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Distribution and Diversity of Grotto and Oklahoma Salamanders in Oklahoma Ozarks

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Principle Investigator: Dr. Ronal M. Bonett
                              Department of Biological Science
                              University of Tulsa
                              Tulsa, OK 74104

A. ABSTRACT

The Oklahoma Salamander and Grotto Salamander are endemic amphibians of the Ozark Plateau, and much is still unknown about their distribution, diversity, and biology. For this project we surveyed surface and subterranean habitats for these species throughout the Oklahoma Ozarks. We also analyzed genetic and ecological data to test for significant conservation units and understand the habitat requirements for these species across their life histories. We added 29 new localities for Oklahoma Salamanders and 16 new localities for Grotto Salamanders throughout the Oklahoma Ozarks. Genetic data showed significant geographic structure and diversity for both species. Ecological analyses showed that paedomorphic (permanently aquatic) populations of Oklahoma Salamanders and larval Grotto Salamanders occur in surface streams with porous chert gravel streambeds and stable thermal regimes. Metamorphosing populations of Oklahoma Salamanders occur in streams with more unstable temperatures, but more moist peripheral habitats. These analyses provide important information for conservation planning for Oklahoma and Grotto Salamanders in Oklahoma.

B. BACKGROUND

The **Oklahoma Salamanders**, *Eurycea tynerensis*, is a small plethodontid species endemic to the Ozark Plateau. All individuals of this species have stream-dwelling larvae, while adults exhibit alternative life histories (metamorphic and paedomorphic), and most populations are exclusively metamorphic or paedomorphic (Bonett and Chippindale, 2004; 2006; Emel and Bonett, 2011). The alternative life history modes of *E. tynerensis* were previously considered separate species (Petranka, 1998). The name *E. tynerensis* was restricted to paedomorphic individuals in the western Ozark Plateau (Figure 1), while metamorphic individuals were considered to be part of a more widespread species, *E. multiplicata* (specifically the Ozark subspecies *E. m. griseogaster*). However, recent phylogenetic analyses showed that *E. tynerensis* (paedomorphic individuals) and *E. m. griseogaster* (metamorphic individuals) form a well supported monophyletic group, yet neither taxon is itself monophyletic, and many adjacent populations of metamorphic and paedomorphic individuals are genetically identical (Bonett and Chippindale, 2004).

As a consequence of the historical taxonomy, conservation efforts focused only on the paedomorphic (permanently aquatic) populations (Cline and Tumlison, 2001; Tumlison et al., 1990). Intraspecific alternative life history strategies such as those exhibited by *E. tynerensis*, can

provide important variation for the long-term persistence of a lineage. Although, species that exhibit alternative life history modes present a unique conservation case, because each life history mode may have different habitat requirements and may be vulnerable to different environmental perturbations. The Ozark Plateau is relatively arid, so most small to medium sized streams are seasonally ephemeral at the surface. Paedomorphic *E. tynerensis* require permanent aquatic habitat, and have been found to inhabit streams with coarse gravel beds composed of Ordovician/Silurian chert rock, allowing them to follow the water level down through the interstitial spaces into the ground water during dry summer months (Bonett and Chippindale, 2006; Tumlison et al., 1990; Tumlison and Cline, 2003). However, metamorphic individuals typically live in streams with compact streambeds where they do not have continuous access to water, yet require moist, forested habitat surrounding the stream (Bonett and Chippindale, 2006). Metamorphic populations of *E. tynerensis* in Oklahoma have never been specifically surveyed, and outside of some museum and collection records, the distribution of this life history mode in the Ozarks of Oklahoma is unknown. Identification and conservation of such habitats will immediately preserve metamorphic populations of *E. tynerensis* and other aquatic species that utilize moist ravines. Furthermore, the preservation of ecologically diverse regions (that include paedomorphic and metamorphic habitats) will allow for shifts between life history modes during climactic fluctuations. Therefore, surveys and a comprehensive assessment of known metamorphic populations of *E. tynerensis* need to be compiled with the known distribution of paedomorphic populations in Oklahoma to fully understand the species' distribution. Furthermore, analyses of habitat requirements for metamorphic populations, such as stream characteristics, surrounding vegetation, humidity, soil temperature, etc. need to be combined with previous habitat analyses of paedomorphic populations (Cline and Tumlison, 2001; Tumlison et al., 1990; Tumlison and Cline, 2003) to fully understand the habitat requirements of the species, and conserve habitats for both paedomorphic and metamorphic life history modes.

Recent genetic work on Oklahoma Salamanders also revealed the presence of a very divergent lineage in Sequoyah County based on mitochondrial DNA and one nuclear gene (Bonett and Chippindale, 2004; Emel and Bonett, 2011). This lineage is only known from three primarily metamorphic localities that are approximately 20 km apart. If this lineage does indeed warrant species level recognition then it could be the only truly Oklahoma endemic amphibian, as well as it would have one of the smallest distributions of any North American amphibian. Further genetic analyses (in terms of more genes and specimens) need to be performed to provide a robust test of the distinctness of this lineage. Also, much more intense field survey work is needed to map the distribution and habitat requirements of this putative species.

The **Grotto Salamander**, *Eurycea spelaea*, (formerly *Typhlotriton spelaeus*) is a primarily subterranean species also endemic to the Ozark Plateau (Figure 2; Petranka, 1998; Bonett and Chippindale, 2004). Adult Grotto Salamanders are obligate cave dwellers and exhibit several subterranean adaptations including loss of pigment and degenerate eyes (Petranka, 1998). However, larval Grotto Salamanders have pigment and functional eyes (Petranka, 1998) and are often found in surface streams and springs (Brandon, 1971; Rudolph, 1978; Fenolio, 2003). While wet caves are necessary for adult life, lotic surface habitats may be important for rapid growth and development of Grotto Salamander larvae due to the abundance of invertebrate prey (Fenolio, 2003).

There are estimated to be approximately 500 to 1000 caves in the Ozarks of Oklahoma (G.O Graening, pers. com.) and Grotto Salamanders are only documented at less than 40 of these caves. Records are particularly scarce from northern (Ottawa and Craig) and southern (Sequoyah) counties indicating a need for additional surveys in these regions. Furthermore, the

distribution and ecology of larval *E. spelaea* in spring and stream habitats is limited throughout their distribution. Since many subterranean habitats are inaccessible, mapping the distribution of surface larvae may be the best way to achieve a comprehensive understanding of the distribution of Grotto Salamanders. Furthermore, monitoring surface populations of larvae may prove to be the most effective way to track the status of this species throughout much of the distribution. Studies of the larval ecology of Grotto Salamanders have been limited to two analyses performed at the same cave (Delaware County, Rudolph, 1978; Fenolio, 2003). Several ecological parameters need to be analyzed in order to understand the habitat requirements of larval Grotto salamanders and effectively conserve the surface habitats. The most important parameters to understand are the downstream distributional limits of Grotto Salamander larvae from a groundwater outlet, because it is necessary to know how much larval habitat downstream of a cave (or other groundwater opening) needs to be protected. Information on basic parameters such as stream sediment type, temperature, etc, are important for assessing habitat requirements, monitoring, and restoration of surface habitats for Grotto Salamander larvae.

Grotto and Oklahoma Salamanders are lungless salamanders of the Family Plethodontidae. The Plethodontidae is the most diverse group of extant salamanders with 394 currently recognized species (Amphibiaweb, 2016). This family includes many highly divergent and reproductively isolated cryptic species that are difficult to distinguish based on morphology, and have been revealed through the examination of molecular markers. Currently only a single species of Grotto salamander is recognized across the Ozark Plateau, although historically up to three species of Grotto salamanders were recognized: *E. spelaea* (formerly *T. spelaeus*), *T. nereus* (Bishop, 1944), and *T. braggi* (Smith, 1968). The latter two taxa were synonymized with *E. spelaea* (*T. spelaeus*) because the morphological characteristics used to define them were not discrete (Brandon and Black, 1970). However, recent molecular phylogenetic studies that included Grotto Salamanders revealed multiple geographically structured, deep genetic subdivisions indicating the presence of multiple species across the Ozark Plateau (Bonett and Chippindale, 2004). Based on mitochondrial DNA, there appear to be a few divergent populations of Grotto Salamanders around the periphery of the distribution in Oklahoma. Additional genetic surveys and analyses are necessary to document the distribution of divergent genetic lineages in the Oklahoma Ozarks, and test if these lineages represent distinct reproductively isolated species. In addition to habitat protection, conserving genetic diversity is essential to the long-term health of populations and preservation of species. Therefore, mapping genetic divergence and diversity of Grotto Salamanders is needed to provide information on the regions of highest genetic diversity, which should be the highest conservation priority.

C. OBJECTIVE(S)

We will survey the subterranean and surface distribution of grotto salamanders, and Oklahoma salamanders in the Ozark Plateau to address an information need identified in the Oklahoma Comprehensive Wildlife Conservation Strategy. We will use DNA sequencing and single nucleotide polymorphism (SNP) genotyping to map genetic divergence and diversity with these taxa and test for undescribed taxa. The distribution, life history, genotype, and ecological data collected during this project will be provided in the performance reports, and will be compiled with existing data to deliver a comprehensive assessment of grotto and Oklahoma salamanders.

D. APPROACH

Participants

The participants of this project include: The PI, Dr. Ronald M. Bonett, who is an Associate Professor of Biological Science at the University of Tulsa and Co-PI, Dr. Dante Fenolio, who is the Manager of Conservation and Research at the San Antonio Zoo. Additional personal who contributed to fieldwork, labwork, or analyses included: Dr. Michael Treglia (Postdoc at the University of Tulsa), Dr. Samuel Martin (former doctoral student at the University of Tulsa), Dr. Sarah Emel (former master's student at the University of Tulsa), John Phillips (current doctoral student at the University of Tulsa), Austin Boardman, Grant Robison, Garrett Klutts, Bradley Harris, Jake Collums (former undergraduate students at the University of Tulsa).

Distribution Surveys of Oklahoma and Grotto Salamanders in Oklahoma

During the winter/spring of both 2012 and 2013, two experienced surveyors searched Oklahoma Ozark streams for *Eurycea tynerensis* and *E. spelaea* for between ten and thirty minutes per site, depending on the extent of habitat available and accessible. Searches included all suitable habitats for *Eurycea* larvae and adults, from the center of the stream out to banks within a half-meter of the margin of the wet channel; salamanders rarely occur far from the stream due to their dependence on moist or wet refuges. Search methods varied based on the types of habitat present, but included visual searches under rocks and other cover objects at stream sites with primarily clastic substrates, and shoveling gravel from stream margins onto a screen for inspection at stream sites with substrates of loose, chert gravels. Surveyors also visually scanned the submerged streambed for active salamanders, though salamanders were most commonly found under cover during daylight hours, especially at sites with potentially predatory fish species present. Many sites were repeatedly visited for ecological analyses (2011 to present).

Cave surveys were conducted winter/spring from 2012 to 2014. Surveys were primarily to target *E. spelaea* and typically included visual inspection of cave walls and waterways by at least two experienced surveyors, as well as dip netting in the waterway. The maximum amount of traversable distance was surveyed in each cave. At most sites this was between 25 and 100 meters of searchable distance. In addition, many karst features such as "windows" into the subterranean karst system were also examined.

Genetic Analyses of Oklahoma and Grotto Salamanders in Oklahoma

We collected tissue samples of *E. tynerensis* and *E. spelaea* from throughout their distribution across the Oklahoma Ozarks (Figures 1 and 2). This included 29 new localities for *E. tynerensis* and 16 new localities for *E. spelaea* to add to our existing tissue collection from this region. Tail tips were collected in the field or salamanders were returned alive to the University of Tulsa. These specimens were euthanized in a 1% solution of MS-222 and liver tissue was harvested for genetic analysis and vouchers were used for other morphological studies. All salamanders were handled according to IACUC procedures (TU-0029). For *E. tynerensis* life history status (paedomorphic or metamorphic) was determined in the laboratory or the field by the presence of aquatic features such as external gills and caudal fin on salamanders that exhibited well-developed testicular lobes in males or mature oviductal eggs in females (paedomorphic); or salamanders that had entirely reabsorbed external gills and caudal fin (metamorphic). In some cases, paedomorphosis was further confirmed when females were raised to reproductive maturity in the lab and oviposited fertilized eggs.

DNA was extracted from tissues using Qiagen DNeasy Blood and Tissue extraction kits. 830 to 1118 base pairs (bp) of the mitochondrial DNA gene Cytochrome b (*Cytb*) was amplified from both *E. tynerensis* and *E. spelaea*. *Cytb* exhibits a high degree of variation in salamanders and is commonly used for studies of phylogeography and species diversity (e.g. Chippindale et al. 2000; Martinez-Solano et al. 2007; Moritz et al. 1992), including phylogeographic studies of *E. tynerensis* (Bonett and Chippindale 2004). Amplification was performed by polymerase chain

reaction (PCR) using a variety of primers and standard methods (e.g. Emel and Bonett 2011). Products were checked on 1% agarose gels, and unincorporated dNTPs and primers were removed from PCR products using ExoSAP-IT (USB Corp). Sequencing reactions using Big Dye Version 3.1 (Applied Biosystems Inc.) were performed with either PCR primers or internal sequencing primers designed for *Eurycea*. Sequencing reactions were purified by centrifugation through columns of Sephadex G-50 (Invitrogen Corp.) in 96-well plates to remove unincorporated dye terminators, and sequenced on an ABI 3130xl capillary sequencer at The University of Tulsa. All sequences were edited and aligned using Sequencher v 4.8.

       For genetic analyses of both species, it is important to place the Oklahoma specimens in the context of specimens from throughout the rest of the range of each species in the Ozarks (i.e. Arkansas, Missouri, and also Kansas for Grotto Salamanders). Therefore, in addition to Oklahoma only analyses, we also show broad scale analyses with additional sequences of *E. tynerensis* and *E. spelaea* from across their distributions. Some of these were recently collected sequences from ongoing studies, and others were previously published sequences on GenBank (Bonett and Chippindale 2004, 2006; McKnight and Nelson 2007). *Cytb* alignments were trimmed to 672 bp for *E. tynerensis* and 850 bp for *E. spelaea* in order for all sequences in the alignment to be of the same length. The alignments were unambiguous and had no gaps/missing data. Genetic divergence among populations was measured by calculating uncorrected pairwise sequence divergence (uncorrected P) in PAUP* (Swofford, 2001). Unweighted Pair Group Method with Arithmetic Mean (UPGMA) analyses of genetic distance based on uncorrected P were also performed in PAUP*.

       Mitochondrial DNA markers such as *Cytb* (described above) have great utility in phylogenetics for understanding population and species-level relationships. However, mitochondrial markers also have limitations. The mitochondrial genome is a single molecule that is maternally inherited, and can be passed among lineages through hybridization (Hurst and Jiggins, 2005). Therefore, a phylogeny based on a mitochondrial marker, could be a good proxy for population/species level relationships, or it could be simply a phylogeny of the mitochondrion, and considerably different than the history of the species. It is ideal to test mitochondrial DNA patterns with independent markers from the nuclear genome. Nuclear DNA markers are more slowly evolving and are more difficult to isolate, but have an important advantage in that randomly selected nuclear markers each represent independent estimates of the population history. We used a recently developed method called anchored sequence capture (Faircloth et al., 2012), in which many independent genes can be simultaneously isolated from the nuclear genome and sequenced using next generation sequencing capacities. We adapted this method for salamanders of the genus *Eurycea* and used it to isolate and sequence up to 200 genes from *E. tynerensis* and 251 genes for *E. spelaea*. Our protocol will be described in detail in Phillips et al. (in prep.). Briefly, Genomic DNA was extracted using Qiagen DNeasy extraction kit, and sheered using a Covaris ultrasonicator. Illumina adapter indices were added to the fragmented DNA following Illumina TruSeq DNA LT Protocol. The concentrations of indexed DNAs were determined using a Qbit and equal proportions were pooled for sequence capture.

       A partial *Eurycea tynerensis* transcriptome (sequences of expressed genes) based on a diversity of tissues was produced through Roche 454 and Illumina HiSeq sequencing. Coding regions and exons were annotated with the *Xenopus* (*Silurana*) *tropicalis* exome. A panel of 100 bp RNA "baits" were designed from a partial *Eurycea tynerensis* transcriptome and synthesized by MYcroarray. Sequences were captured during several runs and conditions were modified slightly each time. Captured libraries were sequenced with 300 or 500 cycle version 2 cartridges on an Illumina MiSeq at the University of Tulsa. De novo assembly with high stringency was used in CLC Genomic Workbench to determine continuous genomic regions, which included

one or more exons (anchoring regions) and RNA bait sequences. These regions were then used as a genomic template to map the reads of each sample. Positions with greater than 5x coverage were included in the alignment, and positions with an alternative nucleotide of greater than 25% frequency were scored with ambiguity codes (likely heterozygous positions). For *E. tynerensis*, 200 regions (genes) had high capture efficiency, high data coverage, and clearly captured orthologous genes across all specimens. These genes totaled 101,354 aligned positions for *E. tynerensis*. For *E. spelaea*, 251 regions had high capture efficiency, high data coverage, and clearly captured orthologous genes across all specimens. These totaled 200,574 aligned positions. Genetic divergence among populations was measured by calculating uncorrected pairwise sequence divergence (uncorrected P) in PAUP* (Swofford, 2001). UPGMA analyses of genetic distance based on (uncorrected P) were also performed in in PAUP*. Similar to *Cytb* analyses we included analyses with Oklahoma only specimens, as well as analyses with specimens from outside of the state, to put the data in a broader context. Mitochondrial and nuclear DNA trees were compared to test for congruence in these data sets.

Ecological Analyses
We analyzed ecological variables at different scales, from landscape to experiments within streams. We primarily analyzed ecological variables among 20 select western Ozark streams to assess ecological associations of life history patterns (Figure 3). We also conducted some *in situ* enclosure experiments to test habitat preferences within streams. Our ecological analyses (described below) are organized by ecological variable analyzed: stream temperature, climate, and predator presence, and streambed substrate. To evaluate associations between life history strategy and environmental variability, we used multivariate analyses comparing paedomorphic and metamorphic sites based on stream temperature and local climate characteristics. Unless otherwise stated, all analyses were conducted using specified packages within R version 3.1.3 (R Core Team, 2014). For each stream, we calculated the overall maximum, mean, and minimum temperatures, and the average daily variance in temperature.

     Stream Temperature: Our stream temperature (as well as some climate, streambed substrate, and predator presence) analyses focused on 18 Oklahoma Ozark streams. The analyses presented here also include data from two additional western Arkansas streams which were included to increase the number of metamorphic *E. tynerensis* streams from the western genetic group (see genetic results). In total, eight of the streams had paedomorphic and 12 had metamorphic populations of *E. tynerensis*. Several of the sites have been visited annually for more than a decade. These sites had streams of similar size (first order), and previous work indicated they had a higher likelihood of having at least several months of persistent surface water. *Eurycea spelaea* co-occurred with *E. tynerensis* at three of the Oklahoma Ozark streams (Figure 3), and these streams were also analyzed separately compared to other streams where Grotto Salamanders were not found.

     We used Thermochron DS1921G iButton dataloggers (Maxim Integrated Products) to monitor stream temperatures at these 20 sites. The loggers were set to record temperature every two hours, and were water-proofed with Plasti Dip (Plasti Dip International, Blaine, MN), a black rubberized coating, as described by Grayson and Dorcas (2004). Preliminary work revealed strong currents and flood events could displace dataloggers, making them difficult to retrieve, thus we simultaneously deployed two dataloggers at each site to minimize the potential for data loss. We replaced the loggers and downloaded data every 30-60 days, excluding data from days on which we replaced loggers. At each site, both loggers were in close vicinity of one-another, and differences in temperature recorded were consistently small (≤ 0.5 °C). When only one logger was retrieved, we used the data from that logger, and when both were retrieved, we

averaged their measurements for analyses. Our longest consistent temperature data set for the 20 streams spanned two seasons (from 27 January 2014 through 24 April 2015), excepting a four month period, July through October 2014, when surface water was low or absent at most sites, as is typical in our study region. These were the data used for the analyses presented below. We also collected more fragmentary stream temperature data for subsets of these sites from 2010-2015 (not included in our analyses, but figured below), and the patterns are consistent. For the two season data (27 January through 24 April) we used a metric of inter-annual variability, and also calculated the average absolute difference in the mean daily temperature for each site by day, from 2014 to 2015. To visualize multivariate differences in metamorphic and paedomorphic sites based on these metrics, we ordinated sites using a Principal Component Analysis (PCA) on the correlation matrix of the data, using the 'vegan' package (Oksanen et al., 2013). As a quantitative assessment of differences in stream temperatures between paedomorphic and metamorphic sites, we used a MANOVA on the same variables (normalized to have means of 0 and variances of 1). We report results based on type III sum of squares, as our samples were unbalanced (eight paedomorphic and 13 metamorphic sites), calculated using the 'car' package (Fox and Weisberg, 2011).

Climate: We obtained long-term climate data (Bioclim variables) from the Worldclim dataset (Hijmans et al., 2005). As with the stream temperature data, we conducted a PCA on the Bioclim variables, focused on metrics characterizing temperature and precipitation regimes of the terrestrial environment (Table 1). We conducted the PCA on the correlation matrix of the Bioclim variables, following the same procedures described for the stream temperature data. Preliminary analyses indicated the data did not meet the assumptions required for a MANOVA, thus we used a non-parametric analogue, PERMANOVA (Anderson, 2001), implemented in the vegan package. This analysis is based on comparisons of multivariate distances among sites to randomized permutations, to compute a pseudo-F statistic and respective P-value; it is unaffected by underlying correlation structures of the data (Anderson and Walsh, 2013). We performed this test using Euclidean distances of the Bioclim variables, normalized to have means of 0 and variances of 1, with 9,999 permutations of the data. PERMANOVA has been documented as being sensitive to heteroscedasticity (Anderson and Walsh, 2013), thus we confirmed our data exhibited multivariate homoscedasticity as described for the residuals of stream temperature analyses.

Predator Presence: We sampled all 20 sites for potential aquatic predators (fishes and crayfishes) of *E. tynerensis* (AmphibiaWeb, 2016; Martin et al., 2012). For sites where it was feasible, we sampled using a seine net within 25 m of the 20 datalogger locations; for smaller, narrow streams, we surveyed visually and used dip nets. To test for associations between paedomorphosis in *E. tynerensis* and predator presence we used a chi-square analysis. We tested these relationships separately for crayfish and predatory fish. Given our small sample size, we used 1,000 Monte-Carlo randomizations to compute a p-value.

Streambed Substrate: The presence of chert gravel was previously found to be strongly associated with paedomorphic life histories of *E. tynerensis*, whereas metamorphic life histories were associated with more clastic substrates. We analyzed this variable across the range of the species as well among the 20 select sites for ecological analyses. Additionally, for paedomorphic populations we specifically tested for substrate size selection within streams for populations that differ in body size.

To quantify the substrate size (space) preferences of *Eurycea tynerensis* and other benthic stream species, including potential prey and predators, we conducted a substrate enclosure experiment in a small Ozark stream (Council Hollow, Ottawa County, Oklahoma, USA, Martin et al. 2012). This stream has a dense population of paedomorphic *E. tynerensis*, prey such as

isopods and amphipods, and potential salamander predators such as darters (Percidae) and crayfish (Cambaridae). We prepared 18 enclosures from ~10 liter buckets, which we filled with gravel from dry gravel bars in one of three sizes: small: fit between 4 mm and 12 mm sieve (average volume 0.2 cm^3); medium: slightly larger than 12 mm sieve (average volume 1.7 cm^3); large: "palm-sized" (average volume 13.6 cm^3). Medium gravel was 8.5 times larger than small, and large was 8 times larger than medium. This totaled six buckets filled with each gravel size (small, medium, or large). We sank these into a relatively uniform section of streambed with weak current (Figure 4).

After 16 days, we removed the enclosures one row at a time (simultaneously by three researchers), starting downstream and working up to avoid disturbing enclosures downstream. Enclosures were immediately placed into larger (~20 liter) buckets to prevent escapes. All invertebrates and fishes were removed and preserved in 70% ethanol. Salamanders found in enclosures and additional collected individuals were maintained alive for subsequent laboratory experiments. We also collected live isopods and amphipods representing a range of size classes to determine maximum consumable size by *E. tynerensis* (see below). All vertebrates were handled according to IACUC procedures (TU-0029 and TU-0037). We used one-way analyses of variance (ANOVAs) with Fischer's least significant differences (LSDs) to test for significant differences in the average number of individuals recovered from each substrate size class. These substrate preference analyses were performed for *E. tynerensis*; for their potential predators, crayfish (Cambaridae: *Orconectes neglectus*) and darters (Percidae: *Etheostoma*); and for potential prey, isopods (Asellidae: *Caecidotea*) of edible size and amphipods (Gammaridae: *Gammarus*). The results of this experiment were also published in Martin et al. (2012).

Both paedomorphic and metamorphic populations of *E. tynerensis* vary tremendously in body size. Therefore, we repeated the enclosure array described above in parallel at two sites that differ in body size. Council Hollow (Ottawa Count, small bodied) and Malloy Hollow (Adair County, large bodied). This analysis was conducted to test whether larger bodied salamanders require larger substrate size (i.e. larger interstitial spaces). Specifically, whether there would be a shift in substrate size selection from medium to large gravel in Mallow Hollow where adult body size of *E. tynerensi*s is 2 to 3 times larger (in body volume) than Council Hollow. We also conducted preliminary substrate size analyses across the distribution of paedomorphic *E. tynerensis* in Oklahoma and analyzed the results with phylogenetic comparative analyses.

E. RESULTS AND DISCUSSION

Distribution Surveys of Oklahoma and Grotto Salamanders in Oklahoma
We systematically surveyed 74 surface streams throughout the Oklahoma Ozarks between Spring 2012 and 2013. In addition, we also spot-checked dozens of other streams during the course of the study (2011 to 2015), including some new sites and known localities. Our systematic surveys identified Oklahoma Salamanders at 55 localities. 29 of these are new locality records (Figure 5). 34 sites were metamorphic, 10 were new paedomorphic (Figure 6), and the life history could not be determined at the other 10 sites. Larval *E. spelaea* were located in 10 surface streams/springs, and 5 of these were new site records. 19 surface streams/springs that were systematically surveyed did not yield *E. tynerensis* or *E. spelaea*. 26 caves and 7 other karst features were examined, and of these 20 caves contained *E. spelaea*. In total, at least 16 *E. spelaea* sites were new locality records for this study (Figure 7). These surveys significantly expand the number of known localities for *E. tynerensis* and *E. spelaea* in Oklahoma, especially metamorphic *E. tynerensis* localities. As we move forward a representative set of localities

should be continuously monitored in a systematic fashion to understand distributional and abundance changes over time (discussed also below).

Genetic Analyses of Oklahoma and Grotto Salamanders in Oklahoma
Eurycea tynerensis: Phylogenetic analyses based on *Cytb* (672 bp, 387 individuals) revealed three divergent lineages that correspond to the eastern, western, and southwestern portions of the *E. tynerensis* distribution (Figure 8, this study, also Bonett and Chippindale 2004; Emel and Bonett 2011), with very little geographic overlap of divergent haplotypes. Two of these lineages occur in Oklahoma (Western and Southwestern). The uncorrected pairwise sequence divergence (P) averages $9.97 \pm 0.05\%$ between the Western and Southwestern lineages. Our analyses of 255 Oklahoma samples from 61 localities shows that the Western lineage occurs in all Oklahoma Ozark counties, while the southwestern lineage occurs primarily in Sequoyah County with a few sites in Adair County (Figure 9), and is potentially an endemic lineage to Oklahoma. We have now documented the southwestern lineage at 12 localities, which likely well describes the distribution of this mitochondrial DNA clade. Prior to this study we only knew of 3 localities where his divergent haplotype occurred. Nuclear sequence data from 200 genes including up to 101,354 bp per sample show that representative populations from the mitochondrial DNA group also form a clade (Figure 10). However, at least based on this analysis the southwestern group is phylogentically nested inside of the western group, rather than sister to it. The nuclear data also reveal several other genetic groups of equal divergence within the western mitochondrial group. In other words, instead of *E. tynerensis* including three major geographically structured genetic lineages (as suggested by *Cytb*), there are several geographically structured genetic groups based on nuclear DNA. This is one of the first phylogeographic studies of a salamander with this much data applied to the problem of species boundaries, so there is not a standard for yet interpreting what the date mean in terms of biological species recognition. More fine scale nuclear assessments are needed at the boundaries of these subclades to determine if these groups are indeed reflective of restricted gene flow.

In Oklahoma, paedomorphic populations are in the more northern localities (Ottawa, Delaware, northern Adair and Cherokee Counties), while metamorphic populations are primarily found in southern localities (Sequoyah, southern Adair and Cherokee Counties). Mapping life history (and stream substrate) with respect to the *Cytb* clades shows that the proportion of metamorphic to paedomorphic populations varies among the western, southwestern, and eastern mitochondrial clades. The southwestern clade contains 12 known localities, only two of which are known to contain both paedomorphs and metamorphs. All other localities appear to be metamorphic. This may be in part due to their southern distribution and minimal overlap with chert gravel streams (see ecological results below). Within the western clade, paedomorphic and metamorphic localities are found in an approximately even ratio across the range. However, considering geographic area paedomorphosis is much more common in Oklahoma (Figure 6).

There are strong ecological correlations of life history mode in this species (see ecological results) so it may not be surprising that most populations are either paedomorphic or metamorphic with few sites with mixed life histories. One interesting finding from our fine-scale surveys is the identification of a few localities of mixed life histories in Oklahoma. In one case, a population appears to exhibit facultative paedomorphosis. That is, individuals can reproduce while remaining in their larval phase, but metamorphose when conditions are ideal to do so. We observed this with individuals from Tulley Hollow in the lab, with females laying viable eggs as paedomorphs, and then subsequently metamorphosing. In another case, in populations in the western tributaries to Lake Tenkiller we find areas where paedomorphs and metamorphs seem to co-occur in a stream with restricted gene flow based on *Cytb* and some nuclear genes. We are

now performing fine-scale within population genetic analyses based on more nuclear genes (anchored sequence capture, described above) to test whether this pattern persists with a host of additional independent markers. This also presents an important test case for understanding the genetics of species boundaries and species recognition in salamanders.

For the long-term conservation of *E. tynerensis* it is key to consider the preservation of genetic diversity and life history (Emel and Bonett 2011). This is because genetic diversity is critical to maintaining diversity of the species, a pillar of the field of conservation genetics. Within Oklahoma salamanders in Oklahoma, there are at least two divergent mitochondrial clades and several nuclear DNA clades. Conservation strategies focused on these species should consider preserving habitat that encompasses representatives of each of these genetic groups. At the same time, life history variation is key for the species to sustain itself in the face of changing environmental conditions. We are continuously finding evidence of a heritable basis to life history in Oklahoma salamanders (at least over one generation). Many populations that have offspring raised in the lab exhibit similar morphologies and life histories as their parents. If this life history variation is genetic, then this is indeed genetic variation (for life history) that would be important to preserve in the population. This variation can be observed though laboratory cultivation and crossing experiments as well as determining the genetic differences responsible for life history variation among populations. Understanding the heritability of this variation and mapping it on the landscape is an important component for future conservation research for this species.

Eurycea spelaea: Our broader scale phylogenetic analyses based on *Cytb* (850 bp) revealed three divergent lineages that correspond to the Salem and subdivisions within the Springfield Plateau (Figure 11; this study; Phillips et al., in prep.; Bonett and Chippindale, 2004). All 17 Oklahoma populations of Grotto Salamanders that have been sequenced (covering most of the distribution) are within the western clade of Grotto Salamanders. This is complimented by nuclear sequence data from 251 genes including up to 200,574 basepairs per sample show strong concordance with *Cytb*, in that there are three genetic groups that fall in the same geographic areas. This means that taxonomic revision of the species is likely and historical names may be resurrected. However, even if the taxonomy of Grotto Salamanders across their range has changed, all populations in Oklahoma will still likely be referred to as *E. spelaea*. Changes would only affect Arkansas and Missouri populations. Nevertheless, *Cytb* shows that the most western populations of Grotto Salamanders within the western clade (Hulbert and Pipe Springs) are 4.5 to 7.8% divergent and sister the rest of the western clade. Nuclear DNA (which is much more slowly evolving) shows 1.5 to 0.7% divergence among western populations in Oklahoma. Even though only one of the primary genetic groups occurs in Oklahoma, there is still considerable geographically structured genetic diversity within this clade and this diversity should be considered in the long-term management of the species.

Ecological Analyses of Oklahoma and Grotto Salamanders in Oklahoma
Stream Temperature: In stream temperature comparisons of paedomorphic and metamorphic *E. tynerensis* show that metamorphic localities have colder winter temperatures and warmer temperatures in late spring compared to paedomorphic localities (Figure 14). Metamorphic localities average from 4°C in winter to 20°C in early summer, where as paedomorphic localities on average range from as 10°C in the winter to ~16°C in the early summer. Smaller fragmentary subsets of the data from April 2010 through September 2015 (Figure 15). The PCA of stream temperature characteristics by study site showed clear differences between sites with paedomorphic and metamorphic *E. tynerensis* (Figure 16). Only the first principal component had an eigenvalue greater than one, and thus explained a higher portion of variance in the data

than individual variables, encompassing 74.18% of variance in the dataset. Study sites with paedomorphic populations tended to cluster on the negative side of this axis, with higher minimum and mean temperatures, while sites with metamorphic populations tended to cluster towards the positive side of this axis, with higher maximum temperatures and greater daily and inter-annual variance in temperature. The MANOVA indicates these differences in sites by life history strategy are significant (Pillai's Trace = 0.653; $F_{5,15}$ = 5.642, P = 0.004; ηp2 = 0.131). The moving-window analyses (Figure 14) indicated water temperatures in metamorphic and paedomorphic sites were significantly different in the fall through early spring (November through March), and in late spring/early summer (mid-May through June). In general, paedomorphic *E. tynerensis* had more stable temperatures than metamorphic *E. tynerensis*. This may be due to the porosity of chert gravel streambeds allowing greater connectivity with stable ground water temperatures. Larval *E. spelaea* co-occur in surface streams with paedomorphic *E. tynerensis*, as well as in springs without *E. tynerensis*. By comparison surface-dwelling populations of larval *E. spelaea*, which will be later tied to subsurface habitats as adults, occur in streams with even more stable temperatures than paedomorphic *E. tynerensis* (Figure 17). Larval *E. spelaea* streams ranged from 13°C in the winter to 15°C in early summer. This may also be due the porosity of the chert substrate, but also due to their proximity to groundwater effluents.

   Climate: The PCA of climate data also showed clear differences between sites with paedomorphic and metamorphic *E. tynerensis* (Figure 15), with eigenvalues > 1 for the first three PCs, explaining 55.91%, 30.03%, and 8.58% of the variance in the dataset respectively. In particular, the first PC shows paedomorphic populations are associated with higher seasonality in temperature and precipitation, with warmer wet seasons, whereas metamorphic populations are generally associated with greater precipitation and warmer dry-season temperatures. The second PC also shows some association between metamorphic populations and warmer temperatures. The PERMANOVA shows multivariate differences between paedomorphic and metamorphic sites are significant (pseudo-F = 6.45, P = 0.0003; R^2 = 0.253).

   Predator Presence: Crayfish were present at all eight paedomorphic sites and 11 of the 13 metamorphic sites, and predatory fish were present at all eight paedomorphic sites and seven of the 13 metamorphic sites. The chi-square tests showed no significant associations between life history mode and presence of crayfish (X^2 = 1.3603, P = 0.4885) or predatory fish (X^2 = 5.1692, P = 0.05195). Given that there was no significant association between predator presence and life history mode at individual sites, we excluded predator presence from the phylogenetic logistic regression. In enclosure experiments conducted in Council Hollow. Paedomorphs significantly selected medium size gravel (discussed more below), whereas potential predators such as fishes and crayfishes preferred large gravel. Fish and crayfish are present in most sites with larval *E. spelaea*. Larval *E. spelaea* and paedomorphic populations of *E. spelaea* living on chert may use this substrate as a refuge to avoid predators (see below).

   Streambed Substrate: We know of only a few locations where metamorphs occur on chert and in three of these locations they co-occur with paedomorphs. This is consistent with correlations between several stream substrate variables and life history mode in *E. tynerensis* (Bonett and Chippindale 2006, and Emel and Bonett, 2011). Among the 20 streams where we performed ecological analyses, there was a perfect association between paedomorphosis and presence of chert gravel. This is by far the most correlated variable in the ecological data set. However, it may be that by creating a porous streambed, the chert rocks are allowing for: 1) paedomorphic *E. tynerensis* to access a more stable subsurface environment, or 2) surface stream temperatures to be stabilized due to the more pervasive connection with ground water. Under this scenario it would be the stable stream conditions created by the chert gravel that promotes paedomorphosis. Alternatively, the chert rocks themselves may be causing salamanders to

develop paedomorphosis. We are currently conducting experiments in the lab to test if substrate and/or water temperature influences the developmental timing of *Eurycea tynerensis*. All sites with larval *E. spelaea* in Oklahoma were either on chert gravel (similar to paedomorphic *E. tynerensis*) or were on limestone bedrock pools extending from caves. We did not find localities where larval *E. spelaea* were on clastic gravel substrates.

In field enclosure experiments at Council Hollow *E. tynerensis* primarily colonized a single size class of gravel, and there was little size variation among individuals, preventing us from testing for relationships between body size and space size. Salamander snout-vent lengths averaged 27.75 ± 3.73 mm. The distribution of salamanders was nonrandom among substrate sizes ($F_{2,15} = 14.05$, $P < 0.001$), with a significant preference for medium gravel over small and large ($P < 0.001$ and $P = 0.001$, respectively), and no difference between small and large gravel ($P = 0.298$; Figure 18). This pattern was identical when repeated at Council Hollow, *E. tynerensis* preferred medium sized gravel. Surprisingly, medium sized gravel was also the preferred substrate size of *E. tynerensis* at Malloy Hollow as well (Figure 19). Even though Malloy Hollow *E. tynerensis* are more than a few times larger than Council Hollow *E. tynerensis*, they did not shift to a larger substrate size. This may be due to the fact that even though there is a 2 to 3x difference in body size among these populations, our different rock size categories (small, medium, large) were 8x different. Further tests of substrate size and *E. tynerensis* body size using enclosures, would need to use more fine scale differences in substrate centered around our medium gravel size.

The implications of the ecological analyses presented here are that paedomorphic *E. tynerensis* require chert gravel streams with relatively stable temperature regimes. Metamorphic *E. tynerensis* typically occur on clastic substrates but require climatically stable peripheral environments, presumably for after metamorphosis. *Eurycea spelaea* also occur on chert or limestone bedrock substrates outside of caves in streams with very stable temperature regimes. Both paedomorphic populations of *E. tynerensis* and *E. spelaea* both utilize chert substrates, which may function as a refuge from predators and to access subsurface streams. However, this substrate is utilized as a refuge when chert particles are of medium to large sizes with sufficient interstitial spaces. Chert streambed s with large interstitial spaces also allow for stable subsurface water to modulate surface stream temperatures. Streambeds with very small and highly compacted chert substrate are unlikely to be functionally useful to these species and may also break the connections between surface and subsurface streams. Therefore, the maintenance of paedomorphic populations of *E. tynerensis* and surface larval populations of *E. spelaea* (at any distance from a cave) may require the maintenance of this critical streambed habitat. Presumably erosion contributes to the deposition of fine particles that could fill in interstitial spaces and eliminate this critical habitat.

F. SIGNIFICANT DEVIATIONS:

The field of genomics has greatly transformed over the past 5 years with the advent of methods to collect large amounts of data (next generation sequencing). It is now becoming the standard to sequence hundreds or thousand of regions of the genome to address population genetic and phylogenetic questions. When submitting the original version of this proposal (December 2010), I proposed to analyze nuclear sequence data by developing assays for 20 single nucleotide polymorphisms (SNPs) from 20 genes. This was one of the best approaches at the time but ultimately this would have only provided 20 pieces of information per sample. Therefore, we transitioned our nuclear sequencing aims to next generation sequencing methods, which allowed us to collect data for thousands of SNPS from more than 200 genes. Although it took

considerable development, we moved in this direction because ultimately the next generation sequencing approaches will be best to apply to this (and all) systems.

G. PREPARED BY: _____

Dr. Ronald M. Bonett
Department of Biological Science
University of Tulsa

H. DATE: 12 February 2016

I. APPROVED BY: _____

Wildlife Division Administration
Oklahoma Department of Wildlife Conservation

_____

Andrea Crews, Federal Aid Coordinator
Oklahoma Department of Wildlife Conservation

J. LITERATURE CITED:

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Table 1. List of Bioclim variables (obtained from the Worldclim dataset), used to analyze relationships between life history mode and climate for *E. tynerensis*.

Bioclim Abbreviation	Description
Bioclim4	Temperature Seasonality
Bioclim5	Max Temperature of Warmest Month
Bioclim6	Min Temperature of Coldest Month
Bioclim8	Mean Temperature of Wettest Quarter
Bioclim9	Mean Temperature of Driest Quarter
Bioclim10	Mean Temperature of Warmest Quarter
Bioclim11	Mean Temperature of Coldest Quarter
Bioclim12	Annual Precipitation
Bioclim13	Precipitation of Wettest Month
Bioclim14	Precipitation of Driest Month
Bioclim15	Precipitation Seasonality
Bioclim16	Precipitation of Wettest Quarter
Bioclim17	Precipitation of Driest Quarter
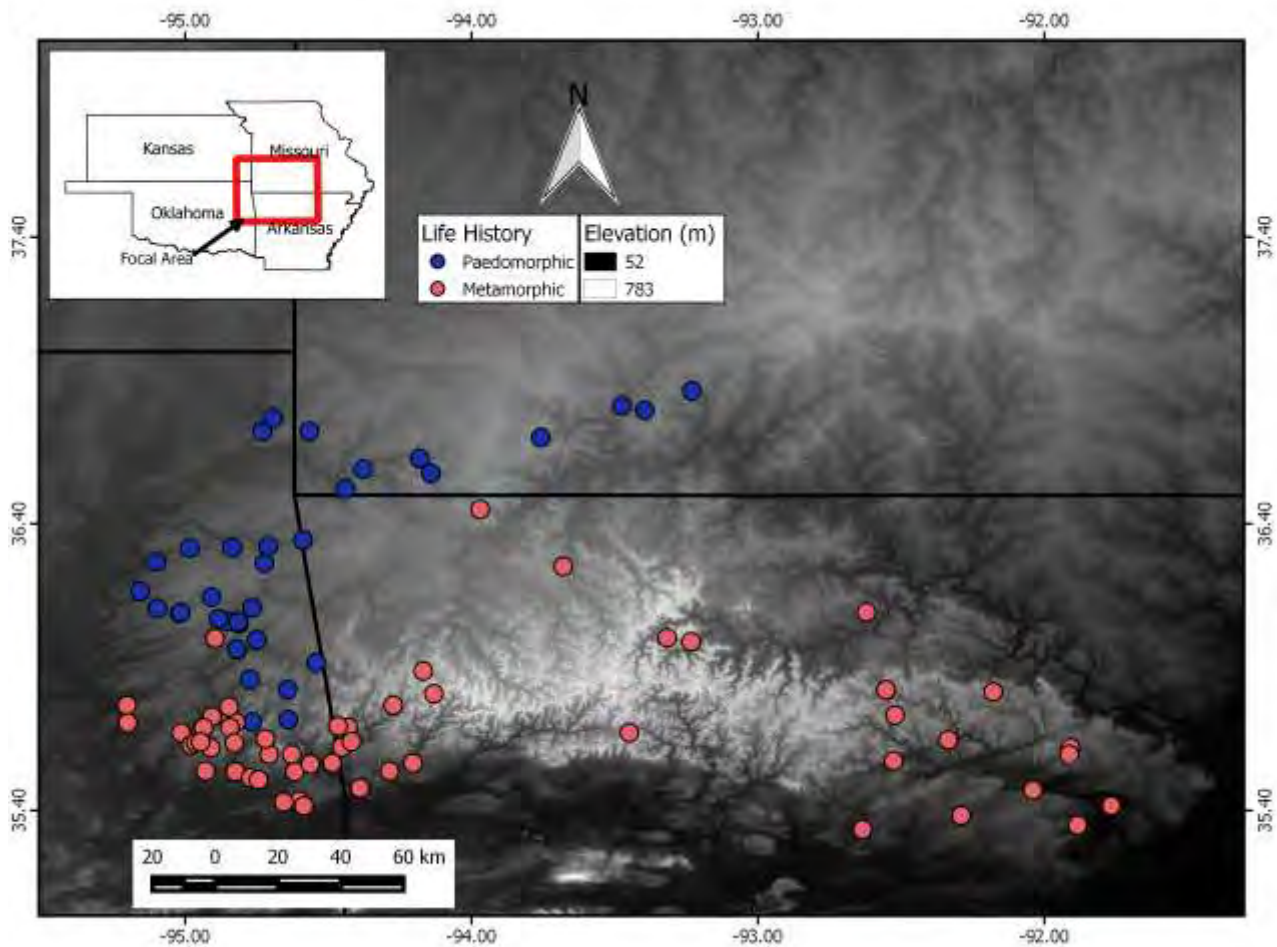
Figure 1. Dot map of the Ozark Plateau that shows the distribution of Oklahoma Salamanders (*Eurycea tynerensis*). Red dots are metamorphic localities and blue dots are paedomorphic localities. This plot covers most of the distribution of the species and their alternative life histories, however, there are more known localities, especially in Arkansas and Missouri. We only plotted localities from our fieldwork (recent and past) where we have evidence of the life history strategy exhibited. The few known facultative paedomorphic locations are not shown, as well the disjunct population in the northern Ozarks (Pulaski Co., MO). New Oklahoma locality records from this study are show in maps presented below (Figure 5).
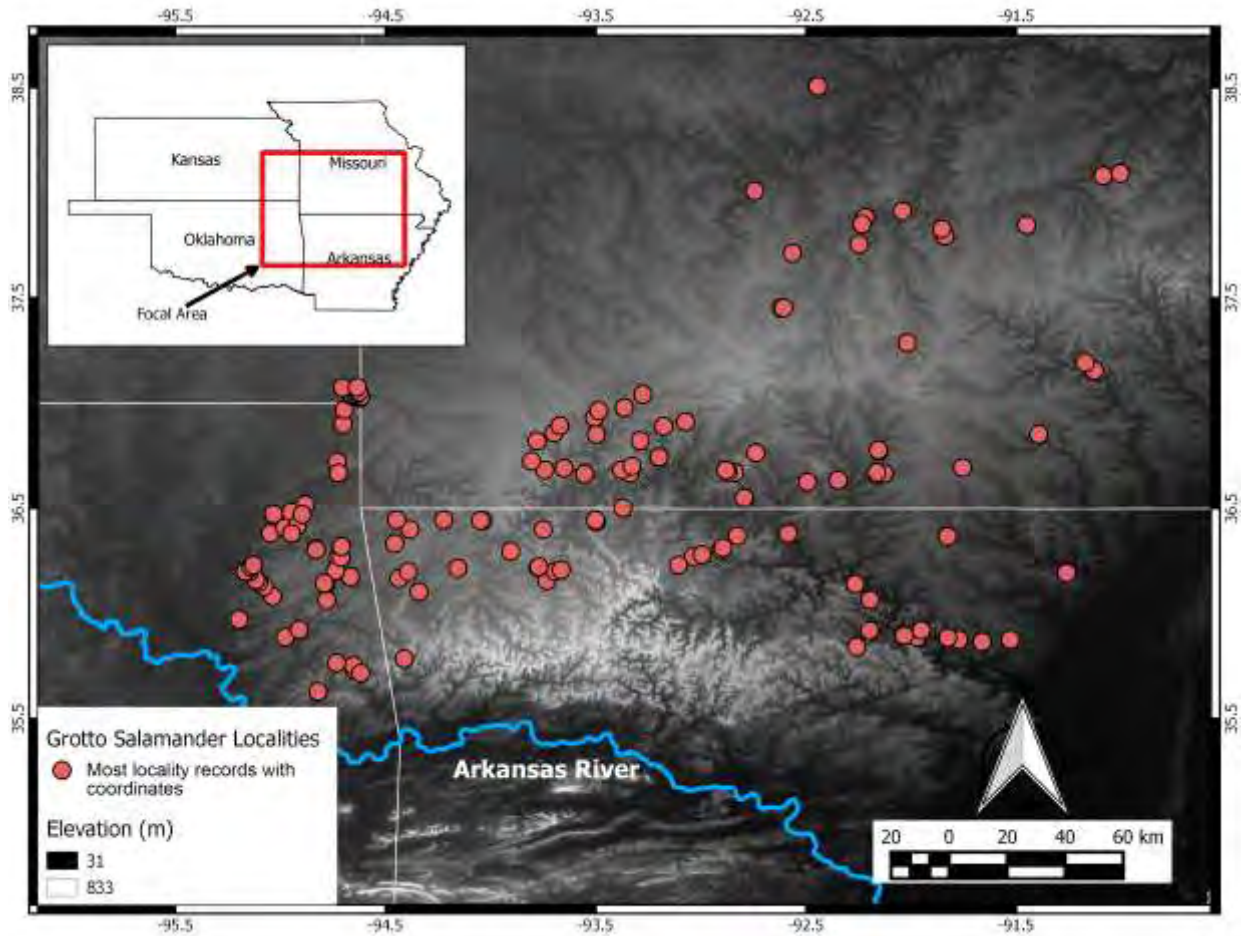
Figure 2. Dot map of the Ozark Plateau that shows the distribution of Grotto Salamanders (*Eurycea spelaea*). This plot covers most of the distribution of the species, however, there are more known localities that are not plotted because they were not georeferenced in databases. New Oklahoma locality records from this study are show in maps presented below (Figure 7).
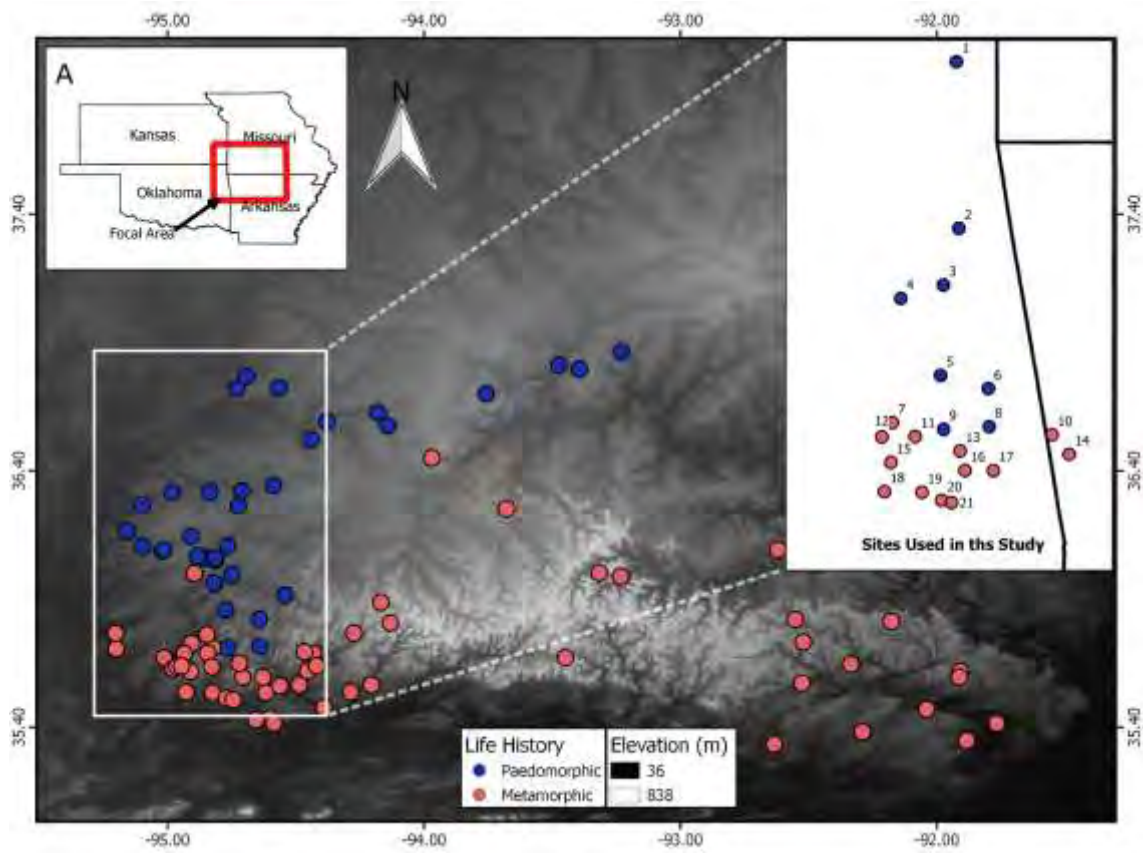
Figure 3. Map of study area, with locations of known populations of *E. tynerensis* and their life histories. The specific study sites are shown and numbered in the inset map (upper right). *E. spelaea* larvae co-occur with *E. tynerensis* three localities (map number 1, 2 and 8).
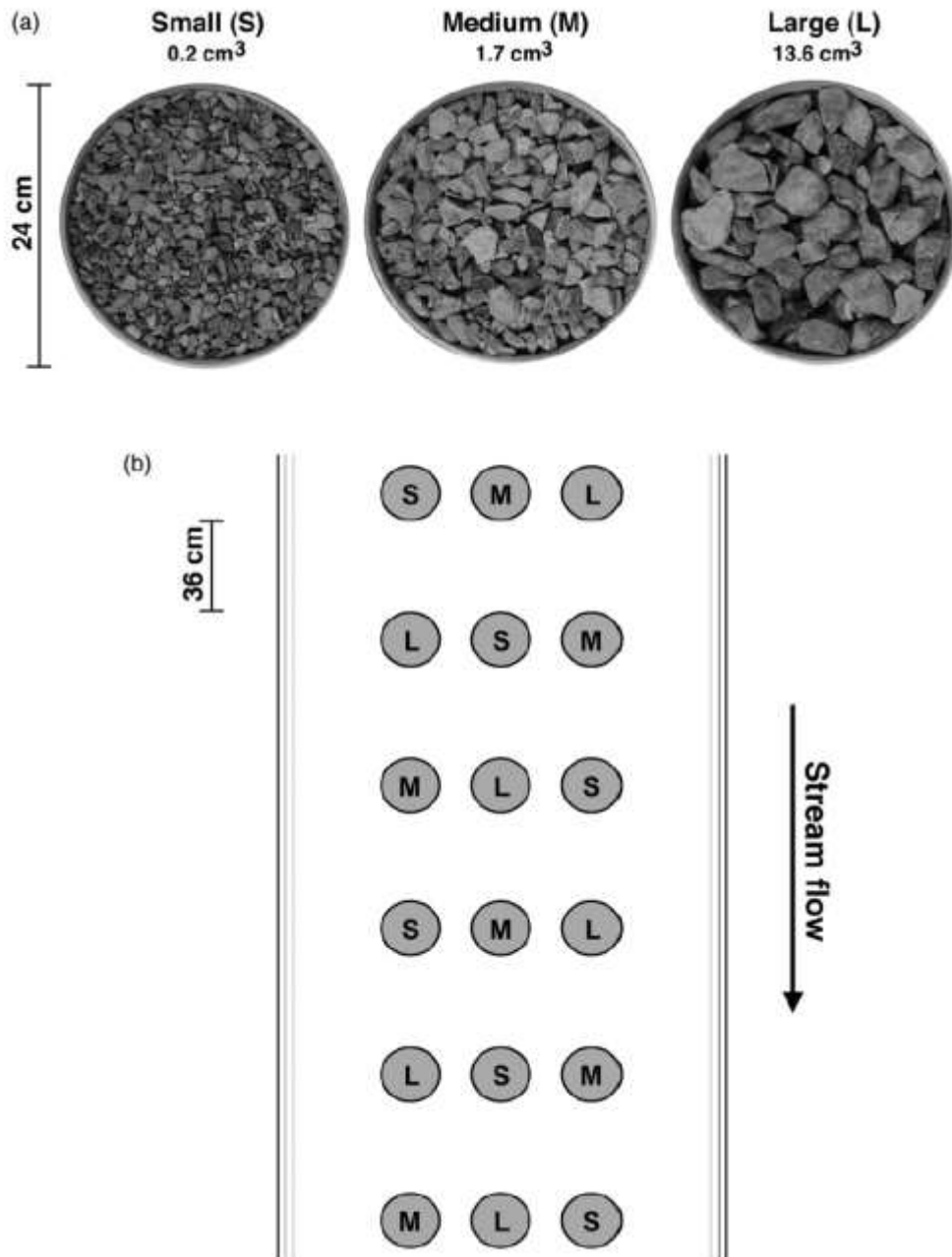
Figure 4. Substrate enclosures and stream experiment layout (Martin et al., 2012). a) Three substrate size classes (small, medium, and large) in ~10 liter bucket enclosures used in this study (scale 24 cm). The average volume of particles in each size class is shown above the enclosures. b) Layout of enclosures (gray circles) in the streambed (approximately to scale), with size of substrate listed in each circle. Vertical rows of parallel black lines represent the stream margins, and arrow indicates direction of current in the stream. Each enclosure had twenty holes (25 mm diameter) drilled on the sides. The tops of the submerged enclosures were level with the surrounding streambed, and holes were positioned facing up and downstream.
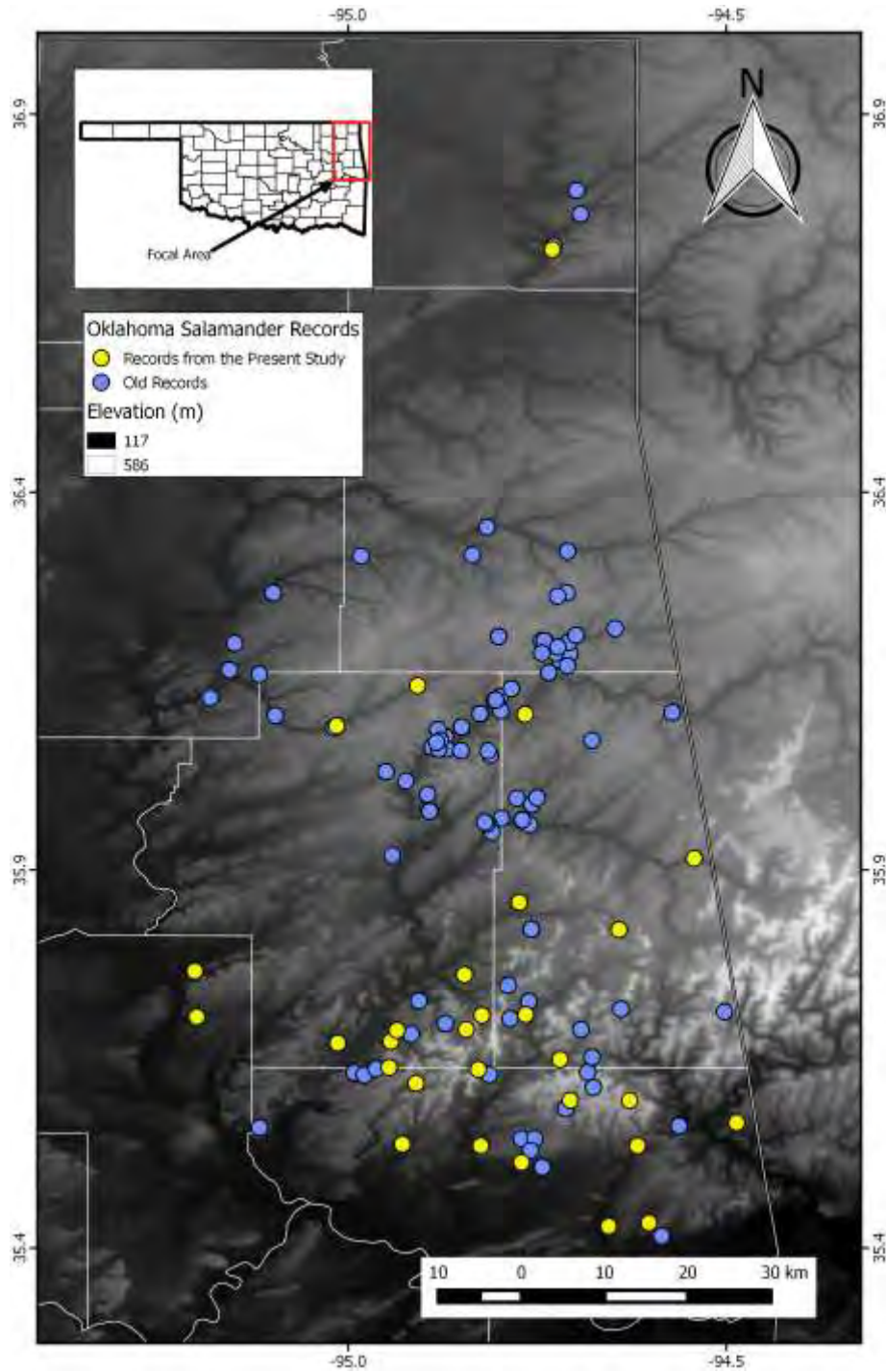
Figure 5. Dot map of the distribution of *E. tynerensis* in Oklahoma. Blue dots represent old records, which includes georeferenced points on VertNet and also specimens from our previous work in this region. Yellow dots represent new locality records from our stream/spring surveys during the course of this study.
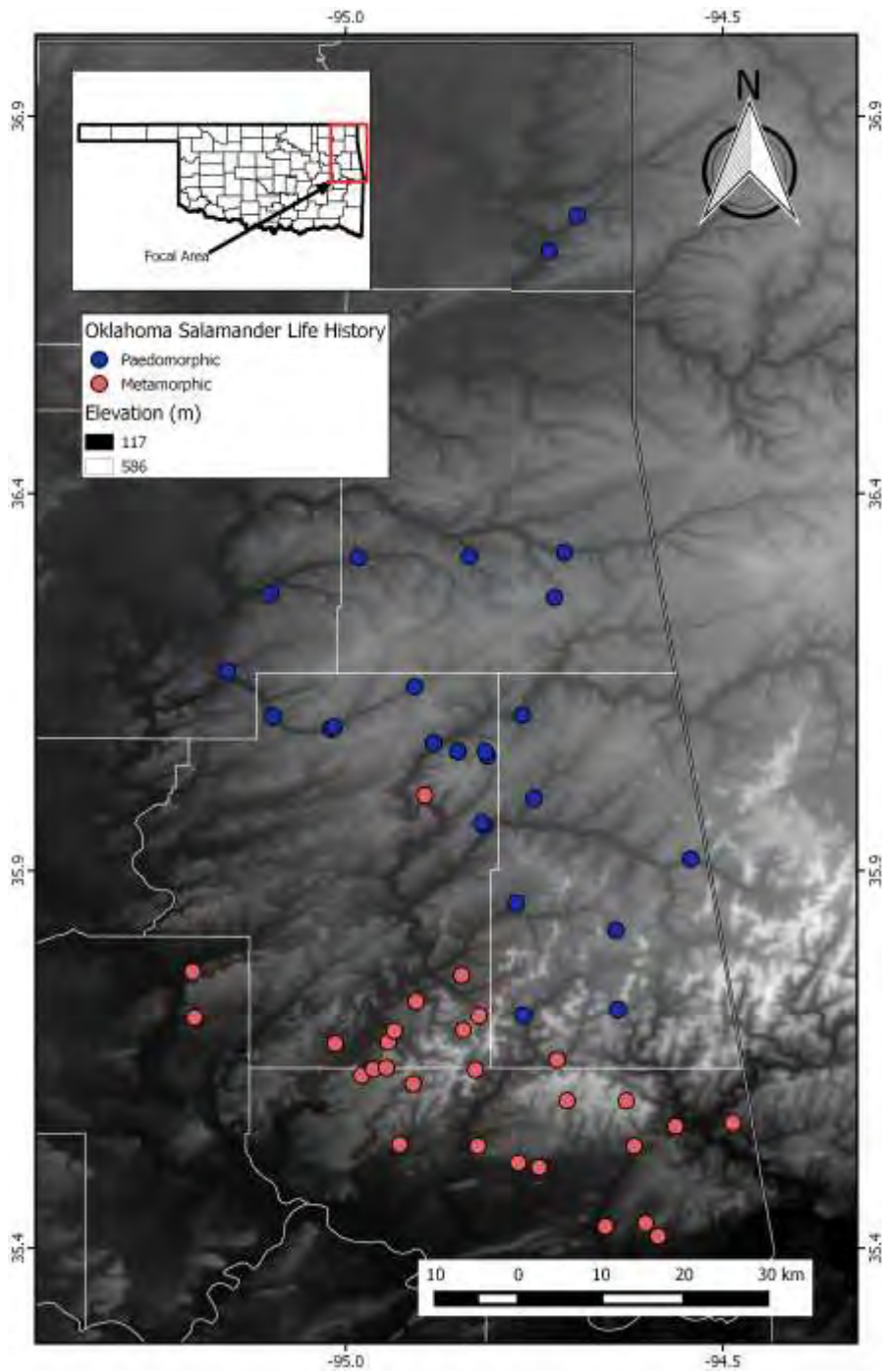
Figure 6. Dot map of the distribution of alternative life histories of *E. tynerensis* in Oklahoma. Blue dots represent paedomorphic localities and red dots indicate metamorphic localities. We only included georeferenced localities where we determined life history from our surveys at the sites. Facultatively paedomorphic sites are not included.
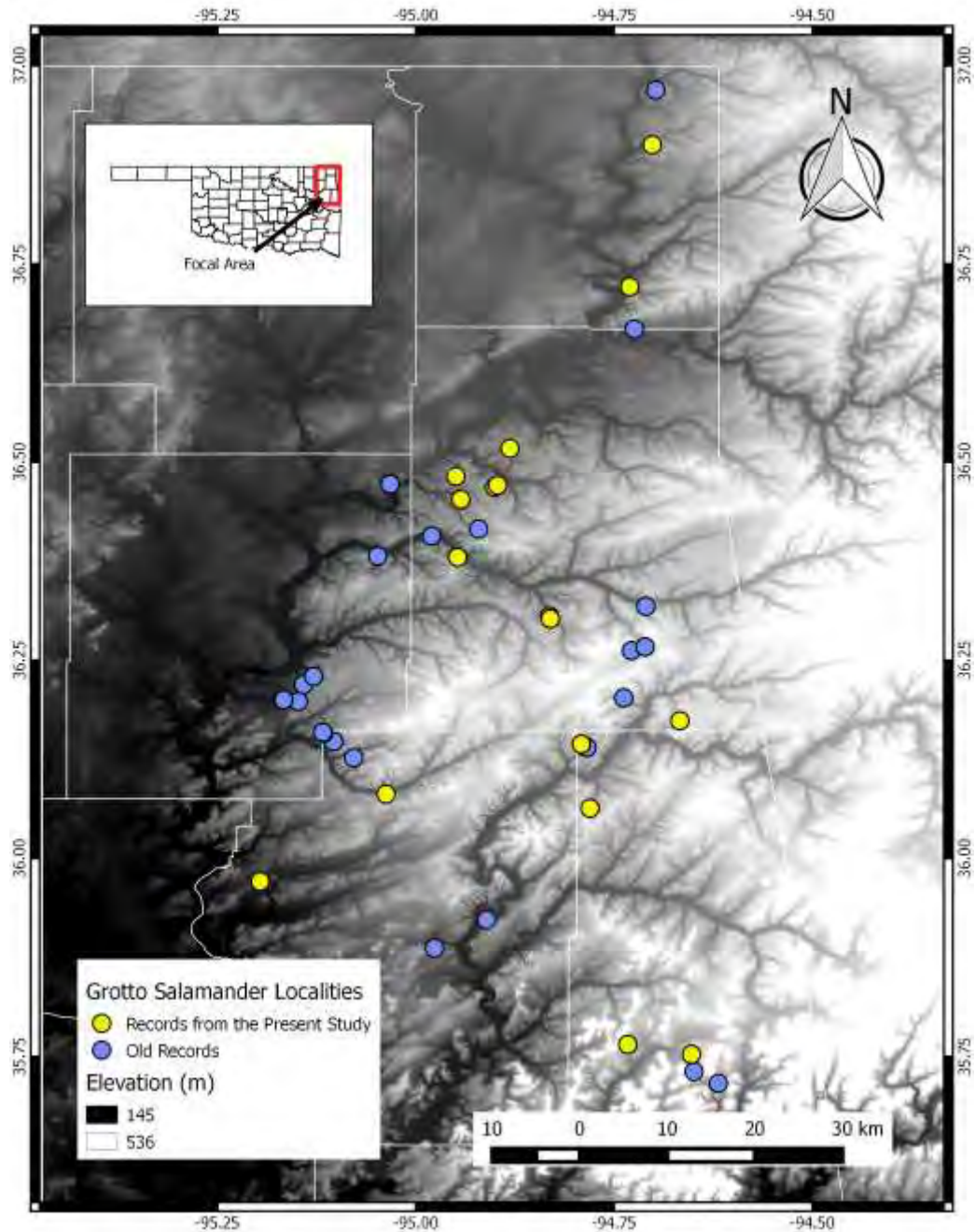
Figure 7. Dot map of the distribution of *E. spelaea* in Oklahoma. Blue dots represent old records, which includes georeferenced points on VertNet and also specimens from our previous work in this region. Yellow dots represent new locality records from our stream/spring surveys during the course of this study. There are more *E. spelaea* localities, especially in caves, although these sites are not accompanied by georeference (only vague locality information).
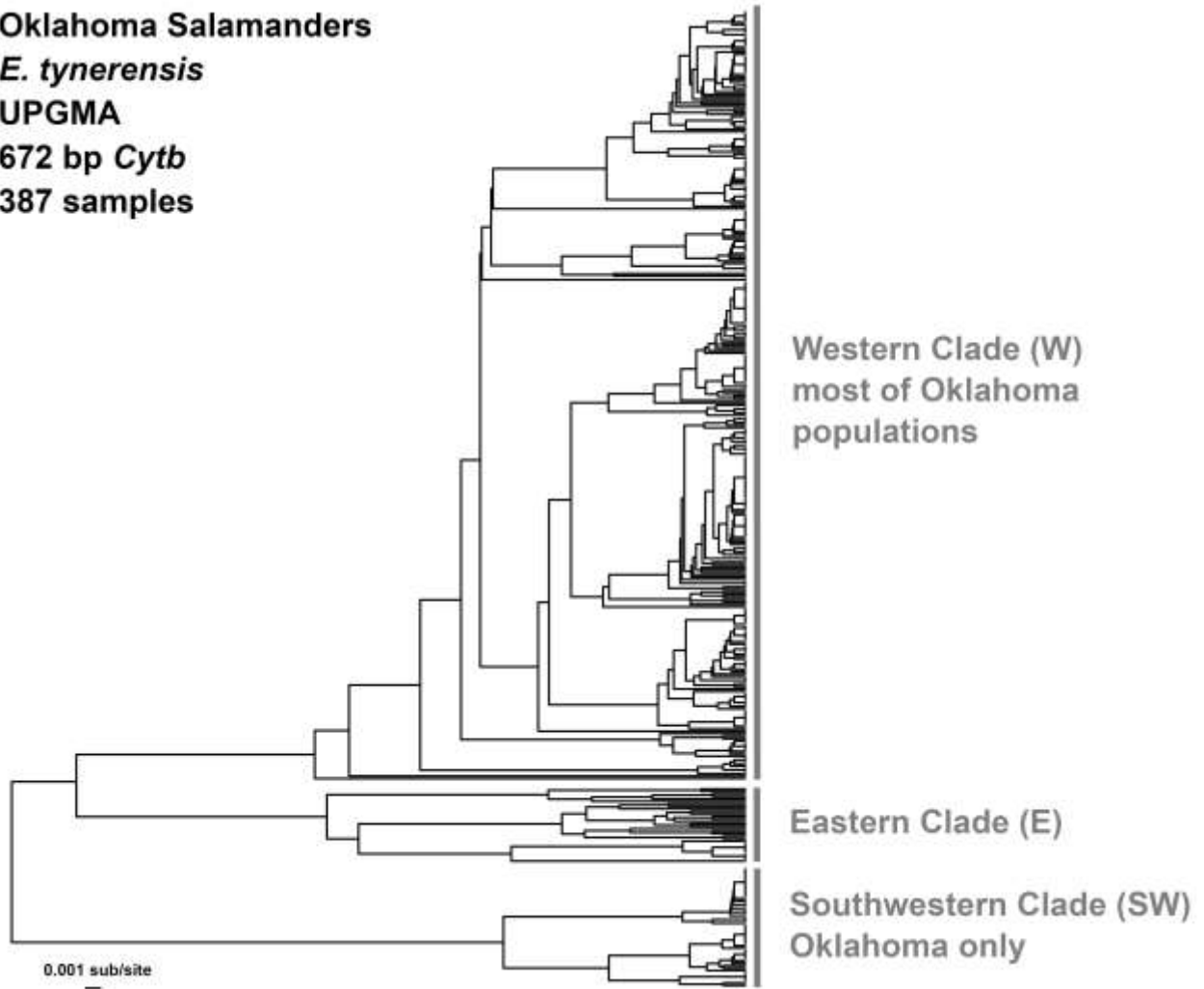
Figure 8. Results of UPGMA analyses of the mitochondrial gene *Cytb* from across the distribution of *E. tynerensis*. This phylogeny includes all of the *E. tynerensis* Oklahoma samples sequenced for this study and all *Cytb* data collected for this species (present and past). All Oklahoma sample are within the western and southwestern clades. See also Figure 9 for more detailed annotations of the populations in the phylogeny with Oklahoma only samples.
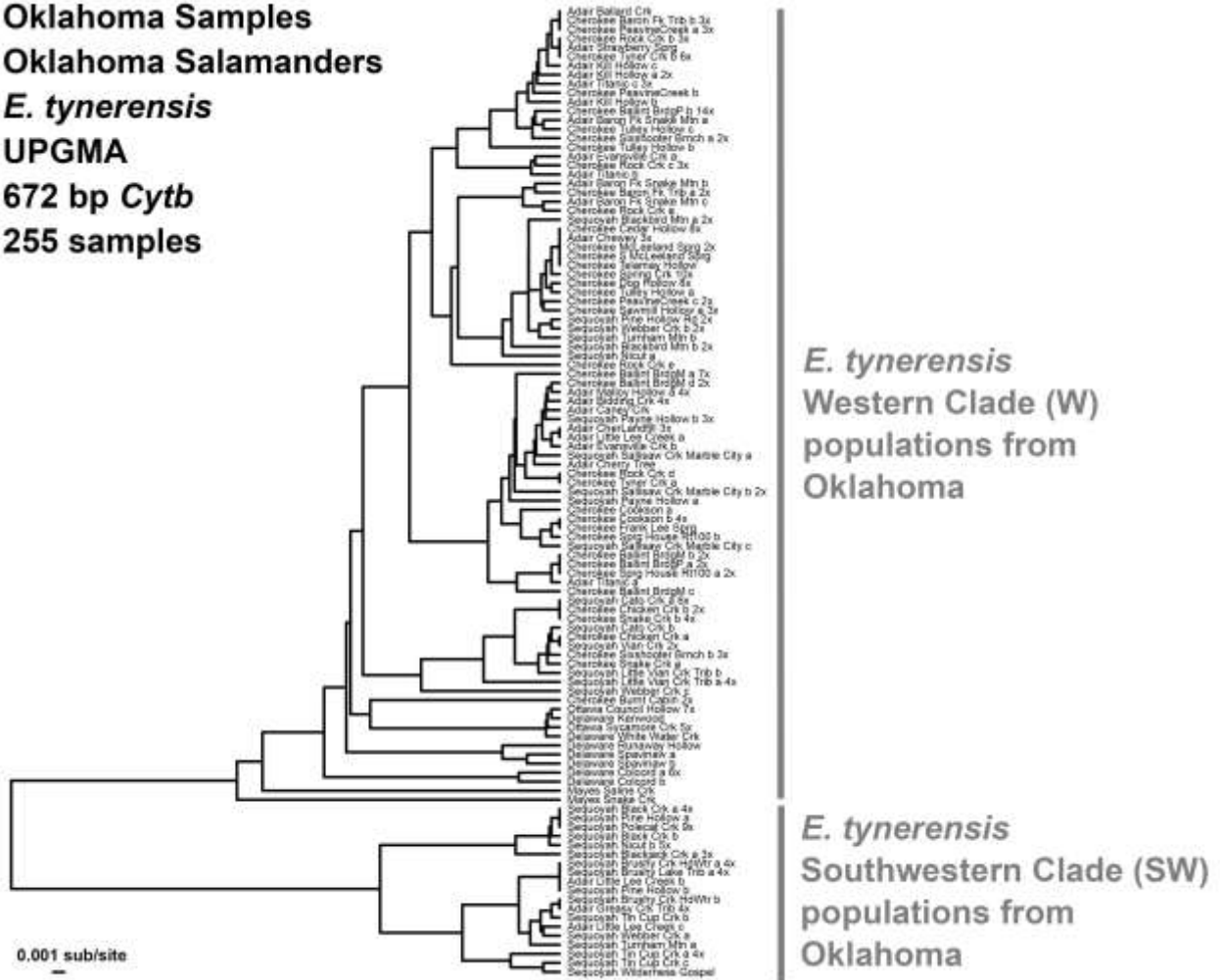
Figure 9. Results of UPGMA analyses of the mitochondrial gene *Cytb* from Oklahoma only populations of *E. tynerensis*. This phylogeny includes all of the *E. tynerensis* Oklahoma samples sequenced for this study in the context of all *Cytb* data collected for this species (present and past). All Oklahoma sample are within the western and southwestern clades (see Figure 8). The tips of the tree are labeled as: County - Locality Name – Haplotype Letter – Number of Individuals. Localities with individuals that have multiple different haplotypes are labeled with letters. In the interest of space, for localities with multiple individuals with similar haplotype (less than 3 mutations difference), we collapsed these down to a single tip (label) on the tree and indicate the number of individuals (#x) that this haplotype represents from the locality. For example, if three individuals from a locality had similar haplotypes then one representative is included on the phylogeny and 3x is appended to the end of the name.

Figure 10. Results of UPGMA analyses of the 200 nuclear genes from 27 Oklahoma populations of *E. tynerensis*. *Cytb* clades (W and SW) are indicated on the right of the phylogeny as a comparison of the two data sets (see also Figure 9). Note that, at least in this analysis, SW populations do form a clade (similar to *Cytb*), but they are nested inside of the western *Cytb* clade. Several other populations form subclades that are equal or greater divergence than the SW clade.
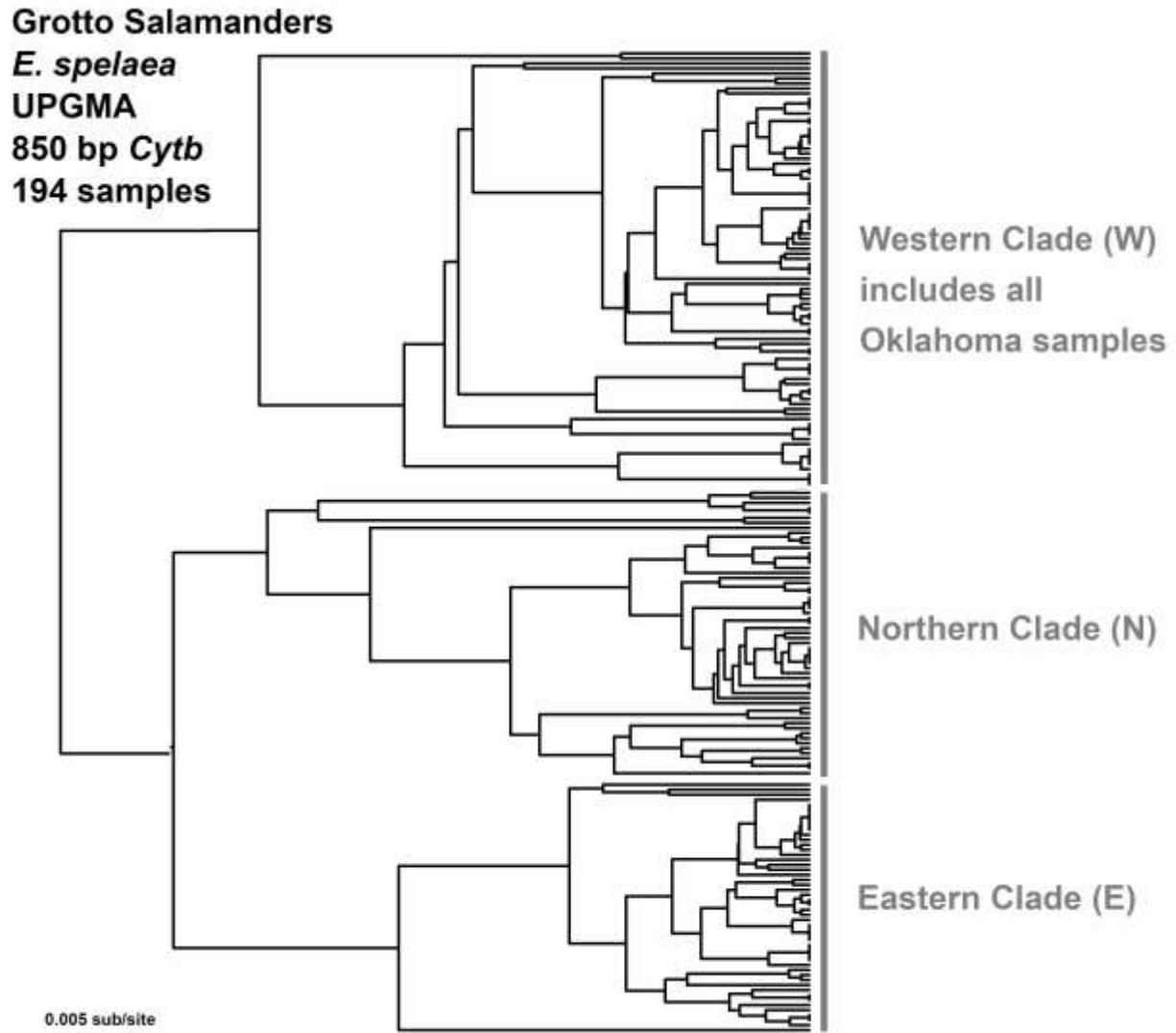
Figure 11. Results of UPGMA analyses of the mitochondrial gene *Cytb* from across the distribution of *E. spelaea*. This phylogeny includes all of the *E. spelaea* Oklahoma samples sequenced for this study and all *Cytb* data collected for this species (present and past). All Oklahoma sample are within the western and southwestern clades. See also Figure 9 for more detailed annotations of the populations in the phylogeny with Oklahoma only samples.
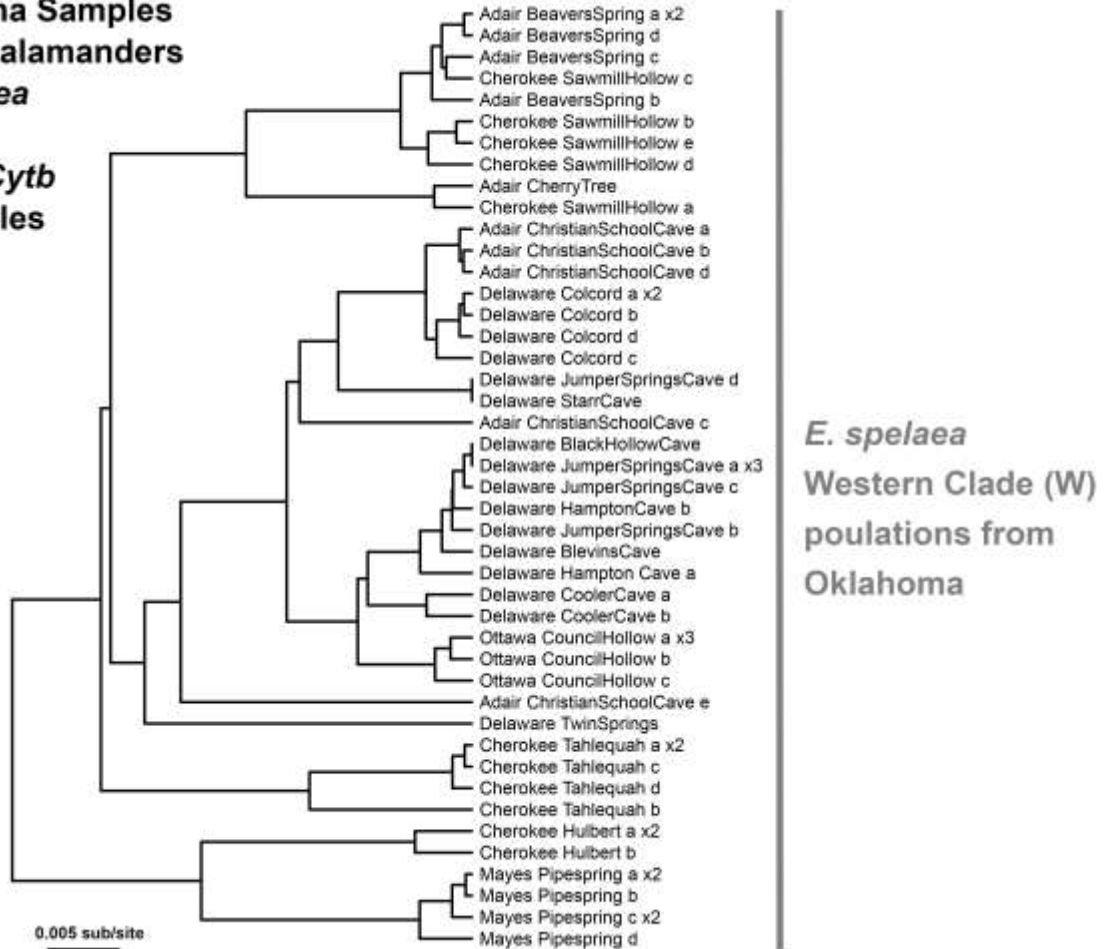
Figure 12. Results of UPGMA analyses of the mitochondrial gene *Cytb* from Oklahoma only populations of *E. spelaea*. This phylogeny includes all of the *E. spelaea* Oklahoma samples sequenced for this study in the context of all *Cytb* data collected for this species (present and past). All Oklahoma sample are within the western clade (see Figure 11). The tips of the tree are labeled as: County - Locality Name – Haplotype Letter – Number of Individuals. Localities with individuals that have multiple different haplotypes are labeled with letters. In the interest of space, for localities with multiple individuals with similar haplotype (less than 3 mutations difference), we collapsed these down to a single tip (label) on the tree and indicate the number of individuals (#x) that this haplotype represents from the locality. For example, if three individuals from a locality had similar haplotypes then one representative is included on the phylogeny and 3x is appended to the end of the name.
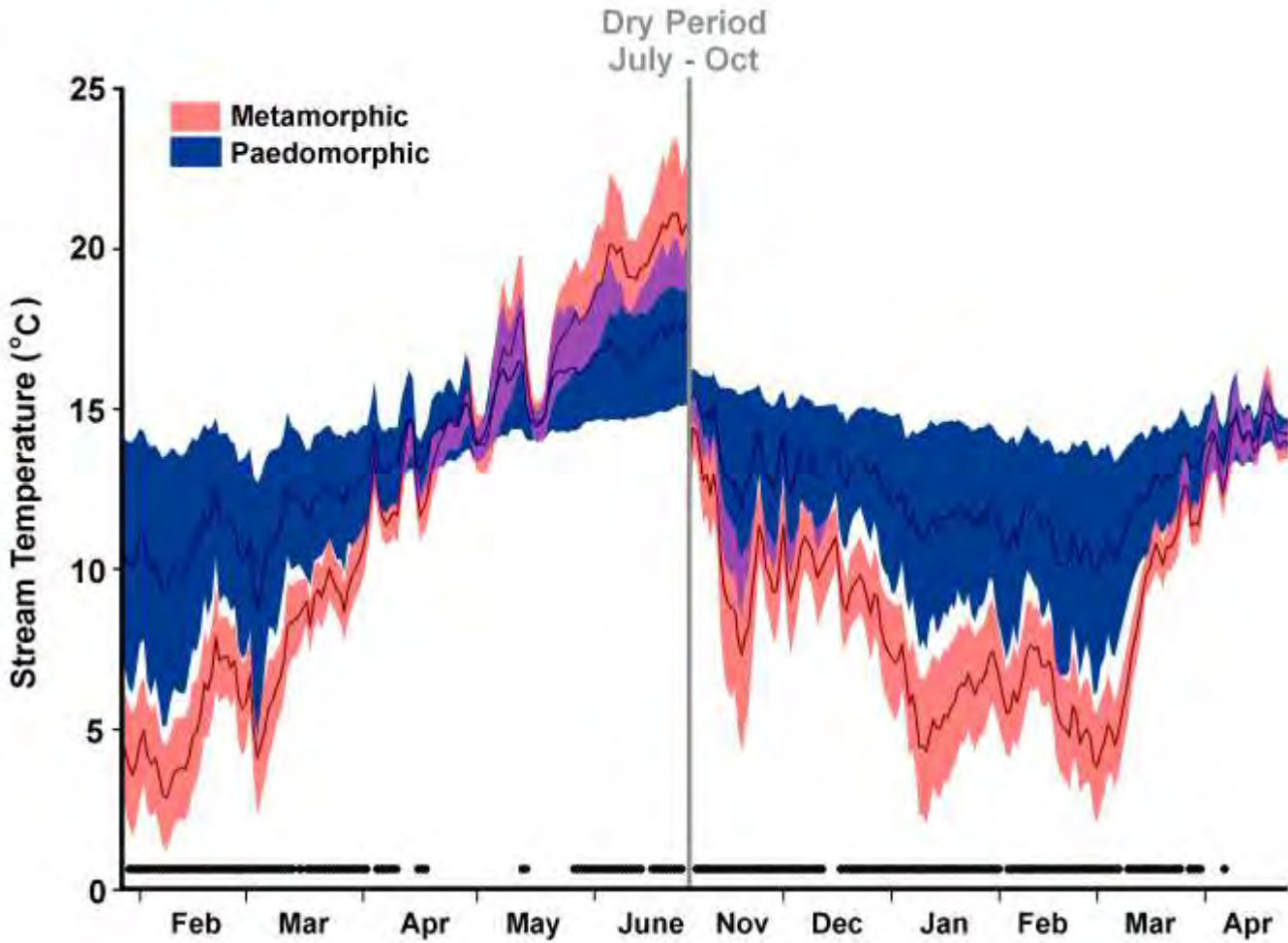
Figure 13. Results of UPGMA analyses of the 251 nuclear genes from 9 Oklahoma populations of *E. spelaea*. *Cytb* clade (W) is indicated on the right of the phylogeny. Most populations show modest divergence, with the deepest divergence between northern and southern localities.
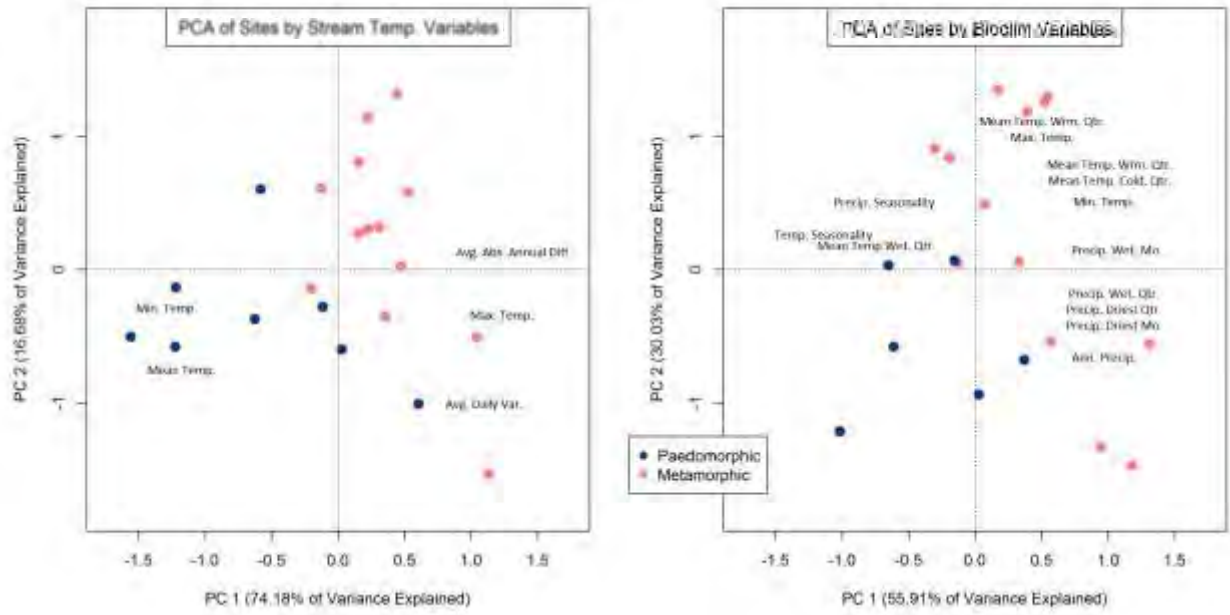
Figure 14. Daily mean of stream temperatures, averaged across paedomorphic and metamorphic sites for 2014 and 2015 (+/- 1 SD). Dots across the bottom of the figure indicate times during which metamorphic and paedomorphic sites had significantly different daily average temperatures based on Wilcoxon tests described in the text.
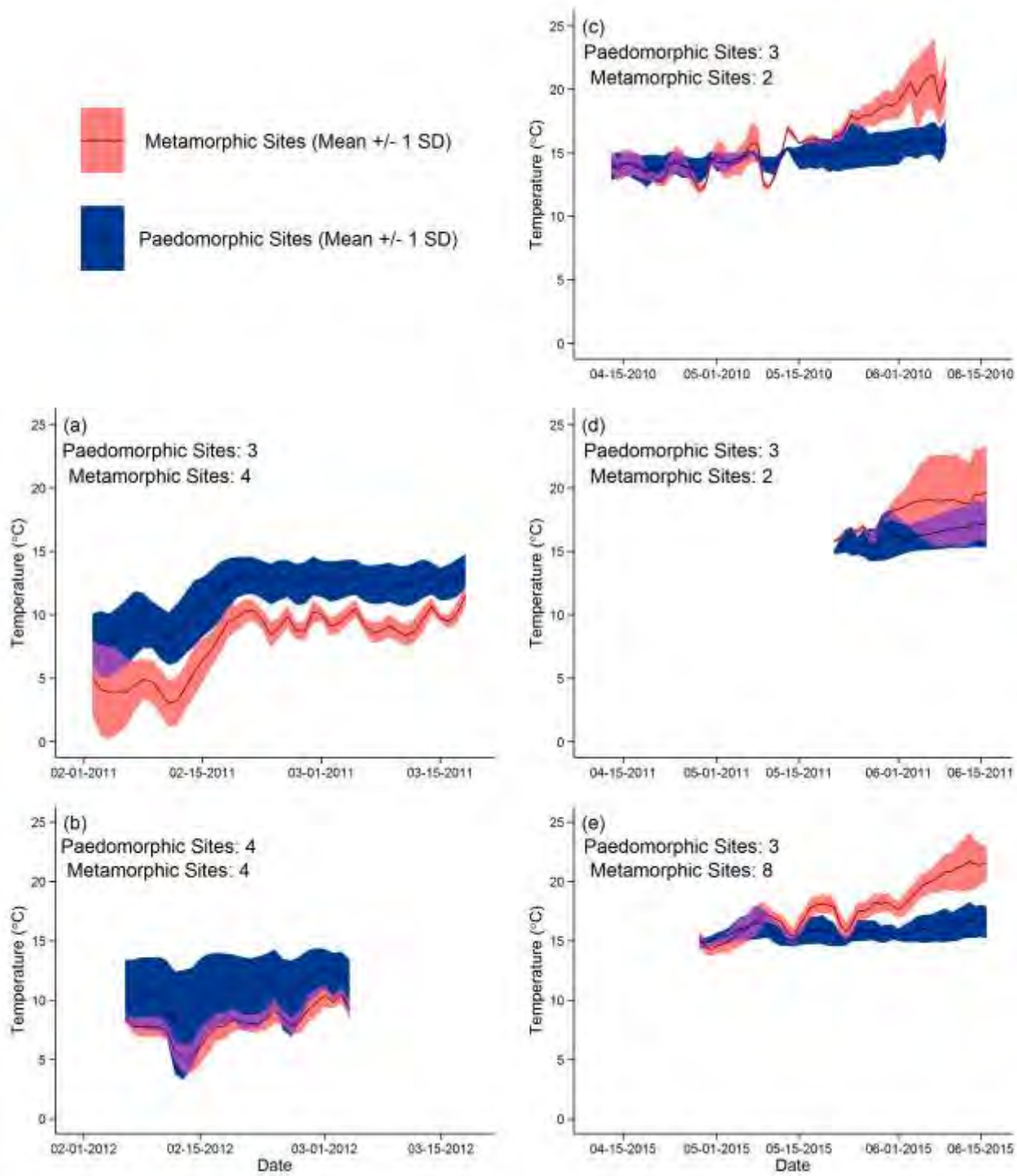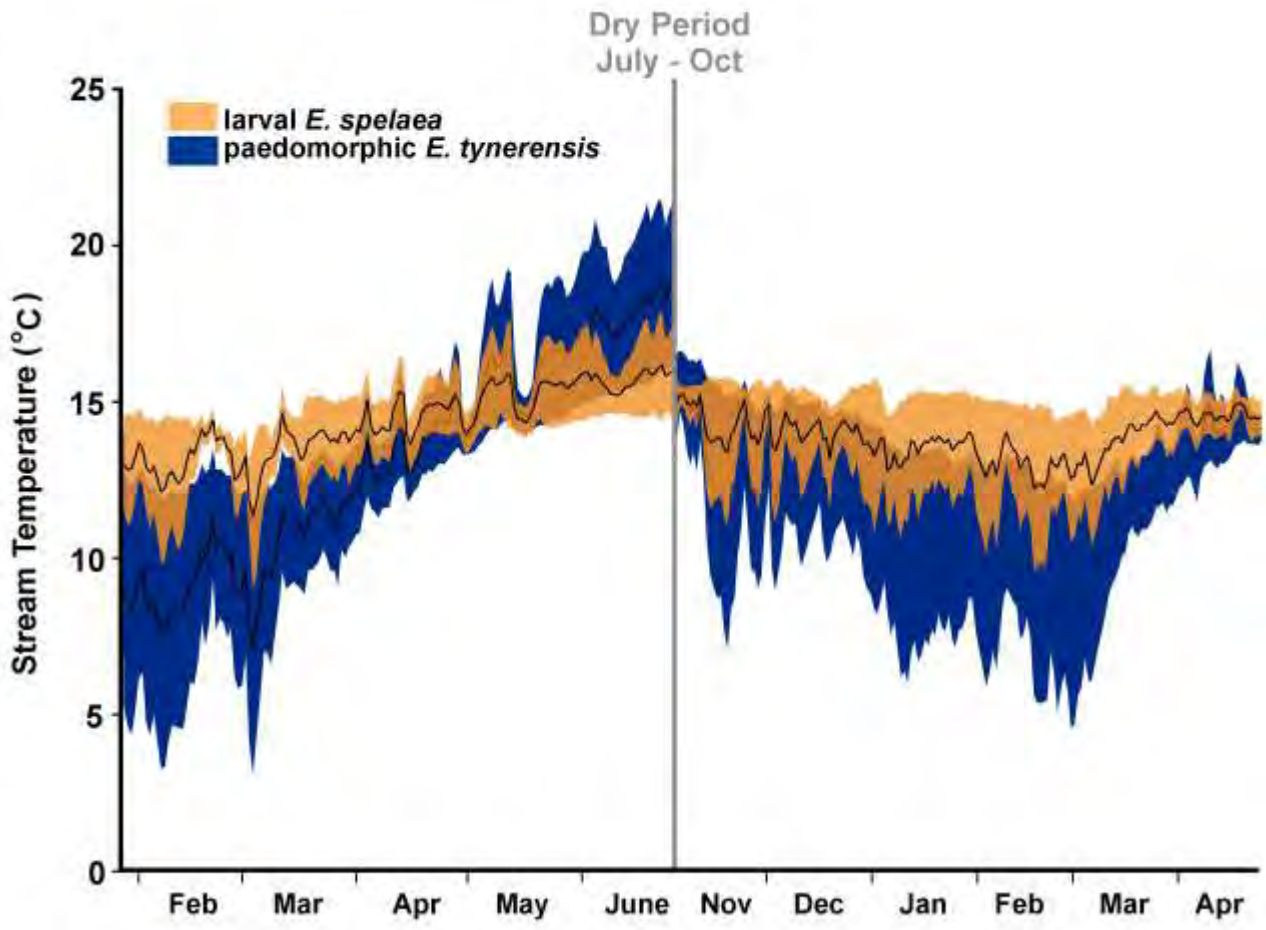
Figure 15. Graphs of fragmentary stream temperature data, ranging from April 2010 through September 2015.
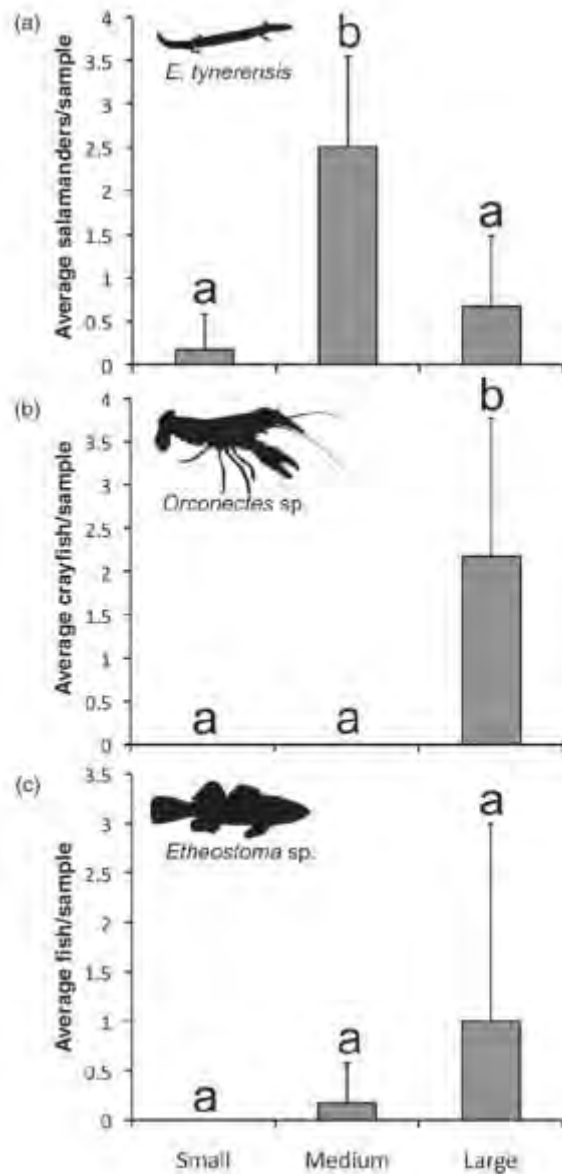
Figure 16. Biplots of the principal component analyses, with sites ordinated by stream temperature data (left panel) and by climate variables (right panel).

Figure 17. Daily mean of stream temperatures, averaged across *E. spelaea* localities compared to other paedomorphic E. *tynerensis* localities without *E. spelaea* for 2014 and 2015 (+/- 1 SD).

Figure 18. Substrate preferences of vertebrates and crayfish recovered from enclosures containing three substrate sizes. Average number of salamanders (A), crayfish (B), and darters (C) found in each enclosure containing a given substrate size class. Data were analyzed by ANOVA and unique lower case letters (a, b, or c) indicate statistically significant group means based on Fisher's LSD ($p < 0.05$). Figure from Martin et al. (2012).
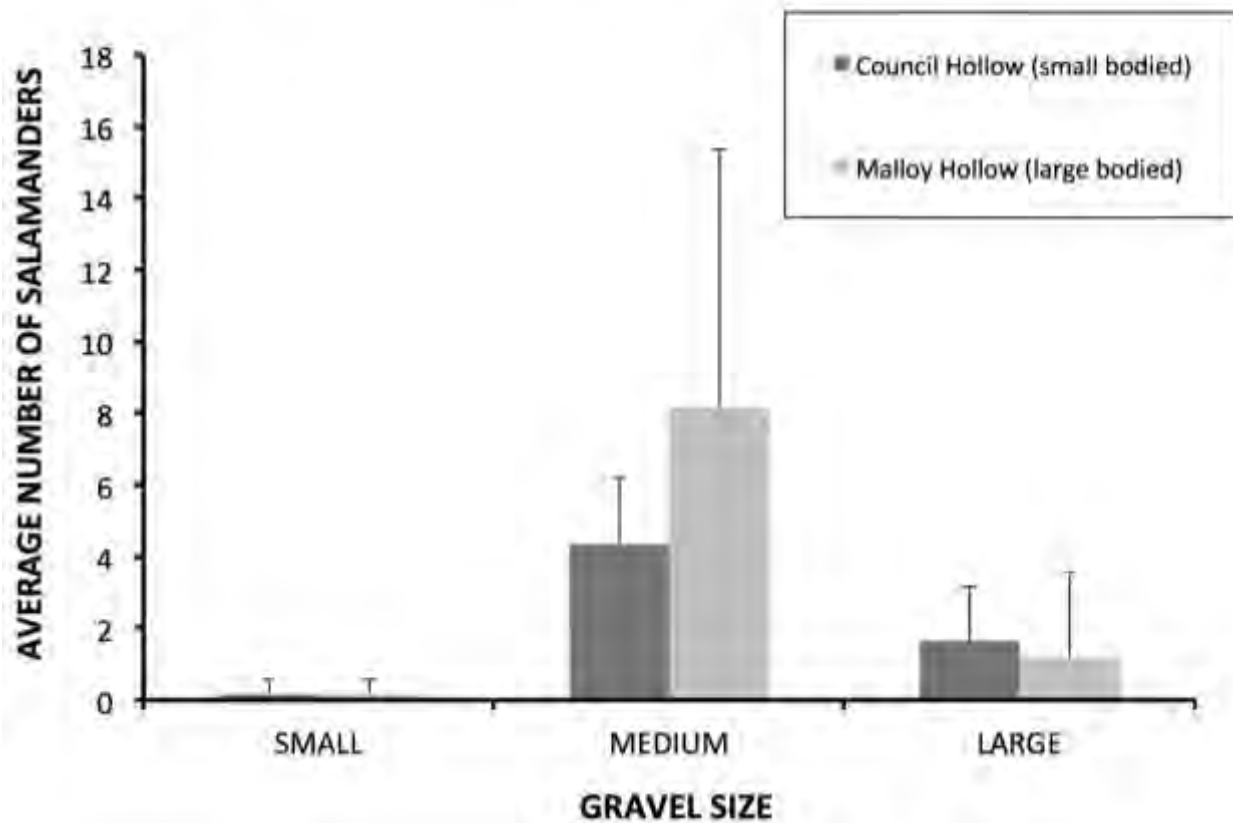
Figure 19. Substrate preferences of *E. tyeneresis* in enclosures of three different substrate sizes (small, medium and large). The experiment was conducted simultaneously (May 2012) at two locations, Council Hollow and Malloy Hollow, with small bodied and large bodied *E. tynerensis* respectively (see approach and Figure 4). Bars show averages and whiskers are standard deviations. Both small and large bodied populations preferred our medium gravel size (average 1.7 cm^3) compared to our small or large gravel enclosures.