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DEVELOPMENT AND VALIDATION OF PCR PRIMERS FOR DETECTING *AMBYSTOMA TEXANUM* (SMALLMOUTH SALAMANDER) FROM EDNA SAMPLES

Elizabeth K. Strasko*, Rebecca R. Piche, Ben F. Brammell

Department of Science and Health, Asbury University, Wilmore, KY 40390
*Corresponding Author email: elizabeth.strasko214@topper.wku.edu

Abstract.—The smallmouth salamander (*Ambystoma texanum)* is a widely distributed member of the family Ambystomatidae found throughout much of the central United States. We developed primers targeting a 147 base pair fragment of the mitochondrial cytochrome b region of the smallmouth salamander and tested these primers in silico, in vitro, and in vivo in laboratory-based studies. We believe these validated tools will be widely useful in the detection of *A. texanum* in breeding ponds, particularly in habitats where multiple species of *Ambystoma* occur.

Key Words.—conservation, eDNA, salamander, Ambystomatidae, *Ambystoma texanum*

Environmental DNA (eDNA) has rapidly become a firmly established method for detecting organisms of research and conservation interest and promises to greatly increase the ease, efficacy, and scope of ecological studies (Dysthe et al. 2018; Mauvisseau et al. 2019; Sawaya et al. 2019). Since its inception in 2008 eDNA has been used to detect rare and endangered species of fish (Laramie et al. 2015; Paine et al. 2021), amphibians (Spear et al. 2015; Pierson et al. 2016; Witzel et al. 2020), crustaceans (Ikeda et al. 2016; Rusch et al. 2020), and mussels (Rusch et al. 2020; Coghlan et al. 2021; Schmidt et al. 2021), in some cases detecting cryptic species where traditional methods were unsuccessful (Sigsgaard et al. 2015; Gargan et al. 2017). Additionally, eDNA has been widely used in the detection of invasive species including Asian carp (Jerde et al. 2011) and many other species of fish (Adrian-Kalchhauser and Burkhardt-Holm 2016; Robson et al. 2016), molluscs (Klymus et al. 2017; Xia et al. 2018), crustaceans (Carim et al. 2016; Dougherty et al. 2016), amphibians (Dejean et al. 2012; Secondi et al. 2016), and even reptiles (Piaggio et al. 2014). The number of eDNA studies has increased at an exponential pace. Single species eDNA assay studies increased from one in 2008 to 56 in 2019 (Xia et al. 2021), eDNA studies focusing on fish ecology increased from one in 2011 to nearly 90 in 2021 (Xing et al. 2022), and fifty eDNA review articles were published in 2020 and 2021, comprising nearly 50% of the 105 of eDNA review articles published since the inception of the technique (Hinz et al. 2022). Clearly eDNA promises to expand the questions scientists are able to address in ecological studies.

Recent works have highlighted the need for carefully tested assays for use in species specific marker studies (Klymus et al. 2020; Xia et al. 2021). Such studies rely on the development of primers that recognize the target, but not sympatric non-target species. A well-defined, species-specific primer pair will present a minimum of two mismatched DNA base pairs with sympatric species sequences (Wilcox et al. 2013), ensuring that the primer does not bind to non-target species. Recent studies have emphasized the need for thorough vetting of eDNA primers using as many local sequences as available given the potential for

false positives (amplification of a sympatric species' DNA) and false negatives (failure to amplify target species DNA) at any given location (Wilcox et al. 2013; Kaganer 2021; Bell et al. 2022). In vitro testing against sympatric species ensures specificity and provides assurance that false positives should not occur. Only 30.4% of specific marker studies published between 2008 and 2019 use previously developed primers (Xia et al. 2021), likely a result of lack of availability of these tools and an indication of the time and effort required to develop and thoroughly vet these molecular tools. Carefully validated species-specific markers that have been rigorously screened to prevent both false positives and negatives hold significant value that extends well beyond their time and location of origin.

As global amphibian communities continue to decline at alarming rates (McCallum 2007; Collins 2010; Fisher and Garner 2020), the need for methods enabling rapid identification of the presence/absence of specific organisms will continue to increase. Collins (2010) and McCallum (2007) suggest that all amphibians are in need of monitoring regardless of current conservation status. Molecular assays that enable the rapid detection of fossorial species such as smallmouth salamanders would provide great benefit to conservationists and ecologists studying these organisms.

The smallmouth salamander is a cryptic, fossorial salamander found throughout much of the central United States (Kraus and Petranka 1989; Petranka 1988). *A. texanum* was initially classified as having two forms: a widely distributed ephemeral pond breeding form and a stream breeding form with a more restricted range (Garcia et al. 2003). The stream breeding form was eventually recognized as the streamside salamander *(Ambystoma barbouri)* (Kraus and Petranka 1989), hypothesized to have diverged from *A. texanum* during the late Pleistocene (Kraus and Petranka 1989; Petranka and Sih 1987). Differentiation of adults of these species using external characteristics is difficult (Brian T. Miller and Joyce L. Miller 2019). Although the larvae of *A. barbouri* are reported to be darker than *A. texanum* (Garcia et al. 2003), both species display cryptic, background color change responses (Garcia and Sih 2003), making them difficult to differentiate as adults. Although *A. texanum* and *A. barbouri* are mostly allopatric, several zones of parapatric and sympatric speciation do occur (Brian T. Miller and Joyce L. Miller 2019). The molecular tools provided by this work will facilitate quantification of the presence of *A. texanum*, including most habitats where *A. texanum* and *A. barbouri* occur sympatrically. These assays have been designed based on locally obtained sequences and validated in silico and in vitro as recommended (Klymus et al. 2017; Langlois et al. 2020; Xia et al. 2021) providing ready-made tools to facilitate future conservation efforts.

METHODS AND MATERIALS

Tissue collection of target and non-target species. – We collected tissue from adult streamside salamanders and Eastern newts *(Notophthalmus viridescens)* (KYDFW Permit# SC2111188). Tissue for the smallmouth and other sympatric species was generously donated by collaborators. All information concerning origin of species used in laboratory testing is found in Table 2, Supplemental Data. We extracted tissue DNA using a DNeasy blood and tissue kit (Qiagen) according to the provided protocol. Tissue was lysed overnight at 56 °C in proteinase K and eluted twice (400 µl total) to increase DNA yield.

Sequencing of target species. – We amplified portions of cytochrome b (cyt b) from both *A. texanum* and sympatric species using published primers (Roe et al. 1985) (Table 2, Supplementary Data). Sequences were run in duplicate or triplicate and edited prior to Gen Bank submission, all sequencing was completed by ACGT (ACTG inc.com).

Assay development and testing. – We aligned published cyt b sequences with nineteen potential sympatric Kentucky salamander species using MegaX and Clustal W. Accession numbers of cyt b sequences used in alignments are found in Table 2. We designed F and R primer pairs using PrimerQuest software (IDT) and aligned these with sympatric or potentially sympatric species to verify specificity (Table 2). All primers have at least 2 mismatches in the F or R primer. We also designed probes (Table 1, Supplemental Data) and tested them in silico but not in vitro.

We evaluated F and R primers via a temperature gradient approach to determine optimal annealing temperature (53.1 – 61.6 ºC) (Figure 1, Supplemental Data). For in-vitro testing we ran end-point PCR on tissue extracts with six sympatric or potentially sympatric *Ambystoma* species and *Notophthalmus viridescens* collected in various localities in Kentucky (Table 5). Tissue extracted DNA concentration was quantified using a Qubit™2.0 (Life Technologies, Carlsbad, CA, USA) and DNA from all species diluted in nuclease free water to a concentration of 10 µg/ml. 25 µl reactions included: 12.5 µl GoTaq Master Mix (Promega, Madison, Wisconson, U.S.A.), 9.5 µl nuclease free water, 2 µl tissue extracted DNA and 2.0 µl of F and R primers (reaction concentration = 1.6 µm). Cycling conditions consisted of an initial denaturation stage of 95.0 ºC for 2 minutes followed by 40 cycles of 95.0 ºC for 45 s, 57.0 ºC for 60 s, and 72.0 ºC for 60 s.

Laboratory water tests. – We tested the validity of our primers through water exposure studies in the laboratory using two smallmouth salamanders: one larva and one adult (both collected in Logan County, KY). 500 mL of deionized water was added to a plastic Tupperware container containing each salamander. The larval salamander's enclosure was left on a flat surface, while the adult's was tilted to allow for resting partially out of the water. The bottom of this tilted container had a paper towel to facilitate traction, but the water still reached the bottom all around the base of the container. These containers were left for 96 hours based on previous studies (Maruyama et al. 2014; Takahara et al. 2012) in a room maintained at 68 °C. Following removal of salamanders exposed water was diluted 20:1 in DI water to approximate in situ concentrations (Davy et al. 2015). Water samples were processed through a 47mm diameter glass microfiber filter (VWR, 0.42mm thickness and 0.7 µm pore size) in a manner similar to previous studies (Eichmiller et al. 2014; Guivas and Brammell 2020; Jerde et al. 2011).

DNA Extraction. – eDNA extraction was performed using a DNeasy blood and tissue kit (Qiagen – Valencia, CA, USA) in the manner described by Guivas and Brammell (2020). Briefly, entire filters were cut into 30–40 pieces and incubated at 56°C overnight in 720 µl ATL buffer and 80 µl Proteinase K. Final elutions were performed twice into a total of 400 µl of AE buffer, and the extracted DNA was stored at −20 °C until analysis.

Amplicon Sequencing. – Amplicons obtained from the larval water test were sequenced in duplicate with both the forward and reverse primer to generate a consensus sequence including the entire amplicon. Sequencing was conducted by ACGT (ACGTinc.com – Wheeling, IL, USA).

RESULTS

In silico testing. – Smallmouth salamander forward and reverse primers have a minimum of two mismatches with all species and four or more mismatches with all species except streamside salamanders (Table 2). When the probe is considered, five or more mismatches exist with all 19 potential sympatric species (Table 2); therefore, amplification of sympatric species is highly unlikely. Additionally, when compared to published smallmouth salamander sequences from other portions of the range, the number of mismatches in only the forward and

TABLE 1. Primers developed to detect smallmouth salamander (*A. texanum*), designed based on sequence from Kentucky (Butler Co.) collected *A. texanum* specimen (Acc# OM236537). Bold, blue bases indicate location mismatches with streamside salamander (*A. barbouri*) (Table 2).

Amplicon length (BP)	Oligo	Sequence (5'-3')
147	F	TCAATGAATTTGAGGCGGATTT
	R	CCTGT**A**GG**G**TTATTAGATCCTGTT

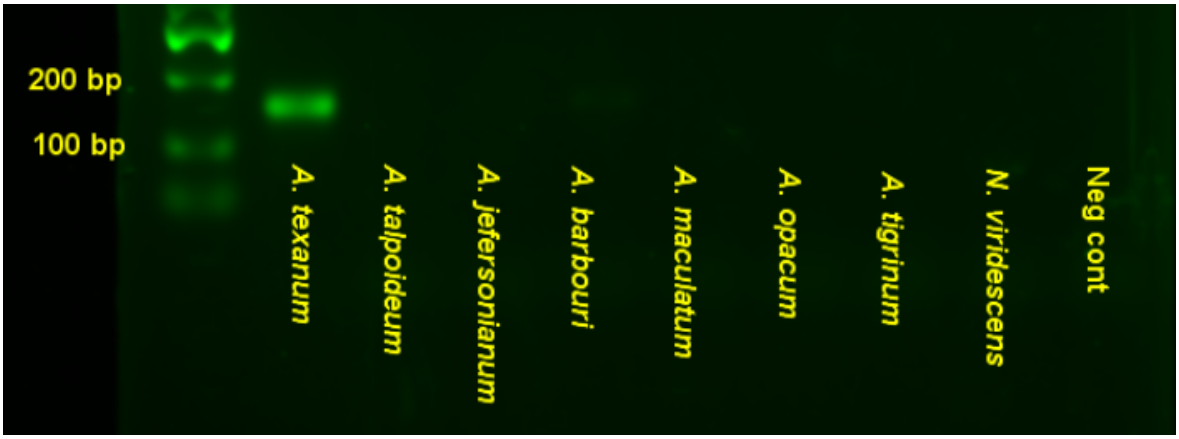

FIG. 1. Tissue extracted DNA specificity tests for smallmouth salamander (*A. texanum*) primers using tissue extracts of six sympatric or potentially sympatric species of *Ambystoma* species and eastern newt (*Notophthalmus viridescens*). 25 µl reactions included: 12.5 µl GoTaq Master Mix (Promega), 9 µl nuclease free water, 2 µl tissue extracted DNA and 1.5 µl of F and R primers. Cycling conditions consisted of an initial denaturation stage of 95.0ºC for 2 minutes followed by 40 cycles of 95.0ºC for 60 s, 57.0ºC for 60 s, 72.0ºC for 60 s. Ladder displayed is Hyper 25 BP ladder (Bioline).

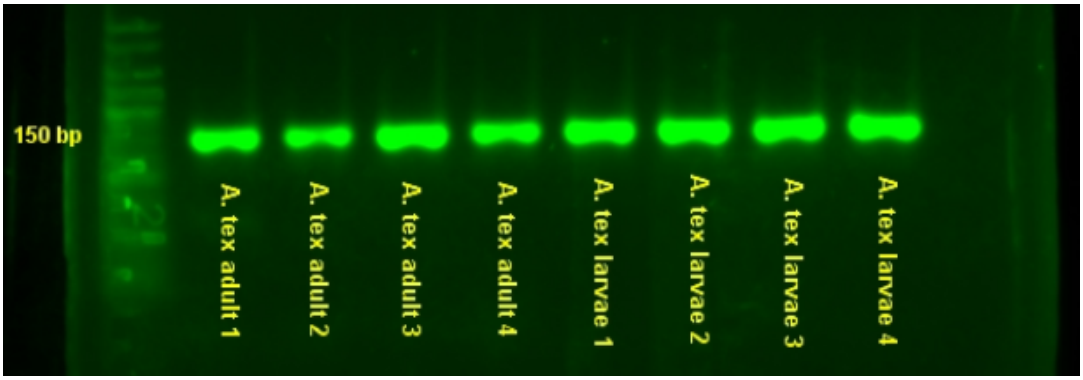

FIG. 2. Filtered water extracted DNA reactions run with smallmouth salamander (*A. texanum*) primers. Lanes 2 – 5 are replicates of the adult water test and Lanes 6 – 9 are replicates of the larval water test. 25 µl reactions included: 12.5 µl GoTaq Master Mix (Promega), 6.75 µl nuclease free water, 3.75 µl filtered water extracted DNA and 2.0 µl of F and R primers. Cycling conditions consisted of an initial denaturation stage of 95.0ºC for 2 minutes followed by 55 cycles of 95.0ºC for 30 s, annealing temperature of 56.5ºC for 30 s, 72.0ºC extension for 30 s with a final extension of 72.0ºC for 5 minutes. Ladder displayed is Hyper 25 BP ladder (Bioline).

reverse primer is zero except for a published sequence from Texas (EF036664.1) although mismatches (up to two) are found in the probe for some of these sequences (Table 3). A comparison of these primers with streamside salamander sequences from various portions of its range indicate a minimum of four mismatches when the probe is considered but 5/13 sequences do have a single mismatch with only the forward and reverse primer (Table 4).

In vitro testing. – Endpoint PCR reactions followed by gel electrophoresis successfully amplified cytochrome b from smallmouth salamander but not sympatric salamander species (Figure 1) following 40 cycles.. Additionally, the amplicon produced with the smallmouth salamander DNA migrated according to the expected size (147 BP, Figure 1).

Laboratory water tests. – Diluted water extracted DNA from both the larvae and adult exposure trials produced strong bands that migrated according to size (Figure 2). Amplicons from the larval tank test were sequenced and produced an amplicon 100% similar to the complete smallmouth salamander cyt b sequenced in this study (Table 3, Supplemental Data).

TABLE 2. Mismatches in smallmouth salamander (*A. texanum*) oligos and sympatric or potentially sympatric Kentucky salamander species. FP = forward primer, RP = reverse primer, P = probe, % sim. = percent similarity of the smallmouth salamander cyt b sequence obtained in this project (Acc# OM236537) to the sequence indicated by the accession # in the table, Symp. = the species does or may occur in the study area, In vitro = the primers were screened in laboratory tissue tests with this species.

Sympatric species	FP mismatches	RP mismatches	P mismatches	% sim.	Seq. accession #	Symp.	In vitro
Ambystoma texanum	0	0	0		OM236537	-	-
Ambystoma talpoideum	4	3	2	84.2	NC_039182.1	Y	Y
Ambystoma barbouri	0	2	3	93.3	OL456142	Y	Y
Ambystoma opacum	3	2	4	85.3	KT780868.1	Y	Y
Ambystoma jeffersonianum	2	2	1	86.8	MZ962318	Y	Y
Ambystoma maculatum	5	3	3	84.1	EF036637.1	Y	Y
Ambystoma tigrinum	3	4	2	88.2	OL456143	Y	Y
Notophthalmus viridescens	2	4	4	80.6	AY691731	Y	Y
Eurycea cirrigera	3	2	3	78.1	NC_035494.1	Y	Y
Eurycea lucifuga	1	3	5	79.0	KT873718.1	Y	N
Eurycea longicauda	3	3	5	77.4	AY528403.1	Y	N
Eurycea bislineata	1	4	2	78.4	AY528402	Y	N
Desmognathus monticola	10	13	12	83.8	MK029465.1	N	N
Desmognathus ochrophaeus	3	13	13	79.8	EU314289	N	N
Desmognathus welteri	1	12	13	82.0	EU314293	N	N
Desmognathus conanti	1	13	11	82.0	EU314275.1	Y	N
Pseudotriton ruber	4	3	3	78.7	AY728220	Y	N
Pseudotriton montanus	3	4	3	77.7	KR054760.1	N	N
Gyrinophilus porphyriticus	3	2	5	77.9	AY728230	Y	N
Hemidactylium scutatum	5	5	7	77.2	AY728231	Y	N

DISCUSSION

As anticipated, of the six sympatric Ambystomid species tested, streamside salamander cyt b sequence used for in vitro testing in this study was 93.3% similar to the smallmouth salamander sequence (Table 2). The sufficiency of two mismatches (Table 2) to distinguish these species is similar to that reported by Wilcox et al. (2013) who noted greatly reduced amplification of non-target DNA with one mismatch and nearly no amplification when two mismatches total were present in both F and R primers. Interestingly, the significance of the proximity of a mismatch to the 3' end of the primer has been noted in enhancing specificity (Stadhouders et al. 2010; Whiley and Sloots 2005; Wright et al. 2014). The mismatches between our smallmouth salamander R primer and streamside salamander sequence (OL456142) occur at the 6th and 9th base (Table 1) from the 5' end of the primer (24 BP total) but were still sufficient to produce specificity (Fig. 1). Furthermore, we note that the addition of the probe (Table 3, Supplemental Data) tested in this study in silico (three mismatches, Table 2) but not in vitro, would provide an additional level of security in preventing the possibility non-specific binding with streamside salamander DNA.

This work underscores the importance of local sequences in assay validation, consistent with recent studies (Czechowski et al. 2021; Goldberg et al. 2016). Variation in mitochondrial genes, and cytochrome b in particular, is consistently observed throughout the range of salamander species (Kuchta et al. 2016; Page et al. 2020; Sweet and Jockusch 2021), creating the potential for both Type I and II errors in eDNA surveys. We utilized locally collected specimens for our in vitro testing and sequenced five of the seven species utilized (Table 2, Supplemental Data) as well as our target species, smallmouth salamander. As expected, our primers work well for published smallmouth salamander sequences from the central portion of their range but not with a published smallmouth salamander sequence from Texas (Table 3), presumably because they are designed based on a specimen collected from Kentucky (Table 1). When our smallmouth salamander primers are compared with published streamside salamander sequences, all sequences compared have a minimum of two mismatches when the probe is included and 12/13 have four or more mismatches (Table 4), indicating they should be sufficient to distinguish smallmouth from streamside in most or all areas of sympatry.

salamanders possessed a cytochrome b sequence most similar to smallmouth salamanders. The streamside

TABLE 3. Comparison of mismatches between smallmouth salamander oligos (including probe not tested in vitro) and published smallmouth salamander sequences (Bi and Bogart, 2010; Robertson et al., 2006).

	Collection locality	Butler Co, KY	Clarke Co., OH	Erie Co., OH	Montgomery Co., OH	Washington Co., OH	Jay Co., IN	Jennings Co., IN	Wabash Co., IN	Mclennon Co., TX	Essex Co., ON	Essex Co., ON	Essex Co., ON
	Gen Bank Accession Number	OM236537	GU078506.1	EF036641.1	GU078471	EF036656.1	EF036657.1	EF036660.1	EF036659.1	EF036664.1	EF036643.1	EF036648.1	EF036644.1
F	TCAATGAATTTGAGGCGGATTT	0	0	0	0	0	0	0	0	1	0	0	0
R	CCTGTAGGGTTATTAGATCCTGTT	0	0	0	0	0	*	0	0	3	0	0	0
P	ACCCGATTCTTTGCCTTCCACTTCT	0	0	0	0	2	1	0	0	2	2	2	2

*Primer falls outside published sequence.

TABLE 4. Comparison of mismatches between smallmouth salamander oligos (including probe which was not tested in vitro) and published streamside salamander sequences (Bi and Bogart, 2010; Robertson et al., 2006).

	Collection locality	Madison Co, KY	Jessamine Co., KY	Fayette Co., KY	Franklin Co., KY	Mercer Co., KY	Anderson Co., KY	Oldham Co. KY	Hamilton Co., OH	Warren Co., OH	Montgomery Co., OH	Butler Co., OH	Rutherford Co., TN	Livingston Co., KY
	Gen Bank Accession Number	OL456142	GU078501	GU078484	GU078482	GU078496	GU078478	GU078490	GU078470	GU078512	GU078474	GU078511	GU078495	GU078504
F	TCAATGAATTTGAGGCGGATTT	0	0	1	0	0	1	1	0	1	0	0	0	1
R	CCTGTAGGGTTATTAGATCCTGTT	2	2	2	1	1	0	0	2	1	2	2	1	*
P	ACCCGATTCTTTGCCTTCCACTTCT	3	3	3	3	3	4	4	2	3	3	2	1	3

*Primer falls outside published sequence.

Although tools for monitoring all amphibian species have value, one of the primary advantages of tools enabling eDNA detection of smallmouth salamanders is the deployment of these tools in the detection of this fossorial species in ephemeral breeding ponds where they may occur with other Ambystomid species and field identification is difficult. The smallmouth salamander range extends throughout much of the central United States (Kraus and Petranka 1989; Petranka 1988) (Figure 3) where it occurs sympatrically with as many as four other Ambystomid species (Niemiller and Reynolds 2011). As previously noted, the streamside salamander is

particularly difficult to distinguish from the smallmouth salamander and these two salamanders occur sympatrically in several portions of their range (Figure 3). The use of these primers presented here should enable the conduction of eDNA studies distinguishing these two species, particularly when used in conjunction with recently published tools for the eDNA detection of streamside salamanders (Witzel et al. 2020).

TABLE 5. Salamander specimens used in in vitro specificity testing. All localities are in the state of Kentucky, the county in which the individual was collected appears in table.

Species	Collection locality	DNA conc. (µg/ml)
A. tigrinum	Warren	680
A. maculatum	Rowan	133
A. barbouri	Madison	449
A. opacum	Hart	261
A. jeffersonianum	Powell	154
A. talpoideum	Logan	354
A. texanum	Butler	261
N. viridescens	Powell	189

Recent works have emphasized the need for thorough specificity testing validation (Goldberg et al. 2016; Klymus et al. 2020; Loeza-Quintana et al. 2020). The assays presented here and tested, both in silico, in vitro, and in laboratory water exposure tests should serve as valuable tools enabling the detection of this widespread salamander species.

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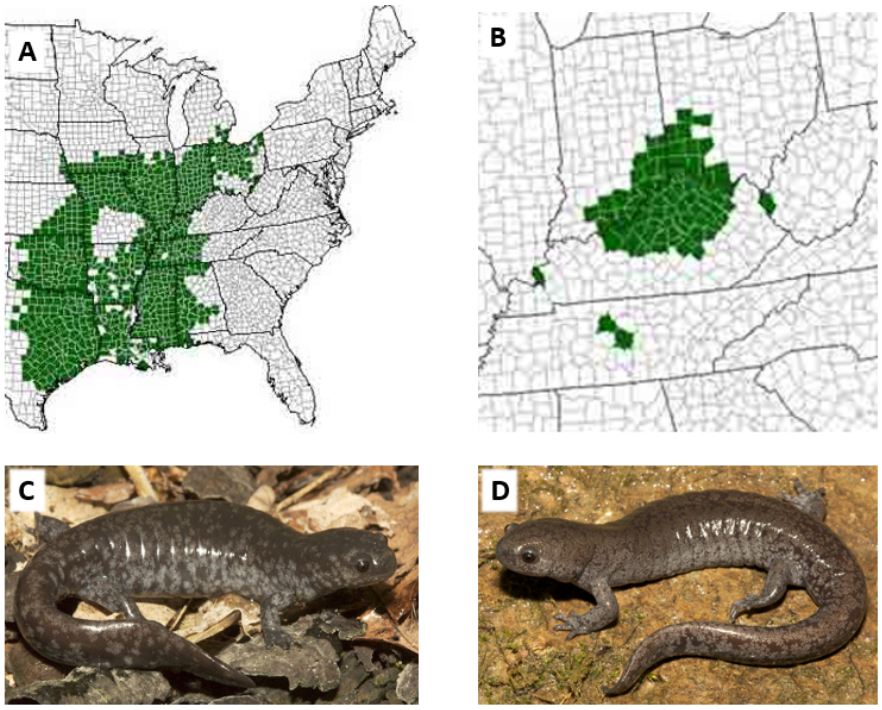

FIG. 3. Ranges and photos of smallmouth and streamside salamanders. (A) Range of smallmouth salamanders (courtesy of USGS and Ohio Amphibians.com), (B) Smallmouth salamander from central Indiana (photo courtesy of Todd Pierson), (C) Range map of streamside salamanders (courtesy of USGS and Ohio Amphibians.com), (D) Streamside salamander from southeastern Indiana (photo courtesy of Todd Pierson).

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Supplemental Data

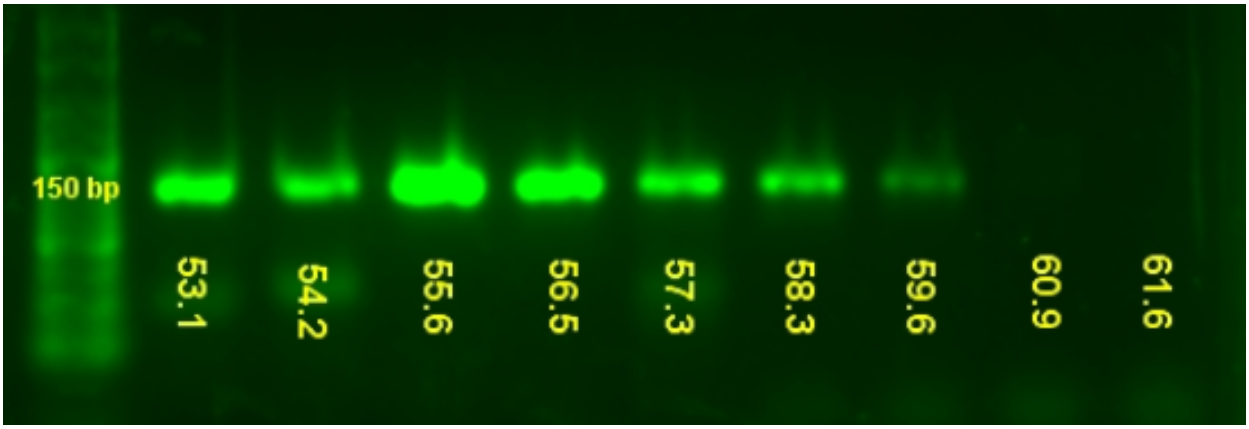

FIG. S1. Gradient reaction run to optimize annealing temperature. Template DNA consisted of water extracted DNA from smallmouth salamander tank tests. 25 µl reactions included: 12.5 µl GoTaq Master Mix (Promega), 6.75 µl nuclease free water, 3.75 µl filtered water extracted DNA and 2.0 µl of F and R primers. Cycling conditions consisted of an initial denaturation stage of 95.0ºC for 2 minutes followed by 55 cycles of 95.0ºC for 30 s, annealing temperature shown on figure for 30 s, 72.0ºC extension for 30 s with a final extension of 72.0ºC for 5 minutes. Ladder displayed is Hyper 25 BP ladder (Bioline).

TABLE S1. Forward and reverse primer pairs and probe developed for smallmouth salamander. Probe sequence is included here but was not tested in vitro or in laboratory water exposure tests.

Amplicon length (BP)	Oligo	Sequence (5'-3')
147	F	TCAATGAATTTGAGGCGGATTT
	R	CCTGTAGGGTTATTAGATCCTGTT
	P	TGTAGCCCATATTTGCCGAGACGT

TABLE S2. Ambystomid species and Eastern Red-Spotted Newt used in in vitro specificity test. All specimens were collected in Kentucky.

Species	**Collection locality**	**Cyt b sequenced**	**Length**	**G.B. accession #**
Ambystoma texanum	Butler Co.	Y	744	OM236537
Ambystoma talpoideum	Logan Co.	N	-	-
Ambystoma jeffersonianum	Powell Co.	Y	749	MZ962318
Ambystoma barbouri	Madison Co.	Y	935	OL456142
Ambystoma maculatum	Rowan Co.	N	-	-
Ambystoma opacum	Powell Co.	Y	720	KT780868.1
Ambystoma tigrinum	Warren Co.	Y	782	OM289824
Notophthalmus viridescens	Powell Co.	Y	272	MZ962319

TABLE S3. Amplicon produced with *A. texanum* primers from water samples taken during the laboratory tanks tests. Bold blue bases represent F and R primers.

Length	Sequence
147 BP	**TCAATGAATTTGAGGCGGATTT**TCAGTTGACAAAGCTA CCTTAACTCGATTCTTTGCCTTCCACTTCTTATTTCCATTC TTAATTGCAGGAACAAGCATTATTCATCTCCTTTTTCTTCA CGA**AACAGGATCTAATAACCCTACAGG**

OBSERVATIONS ON NESTING LOCATION AND BROODING BEHAVIOR OF THE COMMON FIVE-LINED SKINK (*PLESTIODON FASCIATUS*) IN MIDDLE TENNESSEE

Brian T. Miller

Department of Biology, Middle Tennessee State University, Murfreesboro, Tennessee, USA
Corresponding Author email: *brian.miller@mtsu.edu*

Abstract.—Reproductive activities of Common Five-lined Skinks (*Plestiodon fasciatus*) have been documented throughout much of the range of the species, but information on nests or brooding behavior for populations inhabiting Tennessee is lacking. On 16 June 2019, I found a female brooding eight eggs in a nest underneath weathered cardboard on a gravel driveway in southern Cannon County, Tennessee. I photographed the brooding female and her eggs 16 times during the incubation period, which included five times after hatching commenced. The female was positioned alongside her clutch with her tail or other body part on top of or otherwise in contact with one or more of the eggs each time I examined the nest. The female invariably, but only temporarily, abandoned her nest soon after I removed the cardboard and began flash photography. Although always within the same area underneath the cardboard, the eggs were positioned differently each time I observed them. The eggs began to hatch on 6 July, and all had hatched by the morning of 7 July, at which time the female was in the nest with her hatchlings. At least three hatchlings were in the nest the morning of 8 July. This is the first record of Common Five-lined Skinks nesting in gravel, and supports reports from other regions that a brooding female (1) routinely repositions eggs, (2) has a strong bond to the nest even after repeated disturbances during a three-week period, (3) and her hatchlings might remain in the nest a day or two after all eggs have hatched.

Key Words.—clutch size, egg repositioning, hatching date, hatchlings, incubation, nest guarding behavior, reproduction.

The Common Five-lined Skink (*Plestiodon fasciatus*) typically lays eggs in decaying logs and stumps (Hecnar 1994; Trauth et al. 2004; Jensen et al. 2008; Niemiller et al. 2013) from late spring to early summer (Noble and Mason 1933). After laying, females brood their eggs (Fitch, 1954; Mitchell 1994; Trauth et al. 2004). Brooding refers to any behaviors of a parent attending to eggs and young in the nest (Peters 1964), and brooding female Common Five-lined Skinks protect eggs from predators, and help maintain proper moisture in the nest (Noble and Mason 1933; Fitch 1954; Hecnar 1994). Although well-documented in other regions, relatively little information has been published on nesting behavior of the Common Five-lined Skink in Tennessee. Here, I report on an unusual nest site, clutch size, and brooding behavior of a female Common Five-lined Skink observed during a three-week period in middle Tennessee.

METHODS AND MATERIALS

I found a female Common Five-lined Skink brooding eggs underneath weathered cardboard while I was clearing debris from along the edge of my gravel driveway in southern Cannon County, Tennessee on 16 June 2019. I replaced the cardboard over the nest and returned with a

digital camera to photograph the eggs and brooding female later that day. I also examined and photographed the brooding female on the following dates: 17, 18, 21, 24, 25, 26, 29 June, and 6, 7, 8 and 9 July. I used digital images to document the position of the female and her eggs throughout the incubation period. Fitch (1954) reports that females often desert their nest when disturbed, and he cautions that repeated disturbances might cause a female to permanently abandon her eggs; consequently, I never touched the female or her eggs, and I photographed them as quickly as possible, usually replacing the cardboard within 30 secs.

RESULTS

Although I attempted to limit the duration of my observations of the brooding female, she was noticeably disturbed each time I lifted the cardboard from her nest, and she almost always sought refuge in lawn adjacent to the driveway soon after I began flash photography; however, she invariably returned to her nest and resumed brooding activities, sometimes within 30 mins after my initial disturbance (Fig. 1). Each time I examined the nest, the female was positioned alongside the clutch with her tail or other body part on top of or otherwise in contact with one or more of her eggs, which she apparently repositioned between my visits, including those few instances when I checked on the position of the female within an hour of a previous visit (Fig. 1C, 1D). For example, two of seven eggs visible at 0448 h on 18 June (Fig. 1C) were in a different position 40 min later (Fig. 1D), and at least four of the eggs were repositioned within a four-hour interval on 24 June (Fig. 1F, 1G). Based on egg counts made during all encounters, no eggs were added, ingested, or otherwise removed from the nest throughout the duration of my observations.

The eggs began to hatch before 0525 h on 6 July (Fig. 1K, 1L), 21 days after my initial discovery of the nest. Based on later examination of shell fragments, all eggs had hatched by 0510 h on 7 July, at which time the female and six hatchlings remained in the nest (Fig. 2). I checked the nest twice on 7 July (0510 h and 1745 h). The female was with six hatchlings in the morning, but she almost immediately abandoned her hatchlings, which made it impossible for me to obtain a focused photograph. When I rechecked the nest about 12 h later, she had returned and was positioned adjacent to the collapsed egg shells and hatchlings; unfortunately, the hatchlings quickly scampered out of the nest when I lifted the cardboard (Fig. 2B). Three hatchlings were in the nest the morning of 8 July (0711 h) (Fig. 2C), but on 9 July neither the female nor any hatchlings were in the nest, and I removed the decaying cardboard from my driveway.

DISCUSSION

A variety of nesting sites have been reported for the Common Five-lined Skink, including cavities in sawdust piles (Mount 1975), excavated cavities in soil underneath rocks (Fitch 1954), within woody debris (Trauth et al. 2004), beneath bark of standing tree stumps (Corrington 1929), and, most commonly, in cavities of decaying logs and stumps (Hecnar 1994; Trauth et al. 2004; Jensen et al. 2008; Niemiller et al. 2013). Females apparently select sites with environmental parameters, such as temperature and moisture content, that are relatively stable (Hecnar 1994). Gravel is likely not ideal for nesting because it exhibits relatively large thermal variation and lower levels of moisture compared to other substrates (Mitchell and Janzen 2019); however, the weathered cardboard under which the eggs were laid likely served to stabilize humidity in the nest and prevent the eggs from experiencing extreme temperature fluctuations. Furthermore, in addition to guarding eggs from predators (Noble and Mason 1933), brooding female Common Five-lined Skinks from other areas also monitor and regulate moisture levels of their clutch by adjusting their body position

FIG. 1. A female Five-lined Skink (*Plestiodon fasciatus*) brooding eggs underneath a cardboard box on a gravel driveway in southern Cannon County, Tennessee during June and July 2019. Eight egg were present from discovery until hatching, although one egg is sometimes obscured beneath the body of the brooding female. (A) 16 June, 1357 h. (B) 17 June 0523 (C) 18 June 0448 h. (D) 18 June 0528 h. (E) 21 June 0506 h. (F) 24 June 0945 h. (G) 24 June 1314 h. (H) 25 June 0903 h. (I) 26 June 0730. (J) 29 June 0608 h. (K) 6 July 0524 h; hatching had begun. (L) 6 July 0521h.

to maintain contact with eggs (Hecnar 1994), and routinely reposition eggs to prevent them from adhering to the substrate or to other eggs (Fitch 1954). Although I neglected to record temperature and humidity conditions of the nest, I assume the female I observed facilitated normal growth and development of the embryos by maintaining almost constant contact with her clutch and frequently repositioning the eggs.

No data exist on average clutch size for populations of Common Five-lined Skinks in Tennessee. Niemiller et al. (2013) indicate that females typically lay four to 14 eggs per clutch,

but this range of typical clutch size is estimated from reports from neighboring states (M.L. Niemiller, pers com.). Fitch (1954) indicates that females in Kansas typically lay 9, 10, or 11 eggs, with a mean of 8.82 for 34 natural nests (the mean increases to 9.5 when counts of ovarian and oviducal eggs are included). Cagle (1940) reports a mean of 9.2 for 26 nests in Illinois (min 6, max 15), and Conant (1951) a mean of 10 for five nests in Ohio (7 to 13). Groves (1982) reports on five clutches of eggs (6, 6, 7, 11, and 8 eggs; $\bar{x} = 7.6$), including one clutch from Virginia and the other four from North Carolina. Based on the size of each of these clutches, mean clutch size of southeastern populations might be smaller than more northerly populations, as suggested by Smith (1946), but discounted by Fitch (1954). Furthermore, variation in clutch size could in part be associated with body size of the female: larger females lay more eggs than smaller females (Ruthven 1911; Fitch 1954). Regardless, I am unaware of any reports of clutch size of natural nests in Tennessee. I did document with photographs several natural nests in southern Cannon County during the early 1990s, but I found only one of these nests early in the incubation period when I am confident that no egg loss, either from predators or the brooding female, had occurred. This clutch, depicted in the species account for the Common Five-lined Skink in Niemiller et al. (2013), also consisted of eight eggs.

Rate of development and, hence, duration of incubation, is dependent on environmental temperatures to which the eggs are exposed (Fitch 1954). Consequently, incubation periods vary widely, even in those situations in which captive females brood eggs in the laboratory, presumably with controlled and relatively stable environmental temperatures. For example, Noble and Mason (1933) report incubation periods of 27, 29, 29, 36, 41, and 47 days for six clutches of eggs from females collected from the same locality and housed in a laboratory. The eggs I found hatched 21 to 22 days after I discovered them; however, I cannot determine precisely the duration of the incubation period because I am not certain when the female laid her eggs. Fitch (1954) suggests that that newly laid eggs are white, but become soiled and darken to a mottled tan color within a day or two after being moved around in their nest cavity, which is primarily in soil underneath rocks in Kansas. Although laid in gravel and never contacting soil, the eggs I monitored never appeared bright white or clean. Based on the dull coloration of the eggs, I assume that the female had laid them at least a few days before I discovered the nest. Furthermore, the clutch consisted of eight eggs when discovered and no eggs were added,

FIG. 2. A brooding female Five-lined Skink (*Plestiodon fasciatus*) and her hatchlings underneath a decaying cardboard box on a gravel driveway in southern Cannon County, Tennessee. (A) 7 July 0510 h. (B) 7 July 1749 h. (C) 8 July0651 h.

indicating that the clutch was complete when I discovered the nest. Thus, I suspect that the eggs were laid sometime during the first or second week of June. Regardless, an incubation period of 22 to 32 days is not unreasonable for natural nests in middle Tennessee. Mitchell (1994) reports incubation period for two clutches of eggs in Virginia as 22 and 32 days, and Cagle (1940) notes that hatching occurred on 23 and 24 July in a clutch of eggs laid on 30 June (thus a 23– or 24–day incubation period). Fitch (1954) does report incubation periods of more than 40 days for several nests in Kansas, but he suggests that periods might be lower in warmer and drier years.

Although primarily discussing brooding behavior, Noble and Mason (1933) indicate that hatchlings seldom remain in the nest for more than a "few hours", but they are not certain if this is a natural response or a result of being disturbed frequently. They also state, however, that one of the seven females they observed stayed with her young for two days after they hatched, so their meaning of a few hours is vague. I did not observe the female facilitate hatching as suggested by Mount (1975), but she did remain with her nest until all eggs had hatched. My observations support the suggestions of others that a female will remain in her nest until all eggs have hatched and that some hatchlings linger in the nest for at least 24 h. Furthermore, my observations indicate that a female Common Five-lined Skink develops a strong bond with her eggs, and that she might not completely abandon them even after repeated disturbances during a three-week period. Fitch (1954) cautions that repeated disturbances might cause a female to permanently abandon her eggs, but more data on the amount, frequency, and intensity of disturbance is required to better understand variables involved in severing the bond between a mother and her eggs. Lastly, this is the first record of Common Five-lined Skinks nesting in gravel and highlights the dearth of published information on basic natural history for one of the more common species of lizards in Tennessee.

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AMBYSTOMA MACULATUM (Spotted Salamander). **BREEDING FREQUENCY**. Previous studies have explored the breeding strategies of *Ambystoma maculatum* (Spotted Salamander) but there is a knowledge gap concerning the breeding frequency of each sex (Husting 1965, Copeia 3:352-362; Harris 1980, Copeia 4:719-722). Finkler and Cullum found that female salamanders have a much higher metabolic cost associated with breeding than males (Finkler and Cullum 2002, Copeia 3:824–829). Because males and females invest different amounts of energy into breeding, we hypothesize that there will be a difference in the breeding frequency of male and female Spotted Salamanders and that breeding frequency will be positively associated with body size. Understanding the breeding frequencies of males versus females is a key parameter in population models, and thus, describing this parameter will reduce bias in population estimates taken from breeding sites and reduce overestimates of population growth in population projection models. Reducing bias in either of these cases improves our ability to accurately identify threats to population stability.

We used data from a vernal pool surrounded by a drift fence and located on the southern Cumberland Plateau in Sewanee, TN (35.223898°N, -85.971171°W). This drift fence contains 52 evenly spaced pitfall traps. This fence is opened from January to March annually and provides a complete census of individuals moving in and out of the pond during the years sampled, starting in 2015. Each salamander that came to the pond to breed received a year specific visible elastomer implant mark, was measured (snout-vent length and mass), and their spot pattern was quantified (Chase et al. 2015, Herp. Rev. 46:192-196). Despite having 7 years of data collection, we only used individuals captured at the breeding site in 2019, 2020, and 2021 to develop individual breeding frequencies during the 7-year period because of the information necessary to accurately identify individuals. Individuals were identified via spot patterns and capture histories as well as checked for consistency in length and mass measurements. We calculated breeding frequencies by dividing the number of years that an individual bred by the number of years between the last and first captured year. These data were separated into male (N = 357) and female (N = 204) salamanders. We assumed that all individuals traveling to the pond were investing energy in the breeding migration because they intended to engage in breeding activity, but we have no way to determine if these activities were successful.

Using a two-way ANOVA to evaluate the effect of body length and sex on breeding frequency, we discovered that body length and sex significantly affect breeding frequency ($F = 46.83$, $df = 1,652$, $p < 0.001$; $F = 7.302$, $df = 2,652$, $p < 0.001$; respectively), but there was not a significant interaction between body length and sex in their effect on breeding frequency ($F = 1.76$, $df = 2,652$, $p = 0.173$). We found that the probability of males traveling to breeding sites was 1.19 times higher than females (males, 0.8 ± 0.012; females, 0.67 ± 0.014). We postulate that differences in breeding frequency between the sexes is due to the energetic cost of female reproduction versus the less costly male reproduction as supported in Finkler and Cullum 2002. Alternatively, scramble competition at breeding sites may require more frequent breeding activity by males to be successful.

We also observed that larger individuals had lower breeding frequencies. There are several possible hypotheses for why smaller salamanders reproduce more frequently. (1) Spotted Salamanders reproduce more often when they are young. (2) Larger individuals have a greater reproductive output or success than smaller individuals during any single breeding season, therefore they reproduce less frequently (Rauch et al. 2014. Behavior. 151:1869-1884). (3) Spotted Salamanders could have a strong negative correlation between body size and survival appearing as reduced breeding frequencies as large individuals experience mortality through time.

Although many Ambystomatid populations are male biased at the breeding site and males remain longer at breeding sites, the relationships between breeding populations and total populations are unclear (Lannoo. 2005. Amphibian Declines: The Conservation Status of United States Species). Return probabilities here are like those observed in Kentucky and Rhode Island, but 2-3 times higher than observed in areas of Michigan, Missouri, and New York. The factors contributing to this variation are unclear. The results from this project suggest that there are sex and size-specific differences that contribute to these biases and that significant geographic variation indicates that these relationships should be established at each field site before proceeding with population modeling.

CATHERINE J. BRATTON (e-mail: brattcj0@sewanee.edu), **OLIVIA WOODY** (e-mail: woodyoe0@sewanee.edu); **KEVIN FOUTS** (e-mail: klfouts@sewanee.edu), and **KRISTEN CECALA** (e-mail: kkcecala@sewanee.edu) Department of Biology, Sewanee: The University of the South, 735 University Avenue, Sewanee, Tennessee 37383, USA.

STORERIA DEKAYI (**Brownsnakes**). **REPRODUCTION AND NEONATAL DEFENSE.** Although documented in other regions, no information has been published on the reproductive ecology or natural history of Brownsnakes (*Storeria dekayi*) in Tennessee. While weeding a garden in southern Cannon County, Tennessee, USA in late July 2022, I found a pregnant female Brownsnake with a total length (TL) of 228 mm. To gather information on timing of birth, litter size, and TL of neonates, I placed the female snake in a small mesocosm constructed with soil and mulch from the garden. I checked the mesocosm daily and, although they could have been born anytime during the previous 24 hours, I discovered five neonates at 0500 h on 7 August (Fig. 1A). The neonates were darker and their neck band was lighter and more distinct compared to those features of their mother (Fig. 1B, C). The mean TL of the neonates was 78 mm, but more than 15 mm separated the smallest (70.5 mm) and largest (89.5 mm) individuals.

A litter of five snakes is below average for the species ($\bar{x}$ = 13, n = 169; Ernst and Ernst 2003. Snakes of the United States and Canada, Smithsonian Books, Washington, D.C., USA), but litters with as few as three young have been reported from other regions (e.g., Virginia, Mitchell 1994. The Reptiles of Virginia, Smithsonian Institution Press, Washington, D.C., USA). The mean TL of the neonates comprising this litter is smaller than the mean snout-vent length (SVL) of 97 mm for 109 neonates reported by Ernst and Ernst (2003, *op. cit.*), although I suspect smaller neonates occur, as they reported neonates with SVL <70 mm. The date of birth I report is within the range of typical dates reported from throughout the species' range (early June to late September; Ernst and Ernst 2003, *op. cit.*) and coincides with dates when I find neonates in southern Cannon County (late July through early September; unpublished data).

While I was photographing the neonates, one of them engaged in aggressive defensive behaviors (Fig. 2). This individual spread its neck and flattened its head and, with mouth agape, began to sway back and forth, first at my camera and then at my left hand, which I waved to the side to better photograph the gaping behavior (Fig. 2A). I slowly lowered my hand towards the neonate, which then lunged with mouth agape (Fig. 2B) several times. Although they attempted to escape by burrowing beneath mulch, none of

the other four neonates showed any signs of aggression. Furthermore, neither the neonates nor the female used other passive defense behaviors I witness routinely in adults, such as regurgitation of stomach contents, defecation, and release of a foul-smelling musk from anal glands. In addition to these behaviors, Brownsnakes throughout their range (e.g., Kansas, Michigan, Virginia) also occasionally feign death when threatened (Liner 1977. Trans. Kansas Acad. Sci. 80:81–82; Hayes 1987 Herpetol. Rev. 18:16–17, Mitchell 1994, *op. cit.),* a behavior not yet reported for individuals in Tennessee.

Brian T. Miller Department of Biology, Middle Tennessee State University, Murfreesboro, Tennessee, USA 37132. (e-mail: brian.miller@mtsu.edu).

FIG. 1. (A) A litter of five Northern Brownsnakes (*Storeria dekayi*) found on 7 August 2022 in southern Cannon County, Tennessee. (B) Head and neck of a neonate, depicting the dark ground color of the body scales, dark coloration of the head blotch, distinct off-white neck band and black collar. (C) The mother and one of her offspring depicting contrasting colorations of the body, neck band, and collar.

FIG. 2. A neonatal Northern Brown Snake (*Storeria dekayi*) in a defensive pose (A) and after a strike with the anterior half of its body fully outstretched (B).

***EURYCEA WILDERAE* (Blue-Ridge Two-Lined salamander). MESOHABITAT PREFERENCE.** Streams are heterogeneous environments with consistent habitat characteristics at the meso-scale like riffles, runs, and pools that are driven primarily by flow and channel characteristics ranging from fast, turbulent flow to slow-moving or even stagnant reaches, respectively. Flow in these reaches also determines sedimentation rates with low frequency of sand and silt in riffles to high frequency of the same materials in pools. Two variables that are predicted to affect larval salamander survival are sedimentation and flow. Therefore, assessing mesohabitat preferences can provide important insight into the habitat necessary for successful completion of larval salamander life history. We used active dipnet surveys to capture larval Blue-Ridge Two-Lined Salamanders (N = 209) between 21 September – 7 November 2014 over three capture events in a 50 m stream reach on the campus of the University of the South in Sewanee, TN (35.204669°N, -85.925149°W). For each capture, we recorded their distance from a downstream culvert and the meso-scale habitat type for each capture. We performed a t-test to determine if the density of Blue-Ridge Two-Lined Salamander larvae differed between runs and pools. Riffles were not observed in this reach. Overall, we observed no preference for either habitat type with captures being representative of the overall availability of each habitat type ($t = 0.269$, $df = 49$, $p = 0.789$). However, salamanders did not use the habitat evenly with some densities of 10-18 captures per m^2 while other areas had no captures over three sampling occasions. This stream has substrate that is primarily sand and gravel making downstream drift likely at lower velocities than streams with coarser substrate (Barrett et al. 2010. Biol. Cons. 143: 1998-2005). Therefore, we expected that larvae may show a preference for habitat with slower velocities like pools, but we did not observe this pattern. These data also support conclusions of others that Blue-Ridge Two-Lined Salamander larvae are not deterred from occupying areas with high sedimentation rates like pools (Keitzer et al. 2019 Freshwater Biol. 57: 1535-1544). More research is necessary to understand what factors contribute to habitat selection in larval salamanders and what drives high density patches.

CLAIRE SANDERS, University of the South, 735 University Avenue, Sewanee, Tennessee 37383, USA (e-mail: sandecm0@sewanee.edu); **SAUNDERS S. DRUKKER** (e-mail: ssd50@txstate.edu), Texas State University, 601 University Dr, San Marcos, Texas 78666, USA; **KRISTEN K. CECALA**, University of the South, 735 University Avenue, Sewanee, Tennessee 37383, USA (e-mail: kkcecala@sewanee.edu)

What's in a 'Game'? A new way of teaching evolution.

Jacob Botello
Auburn University, Biological Sciences, 350 S College St, Auburn, AL 36830

Teaching evolution by natural selection represents a special challenge for educators in biology because of its natural complexity as well as rampant misconceptions in popular culture. Using the Unity engine, we have developed a free, browser-based video game (2D platformer) as an intervention to supplement instruction of evolution by natural selection in university classrooms. Within this game, students control a population of organisms subject to the forces of selection, set narrow-sense heritability, and observe the response to selection at the population level. We aim to investigate the effects of this intervention on student performance outcomes, student engagement, and general attitudes toward science. To do this, we plan to assess students' knowledge of evolution by natural selection in pre- and post-gameplay surveys composed of questions from the concept assessment of natural selection (CANS), questions evaluating attitudes toward science from the Scientific Attitude Inventory (SAI), and additional questions which assess student engagement.

Bog Turtle Movement and Demographics in Northeast Tennessee

Timothy Calhoun
University of Tennessee, Knoxville, Department of Forestry, Wildlife, and Fisheries; 2621 Morgan Cir, Knoxville, TN 37996

The bog turtle (*Glyptemys muhlenbergii*) is a federally threatened freshwater turtle species found primarily in wetlands within the Appalachians. There is only one known natural meta-population of these turtles in Tennessee, a handful of wetlands within one valley in northeast Tennessee. In recent decades the Nature Conservancy has worked to restore and expand these wetlands. This summer (May – August 2022), I conducted capture-mark-recapture surveys at two of these wetlands. I used live traps, camera traps, and random walk probing surveys to capture individuals. I also attached radio transmitters to six individual turtles to assess movement through the wetland using a passive telemetry grid of receiver nodes installed at one of the wetlands. I captured 19 individuals and found a diverse age structure including a 2-year-old juvenile and adults over 30 years old. Additionally, I found individuals had dispersed into the restored wetland areas where previously there had been no records of captures. I am currently conducting deeper analysis of this data using spatially explicit capture recapture modeling to assess population density.

Thermoregulation of pregnant and non-pregnant *Nerodia sipedon* in Middle Tennessee

Alexis Hamous, Vincent Cobb
Middle Tennessee State University, 1301 E Main St, Murfreesboro, TN 37132

Snakes of several species select and maintain higher body temperatures (Tbs) during pregnancy. The leading hypothesis for this behavior is that the rate of embryonic development is facilitated by warmer temperatures and there are optimal temperature ranges for successful embryonic development. Therefore, maintaining a warmer and relatively constant body temperature could be more beneficial than costly to a pregnant snake. In 2021 we used radiotelemetry and temperature loggers in gravid and non-gravid *Nerodia sipedon* in Middle Tennessee to continuously record their body temperatures throughout the typical gestation period. With a sample size of 10 (6 gravid, 4 non-gravid) and total of 6240 Tbs (3744 gravid, 2496 non gravid) in 2021, we found that overall 24-hour mean Tb for all snakes was 27.1 ±0.03°C. The effect of pregnancy did not lead to differences in 24-hour mean snake body temperature (non-gravid snakes =27.0 ±0.05°C, gravid snakes = 27.2 ±0.04°C). Even after using a generalized additive mixed model where body temperature was the response variable and pregnancy status was the main predictor variable and accounting for time of day (in hours), air temperature, water temperature, month, snake ID (individual snakes) and site as random variables, we found that pregnancy had no effect on body temperature. Therefore, our study with *N. sipedon* does not appear to support the idea of snakes maintaining higher body temperatures during pregnancy.

What are the genomic causes of aging variation? An examination of divergent garter

snake ecotypes.

Randy L. Klabacka, Anne M. Bronikowski, Suzanne E. McGaugh, Dawn Reding, Andrew Lithio, Daniel Nettleton, Laurie S. Stevison, Jessica Judson, Tonia S. Schwartz

Auburn University, Biological Sciences, 350 S College St, Auburn, AL 36830

Identifying the genetic mechanisms that contribute to senescence is necessary to understand the targets of natural selection in facilitating divergence in life history strategies. Examination of genetic dissimilarity in natural populations with variation in senescence provides a window to genes and gene networks involved with senescence. Two divergent ecotypes of terrestrial garter snakes (*Thamnophis elegans*) are present in populations around Eagle Lake in Northeastern California (USA). These ecotypes are on opposite ends of the "pace of life" spectrum, with one ecotype having a higher rate of senescence, shorter lifespan, and higher metabolic rate compared to the other. Targeting gene networks associated with senescence in model organisms, we sought to identify patterns in gene expression and sequence variation associated with senescence. Between ecotypes, we identified networks with enriched expression and genes with significant sequence variation. We also examined the functional implications of sequence variation (e.g., effect of mutation on peptide sequence and protein function). Our results largely corroborate those found in model systems, with variation in the same gene networks (and some of the same genes) associated with variation in senescence.

Comparison of Survival Rate from Two Field Seasons of Reintroduced Zoo-raised Eastern Hellbenders

Marley E. Machara, William Sutton, Sherri Doro Reinsch, Dale McGinnity, Rebecca Hardman, Brian Flock

Tennessee State University, 3500 John A Merritt Blvd, Nashville, TN 37209

Zoo reintroduction programs are becoming necessary to re-establish historic ranges or bolster wild populations of species whose numbers have declined due to anthropogenic disturbances. The Eastern Hellbender (*Cryptobranchus alleganiensis alleganiensis*) is an ideal candidate for captive reintroduction due to their fragmented and aging populations across their historic range. In Tennessee, Hellbender populations have declined considerably, especially in stream habitats within the Interior Plateau ecoregion. Due to these declines, our study evaluated the potential of re-introductions to bolster declining populations over the course of two field seasons. During June and July 2021, we released 6 year-old, zoo-raised hellbenders (N=29, Cohort 1 [13], Cohort 2 [16]). During May 2022 we released 4 and 6 year-old hellbenders (N=34, 6 year-olds [15], 4 year- olds [19]). We used a combination of radiotelemetry and PIT-tag technology to assess hellbender movements, habitat use, and survival. We hypothesized that 2022's release would have greater survival rates than 2021, as the 2022 release animals were exposed to earlier environmental conditioning. We conducted survival analysis to compare the survival rates of the two field seasons after the same number of days in the field. After analysis we found that the survival rate for all the 2022 cohorts was higher than the 2021 cohorts. These results suggest exposure to release conditions such as local water and food items as soon as possible, as well as earlier release dates, is key to the success of released captive-reared animals.

Xenobiotic estradiol-17ß alters gut microbiota of hatchling American Alligators (*Alligator mississippiensis*)

Kaitlyn M. Murphy, Madison M. Watkins, John W. Finger Jr., Meghan D. Kelley, Ruth M. Elsey, Daniel A. Warner, Mary T. Mendonça

Auburn University, 330 Funchess Hall, Auburn, AL 36849

Environmental estrogens pose serious concerns for ecosystem and wildlife population sustainability through their influence on a broad range of organismal functions. The gut microbiome is highly vulnerable to environmental influence, yet the specific effects of estrogens on gut homeostasis are unknown because they are poorly studied in wildlife populations. To determine the influence of environmental estrogens (i.e. xenoestrogens) on the diversity and abundance of gut microbiota, we randomly assigned 23 hatchling American alligators (*Alligator mississippiensis*) to three treatments (control, low, and high estrogen concentrations). We predicted that xenoestrogen exposure would decrease microbial diversity and abundance within the digestive tract and that this effect would be dose-dependent. Microbial samples were collected following diet treatments and microbial diversity was determined using 16S rRNA gene-sequencing. Individuals in estrogen-treatment groups had decreased microbial diversity, but a greater relative abundance of operational taxonomic units than those in

the control group. Additionally, this effect was dose-dependent, whereby as individuals were exposed to more E2, their microbiota became less diverse, less rich, and less even. Findings from this study suggest that estrogen contamination can influence wildlife populations at the internal, microbial-level, which may lead to future deleterious health effects.

Among- and within-population variation in reaction norms of brown anole embryos

Mike C Norris, Joshua M. Hall, Daniel A. Warner
Auburn University, 330 Funchess Hall, Auburn, AL 36849

Developmental plasticity refers to the capacity of a single genotype to express more than one phenotype in response to early-life developmental environments. Phenotypic responses to the environment are quantified as reaction norms (e.g., shape of the relationship between the environment and phenotype), which often vary among individuals and populations. Importantly, variation in reaction norms among individuals (i.e., genotypes) within populations provides an opportunity for natural selection to operate on plasticity. Moreover, differences between populations could indicate local adaptation to environmental conditions. In this experiment we examined variation in plasticity within and between populations in the brown anole lizard (*Anolis sagrei*). Lizards were collected from two island populations in the Matanzas River (Crescent Beach, Florida), and their eggs (n=413) were incubated under one of two treatments that mimicked nest sites found in shaded versus open habitat). After hatching, we measured offspring morphology, sprint speed and desiccation rates. Preliminary results demonstrate considerable plasticity in developmental rate and offspring morphology, and the shape of the reaction norms differ among family groups (i.e., genotypes) within populations.

Interactive Effects of Phenological Shifts and Experimental Warming on Larval Wood Frogs

Reese Sloan, Jon M. Davenport
Appalachian State University, Department of Biology, Boone NC 28608

Climate change is an anthropogenic-induced shift to warmer average temperatures globally. As temperature increases, so does variability in precipitation, which directly affects the timing of phenological events. In response many amphibian species have begun to breed earlier. Many larval amphibians experience faster growth in response to higher temperatures and faster drying of temporary ponds. To measure the interactive effects of hatching phenology and temperature on larval wood frogs (*R. sylvatica*), we performed a mesocosm experiment. We deployed 2 treatment levels of hatching phenology (early and late) and temperature (ambient and +1ºC). Tadpoles in late hatching treatments metamorphosed faster than those in early hatching treatments. Mean size at metamorphosis was reduced in +1ºC treatments compared to ambient treatments. Across treatments, survival was highest in +1ºC treatments compared to ambient treatments. There were no interactive effects between hatching time and temperature on growth, time to metamorphosis, or survival. Larval wood frogs experienced faster growth in response to higher temperatures; however, there was a tradeoff in smaller size at metamorphosis. Exposure to freezing temperatures early in the larval period likely decreased survival and resulted in delayed metamorphosis. Responses to environmental variation can lead to plastic responses but could be difficult to predict without further research.

Investigating summer occupancy of small terrestrial salamanders (genus Plethodon) in the Southern Appalachian Mountains

Elyssa Winterton, Jon Davenport, Zachary Farris
Appalachian State University, 565 Yosef drive. Boone, NC 28607

As habitats become more fragmented and amphibian assemblages change, understanding what environmental factors determine the detection and occupancy of Plethodon salamanders is becoming increasingly important. Previous research has found that salamander habitat requirements may be restricted to specific environmental parameters. We investigated the regional occupancy of *Plethodon cinereus* and *P. richmondi* in the southern Appalachian Mountains, on 47 plots surveyed three times during the months of June, July, and August of 2021. We evaluated 16 covariates to explain both occupancy probability and detection probability for each species. Detection probability for both *P. cinereus* and *P. richmondi* was best explained by Julian date with detection becoming more likely earlier in the field season. The top occupancy model for both *P. cinereus* and *P. richmondi* was aspect. Specifically, occupancy has a negative association with northness. Meaning that higher occupancy occurs on southern facing slopes and lower occupancy on north facing slopes. Across all sites

occupancy was 0.35 + 0.09 for *P. richmondi* and 0.35+ 0.08 for *P. cinereus*. Ultimately, detection and occupancy of both species appear to be influenced by date surveyed and slope direction most heavily. This data will serve as the foundation for future models across seasons and to understand how these species coexist.

Professional Oral Presentations

eDNA Sampling for the Streamside Salamander (*A. barbouri*) at a Sumner County, Tennessee Mitigation Site

Anthony Brais
Resource Environmental Solutions, 103 Continental Place, STE 202 Brentwood, TN 37027

The state endangered streamside salamander (*A. barbouri*) is known from a stream & wetland mitigation site in Sumner County, Tennessee. Individuals are difficult to locate given restricted surface activity within intermittent streams during the Dec.– Apr. breeding season. Environmental DNA (eDNA) passively surveys for presence / absence. Samples were collected at 14 locations in Mar. 2022 to establish baseline reach occupancy. Four control samples were collected at a reference location in Mar. 2022. At each sample point, we attempted to extract a ~1000 mL sample volume through a SterivexTM filter. Samples were analyzed by a third-party lab against a Tennessee specific *A. barbouri* assay from Witzel et al. 2020. Surveys were conducted at each sampling point to identify adults, larvae, or egg masses. Preliminary eDNA results failed to detect A. barbori at the mitigation site; a positive detection occurred at the control site. Adults, larvae and egg masses were observed at both sites. Water quality allowed extraction of 1000 mL per sample at the control site. Increased turbidity prevented collection of 1000 mL from all samples at the mitigation site. Lower concentrations of DNA may have been present due to late season sampling or a less robust population of *A. barbouri* given current agricultural land use. Additional sample volume may have been required to achieve positive detection at the mitigation site. Work is ongoing to determine if genetically distinct clades of *A. barbouri* within Tennessee may have influenced detection results.

Multi-Species Survey and Ecosystem Conservation within The Elephant Sanctuary

Jesse Eaker,
Tennessee Wildlife Resources Agency, 5107 Edmondson Pike Ellington Agricultural Center Nashville, TN 37211

Conservation Opportunity Areas (COA's) as identified by The State of Tennessee's State Wildlife Action Plan (SWAP) identifies lands and waters significant for restoring species of greatest conservation need (GCN). The Elephant Sanctuary in Tennessee consists of 3,000 acres that lie within the Western Highland Rim COA. This property provides a significant amount of land that serves as a sanctuary to not only the resident elephants, but to wildlife native to the Western Highland Rim COA. Several species of greatest conservation need have been observed or are suspected to be within the sanctuary, including the four-toed salamander (*Hemidactylium scutatum*), eastern hog-nosed snake (*Heterodon platirhinos*), pygmy rattlesnake (*Sistrurus miliarius*) and eastern pine snake (*Pituophis melanoleucus*). Tennessee Wildlife Resources Agency has implemented an ongoing species inventory project in 2020 to document native wildlife within The Elephant Sanctuary in Tennessee. Between June 2020 and September 2022, 511 individuals representing 42 species of herpetofauna have been identified.

The Protobiome: Identifying Amoebae and Other Protozoans on Salamander Skin

Aubree J. Hill, Nathan Owens, Emily Lannom, and John Gunderson
Tennessee Technological University, Department of Biology, Cookeville, TN 38505

During previous work, high-throughput DNA sequencing was used to characterize the structure of the skin-bacterial microbiome of salamanders. This work, along with other similar studies, identified chlamydia as relatively abundant members of the skin-bacterial community. Furthermore, ecological interaction networks pinpointed chlamydia as influential members of the community. Because chlamydia are obligate intracellular symbionts, interactions involving chlamydia must be explained by the presence and ecological influence of their eukaryotic hosts (e.g., amoebae). However, the presence of such protozoans in the cutaneous microbiome has not been explored. Protozoans are known to play important roles in the soil rhizosphere and the mammalian intestinal tract. We hypothesize that skin-dwelling protozoans play similar roles in the salamander microbiome and, therefore, could impact host health and response to invading pathogens such as *Batrachochytrium*

salamandrivorans. Here we report preliminary findings of an ongoing endeavor to identify members of the so-called "protobiome" using traditional microbiological isolation techniques, PCR amplification of 18s rRNA genes using a peptide nucleic acid (PNA) to block amplification of salamander genes, and high-throughput DNA sequencing.

Tennessee Snake Identification and Education Facebook Group: What We Are Doing & What We Are Learning

Lisa Powers
Froghaven Farm, Bon Aqua, TN

The popularity of internet social media sites makes getting answers both quick and easy. There are many groups on Facebook that address herps; snakes in particular. The Tennessee Snake Identification and education FB group is one of the largest and most popular snake ID groups on FB. We not only identify the snakes and other herpetofauna from our region, we are able to provide education, promote conservation and help ease the fears of many. We also have the ability to give back to science via our interface with the public. I will present some of the things we do that make us different from the other groups, how we help the public, what we are learning and how we contribute to the scientific community.

Venomous Snake Research in Tennessee: The Past, Present and Future

Lisa Powers
Froghaven Farm, Bon Aqua TN

Tennessee has 4 endemic species of venomous snakes that are of medical significance to humans. They are the Eastern Copperhead (*Agkistrodon contortrix*), the Northern Cottonmouth (*Agkistrodon piscivorus*), the Pygmy Rattlesnake (*Sistrurus miliarius*) and the Timber Rattlesnake (*Crotalus horridus*).Tennessee scientists and research institutions have a long history of studying these venomous snakes in Tennessee, including their basic biology and life histories, distribution and ranges, ecological niches, diseases and conservation needs. In the past, researchers were under fewer restrictive policies as set out by the Tennessee Wildlife Resources Agency (TWRA). Have the newer policies currently in use made the researchers and public any safer? Do they unduly restrict researchers and put the safety of the researcher and the snakes in jeopardy? How do these policies affect the treatment of snakebite for researchers currently required to maintain their own antivenin? Do these restrictions further put the public and our wild snake populations in danger because of the lack of current information due to the difficulties imposed by these restrictions? Do we need to re-examine these policies and propose fact-based information and suggestions that increase safety for the researcher, proper handling and care of the snakes, emergency protocols for snakebite treatment and proper safety precautions to protect the public, our scientific community and our venomous snakes?

The Good, the Bad, and Myatt Drive: Updates on recent *Ambystoma barbouri* surveys and a look at the fate of a fossorial amphibian in the urban interface.

David I. Withers
Tennessee Natural Heritage Program, Division of Natural Areas (DNA), Department of Environment and Conservation, William R. Snodgrass TN Tower, 2nd Floor 312 Rosa L. Parks Avenue, Nashville, TN 37243

The state endangered streamside salamander (*Ambystoma barbouri*) has been of intense conservation interest since it was first reported in Murfreesboro in 1996. The presence of the species in a rapidly developing part of middle Tennessee caused speculation as to its ability to cope with evolving suburban and urban habitats. A denizen of often undervalued seasonal channels, occupied sites frequently were reimagined into neighborhoods, commercial centers, and industrial properties. Due to its apparent rarity amid the growing shadow of the urban interface, the USFWS and TWRA broadened staff participation in surveys, outreach, and environmental review for the species, and have supported seasonal breeding and development inventories by DNA staff. Recent surveys have focused on potential breeding habitats in the Central Basin and a portion of the Highland Rim, all within a ten-county region dominated by Nashville. Surveys have primarily been undertaken in Davidson, Rutherford, Wilson, Sumner, and Robertson counties. With help from several collaborators new locations and novel drainages have been discovered, including the Barren River system. In urbanized Davidson County new populations have been found in the Dry Creek, Pages Branch, and Ewing Creek systems. A site discovered in the Myatt Drive industrial corridor revealed some unusual habitat

constructs and resilience that may enable this once robust population to recover and flourish at the newly developed tract. A review of some unusual finds 2020-2022 provides hope for the species even in the most challenging environments.

Student Poster Presentations

Interactions between skin bacteria and the snake fungal disease pathogen (*Ophidiomyces ophiodiicola*) across a nutrient gradient mimicking host skin chemistry

Ori Bergman , Alexander S. Romer, Emily K. Stone, Claire Matzek, Donald M. Walker
Middle Tennessee State University, 1672 Greenland Drive, Murfreesboro, TN 37132

A salient question in microbiome community ecology is whether bacteria interact with fungi in response to specific host chemistries. The snake skin microbiome can be shaped by factors such as dysbiosis induced by a fungal pathogen (*Ophidiomyces ophiodiicola*), disease, and interactions with fungistatic bacteria in the skin microbiome. We explored interactions between bacterial members of the snake skin microbiome and *O. ophiodiicola* across a nutrient gradient mimicking host skin chemistry, hypothesizing that bacterial species would respond differently to *O. ophiodiicola*-modified skin chemistry as it secretes exoenzymes such as keratinases to metabolize host skin. We postulated that as the fungal pathogen modifies the skin environment, a bacterial growth response will be more pronounced. We modeled a time series interaction between bacteria and *O. ophiodiicola* on keratin minimal media as it is the most abundant carbon source composing host snake skin. Bacterial growth curves were measured using absorbance (OD620nm) over a 48 hour time period in response to three different fungal treatments or a control. Preliminary results suggest that both competitive and cooperative interactions explain the growth of 40 different bacterial species across a gradient of fungal metabolism and exoenzyme/metabolite production. These results will inform the broader scientific community on bacterial-fungal interactions in response to host chemistry in non-mammalian systems.

Use of eDNA in the detection of semiaquatic salamanders in eastern Kentucky streams.

Sara A. Brewer, Florene G. Bell, Angie F. Flores,Chi Jing Leow, Ben F. Brammell Thomas A. Maigret, Kenton L. Sena, Ben F. Brammell
Asbury University, 1 Macklem Drive, Wilmore, KY 40390

Environmental DNA (eDNA) utilizes DNA organisms release into the environment to detect their presence and provides an efficient, non-invasive method to determine organism presence/absence. Recent works have emphasized the need for high quality, carefully tested assays for use in species-specific marker studies and the need to validate these oligos in silico, in vitro, and in situ. We developed species specific qPCR assays for three widely distributed species of semiaquatic North American salamanders: Northern red salamanders (*Pseudotriton ruber*), Spring salamanders (*Gyrinophilus porphyriticus*), and Mud salamanders (*Pseudotriton montanus*). Primers and probes were designed based on sequences obtained from locally collected specimens and screened in silico for specificity against nineteen salamander species that occur sympatrically with these species in various parts of their range. Water samples were collected from field sites in eastern KY and used in field validation of these assays. This project provides thoroughly vetted tools that should be useful for future monitoring or range delineation studies of these species.

Adaptive Plasticity of Coloration in Response to Environmental Change

Karissa M. Coffield
Murray State University, 1375 Chestnut St, Murray, KY 42071

When rapid environmental changes occur, different selective forces can create phenotypic trade-offs in which a trait can provide fitness benefits or costs under different environmental conditions. Amphibians are particularly vulnerable to environmental change, and previous research has revealed that some species will plastically respond to variation in temperature and ultraviolet radiation (UVR) by altering their coloration. Divergent selection on coloration may change with elevation and climate induced shifts in temperature because high temperatures are likely to result in lighter color morphs, but as UVR exposure increases darker color morphs will be more common. I will evaluate the adaptive plasticity of coloration in *Ambystoma mavortium*, the tiger salamander, by testing the following hypotheses: 1) increased UVR levels will more strongly affect color plasticity than temperature; 2) older individuals will converge on similar coloration because color plasticity is more important to larval fitness; and 3) coloration affects an individual's fitness. I will compare variation in coloration metrics of wild *A.*

mavortium present at different developmental stages along an elevational gradient in western Colorado. Individuals will be photographed, and coloration metrics will be quantified using ImageJ to compare differences in coloration. The information I gather in this study can be used to further understand both phenotypic shifts organisms face under environmental changes and the consequences of those shifts.

Incubation moisture influences embryo physiology in the Eastern Fence Lizard (*Sceloporus undulatus*)

Lydia C. Dudley, Haley Oakley, Joshua M. Hall
Tennessee Tech University, 1100 North Dixie Avenue, Cookeville, TN 38505

The effects of moisture and heat stress on embryo development have been extensively studied separately in vertebrate ectotherms but combined effects represent a critical knowledge gap given that climate change will alter both temperature and rainfall simultaneously. To understand the interactive effects of moisture and temperature on embryo development, we incubated eggs from Tennessee populations of the Eastern Fence Lizard (*Sceloporus undulatus*) at two moisture concentrations (- 150 kPa and –550 kPa) and measured fitness-relevant phenotypes of embryos and hatchlings. We then incubated eggs in a 2 by 2 factorial design of moisture and temperature. One temperature treatment was suitable for development and the other induced thermal stress. Eggs in the moist treatment absorbed more water but developed more slowly than those in the dry treatment; however, there were no treatment differences in hatchling body size due to moisture. Thus, our moisture treatments influenced physiology but not morphology. The factorial experiment is still underway; however, current data indicate that interactions between moisture and temperature will result from moisture-induced changes in physiology related to water uptake. These foundational studies will establish Tennessee *S. undulatus* populations as a model for understanding the combined effects of altered temperature and rainfall patterns due to climate change. Such knowledge will benefit reptiles of Tennessee and beyond by enhancing our understanding of ectotherm responses to global change.

Student-led surveillance for *Batrachochytrium salamandrivorans*

Xavier A. Jackson, Aubree Hill
355 Mountain View Drive

Batrachochytrium salamandrivorans (Bsal), a deadly fungal pathogen causing declines in salamander populations, is currently spreading rapidly throughout Asia and Europe with the help of the exotic pet trade. Because it is difficult to tell if an animal is infected based on visual inspection in the field, biologists typically test skin swab samples using quantitative polymerase chain reactions (qPCR) to determine infection status. Collecting these samples is crucial for pathogen monitoring. Thus, our goal was to collect skin swabs from amphibians, to help monitor for the presence of Bsal in a global hotspot for biodiversity. Our team of undergraduate Biology students from Tennessee Tech University collected 30 swabs from species known to be susceptible to infection or that are carriers of Bsal. We were careful to keep swab tips sterile and prevent cross-contamination. After swabbing, we recorded snout-to-vent length, species of each animal, and returned them to the exact location of capture. Swabs were placed on ice and shipped to the National Wildlife Health Center for DNA extraction and qPCR. None of the samples collected at our site tested positive for Bsal, and thus far, no other surveys conducted in wild populations of N. American salamanders have confirmed the presence of Bsal. However, continued large-scale monitoring efforts, such as those conducted by the Student Network for Amphibian Pathogen Surveillance (SNAPS), will be critical for early detection of Bsal and conservation of ecologically vital biodiversity of the Western Hemisphere.

Development and validation of qPCR assays for detection of four toed salamanders (*Hemidactylium scutatum*).

Brendan C. Jeffrey, Sara A. Brewer, Florene G. Bell, and Ben F. Brammell
Asbury University, 1 Macklem Drive, Wilmore, KY 40390

Environmental DNA (eDNA) has rapidly become a firmly established method for detecting organisms of research and conservation interest and promises to greatly increase the ease, efficacy, and scope of ecological studies. Recent works have highlighted the need for carefully tested assays for use in species specific marker studies and thorough vetting of eDNA primers using as many local sequences as available. We developed species specific primers for use in qPCR eDNA detection of *Hemidactylium scutatum* and tested these primers in silico and in vitro against sympatric species to ensure specificity. Additionally, we tested these primers against *Hemidactylium scutatum* species

collected in New York and note that they successfully detect specimens from more northern portions of the range of this species. The assays presented here and tested, both in silico and in vitro, should serve as valuable tools enabling the detection of this widespread salamander species.

Quantifying Habitat Parameters for the Southern Appalachian endemic, *Plethodon welleri*

Rosemary G. Ronca, Jon M. Davenport
Appalachian State University, 572 Rivers St, Boone, NC 28608

Plethodon welleri is a small-bodied salamander species that is endemic to select mountain tops in the Southern Appalachian Mountains and considered threatened across its entire range. Initial descriptions indicated that this species was a high-elevation, spruce-fir specialist only found above 1500m. However, recent observations have documented populations as low as 620m. The goal of this study is to assess environmental parameters for habitat preferences and build a comprehensive dataset on species detection and occurrence. To do this, we conducted surveys across North Carolina and Tennessee. Species surveys were combined with environmental parameters to predict population presence. Preliminary data from the 2021 field season indicates that the top environmental covariates for *P. welleri* during summer months was the interaction between humidity and elevation. Specifically, occupancy in the summer months was typically found at higher elevations due to cooler temperatures and lower evaporative rates. In the fall, occupancy was most influenced by the temperature, as populations retreat below ground with freezing temperatures. We expect that the most influential environmental covariates will change by season, as lower temps allow populations to emerge with less threat of desiccation. This study will provide foundational data for this species and other salamander species in this region. Having access to baseline knowledge for threatened species is necessary for the development of effective conservation strategies and comprehensive management plans.

Assessing the health and behavioral effects of turtle racing on eastern box turtles (*Terrapene carolina*)

Michelle K Weaver, John Hewlett, Sharon L. Deem, Jamie Palmer, Maris Brenn-White, Kathleen Apakupakul, Alex Heeb, Christine Light, Christine Casey, and Andrea Darracq
Murray State University, 1375 Chestnut St Murray Ky 42071

Turtle racing involves the public capturing and using largely wild-caught box turtles (*Terrapene* spp.) to compete in races. Turtle races are generally legal in most states because of a lack of regulations and/or funding to enforce regulations that may exist. Currently we lack information on the potential effects (e.g. disease transmission, survival, movements, and general health) of races on the turtles that could help support the implementation and enforcement of regulations. Our objective was to understand the short-term and long-term health and behavioral effects of turtle races on eastern box turtles (*Terrapene carolina*). To address this, we collected 29 box turtles from a race held in Kentucky. We completed physical exams and collected blood samples and oral and cloacal swabs from each turtle. We quarantined the turtles for 2 weeks and then released 19 turtles (weight > 400 g) onto a national wildlife refuge in western KY following negative PCR test results for ranavirus (FV3) and the attachment of a transmitter. We also captured 8 free-living box turtles from the same release location, followed the same methods, and released them with a transmitter at their collection site. We have been tracking the turtles since release and have completed health evaluations. We will continue to monitor the turtles for up to 2 years. No turtles at the race tested positive for ranavirus and we are currently completing laboratory analyses associated with testing for other diseases. We present here the preliminary data on health metrics, disease surveillance, movement, and survival.

Does stress physiology mediate disease resistance?

Megan E. Zerger, Howard H. Whiteman
Murray State University, 1375 Chestnut St, Murray, KY 42071

Panzootic pathogenic fungi *Batrachochytrium dendrobatidis* (Bd) causes the deadly disease chytridiomycosis (chytrid), a primary driver of global amphibian declines and extinctions. The pathogenesis of chytrid is still unclear, as certain species and individuals within a species are differentially affected. Prolonged stress hormone activity deleteriously affects many of the same physiological processes as Bd infections; therefore, elevated stress in response to Bd

may help explain the susceptibility and lethality of this devastating disease. Corticosterone (CORT) is the primary glucocorticoid released by amphibians in response to stress. Life history morph (terrestrial vs. aquatic), sex, and body condition are traits that may influence CORT production within salamander populations because of differences in physiology and environmental conditions. Thus, the objective of our study was to assess how CORT concentration and Bd infection status varies within Arizona tiger salamander (ATS; *Ambystoma mavortium nebulosum*) populations in the Gunnison Valley of Colorado. We used non-invasive sampling methods to collect baseline and elevated CORT from paedomorph and metamorph ATS in June and July 2021. In June and July 2022, we repeated these methods while additionally sampling individuals for dermal Bd spores. This study will provide a greater understanding of the pathogenesis of Bd, and how stress hormones can be used to assess population health and disease susceptibility. Future research utilizing this method could benefit the conservation of threatened species.

O. Ray Jordan: Winner of the 2022 Tennessee Herpetological Society Bob Hatcher Conservation Award

O. Ray Jordan completed a Master of Science degree at the University of Arkansas in 1962 and became a faculty member in the Department of Biology at Tennessee Tech University in 1965. He served in this role for 44 years until his retirement from academia in 2009. He taught a variety of courses including Herpetology, Ornithology, Comparative Anatomy, and several freshman-level classes for majors and non-majors. He was well known as an understanding and passionate instructor, and he was very popular with students and faculty at TN Tech.

During Ray's time teaching Herpetology, his students contributed hundreds of specimens to the TN Tech Herpetology collection. Most of these were collected in the 1960's and 70's. These efforts provided countless opportunities for TN Tech students to explore the habitats of the upper Cumberland and surrounding areas, learning the herpetofauna of Tennessee in the process. Importantly, 280 of these specimens were examined by Dr. Floyd Scott of Austin Peay State University during the 1990's and their data were accessioned into the David H. Snyder Museum of Zoology and used in the creation of the Tennessee Amphibian and Reptile Atlases. As a result, many of these specimens became vouchers for county records in Tennessee.

Ray served as the advisor for 11 graduate students at Tech, and most of them studied various aspects of amphibian or reptile ecology, including work with copperheads, Ambystomatid salamanders, green anoles, as well as various projects of community structure, habitat variation, biodiversity and abundance of herpetofauna on important public lands such as the Arnold Airforce Base and Cherokee National Forest. His breadth of knowledge concerning biology was well known and respected and he often served on committees for students studying diverse taxa. His own work occasionally ventured outside the realm of herpetology as best exemplified by his involvement in the restoration program of Bald Eagles in Tennessee during the late 1980s and early 1990s. In particular, he was instrumental in creating the restoration plan for Bald Eagles at Dale Hollow Lake.

Ray enthusiastically participated in education and outreach efforts outside of the university. He established a live animal collection of reptiles and amphibians at TN Tech, and used these animals extensively for education and to advocate for the conservation of Tennessee's herpetofauna. This live collection is still heavily used today in teaching, research, and outreach efforts by existing faculty and students of the Tennessee Tech Biology Department; thus, it is impossible to recount the number of people, of all ages, who have benefited from the use of these animals. Ray readily accepted invitations to display the animals and to educate the public about their importance at public events across Middle Tennessee. In addition to 3-5 formal presentations per year at special events at the university and local state parks, Ray frequently visited local schools. His presentations were entertaining, as well as educational, and he was well known throughout the Upper Cumberland as the "snake man". Ray also led wild flower walks

at several events, and a section hiking trail at Standing Stone State Park is named in his honor.

Ray was actively involved in service at Tennessee Tech and received the Outstanding Service Award at the University during his tenure. He served on numerous university committees including the Faculty Senate, Institutional Animal Care and Use Committee, and the Teacher Education Committee. He also served as the faculty advisor of the Beta Beta Beta Biology Honors Society. Ray was passionate about education at all levels, and he served as Tech's Representative on the Tennessee Education Association for many years. He also was actively involved in community service and was a member of the Board of Directors of the Putnam County Clean Commission and the Cookeville's Lion's Club for several years, as well as a number of other civic organizations.

Finally, Ray was a charter-founding member of the Tennessee Herpetological Society, and he was actively involved in the organization throughout his career, serving as an officer on several occasions. In fact, Ray was instrumental in the creation of the Chadwick Lewis Memorial Award which has supported the research of more than a dozen students conducting research on Tennessee's herpetofauna since its inception in 2010. Chadwick Lewis completed his master's degree at TN Tech with Ray Jordan serving as his thesis advisor. Under Ray's leadership, Chad conducted a study of seasonal and elevational distributions of plethodontid salamanders on Unaka Mountain in Cherokee National Forest.

O. Ray Jordan is an excellent candidate for the Bob Hatcher Award because his career exemplified in teaching, research, and service a lifetime commitment to the study and conservation of Tennessee's reptiles and amphibians. His greatest contribution to the conservation of Tennessee's herpetofauna is probably his tremendous effort to educate students and the public about reptiles and amphibians with his coursework, specimen collection, and outreach efforts. He established a legacy of excellence in herpetological education at TN Tech that continues to this day and will certainly do so for decades to come.

Joshua M Hall and Daniel Combs
Tennessee Tech University

28th Annual Meetings of the Tennessee Herpetological Society
29 - 30 September 2022
Austin Peay State University, Clarksville, TN

Business Meeting Notes
Recorded by Jessica Grady

Award Recipients
Congratulations to the 2022 recipient of the Chad Lewis Memorial Grant: **Julia Thulander** from Tennessee Tech University. She will use the funds to study thermal adaptation of *Ambystoma barbouri* during early development.

Three students received Niemiller Travel Awards this year: **Timothy Calhoun** from the University of Tennessee at Knoxville, **Kaitlyn Murphy** from Auburn University, and **Lydia Dudley** from Tennessee Tech University.

The award for best student poster was given to **Ori Bergman** from Middle Tennessee State University for presenting results on the interactions between skin bacteria and the snake fungal disease pathogen (*Ophidiomyces ophiodiicola*) across a nutrient gradient mimicking host skin chemistry.

The award for best student oral presentation was presented to **Marley Machara** of Tennessee State University for presenting results on the comparing survival rate from two field seasons of reintroduced zoo-raised Eastern Hellbenders.

The Bob Hatcher Conservation award was given to **O. Ray Jordan**, retired faculty from Tennessee Tech University.

Awards Committee Report
Stephen Nelson informed the society of the committee's plans to add verbiage to the Chadwick Lewis Grant application encouraging historically underrepresented groups to apply. Per decision at the previous meeting, the grant is now a $1000 award.

Outreach and Social Media Committee
Lisa Powers and Carlin Frost continue to be actively involved in the society's social media presence. Please follow and interact with the Tennessee Herp Society on Facebook, Instagram (@tnherpsociety), and Twitter (@TennesseeHerper).

Treasurer's Report
As of the meeting date, the balance in the checking account was $7,084.69 and the investment balance was $25,572.52. These balances reflect the movement of funds from the checking account to the investment account, as approved at the previous meeting.

New Business
Lisa Powers requested the formation of a new committee to consider the effects of current laws regarding venomous snakes on research progress. The motion was approved and Lisa Powers, Aubrey Hill, Danny Bryan, and Bill Sutton were appointed to the committee.

Kristen Cecala, *in absentia*, requested to pass the responsibility of editor for the Tennessee Journal of Herpetology to a successor. A motion was approved to appoint co-editors to replace her. Matt Grisnik and Joshua Hall were appointed as co-editors, and they will assume responsibility for the journal in January of 2023.

Elections
Vice President: Stephen Nelson
Treasurer: Chris Ogle
Middle TN Representative: Matt Grisnik
East TN Representative: Scott Dykes

Check the website for more information on the 2023 THS Meeting!