5-2020

Chronoecology of a Cave-dwelling Orb-weaver Spider, Meta ovalis (Araneae: Tetragnathidae)

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Recommended Citation

Steele, Rebecca, "Chronoecology of a Cave-dwelling Orb-weaver Spider, Meta ovalis (Araneae: Tetragnathidae)" (2020). *Electronic Theses and Dissertations.* Paper 3713. https://dc.etsu.edu/etd/3713

Chronoecology of the Cave-dwelling Orb-weaver Spider, *Meta ovalis* (Araneae: Tetragnathidae)

A thesis

presented to

the faculty of the Department of Biological Sciences

East Tennessee State University

In partial fulfillment

of the requirements for the degree

Master of Science in Biology

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by

Rebecca Steele

May 2020

Dr. Thomas C. Jones, Chair

Dr. Darrell Moore

Dr. Blaine Schubert

Keywords: Circadian, *Meta ovalis*, Ecology, Spider, Cave

ABSTRACT

Chronoecology of the Cave-dwelling Orb-weaver Spider, *Meta ovalis* (Araneae: Tetragnathidae)

by

Rebecca Steele

Circadian clocks enable coordination of essential biological and metabolic processes in relation to the 24-hour light cycle. However, there are many habitats that are not subject to this light cycle, such as the deep sea, arctic regions, and cave systems. This study analyzes the circadian pattern of isolated populations of a subterranean spider, *Meta ovalis* from two Tennessee caves and five Indiana caves. Locomotor activity was recorded with TriKinetics LAM50 Locomotor Activity Monitor under a 12-hour light (L), 12-hour dark (D) (LD 12:12) cycle preceding total darkness (DD). Significant differences were found within and among populations found in Tennessee cave systems in average free running period and onset of locomotor activity. Selection, drift, and genetic fixation are explored as the causes of variation in the present study, using *M. ovalis* as the model organism. All five caves in Indiana show little variation, whereas both Tennessee caves show large interindividual variation.

ACKNOWLEDGEMENTS

This research could not have been completed without the support, guidance, and constant encouragement from my family, friends, committee, dedicated cavers, and science enthusiasts. I would like to express my deepest appreciation for my committee for pushing me and keeping an adventurous attitude during this process, specifically my committee chair, Dr. Thomas “TJ” Jones, for his sense of humor, advice, and feedback, as well as the time he took out of his schedule to help collect in the field. His unending enthusiasm for ecology is infectious, and really made my time at ETSU enjoyable.

I am indebted to the Asheville, NC Flittermouse Grotto for their help and networking, Dr. Jerry Lewis, Keith Dunlap, and the Indiana Karst Conservancy for their guidance and help with collecting in Indiana. I would like to thank the many others that helped in the field, Clinton Elmore, Dr. Rebecca Wilson, Jake Hanson, and Guthrie Jones. You all made this research more enjoyable by being present.

Above ground, I would like to deeply thank members of our lab, for helping with every aspect of this project, Raven Ragsdale’s genuine kindness, and Shae Crain’s neurotic dedication to science helped sustain a positive atmosphere in which to do science. I would also like to express my deepest gratefulness to Dr. Tom Kwasigroch, Dr. Caroline Abercrombie, and Robert Becker for allowing me to pursue this degree while working full-time. Their continuing support and mentorship kept me on track and allowed me to stay focused on the end goal.

Additionally, I would like to thank my mom and dad for their unwavering support, my amazing twin sister, Michelle Steele for continuing to be present and supportive during my unending pursuit of education, for going on adventures, documenting them, and keeping me sane

during the whole process. Lastly, I would like to acknowledge Dr. Dave Ryan who is my biggest supporter, for his love, tolerance, and statistical skills.

My friends, sister, and Dave have blessed me with a life of joy, happiness, and optimism, shining the brightest light into my life, even in the hours of darkness.

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CHAPTER 1. INTRODUCTION

Circadian Clocks

Circadian clocks are endogenous pacemakers that are rhythmic and predictable. Circadian rhythms are oscillations in behavior or physiology, and can entrain to the day night cycle, can compensate for temperature changes, and persist under constant conditions (Dunlap et al. 2004). Most organisms have a periodicity of about (circa) a day (diem), and terrestrial organisms are exposed to differing light and temperature cycles in concordance with sunrise and sunset. This external cue syncs the endogenous clock with the external day, allowing for control of physiological, behavioral, and metabolic processes in anticipation of the changing external environment, likely increasing fitness (Foster and Kreitzman 2005). Many studies have been conducted on organisms that live in a natural light:dark (LD) cycle. One such organism is the highly studied *Drosophila melanogaster*. Using *Drosophila* mutants in 1971, Konopka et al. determined the mutations in the X chromosome could make changes in the circadian clock period (Konopka and Benzer 1971). The clock is based on transcription-translation feedback loops, which results in oscillations in expression of clock-related genes about every 24 hours. The clock is temperature compensated, such that variation in temperature common to most environments has a reduced effect on the rate of circadian oscillations compared to most metabolic processes (Huang et al. 1995). Circadian rhythms have been studied in depth using gene “knock-out” models, as well as by studying organisms that have naturally occurring genetic mutations in their circadian genes (Huang et al. 1995; Bittman 2014).

This molecular mechanism in most animals, however, is not a stand-alone time keeper. It is routinely modified and reset, known as entrainment, based on external cues, most notably exposure to light, temperature variation, and food availability (Refinetti 2015). Other factors that can entrain the biological clock are feeding, activity of other organisms, social contact, and sound (Refinetti 2016). This ability to be aware of, and entrain to external cues is one of the main features of endogenous biological clocks. These cues are commonly referred to as Zeitgebers, from the German word, meaning "time giver". When all Zeitgebers are removed, the organism is in a constant environment, and can only respond to its endogenous clock. Therefore, any behaviors or physiology which continue to oscillate with a period of about 24 h reflect the internal clock in a pattern known as "free-running". The most frequently studied Zeitgeber is light intensity, as it is the most easily detectable and reliable external source of information for most organisms (Menzel 1979; Erkert 2004).Temperature can also entrain organisms' activities, such as eclosion in flies (Zimmerman et al. 1968), locomotor and transcriptional rhythms in crickets (Kannan et al. 2019), and thermal entrainment in the plant *Arabidopsis* (Avello et al. 2019). Taken together, this suggests that there is an advantage to having multiple cues that circadian clocks can entrain to in the environment.

Caves and Other Arrhythmic Environments

Studies have examined and found circadian rhythms in organisms living in arrhythmic environments (A D Beale et al. 2016; Abhilash et al. 2017), such as caves, deep sea, and polar environments. These studies strive to understand how these rhythms have adapted without daily photic cues. Out of the common arrhythmic environments, caves have the potential to be one of the most stable. Caves lack light, many have relatively steady temperatures, and fairly high humidity levels, although some in arid regions have very low humidity (Poulson and White 1969). Caves have three defined zones that are based on the amount of light they receive; the entrance, twilight, and dark zones, as well as three classifications of animals that reside within caves (Culver and Pipan 2009a). Trogloxenes are animals such as bats that use caves, but are not dependent on them. Troglophiles are organisms that live within caves for most, if not all, of their lives but have no specific physiological adaptations for cave life. Troglobites live in the depths of the cave, and are unable to survive outside of the cave. The spatial distribution in terms of linear distance from the entrance of the cave seems to be related to the level of adaptation. Less adapted species are found closer to entrances, while more adapted species are found in the dark zone. Caves provide a unique opportunity to study the adaptive significance of the biological clock. Dozens of cave dwelling arthropod species and 14 cave dwelling vertebrate species have been the focus of locomotor activity and circadian studies (M Friedrich 2013). The species that have been studied have been defined into three broad classifications based on the extent of visual system retention. Macrophthalmic species retain full visual systems, microphthalmic species have mild to significant loss of visual systems, and anophthalmic species have no trace of a visual system remaining (see Friedrich 2013). Studies that have focused on circadian or locomotor activity of cave dwelling species have found subterranean fish have varying degrees of rhythmicity, correlated with the degree of cave specialization. The more specialized the fish is to subterranean life (e.g. reduction or loss of eyes), the less circadian rhythmicity was detected (Trajano et al. 2009). Others have tested non-photic Zeitgebers to determine if these organisms are receiving time-cues exogenously. A species of cave beetle, *Speonomus diecki* was exposed to temperature cycles of 2.6 °C amplitude, which it entrained to, but the beetle was not allowed to free run after entrainment (Pasquali and Sbordoni 2014). Most studies indicate retention of the molecular components of the clock, even with physiological adaptations for subterranean life. (Erckens and Martin 1982; Andrew David Beale et al. 2016). See Table 1 for details.

Spiders in Subterranean Habitats

Spiders are among the most wide-spread organisms on the earth, inhabiting every continent, except for Antarctica, and colonizing every habitat except for sea and air. They are one of the most diverse groups of arthropods, comprising more than 46,500 known and described species, dating back to around 300-360 million years ago (Rainer 1996; Selden et al. 2014). They are among the most successful groups of organisms as far as adaptation and evolutionary colonization, and have wide physiological and morphological diversity (Rainer 1996). Both the smallest spider ever described (*Anapistula ataecina* approx. 0.4 mm.) (Cardoso and Scharff 2009) and the largest (*Hederopoda maxima* approx. 30cm) (Jaeger 2001) are cave dwelling spiders, aptly showing the morphological diversity of subterranean spiders. Around 1,000 spider species have been classified as strongly bound to hypogean habitats (Christman and Culver 2001). There are at least 48 families out of 113 described families with species that show adaptations for subterranean life (Mammola and Isaia 2017). These adaptations include morphological, physiological, and behavioral adaptations displayed by subterranean spiders. Morphologically, decreasing or loss of cuticular pigments, thinning of integuments, elongation of legs, heavy spination, and the recession or loss of eyes can be seen among troglobitic spiders (Cardoso and Scharff 2009; Marroquín 2014; Yao et al. 2016; Mammola and Isaia 2017). Pigment changes can also be correlated to habitat size. In a central Texas population of *Kryptonesticus eremita* individuals show variability in pigmentation patterns in a single population. This variability was inversely correlated with distance from the entrance of the cave (Vink and Dupérré 2011). In another study, two different species of *Tayshaneta* were found with variation “from darkly pigmented, large-eyed individuals to lightly pigmented, reduce-eyed forms to depigmented, blind individuals" (Ledford et al. 2011). Thus, morphological variation among individuals can be seen in single cave populations.

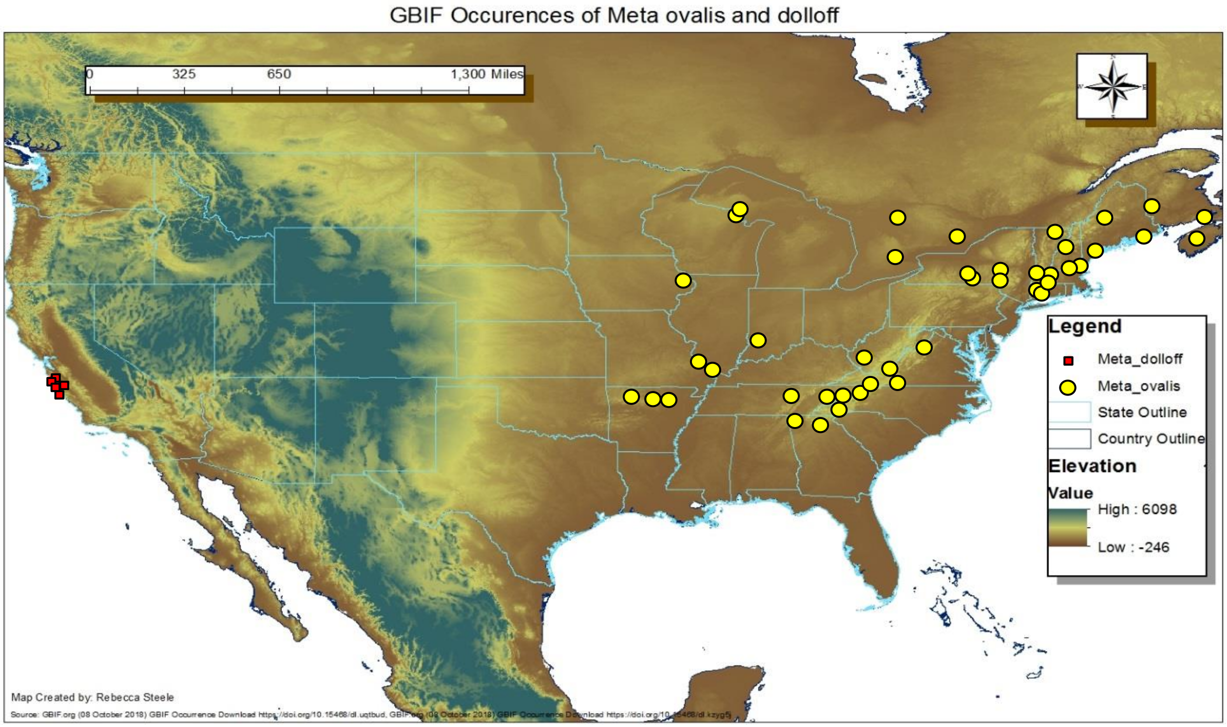
Ecologically, cave populations are similar to island populations, being effectively isolated from the terrestrial environment and can experience adaptations commonly found in remote island populations (Snowman et al. 2010). Physiological adaptations seen in island populations include reduction of metabolism, reduction in fecundity, delayed maturity, and a tendency to lay fewer, larger eggs (Deeleman-Reinhold and Deeleman 1980; Gertsch 1992). It is not clear, however, if these adaptations are in response to environmental effects such as having fewer resources and limited habitat or if is genetic, such as the case with K-selected species. Further studies are needed. Several studies suggest that subterranean spiders have specialized their physiological tolerance to the narrow temperature range and relative constant humidity of their habitats by having a thinner cuticle than their epigean counterparts (Espinasa et al. 2016). The thinning of the cuticle makes these spiders more prone to desiccation. *Lycosa howarthi* showed a particular sensitivity to saturation deficit (Howarth 1980; Howarth 1983; Hadley et al.). Cave dwelling spiders have differing tolerances for cold temperature based on where they reside within the cave. Troglophiles retain their thermotolerance or thermoregulatory mechanisms, while troglobites seem to have lost their ability to withstand temperature fluctuations (Novak et al. 2014).

Available information on behavior of cave adapted spiders is extremely sparse (Doran et al. 2001; L M Carver et al. 2016). Circadian rhythms are still present in some cave spiders (Soriano-Morales et al. 2013; Guadanucci et al. 2015a), however, other troglobiotic species have lost their circadian rhythms (Saunders 2002).

Spiders are generally found in areas that have a high concentration of prey items where they can maximize food intake (Howarth 1980; Mammola et al. 2016; Mammola and Isaia 2016; Resende and Bichuette 2016). Caves, however, have a general trend of low prey availability (Gunn 2004; Culver and Pipan 2009b), so spiders have had to evolve unusual foraging strategies in order to thrive. Many are more opportunistic in the food they will consume (Gibert and Deharveng 2002), and some have been known to eat fish, amphibians, and reptiles (Horstkotte et al. 2010; Rasalan et al. 2015). *Meta menardi* is a well-studied European cave dwelling spider, with a diet consisting mainly of arthropods (Mammola and Isaia 2014; Mammola et al. 2016; Mammola and Isaia 2016; Resende and Bichuette 2016). For *M. menardi*, spiderlings live on the ceiling of the cave, seemingly balancing their shrinking yolk against the risk of leaving the cave and finding an additional food source. Some unknown cue tells them to migrate to live outside of the cave as juveniles, where there is a higher density of small prey items, and a chance for dispersal to other caves (Smithers 2005a), then as adults, they migrate back into the cave (Chiavazzo et al. 2015). There is more known about the early life of *M. menardi* than there is about their adult lives, and circadian rhythms (Smithers 2005b).

Meta ovalis (Araneae: Tetragnathidae), the cave-dwelling orb weaver, is found in caves, basements, abandoned mines, and deep ravines in the United States (Yuri and Seppo 1992). They are found along the eastern coast of the United States and Canada, running from Newfoundland and Labrador south to Georgia, and west to Arkansas (Figure 1.1). It is one of two species of this genus in North America, the other being *Meta dolloff*, located only in two counties in California, and listed on the 2000 IUCN Red List of Threatened Animals (Mammola and Isaia 2014). *M. ovalis* has not been extensively studied, and little, if anything is known about this organism's life cycle, lifespan, or circadian pattern. Of what is known from the literature, *M.*

ovalis has been observed preying on a troglobiotic millipede (Slay et al. 2009), and can potentially transfer entomopathogenic bacteria to co-dwelling cave crickets (Yoder et al. 2009).


GBIF Occurences of Meta ovalis and dolloff

Map Created by: Rebecca Steele

Source: GBIF.org (08 October 2018) GBIF Occurrence Download https://doi.org/10.15468/dl.uqtbud, GBIF.org (08 October 2018) GBIF Occurrence Download https://doi.org/10.15468/dl.kzyg5j

Figure 1.1: Map of the United States showing user-reported occurrences of Meta ovalis *and* Meta dolloff, *according to GBIF.com*

Circadian Studies of Spiders

Few studies analyze the locomotor activity rhythms in cave dwelling spiders, and none thus far have focused on *Meta ovalis*. One study analyzed locomotor activity under laboratory light:dark (LD) and constant dark (DD) conditions for spiders in the families *Ctenidae* and *Dipluridae* collected from Los Riscos Cave in Mexico. The researchers found that individuals from both families were able to entrain to an LD cycle, and free run through DD, both showing a periodicity close to 24 hours (Soriano-Morales et al. 2013). These particular individuals inhabit the twilight and dark zones, with few, if any found in the entrance zone. In the largest subterranean population of mygalomorph spiders (*Trechona* sp.), spiders located in the entrance zone were found to be actively foraging during the night, and either resting during the day, or not present in their webs. The spiders observed in the twilight and dark zones were found to be active for longer periods of time. Those in twilight showed an intermittent pattern, active before dusk, through the night, and returning to their retreats after dawn. The individuals in the dark zone stayed outside of their retreats throughout the night and day (Guadanucci et al. 2015b). This shows the variation of cave-dwelling spiders in circadian activity. We hypothesize *Meta ovalis* will show individual variation within the same cave, but also between isolated sites.

Spiders are able to rapidly entrain to new LD cycles (Ortega-Escobar 2002). This rapid entrainment, along with wide variation of circadian periods, both among and within species show the circadian diversity in various spider species (Moore et al. 2016; Jones et al. 2018; Garmany et al. 2019). This variation within a species of chronobiological measures are examined in the present study using *M. ovalis* as the model organism due to isolated populations, extreme habitat, and potential for local adaptation and/or drift in circadian period.

Abiotic and Biotic Considerations

Several variables need to be considered when analyzing activity patterns of cave inhabitants. Abiotic considerations include moisture content, running water, temperature, light penetration, minerals, and layout of the geology. Biotic considerations include plants and animals. All of these could potentially have an impact on locomotor activity cycles in the cave-dwelling spider, *Meta ovalis*, and cause potential differences between caves in circadian measures.

Although the circadian clock is temperature compensated, temperature fluctuations can be used for entrainment (Erkert 2004; Novak et al. 2014; Avello et al. 2019), light exposure has been shown to have strong effects on FRP in numerous studies (Menzel 1979; Saunders et al. 2002; Soriano-Morales et al. 2013; Refinetti 2015; Tao et al. 2017), but entrainment with humidity alone has thus far only been shown in plants (Mwimba et al. 2018).

Biotic influences on circadian period and locomotor activity are numerous, but can reduced to three main groups of influence: prey, predator, and other. The activities of prey items such as flying or crawling insects could have an effect on activity cycles of *M. ovalis*, especially if those food items are not bound to the hypogean habitat. Troglophilic spiders in the *Meta* genus have prey items that mostly come from epigean habitats, rarely catching hypogean prey (Slay et al. 2009), but other troglobiotic species in the *Nesticus* genus prey on mostly troglobiotic items, such as millipedes, springtails, and beetles (Linnea M Carver et al. 2016). This variation of preferred prey items has the potential to change the spider's activity pattern, based on prey availability. Predation also can have an effect on the spider's activity, but the extent of predation of subterranean spiders is unknown. Generally, spiders are predated upon by wasps, birds, frogs, lizards, toads, and some species of bat (Feldhamer et al. 2009). In the cave ecosystem, most of these predators are sparse, with bats sporadically inhabiting the same caves as the spiders, although there is no evidence that bats predate upon *Meta ovalis*. Other forms of entrainment could come in the form of other animals' daily activities, such as the movement of bats in and out of caves. This movement can act as a Zeitgeber, cuing the spiders in to dawn and dusk. Each cave is unique in each of these factors and the degree to which they are present or absent within the cave.

Impact of Geology

In addition to other abiotic considerations, geology (i.e. type of bedrock, and cave layout) is a potential source of change in activity cycles of *M. ovalis*. These spiders tend to spin their webs on vertical surfaces or on the ceiling, so layout of the cave will have an impact on available space to inhabit, but also the amount of light on each potential location. *Meta ovalis* can be found throughout the cave, including near the entrance(s), twilight zone, and dark zone, and from the ceiling to just above the floor (Rector 2009).

Although caves can provide relatively stable environments, with little fluctuation in temperature, and moisture, caves (and locations within caves) are variable in these measures (Culver and White 2012). The direction the entrance is facing, altitude, airflow, presence of water, exposure of entrance, and configuration of passages all have an impact on the amplitude of fluctuation. An additional difference between cave locations is the amount and type of water present. For example, in this study, one Tennessee cave in this study has a shallow stream running through it, the other has a strong flow of water averaging three feet deep, with a small waterfall near the entrance. Indiana caves visited had either very little water, or shallow streams running through the main passages. These water features have seasonal changes, and the potential for flooding. Season and amount of water can change activity patterns of all biota involved in the cave food chain.

CHAPTER 2. MANUSCRIPT

Journal of Biological Rhythms

Chronoecology of a Cave-dwelling Orb-weaver Spider, *Meta ovalis* (Araneae: Tetragnathidae)

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Abstract The predominant Zeitgeber for most circadian systems is the natural 24-hour light cycle. However, there are many habitats that are not subject to this light cycle, such as the deep sea, arctic regions, and cave systems. Here we describe entrainment profiles and circadian rhythms of locomotor activity in the cave dwelling spider, *Meta ovalis*. We compare populations collected from seven cave systems: two in Tennessee, and five in Indiana. Spiders collected from Indiana caves all had free running periods (FRPs) close to 24 h (23.93 ± 0.13, n=69). Spiders from one of the Tennessee caves showed unusually high variation among individuals (23.71 ± 2.19, n=22), while the other Tennessee cave individuals had unusually long FRPs (26.89 ± 1.71, n=25). Overall, this study found Indiana populations consistent with circadian theory, having free-running periods close to 24 hours with little variance among individuals. Tennessee populations, however, exhibited high interindividual variation in FRP with high deviation from 24 hours which is similar to other species of spider studied this far. These results are discussed in the context of extreme habitat, potential for local adaptation or genetic drift between populations.

Most terrestrial organisms are exposed to daily light and temperature cycles in concordance with the solar cycle, however, there are several organisms that live in arrhythmic environments such as caves, deep sea, and arctic regions. Several organisms inhabiting arrhythmic environments retain circadian rhythms (A D Beale et al. 2016; Abhilash et al. 2017). Others exhibit cyclical patterns only during certain seasons (Lu et al. 2010). Generally, organisms inhabiting typical terrestrial environments show little variation deviating from 24 h among and within species, with most species presenting less than 30 minute total range in recorded free running periods (FRPs) (Aschoff 1981; Ralph R. and Menaker 1988). There is a total range of about 22 to 26 hours in organisms that have been studied including humans, golden hamsters, crickets, and mice (Loher 1974; Aschoff 1981; Ralph R. and Menaker 1988; Czeisler et al. 1999; van der Veen et al. 2012). Spiders, however, exhibit a range of 18.7 ± 0.13 h (*Cyclosa turbinata)* to 29.1 h (*Meta ovalis*), pushing some species far out of resonance with the 24 hour day (Moore et al. 2016; Moore et al. 2017; Jones et al. 2018; Garmany et al. 2019). Typically, organisms that do not "resonate" with the solar cycle, (i.e. they have an internal period that is out of phase with the external environment) will have reduced fitness (Spoelstra et al. 2016), but these abundant wild-collected spiders, do not seem to have a reduction in fitness, as shown by their abundance and intact reproductive ability. Additionally, typical within-species range of FRP has little variability in animals, with a coefficient of variation (%CV) < 1.5. Spiders have a %CV up to 12.34 (Mah et al. in press). Contrary to prevailing evidence for small interindividual variability, FRP close to 24 h, and stability of circadian rhythms, some spider species do not exhibit these patterns, despite inhabiting environments that experience daily variations in light and temperature. This study focuses on the circadian pattern of locomotor activity in *Meta ovalis*, a cave dwelling spider that inhabits an arrhythmic environment, to make comparisons with circadian patterns in other subterranean species.

Circadian rhythms are thought to be retained in organisms that live in arrhythmic environments to enable coordination of metabolic processes (Bloch et al. 2013). Many spider species have been classified as strongly bound to subterranean habitats (Christman and Culver 2001), with some showing adaptations for subterranean life (Mammola and Isaia 2017). From an ecological perspective, cave populations are similar to island populations, being effectively isolated from the terrestrial environment and often show adaptations commonly found in remote island populations (Snowman et al. 2010). However, caution should be displayed in this thought process, as there are interconnected passages humans do not have access to, but smaller organisms do. Studies on behavior of cave-adapted spiders are sparse (Doran et al. 2001; Rector 2009; Reddell 2012; L M Carver et al. 2016). Circadian rhythms are still present in some cave spiders (Soriano-Morales et al. 2013; Guadanucci et al. 2015a), however, over 40 troglobiotic vertebrate and arthropod species have seen weakening in their circadian rhythms, correlated with reduced visual function (Saunders 2002; M. Friedrich 2013). Exposure to light is perhaps the major variable impacting the FRPs of cave dwelling species. One study in cave-dwelling millipedes has shown a significant difference in FRP before and after exposure to light (Koilraj et al. 2000). Consequently, in this study, we collected under red light on the initial trip to investigate the FRP pre- and post-exposure to light.

This study aims to expand our knowledge of circadian rhythms in cave dwelling organisms, focusing on a species that has not been studied previously from a chronobiological perspective. Our spiders were collected from isolated populations of the same species (*Meta ovalis*) in two Tennessee and five Indiana caves. Our findings will add to what is known about spiders inhabiting caves and provide a unique opportunity to increase understanding of circadian variation among populations.

MATERIALS AND METHODS

Study Species

Meta ovalis (Araneae: Tetragnathidae), the cave-dwelling orb weaver, is found in caves, basements, abandoned mines, and deep ravines in the United States (Yuri and Seppo 1992). They are found along the eastern side of the United States and Canada, running from Newfoundland and Labrador south to Georgia, and west to Arkansas (Fig. 1). It is one of two species of this genus in the United States, the other being *Meta dolloff*, which is found only in two counties in

California, and listed on the 2000 IUCN Red List of Threatened Animals (Mammola and Isaia 2014). *Meta ovalis* has not been extensively studied, and little, is known about this organism's life cycle, lifespan, or circadian rhythms. There are limited data on locomotor activity rhythms in cave dwelling spiders in general and none thus far have focused on *Meta ovalis*.

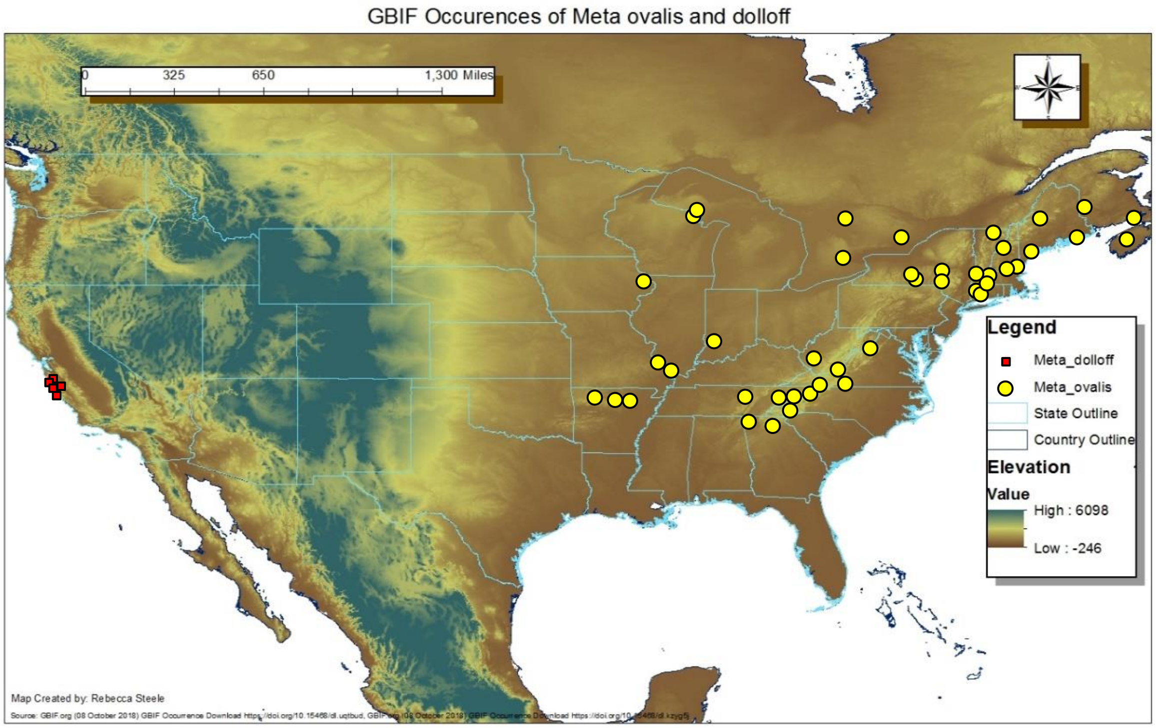

Figure 2.1: Map of the United State and Canada showing occurrences of Meta ovalis *and* Meta dolloff*, according to user generated data from GBIF.com*

Scientific Permits

Work in Tennessee was permitted by The Tennessee Department of Environment and Conservation (permit no. 2018-033). Work in Indiana was permitted for Bronson, Sullivan, and Elrod Caves through the Indiana Karst Conservancy, Indiana Nature Preserves, and Indiana Department of Natural Resources. Work in Riggs Shelter Cave and Salamander Cave in Indiana was permitted by private landowners.

Data Collection

The study species was collected from two caves in northeast Tennessee, five caves in central Indiana, and one cave in western North Carolina between April 2018 and February 2020. All individuals were placed into the experimental apparatus (see below) within 24 hours of collection. Care of animals followed ASAB/ABS guidelines: the spiders were either released at the cave of collection at the depth they were collected from, or were humanely dispatched and donated to the East Tennessee State University arachnid collection. The spiders were a mixture of sub-adults and adults. If they were mature, sex was noted. To record locomotor activity, individuals were placed in either 25 mm or 50 mm diameter X 100 mm length, clear tubes loosely capped to allow airflow. These were then inserted into locomotor activity monitors (model LAM 25 or LAM 50, TriKinetics Inc., Waltham, Massachusetts). During the experiment, monitors were kept in environmental control chambers (18 ± 0.5 °C) under a light-dark cycle of 12 hours of light and 12 hours of dark (LD 12:12) for five days, followed by seven to ten days of constant darkness (DD). For the first collection trip, individuals were collected from Sculpture Cave in Washington County, TN under dim red light, transported in a light-tight container, and transferred to activity monitors under dim red light (Jones et al. 2011). These individuals were first exposed to DD conditions for seven days to record the initial free-run before exposure to light, then exposed to LD 12:12 for five days, then again to DD for an additional seven days. This protocol was used on the initial trip to determine if exposure to light would have an effect on the FRP of the animal before and after light exposure. A paired samples T-Test ($t = -1.674$, $p > 0.1$) showed there was no significant difference between the initial FRP before light exposure, and the second FRP after exposure; therefore, all additional individuals were collected under normal caving headlamps with 40-500 lux. The lights used in the environmental chambers were two horizontally mounted 32 W fluorescent tubes providing approximately 1400-1600 lux at the level of the monitors. Lights were programmed to turn on at 08:00 h and off at 20:00 h during the LD cycle. Containers of water were placed in the chambers to maintain high humidity throughout the study. Activity bouts, compiled in 1-min intervals, were recorded by the monitors whenever the animal crossed an array of infrared beams bisecting each tube. Clocklab Analysis 6 Software (Actimetrics, Wilmette, IL, U.S.A.) was used to generate actograms, determine the phase angle of activity onset relative to the lights-off transition, and to identify significant ($p < 0.001$) circadian and ultradian periodicities. We used Lomb-Scargle periodograms to determine significant periodicities because this method is well suited for sparse data with frequent or large gaps. (Van Dongen et al. 1999). The most stringent p-value of 0.001 was used when determining significant periodicities. Onset of activity after photophase (min) was calculated by hand for each individual for each day of LD to analyze delay of activity after scotophase.

RESULTS

Onset of Locomotor Activity During LD

Onset of locomotor activity was recorded by calculating the delay of the first bout of locomotor activity for each individual after lights out at 20:00 per day of the LD cycle. A one-way between subjects ANOVA was run to examine the main effect of site and sex on onset of activity after lights out. There was a significant main effect of site ($F_{7, 135} = 10.56$, $p < 0.001$, Fig. 2), sex ($F_{2,135} = 16.56$, $p < 0.001$), and interaction of site and sex ($F_{12,135} = 3.82$, $p < 0.001$). Post-hoc comparisons using the Tukey HSD test showed that the onset of activity after lights out at 6-18 Sculpture Cave was significantly later ($p < 0.001$) than Quaker Knobs (M = 1.15 h., SD = 0.5

h.) and Elrod Cave (M = 0.95 h., SD = 0.23 h Additionally, 4-19 Sculpture Cave was significantly later ($p < 0.001$) than Elrod Cave (M = 0.95 h., SD = 0.23 h.). A one-way between subject ANOVA showed a significant main effect of sex ($F_{2,42} = 21.57$, $p < 0.001$). Post-hoc comparisons using the Tukey HSD test revealed a significant difference between mature females and sub-adults at 4-19 Sculpture Cave (Table 1, Fig. 3).

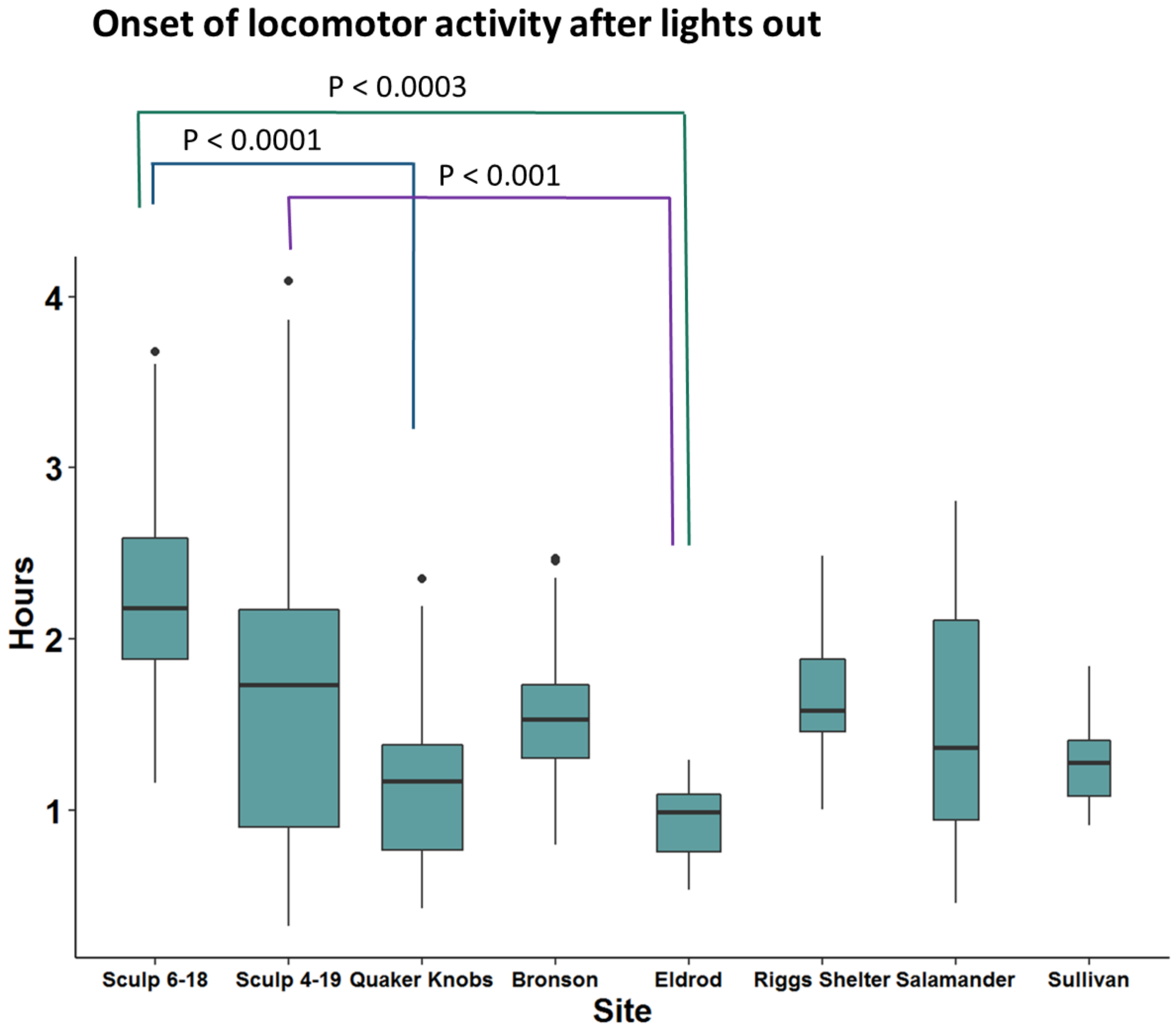

Figure 2.2: Comparison of onset of locomotor activity during the LD phase across sites. Statistically significant relationships are shown with connecting lines. Sculp 6-18 (TN) significantly higher than Quaker Knobs (TN) and Elrod (IN). Sculp 4-19 (TN) is significantly different from Elrod (IN)

Average Onset of Activity (min) per Individual			
Sex/Age	**Mean**	**SD**	**N**
Female	106.115	48.561	71
Male	85.032	32.165	25
Sub-adult	74.411	40.420	56

Table 1: Delay in onset of locomotor activity, averaged of individual spiders in minutes for each day of LD cycle. A significant difference was found between mature adult females and sub-adult specimens

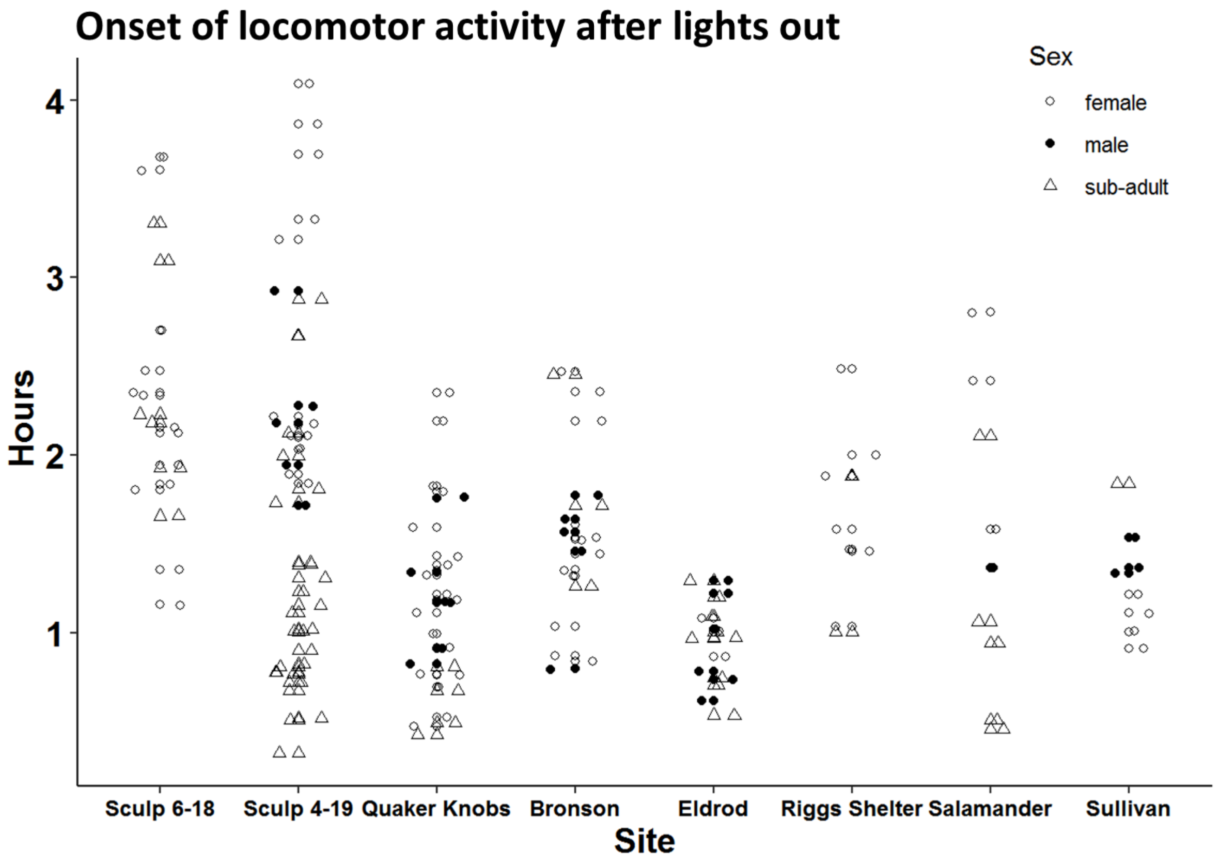

Figure 2.3: Scatterplot showing effect of sub-adults on the April visit to Sculpture Cave. Females are shown by open circles, males by filled circles, and sub-adults by open triangles. Sub-adults generally start locomotor activity more quickly in scotophase than adults, this difference most notable in 4-19 Sculpture Cave

Free-Running Period

The endogenous free running period was calculated with Lomb-Scargle periodograms to determine significant peaks. A one-way between subjects ANOVA was run to examine the main effect of site and sex on free-running period (Fig. 4). There was a significant main effect of site ($F_{7,136} = 9.76$, $p < 0.001$). Post-hoc comparisons using the Tukey HSD test showed that the FRP of Sculpture 6-18 (M = 23.80 h., SD = 2.19 h.) was significantly shorter ($p < 0.01$) than Sculpture 4-19 (M = 25.48 h., SD = 2.39 h.) and Quaker Knobs (M = 26.85 h., SD = 1.54 h.). There was no main effect of sex or interaction of site and sex on free-running period ($F_{2,136}$ = 1.341, $p = 0.256$ and $F_{12,136} = 0.420$, $p = 0.954$, respectively).

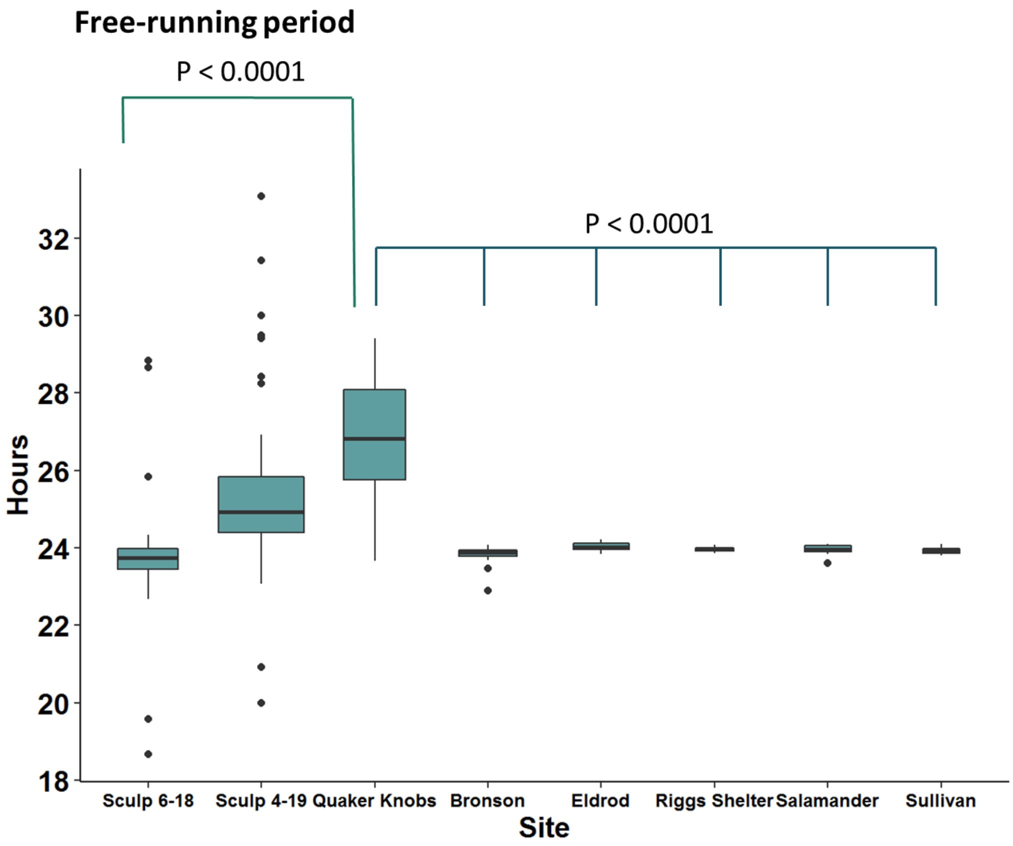

Figure 2.4: Box plot showing variation in free running periods between isolated caves. Both visits to Sculpture cave show a large amount of variation, Quaker Knobs cave individuals showed a longer than 24 h FRP (26.85 h.), and all the Indiana caves show FRPs close to 24 h. with little variation

The average FRP for each site is shown in Table 2. 4-19 Sculpture cave showed longer FRPs than 6-18. Quaker Knobs had a longer FRP than did each of the caves from Indiana (Fig. 5).

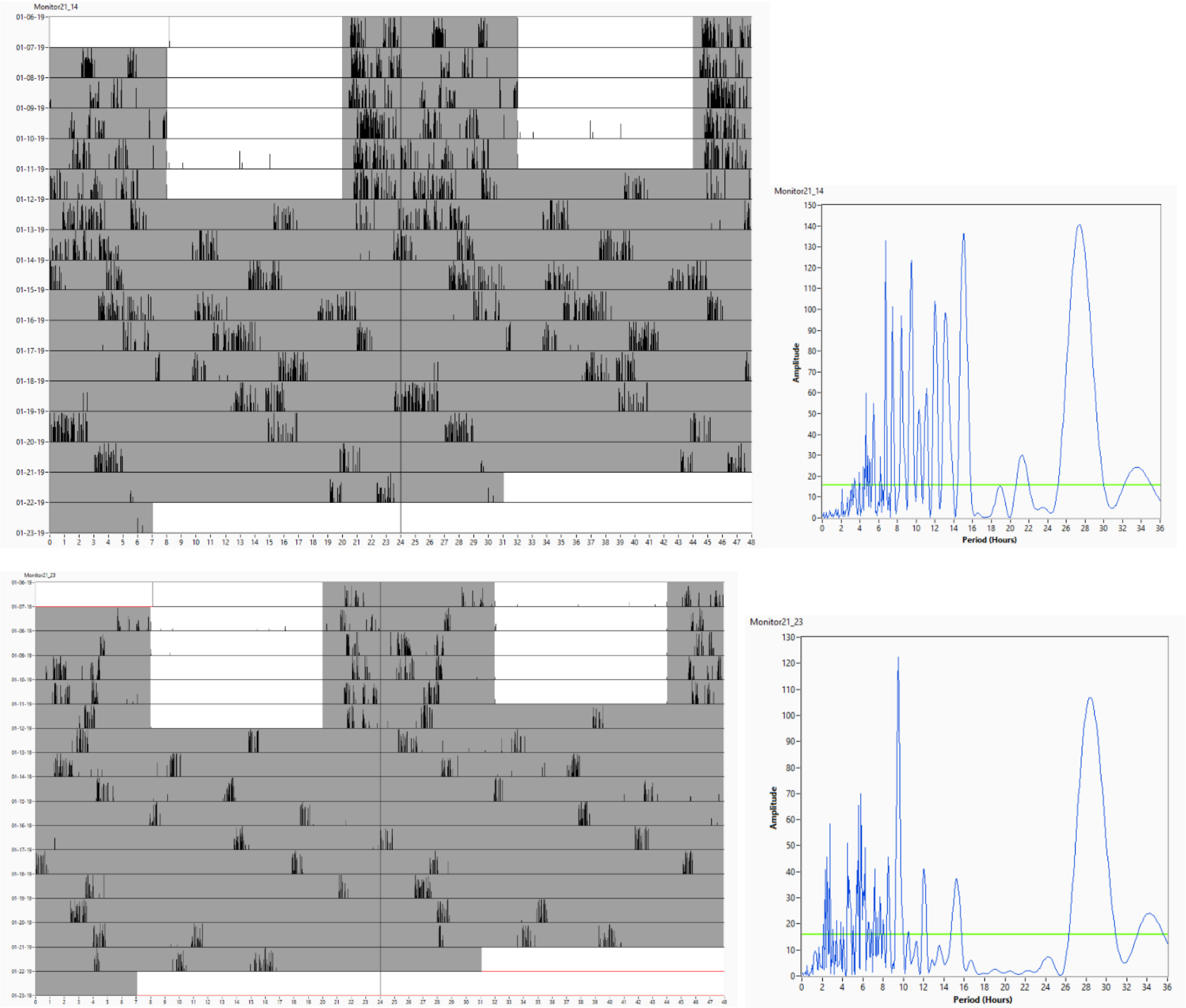

Figure 2.5: Actogram showing locomotor activity of individual 14 (top) and individual 21 (bottom) from Quaker Knobs cave. White areas represent lights on, gray shaded areas represent lights off (left). Associated periodogram analysis (right) reveals individual 14 and 21 have endogenous FRPs of 26.12 h and 28.13, respectively

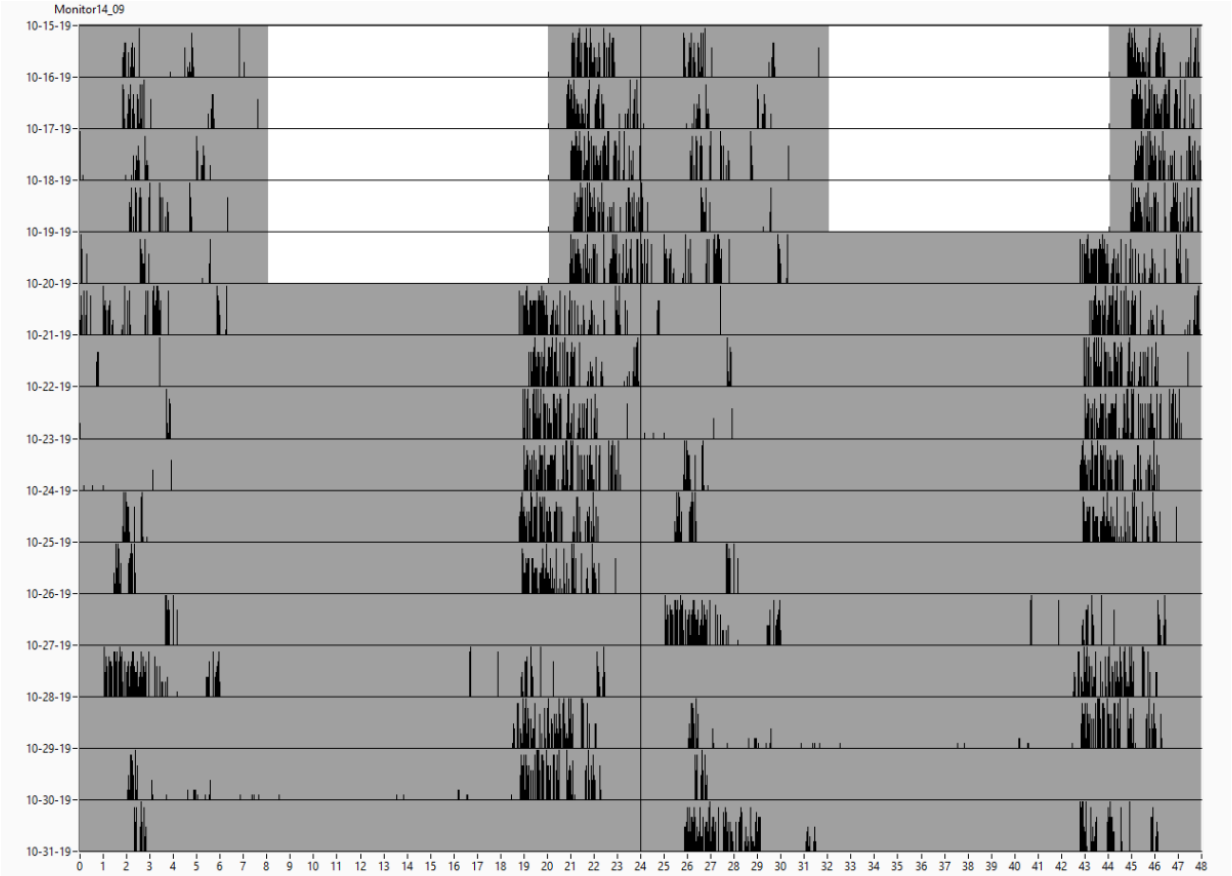

Figure 2.6: Representative actogram of an individual from Indiana showing 24-h FRP

SITE	June 2018 Sculpture	April 2019 Sculpture	Quaker Knobs	Bronson	Elrod	Riggs Shelter	Salamander	Sullivan
AVERAGE FRP (h)	23.80 ± 2.19	25.48 ± 2.69	26.89 ± 1.71	23.83 ± 0.26	24.03 ± 0.11	23.95 ± 0.07	23.96 ± 0.14	23.90 ± 0.08

Table 2: Average free running periods for each individual cave

DISCUSSION

We found differences among and within isolated populations of a single species of subterranean spider. We show large variation in FRPs of locomotor activity between individuals at one southern Appalachian site with many individuals exhibiting remarkably long (e.g. 29.33 h) or short (e.g. 19.58 h) endogenous circadian periods. Some of this within-site variation can be accounted for by the larger number of sub-adults collected during the winter; however, the variation can still be seen in fully mature specimens. Additionally, differences in free running period were found based on time of year the spiders are collected, with one site being sampled twice and showing a significant increase in FRP at the end of winter, and one site showing longer than 24-hour FRPs in January. Ecologically, this could be an adaptive strategy to take advantage of the more temperate habitat within the cave, allowing for a longer period per day of foraging a scarce winter food supply, or for reproductive advantage (L M Carver et al. 2016; Mammola and Isaia 2018). We found individuals at another Tennessee cave, Quaker Knobs, which show an average FRP longer than all the other sampled caves. The longer clock could be selective, allowing individuals to benefit from being released from the constraints of living in a natural light dark cycle. Another hypothesis for the longer endogenous period shown at Quaker Knobs is that the individuals have experienced the genetic effects of a small population size, and overall heterozygosity has been reduced in this population, with the longer clock being close to fixed within the population. Spiders from Indiana caves all show free-runs close to 24-h, with very little variation within and among sampled populations. This similarity between caves in Indiana could be the result of strong selection for a 24-hour period, or there is a large amount of genetic diversity due to migration between the populations. The sampled caves within Tennessee are 30 miles (~48 km) apart, some caves in Indiana were within half a mile of each other, and the maximum distance between caves in Indiana was 20 miles (~32 km). It should be noted, however that these caves likely have interconnected passages the spiders and other organisms can use, therefore distance between entrances for humans is not an ideal measure.

Traditional circadian theory, and prevailing evidence suggests that the majority of organisms exhibit endogenous periods that are close to 24-hours, with a small range, and very little interindividual variation (Aschoff 1981). This study adds mounting evidence that some spider species create their own circadian rules, and do not follow traditional theory (Moore et al. 2016; Garmany et al. 2019). These subterranean spiders show a large range of endogenous periods, interindividual variation, and endogenous periods that are out of phase with the external day. Since these spiders are not subject to cyclical light patterns, this potentially frees them from entrainment constraints, allowing them to drift and adaptively take advantage of fluctuations within their particular habitat. Further research should include more observation to determine effect of predation, seasonal influences, and geologic differences.

Natural variation among and within each cave system has the potential to have an impact on the natural rhythms of cave dwellers. Although generally stable environments, temperature, humidity, other biota, presence of running water, and geological layout are variables that are not accounted for. Each cave is a unique habitat that has the ability to produce local adaptation of the species that dwell there. Future studies should consider recording environmental variables to be included in the analysis, as well as field observations. Removing any organism from its habitat may cause behavioral changes that will skew results.

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APPENDICES

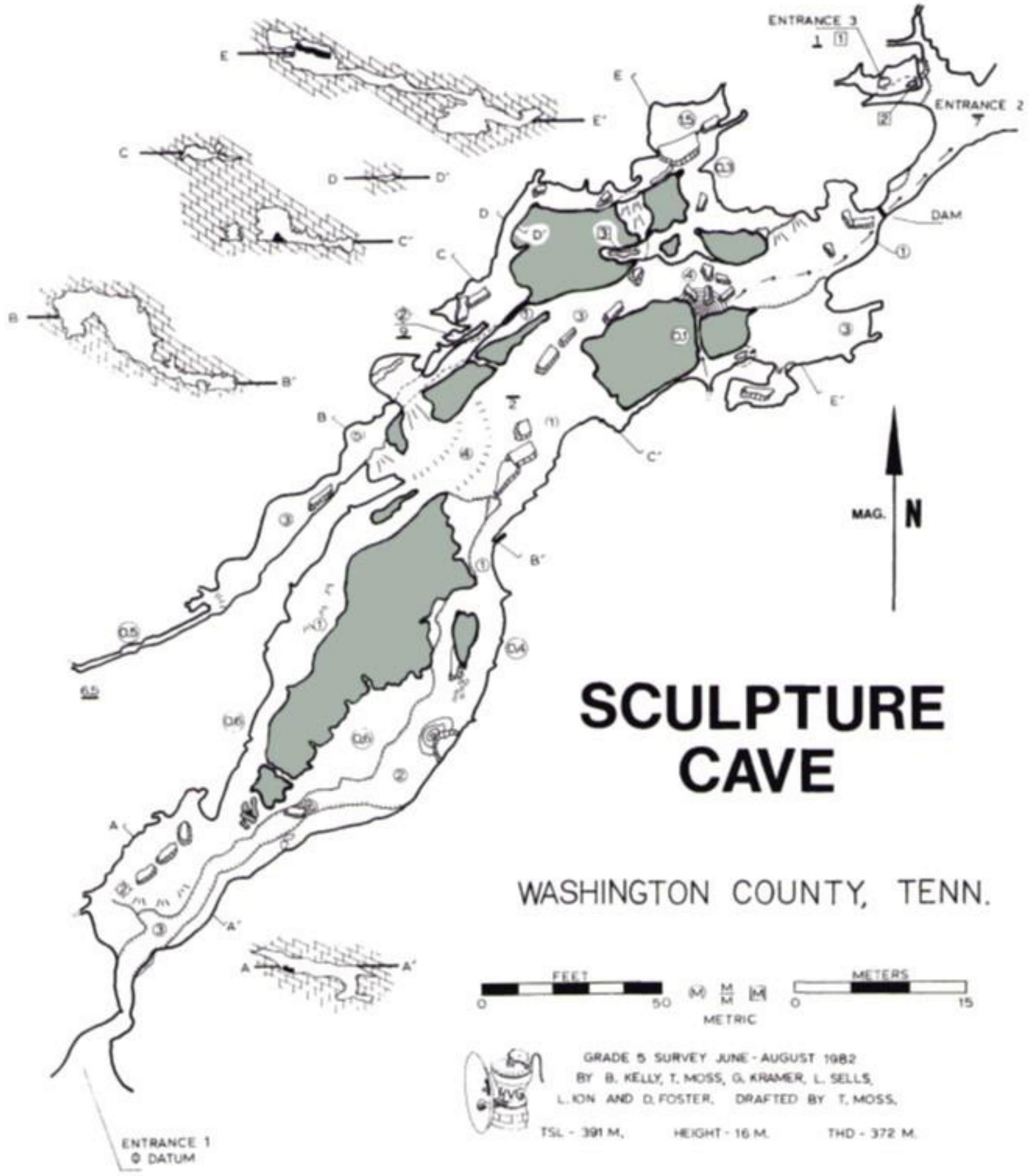
Appendix A: Additional Tables

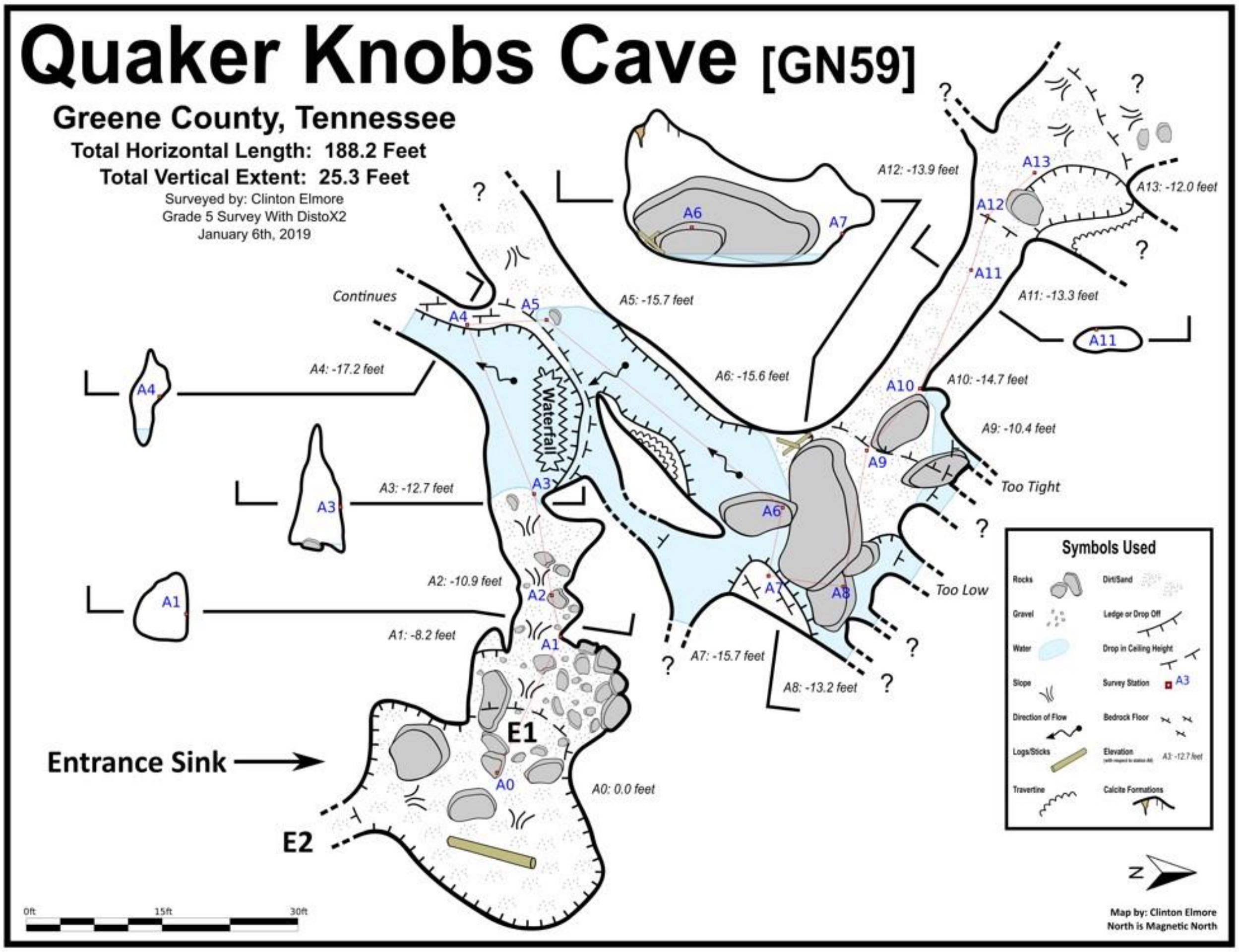
Table 1: Table showing review of papers studying locomotor activity in LD and DD.

PAPER	AUTHOR AND YEAR	ORGANISM	RHYTHMICITY IN LD	RHYTHMICITY IN DD	CONCLUSION
Evolution of time-control mechanisms in subterranean organisms	Trajano, E. et al. 2011	Cave fish *Teleostei: Siluriformes, characiformes*	Strong, significant rhythms	*S. typhlops* (strongly troglomorphic): weak or no rhythmicity *P. kronei* (moderate troglomorph): moderate to strong rhythmicity	External, environmental selection
Circadian locomotor activity and entrainment in cave spiders	Soriano-Morales, S. et al.	Cave spiders *Dipluridae* and *Ctenidae*	Strong rhythms	*Dipluridae*: most show concise activity ~24 hr *Ctenidae*: spread out activity close to 24 hour	When exposed to DD, then LD: Average FRP gets shorter (DD: 25.18 ± 0.75 h, LD: 24.12 ± 0.29h) Diplurids displayed diversity in activity unimodal, bimodal, and arrhythmic
Presence of circadian rhythms in the locomotor activity of a cave dwelling millipede	Koilraj, J. A. et al. 2000	Cave dwelling millipede *Glyphiulus cavernicolus sulu*	Moderate rhythms	No LD prior: 56.5% (n=23) showed rhythms (25.7 ± 3.3 h) 43.5% showed no clear rhythm LD prior to DD: 33.7% (n=9) showed minor rhythmicity, average FRP 24.04 ± 0.8 h	These organisms still retain ability to respond to LD
Comparative study on free-running locomotor activity circadian rhythms in Brazilian subterranean fishes	Trajano, E. et al. 2009	Brazilian subterranean fish *Teleostei: Siluriformes; Characiformes*			Presence and robustness of activity varies according to the degree of specialization to subterranean life: More specialized, less circadian activity detected
Daily pattern of locomotor activity of the synanthropic spiders	Solís, R. et al. 2018	*Loxosceles laeta Scytodes globula*	Strong, mostly nocturnal rhythms, 73.6% of all activity, unimodal Strong, strictly nocturnal 95.9% of all activity, bimodal activity		
Circadian rhythms of locomotor activity in *Lycosa tarentula* and the pathways of ocular entrainment	Ortega-Escobar, Joaquín	*Lycosa tarentula*	Mostly nocturnal activity, some diurnal	Strong, robust rhythms averaging 24.1 h	Varying light intensities used able to entrain to as low as 1 lx. LL caused arrhythmic patterns. All eyes able to detect and entrain to light except anterior median eyes. Placed in DD first, then LD- All entrained within 1-2 days

Appendix B: Cave Maps

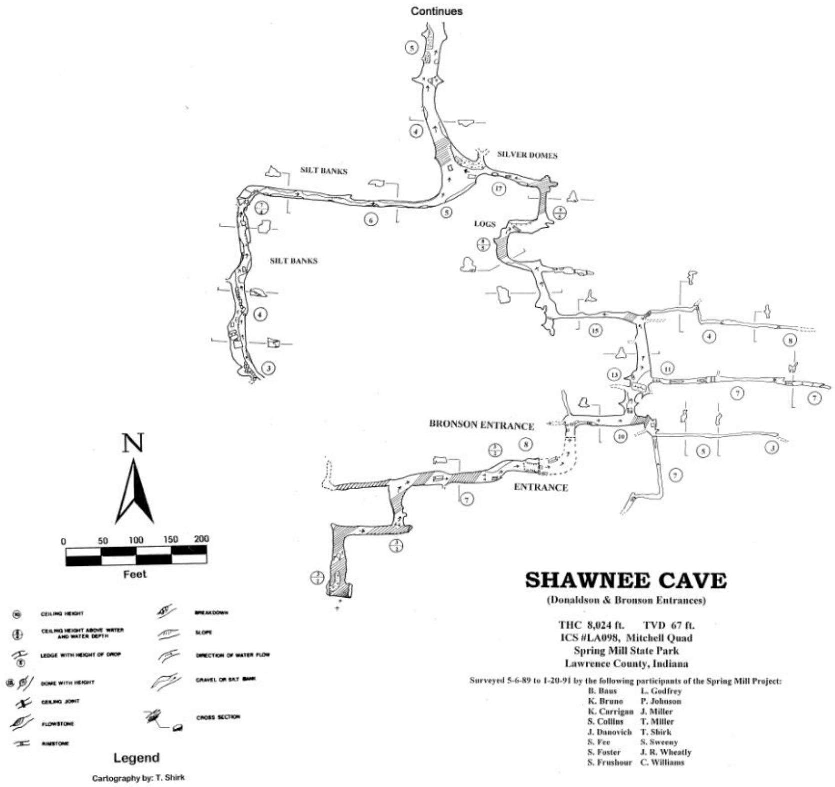
Tennessee Caves

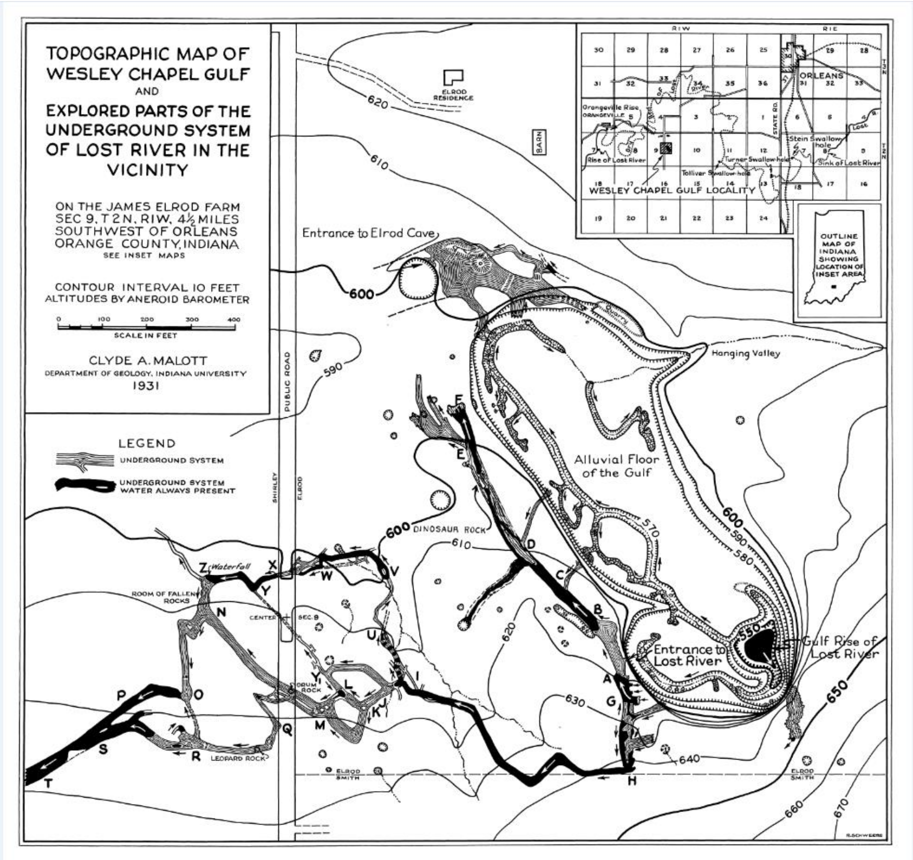


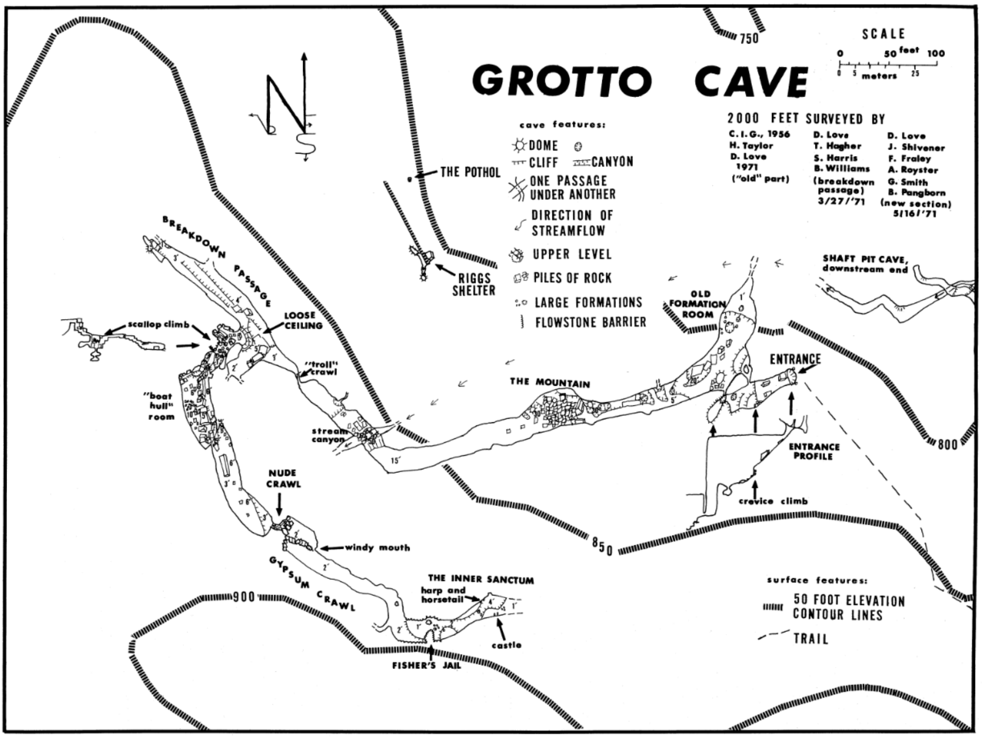


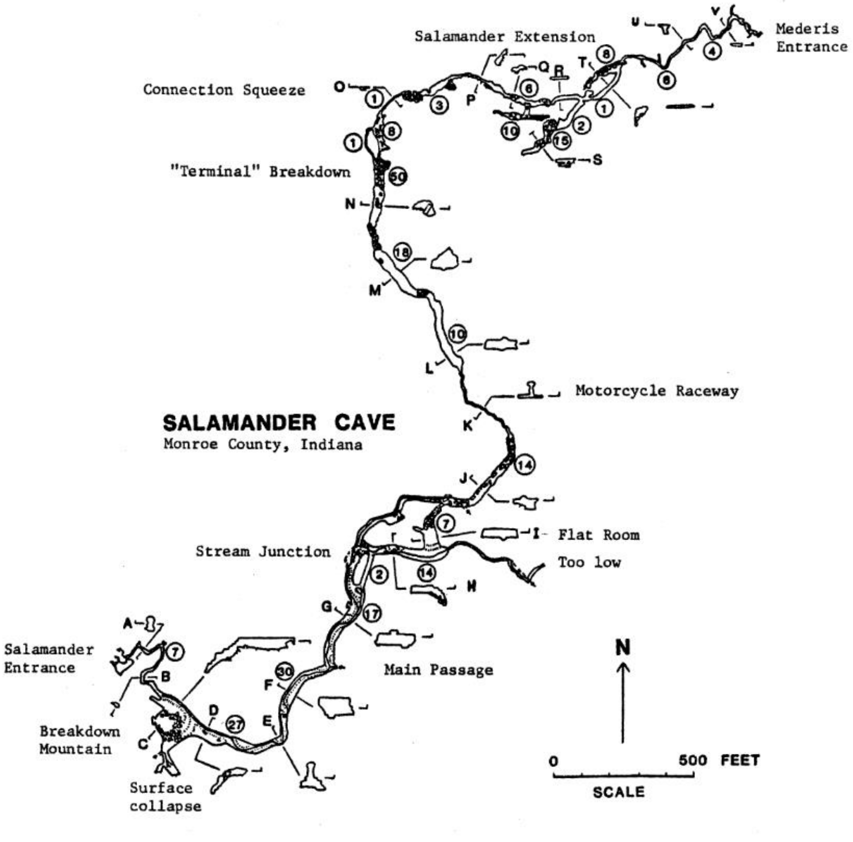
Indiana Caves

Listed in order: Bronson, Elrod, Riggs Shelter, Salamander, Sullivan

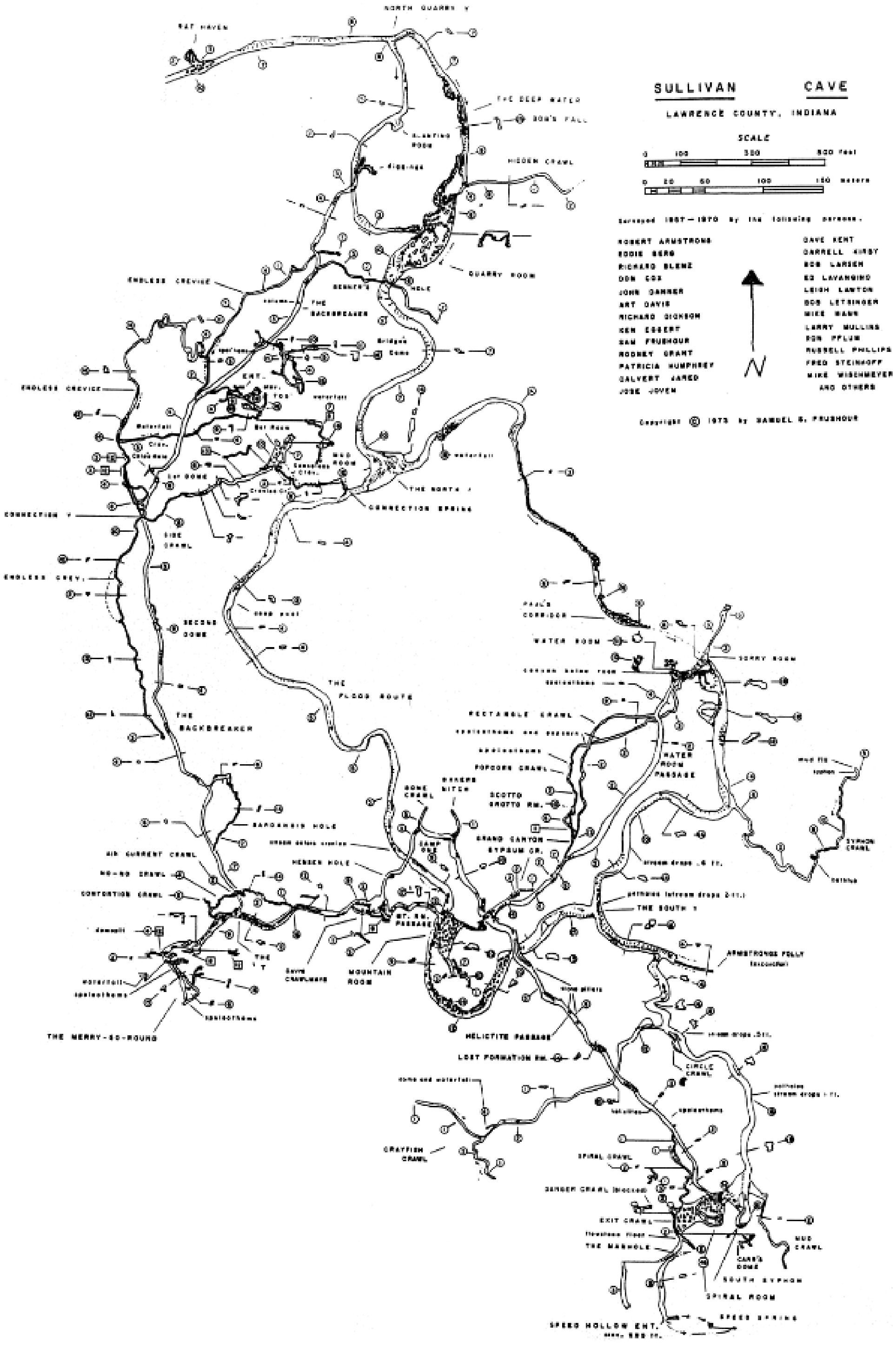

Continues
SILVER DOMES
SILT BANKS
LOGS
SILT BANKS
BRONSON ENTRANCE
ENTRANCE
N
0 50 100 150 200
Feet
CEILING HEIGHT
CEILING HEIGHT ABOVE WATER AND WATER DEPTH
LEDGE WITH HEIGHT OF DROP
DOME WITH HEIGHT
CEILING JOINT
FLOWSTONE
RIMSTONE
BREAKDOWN
SLOPE
DIRECTION OF WATER FLOW
GRAVEL OR SILT BANK
CROSS SECTION
Legend
Cartography by: T. Shirk
SHAWNEE CAVE
(Donaldson & Bronson Entrances)
THC 8,024 ft. TVD 67 ft.
ICS #LA098, Mitchell Quad
Spring Mill State Park
Lawrence County, Indiana
Surveyed 5-6-89 to 1-20-91 by the following participants of the Spring Mill Project:
B. Baus L. Godfrey
K. Bruno P. Johnson
K. Carrigan J. Miller
S. Collins T. Miller
J. Danovich T. Shirk
S. Fee S. Sweeny
S. Foster J. R. Wheatly
S. Frushour C. Williams


TOPOGRAPHIC MAP OF WESLEY CHAPEL GULF AND EXPLORED PARTS OF THE UNDERGROUND SYSTEM OF LOST RIVER IN THE VICINITY
ON THE JAMES ELROD FARM SEC 9, T 2 N, R 1 W, 4½ MILES SOUTHWEST OF ORLEANS ORANGE COUNTY, INDIANA
SEE INSET MAPS
CONTOUR INTERVAL 10 FEET ALTITUDES BY ANEROID BAROMETER
0 100 200 300 400
SCALE IN FEET
CLYDE A. MALOTT
DEPARTMENT OF GEOLOGY, INDIANA UNIVERSITY
1931
LEGEND
UNDERGROUND SYSTEM
UNDERGROUND SYSTEM WATER ALWAYS PRESENT
ELROD RESIDENCE
BARN
PUBLIC ROAD
SHIRLEY
ELROD
Entrance to Elrod Cave
Hanging Valley
Alluvial Floor of the Gulf
Entrance to Lost River
Gulf Rise of Lost River
DINOSAUR ROCK
Waterfall
ROOM OF FALLEN ROCKS
CENTER
SEC. 8
DRUM ROCK
LEOPARD ROCK
ELROD SMITH
620
610
600
590
570
580
550
630
640
650
660
670
A B C D E F G H I K L M N O P Q R S T U V W X Y Z
R 1 W
R 1 E
ORLEANS
Orangeville Rise
ORANGEVILLE
Rise of Lost River
STATE RD.
Stein Swallow hole
Sink of Lost River
Turner Swallow hole
Tolliver Swallow hole
WESLEY CHAPEL GULF LOCALITY
OUTLINE MAP OF INDIANA SHOWING LOCATION OF INSET AREA
R. SCHWEERS





Tape and compass survey by members of the Bloomington Indiana Grotto, Central Indiana Grotto, and Western Indiana Grotto at various times between 1961 and 1984. Compiled and revised by William L. Wilson, October, 1984.


SULLIVAN CAVE
LAWRENCE COUNTY, INDIANA
SCALE
0 100 300 500 feet
0 20 60 100 150 meters
Surveyed 1967 — 1970 by the following persons.
ROBERT ARMSTRONG
EDDIE BERG
RICHARD BLENZ
DON COX
JOHN DANNER
ART DAVIS
RICHARD DICKSON
KEN EGGERT
SAM FRUSHOUR
RODNEY GRANT
PATRICIA HUMPHREY
CALVERT JARED
JOSE JOVEN
DAVE KENT
DARRELL KIRBY
BOB LARSEN
ED LAVANGINO
LEIGH LAWTON
BOB LETSINGER
MIKE MANN
LARRY MULLINS
DON PFLUM
RUSSELL PHILLIPS
FRED STEINHOFF
MIKE WISCHMEYER
AND OTHERS
N
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NORTH QUARRY Y
BAT HAVEN
THE DEEP WATER
BOB'S FALL
SLANTING ROOM
HIDDEN CRAWL
QUARRY ROOM
BENNER'S HOLE
ENDLESS CREVICE
THE BACKBREAKER
ENT.
BAT ROOM
MUD ROOM
THE NORTH Y
CONNECTION SPRING
CONNECTION Y
SIDE CRAWL
ENDLESS CREV.
SECOND DOME
THE FLOOD ROUTE
THE BACKBREAKER
PAUL'S CORRIDOR
WATER ROOM
SORRY ROOM
RECTANGLE CRAWL
POPCORN CRAWL
WATER ROOM PASSAGE
SCOTTO GROTTO RM.
DOME CRAWL
BAKERS HITCH
GRAND CANYON
GYPSUM CR.
CAMP ONE
SARDANGIS HOLE
AIR CURRENT CRAWL
NO-GO CRAWL
CONTORTION CRAWL
HENSEN HOLE
ST. RM. PASSAGE
THE T
MOUNTAIN ROOM
THE MERRY-GO-ROUND
THE SOUTH Y
ARMSTRONGS FOLLY
SYPHON CRAWL
HELICTITE PASSAGE
LOST FORMATION RM.
CIRCLE CRAWL
CRAYFISH CRAWL
SPIRAL CRAWL
DANGER CRAWL (blocked)
EXIT CRAWL
THE MANHOLE
MUD CRAWL
CARR'S DOME
SOUTH SYPHON
SPIRAL ROOM
SPEED HOLLOW ENT.
SPEED SPRING

VITA

REBECCA STEELE

Education:	M.S. Biology, East Tennessee State University, Johnson City, Tennessee, May 2020 B.S. Sociology, East Tennessee State University, Johnson City, Tennessee, 2012 Public Schools, Johnson City, Tennessee
Professional Experience:	Gross Anatomy Laboratory Coordinator, Quillen College of Medicine; Johnson City, Tennessee, 2016-Current Annual Contracted Educator, Virginia Commonwealth University, Richmond, VA 2018- Current
Publications:	Conference Papers (Abstract-Reviewed) Mottern, John L., Steele, Rebecca N., Becker, Robert M., Abercrombie, Caroline L. “Popliteal Vascular Entrapment Syndrome: Accessory Slip of the Medial Head of the Gastrocnemius,” Proceedings of American Association of Clinical Anatomists, July 8-12, 2018.
Honors and Awards:	Caduceus Award, M1 Outstanding Course of the Year: Medical Human Gross Anatomy and Embryology 2018 Runner-up Three Minute Thesis (3MT) Competition 2018 James H. Quillen Scholarship Awardee, 2019-2020

Presentations and Invited Lectures:	Poster Presentation, “Popliteal Vascular Entrapment Syndrome: Accessory Slip of the Medial Head of the Gastrocnemius,” American Association of Clinical Anatomists July 2018. Poster Presentation, “Chronoecology of the Cave Dwelling Orb-Weaver Spider, Meta ovalis”, International Congress of Arachnology, Christchurch, New Zealand February 2019. Oral Presentation, “Circadian Strategy of a Cave Dwelling Spider, Meta ovalis” American Arachnological Society June 2019. Oral Presentation, “Differences in Circadian Strategy of a Cave Dwelling Spider, Meta ovalis between geographically distinct locations” Flittermouse Grotto October 2019.
Professional Training:	Leadership Training, East Tennessee State University, August 2018 “Boss, Bully, or Buddy: How to be a Leader”
Community Service/ Volunteer:	Dawn of Hope, Annual Spooktacular Fundraiser Volunteer, Johnson City TN, October 2017-Present Ashely Academy. Annual Science Fair Judge, Johnson City TN, March 2019-current Alternative Spring Break, Volunteer, Common Ground Relief, New Orleans LA, March 9-16 2019