RESEARCH ARTICLE

Incipient regressive evolution of the circadian rhythms of a cave amphipod

Luis Espinasa[1], Emily Collins[2], Anthony Finocchiaro[1], Joseph Kopp[1], Jenna Robinson[1], Jennifer Rutkowski[1]

1 *School of Science, Marist College, 3399 North Rd, Poughkeepsie, New York 12601, USA* **2** *Rensselaer Polytechnic Institute, 110 Eighth Street, Troy, NY 12180, USA*

Corresponding author: *Luis Espinasa* (luis.espinasa@marist.edu)

Abstract

The habitat of cave-adapted organisms is characterized by complete darkness and in some instances, an apparent lack of environmental distinction between day and night. It is unclear if cave-adapted organisms retain circadian rhythms that can be light-entrained. *Stygobromus allegheniensis* (Allegheny Cave Amphipod) is an eyeless troglobitic crustacean found in caves located in the Northeastern region of the United States. Two cave populations were examined for evidence of light-entrained circadian rhythms. The first population inhabits a small tectonic cave (Ice Caves, Sam's Point Preserve, NY) and the second (Clarksville Cave, Clarksville, NY) inhabits a long cave system in limestone rock. Experiments conducted in both the field and the laboratory suggest that the capacity to exhibit motor rhythms has been conserved in at least some individuals of both populations. Nonetheless, their motor activity rhythms have high variability of period length between individuals and do not appear to be light-entrainable. It is thus proposed that in this species, light-entrainable circadian rhythms controlling motor activity have undergone incipient regressive evolution.

Keywords

Introduction

In the evolutionary history of organisms, the loss or reduction of ancestral characters is a common event that occurs when a character is no longer needed for survival. Examples of this phenomenon in nature include the regression of pelvic and posterior appendages in whales (Bejder and Hall 2002), teeth in birds (Chen et al. 2000), and eyes and dermal pigmentation in cavefish (Jeffery 2001; McCauley et al. 2004). Cave organisms are an excellent model in which to study regressive evolution. Species from many different lineages have undergone parallel evolution and have developed similar traits such as blindness, depigmentation and the loss of circadian rhythms due to the constraints of living in continuous darkness.

Despite the scientific community's interest in chronobiological research in species that do not experience day and night cycles, and the fact that cave-dwelling animals represent a powerful model for understanding the evolution of biological rhythms, few studies have been conducted in this field. Pasquali and Sbordoni (2014) and Beale and Whitman (2015) reviewed the literature and suggest that circadian activity tends to degenerate in cave obligate organisms, as indicated by comparisons of epigean and troglophilic species to troglobitic ones. However, the data have shown great variability. To contribute to a better understanding of this important aspect of regressive evolution, we have studied motor rhythms in the eyeless cave amphipod, *Stygobromus allegheniensis* Holsinger, 1967.

Stygobromus allegheniensis is a completely depigmented and eyeless amphipod, with the largest individuals reaching 2 cm long (Figure 1). Despite the complete regression of ocular structures, this species is able to detect light (Espinasa et al. 2015). Individuals from this species exhibit nyctophilia, a distinct preference for darkness over illuminated conditions. Furthermore, their non-ocular receptors appear to be tuned to specific wavelengths of light, as specimens do not respond to red light, but actively avoid green light (Espinasa et al. 2015).

This species is located within caves found in Maryland, Pennsylvania, and New York, covering a distance of approximately 596 km from North to South, making it one of the largest ranges of any troglobiont in this genus (Holsinger 1967). Despite its wide range, no significant morphological variation among populations has been found (Holsinger 1967), and DNA sequencing of Ice Caves and Clarksville Cave specimens supports that these distant populations belong to a single species (Cahill et al. 2015). The Ice Caves are found along the Shawangunk Ridge, at Sam's Point within the Minnewaska State Park, New York, USA. Clarksville Cave is a popular horizontal stream cave with about 1.5 km of passage in Albany County, New York. Colonization of these caves by the amphipods occurred less than 12,000 years ago as both caves were covered by glacial ice sheets during the Pleistocene Epoch (Espinasa et al. 2015).

While the regression of morphological structures may occur over time in the absence of specific selective pressures, it is unclear if the physiological processes associated with the degenerated organs are maintained throughout these processes of regression.

Figure 1. Adult and juvenile specimens of *Stygobromus allegheniensis* from Ice Cave #1 at Sam's Point Preserve. As is typical of cave-adapted organisms, this species is depigmented, has long appendages, and is fully eyeless. Nonetheless, it can detect light and actively avoids it.

For example, it is unclear if light-entrained circadian rhythms still coordinate diverse, complex physiological processes including behavior when ocular structures are regressed in cave environments (Espinasa and Jeffery 2006). In *S. allegheniensis*, regression of the eyes has occurred, but remains able to detect and react to light. It is thus unclear if other responses to light, such as a light-entrainment of circadian rhythms, are still present. Therefore, the purpose of this study is to analyze if light entrainement of circadian motor rhythms has experienced regressive evolution in *S. allegheniensis*.

Methods

Experiments were conducted both in the field and in the laboratory using collected specimens of *S. allegheniensis.* Two cave populations were studied: a) Sam's Point Ice Cave #1. Total length = 138 m. Minnewaska State Park Preserve, near Sams Point Rd., Cragsmoor, NY. October 24-25, 2015 for laboratory experiments and September 8–11, 2016 for field experiments. b) Clarksville Cave. Total length = about 1,500 m. Clarksville, Albany County, NY. June 23–25, 2016.

Field experiments

Three specimens were analyzed at each of both localities. Specimens were kept throughout the experiment in their natural environment inside of the caves and under continuous darkness. Each amphipod was placed in separate 10 cm wide petri dishes with water from the cave to a height of 3 cm. Water temperature was 14 °C. Specimens were left in the dishes for 24 hours to acclimatize prior to data collection. A DCR-SR42 Sony Digital camera with night vision was used to record the specimens continuously for 36 hrs. To measure motor activity in the field, each petri dish was divided into four quadrants. The video was fast-forwarded to each minute and data was recorded over ten-minute intervals to measure how many instances each specimen crossed into a new sector after a one-minute period. Thus, for each ten-minute interval of footage analyzed, a maximum of ten movements could be recorded. The number of movements to new quadrants for each ten-minute interval were then profiled on a graph. In order to avoid behavioral responses to light inside the cave, all activities of researchers, including the collection of the specimens, were done using night vision cameras and/or red lights as previous studies have shown that red light is not detected in this species (Espinasa et al. 2015). The inability of *S. allegheniensis* to react to red light was confirmed at the end of the experiment by targeted illumination with a red or green laser pointer. An immediate avoidance reaction was observed in response to the green laser, whereas no such reaction was observed in response to the red laser.

To determine if the periods of activity and periods of rest were randomly distributed through time, the goodness of fit of the Poisson distribution were tested using the G statistic. When random distribution is rejected, a variance lower than the mean supports a uniform distribution. A variance higher than the mean supports that periods of rest versus activity are clustered (Zar 2007).

Laboratory experiments

To test whether periods of activity or rest observed in the field followed light-entrainable circadian rhythms, specimens were brought to the laboratory and kept in darkness until the experiments were performed 60 hours post collection. Specimens were placed in separate 10 cm wide petri dishes with water to a height of 3 cm. Water from collection site was used. Two 100W light bulbs positioned one meter above the tanks were used for illumination during light periods. Previous studies (Espinasa et al. 2015) performed under similar illumination conditions have shown that the temperature difference between the beginning of the experiment and the end of the experiment is not significantly different. Data analyses were done with the customary experimental set-ups for studying circadian rhythms, which include: 1) Subjecting specimens to continuous conditions (such as constant darkness) to test if motor activity follows a 24 hour rhythmicity. 2) Subjecting specimens to conditions of light-dark-light-dark-dark (LDLDD 14:10:14:10:14) as organisms following a light-entrained circadian rhythm

are expected to behave in the last second dark period in similar ways to those of the light period as the internal clock is anticipating a period of illumination (Espinasa and Jeffery 2006). 3) Transferring from a day/light, night/dark periodic environment to a day/dark, night/light environment to see if behavior adjusts to the new illumination pattern. Thus, three specimens from the Ice Caves were subjected to the following conditions: Five half-cycles (60 hrs) of darkness, followed by two cycles of light/dark during normal day/night schedules, followed by two cycles of dark/light during reverse day/night schedules, followed by two half-cycle of darkness (Figure 2). One cycle is twenty-four hrs. Three different specimens from the Ice Caves were also subjected to four cycles of continuous light. From Clarksville Cave, four specimens were subjected to six half-cycles of darkness, followed by two cycles of light/dark during normal day/night schedules, followed by one cycle of dark during the day. One cycle is twenty-four hours. Recording of movements and statistical analyses were performed as above.

Results

Laboratory experiments

Specimens subjected to continuous darkness in the laboratory showed periods of rest in which they were observed positioned on their side with little to no movement, followed by periods of active movement where specimens were crawling throughout the petri dish (Figures 2 and 3). Data suggests that the periods of activity and periods of rest were not randomly distributed through time (P<0.001 for all individuals), but instead are clustered. These periods of activity and rest did not occur in a circadian pattern. The periods of rest varied greatly between all individuals of both localities. In two specimens (Figure 3A, D) the periods of rest and activity were short (4 hours or less) and intermittent, making it difficult to discern rhythmic periodicity. Three specimens had rest periods of about 12 hours (Figure 3C, F–G), and two individuals had rest periods of 25 hours or more (Figure 3B, E). Rest periods were also qualitatively different between individuals. Some specimens' rest periods were intermittently interrupted
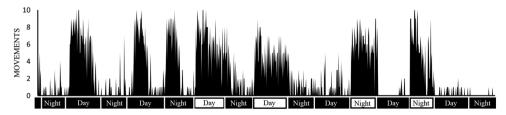

Figure 2. Experimental protocol and representative motor rhythms of one individual. Ice Cave individuals were subjected in the laboratory to the following conditions: Five half-cycles of darkness, followed by two cycles of light/dark during normal day/night schedules, followed by two cycles of dark/light during reverse day/night schedules, followed by a half-cycle of darkness. Black boxes indicate dark conditions while white boxes represent illuminated conditions. Movements were evaluated for each 10-minute period.
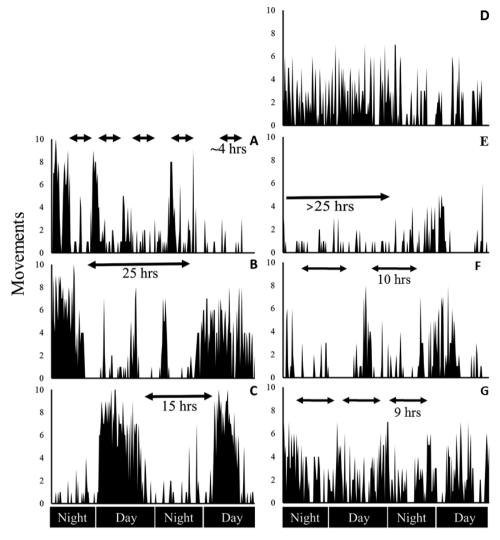
Figure 3. Variability in motor rhythms while in continuous darkness in three Ice Cave individuals (**A–C**) and four Clarksville Cave individuals (**D–G**) tested in the laboratory. Black boxes indicate periods while in darkness.

by short bouts of activity (Figure 3B, E), making it difficult to demarcate periodicity, while in other specimens, particularly specimen C from the Ice Caves (Figure 2 first five half-cycles of darkness and Figure 3C), the difference between rest and inactivity was easily delineated.

The periods of rest were neither synchronized among the individuals, nor synchronized to day and night schedules. For example, the individual depicted in Figure 2 was out of synchronization with the day/night cycles despite having a cycle close to 24 hrs. In this individual, during the first 60 hours of continuous darkness the first two periods of activity coincided with daytime hours, but by the third period it had
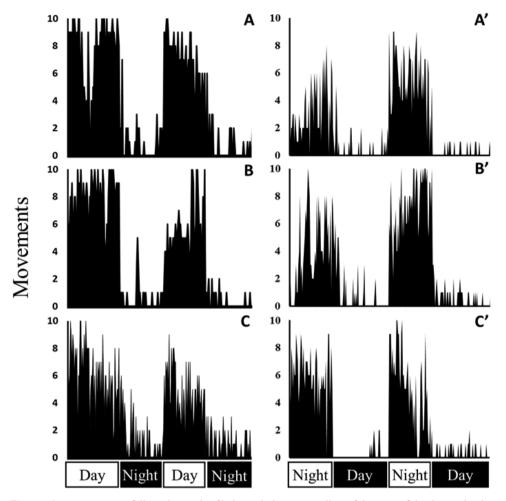
Figure 4. Motor activity followed periods of light or darkness regardless of the time of the day. Individuals on the left (**A–C**) are the same as individuals on the right (**A'–C'**). Black boxes indicate periods while in darkness and white boxes indicate illuminated conditions.

shifted enough to coincide with nighttime. In the other specimens with motor cycles longer or shorter than 24 hours, this lack of synchronization with day/night schedules also occurred.

When the specimens were subjected to LD 14:10 cycles, activity/rest patterns matched illumination (Figure 4), regardless of the time of day or night. Specimens on a LD cycle where light coincided with daytime and dark coincided with nighttime (Figure 4A–C) or on a DL cycle where dark coincided with daytime and light coincided with nighttime (Figure 4D–F), they followed the illumination patterns and not the time of the day. Statistical analysis also support that periods of activity versus rest were not randomly distributed through time (P<0.001 for all individuals), but instead were clustered. While this pattern first gives the appearance of motor rhythms
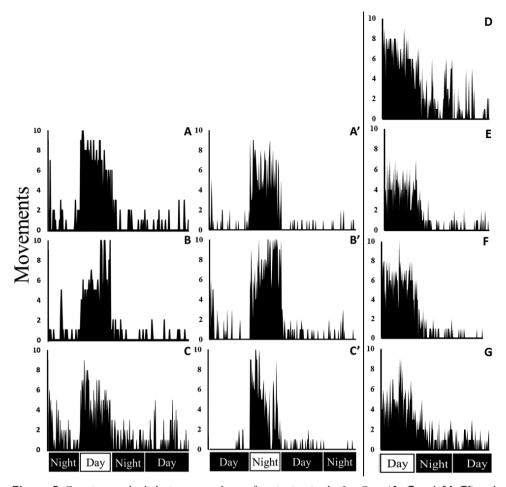
Figure 5. Entrainment by light is apparently not functioning in the Ice Cave (**A–C** and **A'–C'**) and Clarksville Cave (**D–G**) populations. In *S. allegheniensis*, the second dark period lacks the anticipation and synchronization of a period of activity, which is a hallmark of organisms possessing a light-entrained circadian rhythm. Black boxes indicate periods while in darkness and white boxes indicate illuminated conditions.

regulated by a light-entrained circadian rhythm, further experiments fail to support this conclusion. When *S. allegheniensis* from both cave populations were subjected to cycles of LDD 14:10:14, activity in the second dark period did not replicate those of the Light period as when the internal clock is anticipating a period of illumination. Instead, the organisms behaved similarly in both dark periods (Figure 5). This result was further supported when specimens were subjected to continuous light. Specimens continued to be active with few and momentary moments of rest (Figure 6). In the case under continuous illumination, data support that the periods of activity versus rest were not randomly distributed (0.05<P<0.01), but instead they were uniformly distributed through the time assessed for two of the three individuals.
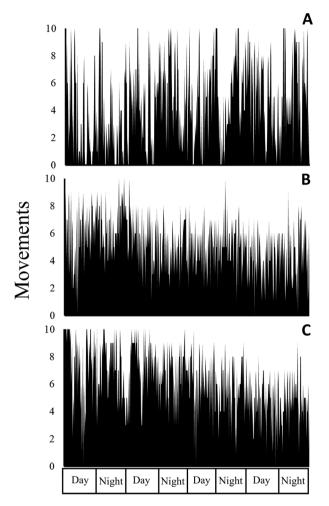
Figure 6. *Stygobromus allegheniensis* has continuous light avoidance behavior which does not appear to follow circadian rhythmicity. White boxes indicate illuminated conditions.

Field experiments

Experiments performed directly in Clarksville Cave and in the Ice Cave confirmed that in their natural environments, specimens behaved similarly in both the laboratory and the natural setting. While in continuous darkness, the specimens' periods of activity and rest did not follow a circadian rhythm (Figure 7). The length of the periods of rest varied greatly among individuals. For most individuals the rest periods are intermittently interrupted by short bouts of activity making it difficult to demarcate periodicity. In only one of the six individuals is there an apparent rest period of 12 hours (Figure 7E). The periods of rest were not synchronized among the individuals. Furthermore, the individual with the 12 hour rest (Figure 7E), periods of activity were not synchronized to day and night schedules.
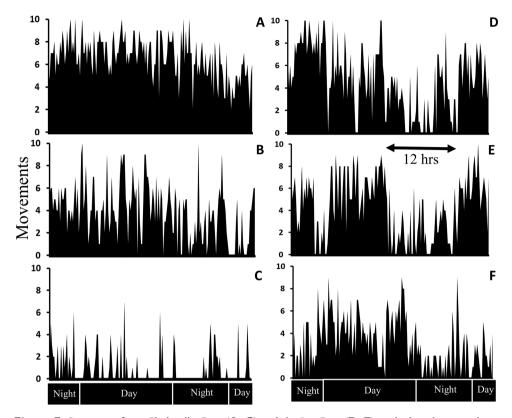
Figure 7. Specimens from Clarksville Cave (**A–C**) and the Ice Cave (**D–F**) studied in the natural environment of the cave. Under continuous darkness, most specimens had periods of activity with no clear indication of periodicity. Only in one of them (**E**) there was an apparent 12 hour rest period. Black boxes indicate periods while in darkness.

Discussion

To better understand the regressive evolution of circadian rhythms, we have studied the motor rhythms of eyeless cave amphipods who in their natural environment are submitted to continuous darkness, and thus reduced environmental distinction between the day and the night. Results from both the field and the laboratory suggest that *S. allegheniensis* has a degenerated expression of circadian rhythms. While specimens from the two cave populations have periods of intense movement followed by periods of apparent rest where specimens lay on their side with no body movements for extended periods, the periodicity of their motor rhythms are not synchronized to day and night cycles. This lack of synchronization would be expected in an environment that lacks distinction between day and night and in specimens with motor rhythms controlled by internal clocks that are not entrained by the environment. Furthermore, as it would be expected from an environment where selection for circadian rhythms is reduced, there is high variability in the length of the motor rhythms observed within a population.

Some individuals appear to have motor-activity cycles that follow rhythms shorter than 24 hours while others follow rhythms that are longer (Figure 3). Most relevant, however, in seven out of thirteen (53.8%) specimens tested under continuous darkness for at least 36 hours there were no clear periods of rest of at least 6 hours followed by a period of activity of an equivalent length. This suggests that the motor periodicity may have been completely lost in some individuals (Figures 3A, D, 7A–D, F).

Despite the absence of functional eyes, experiments both in the field and in the laboratory show that light can generate motor activity. This is in agreement with Espinasa et al. (2015), which showed that individuals from this species actively try to avoid light. Nonetheless, the motor rhythms do not appear to be influenced by a light-entrained circadian rhythm. All specimens under conditions of light:dark:dark (LDD) failed to show activity in the second dark period that would normally be expected to mimic the activity observed in the light period (Figure 5). The anticipation and synchronization of a period of activity is lacking, which is a hallmark of organisms possessing a light-entrained circadian rhythm (Espinasa and Jeffery 2006). Likewise, specimens under continuous light conditions showed non-stop activity (Figure 6). Based on these results, light appears to generate continuous avoidance activity, but does not appear to be able to entrain circadian motor rhythms. It appears that in *S. allegheniensis*, light-entrainable circadian rhythms have undergone regressive evolution despite the retained ability to detect light.

Unfortunately, there are few circadian studies that have been performed on cave adapted animals for comparison. For a review of bibliography, see Beale and Whitmore (2015). One of these studies was on the cave amphipod, *Niphargus puteanus* Koch, 1836. Under continuous darkness (DD) regimes, the animals were active without clear pauses of rest, whereas light/dark (LD) conditions of various period lengths induced activity rhythms that usually disappeared after transfer back to constant conditions (Knight and Johns 2015). It appears that in *N. puteanus* there may not be motor rhythms when in complete darkness.

Beale and Whitman (2015) concluded that there is a vast array of circadian phenotypes in cave-adapted animals. Some retain partially functioning oscillators, some show highly variable rhythms between individuals within populations, and some show an absence of circadian rhythms all together. Our data suggest that *S. alegheniensis* may still have some partially functioning oscillators with highly variable rhythms between individuals, but which are not light-entrainable. It appears that while *S. alegheniensis* has undergone incipient regressive evolution of circadian rhythms, it has not reached the levels experienced by other cave adapted species where motor activity periodicity is completely lost. One hypothesis that could explain this level of regressive evolution is that *S. alegheniensis* may have a young and comparatively short cavernicole evolutionary history. The cavernicole habitat available for *S. alegheniensis* became available only when the glacial ice sheets retreated during the Pleistocene Epoch about 12,000 years ago (Espinasa et al. 2015). By comparison, some troglobites in more tropical areas unaffected by glaciation may have had millions of years for adaptation after colonization of the underground environment. Another possible explanation for *S. alegheniensis* is

that this species is not strictly limited to the cave environment, but can also survive in the hyporheic zone under the gravel of surface creek beds. While still an underground environment, this habitat can be influenced by day and night periodicity.

Conclusions

We report a case of incipient regressive evolution of circadian rhythms in *Stygobromus alleghaniensis*, an eyeless troglobitic crustacean found in caves located in the Northeastern region of the United States. While some individuals collected from two caves may display motor rhythms, they are no longer light-entrainable.

Acknowledgments

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