the white balance tool, I selected the region above the 7.5 cm tick mark on the non-reflective white ruler in each picture. After white balancing, I used the color sampler tool with an 11 x 11-pixel area and selected the same part of the non-reflective white ruler above the 7.5 cm tick mark to collect the average red, green and blue (RGB) data for the pixel area. I then averaged the RGB data for this white point across all 32 photos and determined the average RGB respective values across all pictures to be 164.74, 164.66, and 164.75, respectively. I then equalized the exposure of each photo by creating a new exposure layer and adjusted the exposure so that the white point in each photo was 165, 165, 165, respectively, on the RGB scale. After the exposure was equalized, I used the color sampler tool to collect CIELAB color metric data from each salamander

(Robertson 1977). In the CIELAB color metric, “L*” values represent lightness, “a*” values represent the green-red color component, and “b*” values represent the blue-yellow components (Robertson 1977). Higher L*, a* and b* values represent greater light, red, and yellow coloration, respectively, while lower values represent greater dark, green, and blue coloration, respectively. I collected CIELAB color metrics from five different locations on each salamander: the head behind the eyes, the middle of the dorsal surface of the back, the dorsal surface at the base of the tail posterior to the cloaca, and the dorsal surface of a front and back foot. I used a 51 x 51-pixel area for sampling from the head, back and tail and a 31 x 31-pixel area for sampling from both feet.

Environmental Conditions

I collected water samples at BC, DG, SC, and HC monthly from July 2023 to April 2024 to analyze for metals. I collected samples (50 ml) from every odd-numbered transect (transects 1, 3, 5, 7, and 9, approximately 60 meters apart). I filtered the samples using 0.45 µm filter paper and acidified them (pH < 2) using concentrated nitric acid to minimize metal precipitation before storing them at room temperature until analysis. For their analysis, I used a PerkinElmer (Waltham, MA) NexION 2000 inductively coupled plasma mass spectrometer (ICP-MS) equipped with a quartz cyclonic spray chamber ionizer to determine magnesium (Mg), calcium (Ca), iron (Fe), cobalt (Co), nickel (Ni), zinc (Zn), chromium (Cr), and aluminum (Al) concentrations. I used ten standards with concentrations ranging from 10 ppb to 12,500 ppb for each metal to create a calibration curve, with a 2% nitric acid solution as a blank. At each transect at BC, DG, SC, and HC, I took canopy coverage photographs approximately one meter above the water on June 19 2024 using a Canon EOS 30D digital camera equipped with an 8 mm 1:3:5 EX DG fish-eye lens by SIGMA (Burbank, CA). I used a Canon PowerShot SX530 HS

camera equipped with 0.21X Digital King fish-eye lens made by Toda Seiko, (Tokyo, Japan) to take the canopy pictures at LB approximately one meter above the water on September 16 2024. Canopy openness was estimated using Gap Light Analyzer software by the Cary Institute of Ecosystem Studies (Millbrook, NY). I used a YSI (Yellow Springs, OH) 650 Multiparameter Display System equipped with a 600 QS Multiparameter Water Quality Sonde to record water temperature, dissolved oxygen (DO%), and water conductivity, and an Eutech Instruments (Vernon Hills, IL) pHTestr 2 to record pH at each of the ten transects in all five streams, with measurements collected on June 11 2024 for BC and DG and on June 18 2024 for SC, HC, and LB.

I sampled aquatic macroinvertebrates at BC, DG, SC, and HC to quantify food availability for seal salamanders. I conducted two surveys for each stream, one in June 2023 and the second in November 2023. I conducted the surveys by collecting ten rocks, ranging in size from 0.20 to 0.35 m, along each perpendicular transect in each stream. Immediately after collection, I collected macroinvertebrates off the rocks and identified them using a key from the Stream Monitoring Information Exchange (SMIE) project (Traylor 2004). I identified aquatic macroinvertebrates in the field during the June survey and in the lab for the November survey after they were preserved in 70% ethanol. From aquatic macroinvertebrate abundance and species richness, I calculated Shannon diversity index. I then calculated Pielou's evenness index by dividing the Shannon diversity index by the natural log of species richness. Lastly, since lower aquatic macroinvertebrate tolerance values indicate better overall stream health (Chang et al. 2021), I calculated stream tolerance for each of my sampled streams, which is a weighted average of the tolerance values for the sampled aquatic macroinvertebrates using the published SMIE tolerance values (Traylor 2004). Because the LB stream was added to the study part way

through, I was only able to record environmental data including pH, dissolved oxygen, and canopy cover at the stream, but not heavy metal concentrations and aquatic macroinvertebrate community data.

Statistical Analysis

I conducted all statistical analyses using R 4.4.1 (R Core Team 2024). Waterborne hormone sample concentrations from the ELISA kits were standardized by dividing by the water bath volume, mass of each specimen, and SVL of each specimen. Mass and SVL were used to account for variation among salamanders due to surface area associated with size. Water bath volume was used to account for differences in CORT concentrations due to varying water bath sizes. Plasma sample concentrations were standardized by dividing by the plasma volume that was resuspended for ELISA kit analysis. I transformed CORT concentration data using a natural log transform to meet the assumptions of ANOVA, tested using Shapiro-Wilk normality and Levene tests. I used a Pearson correlation to compare plasma CORT concentration and waterborne CORT concentration to validate waterborne hormone analysis. I used a one-way Analysis of Variance (ANOVA) to analyze differences in waterborne CORT concentrations among populations. I created a Principal Components Analysis (PCA) coupled with Permutational Multivariate Analysis of Variance (PERMANOVA) to compare all environmental data among the sites, except for metal analysis.. I used repeated measures ANOVAs to analyze metal concentrations among the streams. I used linear mixed effect models to separately compare L*, a*, and b* among the populations and body parts, with site and body part being fixed effects while individual ID was a random effect.

RESULTS:

Waterborne Hormone Analysis

There was a significant positive Pearson correlation between plasma CORT concentration and waterborne CORT concentration in the 18 paired CORT samples ($p = 0.05$, $r = 0.462$; Figure 3). There was no significant difference among the populations in mean waterborne CORT concentrations ($F_{4, 43} = 0.763$; $p = 0.555$; Figure 4).

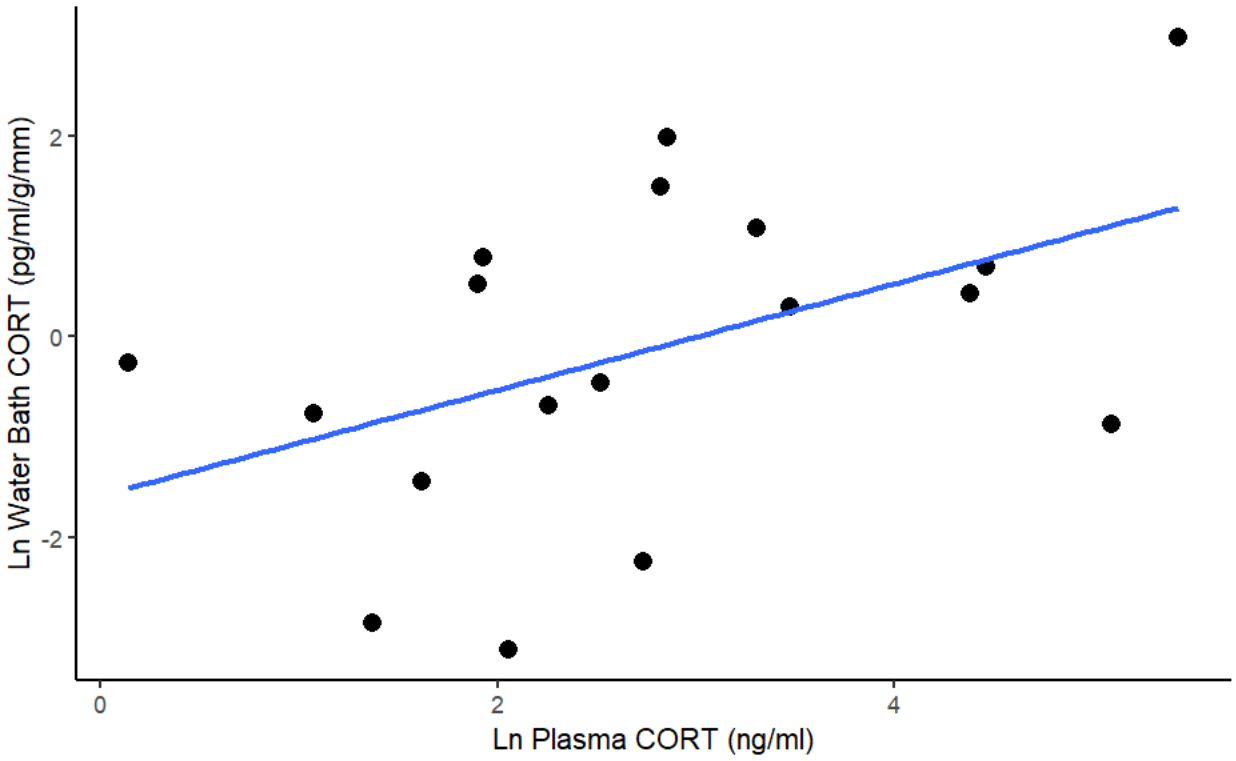

Figure 3. Correlation between Waterborne CORT Concentration and Plasma CORT Concentration in seal salamanders (n = 18)

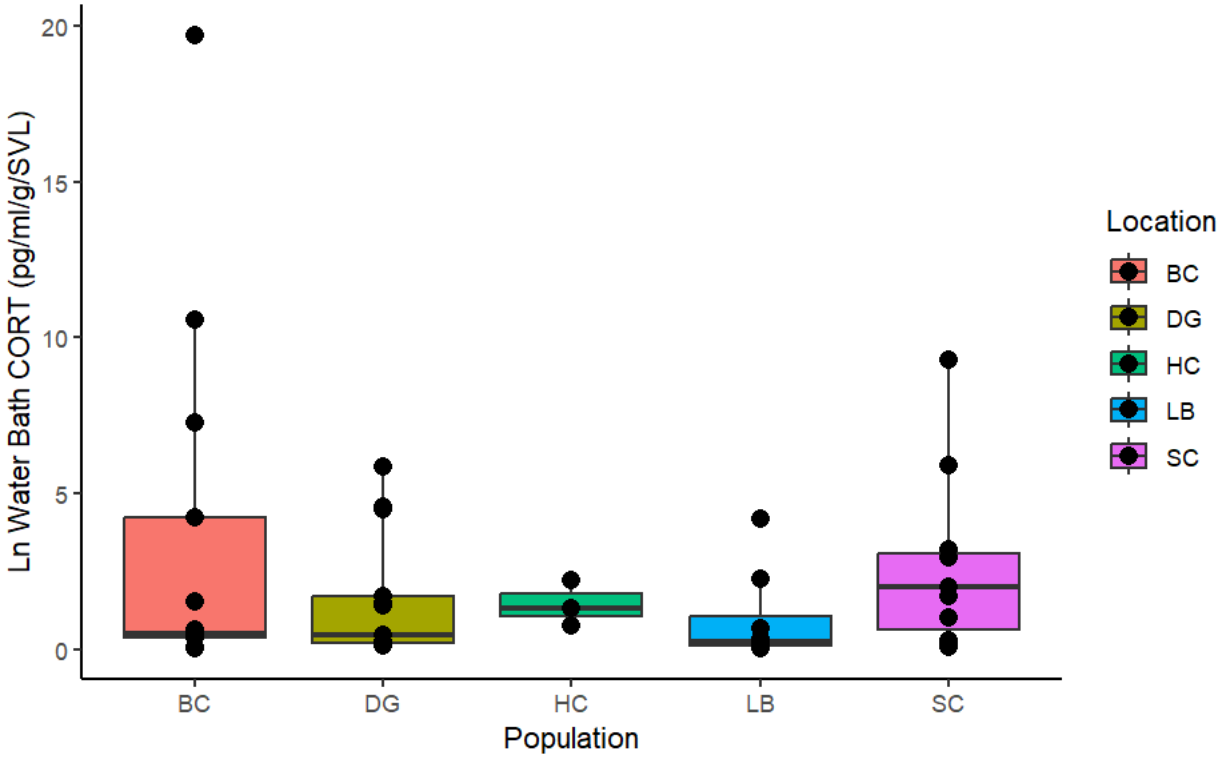

Figure 4. Comparison of Waterborne CORT concentration among the Buck Creek Serpentine Barrens (BC), Deep Gap near Buck Creek (DG), Hurricane Creek (HC), Shooting Creek (SC), and Long Branch (LB) seal salamander populations (n = 46). Boxes represent the interquartile range, with whiskers showing the outer quartiles.

Color Analysis

I found no evidence of differences in any of the three color measures among the four populations (L* $F_{3, 24} = 1.47$ $p = 0.25$; a* $F_{3, 24} = 1.84$, $p = 0.166$; b* $F_{3, 24} = 1.44$, $p = 0.25$; Fig. 5). However, there was a significant body part by population interaction for lightness (L* $F_{12, 96} = 2.55$, $p = 0.006$; a* $F_{12, 96} = 1.61$, $p = 0.10$; b* $F_{12, 96} = 1.50$, $p = 0.13$; Fig. 5) as well as significant differences in coloration among the body parts (L* $F_{4, 96} = 66.39$, $p < 0.0001$; a* $F_{4, 96} = 32.45$, $p < 0.0001$; b* $F_{4, 96} = 52.30$, $p < 0.0001$; Fig. 5), with front and back foot L*, a*, and b* values being greater than head, back, and tail values. One-tailed ANOVAs investigating the interaction between body part and site for L* suggest the interaction specifically applies to head lightness (Head $F_{3, 28} = 3.04$, $p = 0.045$; Back $F_{3, 28} = 2.20$, $p = 0.11$; Tail $F_{3, 28} = 0.54$, $p = 0.66$;

Front foot $F_{3, 28} = 1.93$, $p = 0.15$; Back foot $F_{3, 28} = 2.45$, $p = 0.084$), with the BC population displaying lighter head colorations than DG, LB, and SC. Although it appears different in Figure 5, the back L* among populations was not different due to its greater effect size and larger variance.

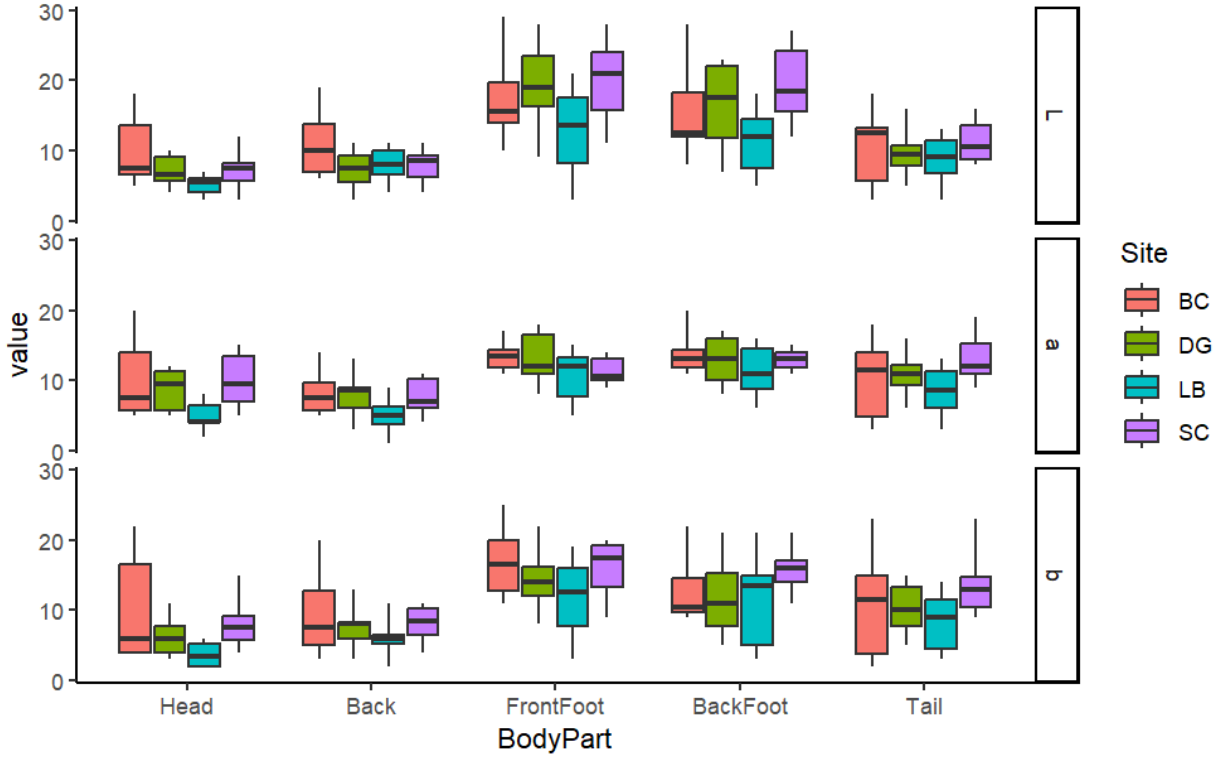

Figure 5. CIELAB L*, a*, and b* color metrics of seal salamanders by population and body part. Boxes represent the interquartile range, with whiskers showing the outer quartiles.

Environmental Conditions

I detected a significant difference in DO%, pH, temperature, conductivity, and canopy openness among the five streams ($F_{4, 45} = 9.396$, $p < 0.0001$; Fig. 6). PCA Dimension 1 and 2 represented 58% and 19.7% of the variation, respectively. BC was widely separated from the other streams along PCA Dimension 1, which was strongly correlated with conductivity and canopy openness, partially correlated with pH and temperature, and partially negatively

correlated with dissolved oxygen. PCA Dimension 2 was partially positively correlated with dissolved oxygen and pH and partially negatively correlated with temperature. On Dimension 2 BC had an intermediate score, DG had a positive score, and the remaining sites had moderately low scores. All five streams had strong clustering among transects, but BC had high spacing among transects within its cluster.

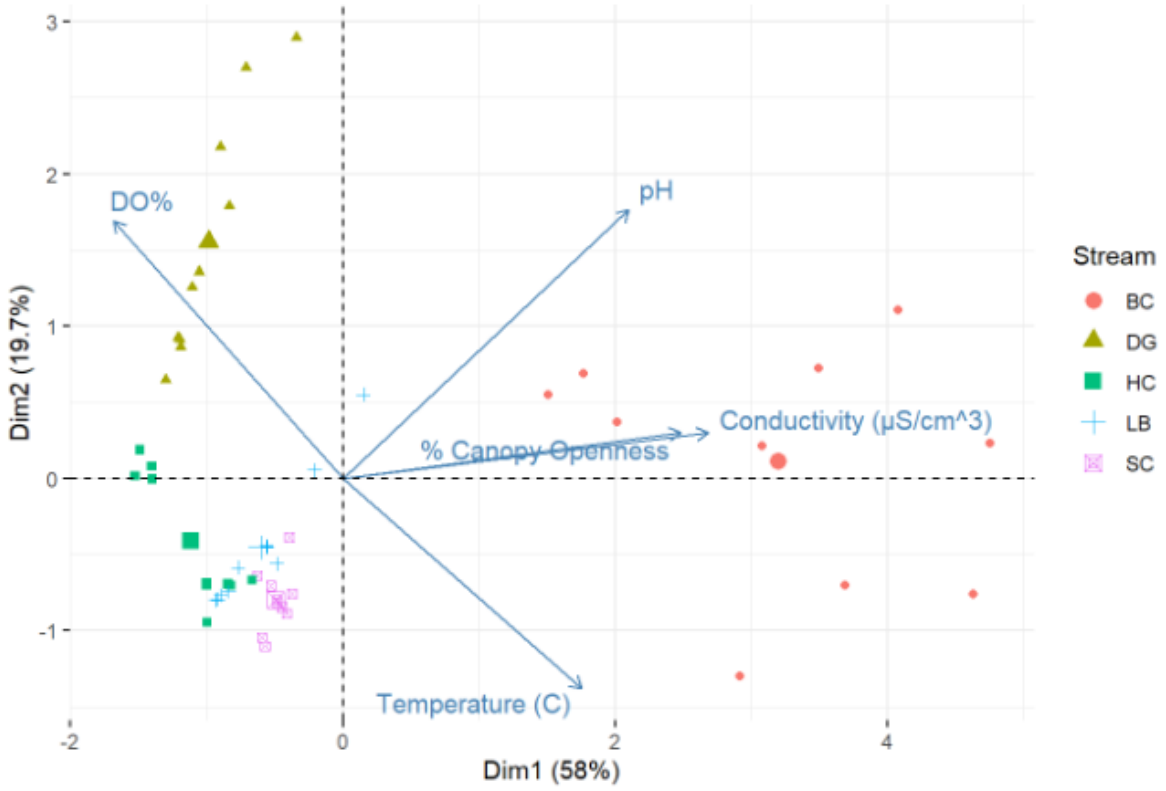

Figure 6. A biplot of PCA scores for environmental samples, including the percentage of dissolved oxygen in stream waters (DO%), pH, temperature, conductivity of stream waters, and the percentage of canopy openness, taken from the five streams. The relationship of each environmental variable with each PCA dimension is shown with a vector. The centroid for each stream is represented by a larger symbol with the same shape.

Large differences between the BCSB and control streams were documented in aquatic macroinvertebrate abundance in both Summer and Fall (Figure 7), with differences in diversity, evenness, richness, and tolerance detected as well (Table 1). I found only 3 aquatic macroinvertebrates in BC in the Summer and none there in Autumn. I found two orders of

magnitude more macroinvertebrates in each of the other streams, pooled across my two surveys (Figure 7; Table 1). Species richness was higher at HC, SC, and DG, with only three species found at BC across both surveys while over total 14 species were found at the other three streams (Table 1). Because the calculation for species evenness incorporates both species abundance and species richness, species evenness was highest at the BC stream, followed by the SC, HC, and DG streams, respectively (Table 1). However, Shannon diversity index was lowest at the BC stream, with the SC stream having the highest Shannon diversity index (Table 1). Stream tolerance index was highest at the BC stream, with SC, HC, and DG having lower stream tolerance indices, respectively (Table 1, Appendix Table 9).

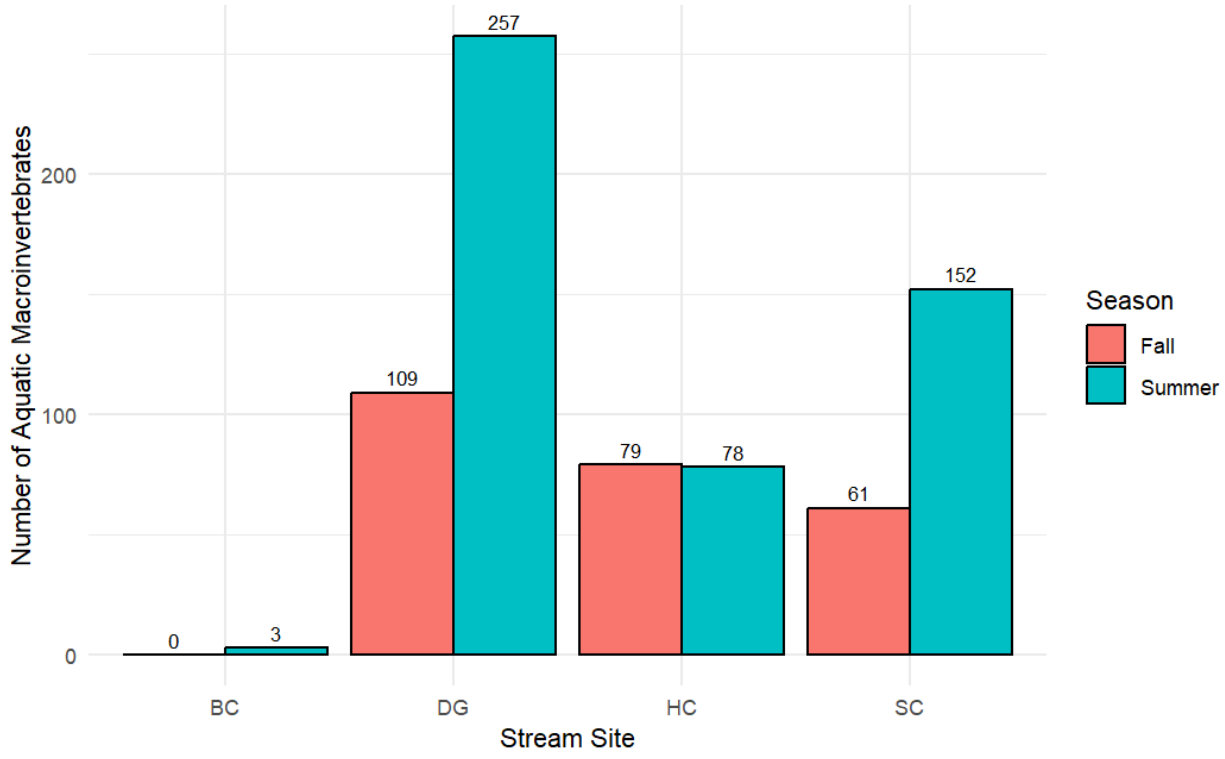

Figure 7. Macroinvertebrate abundance at each sampled stream during both surveys. Values above each bar represent the total aquatic macroinvertebrate abundance for that site and season.

Table 1. Aquatic Macroinvertebrate Diversity, Evenness, Richness, Abundance, and Mean Tolerance of each of the four sampled streams.

Site	Shannon Diversity Index	Evenness	Richness	Abundance	Tolerance
BC	1.10	1.000	3	3	3.60
DG	1.48	0.561	14	266	2.47
HC	2.09	0.697	20	157	2.90
SC	2.34	0.781	20	213	3.41

Water concentrations of magnesium, calcium, iron, and aluminum varied significantly among the streams (Table 2). Magnesium was comparable among the three control streams, but was highest at the BC stream every month despite fluctuating temporally (Figure 8). Magnesium concentrations in the BC stream further increased in October and November and saturated the ICP-MS. The saturated values were input as 20,000 ppb for statistical analyses and Figure 8. Calcium fluctuated more than magnesium throughout the ten-month sampling period in all four streams, but the BC stream had lower calcium concentrations than the other three streams, except for October and November (Figure 8). Iron fluctuated monthly but remained generally similar in all streams except BC, where concentrations increased greatly in November and January (Figure 8). Although aluminum was different among the four streams, it had the least variation between BC and the other streams (Figure 8). Co, Ni, Zn, and Cr concentrations were less than 10 ppb in each stream across the entire sampling period.

Table 2. ANOVA summary table for repeated measures ANOVAS of magnesium (Mg), calcium (Ca), iron (Fe), and aluminum (Al) among the streams

	Mg	**Ca**	**Fe**	**Al**
Site	$F_{3, 17} = 4240.05$ $p < 0.001$	$F_{3, 156} = 60.22$ $p < 0.001$	$F_{3, 156} = 9.17$ $p < 0.001$	$F_{3, 17} = 4240.05$ $p < 0.001$
Month	$F_{9, 141} = 156.33$ $p < 0.001$	$F_{9, 156} = 51.51$ $p < 0.001$	$F_{3, 156} = 7.81$ $p < 0.001$	$F_{9, 141} = 156.33$ $p < 0.001$
Site:Month	$F_{27, 156} = 4.61$ $p < 0.001$	$F_{27, 156} = 4.61$ $p < 0.001$	$F_{3, 156} = 3.17$ $p < 0.001$	$F_{27, 141} = 112.66$ $p < 0.001$

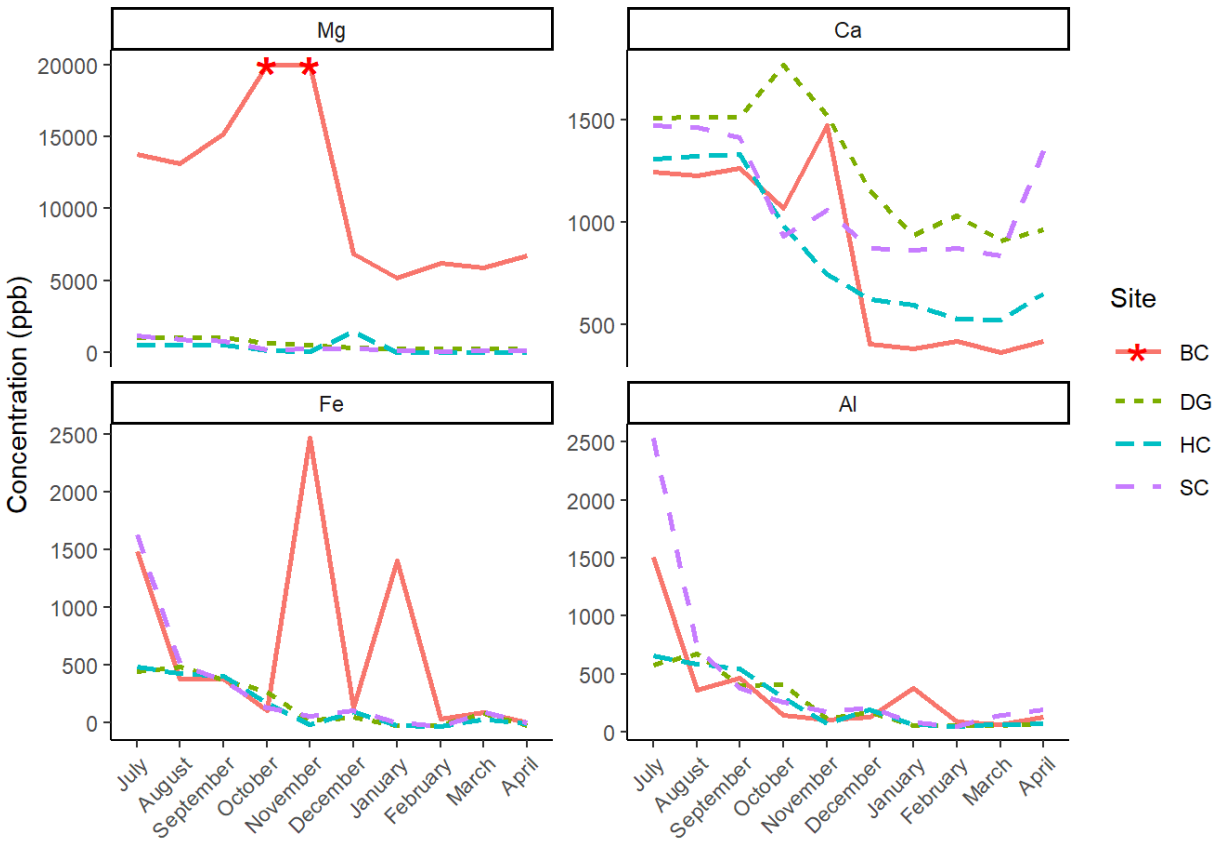

Figure 8. Magnesium (Mg), calcium (Ca), iron (Fe), and aluminum (Al) concentrations from the four sampled streams for water samples collected monthly approximately sixty meters apart between July 2023 and April 2024. Lines connect means. Samples from BC from October and November saturated the instrument and were set to 20,000 ppb (marked with stars).

DISCUSSION:

Waterborne Hormone Analysis

I observed a moderate positive correlation between plasma CORT concentration and water bath CORT concentration in seal salamanders, supporting the use of waterborne hormone analysis for the species in future studies investigating their response to potential stressors. This discovery also allows for new studies that require sequential sampling of the same individuals to be conducted. Waterborne hormone analysis can also be conducted to investigate future threatened salamander populations since many salamander species are predicted to have population declines in the short- and long-term future (Luedtke et al. 2023). To my knowledge, no other studies have attempted to validate waterborne hormone analysis for a species in *Desmognathus.* The viability of waterborne hormone analysis may extend to other species in the genus that are closely related to seal salamanders. Phylogenetic grouping of the success of waterborne hormone analysis has been observed in *Eurycea*, where the technique has been validated for multiple aquifer-dwelling species (Gabor et al. 2013; Gabor et al. 2016). An ACTH challenge would further support the validation of waterborne hormone analysis in seal salamanders. Since ACTH is a precursor hormone to CORT along the HPA/I axis, salamanders treated with ACTH should have elevated plasma and waterborne CORT. However, if waterborne CORT does not increase with ACTH treatment, the HPA/I axis is dysregulated, indicating homeostatic overload with hormone levels above the reactive range (Gabor et al. 2016).

Although waterborne CORT analysis was validated as a minimally invasive technique to measure stress in seal salamanders, the application of the technique has limitations. Firstly, to collect hormone samples, individuals must be confined to a water bath, which may introduce stress. The combination of confinement and agitation increased aquatic salamanders stress in

prior studies (Gabor et al. 2016). However, without agitation, the effects of confinement are likely to be minimal. Confinement had no effects on testosterone or CORT concentrations in breeding male *Bufo spinosus* after 13 hours in captivity (Brischoux et al. 2018). Furthermore, the potential effects of confinement were minimized in our study by using water bath volumes that were proportional to each salamander's size. Although precautions can be incorporated to minimize the effects of confinement on stress, it is also useful to understand the response time of the HPA/I axis. The HPA/I axis response time to stress in plethodontid salamanders is variable, ranging from 30 minutes to over two hours. In Ocoee salamanders (*Desmognathus ocoee*), plasma CORT concentrations were not elevated until after 30 minutes of restraint stress in males and 60 minutes of restraint stress in females (Woodley and Lacy 2010). *Eurycea nana* showed elevated CORT concentrations one hour after exposure to chemical cues from a fish predator (Davis and Gabor 2015). *Eurycea tonkawae* had elevated CORT release rates within 90-120 minutes during a water bath while *Eurycea sosorum* had no change in CORT release rate over a 120 minute period (Gabor et al. 2016). Because most plethodontid stress responses occur within 30-120 minutes of a stressor, it is unlikely that capture or confinement affected the paired blood and waterborne hormone samples used to validate waterborne hormone analysis, making these samples representative of their baseline stress CORT concentrations. The four seal salamanders transported to Auburn University, however, were likely stressed from transportation and captivity. Lengthy exposure to a stressor may cause an increase in CORT production. *Eurycea nana* were found to have significantly higher CORT release rates 24 hours after field collection than when initially collected (Gabor et al. 2013). However, the four salamanders transported to Auburn University did not have drastically higher or lower plasma or waterborne CORT concentrations than the field sampled salamanders.

Waterborne CORT concentrations didn't differ significantly among the four populations. Without a difference in baseline CORT concentration among the populations, there is unlikely to be any physiological difference in baseline stress between the BCSB and neighboring populations, suggesting that the abnormal coloration of BCSB *D. monticola* is not associated with CORT. However, there is a possibility that chromatophore aggregation is a stress response independent of CORT in seal salamanders. In a study investigating stress response in *D. ocoee*, it was found that decreases in locomotory behavior were stress-induced, but were not associated with changes in plasma CORT concentrations (Bliley and Woodley 2012; Woodley 2017). Although decreased locomotory behavior was likely predator-avoidance behavior in response to stress, other stress responses may occur independently of CORT and may even be associated with other hormones. Rapid yellow color change was observed in *Litoria wilcoxii* frogs during amplexus and after toe clipping, but not after more stressful events (intense handling and ACTH injection), suggesting the species' color change may be linked to a more rapid stress response, such as epinephrine or norepinephrine instead of the HPA/I axis (Kindermann et al. 2013). Additionally, another glucocorticoid hormone, such as cortisol, could be linked to chromatophore aggregation in seal salamanders. Although less concentrated than CORT in most amphibians, cortisol and other glucocorticoids are also found in amphibians, and may be more responsible for coloration in seal salamanders than CORT.

The unique coloration of the BCSB population of seal salamanders could also be unrelated to stress entirely. There are two major types of skin color change in vertebrates. Physiological color change can occur rapidly with the movement of pigment within chromatophores, while morphological color change occurs over long periods of time and involves changes in the number of pigment cells and the amount of pigment within those

pigment cells (Kindermann and Hero 2016). Movement of pigments in vertebrates can be stimulated by hormones, neurotransmitters, or environmental cues such as light exposure, temperature, or chemical exposure, with the speed of pigment movement dependent on the type of stimulation (Aspengren et al. 2009; Nilsson Sköld et al. 2013). Sex hormones, which are steroids like glucocorticoids, were documented to stimulate slower color change, alter chromatophore distribution, and alter pigment synthesis (Richards 1982). Inversely, melanosome pigment dispersion and aggregation are rapidly stimulated by neuro-hormones within melanophores (Nielsen 1978; Baker 1993). With this context, the aberrant coloration found in the BCSB seal salamander population could be a result of morphological color change altering the quantity of pigment and/or pigment cells instead of physiological color change that effects the distribution of pigment. This aligns with a previous study investigating the abnormal yellow patches of the BCSB seal salamander population. They discovered that the melanocytes in the yellow patches contained less melanosome pigment, which suggests morphological color change, not physiological color change, is occurring within the population (Harmon 2018).

Color Analysis

Interestingly, there was only a significant difference in brightness (L*) on the head region among the populations, with the BC population having lighter colorations. There were no significant differences in L*, a*, or b* for the remaining body parts among the populations. This lack of difference in coloration between BC and the neighboring streams (DG, SC, and LB) at other body parts is likely due to the inconsistency of the unique yellow coloration found at the BCSB. Although the yellow coloration of seal salamanders is well documented at the BCSB, the coloration is not ubiquitous within the population. Most seal salamanders found at the BCSB have less yellow coloration than the individual shown in Figure 1. My study included individuals

that represent the entire BCSB population, not just those with the yellow coloration. Out of the eight seal salamanders sampled from the BCSB, only four salamanders displayed visible yellow coloration before color analysis was conducted. Because my sample pool from the BCSB was representative of the entire population, the coloration data for the yellow-colored individuals was likely masked by the typically colored seal salamander coloration data. Additionally, there were also significant differences in L*, a*, and b* among body parts, documenting the heterogeneity of coloration of seal salamanders across their body.

Environmental Conditions

Although the BCSB seal salamanders don't display a different baseline CORT concentration compared to their neighboring populations, a significant difference in environmental conditions was observed among the five streams. This suggests the BCSB seal salamander population tolerates atypical environmental conditions for the species, which may contribute to their abnormal coloration. Canopy openness, conductivity, temperature, and pH were all higher in the BCSB stream than the neighboring typical Southern Appalachian streams, DG, HC, SC, and LB. On the PCA biplot in Figure 5, all four typical streams had strong clustering among all their respective transects, suggesting high similarity among transects. This contrasts with the BCSB stream that had high variance among its transects, indicating the stream has high environmental variability over short stream distances. Additionally, all four typical Southern Appalachian streams occur on the left side of the PCA biplot while the BCSB stream occurs on the right side of the biplot, suggesting large differences between the typical Southern Appalachian streams and the BCSB stream within Principal Component 1 (PC1). Conductivity and canopy openness are strongly correlated with each other and PC1. This is due to the unique serpentine barrens ecosystem that directly effects both variables by having higher metal

concentrations and less woody vegetation. Expectedly, temperature and DO% are inversely related since colder water temperatures are well known to hold higher quantities of dissolved oxygen. In general, the water chemistry and canopy coverage in the BCSB stream greatly contrasts those found at typical Southern Appalachian streams, suggesting a plethora of potential causes of the aberrant appearance observed in the BCSB seal salamander population.

The concentration of heavy metals also varied among the four sampled streams. BC had the highest average Mg concentration out of all four streams across every month in the ten-month sampling period. The BCSB stream also had fluctuating average Ca concentrations but maintained a lower average Ca concentration than the other streams, except for the months of October and November. Fe concentrations fluctuated at BC, but remained minimal at DG, HC, and SC. The elevated levels of Mg and Fe and the low levels of Ca observed at the BCSB were expected due to the composition of serpentine soil containing elevated Mg and Fe and low concentrations of Ca (Brooks 1987). The increased concentrations of Mg, Ca, and Fe observed at BC in October and November coincide with a severe drought observed at the BCSB. During this drought, BC was nearly completely dry while the neighboring streams remained visibly unaffected. In October and November, only three of the five transects at BC had surface water for sample collection. With less water in the streambeds but similar amounts of Mg, Ca, and Fe present, the concentrations of these metals increased. With the frequent stream drying and near-drying that occurs at the BCSB, the seal salamanders and aquatic macroinvertebrate community found within the BC stream must often withstand extreme concentrations of these metals in the stream waters that do not persist throughout the year. However, drought conditions at the BCSB allowed Ca concentrations within the BC stream to reach levels comparable to the other streams, which may allow the BCSB seal salamanders to obtain ample Ca to maintain their physiological

health and ameliorate the negative impacts of high Mg. This requirement to tolerate extreme Mg and Fe concentrations at the BCSB may have a direct or indirect effect on the health of seal salamanders, resulting in their unique coloration. Overall, the metal concentrations observed in the BCSB stream greatly contrast with those of the surrounding Southern Appalachian streams, suggesting the BCSB seal salamanders must endure unique chemical conditions to persist.

In addition to the chemical differences, the macroinvertebrate communities also varied among the four sampled streams (BC, DG, HC, and SC). Diversity, richness, and abundance were all lowest at BC while evenness and tolerance were highest at BC out of all four streams. The low sample size of aquatic macroinvertebrates at BC compared to the other streams can directly impact the evenness and richness measurements of a community since both require substantial datasets to accurately represent a community. There are also likely aquatic macroinvertebrate species in BC that were not detected. The aquatic macroinvertebrate species found in BC also likely occur in differing population sizes, discrediting the 1.000 evenness index found with the low sample size. Because of the unlikely 1.000 evenness index I received, it is also unlikely that the diversity index of 1.10 is accurate for the stream since each species was only documented once at BC. However, streams with heavy metal pollution have been documented to have lower diversity indices (Rico-Sánchez et al. 2022).

Although some of the aquatic macroinvertebrate community data are difficult to interpret due to the small sample size at BC, there is some useful information to gain. The sheer difference in abundance between BC and the neighboring streams indicates that the BCSB seal salamander population likely must rely on other food sources to survive. Anecdotally, this nutrient demand is likely met by terrestrial macroinvertebrates that fall into stream waters at the BCSB since terrestrial macroinvertebrates seem more plentiful. In addition to the abundance, the tolerance

index calculated for BC may be representative of the larger aquatic macroinvertebrate community. The only species found at the BCSB were those with higher tolerance values, such as adult riffle beetles and net-spinning caddisflies while species with lower tolerance values, such as gravel coffin case caddisflies, fragile detritivores, and small head caddisflies were observed in all three neighboring streams. This suggests that the stream health is poorer at BCSB than the control streams, likely due to the presence of higher metal concentrations in stream waters. Although few, if any, studies have been conducted investigating aquatic macroinvertebrate communities at serpentine barrens, similar community patterns can be observed in areas with heavy metal pollution due to industrial mining. Mayfly richness and abundance, caddisfly richness and abundance, and chironomid richness were all significantly reduced by heavy metal pollution from mining activities in Japan (Iwasaki et al. 2009). High concentrations of heavy metals also influenced the presence of several sensitive aquatic macroinvertebrate families in Mexico, with lower diversity indices being associated with pollution and salinity (Rico-Sánchez et al. 2022).

Although the cause of the aberrant yellow coloration observed in the BCSB seal salamander population has not been determined, there have been a few discoveries to come from this study. Firstly, waterborne hormone analysis was determined to be a viable non-invasive technique for sampling CORT in seal salamanders. Our study is the first to our knowledge to attempt this validation for a species within *Desmognathus*, and it may suggest the use of the technique in other species within the genus. Secondly, high canopy openness, water temperature, conductivity, and concentrations of Mg and Fe persist in the BCSB stream waters year-round that local wildlife must be able to tolerate. Lastly, the aquatic macroinvertebrate community at the BCSB differs from that of other, nearby Southern Appalachian streams, having lower

abundance, diversity and tolerance index, which may indicate that food shortages occur for the BCSB seal salamander population. These stressors found at the BCSB may directly or indirectly contribute to the aberrant coloration of the BCSB seal salamanders by causing a morphological or physiological color change independent of CORT. This study provides valuable insight on the effects of serpentine barrens on vertebrate inhabitants and the aquatic macroinvertebrate community, pushing the scientific community one step closer to understanding this unique salamander population and the biodiversity found in western North Carolina.

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APPENDIX:

Table 3. Paired plasma and water bath CORT concentrations from samples used for waterborne hormone analysis validation.

Sample ID	Plasma [CORT] (ng)	Water Bath [CORT] (pg/ml/g/mm)
AUBURN BC1	5.040	0.235
BC2	17.421	7.293
BC3	228.789	19.674
BC4	162.718	0.415
BC5	12.401	0.633
BC6	79.981	1.538
AUBURN DG1	7.812	0.044
DG2	16.856	4.484
DG3	15.446	0.106
DG4	2.935	0.466
AUBURN HC1	9.559	0.501
HC2	32.349	1.343
HC3	1.151	0.767
HC4	6.862	2.207
AUBURN SC1	3.942	0.057
SC2	6.685	1.695
SC3	86.834	1.991
SC4	27.323	2.958

Table 4. Waterborne hormone samples used in combination with those in Table 2 to compare baseline CORT concentration among seal salamander populations.

Sample ID	Water Bath [CORT] (pg/ml/g/mm)
BC1	4.235
BC2	10.556
BC3	0.468
BC4	0.045
BC5	0.516
BC6	0.027
BC7	0.076
BC8	0.382
DG1	1.492
DG2	0.248
DG3	0.189
DG4	0.262
DG5	5.859
DG6	0.172
DG7	1.719
DG8	4.595
DG9	0.123
DG10	1.409
SC1	0.064
SC2	9.285
SC3	1.024
SC4	3.211
SC5	0.276
SC6	5.925
SC7	0.175
SC8	2.021
LB1	0.143
LB2	2.266
LB3	0.123
LB4	0.320
LB5	0.045
LB6	0.675
LB7	4.171
LB8	0.160

Table 5. ANOVA summary of test for comparing baseline CORT concentrations among seal salamander populations.

	Df	Sum Sq.	Mean Sq,	F value	P value
Location	3	3.02	1.008	0.348	0.791
Year	1	2.18	2.181	0.754	0.390
Location:Year	3	9.62	3.205	1.108	0.356
Residuals	44	127.34	2.894		

Table 6. ANOVA summary table for comparing L* among sites, body parts, and treatments.

	Sum Sq.	Mean Sq.	NumDF	DenDF	Statistic	P value
Site	43.447335	14.482445	3	24	1.4715440	0.2473315
Treatment	1.208850	1.208850	1	24	0.1228298	0.7290428
BodyPart	2613.600000	653.400000	4	96	66.3911940	0.0000000
Site:Treatment	4.706566	1.568855	3	24	0.1594095	0.9225649
Site:BodyPart	301.500000	25.125000	12	96	2.5529213	0.0056732
Treatment: BodyPart	4.225000	1.056250	4	96	0.1073243	0.9797064
Site:Treatment: BodyPart	61.075000	5.089583	12	96	0.5171465	0.8990234

Table 7. ANOVA summary table for comparing a* among sites, body parts, and treatments.

	Sum Sq.	Mean Sq.	NumDF	DenDF	Statistic	P value
Site	30.4237329	10.1412443	3	24	1.8449108	0.1659954
Treatment	0.3061895	0.3062895	1	24	0.0557207	0.8153950
BodyPart	713.587500	178.396875	4	96	32.4542354	0.0000000
Site:Treatment	0.0918868	0.0306289	3	24	0.0055721	0.9994108
Site:BodyPart	106.1125000	8.8427083	12	96	1.6086792	0.1018423
Treatment: BodyPart	18.1875000	4.5468750	4	96	0.8271745	0.5110627
Site:Treatment: BodyPart	24.8125000	2.0677083	12	96	0.3761607	0.9690125

Table 8. ANOVA summary table for comparing b* among sites, body parts, and treatments.

	Sum Sq.	Mean Sq.	NumDF	DenDF	Statistic	P value
Site	28.7585090	9.5861697	3	24	1.4437909	0.2548061
Treatment	0.0802944	0.0802944	1	24	0.0120933	0.9133480
BodyPart	1389.02500	347.256250	4	96	52.3009102	0.0000000
Site:Treatment	3.7050136	1.2350045	3	24	0.1860063	0.9048693
Site:BodyPart	119.3750000	9.9479167	12	96	1.4982742	0.1380805
Treatment: BodyPart	14.400000	3.600000	4	96	0.5422027	0.7051011
Site:Treatment: BodyPart	100.600000	8.3833333	12	96	1.2626294	0.2534749

Table 9. PERMANOVA summary table for test of differences among multivariate centroids for the five streams based on the Mahalanobis distance matrix.

	Df	SS	R^2	F value	P value
Stream	4	111.5	0.455	9.396	0.0001
Residual	45	133.5	0.545		
Total	49	245.0	1.000		

Table 10. Species and abundance breakdown for both aquatic macroinvertebrate surveys conducted at the BCSB stream and typical Southern Appalachian streams.

Season	Taxa	BC	DG	HC	SC
Fall	Black Fly Larvae	0	0	1	0
Fall	Burrowing Mayfly	0	0	0	1
Fall	Chironomid Midge	0	1	2	2
Fall	Coiled Right Face Snail	0	34	0	11
Fall	Fat Headed Cranefly	0	0	0	1
Fall	Filer Mayfly	0	0	0	2
Fall	Flattened Scraper	0	21	28	3
Fall	Fragile Detritivore	0	4	4	0
Fall	Giant Shredder	0	0	1	0
Fall	Gravel Coffin Case Caddisfly	0	25	13	1
Fall	Isopod	0	0	0	2
Fall	Larval Riffle Beetle	0	0	1	1
Fall	Net Spinning Caddisfly	0	9	16	17
Fall	Oligochaete Worm	0	0	1	0
Fall	Roach Shredder	0	1	1	5
Fall	Roundheaded Swimmer	0	0	1	0
Fall	Small Head Caddisfly	0	2	2	7
Fall	Sowbog	0	0	0	2
Fall	Spiny Turtle Mayfly	0	1	0	1
Fall	Square Log Cabin Caddisfly	0	0	3	0
Fall	Water Penny	0	11	5	5
Summer	Adult Riffle Beetle	1	0	0	0
Summer	Burrowing Mayfly	0	0	0	1
Summer	Chironomid Midge	0	1	1	8
Summer	Coiled Right Face Snail	0	53	0	28
Summer	Crayfish	0	0	1	0
Summer	Filter Mayfly	0	0	1	0
Summer	Flattened Scraper	0	5	24	22
Summer	Fragile Detritivore	0	10	5	2
Summer	Giant Shredder	0	1	0	2
Summer	Gravel Coffin Case Caddisfly	0	167	26	40
Summer	Net Spinning Caddisfly	1	0	4	20
Summer	Predatory Beetle Larva	0	0	2	0
Summer	Roach Shredder	0	1	0	0
Summer	Roundheaded Swimmer	0	1	2	2
Summer	Sand and Stick Case Caddisfly	0	0	2	2
Summer	Sand and Mineral Case Caddisfly	0	1	5	1
Summer	Small Head Caddisfly	0	0	3	5
Summer	Vegetated Case Caddisfly	0	17	2	12
Summer	Water Penny	1	0	0	7

Table 11. ANOVA summary of tests for differences in stream magnesium concentrations.

	Sum Sq.	Mean Sq.	NumDF	DenDF	Statistic	P value
Site	3806294005	1268764668	3	17.19857	4240.0499	< 0.001
Month	421025949	46780661	9	141.78928	156.3350	< 0.001
Site:Month	910184006	33710519	27	141.26358	112.6563	< 0.001

Table 12. ANOVA summary of tests for differences in stream calcium concentrations.

	Sum Sq.	Mean Sq.	NumDF	DenDF	Statistic	P value
Site	6774973	2258324.4	3	156	60.220048	< 0.001
Month	17386785	1931865.0	9	156	51.514744	< 0.001
Site:Month	4670441	172979.3	27	156	4.612633	< 0.001

Table 13. ANOVA summary of tests for differences in stream iron concentrations.

	Sum Sq.	Mean Sq.	NumDF	DenDF	Statistic	P value
Site	7325300	2441766.6	3	156	9.170698	< 0.001
Month	18722607	2080289.7	9	156	7.813076	< 0.001
Site:Month	22807762	844731.9	27	156	3.172613	< 0.001

Table 14. ANOVA summary of tests for differences in stream aluminum concentrations.

	Sum Sq.	Mean Sq.	NumDF	DenDF	Statistic	P value
Site	3806294005	1268764668	3	17.19857	4240.0499	< 0.001
Month	421025949	46780661	9	141.78928	156.3350	< 0.001
Site:Month	910184006	33710519	27	141.26358	112.6563	< 0.001

Table 15. Mg, Ca, Fe, and Al concentration data from BC, DG, HC, and SC transects from July 2023 to April 2024. Co, Ni, Zn, and are not included due to their concentrations being below the calibration curve's lowest concentrated standard (10ppb).

Sample ID	Mg (ppb)	Ca (ppb)	Fe (ppb)	Al (ppb)
JULBC1	12789.364	1240.731	746.229	525.795
JULBC3	13060.661	1228.937	608.272	524.492
JULBC5	13909.155	1221.233	719.906	783.738
JULBC7	14528.020	1275.720	3133.024	3647.946
JULBC9	14709.825	1245.246	2226.502	2035.977
JULDG1	1101.118	1561.318	466.567	655.640
JULDG3	1012.730	1478.077	476.341	686.570
JULDG5	1003.444	1491.198	476.531	627.490
JULDG7	950.602	1479.244	391.695	444.420
JULDG9	1033.909	1525.435	408.486	495.050
JULHC1	504.041	1302.797	530.170	708.259
JULHC3	518.208	1315.862	461.570	596.144
JULHC5	565.315	1308.996	603.165	1081.867
JULHC7	476.753	1297.260	360.375	340.316
JULHC9	499.795	1320.576	473.899	582.163
JULSC1	1107.963	1396.687	551.901	880.024
JULSC3	800.582	1440.093	494.704	724.970
JULSC5	1092.279	1457.343	2445.772	3848.295
JULSC7	797.736	1396.971	547.037	919.704
JULSC9	2167.168	1656.500	4112.974	6277.395
AUGBC1	12911.708	1275.911	461.526	567.802
AUGBC3	13076.220	1218.206	357.422	295.582
AUGBC5	12999.588	1212.130	362.569	333.353
AUGBC7	13093.180	1212.939	363.094	334.462
AUGBC9	13462.111	1205.096	348.103	265.246
AUGDG1	1030.001	1525.028	459.684	536.632
AUGDG3	1100.316	1528.778	725.130	1348.391
AUGDG5	1003.729	1487.810	361.043	349.465
AUGDG7	969.127	1491.003	363.294	356.176
AUGDG9	986.602	1516.908	503.697	776.947
AUGHC1	576.904	1307.963	482.103	665.783
AUGHC3	497.448	1325.224	362.660	350.330
AUGHC5	504.158	1335.370	374.199	362.766
AUGHC7	586.235	1327.142	540.446	1141.923
AUGHC9	497.595	1318.238	387.828	427.166
AUGSC1	1285.707	1460.713	643.810	938.218
AUGSC3	842.289	1447.822	501.477	717.774
AUGSC5	819.946	1469.992	473.791	643.135
AUGSC7	764.751	1467.049	529.815	954.485
AUGSC9	696.630	1458.643	373.617	377.773

SEPBC1	15160.057	1268.748	341.310	331.804
SEPBC3	14675.129	1234.574	469.629	912.886
SEPBC5	14799.452	1238.801	356.981	317.773
SEPBC7	15701.413	1221.140	361.071	302.180
SEPBC9	15627.783	1350.974	378.703	470.227
SEPDG1	977.124	1530.294	339.202	287.761
SEPDG3	1019.616	1525.960	423.136	673.726
SEPDG5	1025.402	1521.430	349.938	320.239
SEPDG7	1011.378	1502.625	350.686	345.998
SEPDG9	992.184	1485.622	369.205	361.106
SEPHC1	672.186	1366.826	581.746	1382.792
SEPHC3	468.477	1302.137	365.994	374.709
SEPHC5	491.095	1344.349	343.577	293.616
SEPHC7	477.958	1339.620	342.221	316.081
SEPHC9	463.067	1287.977	362.196	373.012
SEPSC1	921.314	1424.790	382.387	475.679
SEPSC3	783.020	1433.983	343.147	315.459
SEPSC5	685.549	1410.718	342.174	310.968
SEPSC7	717.681	1391.956	373.481	444.510
SEPSC9	658.069	1395.366	364.974	360.212
OCTBC1	S	913.882	243.609	253.511
OCTBC6	S	1244.502	47.412	93.618
OCTBC8	S	1037.327	25.378	84.866
OCTDG1	600.955	1748.980	82.398	221.105
OCTDG3	646.479	1808.001	259.779	631.026
OCTDG5	661.013	1691.525	315.876	380.988
OCTDG7	810.868	1831.618	540.131	604.524
OCTDG9	593.967	1750.217	123.124	199.768
OCTHC1	127.296	1072.607	215.544	375.852
OCTHC3	137.213	868.588	126.024	207.846
OCTHC5	171.673	1135.673	259.198	456.098
OCTHC7	149.518	947.842	57.655	180.930
OCTHC9	148.275	880.717	187.095	281.225
OCTSC1	431.957	1115.040	374.289	685.854
OCTSC3	261.232	1089.513	85.299	150.474
OCTSC5	276.815	1154.306	141.405	272.116
OCTSC7	266.892	1137.235	71.337	170.872
OCTSC9	-176.154	155.983	-54.013	23.649
NOVBC1	S	1046.704	6305.132	94.584
NOVBC5	S	1736.510	841.497	133.112
NOVBC9	S	1634.787	256.133	93.237
NOVDG1	548.668	1671.387	55.093	185.131
NOVDG3	634.710	1562.987	-12.189	65.701
NOVDG5	580.333	1602.444	53.465	154.832

NOVDG7	531.449	1453.064	-22.941	59.358
NOVDG9	451.654	1311.227	9.176	83.100
NOVHC1	41.097	717.499	-18.520	71.343
NOVHC3	31.307	702.513	-22.485	62.317
NOVHC5	103.092	699.712	2.425	69.137
NOVHC7	59.330	886.101	-6.124	90.262
NOVHC9	20.925	712.248	-27.992	60.844
NOVSC1	302.305	950.672	140.611	240.017
NOVSC3	180.327	903.382	-15.662	76.821
NOVSC5	169.370	914.670	7.511	101.445
NOVSC7	225.472	1317.194	11.212	154.638
NOVSC9	369.197	1213.680	122.088	309.563
DECBC1	6800.607	409.616	110.165	115.288
DECBC3	6955.358	427.608	207.170	156.509
DECBC5	6840.934	388.473	78.537	99.555
DECBC7	6811.790	424.458	123.176	165.005
DECBC9	6895.044	379.822	108.660	118.607
DECDG1	461.215	1260.046	313.031	590.544
DECDG3	337.076	1157.745	-23.945	58.778
DECDG5	325.756	1124.328	-25.071	48.387
DECDG7	327.505	1161.017	-6.980	88.056
DECDG9	321.035	1065.017	-15.300	60.260
DECHC1	17.504	613.274	-7.802	79.642
DECHC3	18.757	629.060	-13.989	82.953
DECHC5	8.418	622.718	-26.473	56.726
DECHC7	6897.379	424.379	208.387	188.560
DECHC9	194.257	817.890	299.382	540.563
DECSC1	628.204	866.676	584.539	744.331
DECSC3	163.751	864.448	-6.536	88.921
DECSC5	137.313	864.447	-28.245	68.438
DECSC7	138.519	872.691	-26.884	59.622
DECSC9	145.862	888.425	-4.161	68.184
JANBC1	5127.111	374.739	219.510	152.289
JANBC3	5146.316	360.024	1994.849	519.043
JANBC5	5183.813	357.789	1315.812	362.749
JANBC7	5237.136	344.887	1230.023	324.252
JANBC9	5275.848	478.283	2259.928	539.332
JANDG1	277.017	950.914	-9.312	65.077
JANDG3	258.365	937.793	-25.317	52.818
JANDG5	274.882	964.549	-23.545	65.109
JANDG7	263.593	917.841	-27.222	47.977
JANDG9	260.044	914.269	-28.934	48.572
JANHC1	-2.895	590.351	-24.273	61.015
JANHC3	0.328	589.200	-25.218	62.563

JANHC5	2.359	713.622	-26.959	61.138
JANHC7	-10.260	554.664	-19.048	64.413
JANHC9	-18.174	534.226	-27.610	57.305
JANSC1	137.505	796.795	-16.751	63.591
JANSC3	152.401	887.502	2.859	128.968
JANSC5	114.926	815.770	54.283	68.890
JANSC7	127.071	962.615	-6.590	71.460
JANSC9	109.218	851.731	-24.882	61.798
FEBBC1	5804.715	428.461	4.425	61.367
FEBBC3	5893.903	372.807	16.033	66.800
FEBBC5	7803.528	556.320	71.447	162.333
FEBBC7	5886.975	358.951	30.099	69.895
FEBBC9	5574.103	375.142	15.048	69.041
FEBDG1	257.118	1013.338	-29.939	52.677
FEBDG3	245.200	1002.217	-33.654	46.688
FEBDG5	273.868	1202.524	-10.536	88.519
FEBDG7	232.252	910.481	-31.388	50.973
FEBDG9	238.258	1032.800	-31.881	55.057
FEBHC1	-22.125	547.896	-34.609	47.249
FEBHC3	-30.898	528.277	-28.078	59.388
FEBHC5	-33.136	505.872	-34.749	49.364
FEBHC7	-28.491	525.711	-34.426	50.695
FEBHC9	-30.110	521.301	-34.786	48.741
FEBSC1	84.463	814.611	-30.843	58.329
FEBSC3	98.378	866.710	-32.264	56.838
FEBSC5	99.420	932.826	-28.027	60.951
FEBSC7	108.973	908.374	-38.918	28.230
FEBSC9	86.989	833.957	-34.684	43.962
MARBC1	5737.617	375.202	0.413	50.175
MARBC3	5992.561	356.297	104.128	84.571
MARBC5	5852.234	361.859	105.060	61.855
MARBC7	5947.802	354.779	152.411	58.993
MARBC9	5894.699	358.097	58.704	50.880
MARDG1	229.521	933.813	16.226	47.976
MARDG3	226.778	931.598	17.335	49.095
MARDG5	265.967	913.838	167.510	68.854
MARDG7	257.205	886.400	117.974	57.442
MARDG9	242.219	882.387	84.693	53.284
MARHC1	-10.368	539.974	114.087	102.990
MARHC3	-29.873	516.967	9.415	53.377
MARHC5	-28.647	511.927	-6.316	46.826
MARHC7	-21.385	514.485	8.496	53.963
MARHC9	-27.615	527.885	11.801	55.949
MARSC1	125.697	819.566	44.948	85.665

MARSC3	99.552	825.035	18.606	76.030
MARSC5	115.724	926.307	133.962	186.807
MARSC7	82.148	819.072	22.569	80.621
MARSC9	190.020	784.935	234.455	294.809
APRBC1	6516.299	429.226	-3.440	55.328
APRBC3	6900.081	418.964	6.427	55.379
APRBC5	6723.166	390.869	3.732	53.608
APRBC7	6714.436	382.386	-12.899	118.525
APRBC9	6892.652	462.404	-1.062	353.502
APRDG1	259.804	1022.312	-14.118	123.957
APRDG3	257.354	996.651	-16.853	64.935
APRDG5	179.157	842.679	-28.889	67.644
APRDG7	257.378	964.492	-26.377	49.780
APRDG9	266.023	982.566	-26.921	49.997
APRHC1	-13.987	623.816	-4.856	60.287
APRHC3	-24.639	574.971	-18.000	62.481
APRHC5	-16.299	607.633	-12.759	69.519
APRHC7	-2.181	701.734	-22.299	67.721
APRHC9	20.241	741.713	14.967	114.644
APRSC1	119.638	940.780	-8.998	119.022
APRSC3	100.517	873.835	-14.762	371.069
APRSC5	108.047	914.524	-6.851	190.008
APRSC7	102.652	851.611	-21.070	106.632
APRSC9	166.780	3151.340	33.019	189.318