

Evidence of ancestral nocturnality, locomotor clock regression, and cave zone-adjusted sleep duration modes in a cave beetle

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Abstract

The small carrion beetle *Ptomaphagus hirtus* is an abundant inhabitant of the exceptionally biodiverse Mammoth Cave system. Previous studies revealed negative phototaxis and the expression of biological clock genes in this microphthalmic cave beetle. Here we present results from probing *P. hirtus* for the entrainment of locomotor rhythms using the TriKinetics activity monitor setup. Although curtailed by low adjustment frequency of animals to the test environment, the data obtained from successfully monitoring two animals in constant darkness (DD) and six animals exposed to 12 hour light-dark cycles (LD) revealed a strong effect of light on locomotor activity in *P. hirtus*. In LD, activity was prevalent during the artificial night phases while close to absent during the presumptive day phases, suggesting conserved nocturnality. Upon transitioning LD animals to constant darkness, none displayed detectable evidence of free-running activity rhythms, suggesting complete regression of the central circadian clock. Equally notable, overall locomotor activity of the two DD-monitored animals was about three-fold lower compared to LD animals due to longer rest durations in the former. We, therefore, propose the existence of cave zone-specific energy expenditure modes that are mediated through light schedule responsive modification of sleep duration in *P. hirtus*.

Keywords

Cave adaptation, circadian clock, Coleoptera, Mammoth Cave, microphthalmic, sleep, TriKinetics activity monitor

Introduction

One of the most fundamental discoveries in behavioral biology is the near-universal capacity of organisms to track circadian light level changes to the effect that the onset and termination of time-sensitive biological activities can occur in a stable, anticipatory manner, unmitigated by daily variation of light or temperature. Key evidence of this capability is the stunning continuation of behavioral rhythms in the absence of daily light level change as the zeitgeber, which has been documented in plants and animals alike (Bell-Pedersen et al. 2005). Pioneering studies in *Drosophila* defined the complex molecular machinery which facilitates this capacity in animals (Konopka and Benzer 1971; Patke et al. 2020). The critical significance of the biological clock for survival fitness and reproduction is documented by its regulatory sophistication, deep evolutionary conservation (Jindrich et al. 2017; Nikhil and Sharma 2017; Tarrant et al. 2019), and the large number of diverse fitness-reducing consequences of biological clock misregulation (Patke et al. 2020). At the same time, the question of exactly how the biological clock affects evolutionary fitness in natural populations continues to be a subject of ongoing research (Horn et al. 2019).

Cave-dwelling animals have long had a special place in biological clock research. This is for the intuitive prediction that adaptation to constant darkness should lead to the eventual evolutionary loss of the biological clock together with the well-documented dramatic loss of light perception-related traits such as eyes (Poulson and White 1969; Abhilash et al. 2017). Cave species are, if at all, only exposed to the diurnal changes of low-intensity light in the twilight zone areas of open caves (Beale and Whitmore 2016). Also temperatures stay relatively constant in this subterranean niche, usually fluctuating only by a few degrees Celsius within a year (Badino 2004). Thus, given the overall ecological constancy of cave environments, biological clocks seem dispensable in caves and destined to neutral evolutionary decay. And yet, studies designed to verify the “clocklessness” of cave species encountered a surprisingly widespread persistence of the biological clock in cave-dwelling organisms (Friedrich 2013; Beale et al. 2016). One of the best-studied examples is the Somalian cavefish *Phreatichthys andruzzii*. Although completely eyeless, *P. andruzzii* displays light avoidance by virtue of deep brain photoreceptor cells (Tarttelin et al. 2012). However, this residual light sensitivity notwithstanding, *P. andruzzii* is unresponsive to circadian activity entrainment by light despite the conservation and expression of an intact biological clock machinery gene repertoire (Cavallari et al. 2011). Regularly timed daily feeding, however, entrains anticipatory foraging behavior. Based on comparison with surface species, the behavioral entrainment by food provisioning represents an ancestral trait of *P. andruzzii* (López-Olmeda and Sánchez-Vázquez 2010), which remained conserved together with the biological clock gene complement. Equally remarkable, the molecular clock of *P. andruzzii* also supports the metabolic synchronization of cells, although at an expanded period of 30 hours (Cavallari et al. 2011). This example of what is known as “peripheral clocks”, i.e. the autonomous activity of the biological clock machinery in a variety of tissues beside and independent of the circadian pacemaker neurons in the brain, is likewise well-documented in insects (Plautz et al. 1997).

Cave species like *P. andruzzi*, thus, raise the question of whether peripheral clock fitness benefits might continue to enforce conservation of the biological clock in cave environments (Lamprecht and Weber 1985; Vaze and Sharma 2013; Beale et al. 2016; Abhilash et al. 2017). In further support of this possibility, synchronized metabolic transcriptomes have been proposed to benefit the growth rates of fish larvae (Yúfera et al. 2017). Thus, while it is reasonable to predict that the circadian clock will regress in the absence of extrinsic advantages such as in the constant environments of caves (Shindey et al. 2017), the biological clock machinery itself may remain conserved due to its intrinsic advantages (Beale and Whitmore 2016). Cave species thus constitute an important resource to elucidate which functions of the biological clock are of intrinsic fitness significance besides optimizing the circadian behavior of surface species (Beale and Whitmore 2016).

In previous work (Friedrich et al. 2011), we performed an analysis of global gene expression in the adult head of the cave beetle species *Ptomaphagus hirtus*, an approximately 3 mm long small carrion beetle that is endemic to the highly biodiverse Mammoth Cave system in Kentucky (Packard 1888; Peck 1975; Culver and Hobbs 2017). This effort not only uncovered the expression of a large number of phototransduction genes but also of homologs of all insect core circadian clock genes, including *period* (*per*), *timeless* (*tim*), *Clock* (*Clk*), and *cycle* (*cyc*). Behavioral evidence of photophobic response to light produced further evidence of a highly reduced yet functional visual system, leading to the recategorization of *P. hirtus* as a microphthalmic cave beetle, in which the ancestral compound eyes have been reduced to relict eyelets with single lenses. Here we present the findings of efforts to probe for a functional circadian clock in *P. hirtus*, a scavenger, by testing for the light-entrainability of activity rhythms. While handicapped by a poor adjustment rate of laboratory-cultured animals to the experimental approach, our findings produced strong evidence that the circadian clock has regressed in *P. hirtus*, despite the central nervous system expression of biological clock genes. In addition, we present evidence of deep cave and twilight zone-attuned activity states in *P. hirtus* that may be the adaptive outcomes of nutrient abundance differences between the two ecological theaters.

Methods

Animal culture

P. hirtus adults were collected in March 2013 from White Cave entrance following guidelines defined in National Park Service permit MACA-2015-SCI-0019. Animals were cultured in a light-insulated cave laboratory room. Animals were housed in 60mm polystyrene Petri dishes supplied with a 50 millimeter deep bottom layer of cave soil. The Petri dishes were sealed off at the edges with parafilm. Culture dishes were placed in a Styrofoam box with a layer of moist paper towel at the bottom. The Styrofoam boxes were housed in an incubator at a temperature ranging between 10–12° degrees Celsius. Cultures were fed every two weeks with Fleischmann's Yeast pellets. All animal

handling was conducted under low-intensity red light, given the lack of light stress protective eye pigmentation in the eyelets of *P. hirtus* (Friedrich et al. 2011). Locomotor activity tests were conducted with offspring animals in 2017.

Monitoring of locomotor activity

Circadian activity was monitored using the Trikinetics Activity Monitor (TAM) (Trikinetics Inc., Waltman, MA, USA) placed in a Thermo Scientific Precision Low Temperature Incubator set at 11° degrees Celsius. Moist paper towel was secured to the bottom of the box to maintain humidity. Holes were poked in the plastic caps of the capillaries with a thumbtack for air circulation. Activity data were binned in 30-min intervals. For trials in constant darkness (DD), single beetles were placed into individual monitor capillaries and monitored for up to 14 days without the provision of food. For 12 hour light/12 hour dark regimen trials (LD), beetles were given a 5-hour acclimatization period in darkness, followed by the onset of 12h:12h light/dark cycles. RL5-W4575 White 75 Degree 4500 mcd LED lights (Super Bright LEDs Inc.) were used as light source powered at 2.5V and fixed 3 inches above the TAM, resulting in exposure of test animals to an approximate light intensity of 5×10^{16} photons/cm²/second during the 12 hour light phases.

Data analysis

Google spreadsheets and Actogram (Schmid et al. 2011) were used for the preparation of periodograms and actograms. The online implementation BoxPlotR (Spitzer et al. 2014) was used to generate summary box plots of activity intensities, activity periods, and rest periods. Nonparametric analyses of variance were conducted using the Kruskal Wallis Test Calculator hosted by Statistics Kingdom (2017): <https://www.statskingdom.com/kruskal-wallis-calculator.html>.

Results

Arrhythmic locomotor activity of *P. hirtus* in constant darkness

In addition to serving as the main tool in *Drosophila melanogaster* biological clock studies, the TAM setup has found increasing application for studying a broader range of insects (Bahrndorff et al. 2012; Giannoni-Guzmán et al. 2014; Pavan et al. 2016; Wang et al. 2021). Given the similar body sizes of *P. hirtus* and *D. melanogaster*, we explored the suitability of the original *Drosophila*-optimized TAM setup to monitor locomotor activity of adult *P. hirtus*, starting experiments in the light condition of deep cave zones, i.e. DD. Of a total of 28 individuals tested under these conditions in three separate trials, most perished within 2–5 days, after displaying high levels of locomotor activity (Suppl. material 1). Two individuals, however, completed an observation time span of 14 days alive (Fig. 1a, b and Suppl. material 2). Actogram inspection revealed

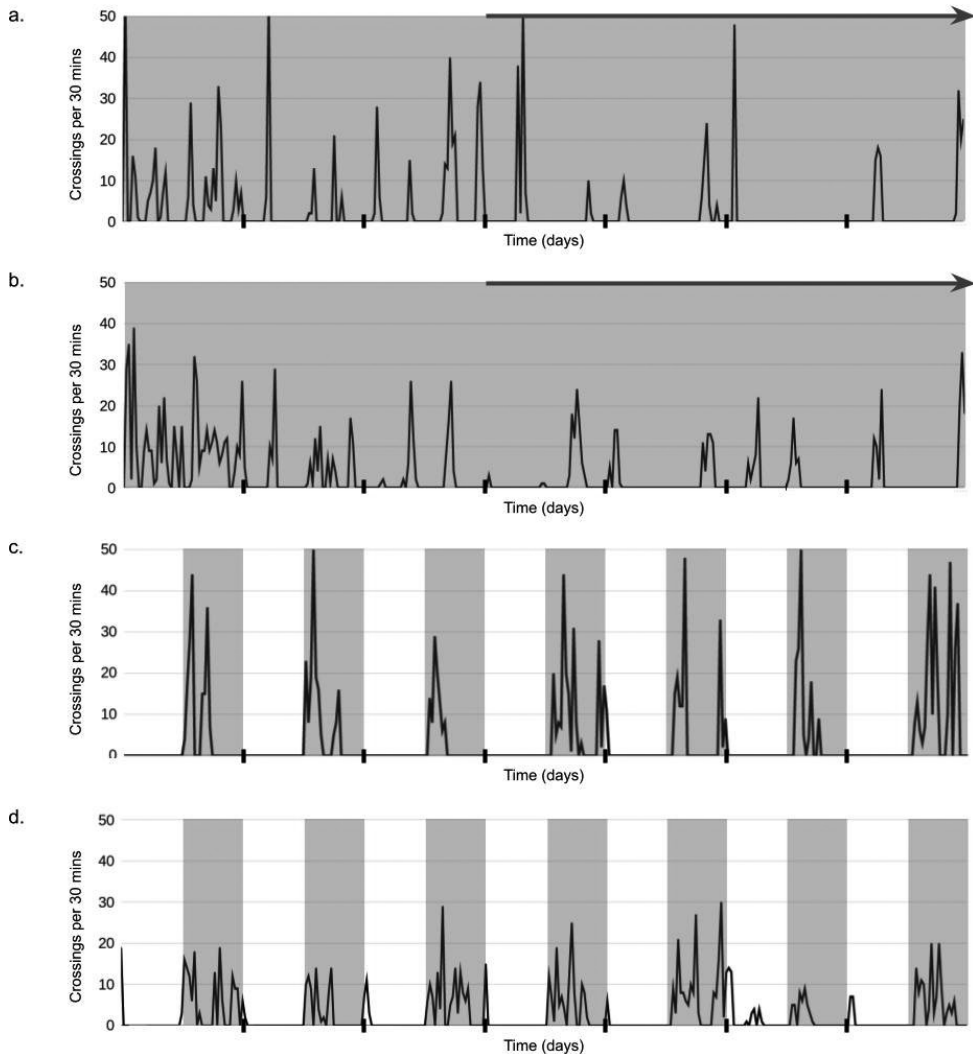


Figure 1. Initial 7-day activity profiles of *P. hirtus* adults under DD and LD. Graphs represent the first seven days of locomotor activity monitoring. Light-off phases indicated by grey overlays **a, b** adjusting animals in DD. Top right arrows indicate start of analyzed time span after the 3-day time window arbitrarily defined as adjustment phase **c, d** examples of adjusting animals in LD.

a decline of locomotor activity during the first three days of the trials. On day 1, individual locomotor activities averaged 29.4 and 18.2 crossings per hour, compared to an average of 14.5 and 11.2 crossings per hour on day 2, and followed by 5.2 and 3.3 crossings per hour on day 3. As the latter numbers were close to the averages of 4.3 and 8.3 crossings per hour observed on day 4, we concluded that the two animals had completed an initial phase of adjustment to the TAM capillary environment by the third day of monitoring. Activity patterns were therefore analyzed starting from day 4 of the observation period (Fig. 1a, b).

For the two adjusted animals monitored at DD, individual average locomotor activity levels amounted to 2.9 and 2.8 crossings per hour over 11.5 days of observation time following the adjustment phase. Further inspection of the corresponding temporal activity plots revealed that these low overall locomotor activities were the result of long resting phases interspersed by short activity bouts (Fig. 1a, b). The latter measured on average 1.6 (+/-0.8) and 1.3 (+/-0.7) hours for each animal in contrast to average resting phase durations of 9.8 (+/-6.5) and 13.8 (+/-10.4) hours. Peak resting phases exceeded 30 hours (Suppl. material 2).

Actogram analyses suggested a random distribution of activity bouts between resting periods (Suppl. material 3). Consistent with this, tests for periodicity in activity distribution failed to detect significant rhythmicity.

Presumptive night concentrated activity of *P. hirtus* exposed to 12h LD cycles

To test whether locomotor activity was affected by light in *P. hirtus*, we monitored animals exposed to 12:12 LD. Overall, the adjustment rate to the TAM environment was slightly higher compared to the tests conducted in DD, with six out of 28 animals tolerating the test tube environment for long-term, producing data for more than 20 days (Suppl. material 4). The activity profiles of adjusted LD animals differed conspicuously from that of the DD animals. Locomotor activity was concentrated in the artificial night (D) phases with an average of 15.6 tube crossings per hour (+/-3.3), which compared to only 1.3 (+/-0.7) in the L phases (Fig. 1c, d) Suppl. material 4). 80% of the L-phases were characterized by non-detectable locomotor activity (Suppl. material 4).

Higher locomotor activity in the LD-monitored animals

Given the activity pattern differences between DD- and LD-monitored animals, we asked whether the long-term LD-adjusted animals had expended more locomotor activity than the two animals monitored in constant darkness. Comparing the daily numbers of tube crossings revealed that the six LD-adjusted animals had performed an average of 191.3 (+/-36.3) tube crossings per day compared to 66.8 (+/-2.0) crossings by the two DD-adjusted animals (Suppl. materials 2, 4). These numbers revealed a close to threefold higher energy expenditure of the LD animals compared to the two DD-monitored animals.

Longer resting breaks of DD-monitored animals compared to 12h L/D-adjusted *P. hirtus*

The difference in daily activity amounts between the LD- and DD-adjusted animals could have been due to differences in activity intensities, activity phase durations, rest phase durations, or a combination of the three variables. To evaluate their relative impacts, we compared their ranges and averages between the LD- and DD-adjusted animals (Fig. 2). Given the preponderance of locomotor activity displayed by the LD-

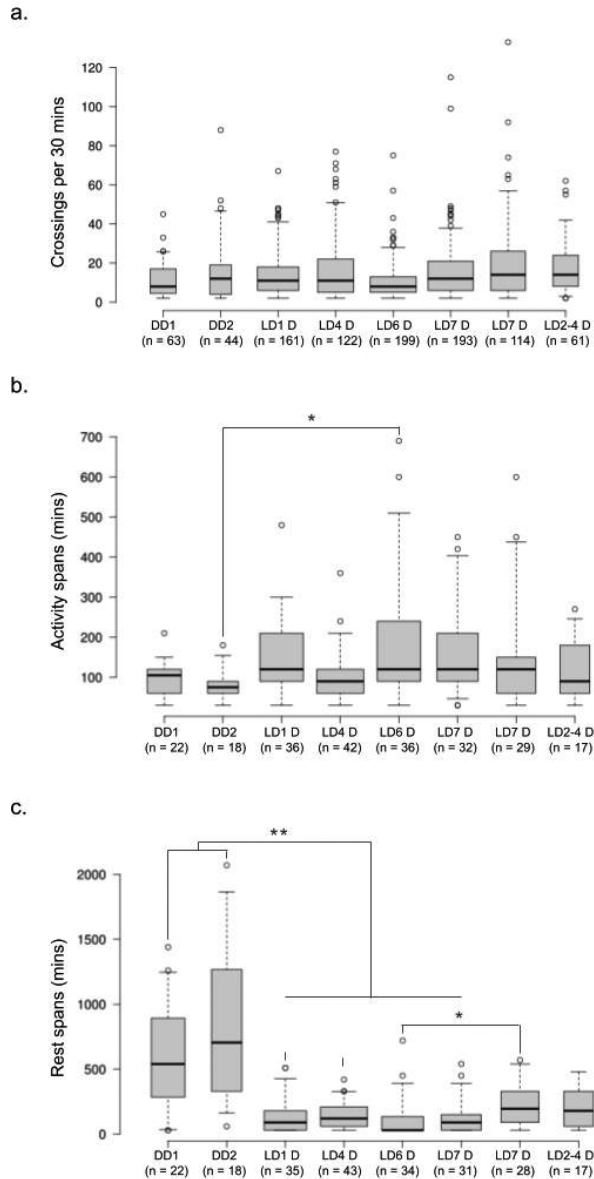


Figure 2. Analyses of rest-activity in long-term DD- and LD-adjusted animals **a–c** box plots, visualizing the relative numbers of observations as widths of boxes median values as back bars inside boxes. Altman whiskers extending from 5th to 95th percentile. Each comparison shows the results for the two long-term DD-adjusted animals (DD1 and DD2) followed by the results for the six long-term DD-adjusted animals (LD1, LD4, LD6, LD7, LD8, and LD2-4 in the D-phase **a** comparison of activity intensities measured as the number of tube crossings per discrete activity bout. Time intervals with less than 2 tube crossings were excluded. Numbers of bouts per animal is given in parentheses. Kruskal-Wallis ANOVA detected no statistically significant differences after adjustment for multiple comparisons **b** comparison of activity bout durations with numbers of bouts per animal given in parentheses **c** comparison of resting period durations with numbers of resting periods per animal given in parentheses.

adjusted animals in the presumptive night phases, we focused these comparisons on the D-phase activity profiles of the LD-adjusted animals.

The mean activity intensities of the two DD-adjusted animals were 11 and 16 tube crossings per half hour (Fig. 2a). These values fell within the range of mean D-phase activity intensities in the LD-adjusted animals, which spanned from 11 to 20 tube crossings per half hour (Fig. 2a and Suppl. material 5). None of the activity intensities were detected as significantly different from other tested animals.

The mean activity bout durations of the DD-adjusted animals were 94 (+/-50) min and 80 (+/-42) min (Fig. 2b). These numbers were closely below the lowest mean of 103 (+/-42) min in the LD-adjusted animals and close to twofold lower than the maximum mean of 164 (+/-110) min in the LD-adjusted animals (Fig. 2b and Suppl. material 5). There was also a conspicuous difference in the spread of maximum activity phase durations in the LD-adjusted animals, reaching up to 690 min in contrast to the maximum activity durations of 180 min and 210 min of the two DD-phase-adjusted animals (Fig. 2b, Suppl. material 5). These findings did indicate a possible contribution of activity bout duration to the net activity differences between LD- and DD-adjusted animals. However, only one DD-adjusted vs LD-adjusted animal comparison was detected as marginally significant (Fig. 2b).

The average resting period durations differed most prominently between the DD- and LD-adjusted animals. While the former were characterized by mean resting phase durations of 586 min and 828 min, mean resting phase lengths among the LD-adjusted animals ranged between 107 min to 231 min (Fig. 2c and Suppl. material 5). The maximum resting phase durations of 1,440 min and 2,070 min of the two DD-adjusted animals compared to 720 in the LD-adjusted animals (Fig. 2c, Suppl. material 5). Both DD-adjusted animals differed from four of the six LD-adjusted animals with high statistical significance (Fig. 2b). Taken together, these findings identified the variation in resting phase durations as the key variable underlying the net activity differences between LD- and DD-adjusted animals.

Lack of free-running activity rhythm

To probe whether the nocturnal locomotor activity rhythm of LD-cultured animals was entrainable in *P. hirtus*, we transitioned the LD-initiated animals into DD after 17 days. Without exception, all six animals discontinued their circadian activity rhythms, displaying stochastically occurring activity bouts (Fig. 3). Comparing the periodograms covering the DD period of seven days did not produce evidence of shared rhythms. The lack of evidence of free-running locomotor rhythms led us to conclude that the circadian locomotor clock has completely regressed in *P. hirtus*.

Discussion

The high lethality of tester animals in the TAM, as utilized in our experiments, confirms the systematic challenge of working with cave species (Mammola et al. 2021) and

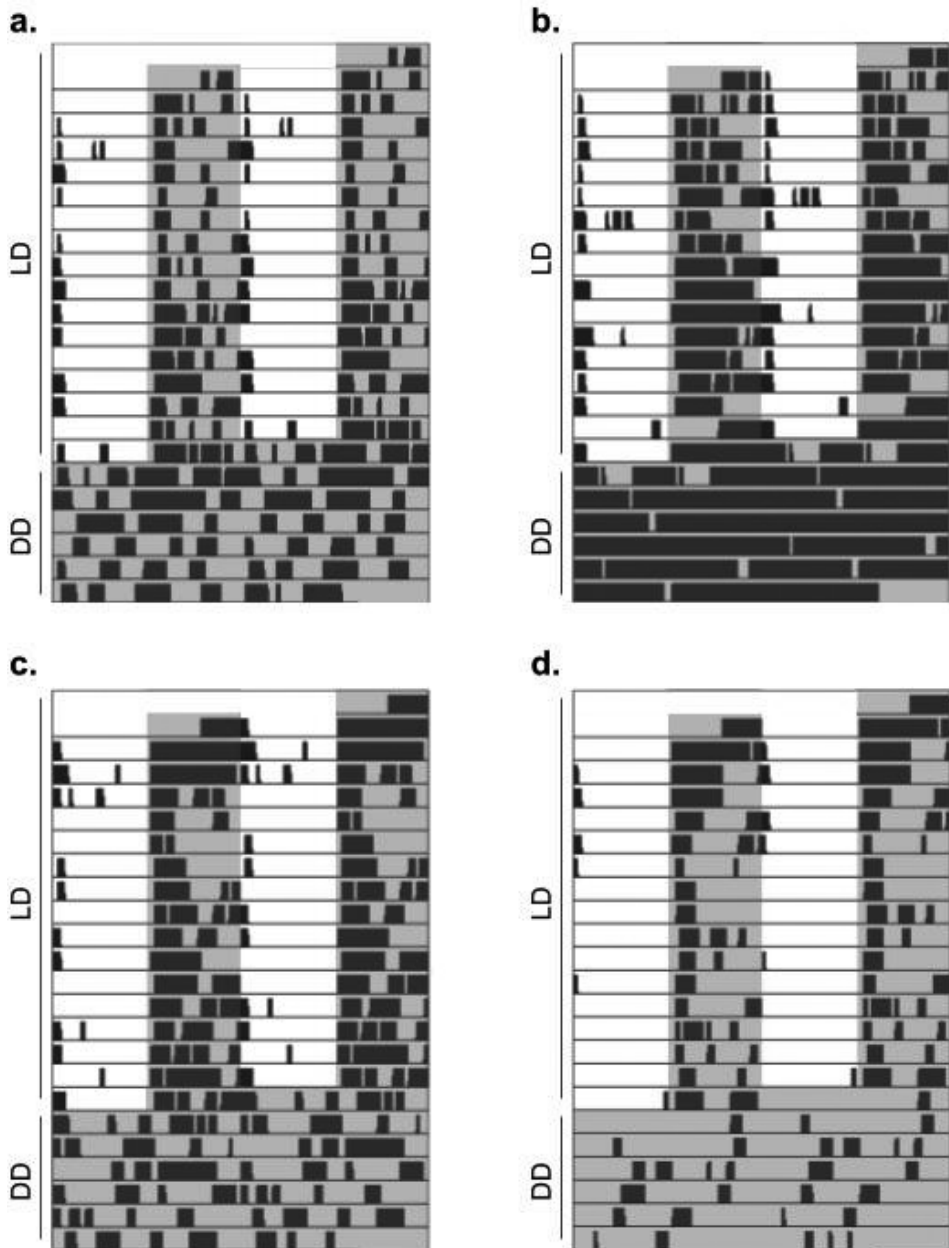


Figure 3. Lack of free-running circadian rhythm in LD-entrained animals. Actograms for LD animals 4, 6, 7, and 8 generated with Actogram (Schmid et al. 2011) at default settings. Each row represents two days, shifted by one day forward in relation to the previous row. Grey overlays indicate light-off periods.

reveals possible limitations of the TAM setup despite its well-documented versatility in surface species studies (Bahrndorff et al. 2012; Giannoni-Guzmán et al. 2014; Pavan et al. 2016; Wang et al. 2021). Future efforts will need to explore how food provisioning

and stringent humidity controls affect *P. hirtus* viability in the confinement of the TAM test tubes. In preliminary experiments, we find that *P. hirtus* adults can be cultured for over two months on moist filter paper on a weekly feeding schedule (MF, unpublished). Given the abundance and relatively easy accessibility of *P. hirtus* in the Mammoth Cave system, it should be possible to develop successful laboratory as well as field-based long-term monitoring approaches of *P. hirtus* locomotor activity.

Further evidence that *P. hirtus* is sensing light

While very limited in trial number and likely impacted by artificial stressors, our observations gained from monitoring two animals in DD and six animals in LD yield new compelling insights into the role of light in the biology of *P. hirtus* and provide preliminary evidence of possibly adaptive deep cave- vs twilight zone rest-activity (RA) modes in this microphthalmic cave beetle species.

The first strongly supported finding of our study is that locomotor activity is affected by exposure to light in *P. hirtus*, given the highly nocturnal activity patterns of the six monitor-adjusted LD animals. This is further supported by the contrasting activity patterns of the two DD-adjusted animals. It is also consistent with the previously reported light avoidance of *P. hirtus* as well as the transcriptomic and structural evidence of a reduced but functional visual system (Friedrich et al. 2011). Given the likely lack of extraretinal light perception in *P. hirtus* based on the failure to detect homologs of extraretinal opsins in the available transcriptome data (Friedrich et al. 2011), it is most likely that the locomotor activity response to light is mediated through the *P. hirtus* eyelets.

Laboratory evidence that the locomotor clock has regressed in *P. hirtus*

As the second strongly supported finding of our study, our results leave little doubt that *P. hirtus* lacks the capacity to maintain a free-running circadian locomotor rhythm instructed by light as the zeitgeber. The previously reported expression of the biological clock gene network in the adult head may thus represent the activity of central pacemaker neurons responding to different zeitgeber sources. Alternatively, core clock gene expression may be associated with different outputs. In *Drosophila*, for instance, the expression of *Clk* and *cyc* in specific pacemaker neurons controls non-circadian rest-activity (RA) patterns and their maintenance throughout life history (Keene et al. 2010; Vaccaro et al. 2017).

Transcriptome-based studies in samples of independently evolved cave populations of the Mexican cavefish *Astyanax mexicanus* revealed the parallel regression of both core clock gene regulation and target gene regulation (Mack et al. 2020), despite the conservation and non-retinal expression of a considerable number of opsin genes (Simon et al. 2019). Unlike in *P. hirtus*, however, locomotor rhythms are short-term entrainable (Espinasa and Jeffery 2006; Carlson and Gross 2018).

Interestingly, cave populations of Asian loach (Family Balitoridae) species were found to display higher frequencies of conserved circadian clock penetrance and expressivity (Pati 2001; Duboué and Borowsky 2012). Together with the conserved

food provision-entrainable clock of *P. andruzzi* (Cavallari et al. 2011), these findings indicate different trajectories of central clock conservation in fish and beetles. This may be explained by the shorter time frame of cave colonization and the continued interbreeding with surface fish in the case of *A. mexicanus*. Further light on the generality of this difference could be gained by comparative studies of the considerable number of cave-dwelling congeners of *P. hirtus* in the central and southeastern United States of America (Leray et al. 2019) and the large numbers of subterranean beetle species in the Palearctic and Australia (Leys et al. 2003; Ribera et al. 2010).

Conserved nocturnality: Ancestral adaptive response to predation pressure in the twilight zone?

Another strongly supported finding of our study is the D-phase activity preference of *P. hirtus*, when cultured in LD. This finding suggests that *P. hirtus* is a nocturnally active occupant of twilight zones in the open cave system of Mammoth Cave National Park. Our observations during collections confirmed the presence of *P. hirtus* in twilight areas, but we did not explore RA patterns in the field. It is interesting to note, however, that *P. hirtus* displayed a similarly strong level of photophobia as its close relative *P. cavernicola* in light-dark choice tests (Friedrich et al. 2011). *P. cavernicola* is a facultative cave species, equipped with fully developed compound eyes (macrophthalmic) and flight-facilitating hind wings, and occupies a wide dispersal area in the Southeast of the United States (Peck 1984). While *P. cavernicola* has not yet been explicitly tested for nocturnality, this seems very likely given its strong avoidance of light. It seems reasonable, therefore, to speculate that nocturnality is an ancestral shared trait of *P. hirtus* and *P. cavernicola*. Ultimate evidence to probe this idea, however, will require comparative studies in both, closely-related cave- and surface species.

As the twilight zones of open caves constitute continuous links between surface and completely light-secluded deep cave space areas, it is further tempting to speculate that nocturnality originated in response to predation pressure at daylight. In further support of this, similar “nocturnal” activity profiles have been reported in all tested microphthalmic cave beetle species so far (Lamprecht and Weber 1977; Rusdea 1990; Friedrich 2013b). Also the phylogenetically more distant anophthalmic cave-dwelling amphipod *Niphargus puteanus* has been found to display a nocturnal activity profile when exposed to LD cycles (Gunzler 1965). Taken together, these data support the idea that nocturnality or crepuscular behavior are pre-adaptive traits for cave colonization (Romero 2011).

An obvious task towards gaining a better understanding of the significance of nocturnality in *P. hirtus* would be to elucidate the identity and biology of its assumed predators. Besides *P. hirtus*, Mammoth Cave is populated by six ground beetle species of the genera *Neaphaenops* and *Pseudoanophthalmus*, which are predators of small invertebrates, which might include larval or adult *P. hirtus* (Barr 1966, 1967; Culver and Hobbs 2017). Indeed, the most abundant of these, *Neaphaenops tellkampfi*, which is completely devoid of a visual system (Ghaffar et al. 1984) and highly specialized to prey on cave cricket eggs (Kane and Norton 1975), was found to consume *P. hirtus* larvae in laboratory culture

(Griffith 1990). In addition, it is possible that the massively abundant nymphal stages of the cave cricket *Hadenoeus subterraneus*, a likely omnivore (Levy 1976; Studier et al. 1986; Helf 2003), might exert daytime predation pressure on *P. hirtus* in twilight zones. *H. subterraneus* adults possess well-developed compound eyes, populate the cave ceilings, conduct nocturnal foraging dispersal into the surface environment, and have been reported to maintain a long-term free-running nocturnal activity rhythm (Reichle et al. 1965). The activity patterns of juvenile *H. subterraneus*, however, which populate cave floors reaching into the twilight zone at high frequencies, have not been explored yet. Last but not least, it is furthermore likely that surface species disperse into peripheral sections of the twilight zones, maintaining the ancestral predation pressure on nocturnality.

More resting in constant darkness: A cave-adaptive outcome of nutrient scarcity?

The final notable finding of our study is that LD-monitored *P. hirtus* displayed about three-fold higher locomotor activity than the two monitor-adjusted DD animals, mostly due to longer resting periods as opposed to differences in activity levels or activity bout durations. While preliminary, given the small sample size of our study, these findings raise the possibility that *P. hirtus* engages in light-contingent rest phase modes that may be adaptively optimized to cope with the contrasting nutrient provisions of the deep cave and twilight zone environments.

A rich body of studies in *Drosophila* confirmed that prolonged resting periods are generally reflective of physiologically and neurologically conserved sleep-like states in insects (Anafi et al. 2019; Beckwith and French 2019; Shafer and Keene 2021). Moreover, studies in vertebrate and invertebrate species converge on supporting the model that the amount of sleep and its circadian distribution are subject to regulatory mechanisms that are independent of the central clock (Borbély 1982; Borbély and Tobler 1996; Duboué et al. 2011; Duboué and Borowsky 2012). Of further potential relevance is that core biological clock genes regulate sleep frequency in *Drosophila*, as mentioned above (Keene et al. 2010). In light of these data from other species, it is reasonable to speculate that the RA patterns of *P. hirtus* are the outcome of conserved clock gene expression and yet independent of a central clock.

As a scavenger species, *P. hirtus* likely has to invest energy in foraging. The nutrient-poor environment of deep cave zones may enforce a more conservative energy investment strategy in the form of a lower food search frequency compared to the more nutrient-rich twilight zones. In the latter, *P. hirtus* likely enjoys higher probabilities of successful resource discovery, translating into a higher energy budget for sustaining foraging mobility and reproduction. It is therefore conceivable that *P. hirtus* may be characterized by cave zone ecology-adjusted sleep duration states.

Light regimen contingent sleep duration states have been reported in a number of cave-adapted fish species. In contrast to *P. hirtus*, however, cavefish species so far analyzed are characterized by higher amounts of energy expenditure in constant darkness due to the shortening of rest phases. This is true for the diverse cave populations of *A. mexicanus* as well as cave-adapted loach species (Duboué et al. 2011; Duboué and Borowsky 2012;

Carlson and Gross 2018). This difference between *P. hirtus* and cave-adapted fish may be due to different trophic structures of the aquatic subterranean environments of the cavefish populations and the terrestrial deep cave zone populated by *P. hirtus*.

Drosophila has been found to respond to feeding scarcity with activity increase (Lee and Park 2004; Meunier et al. 2007; Keene et al. 2010; Yang et al. 2015). However, fruit flies can also be subjected to selection for starvation resistance, which results in activity reduction due to sleep phase extension (Masek et al. 2014; Slocumb et al. 2015; Miura and Takahashi 2019). It is, therefore, tempting to speculate that the proposed sleep phase extension state of *P. hirtus* in constant darkness may represent the result of long-term selection to tolerate food scarcity. Equally intriguing, suppressing flight capacity has been found to extend sleep duration in *Drosophila* (Melnattur et al. 2020). This raises the fascinating possibility that the adaptive sleep-extended state in the flightless *P. hirtus* may have originated as an integrated outcome of the complete regression of flight capacity. Taken together, our laboratory experiments with *P. hirtus* uncovered compelling but preliminary evidence of two new paradigms of cave adaptation, which invite further study in this and other cave species systems.

Conclusions

Given the small number of successfully TAM-monitored animals the findings of our study constitute preliminary evidence that the central locomotor clock has regressed in *P. hirtus*. Our findings further suggest that *P. hirtus* is nocturnally foraging in twilight areas of the Mammoth Cave system and may switch to longer resting periods in the deep cave areas to meet the challenge of more limited food resources.

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Supplementary material I

Activity logs of select non-adjusting DD animals

Authors: Sonya Royzenblat, Jasmina Kulacic, Markus Friedrich

Data type: Trikinetics Activity Monitor output

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Link: <https://doi.org/10.3897/subtbiol.45.100717.suppl1>

Supplementary material 2

Activity logs of the two long-term adjusted DD animals

Authors: Sonya Royzenblat, Jasmina Kulacic, Markus Friedrich

Data type: Trikinetics Activity Monitor output

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Link: <https://doi.org/10.3897/subtbiol.45.100717.suppl2>

Supplementary material 3

Actograms of the long-term adjusted LD animals

Authors: Sonya Royzenblat, Jasmina Kulacic, Markus Friedrich

Data type: Trikinetics Activity Monitor output

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Supplementary material 4

Activity logs of the long-term adjusted LD animals

Authors: Sonya Royzenblat, Jasmina Kulacic, Markus Friedrich

Data type: Trikinetics Activity Monitor output

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Supplementary material 5

Activity stats comparisons of monitor-adjusted LD animals in D phase with monitor-adjusted DD animals

Authors: Sonya Royzenblat, Jasmina Kulacic, Markus Friedrich

Data type: Trikinetics Activity Monitor output

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