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THE CULTURABLE SKIN MICROBIOME OF THE OCOEE SALAMANDER, *Desmognathus ocoee*

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Abstract.—The Ocoee Salamander (*Desmognathus ocoee*) is a streamside salamander endemic to the southeastern USA. Like other salamander species, *D. ocoee* hosts a diverse assemblage of microorganisms on its skin. In this study, we characterized the culturable cutaneous microbiome of six *D. ocoee* individuals using standard microbiological techniques and Sanger DNA sequencing. We isolated 41 bacterial colonies that occurred primarily in the bacterial phyla Proteobacteria (n = 27) and Bacteroidetes (n = 9). A SIMPER analysis indicated that skin bacterial communities on individual salamanders were 42.5% similar to one another. A Kruskal-Wallis test indicated that the individual salamanders did not differ from one another at the bacterial rank of family. This work documents foundational knowledge on the microbiome hosted by the skin of *D. ocoee*.

Key words. — Amphibian, Bacteroidetes, direct colony PCR, probiotics, Proteobacteria, streamside salamander

Global biodiversity is currently experiencing its sixth major extinction event (Gibbons et al. 2000; Wake and Vredenburg 2008), and this represents a major challenge in the field of conservation biology (Thomsen and Willerslev 2015). Currently, there is scientific agreement on the importance of this issue, yet the Earth's biodiversity remains under described, making conserving biodiversity on Earth especially challenging (Hawksworth 1991; Stork 1993; Pons et al. 2006; Schmit and Mueller 2007).

The biodiversity of salamanders of the southeastern United States ranks among the highest in the world (Wake 1991) and is considered a hotspot of salamander biodiversity. Populations of amphibians are suffering from anthropogenic effects like habitat loss, pollution, and emerging fungal pathogens (Daszak et al. 2003). Relative to other pathogens, the chytrid fungus *Batrachochytrium dendrobatidis (Bd)* has been responsible for the greatest number of amphibian-population declines worldwide

during the last decade (Belden and Harris 2007). The discovery in 2013 of an especially virulent chytrid species in Asia and Europe, *B. salamandrivorans* (*Bsal*), has led to increasing concern for the welfare of salamander populations (Martel et al. 2013, 2014). If *Bsal* should arrive in the southern Appalachian Mountains, it could extirpate vast numbers of salamander species in a global hotspot for biodiversity (Martel et al. 2013; Stokstad et al. 2014).

The skin of amphibians is an interesting interface between the organism and its environment; for most species, it must be kept moist and therefore is excellent habitat for microorganisms, including fungal pathogens (Walke et al. 2014). Amphibians have been documented to have a core skin microbiome made of resident bacterial communities that are in part influenced by environmental microbes (Fitzpatrick and Allison 2014; Kueneman et al. 2014; Loudon et al. 2014; Walke et al. 2014). Several bacterial members in the core microbial community have been documented to provide resistance against the chytrid fungus *Bd* (Lauer et al. 2007; Harris et al. 2009; Becker and Harris 2010; Bletz et al. 2013; Antwis et al. 2014).

The Ocoee Salamander (*Desmognathus* ocoee) is endemic to the southeastern United States where it is relatively abundant (Petranka 1998; Fig. 1). While often found in seepages and streambeds of larger streams, *D. ocoee* will move away from the streambeds and become part of the terrestrial-salamander community under moist conditions (Hairston 1987; Petranka 1998). The purpose of our study was to produce foundational work on the host-microbe associations of *D. ocoee* by characterizing the culturable skin microbiome. We compared individual salamander microbial assemblages to one another and determined bacterial taxa contributing to community similarity.

METHODS AND MATERIALS

Isolation of skin microorganisms—. Six D. ocoee salamanders were collected by C. Camp in 2015 in Sosebee Cove (Blairsville, GA). Individuals were briefly washed with sterile water to remove transient microbes and swabbed as in Walker et al. (2015). Freshly collected swabs were streaked out in triplicate onto quadrant one of a Luria agar Petri plate (90 mm) using a standard microbiological streak-plating technique. Sterile inoculating loops were then used to complete the streak-plate technique in quadrants 2–4. The primary plates were incubated at 30 °C and all morphologically distinct colonies isolated into pure culture for six days.

Microbial genotyping—. Independent fresh bacterial colonies (\approx 24 hours) were aseptically transferred with a sterile needle into 0.2 mL PCR tubes and diluted with 100 µl sterile water. An approximately 1,300 bp fragment of the bacterial 16S rRNA gene was amplified using the following reaction components (25 µl total

volume): 12.5 µl BioMix Red (Bioline), 2.5µl (10 mM) of both forward and reverse primers AGAGTTTGATCCTGGCTCAG (8F· and 1492R: GGTTACCTTGTTACGACTT; Lane 1991), 5 µl molecular grade water, and 2.5 µl bacterial cell suspension. PCR amplification conditions were as follows: an initial incubation step at 94 °C for 5 minutes followed by 35 cycles of 94 °C for 30 seconds, 50 °C for 45 seconds, 72 °C for 90 seconds, and a final extension of 10 PCR amplicons were minutes at 72 °C. visualized on a 2% gel for correct amplicon size and purified by combining 3 µl of ExoSapIT (ThermoFisher Scientific) with 13 µl of PCR amplicon and incubating at 37 °C for 15 minutes followed by 80 °C for 15 minutes. Purified PCR product was sent to the University of Chicago Sequencing DNA Facility for Sanger sequencing.

Sequence processing and taxonomic assignment—. Geneious v. 7.1 was used to construct contigs from the forward and reverse reads. Taxonomy was assigned using UCLUST (Edgar 2010) in QIIME (Caporaso et al. 2010) by comparison to the Greengenes v 13.8 database at 97% sequence similarity. A SIMPER analysis in the software Primer7 was used to determine overall community similarly among salamanders at the taxonomic rank of bacterial family. Statistical differences between skin communities at the rank of bacterial family were using a Kruskal-Wallis determined test preformed in GraphPad Prism (Prism 6, Graphpad). Since 12 of 41 isolates were unidentified at the genus rank, we chose to analyze communities at the bacterial family level to allow for comparison between all isolated taxa.

RESULTS

A total of 41 bacterial isolates were obtained from six D. *ocoee* salamander individuals (Table 1). The minimum number of isolates from a single salamander was one, and the maximum was 10. The most abundant bacterial phyla were the Proteobacteria (n = 27) and Bacteroidetes (n = 9). The most commonly isolated classes were the Gammaproteobacteria (n = 22) and the Flavobacteria (n = 9). The SIMPER analysis indicated that skin bacterial communities from individual salamanders were 42.5% similar to one another at the rank of bacterial family. The families Enterobacteriaceae, Weeksellaceae, and Pseudomonadaceae contributed 36.8%, 22.3%, and 20.1% to the shared similarity among salamanders, respectively (79.3% total community similarity). A Kruskal-Wallis test indicated that the individual salamanders did not differ from one another at the bacterial rank of family (H (5) = 6.835, P = 0.2332).

DISCUSSION

The main objective of this research was to characterize the culturable microbes that associate with the skin of D. ocoee. We found that there were no significant differences in culturable microbes between individuals at the rank of bacterial family, and that three bacterial families, Enterobacteriaceae, Weeksellaceae, and Pseudomonadaceae, contributed to overall community similarity. Current research suggests that the skin microbial community of amphibians is most influenced by host-species identity, with site explaining additional variation in composition (McKenzie et al. 2012; Kueneman et al. 2014). The skin microbiome of salamanders removed from their natural habitat and housed in sterile conditions results in community shifts. suggesting that environmental microbes influence the microbiome (Loudon et al. 2014).

The culturable cutaneous microbiome of *D.* ocoee is similar to previously studied salamander species. The most commonly cultured bacterial species found in this study were within the phyla Proteobacteria (n = 27) and Bacteroidetes (n = 9), both of which have been described as core or dominant phyla in other aquatic and terrestrial amphibian species (McKenzie et al. 2012; Kueneman et al. 2014;



FIG. 1. Our study organism, the Ocoee Salamander (*Desmognathus ocoee*).

Becker et al. 2015). The classes most commonly cultured this study were in Gammaproteobacteria *(n* = 22) and Flavobacteria (n = 9). Species within the class Gammaproteobacteria have been commonly found in the cutaneous microbiome of amphibians, with the genus Pseudomonas often found on amphibian skin and in the environment (Kueneman et al. 2014; Loudon et al. 2014). Bacteria from the class Gammaproteobacteria are often cited as having antifungal activity against Batrachochytrium dendrobatidis (Bd), the causative agent of chytridiomycosis (Lauer Chrvseobacterium in the et al. 2007). Bacteroidetes, a common isolate in our study, has also been cited for having antifungal activity (Lauer et al. 2007).

The bacterial species *Janthinobacterium lividum* has been bioaugmented in several studies and used as a probiotic to treat chytridiomycosis (Brucker et al. 2008; Becker et al. 2009; Harris et al. 2009). This species is commonly found in the salamander skin microbiome (Lauer et al. 2007); however, we did not isolate this species during our study. The use of culture-dependent methods often fails to

Salamander	Phylum Class Order Family		Family	Genus			
1	Firmicutes	Bacilli	Bacillales	Bacillaceae	unknown		
1	Proteobacteria	Alphaproteobacteria	Rhizobiales	Brucellaceae	Pseudochrobactrum		
2	Actinobacteria	Actinobacteria	Actinomycetales	Microbacteriaceae	Leucobacter		
2	Firmicutes	Bacilli	Bacillales	Paenibacillaceae	Paenibacillus		
2	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	unknown		
2	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas		
2	Proteobacteria	Betaproteobacteria	Burkholderiales	Alcaligenaceae	Achromobacter		
2	Proteobacteria	Alphaproteobacteria	Rhizobiales	Brucellaceae	Pseudochrobactrum		
2	Proteobacteria	Betaproteobacteria	Burkholderiales	Oxalobacteraceae	unknown		
2	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas		
2	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	unknown		
2	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas		
3	Bacteroidetes	Flavobacteria	Flavobacteriales	Weeksellaceae	Chryseobacterium		
3	Bacteroidetes	Flavobacteria	Flavobacteriales	Weeksellaceae	Chryseobacterium		
3	Bacteroidetes	Flavobacteria	Flavobacteriales	Weeksellaceae	Chryseobacterium		
3	Bacteroidetes	Flavobacteria	Flavobacteriales	Weeksellaceae	Chryseobacterium		
3	Firmicutes	Bacilli	Bacillales	Bacillaceae	unknown		
3	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Enterobacter		
3	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas		
3	Proteobacteria	Alphaproteobacteria	Rhizobiales	Brucellaceae	Pseudochrobactrum		
3	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas		
3	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	unknown		
4	Bacteroidetes	Flavobacteria	Flavobacteriales	Weeksellaceae	Chryseobacterium		
4	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas		
4	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	unknown		
4	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	unknown		
4	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas		
4	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas		
4	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas		
4	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	unknown		
4	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	unknown		
4	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	unknown		
5	Bacteroidetes	Flavobacteria	Flavobacteriales	Weeksellaceae	Chryseobacterium		
5	Bacteroidetes	Flavobacteria	Flavobacteriales	Weeksellaceae	Chryseobacterium		
5	Bacteroidetes	Flavobacteria	Flavobacteriales	Weeksellaceae	Chryseobacterium		
5	Firmicutes	Bacilli	Bacillales	Bacillaceae	Bacillus		
5	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	unknown		
6	Bacteroidetes	Flavobacteria	Flavobacteriales	Weeksellaceae	Chryseobacterium		
6	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas		
6	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Serratia		
6	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Citrobacter		

TABLE 1. Bacterial isolates from the skin of Ocoee salamanders based on taxonomic assignment using UCLUST in QIIME by referencing the GreenGenes v13.8 database.

the majority of bacterial species within the microbiome, and therefore may explain the inability to identify J. lividum on the skin of D. ocoee salamanders. It has been estimated that fewer than 0.1% of bacterial species can be cultured in laboratory conditions, severely limiting the effectiveness of culture-dependent methods (Torsvik and Ovreas 2002). Nextsequencing technologies generation have increased the resolution at which we can observe complex microbial communities (Claesson et al. 2011). This study has provided foundational work to identify the culturable microbiome of the stream side *D. ocoee* salamanders, which can now be compared to next-generation sequencing data.

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DETERMINING CREVICE PREFERENCES OF LUNGLESS SALAMANDERS ON THE SOUTHERN CUMBERLAND PLATEAU OF TENNESSEE

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Abstract.—Lungless salamanders in family Plethodontidae rely on cutaneous respiration that influences habitat selection. Refuge from warm, dry microclimates on the Cumberland Plateau may be found in crevices within sandstone and limestone bluffs where plethodontids are frequently found. The objective of our study was to describe patterns of crevice occupancy by plethodontid salamanders on the southern Cumberland Plateau. We surveyed 6.5 km of bluff habitat and measured geographical and micro-environmental factors at capture and random locations. We found seven species of plethodontid salamanders using crevice habitats, but only three species were abundant enough for statistical analyses. Green salamander (*Aneides aeneus*) occupancy was poorly explained by most of our measured variables, although it was positively associated with warmer temperatures. Northern slimy salamander (*Plethodon glutinosus*) occupancy was primarily associated with cooler temperatures and the presence of water. Cave salamander (*Eurycea lucifuga*) occupancy was positively associated with forest cover. Lungless salamanders of the Cumberland Plateau may use crevice habitat facultatively as one mechanism to avoid warm and dry conditions in the summer.

Key Words.—Crevice, Cumberland Plateau, Distribution, Microclimate, Occupancy, Plethodontidae, Green Salamander

Understanding factors driving distributions and habitat use of species is critical for understanding how species may respond to habitat loss and climate change (Parmesan 2006; Pounds et al. 2006; Rosenzweig et al. 2008). Both environmental changes yield large and small-scale change that can affect different demographic processes that culminate in species declines and shifts in their distributions (Aitken et al. 2008; Chen et al. 2011). Amphibians have behavioral traits and demographic rates that are correlated with local climate (Spotila 1972; Grover 1998; Peterman and Semlitsch 2013). Therefore, changes in microclimate from climate change or from forest loss could have strong effects on the future success of populations (Marsh and Beckman 2004; Pounds et al. 2006).

Plethodontid salamanders are from a diverse family that comprise most of the salamander diversity of the United States (Lanoo 2005). All the members of this family are lungless and

respire cutaneously (Feder and Londos 1984). Skin permeability necessary for cutaneous respiration comes with an increased risk of desiccation (Spotila 1972; Feder and Londos 1984; Grover 1998). Therefore, environmental factors including temperature, water, and relative humidity can affect behaviors such as foraging and habitat selection (Spotila 1972; Feder 1983; Grover 1998; Rossell et al. 2009). Specifically, salamander activity is directly correlated with moisture content in the air, where salamanders exposed to higher relative humidity spend more time outside of refugia than those in lower humidity (Feder and Londos 1984). Standing water in forest floor refugia is positively associated with the colonization and presence of two terrestrial plethodontids (Grover 1998). Similarly, terrestrial movement of amphibians is governed by habitat heterogeneity and availability of refugia from high temperatures and low moisture (Ash 1997; Grover 1998; Marsh and Beckman 2004).

Spatial and temporal gradients of climate also affect salamander performance and influence factors affecting fitness and population viability including body size and density (Peterman and Semlitsch 2013; Caruso et al. 2014).

The southern Cumberland Plateau is characterized by thin, nutrient-poor soils with low moisture retention (Smalley 1982), perhaps contributing to low salamander densities in the region (McKenzie and Cecala in review; Kirchberg et al. 2016). Therefore, microhabitats that buffer extreme temperatures and low humidity may be particularly important for the ecology of plethodontid salamanders in this ecoregion. One habitat type that could perform this function is the steep sandstone bluffs that border the plateau. Vertical and horizontal crevices on bluffs provide refuge for a range of species during the warm seasons (Petranka 1998). For example, green salamanders (Aneides aeneus) are highly dependent on these crevice refugia and have been shown to use these crevices to minimize cutaneous water loss and regulate body temperature (Rossell et al. 2009). For this species, canopy cover positively affects the suitability of crevice habitat for green salamanders and could indicate habitat suitability for other lungless salamanders as well (Rossell et al. 2009; Tilghman et al. 2012). Descriptions of the diversity of plethodontids that use this habitat and predictors of habitat use are largely unavailable.

In this study, we sought to determine which species regularly use crevice habitat and evaluate what environmental factors are associated with their occupancy. Specifically, we evaluated the relationships between climatic factors, forest cover, and distance to subsurface habitats to occupancy of plethodontid salamanders. We predicted that salamander occupancy will be positively associated with cooler temperatures, higher canopy cover, and higher relative humidity.

METHODS AND MATERIALS

Study Region-. The Cumberland Plateau consists of relatively flat oak-hickory forests (e.g., White Oak, Quercus alba; Chestnut Oak, Q. montana; Northern Red Oak, Q. rubra; Pignut Hickory, *Carya glabra*; Shagbark Hickory, *C. ovata*, and Sand Hickory, *C.* pallida) bordered by sandstone outcrops and bluffs before falling away into coves with Sugar Maple (Acer saccharum), Red Maple (A. rubrum) and Tulip-Poplar (Liridiodendron tulipifera) dominated forests (Kuers 2007). Specifically, this study took place on the 13,500acre campus of the University of the South in Franklin County, Tennessee, that encompasses over 20 km of sandstone bluff. Fractured sandstone bluffs provide crevice habitat that the authors have observed to be used by invertebrates and vertebrates (e.g. camel crickets [Rhaphidophoridae], centipedes [Scolopendra copperhead snake [Agkistrodon spp.], chimney [Chaetura contortrix], swifts pelagica], green salamanders [A. aeneus] and long-tailed salamanders [Eurycea longicauda]).

Field Data Collection-. We recorded the occupancy and identity of all salamanders and other taxa when we were confident in our species identification. Field surveys were conducted from 11 May - 28 July 2015. We haphazardly selected 10 transects of 100 m to account for potential spatial variation. Transects were selected by proximity to cove access points, which were typically old roads (Smith and Williamson 2008). We surveyed bluff stone faces and all crevices along transects located 0.5-1.9 m above the ground using a flashlight to illuminate deep crevices. We collected a series of variables from locations where salamanders were found as well as random points where they were absent for comparison. To locate random points, we randomly selected 10 distances from the start point for each transect, and we surveyed crevices located at those points. At each location that a salamander was observed and at all

random points, we recorded GPS location, canopy cover using a spherical densiometer, temperature of the crevice using an infrared thermometer, relative humidity using psychrometer, and presence of standing water like small puddles or pools. If individuals were found outside of crevices, we collected all the same data except the presence of standing water. At random points, we searched extensively to confirm salamander absence. For each detected salamander, we identified them to species but did not extract salamanders from the crevices. Daily mean ambient air temperature was provided by the historic weather data archive for University The South of the (www.sewanee.edu/offices/oess/thedomain/resources/historic-weather-data/) from a weather station located on the Cumberland Plateau within 5 km of our study transects.

Landscape Data Collection—. We used South Cumberland Conservation Action Plan database layers to delineate bluff lines (Hollingshead et al. 2010). National Land Cover Data from 2011 was used to assess the percent forest within a 150 m radius of each point (Homer et al. 2015). Forest was determined by combining categories of deciduous, evergreen, and mixed forest. We also quantified Euclidean distance to the nearest documented cave.

Data Analysis-. We used multi-model inference to assess the habitat variables that predict occupancy by different species of occupying salamanders sandstone bluffs. developed Models were using logistic regressions including salamander detections (1) or random points (0) as our response variable and habitat predictors (e.g., temperature, relative humidity, canopy cover, forest cover, permanent water, or distance to nearest cave). For each species, we assessed relative support for single factor models using Akaike's Information Criteria corrected for small sample size (AIC_c) to distinguish among models (Akaike 1973). Larger AIC_c weights indicate higher likelihood

of a model to describe the data relative to other models (Burnham and Anderson 2002). Model rankings as well as coefficients and their associated variability were used to assess important relationships. Daily mean ambient air temperatures were compared to daily mean crevice random point temperatures in a twosample t-test assuming unequal variance. All analyses were conducted in R (R Development Core Team, 2015).

RESULTS

We detected 7 species of salamander using crevices. We found 18 green salamanders, 20 cave salamanders (E. lucifuga), and 12 northern slimy salamanders (Plethodon glutinosus) samples that we deemed sufficiently large to analyze further. All of these individuals were found in crevices except 2 green salamanders found on moss on the sandstone bluffs. Other amphibian and reptile species found to use crevices along the bluff include spotted dusky salamanders (Desmognathus conanti; n = 4), long-tailed salamanders (n = 1), red-spotted newts (Notophthalmus viridescens; n = 1), northern zigzag salamanders (*P. dorsalis;* n = 3), eastern box turtles (*Terrapene carolina*; n = 1), and ring-necked snakes (Diadophis punctatus; n = 4). We also surveyed 158 random points. Bluffs on this landscape remained primarily forested (97.2 \pm 0.5%; SE), cool (20.97 \pm 0.15 °C), and humid $(77.7 \pm 0.54\%)$. Mean temperature for all random points was $21.4 \pm$ 0.17 °C and was significantly lower than the mean ambient temperature for the time period we surveyed (23.15 ± 0.46 °C; $t_{df=40}$ = -4.8, P <0.001; Fig. 1).

We found that there was little support for one factor being more important than the others in predicting green salamander occupancy though it may be positively associated with temperature based on the estimate and associated standard error of the relationship between occupancy and temperature (Table 1; 0.24 ± 0.25). Northern slimy salamander occupancy was best predicted

by temperature, and occupancy was predicted to decline by 0.77 for every one-degree increase in temperature. Temperature was 3.4 times better at predicting slimy salamander occupancy than the next highest ranked variable, forest cover, but evaluation of effect sizes suggest that occupancy is also more likely in less shaded and high humidity crevices closer to caves (Table 1). Finally, cave salamanders were positively associated with forest cover, which was 3.6 times more likely than distance to cave to influence their distribution. Cave salamanders were also more likely to occupy crevices closer to caves. Unlike the other two species, they were not associated with climatic variables (Table 1).

TABLE 1. Model ranking results for single predictor logistic models comparing capture locations to random points. Models are ranked from highest to lowest support, and estimates with standard errors (SE) are presented.

	AIC _c	ΔAIC _c	AIC _c weight	Estimate	SE		
Green salamanders							
Temperature	121.64	0.00	0.20	0.24	0.25		
Distance to cave	121.84	0.21	0.18	0.22	0.27		
Relative humidity	121.90	0.26	0.18	0.19	0.23		
Canopy cover	122.06	0.43	0.16	0.22	0.36		
Native forest cover	122.37	0.73	0.14	0.11	0.29		
Permanent water	122.53	0.90	0.13	0.02	0.25		
Northern slimy salaman	ders						
Temperature	87.05	0.00	0.49	-0.77	0.32		
Canopy cover	89.51	2.45	0.14	-0.37	0.20		
Relative humidity	89.71	2.65	0.13	0.50	0.26		
Distance to cave	89.79	2.74	0.12	-0.66	0.41		
Native forest cover	91.15	4.10	0.06	0.81	0.84		
Permanent water	91.31	4.26	0.06	0.41	0.30		
Cave salamanders							
Native forest cover	123.35	0.00	0.67	3.31	2.96		
Distance to cave	125.89	2.54	0.19	-0.65	0.31		
Permanent water	128.22	4.87	0.06	-0.44	0.26		
Temperature	128.31	4.96	0.06	-0.41	0.24		
Canopy cover	131.11	7.76	0.01	-0.08	0.20		
Relative humidity	131.26	7.91	0.01	0.01	0.24		



FIG. 1. Mean temperatures (\pm standard error) for local air temperature were higher than random points. Temperatures are also plotted for green salamanders and northern slimy salamanders which demonstrated associations with temperature.

DISCUSSION

Many species are found to use crevice habitat on the southern Cumberland Plateau. Acting as a buffer, these crevices provide refuge from extremes in temperature and humidity. In our study, we found that random crevice sites 17 °C cooler than ambient were air temperatures, and that salamanders selected temperatures equivalent or cooler than random crevices (Fig. 1). Avoiding thermal extremes is important for lungless amphibians to avoid desiccation and can result in decreased surface activity necessary to complete their life histories (Spotila 1972; Feder and Londos 1984). Though recent evidence suggests that green salamanders use crevice habitat extensively but not exclusively (Waldron and Humphreys 2005; Rossell et al. 2009), facultative use of crevices in warm seasons (April-October) may facilitate increased activity of lungless salamanders. Additional research is needed to determine the spatial and temporal extent of habitat use of species that use bluffs facultatively. Similarly,

research determining how far individuals move to exploit this habitat and what types of behaviors salamanders use crevices for would further our understanding of the importance of crevice habitat to lungless salamanders on the southern Cumberland Plateau.

Unlike associations of other species, we observed that green salamanders were positively associated with crevice temperatures. Despite this association, they were rarely found on rock faces where ambient air temperatures would be warmer (Fig. 1). This habitat preference could also result from selection of drier habitats that would be positively associated with warmer temperatures. Recent work suggests that green salamanders prefer deeper crevices, but the relationship between crevice morphology and microclimate is unknown (Smith et al. 2017). Smith et al. (2017) also found a positive association with forest cover that was not observed in the present study. One potential explanation may be the well-forested nature of our study area. Secondly, this association could be a result of competitive interactions. Others have found vertical stratification of rock crevice use when they co-occur with slimy salamanders (Cliburn and Porter 1986, 1987). Though we did not measure crevice height, slimy salamanders were rarely found more than 0.5 m off the ground whereas green salamanders were found up to at least 1.2 m. If slimy salamanders prefer cooler temperatures, it could also be that slimy salamanders exclude green salamanders to higher, warmer crevices. Consistent with this hypothesis, our surveys and others (Rossell et al. 2009) did not find other species co-occupying crevices, and studies have reported territorial behaviors of green salamanders towards conspecifics (Cupp 1980). Similar to recent research, we also suggest that green salamanders may use far more terrestrial and arboreal habitat in addition to rock crevices (Waldron and Humphreys 2005). Notably, we found 5 green salamanders (4 adults; 1 juvenile, 23 mm SVL) during this study period considerable distances from rock crevices (0.47 - 0.61 km), either on

trees, under cover on the forest floor, or crossing the road.

Climbing behaviors have previously been documented in closely related slimy salamander species, but climbing was most common during wet conditions and typically observed when in competition with other large species (Cliburn and Porter 1987; McEntire 2016). In the absence of another large plethodontid species, it is unclear how northern slimy salamanders use crevices, but their associations with climatic variables suggest a potential mechanism for crevice use. Relative humidity and temperature have previously been linked to dehydration rates in plethodontid salamanders and cessation of terrestrial activity (Spotila 1972; Feder 1983; Feder and Londos 1984). Opportunistic use of may crevices provide northern slimy opportunities salamanders additional for foraging or reproduction by allowing them to remain surface-active for longer periods of time. This hypothesis is supported by finding northern slimy salamanders in lower crevices where crevices were more likely to have small pools of standing water. Furthermore, there was a weak negative relationship between northern slimy salamander occupancy and canopy cover (Table 1). More variable climate experienced in areas with less canopy cover (Chen et al. 1999; Carlson and Arthur 2000) could encourage crevice use as a mechanism for avoiding dry forest floor conditions similar to forest floor seeps that also attract terrestrial salamanders (Grover 1998). Alternatively, crevices could be used by adult females for brooding as we found both large individuals and juveniles using crevice habitat (Trauth et al. 2006). Regardless of the mechanism resulting in crevice use, more information is necessary to evaluate whether individuals move to exploit crevice habitats or if proximity to rock crevices increases salamander body condition and density.

As their name implies, cave salamanders are most often associated with caves or limestone areas with access to subsurface habitat (Hutchison 1958; Petranka 1998; Camp and Jensen 2007). Our models corroborate these patterns with the highest occupancy estimates closest to known caves. We also note a positive association with forest cover and proximity to streams. As a stream-breeding species, these results may indicate that cave salamanders require intact forests for annual breeding migrations to streams (Petranka 1998; Semlitsch et al. 2009). Crevice habitat close to streams at high elevations on the Cumberland Plateau may increase the elevational range of the species from limestone layers up to the sandstone layers that overlie them.

Little is known about the strength of interspecific interactions in driving patterns of crevice use. Many other salamander and nonsalamander species were observed in these crevices. Alongside plethodontid salamanders, wood mice (Apodemus sylvaticus), an eastern box turtle, and ring-necked snakes were found. As predators of salamanders, ring-necked snakes may opportunistically use crevices during foraging and could affect how salamanders distribute themselves. Furthermore, other species including dusky salamanders or longtailed salamanders may have spatially or temporally restricted impacts on crevice-using salamanders when either close to a stream or during cooler seasons respectively. Another limitation of our study is the inability to assess how detection of species may have changed our inferences about their distributions due to the lack of repeated observations. Despite these changes. visual evaluation of residuals demonstrated no consistent spatial pattern suggesting that spatial bias is unlikely though there may have been temporal changes in detection rates (Bailey et al. 2004).

Terrestrial and stream-breeding salamanders regularly use sandstone bluff habitat at the edge of the Cumberland Plateau during the warm season. Microclimate conditions provide the clearest link to salamander occupancy patterns and suggest a potential mechanism for crevice use — to avoid high temperatures and low humidity. Facultative use of these refuges could promote additional foraging activities improving individual fitness and potentially providing a region of elevated densities relative to forest floors where salamander communities appear to be at low densities relative to other eastern North American forests (McKenzie and Cecala *in review*). More research is necessary to evaluate the significance of crevice habitat to the ecology of terrestrial salamanders on the Cumberland Plateau.

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HERPETOLOGICAL SURVEYS IN TENNESSEE WILDLIFE RESOURCES AGENCY'S ADMINISTRATIVE REGION IV FROM 2016

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Abstract.—Wildlife Surveys are conducted year-round by Tennessee Wildlife Resources Agency biologists for all classes of wildlife. Specifically, Diversity Biologists are tasked with inventory and monitoring Amphibians, Reptiles, Birds, and Mammals. Several techniques are utilized to achieve this. I am presenting an update of the herpetological work and species captured within TWRA's administrative Region IV in 2016.

Key Words.-Greatest Conservation Need, Inventory

Since 2004, the Tennessee Wildlife Resources Agency (TWRA) has been surveying nongame species on numerous Wildlife Management Areas (WMA's), State Parks and other public lands. Recently, these inventories have focused on vertebrate species of Greatest Conservation Need (GCN). Within each of TWRA's four administrative Regions, one Wildlife Manager 1 has been tasked with the responsibility of conducting inventories. This monitoring is conducted to "gain a long-term understanding of trends in populations or ecosystem health, provide greater understanding of species responses and needs relative to problems changing environmental and conditions. assess and the results and effectiveness of conservation actions" (TWRA 2015).

METHODS AND MATERIALS

Study Site—. Survey sites represented a mixture of wetland, ponds, streams, riparian areas, rock outcrops, native warm season grass fields, old fields, Northern hardwood forests, Southern hardwood forests, and grassy balds. Survey sites included Henderson Island Wildlife Refuge in Jefferson County, Kyker Bottoms WMA in Blount County, Lick Creek Bottoms WMA in Greene County, Nolichucky WMA in

Greene County, Buffalo Springs WMA in Grainger County, Forks of the River WMA in Knox County, North Cumberland WMA Royal Blue Unit in Campbell County and Tackett Creek Unit in Claiborne County, Roan Mountain State Park in Carter County, the Unaka Mountain portion of the Cherokee WMA and National Forest in Unicoi County, and some private property in Campbell County.

I conducted on 11 sites in East Tennessee, from January to September 2016. I utilized coverboard arrays and tin transects to capture the majority of species. Coverboard arrays typically consisted of 0.6 meter by 0.6 meter pieces of plywood arranged in a 5 board by 5 board grid approximately 3 meters apart, and placed on bare ground by raking leaves and other debris away. Tin transects were 0.6 meter by 1.2 meter pieces of metal placed on top of the grass, and arranged in a straight line transect approximately 10 meters apart. I conducted Visual Encounter Surveys (VES) that were either timed or area constrained. To locate herpetofauna, I turned over logs or other cover objects, inspected rock crevices, and sifted through leaf litter. Given the variety of activity preferences, I conducted VES during both day and night. I also used pitfall traps, reptile box traps, and funnel traps with drift fence arrays of various designs to trap herpetofauna. On 7–9 June 2016 a BioBlitz was

conducted at Tackett Creek in Claiborne County. A BioBlitz is a high intense team effort over a designated time period to document as many species as possible (Karns et al. 2006; Lundmark 2003). This BioBlitz lasted 3 days and 2 nights, and a total of 13 biologists, managers, technicians, and volunteers assisted with the effort. The BioBlitz was for all species of wildlife, but the techniques that were utilized to capture herpetofauna were VES, tin transects, and coverboard arrays. Date, sex, age, weight (WT), snout-to-vent length (SVL), total length (TL) or carapace length (CARL), latitude, and longitude were recorded for each specimen captured. No specimen was intentionally vouchered, but if one was found dead, then it was collected and is now housed for educational purposes by TWRA Region IV.

RESULTS

In total, 34 different species were captured in Region IV in 2016 (See Table 1). The Tackett Creek Unit of North Cumberland WMA had the most species documented with twelve, and six of those being snake species which was the most of any area. The Eastern black kingsnake (*Lampropeltis nigra*) found at Buffalo Springs WMA in Grainger County may represent a County Record, but this needs to be verified.

DISCUSSION

The highest intense efforts put forth this year for herpetological studies were in tin transects at the Tackett Creek Unit of North Cumberland WMA and in visual encounter surveys for salamanders in the Unaka Mountain portion of Cherokee WMA and National Forest, and this shows in the data. Of special note are the following species of Greatest Conservation Need (GCN) that were captured: green Wehlre's salamander (Aneides aeneus), salamander (Plethodon wehlrei), Weller's salamander (Plethodon welleri). and Yonahlossee salamanader (Plethodon

yonaholssee). The green salamander found at Tackett Creek represents a new site for this species and warrants further study. The Wehlre's salamander was found from the only known location of the species in the state of Tennessee. Future efforts by TWRA Region IV include, more intense surveys for green salamanders at known sites, surveying for new sites of green and Wehlre's salamanders, and swabbing of all snakes for Snake Fungal Disease (*Ophidiomyces ophiodiicola*) in conjunction with a study by Tennessee Tech University.

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Name na maculatum teneus athus fuscus athus marmoratus athus orestes athus quadramaculatus cirrigera n ventralis n glutinosis n cylindraceus n wahrlei	X Henderson Island	Kyker Bottoms	X X Buffalo Springs	Forks of the River	Lick Creek & Nolichucky	Royal Blue Unit	X Tackett Creek Unit	Roan Mountain SP	X X Unaka Mountain	X Private Property
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TABLE 1. Results of biodiversity surveys in TWRA Administrative Region IV in 2016.

ANAXYRUS **AMERICANUS** (American Necrophilia, the Toad). NECROPHILIA. attempt to mate with the corpse of conspecifics, has been reported in diverse lineages of anurans, including bombinatorids (Bombina variegata, Sinovas 2009 Herpetol. Rev. 40:199), ranids (Rana boylii, Bettaso et al. 2008 Herpetol. Rev. 39:462; and R. cascadae, Garwood and Anderson 2010. Herpetol. Rev. 41:204), hylids (Osteopilus septentrionalis, Meshaka 1996. Florida Scientist 59:74-75), and bufonids (Anaxyrus terrestris, Meshaka 1996, op. cit.; A. woodhousii, Jennier and Hardy 2015. Herpetol. Rev. 46:73; Bufo bufo, Ayres 2010 Herpetol. Rev. 41:192–193; Duttaphrynus melanostictus, Patel et al. 2016. IRCF Reptiles & Amphibians 23:32-33; Incillius nebulifer, Overviddes and Zaidan 2013. Herpetol. Rev. 44:655-656; Rhinella marina, Lewis, 1989. Cane Toads: An Unnatural History. Doubleday. New York, New York. 98 pp.; and R. proboscidea, Izzo et al. 2012. Journal of Natural History 46:2961-Male R. proboscidea apparently can 2967). extract and fertilize oocytes from dead females, suggesting a possible evolutionary benefit of engaging in amplexus with a gravid female corpse (Izzo et al. 2012 op. cit.). Although widespread bufonids, apparently among necrophilia has not been reported previously in the American Toad (A. americanus).

On 25 March 2017 at approximately 1100 h, I found an amplexed pair of American Toads floating near the surface of a small garden pond in southern Cannon County, Tennessee, USA. The female was dead, bloated, and beginning to decay, but the male appeared healthy. The male did not relinquish his grip on the female when I removed the amplexed pair from the pond and placed them on the ground for photography (Fig. 1); rather, the male visibly tightened his grip and uttered a release call when touched. Rather than removing the male from the carcass, I returned the toads to the pond and checked them periodically to determine when the male would abandon the carcass. I last observed the male amplexed to the carcass at 0400 h on 27 March. At that time, the female was bloated and floating with the male still positioned on top of her while they floated at the surface. By 1530 h of that same day, the male had abandoned the carcass. which had apparently burst and had sunk to the bottom of the pond. I do not know when amplexus began, but from the time of discovery, the male was amplexed with the carcass for at least 41 h. The known duration of necrophilia reported here is similar to that reported for A. woodhouseii (Jennier and Hardy 2015, op. cit.).

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Fig. 1. A male American Toad (*Anaxyrus americanus*) engaged in amplexus with the corpse of a conspecific female on 25 March 2017 in southern Cannon County, Tennessee, USA.

DESMOGNATHUS MARMORATUS (Shovel-nosed Salamander).

TERRESTRIALITY. On 9 November 2017, I observed a young, metamorphosed Desmognathus marmoratus under a rock approximately 0.3 m from the edge of Cold Springs Creek in Haywood County, North Carolina (35.7450°N; 83.0079°W). The stream was high and turbulent, and it is possible that the salamander was driven from its aquatic refuge due to high flow or low oxygen. Bishop (1941) described this species as "[e]ssentially aquatic but occasionally on land" (A Handbook of Salamanders: The Salamanders of the United States, of Canada, and of Lower California, Comstock Publishing Company, Ithaca, New York. 555 pp.). One has been discovered ~ 1.5 m from a stream following a heavy rain (Niemiller pers. comm.), and one has been discovered sitting out of the water, upon a rock in a stream (Tilley pers. comm.). Southerland (1986) found *D. marmoratus* in pens that they should not have been able to enter without climbing and inferred that this species must make brief movements out of the water (Herpetol. Rev. 17:45). Published documentation of terrestriality in D. marmoratus is rare, but these direct and indirect observations of terrestrial movement in this species suggest that the behavior is infrequent but possible.

Submitted by **TODD W. PIERSON**, Department of Ecology and Evolutionary Biology, University of Tennessee, Knoxville, TN 37996 (e-mail: tpierso1@vols.utk.edu).

GYRINOPHILUS PORPHYRITICUS (Spring Salamander). COURTSHIP. Extensive collections and examinations of reproductive anatomy suggest that *Gyrinophilus porphyriticus* reproduces between late fall and early spring both in the southern Appalachians (Bruce 1972. Herpetologica. 28:230–245; Bruce 1978. Herpetologica. 34:53–64) and further north in its range (Dieckmann 1927. Biol. Bull.

53:258-274,276-280; Bishop 1941. N. Y. State Mus. Bull. 324:1-365). Successful courtship trials in captivity have been conducted between November and March (Beachy 1996. Copeia. 1996:199-203; Beachy 1997. Herpetologica. 53:289-296). However, to our knowledge, there exist no published accounts of definitive courtship behaviors observed in the field, although Bishop (1941) described one brief observation of an interaction that could alternatively be interpreted as agonistic behavior. From approximately 2015 to 2300 on 5 December 2017, we observed 14 adult Gvrinophilus porphyriticus surface-active in a light rain along the Little Pigeon River and its tributaries (from approximately 35.7384°N; 83.4163°W to 35.6948°N; 83.3898°W) in Great Smoky Mountains National Park in Sevier County, Tennessee. At approximately 2200 h, we observed two adults in close proximity on the forest floor, approximately 1 m from a small, muddy spring. One appeared to be following the other, but we observed no specific courtship behavior. We left them undisturbed and returned at approximately 2300 h to find them approximately 1 m from where we last saw them, with one following directly behind the other. We suspect that we witnessed a male pursuing a female or a female being led in the early stages of tail-straddle walk, but we did not interrupt their activity to confirm sex of the animals. This research was conducted under GRSM-2017-SCI-1197, permits **TWRA** Scientific Collection Permit 1213, and University of Tennessee Knoxville IACUC 2372-0616.

Submitted by **TODD W. PIERSON**, (e-mail: tpierso1@vols.utk.edu); **EVIN T. CARTER**, (e-mail: ecarte19@vols.utk.edu), and **ALEX FUNK**, Department of Ecology and Evolutionary Biology, University of Tennessee, Knoxville, TN, 37996, (e-mail: afunk4@vols.utk.edu).

APALONE SPINIFERA (Spiny Softshell). USA: TENNESSEE: Polk Co.: Hiwassee River, Cherokee Forest (35.21951°N National 84.51798°W; WGS84). 28 May 2016. Stephen K. Nelson and Marcy Dochtermann. Verified by A. Floyd Scott. David H. Snyder Museum of Zoology, Austin Peay State University (APSU 19778, color photo). A. spinifera has previously been reported from Polk County (Powers, L. and M. L. Niemiller. 2013. Spiny Softshell (Apalone spinifera). In M. L. Niemiller, R. G. Reynolds, and B. T. Miller (eds.), The Reptiles of Tennessee. pp. 309-312. University of Tennessee Press, Knoxville. 366 pp.). However,

this record represents the first vouchered record to be published as well as the first locality reported for Polk County (Redmond and Scott 2008. Atlas of Reptiles in Tennessee. The Center for Field Biology, Austin Peay State University, Clarksville, Tennessee. Internet version, available at http://apsu.edu/repatlas/ [updated 11 February 2016]; accessed 15 January 2017). An adult male found swimming. Tennessee Wildlife Resource Agency permit #3620.

Submitted by **Stephen K. Nelson** (e-mail: snelson@zooknoxville.org) and **Marcy Dochtermann**, Zoo Knoxville, Knoxville, Tennessee 37914, USA.

23rd Annual Meeting of the Tennessee Herpetological Society

Middle Tennessee State University September 28-29, 2017 Business Meeting Notes Recorded by Stephanie Chance

Chad Lewis Memorial Grant Recipient:

Congratulations to Nicole Witzel for being awarded the 2017 Chad Lewis Memorial Grant for eDNA work on Streamside Salamander distributions.

Past Business:

Assessment of the Salamanders of Tennessee website transfer and the THS website. Discussion continued about formatting and domain names, use of the TWRA Watchable Wildlife Page and whether or not to redesign the THS website. A new website could include species accounts for all TN herpetofauna that could include member photos, species accounts, and range maps. Data Driven, a Nashville Company, was contacted about making revisions to the site. Motion was made by Josh Campbell to form a Public Relations Committee for website and Facebook communication. Volunteers included: Lisa Powers, Steven Nelson, Dustin Thames, and Todd Pierson.

Conservation Committee:

Nothing new to report from the committee. Questions were raised about Bluegrass Underground. Dr. Matthew Niemiller has been contacted to survey the cave, and The Nature Conservancy is heavily involved in the project. Though no impacts are expected for the Tennessee Cave salamander, discussion progressed about whether THS should issue a letter expressing concern and/or thanking them for their conservation effort.

Chad Lewis Memorial Grant Committee

The committee is considering increasing the number of awards – potentially developing categories for graduate, undergraduate, and even high school awardees. Discussion was had about use of the CD currently and in the future. More promotion of the grant is needed. Discussion was also had about how to minimize conflict with university grants offices about indirect costs including calling it a scholarship rather than a grant or awarding the grant directly to the student. The Committee will devise a plan for review understanding that the decision has impacts on tax-exempt purchases. Clarification is needed for whether the grant is awarded for previous work or for upcoming work.

Website:

The THS domain is paid for 2 additional years. The Frogs of Tennessee page was moved to the THS site, and Lisa Powers is currently migrating the salamanders page. Lisa continues to work on the Snakes of Tennessee page, but is in need of species descriptions. Volunteers are welcome. Turtles and lizards are also mostly complete. More reports are coming for non-native species (e.g. Mediterranean Gecko). The Facebook page for snake identification is more efficient than emails to the web site. Lisa welcomes feedback on the website.

Publication/Newsletter Committee:

We continue to request new submissions. The Tennessee Journal of Herpetology may be found at:

http://tnherpsociety.wixsite.com/home.

Treasurer's Report:

Members approved last year's report. Balance in the checking account is \$13,940 and the CD balance is \$12,138. Participate and support the auction to support our Chad Lewis Memorial Grant.

New Business:

New Elections: Vice President: David Withers Treasurer: Chris Ogle East TN Representative: Steven Nelson Middle TN Representative: Danny Bryan

New Hats: Josh Campbell is searching for a company to produce new THS hats. EcoWear is an option, but they require an artist to digitize a picture for \$150 and a minimum order of 48 hats at \$10/hat. If anyone has other leads, please let Josh know.

Travel Award Committee: Matthew Niemiller and Denise Kendall wish to support an annual \$100 travel award to support student attendance at the annual THS meeting. A new committee, the Travel Award Committee, was formed to administer this annual award.

2019 Annual THS Meeting: Hosts are requested for the 2019 meeting in east Tennessee. Rainforest Adventures and the Grey Fossil Museum were suggested as potential locations.

2018 Annual Meeting of the Tennessee Herpetological Society Lichterman Nature Center East Memphis

We hope to see you there!