Baseline monitoring and molecular characterization of the state endangered Enigmatic Cavesnail, *Fontigens antrocetes* (Hubrict 1940)

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Interim Report to:

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ILLINOIS NATURAL HISTORY SURVEY
PRAIRIE RESEARCH INSTITUTE

Baseline monitoring and molecular characterization of the state endangered Enigmatic Cavesnail, *Fontigens antroecetes* (Hubrict 1940)

(interim report)

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Introduction

Fontigens antroecetes (Hubricht 1940), the Enigmatic Cavesnail[1] (Figure 1), is a minute (shell 2.5-4.5 mm tall) troglobiontic[2] aquatic snail (Gastropoda: Mesogastropoda: Hydrobiidae). In Missouri it has been reported from several caves in Perry County (where it co-occurs with other species of *Fontigens* and another cave hydrobiid, *Amnicola stygia* Hubricht 1971), a single cave in St. Louis County (Hershler *et al.* 1990, Peck and Lewis 1978), and it may also occur in Cape Girardeau County (Wu *et al.* 1997). The type locality for *F. antroecetes* is in Illinois (Stemler Cave, St. Clair County) (Hubricht 1940) (Figure 2). The Illinois population has been confirmed as extant by Lewis et al. (2003) and Taylor (unpublished, in 2004, see Figure 2). Recently, this snail was listed as State Endangered by the Illinois Endangered Species Protection Board (ESPB).

Like most endangered cave invertebrates, the first thing that we learn in attempting to recover the species is that we know virtually nothing about it. This is the case for *Fontigens antroecetes* – we know only have information on the distribution and morphology of the species. Population size, microhabitat preferences, reproductive rates, or connectivity among populations (likelihood of gene exchange) are unknown.

With continuing rapid growth in the greater St. Louis metropolitan area, which extends deep into Monroe and St. Clair counties, Illinois, there are ever increasing

[1] This common name apparently coined by Wu *et al.* 1997

[2] ***troglobiont*** – formerly, this would have been called a *troglobite* – a species which does not exist outside of caves, the upper hypogean [below ground]zone, or superficial underground compartments (after Humphreys 2000). Some authors prefer to use the term *stygobite* for an aquatic troglobite. More recently, Sket (2008) has proposed new terminology, under which the Enigmatic Cavesnail is termed a *troglobiont* – a species "strongly bound to hypogean habitats." Under Sket's (2008) classification, other organisms encountered in subterranean environments might be termed *eutroglophile*s ("essentially epigean species able to maintain a permanent subterranean population"), *subtroglophile*s ("species inclined to perpetually or temporarily inhabit a subterranean habitat but is intimately associated with epigean habitats for some biological functions"), or *trogloxene*s ("species only occurring sporadically in a hypogean habitat and unable to establish a subterranean population")

threats to the shallow groundwater in Illinois' sinkhole plain. High levels of fecal coliforms and other contaminants have already been well documented in Illinois caves (Taylor et al. 2000c). In addition, the population of the Illinois Cave Amphipod (*Gammarus acherondytes*, an aquatic cave species listed as Endangered at the federal level) in Stemler Cave is thought to have been extirpated. As this is the sole Illinois locality for the Enigmatic Cavesnail, there is reason for grave concern for the long term persistence of this snail population.

Figure 1. *Fontigens antroecetes* (Hubricht 1940) in Stemler Cave, St. Clair County, Illinois, in 2004.

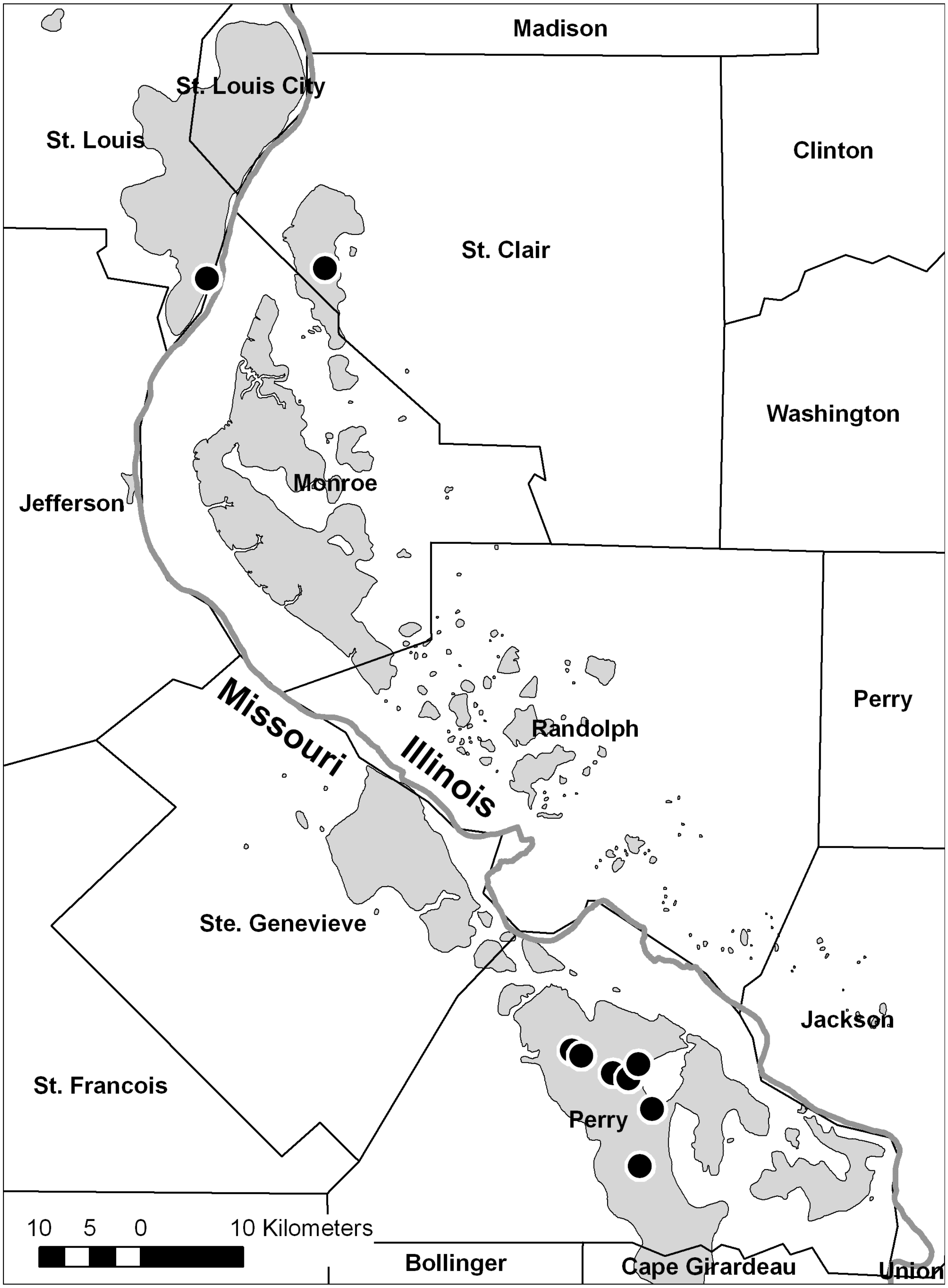

Figure 2. Known distribution of *Fontigens antroecetes* (Hubrich) based on Hershler *et al*. (1990). Shaded areas correspond to distribution of karst, based on Panno *et al*. (1999).

Objectives

Given the above facts, we felt it was important to obtain baseline data on the Illinois population of *F. antroecetes*, including abundance, evaluation of microhabitat preferences, and characterization of genetic diversity:

1. Abundance and microhabitats.

Basic information on snail abundance for *F. antroecetes* is unavailable. We proposed to use a quadrat sampling approach to carry out baseline population monitoring, and to characterize microhabitat availability in these quadrats. This information will:

A. Establish baseline information on population densities.
B. Establish baseline data on microhabitat utilization.
C. Evaluate the variability in densities by microhabitat in Stemler Cave.
D. Evaluate the variability in densities over time (i.e., over multiple monitoring events).

2. Genetic diversity.

Estimating genetic diversity of the Stemler Cave population of *F. antroecetes* will facilitate better management and recovery of the species in several ways:

A. In a scenario where this population is extirpated, it will allow us to identify which of the remaining populations would be the most appropriate source for reintroduction (i.e., genetically most similar).
B. It will allow us to assess whether there has been a genetic bottleneck in this population.
C. Using the genetic data, it may be possible to estimate the effective poulation size in Stemler Cave.[3]
D. The genetic data could help determine whether or not the Stemler Cave population is con-specific with the Missouri populations currently recognized under the same name.

Methods

We used a quadrat approach to quantify snail densities. The quadrat was constructed of PVC pipe, with dimensions of 0.316 m x 0.316 m, or an area of 0.1 m^2. Within Stemler Cave, we defined five sampling areas, labeled A through E, at locations increasingly remote from the entrance (Figure 3). These areas were selected to have sufficient

[3] "Effective Population Size" can be estimated using mitochondrial DNA sequences, see Buhay and Crandall (2005), Sbordoni et al. (2005), and Culver and Pipan (2009, p. 198).

water flow to allow sampling, and water shallow enough to make sampling feasible. We placed the quadrate haphazardly in appropriate habitats to obtain preliminary estimates of snail density.

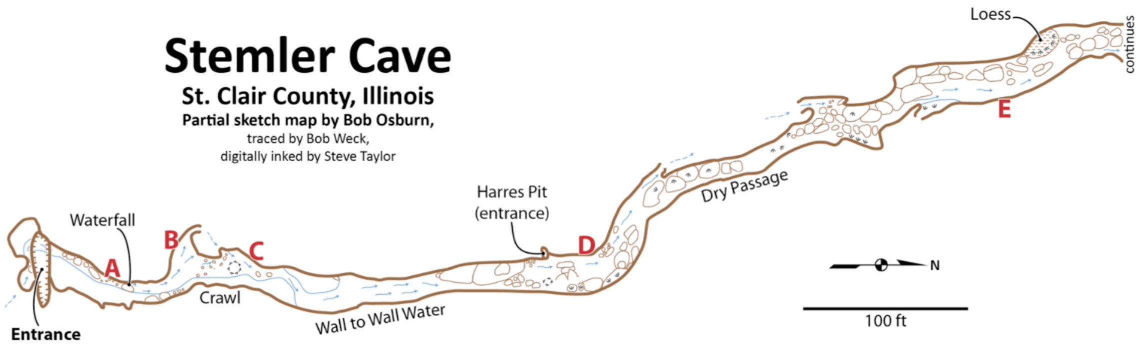

Figure 3. Partial map of Stemler Cave, showing location of sampling stations.

Within each quadrat, we estimated substrate composition percentages for the following categories:

bedrock	host rock containing cave
breakdown	> 80 cm
rock	> 18 to <= 80 cm
cobble	> 7 to <= 18 cm
gravel	> 2 to <= 7 cm
sand	<= 2 cm
silt	smooth, not grainy

In addition, we measured water depth and flow rate (with a flow meter) at each quadrat. Finally, we examined each substrate item within each quadrat from bedrock down to gravel, scoring the number of individuals, by taxon, on the top, bottom, and side of each item in the substrate (i.e., each rock, cobble, etc.).

In addition to Stemler Cave, we also surveyed several other Illinois caves, representing major cave drainage basins in the Salem Plateau. For comparison, we also included data collected earlier from caves in southeastern Missouri, focusing especially on localities where *Fontigens antroecetes* has been reported to occur. Vouchers of cavesnails (Hydrobiidae) were occasionally collected, and these will be used in upcoming molecular analyses.

Results

Ten different caves were evaluated in the present study (Table 1). In the six Illinois caves, *F. antroecetes* was detected only at Stemler Cave. In Missouri, four caves were sampled, with hydrobiid snails recovered from all of these caves. Species level identification of this material awaits molecular studies, as multiple *Fontigens* species are possible from these localities.

Table. 1. Sites sampled for hydrobiid snails. Species level identification of Missouri material awaits molecular analyses.

State	County	Cave	Date	Hydrobiids?
Illinois				
	Monroe County			
		Spider Cave	8 July 2011	no
		Fogelpole Cave	9 July 2011	no
		Pautler Cave	9 July 2011	no
		Wanda's Waterfall Cave	10 July 2011	no
	St. Clair County			
		Brown's Cave II	1 August 2011	no
		Stemler Cave	29 September 2009	*Fontigens antroecetes*
			27 March 2010	*Fontigens antroecetes*
			10 April 2010	*Fontigens antroecetes*
			2 July 2010	*Fontigens antroecetes*
			31 October 2010	*Fontigens antroecetes*
			11 November 2010	*Fontigens antroecetes*
			02 April 2011	*Fontigens antroecetes*
			20 July 2011	*Fontigens antroecetes*
Missouri				
	Perry County			
		Mertz Cave	16 March 2010	yes
		Klumps Cave	16 March 2010	yes
		Running Bull Cave	16 March 2010	yes
	St. Louis County			
		Cliff Cave	15 March 2010	yes

At Stemler Cave, eight site visits were conducted as a part of this study, with *F. antroecetes* being detected on all sampling dates. The remainder of the results will focus on the Stemler Cave material obtained from quadrat sampling.

At Stemler Cave, 85 quadrat samples have been scored over the eight site visits. Within these quadrats, 581 gravels, cobbles and rocks were examined. Of the 215 *F. antroecetes* observed in quadrats, 74.9% were found on the bottoms of rocks, cobbles, and gravels, and 22.3% on the sides (Figure 4). In addition, several other aquatic invertebrates were frequently encountered including Isopoda (*Caecidotea brevicauda*), Amphipoda (primarily *Gammarus troglophilus*, but occasionally *Crangonyx forbesi*), the cave adapted flatworm *Sphalloplana hubrichti*, and an unidentified snail of the genus *Physella* (Physidae) (Figure 4).

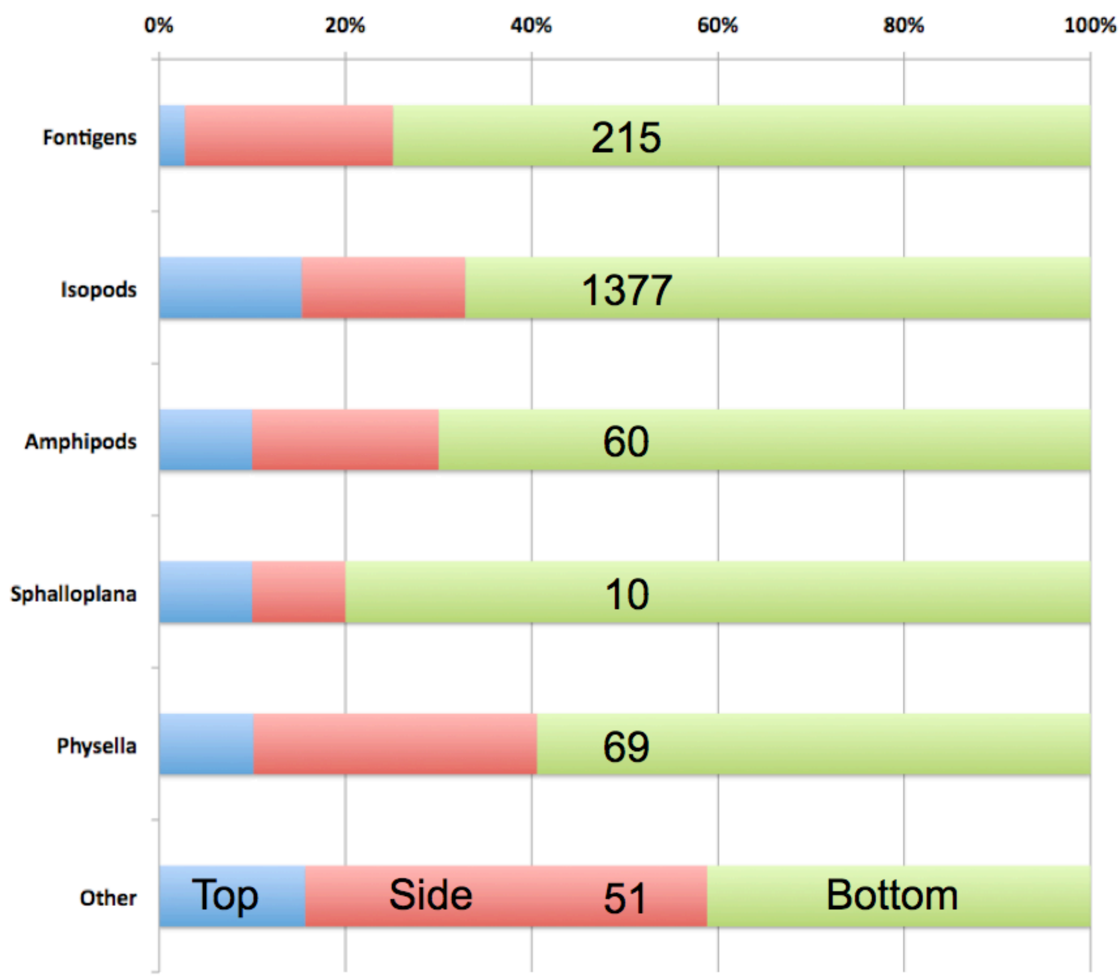

Figure 4. Spatial location of animals on rocks, cobbles, gravels in Stemler Cave (St. Clair County, Illinois) in quadrat samples assessed over 8 sampling occasions between 29 September 2009 – 20 July 2011. Number in center indicates total number of individuals for each taxon, with three shaded areas representing top (=blue), side (=red) and bottom (=green) of substrate item.

All of these taxa showed a similar pattern of being more frequently encountered on the bottom surface of rocks, cobbles and gravels.

Fontigens antroecetes was absent from about one third of the quadrat samples and present in low numbers in most samples, with fewer samples having higher densities of individuals, up to 16 in a single quadrat (Figure 5, left). There was no clearly discernable pattern for type of substrate (whole quadrat characterization) in relation to number of snails in quadrat, though snails were more likely to be present when rocks, cobbles, or gravels were abundant (Figure 5, right).

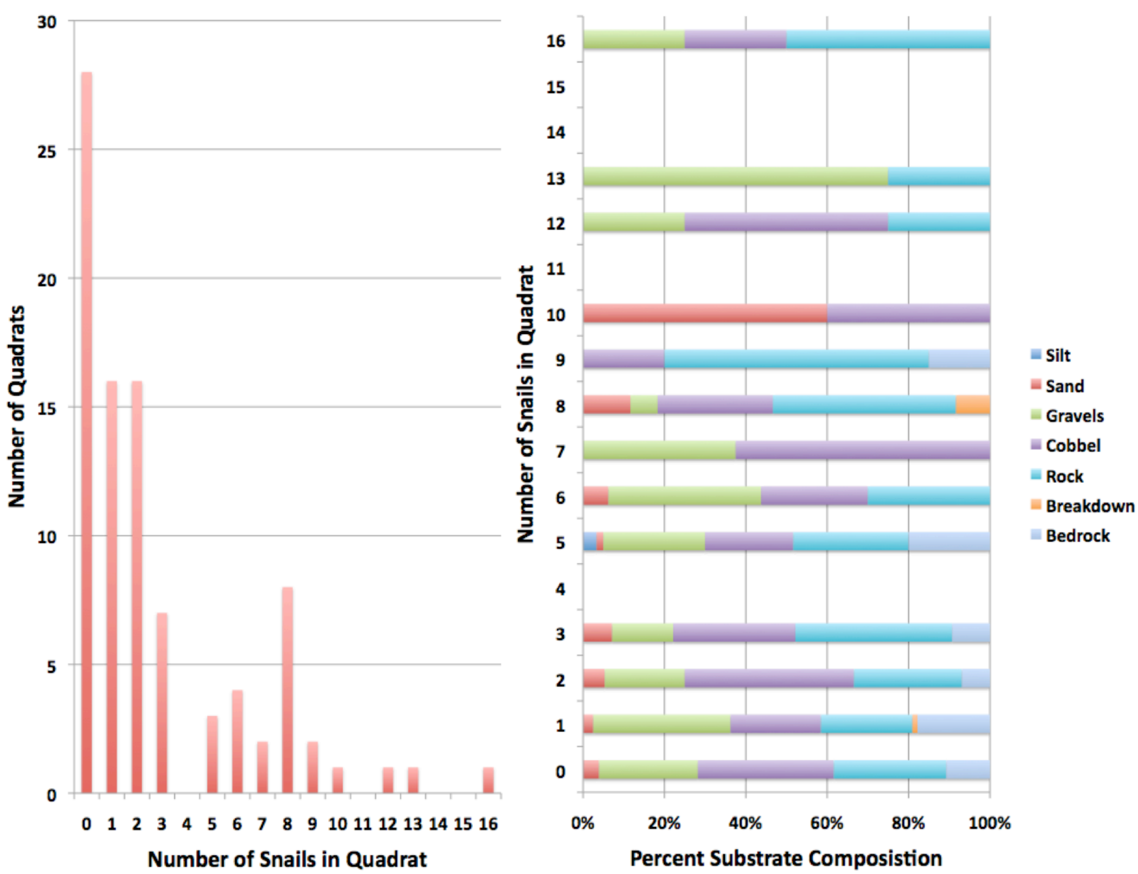

Figure 5. Numbers of snails per quadrat (left) and quadrat substrate composition in relation to numbers of snails per quadrat (right), Stemler Cave, St. Clair County, Illinois, in quadrat surveys from 29 September 2009 to 20 July 2011.

Snail densities varied by sample location, with those at the site closest to the entrance (A) being markedly lower than those from the other sample locations (B-E) (Figure 6). In addition to being closer to the entrance, the flow rate and amount of available habitat in stream segment A varied among site visits. In this portion of the cave, much of the cave stream may flow inaccessibly behind/beneath the west wall rather than through the cave passage. Densities varied from $0/m^2$ to $160/ m^2$, averaging $25.3/m^2$.

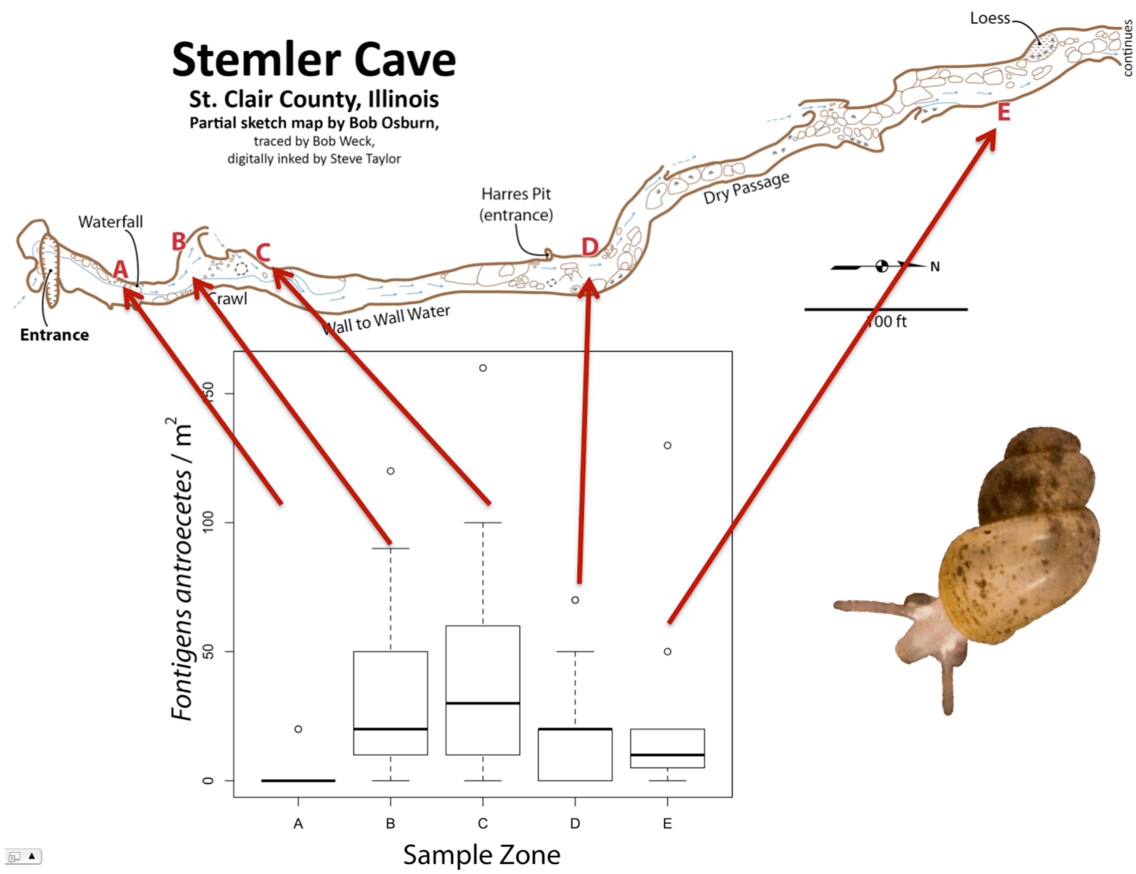

Figure 6. Density estimates for *Fontigens antroecetes* at five sampling stations in Stemler Cave, St. Clair County, Illinois, based on quadrat surveys from 29 September 2009 to 20 July 2011.

There was no obvious correlation between water depth and snail density, nor between percent cover of cobbles + rocks and snail density, however, snail density was negatively correlated with flow rate (Figure 7). When flow rates were high, snail densities were always low, but when flow rates were low, snail densities were variable, but sometimes quite high (Figure 7).

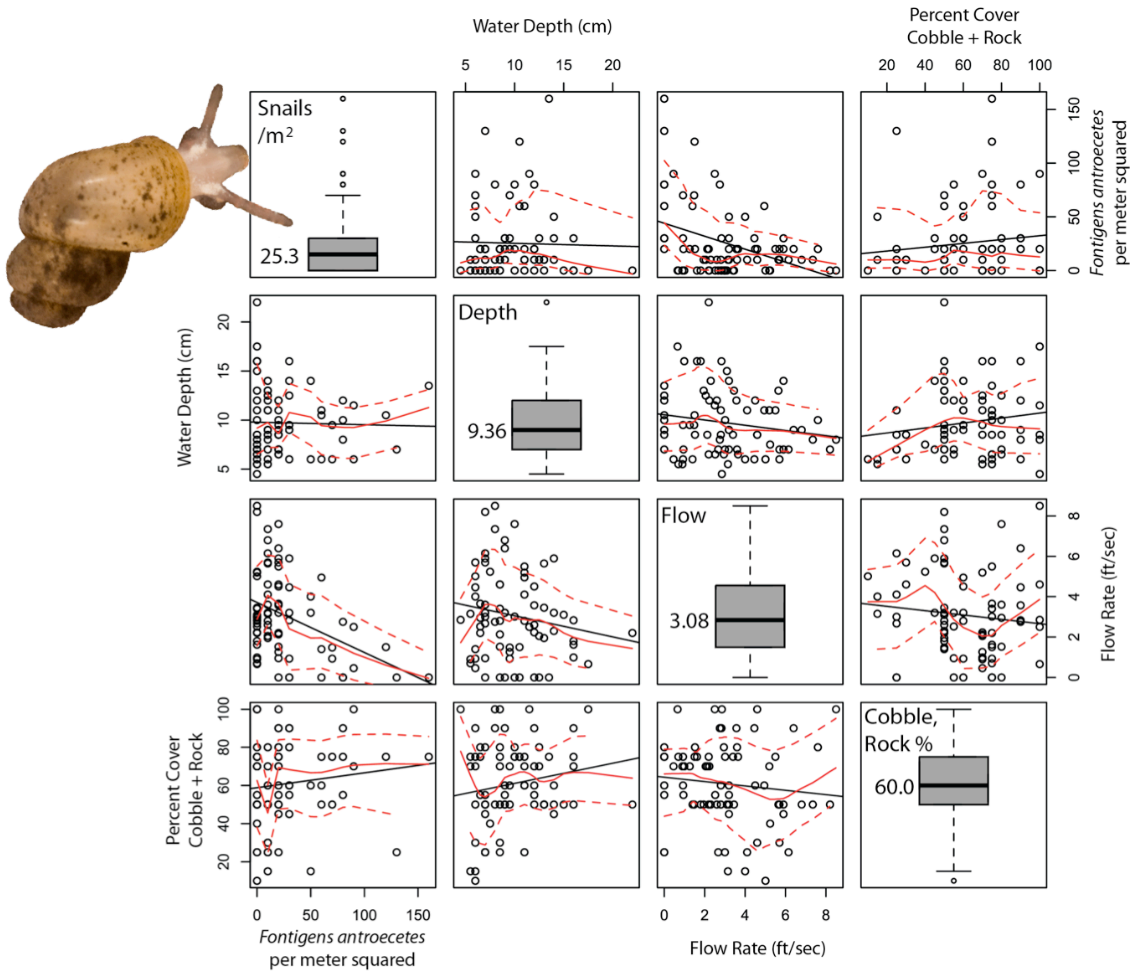

Figure 7. Relationship between snail densities in quadrats and three environmental variables in Stemler Cave, St. Clair County, Illinois, based on quadrat surveys from 29 September 2009 to 20 July 2011. Black straight line is best-fit linear regression, red line is loess smooth, with dashed lines as confidence limits. Diagonal gives box plots and mean values for parameters.

Through the course of the fieldwork, we also observed some very small individuals of *Fontigens antroecetes* (Figure 8), as well as snail egg masses. Unfortunately, the presence of another relatively common snail, *Physella* sp., meant it was not feasible to unambiguously associate egg masses with *F. antroecetes*. Nonetheless, some, if not most, of the egg masses were likely those of *F. antroecetes*, as it is the dominant snail in this cave system (Figure 4).

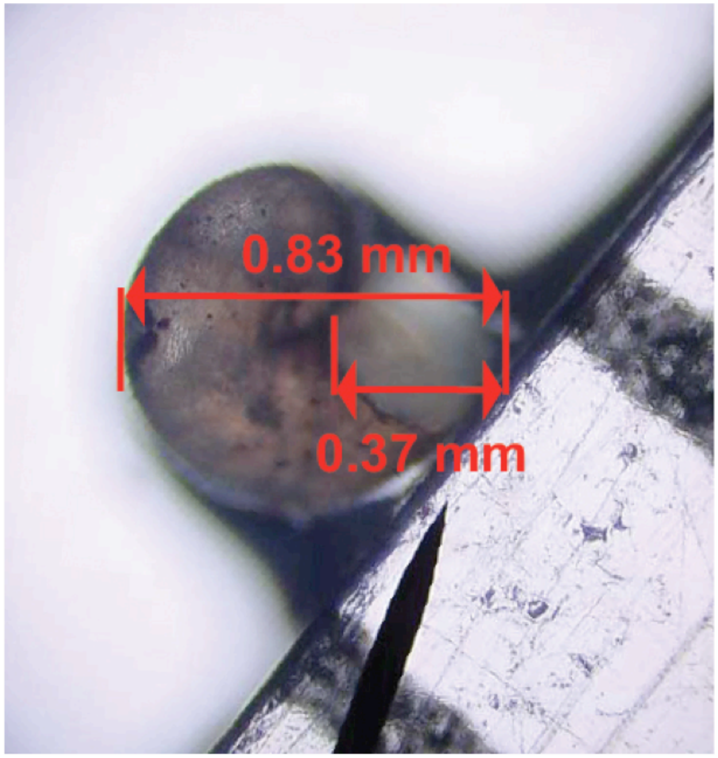

Figure 8. Photomicrograph of a small specimen of *Fontigens antroecetes* from Stemler Cave, St. Clair County, Illinois, showing shell width (0.83 mm) and aperture width (0.37 mm).

Snail densities in quadrats also varied across sample dates (Figure 9). Densities were highest on the first sample date, although only two quadrats were censused on that date, and these values were within the range of variability seen on later dates. In addition, the 29 September 2009 census did not include samples nearest to the entrance, where we typically encountered lower densities of individuals (Figure 6). The two subsequent sample periods (27 March 2010, 10 April 2010) had snail densities that were markedly lower than the overall average. From 2 July 2010 through 20 July 2011, snail densities averaged close to the overall snail density estimate of 25.3 snails/m^2 (Figure 9). Small snails were observed primarily between late September and early April, with egg masses being most abundant in July (Figure 9).

From earlier sampling in caves of the Salem Plateau, including Stemler Cave (Taylor et al. 2000c) we know that flooding is most frequent in the spring months, especially April and May, with lower flow levels occurring from November through January. In addition, this earlier work shows that cave stream water temperatures tend to be warmest in July and August, and coldest in January and February. These data, along with the seasonal presence of egg masses and small snails, affords an opportunity to speculate on the life history of this species – we caution that this is only informed speculation, and that further sampling and future studies may yield a very different picture.

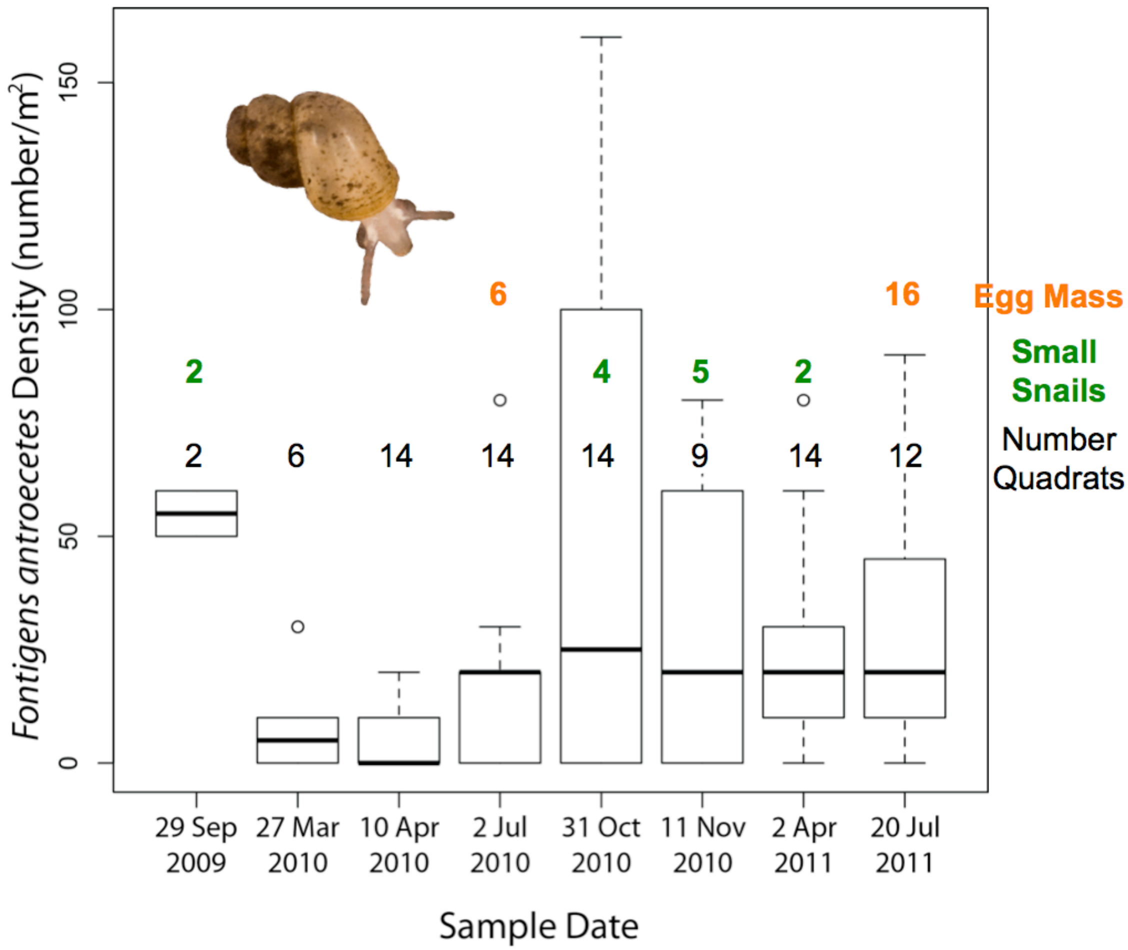

Figure 9. Densities of snails in quadrats across sample dates at Stemler Cave, St. Clair County, Illinois, along with counts of small snails and egg masses observed in quadrats.

The hypothesized life history pattern for the species, based on the limited data available, is as follows (Figure 10): Spring flooding brings extra nutrients into the cave system, allowing larger individuals to gain sufficient energy for reproduction. Breeding may occur in May and June, with egg masses produced in July, when warmer temperatures facilitate rapid development and emergence of young. Young snails feed and grow through the late summer and fall, when water levels begin to drop and temperatures turn colder. It is unclear from our data whether the species life cycle lasts more than a year - many cave-adapted species tend to live longer than their surface relatives. It is possible that the young take several years to sexually mature. Our data do not record sizes of individuals with enough specificity to inform these aspects of the species' life history.

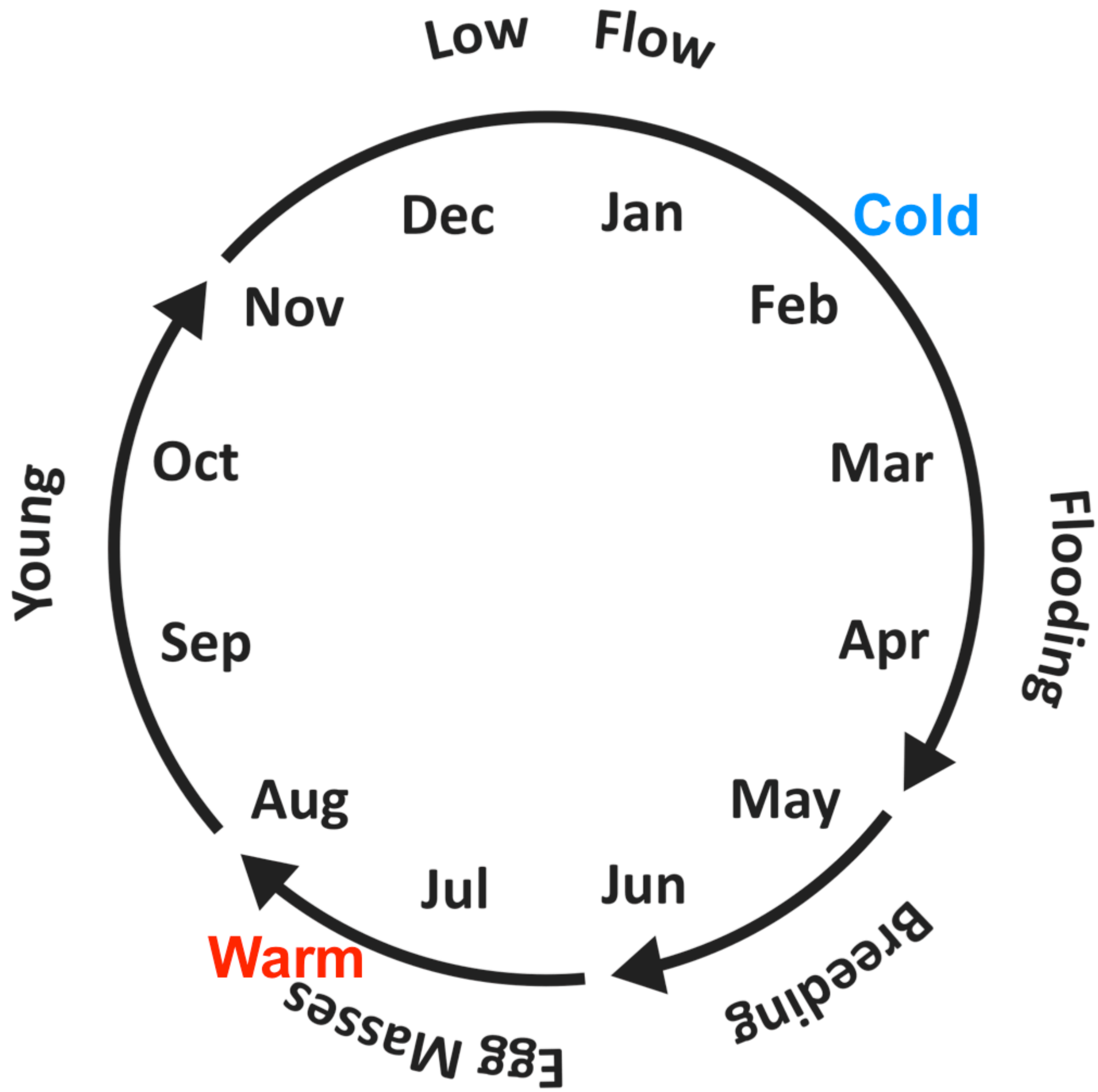

Figure 10. Hypothesized life history of *Fontigens antroecetes* based on limited data from Stemler Cave, St. Clair County, Illinois, quadrat surveys from 29 September 2009 to 20 July 2011. This information is hypothesized on fairly limited data, and is subject to revision with further study.

Molecular analyses

Part of the impetus for molecular analysis is the potential for barriers to interbreeding among populations of the enigmatic cavesnail. Both geographic distance (between the northern populations and the Perry County, Missouri populations) and physical barriers (Mississippi River, discontinuities in karstic bedrock) could lead to reproductive isolation in this species (Figure 2). In addition, of the nine species in the genus *Fontigens*, three co-occur in southeastern Missouri (Figure 11).

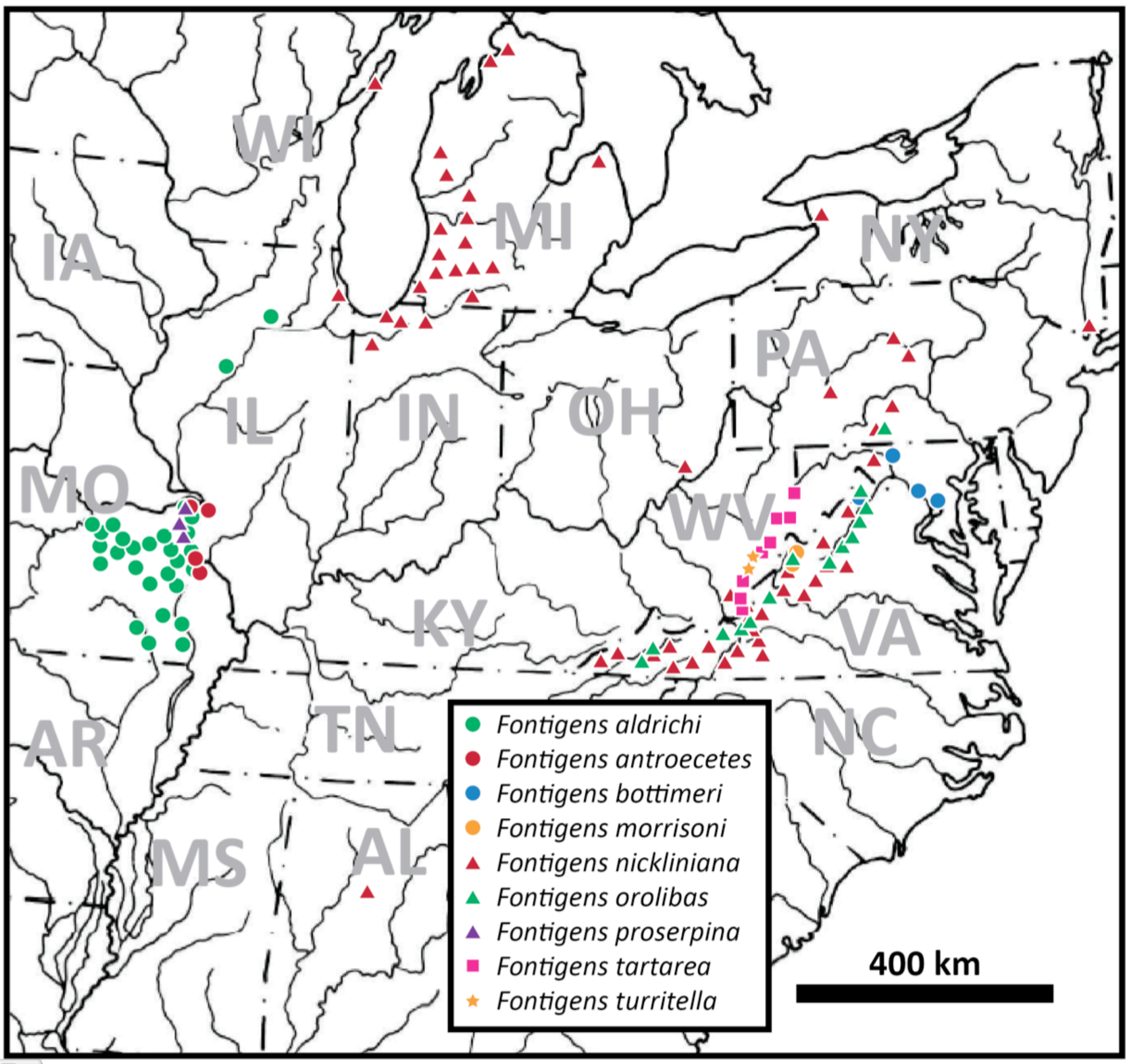

Figure 11. Distribution of the species in the genus *Fontigens*, modified from Hershler et al. (1990).

We hope to use the molecular analyses in this study to identify distinct evolutionary lineages by determining mtDNA diversity within and among caves. We have extracted genomic DNA from whole specimens and are attempting to utilize minimal amounts of tissue so that shell morphology can be preserved for shape analysis. After evaluation of various commercially available kits for extraction, including Qiagen DNeasy Tissue Kit and Qiagen Micro Kit, it appears that the InstaGene Kit from BioRad yields high quality genomic DNA. The InstaGene Kit is based on a Chelex extraction method, initially developed for forensic analyses. This Kit allows retrieval of minute amounts of DNA from samples. However, because of its efficiency to extract traces of DNA, contamination of a sample with non-target DNA (i.e., other organisms living in the environment, human DNA) is a potential problem.

Target genes were amplified using PCR, followed by purification and sequencing. Sequences were manually aligned and ambiguous base calls verified by comparing redundant sequences generated for each individual. Preliminary analyses include standard phylogeographic approaches yielding percent sequence divergence (within- and among-caves) and unrooted neighbor-joining clustering (Jukes-Cantor Distance).

We obtained ~850 base pairs of the mtDNA CO1 gene (commonly used for "DNA Barcoding"), and selected a few individuals per cave for preliminary analysis. Due to the species being state endangered in Illinois, we are waiting to analyze Stemler Cave specimens until methods are optimized. For the preliminary samples, we sequenced each individual 5-6 times.

We experienced difficulties obtaining unambiguous sequences, recording up to 40% sequence divergence in preliminary analyses. Possible explanations for these findings include:

- numts (nuclear copy of mtDNA)
- contamination with non-snail DNA (human, parasites, biofilm, etc.)
- heteroplasmy (multiple mtDNA genomes within a single individual)

We will continue to pursue the genetic analyses problems, and will provide an update on the molecular aspects of this study at a later date.

Conclusions

Fontigens antroecetes reaches its highest densities in situations where flow rate is low.

Because suspended particles tend to drop out at lower flows, this snail may be particularly vulnerable to problems associated with sedimentation.

It is highly likely that in Illinois this snail is restricted to the Stemler Cave drainage basin.

Snail densities are approximately 20-25/m^2 in suitable habitat at Stemler Cave.

As for other cave hydrobiids (Ashley 2003, Slay and Taylor 2007, 2011), quadrat counts appear well suited for monitoring.

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