

**Assessing Site Occupancy and Microhabitat Use of the Hellbender salamander
(*Cryptobranchus alleganiensis*) in Tennessee**

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Master of Science

by

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May 2018

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ABSTRACT**Assessing Site Occupancy and Microhabitat Use of the Hellbender salamander
(*Cryptobranchus alleganiensis*) in Tennessee**

May 2018

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Precise monitoring of species distributions across its range and within its habitat is important for the development of effective conservation plans. Amphibian declines have been reported since the 1980s, and lack of data on distribution and habitat requirements often hinders conservation efforts. The hellbender salamander (*C. alleganiensis spp.*) is no exception to the trend. Although Tennessee provides areas of high-quality habitat for *C. alleganiensis*, standardized state-wide assessment of *C. alleganiensis* distribution in Tennessee has been limited to known populations and microhabitat use differences among life stages have not been well documented. I used the Maximum Entropy Algorithm to create a predictive habitat model to guide sampling efforts in Tennessee and used environmental DNA and occupancy modeling to ground truth my model and

evaluate the status of the target species. I evaluated microhabitat use of larval, juvenile, and adult life stages at three streams in east Tennessee by comparing sites occupied by *C. alleganiensis* to random sites within each stream. Overall, my analysis suggests that available habitat for *C. alleganiensis* within its range in Tennessee is significantly limited, and ecoregion has a strong effect on detection probability and occupancy, with the Blue Ridge ecoregion having the greatest detection probability and occupancy rate compared to other ecoregions. Finally, my analysis indicated a strong difference in microhabitat use among life stages and identified microhabitat covariates that are potentially important for long-term *C. alleganiensis* conservation as means of providing guidance for stream restoration practices that may mitigate sedimentation and habitat degradation in impacted streams.

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(*Cryptobranchus alleganiensis*) in Tennessee**

May 2018

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DEDICATION

To my father and mother, Jeronimo and Lilian, for the all the love, guidance, respect, trust, and for understanding my restless soul. To my mentors for their patience, encouragement, and for teaching me how to transform knowledge into wisdom. Lastly, to all my friends and loved ones who have helped me through my journey. Thank you.

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CHAPTER I

THREATS TO THE HELLBENDER SALAMANDER (*CRYPTOBRANCHUS ALLEGANIENSIS*)

The main threats to earth's biodiversity include overexploitation, habitat destruction, the introduction of exotic species, diseases, and pollution. All these threats are directly or indirectly related to anthropogenic factors and land-use activities (Wilcove 1998). Conversion of land for agricultural purposes, urbanization, and large- and small-scale land alterations (i.e., dams, impoundments) are the leading threats to species that are either listed or proposed for listing under the Endangered Species Act (Wilcove 1998). Large-scale agricultural practices have been linked to the decline of a greater number of endangered organisms than any other anthropogenic factor (Wilcove et al 1998). As urbanization, land alteration, and large scale agricultural practices continue to grow, anthropogenic disturbances will continue to represent one of the greatest challenges for the future of biodiversity conservation.

Although land-use changes may impact species differently, species with specific physiological and habitat requirements are often more sensitive to changes in habitat characteristics and quality, and often display species-specific responses. Class Amphibia is represented by frogs, salamanders, and caecilians. Beginning in the early 1980s, amphibian species world-wide began experiencing devastating population declines and extinctions (Collins 2010). Habitat destruction, diseases, over-exploitation, and exotic species introduction, have been the main drivers of amphibian declines (Collins and Crump 2009; Collins 2010). Amphibians have several physiological and morphological characteristics that make them particularly susceptible to changes in habitat availability

and quality. Amphibians have highly permeable skin that serves as the main pathway for water absorption, osmoregulation, gas exchange, and nitrogen excretion which influence almost all aspects of amphibian ecology and behavior (Wells 2010). For example, warmer temperatures can increase water evaporation rates in terrestrial amphibians, which can impact breeding success by influencing movement capability during breeding events (Blaustein et al., 2010; Wells 2010). Furthermore, amphibians often have small home ranges, low vagility, and a narrow habitat tolerance (i.e., limited to areas with high moisture level essential for survival and breeding), which can limit their ability to disperse and adapt to changing environments (Blaustein et al. 1994; Crushman 2010). Amphibian eggs are vulnerable to environmental contaminants due to lack of a shell (i.e., the wall of anamniotic eggs is composed of a gelatinous membrane), which can increase their exposure to environmental pollutants (Blaustein et al. 2003; Barrett and Guyer 2008; Burlibaşa et al., 2011). Because of their morphological and physiological characteristics, and susceptibility to environmental variables, amphibians are good indicators of ecosystem health (Duellman and Trueb 1986; Southerland et. 2004; Burlibaşa et al. 2011).

The Hellbender (*Cryptobranchus alleganiensis*) is a fully-aquatic salamander and the only representative of the “giant salamander” family (Cryptobranchidae) in the United States, reaching up to 74 cm in total length. The historical geographic range of this species once extended from southern New York, south to north Alabama, north Georgia, and northeast Mississippi with disjunct populations in the Ozark Mountains of Missouri and Arkansas (Figure 2; Petranks 1998; Niemiller and Reynolds 2011). *C. alleganiensis*

populations across the range have experienced drastic population declines associated with a complex interaction of factors such as habitat degradation, accelerated sedimentation, wildlife trade, pollutants, and infectious diseases (Wheeler et al., 2003). Despite research completed over the past four decades, important challenges remain in the field of *C. alleganiensis* conservation. Conservation efforts for this species are often hindered by the lack of accurate data on species distribution, habitat requirements, and causes of population declines. Therefore, further evaluation of these challenges is critical for the future of *C. alleganiensis* conservation.

CHAPTER II
USING SPECIES DISTRIBUTION MODELING, ENVIRONMENTAL DNA, AND OCCUPANCY
MODELING TO EVALUATE THE STATUS OF THE HELLBENDER SALAMANDER
(*CRYPTOBRANCHUS ALLEGANIENSIS*)

Introduction

Accurate prediction of species distributions is essential for proper management and development of effective long-term conservation plans (Loiselle et al. 2003; Wilson et al. 2005). For example, distribution data is often used for delineation of priority research and management areas allowing for optimal use of resources (e.g., funding and field survey time). It also enables development of predictive habitat models which are effective tools for studying species that lack extensive distribution data (Wilson et al. 2005; Newbold 2010; Barve et al. 2011). However, distribution data is often only available in museum and private collections and natural history literature, which is often inaccurate and biased (e.g., species can be incorrectly classified, and field sampling can be biased towards areas that are easier to sample). While presence-only data is available for many taxa, true absence data (i.e., evidence of absence is not due to failure of detection) is rare, particularly for species located in areas that are hard to access or that are difficult to detect (Phillips et al. 2006a, Elith et al. 2011). Therefore, further evaluation of challenges associated with imperfect detection and quality of distributional data is critical for the long-term conservation of species diversity at both local and regional scales. Accurate distribution data is important when evaluating population trends

(i.e., fluctuation in population size) associated with species in rapid decline or those threatened with extinction (Green 2003, Beebee and Griffiths 2005).

Amphibians can be used as a representation to demonstrate how lack of distribution data can impair conservation efforts (Lannoo 2005; Collins and Crump 2009). Since the 1980s, global amphibian declines and extinctions have been reported, exemplifying an ongoing crisis of global biodiversity decline across many taxa (Collins and Storfer 2003). These massive amphibian declines are a consequence of the interaction between several factors such as habitat loss and fragmentation, introduction of non-native species, climate change, and emerging infectious diseases (Collins and Storfer 2003; Beebee and Griffiths 2005). Efforts to better understand conservation challenges and remediate causes of amphibian declines are often impaired by the lack of baseline data on species distribution and historical presence (Collins and Crump 2009). For example, researchers may fail to detect areas where amphibian extinctions occurred because baseline presence data was never available for those areas (Collins and Crump 2009). In addition, the collection of distribution data may be hampered by difficulties locating individual species due to factors that include rarity, behavioral adaptations (e.g., fossorial, arboreal, aquatic habitat restrictions), researcher experience, and sampling season (Beebee and Griffiths 2005; Lannoo 2005). Therefore, it is essential that researches and managers explore different methods to acquire high quality distribution data that facilitates the development and implementation of effective conservation efforts.

Species distribution models, or SDMs, are an important tool to evaluate the potential distribution and/or habitat suitability for a target species (Hirzel et al. 2001,

Fernandez et al. 2003; Graham et al. 2004). Species distribution models are analysis techniques that use species distribution data (i.e., presence and/or absence data), combined with environmental variables (i.e., predetermined environmental factors that are known to be associated with the target species) of known locations to estimate the potential distribution across the species' geographic range and the environmental conditions required to maintain viable populations (Rissler and Apodaca 2007; Elith and Leathwick 2009). A variety of SDM techniques are available, however, these analysis methods have different statistical assumptions and data requirements. As many historical species-occurrence datasets lack information on true absences, presence-only SDMs can be a valuable tool to estimate distribution patterns of target species (Phillips et al. 2006a). The Maximum Entropy Algorithm (MaxEnt; Phillips et al. 2017) is an example of one such method and represents a machine-learning approach that estimates a given species' geographic distribution by combining both presence-only data (i.e., known occurrences only) and predetermined environmental covariates (Phillips et al. 2006a; Ficetola et al. 2007). MaxEnt is commonly used because it does not require true absence data, it is less sensitive than other approaches to the number of presence locations required to estimate species distributions, and it is not strongly influenced by spatial autocorrelation associated with presence data (Phillips et al. 2006a; Ficetola et al. 2007; Baldwin 2009). In addition, research comparing traditional SDM approaches (i.e., BIOCLIM, GARP, or DOMAIN) indicate that MaxEnt is an effective method when using presence-only data and often performs as well as several traditional tools that use both presence and absence data (e.g., BIOCLIM, GARP, DOMAIN; Philips et al. 2004; Elith et al. 2006; Hernandez

et al. 2006). However, there are still challenges with presence-only models, and management decisions (e.g., delineation of high conservation priority areas) based on MaxEnt models should be decided based on multiple lines of evidence (Reddy and Devalos 2003; Kremen et al. 2008; Ortega and Peterson 2008; Baldwin 2009; Kramer-Schadt et al. 2013).

Ground-truthing (i.e., evidence confirmed through actual observation during a field check or experimentation) is a source of additional evidence that can be used to validate model results and inform management decisions (Greaves et al. 2006). Habitat modeling studies often fail to conduct field surveys to verify occurrence within predicted areas due to cost or time restrictions (Jimenez-Valverde 2008; Rebelo and Jones 2010). Other techniques besides systematic field surveys can be used for on-site validation of distribution models and enhancement of distribution datasets. Environmental DNA, or eDNA, is an example of such sampling method and refers to genetic material (e.g., nuclear or mitochondrial DNA) that is naturally released into the environment by organisms inhabiting such locations (Bohmann et al. 2014; Davy et al. 2015). Environmental DNA can be extracted from cellular (e.g., feces, mucous, shed skin and fur) or extracellular sources (e.g., DNA diluted in aquatic systems) in the environment (Bohmann et al. 2014; Barnes and Turner 2016). Determining presence or absence of a species using eDNA typically consists of capturing and extracting DNA from a source, followed by the amplification of the target DNA using a polymerase chain reaction (PCR) or quantitative polymerase chain reaction (qPCR; Bohmann et al. 2014; Deiner et al. 2015; Spear et al. 2015). Environmental DNA has been used as a noninvasive sampling

tool to identify biodiversity and evaluate genetic diversity in both terrestrial and aquatic systems (Waits and Paetkau 2005; Spear 2015). Recent work done with amphibians, reptiles, and fish indicate that eDNA can be a reliable tool for monitoring rare, threatened, and secretive species, as well as invasive species. For example, Goldberg et al. (2011) showed that eDNA techniques are an effective tool for detection of Rocky Mountain tailed frogs and Idaho giant salamanders in freshwater ecosystems. Moreover, Jerde et al. (2013) used eDNA to evaluate the status of two invasive carp species in the Great Lakes. Although eDNA concentration and quality are affected by DNA production, transport, and degradation rates (Bohmann et al. 2014; Goldberg et al. 2014), these approaches can provide a more rapid, cost-effective, less labor intensive, and potentially more sensitive surveying tool compared to standard survey methods (Goldberg et al. 2011, 2014; Spear et al. 2015; Davy et al. 2015). In addition, it offers a non-invasive survey method that is less disruptive and reduces stress caused by handling and habitat disturbance, which may be preferred when working with rare and threatened species (Bohmann et al. 2014; Davy et al. 2015).

The accuracy of presence and absence data gathered through field surveys is dependent on the researcher's ability to accurately determine if a location is occupied (i.e., detection) by the target species or not (i.e., non-detection). Studies rarely have perfect detection (i.e., detection is equal to 1) due to population density, species-specific behavior and movement and random variations due to sampling approaches (Gu and Swihart 2004; Mordecai et al. 2011). Non-detections are a result of the species being not present (i.e., true absence) or the researcher failed to detect the species even when present

(i.e., false negative; MacKenzie et al. 2002). If imperfect detection (i.e., detection is less than 1) is not taken into consideration, site occupancy can be underestimated, and predictions of colonization and extinction rates can be biased (MacKenzie et al. 2003). This is particularly important for rare and threatened species, because underestimation of occupancy could hinder conservation efforts (Kerr et al. 2000). In addition, detection is significantly more difficult due to low population densities, or major population declines (Gu and Swihart 2004; Jerde et al. 2011). Therefore, field survey results should be further analyzed using an occupancy modeling approach which enables the incorporation of imperfect detection into occupancy estimates.

Hellbender salamanders (*Cryptobranchus alleganiensis*) inhabit streams and rivers in the midwest and eastern United States and are the only salamander in the family Cryptobranchidae found in North America (one of three species known globally). Two subspecies are found in the Americas. The Ozark Hellbender (*Cryptobranchus alleganiensis bishopi*) inhabits the Ozark mountain streams of Missouri and Arkansas in the Midwest, while the Eastern Hellbender (*Cryptobranchus alleganiensis alleganiensis*) inhabits streams and rivers of the Ohio, Tennessee, Cumberland, and Mississippi River drainages in the Eastern United States with a disjunct population in Missouri. Both subspecies have experienced population declines throughout their historical geographic ranges predominantly due to habitat degradation, siltation, contaminants, infectious diseases, illegal trade and poaching, and direct mortality upon human interaction (Williams et al. 1981; Wheeler et al. 2003; Foster et al. 2009, Freake and DePerno 2017). As a result, *C. a. bishopi* has been listed as an endangered species under the Endangered

Species Act (USFWS 2011a), and both subspecies have been listed as near threatened by the International Union for Conservation of Nature (IUCN; Hammerson and Phillips 2004) and included in the appendix III of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES; USFWS 2011b). In Tennessee, the focal region of my study, *C. alleganiensis* is categorized as a nongame species with “greatest conservation need” under the State Wildlife Action Plan (TNSWAP 2015) due to lack of information on distribution, limiting factors, and biological and ecological requirements for the long-term conservation of the species in the state. In addition, *C. alleganiensis* is currently under review in the Candidate and Petition Process to be listed as Federally Threatened (i.e., species is likely to become endangered within the foreseeable future) under the Endangered Species Act (USFWS 2010, ECOS).

Despite research completed over the past four decades, important challenges remain in the field of *C. alleganiensis* conservation. The lack of accurate data on current and potential *C. alleganiensis* distribution and identification of causation behind population declines continues to hinder effective conservation efforts. Therefore, the objectives of my research are to 1) provide a predictive habitat model to guide sampling efforts and depict areas of highest conservation concern in Tennessee, 2) use environmental DNA to ground truth my habitat model and evaluate model accuracy, and 3) use occupancy modeling to further evaluate the status of *C. alleganiensis* within its range in Tennessee. My collective research goal is to use a multi-level sampling approach to provide an intensive evaluation of *C. alleganiensis* populations throughout Tennessee, identify factors determining and limiting site occupancy, and provide range wide baseline

data that can be used to guide future surveys and selection of high priority conservation areas for possible relocation and habitat restoration. I expected Strahler stream order to be an important determinant of site suitability, and areas of greatest site occupancy to be in areas with historically and current lower landscape development (i.e., agriculture and urbanization). Streams suitable for relocations were expected to be located across the range, with highly suitable streams located in the eastern portion of the study area. Finally, I expected a high detection probability, with Julian date having the strongest effect on detection.

Material and Methods

Development of the Species Distribution Model

Study area

The study area consisted of the known range of *C. alleganiensis* in Tennessee (Petranka 1998; Figure 1.1). The study area includes the Upper, Middle, and Lower Tennessee River, and Cumberland River basins which drain to the Gulf of Mexico through the Ohio and Mississippi Rivers. The greater (i.e., upper, middle, and lower) Tennessee River basin crosses the Blue Ridge, Ridge and Valley, Southwestern Appalachian, and Interior Plateau ecoregions. These ecoregions possess temperate to subtropical climates, and are predominately covered by forest, with agriculture being a major form of land-use across most of the middle and west Tennessee ecoregions (Hampson et al. 2000; Abell et al. 2008). Several topographic regions make up the ecoregions across the *C. alleganiensis* range, from lower elevation (approximately 300m)

uplands areas at the Western Highland Rim in the west to high mountain ranges (above 1,500 m) at the Unaka Mountains in the east (Starnes and Etnier 1986; Abell et al. 2008).

Occurrence data

Species occurrence data consisted of 153 museum records acquired from seven main sources (Figure 1.2, Step 1A), including the Biodiversity Information Serving Our Nation (BISON) database (www.bison.usgs.ornl.gov), Global Biodiversity Information Facility (GBIF) database (www.gbif.org), Conservation Fisheries database, Tennessee Species Wildlife Action Plan (SWAP) database, Tennessee Herpetofaunal Atlas (administrated by Austin Peay University), VertNet (www.vertnet.org) database, and the Tennessee Valley Authority (TVA). All points used were located within the known *C. alleganiensis* range in Tennessee, and I selected occurrence data recorded from 1950 to 2015 to ensure that points within Tennessee Valley Authority (TVA) impoundments were likely to be removed. Finally, I removed duplicate presence points and points located outside streams and rivers in attempt to decrease sampling bias and the inclusion of inaccurate occurrence points, respectively.

Maximum entropy modeling technique

I used the MaxEnt software to create an initial SDM for *C. alleganiensis* in Tennessee (Figure 1.2 - Step 1B). The MaxEnt algorithm evaluates the suitability of each raster cell as a function of environmental covariates (i.e., covariates that are believed to be ecologically-relevant to *C. alleganiensis*) and presence-only data (i.e., known occurrences only) and classifies the suitability of each raster cell using a logistic scale from zero (i.e., least suitable) to one (i.e., most suitable; Phillips and Dudik 2008; Elith et

al. 2011). I used a ten-fold replicate cross-validation approach to obtain a better estimate of model variance (Messerman 2014; Sutton 2015) and applied a 10-percentile training presence logistic threshold (hereafter referred as F10 threshold) to generate a binary (i.e., suitable vs. not suitable) map, which means that the model is required to correctly identify 90% of the training data (i.e., model must have a sensitivity of at least 90%; Freeman and Moisen 2008). Although the selection of a threshold is often arbitrary, this threshold provides a more conservative approach and avoids overfitting compared to other thresholds such as the minimum training presence threshold (Freeman and Moisen 2008; Tinoco et al. 2009; Jarnevich and Reynolds 2011). I used natural-breaks approach in GIS to divide the resulting suitable areas (areas with suitability index greater than the minimum suitability score based on the F10 threshold) into four categories: low, medium, high, and very high suitability. The fit of the resulting model was evaluated using the Area Under the receiving operating characteristic Curve (AUC; Figure 1.3) value and True Skill Statistic (TSS). The AUC value was obtained from the cross-validation of 10 sub-samples replicates and the average TSS was calculated based on recommendations in Allouche et al. (2006). Area Under the Curve values represent the probability that a random presence point is ranked higher than a random background point (i.e., pseudo-absence), and I considered the model informative when the AUC value was above 0.75 (Elith 2002; Phillips 2008; Baldwin 2009). True Skill Statistics results ranges from -1 to +1, where values below 0.2 represent poor/as good as random model performance, and values above 0.7 represent very good/excellent model performance (Monserud and Leemans 1992, Allouche et al. 2006).

Background data were used to provide a sample of conditions available for comparison against presence points. Targeted-group background data, or bias files, can be often used to improve model results by reducing the effect of sampling bias; however, to my knowledge, no other species has been sampled in a similar manner or effort and therefore, target-group background data for *C. alleganiensis* would not represent true absences (Phillips et al. 2009). Because I lacked validated non-detection data and targeted-group background, I used approximately 10,000 randomly generated pseudo-absences restricted to streams and rivers throughout the range of *C. alleganiensis* in Tennessee (Phillips et al. 2009, Kramer-Schadt et al. 2013).

Environmental data

I used the following variables to create the initial *C. alleganiensis* SDM: elevation, stream flow direction, geology, hillshade, landcover type, mean annual precipitation, slope, Strahler order, and temperature (Figure 1.2, Step 1A; Table 1.1) at a 30m x 30m cell size. All environmental data used was hypothesized to be ecologically-relevant to *C. alleganiensis* and were delimited to the species range within Tennessee. Geology, flow direction, and Strahler stream order were included based on results from a similar landscape-scale study of *C. alleganiensis* in North Carolina (Messerman 2014). I used the National Hydrography Dataset Plus version 2.1 (NHDPlusV2; Horizon Systems Corp. 2012) to calculate stream flow direction, stream flow accumulation, and Strahler stream order for all streams within the study area (Table 1.1). I used a geological layer developed by the United States Geological Survey for the state of Tennessee with geologic units, structure features, characteristics, and age (USGS-Schruben et al. 1994). I

used several NEDSnapshots (5a, 5d, 6a, 8b, and 3f) from the National Hydrography Dataset Plus Version 2.1. (NHDPlusV2, Horizon Systems Corporation 2012) to create an elevation mosaic for Tennessee, which was used to calculate slope. In addition, I used the National Land Cover Database (NLCD; 2001 – 2011; USGS 2011) to calculate percent of the following land cover types: barren land, cropland, developed land, forested land, pasture land, shrub land, water per catchment, and wetlands per catchment. Finally, I used annual precipitation and average annual temperature data from 1960 to 1990 (Hijmans et al. 2005; WorldClim Version 1.4; Table 1.1).

Model Validation through environmental DNA

Field surveys using environmental DNA

All sites used in this study were sampled using environmental DNA sampling techniques (described below). To increase the inference power of this study, I collaborated with Dr. Michael Freake and Dr. Stephen Spear to increase the number of sampled sites by incorporating their data on 69 sites sampled between 2012 – 2015. In 2016, after I constructed the species distribution model, I selected an additional 219 sampling sites within suitable streams across the study area. Based on *C. alleganiensis* small home range sizes and uncertainties associated with eDNA sampling techniques, I selected sites that were at least 5 km apart to increase site independence (Peterson and Wilkinson 1996, Olsen et al. 2012). To facilitate further analysis via an occupancy approach, I selected 29 sites where I took repeated samples for a total of three sample events per site (i.e., each of the 29 sites was sampled on June, July, and August 2016). These repeated sampling sites were stratified throughout the known range of *C.*

alleganiensis in Tennessee as much as possible to control for potential influences of ecoregion and stream geology on detection probabilities.

At each site I filtered 1L of surface water through a 0.45 um cellulose nitrate filter (Whatman international, Ltd.) attached to a 1L Nalgene Polypropylene Vacuum Flask (Fisher Scientific, Hampton, NH; cat. #:10 182 50B,) as described in Spear et al. (2015). A hand pump (Fisher Scientific, Hampton, NH; cat. #: S90724A) or electric vacuum pressure pump (GAST-31.2L/min #XX5500000; EMD Millipore, Billerica, MA) was used to vacuum water samples through the filters. All samples were collected before I entered the water or up-stream of entry point to reduce the risk of contamination. Each filter sample was removed using forceps and stored in 2.0mL microcentrifuge tubes filled with 1 mL 95% ethanol and placed into a -20 °C refrigerator until subsequent DNA extraction. All field equipment used between sites (e.g., boots, tubes, Nalgene bottles, forceps) were treated with 50% bleach solution (i.e., 50% commercial bleach, 50% deionized water) for five minutes to ensure no cross-contamination of eDNA or transfer of pathogens between sites. Finally, I filtered 1 L of deionized water as a negative control (i.e., field negative control) after every three sites to account for possible contamination between sites (i.e., *C. alleganiensis* being transported from one site to another).

Laboratory methods

I extracted, purified, and amplified DNA from filters using modified protocols reported in Goldberg et al. (2011) and Spear et al. (2015). I used a DNeasy Blood and Tissue Kit (Qiagen, Inc.) and followed the standard protocols except for the use of a QiaShredder (Qiagen, Inc.) spin column after the lysis buffer step. I amplified DNA using

a qPCR protocol via a species-specific reverse and forward primers and probe which amplified a 104 bp region of mitochondrial *Cytochrome-B* as described by Spear et al. (2015) and Franklin (2016). These primers and probes were developed based on DNA from individuals collected across the southeastern United States as described by Sabatino and Routman (2009). Because *C. alleganiensis* are not closely related to any North American salamanders, I did not test for non-target amplification (Spear et al. 2015). As described in Spear et al. (2015), I ran 15 µl qPCR reactions with 2.85 µl nuclease free water, 7.5 µl QuantiTect Multiplex PCR Mix (Qiagen, Inc.), 0.75 µl probe and primer mix, 0.6 µL of TaqMan® Exogenous Internal Positive Control 10X Exo IPC Mix (Applied Biosystems), 0.3 µL of TaqMan® Exogenous Internal Positive Control 50X Exo IPC DNA (Applied Biosystems), and 3 µL of sample DNA filter extract on an Applied Biosystems 7000 Fast Real-Time PCR system (Applied Biosystems). The qPCR cycling protocol consisted of 15 minutes at 95 °C, and 50 cycles of 94 °C for one minute, and 60 °C for one minute. All laboratory processes were conducted in a facility dedicated to low-copy DNA extractions and PCR procedures at The Wilds, Cumberland, OH, and Tennessee State University, Nashville, TN. Finally, I used Applied Biosystems 7000 software v2.0.6 (Applied Biosystems, Inc) to analyze amplification results. I ran all samples in triplicates using a 96-well PCR plate, with a single nuclease-free water negative control (Applied Biosystems) per plate. I considered the result a detection if at least one of three replicates amplified during qPCR. Samples that amplified only once were re-run to ensure that amplification was not due to contamination.

Occupancy Modeling

Statistical analysis

I followed occupancy modeling techniques described by MacKenzie et al. (2002, 2003) to create a single-season, single species, hierarchical occupancy model using the Unmarked package in RStudio (RStudio Team 2015, Boston, MA; Fiske and Chandler 2011). I considered each sampling site to be independent (i.e., sites were at least 5 km apart) and assumed that no colonization or extinction happened between 2012 and 2016 because *C. alleganiensis* are long lived (individuals can live 20+ years) and have small home ranges (Taber et al. 1975). I used a total of 284 sites sampled during seven sampling events: 2012, 2013, 2014, 2015, 2016A(June), 2016B(July), 2016C(August), where 29 sites were sampled three times in 2016 (June, July, and August). At each sampling site, *C. alleganiensis* can be present and detected, present and undetected, or absent (Grant et al. 2009). Therefore, how effectively I detect *C. alleganiensis* at each sampling site (i.e., detection probability, p) influences the accuracy of occupancy estimates (Ψ) for all sampling sites. I evaluated how linear and quadratic Julian date patterns may impact detection probability (i.e., observation-level covariate) and how elevation, geology, slope, Strahler order, land cover, and ecoregion (i.e., site-level covariates) may explain site occupancy (Table 1.6). I distinguished between detection (1; successful amplification of *C. alleganiensis* DNA) and non-detection (0; sample did not contain *C. alleganiensis* DNA or amplification was not successful). I developed a total of 29 occupancy models for *C. alleganiensis* (Table 1.6). I selected the best models based on Akaike's information criterion (AIC) adjusted for small sample sizes (AIC_c; e.g.,

Burnham and Anderson 2002) and considered a model potentially informative when the ΔAIC_c value was <2 (Burnham and Anderson 2002). I calculated model averaged beta coefficients and standard errors for all top supported models and plotted the top supported model using the ggplot2 package in RStudio (RStudio Team 2015, Boston, MA). Furthermore, because ecoregion was a strong covariate within all top supported models, I performed a *post hoc* occupancy analysis to further evaluate the simultaneous effect of ecoregion on occupancy and detection (equation = $p(ecoreg_3)$, $\psi(ecoreg_3)$).

Results

MaxEnt modeling

The MaxEnt model performed significantly better than random as indicated by the AUC value of 0.941 (STD = 0.023; Figure 1.3), which means that the training data was correctly classified approximately 94% of the time, along with a TSS value of 0.77. According to the TSS analysis, the model had an average sensitivity and specificity of 89% and 87% respectively, based on 10 replicates. Strahler stream order had the highest contribution to the model (85.4%), followed by geology (7.4%), and mean annual precipitation (2%; Table 1.2). In addition, the jackknife analysis of regularized training data indicated that Strahler stream order was the most informative variable, followed by geology, and landcover (Figure 1.4).

After applying the F10 threshold, the total suitable stream area for *C. alleganiensis* within the study area represented 12.2% ($\sim 422 \text{ km}^2$) of the total stream area ($\sim 3,463 \text{ km}^2$) present within the study area (Table 1.3). Of the 422 km^2 of suitable streams, low suitability streams constituted 48.6% ($\sim 205 \text{ km}^2$), medium suitability

streams constituted 24.4% ($\sim 103 \text{ km}^2$), high suitability streams constituted 24.6% ($\sim 104 \text{ km}^2$), and very high suitability streams constituted 2.4% ($\sim 10 \text{ km}^2$) of the area (Table 1.3). The Interior Plateau ecoregion holds the largest portion of all suitable stream area ($\sim 225 \text{ km}^2$, 53%), followed by the Ridge and Valley ($\sim 97 \text{ km}^2$, 23%), Blue Ridge ($\sim 55 \text{ km}^2$, 13%), Southwest Appalachian ($\sim 33 \text{ km}^2$, 8%), Southeastern Plains ($\sim 8 \text{ km}^2$, 2%), and Central Appalachian ecoregions ($\sim 5 \text{ km}^2$, 1%; Table 1.3). When controlled for ecoregion area, Interior Plateau has the greatest proportion of high suitability streams with $\sim 31\%$ of all suitable streams within the ecoregion classified as highly suitable, followed by the Ridge and Valley ($\sim 23\%$), Blue Ridge ($\sim 14\%$), Southeastern Plains ($\sim 13\%$), Southwest Appalachians ($\sim 8\%$), and Central Appalachian ecoregions (3%; Table 1.3). When considering only the highest suitability category, Southwest Appalachian has the greatest proportion of very high suitability streams with approximately 10% of all suitable streams within the ecoregion classified as very high suitability, followed by Blue Ridge (9%), Ridge and Valley (2%), and Interior Plateau (1%) ecoregions, with Central Plains and Southwest Appalachian ecoregions having close to none or zero streams classified as very high suitability (Table 1.3).

Field surveys and model validation

Between 2012 and 2015, Dr. Michael Freake and Dr. Stephen Spear sampled a total of 65 sites. In 2016, I sampled a total of 219 sites. Predicted low suitability areas had the lowest proportion of detections (13.2%) and highest proportion of non-detections (86.8%), while very high suitability areas had the highest proportion of detections (46.3%) and the lowest proportion of non-detections (53.7%; Table 1.4). The Blue Ridge

ecoregion had the greatest number of detections (34), followed by the Interior Plateau (17), Ridge and Valley (10), and Southwest Appalachian ecoregions (4), for a total of 65 detections across the study area (Table 1.5). All field and lab controls were negative, which indicated that there was no contamination during field sampling or lab procedures.

Occupancy model

The top supported occupancy models were 19 ($AIC_c = 383.41$; $k = 5$, $\Delta AIC_c = 0.00$), 6 ($AIC_c = 384.91$; $k = 6$, $\Delta AIC_c = 1.49$), 29 ($AIC_c = 384.99$; $k = 6$, $\Delta AIC_c = 1.57$), and 24 ($AIC_c = 385.07$; $k = 5$, $\Delta AIC_c = 1.65$; Table 1.6). These models included ecoregion (Blue Ridge $\beta = 3.04 \pm 0.71$; Interior Plateau $\beta = -0.75 \pm 1.02$; Southwest Appalachian $\beta = 1.03 \pm 0.76$; Ridge and Valley $\beta = 1.12 \pm 0.54$) and open water ($\beta = -0.41 \pm 0.26$) effects on occupancy and ecoregion (Blue Ridge $\beta = 2.26 \pm 0.36$; Interior Plateau $\beta = -0.27 \pm 0.84$; Southwest Appalachian $\beta = 0.9 \pm 0.64$; Ridge and Valley $\beta = 0.91 \pm 0.45$) and survey ($\beta = 0.14 \pm 0.18$) effects on detection. Within all four top supported models, ecoregion had a strong effect on both occupancy and detection, and probability of detection was not influenced by quadratic or linear Julian date term. The *post hoc* results included the effect of ecoregion on occupancy probability (Blue Ridge $\beta = 2.06 \pm 0.68$ Interior Plateau $\beta = -1.09 \pm 0.47$; Southwest Appalachian $\beta = 0.72 \pm 1.10$; Ridge and Valley $\beta = 1.31 \pm 1.10$), and detection probability (Blue Ridge $\beta = 1.11 \pm 0.56$; Interior Plateau $\beta = -0.30 \pm 0.46$; Southwest Appalachian $\beta = 0.47 \pm 1.07$; Ridge and Valley $\beta = -0.01 \pm 0.78$).

Estimates of naïve occupancy (naïve Ψ) are derived from the number of detections over the total number of sampled sites (i.e., percent of total sites where C).

alleganiensis DNA was detected), without taking into consideration detection probability (p ; Table 1.3). Within the entire study area, I detected *C. alleganiensis* DNA at 65 sites out of 284 sites (overall naïve $\Psi = 22.9\%$). The Blue Ridge ecoregion had the greatest naïve Ψ (48.6%), followed by the Ridge and Valley (naïve $\Psi = 25.6\%$), Southwest Appalachian ecoregion (naïve $\Psi = 25\%$), and Interior Plateau (naïve $\Psi = 10.9\%$; Table 1.5). According to the top model (19; Table 1.6), the detection probability (p) over the entire study area was $56.4 \pm 0.06\%$. When corrected for overall detection probability, occupancy was greatest within the Blue Ridge ecoregion ($82.1 \pm 0.10\%$), followed by Ridge and Valley ($40.6 \pm 0.12\%$), Southwestern Appalachians ($38.1 \pm 0.17\%$), and Interior Plateau ecoregions ($18.3 \pm 0.05\%$; Figure 1.5). The *post hoc* analysis indicated that detection probability was greatest within the Blue Ridge ($69.2 \pm 0.07\%$), followed by the Southwest Appalachians ($54.2 \pm 0.24\%$), Interior Plateau ($42.6 \pm 0.11\%$), and Ridge and Valley (42.2 ± 0.15 ; Figure 1.6). When I corrected Ψ for detection probability by ecoregion, the Blue Ridge had the greatest corrected Ψ ($72.4 \pm 0.10\%$), followed by the Ridge and Valley (corrected $\Psi = 55.5 \pm 0.24\%$), Southwestern Appalachians (corrected $\Psi = 40.9 \pm 0.24\%$), and Interior Plateau (corrected $\Psi = 25.1 \pm 0.10\%$; Figure 1.6).

Discussion

My results indicate that the predicted suitable area available for *C. alleganiensis* within its range in Tennessee is alarmingly small, representing only 12.2% of all the stream area within the state. With limited availability of suitable habitat for *C. alleganiensis* within their range in Tennessee, and the growing threats to the integrity of their habitat, the need for additional biological information on the species is apparent

(Alig et al. 2003). My results indicate that ecoregion is a strong determinant of *C. alleganiensis* occupancy across the known geographic range in Tennessee, which also greatly impacts detection probability. Ecoregion-specific geomorphology and topography influence stream hydrogeology, which governs the type of river substrate, and influences stream hydrological patterns and species distribution (Cleland et al. 1997; Gordon et al. 2004; Quinn et al. 2013). For example, higher stream orders have been shown to be correlated with higher species diversity, and heterogeneous substrate with less gravel (Harrel et al. 1967; Platts 1979). Notably, my results support the hypothesis that Strahler stream order was an important contributor to stream suitability with the highest percent contribution to the habitat model, followed by geology. Specifically, lotic environments with stream orders 4 – 6 with granitic and sedimentary substrates explained a majority of *C. alleganiensis* presence. Ecoregion may be a strong determinant of *C. alleganiensis* occupancy because the species is fully aquatic and has a strong relationship with substrate composition and hydrology, which are determined by ecoregion-specific hydrogeology (Nickerson et al. 2003; Quinn et al. 2013). The effect of ecoregion on detection could be explained by the relationship between abundance and detection (Dejean et al. 2011). Because the amount of DNA in the environment is also a function of a species abundance, density, biomass, and occupancy (Pilliod et al. 2013), ecoregions that provide higher quality habitat and have higher greater *C. alleganiensis* abundance could have higher detection probabilities compared to ecoregions with low species abundance (Buxton et al. 2007). Franklin (2016) indicated that eDNA detection probability of *C. alleganiensis* in North Carolina increased with eDNA concentration,

abundance, and animal size. Compared to results from Franklin (2006), where detection probability was approximately 90%, I reported significantly lower detection estimates of approximately 55%. I believe the difference in detection estimates between studies can be explained mainly by the difference in size of the study area, along with disparities among ecoregions surveyed. The *C. alleganiensis* range in Tennessee is considerably larger, and the overall detection probability was derived from areas with a large gradient of habitat quality and species abundance, which likely decreased overall detection estimates.

The effect of ecoregion and occupancy could also serve as a proxy for the effect of land use practices on habitat quality within a given ecoregion. Jachowski (2016) suggested that even though *C. alleganiensis* occupancy in Virginia was explained mainly by geology and physiography, agriculture and development could have a negative effect on occurrence of the target species. Urbanization and agricultural practices affect water quality, increase sediment input into waterways, and significantly disrupt hydrological patterns (Malmqvist and Rundle 2003; Muenz et al. 2006; Roy et al. 2009; Henley et al. 2010). The data supported the hypothesis that *C. alleganiensis* occupancy was greater within ecoregions with lower historical landscape changes (i.e., conversion of forest to agriculture and urban areas). Congruent with results presented by Jachowski (2016), the Blue Ridge ecoregion within the *C. alleganiensis* range in Tennessee had the greatest occupancy rates compared to the Ridge and Valley, Southwestern Appalachian, and Interior Plateau ecoregions. The Blue Ridge ecoregion historically has experienced the smallest net percent change in forest cover between 1973 and 2000, while Interior Plateau and Ridge and Valley experienced higher net percent change in forest cover mainly due

to agriculture and mechanical disturbance of forest (Brown et al. 2005; Drummond and Loveland 2010). These differences in historical land-use alterations between ecoregions could have created a gradient of habitat quality across ecoregions, which over time translated into different occupancy rates across ecoregions (Bryce et al. 1999; Jachowski 2016). This historical land use pattern may also explain why the Blue Ridge ecoregion contains the greatest proportion of very high suitability streams and the greatest occupancy rate compared to all other ecoregions. My results indicated that the Southeastern Plains had a greater proportion of very high suitability stream area than the Blue Ridge, however; I believe that the area of the Southeastern Plains ecoregion within the *C. alleganiensis* range was not an accurate representative of the ecoregion because it represents only a small portion of the overall ecoregion. Collectively, the data suggests that the Blue Ridge ecoregion holds the best habitat for *C. alleganiensis* within its range in Tennessee, followed by the Interior Plateau and Ridge and Valley. Within all ecoregions, it is important to consider current land use and its possible impact on future *C. alleganiensis* populations. For example, in a separate analysis investigating the percent of agricultural and urban development at a HUC3 watershed level within the *C. alleganiensis* range in Tennessee, the Ridge and Valley and Interior Plateau had the highest percent of watersheds with over 50% of land use dedicated to agriculture and development (unpublished data). Furthermore, Alig et al. (2003) predicted a population growth of 45% by 2050 within the South-Central region which includes Tennessee. Therefore, it is my prediction that the Interior Plateau and Ridge and Valley ecoregions will continue to undergo high levels of development, which will likely include complex

interactions of anthropogenic factors that will continue to influence stream habitat quality in high-development areas. However, the Blue Ridge ecoregion may not be as susceptible to disturbance and development due to the large amount of protected forests within the ecoregion and a low population density compared to other ecoregions (Bryce et al. 1999). Conservation efforts focusing on relocation and repatriation within Interior Plateau and Ridge and Valley should consider historical, current, and predicted catchment-wide land-use practices and protection status of target areas (e.g., private land vs. national forest; Jachowski 2016).

I predicted the potential distribution of *C. alleganiensis* within its range in Tennessee with high accuracy. The AUC value of 0.94 and TSS value of 0.77 indicated great model performance. Even though AUC is frequently used to evaluate model performance, there are several issues with using only AUC to evaluate the performance of presence-only models. For example, AUC weights commission and omission errors equally, and is also sensitive to the extent of territory; two characteristics that are not desired when evaluating presence/absence model results (Lobo et al. 2008). In addition, AUC summarizes model performance over all possible thresholds, which is not optimal when trying to create a binary map (i.e., presence vs. absence) where the selection of a specific threshold is necessary (Lobo et al. 2008). Overall, AUC is a useful tool to determine if the model is efficient at differentiating presence and random points, but it does not provide information on overall model accuracy, sensitivity, specificity, and omission and commission errors (Lobo et al. 2008, Phillips and Elith 2010, Warren and Seifert 2011). Therefore, I used TSS to further analyze model performance. The TSS is a

suitable alternative to AUC when evaluating presence/absence model predictions because it is independent of prevalence (i.e., it is not sensitive to extent of territory), and incorporates a selected presence/absence threshold into model accuracy analysis (i.e., threshold dependent), while still accounting for omission and commission errors (Allouche et al. 2006).

Even though predictive habitat models are a useful tool for conservation across multiple taxa, caution must be adopted when transcribing model results into applied management. Because I created the MaxEnt model using *C. alleganiensis* occurrence data (1950 - 2016) from a different period than some of the environmental covariates (e.g., land use cover 2001 - 2010), the effect of these covariates on the target species may not be fully represented. In addition, the MaxEnt model does not take into consideration small scale biotic, abiotic, and inter- and intra-specific relationships that are important determinants of a species distribution (Barve et al. 2011). For example, sites that were suitable for *C. alleganiensis* in the early 1900s and were dammed during the 1930s are no longer suitable habitat. However, I used multiple lines of evidence such as field surveys and occupancy modeling to increase the quality of the model performance examination. Overall, my predictive habitat model can be a useful conservation tool for future identification of priority conservation areas for protection, management, and translocation/repatriation of captive-reared individuals.

My results verified that environmental DNA was an effective approach to validate the habitat model, evaluate model prediction accuracy, and contribute to *C. alleganiensis* distribution data within its range in Tennessee. When I evaluate the percent of detections

and non-detections within each model suitability category, the overall trend shows an increase in percent detection from low suitability to high suitability stream reaches (13.2% to 22.9%, respectively), and a decrease of percent non-detections from low to high suitability stream reaches (86.8% to 77.1%, respectively). My results indicate that Julian date did not have an impact on detection probability in the field. These results did not meet my expectations, because *C. alleganiensis* eDNA concentrations in the field has been shown to fluctuate with season (i.e., increase eDNA available in the water during breeding season), which can affect detection probability (Spear et al. 2015; Franklin 2016). Therefore, I expected Julian date, particularly around breeding season, to be correlated with probability of detection. These results were likely affected by the extent of the study area which includes a range of abundance influenced by habitat quality. Overall, these findings indicate that I can successfully use eDNA sampling approach to detect *C. alleganiensis* anytime during their active season (June – September), which increase my ability to make inferences on population dynamics over longer time periods.

Even though environmental DNA provides several benefits for sampling rare and elusive species, there are weaknesses with the sampling method that must be taken into consideration for proper interpretation of sampling results. For example, eDNA sampling methods often have imperfect detection, and detection can be influenced by the rate of DNA input and output within the system, degradation to UV light and water pH, rate of transport, and target species abundance (Dejean et al. 2011; Goldberg et al. 2014; Buxton et al. 2017). In addition, it is unclear how distance between DNA source and sampling location can impact detectability (Goldberg et al. 2014). However, through this eDNA

sampling approach, I were able to sample over 200 sites in a significantly shorter time frame compared to typical field surveys which often require several sampling hours and several researchers (Freake and DePerno 2017). Similar work completed by Schmidt et al. (2013) suggests that studies using environmental DNA and occupancy modeling can be more informative when sampling effort is focused on visiting more sites, with fewer repeated visits at each site. Furthermore, I were able to sample across ecoregions and watersheds, and identify five streams that were previously unknown *C. alleganiensis* occurrence locations. Therefore, I consider eDNA sampling approach an important conservation tool, especially when attempting to detect rare or elusive species over a large geographic range where limited research resources would make the use of typical sampling techniques almost infeasible

Overall, my study illustrated the benefits of using a multi-level sampling approach to investigate the status of a rare stream-dwelling organism over a large geographic area. By using a combination of species distribution modeling, environmental DNA sampling techniques, and occupancy modeling, I were able to identify and evaluate potential factors influencing occupancy of *C. alleganiensis* in Tennessee and provide baseline information on the status of the target species and areas of high conservation concern. Above all, I were able to estimate the area of suitable streams available for the target species and draw attention to the significantly small quantity of high and very high suitability streams available. A large portion of suitable streams located within ecoregions under high levels of development will most likely continue to be affected by anthropogenic factors. To increase the inference of this study, future research should

focus on selecting high and very high suitability streams with positive detections within the Interior Plateau and Ridge and Valley ecoregions and conduct physical surveys to assess the occurrence and abundance of *C. alleganiensis*. This information would not only help further ground-truth my results, but it would also provide baseline population demographics and facilitate long term assessment of the effects of land use changes on different *C. alleganiensis* populations among ecoregions. Future conservation of the species may rely on prioritizing populations and areas of highest conservation need. Therefore, demographic and distribution data will be essential if future conservation strategies include targeted stream restoration, watershed protection, population augmentation, and relocation/repatriation of individuals into priority conservation areas.

Tables

Table 1.1 Environmental covariates used to develop a species distribution model (SDM) for C. alleganiensis within its range in Tennessee using Maximum Entropy Algorithm. All covariates are believed to be ecologically-relevant to the target species. Covariate descriptions and sources are listed.

Covariate	Description	Source
Elevation	Digital Elevation Model (DEM) representing the distance of target surface above sea level	NHDPlusV2
Stream flow direction	Direction of water flow derived from DEM	NHDPlusV2
Geology	Categorical covariate with geological units and their characteristics	USGS
Hillshade	Representation of the target surface taking into consideration altitude and azimuth	NHDPlusV2
Landcover	National Land Cover Database (NLCD) representing land cover categories and their characteristics from 2001 – 2011.	USGS
Precipitation	Average precipitation in millimeters within <i>C. alleganiensis</i> range in Tennessee from 1960 - 1990	WorldClim
Slope	Slope of target surface	NHDPlusV2
Strahler order	A hydrological unit representing stream size based on the order of tributaries	USGS
Temperature	Average temperature in Celsius within <i>C. alleganiensis</i> range in Tennessee from 1960 - 1990	WorldClim

Table 1.2 MaxEnt output representing relative environmental covariate contribution to the MaxEnt model.

Covariate	Percent contribution (%)
Strahler order	85.4
Geology	7.4
Precipitation	2
Temperature	1.5
Landcover	0.9
Elevation	0.9
Slope	0.8
Hillshade	0.6
Stream flow direction	0.5

*Table 1.3 Suitable stream area (km²) per suitability category (i.e., low, medium, high, and very high) within each ecoregion (Central Appalachians, Blue Ridge, Southwest Appalachians, Ridge and Valley, Southeastern Plains, and Interior Plateau) and respective percent of each suitability category per ecoregion after accounting for ecoregion area. The “Total” column represents total suitable stream area per ecoregion (km²). The “Total” row represents the total stream area per suitability category and the total area (km²) of suitable stream within the *C. alleganiensis* range in Tennessee (422.39 km²).*

Ecoregion	Suitable stream area per suitability category (km^2)					
	Low	Medium	High	Very high	Total (km^2)	
Central Appalachians	3.09	1.74	0.13	0.00	4.96	
Blue Ridge	29.72	12.07	7.80	5.02	54.60	
Southwest Appalachians	19.33	11.27	2.75	0.13	33.48	
Ridge and Valley	46.75	25.59	22.63	1.91	96.89	
Southeastern Plains	3.59	2.24	0.99	0.76	7.58	
Interior Plateau	102.19	50.64	69.94	2.11	224.88	
Total (km^2)	204.68	103.54	104.25	9.93	422.39	Total (km^2)
	Percent of each suitability category per ecoregion (%)					
Central Appalachians	62.39	35.03	2.58	0.00		
Blue Ridge	54.43	22.10	14.29	9.19		
Southwest Appalachians	57.73	33.65	8.22	0.39		
Ridge and Valley	48.26	26.41	23.36	1.97		
Southeastern Plains	47.36	29.51	13.09	10.04		
Interior Plateau	45.44	22.52	31.10	0.94		

Table 1.4 Number of detection and non-detections within each stream suitability category (i.e., low, medium, high and very high), and the total number of sites sampled per category. The lower portion of the table represents the percent of detections and non-detections per category. This matrix could be used to help analyze model performance.

	MaxEnt Suitability Category				
	Low	Medium	High	Very high	Total (#)
Detection (#)	9	12	19	25	65
Non-detection (#)	59	66	65	29	219
Total (#)	68	78	84	54	284
Detection (%)	13.2	15.4	22.6	46.3	22.9
Non-detection (%)	86.8	84.6	77.4	53.7	77.1

Table 1.5 C. alleganiensis detection and non-detection within each ecoregion (Central Appalachians, Blue Ridge, Southwest Appalachians, Ridge and Valley, Southeastern Plains, and Interior Plateau) found within the target species range in Tennessee. The “Total” column represents the total number of sites sampled at each ecoregion. The “Total” row represents the total number of detections and non-detections across all ecoregions.

Ecoregions	Detection	Non-detection	Total	Naïve Occupancy (%)
Blue Ridge	34	36	70	48.6
Interior Plateau	17	139	156	10.9
Ridge and Valley	10	29	39	25.6
Southwestern Appalachian	4	12	16	25
Central Appalachian	0	1	1	0
Southeastern Plains	0	2	2	0
Total	65	219	284	-

Table 1.6 A list of a-priori developed occupancy models that may explain C. alleganiensis occupancy across sampled sites in Tennessee.

Model	Model Abbreviation
1 Detection(Null), Occupancy (Null)	$p(\cdot), \Psi(\cdot)$
2 Detection (Julian Day), Occupancy (Null)	$p(\text{survey}), \Psi(\cdot)$
3 Detection (Julian Day), Occupancy (Elevation)	$p(\text{survey}), \Psi(\text{ELEV_STD})$
4 Detection (Julian Day), Occupancy (Geology)	$p(\text{survey}), \Psi(\text{geology})$
5 Detection (Julian Day), Occupancy (Strahler order)	$p(\text{survey}), \Psi(\text{strahler})$
6 Detection (Julian Day), Occupancy (Ecoregion)	$p(\text{survey}), \Psi(\text{ecoreg_3})$
7 Detection (Julian Day), Occupancy (% Agriculture within 100m buffer around presence point)	$p(\text{survey}), \Psi(\text{X83_STD})$
8 Detection (Julian Day), Occupancy (% Forest within 100m buffer around presence point)	$p(\text{survey}), \Psi(\text{X44_STD})$
9 Detection (Julian Day), Occupancy (% Open Water within 100m buffer around presence point)	$p(\text{survey}), \Psi(\text{X11_STD})$
10 Detection (Julian Day ² + Julian Day), Occupancy (Null)	$p(\text{survey}^2 + \text{survey}), \Psi(\cdot)$
11 Detection (Julian Day ² + Julian Day), Occupancy (Elevation)	$p(\text{survey}^2 + \text{survey}), \Psi(\text{ELEV_STD})$
12 Detection (Julian Day ² + Julian Day), Occupancy (Strahler order)	$p(\text{survey}^2 + \text{survey}), \Psi(\text{strahler})$
13 Detection (Julian Day ² + Julian Day), Occupancy (Ecoregion)	$p(\text{survey}^2 + \text{survey}), \Psi(\text{ecoreg_3})$
14 Detection (Julian Day ² + Julian Day), Occupancy (% Agriculture within 100m buffer around presence point)	$p(\text{survey}^2 + \text{survey}), \Psi(\text{X83_STD})$
15 Detection (Julian Day ² + Julian Day), Occupancy (% Forest within 100m buffer around presence point)	$p(\text{survey}^2 + \text{survey}), \Psi(\text{X44_STD})$

16	Detection (Julian Day ² + Julian Day), Occupancy (% Open Water within 100m buffer around presence point)	$p(\text{survey}^2 + \text{survey}), \Psi(\text{X11_STD})$
17	Detection (Null), Occupancy (Elevation)	$p(\cdot), \Psi(\text{ELEV_STD})$
18	Detection (Null), Occupancy (Strahler order)	$p(\cdot), \Psi(\text{strahler})$
19	Detection (Null), Occupancy (Ecoregion)	$p(\cdot), \Psi(\text{ecoreg_3})$
20	Detection (Null), Occupancy (% Agriculture within 100m buffer around presence point)	$p(\cdot), \Psi(\text{X83_STD})$
21	Detection (Null), Occupancy (% Forest within 100m buffer around presence point)	$p(\cdot), \Psi(\text{X44_STD})$
22	Detection (Null), Occupancy (% Open Water within 100m buffer around presence point)	$p(\cdot), \Psi(\text{X11_STD})$
23	Detection (Null), Occupancy (% Open Water, % Wetland, % Agriculture, % Shrub, % Forest, % Barren Land within 100m buffer around presence point)	$p(\cdot), \Psi(\text{X11_STD} + \text{X91_STD}$ $+ \text{X83_STD} + \text{X60_STD} + \text{X44_STD} + \text{X31_STD})$
24	Detection (Ecoregion), Occupancy (Null)	$p(\text{ecoreg_3}), \Psi(\cdot)$
25	Detection (Ecoregion), Occupancy (Elevation)	$p(\text{ecoreg_3}), \Psi(\text{ELEV_STD})$
26	Detection (Ecoregion), Occupancy (Strahler order)	$p(\text{ecoreg_3}), \Psi(\text{strahler})$
27	Detection (Ecoregion), Occupancy (% Agriculture within 100m buffer around presence point)	$p(\text{ecoreg_3}), \Psi(\text{X83_STD})$
28	Detection (Ecoregion), Occupancy (% Forest within 100m buffer around presence point)	$p(\text{ecoreg_3}), \Psi(\text{X44_STD})$
29	Detection (Ecoregion), Occupancy (% Open Water within 100m buffer around presence point)	$p(\text{ecoreg_3}), \Psi(\text{X11_STD})$

Figures

Figure 1.1 Map representing the ecoregions found within the *C. alleganiensis* range in Tennessee, as well as 293 eDNA sampling sites surveyed between 2012 – 2016.

Map of ecoregions and eDNA sampling sites within the *C. alleganiensis* range in Tennessee

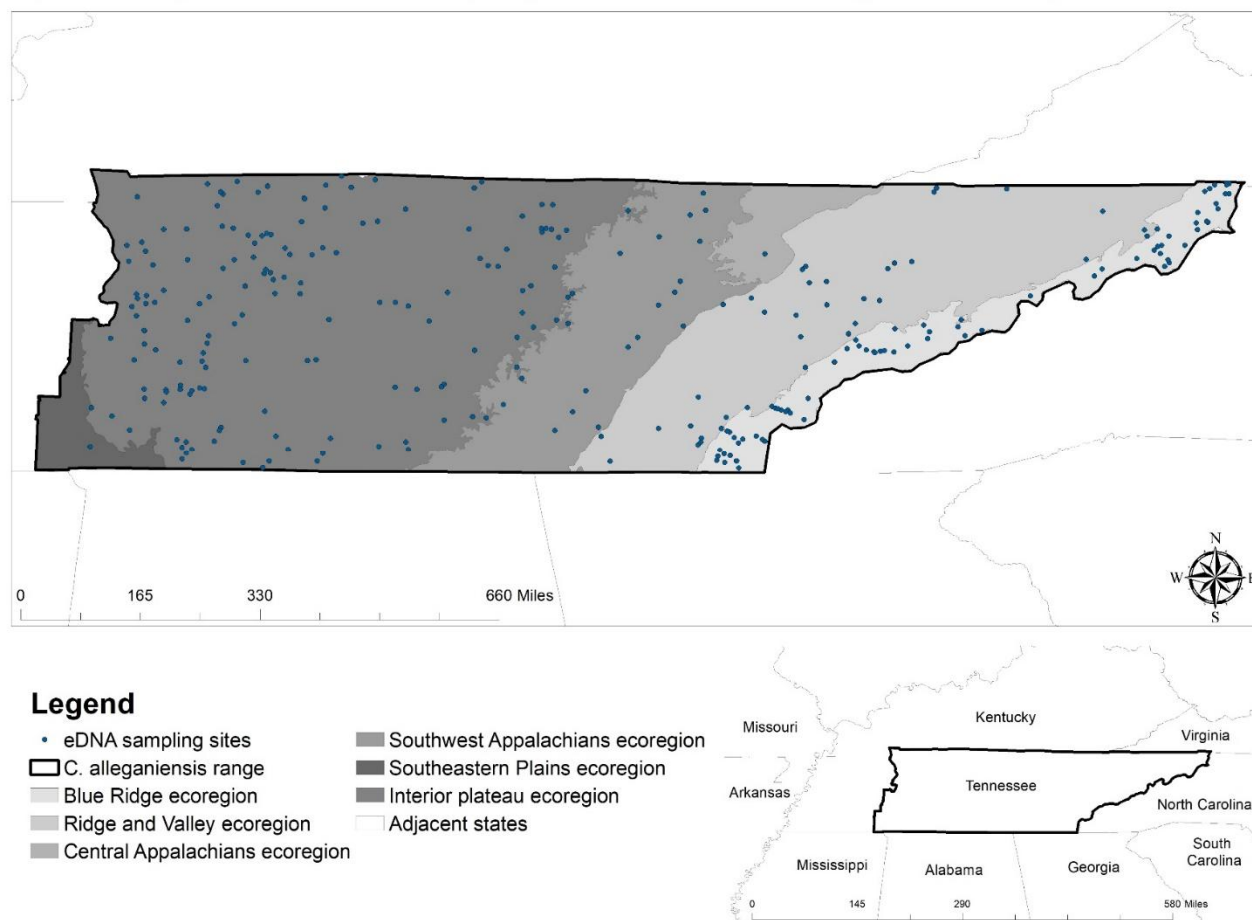


Figure 1.2 Flow chart explaining my study design. My study is composed of three main steps (Species Distribution Model, Field Sampling, and Occupancy Modeling) that were completely in the order. Each main step was composed of three steps (A, B, and C) that were completely in order. “Validation steps” explain that Step 2 was used to validate Step 1, and Step 3 was used to validate Step 2. The “Final Goal” represents three overall objectives that I aimed to accomplish through my study.

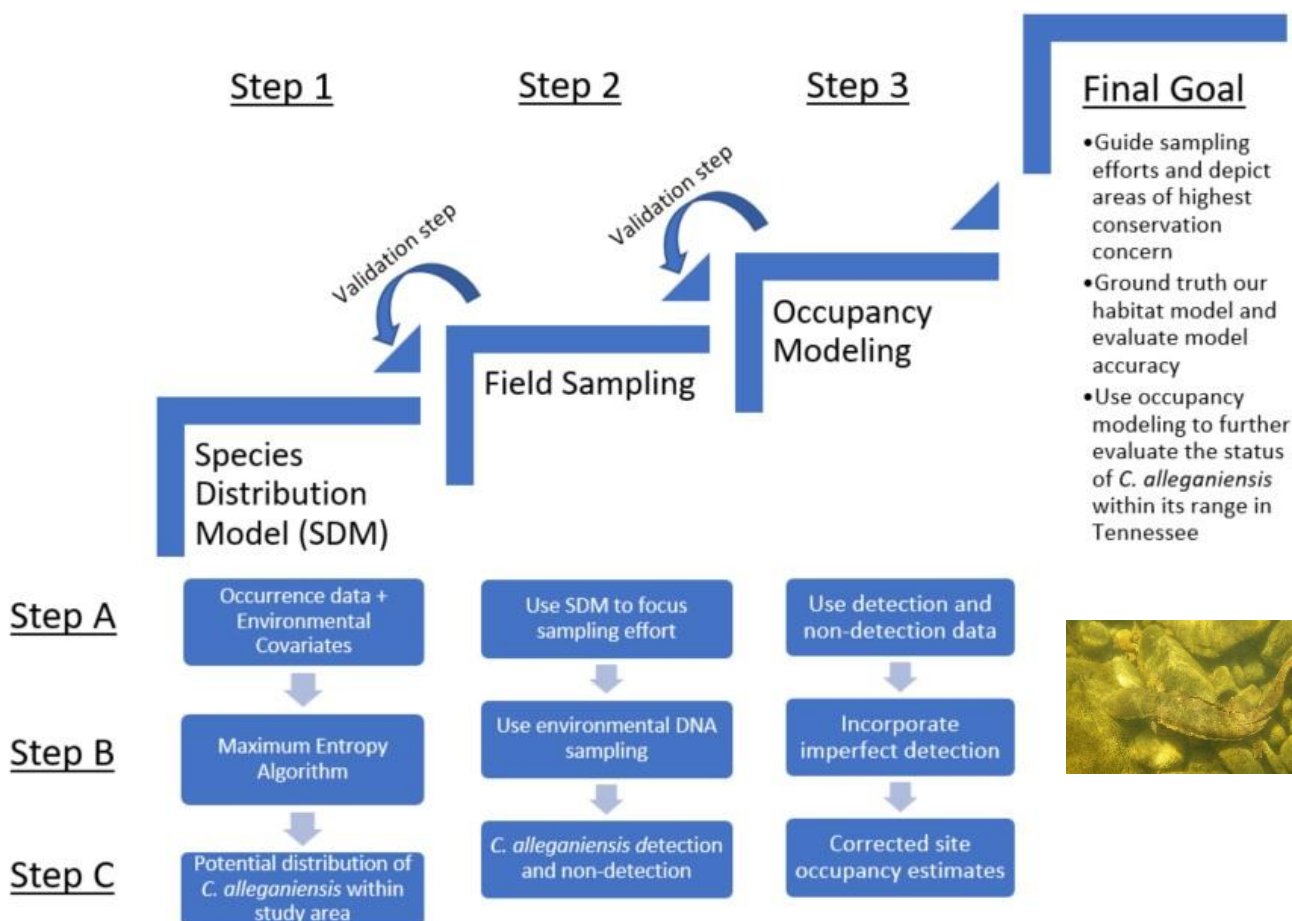


Figure 1.3 Receiving operating curve (ROC) and Area Under the Curve (AUC) estimates for the MaxEnt model. The ROC represents the relationship and tradeoff between true positives (i.e., sensitivity or $1 - \text{omission rate}$), and false positives (i.e., commission error or $1 - \text{specificity}$). Sensitivity is a measure of true positives, while specificity is a measure of true negatives. Omission errors represent false negatives, and commission errors represent false positives. A higher AUC value (i.e., closer to 1) and a curve located close to the top left corner, indicates a good model performance.

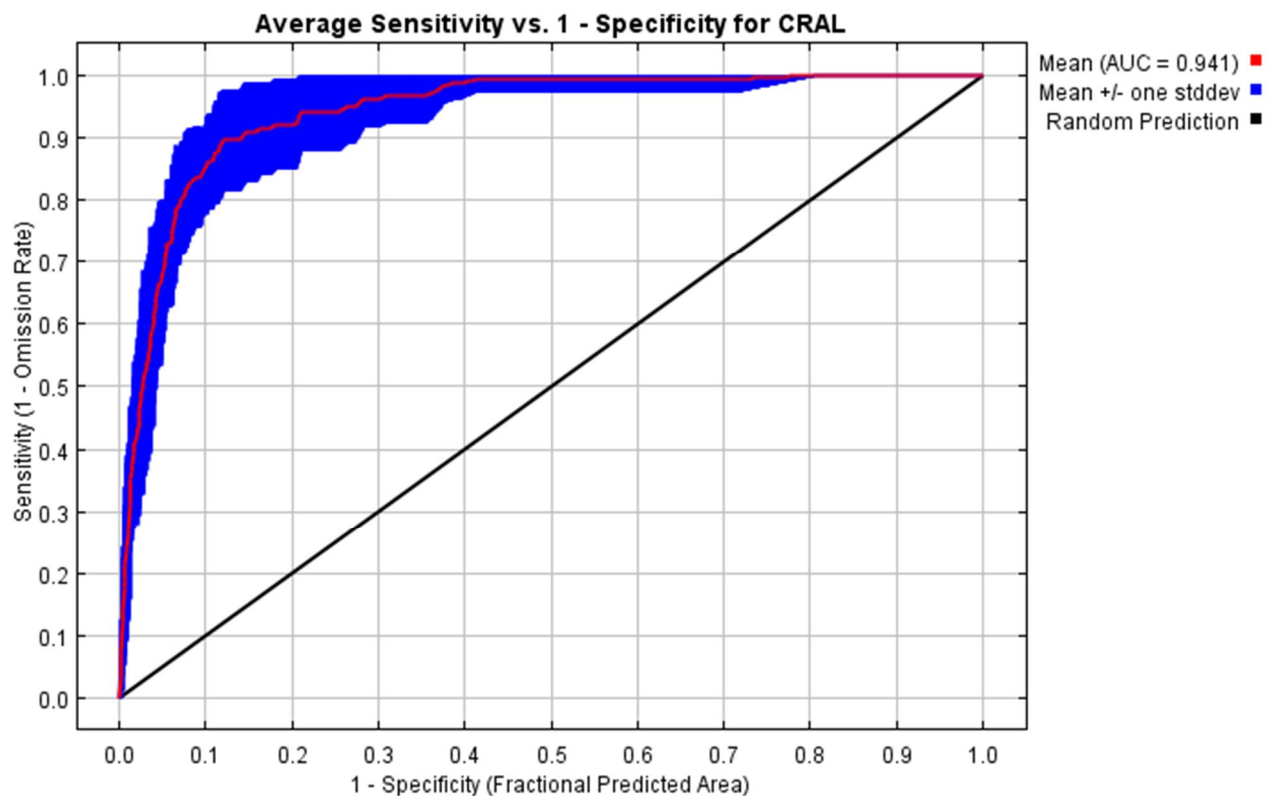


Figure 1.4 Jackknife test of variable importance of nine covariates used for the MaxEnt model. Covariates with the highest regularized training gain when used in isolation (i.e., dark blue) are considered to contribute the most information to the model compared to other covariates. Covariates with the lowest gain when omitted (i.e., light blue) are considered to have the most unique information compared to other covariates.

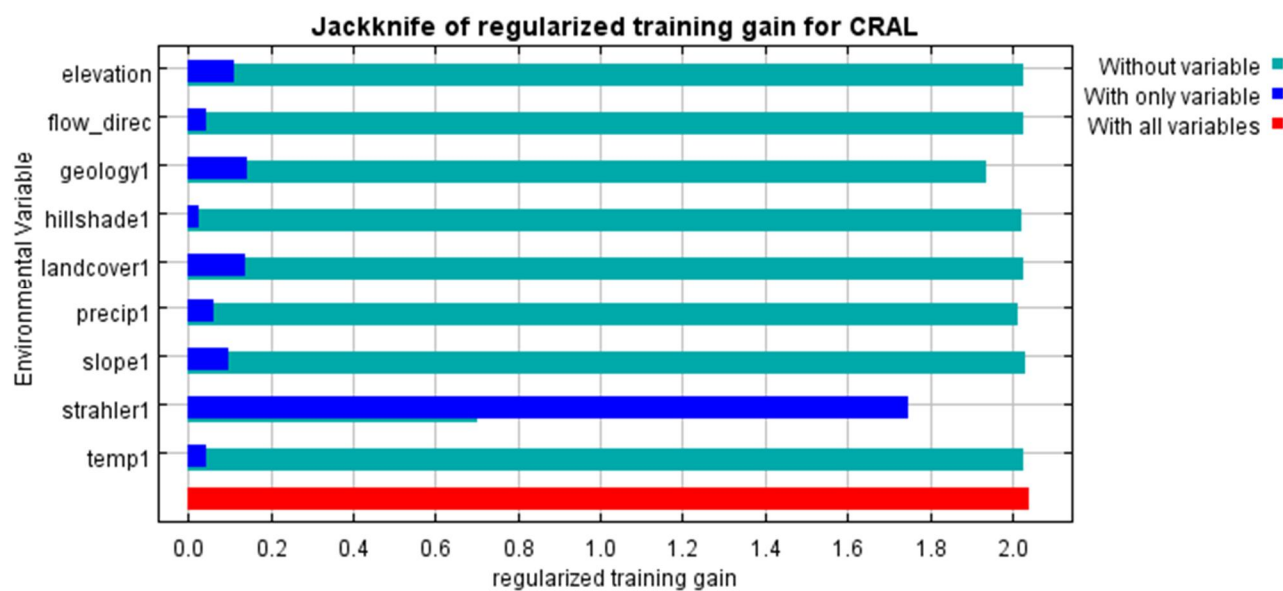
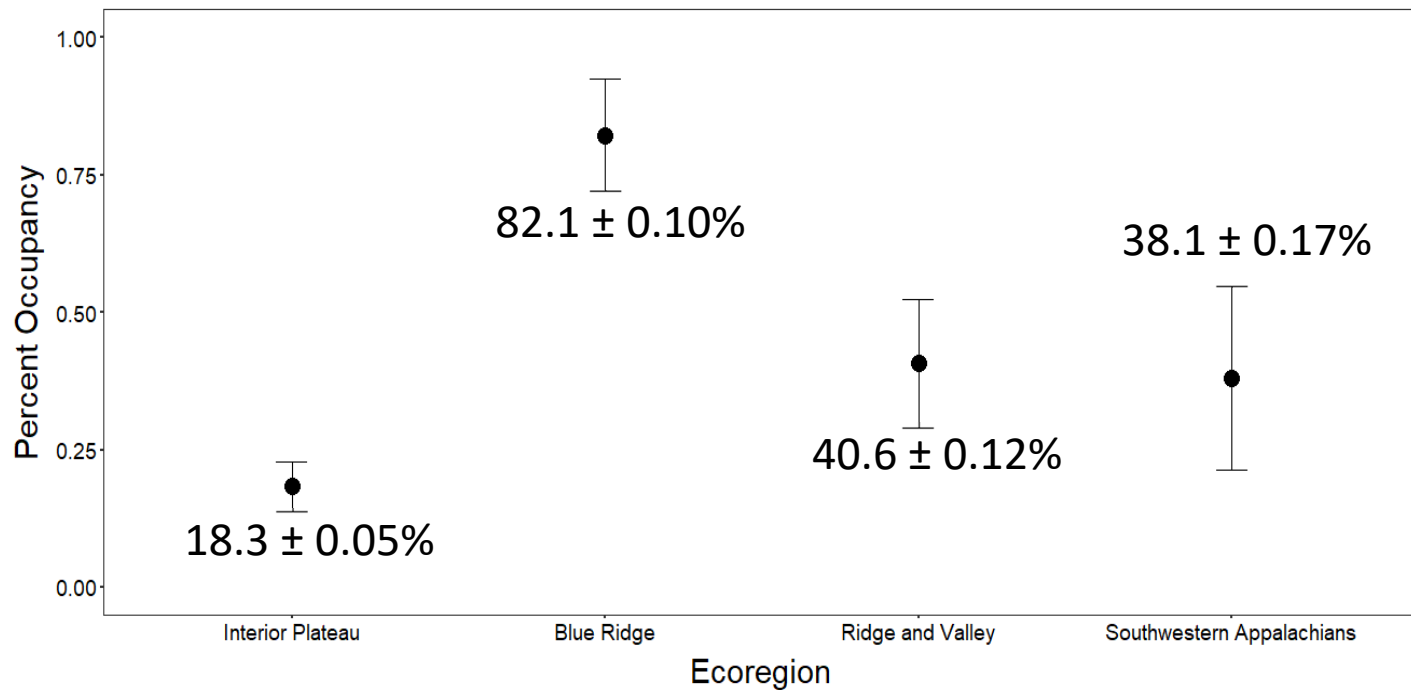
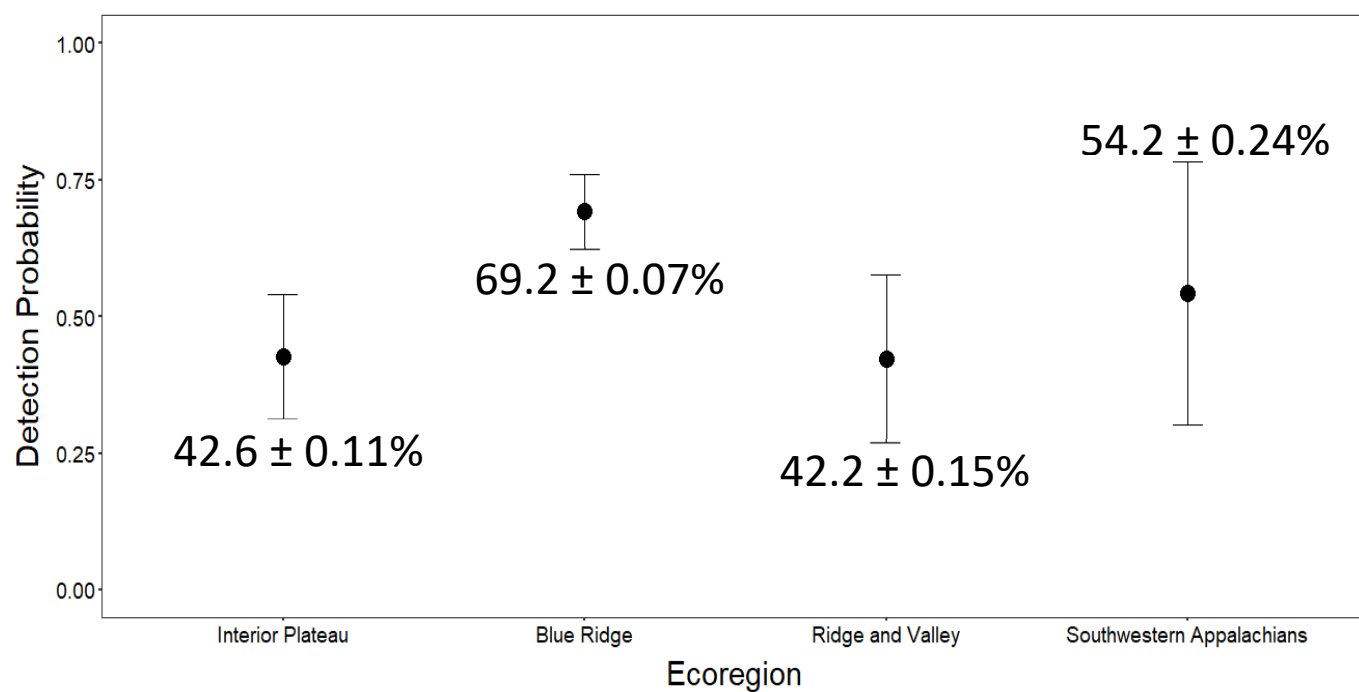
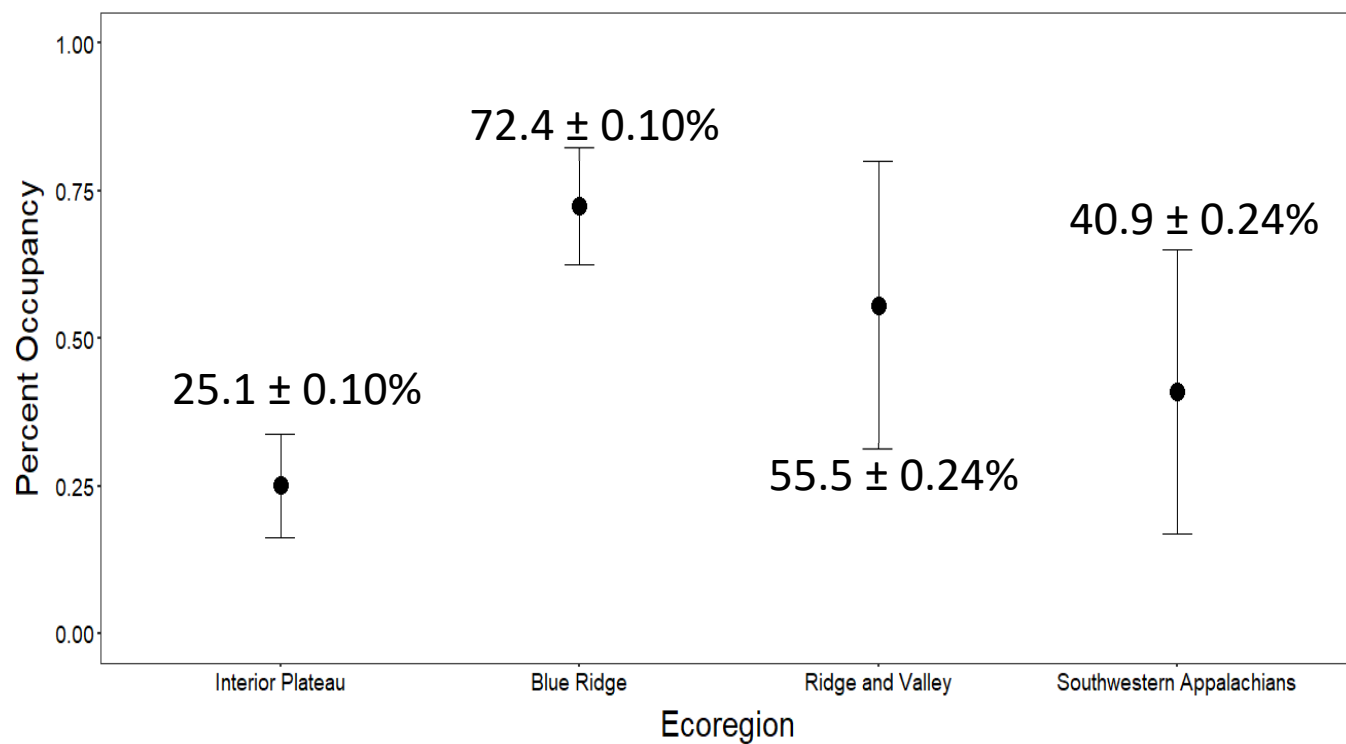


Figure 1.5 Corrected occupancy estimates results from the top supported model (19) which includes ecoregion effect on occupancy and constant detection (p) of 58.7% across the *C. alleganiensis* range in Tennessee.



*Figure 1.6 Detection probability and corrected occupancy estimates from the post hoc analysis which includes simultaneous effect of ecoregion on occupancy and detection across the *C. alleganiensis* range in Tennessee.*





CHAPTER III
MICROHABITAT USE BY THE HELLBENDER SALAMANDER (*CRYPTOBRANCHUS*
***ALLEGANIENSIS*) IN EAST TENNESSEE**

Introduction

Amphibian population declines across the United States and worldwide have been linked to introduced species, over-exploitation, habitat destruction and fragmentation, environmental contaminants, climate change, and emerging infectious diseases (Blaustein et al. 1994; Lannoo 2005; Gallant et al. 2007; Collins and Crump 2009). Agriculture and landscape alteration (e.g., urbanization, construction of dams, and impoundments) represent primary forms of habitat degradation and are leading threats to species that are either listed or proposed for listing under the Endangered Species Act (Wilcove 1998; Malmqvist and Rundle 2003). In addition, urban development is often responsible for high levels of local extinction rates, loss of biodiversity, habitat homogenization, and replacement of native and rare species with non-native species (McKinney 2002). As these threats increase in occurrence, anthropogenic disturbances will continue to represent one of the greatest challenges for future biodiversity conservation.

Land-altering practices have severely impacted habitat integrity of freshwater ecosystems (Malmqvist and Rundle 2003; Muenz et al. 2006; Henley et al. 2010). In the early 1990s, approximately 35% of freshwater amphibians and fishes, and 73% of freshwater mussels in North America were considered vulnerable, imperiled, or endangered due to habitat degradation (Henley et al. 2010). Furthermore, Ricciardi and Rasmussen (1999) suggested that in North America, extinction rates for mussels,

crayfishes, fishes, and amphibians could be five times higher than species losses in any terrestrial habitat. This indicates that freshwater biodiversity faces threats on multiple levels (Lannoo 2005; Dudgeon et al. 2006). Land alteration through intensive agriculture can cause physical changes in river and stream channels, disrupt flow, and disturb aquatic habitat through changes in chemical concentrations and sediment loads (Schultz et al. 1995; Malmqvist and Rundle 2003). Many agricultural practices can degrade riparian zones, which are critical for the control of sediment input and maintenance of surface water quality and biotic integrity (Roth et al. 1996; Stevens and Cummins 1999). Sedimentation (i.e., the act of sediment filling interstitial spaces between rocks on the substrate of streams and rivers), facilitated through agricultural practices and deforestation, represents one of the major contributors to habitat degradation in freshwater systems and has been linked to declines in amphibian populations (Welsh and Ollivier 1998; Muenz 2006; Barrett and Guyer 2008). Furthermore, elevated levels of sedimentation impact community structure, disrupt local food webs, and negatively affect population demographics of local fauna and flora (Henley et al. 2010).

Hellbenders (*Cryptobranchus alleganiensis*) are fully-aquatic salamanders that inhabit streams and rivers in the central and eastern United States. The family Cryptobranchidae is represented globally by two genera and three species and in North America is represented by the Eastern Hellbender (*Cryptobranchus alleganiensis alleganiensis*) and the Ozark Hellbender (*Cryptobranchus alleganiensis bishopi*) subspecies. *C. alleganiensis* are habitat specialists and prefer cool, fast-flowing, well-oxygenated streams and rivers with a heterogeneous rock substrate (i.e., small, medium, and large size rocks; Nickerson and Mays 1973; Humphries and Pauley 2005; Burgmeier

et al. 2011a). Species presence has also been correlated with reduced amounts of organic and fine sediment (e.g., sand and silt), lower conductivity, and higher pH (Nickerson et al. 2003; Keitzer et al. 2013; Pugh et al. 2015). As a benthic species, *C. alleganiensis* use interstitial spaces between rocks on stream substrate for shelter and reproductive sites (Nickerson and Mays 1973). Although once abundant, both *C. alleganiensis* subspecies have experienced major declines across their historical range (Williams et al. 1981; Wheeler et al. 2003; Foster et al. 2009). Impoundment, siltation, aquatic contaminants, and infectious diseases are suggested to be contributors to these declines (Nickerson and Mays 1973; Trauth et al. 1992; Nickerson et al. 2002; Burgmeier et al. 2011b). Because sedimentation decreases availability of interstitial spaces between objects on the stream substrate, it is likely that sedimentation represents a major threat to remaining *C. alleganiensis* populations across their range. Finally, *C. alleganiensis* have delayed sexual maturity (i.e., 5 – 8 years for males), and have limited capability of withstanding chronic high larval mortality. Therefore, to better protect this rare, long-lived species, it is important to understand how early life stages use in-stream habitat resources.

Despite over 30 years of research conducted across the species range, most studies on habitat use have focused on general habitat requirements, status, and demographics of adult *C. alleganiensis*. Adult individuals are often more abundant than larvae and easier to detect during surveys. In addition, larvae and subadults have been known to use different microhabitat than adults, which increases the difficulty of detecting these life stages using standard snorkeling surveys (e. g., lifting large rocks with a log peavey). Therefore, researchers still do not fully understand *C. alleganiensis* larval ecology, habitat use and selection, and how larvae and subadults use available habitat

compared to adults. The purpose of my study was to 1) identify which microhabitat attributes correlated with presence of adult, subadult, and larval *C. alleganiensis*, 2) identify which features were positively correlated with increase probability of habitat use, and 3) evaluate shifts in microhabitat use between adult, subadult, and larval *C. alleganiensis*. Based on my study objectives, I predicted ontogenetic differences in microhabitat use, with adult *C. alleganiensis* using sites with larger, unembedded rocks, and larvae using sites with low levels of fine sediment and comparatively higher levels of gravel, pebble, and cobble.

Material and Methods

Field Methods

Study area

All surveys occurred at streams located in eastern Tennessee (Fig.1). The exact stream locations have been omitted due to conservation concerns and to avoid illegal collection of animals and destruction of stream habitat. All three streams are located within public lands protected and managed by the National Parks Service and United States Forest Service in the Blue Ridge Mountains range of Tennessee. These streams were selected because previous survey work has indicated that all *C. alleganiensis* life stages are present at these streams (M. Freake, unpublished data).

Snorkeling surveys

At each stream, prior to performing habitat surveys, I conducted snorkeling surveys where I lifted all rocks above 10 cm within a delineated stream section (i.e., generally 75–100 m in length) and searched for *C. alleganiensis* under cover objects. All

cover objects were returned to the original locations to minimize habitat disturbance. I also recorded animals that were seen but not captured. Exact capture locations occupied by *C. alleganiensis* within the stream section were classified as “used sites.” I randomly selected “random sites” based on random distances and directions within the stream section and assumed that random sites were not occupied. For each individual captured, I recorded snout–vent length (SVL; mm), total length (TL; mm), weight (g), and PIT-tagged individuals that had not been previously marked. After measurements were taken, individuals were released at the site of capture. I used TL as a representation of life-stage, and individuals below or equal to 12.5 cm were considered larval *C. alleganiensis*, individuals between 12.6 cm and 29 cm were considered subadults, and individuals above 29 cm were considered adults (Peterson et al. 1983; Jachowski 2016).

Microhabitat characterization

At both used and random sites, I quantified substrate size and composition based on methodology similar to Welsh and Ollivier (1998; Table 1). I used a 0.75 m x 0.75 m quadrat divided into 25 squares of equal size (i.e., each square represents 4% of the total 100%). At used sites, the plot center was the cover rock used by the individual captured. I used the quadrat sampler to estimate the percent cover of the following substrate categories: fine sediment (<0.06 mm), sand (0.06–2.0 mm), gravel (2.1–32.0 mm), pebble (32.1–64.0 mm), cobble (64.1–256.0 mm), large rock (>256.1 mm), bedrock, woody debris, vegetation, and algae (Table 1). To determine the total percentage of each substrate category within the quadrat, I estimated the percent of each substrate category within each square and summed the percent of each category over the 25 quadrat squares.

In addition to within-quadrat substrate measurements, I measured stream width (m), distance of cover rock to bank (m), average water velocity (m^3/s), and average water depth (mm; Table 1). I used a standard measuring tape and a water velocity current meter to measure water depth and water velocity at each side of the quadrat, respectively. Because water depth ranged widely between sampling locations, I measured water velocity at the middle of the water column at each location. I determined mean water depth and velocity by averaging among the four estimates taken at each quadrat. I recorded the number of rocks above 50 cm in length, and the embeddedness of rocks above 25 cm in length present in the quadrat. I determined the volume of the cover object (i.e., rock used as shelter) at used sites. At random sites without cover objects, I determined the volume of the largest object closest to the center of the grid. I also quantified the percent of low embedded rocks (i.e., rocks that had less than 50% of its surface embedded in the substrate) and high embedded rocks (i.e., rocks that had more than 50% of its surface embedded in the substrate) by counting the total number of large rocks (rocks > 256.1 mm) within the quadrat and dividing each embeddedness category by the total number of large rocks. Lastly, because Stream 1 was the only stream where I captured all *C. alleganiensis* life stages, (i.e., larval, subadult, and adult), I calculated average percent of substrate covariates and stream characteristics at random and used sites for each life stage at Stream 1, and for non-adults (i.e., larval and subadult) and adults among all streams (Table 3, 4).

Data Analysis

Among Stream Analysis

Generalized Linear Mixed Model (GLMM)

Because I did not find larval *C. alleganiensis* in Streams 2 and 3, I was unable to assess microhabitat use for this life stage for all streams and merged larval and subadult age groups (hereafter non-adults) to allow for comparisons among all three streams. I developed six a-priori habitat models based on known *C. alleganiensis* habitat requirements and natural history (#1 sedimentation, #2 cover structure, #3 location within stream, #4 substrate, #5 stream size, #6 life stage, #7 global; Table 5). A multi-hypothesis approach is better suited for analysis of “biologically-relevant” research hypotheses, rather than haphazard testing of all possible variables (Burnham and Anderson 2002). I used a GLMM with a binomial data distribution and logit link function via the lme4 package (Bates et al. 2015) in RStudio (RStudio Team 2015, Boston, MA) to evaluate how microhabitat features within each model correlated with *C. alleganiensis* presence of non-adult (i.e., larval and subadult) and adult *C. alleganiensis* at all three streams. As vegetation and algae were absent in all three streams, these variables were not included in the analysis. The sample size for percent fine sediment, woody debris, and bedrock was not adequate for analysis; therefore, these covariates were also removed from subsequent analyses. I found that high and low percent rock embeddedness were highly correlated, therefore I chose to include low rock embeddedness and exclude high rock embeddedness to avoid over-fitting in subsequent analyses.

In this analysis, I considered habitat use (i.e., used sites versus random) as the response variable, microhabitat variables as fixed effects, and stream site as a random

effect. The inclusion of study stream as a random effect is important to control for non-independence of repeated microhabitat samples within individual streams. I used Akaike's Information Criterion (AIC) adjusted for small sample size (AIC_c; e.g., Burnham and Anderson 2002) to evaluate habitat use differences among used and random sites for adult and non-adult *C. alleganiensis*, and to identify the model(s) that best explain microhabitat use. I chose AIC_c as a model comparison technique because it compares multiple competing models at the same time given the existing data, while accounting for model selection uncertainty and number of covariates (Symonds and Moussalli 2011). I also evaluated relative fit of each variable from the top-ranked model that represented probability of microhabitat use by adult and non-adult *C. alleganiensis* among all three streams.

Probability of Habitat Use

For both adults and non-adults, I used the Predict function in the lme4 package in RStudio (RStudio Team 2015, Boston, MA) to plot probability plots for variables within top-ranked models (ΔAIC_c values < 2.0) that had confidence intervals that did not overlap zero.

Within Stream Analysis

Discriminant Function Analysis (DFA)

As Stream 1 was the only stream where I captured all *C. alleganiensis* life stages, (i.e., larval, subadult, and adult), I used discriminant function analysis (DFA) via SPSS (IBM Corp, Version 24, Armonk, NY) to further describe microhabitat differences among life stages within a single stream. Discriminant function analysis is often used to explain the difference or similarity among well-defined groups (i.e., life stages) based on

a set of explanatory variables (McGarigal et al. 2000). Prior to analysis, I conducted a correlation analysis, normality test, and standardized all data using z-scores. All covariates besides percent large rock and average water depth were not normally distributed. Therefore, I performed a natural log transformation to normalize covariates to satisfy normality requirements. DFA results were considered statistically significant when $p < 0.05$.

Results

Capture Data

I sampled Stream 1 in 2015, and Stream 2 and 3 in 2016. I captured a total of 60 individuals: 35 individuals (9 larvae (<12.5 cm), 7 subadults (12.5–29 cm), 19 adults (>29 cm)) from Stream 1, 7 individuals (2 subadults, 5 adults) from Stream 2, and 11 individuals (3 subadults, 8 adults) from Stream 3. I observed an additional five adults on Stream 2 and two adults on Stream 3, but they were not captured because they were located under potential nest rocks and I did not want to disturb a potential breeding location. I determined the sex of 8 individuals (4 females, 4 males) captured at Stream 1. Stream 2 and 3 were not sampled close to, or during, breeding season (early to mid-September in these streams; Freake, unpublished data) and animals did not display cloacal swelling.

Generalized Linear Mixed Model and Probability of Site Use

Overall, the cover structure model best explained *C. alleganiensis* presence among all three streams for both adults ($\omega_i = 1.0$) and non-adults ($\omega_i = 0.98$; Table 6). For

adults, the large rock model covariate was highly associated with microhabitat use ($\beta = 2.54 \pm 0.65$; 95% C.I.: 1.27, 3.81; Fig.2A), followed by the low embeddedness covariate ($\beta = 1.95 \pm 0.55$; 95% C.I.: 0.87, 3.03; Fig.2B) and the number of rocks above 50 cm covariate ($\beta = 0.75 \pm 0.36$; 95% C.I.: 0.05, 1.45; Fig.2C; Table 7). Similarly, for non-adults the large rock model covariate was highly associated with microhabitat use ($\beta = -0.13 \pm 0.41$; 95% C.I.: 0.93, 1.21; Fig.3A), followed by the low embeddedness covariate ($\beta = 1.95 \pm 0.65$; 95% C.I.: 0.68, 3.22; Fig.3B; Table 7). The probability of microhabitat use by adult *C. alleganiensis* increased considerably at 50% to 80% large rock cover (Fig.2A). Microhabitat use probability by non-adults increased substantially from 38% to 85% large rock cover (Fig.3A).

Discriminant Function Analysis

Function 1 was the only significant function (Wilk's $\lambda = 0.125$, $P < 0.001$) and explained 75.8% of the variance among groups. Used microhabitat sites for subadults were most correctly classified (100%), followed by random sites (81.3%), sites used by adults (78.9%), and sites used by larvae (66.7%). Overall, 80.4% of original grouped cases were correctly classified. According to Function 1, volume of cover rock, number of rocks above 50 cm, and percent of low embedded rocks were the most important variables to discriminate life stage distributions, especially for adults and subadults (Table 8). These variables were negatively correlated with percent gravel, distance to bank, and percent sand, respectively (Table 8). The DFA plot indicated that the larval life stage was positively correlated with a greater percent of gravel and sand, and greater distance to bank when compared to subadults and adults (Fig.4A, B). Microhabitat use for both the subadult and adult life stages was positively associated with greater volume

of rock cover, greater number of rocks above 50 cm, and greater percent of low embedded rocks (Fig.4A, B). My results indicate that volume of rock cover, greater number of rocks above 50 cm, and greater percent of low embedded rocks are possible limiting microhabitat factors for subadults and adults. In addition, percent gravel and sand, and distance to bank are possible limiting microhabitat factors for larval *C. alleganiensis*. It is important to note that sites used by larvae and random sites shared similar covariates, whereas other groups did not share similar covariates (Fig.4B).

Discussion

Collectively, my microhabitat analysis revealed that microhabitat use requirements differed among *C. alleganiensis* life stages. According to my results, the cover structure model best explained *C. alleganiensis* presence across all three streams for both adults and non-adults. For adults, the large rock model term had greatest support followed by the low embeddedness and cover above 50 cm model term. In addition, microhabitat use probability for adult *C. alleganiensis* increased rapidly after large rock cover approached 45%, and the percent low-embeddedness of large rocks approached 60% within the 0.75 m² microhabitat plot. For adults, the probability of microhabitat use dropped below 10% when percent of low embedded rocks declined below 30%, and a minimum of three rocks above 50 cm were necessary to achieve a microhabitat use probability of at least 60% for adults. These results indicate that within stream and rivers that are suitable for *C. alleganiensis*, accessibility to interstitial spaces under large rocks may be the most important limiting factor for adult *C. alleganiensis* presence, compared to other physical (i.e., not including chemical and biotic) habitat characteristics such as water velocity, water depth, distance to bank, and stream width. Notably, the habitat use

summary showed that across all three streams, adults used sites that had on average greater percent large rock cover, greater percent low embedded rocks, and a greater number of rocks above 50 cm compared to non-adults. Pugh et al (2015) and Humphries and Pauley (2005) have reported similar findings from study streams in North Carolina and West Virginia, respectively. My results are congruent with accepted expert opinion that *C. alleganiensis* life history is highly dependent on rock cover and interstitial spaces under rocks (Nickerson and Mays 1973; Peterson and Wilkinson 1996; Bodinof et al. 2012).

Similarly, for non-adults, the large rock model term had greatest support, followed by the low embeddedness model term. Microhabitat use probability for non-adult *C. alleganiensis* increased rapidly after large rock cover approached 50% and the percent of low embedded rocks approached 55% in the 0.75 m² microhabitat plots. However, the probability of microhabitat use seemed to plateau around 70% even with 100% low embedded rocks, and microhabitat use approached 0 when percent of low embedded rocks fell below 30%. Notably, the habitat use summary showed that across all three streams, non-adults used sites that had on average greater percent large rock cover and greater percent of low embedded rocks than random sites, but lower than sites used by adults. Compared to adults, non-adults require a greater percent large rock cover to reach similar probability of use. This does not indicate that larval and subadult *C. alleganiensis* are using larger rocks compared to adults. I classified large rocks as any rock larger than, or equal to, 25.6 cm; which means that non-adults could be using sites with a higher percent of large rocks, but not necessarily using rocks as large as rocks used by adults. Freake and DePerno (2017) showed that adult *C. alleganiensis* used rocks

significantly larger than non-adults, and that rocks used by adults averaged 100.9 cm in length. Therefore, my results indicate that percentage large rock is still highly correlated with non-adult *C. alleganiensis* presence. In addition, as the amount of cover rocks greater than 50 cm was not a supported model term for non-adults, cover rock size for non-adults likely falls within 26 cm and 50 cm.

I used DFA to evaluate population-level differences in microhabitat use and to assess how microhabitat features were correlated with in-stream distribution patterns of larval, subadult, and adult *C. alleganiensis* at Stream 1. Overall, my DFA analysis showed clear separation in microhabitat use among life stages. Specifically, presence of *C. alleganiensis* larvae was positively correlated with greater percent gravel and sand, and greater distance to bank when compared to adults. I expected a negative relationship between larvae presence and higher percent sand because small particles fill in interstitial spaces between substrate structures and can decrease microhabitat available for larvae (Nickerson and Mays 1973; Nickerson et al. 2003). The habitat use summary showed that larvae used microhabitats with lower percent gravel, pebble, and cobble, and greater distance to bank compared to random sites. As the percent of gravel and sand cover decreased, and volume of rock cover, number of rocks above 50 cm, and percent of low embedded rocks increased, microhabitat characteristics transitioned from larval to subadult and adult habitat. Similar results have been reported by Keitzer et al (2013), where abundant gravel substrate had a negative impact on microhabitat use of adult *C. alleganiensis* in West Virginia. This relationship could be influenced by the fact that my habitat sampling only accounts for the substrate that is on top of the stream or river bed. Therefore, all the gravel that is present under large rock is not accounted for, but may still

be used as habitat by subadult and adult *C. alleganiensis*. In addition, stream substrate at sampled streams is composed mainly of metamorphic sandstone and siltstone rocks, which produce greater pebble and cobblestone cover, rather than gravel (Freake and DePerno 2017). Both the subadult and adult life stages were positively associated with greater volume of rock cover, higher number of rocks above 50 cm, and higher percent of low embedded rocks. In addition, the data indicates that subadults and adults were using sites with larger rocks compared to larvae, and with lower percent sand, gravel, and pebble. This is congruent with work published by Freake and DePerno (2017) where they found a linear relationship between body size and cover rock use.

The DFA plot demonstrated clear separation between used and random sites for all life stages. This further supports the trend that sites occupied by *C. alleganiensis* of all life stages are selecting microhabitat attributes compared to what is randomly available in a given stream. Overall, the habitat use summary showed that random sites had greater percent gravel and sand, and were further from the bank compared to sites used by subadult, and adult *C. alleganiensis*. The small overlap between the random site and larval group cluster, and between the adult, larval, and subadult group cluster can be explained by the fact that my analysis does not perfectly explain habitat use differences. Also, I assigned animals to each life stage according to pre-determined sizes, and there is most likely some overlap in habitat use among individuals of similar sizes at the life stage thresholds.

Lack of knowledge on habitat requirements and distribution of rare, threatened, and endangered species can halt conservation and management efforts (Thompson 2004). Data on species habitat requirements are essential for conservation at both local (i.e., site

mitigation and habitat restoration) and landscape (i.e., watershed and landscape conservation) spatial scales. The data support the view that *C. alleganiensis* have a complex relationship with their microhabitat, and all life stages may be affected by small changes in substrate structure and availability of interstitial space. For example, an increase of only 10% (from 50% to 60%) in large rock cover increased the probability of microhabitat use by adult and larval *C. alleganiensis* by approximately 30% (from 38% to 65%) and 10% (from 46% to 56), respectively. My study highlights important microhabitat features correlated with *C. alleganiensis* presence, and provides baseline information on microhabitat use of each *C. alleganiensis* life stage within sampled streams in eastern Tennessee.

As anthropogenic changes will continue to impact freshwater ecosystems and the respective biodiversity, knowledge of *C. alleganiensis* microhabitat use is essential for future conservation efforts (Strayer et al. 2010). Landscape scale conservation is essential for the protection of remaining *C. alleganiensis* populations; however, local-scale conservation efforts, such as translocation and repatriation of captive-reared individuals, have been used and are still considered a possible strategy to strengthen existing and re-establish locally extinct *C. alleganiensis* populations, respectively (Bodinof et al. 2012; Jachowski 2016). When developing local-scale conservation strategies (e.g., translocation target sites, stream restoration), I suggest considering microhabitat suitability by evaluating microhabitat, especially large rock availability, and overall rock embeddedness.

Although the study sites represent a geographically-restricted sample of *C. alleganiensis* microhabitat use, study streams were selected for the sole purpose of

evaluating microhabitat use differences among larval, subadult, and adult *C. alleganiensis*. As stream systems that support populations that contain all *C. alleganiensis* life stages are very limited in Tennessee (especially for *C. alleganiensis* larvae) and are primarily restricted to the Blue-Ridge ecoregion of east Tennessee, I was unable to establish study sites in streams in additional ecoregions. However, these microhabitat data can be used as a starting point for restoration of degraded stream habitats when conservation, translocation, and repatriation efforts for *C. alleganiensis* are planned or currently underway. Because my results are limited to microhabitat use in high-quality stream systems and describe microhabitat differences between used and random microhabitat sites, future research can increase inference by completing habitat sampling transects at discrete distances along stream sections in stream systems where *C. alleganiensis* are declining or are no longer present. Microhabitat sampling should be completed across a gradient of stream disturbance and the resulting microhabitat data paired with capture history and demographic data to better define microhabitat thresholds where in-stream microhabitat is no longer sufficient to provide required microhabitat features for all *C. alleganiensis* life stages.

Tables

Table 2.1 Substrate categories quantified using a 0.75cm x 0.75cm quadrat during microhabitat surveys at three different C. alleganiensis streams in east Tennessee, U.S.A.

Habitat variable	Code	Variable description
Percent fine sediment	fine_sediment	Presence (%) of fine sediment (<0.06mm) within plot
Percent sand	sandy	Presence (%) of sand (0.06–2.0mm) within plot
Percent gravel	gravel	Presence (%) of gravel (2.1–32.0mm) within plot
Percent pebble	pebble	Presence (%) of pebble (32.1–64.0mm) within plot
Percent cobble	cobble	Presence (%) of cobble (64.1–256.0mm) within plot
Percent large rock	large_rock	Presence (%) of large rocks (>256.1mm) within plot
Percent bedrock	bedrock	Presence (%) of bedrock within plot
Percent woody debris	woody_debris	Presence (%) of leaves of small woody debris within plot
Percent vegetation	vegetation	Presence (%) of underwater vegetation within plot
Percent algae	algae	Presence (%) of algae within plot
Stream width	stream_width	Stream width (m) measured at the center of the sampled site
Distance to bank	distance_to_bank	Distance (m) of cover rock to the nearest bank
Average water velocity	vel_average	Average water velocity (m ³ /s)
Average water depth	wd_average	Average water depth (cm)

Volume of cover rock	cover_volume	Volume (cm ³) of cover rock on a used site or potential cover rock on a random site
Low Embeddedness	low_embed	Percent of rocks above 25cm within embeddedness categories 1 and 2 within plot
High Embeddedness	high_embed	Percent of rocks above 25cm within embeddedness categories 3 and 4 within plot

Table 2.2 Covariates and their average percent representation within a sampling quadrat at used and random plots among three different C. alleganiensis streams in east Tennessee, U.S.A. Please see Table 2.1 for a description of microhabitat covariate codes.

Habitat Variable	Used	Random
sandy (%)	2.62	3.36
gravel (%)	3.20	6.51
pebble (%)	11.47	21.49
cobble (%)	17.25	21.18
large_rock (%)	62.20	32.33
stream_width (m)	17.95	15.12
distance_to_bank (m)	5.40	5.38
vel_average (m ³ /s)	0.32	0.30
wd_average (m)	0.60	0.49
cover_volume (cm ³)	66.93	46.10
low_embed (%)	72.00	43.00
high_embed (%)	28.00	39.00
cover above 50 (#)	1.28	0.67

Table 2.3 Average percent presence of eight substrate covariates and average values of seven stream characteristics at random sites and sites used by non-adult (i.e., larval and subadult), and adult C. alleganiensis among three streams in east Tennessee. Please see Table 2.1 for a description of microhabitat covariate codes.

Covariates	Random	Non-adult	Adult
		Used	Used
sandy (%)	3.3	4.6	1.5
gravel (%)	6.5	3.8	2.9
pebble (%)	21.5	12.0	11.1
cobble (%)	21.2	18.7	16.5
large_rock (%)	32.3	59.2	63.8
stream_width (m)	15.1	20.1	16.8
distance_to_bank (m)	5.3	6.4	4.8
vel_average (m ³ /s)	1.0	1.1	1.0
wd_average (m)	48.9	66.7	56.9
cover_volume (cm ³)	46.1	37.8	82.6
low_embed (%)	42.9	67.0	74.8
cover above 50 (#)	0.7	0.8	1.5

Table 2.4 Average percent presence of eight substrate covariates and average values of seven stream characteristics at random sites and sites used by larval, subadult, and adult C. alleganiensis at Stream 2.1 located in east Tennessee. Please see Table 2.1 for a description of microhabitat covariate codes.

Covariates	Random	Larva	Sub-adult	Adult
		Used	Used	Used
sandy (%)	2.8	4.7	1.0	2.0
gravel (%)	15.9	6.9	2.4	5.9
pebble (%)	15.9	13.6	11.4	6
cobble (%)	25.6	12.7	15.1	15.4
large_rock (%)	34.6	61.1	66.1	68.6
stream_width (m)	23.1	27.2	18.9	6.7
distance_to_bank (m)	8.0	9.1	5.4	6.7
vel_average (m ³ /s)	1.4	1.3	1.2	1.3
wd_average (m)	58.4	62.8	80.7	61.1
cover_volume (cm ³)	6.4	21.5	73.7	97.6
low_embed (%)	47.6	58.9	76.7	71.7
cover above 50 (#)	0.6	0.6	1	2

Table 2.5 Justification for habitat models used to evaluate the effect of different habitat variables on the presence and absence of C. a. alleganiensis among three streams in east Tennessee. Please see Table 2.1 for a description of microhabitat covariate codes.

Model	Covariate Codes	Justification
Sedimentation	low_embed + gravel + pebble	Sediment fills in interstitial spaces between substrate structures and can decrease microhabitat availability (Nickerson and Mays 1973; Nickerson et al. 2003).
Cover structure	cover_above_50 + large_rock + low_embed + cover_volume	Rock availability and interspatial space can determine shelter quality, along with breeding and nesting success (Keitzer et al. 2013; Quinn et al 2013).
Location within stream	distance_to_bank + wd_average + vel_average	<i>C. alleganiensis</i> are not evenly distributed across the habitat. For example, Burgmeier et al. (2011) indicated that <i>C. alleganiensis</i> in Indiana use runs more often than pools and riffles.
Substrate	pebble + cobble + large_rock	Stream substrate can influence <i>C. alleganiensis</i> presence. Pugh et al. (2015), indicated that <i>C. alleganiensis</i> presence is correlated with greater particle size and bedrock, and reduced percentages of fine sediment.
Stream size	stream_width + vel_average + wd_average	Stream order and hydrological patterns can influence species diversity and distribution (Harrel et al. 1967; Platts 1979; Gordon. 2004)
Life stage	distance_to_bank + wd_average + cover_volume + gravel	Different <i>C. alleganiensis</i> size classes use different areas within the substrate. For example, larvae tend to prefer gravel substrate located closer to the stream edge (Nickerson et al. 2003).
Global	low_embed + gravel + cover_above_50 + large_rock + cover_volume + distance_to_bank + wd_average + vel_average + pebble + cobble + stream_width	Global model

Table 2.6 Predictive model describing microhabitat relationships among three streams in east Tennessee for larval, subadult, and adult *C. a. alleganiensis*. The log-likelihood (-LL), number of covariates (K), AIC values (AIC_c), change in AIC values (ΔAIC_c), and model weight (ω_i) are shown below. Please see Table 2.1 for a description of microhabitat covariate codes.

Life Stage	Model	Covariates	-LL	K	AIC_c	ΔAIC_c	ω_i
Adult	Cover Structure	cover_above_50 + large_rock + low_embed + cover_volume	24.91	6	63.00	0.00	1.00
Non-adult (larval and subadult)	Cover Structure	cover_above_50 + large_rock + low_embed + cover_volume	21.15	6	55.88	0.00	0.98
Non-adult (larval and subadult)	Global	low_embed + gravel + cover_above_50 + large_rock + cover_volume + distance_to_bank + wd_average + vel_average + pebble + cobble + stream_width	14.71	13	63.32	7.45	0.02

Table 2.7 Parameter estimates, SEs, and 95% CIs for microhabitat variables used in the highest supported model (i.e., cover structure model) for both adult and non-adult (i.e., larval and subadult) *C. alleganiensis*. The cover structure model best described microhabitat use between used and random microhabitat sites for adult and non-adult *C. alleganiensis*. Please see Table 2.1 for a description of microhabitat covariate codes.

Variable	ADULTS		NON-ADULTS	
	$\beta \pm \text{SE}$	95% CI	$\beta \pm \text{SE}$	95% CI
cover_above_50	0.75 ± 0.36	0.05 to 1.45	-0.13 ± 0.41	0.93 to 1.21
large_rock	2.54 ± 0.65	1.27 to 3.81	2.86 ± 0.78	1.33 to 4.39
low_embed	1.95 ± 0.55	0.87 to 3.03	1.95 ± 0.65	0.68 to 3.22
cover_volume	-0.35 ± 0.40	0.43 to 0.43	-0.61 ± 0.59	-1.77 to 0.55

Table 2.8 Standardized canonical discriminant function coefficients for the first two discriminant functions used to determine which variables discriminate *C. alleganiensis* life stage (larval, subadult, and adult) distribution within Stream 2.1. Values in **BOLD** have the highest weight within each function and were used to produce Figure 2.4A, 2.4B. Please see Table 2.1 for a description of microhabitat covariate codes.

Variable	Function 1	Function 2
sandy	-.313	.370
gravel	-.532	.705
pebble	-.134	-.442
cobble	-.023	.135
stream_width	-.037	1.143
distance_to_bank	-.499	.076
vel_average	.235	-.031
cover_volume	.798	-.244
low_embed	.254	.155
cover_above_50	.348	.446
large_rock	-.106	.227
wd_average	.195	.068

Figures

Figure 2.1 Location of stream sampling sites in east Tennessee, U.S.A. The dashed line represents the historical geographic range for *C. alleganiensis* within the state.

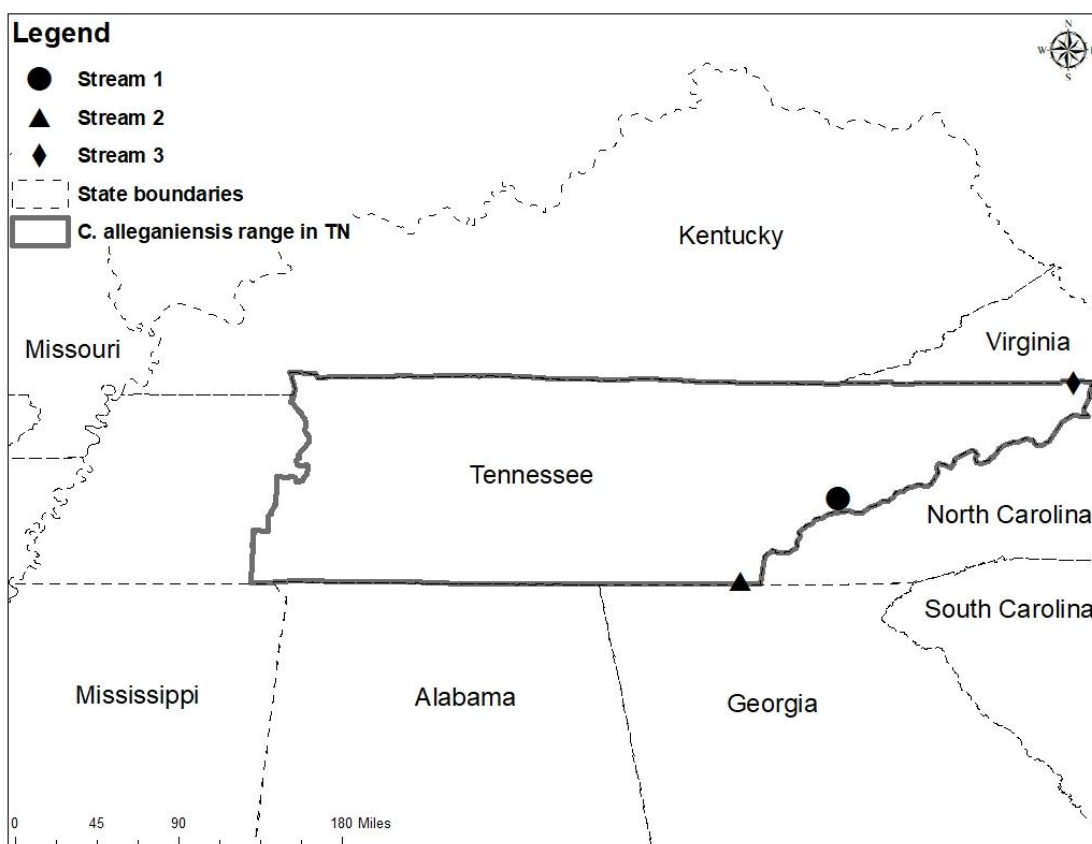


Figure 2.2 The effect of percent large rock (rocks above 25 cm; Plot A), percent of low embedded rocks (i.e., rocks that are not embedded at all, or slightly embedded; Plot B), and number of rocks above 50 cm (Plot C) on probability of microhabitat use by adult *C. alleganiensis* across all three streams. Dashed line represents 95% C.I.

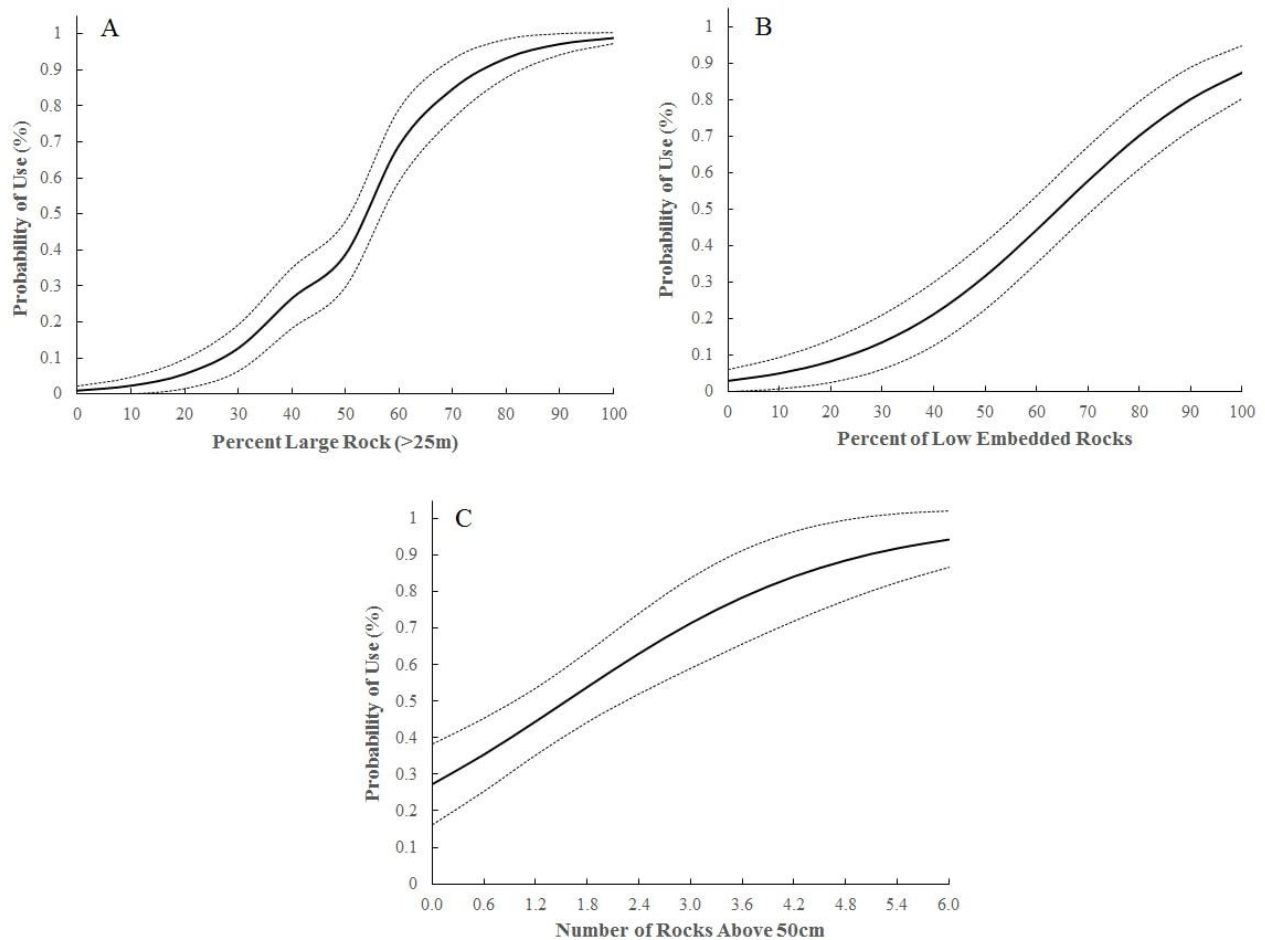


Figure 2.3 The effect of percent large rock (Plot A), and percent of low embedded rocks (i.e., rocks that are not embedded at all, or slightly embedded; Plot B) on the probability of microhabitat use by non-adult (i.e., larval and subadult) *C. alleganiensis* across all three streams. Dashed line represents 95% C.I.

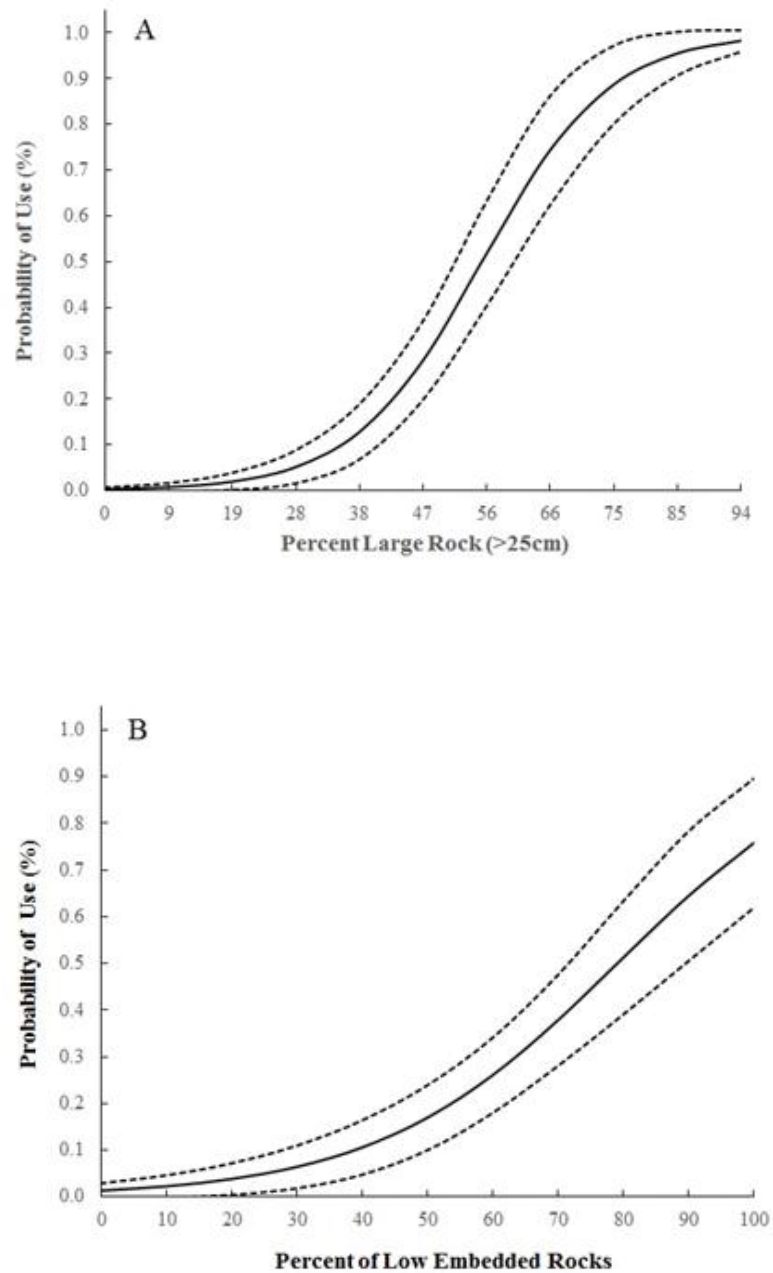
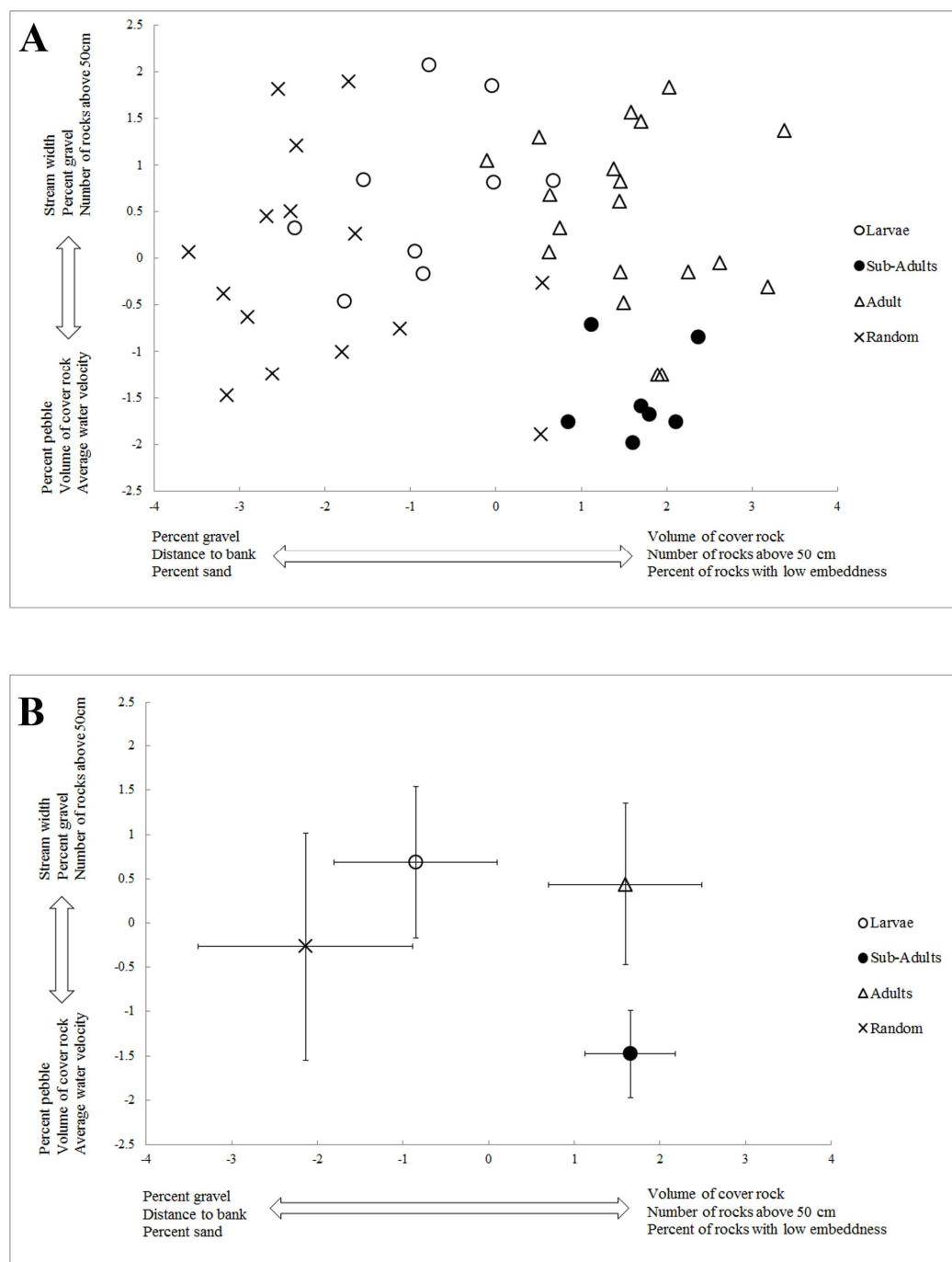


Figure 2.4 Discriminant Function Analysis diagram illustrating differences in distribution patterns among all life stages and random sites according to different habitat covariates (Plot A), and their respective centroids (Plot B). Overlapping groups share covariates, whereas groups that do not overlap do not share covariates.



CHAPTER IV

CONCLUSION AND FUTURE RESEARCH

Overall, I used a multi-spatial scale approach to address unanswered questions regarding *C. alleganiensis* conservation in Tennessee. Although *C. alleganiensis* is considered a species of special concern in Tennessee, standardized state-wide distribution assessments have been limited to known populations, and status of other *C. alleganiensis* populations remains unknown. Through development of a species distribution model to focus my sampling efforts, I was able to effectively evaluate the distribution of the target species throughout the state using environmental DNA sampling techniques.

Furthermore, through occupancy modeling, I identified factors that were potentially strong determinants of species occupancy and assessed the detection probability of my sampling method. Through this multi-level sampling approach, my goal was to provide a robust assessment of the distribution of the target species in Tennessee and identify areas that may be important for conservation of the species and should be considered when developing long term conservation and management plans. Finally, I was able to estimate a gradient of ontogenic differences in habitat use between adult, subadult, and larval *C. alleganiensis* life and evaluate microhabitat covariates associated with species presence on a local level. Future conservation of the target species may rely on prioritization of populations and areas of highest conservation need. Therefore, my overall goal was to provide baseline data on microhabitat use that can be used to guide stream restoration efforts and improve habitat quality assessment prior to reintroduction/relocation. On a landscape level, future research should focus on further assessment of land use impacts on *C. alleganiensis* populations via continued physical surveys across the state to

determine population demographics. On a microhabitat level, future research should focus on conducting additional microhabitat surveys across a habitat-quality and species abundance gradient as a means to identify microhabitat thresholds for each *C. alleganiensis* life stage and to better assess the impact of habitat degradation on population declines.

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VITA

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EDUCATION

2015 - 2018	Tennessee State University, Department of Agricultural and Environmental Sciences, M.S. in Environmental Sciences (Summa Cum Lade)
2009 - 2013	Virginia Tech University, Department of Fish and Wildlife Conservation, B.S. in Wildlife Sciences (Magna Cum Lade).

PROFESSIONAL EXPERIENCE

2014	Hellbender Field Technician, Wildlife Ecotoxicology and Physiology Ecology Program, Department of Fish and Wildlife Conservation, Virginia Tech, VA.
2013 - 2014	Amphibian Technician, Eglin Air Force Base, FL.
2013	Invasive Species Technician, U.S. Fish and Wildlife Service, Back Bay National Wildlife Refuge, VA.
2011 – 2013	Crew Leader, Southern Appalachian Silviculture and Biodiversity Study, Department of Fish and Wildlife Conservation, Virginia Tech, VA.

PROFESSIONAL EXPERIENCE

Zemmer, S. A., Wyderko, J., Da Silva Neto, J. , Cedillos, I., Clay, L., Benfield, E. F., & Belden, L. K. 2017. Seasonal and Annual Variation in Trematode Infection of Stream Snail <i>Elimia proxima</i> in the Southern Appalachian Mountains of Virginia. <i>Journal of Parasitology</i> , 103: 213-220.
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Neto, J. G. D. S., Gorman, T. A., Bishop, D. C., & Haas, C. A. 2014. Population

Demographics of the Florida Bog Frog (*Lithobates okaloosae*). Southeastern naturalist, 13: 128-137.

GRANTS & AWARDS

2017	2 nd place - Ecology and Environmental Technology Student Oral Presentation, 127 th Meeting of Tennessee Academy of Science, Martin, TN.
2017	ASIH Frederick H. Stoye Award (\$300) - General Herpetology, JMIH, Austin, Texas.
2017	Outstanding Masters Student of The Year, Department of Agricultural and Environmental Sciences, Tennessee State University, Nashville, TN.
2016	Honorable Mention in the Ecology and Environmental Science Student Oral Presentation, 126 th meeting of the Tennessee Academy of Science, Clarksville, TN.
2016	CIG Jennifer Elwood Hellbender Conservation Grant Recipient (\$1000.00).
2016	1 st place - Graduate Science Oral Presentations, 38 th Annual University-Wide Research Symposium, Tennessee State University, Nashville, TN.
2015	2 nd place - Ecology and Environmental Technology Student Oral Presentation, 125 th meeting of the Tennessee Academy of Science, Murfreesboro, TN.

2012 Fralin Summer Undergraduate Research Fellowship (\$4,000.00), Virginia Tech,
VA.

PROFESSIONAL AFFILIATION

2017 – Present American Society of Ichthyologists and Herpetologists.

2017 – Present Herpetologists' League.

2017 – Present Reviewer for the journal Herpetologica.

2016 Section Chair - Ecology and Environmental Technology, Tennessee Academy of
Science.

2011 – Present National Member of The Wildlife Society.