Summer 7-7-2016

# Conservation Genetics and Mark-Recapture Monitoring of the Rare Pigeon Mountain Salamander (Plethodon petraeus) within a Highly Restricted Range

Kate Donlon

# Conservation Genetics and Mark-Recapture Monitoring of the Rare Pigeon Mountain Salamander (*Plethodon petraeus*) within a Highly Restricted Range

Thesis submitted by: Kate Donlon

Kennesaw State University

College of Math and Science

Department of Ecology, Evolution, and Organismal Biology

Major Professor: Thomas McElroy

Date: July, 7th 2016

**Abstract**

Globally, amphibian species are experiencing declines at an alarming rate largely due to habitat loss, disease and climate change. Species with limited ranges are at an elevated risk of a significant decline in population numbers and extinction because of the inability to avoid and recover from these impacts. Long-term management plans are critical for conservation of species with small ranges; however, the knowledge required to develop effective plans is absent from the literature for many species. One such species is the Pigeon Mountain Salamander. The distribution of the Pigeon Mountain Salamander, *Plethodon petraeus*, is restricted to roughly 17 kilometers along the eastern flank of Pigeon Mountain in northwest Georgia. Consequently, *P. petraeus* is highly vulnerable to the impacts associated with amphibian declines, a fact that placed the salamander on the list of rare and protected species in Georgia. The distribution of *P. petraeus* is highly correlated with patchily distributed rocky outcrops, which provides an efficient management target. However, the development of an effective, long-term management plan requires an understanding of genetic population structure, gene flow, and habitat use patterns. Robust design mark-recapture methods and population genetics with cross-amplified microsatellites were used to further our knowledge of how this species is distributed. Mark recapture results indicated high site fidelity of recaptured salamanders and abundance estimates (average number of total salamander abundance in a single plot, 57.8) within two 25 x 25 meter study areas. Population genetic results revealed four distinct populations across the known range of *P. petraeus* and significant isolation by distance genetic structuring.

Keywords: *Plethodon petraeus*, Pigeon Mountain Salamander, conservation genetics, microsatellite, mark-recapture, visual implant elastomer.

**ACKNOWLEDGEMENTS**

The research detailed below included lab and field work that would not have been possible without the help of advisors, friends, and student assistants with a sense of adventure. Many obstacles stood between the objectives of the project and its completion, including thickets of poison oak, countless numbers of ticks, and several Georgia thunderstorms. I am especially thankful for those who braved these conditions at all hours of the night to assist with various field components. It would not have been possible without their help.

I am grateful to the Department of Ecology, Evolution, and Organismal Biology for awarding me a teaching assistantship to allow me to pursue a Master's degree. I would like to thank Dr. McElroy for having confidence in this project and am especially grateful for taking me on as his as his first graduate student. I would also like to thank Dr. Ensign and Dr. McNeal for serving as the members of my committee and their much-appreciated mentorship. I am especially indebted to J.J. Apodaca for introducing me to the field of conservation genetics and John Jensen with Georgia Department of Natural Resources for sharing his time and knowledge about the Pigeon Mountain Salamander with me and making this research possible. I must also thank Josh Aldridge and the other Department of Natural Resources staff at the Crockford-Pigeon Mountain Wildlife Management Area for making their resources and facilities available as I completed fieldwork and Malcolm Hodges of The Nature

Conservancy, who helped grant access to private lands. Lastly, a special thanks to family and friends who have supported me both financially and emotionally during the duration of my degree as I pursued my passion and furthered a career in science.

This research was funded by the Foundation for the Conservation of Salamanders and the Department of EEOB. The field portion of this research was conducted under Georgia Wildlife Resources Agency Permit 29-WJH-14-252, and research at Kennesaw State University was conducted in accordance with the approved IACUC protocols.

## FIGURES AND TABLES

### CHAPTER 2

*Figures*



### CHAPTER 3

*Figures*



*Tables*

**CHAPTER 4**



**CHAPTER 5**

**TABLE OF CONTENTS**

5. POPULATION GENETICS

# CHAPTER 1

# INTRODUCTION

## Study Organism

360 million years ago the ancestors of modern amphibians first evolved. This ancient group of vertebrates reached their peak diversity around 200 million years ago (Petranka 2010). Today there are over 4600 known species of classified amphibians including frogs, caecilians, and salamanders. Around 10% of this total is made up of species of salamanders, and the region in the world with the greatest diversity of salamander is the southeastern United States. Seven families, 19 genera, and over 75 species are represented (Petranka 1998). To date, Georgia is known to be home to a total of 57 species (Jensen et al. 2008). Two of these are endemic to the state- *Plethodon savannah*, commonly known as the Savannah Slimy Salamander, and *P. petraeus*, commonly known as the Pigeon Mountain Salamander.

*P. petraeus* is a recently discovered species. The first specimen was documented from Pettijohn Cave in 1972 by Carol Ruckdeschel of Georgia DNR (Jensen et al. 2002) The new species was later described in 1986 (Wynn et al. 1988). The species is a large Plethodontid salamander that is fully terrestrial. Individuals are easily distinguished from other salamanders within their range due to distinct toes and feet, elongated legs and dorsal coloration. Specifically, the fourth toe on the front

feet and the fifth toe on the hind feet are longer than in species of similar size and are distinctive webbing is present between all digits (Wynn et al. 1988). The coloration of Pigeon Mountain Salamanders is black with an irregular reddish brown dorsal pattern that extends the length of the body. White iradospore spots or brassy flecks are often common along the body. Recent molecular phylogenetic studies indicate that *P. petraeus* falls within the *Plethodon glutinosus* group of salamanders (Highton et al. 2012).

Since its discovery, the salamander has been documented in 11 locations within its highly restricted range (John Jensen, personal correspondence). All of the known locations of the species are caves, outcrops, and rocky areas on eastern slopes of Pigeon Mountain in Walker and Chattooga counties in northwest Georgia (Jensen et al. 2002; Wynn et al. 1988). The specific scientific name, *petraeus,* is Greek meaning among rocks or rock dwelling and was chosen for this species based on its affinity for these locations. This habitat provides sufficient moisture year round for survival and reproduction but is not continuous throughout its small range, resulting in a patchy distribution of the species (Jensen et al. 2002). As a lungless, terrestrial salamander, the species requires damp microenvironments such as rock crevices provide to maintain moist skin for gas exchange. Based on the brooding preferences of members of the closely related slimy salamander complex and other crevice dwelling species, it is presumed that the *P. petraeus* females lay egg clutches within caves and rock crevices. No clutch has been seen in the wild, but in 2014 a female *P. petraeus* in

captivity at the Toledo Zoo laid a small clutch of eggs in part of an enclosure that is blacked out and always dark. Although possibly an artifact of captivity, they were laid right at the water's edge, with some of the eggs actually resting in the water. It is uncommon for *Plethodon* species to lay eggs in water (Petranka 2010). The female tended them for a day or two before abandoning the non-viable eggs (Tim Herman, personal correspondence).

Even though it is a rare species based on its limited distribution, *P. petraeus* is abundant where it is found and even outnumbers sympatric salamanders at some locations (Jensen 2000; Jensen et al. 2002; Wynn et al. 1988). Finding individuals foraging on the forest floor is rare and only observed during or after a recent rainfall when the leaf litter is wet (Jensen et al. 2002, personal observation). Due to the generally low dispersal rate of terrestrial salamanders (Ousterhout & Liebgold 2010; Liebgold et al. 2011) it is possible that *P. petraeus* does not exist as a continuous, connected population throughout its range. Gene flow between habitats could be limited not only by distance between the patchily distributed habitats but by competition with a sympatric species. The Northern Slimy Salamander, *Plethodon glutinosus*, is distributed widely on the forest floor but is a poor climber compared to the Pigeon Mountain Salamander (Marshall et al. 2004). When the leaf litter dries, the competition for refuge under cover objects such as rocks and logs where moisture levels remain high would increase. The more aggressive *P. glutinosus* has been shown to win territory disputes and evict *P. petraeus* individuals from cover

objects in a laboratory study. The absence of available cover objects for *P. petraeus* to use during movements across the forest floor could effectively interrupt movement between distant patches (Marshall et al. 2004).

**Study Area**

The study area was located on Pigeon Mountain at approximately N 34° 39' 41" W 85° 21' 17" in Walker and Chattooga counties in Northwest Georgia, USA. The mountain is the southernmost extension of the Cumberland Plateau into Georgia. The Cumberland Plateau is characterized by karst geology, and Pigeon Mountain has numerous cave entrances and extensive sandstone and limestone outcroppings. A mesic deciduous forest composed primarily of oak and hickory trees covers the majority of the landscape. The mountain is within the boundary of the 20,657 acre Crockford-Pigeon Mountain Wildlife Management Area (CMWMA). It is well known by recreationists and naturalists for its cave systems and several rare species of flora and fauna, including the Pigeon Mountain Salamander. All known locations of *P. petraeus* exist in or around this Wildlife Management Area (WMA). All sites to be included in this study are found within the WMA other than the most recently discovered location. This site is the most southern known location and is on a private land track accessible through an agreement with the landowners and The Nature Conservancy of Georgia (TNC).

The habitat that *P. petraeus* can be found occupying tends to be limestone or sandstone outcrops and other rock formations, including caves and cliffs. During and after rain *P. petraeus* individuals are commonly found on the forest floor in areas where boulders and rocks are scattered.

**Integration of the Thesis**

Globally, amphibian species are experiencing declines at an alarming rate (Houlahan et al. 2000; Grant et al. 2016; Mendelson et al. 2006) largely due to habitat loss, disease, and climate change (Stuart et al. 2004). Species with limited ranges are at an elevated risk of a significant decline in population numbers and extinction because of the inability to avoid and recover from these impacts (Bayer et al. 2012). Long-term management plans are critical for conservation of species with small ranges; however, the knowledge required to develop effective plans is absent from the literature for many species. The Pigeon Mountain Salamander has one of the smallest ranges of any terrestrial vertebrate in North America. Consequently, *P. petraeus* is highly vulnerable to the impacts associated with amphibian declines, a fact that placed the salamander on the list of rare and protected species in Georgia. The distribution of *P. petraeus* is highly correlated with patchily distributed rocky outcrops, which provides an efficient management target. However, the development of an effective, long-term management plan requires an understanding of natural history and population genetics.

It is not difficult to see how the goals of integrated biology can be applied to the conservation of rare and endangered species. Conservation biology and the applied field of resource management face a multitude of hurdles, beginning with research that could lead to policy and lawmaking at state and federal levels. Incorporating many disciplines across biology and those relevant outside science, including economic, education and government policy, is necessary due to the complexity of conservation issues. The inherently integrated nature of conservation makes this project suitable research to complete for a Master's of Science in Integrated Biology. Here we present research that uses methods within the fields of ecology and population genetics to advance the scientific knowledge of *P. petraeus* for the purpose of conservation.

**Objectives**

Due to the limited range of Pigeon Mountain Salamanders this rare species is more vulnerable to perturbations in its environment. Although studies have investigated several different aspects of this species' life history, much is still unknown about the species, and further research will benefit its conservation. The goal of this study is to delineate the genetic structure and diversity of *P. petraeus* using genomic DNA profiles and establish a mark recapture study to estimate local population size and monitor contemporary movement patterns within a selected location. To achieve these goals we: a) identify polymorphic microsatellite markers for *P. petraeus* via cross amplification, b) determine the genetic structure of *P. petraeus* with genomic

DNA profiles from polymorphic microsatellite loci to delineate population structure and c) investigate species abundance and dispersal by establishing a mark recapture program using Visual Implant Elastomer (VIE) tag methods. Based on the current scientific record about *P. petraeus* and the results of population genetics studies of other *Plethodon* species, we predict that genetic structure exists within the range of the species. The outcome of this project is significant for the conservation of this rare endemic species and will contribute to future management planning.

## CHAPTER 2

## Visual Implant Elastomer (VIE) Tagging Trial in a large terrestrial salamander, *Plethodon glutinosus*.

### Introduction

Mark-recapture studies allow researchers to monitor a species of interest and estimate population level trends such as abundance and survivorship. Effectively marking individuals is necessary for the success of any mark-recapture study. Visible Implant Elastomer (VIE) tags, originally developed to tag fish (Northwest Marine Technology Inc., Shaw Island, Washington), are now used to mark a number of small animals including salamanders in the genus *Plethodon* (Heemeyer & Homyack 2007). VIE tags are often favored over other marking techniques because the are easily readable, have minimal or no negative impact on species' health or survival, and are durable.

Non-invasive mark-recapture techniques are important in conservation work, especially when working with threatened and endangered species. The use of VIE marking is an effective and less invasive alternative to toe clipping amphibians (Sapsford et al. 2014). This technique was judged too invasive for use in *P. petraeus* due to this species' specialized toes. Their elongated toes and broadened toe pads, compared to other terrestrial salamanders in the genus *Plethodon*, may facilitate

climbing and foraging within the crevices of rock where they are commonly found (Wynn et al. 1988).

Prior to utilizing VIE tags in *P. petraeus*, a preliminary study of tag retention and visibility was conducted using captive Northern Slimy Salamanders, *Plethodon glutinosus*. This species was selected as model because it is common in Georgia and similar in size and coloration. Adults of both species have darkly pigmented coloration that can make VIE tag detection difficult.

The objectives of this study were to a) assess the retention of VIE tags in juvenile and adult Northern Slimy Salamanders, b) compare the tag readability in adults across time, three colors and three tagging locations. Results were used to determine if VIE tagging might be a viable technique for marking related species, notably *Plethodon petraeus*.

## Methods

### *Collection and Animal Care*

Northern Slimy Salamanders were collected in October 2015 and housed at Kennesaw State University, Kennesaw, Georgia, USA through April 2016. We hand

captured 15 juvenile (SVL < 40 mm) and 19 adult salamanders in the Crockford-Pigeon Mountain Wildlife Management Area, Walker County, Georgia. Prior to tagging, individuals were weighed and measured (SVL) and assigned an identification number (1 to 25). Within age categories, juvenile or adult salamanders were randomly assigned one of three dorsal tag placement patterns. Six ventral tagging locations were utilized, one tag on each side of the body at the anterior body location, posterior body location, and at the base of the tail on either side of the cloaca (Fig 1.) Three tag placement patterns were applied by rotating colors at tagging locations so replicate tags of each color at each location were present (Fig. 2).

Salamanders were individually housed in Sterlite 6Q plastic containers with a dampened paper towel and Zoomed moss as substrate. Enclosures were kept on shelves in a temperature-controlled room held between 14-17 degrees Celsius. Enclosures were cleaned on a biweekly schedule. 1-2 crickets were offered as food by adding them to salamander enclosures for 24 hours once a week. Uneaten crickets were removed after the allotted foraging time.

The enclosures were closed with the plastic lid that came with the containers; however, within the first week of the experiment, this was found to be an inadequate method for keeping in juvenile and even small adult salamanders. The slightest gap between the lid and the rim of the enclosure created an opportunity for

salamanders to escape. Further losses were minimized by wrapping rubber bands around the containers and lids to decrease the chance of space for a salamander to squeeze between the two. This prevented further escapes by adults but not juveniles. To prevent more juveniles from escaping, they were transferred to empty pipette tip boxes, roughly 9 cm x 5 cm enclosures. The lids of these containers overlapped with their the walls preventing further escapes but did not have a tight seal so air could still enter. This change prevented further losses of juvenile salamanders from the study. In total, two juveniles and five adults escaped over the course of the study, so sample sizes are smaller in later time periods.

*VIE Tagging and Monitoring*

One researcher (KCD) injected VIE tags using a 1 cc insulin syringe in the six locations underneath under the ventral dermis of each salamander (Fig 1). Exact placements varied slightly due to the amount of the elastomer injected and the size of the salamander. Adults and juveniles were anaesthetized prior to injections in a concentration of 500 mg/L or 250mg/L of Tricaine methanesulfonate (MS-222) in de-chlorinated water, respectively. To achieve a neutral pH so not to irritate the salamander's skin, the solution was buffered with sodium bicarbonate (Osbourn et al. 2011). Salamanders were placed in the anesthetic bath until unresponsive to gentle prodding and unable to right themselves when placed on their backs. Once tagged, salamanders were placed in water baths to recover from the anesthetic.

Tag visibility under UV light was first recorded immediately after the tags were placed and then weekly for the first month. After a month with no tag loss the monitoring schedule was changed to once a month to reduce the amount of handling stress on the salamanders. The visibility of VIE tags was assessed based on their difficulty to read using the 1- 4 scale as described in Heemeyer et al. (2007). A score of 4 indicated a tag that could be "easily read" by the observer under UV light with no manipulation of the salamander's body. On this scale tag visibility decreases from 4 to an "absent" or not visible tag represented by a score of 1. Three separate researchers who had been trained to observe VIE tags recorded results over the course of the study.

*Data Analysis*

We examined the effects of age cohort and time on tag visibility across months. The first measurement taken after tagging was used in analysis to represent the first monitoring period since multiple reading were taken during this month. Due to a significant difference in tag readability between age cohorts, we examined the outcome of VIE color, tag placement, and time interactions within tagged adults. The Kruskal-Wallis test, which is a non-parametric alternative to the One-Way ANOVA test that follows the assumption of normality of the residuals, was used for in the

analysis because of the numbered ordinal nature of the tag visibility. Although the data could not fit the parametric assumption of normality for ANOVA, it has been suggested that parametric tests can be a valid approximation of trends. We also preformed a one-way repeated-measure ANOVA on the ranked data (Conover 1999). Results were compared to the non-parametric analysis for similarities of results. For each analysis a p-value of $< 0.05$ was considered significant.

**Results**

After of results between the parametric and non-parametric tests closely fit each other. The results presented are from the parametric version of the analysis. The mean visibility of tags was greater in juveniles than adults (Average diff. = 0.290, $p = 0.035$) (Fig. 3). There was not a significant difference between mean tag visibility when comparing the start and the end of the study within age categories (juveniles, $p = 0.501$, adults, $p = 0.421$) (Fig. 4). Migration of VIE marks occurred in only two juvenile salamanders (13% of individuals and less than 1% of placed tags). The movement that occurred was from anterior body tagging locations. The tags were still visible but had moved to the posterior body position on the same side as the placement.

For the analysis of adult marked salamanders, the influence of color and tag placement was investigated. There was no difference in the mean readability of tags between color ($p = 0.532$) and location ($p = 0.059$) (Fig 5 and 6). However, there was a significant difference in orange tag readability between initial and final monitoring periods (Red; $p = 0.758$, Yellow; $p = 0.078$, Orange; $p = 0.001$). Tail average readability was the only parameter that actually increased over the course of the study but the difference was not significant.

The visibility of tags varied with the interaction of color and tag location. The three VIE colors significantly differed within tagging locations (Anterior; $p < 0.001$, Posterior; $p < 0.001$, Tail; $p < 0.001$) and each single color was significantly different across each tagging location (Red; $p = 0.005$, Yellow; $p < 0.001$, Orange; $p < 0.001$) (Fig. 7).

**Discussion**

The results of our experiment suggest that VIE tags are appropriate for use in *P. glutinosus* for up to six months and therefore may be viable in other large terrestrial woodland salamanders within the slimy salamander phylogenetic complex. It is possible, however, that tag movement may increase in field experiments. The salamanders used in the study did not have the opportunity to move as they may have if they had been returned to their location of capture after tagging. Species

within the genus *Plethodon* can be found in a variety of habitats and may burrow underground, climb vegetation or rocks, and squeeze into crevices or under cover objects. The movement of a salamander through its natural habitat may put pressure on tags causing them to migrate that was not replicated in captivity.

Tags placed in adults were less visible than tags in juvenile salamander (Fig.3). The darker ventral pigmentation of adults made detection more difficult. However, the mean tag readability never dropped below a measure of 3, easily visible under VI light. Overall readability of tags regardless of color or tagging position did not change significantly over the course of the study within juvenile or adult groups (Fig. 4). Despite the finding that VIE colors varied significantly within and across tagging locations, no clear pattern of a single more visible color or easier read tagging location was identified. The variation that is present might be due to researchers experience and consistency with VIE tag placement. The researcher who injected the salamanders for the study had moderate experience with the technique (< 100 salamanders previously tagged) so these results could be comparable to a biologist who has been previously introduced to method but is still becoming proficient.

Tags were easier to see in juveniles due to lightly pigmented ventral dermis. However, tag movement only occurred in juveniles. If juvenile terrestrial salamanders are to be included in a mark-recapture study, researchers should take

this into consideration. Extra effort could be taken to design a coding system that would make the identification of moved tags easily recognizable. For instance, the number of VIE tags used per individual could be kept constant. Also, the insertion of two tags at the same location could be avoided in juveniles. If two tags are visible at the same location it would be obvious one had become displaced.

If there is concern about tag migration, preferential usage of the tail location for tagging could be considered. The risk of inserting a tag too deep under the skin and into the body cavity is not present when tagging into the muscle at the base of the tail. However, the long-term results of visibility and retention of tags placed at this location should be investigated in juveniles and adults. Future research should also monitor the readability and retention of tags in juveniles as they grow into adults and their ventral dermis darkens. Also, tags placed in juveniles tended to be much smaller than those placed in adults. This could result in a decrease in detection ability as they grow.

Overall, this study offered additional support for the use of florescent colored VIE tags in the genus *Plethodon* and their success in juvenile salamanders and individuals with dark pigmentation. These results are similar to other studies that investigated the retention rates and visibility of marks. For example, when the same colors were used to tag Redbacked Salamanders over the course of a year the average readability stayed consistent over time and stayed within a readability

score between 3-4 (Heemeyer & Homyack 2007). VIE tags have also been shown to be less detrimental than toe clipping of terrestrial salamanders to create unique patterns used for identification (Perry et al. 2011; Johnson et al. 2009). In a field trial, recaptured Western Red-backed Salamanders that had had toes clipped had gained significantly less weight than recaptured VIE tagged salamanders, suggesting toe clipping interfered with foraging behavior (David and Ovaska, 2001). Despite the potential draw backs of VIE tagging, including a risk of loss of tags or migration, relatively extensive handling time of salamanders for tags to be placed (5+ minutes per individual including anesthesia) and costs associated with the purchase of VIE materials, this technique has been shown to be a effective when it is necessary to identify individuals within a population.

**CHAPTER TWO FIGURES AND TABLES**

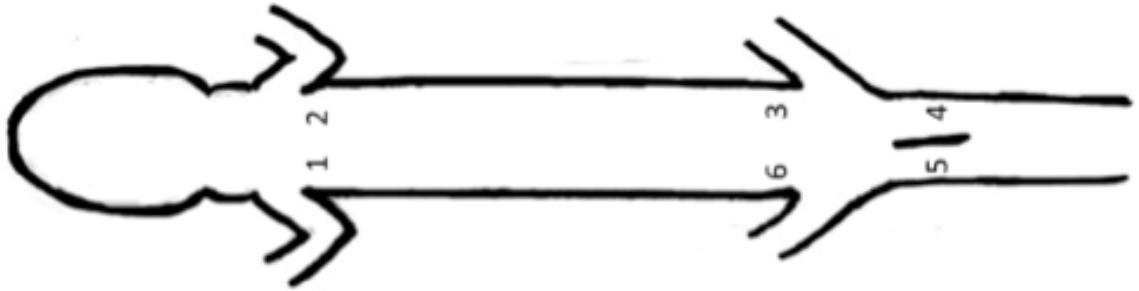



**Figure 1.** Six ventral tagging locations. Anterior tags (1 & 2) were placed behind front legs. Posterior tags (3 & 6) were placed in front of hind legs. Tail tags (4 & 5) were placed on either side of the cloaca at the base of the tail.

a.

b.

c.

**Figure 2.** Tagged juvenile salamanders. The three tagging arrangements are represented on the three individuals, from anterior to tail tagging locations, a. yellow-orange-red, b. orange-red-yellow, c. red-yellow-orange.

**Chapter 2: Figures cont…**

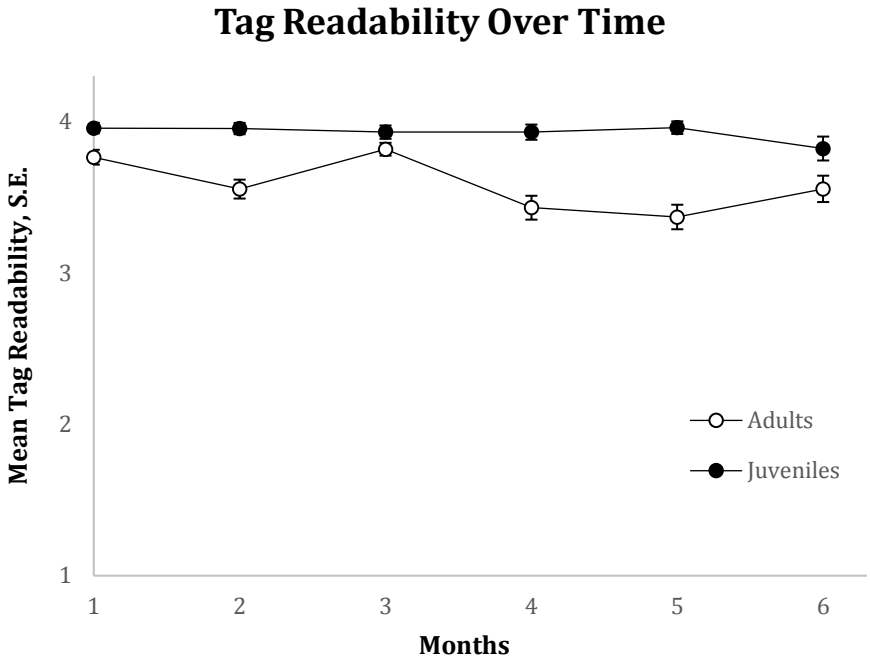



**Figure 3.** Readability of VIE marks. Readability of VIE marks was greater in marked juveniles (dark circles) than adults (open circles). Readability ranged from 1 (mark absent) to 4 (mark easily under florescent light).

**Chapter 2: Figures cont…**

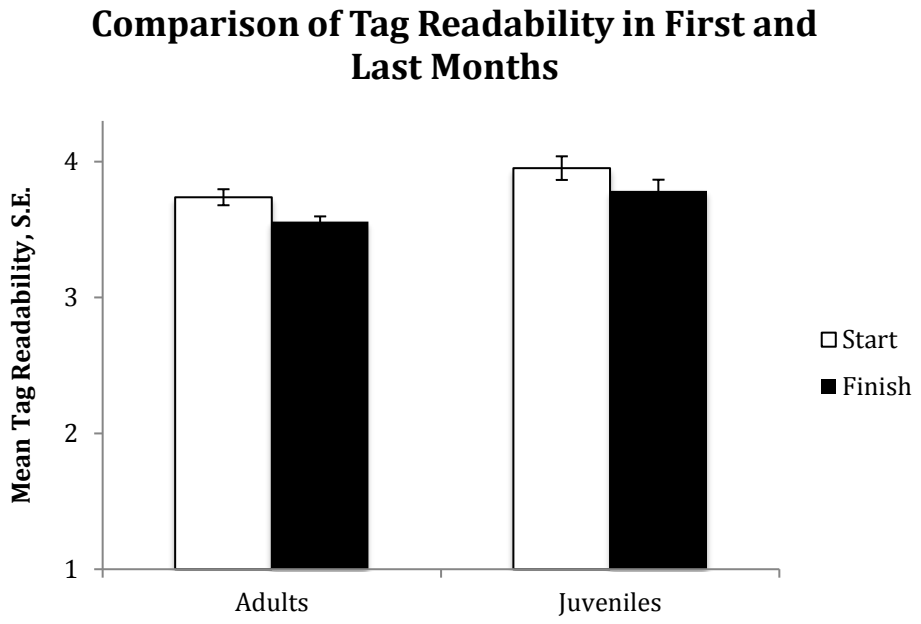



**Figure 4.** Readability of all VIE marks in *P. glutinosus* adults and juveniles compared from the beginning of the study to six months post placement. There was not a significant difference between mean tag readability between the first and last observations made for either juveniles or adults. Readability ranged from 1 (mark absent) to 4 (mark easily under florescent light).

**Chapter 2: Figures cont…**

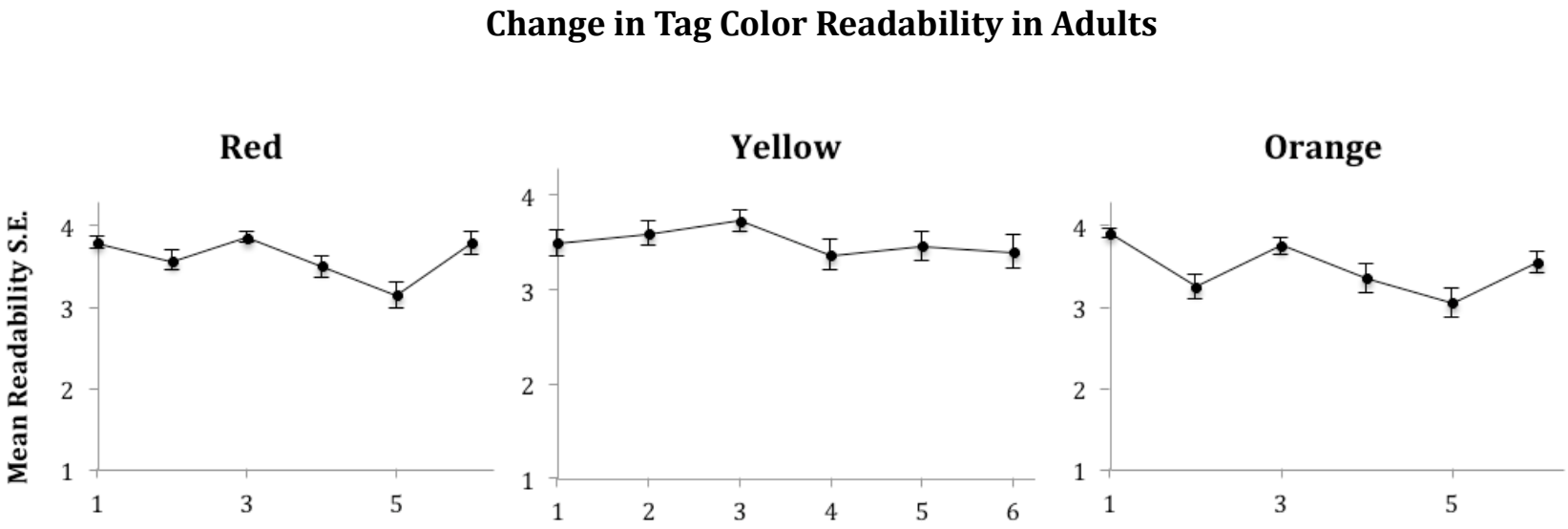



**Figure 5.** Readability of three different florescent colored VIE marks in *P. glutinosus* adults observed up to six months post-tagging. There was not a significant difference between mean tag readability of the three colors. Readability ranged from 1 (mark absent) to 4 (mark easily under florescent light).

**Chapter 2: Figures cont…**

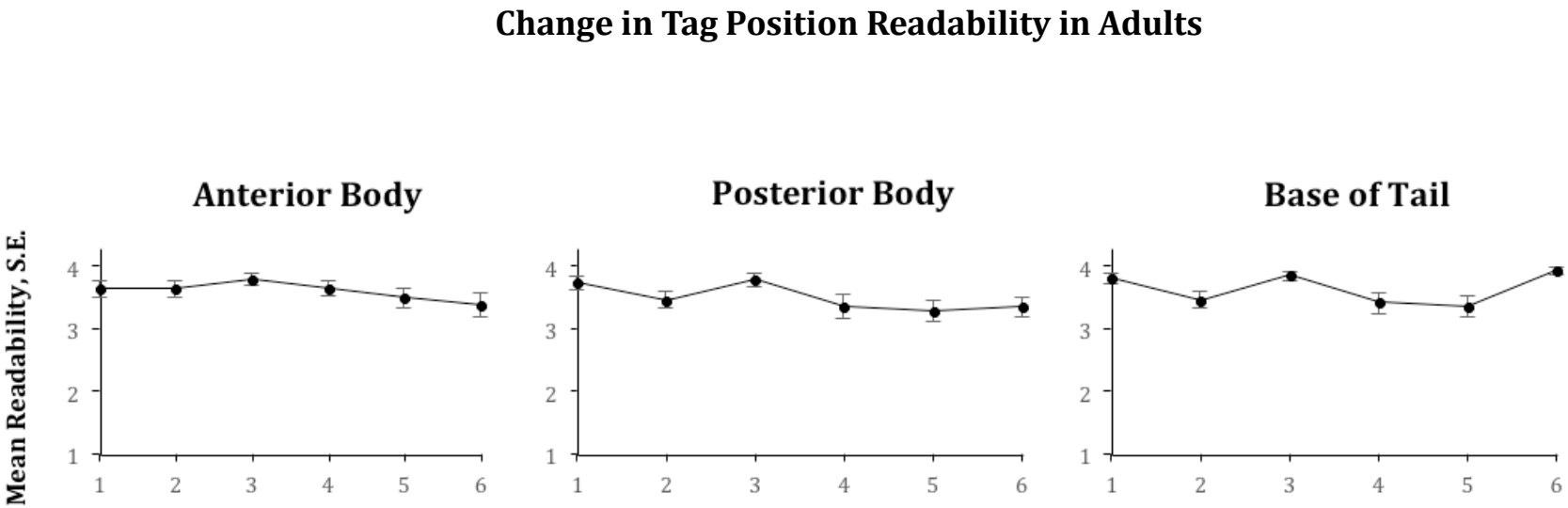



**Figure 6.** Readability of VIE marks in *P. glutinosus* adults at three tagging locations observed up to up to six months post-tagging. There was not a significant difference between mean tag readability of the three colors. Readability ranged from 1 (mark absent) to 4 (mark easily under florescent light).

**Chapter 2: Figures cont…**

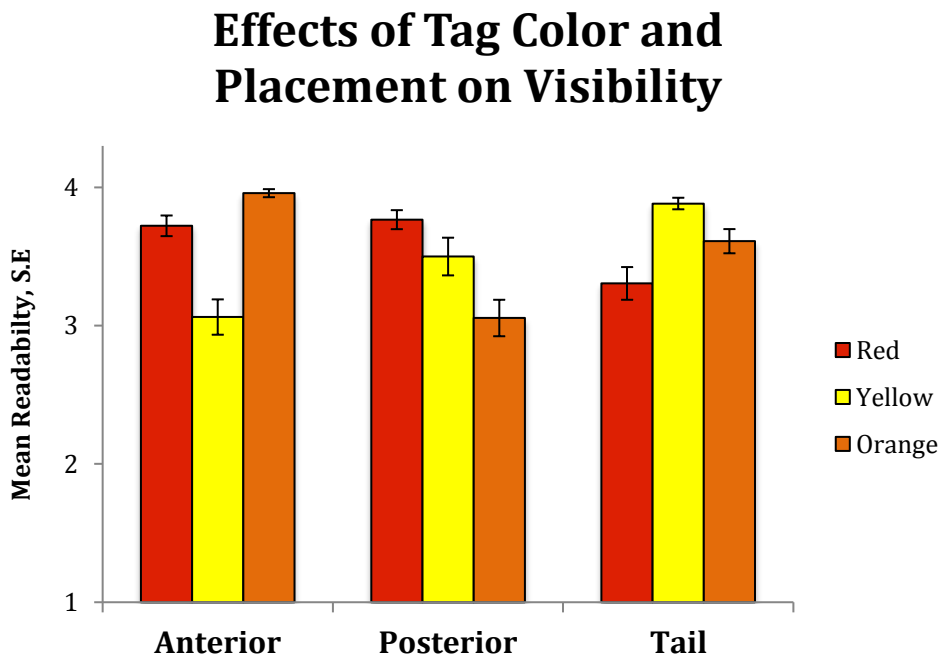



**Figure 7.** Readability of three florescent colors used for VIE tags placed at three tagging locations in *P. glutinosus* adults at month six. Within tagging locations, the three colors are significantly different. The same colors across all tagging locations are also significantly different. Despite this variation, no clear pattern regarding a less visible color or less effective tagging location was obvious. Readability ranged from 1 (mark absent) to 4 (mark easily under florescent light).

# CHAPTER 3

## Mark-Recapture Monitoring of Terrestrial Salamanders within the Crockford-Pigeon Mountain Wildlife Management Area.

### Introduction

Global amphibian populations are declining at an alarmingly high rate (Houlahan et al. 2000; Mendelson et al. 2006). In North America, amphibians are experiencing declines individuals from metapoplations at an estimated rate of 3.79% annually (Grant et al. 2016). Establishing monitoring programs for at risk species should be a priority to detect decreases in populations and identify underlying causes. Although often time consuming and difficult for cryptic amphibian species, estimating population size should be a goal for monitoring projects. This basic quantitative measure is often required for the conservation planning of rare, threatened and endangered species. Effective monitoring can allow managers to appropriately implement recovery plans and monitor the long-term effects on the population (Seber 1986; Williams et al. 2002). However, many amphibian-monitoring studies focus on species detection and richness. Accurate estimates of population size and structure, such as age and sex, before a population is impacted could be valuable knowledge for conservationists and provide benchmarks for management plans. Also, long-term monitoring could detect early declines in a population allowing managers to respond quickly to a conservation need (Bell et al. 2004).

The southeastern United States is considered a "hotspot" of salamander diversity. Many of the species of salamander that make up this biodiversity are terrestrial

woodland salamanders. These species tend to have direct development (without a larval stage), have lower fecundity when compared to other amphibians, are slow to mature, and are relatively long lived (Petranka 2010). These life history traits, combined with their sensitive permeable skin that is used for both respiration and osmogrulation, make them susceptible to environmental perturbations. This makes them excellent bio-indicators of habitat quality but also particularly vulnerable to population declines (Welsh & Droege 2001). Across the southeast, several species of salamander are protected at the state and federal level.

The Pigeon Mountain Salamander, *P. petraeus*, is one of these species of conservation concern and protected by the state of Georgia due to its small range (<20 km distribution). This large terrestrial species is known to be abundant at several locations across its small range but only has been found along the southeast-facing slope of the southernmost extension of the Cumberland Plateau into Northwest Georgia. A habitat preference has clearly been established in the literature and even in the species chosen Latin name, *petraeus*, meaning 'among the rocks'. Due to the patchy availability of rocky habitats throughout its range, the interconnectedness of populations depends on the species dispersal capability. A more complete understanding of how the species utilizes the rocky environment it inhabits and the full extent of its abundance would be useful when considering long term management and conservation goals.

Ongoing monitoring has taken place for *P. petraeus* within the openings of two caves the species is known to occupy (Camp & Jensen 2007). Population size estimates can be made with this type of data. However, estimates made from simple count data have been shown to be inaccurate, particularly when extrapolated across varying habitat types (Slade & Blair 2000). The Pigeon Mountain Salamander is found in a variety of habitats other than cave entrances such as cliff faces, outcrops, rocky ground cover, and woody debris. A comprehensive understanding of how this species is currently distributed across various habitats and its abundance at these locations will help in future conservation planning for this species.

Accurate estimates of populations can be difficult for species that are semi-fossorial and therefore may be regularly unavailable for sampling. Plethodontid salamanders are notoriously cryptic and generally have low detection probabilities (Bailey et al. 2004). Estimation of population parameters for terrestrial salamanders therefore requires intensive sampling designs. Due to the large proportion of salamanders unavailable for sampling at any given time an indirect method of estimating abundance needs to be implemented. The prevailing sampling method used for estimating population abundance of salamanders is the robust design (Bailey et al. 2004b). This capture-recapture method involves a combination of both open and closed models, with sampling over both long term (open) and short term (closed) time frames, also referred to as primary and secondary sampling periods respectively (Pollock 1982) (Table 1).

This method has been demonstrated to be the most flexible approach and provides more precise estimates of population density than traditional count methods (Bailey et al. 2004). Sampling between primary periods is assumed to be open. The concept of open time period refers to the possibility of salamander emigration and immigration to the survey area. Therefore, primary sampling periods for terrestrial salamander species can be separated by a few weeks, months, or even years. Across secondary sampling events within a primary period no movement from or to the study area is assumed. For this assumption to be met for salamanders within the genus *Plethodon*, secondary sampling occasions are planned within one or two days of each other. Therefore, capture and recapture probabilities can be assumed to be constant during secondary sampling periods and vary between primary periods (Kendall et al. 1997).

Due to this sampling designs statistical strength, robust design mark recapture studies have been useful in furthering our understanding of terrestrial salamander ecology. Not only is it effective at determining abundance, the robust design can be used to estimate other parameters including rates of temporary emigration and capture and recapture probabilities across age, sex, or other classes of interest (Bailey et al. 2004; Wen et al. 2013; Kendall et al. 1997). In regards to the Pigeon Mountain Salamander, a protected species of conservation concern, there is still much that is unknown about its life history and ecology. The application of not only an intensive mark recapture study but also one that utilizes the robust design has

the potential to greatly expand the knowledge of the species and inform management.

*Objectives*

We conducted a mark-recapture trial using a robust design sampling structure to investigate several species-specific population estimates for *P. petraeus* including abundance, capture probability, and movement. Across all species, we also estimated total salamander abundance, capture probability, and made comparisons of microhabitat use.

## Methods

*Study Area*

Fieldwork was conducted during the Fall of 2015 and Spring 2016 within the Crockford-Pigeon Mountain Wildlife Management Area in Walker County, Georgia, USA. Two 25 x 25 meter plots were established approximately 25 meters apart on a NW-facing slope of a ravine (Fig. 1). Plot 1 was established in 2015, and Plot 2 was added to the study in 2016. Each plot was divided into 25, 5 x 5 meter quadrants. The vegetation at the site was a mature mixed deciduous forest containing. Across both plots the forest floor was covered with leaf litter, rocks, rock outcrops, and woody debris of various sizes from decaying entire trees to stumps, logs, branches, and bark. Notable differences in the terrain included a small drainage that ran through the center of Plot A. This remained dry most of the year except in the late

winter and immediately after heavy rain events. The survey area was selected based on known occurrences of *P. petraeus* from the location and the heterogeneity of the terrain. The mixture of ground cover is characteristic of the several terrestrial salamander microhabitats found on Pigeon Mountain.

*Search and capture techniques*

Nighttime searches for salamanders were conducted within study Plot 1 between October 15th and 28th in 2015 and within both Plots 1 and 2 between March 23rd and May 11th in 2016. Primary periods were separated by a minimum of 10 days. Plot 1 was searched a total of 11 secondary periods and Plot 2 was searched a total of 8 secondary periods (Table 1). Due to the relatively small range of salamanders in the genus *Plethodon*, secondary sampling periods for their mark recapture studies tend to be three to four secondary events taking place within a week during the primary period (Bailey et al. 2004).

Pigeon Mountain Salamanders were collected under a Georgia Natural Resources collection permit, 29-WJH-14-252. All salamander species were included in the study except Green salamanders, *Aneides aeneus*, another species protected by the state of Georgia. We did not have a collection permit for *A. aeneus* so we only recorded their presence when individuals were observed within a plot.

Searches began 30 minutes to two hours after sunset and continued for 2-3 hours per plot depending on the number of salamanders available for capture that night. The plot that was surveyed first alternated every secondary sampling event. Prior to the start of each survey the ground, air and rock face temperatures were recorded

along with the weather conditions and observation of leaf litter being dry or wet. Approximately 5 minutes of searching was done within each 5 x 5 meter quadrant. This excluded time spent actively attempting to capture salamanders and record data. Salamanders were captured by hand and placed individually in plastic sample bags with a handful of leaf litter. To minimize habitat disturbance, cover objects were not flipped in the search for salamanders. Only salamanders utilizing the forest floor or partially exposed in a burrow or rock crevice were available for capture. Salamanders visible in rock crevices were removed using twigs or chopsticks. If a salamander was observed with only its head protruding from a hole or burrow, an attempt to lure out the salamander was made by wiggling the end of thin stem of grass or leaf in front of its' snout. This movement simulated the presence a prey item and would often prompt a feeding response making it possible to lure the salamander out of its burrow.

The exact location of each captured salamander was marked with a labeled piece of flagging tape. Beginning in 2016, the capture locations were recorded using the distance and bearing of the flag to the southwest corner of the quadrant in which the capture occurred. Using this information, the distance between initial and subsequent recaptures could be calculated. At the time of capture, the habitat type the salamander was found utilizing (e.g. rock, forest floor, vegetation, woody debris) was recorded. A chi-square test for independence was conducted to identify significance in habitat use difference between species.

Once surveys of plots were completed, salamanders were transported to the ranger station on the Wildlife Management Area and transferred to individual plastic containers (9 cm x 5 cm) that contained a piece of paper towel dampened with natural spring water. Salamanders were held between 12 to 24 hours before being returned to their location of capture. Any feces deposited by salamanders were collected and stored in 95% EtOH for future use in a DNA barcoding dietary analysis project.

*VIE tagging*

In order for a mark-recapture study to be successful, individuals within the research population must be able to be distinguished from one another. Captured animals were given unique codes with florescent Visual Implant Elastomer (VIE) tags (Northwest Marine Technology, Inc. Shaw Island, Washington, USA). Three colors, yellow, red and orange, were used as tags and were placed at six ventral locations, behind front legs, in front of the hind legs and at the base of tail, on either side of the cloaca. We assessed the retention rate of tags by keeping the number of tags per individual at a constant number of three. If an individual was observed with fewer than three marks, it was be clear that a tag had been lost.

Prior to tagging, the body mass of each salamander was measured to the nearest 0.1 gram. Salamanders were anesthetized prior to injections in a solution of 500 mg/L or 250mg/L of Tricaine methanesulfonate (MS-222) and de-chlorinated water depending on their mass, less than 3 grams or greater than 3 grams respectively. To achieve a neutral pH so not to irritate the salamander's skin (Grant 2008), the solution was buffered with sodium bicarbonate. Salamanders were placed in the anesthetic bath until unresponsive to gentle prodding and unable to right themselves when placed on their backs. While immobilized, snout-vent-length (SVL), sex (male, female or unknown), relative age (adult or juvenile, SVL > or < 45 mm respectively for *P. petraeus*) and any distinguishing features (e.g., scars, bite marks, regenerating limbs or tail) of individuals were noted. Once tags were injected and observations made, salamanders were placed in water baths to recover from the anesthetic and returned to temporary enclosures.

*Abundance*

The primary goal of our mark-recapture analysis was to estimate abundance. Mark-recapture data was analyzed using Program MARK, a mark-recapture program that can estimate parameters including population size using numerical maximum likelihood techniques (White & Burnham 1999). Due to a small data set, a general model with few parameters was utilized for data analysis. We analyzed our robust design mark-recapture data using a random emigration model. This decision was

based on the results from previous model fitting studies for Plethodontid salamanders that used datasets spanning multiple years and thousands of individuals. This work consistently showed random emigration models being the best fit for woodland terrestrial salamander mark-recapture data (Bailey et al. 2004). Random movement models equate the probability that an animal is unavailable for sampling and present during the previous primary period with the probability of being unavailable for sampling and absent during the previous primary period. A random temporary emigration model establishes that the chance of emigrating or immigrating is equal (Kendall et al. 1997). Under the constraints of this model, there would be equal chance an individual would be available or absent any given sampling period.

For these analyses, our mark-recapture data was structured in multiple data sets. Estimates were generated for total salamander abundance (including all species except *A. aeneus*) and specifically for *P. petraeus* abundance. Estimates were then made for both plots and separately for plots one and two. Estimates of total salamander abundance and *P. petraeus* abundance for plot one are the only estimates that contain data from 2015 since the second plot was not added to the study until 2016. We were unable to make species-specific estimates for other species due to low numbers of captures or recaptures.

## Results

*Abundance*

A total of 98 salamanders were captured throughout the duration of the study from the two plots (Table 2.). This included three species within the genus *Plethodon*- *P. petraeus*, *P. glutinosus,* and *P. ventralis*- and two other species- *E. lucifuga* and *A. aeneus* (Table 2). In all, 93 salamanders were marked with VIE tags and 47 (20 females, 11 males, 16 juveniles) of these were Pigeon Mountain Salamanders. Twelve (six females, two males and four juveniles) *P. petraeus* were recaptured; however, two individuals were recaptured twice, bringing the number of recapture events for the species to 14. Four other individuals were recaptured, including a juvenile Northern Slimy Salamander and three Cave Salamanders. Three *P. petraeus* (one female, one male, and one juvenile) were tagged in Fall 2015 and recaptured in Spring 2016.

Captures were highest in Plot 1 during October 2015. Relative captures of *P. petraeus* were consistently higher than any other species except during the April primary periods when more *P. glutinosus* were captured (Fig. 2). During each primary period there was at least a single secondary period that corresponded with a rain event and wet leaf litter. On these occasions, numbers of captures peaked (Fig 3). Through time, captures of new, unmarked individuals did decrease. However, new individuals continued to be captured for the duration of the study. Also, the

trend of decreasing new individuals followed a general decrease in the total number of salamanders being captured in each primary period. The exception occurred in Plot 2 during the final primary period when more recaptured salamanders were sampled than unmarked ones (Fig 4 and 5).

The random movement model was used to make several estimates of salamander abundance, including total salamander abundance and species-specific abundance for *P. petraeus* within and across the two study areas, Plot 1 and Plot 2. There was considerable variation in abundance estimates between plots and across primary sampling periods (Table 1 and 2). Salamander abundance estimates were consistently estimated to be lower in Plot 2. The April primary period in Plot 2 is notably low for both estimates of abundance. This was due to low capture and recapture numbers within April. There were zero *P. petraeus* recaptures during this primary period. Also, this primary only consisted of two secondary periods compared to the three within the other primary periods.

*Capture probability*

The inconsistency of recaptures also affected the estimates of capture probability (Fig. 6 and 7). The majority of capture probabilities fell between 1% and 25% but the low abundance estimate and recaptures in the April primary period, 7$^{th}$ and 8$^{th}$ secondary periods, influenced the capture probability. This can be seen most notably in the capture probability estimates for Plot 2. Only three unmarked *P.*

*petraeus* salamanders were captured during this primary period, during the evening of the 7th secondary period. This caused the population estimate to be 3 individuals and resulted in a 100% capture probability for the 7th secondary period and a 0% capture probability on the 8th secondary period. The remaining capture probabilities are comparable with other terrestrial woodland salamanders. Similar species have encounter probabilities of 10% or less (Bailey et al. 2004).

*Habitat Preference*

Microhabitat use by species was not evenly distributed across habitat type. There was a significant difference in habitat use per species (Fig. 8). The three species that primarily utilized rock surfaces were one species of each genus represented in the study: *P. petraeus*, *E. lucifuga,* and *A. aeneus*. *P. glutinosus* and *P. ventralis* were routinely encountered on the forest floor.

*Movement of Plethodon petraeus*

12 Pigeon Mountain Salamanders were recaptured at least once during the study. Beginning in Spring 2016, we were able to precisely record the capture location of 10 Pigeon Mountain Salamanders from March to April of 2016. Of these 10 individuals, nine individuals were recaptured a single time and one (individual 6) was recaptured twice. There was an average distance between capture locations of 2.29 meters (Table 5). All 12 Pigeon Mountain Salamanders recaptured were found

within the same or adjacent 5 meter plot. The other recaptures, 3 Cave Salamanders and 1 Northern Slimy Salamander were also found within the same or adjacent plot.

**Discussion**

This study demonstrated the successful implementation of VIE tagging and a robust design mark recapture monitoring project of terrestrial salamander species on Pigeon Mountain. Marked individuals were recaptured across secondary and primary sampling periods, including over winter. No recaptured salamanders had lost tags. Tag migration did occur almost immediately after tagging one juvenile *P. glutinosus*. However, this was noted before its release. We were successfully able to construct capture histories for 93 salamanders over an eight-month study.

Abundance estimates for all salamanders tagged in 2016 within the two 25 x 25 meter plots ranged from 51.6 - 159.8 individuals and 53.6 – 198.1 for *P. petraeus*-specific estimates (Table 3). Due to difference in capture probability between species and the small sample size, comparisons of abundance between species could not be drawn. A larger sample size is needed to increase the accuracy of community and species abundance estimates. However, average plot-specific estimates provided a more reasonable estimate and ratio of *P. petraeus* to other species (Plot 1: 48.4 *P. petraeus*/80.6 total species, Plot 2: 16.3 *P.petraeus*/39.3 total species) (Table 4). These ratios suggest that, 60% and 41.5%, of the total salamander abundance consists of *P. petraeus* within Plot 1 and 2 respectively. This finding supports previous observations that *P. petraeus* often outnumbers others species in

locations were it is found. Also, these estimates are comparable to abundance estimates for woodland salamanders made in the Great Smoky Mountain National Park using the robust design and equal capture probability model. Within two 15 x 15 meters plots established in the National Park, Bailey (2004) detected total salamander abundances of 92 and 264 for all species present

In addition to initial estimates of abundance, evidence was provided for species-specific segregation across habitat type. The majority of *P. glutinosus* captures occurred on the forest floor. Notably, in similar rocky habitat not occupied by *P. petraeus*- the northwest facing slope of Pigeon Mountain for example- it is common to find slimy salamanders utilizing rock crevices. These observations add support to the hypothesis of interspecific competition between these two closely related species and habitat partitioning (Marshall et al. 2004). Seemingly similar habitat partitioning has been documented within overlapping ranges of Red-back Salamander, *Plethodon cinereus*, and related species with much smaller ranges (Farallo & Miles 2016). Farallo and Miles hypothesized that this separation may limit hybridization and may have important implications regarding how climate change could influence microhabitats differently.

Also, the first investigation into site fidelity and home range of Pigeon Mountain Salamanders was included as part of the mark recapture sampling. The average distance between captures and recapture locations was 2.29 meters (Table 5). The results of this study are similar to the findings of other studies showing movement

and dispersal within the genus *Plethodon* is limited and species have strong site fidelity (Mathis 1991). However, this study contained few individuals and not enough data to investigate age or sex based dispersal differences or make comparisons across species about average home ranges. When capture numbers are relatively low and researchers are not managing hundreds of individuals per night, it is easy to record and precisely measure the exact location of captures and recaptures. Due to the significance of movement and dispersal to resource allocation and gene flow further studies should be undertaken to better understand Plethodontid salamander movement (Sinsch 2014).

*Natural History Notes*

Described as a crevice-dwelling salamander, climbing behavior in in this species is well documented on rocky outcrops, cliff faces, and cave entrances (Jensen et al. 2002). Despite morphological adaptations for climbing and ample evidence of utilizing vertical exposed rock there have been no reports of individuals on trees. During a collection period, an adult *P. petraeus* was observed 45 cm above the ground on the side of a hardwood tree. During this encounter, the individual was seen consuming an ant, a large component of this species' diet (Jensen 2000). Although it was not raining at the time of the observation, the location had received rainfall within the previous 24 hours. This observation adds to the increasing documentation of facultative use of vegetation by plethodontid salamanders for foraging (McEntire 2016).

Other notable observations during sampling periods included a Cave Salamander sharing a crevice with a green anole, *Anolis carolinensis*. Cave Salamanders and Pigeon Mountain Salamanders are frequently observed inhabiting the same rock crevices; however, this type of interspecific interaction had not been observed by the researcher before. Also, although Green Salamanders and Pigeon Mountain Salamanders utilize similar habitat types and share similar morphological adaptations (broad toe pad, long limbs, dorso-ventrally flattened (Wynn et al. 1988) they are not observed on Pigeon Mountain in close proximity to one another. The first personal occurrence of this was observed when a neonate juvenile green salamander was found occupying a rock face roughly 20 centimeters away from an adult Pigeon Mountain Salamander.

*Conservation Implications*

The declines in amphibians observed in North America are not driven by any one cause but a combination of threats and the effect of stressors at regional scales (Grant et al. 2016). With the variability of threats such as disease, habitat alteration, and climate change and the inconsistency at which species respond, an emphasis should be placed on developing local conservation strategies. Mark-recapture monitoring can be a valuable tool in identifying population changes and can help optimize conservation actions by bringing greater quantitative accuracy into the development and application of management plans.

From a management perspective, monitoring should be economically and logistically feasible. An annual robust design study with multiple sample sites would require a large amount of effort, time, and financial resources. The potential knowledge gained must be weighed against the overall cost of the study. In the case of this project, we had the opportunity to study not only the Pigeon Mountain Salamander but also several other species within the same plots. Pigeon Mountain is an amphibian biodiversity hotspot for the state of Georgia and the southeast. In total, 16 species of salamander are known from the mountain, five of which were commonly found utilizing the terrestrial habitats within mark-recapture plots that were established. These observations included two protected species, the Pigeon Mountain Salamander and Green Salamander. The potential for increasing our understanding of not only the Pigeon Mountain Salamander but community level population dynamics for multiple species is a great advantage of implementing of mark-recapture monitoring with a robust sampling design.

Expansion of the study spatially and temporally would increase sample size and the ability to generate more accurate estimates of salamander abundance. Successful long-term mark-recapture studies with terrestrial woodland salamanders have included over a dozen similarly sized plots over several sampling years (Bailey et al. 2004; Connette & Semlitsch 2013). Increasing the scale of the study, and therefore sample size, would allow for the opportunity to investigate survival rates and recapture probabilities along with other parameters of interest to managers, such as

age and sex specific parameters and habitat or environmental variables that influence detection.

## CHAPTER THREE FIGURES AND TABLES

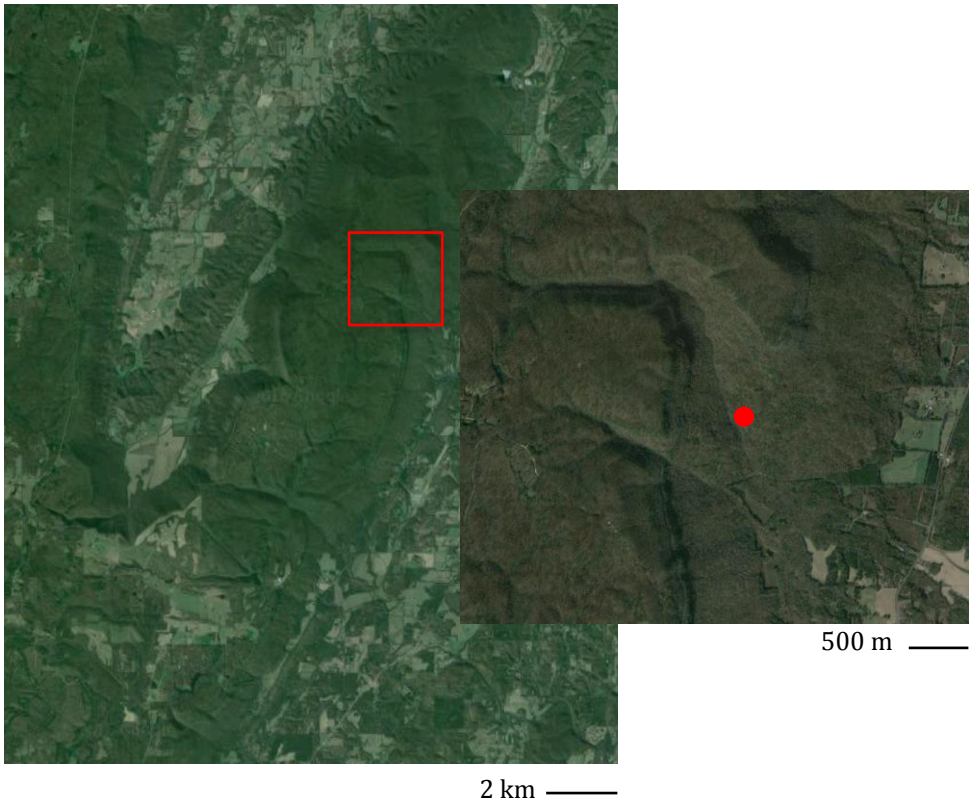



**Figure 1.** Map of approximate location of mark recapture plots within the Crockford-Pigeon Wildlife Management Area.

**Chapter 3: Figures cont…**

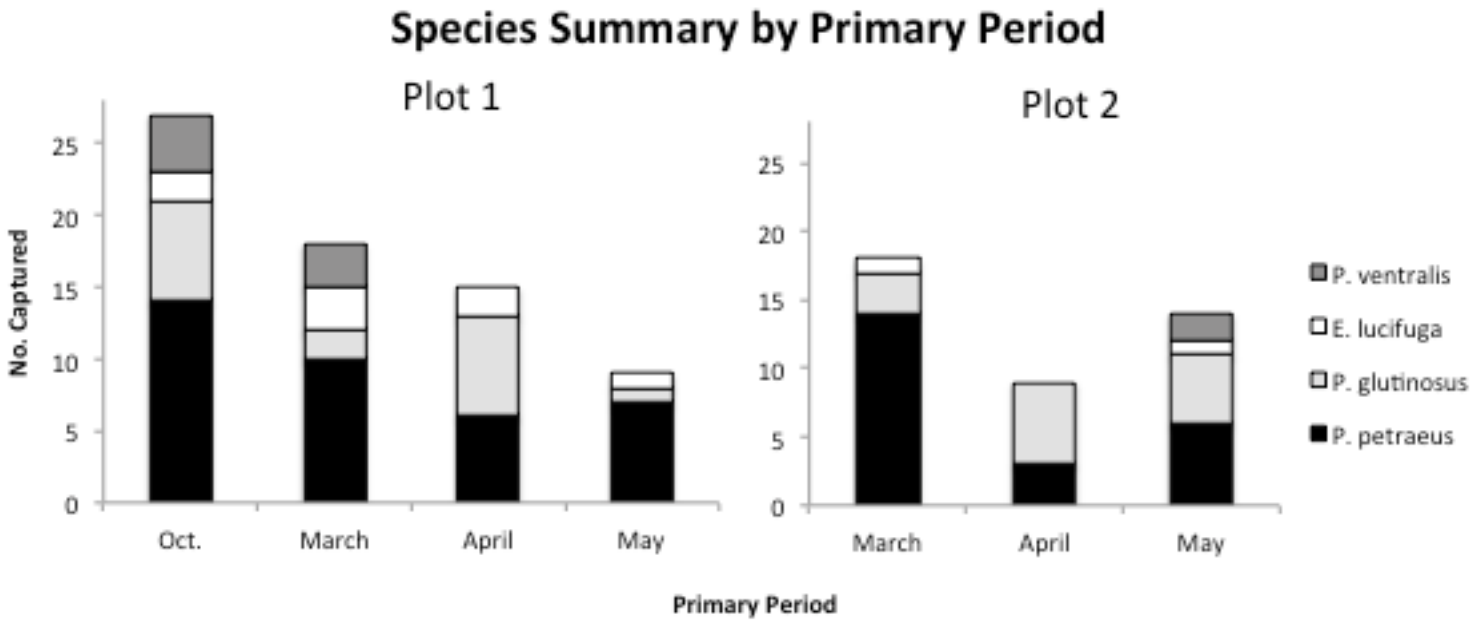



**Figure 2**. Summary of captured salamanders within the study plots by species. The majority of captures during each primary period were *P. petraeus*, except in Plot 2 during the April primary period.

**Chapter 3: Figures cont…**

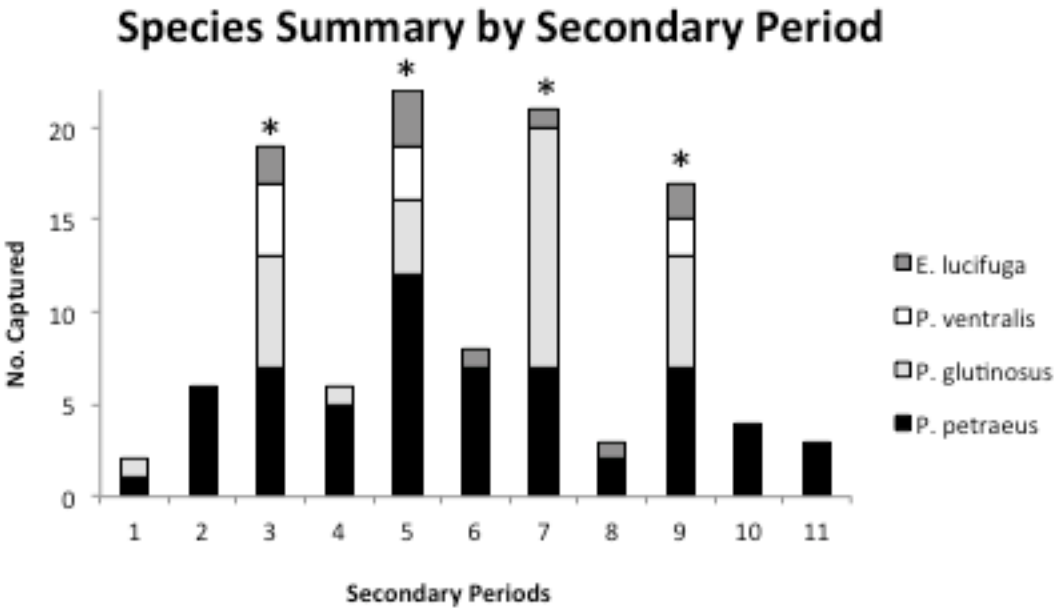



**Figure 3.** Summary of captured salamanders within the study plots by species. *P. petraeus* were captured during every secondary sampling period. Secondary sampling periods 3, 5, 7 and 9 corresponded with rain or wet leaf litter and significantly greater number of salamander captures. Secondary periods 1-3 took place only in Plot 1 during Fall 2015.

**Chapter 3: Figures cont…**

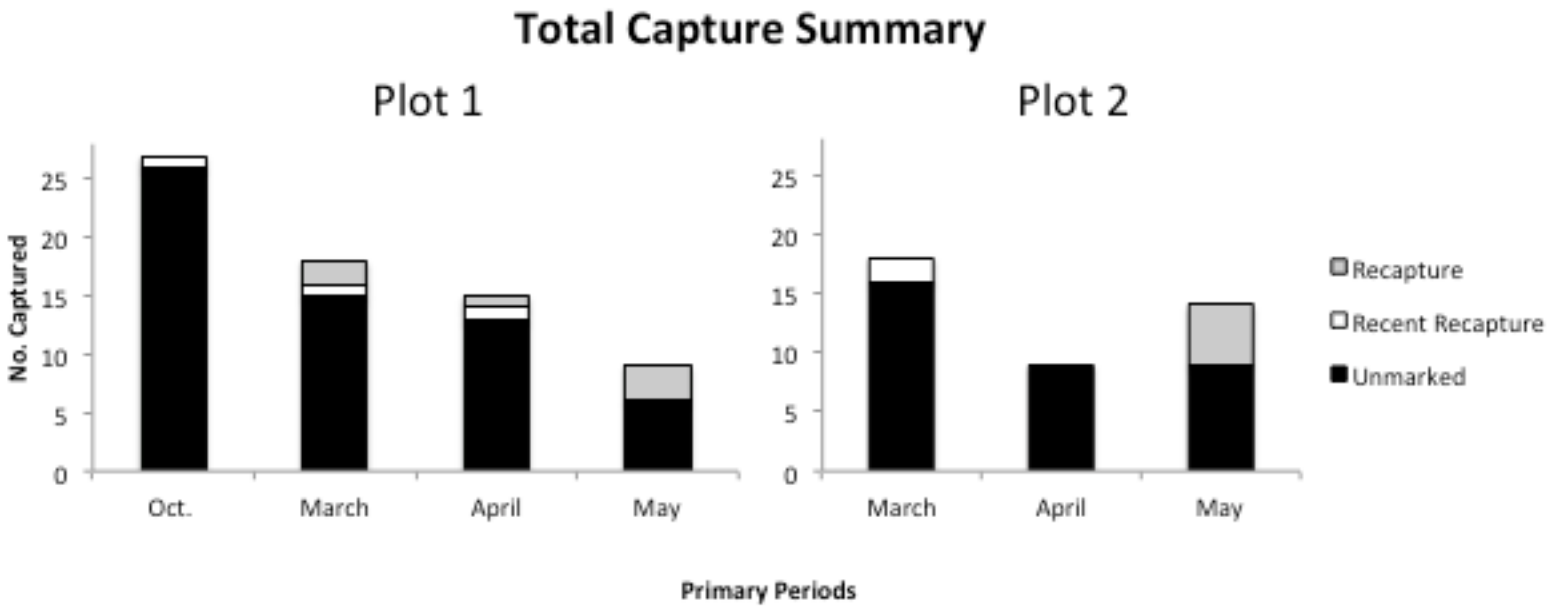



**Figure 4.** Capture summary for all salamander species captured from Oct. 2015 to May 2016. Bars represent total numbers of salamanders captured, broken down into naive, unmarked individuals (black bars), recaptures from previous primary sampling intervals (grey bars), and recent recaptures within primary sampling intervals (open bars).

**Chapter 3: Figures cont…**

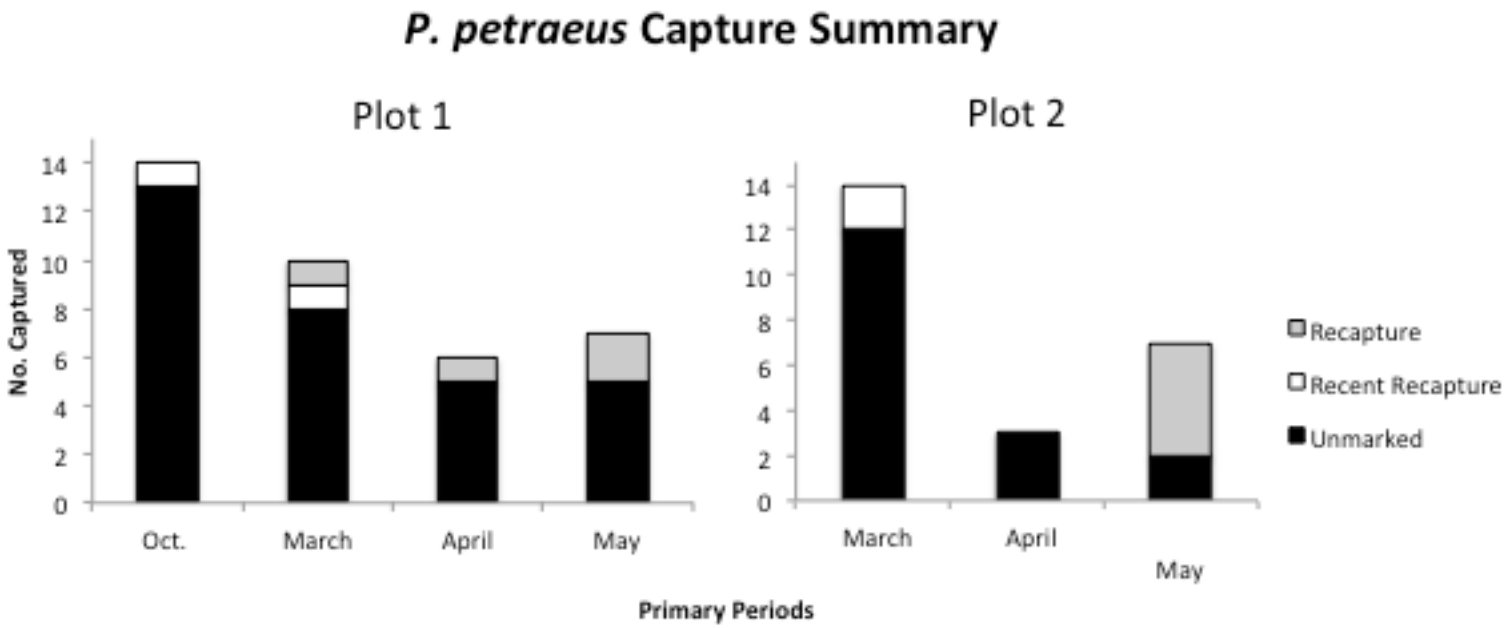



**Figure 5.** Capture summary for Pigeon Mountain Salamanders captured from Oct. 2015 to May 2016. Bars represent total numbers of salamanders captured, broken down into naive, unmarked individuals (black bars), recaptures from previous primary sampling intervals (grey bars), and recent recaptures within primary sampling intervals (open bars).

**Chapter 3: Figures cont…**

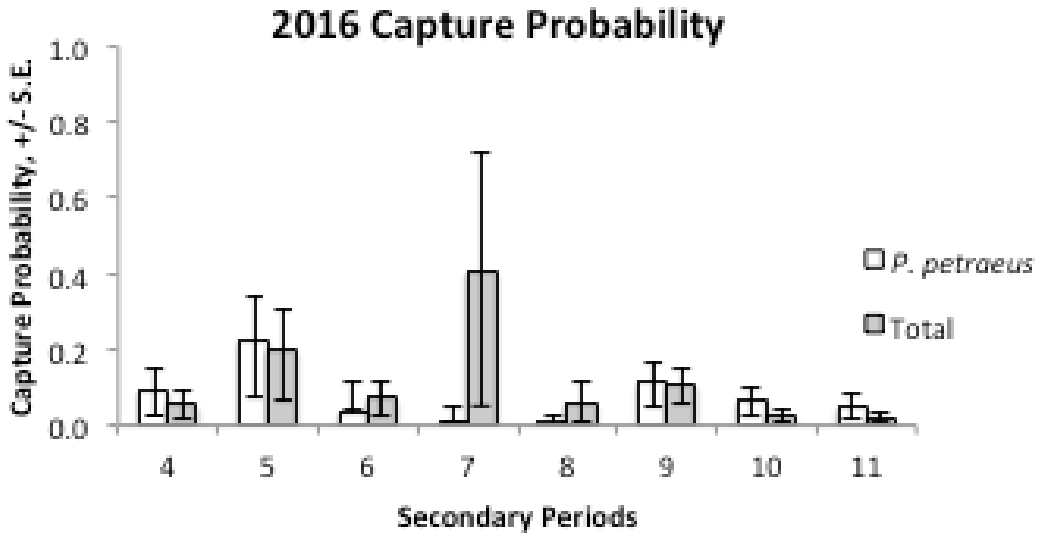



**Figure 6**. Variations in capture probabilities for *P. petraeus* and total individuals captured from both Plots in 2016.

**Chapter 3: Figures cont…**

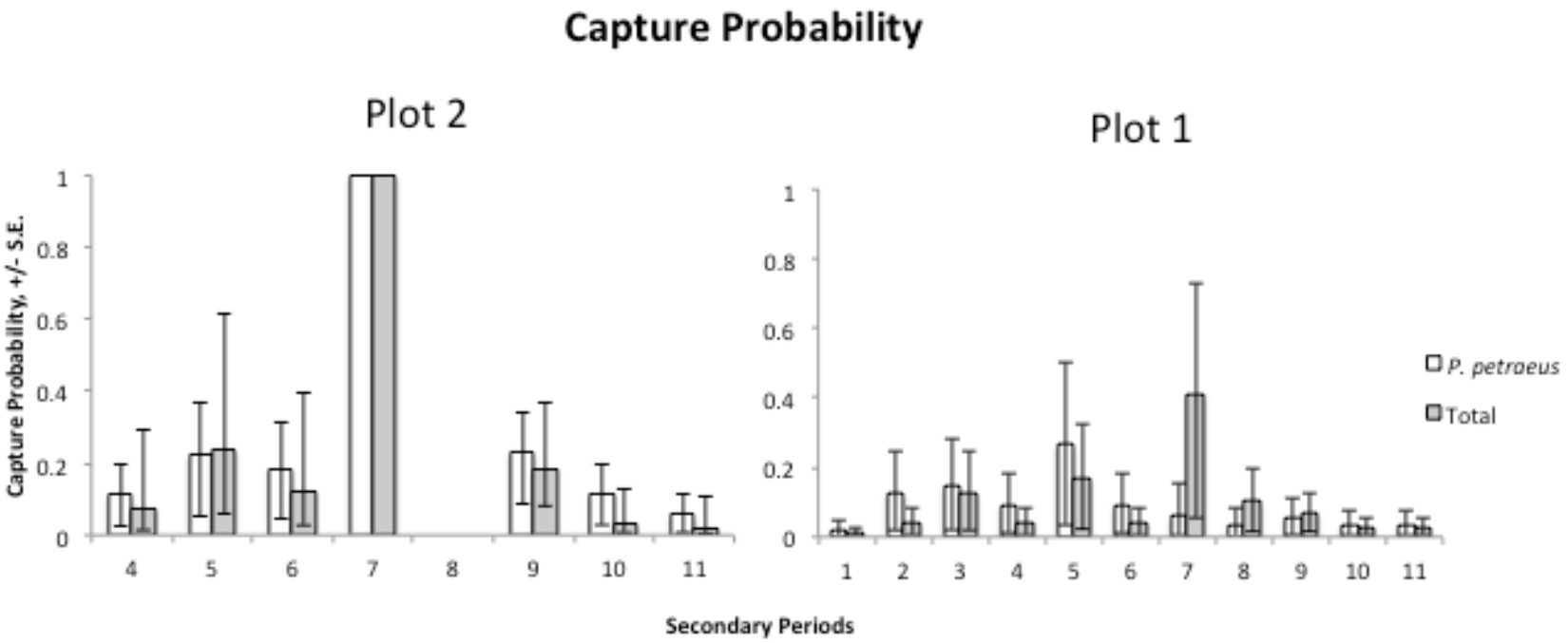



**Figure7**. Variations in capture probabilities for *P. petraeus* and total individuals captured in Plot one from Oct. 2015 to May 2016 and Plot 2 in 2016.

**Chapter 3: Figures cont…**

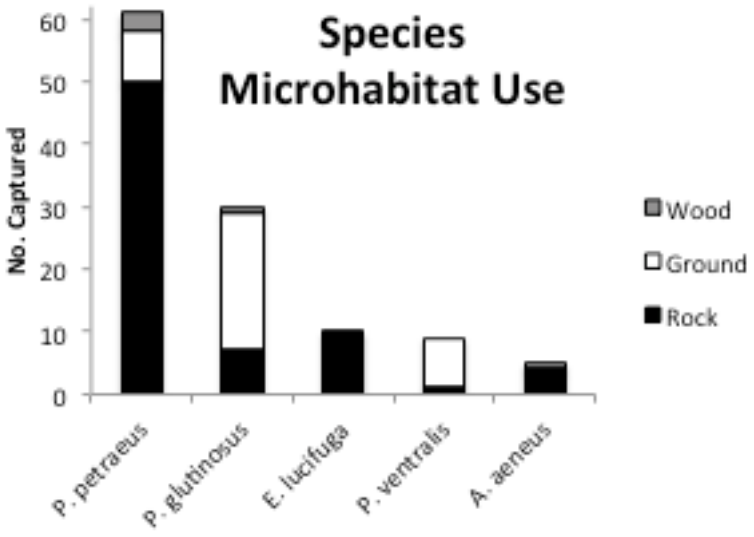



**Figure 8.** Habitat use for captured salamander species Oct. 2015 to May 2016. Bars represent total numbers of salamanders captured, broken down into habitat type they were using at moment of capture, rock (black bars), wood or woody debris (grey bars), and ground or leaf litter (open bars).

## Chapter 3: Tables

**Table 1.** Number of surveys within the two-tiered structure of the Robust Design for two mark recapture plots.

| **Plot** | | 1 | 1 & 2 | | |
|---|---|---|---|---|---|
| **Year** | | **2015** | **2016** | | |
| **Sampling Period** | **Primary** | **Oct.** | **March** | **April** | **May** |
| | Secondary * | 3 | 3 | 2 | 3 |

* No. of times the study area was surveyed within the primary period

**Table 2.** Total number of tagged salamanders by species and number of recapture events

| | **Captures** | **Recaptures** | |
|---|---|---|---|
| **Species** | **Tagged** | **Total** | **Overwinter** |
| *P. petraeus* | 47 | 12 | 3 |
| *P. glutinosus* | 30 | 1 | 0 |
| *P. ventralis* | 9 | 0 | 0 |
| *E. lucifuga* | 7 | 3 | 1 |
| *A. aeneus** | 5 | --- | --- |
| **Totals** | **98** | **16** | **4** |

*Observations of *A. aeneus*. Individuals were not captured and tagged.

## Chapter 3: Tables cont…

**Table 3.** Population estimates for all species across sampling locations and primary periods.

| Sampling Area | Primary Period | N | S.E. | 95% C.I. | |
|---|---|---|---|---|---|
| Plot 1 and 2 | March | 110.0 | 54.0 | 55.3 | 298.8 |
| | April | 51.6 | 38.5 | 26.9 | 233.0 |
| | May | 159.8 | 57.7 | 85.1 | 325.8 |
| Plot 1 | October* | 149.5 | 136.6 | 47.4 | 738.2 |
| | March | 71.0 | 62.7 | 25.8 | 347.0 |
| | April | 29.1 | 21.1 | 16.0 | 130.0 |
| | May | 72.6 | 53.4 | 24.2 | 275.1 |
| Plot 2 | March | 41.7 | 23.8 | 21.5 | 136.0 |
| | April | 9.0 | 0.0 | 9.0 | 9.0 |
| | May | 67.3 | 28.9 | 34.0 | 158.6 |

* Fall 2015 sampling period, N (abundance), S.E. (standard error), C.I. (confidence interval).

**Table 4**. Population estimates for *Plethodon petraeus* across sampling locations and primary periods.

| Sampling Area | Primary Period | N | S.E. | 95% C.I. | |
|---|---|---|---|---|---|
| Plot 1 and 2 | March | 53.6 | 24.8 | 29.7 | 143.6 |
| | April | 198.1 | 204.5 | 42.7 | 1070.2 |
| | May | 65.6 | 27.1 | 33.6 | 149.9 |
| Plot 1 | October* | 47.7 | 41.1 | 18.5 | 230.4 |
| | March | 22.5 | 18.0 | 10.9 | 106.6 |
| | April | 64.8 | 95.8 | 12.3 | 553.8 |
| | May | 58.5 | 59.6 | 15.5 | 320.9 |
| Plot 2 | March | 26.9 | 14.7 | 14.9 | 87.0 |
| | April | 3.0 | 0.0 | 3.0 | 3.0 |
| | May | 18.9 | 8.6 | 10.4 | 49.6 |

* Fall 2015 sampling period, N (abundance), S.E. (standard error), C.I. (confidence interval).

## Chapter 3: Tables cont…

**Table 5.** *P. petraeus* recaptures and the distance between initial (0), secondary (1) and tertiary (2) capture locations.

| Individual | Recapture Event | Distance (m) |
|---|---|---|
| 1 | 0 -> 1 | 1.21 |
| 2 | 0 -> 1 | 5.02 |
| 3 | 0 -> 1 | 2.35 |
| 4 | 0 -> 1 | 3.27 |
| 5 | 0 -> 1 | 0.16 |
| 6 | 0 -> 1 | 2.56 |
| 6 | 0 -> 2 | 2.29 |
| 6 | 1 -> 2 | 0.85 |
| 7 | 0 -> 1 | 2.52 |
| 8 | 0 -> 1 | 0.56 |
| 9 | 0 -> 1 | 2.21 |
| 10 | 0 -> 1 | 3.79 |
| **Average Distance** | | **2.29** |
| | SE | 0.41 |

# Chapter 4

## Detection of Polymorphic Microsatellite Loci in The Pigeon Mountain Salamander, *P. petraeus,* by Cross-amplification of Microsatellites within the Slimy Salamander Group.

### Introduction

The assessment of genetic structure, diversity, and gene flow are measures that are often considered by conservation biologists when developing or implementing conservation goals. There is often a need to investigate fine-scale genetic patterns across a species distribution to determine the genetic health of the population, identify unique populations that may require protection, and detect natural or anthropogenic barriers to gene flow, such as, rivers or roads respectively. Therefore, quickly evolving nuclear markers, such as microsatellite loci or single nucleotide polymorphisms (SNPs) are vital tools for the field of conservation genetics. While SNPs are quickly becoming more affordable to develop and increasingly prevalent as a maker in population genetic literature (Seeb et al. 2011), there can be advantages for a researcher to choose microsatellites for projects. For example, challenges still exist in SNP development for non-model organism (Helyar et al. 2011). This is even more evident when the species of interest does not have a sequenced reference genome. Working with salamanders adds to this complexity due to the large genome size.

For conservation genetics questions about genetic diversity and structure, microsatellites are still widely used. The usefulness of microsatellites originates

from the fact they are species-specific; however, they can be highly conserved between closely related species. Cross-amplification has been shown to be a successful method for identifying useful nuclear loci across species within the same genus of salamander (Steele et al. 2008; Spatola et al. 2013; Hendrix et al. 2010). If primers already have been identified for repeat regions for a closely related species, it can be advantageous to screen them in your species of interest. This exploratory process can detect polymorphic loci that can be used in a population genetic study, but cross-amplification comes with a decrease in efficiency. Cross-amplification can identify conserved polymorphic regions across a genus; therefore, this approach can save a research project time and money spent developing new species-specific markers at the onset of a study.

Cross-amplification was used to identify markers for a population genetic study of *P. petraeus*. Primers were previously identified for 27 microsatellites in the Western Slimy Salamander, *Plethodon albagula*, a large terrestrial salamander distributed across the Ozark Mountains and in a separate population in central Texas (Petranka 2010). Not only are these members of the same genus, they belong to the same "group" as *P. petraeus* (Fig. 1). As a member of the same genus as the Pigeon Mountain Salamander, microsatellite regions may be shared across the two species. They have already been shown to cross-amplify in another species within the slimy salamander group, *Plethodon shermani* (Spatola et al. 2013). The same primer pairs developed for microsatellites in *P. albagula* are used here to identify regions in the *P. petraeus* genome that can be used in a conservation genetics study.

## Methods

*Tissue Collection*

Tissue samples of *P. petraeus* were contributed to the study by Glenn Marvin (University of Northern Alabama) and collected from the field by the authors in Fall 2014. Individuals were sampled from Dickson Gulf (N=10) and Pettijohn Cave (N=4), within the Crockford-Pigeon Mountain Wildlife Management Area, Georgia, USA. These collection sites are separated by approximately 3.5 km. 1cm of tail tissue was collected and transported in 95% EtOH or in an empty collection tube on ice until they could be stored at -20 degrees Celsius. DNA was extracted from each tissue using MoBio DNA Extraction Kit for tissue (Carlsbad, CA, USA). Extracted DNA samples were stored in 50 ul elution buffer at -20 degree Celsius.

*Microsatellite Screening*

The 27 microsatellite primer pairs described in Spatola et al. (2013) were screened for amplification of microsatellite regions in the 14 *P. petraeus* samples. PCR cycling profile for initial primer screening consisted of a denaturation of 95 degrees Celsius for 10 min, followed by 35 cycles of 95 degree Celsius denaturing for 45 s, 60 degree C annealing for 45 s, extension at 72 degree C for 45 s, and a final 5 min extension at 72 degree C for 45 s (Spatola et al. 2013). PCR products were visualized on 3% agarose gels.

After visualization of PCR products with gel electrophoresis, only two of the 27 cross-amplified loci amplified non-specific regions. Of the remaining 25 loci, 17

were monomorphic and eight were polymorphic (Table 1). The annealing temperature for two primer pairs, Plal_402 and Plal_542, were optimized due to smearing in PCR product bands (Table 1). The forward primers for the eight loci with multiple alleles were fluorescently labeled with 6-FAM, HEX or NED (Table 1) for capillary electrophoresis on an ABI 310 DNA Analyzer. PCR reactions were done either under the initial cycling conditions or with the optimized annealing temperatures (Table 1). Amplification products were sized on an ABI 310 DNA Analyzer (Applied Biosystems) using a Rox-500 size standard and results were scored using GeneScan software Version (Applied Biosystems).

*Genetic Analysis*

Deviations from Hardy-Weinburg (HW) equilibrium and observed and expected heterozygosities were calculated using GenoDive v2.0 (Meirmans & Van Tienderen 2004). GENEPOP v4.2 (Rousset, 2008) was used to detect linkage disequilibrium (LD) between loci at each population. The Bonferroni correction for multiple comparisons was applied for both tests. Micro-Checker v2.2.3 (Van Oosterhout et al. 2004) was used to check for the presence of null alleles at each locus for all populations, allele drop out, and stuttering.

**Results**

Cross-amplification was successful in 25 of the 27-screened primers, eight of which were polymorphic in *P. petraeus*. Two primer pairs resulted in non-specific PCR products, and the remaining alleles were monomorphic for the samples. The number of alleles per locus for the eight polymorphic loci ranged from three to eight

(mean = 5, Table 1). The observed heterozygosity ranged from 0.143 to 0.929 (mean = 0.641). All loci were in HW equilibrium. No linkage disequilibrium was detected between locus pairs. Analysis of loci PG_43M, Plal_402 and Plal_701, indicated homozygote excess, suggesting that null alleles may be present. This could also be due to the small sample size and low number of alleles sampled. There was no evidence of large allele dropout at any of the loci. Stuttering (an artifact of PCR) may be affecting the detected polymorphism at locus Plal_701 since a single repeat unit differentiates the three alleles detected at this locus.

**Discussion**

The eight cross-amplified polymorphic microsatellites optimized in *P. petraeus* were applied in the broader context of a population genetic study. It will be possible to use these markers to estimate genetic diversity, fine-scale genetic structure across the species' distribution, and estimate gene flow between sampled locations. The use of these loci combined with comprehensive sampling from the known locations of *P. petraeus* will be important for conservation of this protected salamander.

Furthermore, these microsatellite markers will contribute to a better understanding of terrestrial salamanders. Few terrestrial species within the genus *Plethodon* have had microsatellite primers developed at the species level. Despite the increase in population level genetic studies of amphibians over the last decade, there is a notable gap in the literature of terrestrial population genetic studies (Emel & Storfer 2012). The loci identified by Spatola et al. (2013) were successfully cross-

amplified in *P. shermani* after their development and now also in *P. petraeus.* The success of the cross-amplification of these primers developed for *P. albagula* suggests that they may be applicable across species within the genus and specifically among the larger bodied species of the slimy salamander group. It would be appropriate to screen these primers for use in population genetic studies of other terrestrial salamanders, especially when considering projects that may be limited by time and money to develop species-specific markers.

**CHAPTER FOUR: FIGURES AND TABLES**

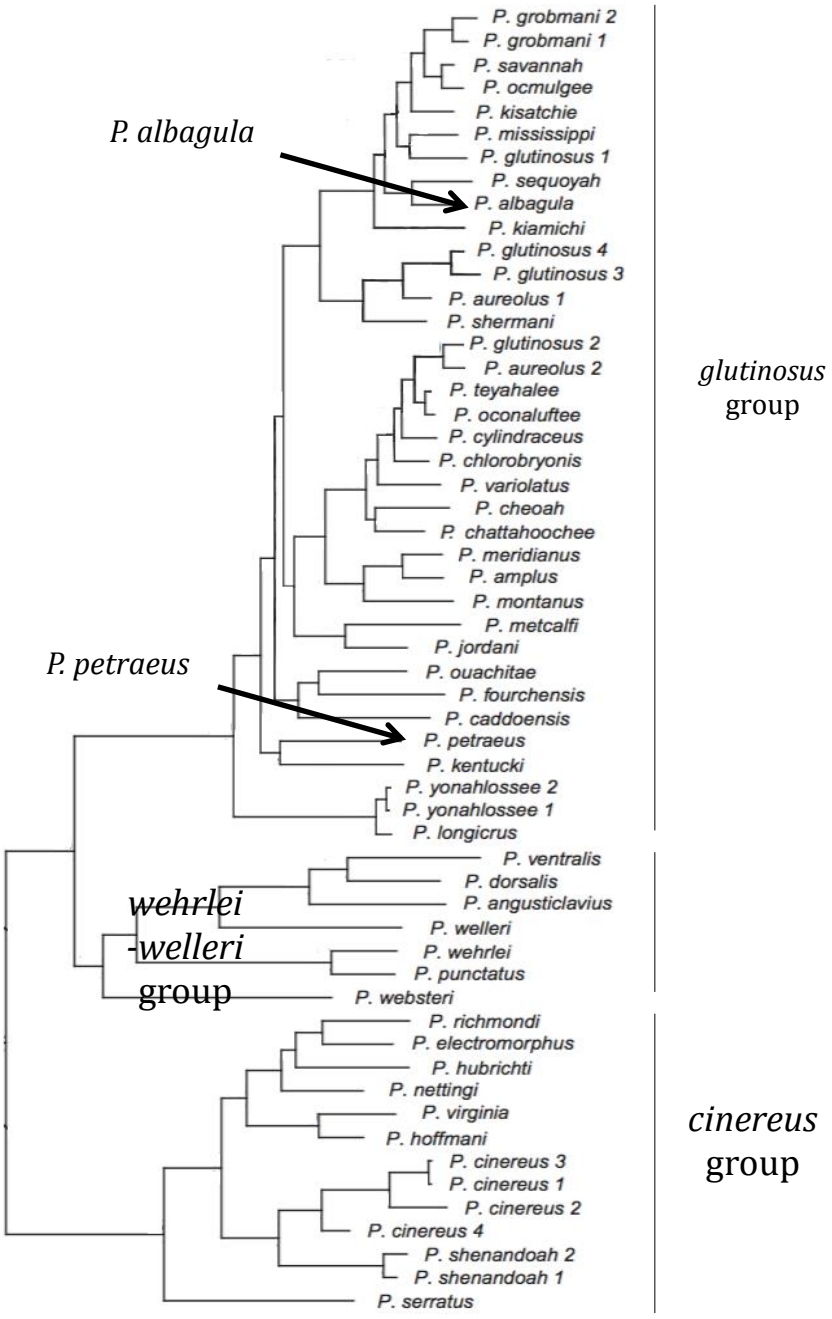



**Figure 1.** Eastern *Plethodon* phylogeny. Combined mtDNA and nuclear DNA *Plethodon* phylogeny (modified from Fisher-Ried and Wiends, 2011). *P. petraeus* is closely related to *P. albagula* and other slimy salamander species.

## Chapter 4: Tables

**Table 1.** Description of eight cross-amplified polymorphic loci for *P. petraeus*. Locus name, primer sequences (5' to 3') (Spatola et al. 2013), florescent dye, and annealing temperature ($T_a$) are listed for each locus. Also indicated are the number of individuals genotyped (n), size range of alleles, number of alleles ($N_a$), and observed (Ho) and expected ($H_e$) heterozygosities for each locus.

| Locus | Primer sequences | Labeling dye | $T_a$ (°C) | Size Range (bp) | N | Na | $H_o$ | $H_e$ |
|---|---|---|---|---|---|---|---|---|
| *PG_3XI* | F: AGCGGTGGATAGTCGTACAC<br>R: ATAGCACATAGGCAGATCAGTC | 6-FAM | 60 | 125-185 | 14 | 4 | 0.929 | 0.635 |
| *PG_43M* | F: AGTCATTGTCAGCTTGCGC<br>R: GGGAGCTTGCATCAGGAAAG | HEX | 60 | 109-161 | 14 | 4 | 0.286 | 0.591 |
| *PG_POG* | F: ACCTGTATTTCACGCTGCAC<br>R: CTGCACCTCTCACCCTACTG | NED | 60 | 246-298 | 14 | 5 | 0.857 | 0.725 |
| *PLAL_084* | F: ACTCCACAAACTCACTACCTG<br>R: TGTGGACCCTATTCTTGGCC | 6-FAM | 60 | 300 - 356 | 13 | 5 | 0.846 | 0.679 |
| *PLAL_127* | F: ATGTCCGAGCTATGAAACCC<br>R: GCACTCGCCTTGACCATTAC | HEX | 60 | 79 - 115 | 14 | 8 | 0.929 | 0.819 |
| *PLAL_402* | F: AGTGGTGAGGGAGATGGATG<br>R: TGGACTGTTGCTTTCTTGTGC | NED | 62 | 119 - 223 | 14 | 8 | 0.5 | 0.852 |
| *PLAL_542* | F: ATGCCTTAGGACCGCAGTAG<br>R: TGGGTTTCCTGGCATACTCC | 6-FAM | 62 | 234 - 274 | 14 | 8 | 0.643 | 0.852 |
| *PLAL_701* | F: CATGCGTACAGGATTAGGTCAG<br>R: CAGTCTGCCTCTTTGTAAGGC | HEX | 60 | 206 - 214 | 14 | 3 | 0.143 | 0.379 |
| | | | | Mean | | 5.625 | 0.641 | 0.691 |

# Chapter 5

## Conservation Genetics of the Rare Pigeon Mountain Salamander (*Plethodon petraeus*) within a Highly Restricted Range

### INTRODUCTION

The magnitude of amphibian declines and recently documented extinctions both at the level of population and species has prompted a need for conservation planning for declining amphibians (Grant et al. 2016; Mendelson et al. 2006). Molecular methods are now the standard for assessing the status of amphibian species due to technological advances that allow for information such as genetic diversity to be obtained in relatively short periods of time (Eastman et al. 2009). The difference between several months and several years in conservation efforts can be meaningful to a project's success. Using population genetics to study the Pigeon Mountain salamander will provide wildlife managers with useful information regarding genetic diversity and population structure across the species small range. The limited range of this salamander increases its risk of extinction because the species is more vulnerable to threats such as disease, habitat loss, and climate change (Houlahan et al. 2000; Velo-Antón et al. 2013). This study is an initial effort to document the genetic diversity and structure of this rare species. Previous work answered questions regarding life history traits (Camp & Jensen 2007; Jensen 2000; Marshall et al. 2004), and the addition of a genetic analysis to the scientific knowledge of the species will greatly aid in its conservation by delineating management units.

There has been great success using molecular markers to measure gene flow and genetic structure in amphibian populations. This approach is considered a key tool in directing the conservation efforts of amphibians as species suffer from global declines (Eastman et al. 2009). From 2001 to 2010 over 550 publications on amphibian populations and genetic studies have been published in scientific journals (Emel & Storfer 2012). The majority of these studies characterize population genetic structure across large ranges and in species that are not direct developing terrestrial salamanders. However, there are other salamanders with limited ranges including the Peaks of Otter Salamander, federally threatened Cheat Mountain Salamander, and federally endangered Shenandoah Salamander (Bayer et al. 2012). Along with the Pigeon Mountain Salamander, these species have some of the smallest ranges of any terrestrial vertebrates in North America. In 2000, a conservation genetic study of the Shenandoah Salamander, *Plethodon shenandoah,* provided mtDNA haplotype evidence that showed no genetic differentiation among three seemingly geographically isolated groups of the species (Carpenter et al. 2001). However, microsatellite data was not included in their analysis.

Finer scale genetic differentiation has been detected in species with widespread ranges but across small distances comparable to the known range of *P. petraeus.* Within a single species, genetic differentiation has been demonstrated to vary widely between distances based on barriers to movement. The widely distributed

Northern Red-backed Salamander, *Plethodon cinereus*, is found from the southeastern United States to as far north as Canada. A microsatellite analysis using six loci from 12 Northern Red-backed Salamander populations spread across roughly 40 km of urbanized Montreal and surrounding islands revealed a high degree of genetic differentiation (Noël & Lapointe 2010). Genetic structure was identified between populations separated by waterways as well as at a smaller spatial scale of roughly 1-2 km for populations located in downtown areas where anthropogenic disturbances limiting dispersal have been in place for hundreds of years (Munshi-South et al. 2013; Noël & Lapointe 2010). Conversely, the same study showed no genetic difference in Northern Red-backed Salamanders 35 km apart in areas connected by forest. However, a separate study of the same species in an undisturbed forest in Virginia, did detect fine scale genetic differentiation in *P. cinereus* across a transect of continuous forest (Cabe et al. 2007). This study also used six microsatellite loci to detect genetic structure between Northern Red-backed Salamanders separated by 200 m along a 2 km transect, and small but detectable genetic differentiation among populations as close together as 200m was detected (Cabe et al. 2007). These researchers hypothesized that the increased aggression of Northern Red-backed salamanders within the central area of their range, the Appalachian Mountains, may have been limiting dispersal factor acting on the population instead of anthropogenic disturbances (Mathis 1991).

Landscape features and habitat variation have also been shown to influence gene flow of a species in the same genus as *P. petraeus*, the Western Slimy Salamander,

*Plethodon albagula* (Peterman et al. 2014). This study compared sampling locations separated by only a few kilometers, comparable to the distances that separate some of the known locations of *P. petraeus*. Although distinct populations were not detected across the range of the study, significant genetic differentiation was observed.

The conflicting results from previous studies make predicting the results of our genetic analysis of *P. petraeus* difficult. If Pigeon Mountain Salamanders are readily dispersing between drainage gulfs then we would not expect to see significant spatial genetic structure across the habitat range. However, if the lack of rocky-outcrop connectivity and interspecific aggression are limiting dispersal, then genetic differentiation within the small range is likely since previous studies of salamanders have demonstrated genetic structure within comparable ranges. Aggression and response to aggression may also be a limiting factor for dispersal. Lab trials have demonstrated that the Northern Slimy Salamander, *Plethodon glutinosus*, which exists sympatrically with *P. petraeus*, is more territorial and aggressive (Marshall et al. 2004). Using an experimental design to test for behavioral interactions between males of both species, Carlos Camp's lab established that individuals of both species defend territories, but intruding *P. glutinosus* are more aggressive and more effective at evicting a resident *P. petraeus* from its territory. On the contrary, intruding *P. petraeus* individuals were less aggressive and not effective at evicting resident *P. glutinosus*. These results led to the hypothesis that direct competition

from slimy salamanders could be affecting *P. petraeus* movement and dispersal within and among suitable habitats on Pigeon Mountain.

*Objectives*

Due to the small range of *P. petraeus*, the species is highly vulnerable to the impacts driving amphibian declines. Fortunately, a large portion of the species known range is already preserved within a WMA, and the species is protected by the state of Georgia through a listing as a rare species. Despite its current level of protection, understanding the species' population genetics can support long-term management plans. The objective of the conservation genetic assessment is to use microsatellite marker data from six sampling locations across the species range to investigate a) genetic diversity b) genetic structure and c) gene flow.

**METHODS**

*Tissue Collection*

Tissue collection was performed under Georgia Department of Natural Resources scientific collecting permit (29-WJH-14-252) Salamanders were collected by hand and standard morphological measurements were recorded including snout-vent-length (SVL), tail-length (TL), tail-width (TW), head-length (HL), head-width (HW) and mass. A 1 cm tail tip was collected from each individual captured. Following tissue collection, salamanders were released at their point of capture. Tissue was placed on ice in the field and transferred to the laboratory for storage in a -20º C freezer prior to DNA extraction.

*Genetic Laboratory Techniques*

Whole genomic DNA was extracted from tissue with the commercial DNA extraction kit MoBio UltraClean Tissue & Cells DNA Isolation Kit (Carlsbad, CA, USA). Extracted DNA samples were stored in 50 ul elution buffer at -20 degree Celsius. Eight previously identified microsatellite loci were amplified from each sample using polymerase chain reaction (PCR). Microsatellite markers have not been developed in *P. petraeus*; however, eight polymorphic loci successfully cross-amplified in Pigeon Mountain Salamanders from 27 microsatellite loci identified within closely related species of slimy salamander (*Plethodon albagula* and *P. glutinosus*) (Spatola et al. 2013). Primers were 5-prime end-labeled with a fluorescent dye (6-FAM, NED or HEX; Applied Biosystems). The amplification products were sized on an Applied Biosystems 3130xl DNA analyzer (Applied Biosystems, Foster City, CA, USA) at the Savannah River Ecology Laboratory. Results were scored using GENEMARKER (v. 1.97; Sofgenetics, State College, PA, USA). We tested for full-siblings within our data set using COLONY and removed related individuals prior to data analysis (Jones & Wang 2010). Following removal of siblings, 103 individuals were included in the data set (mean = #/site; Table 1).

*Statistical Analyses*

Genepop v4.2 (Raymond & Rousset 1995) was used to test microsatellite loci for linkage equilibrium and deviations from Hardy-Weinberg equilibrium (HWE) at each locus and in each population. Micro-Checker v2.20 (Van Ossterhout et al. 2004) was used to test for null alleles in the microsatellite data. We used GENODIVE v2.0

(Meirmans 2006) to test for overall population genetic structure among collection sites relative to genetic diversity within in each collection site. Statistical significance of these estimates was tested and interpreted in an AMOVA framework. The overall correlation of pairwise $F_{ST}$ and distance separating sampling sites was calculated in order to assess the presence or absence of isolation by distance (IBD) for both Euclidean distance and distance along drainages, a more accurate representation of true salamander movement throughout the habitat of Pigeon Mountain (Hutchison & Templeton 1999; Slatkin 1977; Slatkin 1993). Measuring the ridgeline along the southeast facing slope of Pigeon Mountain using a topographic map was done to make an approximation of distance along drainages. Significance of matrix correlations between pairwise $F_{ST}$ and distance separating the sites will be assessed by Mantel test (1000 permutations) (Mantel 1967; Sokal et al. 1986; Meirmans 2006).

A prime objective of this study was to delineate population genetic structure among collection sites for *P. petraeus* throughout the species range. We used the multi-locus clustering software program STRUCTURE v2.3.3 (Pritchard et al. 2000) to assign individuals to populations. This approach infers genetic assemblages by estimating the probability of the observed genetic data given K number of genetic clusters. Each individual's population membership probability (to each cluster) was mapped to provide a visual representation of genetic structure. In this study, we are interested in delineating population boundaries, if present. We ran 10 independent simulations at each value of K between 2 and 6 (exploratory analyses supported K > 1). Each run consisted of a 100,000 step burn-in period followed by 100,000

iterations. The appropriate K value was selected using STRUCTURE HARVESTER (Earl & VonHoldt 2011) to determine the most likely number of populations using the DeltaK criterion (Evanno et al. 2005). Replicate runs were averaged using CLUMPP v1.1 (Jakobsson & Rosenberg 2007) and plotted using DISTRUCT v1.1 (Rosenberg 2003). We used an analysis of molecular variance, AMOVA, to partition genetic variance at different levels of organization: within collection sites, among collection sites, and among clusters (Meirmans 2006). Estimates of effective gene flow (Nm) between populations were estimated using Wright's formula, Fst = 1/(4Nm +1) (Larson et al. 1984; Wright 1943).

**RESULTS**

*Population Genetic Analysis*

The eight loci had 4- 29 alleles (mean = 11.25, S.E. +/- 3.24) across all samples (Table 1). There was no evidence of linkage dis-equilibrium between the eight loci surveyed. There was also no evidence of scoring errors, larger allele dropout, or null alleles detected. All loci and populations were within HWE expectations. Observed heterozygosity at each sample location ranged from 0.543 to 0.679 (mean = 0.607; Table 2). Pairwise estimates of $F_{ST}$ ranged from 0.072 to 0.344 (Table 3).

*Population structure*

The analysis of eight microsatellite loci grouped individuals into four distinct clusters based on the DeltaK values (Fig. 2). The two northernmost sampling locations, NR and NW, were within the same drainage and grouped together.

Although less than a kilometer apart, the two most central locations, PJ and LW, were found to belong to separate groupings. The remaining cluster contains the two southern most localities, AC and NG. Pairwise estimates of $F_{ST}$ ranged from 0.071 to 0.242 (Table 4). An AMOVA confirmed the significance of population structure (Table 5).

*Isolation By Distance*

Our isolation-by-distance analyses (Mantel tests; Mantel 1967) indicated that geographic distances and genetic distance are strongly correlated for *P. petraeus*. Isolation by Euclidean distance resulted showed a strong correlation with genetic distance (r = 0.703. P = 0.006) (Fig 3). This already strong correlation increased when the distance measured was adjusted along drainages (r = 0.884, P = 0.009). These measures represented a pathway containing a more suitable slope and habitat that could facilitate salamander movement compared to linear distances.

*Gene Flow*

Overall, the rates of migration met normal expectations of closer populations having higher migration estimates (Fig 4). Estimates of effective gene flow between populations suggest relatively consistent rates of movement across the landscape. As expected, gene flow is higher across shorter distances between populations but gene flow is not sufficient to homogenize populations.

## DISCUSSION

The results of our analysis revealed the Pigeon Mountain salamander, a species protected by the state of Georgia, has strong patterns of genetic structure across its entire range of less than 20 km. We found clear support for intrapopulation genetic structure and high levels of genetic differentiation across the known range of *P. petraues*. Bayesian clustering identified four distinct populations that were supported by high Fst values between populations and AMOVA results (Table 4 and 5). A high degree of genetic differentiation and multiple populations is somewhat surprising given the species small range. However, natural history characteristics such as the species known patchy distribution, specific habitat preference, and potential interspecific competition give support to these findings. These findings directly support observations of the species being patchily distributed and having limited dispersal. Previous work found evidence of strong site fidelity. The average distance moved between 10 marked and recaptured individuals was 2.26 meters. Further fieldwork needs to be done to determine if *P. petraeus* has high site fidelity and smaller home ranges and dispersal patterns as similar species.

The two populations PJ and LW are the closest together yet are genetically distinct enough to be structured separately. The linear distance between these two collections sites was less than 1 km and they have a genetic distance of 0.052 Fst (Table 5). A road (Rocky Lane) and drainage (McWhorter Gulf) separate PJ from the LW; both are possible barriers to Plethodontid salamander movement. Roads, along

with openings in forest canopy, such as clearings for industrial power lines, have been recognized as reducing salamander dispersal (Connette & Semlitsch 2013a).

*Conservation Implications*

The conservation status of *P. petraeus* is based on its endemism to Pigeon Mountain and highly restricted range. The protection provided to the species includes listing as a rare species by the state of Georgia and the preservation of roughly half of its known range within a wildlife management area (WMA). Our findings show that one of the four detected population falls mostly south of the WMA and the habitat protection that it offers. Efforts to educate private landowners about the biodiversity on their property and benefits of conservation should be pursued along with long-term conservation options such as the establishment of land easements and purchases when possible.

Seemingly suboptimal habitat for *P. petraeus* (areas lacking high density of outcrops and rocks) between known locations should also be considered for conservation. Such habitat could contain individuals at low densities that have not been detected during surveys and could be vital to gene flow between populations. Due to the species' exceedingly linear range along on the southfacing slope of Pigeon Mountain and strong isolation by distance genetic patterning, a single disturbance in habitat connectivity could further reduce natural migration, inhibiting already low levels of gene flow between populations. Also, decease in movement within the distinct populations could lead to fragmentation within current populations. Numerous

conservation genetics studies have shown that the maintenance of continuous suitable habitat is vital for species persistence (Allendorf et al. 2012).

*Future work*

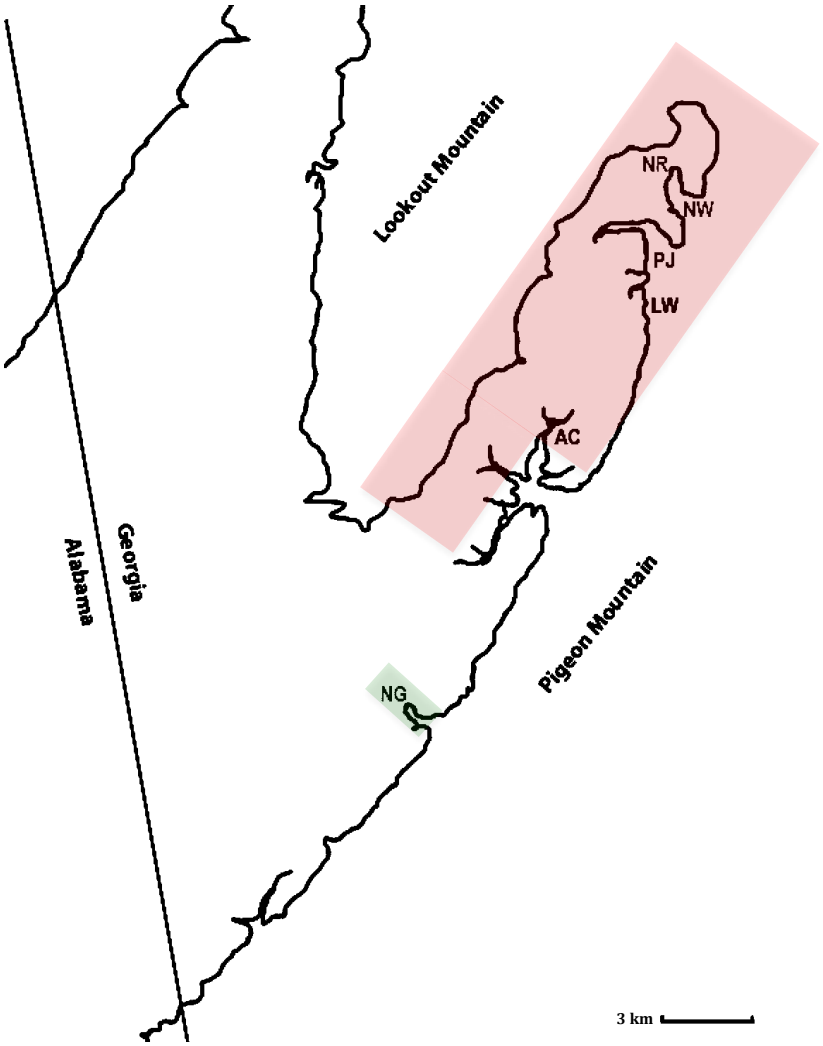
There is also great potential on Pigeon Mountain for a comparative population study within genus and across genus. Three additional species of *Plethodon* are found throughout the range of *P. petraeus*, including *P. glutinosus*. Across genera there are three species- *E. lucifuga*, *A. aeneus* and *P. petraeus*- that utilize rock outcrops and crevices. A comparative population genetic study across the landscape could inform how interspecific competition and differences in dispersal abilities may be influencing rates of gene flow and genetic connectivity (Storfer et al. 2010).

Better understanding the environmental factors that are influencing gene flow across Pigeon Mountain is also important. Variables such as stream cover, temperature, moisture, slope, canopy cover, and frost free periods have been shown to influence salamander gene flow in salamander populations (Emel & Storfer 2015; Apodaca et al. 2012; Peterman et al. 2014). The application of least cost path models to the range of *P. petraeus* would give us a deeper understanding of how the landscape affects gene flow and population structure. Other statistical methods such as occupancy estimation using Bayesian inference could be used to detect habitat that may contain new locations of *P. petraeus*. While sampling the six

locations included in this study, several new locations throughout the current range were recorded, and one site roughly .25 km farther south than their previously known most southern location was identified. Further efforts to know the full extent of the species range should be taken.

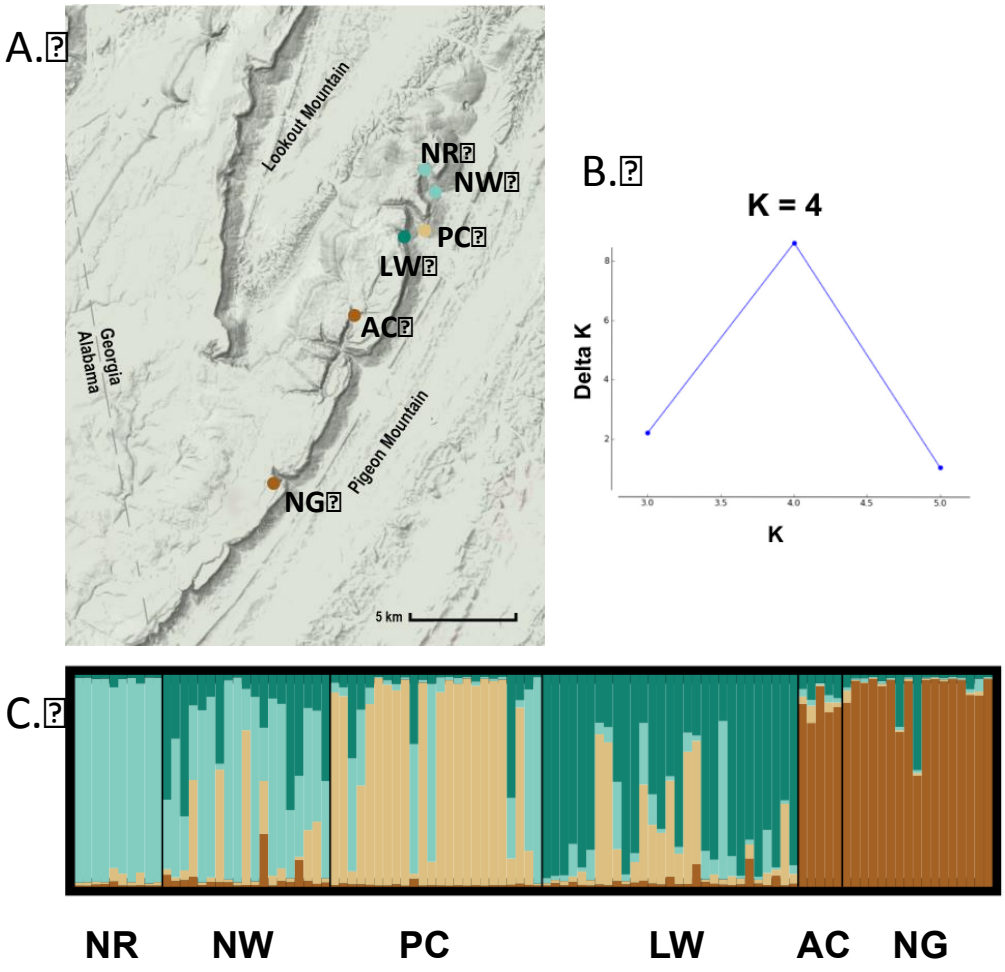
## CHAPTER FIVE: FIGURES AND TABLES

**Figure 1.** Sampling locations across the range of *P. petraeus.* Sampling locations AC, LW, PJ, NW and NR are within the Crockford-Pigeon Wildlife Management Area (red shading roughly approximates land within the WMA). The most southern location, NG, was accessible through a Nature Conservancy Land easement (green shading).



**Chapter 5: Figures cont…**

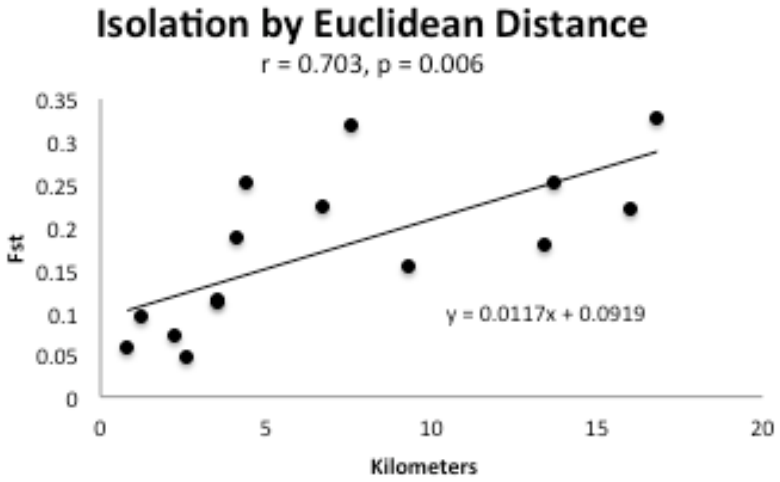
**Figure 2.** STRUCTURE results for four populations of *P. petraeus*. A) Map of the four populations. B) deltaK results for the optimal value of K. C) STRUCTURE bar graph results for the six sampling locations.



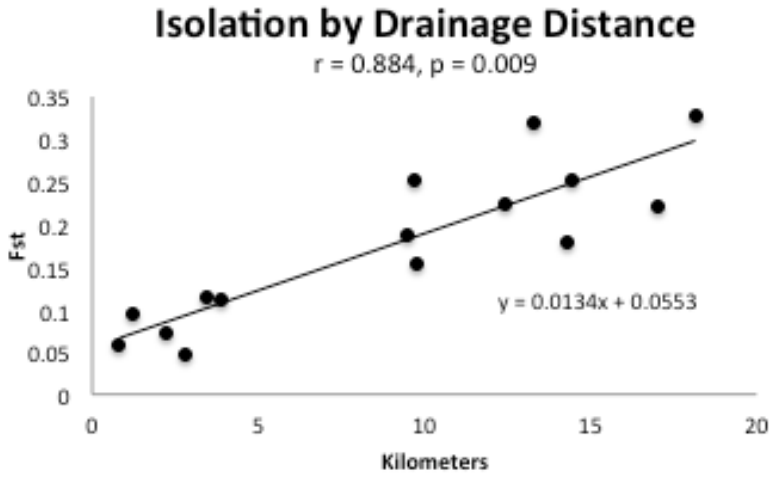
**Chapter 5: Figures cont…**

**Figure 3.** Isolation by distance. The relationship between pairwise genetic distance (Fst) and two measures of distance (Km). A) Linear distance and B) distance measured along southeast ridgeline of Pigeon Mountain representative of preferred habitat use connectivity.

**A.**

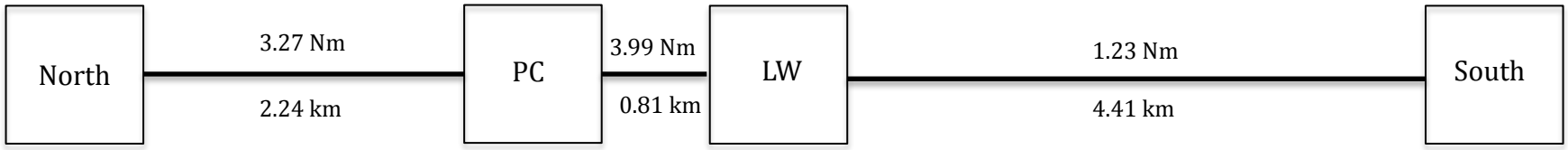



**B.**



**Chapter 5: Figures cont…**

**Figure 4.** Gene flow. Diagram of distance (km, bottom) between four populations and estimates of effective gene flow (Nm, top). The Northern and Southern populations contain two sampling locations, NR/NW and AC/NG, respectively. The distance between populations is measured to the nearest collection site between populations.



**Chapter 5: Tables**

**Table 1.** Genetic diversity per locus data.

| Locus | Allele Range | N | $N_a$ | $N_e$ | $H_o$ | $H_e$ | $F_{is}$ |
|---|---|---|---|---|---|---|---|
| *PG_3XI* | 138-150 | 97 | 4 | 1.878 | 0.693 | 0.473 | -0.457 |
| *PG_43M* | 103-131 | 102 | 8 | 2.965 | 0.73 | 0.675 | -0.113 |
| *PG_POG* | 208-256 | 99 | 7 | 2.061 | 0.488 | 0.526 | 0.045 |
| *PG_084* | 326-354 | 101 | 4 | 2.193 | 0.748 | 0.551 | -0.419 |
| *PLAL_127* | 97-121 | 92 | 7 | 1.998 | 0.413 | 0.515 | 0.173 |
| *PLAL_404* | 156-308 | 100 | 29 | 8.852 | 0.782 | 0.908 | 0.105 |
| *PLAL_542* | 164-280 | 92 | 22 | 5.109 | 0.831 | 0.823 | -0.031 |
| *PLAL_701* | 201-241 | 102 | 9 | 1.504 | 0.351 | 0.342 | -0.026 |
| Overall | | | 11.25 | 3.32 | 0.629 | 0.602 | -0.074 |

Allele range (bp), number of individual amplified (N), number of alleles ($N_a$), observed ($H_o$) and expected ($H_e$) heterozygosities and inbreeding coefficient ($F_{is}$).

**Chapter 5: Tables cont…**

**Table 2** Genetic diversity per sampling location.

| Location | N | $N_a$ | $N_e$ | $H_o$ | $H_e$ | $F_{is}$ |
|---|---|---|---|---|---|---|
| North Rim | 10 | 3.13 | 1.98 | 0.545 | 0.43 | -0.269 |
| Nash Waterfall Cave | 18 | 5.5 | 3.21 | 0.655 | 0.569 | -0.151 |
| Pettyjohn Cave | 24 | 5.25 | 3.266 | 0.599 | 0.618 | 0.03 |
| Lost Wall | 29 | 8.12 | 4.855 | 0.679 | 0.645 | -0.053 |
| Allen Creek | 5 | 3 | 2.349 | 0.543 | 0.562 | 0.034 |
| Neals Gap | 17 | 5.38 | 3.296 | 0.623 | 0.575 | -0.082 |
| Overall | | 5.063 | 3.159 | 0.607 | 0.567 | -0.137 |

Allele range (bp), number of individual amplified (N), number of alleles (Na), observed (Ho) and expected ($H_e$) heterozygosities and inbreeding coefficient ($F_{is}$).

**Chapter 5: Tables cont…**

**Table 3.** Matrix of sampling location Fst (lower left) and P values (upper right).

| | NR | NW | PC | LW | AC | NG |
|---|---|---|---|---|---|---|
| NR | -- | 0.001 | 0.001 | 0.001 | 0.001 | 0.001 |
| NW | 0.093 | -- | 0.001 | 0.001 | 0.001 | 0.001 |
| PC | 0.112 | 0.072 | -- | 0.001 | 0.001 | 0.001 |
| LW | 0.109 | 0.048 | 0.059 | -- | 0.001 | 0.001 |
| AC | 0.318 | 0.22 | 0.247 | 0.184 | -- | 0.001 |
| NG | 0.344 | 0.227 | 0.259 | 0.185 | 0.146 | -- |

**Table 4.** Matrix of population Fst (lower left) and P values (upper right).

| | North | PC | LW | South |
|---|---|---|---|---|
| North | -- | 0.001 | 0.001 | 0.001 |
| PC | 0.071 | -- | 0.001 | 0.001 |
| LW | 0.055 | 0.059 | -- | 0.001 |
| South | 0.231 | 0.242 | 0.169 | -- |

**Table 5**. Results of the analysis of molecular variance for best clustering according to the Evanno Method, K = 4

| Source of Variation | d.f. | Sum of Squares | Variance components | Percentage of variation | *P* value |
|---|---|---|---|---|---|
| Within Population | 99 | 395.215 | 3.992 | 0.833 | -- |
| Among Population | 3 | 73.532 | 0.8 | 0.167 | <0.001 |
| Fixation Index (Fst) | 0.167 | | | | |

## LITERATURE CITED


Allendorf, F.W., Luikart, G. & Aitken, S.N., 2012. *Conservation and the genetics of populations*, John Wiley & Sons.

Apodaca, J., Rissler, L. & Godwin, J., 2012. Population structure and gene flow in a heavily disturbed habitat: implications for the management of the imperilled Red Hills salamander ( Phaeognathus hubrichti). *Conservation Genetics*, 13(4), pp.913–923.

Bailey, L., Simons, T.R. & Pollock, K.H., 2004. Comparing Population Size Estimators for Plethodontid Salamanders. *Journal of Herpetology*, 38(3), pp.370–380.

Bailey, L., Simons, T.R. & Pollock, K.H., 2004. Estimating Detection Probability Parameters for Plethodon Salamanders Using the Robust Capture-Recapture Design. *The Journal of Wildlife Management*, (1), p.1.

Bayer, C. et al., 2012. Conservation genetics of an endemic mountaintop salamander with an extremely limited range. *Conservation Genetics*, 13(2), pp.443–454.

Bell, B.D. et al., 2004. The recent decline of a New Zealand endemic: how and why did populations of Archey's frog Leiopelma archeyi crash over 1996–2001? *Biological Conservation*, 120(2), pp.189–199.

Cabe, P.R. et al., 2007. Fine-scale population differentiation and gene flow in a terrestrial salamander (Plethodon cinereus) living in continuous habitat. *Heredity*, 98(1), pp.53–60.

Camp, C.D. & Jensen, J.B., 2007. Use of Twilight Zones of Caves by Plethodontid Salamanders. *Copeia*, (3), p.594.

Carpenter, R.E., Sites, J.W. & Jung, D.W., 2001. Conservation genetics of the endangered Shenandoah salamander (Plethodon shenandoah, Plethodontidae). *Animal Conservation*, 4(2), pp.111–119.

Connette, G.M. & Semlitsch, R.D., 2013a. Context-dependent movement behavior of woodland salamanders (Plethodon) in two habitat types. *Zoology (Jena, Germany)*, 116(6), pp.325–30.

Connette, G.M. & Semlitsch, R.D., 2013b. Life History as a Predictor of Salamander Recovery Rate from Timber Harvest in Southern Appalachian Forests, U.S.A. *Conservation Biology*, 27(6), pp.1399–1409. Available at: http://dx.doi.org/10.1111/cobi.12113.

Conover, W.J., 1999. *Practical nonparametric statistics*, Wiley.

Earl, D.A. & VonHoldt, B.M., 2011. STRUCTURE HARVESTER: a website and program for visualizing STRUCTURE output and implementing the Evanno method. *Conservation Genetics Resources*, 4(2), pp.359–361.

Eastman, J.M., Spear, S.F. & Storfer, A., 2009. Modern Molecular Methods for Amphibian Conservation. *Bioscience*, 59(7), pp.559–571.

Emel, S. & Storfer, A., 2012. A decade of amphibian population genetic studies: synthesis and recommendations. *Conservation Genetics*, 13(6), pp.1685–1689.

Emel, S. & Storfer, A., 2015. Landscape genetics and genetic structure of the southern torrent salamander, Rhyacotriton variegatus. *Conservation Genetics*, (16), pp.209–221.

Evanno, G., Regnaut, S. & Goudet, J., 2005. Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. *Molecular ecology*, 14(8), pp.2611–20.

Farallo, V.R. & Miles, D.B., 2016. The Importance of Microhabitat: A Comparison of Two Microendemic Species of Plethodon to the Widespread P. cinereus. *Copeia*, 104(1), pp.67–77.

Grant, E.H. et al., 2016. Quantitative evidence for the effects of multiple drivers on continental-scale amphibian declines. *Scientific Reports*, 6, p.25625.

Grant, E.H.C., 2008. Visual Implant Elastomer Mark Retention through Metamorphosis in Amphibian Larvae. *The Journal of Wildlife Management*, 72, pp.1247–1252.

Heemeyer, J.L. & Homyack, J.A., 2007. Retention and readability of visible implant elastomer marks in eastern redbacked salamanders (Plethodon cinereus). *Herpetological Review*, 38, pp.425–428.

Helyar, S.J. et al., 2011. Application of SNPs for population genetics of nonmodel organisms: new opportunities and challenges. *Molecular ecology resources*, 11 Suppl 1, pp.123–36.

Hendrix, R. et al., 2010. Strong correlation between cross-amplification success and genetic distance across all members of "True Salamanders" (Amphibia: Salamandridae) revealed by Salamandra salamandra-specific microsatellite loci. *Molecular Ecology Resources*, 10(6), pp.1038–47.

Highton, R. et al., 2012. Concurrent speciation in the eastern woodland salamanders (Genus Plethodon): DNA sequences of the complete albumin nuclear and partial mitochondrial 12s genes. *Molecular phylogenetics and evolution*, 63(2), pp.278–290.

Houlahan, J.E. et al., 2000. Quantitative evidence for global amphibian population declines. *Nature*, 404(6779), pp.752–755.

Hutchison, D.W. & Templeton, A.R., 1999. Correlation of Pairwise Genetic and Geographic Distance Measures: Inferring the Relative Influences of Gene Flow and Drift on the Distribution of Genetic Variability. *Evolution*, 53(6), p.1898.

Jakobsson, M. & Rosenberg, N.A., 2007. CLUMPP: a cluster matching and permutation program for dealing with label switching and multimodality in analysis of population structure. *Bioinformatics (Oxford, England)*, 23(14), pp.1801–6.

Jensen, J.B. et al., 2008. *Amphibians and reptiles of Georgia*, Athens: University of Georgia Press.

Jensen, J.B. and M.R.W., 2000. Diets of sympatric Plethodon petraeus and Plethodon glutinosus. *Journal of Elisha Mitchell Scientific Society*, 116, pp.245–250.

Jensen, J.B., Camp, C.D. & Marshall, J.L., 2002. Ecology and Life History of the Pigeon Mountain Salamander. *Southeastern Naturalist*, 1(1), p.3.

Johnson, S. et al., 2009. Evaluation of a new technique for marking anurans. *Applied Herpetology*, 6(3), pp.247–256.

Jones, O.R. & Wang, J., 2010. COLONY: a program for parentage and sibship inference from multilocus genotype data. *Molecular Ecology Resources*, 10(3), pp.551–555.

Kendall, W.L., Nichols, J.D. & Hines, J.E., 1997. ESTIMATING TEMPORARY EMIGRATION USING CAPTURE–RECAPTURE DATA WITH POLLOCK'S ROBUST DESIGN. *Ecology*, 78(2), pp.563–578.

Larson, A., Waket, D.B. & Yanevt, K.P., 1984. MEASURING GENE FLOW AMONG POPULATIONS HAVING HIGH LEVELS OF GENETIC FRAGMENTATION HE importance of gene flow as a factor maintaining phenotypic cohesion. *Genetics*, 1984(106), pp.293–308.

Liebgold, E.B., Brodie III D., E. & Cabe, P.R., 2011. Female philopatry and male-biased dispersal in a direct-developing salamander, Plethodon cinereus. *Molecular ecology*, 20(2), pp.249–257.

Marshall, J.L., Camp, C.D. & Jaeger, R.G., 2004. Potential Interference Competition between a Patchily Distributed Salamander (Plethodon petraeus) and a Sympatric Congener (Plethodon glutinosus). *Copeia*, 2004(3), p.488.

Mathis, A., 1991. Territories of Male and Female Terrestrial Salamanders: Costs, Benefits, and Intersexual Spatial Associations. *Oecologia*, (3), p.433.

McEntire, K.D., 2016. Arboreal Ecology of Plethodontidae: A Review. *Copeia*, 104(1).

Meirmans, P.G., 2006. USING THE AMOVA FRAMEWORK TO ESTIMATE A STANDARDIZED GENETIC DIFFERENTIATION MEASURE. *Evolution*, 60(11), pp.2399–2402.

Meirmans, P.G. & Van Tienderen, P.H., 2004. GENOTYPE and GENODIVE: two programs for the analysis of genetic diversity of asexual organisms. *Molecular Ecology Notes*, 4(4), pp.792–794.

Mendelson, J.R. et al., 2006. Confronting Amphibian Declines and Extinctions. *Science*, (5783), p.48.

Munshi-South, J., Zak, Y. & Pehek, E., 2013. Conservation genetics of extremely isolated urban populations of the northern dusky salamander (Desmognathus fuscus) in New York City. *PeerJ*, p.e64.

Noël, S. & Lapointe, F.-J., 2010. Urban conservation genetics: Study of a terrestrial salamander in the city. *Biological Conservation*, 143(11), pp.2823–2831.

Osbourn, M.S. et al., 2011. Use of fluorescent visible implant alphanumeric tags to individually Mark Juvenile ambystomatid salamanders. *Herpetological Review*, 42(1), pp.43–47.

Van Oosterhout, C. et al., 2004. micro-checker: software for identifying and correcting genotyping errors in microsatellite data. *Molecular Ecology Notes*, 4(3), pp.535–538.

Ousterhout, B.H. & Liebgold, E.B., 2010. Dispersal Versus Site Tenacity of Adult and Juvenile

Red-Backed Salamanders (Plethodon cinereus). *Herpetologica*.

Perry, G. et al., 2011. Toe Clipping of Amphibians and Reptiles: Science, Ethics, and the Law. *Journal of Herpetology*, 45(4), pp.547–555.

Peterman, W.E. et al., 2014. Ecological resistance surfaces predict fine-scale genetic differentiation in a terrestrial woodland salamander. *Molecular ecology*, 23(10), pp.2402–2413.

Petranka, J.W., 2010. Salamanders of the United States and Canada. *American Scientist*.

Pollock, K.H., 1982. A Capture-Recapture Design Robust to Unequal Probability of Capture. *The Journal of Wildlife Management*, (3), p.752.

Pritchard, J.K., Stephens, M. & Donnelly, P., 2000. Inference of population structure using multilocus genotype data. *Genetics*, 155(2), pp.945–959.

Raymond, M. & Rousset, F., 1995. GENEPOP (Version 1.2): Population Genetics Software for Exact Tests and Ecumenicism. *J. Hered.*, 86(3), pp.248–249.

Rosenberg, N.A., 2003. Distruct: a program for the graphical display of population structure. *Molecular Ecology Notes*, 4(1), pp.137–138.

Sapsford, S.J. et al., 2014. Visible Implant Elastomer Marking does Not Affect Short-Term Movements Or Survival Rates of the Treefrog Litoria Rheocola. *Herpetologica*, 70(1), pp.23–33.

Seber, G.A.F., 1986. A Review of Estimating Animal Abundance. *Biometrics*, 42(2), p.267.

Seeb, J.E. et al., 2011. Single-nucleotide polymorphism (SNP) discovery and applications of SNP genotyping in nonmodel organisms. *Molecular ecology resources*, 11 Suppl 1, pp.1–8.

Sinsch, U., 2014. Movement ecology of amphibians: from individual migratory behaviour to spatially structured populations in heterogeneous landscapes 1 , 2. *Canadian Journal of Zoology*, 92(6), pp.491–502.

Slade, N.A. & Blair, S.M., 2000. AN EMPIRICAL TEST OF USING COUNTS OF INDIVIDUALS CAPTURED AS INDICES OF POPULATION SIZE NORMAN. *Journal of mammalogy*, 30(4), pp.399–411.

Slatkin, M., 1977. Gene flow and genetic drift in a species subject to frequent local extinctions. *Theoretical population biology*, 12(3), pp.253–62.

Slatkin, M., 1993. Isolation by Distance in Equilibrium and Non-Equilibrium Populations. *Evolution*, 47(1), p.264.

Spatola, B. et al., 2013. Development of microsatellite loci for the western slimy salamander (Plethodon albagula) using 454 sequencing. *Conservation Genetics Resources*, 5(1), pp.267–270.

Steele, C.A., Baumsteiger, J. & Storfer, A., 2008. Polymorphic tetranucleotide microsatellites for Cope's giant salamander (Dicamptodon copei) and Pacific giant salamander

(Dicamptodon tenebrosus). *Molecular Ecology Resources*, 8(5), pp.1071–1073.

Storfer, A. et al., 2010. Landscape genetics: where are we now? *Molecular ecology*, 19(17), pp.3496–3514.

Stuart, S.N. et al., 2004. Status and trends of amphibian declines and extinctions worldwide. *Science*, 306(5702), pp.1783–1786.

Velo-Antón, G. et al., 2013. Tracking climate change in a dispersal-limited species: reduced spatial and genetic connectivity in a montane salamander. *Molecular ecology*, 22(12), pp.3261–3278.

Welsh, H.H. & Droege, S., 2001. A Case for Using Plethodontid Salamanders for Monitoring Biodiversity and Ecosystem Integrity of North American Forests. *Conservation Biology*, (3), p.558.

Wen, Z. et al., 2013. A robust design capture-recapture model with multiple age classes augmented with population assignment data. *Environmental and Ecological Statistics*, 21(1), pp.41–59.

White, G.C. & Burnham, K.P., 1999. Program MARK: survival estimation from populations of marked animals. *Bird Study*, 46, pp.S120–S139.

Williams, B.K., Nichols, J.D. & Conroy, M.J., 2002. *Analysis and Management of Animal Populations*, San Diego, California, USA: Academic Press.

Wright, S., 1943. ISOLATION BY DISTANCE*. *Genetics*, 28, pp.114–138.

Wynn, A.H., Highton, R. & Jacobs, J.F., 1988. A New Species of Rock-Crevice Dwelling Plethodon from Pigeon Mountain, Georgia. *Herpetologica*, (2), p.135.